Methods and Principles in Medicinal Chemistry

Edited by
Eric Francotte and Wolfgang Lindner

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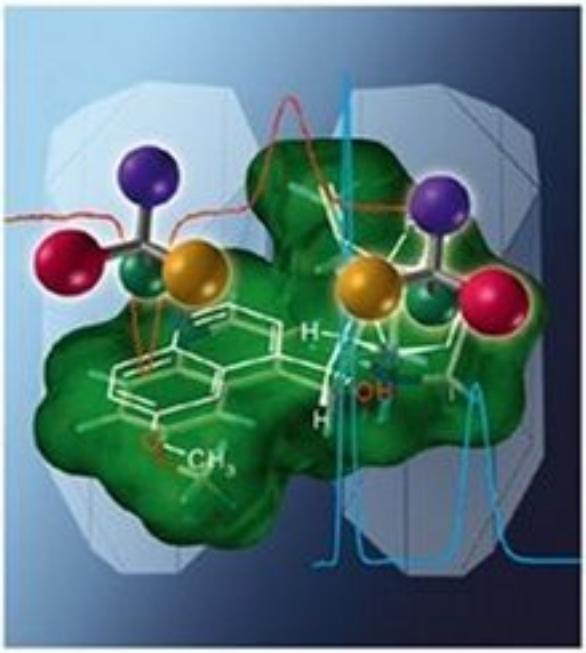
Chirality in Drug Research



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R. Mannhold,
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G. Folkers

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Series Editors

Prof. Dr. Raimund Mannhold
Biomedical Research Center
Molecular Drug Research Group
Heinrich-Heine-Universität
Universitätsstrasse 1
40225 Düsseldorf
Germany
mannhold@uni-duesseldorf.de

Prof. Dr. Hugo Kubinyi
Donnersbergstrasse 9
67256 Weisenheim am Sand
Germany
kubinyi@t-online.de

Prof. Dr. Gerd Folkers
Collegium Helveticum
STW/ETH Zurich
8092 Zurich
Switzerland
folkers@collegium.ethz.ch

Volume Editors

Dr. Eric Francotte
Novartis Institutes for Biomedical Research
Discovery Technologies
WKL-122.P.25
4002 Basle
Switzerland
eric.francotte@novartis.com

Prof. Wolfgang Lindner
Institute of Analytical Chemistry
University of Vienna
Währinger Strasse 38
1090 Vienna
Austria
wolfgang.lindner@univie.ac.at

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Contents

Chirality in Drug Research. Edited by Eric Francotte and Wolfgang Lindner

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Preface

It are the 21 stereocenters in the gycopeptide antibiotics that give always raise to a headache for my students and it takes them some time to appreciate the incredible precision of complementarity in binding of these drugs to their bacterial targets.

The fascinating world of chirality in drug research, where microorganisms have been the masters for a long time, is brought to us in this new volume in the series, edited by Eric Francotte and Wolfgang Linder. There are three major parts to reflect the chiral research and technology of today. The first one is dealing with synthesis, the second with separation and the third one with analysis and modeling.

Here we learn how man created a methodology to get access to a chiral universe, so far property of nature. Research started by copying biological mechanisms or simply make use of natural sources. For instance microorganisms have been, and are still used to produce the desired structures. Today, highly sophisticated concepts of chiral catalysis, rather a pleonasm, are able to introduce almost all desired chirality at all locations in a synthetic molecule. Proof of the concept are numerous multi-step syntheses of complex natural compounds from a immensely broad variety of sources.

Even more impressive is the development of appropriate analytics. Modern NMR technology reveals such perfect complementarity as in the case of glycopeptides at levels of atomic resolution, providing virtual threedimensional models of ligand protein interaction. Bacterial resistance and means to cope with it, can be discussed by studying models of chiral interaction in the computer.

We have not always been in such a lucky position. Chirality and drugs are still related to each other by the tragedy of the sedative drug thalidomide, the "wrong" enantiomer being the cause for thousands of children being born disabled. But it lasted until recently that research showed that it would have been useless to separate the "wrong" enantiomer since the "good" one is immediately metabolized to the "bad" one in our body. The tragedy led to profound and sustainable change in the ways of thinking about individuality of molecules raising the concept of enantiomers being two different chemical entities at least with respect to drugs.

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The historical perspective by Joseph Gal is therefore a perfect introductory chapter for the book.

We would like to express our gratitude to Renate Doetzer and Frank Weinreich from Wiley-VCH for their invaluable support in this project.

Raimund Mannhold, Düsseldorf
Hugo Kubinyi, Weisenheim am Sand
Gerd Folkers, Zürich

A Personal Foreword

Although the origin of chirality in life is still obscure, it is the source of diverse phenomena on the macromolecular and molecular level, governing our environment and the existence of living organisms. The principle of molecular chirality was established over a century ago by van't Hoff and LeBel, but awareness of how this characteristic affects the biological activity of molecules, is much more recent. Likewise, systematic investigation of the biological activity, including pharmacology and toxicology of individual stereoisomers, only recently became commonplace for all new chiral drugs. Due to increased interest in the consequences of chirality on physical and biological properties of molecules, the preparation of pure stereoisomers has become a topic of great importance, and methods of supplying optically pure isomers are being intensively pursued. In this context, there has been a rapid development of stereoselective synthetic methodologies, which have now reached a high degree of diversity and complexity. Developed synthetic approaches include those utilizing chiral building blocks or chiral auxiliaries, highly efficient catalytic processes, stereoselective enzymatic reactions and separation techniques. These different approaches are reviewed in dedicated chapters. Concomitantly, this trend created a rapid increase in the demand for stereoselective analysis techniques, capable of determining precisely the stereoisomeric composition of chiral compounds from synthesis, from biological assays and from pharmacological, metabolic and clinical studies. Among the different methodologies developed for this purpose, gas and liquid chromatographic separation on chiral stationary phases has attracted the attention of numerous research groups and is currently considered the method of choice for analyzing chiral compounds. Other techniques, such as capillary electrophoresis or sensors, have also been found to be useful for specific applications. The state-of-the-art for most of these analytical techniques is described in this book. Physical and chiroptical methods have also been intensively used for investigating and characterizing chiral drugs and are discussed as well. This book is meant to serve as a reference for scientists interested in the chirality-related aspects of chemistry and analysis.

Basle and Vienna
June 2006

Eric Francotte
Wolfgang Lindner

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List of Contributors

Hans-Jürgen Federsel
Global Process R&D
AstraZeneca
151 85 Södertälje
Sweden

Gerd Folkers
Collegium Helveticum
STW/ETH Zurich
8092 Zurich
Switzerland

Eric Francotte
Novartis Institutes for Biomedical Research
Discovery Technologies
WKL-122.P.25
4002 Basle
Switzerland

Joseph Gal
Division of Clinical Pharmacology and Toxicology
University of Colorado School of Medicine
Denver, CO 80262
USA

Nobuyuki Harada
Institute of Multidisciplinary Research for Advanced Materials
Tohoku University
2-1-1 Katahira
Aoba
Sendai 980–8577
Japan

Philipp Krastel
Novartis Institutes for Biomedical Research
Discovery Technologies
WSJ-506.4.01
4002 Basle
Switzerland

Wolfgang Lindner
Institute of Analytical Chemistry and Food Chemistry
University of Vienna
Währingerstrasse 38
1090 Vienna
Austria

Norbert M. Maier
Institute of Analytical Chemistry and Food Chemistry
University of Vienna
Währingerstrasse 38
1090 Vienna
Austria

Chirality in Drug Research. Edited by Eric Francotte and Wolfgang Lindner

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Michael Müller
Institute of Pharmaceutical Sciences
Department of Pharmaceutical and Medicinal Chemistry
Albert-Ludwigs-University Freiburg
Albertstrasse 25
79104 Freiburg
Germany

Frank Petersen
Novartis Institutes for Biomedical Research
Discovery Technologies
WSJ-506.4.01
4002 Basle
Switzerland

Pavel Pospisil
Radiology Department
Harvard Medical School
220 Longwood Avenue
Boston, MA 02115
USA

Silvio Roggo
Novartis Institutes for Biomedical Research
Discovery Technologies
WSJ-506.4.01
4002 Basle
Switzerland

Serge Rudaz
Laboratory of Pharmaceutical Analytical Chemistry
School of Pharmaceutical Sciences
University of Geneva
20 Bd. d'Yvoy
1211 Geneva 4
Switzerland

Kazuhiko Saigo
The University of Tokyo
Graduate School of Engineering
Department of Chemistry and Biotechnology
7-3-1 Hongo
Bunkyo-ku
Tokyo 113
Japan

Kenichi Sakai
Specialty Chemical
Research Laboratory
Toray Fine Chemical Co., Ltd.
Ooe-cho 9-1, Minato-ku
Nagoya 455-8502
Japan

Esther Schmitt
Novartis Institutes for Biomedical Research
Discovery Technologies
WSJ-506.4.01
4002 Basle
Switzerland

Ansgar Schuffenhauer
Novartis Institutes for Biomedical Research
Discovery Technologies
WSJ-506.4.01
4002 Basle
Switzerland

Jean-Luc Veuthey
Laboratory of Pharmaceutical Analytical Chemistry
School of Pharmaceutical Sciences
University of Geneva
20 Bd. d'Yvoy
1211 Geneva 4
Switzerland

Marcel Wubbolts
DSM Research
Department of Advanced Synthesis,
Catalysis & Development
DSM Research
P.O. Box 18
6160 MD Geleen
The Netherlands

Mine Yarim
Faculty of Pharmacy
26 Agustos Campus
Kayisdagi Street 34755
Kadikoy, Istanbul
Turkey

Introduction

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1
Chiral Drugs from a Historical Point of View

Joseph Gal

1.1
Introduction

Chiral molecules are constituents of a large proportion of therapeutic agents. In 1984 Simonyi surveyed a Swedish manual of drugs in clinical use and found that of a total of 666 drugs 355 (53%) had at least one chiral center; 181 drugs (27% of the total) were in use in single-enantiomer form while 174 (26%) were racemic [1]. In1987 Ariens and Wuis estimated that ca. 57% of marketed drugs are chiral (that is, are based on chiral molecules, be they racemic, single-enantiomeric, or some other mixture of chiral stereoisomers) [2]. They also showed that ca. 55% of the chiral drugs were used clinically in the racemic form and the remainder as single-enantiomers. Overall it appears, therefore, that by the end of the century ca. half of the chiral drugs were single-enantiomeric and the other half racemic.

The situation is different today. With rare exceptions new chiral drugs are developed in single-enantiomer form, and new racemic drugs are highly unlikely to appear. This is a profound change in drug development from a stereochemical viewpoint. How did we get here? What are the factors that have influenced the introduction and use of therapeutic agents based on chiral molecules? What is the history of chiral drugs?

1.2
A Word About Words

Before we attempt to answer the above questions, we need to examine briefly the terminology relevant to a discussion of chiral drugs. Specifically, the definition and usage of two important terms need to be clarified. *Chiral* was defined in one recent leading monograph on stereochemistry as follows: "Not superposable ... with its mirror image, as applied to molecules, conformations, as well as macroscopic objects, such as crystals" [3]. Mislow gave a shorter but essentially equivalent definition: "An object is chiral if and only if it is not superposable on its mir-

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ror image; otherwise it is achiral" [4]. Thus, it is clear that *chiral* refers to a spatial property of objects, including molecules. Therefore, the term describes that nature of a molecule which makes it *non-superposable* on its mirror image, and *does not refer to the stereochemical composition of bulk material, i.e., drugs, compounds, substances, etc.* [5]. Thus, "chiral drug" does not tell us whether the drug is racemic, single-enantiomeric, or some other mixture of the stereoisomers. In the present article, therefore, *chiral* will be used strictly according to the definitions cited above, i.e., to refer to the chirality of individual molecules or other chiral *objects*. Thus, "chiral drug", "chiral substance", etc., will be used to indicate that the drug in question is composed of chiral molecules, but the enantiomer composition is not specified by this terminology.

There is however a great and obvious need for a convenient term to refer to chiral substances that are composed of only one of the two enantiomers. Numerous terms for this purpose have been introduced over many years, but the issue remains complex and largely unresolved. The present author recently discussed this issue in detail and introduced a new term for the purpose: *unichiral* [5]. In the present chapter *unichiral* will be used to specify the stereochemical composition of a chiral drug, substance, compound, sample, etc, as stereochemically homogeneous, i.e., consisting of a single-enantiomer (in the context where the term is used and within the limits of measurement) [5].

1.3 Old Chiral Drugs: Natural Remedies 3000BC–1900

For thousands of years, remedies from nature obtained from vegetable, animal, or mineral sources were relied upon for relief from human diseases. Such folk medicine was, by its very nature, inaccurate and unscientific and often had no rational basis. Moreover, the toxicity of many of the products was a serious problem; indeed, some of the pharmacologically active preparations were used as poisons. The advent of the printing press in the 15th century resulted in the wide dissemination of knowledge about natural medications and this in turn produced a considerable increase in the use, and misuse, of such remedies [6]. More rational therapy with purified natural products did not begin until the 1800s.

Despite the problems, however, some of the natural preparations were effective in relieving the symptoms and at times even eliminating the disease. In fact, we know today that the number of pharmacologically active substances produced by nature is large and the spectrum of biological activities of natural products is extraordinarily broad; for example, antimicrobial, antineoplastic, CNS-active, anti-inflammatory, cardiovascular, etc., are only a few of the therapeutic classes of drugs from nature [7].

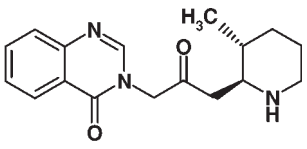
Chirality is a hallmark of many molecules from nature. Indeed, the number of chiral natural molecules is very large and the structural variety they represent is vast. Among such substances – be they small molecules or macromolecules – an overwhelming majority occur in unichiral form. For example, chiral α-amino acids

and the peptides and proteins containing them, sugars and their polysaccharides, steroids, antibiotics, and many other compounds from nature are unichiral. Another important aspect of many chiral molecules from nature is their *homochirality*. This means that related chiral molecules in the same chemical class usually have the same sense of chirality. For example, with rare exceptions α-amino acids occurring in nature consistently have the L configuration; similarly, monosaccharides are of the D configuration. Thus, both *unichirality* and *homochirality* are typical for compounds from nature: most of them occur in enantiomerically homogeneous form, and closely related molecules usually have the same sense of chirality.

In the light of the above, then, it is not surprising that many of the compounds used as therapeutic agents in natural remedies over the centuries and millennia have been chiral and that the vast majority of such substances occur in unichiral form. For thousands of years and until the beginning of the 19th century most such natural remedies were used as crude plant extracts rather than purified active principles. Obviously, in that "pre-scientific" era, the remedies were used without any clue as to the nature or identity of the active ingredient(s) within, let alone any understanding of the chirality of the molecules involved. Recognition of the existence of chiral drugs had to await a better understanding of chemical structure, i.e., the advent of modern organic chemistry and the discovery of molecular chirality (see below).

The number of pharmacologically active agents now known to be present in various old remedies is large [7] and many of these compounds are based on chiral molecules. Information about some of the earliest herbal remedies that contain chiral active ingredients goes back nearly 5000 years. A few examples of old therapies with chiral active ingredients are presented below.

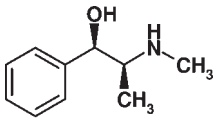
In a book about herbs, the Chinese scholar-emperor Shen Nung described in 2735 BC the beneficial effects of *Ch'ang Shan* in the treatment of "fevers" [8]. This preparation is the powdered root of a plant, *Dichroa febrifuga* Lour. Modern medicinal chemistry has identified several alkaloids with *antimalarial* properties in the plant, and it is therefore clear that the ancient use of Ch'ang Shan in fevers was not entirely without basis. One of the antimalarial compounds from Ch'ang Shan is *februgine* (*β*-dichroine), a relatively simple unichiral compound **1**. Modern attempts to develop these agents as antimalarial drugs failed, due to significant toxicity [8].



1

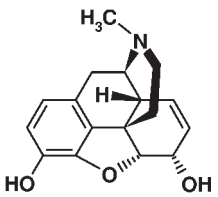
Shen Nung also observed the stimulant properties of another Chinese plant, Ma Huang, now known as *Ephedra sinica* [9]. The chief active ingredient, *ephedrine*, is a sympathomimetic amine, and therefore it is clear in this case also that the use of Ma Huang as a stimulant had a rational basis. The ephedrine molecule is simple and contains two chiral centers; the compound from *ephedra* is unichiral and has the 1*R*,2*S* configuration **2**. Ephedrine was first isolated from Ma Huang in 1887

[10], i.e., more than 4600 years after the effects of the compound were recorded. Ephedrine was introduced into medical practice during the 1920s [11] and for decades was widely used – as a CNS stimulant in narcolepsy, as a bronchodilator, in the treatment of Adams-Stokes syndrome with complete heart block, as a stimulant in some forms of depression, and in some other disorders – but more recently it has been largely replaced in most of these indications by other treatment modalities [12]. Ephedrine has also been widely available in "dietary supplements" for weight loss, increased energy, body building, etc. However, in the early 1990s concern arose over potentially serious adverse effects from such use of ephedrine, including cardiovascular, nervous-system, and other toxic effects, and in April 2004 the U.S. Food and Drug Administration (FDA) banned the sale in the United States of dietary supplements containing ephedrine or closely related compounds [13].



2

Another millennia-old unichiral drug is the opioid agent morphine. *Opioid* refers broadly to all compounds related to opium (a more recent definition states that the term *opioid* includes any compound that interacts with the brain's opioid receptors) [14]. Opium powder is the dried juice from the unripe seed capsule of the poppy *Papaver somniferum* and its name is derived from the diminutive of the Greek word *opos*, i.e. juice. Opium has analgesic, euphoric, and other effects and contains many alkaloids, including morphine **3** and codeine **4**. Poppy juice is mentioned in the writings of the Greek philosopher and naturalist Theophrastus (ca. 371–287 BC), but evidence has been found suggesting that opium may have been known much earlier, to ancient civilizations in Egypt and Mesopotamia (Fig. 1.1) [14, 15]. Within the Arab–Islamic civilization, whose rise began in the 7th century, opium came to be used mainly as a constipant to control dysentery [16]. The arrival of the Islamic armies and their influence in Europe in the 16th century (Constantinople fell to the Ottoman Turks in 1453 and the first siege of Vienna by the Ottoman army took place in 1529) brought opium to Europe. Laudanum, a somewhat purified opium concentrate, was compounded by Paracelsus (Theophrastus Bombastus von Hohenheim, 1493–1541), a Swiss alchemist and physician, and the smoking of opium became openly popular during the 1700s; however, opium may have been extensively but less openly used in Europe in earlier times [17].



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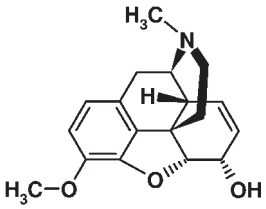


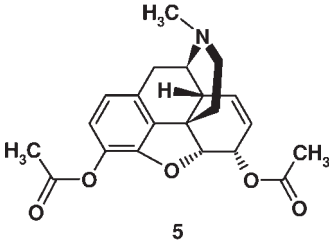
4

Fig. 1.1 Frieze from the palace of Assyrian king Sargon II, in Khorsabad (in modern-day Iraq), depicting two priests. Note the poppy heads carried by the priest on the right. 8th century BC. Musée du Louvre, Paris, Antiquités orientales. Photograph: Service de documentation photographique de la Réunion des Musées Nationaux, Château de Versailles. (Reprinted from Lydia Mez-Mangold, *A History of Drugs*, F. Hoffmann-La Roche & Co., Ltd, Basle, Switzerland, 1971, with permission).

Morphine, the most important alkaloid in opium, was obtained as a purified powder from opium in 1805 by Friedrich Wilhelm Sertürner (1783–1841), a German pharmacist's assistant [18]. He named it *morphium* after Morpheus, the Latin god of dreams, so named by Ovid using a Greek word. Later, the great French chemist and physicist Joseph-Louis Gay-Lussac (1778–1850), who was a strong supporter of Sertürner in his priority claim for the isolation of the substance over French pretenders, renamed the drug *morphine*, against the wishes of Sertürner [19]. The morphine molecule is a pentacyclic tertiary amine with five chiral centers **3** and the natural product is the levorotatory enantiomer.

The invention of the hypodermic needle and syringe in the middle of the 19th century resulted in the widespread use of morphine, and addiction became a common problem. An early – and false – hope to circumvent the addiction liability of morphine was provided by a most unlikely candidate: heroin. This compound, the diacetyl derivative of morphine **5**, is a potent opiate narcotic first synthesized in 1874 via acetylation of morphine, and was introduced into medical practice in 1898 as a cough suppressant [10]. Heroin is a *semisynthetic* drug, i.e., a chemically modified derivative of a natural product, and retains the stereochemistry of mor-

phine. Heroin may have been the first synthetic unichiral drug introduced in clinical medicine.



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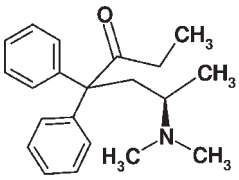
Heroin was actively marketed to physicians by its manufacturer, as an advertisement from ca. 1900 shows (Fig. 1.2). The drug was touted as a "non-addicting" morphine analog that could safely replace morphine and thereby eliminate the latter's addiction problem [20]. This claim turned out to be tragically mistaken and today heroin is the most important abused opioid, with grave social, economic, and medical consequences. Another chiral drug, methadone, a totally synthetic opiate agonist, has been recruited to fight heroin addiction. Methadone was first synthesized, in the racemic form, in the 1940s and was later shown to have stereoselective opioid agonist properties, concentrated nearly exclusively in the (*R*)-(-) enantiomer **6** [21]. Methadone is used in the racemic form in the U.S. as an analgesic and in the treatment of opiate addiction, but in some other countries the pharmaceutical product is the unichiral *levo* form [22].

Perhaps the most fascinating old chiral drug, from a historical point of view, is quinine. Its earliest history is obscure, but it is known that by the early 1600s it was being used by South American natives in Peru, Ecuador, and neighboring regions

Fig. 1.2 Drug advertisement ca. 1900. (Reprinted from Roy Porter (Ed.), *Cambridge Illustrated History of Medicine*, Cambridge University Press, Cambridge, 1996, with permission).

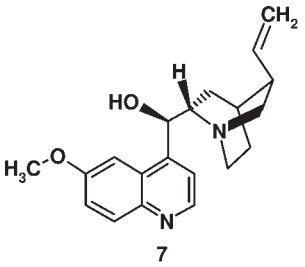


6

as a crude preparation from the bark of the *cinchona* tree (Fig. 1.3) for the treatment of malaria. In 1633 Antonio de la Calancha (1584–1654), an Augustinian monk in Lima, wrote a pamphlet describing the native use and fever-curing powers of cinchona [23] and by the middle of 1600s the extract of "Jesuit's bark" (one of the names cinchona came to be known by) was being used in Europe indiscriminately for a variety of fevers. Cinchona was, however, effective only against malaria, an infectious disease widespread in many regions of Africa and Asia, and even in Europe for centuries. Cinchona was the first effective treatment for malaria, and in 1820 the French pharmacists Pierre Joseph Pelletier (1788–1842) and Joseph Bienaimé Caventou (1795–1877) isolated quinine, the main antimalarial ingredient, from cinchona bark [24]. The quinine molecule contains four chiral carbon centers and the natural product is the levorotatory unichiral compound **7**.

The name *cinchona* was coined by the Swedish botanist Linnaeus (Carl von Linné, 1707–1778) in honor of Doña Francisca Henriquez de Ribera, the fourth *Condesa* (Countess) of Chinchón and wife of the viceroy of Peru, a Spanish colony at the time [24, 25]. According to legend, in 1638 she was cured of malaria by the bark and, impressed with the cure, she took samples of cinchona to Spain, thereby

Fig. 1.3 One of the earliest illustrations of the cinchona tree, from Jonston's *Dendrographias*, published in 1662. (Reprinted from Mark Honigsbaum, *The Fever Trail*, Farrar, Straus and Giroux, New York, 2001, with permission).



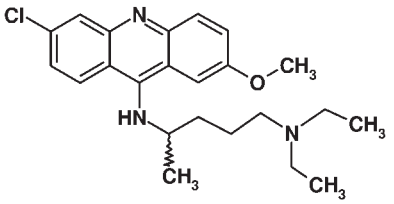
7

launching the European career of the miracle remedy. However, as has been frequently pointed out, there are problems with Linnaeus' nomenclature. First, he misspelled *cinchona*, leaving out the first *h* in the countess' name; second, she died in South America before she could return to Spain [24, 25]. Be that as it may, cinchona has stuck in the official names of several species (e. g., *Cinchona officinalis* L and other species in the *Rubiaceae* family). As for *quinine*, this name is derived from *quina quina* ("bark of barks"), the Spanish spelling of a native Quechua name that was sometimes used for the cinchona tree in Peru, and was given by Pelletier and Caventou to their new substance [26].

After its isolation in 1820, purified quinine quickly replaced the crude cinchona preparations in the treatment of malaria. Supplies of quinine were limited and the need was great, as the drug was in demand for the treatment of malaria not only in Europe but also in various parts of Africa and Asia, where European powers were engaged in establishing or strengthening their colonial control. Chemists in Europe were responding to the need with attempts to synthesize quinine in the laboratory. In England in 1856 an 18-year old chemistry student named William Henry Perkin (1838–1907), working with August Wilhelm von Hofmann (1818–1892), a German professor of chemistry appointed director of the newly established Royal College in London, attempted to synthesize quinine by oxidizing *N*-allyltoluidine with potassium dichromate. The reaction, predictably in hindsight, did not produce quinine, but Perkin's further studies of the reaction led to the discovery of *mauveine*, a purple dye which in turn launched the artificial, "aniline" or "coal-tar", dye industry. The invention of mauveine not only revolutionized the dye and textile industries but also produced an intense stimulatory effect on chemical research in general, on the pharmaceutical industry, and on medicine [27] (Perkin's mauveine is a mixture of two compounds neither of which is chiral).

The need for effective antimalarial drugs has persisted over the nearly two centuries since quinine was first isolated. Modifications of the quinine molecule have produced many useful antimalarial agents, including the chiral drugs quinacrine **8**, primaquine **9**, and chloroquine **10**. These compounds were introduced during the 20th century, in racemic form. Chloroquine (preparation patent issued 1939) was particularly useful inasmuch as it was cheap and effective, but more recently resistance by the malaria parasites to this drug has made it ineffective in many parts of the world where the disease is endemic [28]. Quinine remains a useful antimalarial agent in the treatment of chloroquine- and multidrug-resistant falciparum malaria today, but increasing resistance by the parasites may result in a re-

duction in the drug's importance in the future [29]. Malaria remains one of the great killers, with about 1–1.5 million victims dying of the disease every year, the majority of them African children [30].



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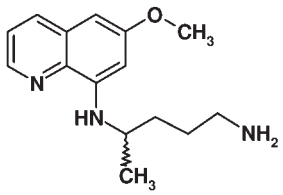

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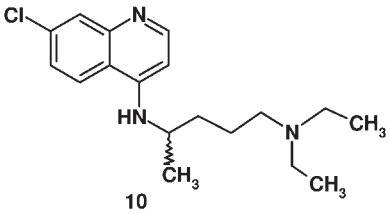

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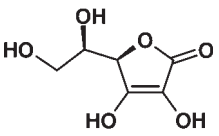
The devastating disease scurvy is caused by insufficient amounts of L-ascorbic acid **11** (vitamin C) in the diet. After the 15th century, exploration, expanding trade, and colonization by European powers required long sea voyages, usually undertaken without foods rich in vitamin C on board. The result was the decimation of ships' crews by scurvy. In a remarkable study in 1747 that can be described as the first serious clinical therapeutic trial, British physician James Lind (1716–1794) (Fig. 1.4), a surgeon in the Royal Navy and the "father of naval hygiene", demonstrated that fruits such as oranges and lemons can reverse and prevent the disease. However, it was nearly 50 years later, in 1795, that the British Admiralty finally took notice of these findings and instituted an appropriate diet on board Royal Navy ships to prevent scurvy [31]. Ascorbic acid was isolated by the Hungarian biochemist Albert Szent-Györgyi (1893–1986) from fruit juices in 1928 and, in part for this work, he was awarded the Nobel Prize in Physiology or Medicine in 1937. In that same year one half of the Nobel Prize in Chemistry went to the English chemist Walter Norman Haworth (1883–1950) for the proof of the structure and synthesis of ascorbic acid.



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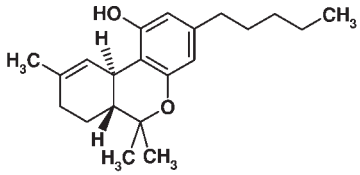
The above examples of old chiral drugs from natural sources are but a handful from a long list of many examples. Others include (some plant origins given in parentheses) tetrahydrocannabinol **12** (marihuana, hashish), digoxin (foxglove,

Fig. 1.4 James Lind, MD. From an engraving by I. Wright from a painting by Sir George Chalmers, Bart. (1783). The engraving forms the Frontispiece to the copy of Lind's *Essay on Diseases incidental to Europeans in Hot Climates* in the library of Haslar Hospital. The signature is from Lind's letter to Sir Alexander Dick. (Reprinted from C. P. Stewart and D. Guthrie (Eds.), *Lind's Treatise on Scurvy*, Edinburgh University Press, Edinburgh,1953).

digitalis lanata Ehrh.), cocaine **13** (*erythroxylon*), cathinone (khat, *Catha edulis* Forsk.), nicotine **14** (tobacco, *Nicotiana tabacum*), atropine (deadly nightshade, *atropa belladonna* L.), reserpine (*Rauwolfia*), colchicine **15** (autumn crocus, meadow saffron), and emetine (ipecac), to name only a few. Each of these chiral compounds has an interesting history but these accounts are beyond our scope here. The chemical structures encompassed by just these relatively few chiral molecules are highly varied. Stereochemically, atropine is an interesting case: this racemic substance is believed not to occur naturally, but its levorotatory form, (*S*)-(–)-hyoscyamine **16**, occurs in several *Solanaceae* plant species and is racemized to atropine during isolation [32, 33]. This facile racemization reaction is the result of the stereochemical lability of the chiral center due to the presence of the adjacent carbonyl group and the *β*-hydroxy group, in combination with its benzylic position.



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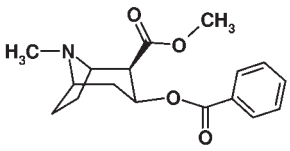

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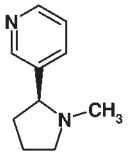

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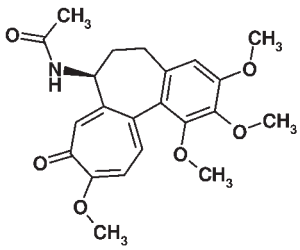

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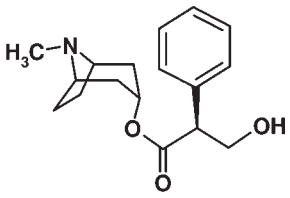


16

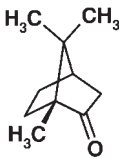
The vast majority of chiral drugs present in the old remedies were unichiral: Mother Nature is not even-handed. All in all, chiral drugs have been of great importance in the development of pharmacotherapy, from the earliest plant remedies of millennia ago to the modern age. Many of these ancient chiral drugs are still in use today, and many new and important drugs have been developed by modifying the molecules of natural products identified in old remedies.

The "pre-science" era of pharmacotherapy based on crude natural remedies came to an end as the 19th century was drawing to a close. The dawn of the modern era of therapeutics did not mean, however, the end of the therapeutic use of natural compounds, chiral or achiral; only the science and technology became different. Beginning with the first decades of the 20th century, natural products were routinely purified from their sources and their chemical structures were elucidated. Chirality, when present, was now recognized.

1.4 Recognition of Chirality in Drugs

The earliest recognition of chirality in drugs was intimately linked to the discovery of molecular chirality. The relevant background work that led to the discovery was accomplished mainly in France during the first half of the 19th century [34]. *Hemihedrism* in crystals – those of quartz – was first reported by René-Just Haüy (1743–1822), a French priest and crystallographer, in 1801 [35]. Circularly polarized light (often referred to as plane-polarized light) was discovered in 1809 by Étienne Louis Malus (1775–1812), and the physicist François Arago (1786–1853) made the first observation of optical rotation by a substance when he studied the effects of quartz crystals on polarized light [34].

French physicist Jean-Baptiste Biot (1774–1862) discovered beginning in 1815 that certain organic compounds rotate polarized light in the noncrystalline state, e.g., in the liquid or solution state. Among these compounds were sucrose, turpentine, camphor, and tartaric acid [34]. Tartaric acid obtained from tartar deposits produced by the fermenting juice of grapes during the wine-making process was discovered by the Swedish pharmacist Carl Wilhelm Scheele (1742–1786) in 1769 [36], and Biot showed that the compound was dextrorotatory [37]. Biot understood that optical rotation by substances in the noncrystalline state was the result of some structural property of the molecules, and he referred to such compounds as *substances moléculairement actives* (molecularly active substances). This realization by Biot of a molecular-structural cause of optical rotation, coupled with his discovery in 1815 of the optical rotation of (+)-camphor **17**, a therapeutic agent, may be considered the earliest scientific hint for chirality in drugs. Camphor, a carminative, rubefacient, and a mild expectorant, is stereochemically a rare example in the field of chiral natural products in that both enantiomers occur in nature. However, (+)-camphor was the only form known in the early 1800s when Biot undertook his studies; (–)-camphor was not discovered until 1853 [38].



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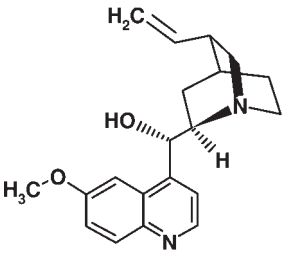
A fuller appreciation of the existence of chiral drugs was achieved a few decades later by the celebrated French chemist (and later microbiologist) Louis Pasteur (1822–1895). Pasteur was familiar with the above-outlined work of Biot on optical rotation by organic compounds. In 1848 he found that the crystals of sodium ammonium tartrate (from *dextro*-tartaric acid) were hemihedral, i.e., there were small facets at alternate corners of the crystals [39]. He recognized that these facets rendered the crystals chiral (Pasteur used *dissymmetric* in the meaning of *chiral* – the latter term was coined by Lord Kelvin only in 1893 [40]). Pasteur then examined the sodium ammonium salt of another, related, acid. That acid had been obtained in 1820 – unexpectedly and on a single occasion – as a side-product during the manufacture of (+)-tartaric acid from tartar at a chemical plant in Thann, Alsace, France [41]. The mysterious new acid intrigued chemists. In 1826 Gay-Lussac obtained a sample for study and named it *racemic acid*, from *racemus*, Latin for cluster of grapes [42]. Racemic acid was found to be identical with (+)-tartaric acid, with the exception that – inexplicably at the time – it did not rotate polarized light, a fact first shown by Biot [43].

Pasteur obtained a sample of the new acid [41, 44] and found – to his initial dismay – that the crystals of sodium ammonium racemate, like those of the corresponding (dextrorotatory) tartrate, were hemihedral (he had predicted that the crystals of the optically inactive acid would not be hemihedral or chiral). To his surprise, however, he observed that there were two different crystals present in the salt of racemic acid. That is, in some of the crystals the hemihedral facets were inclined to the right and some to the left (as in quartz), and Pasteur recognized that the two crystals were related to each other as the two hands, i.e., they were *enantiomorphous*. Pasteur then manually separated the two kinds of crystals and found that they rotated polarized light in solution, the rotations by the two being equal in absolute value (within experimental error) but opposite in direction. The dextrorotatory salt thus obtained was identical in all respects to the corresponding salt of the known (+)-tartaric acid and could be converted to a free acid that was identical in all respects with (+)-tartaric acid, while the levorotatory salt gave an acid that was identical with the natural acid except that it rotated polarized light in the opposite direction. These results led Pasteur to the realization that its crystals were enantiomorphous and the molecules of the two substances in racemic acid must be chiral, due to some 3-dimensional feature of their molecular structure, and that they are mirror-image (i.e., enantiomeric) molecules [39]. This was the discovery of molecular chirality – the year was 1848 and Pasteur had not yet turned 26 years old. The discovery also opened the road toward an appreciation and development of drug chirality.

The first steps on that road were taken by Pasteur himself. In the early 1850s he went on to study many chiral compounds, among them quinine, quinidine **18**, (an antiarrhythmic drug and a diastereoisomer of quinine also obtained from cinchona), etc. He recognized that these molecules were chiral and that the substances isolated from their natural sources were unichiral, and he measured their optical rotation and described their crystal habit [45]. Quinine was already well-known as an antimalarial agent at the time (see above) and we may therefore consider Pasteur's description of this drug as chiral to have been the first clear recognition of molecular chirality in a therapeutic agent. Later, in a lecture in 1860 on the dissymmetry of natural products, Pasteur stated the essence of the matter: ... morphine, codéine, quinine, strychnine, brucine, ...Tous ces principes immédiats sont moléculairement dissymétriques." (... morphine, codeine, quinine, strychnine, brucine, ... All these natural compounds have molecular dissymmetry) [46]. Clearly, Pasteur was the first to appreciate that certain drug molecules are chiral.



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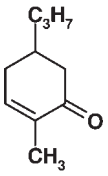
From the chirality standpoint the next fundamental development occurred in 1874, when the tetrahedral carbon atom was proposed as a basis for molecular chirality by the Dutch and French chemists Jacobus Henricus van't Hoff (1852–1911) [47, 48] and Joseph Achille LeBel (1847–1930) [49], respectively, independently and almost simultaneously. The discovery of the "asymmetric carbon atom" (van't Hoff's terminology) finally provided the explanation for the existence of "optical isomers" and for the chiral nature of the molecules of optically active substances, including many drugs. In his original 1874 pamphlet proposing the tetrahedron [47] van't Hoff listed camphor as a chiral molecule, but the structure he gave (**19)** was incorrect.



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Advances in organic chemistry during the second half of the 19th century began the era of the elucidation of the structures of organic molecules, including many chiral molecules. By the early 1880s the 2-dimensional structures (i.e., the connectivity of the atoms) of many relatively simple organic compounds were elucidated, but the structures of more complex molecules were not known. For example, the English edition of *Adolph Strecker's Short Text-Book of Organic Chemistry* (1882), written by Johannes Wislicenus (1835–1902), a leading German chemist of the time, included many naturally occurring chiral drugs, e.g., camphor, codeine, morphine, quinidine, quinine, etc., and optical rotation data was provided for many of them, but their chemical structures were not addressed. Similarly, the second edition of *Watts' Dictionary of Chemistry*, a standard compendium published during the period 1892–1899, had no, or incorrect, structures for the complex drugs mentioned above.

By the end of the 19th century, despite limitations in the elucidation of complex organic structures, many chiral pharmacologically active compounds became available, often in both enantiomeric forms. This in turn led to studies comparing the enantiomers for their pharmacological actions and biological fate.

1.5 Enantioselectivity in Drug Action and Drug Metabolism: The Beginnings

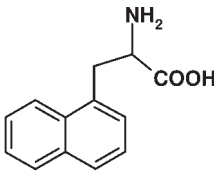
The first observation of biological enantioselectivity was made by Pasteur himself. He found, in 1858, that when solutions of racemic ammonium tartrate were fortified with "organic matter" (i.e., a source of microorganisms) and allowed to stand, the solution "fermented" and (+)-tartaric acid was consumed rapidly while (–)-tartaric acid was left behind unreacted. Eventually the (–)-enantiomer was also metabolized, but considerably more slowly than (+)-tartrate [50]. In later experiments Pasteur showed that the common mold *Penicillium glaucum* metabolized (+)-tartaric acid with high enantioselectivity [51]. He correctly theorized that the enantioselective destruction of tartaric acid by microorganisms involves selective interaction of the tartrate enantiomers with a key chiral molecule within the microorganism [50, 51].

Towards the end of the 19th century the role of chirality in biological activity began to receive serious attention. Two lines of investigation were pursued: one focused on the metabolic fate of chiral compounds while the other examined their pharmacological activity. The first report of enantioselectivity in what may be considered a pharmacological effect appeared in 1886, when (+)-asparagine was found to have a sweet taste while (–)-asparagine was without taste [52]. Pasteur, aware of the finding, interpreted the results as an indication of the presence of a unichiral compound in the nervous system of taste, suggesting that the interactions of the asparagine enantiomers with the chiral biological mediator were different [53]. During the period from the mid-1880s to the mid-1920s many studies comparing the enantiomers of pharmacologically active compounds were carried out, and many examples of enantioselective pharmacological effects were ob-

served. As an example, (–)-hyoscyamine **16** was found to be ca. 12–20 times more potent than the *dextro* enantiomer in a variety of pharmacological effects, e.g., mydriasis in the cat, salivary secretion in the dog, and at cardiac myoneural junctions. Interestingly, (+)-hyoscyamine was the more potent enantiomer in CNS-excitatory effects [54].

By the 1890s stereoselective action by enzymes on substrates was known, in large measure as a result of the monumental work of the great German chemist Emil Fischer (1852–1919) on sugars which spanned the period 1884–1907. Fischer first demonstrated that microbial fermentation of sugars (e.g., by beer yeast) displayed considerable enantioselectivity. Later Fischer extended these studies to the action of enzymes isolated from the microorganisms, and, here too, profound enantioselectivity was found in the reaction of sugars. From his structural and stereochemical studies of sugars as enzyme substrates Fischer concluded that overall shape and stereochemical configuration strongly influence the suitability of a molecule to serve as substrate for an enzyme. He condensed these spatial requirements in the statement that for an enzyme to act on a substrate the two must fit like a lock and its key [55].

It was against this background that a variety of *in vitro* investigations of enantioselectivity in the metabolism of a variety of chiral compounds were undertaken in the late 1800s and early 1900s. Enantioselective enzymatic reactions were shown *in vitro* for many physiological compounds, e.g., amino acids, peptides, lactic acid, etc., but some foreign compounds were also studied. For example, it was found that racemic *β*-(*α*-naphthyl)alanine **20** was enantioselectively metabolized by bacteria, the *levo* enantiomer being consumed while the *dextro* enantioform was untouched [56]. A complex picture of enzymatic stereoselectivity emerged from these studies: depending on the substrates and enzymes, in some cases no enantioselectivity was found while in others one of the enantiomers was selectively acted upon; moreover, in some cases the direction of enantioselectivity changed for the same substrate, depending on the enzyme [57].



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Many i*n vivo* studies of enantioselective metabolism were also carried out in the same period. For example, when (±)-camphor was fed to dogs or rabbits more of the *levo* enantiomer was converted to a glucuronyl conjugate than of the *dextro* enantiomer [58]. When (±)-malic acid was injected subcutaneously into the rabbit larger amounts of (+)-malate appeared in the urine, indicating that (–)-malate (the naturally occurring form) was more extensively metabolized [59].

In 1926 Arthur Robertson Cushny (1866–1926) (Fig. 1.5), a Scottish pharmacologist, reviewed the studies of enantioselective pharmacology and metabolism pub-

Fig. 1.5 Dr. Arthur Robertson Cushny. (Reprinted from V. C. Vaughan, *A doctor's memories*, The Bobbs-Merrill Company, Indianapolis, 1926).

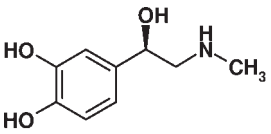
lished during the previous ca. 40 years [60]. The review, which was the first extensive, detailed, and critical discussion of enantioselectivity in pharmacology, reveals a great deal of insight into the nature of chirality and its biological implications. Cushny also made important experimental contributions to the field [60] and was a true pioneer of chirality in pharmacology.

All in all, it is clear that early in the 20th century it was known that drug action and metabolism can be enantioselective. However, this knowledge remained largely in the academic halls of pharmacology, medicinal chemistry, and biochemistry and its broader implications for the creation of safer and more effective drugs were largely ignored until the last 20 years of the century.

1.6 Drug Chirality in the 20th Century

As described above, by the beginning of the 20th century examination of the role of chirality in drug action and disposition had begun and enantioselectivity was found in many cases. Such studies continued at an accelerated rate during the rest of the century. In 1933 Easson and Stedman proposed a fundamental model as the basis for enantioselective drug–receptor interactions [61]. This model was deduced from studies of the pressor effects of the enantiomers of epinephrine which showed a 300 : 1 enantioselectivity, the natural (*R*)-(–) form being the more potent enantiomer **21**. It was concluded that three groups in the molecule – the amino group, the aliphatic hydroxyl group and the electron-rich aromatic ring – interact with three complementary sites on the (chiral) receptor, and it was argued from the 3-dimensional geometry of contact between two chiral entities (the drug and the receptor) that if all three groups of one enantiomer of the drug fit three complementary sites on the receptor, the other enantiomer will not be able to interact fully or in the same manner with the same three bonding sites on the recep-

tor. Thus, the binding of the two drug enantiomers to the receptor can be significantly different, which in turn may produce different biological effects by the enantiomers. The 3-point-interaction model, originally proposed for a specific effect of epinephrine, was later broadened to explain biological enantioselectivity of chiral drugs in general [62], be it in drug–receptor interactions, enzyme–substrate interactions, protein binding, etc. Moreover, the 3-point-interaction model of enantioselectivity has also been used in chromatography to explain enantioselective retention arising from interactions of the chiral analyte molecules with the molecules of the chiral stationary phase or other chiral selectors [63].



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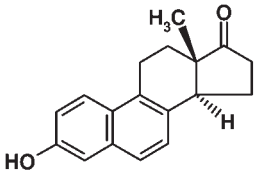
With the advances in organic chemistry in the early decades of the century the more complex drugs began to yield their chemical structures. For example, the structure of morphine was proposed in 1923 [64]; the drug was synthesized in 1952 [65], and its absolute configuration was determined in 1955 [66]. (+)-Morphine was synthesized in 1960 and was shown to differ significantly from the natural (–)-morphine in that it lacks analgesic activity. (+)-Morphine does possess antitussive activity, albeit to a lesser extent than (–)-morphine [67].

The correct connectivity of the atoms of the quinine molecule was determined early in the 20th century by Rabe [68] but without establishing the stereochemistry of the molecule. Attempts were made by several groups over subsequent decades to synthesize quinine stereoselectively, but success was not obtained until 2001 [69], 181 years after the compound was first isolated.

It should be pointed out here that the firm establishment of the *absolute* configuration of chiral drugs, as of other chiral molecules, by an experimental method had to await the famous experiment of 1951 in which Bijvoet et al. determined the absolute configuration of sodium rubidium tartrate using the technique of anomalous X-ray scattering [70]. This milestone in stereochemistry opened the door to the elucidation of the absolute configuration of thousands of compounds, including many drugs.

Chirality continued to occupy pharmacologists and chemists during the remainder of the century. Enantioselectivity in the effects or disposition of chiral drugs was found in a large number of cases, for a large variety of pharmacological effects and chemical structures. To mention a few examples, in 1940 significant biological differences between the enantiomers of sex hormones, e.g., those of the steroid equilenin **22**, were reported [71]; the β-adrenergic-antagonist activity of propranolol, the first commercially successful beta blocker, was determined to be lopsidedly in the (*S*)-(–) enantiomer **23**, and similar selectivity was found in several other, related, β-adrenergic antagonists [72]. Examples of enantioselective toxicity were also found, e.g., levodopa (L-3,4-dihydroxyphenylalanine **24**). Initial clinical

trials in the 1960s of this breakthrough treatment for Parkinson's disease used the racemic mixture but it quickly became clear that unacceptable toxicity was present in the D-enantiomer, and the drug was therefore developed in the unichiral, L, form [73]. By the 1970s a large body of information had accumulated on the role of chirality in drug action and metabolism, and many reviews and monographs on the subject appeared during the last ca. 30 years of the century, for example [74–79]. In 1973 a seminal review of stereoselectivity in drug biotransformations and metabolism was published by Jenner and Testa [80].



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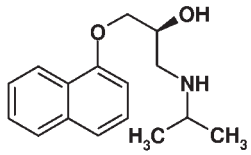

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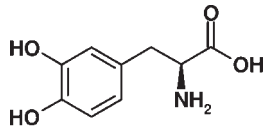

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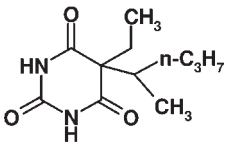
Modern pharmacotherapy came of age during the 20th century. Many new pharmacologically active natural products were isolated and identified, thousands of new compounds (many of them chiral) were synthesized and examined for pharmacological effects and therapeutic potential, and a large number of new drugs were introduced into the armamentarium of the physician. As mentioned above, by 1987 ca. 55% of all clinically used drugs were based on chiral molecules [2]. Many of the new chiral drugs introduced were natural products or semisynthetic derivatives thereof and, as Ariens and Wuis pointed out, a vast majority, ca. 98%, of such drugs were introduced in unichiral form [2]. Atropine was one of the few exceptions. As the racemized derivative of the naturally occurring (–)-hyoscyamine (see above), atropine may be considered a semisynthetic agent and may have been the first synthetic racemic drug introduced into medical practice. The drug was first isolated in 1833, its pharmacological properties studied in the 1880s, and the compound was synthesized in 1901 [81]. A patent for the preparation of atropine sulfate was issued in 1912.

With time, entirely synthetic chiral drugs began to form a major segment of the new therapeutic agents. This trend began slowly early in the century, but by the 1950s the number of such drugs was increasing rapidly. The vast majority of synthetic chiral drugs introduced by 1987, ca. 88%, were racemic, and by the late 1980s roughly a quarter of the drugs on the market were chiral and racemic [1, 2]. Among the earliest entirely synthetic racemic drugs were several anticonvulsant or sedative barbituric-acid derivatives, e.g., pentobarbital **25**, for which a preparation patent was issued in 1916. In this context it is also of interest that the first report of the synthesis of the enantiomers of a barbituric acid and a comparison of their efficacy and toxicity appeared as early as 1928 [82].

It is also noteworthy that some of the chiral drugs introduced as stereochemical mixtures were more complex than the simple racemate. For example, some new agents were marketed as a mixture of two or more racemic mixtures, e.g., labetalol **26** (2 racemates, preparation patent 1971) [83], and cyclothiazide **27** (4 race-



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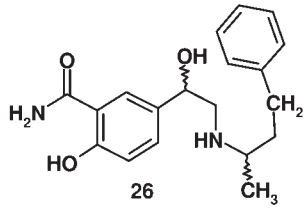
mates, preparation patent 1966) [84]. In such cases it was sometimes claimed that all or most of the stereoisomers contributed therapeutically useful activity, but it is difficult to avoid the conclusion that synthetic considerations and their cost implications weighed heavily in the decision to market the complex mixture. A few other chiral drugs were mixtures of epimers, resulting either from the stereochemical instability of a chiral center within the molecule, e. g., carbenicillin **28** (preparation patent 1964) or from nonstereoselective synthesis, e. g., the prodrug cefpodoxime proxetil (preparation patent 1982).



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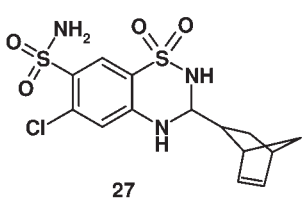

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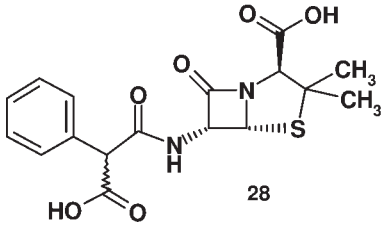

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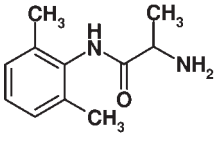
Overall, then, a vast majority of synthetic chiral drugs were introduced during the 20th century in racemic (or, in a few cases, in other stereoisomeric mixture) form, as discussed above. It is relevant in this regard that the clinical use of some racemic drugs was stopped or severely curtailed due to toxicity that became evident only after introduction of the drug on the market, e. g., the antiarrhythmic agent tocainide **29** [85] and the analgesic and anti-inflammatory drug benoxaprofen **30** [86]. One may wonder whether in such cases the adverse effects in question may be enantioselective, i. e., whether a unichiral version (that excludes the more toxic enantiomer) would have been a safer drug.



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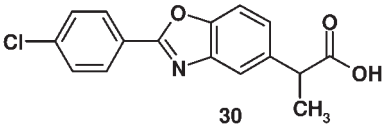

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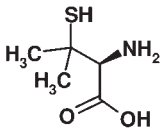
It should be noted, however, that despite the general preference for the marketing of synthetic chiral therapeutic agents in racemic form, a few synthetic chiral drugs *were* introduced in a unichiral form. Such exceptions included the above-mentioned levodopa and also D-penicillamine **31** [78], (–)-timolol **32** [87], methyldopa **33** [88], etc, and it is clear that in most such cases the choice of developing a unichiral form was dictated by overt serious toxicity present predominantly in the other enantiomer.



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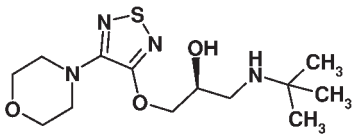

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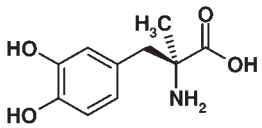

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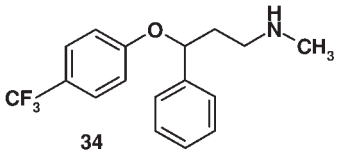
From the above considerations of new-drug development in the 20th century a clear conclusion can be drawn: during most of the century pharmaceutical firms did not make an effort to study the role of chirality in new-drug candidates and did not have a great deal of interest in developing unichiral drugs if nature did not provide them. This lack of interest in chirality from the industry may have been the result of a lack of interest in chirality from governmental drug-regulatory agencies. For example, until 1987 the FDA did not explicitly require the inclusion of information on the enantiomer composition of chiral substances in new-drug applications [89].

A broad and serious examination of the role of chirality in new-drug development only began during the 1980s. The driving force behind this change in attitudes must be ascribed to the advent of enantioselective analytical methods capable of selectively detecting and measuring the individual enantiomers in the presence of each other [90]; to the development of powerful new methods for the synthesis of unichiral compounds [91], and to preparative chromatographic methods for the separation of drug enantiomers on a useful scale for pharmacological testing [92].

The new climate in chiral drugs produced discussions of the merits of the development of unichiral agents vs. racemic mixtures as new drugs, e.g., [78, 93, 94]. A great deal of evidence accumulated in favor of unichiral drugs. The unichiral drug is a single agent instead of a mixture of two distinct drugs, which simplifies the interpretation of the basic pharmacology, therapeutic and toxic effects, pharmacokinetic properties, and the relationship of plasma concentrations to effects. Other advantages may include reduced dosage, reduced drug interactions, and reduced toxicity. This, however, is a complex matter and each drug must be judged

on its own merits [78, 93–95]; indeed, the preference for unichiral drugs is not absolute, and in several cases a unichiral form proved to be less safe than the racemic (or some other) mixture of stereoisomers, e.g., fluoxetine **34** [96], labetalol **26** [97], and sotalol **35** [78]. The explanation for this phenomenon may be a direct pharmacodynamic or pharmacokinetic competition/interaction between the stereoisomers which results in the prevention by one stereoisomer of toxicity by another (as is likely to be the case for labetalol), or a specific protective effect provided by one of the enantiomers in the racemic mixture (as in the case of sotalol) [98]. About 25 years ago a novel concept in this regard was introduced by Tobert et al. on the basis of their studies of the diuretic and uricosuric agent indacrinone **36**: the *non-racemic* mixture of the enantiomers as an optimized drug. The optimum therapeutic effects for indacrinone were obtained with the 4:1 *S*/*R* mixture of the enantiomers [99]. The broad applicability of this concept remains to be determined.



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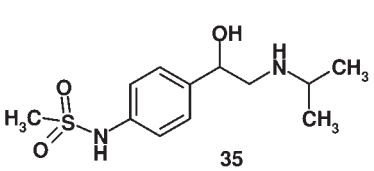

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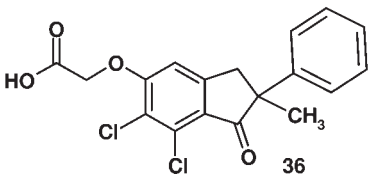

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We end our tour of the history of chiral drugs with the year 1992, when the FDA issued its guidelines governing the development of new chiral drugs [100]. In roughly the same period other regulatory bodies around the world, e.g., the agencies of the European Union, Canada, Japan, Australia, etc., issued similar guidelines (although the various agencies differ somewhat in the level and detail of some of the requirements) [101]. While the new regulations do not ban the introduction of new racemic drugs, their overall effect is in fact the near-total disappearance of racemic substances as new drugs. Henceforth, the overwhelming majority of new chiral drugs will be unichiral. Such drugs are developed as new chemical entities or are obtained, less frequently, via a *chiral switch*, in which a single-enantiomer from an existing racemic drug is developed as a new drug [98]. We have come full circle: After nearly 5000 years of single-enantiomer chiral therapeutics, followed by about 100 years of a strong trend towards racemic formulation, unichiral drugs are again the order of the day.

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Synthesis

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2
Stereoselective Synthesis of Drugs – An Industrial Perspective

Hans-Jürgen Federsel

2.1
Introduction: Historical Overview

Over the past three decades or so, we have learnt to master the stereoselective synthesis of chemical compounds in a way that virtually no one could have imagined before. This knowledge has allowed the construction of molecules with immense structural complexity and architectural diversity. It is entirely justified to claim that many of today's drugs with their intricate stereochemistry would never have succeeded in becoming commercialized had it not been possible to synthesize them, or, more accurately, produce them in large quantities. Making molecules on the laboratory scale is one thing, but it is only on scale-up that the methodology applied faces the ultimate test. Under the currently very stringent requirements on safety, minimal environmental impact, cost of goods and so forth, these demands on a process for commercial operation are challenging to say the least. A high percentage of marketed drugs, as well as those in the development pipeline, are chiral and, therefore, with few exceptions have to be made in enantiomerically pure form. Needless to say this has forced the chemistry community – in academia and industry – to design and optimize routes with high practical utility, capable of addressing difficult target structures to an increasing extent 'dressed-up' with multiple stereogenic centers. Before taking a closer look at the current situation, a brief recap of what has happened over the past more than 100 years in the area of asymmetric synthesis seems well motivated.

First, it is important to stress that asymmetric chemical transformations are not a recent invention; on the contrary, we are now well past the 100th anniversary of the first reported case in the literature by Emil Fischer who in 1889–1890 found that HCN addition to L-arabinose produced two epimeric carbohydrate acids after hydrolysis [1]. Soon after, a second example [2] – NaHg [sodium amalgam] reduction of D-fructose – was published, together with some thoughts on the fundamental importance of these observations for the future. In the first decade of the new century McKenzie et al. published a series of papers titled "Studies in Asymmetric Synthesis", a phrase which, thus, was by no means created only in the re-

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cent past. These papers were, for example, on the Grignard reaction of optically active menthol esters [3] and on the stereoselective oxidation of fumaric acid using $KMnO_4$ in stoichiometric amounts, which after esterification of the substrate with a chiral handle, again the natural product (–)-menthol, gave L-(+)-tartaric acid, albeit of relatively poor optical quality (<20% de; de = diastereomeric excess) [4]. Only 6 years later, in 1913, Bredig and Fiske reported the first catalytic asymmetric process, in which HCN was added to benzaldehyde in the presence of quinine, a stereochemically defined alkaloid, as the catalyst [5]. In this case, the outcome from a stereoselectivity point of view was also far from impressive in today's terms (a few % ee only; ee = enantiomeric excess), but a new field of chemistry had been opened up. An illustration of the start of asymmetric chemistry, as well as of two conceptually different approaches to bring about these kinds of transformations, is provided in Fig. 2.1, where, in both cases, pro-chiral substrates are converted to stereoisomeric products.

Surprisingly, the area was largely dormant for several decades, and it was not until well into the 1960s that the first signs of renewed interest became visible. The in-

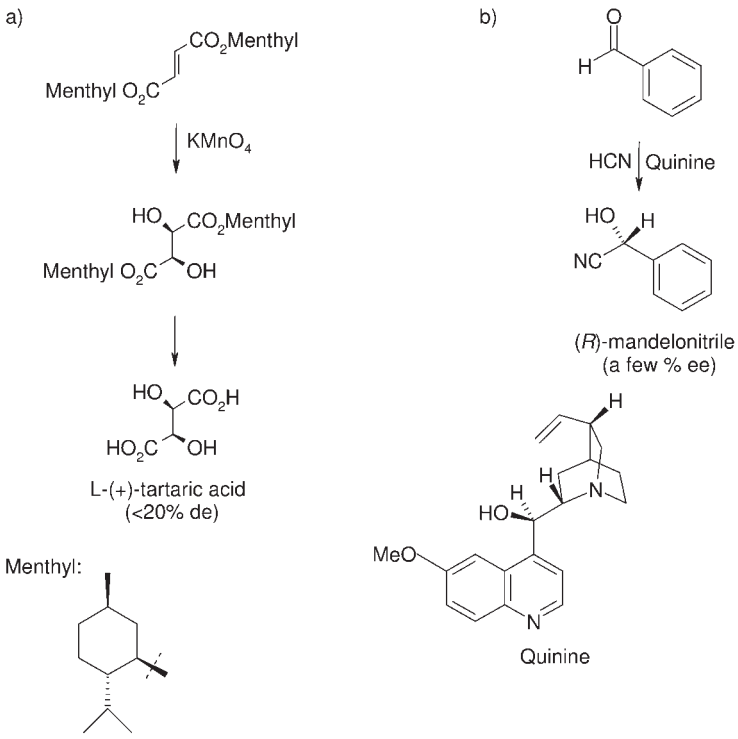

Fig. 2.1 Milestones in the history of asymmetric synthesis.
(a) Auxiliary part of substrate. The McKenzie procedure for di-hydroxylation of fumaric acid ester leading to L-tartaric acid. (b) Auxiliary added as a separate entity. Quinine catalyzed HCN addition to benzaldehyde (according to Bredig and Fiske).

dustrial utility of this technology was clearly demonstrated in 1968 by the landmark discovery of the Monsanto group led by William Knowles, who were able to design and prepare chiral metal complexes that could effect reductions of enamides under homogenous conditions [6]. Shortly after, the commercial production of L-Dopa, a drug that alleviates the symptoms of Parkinson's disease, building on this methodology, was launched and eventually reached ton quantities per annum [7]. Knowles was later honored for this achievement when he shared the 2001 Nobel Prize in chemistry (Fig. 2.2) [8]. However, this approach now seems to have been largely abandoned in favor of a more cost competitive enzymatic process.

Since this pioneering example, the field of asymmetric synthesis has seen remarkable development, and new improvements are still being added to the current arsenal of methodologies. The areas where most applications can be found are the pharmaceutical and fine-chemicals industries [9–13], but superb examples are also seen in agrochemistry [14], with its much tougher price competition and

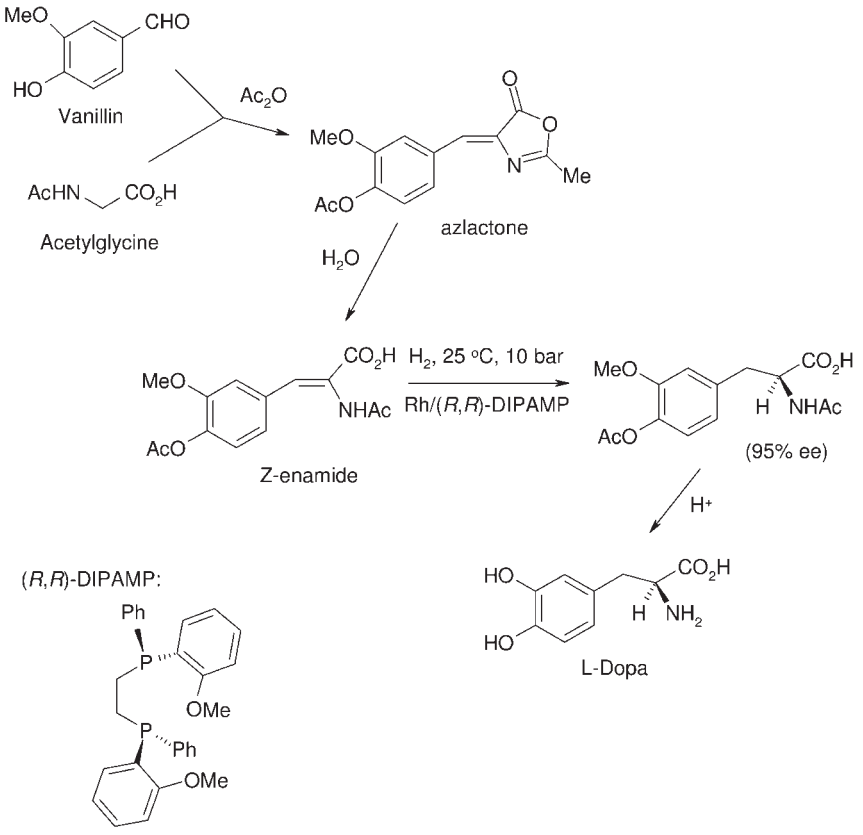

Fig. 2.2 The industrial breakthrough for asymmetric catalysis: Monsanto's L-Dopa process. Performance data for the asymmetric step: TON (turnover number) = 20×10^3, TOF (turnover frequency) = 10^3 h^{-1}.

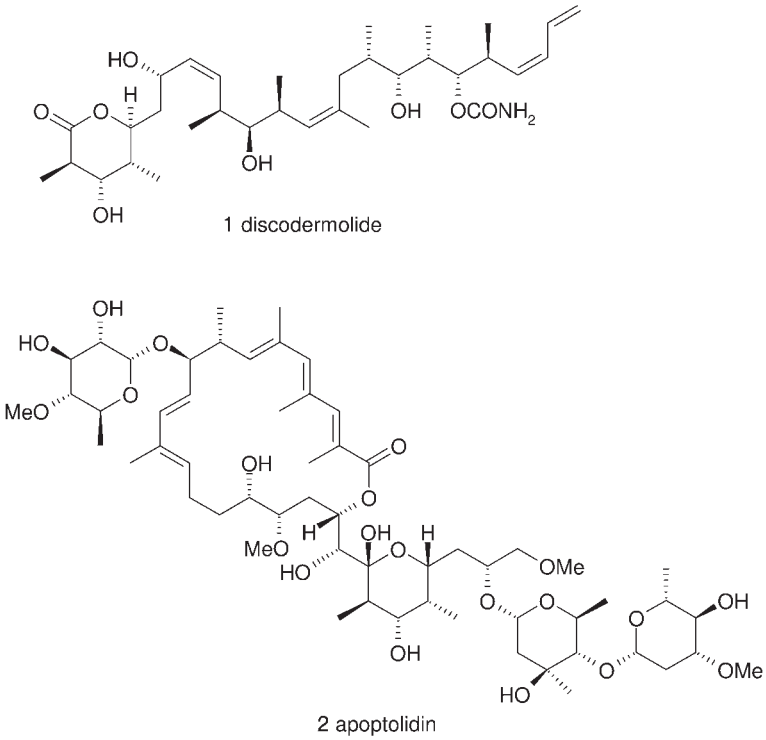

Fig. 2.3 Structures of discodermolide and apoptolidin – two extremely challenging pharmacologically interesting target molecules.

profile of low-cost products, and in the liquid crystal domain [15]. To conclude, exciting demonstrations of how far the technology has advanced since the L-Dopa experiments are provided by the large-scale process development for (+)-discodermolide **1** [16], a polyketide with 13 stereocentres, and the macrocyclic lactone apoptolidin **2** [9a, 17], which has 25 stereogenic centres, both compounds displaying strong cytotoxic and tumor inhibiting effect. Unfortunately, work on the former had to be discontinued due to emerging toxicity problems.

2.2
Asymmetry from an Industrial Scale Perspective

As mentioned previously, the field of asymmetric synthesis began to advance rapidly in the late 1960s. However, the important prior contribution of Nakamura [18] should be noted. In 1941 he demonstrated that a chiral auxiliary (an acid) could be used to alter the surface of a heterogeneous catalyst (Pt black) to successfully reduce an imine bond (C=N) in 18% ee. Other Japanese workers built on these ideas and were able to develop two commercially viable catalytic systems

almost 40 years later – a Ni/tartrate complex [19] and *Cinchona* alkaloids deposited on Pt [20] – that offered product with ees in the high 80s when applied to hydrogenations of various keto-esters, although, in general, only a few such processes could be successfully scaled up for production purposes. Instead, in 1966–1968, the major focus became homogenous counterparts, an area in which the research groups of Nozaki [21], Horner [22], and Knowles [6, 8] have made a lasting imprint with their landmark preparation of metal complexes using different chiral phosphines as ligands. Optical purities gained early on were low (< 15%), but the situation improved rapidly, driven by the discovery of the DIOP ligand by Kagan [23] around 1970. This breakthrough opened up the versatile area of C2 symmetric biphosphines and was instrumental for the launch of L-Dopa production a few years later, where an outstanding quality of up to 95% ee was achieved – a value that still compares favorably with many processes developed much more recently.

The "modern" era of asymmetric organic synthesis could be viewed as beginning in 1980 with the appearance of two seminal papers – one on the epoxidation of allylic alcohols [24] and one on the new family of BINAP ligands [25] – produced by two research teams, one in the USA and one in Japan. The first effort was led by Sharpless, and the latter by Noyori, who have continued to develop a multitude of practically operable transformations with wide generality. The importance of these contributions was recognized by the award of the Nobel Prize [26] in chemistry in 2001 to Sharpless and Noyori, together with Knowles. A good indication of the maturity that these two technologies have reached is the fact that commercial applications have been found, and operation on a large scale has proven to be robust and very successful; two examples are highlighted in Fig. 2.4.

The reaction types just described – oxidation and reduction, respectively – are good illustrations of what has been achieved, and the latter especially dominates the arena of asymmetric processes on an industrial scale. In fact, of the less than 20 full scale chemo-catalyzed reactions known to be running currently, at least half are used for reducing various functionalities, such as C=C, C=O, and C=N groups [7, 29]. At this point, the outstanding contributions of Corey – who, like the others referred to earlier, has been awarded the Nobel Prize (in 1990) for his achievements [30] – should be mentioned. A major part of these concern the oxazaborolidine-catalyzed reduction of keto-functionalities [31], building on the pioneering work by Itsuno and his group in Japan in the early 1980s [32]. Elegant and groundbreaking use of this methodology has been made in the synthesis of compound MK-0417 [33], a forerunner of Trusopt® (MK-0507), the marketed antiglaucoma drug developed by Merck. The current production process of the latter, however, uses a whole-cell based biotransformation (alcohol dehydrogenase in *Neurospora crassa* as the active enzyme) [34], see Fig. 2.5.

Space limitations do not allow every procedure of importance to be mentioned, but one that should be included is the production process for (*S*)-metolachlor, a herbicide marketed under the trade name Dual Magnum® by Syngenta (formerly Ciba-Geigy). Volume-wise, this presently constitutes the largest production process of any kind applying an asymmetric reaction, with its annual capacity of more than 10 000 tonnes [35]. The efficiency obtained in this method based on a planar

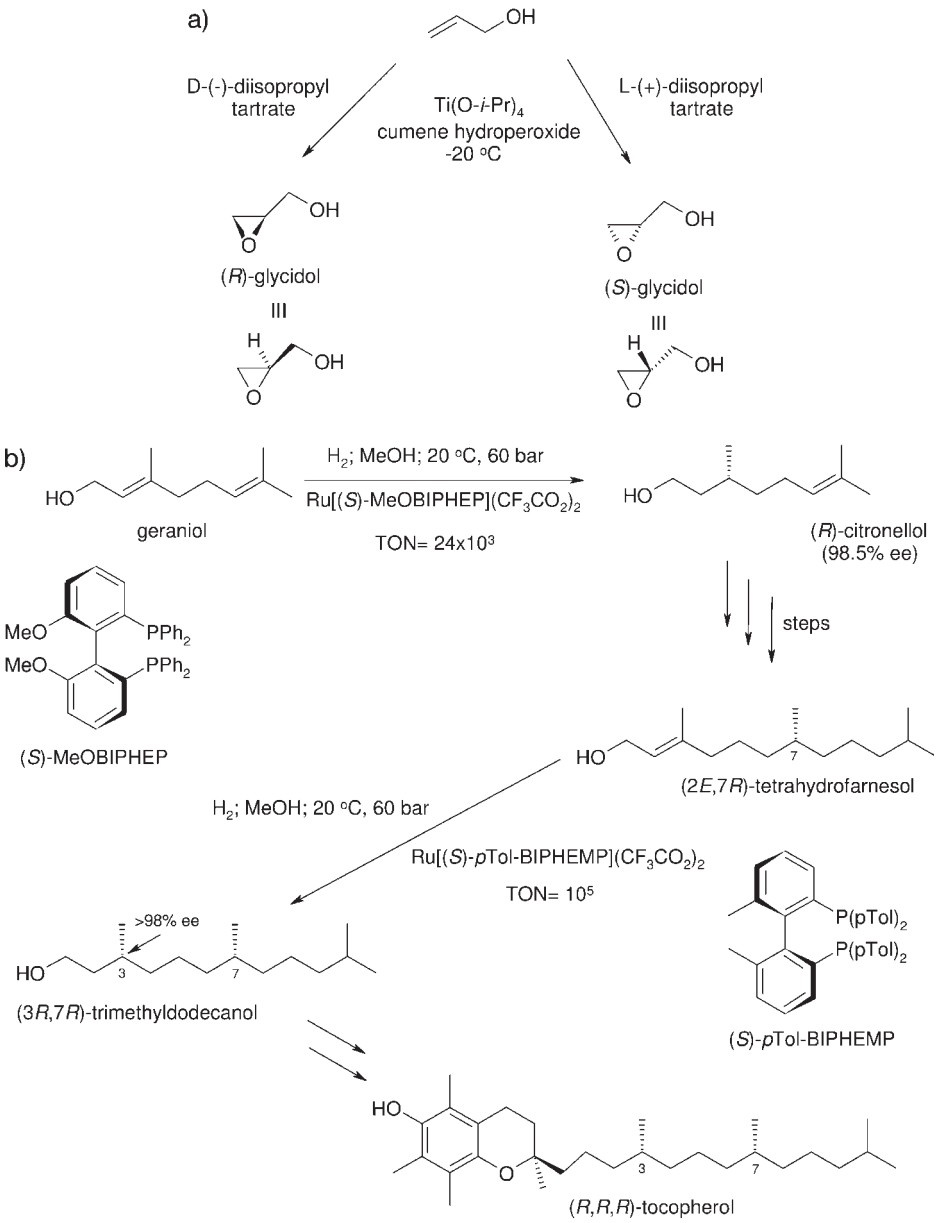

Fig. 2.4 Commercialization of Nobel Prize awarded chemistry: (a) the preparation of both glycidol antipodes using Sharpless titanium catalyzed epoxidation [27] (although there are indications that production (by PPG-Sipsy) has been discontinued); (b) double ruthenium catalyzed hydrogenations using two BINAP congeners as catalysts to a vital building block for the synthesis of (*R,R,R*)-tocopherol, a key Vitamin E constituent, developed by Roche [28].

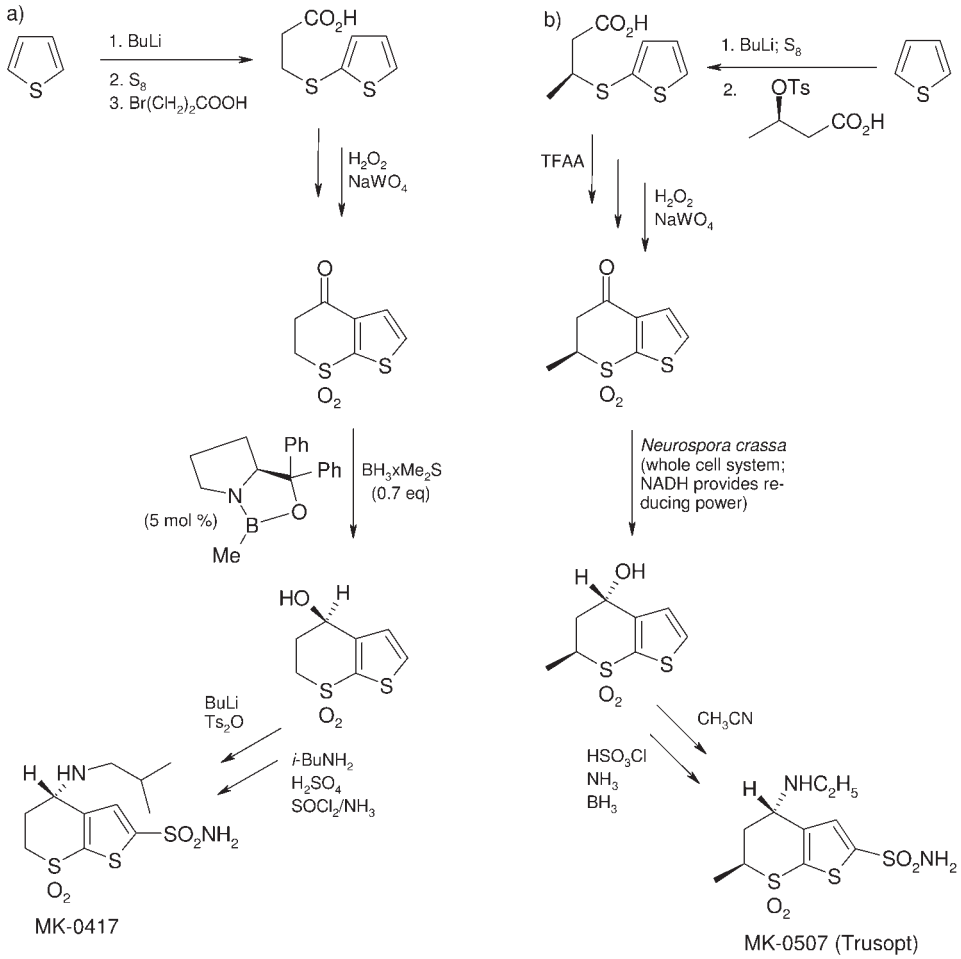

Fig. 2.5 (a) Enantioselective keto-group reduction using oxazaborolidine en route to MK-0417. (b) Commercial manufacture of MK-0507 (Trusopt®) incorporates a biocatalytic reduction step using an alcohol dehydrogenase (*Neurospora crassa*) and operates in a diastereoselective manner as the 6-methyl group is in a fixed (*S*)-configuration.

chiral ferrocene ligand belonging to the josiphos family as the catalytic species is remarkable – expressed as the turnover number (TON), defined as mol of product per mol of catalyst, the figure is $>10^6$. In other words, one single molecule of catalyst has the ability to transform more than one million substrate molecules! Here, we are approaching the performance levels of biological catalysts in the form of enzymes. Figure 2.6 focuses on the catalytic stage of the metolachlor process.

To complete the list of frequently encountered chiral ligands that have demonstrated their versatility on the production scale, the DuPHOS [36] and salen [37] motifs should be mentioned. Members of the former class have shown excellent

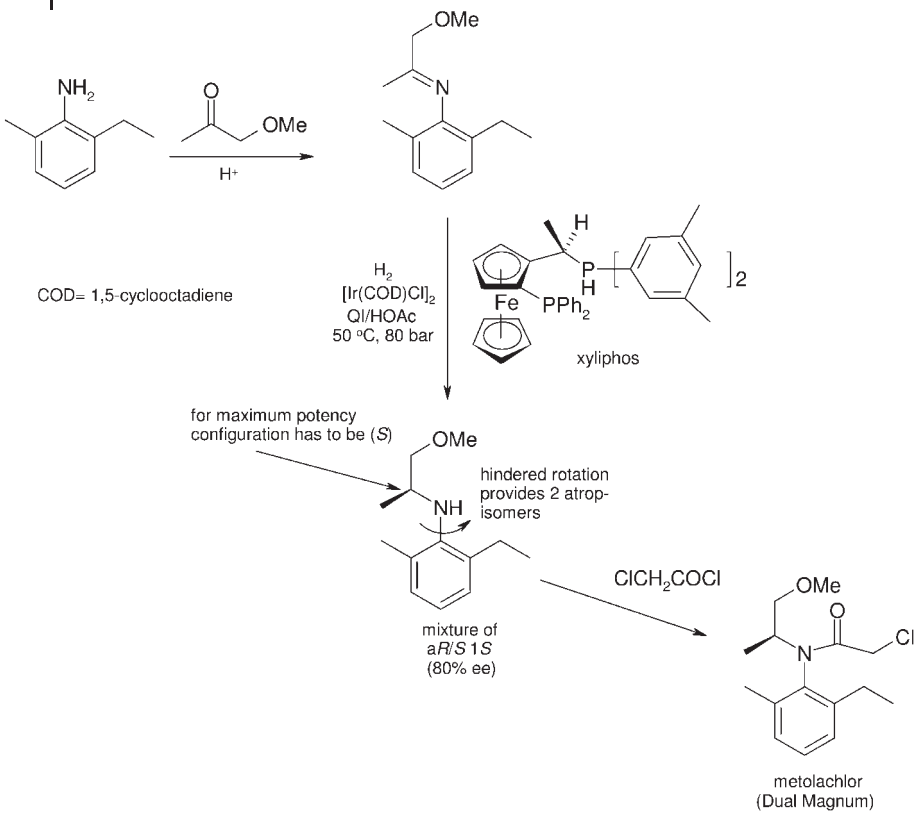

Fig. 2.6 World-record holder in asymmetric catalysis. With 10 000 t or more being produced per annum at an unsurpassed catalytic efficiency and effectiveness, the metolachlor process is unique.

performance in the reduction of variously substituted carbon–carbon double bonds, whereas the latter, when used as the Mn-complex, has achieved a world reputation for epoxidation of nonfunctionalized olefins or, when applied as its cobalt counterpart, for hydrolytic kinetic resolution of racemic epoxides. Figure 2.7 shows one example from each category.

Of course, the literature on the subject of asymmetric synthesis is so vast that a comprehensive coverage is far beyond the scope of this chapter. Instead, a few recent relevant sources are given for those readers interested in gaining a deeper and broader insight [40–43].

Fig. 2.7 Ligands with broad scope and versatility. (a) A concise synthesis of a non-proteinogenic amino acid developed and scaled by Dowpharma using a DuPHOS catalyst [38]. (b) Merck route to *cis*-aminoindanol, a key building block made via salen-catalyzed epoxidation for onwards usage, for example in the synthesis of the anti-HIV compound indinavir [39].

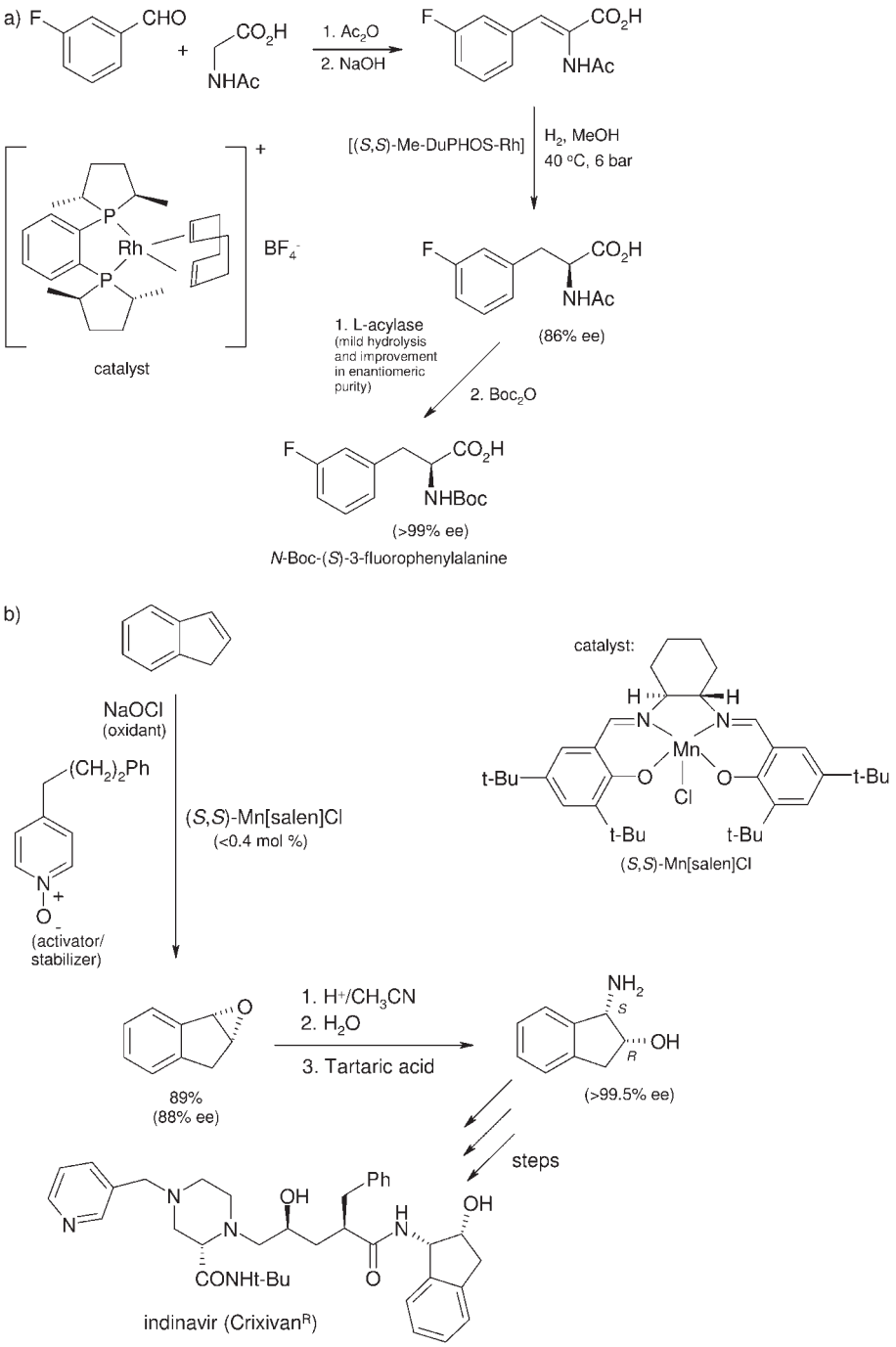

Fig. 2.7 (legend see p. 36)

2.3
Stereoselective Processes in Drug Manufacture – Drivers and Blockers

The development of asymmetric chemistry over the recent decades from being primarily of academic interest to something of considerable industrial significance, amply demonstrated by the examples provided (*vide supra*), brings up the question of how this field is viewed in reality by pharmaceutical and other companies, notably in the fine chemicals sector, today. Thus, has this technology been fully embraced and is it therefore seen as a realistic opportunity when designing new processes? Or does the opposite hold: Reluctance and hesitance prevail because the area is still seen as "alien" to many, with major hurdles to overcome? In other words: Is this now an area that has reached a state of maturity and, furthermore, how far have we actually come in incorporating this approach into the toolbox of methodologies?

Judging by the small number of known commercially operated chemo-catalyzed asymmetric processes already alluded to (Section 2.2), which according to best estimates [7, 13] is under 20, even if there is considerable uncertainty about the absolute figure, then this appears to be a rather exclusive club. When viewed from this perspective, the level of practical utilization is astonishingly low and one could debate the true importance that it represents. If one, however, looks behind the scene another picture emerges. The number of asymmetric reactions in various stages of development is at a pleasingly high figure, maybe an order of magnitude higher than what is currently seen in full-scale production. So in fact there are a large number of projects that incorporate stereoselective transformation in the industrial pipeline, the downside being that only a small fraction of these will ever reach the market. Indeed, with an industrial average of about 90% attrition only 1 out of 10 drugs under development will be launched and, consequently, require the active ingredient to be manufactured at scale [10, 11]. Many contract companies are equally hit by this unfortunate drawback, as they will only be supplying optically pure molecules as long as a particular project stays alive. This is, at least partly, the reason for maintaining a high share of other "competing" approaches seen as easier to handle when process chemists are asked to prepare enantiomerically pure materials. A recent analysis [9c] shows that in 2003 more than 50% of the compounds made in homo-chiral form are achieved either by classical resolution or via building blocks from the chiral pool. About one third utilize asymmetric procedures with chemical catalysts and this distribution is in stark contrast to the picture delivered by the scientific literature dealing with chirotechnology. Journal citations for the period 1994–2003 reveal that as many as 72% represent this particular field as compared to only 18% for the former. An obvious imbalance as seemingly (too?) large resources are spent on the more basic research in an area where there is considerably less interest for taking it further into practical applications and vice versa when dealing with the resolution-chiral pool based methodologies. In reality the situation is much more favorable given the relatively large number of processes under development that use asymmetric synthesis and a look at projections of market size for stereoisomerically defined

products supports this trend further. The global forecast for single enantiomer sales according to the firm Frost & Sullivan [9c] indicates an annual growth of more than 11% from over $8.5bn in 2004 to almost $15bn by the end of 2009 and this, if anything, underpins the need for cost effective and efficient asymmetric processes. In the pharmaceutical sector the data are even more convincing as out of the top 10 marketed drugs, 6 represent single enantiomers at a combined sales volume of $31bn. Moreover, the global drug market has an annual turnover which in 2005 is approaching $600bn, one third of which comes from the optical actives.

With the bulk of knowledge and experience in the field of catalytic asymmetric chemistry – originating from laboratory work and based on plant scale operation – would it not be fair to assume that what we are seeing is an area that has reached a state of maturity? Not really, is the clear and unambiguous view expressed by two leading experts representing, respectively, industry and academia – Blaser [44] and Trost [45] – not ignoring or failing to praise the tremendous advances encountered over recent decades, but instead mentioning the huge opportunities for further development and hinting at the vast amount of molecules that could potentially function as catalytic species as well as the large number of variables influencing the reactivity and selectivity of a reaction. From a strictly manufacturing perspective, a set of seven descriptors can be used to decide the maturity level of a technology area such as this [44]:

- How well defined is it and are the scope and limitations known, i.e. in terms of selectivity, activity, productivity, functional group tolerance?
- What is the number of existing technical applications on scale (pilot, commercial)?
- Is the required equipment readily available?
- In the design of synthetic routes is it routinely and widely considered as an attractive and feasible alternative?
- Are required reagents, notably catalysts and auxiliaries, easily available commercially, from laboratory quantities through to amounts needed for production?
- Can intellectual properties (IP) be handled with third parties and what are the options for, if necessary, gaining a license to operate (royalty payment, profit sharing etc)?
- Is it competitive from an economic point of view compared to other technologies?

The assessment of enantioselective synthesis along these lines reveals that, in certain areas, the current state-of-the-art is actually not far from deserving to be called mature. Thus, the preparedness in industry to take on the challenge to develop an asymmetric process is now much more widespread than before. This is by far not seen as an exclusive territory for a limited number of specialist companies and a clear testimony to this positive trend is the constantly increasing number of publications and patents originating from a steadily growing group of players in the field. The practical implementation of the technology has also experienced a boom in recent years, powered largely by a series of success stories – e.g. the aforementioned (*S*)-metolachlor and the anti-ulcer agent esomeprazole [10, 11, 46] (active component in Nexium®) – which have demonstrated the strengths and capabil-

ities in a very direct manner to a wider "audience". In contrast, other parts of the maturity grid are not so well developed, even for reactions where many successful applications are known, as is the case with hydrogenations. There are multiple reasons for this including: lack of experience of operating catalytic reactions under homogenous conditions, a general uneasiness with organometallic chemistry, and the demand for "extreme" conditions (high pressure, low temperature etc.). Nonetheless, performance speaks for itself and convincing data will continue to accumulate, which will drive this technology forward at a steady, or even accelerating pace.

From the now increasingly long list of asymmetric C–C bond forming reactions of proven ability, two cases have been selected to emphasize that this "core business" in organic synthesis, namely the construction of new molecular scaffolds of sometimes considerable complexity, can be efficiently addressed (see Fig. 2.8). The industrial applications are still in a state of infancy (low volume manufacture, high catalyst loading, pilot scale) as compared to redox transformations [47], but as more projects come through the pipeline that are "confronted" with the vast and versatile range of such reactions, the likelihood of seeing a manifold of products being manufactured commercially in large quantities can only be described as promising.

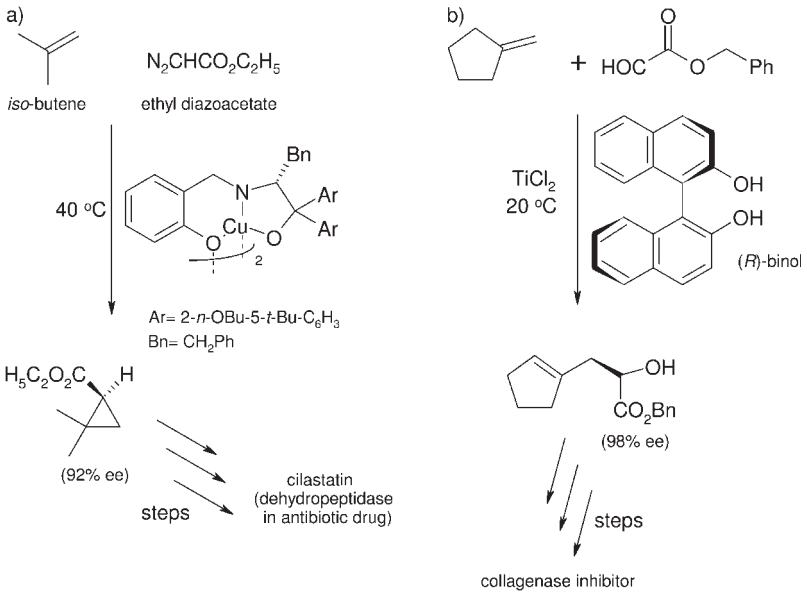

Fig. 2.8 Generation of asymmetric carbon–carbon bonds: (a) cyclopropanation on the commercial scale as a key step to cilastatin, which is used in combination with imipenem in the antibiotic drug Primaxin® (Merck); (b) C-nucleophilic addition to glyoxylate aldehyde-carbonyl affording a highly pure intermediate used for pilot manufacture of a collagenase inhibitor (Roche).

2.4
The Metal – Friend and Foe

Special attention is due to the centerpiece in chemo-catalysis: the metal. As can be seen in previously shown examples this vital component is represented by members from the group of transition or main-group elements. Notable examples are rhodium (Rh), ruthenium (Ru), palladium (Pd), platinum (Pt), cobalt (Co), chromium (Cr), iridium (Ir), vanadium (V), titanium (Ti), and tungsten (W) but less precious ones such as iron (Fe), molybdenum (Mo), copper (Cu), and zinc (Zn) are also quite prevalent.

Many of these metals are actually very expensive and, hence, their use requires a high investment when operating under production conditions. A reaction that uses 1 mol% Pd in a 4 m^3 vessel loaded with 2000 mol substrate will consume a little over 2 kg (20 mol) of palladium metal. Being considered as strategically important the precious metals are traded at day-to-day prices, which over a period of time can vary considerably [48]. With a current price range of $5–10 per g the Pd value in a single production batch amounts to $10–20k or, expressed differently, equals $5–10 per mol of substrate. Thus, there should be enough economic driving force to include a recirculation step in the process, even if the target product in the reaction is of considerably higher value. In practice a recovery rate of >90% is feasible and would mean that the metal share of the total price for the precursor (the metal is delivered either as a soluble complex with certain ligands or in ready-made form) could be reduced by up to 50% [49]. Refining spent catalyst will, no doubt, make the process more cost effective and reduce the environmental burden via effluent streams. An elegant way to address the quest for facile reuse of catalyst has proven to be the attachment of the catalytic species onto a solid support, in close analogy to the well-validated procedure of immobilizing enzymes [50]. These supports could be of silica-gel type, poly(ethylene glycols) [PEGs] or other suitable polymers or dendrimers and one advantage, besides enabling a simple recovery, is the possibility to bind ligands that are dangerous from a handling point of view such as the highly toxic cinchona alkaloids. Applying this principle in the homogenous osmium-catalyzed aminohydroxylation of olefins has shown outstanding yield (~90%) and optical quality with only little degradation (from 99 to 95% ee) after five consecutive runs using recycled catalyst [51]. Further usage of this production-friendly methodology can be expected.

It is easy to become overwhelmed and even fascinated by the performance that catalytic processes in general, and stereoselective ones in particular, display (comparable with the beauty and efficiency of enzymatic transformations). However, considering the use of the final product as an ingredient in a drug product to be given to patients it is a mandatory requirement to remove the remains of the catalyst from the process stream. In particular this is true for the metal component and therefore much effort has to be devoted to designing a suitable work-up to ensure that the regulatory limits are not exceeded. When one realizes that the outgoing stream from a catalytic step can be "contaminated" with 1000 ppm of a transition metal – in some cases even much more – which has to be reduced to only a

few ppm (case by case judgment, but most likely <5–10 ppm), then it becomes clear that this poses quite a challenge. It needs to be stressed, however, that it is not only the absolute figure of residual metal that is important but also this level must be put in the context of the daily dose in man. So a case-by-case analysis has to be performed which will say what amounts of a given metal can be tolerated and what is not acceptable. This already very stringent demand can change to an even tougher one if the metal in question shows extremely toxic properties and in effect this excludes the most "evil" ones such as thallium and mercury from ever being used in the production of pharmaceutical products.

There is a clear distinction between dealing with heterogeneous and homogeneous systems; in the former case the metal is applied as a solid, most likely attached to a support, exemplified by Pd on charcoal, whereas in the latter the metallic species is solubilized by binding it to organic ligands such as acetate, triflate, acetonitrile or dba (*E,E*-dibenzylideneacetone). Whilst the solid catalyst can easily be separated off via simple filtration/centrifugation and it is only in situations where there is a leakage to the solution that one faces a tougher challenge, a work-up of a homogeneous solution to extract traces of metal has to be much more sophisticated and will probably require a multi-step process. A number of ways to ensure efficient removal have been tested and validated [52], for example adsorption onto various complexing agents such as trimercaptotriazine in free form or attached to different polymer resins, activated carbon, glass bead sponges, or even via distillation in cases where the product is heat stable. Approaching the problem from the other side is also possible and builds on the mechanism that the metallic species has to be kept in solution while precipitating the product. This has been achieved by the addition of agents – exemplified by *N*-acetylcysteine or thiourea – capable of solubilizing the metal-containing complex that is formed. A further method available is the use of extractions, where the requirement is that there has to be a significant solubility difference between the components in the liquid phases (notably water–organic solvent). Perhaps the ultimate and somewhat speculative way to tackle the issue would be to design a reaction system where the catalyst precipitates when the transformation is completed, allowing its facile separation and recycling. Indeed, a case where this principle has been proven (on the test-tube scale) is the tungsten-catalyzed hydrosilylation of ketones under solvent-free conditions [53]. The approach builds on the intriguing formation of a metastable liquid clathrate consisting of catalyst and substrate (restricted to aliphatic compounds) towards reaction completion. At the finish, the work-up is simply a matter of decanting off the supernatant leaving the now precipitated catalyst behind and isolating the product in a virtually metal-free (below level of detection) form. With this background it is important to stress the need for a forward-looking strategy when developing full-scale processes for active ingredients so that appropriate procedures are incorporated to eliminate any metallic residues, which could cause unwanted and unacceptable contamination.

2.5
Ligand Development – At the Core of Catalytic Chemistry

A key problem to address is the substrate specificity expressed by the catalyst, which in turn is created by the interaction between three intimately linked species: metal atom–chiral auxiliary–substrate. To optimize and fine-tune this interplay achieving binding properties/geometries such that a high degree of stereochemical differentiation can be ensured is in a sense the essence of asymmetric reactions. Preferably, the design of a catalyst should aim to maximize the acceptance width of substrate architectures to allow accommodation of the broadest possible structural diversity. A number of cases of so-called privileged ligand backbones [54], for example BINAPs, DuPHOS, *Cinchona* alkaloids, and salen complexes have amply demonstrated their wide-reaching strength in this regard, each allowing a versatile range of reactions to be conducted, some of which have been given as examples in earlier paragraphs. Essentially, the parameters that are at one's disposal when designing new catalysts are:

- Type of metal
- Structure and stereochemistry of ligand backbone
- Type of heteroatom and substituent groups attached to ligand backbone
- Properties of catalyst promoters

Bringing these elements together a schematic picture can be drawn which displays a generic illustration of an organometallic catalyst (Fig. 2.9).

As is obvious from this cartoon the variation that one can perceive is enormous and this allows for ample fine-tuning opportunities to reach the desired/optimum performance. The analogy to enzymes is actually not far-fetched as their construction from simple α-amino acids (notably the 20 natural – proteinogenic – ones) can be varied in a myriad of ways by changing the sequential order and relative abundance of the discrete building blocks as well as the size (chain length), so as to tailor-make each for a given task (directed mutagenesis being an excellent example of this principle). This modularity, that is the easy and straightforward crea-

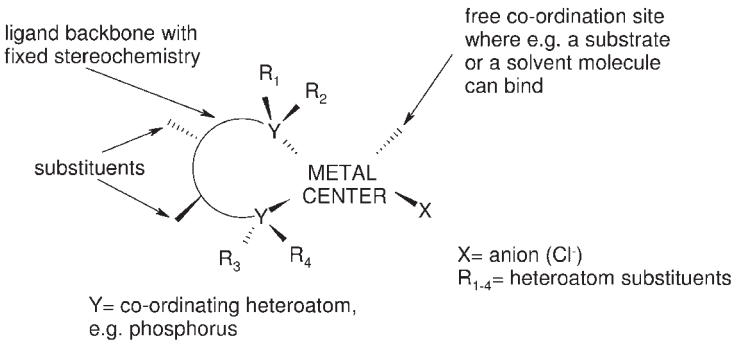

Fig. 2.9 The three-dimensional architecture of an intact metal containing catalyst attached to a chiral ligand with defined stereochemistry.

tion of new members of a given class, is a guiding rule also in the design of man-made catalysts. Around a versatile scaffold, exemplified by bis-phospholanes (DuPHOS type), bi-aryls (BINAP family) and ferrocenes, changes of substituents – electron donors versus acceptors, spatial size, geometric position – can be made which alter the overall stereoelectronic structure to cover an array of properties as broad and diverse as possible [55]. There are many parameters that exert important influence on the stereochemical outcome of reactions; examples are rigidity of ligand backbone, chelate ring size, bulk and basicity of PR_2 groups, and bite angle. In a three-dimensional setting defining the intact catalyst, the latter describes the perceived angle through space between the metal center and its attached ligand atoms. An increased value is believed to augment the stereodiscriminating power, leading to a product with enhanced optical purity [56]. Again a comparison with the enzymatic world is fruitful, as the active site of biological catalysts offers an exquisitely streamlined binding pocket to the substrate, enabling the chemical reaction to be performed in a highly selective manner. Conformational changes in this domain can be brought about by altering the solvent environment or structurally modifying the protein backbone, which will affect substrate affinity and, therefore, chemical selectivity. In a similar vein, studies on the prevalent bi-aryl structural class, which displays the intriguing phenomenon of atropisomerism, a stereosiomeric relationship that is caused by the hindered rotation around aryl–aryl bonds due to through-space steric interactions, have shown analogous results. Thus, using model reactions (H_2-reduction of β-keto esters), the angle between the pseudo-planar aryl groups – referred to as the dihedral angle θ – was seen to exert a very marked influence on the stereoselectivity [57]. Combining these findings with directed manipulation of the electronic characteristics of the ligand by incorporation of different functional groups or substituents to impart varying degrees of electron richness/poorness to the system opens up enormous opportunities for optimization.

The inevitable question arising after all this technical discussion is: How do I find the right catalyst for my specific problem? In principle, two options are available; either you can use a commercially available system or a new one has to be designed and developed [58]. In the former case the task is more of fine-tuning the reaction conditions (temperature, stoichiometry and so forth) to achieve the desired process characteristics in terms of for example productivity, product quality, and economics, together with the crucial need to ensure that there is a reliable supply of catalyst (IP issues solved, royalty or licensing agreements in place) [59]. The latter, in contrast, puts up the challenging demand of screening for hits amongst various collections – from in-house or external sources – to identify classes that can be optimized further [60]. If this approach, however, turns out to be negative then it is a matter of a *de novo* design, meaning that a unique ligand scaffold coupled with an appropriate metal fitting a particular substrate or class of substrates has to be found. A fascinating and pedagogical example describing this methodology is the tremendously successful xyliphos case (member of the josiphos family), where a super-active catalyst was invented for the purpose of bulk production of (*S*)-metolachlor (see Section 2.2) [61]. Ways to support and speed-up

the selection process for ligand and solvent are in high demand, especially in industry, if catalytic methods are to increase their "market share" at the expense of traditional, noncatalyzed syntheses. An approach to this problem holding considerable promise might be the use of cheminformatics tools, notably principal component analysis (PCA). This method uses high-dimensional multivariate datasets where a large number of descriptive parameters (>10 up 50 or 100) – stereoelectronic and electrotopological – are ranked by projecting them onto their most important linear combinations. The graphical presentation emanating from this can then be analyzed for the degree of descriptor-correlation, which, ultimately, will allow predictive conclusions to be made, albeit their quality is strongly dependent on how accurately the model describing the reaction system under scrutiny has been constructed [62]. Moving into this combinatorial catalysis era, virtual ligands (and solvents and so forth) can be screened in a high-throughput fashion eliminating non-value-adding "dead-end" experiments and instead focusing resources on more rewarding leads. Maybe, at some point in time we will be able to take a fast-track "highway" directly to our final destination; the desired catalyst! Figure 2.10 shows a selection of some prominent members of the large and continuously increasing family of catalyst ligands.

We have to accept that there is nothing even close to being a universally useful ligand that can address any synthetic problem. Instead existing classes will be further explored by changing functionalities, substituents, regiochemistry and so forth, to a large extent driven by the mechanistic insights gained when using "old" catalysts for specific transformations. Moreover, entirely new ligand families will see the light of day, partly building on established molecular architectures but also leading into entirely new domains of chemical space. This thrilling development will continue to boost the area of chiral ligands, which over the past decade (from 1992) has seen a 5-fold increase in the hit rate when scanning through the literature [63].

2.6
Asymmetric Reactions – A Rich Reservoir

The performance of several of the reactions alluded to previously (*vide supra*) coupled with the versatility displayed by many catalysts with regard to substrate tolerance motivates the question whether there are any major hurdles left to overcome or if, in fact, every challenge will have a solution? Not surprisingly, there is no clear answer to this. Instead it will depend on the more specific circumstances that apply in a given situation to sort out if a stereocontrolled approach is at all feasible. It does not take much browsing of the current literature to come across work that, mainly from an academic standpoint, focuses on asymmetric synthesis of highly complex target compounds, most frequently originating from various natural sources, and in many cases the total synthesis has been successfully achieved. En route to this goal it is not unusual to discover new reactivities, unexpected synthetic paths or the need to develop a catalyst to effect a particular trans-

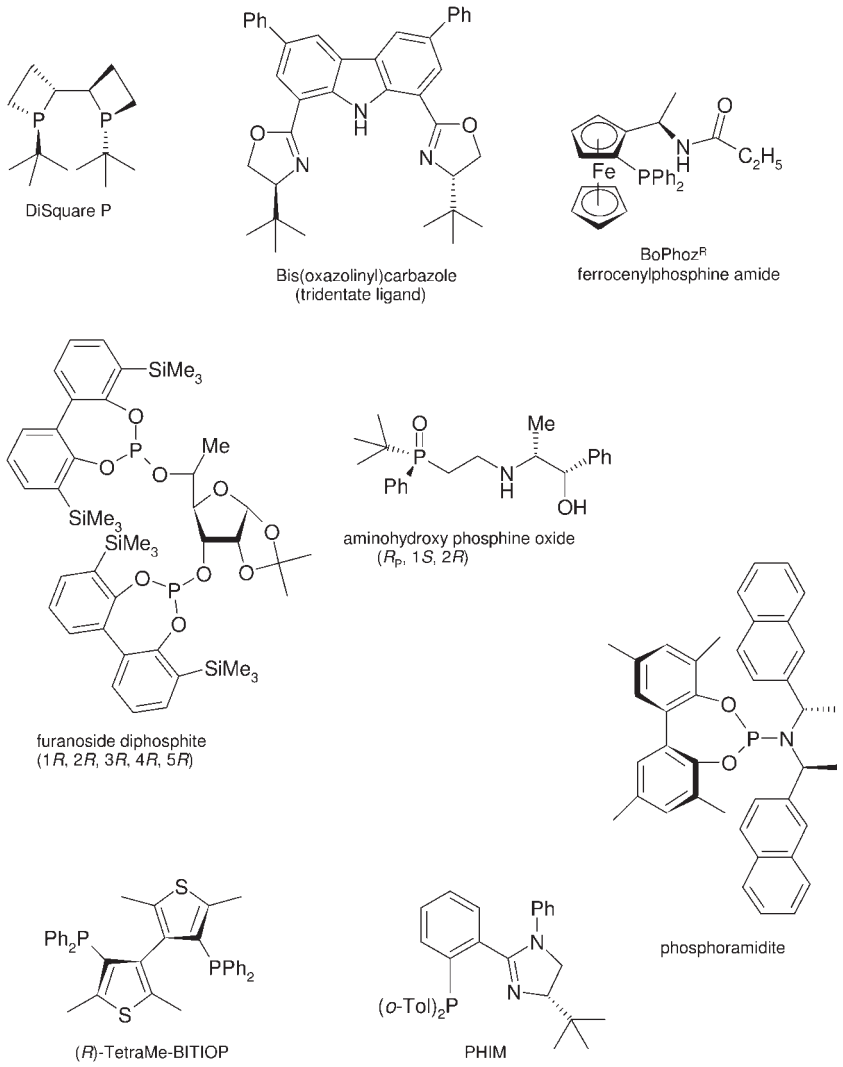

Fig. 2.10 The ligand universe: A stunning breadth of structural variation with members representing various motifs, e.g. atropisomeric biaryls, ferrocene-based bisphosphanes, *P*-chiral bisphosphanes, bisphosphites, monodentate phosphoramidites, *N,P*-ligands.

formation. Looking at it from an industrial angle will, however, bring forward rather different issues that need to be addressed. As already mentioned (Section 2.3) a number of questions of varying importance and relative weight have to be solved before a well-founded decision can be taken to progress the development of a commercially viable asymmetric route, irrespective of whether or not the stereoselective step has already been demonstrated on the laboratory scale!

The number of catalytic asymmetric processes running in full production is small with a preponderance of hydrogenations, most likely due to the historical "heritage" from the renowned L-Dopa synthesis which was commercialized a long time ago. Even if this particular type of reaction is represented by some of the most outstanding performance measures recorded to date – TON $>10^6$ and $>10^5$ tons of material produced per annum – the capabilities at hand go far beyond this "basic" technology, including when it comes to operability at scale. Hence, having access to practically useful and robust protocols to effect oxidations (at carbon as well as sulfur centers), carbon–carbon bond formations, and rearrangements opens up tremendous possibilities to apply asymmetric catalysis to the manufacture of complex target molecules. One effect of this is also that the pool of stereodefined building blocks originally available only from natural sources – for example amino acids, terpenes, sugars, and alkaloids – has been greatly expanded so that there are a variety of versatile small molecules such as C-3 synthons and others at hand. With the very well equipped arsenal of asymmetric methodologies of today a powerful approach to target compounds is possible that allows flexible and efficient construction of molecular frameworks. Three conceptually different strategies to achieve the synthesis of products with defined absolute configuration can be identified [64]:

- Based on existing and available homo-chiral components (building blocks) the desired compound is built up without affecting the stereochemistry. A pertinent example is the synthesis of peptides from their amino acid precursors.
- New stereogenic centers are created via the influence of, and in relation to, existing ones – present either in the substrate or the reagent – to form a product in a diastereoselective fashion. This means that of the newly formed stereogenic center, one of the configurations [(*R*)- or (*S*)-] is made exclusively, or at least in excess.
- A stereoisomer with one or several stereogenic centers is assembled from prochiral or achiral starting materials. This will most probably involve substrate and catalyst control and can, depending on the circumstances, operate in an enantio- or diastereoselective way.

Even a fourth variant is feasible, namely the submission of *meso*-compounds – these contain stereogenic centers but feature internal elements of symmetry and, hence, are achiral – to a desymmetrization, which in principle generates only one stereoisomer. The classical case is the hydrolysis of a diester to form a half-acid/ester as a single antipode. Generation of both optical isomers of synthetically useful 1,2-diol products in good yield and stereoisomeric purity applying this methodology has recently been demonstrated (see Fig. 2.11) [65].

Elegant use of a desymmetrization protocol has recently been reported as a key step in a total synthesis of (+)-cocaine [66]. In this case, submitting a *meso*-dialdehyde to a proline-catalyzed (20 mol%) intramolecular enol-*exo*-aldol reaction afforded a 1 : 1 mixture of the axial-equatorial aldol product in excellent yield (91%). A few concluding final steps – amongst others a chromatographic separation of

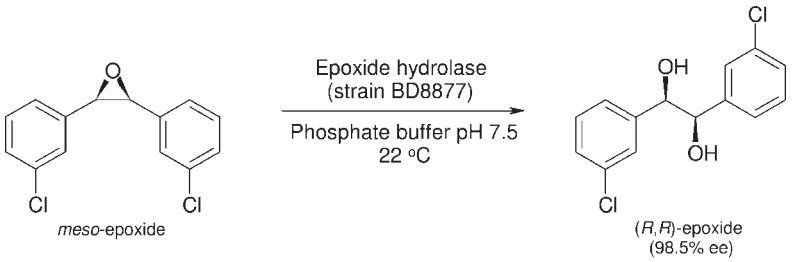

Fig. 2.11 Enzyme-catalyzed desymmetrization of *meso*-epoxides using epoxide hydrolases of microbial origin that were screened for maximum activity. Note that the relative rate (measured as the turnover frequency, TOF) of forming the (*R*,*R*)-stereoisomer is about 250 times higher than for the (*S*,*S*) one.

the axial isomer from its equatorial counterpart – produced the target molecule in a respectable overall yield. Figure 2.12 shows the details.

A strong driver to move into catalytic processing – irrespective of whether asymmetric or racemic – that deserves to be particularly emphasized is the quest for minimizing the environmental burden. The desire, of course, is to obtain quantitative conversion to a single product at virtually zero waste, except for water, which rightly is seen as a friendly solvent in this context. In extremely rare cases a process might approach this utopian state, but in general, industrial chemical production is far from this, especially in the pharmaceutical area. Taking the structural complexity of many drug molecules into account this should not come as a surprise, inasmuch as multiple steps are needed to affect the assembly of reactants under the influence of a broad range of reagents, many of which are used in stoichiometric amounts or even in excess, often running in fairly dilute systems with less pleasant solvents. Therefore, catalysis can offer a step-change to reduce considerably the volumes of byproducts and residual materials, rendering the processes a much "greener" or more benign [67]. To illustrate the current state of the art and indicate promising opportunities for future scale-up along these lines, a number of examples are highlighted that represent various types of reactions and functional group interconversions which are deemed to have potential interest for the manufacture of active pharmaceutical ingredients (APIs).

2.6.1 Reductions

The field of asymmetric hydrogenations has been extensively studied and this has led to a high degree of sophistication in the mechanistic understanding of these reactions. To a large extent this fortunate situation was founded on the almost instantaneous success experienced with the BINAP ligands and their many congeners and follow-ups. From a process chemistry perspective these reactions are very attractive in the sense that the reducing agent, hydrogen gas, is cheap, abundantly available, and environmentally friendly, albeit it has to be handled with cau-

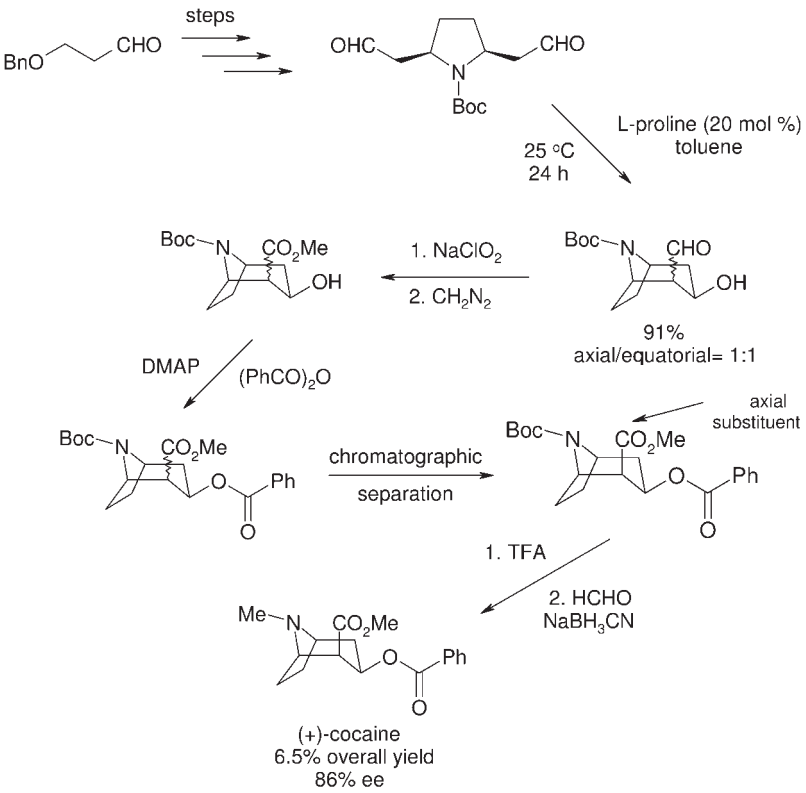

Fig. 2.12 Using desymmetrization to create a bicyclic intermediate in the synthesis of (+)-cocaine, a molecule with a slightly disputable reputation. DMAP = 4-dimethyl-aminopyridine, TFA = trifluoroacetic acid.

tion due to the risk of explosion. Moreover, the transformations – reductions of various double bonds – can utilize an enormous variety of substrates, as the functional groups in question (notably C=C and C=O) are encountered rather frequently. The most recent catalyst development in this area has allowed the precise construction of performance chemicals, for example drug molecules, with a stunning level of control [68].

Findings by Noyori and Ikariya in the mid and late 1990s showed that the combination of BINAP or derivatives thereof with diamines (1,2-diphenylethylenediamine) in the presence of ruthenium gives rise to super-active catalysts that, for example, manage to reduce acetophenone to (*R*)-1-phenylethanol quantitatively with an almost unimaginable efficiency of TON = 2.4×10^6 and TOF = $228 \times 10^3\ h^{-1}$, albeit at a relatively modest optical purity (80% ee) [69]. Considerably higher ee-values (≥98%) were obtained using analogs of this catalyst, however at the expense of severely reduced TONs (10^4–10^5). Building on this, a clear understanding of the structural and electronic effects that govern reactions of this type and the crucial role of the NH_2-group in obtaining high catalytic activity has been es-

tablished. Thus, by introducing bulky electron-donating substituents in the 4- and 4′-positions of the BINAP scaffold as well as changing to more sterically demanding diamines, the conditions are further optimized to furnish product purities of 97.1–99.8% ee from a series of aromatic ketones (see Fig. 2.13) [70]. Another way of performing reductions is by transfer hydrogenation, which is distinguished from the previous hydrogenations in the sense that it relies on sources other than H_2 as reducing agent, namely formic acid or a formate salt. One advantage besides eliminating the need for handling hydrogen gas under pressure is that this chemistry can be performed in neat water in open air and at moderate temperatures. Furthermore, development of the original catalysts, which unfortunately are difficult to separate from the product during work-up, by attaching them to a polymer support, facilitates the situation considerably. This modification allows not only the facile and virtually complete precipitation of the intact catalyst (only 0.4 mol% Ru is leached into the organic phase), but also recycling for renewed use that offers a highly consistent operation without noticeable loss of enantio-

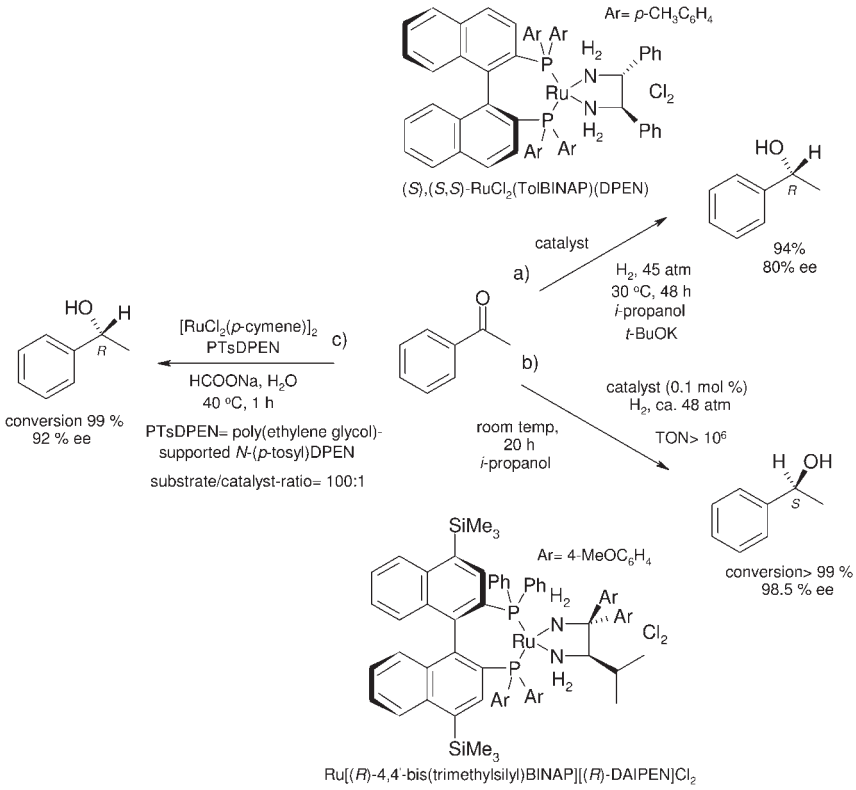

Fig. 2.13 Ketone reduction of unprecedented efficiency, demonstrated with acetophenone as substrate; (a) original BINAP-DIPEN run; (b) using a 4,4′-disubstituted BINAP motif as catalyst; (c) operating under transfer hydrogenation conditions in H_2O and a catalyst on a solid support.

meric purity, even after more than 10 cycles. Combining all these changes into an operating procedure applied to a variety of aromatic ketones, superb performances have been achieved, especially with regard to a considerably enhanced reaction rate, exemplified by acetophenone which is reduced at a 99% conversion in only 1 h at 40 °C, however, at the expense of a somewhat lower product quality (92% ee) [71], details are given in Fig. 2.13. Further applications of this concept using a Ru-containing soluble copolymer of PEG and a norephedrine moiety have been reported [72] with an outcome of a similar order (95% conversion, 81% ee in the acetophenone case).

Much of the basic asymmetric reduction research has been conducted on compounds carrying the *N*-acylated enamine functionality (cf. the L-Dopa process in Section 2.1). The presence of an acyl-group (for example acetyl or benzoyl) has been a prerequisite to achieving good chelation between metal and substrate, which, in turn, is a requirement for selectivity and reactivity. A downside of this, however, is that neither attachment nor removal of this "activating" group is a facile step, and especially the latter transformation may call for relatively harsh conditions which could be harmful to the rest of the molecule. Scanning through a set of available catalysts showed some members of the josiphos ligand family (Sections 2.1 and 2.5) to stand out in a positive sense compared to most of the

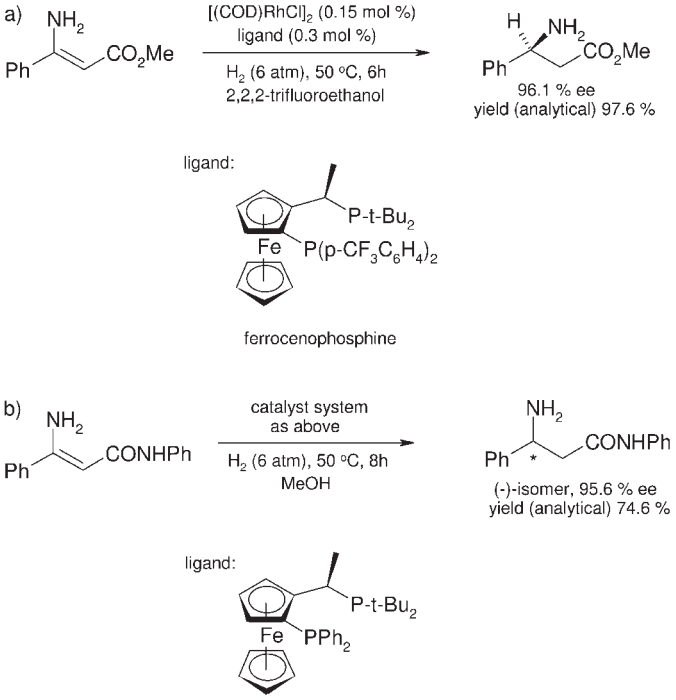

Fig. 2.14 Rh-catalyzed enamine reductions in the absence of an *N*-acyl group; (a) enamine ester substrate, (b) enamine amide substrate.

others. When applying these under appropriately designed conditions (0.3 mol% catalyst, 50 °C, 5–6 bar H_2) the direct hydrogenation of enamine esters and enamine amides has been successfully conducted in excellent yields and optical purities without the need for the addition of an acyl group (see Fig. 2.14) [73].

2.6.2 Oxidations

Compared to reductions the oxidation reactions constitute an area that is still relatively unexplored from a large-scale point of view. In spite of the enormous efforts which have been spent on basic research, applications at scale are still scarce. The reasons for this could be that the asymmetric procedures currently at hand are deemed to be inefficient, that the types of functional group interconversions addressed with oxidations are far less in demand than is the case for reductions, or the existence of competitive stoichiometric methods (mostly based on the use of metal oxides and salts, such as CrO_3 and $KMnO_4$) that are considered to be sufficient for most purposes. Another factor that needs to be included is the intrinsic difficulty in designing a catalyst that is stable under the relatively aggressive oxidative conditions (compare reactivity of [H] and [O]). Nonetheless, the capability of enantioselective oxidations has been unambiguously proven at the manufacturing level in enough cases to make this approach a viable option for commercial production (see Sections 2.2 and 2.3).

The hype experienced over recent years regarding enantiopure sulfoxides does not show any tendency to vanish [74]. Instead, continuing efforts are being made to devise cheaper and more practical procedures. Ways to address this are primarily to drive down the amount of catalyst and to use a less expensive metal and oxidant while maintaining high yield and purity. A decisive step in this direction has now been taken by the construction of a system – inspired by the heme-containing peroxidases – based on iron and hydrogen peroxide which, when applied to a series mainly consisting of rather simple alkyl aryl sulfides, has delivered the corresponding sulfoxide in stereoisomeric purity as high as 96% ee and isolated yields of up to 78% [75]. In order to achieve this outcome, however, it was found that the presence of a carboxylic acid, or even better of a salt thereof, is crucial. Fine-tuning the conditions has revealed that the optimum ratio between acid and iron pre-catalyst should be 1 : 2 and when increasing the amount of the former there is a pronounced tendency to lose optical purity while maintaining the yield at a decent level. On examination of a range of carboxylic acid additives it was obvious that those with electron-donating substituents (giving rise to higher pK_a-values and, consequently, being less acidic), were superior; this is ascribed to their chelating abilities and the possibility to act as a co-ligand. This led to the conclusion that salts of these acids would augment this effect further, which was, in fact, shown by a substantial gain in the optical purity of the product obtained. In Fig. 2.15 an example of this process amenable method operating at room temperature in normal atmosphere is presented.

Interesting development over the last decade of the Baeyer–Villiger oxidation, originally discovered in the late 19th century, has opened up opportunities to con-

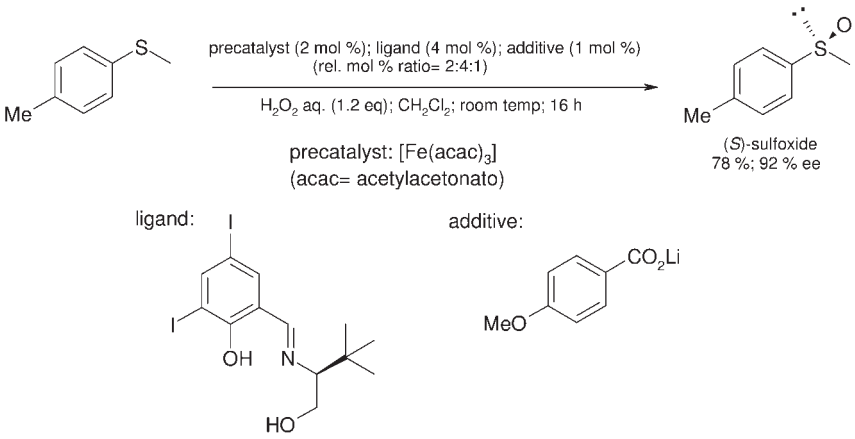

Fig. 2.15 A promising stereoselective sulfide to sulfoxide oxidation using Fe as catalyst (2 mol%) using environmentally benign H_2O_2 as oxidizing agent.

duct this synthetically useful ketone to ester transformation in an enantioselective mode. Besides aiming at an asymmetric version there has been a strong drive to replace the standard terminal oxidant represented by various peracids – for example CF_3COOH, monoperphthalic acid, *m*-CPBA, and CH_3COOH – with hydrogen peroxide. The advantages are manifold and include high oxygen content, H_2O being formed as by-product, safety, cost, and, in comparison to the peracids, there is no need to handle carboxylic acid residues in the outgoing effluent streams. However, drawbacks do exist as the water being generated could hydrolyze sensitive ester-groups, it is actually one of the weakest oxidants which requires the use of an activating catalyst, and there is a risk that oxygen released due to decomposition could create a potentially hazardous situation. Fortunately, most of these downsides can be handled efficiently even on the manufacturing scale making the use of H_2O_2 still very desirable [76]. With a bidentate catalyst of a combined BINAP-salen type using cobalt, the results achieved with a prochiral ketone substrate are of the order of 75% ee at 85–90% yield [77], see Fig. 2.16(a). Further development of this concept with even more complex ligands of concave structure with zirconium as the metal has enabled the improvement of the quality up to 94% ee (depicted in Fig. 2.16 b) triggered by the conclusive formation of a *cis-β*-configurated catalyst chelated by a reaction intermediate [78].

When racemic ketones are used as the starting material the reaction outcome is governed not only by stereoelectronic control but also by chiral recognition. Here the mechanistic aspects get extremely complicated, as it is necessary to account not only for differences in reactivity between the enantiomeric substrates but also to involve the formation of two isomeric lactones due to the varying migratory aptitude exposed by different substituents on the carbonyl group. All in all this results in a product mixture that, theoretically, could contain four components [78, 79]. Generally speaking, better results in the Baeyer–Villiger reaction have

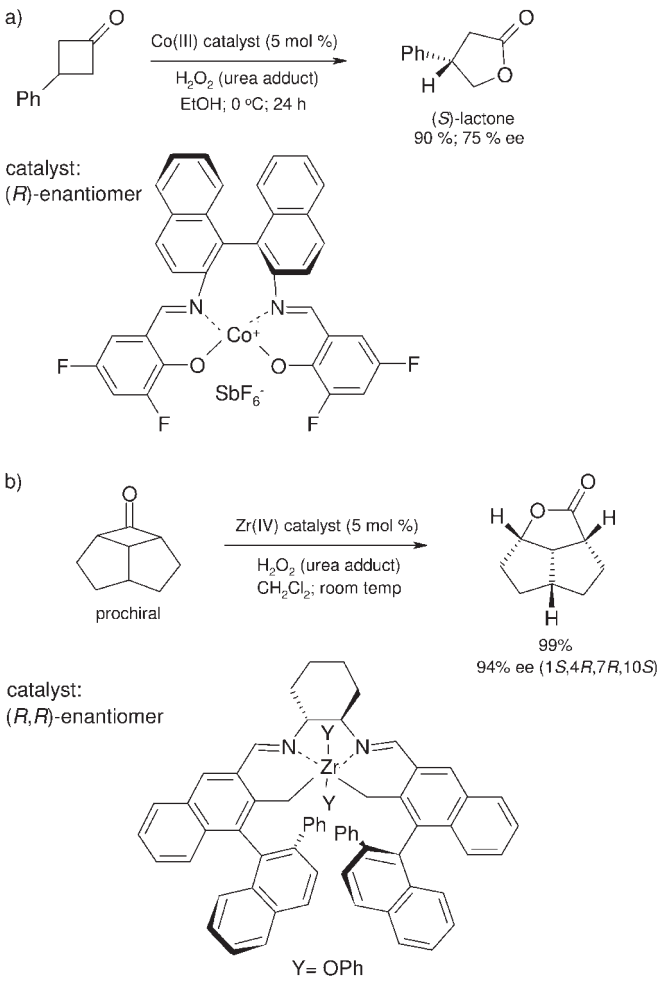

Fig. 2.16 Catalytic enantioselective Baeyer–Villiger oxidation of prochiral ketones, using H_2O_2 (in the form of a urea-adduct) as oxygen equivalent in stoichiometric amounts; (a) 3-phenylcyclobutanone, (b) tricyclic cyclo-butanone.

been obtained when performing it under biocatalytic conditions with enzymes or whole cells [76], but there is still ample room for improvement both of this approach and of the chemocatalysis route.

Not surprisingly, the "classical" Os-catalyzed asymmetric dihydroxylation of olefins (cf. Section 2.2) continues to be of interest. The basic principles, such as type of catalyst, stay relatively the same and instead efforts are concentrated on making the reaction more suitable for operation under process-like conditions. A modification that could improve the operability is to replace the conventional *t*-BuOH/H_2O solvent mixture by ionic liquid containing mixtures, either as a monophasic

(ionic liquid/*t*-BuOH/H_2O) or biphasic (ionic liquid/H_2O) system. After optimization of parameters – such as temperature, concentration, solvent ratios, amount and sort of catalyst – yields and enantioselectivities obtained with the *N,N′*-dialkylimidazolium based ionic liquids [C_4mim] [PF_6] and [C_8mim] [PF_6] were comparable or higher than when running under previously used conditions. The kinetics of the reaction was also improved, especially with the [C_4mim] system and the rate of conversion to the product is notably enhanced. Furthermore, the use of ionic liquids in general has been seen as a good way of effecting the recycling of spent catalyst and in the present case over 90% of the osmium load was retained in this phase. The work-up procedure is also considerably facilitated as the desired product can simply be extracted into a water or organic solvent phase or even be withdrawn via a membrane separation [80]. The development of a proven and firmly established methodology (chemical reaction) focusing on technical aspects, like those mentioned, with the aim being to turn it into something more process-friendly holds much promise for increased use on a manufacturing scale.

2.6.3
Carbon–Carbon Bond Formation

At the very heart of organic synthesis lies the construction of new compounds from simpler starting materials and for that purpose formation of carbon–carbon bonds is a key target. Since the dawn of synthetic chemistry a multitude of transformations have been discovered with the ability to create this crucial linkage utilizing a variety of substrates and reagents under a vast array of conditions, albeit with an outcome in terms of yield that spans the entire range from excellent to poor or even 'lousy'. Nonetheless, in a given situation they might all be useful and adequate to fulfill the task of making a particular molecule of interest. A short and definitely not comprehensive survey of the history highlights some reactions that over the years have been documented as useful tools in C–C bond generation (the year of their first report in the literature is given in brackets): aldol condensation (1838), Strecker synthesis (1850), Friedel–Crafts reaction (1877), Michael addition (1887), Henry reaction (1895), Pictet–Spengler reaction (1911), Mannich reaction (1912), Claisen rearrangement (1912), Diels–Alder cycloaddition (1928), hydroformylation (1938), Nazarov cyclization (1942), and Heck reaction (1972). Common to all these is that they were originally developed in racemic form but today exist in asymmetric versions, taking the utility and scope far beyond the limits of their old origins. With the vigorous and rapid extension of the pool of available catalysts/ligands capable of addressing ever more challenging problems, this "line-extension" is bound to continue. New and rich avenues will be opened up to improved synthetic transformations characterized by higher catalytic efficiencies, broader substrate tolerance, and better yield and enantiomeric purity. Some pertinent examples (from small-scale laboratory work) where carbon–carbon bonds have been formed enantioselectively under catalytic conditions are shown in Fig. 2.17.

Based on this selection of achievements with the, in many cases, outstanding yields and optical purities obtained under conditions which, at least superficially,

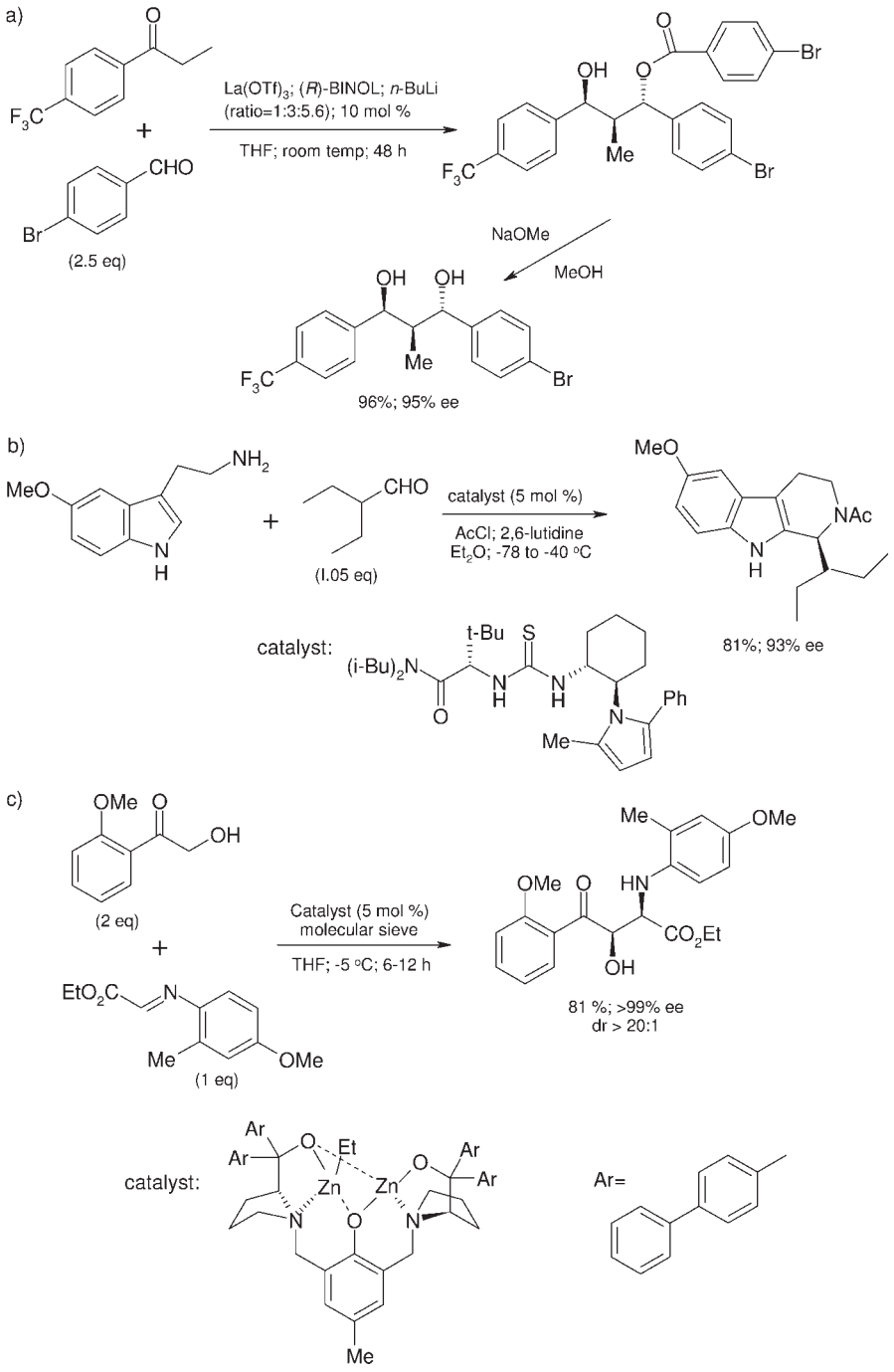

Fig. 2.17 a–c (legend see p. 57)

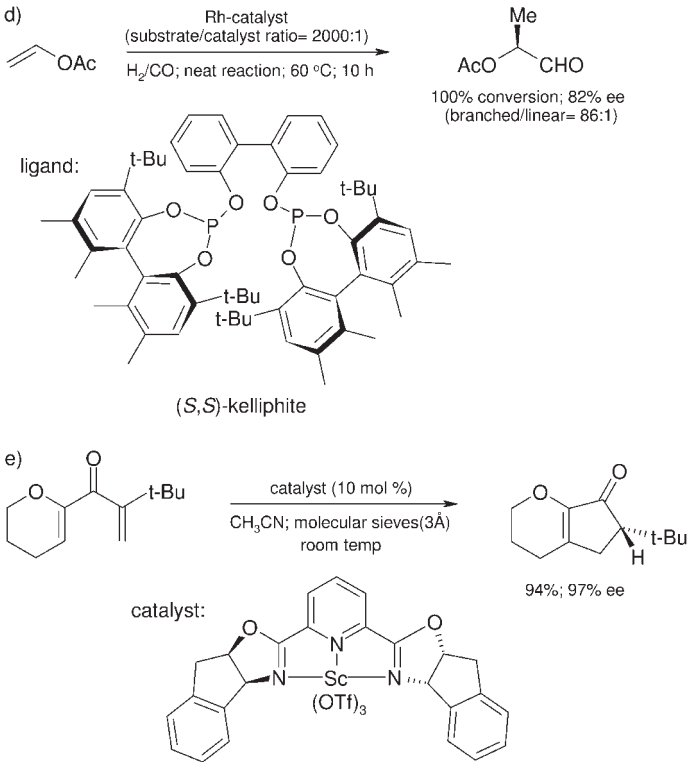

Fig. 2.17 At the centerpiece of organic chemistry: the making of new carbon–carbon bonds in stereoselective fashion using organometallic catalysis. (a) Aldol–Tishchenko reaction [81], (b) Pictet–Spengler reaction [82], (c) Mannich reaction [83], (d) hydroformylation [84], (e) Nazarov cyclization [85].

must be characterized as amenable to scale-up, a fair level of optimism is appropriate with regard to being able to take on increasingly intricate molecules. However, the structural variability offered by organic compounds of even modest size (molecular weight <500) is just breathtaking and therefore the arsenal of methodologies requires to be constantly expanded. In this context much attention has been devoted to ring-closing metathesis (RCM) and the creation of quaternary carbon stereocenters, the former capable of making much sought-after cyclic structures from olefins and the latter to provide the means to achieve a motif encountered fairly frequently but with only limited accessibility. The arena of asymmetric RCM reactions was opened up only quite recently (around the mid-1990s) and it was with the preparation of enantiomerically pure molybdenum complexes by Schrock that their synthetic utility could be demonstrated [86]; see Fig. 2.18a for an illustrative example. In spite of the high yield and optical purities that occasionally can be obtained with these Mo catalysts they also suffer from some notable drawbacks. Thus, due to the sensitive nature of the catalyst–substrate interaction a fine-tuning is required

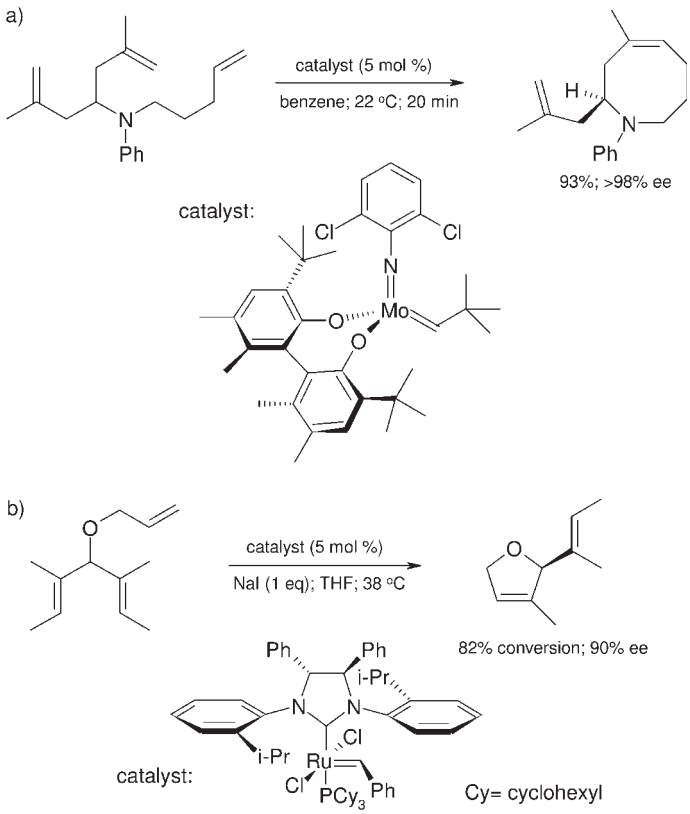

Fig. 2.18 Asymmetric RCM demonstrating the power of a new technology: (a) application of a kinetic resolution protocol using a Mo catalyst leads to the formation of an azocine, an 8-membered *N*-heterocycle; (b) conversion of an achiral triene with a Ru-based system creates a high-grade 2,5-dihydrofuran product.

which, consequently, demands optimization of the reaction. Other severe shortcomings are the lack of a wider functional group tolerance and the need for rigorous air and moisture exclusion, a condition which is almost impossible to guarantee in pilot plant and production settings. Therefore, the newly introduced air-stable ruthenium-based systems of Grubbs could offer an advantage in this regard and expand the scope of this promising transformation. Starting from a *meso*-triene, a desymmetrization RCM affords the resulting dihydrofuran product in 90% ee optical purity (see Fig. 2.18b) [87]. The vigorous development of this exciting field will most certainly increase our understanding of the factors that govern the enantioselectivity [88] and add on new features such as *in situ* generation of the more sensitive catalysts (Mo-type) as well as attachment onto solid supports to allow facile recycling [89]. Also the opposite of RCM, namely ring-opening metathesis (ROM), will see new and successful asymmetric applications in the future, either on its own or

coupled with RCM. With the announcements of the 2005 Chemistry Nobel Prize (see Royal Swedish Academy of Sciences website www.kva.se) to be shared by Schrock and Grubbs together with Chauvin who unraveled the mechanistic cycle of these intriguing reactions, the whole area of metathesis has come even more into the limelight.

When setting out to synthesize compounds that have quaternary carbon stereocenters solely carrying C-substituents, the choice of methods is still fairly narrow. Presently, there are only a few options available that allow this structural arrangement with its destabilizing steric repulsion between substituent groups to be constructed in an asymmetric sense under catalytic conditions. The list comprises Diels–Alder reactions, the combination of chiral carbon nucleophiles with carbon electrophiles, the reaction of allyl-metal intermediates with carbon nucleophiles, and intramolecular Heck reactions as well as the less general approach via desymmetrization [90]. Many impressive results have been reported from all the different variants, but limitations in the universality of reactant tolerability have to be taken into account. Another constraint to be aware of is the relatively high catalyst loading (up to 40 mol%) that is occasionally required to ensure good conversion and product ee. A beautiful assembly of a key intermediate in the total synthesis of (+)-aspidospermidine convincingly illustrates the strength of the Diels–Alder cycloaddition, here connecting an electron-rich diene with an electron-poor prochiral dienophile in the presence of a Cr-salen catalyst (see Fig. 2.19 a) [91]. The combination of two properties – i. e. simultaneously displaying Lewis acid and base properties – leading to bi-functional catalysts capable of dual-activation of both reaction partners (nucleophile and electrophile) would take us close to a perfect catalytic synthesis [92]. This concept allows reaction rates to be high and stereochemical information to be transferred efficiently as the activation occurs in a synergistic regime. A catalyst of this sort is showing clear similarities with Nature's own systems for this purpose – enzymatic processes involving metal-ion containing co-catalysts. One example demonstrating the sort of synthetic transformation made possible through this intriguing type of reaction is given in Fig. 2.19 b [93].

2.7
Retrospect and Prospect

We have witnessed a veritable "Golden Age" of asymmetric chemistry over the last 25–30 years, yet we need to be mindful of the fact that the scientific discipline is actually over a century old. For several decades the development was rather sluggish, but this has now been amply compensated for as new results and achievements are reported at high speed and in quantities that virtually make it impossible to stay abreast with all aspects of the field. Nonetheless, the need for technologies that can convert a substrate molecule into a desired product in high chemical and optical yield is very pronounced – both from academia and in industry – and therefore the large investments made can easily be justified. To operate reactions under catalytic conditions is in demand (not forgetting that about 80 % of all che-

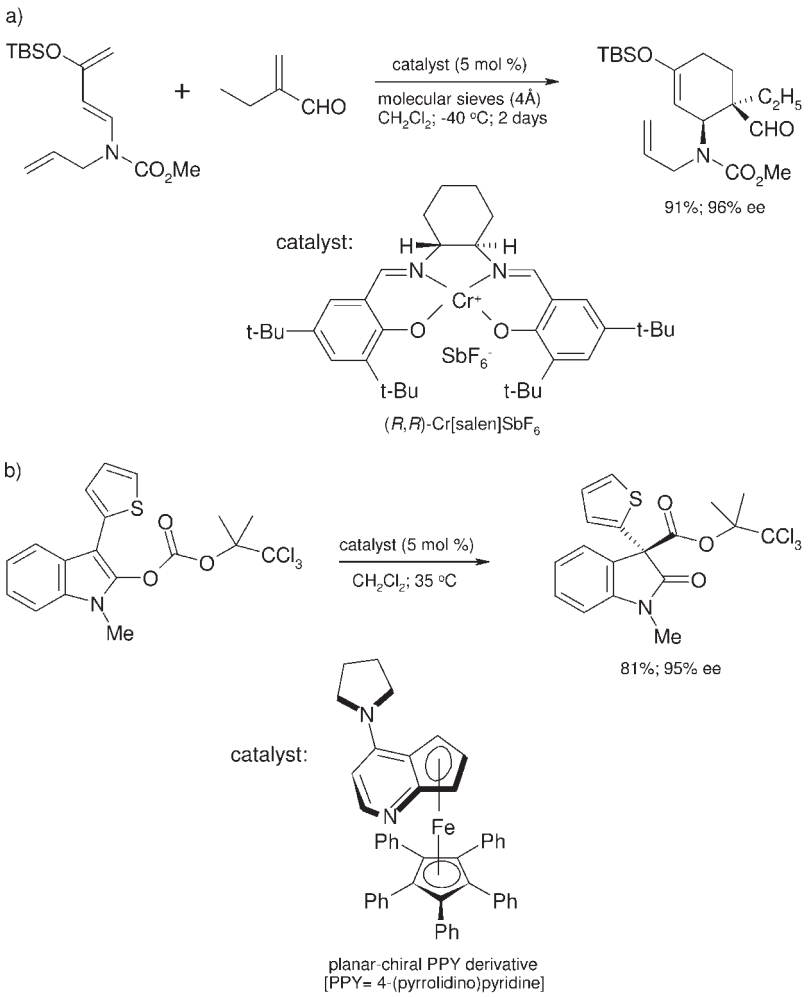

Fig. 2.19 Effective methods to generate all-carbon substituted quaternary stereocenters: (a) Diels–Alder assembly of a complicated building block for the synthesis of (+)-aspidospermidine; (b) intermolecular C-acylation using a planar nucleophilic ferrocene type catalyst.

micals being produced use catalysis for their manufacture), inasmuch as this reduces the environmental burden via outgoing streams considerably – giving a real meaning to the hype around "green chemistry" – and also contributes favorably by driving down the cost. Many of the new tools have been eagerly taken up by industry for further development and it is very rewarding to see the large number of liaisons with academic institutions that in an amazingly quick way have commercialized these synthetic methodologies. The preparedness from an industrial point of view to adopt modern approaches to asymmetric transformations is well docu-

mented and has, furthermore, been demonstrated by a number of full-scale processes that have been taken to production and are operated in a range from a few to more than 10 000 tones per annum. This is not to say that the technical area as such is mature; on the contrary, it is still seen by many as very challenging and risky, where the stakes are high and the outcome uncertain. Inevitably, time will gradually overcome reluctance and resistance, as methods are improved and new success stories are added to the old ones. We should not expect a one-fits-all scenario, where literature data can simply be translated and applied to any problem at large scale. Instead, as a rule of thumb, a lot of adjustment and modification of the original procedure is required, but the mere knowledge that the transformation of interest has been successfully conducted on other substrates is invaluable. The absence of any precedence should not deter us from probing for asymmetric catalysis options, as long as there is a good scientific rationale behind it.

Before closing it would be motivating to ask the question: What will come next? A simplistic answer could be: More of what we have already seen! That is new ligands being prepared – adding to the current list of about 1500 – which when transformed into catalysts show unprecedented results for a given reaction or class of reactions. Moreover, a recently reported heterocombinatorial approach, i. e. mixing together different chiral monodentate phosphorus ligands as opposed to using them separately (homocombination), has, when tested in model reactions (catalytic hydrogenation), proven superior. Thus, in a specific case run under identical conditions the former gave 96% ee whereas the latter resulted in not more than ca. 76% [94]. The potential for further exploration seems virtually endless. We will also see catalytic systems capable of effecting transformations which today we are nowhere near or can only faintly master; for example the stereoselective functionalization of achiral molecules such as the simple and abundantly available hydrocarbons and other un-activated sp^3 C–H bonds – for instance a regio- and stereospecific transfer of butane → (*S*)-2-butanol) [95]. The feat of oxygenating these very strong bonds is actually achieved enzymatically, for example with cytochrome P450 or chloroperoxidase under physiological conditions, and the mechanism is now starting to be unraveled [96]. Another "hot" area receiving much interest is catalysis under metal-free conditions, so called organocatalysis. This conceptually attractive approach explores the boundaries of intermolecular adaptation where laboratory experiments have shown that with addition of the simple amino acid L-proline, analogs thereof or other suitable compounds (20–30 mol%) to aldol, Mannich, Michael and other reactions can generate products with high ee (>90%) [97]. A third option would be to see how far the validated and well-tested application of chiral phase-transfer catalysts [98] can be taken and what use on a larger preparative scale might arise from the slick combination of metal catalysis and enzymes in a one-pot procedure [99]. Finally, the use of free radicals does not seem to be restricted to production of polymers, as has convincingly been shown with the synthesis of some fine chemicals in the recent past. A particularly rewarding approach that has already demonstrated its commercial potential is the reagent-controlled transfer of chirality from the radical initiator, often a tin-based species, onto the substrate, which proceeds with both high yield and optical purity

[100]. In summary it is fair to say that the intricacies of asymmetric synthesis and its vast possibilities will occupy the hands and minds of academic and industrial scientists for many years to come. To freely quote a well-known phrase from a famous politician [101]: *This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning*!

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101 Winston Churchill (1874–1965), British Prime Minister.

3
Aspects of Chirality in Natural Products Drug Discovery

Philipp Krastel, Frank Petersen, Silvio Roggo, Esther Schmitt, and Ansgar Schuffenhauer

3.1
Introduction

Natural products are isolated mainly from plants, fungi, bacteria, and marine macro-organisms. They are also called secondary metabolites, as they are not essential for the life cycle of their producers. In general, they are synthesized at the end of the microbial growth phase. In the case of plant derived products, their production is often coupled to differentiation and seasonal changes influencing the life cycle of a plant [1–3].

The therapeutic use of natural products, mainly administered as herbal preparations, has long been known in human history. It is therefore not astonishing that approximately 80% of the world's population is using plant derived medicines for basic healthcare [4]. The analysis of Farnsworth and Morris of the National Prescription Audit data from 1976 showed that approximately 40% of the US prescriptions in 1973 contained natural products derived from plants and microorganisms [5].

Drug substances from pure natural products together with their derivatives, and synthetic compounds deduced from a natural product precursor represent a major part of today's pharmaceutical market [6, 7]. Although natural products represent less than 1% of all published chemical compounds, they accounted for 35% or $230 billion of the total pharmaceutical sales in 1996 [8]. Between 1990 and 2000, 41 drugs derived from natural products were introduced to the market [9]. Nearly, all of them are chiral with an average of eight chiral centers. Table 3.1 lists some recently launched drugs derived from natural products and Fig. 3.1 shows the structures of the active compounds [10].

In the following, it will be shown that chirality is a key characteristic of natural products, and stereochemistry is often crucial for a specific biological activity (e.g., interaction with a target protein).

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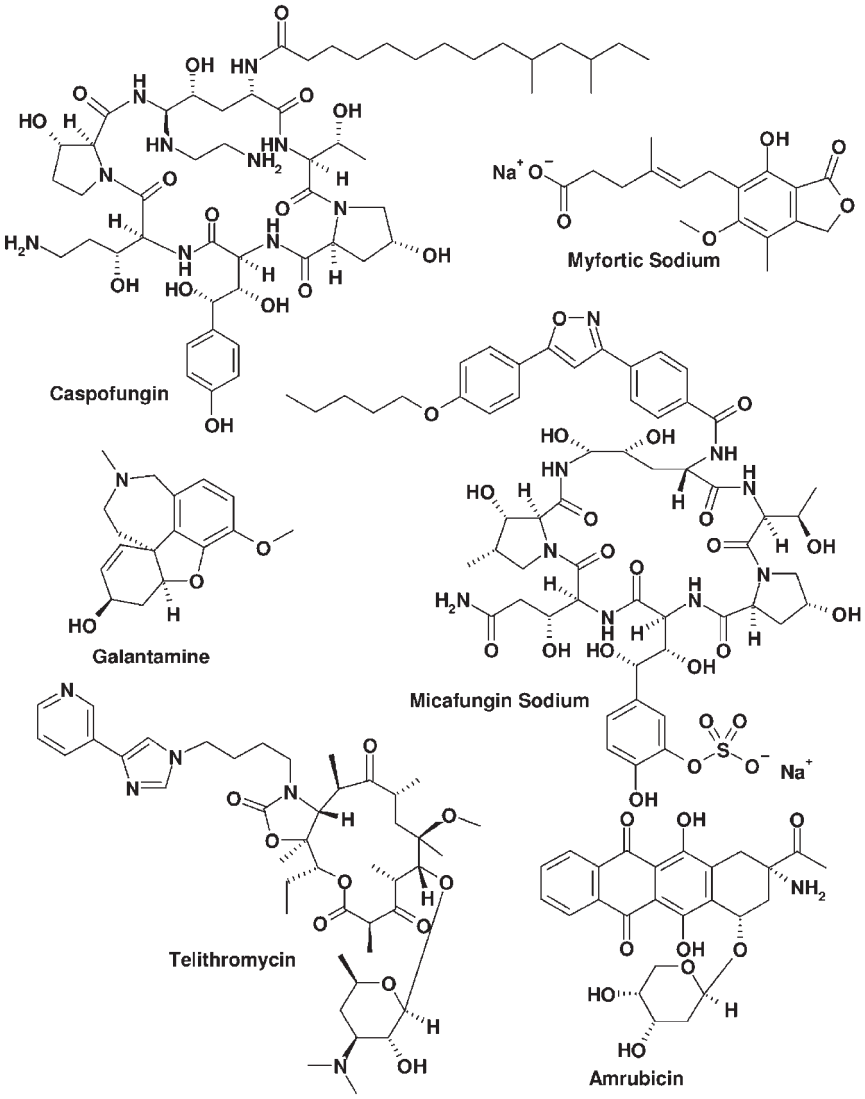


Fig. 3.1 Chemical structures of drugs derived from natural products launched between 2000 and 2003.

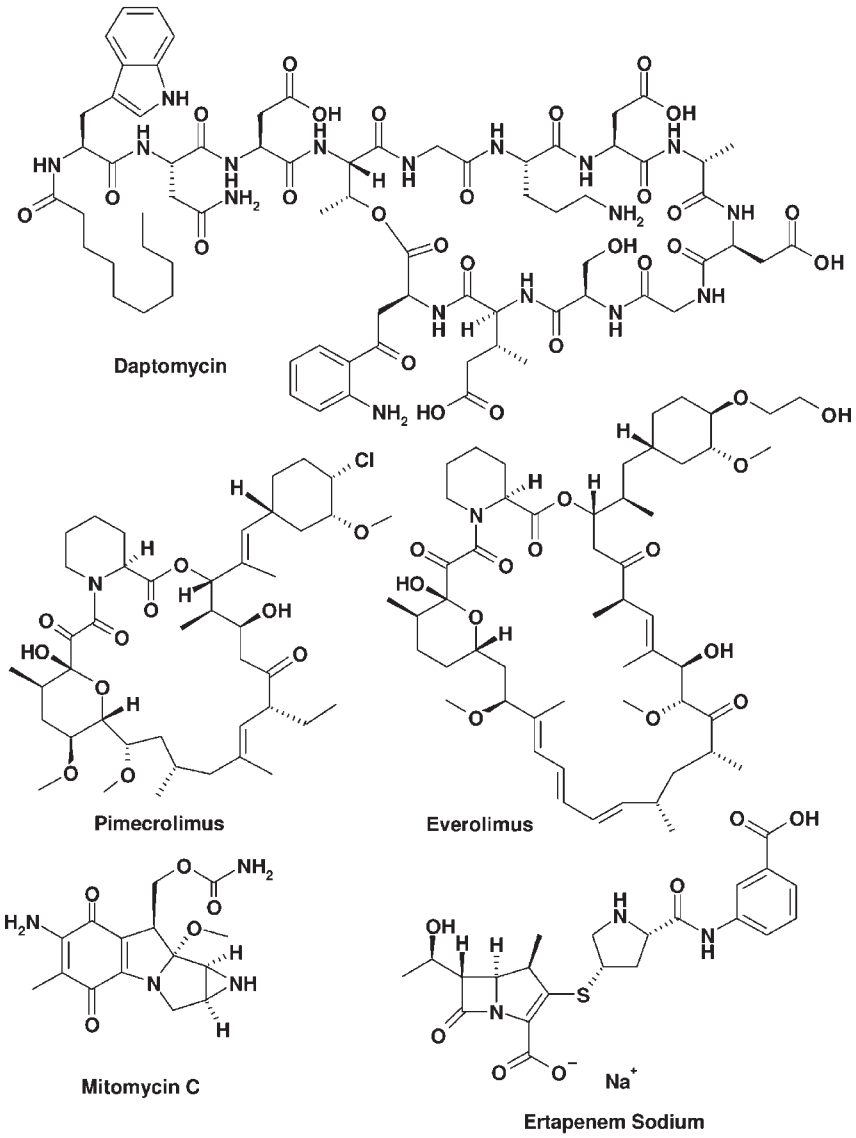


Fig. 3.1 (continued)

Table 3.1 Examples of drugs derived from natural products launched between 2000 and 2003.

Generic name	Trade name	Year introduced	Use	Company	Type of compound	Number of chiral centers	Origin
Ertapenem Sodium	INVANZ	2002	antibacterial	Zeneca	Carbapenem	6	derivative (fungus)
Telithromycin	KETEK	2001	antibiotic	Aventis	Erythromycin	13	derivative (streptomycetes)
Caspofungin Acetat	CANCIDAS	2001	antifungal	Merck	Echinocandin	14	derivative (fungus)
Micafungin Sodium	FUNGUARD	2002	antifungal	Fujisawa	Echinocandin	15	derivative (fungus)
Amrubicin HCL	CALSED	2002	antineoplastic	Sumitomo	Anthracycline	5	synthetic (streptomycetes)
Pimecrolimus	ELIDEL	2002	immunosuppressant	Novartis	Ascomycin	14	derivative (streptomycetes)
Daptomycin	CUBICIN	2003	antibacterial	Cubist	Lipopetide	13	derivative (streptomycetes)
Mycophenolat Sodium	MYFORTIC	2003	transplant rejection	Novartis	Mycophenolic acid	0	natural product (fungus)
Everolimus	CERTICAN	2004	transplant rejection	Novartis	Rapamycin	15	derivative (streptomycetes)
Mitomycin C	MYTOZYTREX	2002	anticancer	SuperGen	Benzoquinone	4	synthetic (streptomycetes)
Galantamine	REMINYL	2000	acetylcholinesterase inhibitor	Jansen	Alkaloid	3	natural product (plant)

3.2
Stereochemical Aspects of Natural Products

Natural products differ from synthetic compounds in various properties. They are structurally very diverse, often exhibit specific biological activities and reveal an astonishing sterical complexity. This is an obvious consequence of the fact that the biosynthesis enzymes are inherently three dimensional and chiral. Furthermore, based on the limited palette of building blocks used for biosynthesis, nature has to generate novelty by branching out common intermediates into diverse scaffolds. Because of their buildup on enzymatic pathways natural products have shown their capability to bind to enzyme or, generally, protein targets and may therefore also interact with disease relevant target proteins [11].

Several statistical studies compare synthetic compounds, combinatorial libraries, drugs and natural products [12–14]. Compared with drugs and synthetic compounds, natural products contain fewer nitrogen, sulfur, or halogen atoms on average, but are considerably more oxygen-rich and contain more hydrogen-bond donors. This divergence probably reflects the fundamental differences between biosynthesis and (combinatorial) synthetic chemistry. Many biosynthetic pathways are based on reagents which have similar numbers of oxygen and nitrogen atoms, such as amino acids, or which contain more oxygen than nitrogen, as for example in polyketide synthesis. Compared to synthetic compounds, natural products contain significantly more rings – often with sp3 hybridized bridgehead atoms – and chiral centers.

Interestingly in terms of *Lipinski's rule of five*, natural products are quite similar to drugs. About 10% of the traded drugs contain two or more violations of the rules, compared to 12% for natural products. Also the average calculated molecular weight is almost identical for marketed drugs and for natural products ($MW \approx 360$) [13].

3.2.1
Chirality Analysis of Natural Products versus Drugs and Synthetics

For a chirality analysis of natural products the Dictionary of Natural Products (DNP) was used as the data source [15]. This database with its 161 278 entries represents the bulk of published natural products and derivatives. By using the biological source information given in DNP and a dump of the ITIS (Integrated Taxonomic Information System) taxonomy database [16], subgroups of compounds were defined, representing the different origins of natural products (plants, animals, fungi, bacteria). For a comparison with marketed drugs, a dataset of 8561 molecules was compiled from the MDDR (MDL Drug data report) and the Comprehensive Medicinal Chemistry database. All compounds of these two databases which had either a tradename in at least one of the databases or were at least in Phase III clinical trials were considered. For synthetics, a representative set of 24 725 compounds was generated by random sampling of a database containing 4.8 million screening samples available from commercial vendors. The number of chiral centers was determined with the PipelinePilot [17] software.

Table 3.2 Average number of chiral centers in synthetics, drugs and natural products. (Natural products are divided into subclasses related to their biological origin.)

Source	Synthetic	Drugs	DNP	Subgroups DNP			
				Plantea	Fungi	Animalia	Monera
Number of compounds	24 728	8561	161 278	64 314	3206	8594	6626
∅ chiral centers	0.39	2.82	5.19	6.03	4.02	4.62	7.24

The average numbers of chiral centers for the different groups are shown in Table 3.2; the percentage distribution for the numbers of chiral centers is shown in Fig. 3.2. About 80 % of the natural products have at least one chiral center, and the distribution drops only slowly for higher numbers. There are 15 % of the compounds with 11 stereocenters or more. From the synthetic compounds, about 70 % are achiral and 20 % have only one stereochemical center; there are almost no compounds having more than three stereocenters. For drugs, the number of chiral centers is lower than for natural products but higher than for synthetics. The average

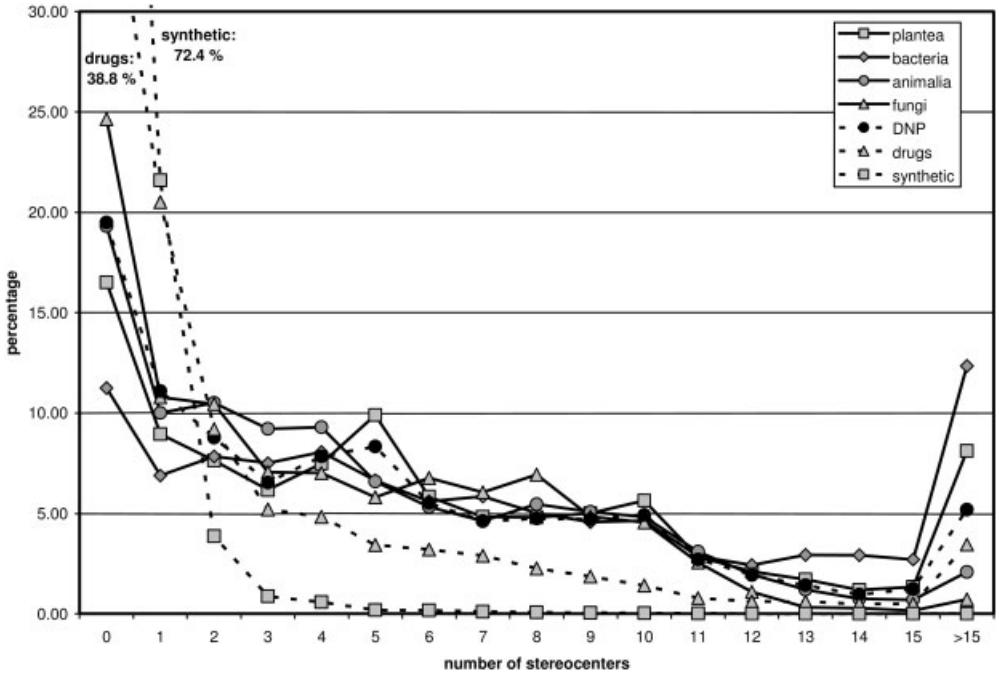


Fig. 3.2 Distribution of the number of chiral centers among synthetics, drugs, and natural products. (Natural products are divided into subclasses related to their biological origin).

number in drug substances is 2.8 compared with 5.2 for natural products and 0.4 for synthetics. About 40% of the drugs have no chiral center, about 20% have one, followed by a broad distribution between 2 and 10 or more stereocenters.

The stereo index, as shown in Fig. 3.3 is the coefficient between the number of stereo centers and the number of non-H atoms. While there are completely achiral natural products, natural products with a low stereo index, meaning that there are at least 27 non-H atoms, are very uncommon. A possible explanation might be that natural products often contain highly substituted or fused ring systems. If these rings are aromatic, they contain no chiral centers at all, if these rings are non-aromatic there will likely be more than one chiral center.

Analyzing the biological sources of the natural products, there are some differences between the various groups. Bacteria – mainly gram negative, such as gliding bacteria and gram positive (e.g., actinomycetes) eubacteria and archaebacteria – show the lowest percentage of achiral molecules (10%); the median of chiral centers is 7.2, 25% of the compounds have more than 11 stereo centers. From the fungal metabolites about 25% have no chiral center; the mean number of chiral centers is 4.0. Fungi are known to produce many small achiral aromatic compounds like quinones, xanthones or pyrones, which originate from the polyketide pathway [18]. This could be one reason for the high percentage of achiral molecules from this source.

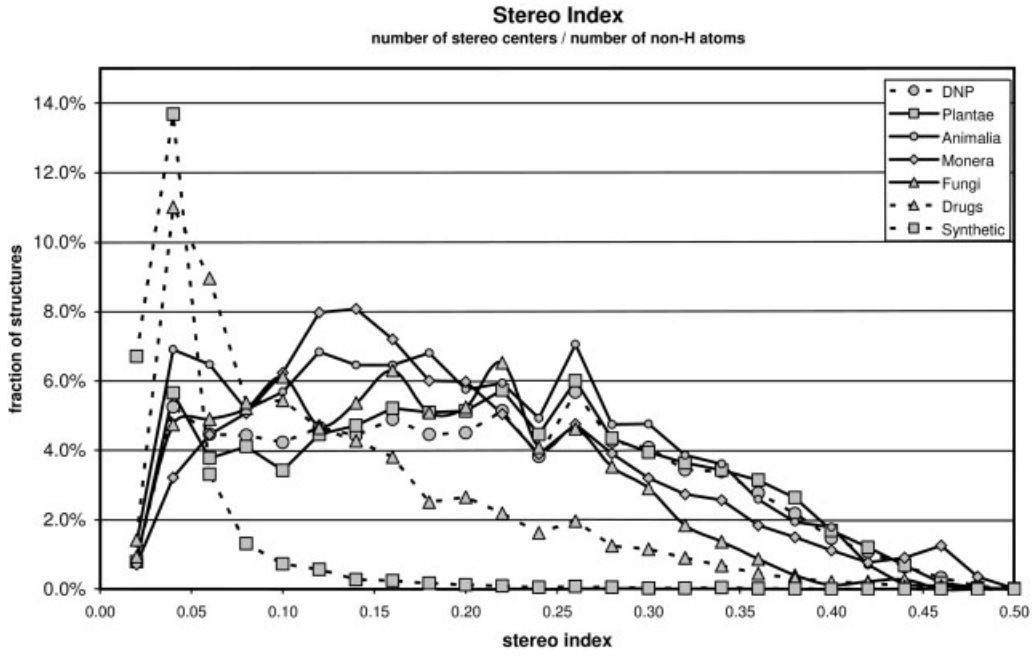


Fig. 3.3 Stereoindex of synthetics, drugs, and natural products.
(Natural products are divided into subclasses related to their biological origin).

Looking at the marketed drugs derived from the natural product pool (this could be the natural product itself, a semi-synthetic derivative thereof, or a derivative generated by total synthesis), the average number of chiral centers is higher than the average of the drug pool. About 40 drugs derived from natural products were launched between 1990 and 2000, and most of them were chiral containing eight chiral centers in average [19].

3.2.2 Determination of the Relative and Absolute Stereochemistry of Natural Products

In synthetic chemistry, for every reaction step the structure of the product is more or less predictable and only a limited number of stereocenters is built up. Starting with molecules from the chiral pool (e.g., amino acids, carbohydrates, hydroxy acids), only the relative stereochemistry of the new centers has to be elucidated and the absolute stereochemistry is given by the stereoinformation of the chiral educt. Looking at natural products the starting point is different. In natural product analysis, initially nothing is known about atom connectivities or stereochemistry. The ability to work nondestructively and with only small amounts of sample is most desirable. NMR spectroscopy has become the most powerful tool for the elucidation of the structure of natural products [20]. Using 2D-NMR methods (e.g., HMQC, HMBC) in combination with modern NMR equipment (e.g., cryoprobes) and computer-assisted methods [21], structure elucidation of tiny amounts of a natural sample is possible. However, for relative and absolute stereochemistry determination the use of NMR spectroscopy is limited. For many natural products no stereochemical information has been published. The integration of virtual screening and high-throughput docking [22] in drug discovery strongly requires stereochemical information of natural products. In recent years many improvements have been achieved in standardized stereochemistry determination. This involves, in many cases, a combination of different methods such as NMR spectroscopy, X-ray, chiroptical methods, total synthesis as well as degradation reactions which lead to the elucidation of the three-dimensional structure.

3.2.2.1 NMR Spectroscopy

The stereochemical analysis of compounds with well-defined conformational properties is presently fairly easy to accomplish. From cyclic compounds with small (three- to six-membered) rings the configuration can be extracted from simple NMR parameters, such as proton–proton (^{1}H–^{1}H) *J*-coupling values and/or nuclear Overhauser effect (NOE) intensities. A much more challenging task is the assignment of the relative configuration of flexible systems, such as macrocyclic compounds or polysubstituted chains. On the one hand this could be solved using derivatives. For example, 1,3-diols, which are often part of polyketide chains, can be converted into acetonides, if enough material is available. The generated ring can be used to determine the relative stereochemistry of both chiral centers [23]. On the other hand, a *J*-based NMR approach has been developed for the determi-

nation of the stereochemistry of acyclic systems [24]. It is based on a detailed analysis of homonuclear (^{1}H–^{1}H) and heteronuclear (^{13}C–^{1}H) $^{2,3}J$- couplings and NOE data and is particularly suitable for acyclic structures having stereogenic carbon atoms bearing hydroxy, alkoxy or methyl substituents. The coupling constants between protons separated by three bonds ($^3J_{HH}$) are directly related to their dihedral angles through the Karplus equation. Likewise, heteronuclear (^{1}H–^{13}C) vicinal coupling constants ($^3J_{CH}$) follow a Karplus-like relationship and can be used to derive additional angular constraints. The $^2J_{CH}$ values can also be useful if the carbon bears an electronegative substituent, such as an oxygen or halogen atom. $^{2,3}J_{CH}$ values have been rather inaccessible for structure elucidation for a long time, thanks to progress in 2D-NMR techniques, hardware improvement and the diffusion high-field magnets these *J*-couplings are nowadays measurable on compounds at the natural abundance of ^{13}C.

For the determination of the absolute stereochemistry by NMR spectroscopy, Mosher derivatives are widely used [25]. This method is based on the formation of (*R*)/(*S*)-MTPA (α-methoxy-α-(trifluormethyl)phenylacetic acid) esters and comparison of the NMR data of both diastereomers. However, it is limited to compounds with amenable functional groups to be derivatized (typically hydroxy groups) and in some cases the conclusions from the NMR experiments are unsafe.

3.2.2.2 **Chiroptical Methods**

Polarimetry is still being used to characterize optically active compounds and as an enantiomeric purity criterion. A prerequisite for the absolute stereochemistry determination with this method is the presence of reference compounds. The application of circular dichroism (CD) spectroscopy for this purpose is a useful tool in natural product analysis [26]. CD is based on the differential absorption of left or right circularly polarized light. CD spectra can be calculated from known geometries and transition moments, which allows the prediction of the absolute stereochemistry. In the 1970s the exciton chirality method (ECCD) was established. If two identical, or nearly identical, chromophors are present in a chiral molecule, coupling between the two oscillating transition dipoles in a symmetric and antisymmetric fashion gives rise to a split of the UV absorption band. The effect of this split absorption is then seen as a split Cotton effect (CE) or a couplet which may be either positive or negative. There are several examples of successful absolute stereochemistry determination such as the michellamines [27] and the thorectandrols [28]. However, there are some limitations of this method. The assignment of Cotton effects retains an empirical component when applied to new structural types and CD depends on the presence of chromophors, or requires the preparation of "Cottonogenic" derivatives.

3.2.2.3 **X-ray Crystallography**

If crystals of a natural product are available, X-ray crystallography is the most efficient way to determine the relative stereochemistry and, in suitable cases, also the

absolute configuration. In 1951, Bijvoet achieved the first experimental determination of the absolute configuration of a chiral molecule from the effects of anomalous X-ray scattering [29]. The method involves using X-rays with a wavelength near the absorption edge of one of the atoms. This results in a phase change for the X-rays scattered by atoms of this type, relative to the phase of the X-rays scattered by the other atoms. Hence the diffraction pattern is no longer truly centrosymmetric, pairs of spots (Bijvoet pairs) in the pattern that are related by the center of symmetry become unequal in intensity. From a known structure, except for the absolute configuration, one can calculate the relative intensities of the Bijvoet pairs for the *R*- and *S*-isomers. In this calculation a parameter x is included (Flack parameter), when this parameter takes the value of 0, the correct stereochemistry has been used for the calculation, if not, x becomes 1 [30]. The phase change generally increases with the atomic mass of the elements present and the wavelength of the radiation used. In the past, heavy atoms (Si or larger) were necessary to use this method reliably. Nowadays, exploiting the high precision and data redundancy achievable with modern X-ray equipment, it is also possible to determine the absolute stereochemistry from structures containing oxygen as the heaviest atom. For example the absolute stereochemistry of a building block for the synthesis of discodermolide was determined using this method [31].

3.2.2.4 **Total Synthesis and Degradation Reactions**

If no crystals are available and spectroscopical methods are not successful in determining the absolute stereochemistry, total synthesis or degradation reactions can be used. In degradation reactions the molecule is split into smaller subunits, which can be compared with molecules of known absolute configuration. For example, the absolute stereochemistry of the amino acids in the marine-derived cyclic depsipetide kahalalide F has been defined by a series of degradation reactions (hydrolysis, ozonolysis, Edman degradation and Marfey derivatization) [32]. If only tiny amounts of the natural product are available (e.g., metabolites from marine invertebrates) and degradation reactions, therefore, are not possible, a total synthesis is the alternative for the elucidation of the absolute stereochemistry. If the relative stereochemistry of the molecule is known, there are only two possible antipodes as target molecules; if the relative stereochemistry is unclear and a number of enantiomers and diastereomers are possible, the synthesis of the correct isomer might be a difficult task. For example, the first synthesis of the microtubule stabilizing agent discodermolide yielded in the non-natural antipode (–)-discodermolide. Three years later the synthesis of the natural (+)-enantiomer was reported [33]. In some cases total synthesis has led to the revision of the previously determined stereochemistry of the isolated metabolite. For example the total synthesis of yanucamide A allowed the determination of the configuration at an ambiguous stereocenter and the stereochemistry of a second center had to be revised [34].

In conclusion, there is no general guideline for the determination of the stereochemistry of natural products. The most efficient way to solve the stereochemical problem depends on the structural class and the availability of methods.

3.3
Mechanisms of Stereochemical Control in Natural Product Biosynthesis

Natural products can be differentiated according to their biosynthetic origin. Characteristic for a biosynthesis are the building blocks used to assemble the core structure and the classes of enzymes catalyzing the condensation of the building units, as well as cyclization and derivatization reactions. Complex natural products with a number of stereocenters are usually produced as pure enantiomers. For example in the Dictionary of Natural Products about 80% of all products are single entries, meaning there is no other molecule with the same connectivities present [15]. In general, stereospecific conformations are necessary for specific biological activities. Thus, the amazing configurative specificity ensures an efficient usage of the cost-intensive secondary metabolite biosynthesis capacities of the producing organisms.

There are several routes to generate chirality in a natural product. (1) It can be attained in a stereospecific biosynthesis step. (2) It is introduced at the beginning of the biosynthesis by chiral building blocks derived from primary metabolism. For example, proteinogenic and non-proteinogenic amino acids in L- and D-configuration are assembled in non-ribosomal peptides. (3) Decorating enzymes can add sugar moieties to the peptide or polyketide backbone resulting in complex structures like the glycosylated antibiotic vancomycin.

Although an increasing number of biosynthesis enzymes and the corresponding genes or gene clusters have been discovered and analyzed in recent years, detailed mechanistic investigations on the stereochemical control in the biosynthesis of complex natural products are rare. The following sections focus on four mechanisms which are relatively well understood. The presented examples comprise natural products typical for microorganisms, like non-ribosomal peptides, macrolide and aromatic polyketides, and the plant-derived terpene taxol.

3.3.1
Origin of D-Amino Acids in Non-ribosomal Peptides

Non-ribosomally processed peptides represent a large class of natural products of microbial origin. Pharmaceutically important examples are antibiotics of the beta-lactam type (e.g., cephalosporin C) and glycopeptides of the vancomycin class as well as the immunosuppressant cyclic undecapeptide cyclosporine (Fig. 3.4).

Non-ribosomal peptides are assembled on very large protein templates called peptide synthetases that exhibit a modular organization, allowing polymerization of monomers in an assembly-line-like mechanism [35]. Modules are comprised of at least three catalytic domains: the adenylation domain for substrate recognition and activation; the thiolation domain for covalent incorporation of thioesters and the condensation domain leading to peptide formation. The largest known peptide synthetase directing the synthesis of the undecapeptide cyclosporin contains 11 modules on a single polypeptide chain and has a mass of about 1500 kDa [36].

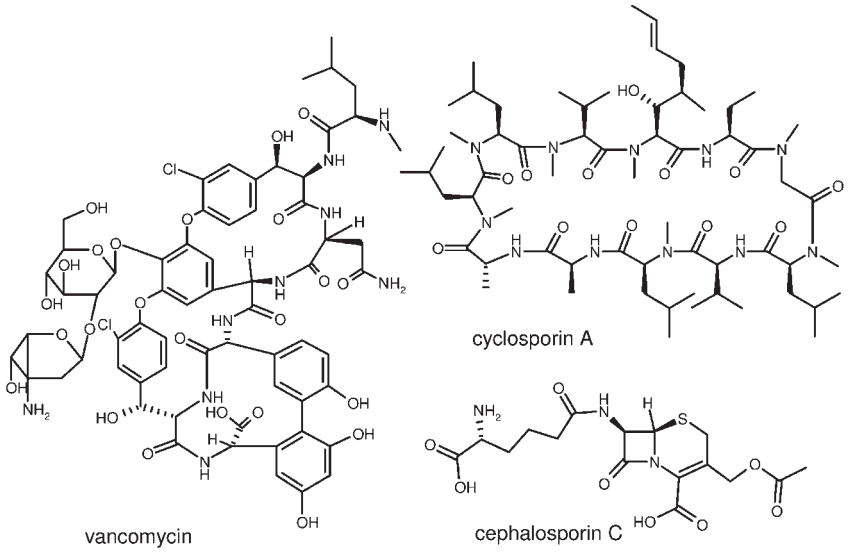


Fig. 3.4 Non-ribosomal peptides derived from microbial origin.

D-amino acids are very frequent components of non-ribosomal peptides and their derivatives. The D-configuration of such peptide residues may, in part, be utilized to slow proteolytic degradation of peptide antibiotics, given that most proteases exhibit selectivity for L-amino acid residues. Conversely, they may also play a functional role in retaining biological activity, e.g., by setting conformers and side chains for subsequent processing steps, such as oxidative cyclizations [37].

Three different mechanisms have been elucidated for the incorporation of D-configurated amino acid residues in non-ribosomal peptides:

1. Direct activation and incorporation of D-amino acids by the gate-keeping adenylation domains of synthetases have been described in some cases. This requires a pool of the corresponding D-amino acid generated by a separate enzyme starting with the abundant L-amino acids. The biosynthesis of the immunosuppressant cyclosporin, which is widely used in transplantation medicine, requires D-alanine as a starter molecule. In the producing fungus, *Tolypocladium inflatum*, a separate enzyme, the alanine racemase, provides D-alanine. The cyclosporin synthetase itself is not able to isomerize L-alanine and does not accept L-alanine as a substrate [38].

2. In most peptides synthetases an epimerization domain that mediates D-amino acid incorporation is embedded within the module. Thus, L-amino acids are substrates of the synthetase and *in situ* epimerization occurs during peptide chain elongation. The first module of gramicidin S synthetase from *Bacillus brevis* is a well studied example [39]. This module recognizes L-phenylalanine, and the tightly bound intermediate is epimerized to the D-Phe-enzyme com-

plex. During the biosynthesis of gramicidin S additional L-amino acids are coupled to the growing peptide chain in four subsequent elongation steps.

3. In the synthesis of the beta-lactam antibiotic cephalosporin, a third mechanism is used to epimerize a stereocenter in the non-ribosomal tripeptide. The biosynthesis of cephalosporins and penicillins starts with three amino acids, L-aminoadipic acid, L-cysteine, and L-valine. After condensation the resulting tripeptide is cyclized by the oxidase isopenicillin N-synthase [40]. A separate epimerase catalyzes the conversion of the L-α-aminoadipyl side chain of the cyclic tripeptide isopenicillin N into the D-α-aminoadipyl side chain of penicillin N. Interestingly, different epimerase systems have evolved in fungal and bacterial cephalosporin producers. In the bacterium *Streptomyces clavuligerus* a single 44 kDa protein catalyzes the pyridoxal phosphate-dependent removal of the amino group followed by reintroduction in the D-configuration [41, 42]. In the fungal cephalosporin producer *Acremonium chrysogenum* the corresponding epimerase system was discovered only recently, partly because the system is entirely different from the bacterial epimerase. The fungal epimerase system consists of two enzymes, CefD1 and CefD2, with similarity to long chain acyl-CoA synthetases and acyl-CoA racemases frequently found in higher eukaryotes. From the similarity to known eukaryotic epimerases it was deduced that isopenicillin N is activated with acyl-CoA, epimerized and an uncharacterized thioesterase hydrolyzes the CoA thioester to release penicillin N. Such a system has not been described elsewhere in the biosynthesis of secondary metabolites [43].

3.3.2 Control of Chirality in Modular Polyketide Synthesis

Macrolide polyketides are a second class of natural products characterized by a modular biosynthesis. Type I polyketide synthases catalyze the assembly of complex natural products from simple acyl-CoA precursors. This biosynthetic process closely parallels fatty acid biosynthesis [44]. In contrast to fatty acid synthases, modular polyketide synthases utilize a wider variety of starter and extender units. Each module contains a ketosynthase domain which catalyzes the formation of a carbon–carbon bond. The acyltransferase domain recruits the chain extension unit, normally from either malonyl-CoA or methylmalonyl-CoA. The acyl carrier protein cooperates in the carbon–carbon bond formation and carries the growing chain to optional domains, e.g., ketoreductase domains for the processing of the keto group. The methyl centers at C-2 and the hydroxy centers at C-3, generated in many newly added chain extension units, can have either *R*- or *S*-configuration. Typically both configurations are present in a reduced polyketide. Despite the variation within a single structure, there are strong position-specific homologies among diverse polyketides pointing to a common ancestral enzyme (Celmer's rule) [45].

The modular organization of type I polyketide synthases was first recognized by sequencing the gene cluster for the biosynthesis of the antibiotic erythromycin

(Fig. 3.5) from the Streptomyces-related bacterium *Saccharopolyspora erythraea* [46]. Now 6-hydroxyerythronolide B synthase serves as a model for studying *in vitro* activities of enzymatic domains within the modules. Experiments with recombinant enzyme revealed a complex interplay between the ketosynthase and ketoreductase domain in achieving stereocontrol. In module 1 the ketoreductase likely determines the configuration of both stereocenters of the first chain extension unit, whereas in module 2 of the 6-hydroxyerythronolide B synthase, the ketosynthase establishes the methyl stereochemistry at C-2 and the ketoreductase the hydroxy configuration at C-3 of the second chain extension unit [47]. Thus, two different modes of cooperation between ketosynthase and ketoreductase domains exist in the biosynthesis of the macrolide erythromycin. The ketoreductase of module 1 was further investigated using a recombinant diketide synthase containing a single ketoreductase domain. The bicyclic ketone (9*R,S*)-*trans*-1-decalone, a known substrate for ketoreductase in fatty acid metabolism, was also found to be an effective substrate for the ketoreductase of the diketide synthase. Only the (9*R*)-*trans*-1-decalone was reduced, selectively and reversibly, to the (1*S*,9*R*)-*trans*-decalol. The stereochemical course of reduction and oxidation is exactly as found for the ketoreductase of animal fatty acid synthase, an additional indication of the close similarity of these enzymes [48].

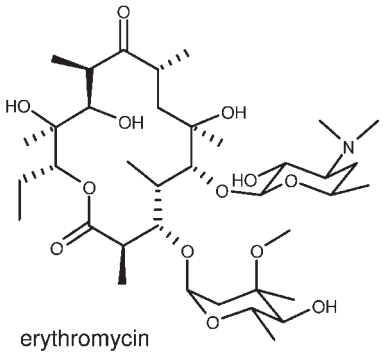


Fig. 3.5 Structure of erythromycin.

3.3.3
Modes of Stereodifferentiating Cyclization

Polyketide and non-ribosomal peptides produced by bacteria and fungi often attain the conformations that establish biological activity by cyclization constraints introduced by tailoring enzymes. This includes heterocyclization of cysteines, serines and threonines in non-ribosomal peptides. The second cyclization constraint is macrocyclization in polyketides, such as the above-mentioned antibiotic erythromycin and the antitumor epothilones. Regio- and stereospecific macrocyclization usually occurs at the end of the polyketide and non-ribosomal peptide assembly lines during chain release by thioesterase domains [49]. However, in the case of antibiotics of the ansamycin class, like the antitubercular drug rifamycin, the final

amide bond formation in macrocyclization occurs by the action of separate amide synthase subunits rather than embedded thioesterase domains [50].

Besides macrolide polyketides, aromatic polyketides are produced by microorganisms from the same acyl-CoA building blocks. They are produced by type II polyketide synthases, which are multienzyme complexes that carry a simple set of iteratively acting activities. In the biosynthesis of aflatoxin B the simple aromatic polyketide norsolorinic acid is converted in a complex manner. Aflatoxin B1 is a potent environmental carcinogen produced by *Aspergillus* species, common molds that infect nuts and grains. The epoxide of aflatoxin B1 is formed in the liver and intercalates readily into double-stranded DNA to give selective adduct formation through the N-7 guanine residues. For the covalent reaction with DNA the absolute configuration of the dihydrobisfuran ring system is essential.

The synthesis of aflatoxins begins with a six-carbon starter unit and seven malonyl units. Cyclization and oxidation leads to norsolorinic acid, the first anthraquinone intermediate in the pathway. The biosynthesis proceeds through three configurationally liable intermediates to racemic versiconal hemiacetal. Subsequent enzymatic cyclization establishes the stereochemistry of the critical ring fusion in (–)-versicolorin B and is catalyzed by versicolorin B synthase. This enzyme selects from two equilibrating enantiomers of versiconal hemiacetal to cyclize the appropriate antipode to optically pure versicolorin B (Fig. 3.6). The rate of enzymic cyclization is limited by the intrinsic rate of epimerization of the substrate. This was shown by varying the amount of versicolorin B synthase, which was isolated from the filamentous fungus *Aspergillus parasiticus*, and given to a fixed concentration of substrate [51].

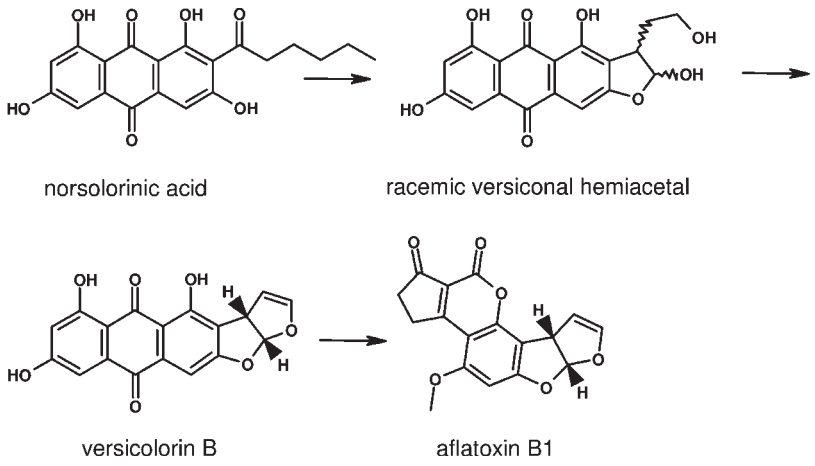


Fig. 3.6 Biosynthesis of aflatoxin B1.

3.3.4
Terpene Biosynthesis in Plants

Another important source of chiral compounds with polycyclic structures are plants. Terpenes or terpenoids comprise the largest class of plant secondary products. All terpenes are derived from the activated 5-carbon building blocks of isopentenyl pyrophosphate (PP) and the isomer dimethylallylPP. These building blocks react to give geranylPP, farnesylPP and geranylgeranylPP as 10-, 15- and 20-carbon precursors of mono-, sesqui- and diterpenes, respectively.

The diterpene taxol is used as a potent chemotherapeutic agent in cancer treatment. The limited supply of the drug from the natural source, the bark of the Pacific Yew (*Taxus brevifolia*), was overcome by a semisynthetic approach. Taxol is presently manufactured by coupling the advanced naturally occurring taxoid 10-deacetylbaccatin III, isolated from needles of the more abundant *Taxus baccata*, to a synthetic side-chain precursor.

The first committed step in the biosynthesis of taxol is the cyclization of geranylgeranylPP to the taxadiene precursor of taxol, a step catalyzed by taxadiene synthase (Fig. 3.7). This remarkable terpene cyclase mediates the formation of three carbon–carbon bonds, three stereogenic centers, and the loss of hydrogen in a single step. Recombinant taxadiene synthase was incubated with specifically deuterated substrates to define the crucial hydrogen migration and terminating deprotonation steps [52]. The experiments suggest that taxadiene synthase is capable of mediating complex olefin cation cyclizations, with absolute stereochemical fidelity, by conformational control alone.

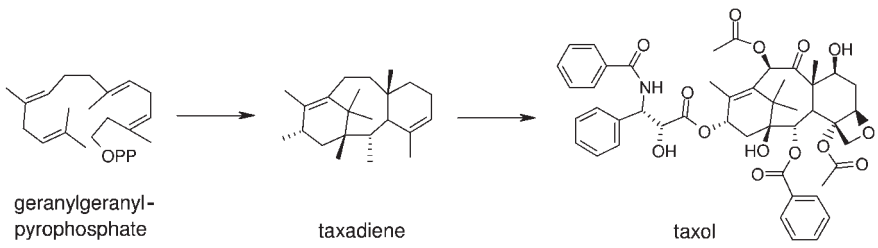


Fig. 3.7 Biosynthesis of taxol via taxadiene as precursor.

Taxadiene undergoes an extended series of oxygenation and acylation reactions to yield taxol. So far, 8 out of 20 enzymatic steps have been *in vitro* catalyzed through cloned cDNAs [53]. Several regioselective CoA thioester-dependent acyltransferases, including a C13-side-chain *N*-benzoyltransferase which predominantly forms one 3′-epimer of 2′-deoxytaxol, are involved in the synthesis. This indicates that there is still a long way to go before taxol could be produced in microorganisms from a reconstituted biosynthesis.

The presented examples show that it is possible to assign some general rules for the introduction of stereocenters in the biosynthesis of natural products. For this, natural products need to be grouped according to their mode of biosynthesis

and classes of building blocks. However, it is evident that the biosynthesis of a complex natural product with several stereocenters requires very specific enzymatic activities. Many exceptions to rules exist and the knowledge gained by studying one biosynthesis cannot be easily transferred to another natural product.

3.4 Biological Activity of Natural Products Related to Stereochemistry

Living organisms use chiral enzymes for the biosynthesis of natural products. The biochemical reactions performed by these enzymes are generally enantioselective. As a consequence, nature normally produces only one stereoisomer of a natural product. This fundamental feature is probably the outcome of an evolutionary selection process for the most economical production of molecules by nature to achieve maximal biological activity in a chiral environment. Natural products are therefore perfect examples to illustrate Pfeiffer's rule: "the lower the effective dose of a drug, the greater the difference in the pharmacological effect of optical isomers" [54]. It also means that in a series of chiral compounds the relative potency (eudismic ratio) of stereoisomers increases with the increasing potency of one isomer (eutomer) compared to another isomer (distomer). This rule can also be exemplified by the finding that enantiomers can have totally different drug properties, such as quinidine (antiarrythmic) and quinine (antimalarial) [55] (Fig. 3.8), and that the derivatization of highly potent natural products can lead to compounds with profoundly altered biological activities and drug actions in different indications.

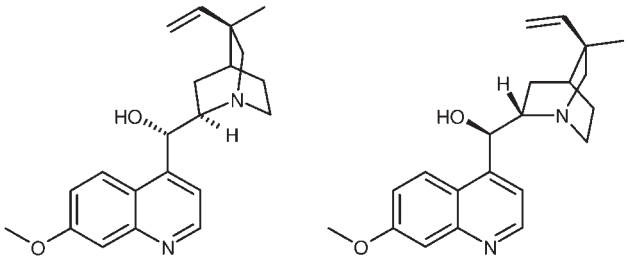


Fig. 3.8 Stereoisomeric alkaloids exhibiting different therapeutic effects.

In the drug discovery process only the naturally available stereoisomer of a natural product is generally tested and promoted to clinical studies. The structures of many natural products are rather complex in comparison to synthetic drugs. A synthetic approach to obtain both enantiomers or defined diastereoisomers is often not feasible and beyond the scope of medicinal chemistry, despite the tremendous progress in the field of stereoselective synthesis during the last 20 years.

Here, those rather rare cases are described where stereoisomers of natural products have been analyzed and compared in biological studies.

In drug discovery nowadays, it is common practice to profile all enantiomers and diastereoisomers of drug candidates, especially for synthetic drugs, if a mixture of isomers is being considered for drug development or if the compounds are prone to isomerization.

The influence of stereochemical differences of natural products on biological systems has already been studied by Pasteur who recognized that *dextro* ammonium tartrate was digested more rapidly by the fungus *Penicillium glaucum* than the *levo* isomer [56]. Ascorbic acid, also known as vitamin C, only exhibits redox activity as the (+)-isomer, whereas the (–)-isomer is inactive and cannot be used to treat scurvy [57].

Epinephrine (adrenaline) was one of the first hormones for which differential effects on blood pressure were reported for the two epimers in the early 20th century [58]. A reassessment of epinephrine activity was made by analyzing the effects of optical isomers of ephedrine and methylephedrine on the spontaneous beating rate of the isolated right atrium of normal and reserpinized rats by investigating direct and indirect actions on α1-adrenoceptors. L-ephedrine, and to a lesser extent D-ephedrine, markedly increased the beating rate of rat right atrium [59].

3.4.1 Natural Products Active on the Nervous System

Many plant metabolites were discovered early on as modulators of neurotransmission affecting the action of adrenaline and other neurotransmitters. The stereoisomers of muscarine, a toxic fungal metabolite isolated from *Inocybe* and *Clitocybe* species, were tested as cholinergic agonists. The naturally occurring (+)-5*S*,4*R*,2*S*-isomer was the most active on muscarinic receptors [60]. A large variety of nicotinic agonists such as nicotine, pilocarpine, lobeline, cytosine, anatoxin A and epibatidine originated from plants, bacteria and amphibia. The activities of the individual stereoisomers have been studied (Fig. 3.9). Pilocarpine, from the leaves of the *Pilocarpus* species, is a potent muscarinic agonist in use for the treatment of glaucoma. It does isomerizes under basic conditions to the inactive isopilocarpine [61]. Epibatidine has been isolated from frog skin (*Epipedobates tricolour*) and is a potent nicotinic receptor agonist, 200 times more active than morphine, and a very effective analgesic [62, 63]. Yohimbine and pseudoyohimbine were used to probe the binding of ^{3}H-clonidine to rat cerebral cortex membranes. The results indicate that yohimbine is an α_2-adrenoceptor antagonist [64]. The stereochemistry of drug action at adrenoreceptors has been described and reviewed comprehensively [65–71].

The natural product willardiine is an AMPA receptor agonist. Substitutions at positions 3 and 5 led to the identification of AMPA and kainate antagonists. UBP291 was the most potent AMPA antagonist in this series with an IC_{50} = 13.7 μM as measured by the dorsal root-evoked ventral root potential. Only analogs with absolute configuration *S* were active, such as the naturally occurring willardiine (Fig. 3.10) [72].

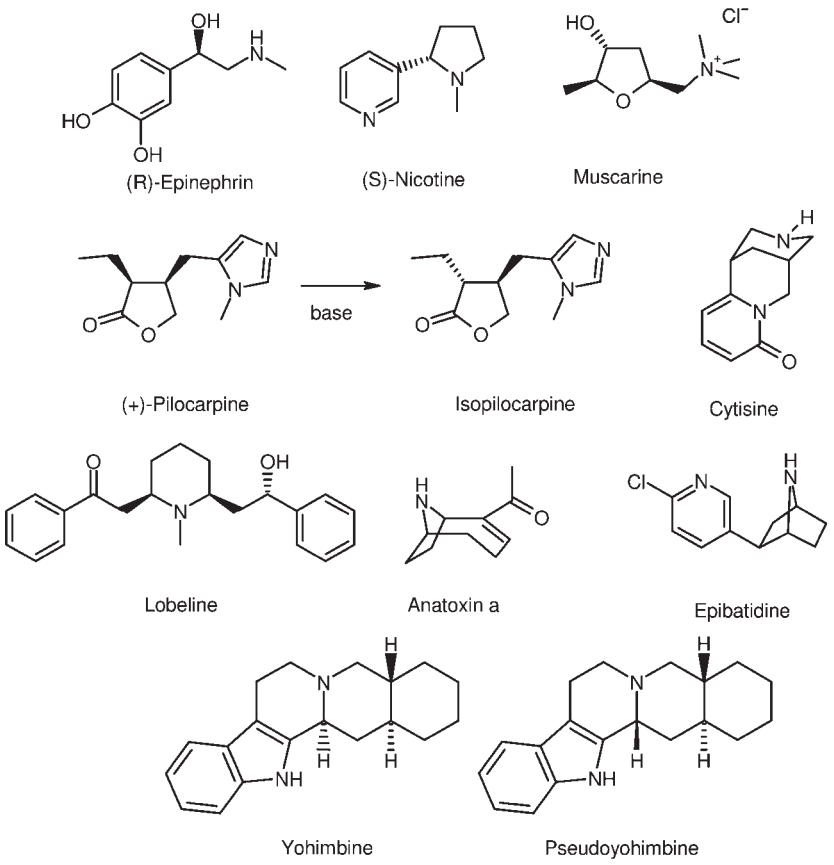


Fig. 3.9 Chemical structures of natural products and analogs with nicotinic, muscarinic, cholinergic and dopaminergic activities.

Acetylcholinesterase (AChE) inhibitors are successfully used as drugs for the treatment of Alzheimer's disease. Physostigmine, also named eserine, has been isolated from the Calabar bean (*Physostigmina Venenosum*). It is classified as a pseudo-irreversible inhibitor because it reacts with acetyl- as well as butyryl-cholinesterase (BChE) to form a carbamylated serine which is hydrolyzed again with a $t_{1/2}$ of 40 min [73]. Physostigmine inhibits AChE with an IC_{50} of 27.9 nM and BChE with an IC_{50} of 16 nM. Its enantiomer is 350 times less active on AChE and 150 times less active on BchE [74].

Antillatoxin is a highly ichthyotoxic metabolite isolated from the marine cyanobacterium *Lyngbya majuscule*; its potency is only exceeded by brevetoxins (Fig. 3.10) [75]. Using cerebellar granule cells, antillatoxin produces neuronal death, which can be prevented with co-incubation of NMDA receptor antagonists or tetrodotoxin, indicating that antillatoxin interacts with voltage-gated sodium channels. This finding is further supported by the allosterically enhanced stabili-

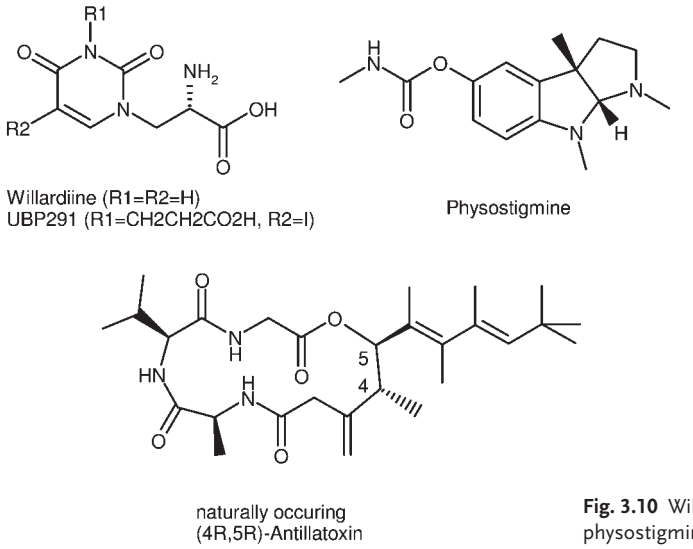


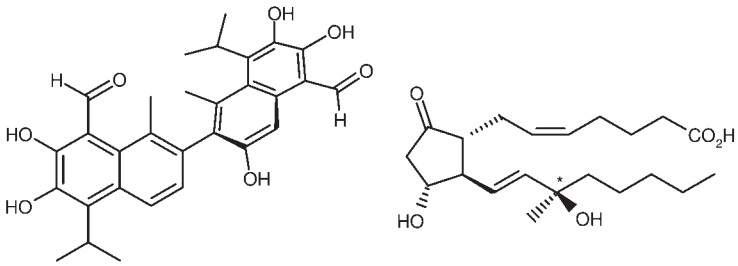
Fig. 3.10 Willardiine, UBP291, physostigmine, antillatoxin.

zation of the open conformation of the sodium channels by antillatoxin when treating intact cerebellar granule cells with batrachotoxin, a known sodium channel opener. The actual binding site could not yet be determined but the results obtained from the studies with batrachotoxin exclude neurotoxin sites 1, 2, 3, and 5. The four possible diastereomers of antillatoxin on C-4 and C-5 have been made and their neurotoxic activities evaluated. The natural 4*R*,5*R*-isomer was the most potent analog with an LD_{50} = 0.18 μM against a mouse neuro 2a cell line, 25–50 times more potent than the next most active 4*S*,5*S*-isomer. The isomers showed the same order of potency in the test systems [76].

3.4.2
Gossypol, a Racemic Natural Product

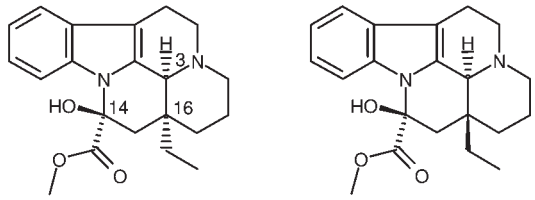
Gossypol is a natural product isolated from the cotton plant *Gossypium herbaceum L.* or *G. Hirsutum L.* and has an axial dissymmetry. It attracted considerable attention as a male antifertility agent but the interest faded due to slow onset of action and the risk of sterility. The plant produces a racemic mixture of gossypol of which only the (–)-enantiomer showed antispermatogenic activity, which also goes along with the cytotoxicity. (+)-Gossypol does not affect sperm count and is also less toxic than its enantiomer (Fig. 3.11) [77–79].

As the example of gossypol shows, it is essential to have a detailed understanding of the stereochemical stability of a chiral center and knowledge about the mode of action of the stereoisomers in order to decide whether to start drug development with a pure active enantiomer or the racemic mixture. The configurational stability has to be analyzed in the manufacturing process, in the final storage form of the drug and in the physiological application.



Gossypol

15(S)-Methylprostaglandin E_2
(Arbaprostil)



3a, 16a-Vincamine

3a, 16b-Vincamine

Fig. 3.11 Gossypol, araprostil and vincamines.

3.4.3
Epimerization of Natural Products *in vivo*

Several prostaglandins have been shown to inhibit gastric secretion in animals [80] and in man [81]. 15(*S*)-Methylprostaglandin E_2 (arbaprostil) is a synthetic derivative of prostaglandin E_2 bearing an additional methyl group in position 15 and having the opposite absolute configuration at this chiral center (Fig. 3.11). The compound is inactive *in vivo* when applied intravenously, but if it is given orally, gastric secretion is inhibited efficiently [82]. Allylic alcohols are known to undergo acid catalyzed racemization *in vitro* upon exposure to mineral acid. Both epimers of arbaprostil showed oxygen exchange at low pH leading to epimerization. The kinetics of this chemical reaction have been studied with ^{18}O-enriched water. A significant percentage of the initially formed epimerized products did not incorporate ^{18}O from the water. This result indicates that, in this case, the departing water molecule ultimately forms a new bond with the carbonium ion from the opposite side [83]. Since it was known that only the 15-(*S*)-epimer showed pharmacological activity, the inactive 15-(*R*) epimer of arbaprostil was selected as drug substance on the premise that this compound would only be converted to a significant degree under conditions of high acidity. With a normal activity of the stomach, it was argued that the acidity should not lead to epimerization. Therefore, arbaprostil would only be activated in the stomach by the conditions for which it was supposed to be active, namely under excess gastric secretion [84].

Vincamine is found in the aerial part of the *Vinca minor* plant and can also be derived from other plant sources such as the *Voaconga* and the *Crioceras longiflorus*. It is known primarily as a vasodilator and is, in general, used as an aid in activities requiring highly focused attention and concentration such as technical writing or computer operation, as increase of neuronal metabolism has been observed as well. All possible isomers of vincamine have been synthesized and their cardiovascular effects have been evaluated [85]. At a dose of 1 mg kg^{-1} *iv* the trans16β-ethyl derivatives showed consistently the strongest vasodilatatory effect measured as femural and internal carotid blood flow in dogs. The series of compounds with the 16α-ethyl configuration were mostly inactive in this model.

3.4.4 Tubuline Stabilizing Agents

Epothilones were the first class of promising compounds to stabilize microtubules in cells, 15 years after the mode of action of taxol was discovered [86, 87]. These 16-membered macrolactones were isolated from the myxobacterium *Sorangium cellulosum*. The anticancer activity raised immediate interest in this new class of lead structures, especially since they were equally active against drug-sensitive and multidrug-resistant human cancer cell lines *in* vitro [88, 89]. A vast number of analogs have been synthesized and a variety of stereochemical questions related to structure–activity relationships have been addressed. Inversion of the stereochemistry at both epoxy carbons results in analogs with the 12*S*,13*R* configuration which are significantly less active than the natural 12*R*,13 *S*-isomers (Fig. 3.12) [90]. Changing the C12,C13 double bond geometry from cis to trans resulted in a loss of activity. *Trans*-epothilone C is about 3–5-fold less active than epothilone C. In the epoxide series the situation is different, where one *trans*-epothilone A isomer is at least equipotent with epothilone A itself, but the other 12*R*,13*R*-isomer is more than 500-fold less potent [91]. The activity pattern for the *trans*-epothilone D isomers is somehow unclear since different results have been reported and the assignment of the absolute configuration has only been elucidated recently [92]. Conformation analysis of epothilones using a molecular modeling approach [93] gave rise to the synthesis of isomers of 14-methyl epothilone B. The conformational changes induced by the methyl group in (14*R*)-methyl-epothilone B were analyzed using NMR, X-ray diffraction and a panel of human tumor cell lines to test cytotoxicity [94]. The calculated conformation was quite similar to that obtained by X-ray diffraction and by NMR in solution; the backbone conformation was not altered by the additional (14*R*)-methyl group. The potent activity of (14*R*)-methyl epothilone B in the range of IC_{50} = 0.7–3.6 nM strongly supports the proposed bioactive conformation; the deoxy (14*R*)-methyl-epothilone D is less active against the cancer cell lines by a factor of about 25. These results are in accordance with the structure of 15N-labeled epothilone A obtained by high resolution NMR analysis in the presence of bovine tubulin [95]. An X-ray analysis of crystallized epothilone A revealed a very similar conformation to that of the studies mentioned before [96].

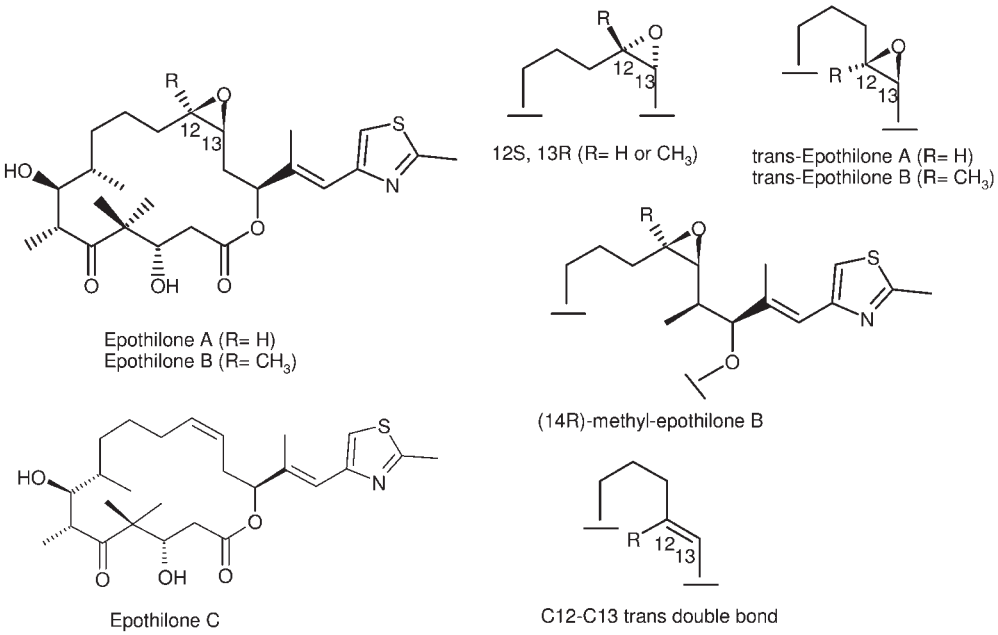


Fig. 3.12 Stereochemistry of natural epothilones and derivatives.

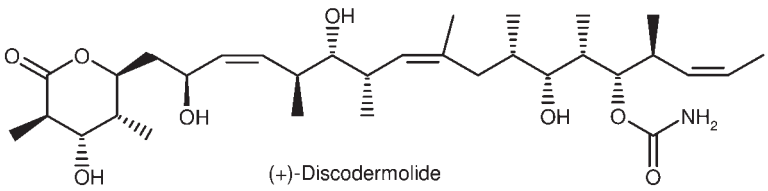


Fig. 3.13 (+)-Discodermolide.

Discodermolide is yet another natural product with microtubule stabilizing properties similar to taxol and competitively inhibits binding of taxol to microtubules (Fig. 3.13) [97–99]. It was originally isolated in small quantities from the marine sponge *Discodermia dissoluta*. A synthesis has been optimized to provide ample material for clinical trials [100]. Discodermolide possesses distinct activities in addition to those of taxol and epothilones; its unique profile could be related to the effects on tubuline initiation and nucleation or it might indicate that the drug has intracellular targets other than the microtubule. Both enantiomers of discodermolide have been made and astonishingly enough both compounds inhibit cell cycle progression. The natural compound (+)-discodermolide stops the cell cycle at the G2/M phase, whereas synthetic (–)-discodermolide seems to block the cells in the S-phase. Both enantiomers of discodermolide have antiproliferative activity, but they act by distinct mechanisms and appear to have distinct cellular targets [101].

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4
Biotransformation Methods for Preparing Chiral Drugs and Drug Intermediates

Michael Müller and Marcel Wubbolts

4.1
Introduction

Macromolecules exhibiting biological activity, such as enzymes, antibodies, receptors or viruses, are composed of chiral monomolecular building blocks, such as amino acids or carbohydrates. Thus, it is not surprising that for many small molecules possessing biological activity it is found that one of the possible (stereo) isomers is active while the other (stereo) isomers are either inactive or exhibit reduced activity. Chiral drugs and drug intermediates can be prepared by biotransformations since many enzymes act in a stereoselective manner.

Several monographs have been published on this topic. In the book *Stereoselective Biotransformations* [1] many examples of the successful application of biocatalysts can be found. In *Asymmetric Catalysis on the Industrial Scale* several enzymatic and nonenzymatic processes are described in detail [2]. The two volume set *Chirality in Industry* gives a more general view of this topic [3]. Additionally, many review articles have been published recently dealing with stereoselective biotransformations [4–11]. Nevertheless, the advantages of biocatalysis are not restricted to stereoselectivity, rather, many different aspects make biocatalysis a highly competitive and sometimes superior method in comparison to nonenzymatic transformations. Biotransformations can be carried out at ambient temperature, atmospheric pressure and at or near neutral pH enabling the performance of multistep processes in one vessel. Enzymes are highly efficient catalysts, much more active than low molecular weight organocatalysts, for example. The use of toxic metal ions is omitted which is highly desirable for pharmaceutical purposes.

Biocatalysts can be immobilized and reused in many cycles, or even in a continuous process. Nevertheless the application of biocatalysts on an industrial scale requires reaction engineering techniques in order to enable the application of high substrate and product concentrations. Additionally, the stability of enzymes under the production conditions may be decreased so that modern molecular biology methods like directed mutagenesis for increased long-term stability are required [12, 13]. Furthermore, the substrates and starting materials applied in biocatalytic

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transformations might be quite different from those in a nonenzymatic process. In particular, if a technical process has to be established, cheap starting materials have to be accessible reliably over a period of years or even decades.

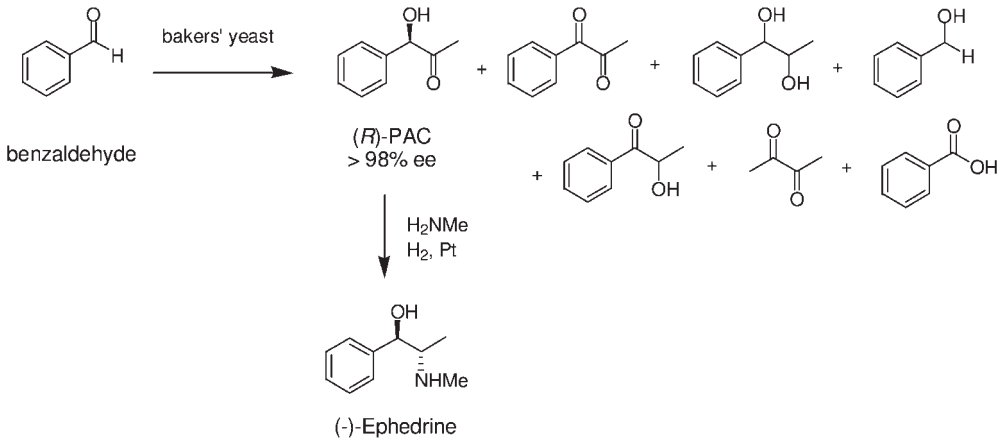
Finally, at different stages of a drug development program, the requirements for volume productivity, costs, development time, and availability of facilities, starting materials or catalysts are quite different. Moreover, intellectual property issues can outweigh any substantial scientific advantages that could have been contributed by a process. Accordingly, biocatalysis has to compete in the development of the most efficient processes that will be used in the production of chiral building blocks for the pharmaceutical industry.

During the last two decades tremendous progress has been made in the field of biocatalysis. This chapter is not intended as a comprehensive review, but rather a presentation of the scope, potential and limitations of the highly interdisciplinary approach of biocatalysis.

4.2
Examples of Established Applications of Biocatalysts in the Synthesis of Pharmaceuticals

4.2.1
Ephedrine Synthesis

The earliest example of the industrial application of a biotransformation for the synthesis of chiral pharmaceuticals was the production of (*R*)-phenylacetylcarbinol ((*R*)-PAC) by fermenting bakers yeast. This transformation is still in use to obtain this compound as a chiral intermediate for the synthesis of (-)-ephedrine (Scheme 4.1) [14].

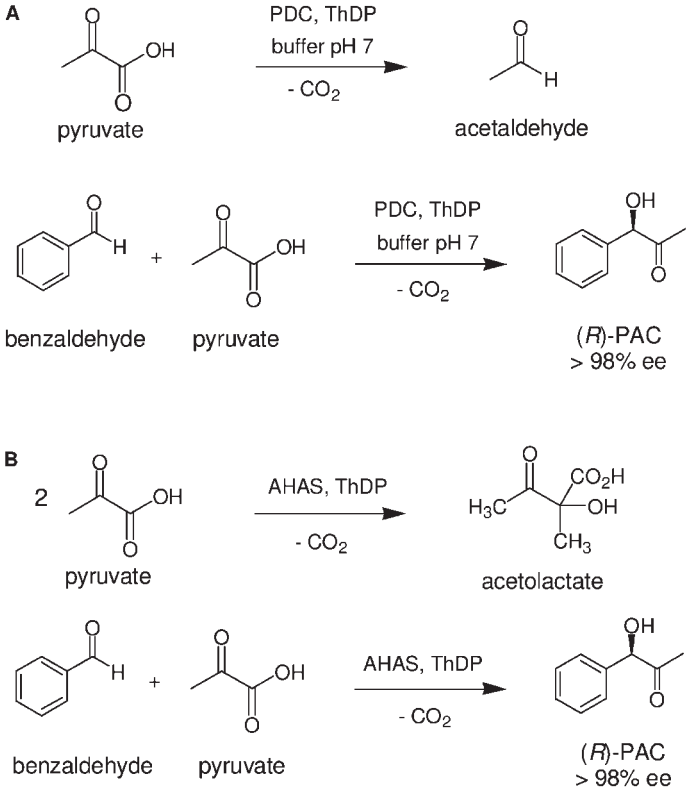


Scheme 4.1 Biocytalytic synthesis of (*R*)-PAC and (–)-ephedrine.

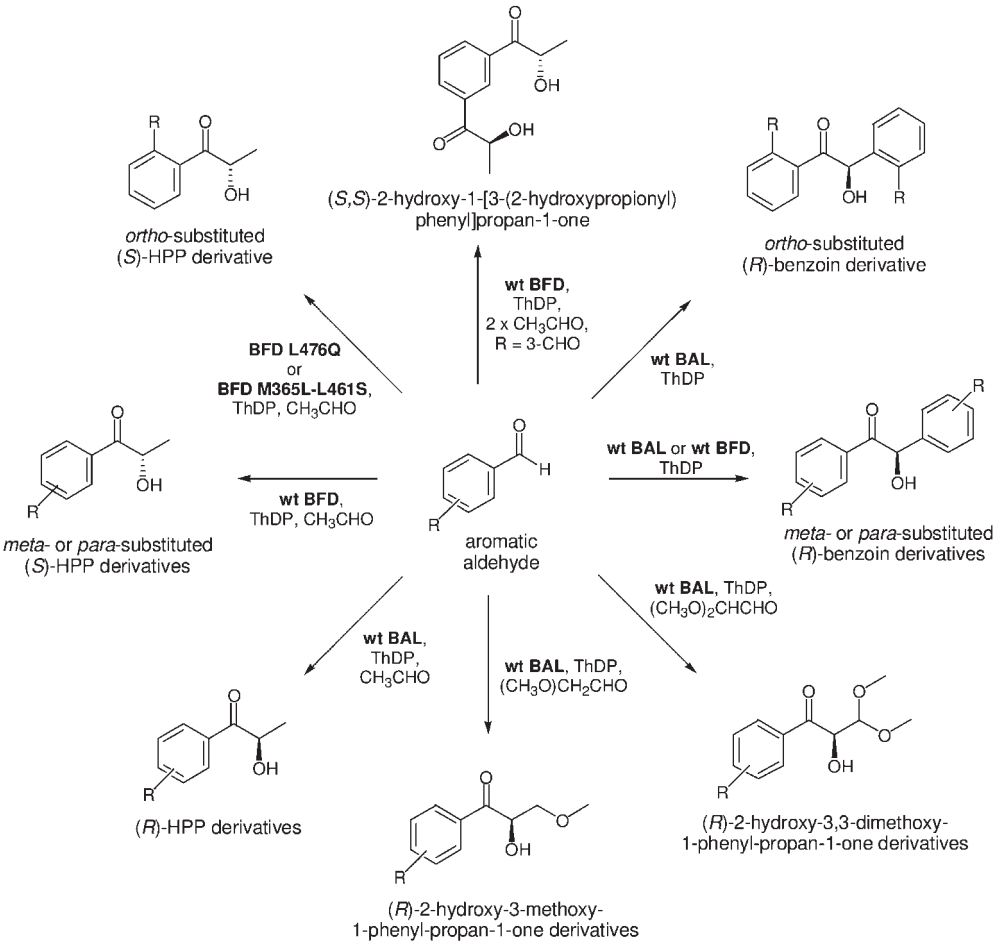
This whole-cell biotransformation is still a subject of research for several reasons: Besides the desired product (*R*)-PAC, several by-products are formed through enzymatic reduction of the product or the substrate benzaldehyde, resulting in the formation of 1-phenylpropan-2,3-diol and benzylalcohol, respectively. Further by-products are acetoin, butane-2,3-dione, 1-phenyl-propane-2,3-dione, benzoic acid and 2-hydroxypropiophenone, leading to a reduced yield of the desired product and difficult product isolation. To circumvent this problem strain improvement and reaction engineering have been used [15, 16]. Application of an isolated enzyme in a two-phase system resulted in improved space–time yield and high product purity [17].

As early as 1930 it was proposed that the production of 2-hydroxy ketones (here (*R*)-PAC) is a side reaction of pyruvate decarboxylase (PDC) [18, 19]. Later, it was shown unequivocally that thiamine diphosphate (ThDP)-dependent PDC catalyzes both the decarboxylation of pyruvate and the carboligation of the intermediate "activated aldehyde" to benzaldehyde (Scheme 4.2 A).

The problems encountered with the bakers' yeast whole cell process may be overcome by the application of isolated enzymes. Pohl's group applied site-directed mutagenesis strategies to tailor PDC for the synthesis of (*R*)-PAC. They



Scheme 4.2 Enzymatic synthesis of (*R*)-PAC.



Scheme 4.3 Chiral 2-hydroxyketones generated using ThDP-dependent enzymes BAL, BFD, and mutants thereof.

showed that the mutant PDC (*Zymomonas mobilis*) W392A has, by a factor of 3–4, higher carboligase activity with respect to the formation of (*R*)-PAC [15, 16].

Besides optimizing a known enzyme with respect to the desired application, screening for new enzymes catalyzing the wanted transformation might give access to altered reaction parameters. Barak, Chipman and coworkers identified acetohydroxyacid synthase (AHAS) from *E. coli* as an efficient catalyst for the formation of (*R*)-PAC starting from pyruvate and benzaldehyde (Scheme 4.2 B) [20].

This example demonstrates that "non-natural" transformation of known enzymes is an extremely helpful tool for organic chemists [21]. Recently, other ThDP-dependent enzymes have been used for the asymmetric synthesis of many different 2-hydroxy ketones (Scheme 4.3) [22, 23].

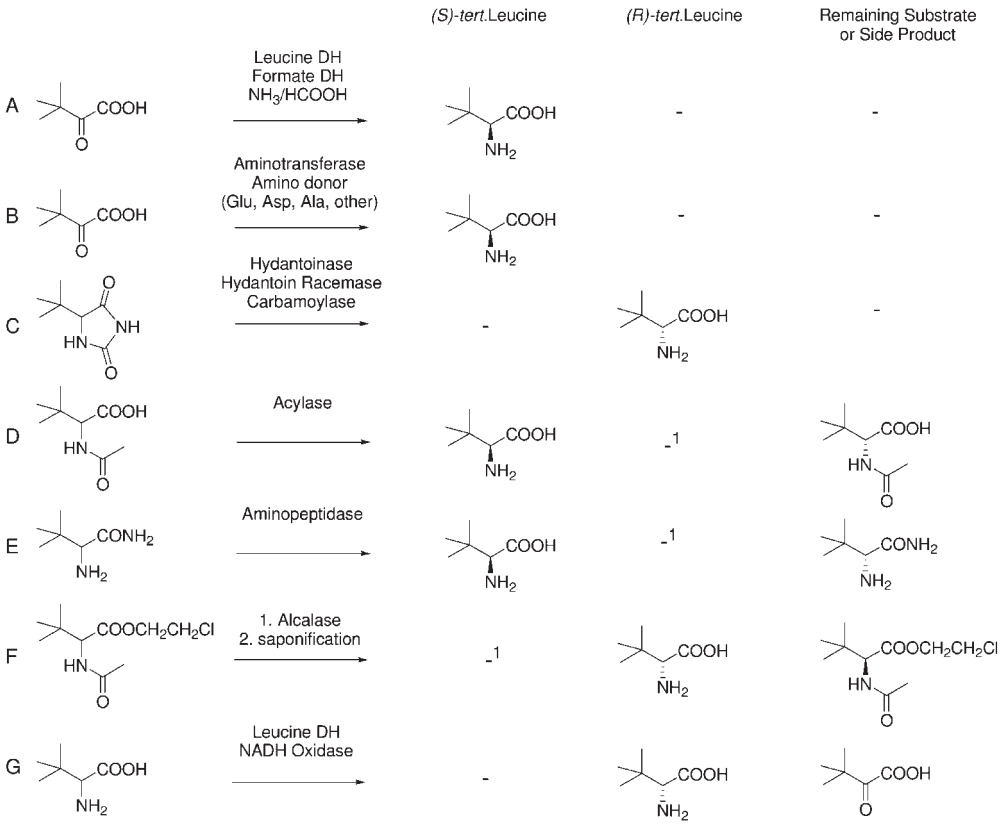
So far, the PDC-catalyzed formation of (*R*)-PAC (whole cell biotransformation) is the only process for the synthesis of chiral 2-hydroxyketones via carboligation used on an industrial scale. The transformations depicted in Scheme 4.3 might provide access to chiral intermediates used in the synthesis of biologically active compounds, like the antifungal Ro 09–3355 [24], the antidepressant bupropion [25], and the analgesic naproxen [26]. So far, such chiral building blocks have mostly been synthesized by racemic resolution using e.g. lipases.

Although the first industrial process using biocatalysts was established 70 years ago, based on enzymatic C–C bond formation, this type of catalysis has only recently been taken up for other transformations (cf. Section 4.2.7).

4.2.2 Amino Acids

Numerous biotranformation processes for the synthesis of amino acids have been described and for the purpose of this chapter, we have restricted the discussion to the unnatural amino acids that are not accessible by fermentation. For this class of amino acids, commercialized biotranformations are either based on asymmetric synthesis starting from a prochiral compound or on (dynamic) kinetic resolutions of a racemate. As an illustration the published processes for (*R*)- and (*S*)-*tert*-leucine are outlined in Scheme 4.4. Both stereoisomers of *tert*-leucine have been used for the synthesis of peptides that serve as protease inhibitors acting against viral infections (e.g. Hepatitis C, HIV), bacterial infections, autoimmune diseases and cancer [27]. This particular amino acid is versatile in these applications since the *tert*-butyl moiety provides resistance against endogenous proteases and can enhance the binding affinity of the peptide to the target protease.

Asymmetric synthesis of (*S*)-*tert*-leucine from the prochiral ketone trimethylpyruvic acid is carried out with leucine dehydrogenase, which carries out a reductive amination reaction. The cofactor NADH is regenerated in a coupled enzyme reaction with formate dehydrogenase (FDH, A in Scheme 4.4). An alternative asymmetric synthesis process utilizes amino-transferases to transfer the amino functionality from an amino donor, such as L-glutamic acid, L-glutamine or L-aspartic acid, to trimethylpyruvic acid (B). In contrast to asymmetric syntheses, resolution processes have a maximum yield of 50% unless the remaining substrate undergoes spontaneous or catalyzed racemization (dynamic kinetic resolution or DKR). A DKR process involving an (*R*)-selective hydantoinase, a hydantoin racemase and an (*R*)-selective carbamoylase has recently been developed (C). Most other resolution processes provide access to both the (*R*)- and the (*S*)-amino acid, either as the direct hydrolysis product or via the slowly reacting remaining substrate (Scheme 4.4D–F). Interestingly, the same leucine dehydrogenase that has been applied for asymmetric synthesis of (*S*)-*tert*-leucine has also been used for the production of the (*R*)-enantiomer in a resolution process starting with racemic *tert*-leucine. Reduced cofactor was destroyed by NADH oxidase (G) to drive the process [28].

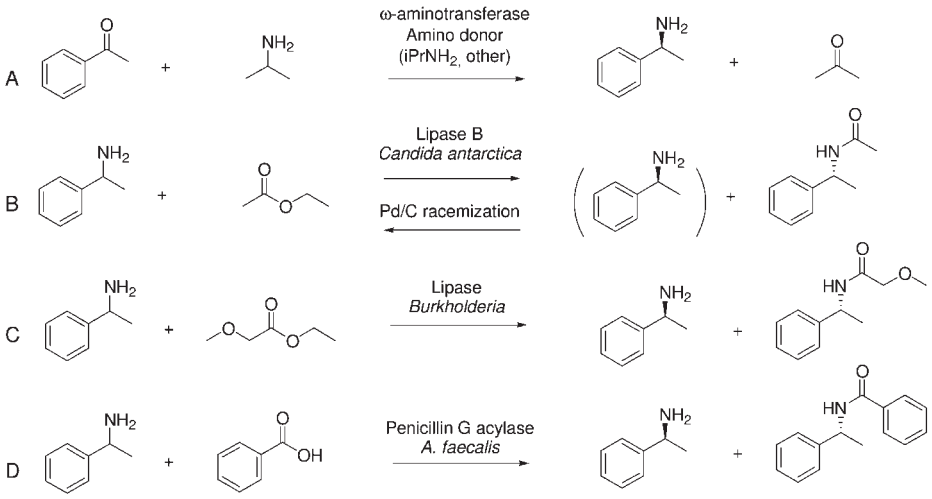


Scheme 4.4 Biotransformation routes to unnatural amino acids: (*S*)- and (*R*)-*tert*-leucine.
[1]Access to the other stereoisomer is possible via the remaining substrate.

4.2.3
Amines

The product class of enantiomerically pure amines is of considerable importance in both pharmaceutical and agrochemical applications. For instance, enantiopure aryl-alkyl amines are utilized for the synthesis of intermediates for pharmaceutically active compounds such as amphetamines and antihistamines. Several chemical as well as biotransformation methods for the asymmetric synthesis/dynamic kinetic resolution [29] or separation of enantiomers of chiral amines have been described. These are illustrated in Scheme 4.5 for (*S*)-α-methylbenzylamine [30].

Aminotransferases typically act on α–amino acids, but some enzymes from this class do not require the amino group vicinal to the carboxylic acid moiety and are called ω-aminotransferases. Celgene (Celgro) has developed enantiocomplementary ω-aminotranferases that accept simple amino donors such as isopropylamine



Scheme 4.5 Asymmetric synthesis (A), dynamic kinetic resolution (B) and resolution routes (C,D) to chiral amines.

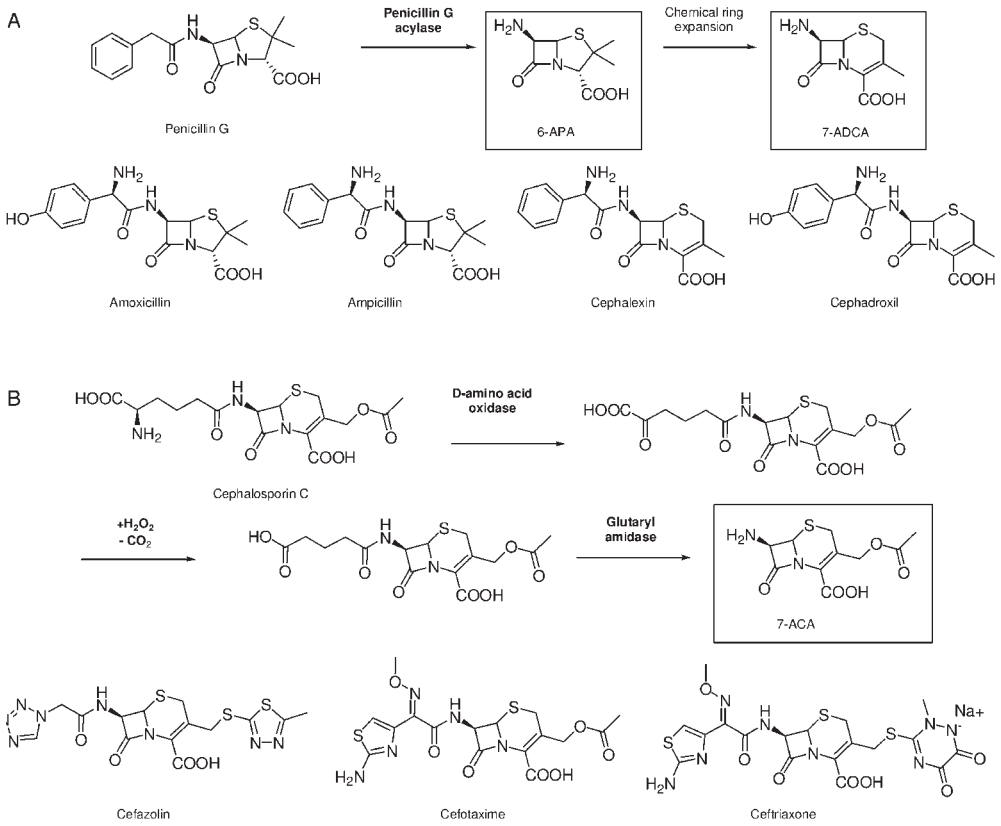
for asymmetric synthesis of either (*R*)- or (*S*)-amines from the corresponding prochiral ketones (Scheme 4.5A). An alternative process, which also has a theoretical yield of 100%, is based on a dynamic resolution of *rac.* α-methylbenzylamine by *Candida antarctica* Lipase B (CAL-B) in the presence of Pd/C. The latter catalyst racemizes the residual (*S*)-α-methylbenzylamine via an oxidation/reduction mechanism and yields 75–77% of the (*R*)-*N*-acetyl-α-methylbenzylamine with 99% e.e. (Scheme 4.5B) [29d].

Nondynamic resolution processes for the production of chiral amines are based on selective *N*-acylation by either lipases from *Burkholderia plantarii* (Scheme 4.5C) or *Alcaligenes faecalis* penicillin G acylases (Scheme 4.5D). The former reaction is optimal with ethylmethoxyacetate as acylating agent [30a], whereas the acylase is most selective with the natural substrate phenylacetic acid [30b].

4.2.4
Penicillins/Cephalosporins

As a result of the build-up of antibiotic resistance, the demand for derivatives from penicillins and cephalosporins rather than for the natural fermentation products has increased. One of the problems in the manufacture of semi-synthetic penicillins and cephalosporins is that these are vulnerable compounds and chemical modification is elaborate and difficult. Modification of the initial fermentation products penicillin G/V and cephalosporin C by the use of enzymes has provided economically feasible routes to semi-synthetic penicillins and cephalosporins [31].

Initially, enzymes were only used to liberate the 6-aminopenicillanic acid (6-APA) nucleus from the fermentation product penicillin G/V from *Penicillium chrysogenum* using Penicillin G acylase (Scheme 4.6A), and 7-aminocephalospora-

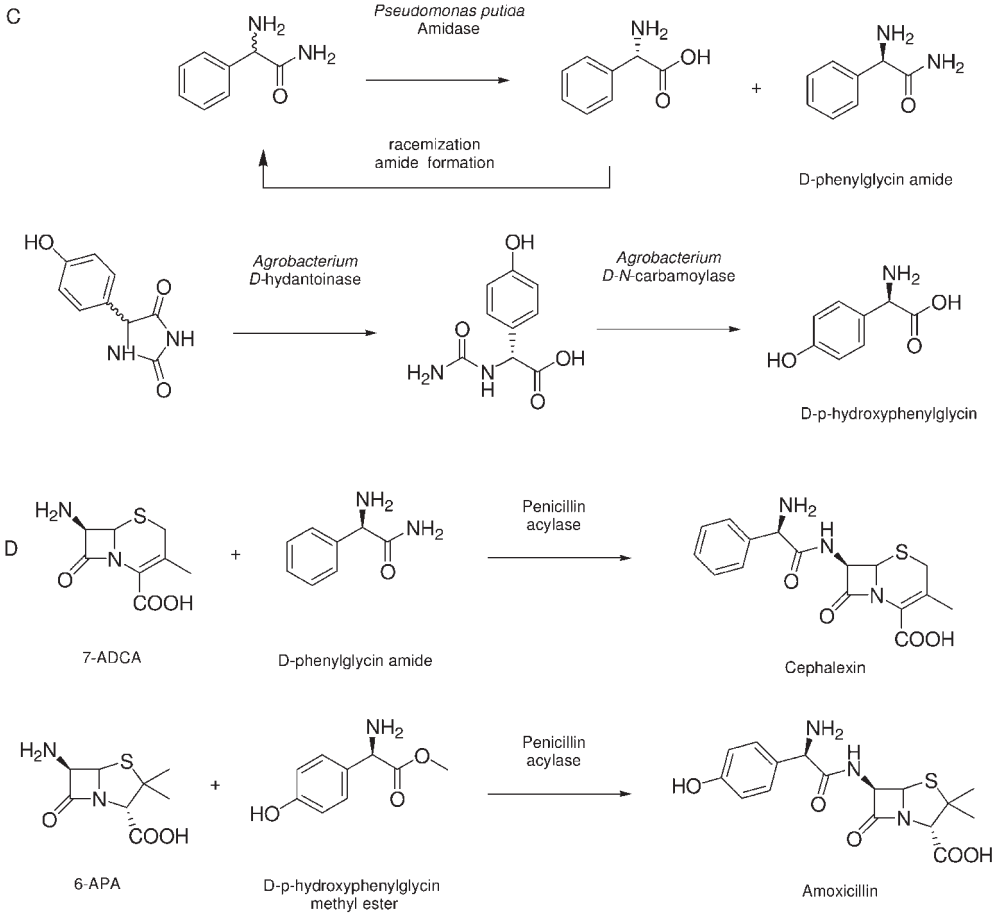


Scheme 4.6 Semi-synthetic antibiotics synthesized via penicillin derived 6-APA, 7-ADCA (A) or cephalosporin derived 7-ACA (B).

nic acid (7-ACA) from cephalosporin C (*Cephalosporium acremonium*) by subsequent use of two enzymes, D-amino acid oxidase and glutaryl amidase (Scheme 4.6B). Chemical ring expansion of 6-APA led to another important nucleus, 7-amino deacetoxy-cephalosporanic acid (7-ADCA) for the synthesis of semi-synthetic cephalosporins (Scheme 4.6A).

In subsequent process generations, penicillin G acylase derived enzymes were also used to couple the synthetic side chains, such as D-phenylglycine (ampicillin, cephalexin) and D-*p*-hydroxyphenylglycine (amoxicillin, cephadroxil) in the form of amino acid amides or esters to 6-APA and 7-ADCA (Scheme 4.6D). Biotransformation routes to the D-phenylglycine and D-*p*-hydroxyphenylglycine side chains were also developed (Scheme 4.6C), but the enzymatic process towards D-phenylglycine amide has been substituted by a classical resolution.

The replacement of the chemical synthesis route for cephalexin, which consisted of ten difficult chemical steps starting from penicillin G, by the enzymatic synthesis or "green" route (six steps) has led to a more sustainable water-based



Scheme 4.6 The side chains D-phenylglycin amide and D-*p*-hydroxyphenylglycin – accessible by bio-transformation (C) – are coupled by penicillin acylases, which alleviates the need for protective group chemistry (D).

process with better economics as well [32]. In addition to the enzymatic catalysis, metabolic engineering of *Penicillium chrysogenum* has been performed to obtain an organism that performs the ring expansion reaction in the cell (see section on metabolic engineering).

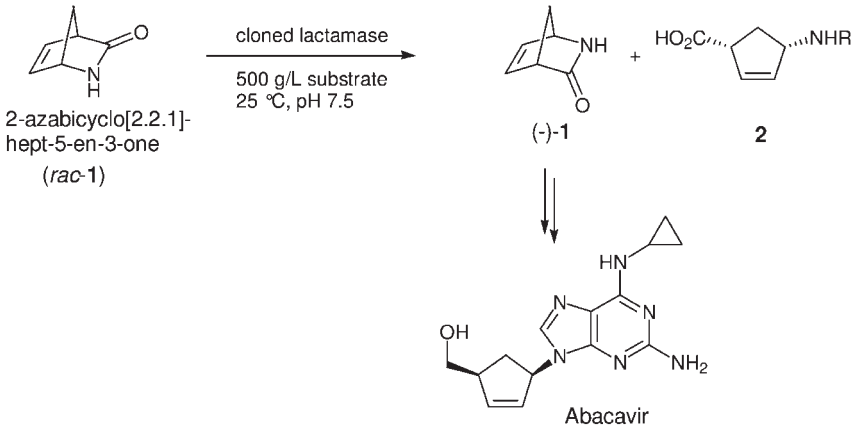
4.2.5
Racemic Resolution Using Hydrolytic Enzymes

Due to the reasons mentioned above (costs, substrate availability, development time, patent issues, etc.) many chiral products are produced first as a racemate. At a later stage, usually, the enantiomers (or stereoisomers) have to be separated in

order to obtain the most potent biologically active isomer. Enantiomeric separation can be achieved by many different methods, e.g. crystallization, chromatographic separation, derivatization with chiral auxiliaries, or kinetic resolution by enzymatic or nonenzymatic methods. The application of hydrolases, especially lipases, in the synthesis of chiral pharmaceuticals has some specific advantages: many hydrolases are commercially available on a large scale and some are already approved for use in humans (known from food processing applications). Hydrolases are often stable under forced conditions, they do not require a cofactor for activity and, hence, many of them can easily be immobilized and reused manifold or in a continuous way. Moreover, since lipases are active at an aqueous/organic interphase, organic solvents can be used so that high substrate and product concentrations can be achieved. However, the maximum theoretical yield of one enatiomer is usually restricted to 50%, usually (cf. Section 4.3.1).

More than 60% of all biotransformations using isolated enzymes are hydrolase-dependent processes. Several reviews have summarized the achievements in this field [33–37]. From the numerous examples published, two will be discussed in order to highlight the potential of enzyme-catalyzed kinetic resolution for the production of chiral pharmaceuticals.

Abacavir, a 2-aminopurine nucleoside analog, is a selective reverse transcriptase inhibitor for the treatment of HIV and hepatitis B virus. The γ-lactam (–)-2-azabicyclo[2.2.1]hept-5-en-3-one [(–)-**1**] is a potential intermediate in the synthesis of Abacavir. A biocatalytic process has been developed for the resolution of *rac*-**1** to yield the desired (–)-**1** and the amino acid **2** using γ-lactamase, either as a whole cell process or applying isolated enzyme (Scheme 4.7). The biocatalytic process was improved to support the demands of multi tonne product supply. A combination of classical molecular biology techniques together with screening methods led to the isolation and expression of a γ-lactamase gene from *Comomonas acidovorans*. The isolated enzyme was characterized with regard to its process performance and proved to be active at 500 g L^{-1} substrate and, moreover, retained half

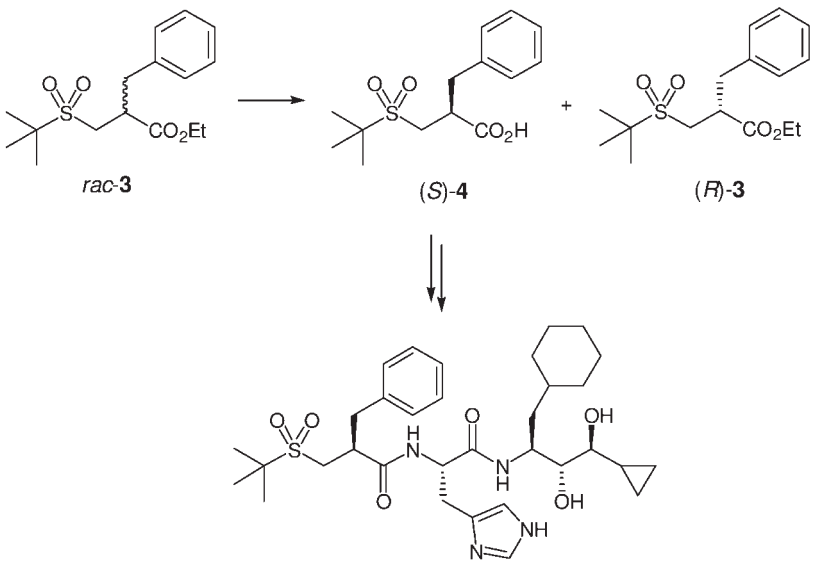


Scheme 4.7 Lactamase-catalyzed racemic resolution at high substrate concentration.

of its activity after incubation at 60 °C for 4 h. A 500 L, 4 day fermentation of the recombinant *E. coli* expressing the lactamase gene yielded approximately 100 g L^{-1} of cell biomass (wet weight), sufficient to resolve about 5 t of *rac*-**1**. Crude cell free extract, after lysis, polyethyleneimine precipitation and centrifugation, was used as a biocatalyst. The biotransformation was optimized with respect to substrate concentration (500 g L^{-1}), enzyme loading, pH (7.5), buffer and temperature (25 °C) resulting in a high volume efficiency. Product isolation was greatly simplified since concentrated enzyme and substrate solution were used. The final step was the recrystallization of the product from the solvent concentrate [38, 39].

The large scale preparation of (*S*)-2 [(*tert*-butylsulfonyl)-methyl]hydrocinnamic acid [(*S*)-**4**], a chiral building block in the synthesis of the potential rennin inhibitor Remikiren (not in use since it failed in the clinical phase), was performed via Subtilisin Carlsberg-catalyzed racemic resolution of the ester *rac*-**3**. The substrate was emulsified at 39–43 °C in 29% concentration in aqueous buffer and the pH was controlled using a 2–4 M NaOH solution. Work-up proved to be the rate-limiting step, nevertheless, the desired product could be isolated in 41.6% yield on a 100 kg scale. The product was obtained with excellent chemical (>99.5%) and enantiomeric (>99%) purity (Scheme 4.8).

Subtilisin Carlsberg, a laundry enzyme, is extraordinarily cheap and stable even at extreme concentrations of substrate and salt at elevated temperature. A major disadvantage of this chemoenzymatic route was the fact that no racemization procedure for the unwanted (*R*)-enantiomer [(*R*)-**3**] could be established [40].

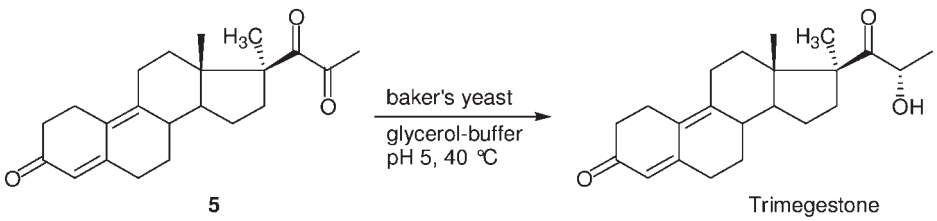


Scheme 4.8 Enantioselective hydrolysis of ethyl sulfopropionate *rac*-**3**.

4.2.6
Oxidoreductases

Asymmetric biocatalytic reduction of prochiral ketones gives access to one enantiomer of the respective secondary alcohol in the theoretical 100% yield, clearly demonstrating that this approach is advantageous in comparison to racemic resolution of chiral secondary alcohols. However, application of isolated oxidoreductases such as alcohol dehydrogenases and carbonyl reductases on an industrial scale is hampered by the fact that many of these enzymes are NAD(P)H-dependent. Usually, the costs for the cofactor are much higher than the costs for enzymes or substrates. Thus, the cofactor has to be applied in catalytic amounts, resulting in the requirement of efficient cofactor regeneration [41]. This problem can be circumvented by application of a whole cell biotransformation, in which the cofactor is regenerated using intracellular reduction or oxidation equivalents, respectively [42].

Bakers's yeast is the most widely employed biocatalyst for asymmetric ketone reductions [43]. A group at Roussel Uclaf described an industrial application of bakers' yeast mediated ketone reduction for the synthesis of trimegestone [44], a progestomimetic compound for the treatment of postmenopausal diseases. The key step of the nine-step synthesis is a chemo-, regio- and stereoselective bakers' yeast reduction of the triketone **5** (Scheme 4.9). This transformation could not be performed efficiently using nonenzymatic methods.

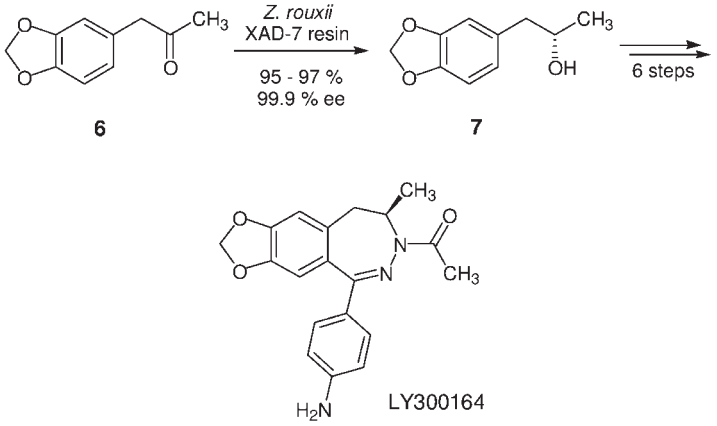


5 Trimegestone

Scheme 4.9 Bakers' yeast catalyzed regio- and stereoselective reduction.

The main disadvantage of this whole cell biotransformation is the high dilution: more than 500 L aqueous medium was used for 1 kg of triketone substrate. Such problems can be circumvented by the application of two-phase systems, where the substrate is solubilized in high concentration in the organic phase. Furthermore, the substrate and product concentration in the aqueous phase is lowered, circumventing toxicity problems.

In an effort to reduce benzylmethylketone **6** Anderson et al. identified that the yeast *Zygosaccharomyces rouxii* could catalyze this transformation efficiently at low substrate concentration (Scheme 4.10). Since the substrate and product were both toxic to *Z. rouxii*, polymeric hydrophobic resins were used to both supply substrate and remove the product from the reaction mixture as it formed. This approach allowed the reaction concentration to be increased from 2 to 40 g L^{-1}. The

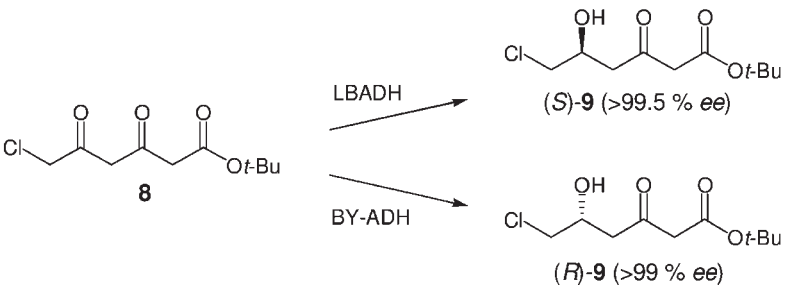


Scheme 4.10 Whole-cell bioreduction using a two phase system.

overall space–time yield was 75 g L^{-1} d^{-1}. Simple reactor design allowed the reaction, product isolation, and resin recycle to be accomplished within one reactor [45, 46]. Advantageously, *Z. rouxii* is used commercially in the production of soy sauce and is classified as a GRAS (generally regarded as safe) organism.

Reduced selectivity in bakers' yeast mediated transformations is most likely due to the presence of several alcohol dehydrogenases possessing opposite enantioselectivity. Stewart et al. have successfully cloned 49 different oxidoreductases from *Saccharomyces cerevisiae* [47]. They could show that the identification of specific alcohol dehydrogenases will result in access to chiral alcohols in high optical purity. Moreover, both enantiomers are accessible sometimes using enzymes from one parental strain [48].

In a collaboration with the group of Müller, Stewart and coworkers identified ADH YGL157w from bakers yeast to reduce selectively the diketone **8** resulting in the formation of enantiopure (*R*)-hydroxyketone (*R*)-**9** (Scheme 4.11) [49]. The enantiomer (*S*)-**9** is accessible via *Lactobacillus brevis* alcoholdehydrogenase (LBADH)-catalyzed reduction of diketone **8** [50]. This example clearly demonstrates the potential and practical use of genetic information resulting from genome sequencing projects.



Scheme 4.11 Enzymatic regio- and enantioselective reduction.

4.2.7 Oxynitrilases

Oxynitrilases or hydroxynitrile lyases (HNL) constitute a group of enzymes that catalyze the reversible addition of HCN to ketones and aldehydes. The natural role of these enzymes is a defence mechanism of higher plants against herbivores, whereby HCN is liberated from cyanoglucosides such as prunasin (almond, cherry, apple) by the action of a glycosidase and a hydroxynitrile lyase.

Hydroxynitrile lyases that are (*R*)-selective from the almond tree (*Prunus amygdalus*), flachs (common flax) as well as (*S*)-selective enzymes from cassava (*Manihot esculenta*), sorghum (*Sorghum bicolor*) and the rubber tree (*Hevea brasiliensis*) have been mostly applied in organic synthesis of chiral intermediates (Scheme 4.12A). For synthetic applications on an industrial scale, however, the availability of these enzymes and their inhomogeniety has long been a bottleneck since cloning and overexpression of the corresponding genes in common hosts such as *E. coli* remains cumbersome. Nevertheless, efficient overproduction of the above enzymes in *E. coli* or the yeasts *Saccharomyces cerevisiae* or *Pichia pastoris* has been successful recently and the first large scale applications of several 100 tpa have been realized [51].

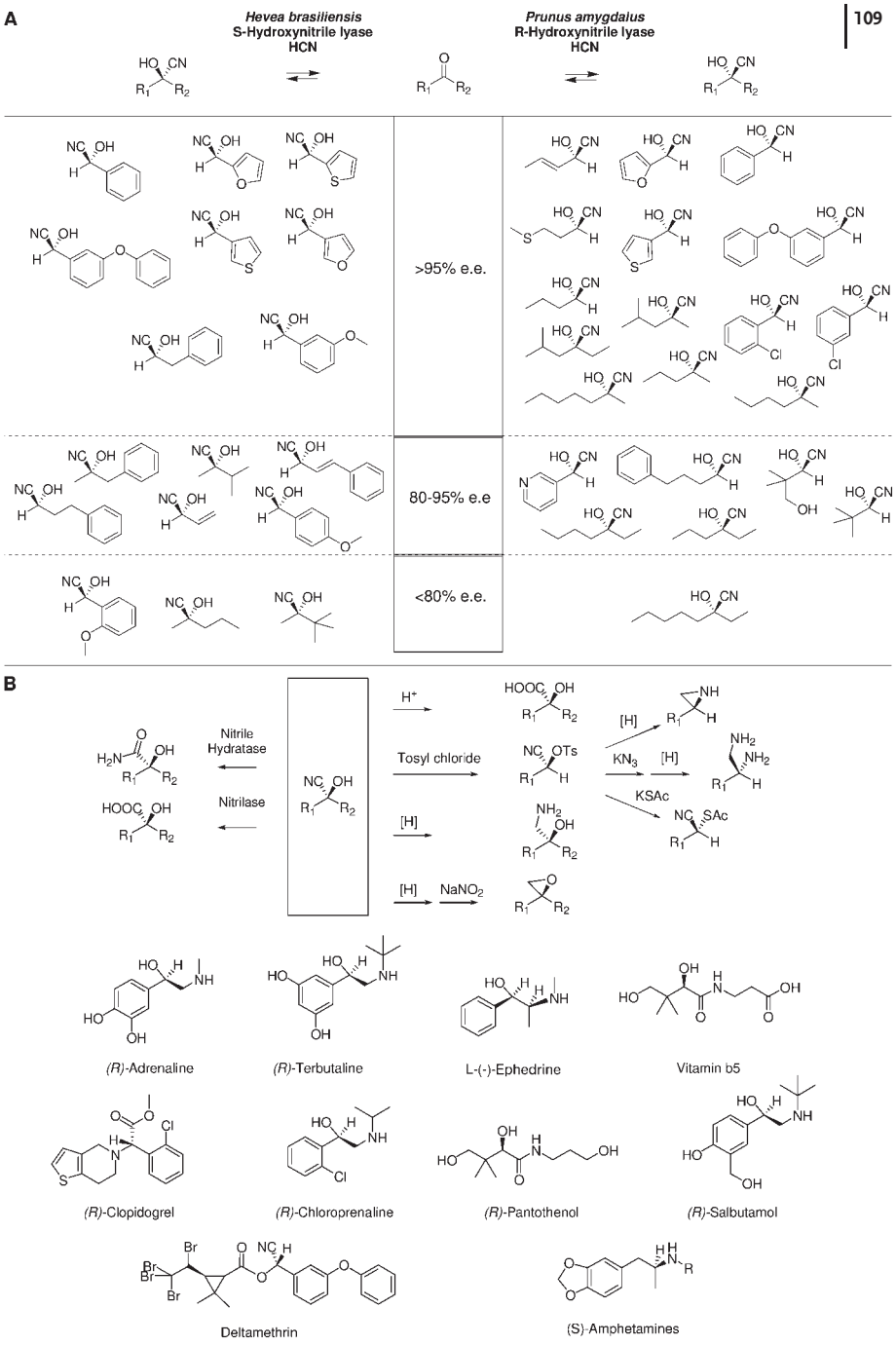
Due to the nitrile and hydroxyl functionality, enantiomerically pure cyanohydrins are useful building blocks for synthesis of chiral drugs and having enzymes available that make either the (*R*)- or (*S*)-enantiomer is very versatile. Scheme 4.12A illustrates the selectivity and substrate scope of *Prunus amygdalus* R-HNL and *Hevea brasiliensis* S-HNL. For most aldehyde and ketone substrates tested, high e.e.s can be reached [51 a, 52].

Enantiomerically pure cyanohydrins can easily be modified chemically or enzymatically (Scheme 4.12B), providing access to chiral α-hydroxy acids, α-hydroxy amides, 2-aminoalcohols, and epoxides. Replacement of the hydroxyl functionality by a better leaving group, such as a sulfonyl moiety (e. g. tosylate), allows the introduction of various other nucleophiles with inversion at the stereocenter [51 a]. The structures of some bioactive molecules that have been synthesized using a biotransformation step with a hydroxynitrile lyase are depicted in Scheme 4.12B [51 a, 52].

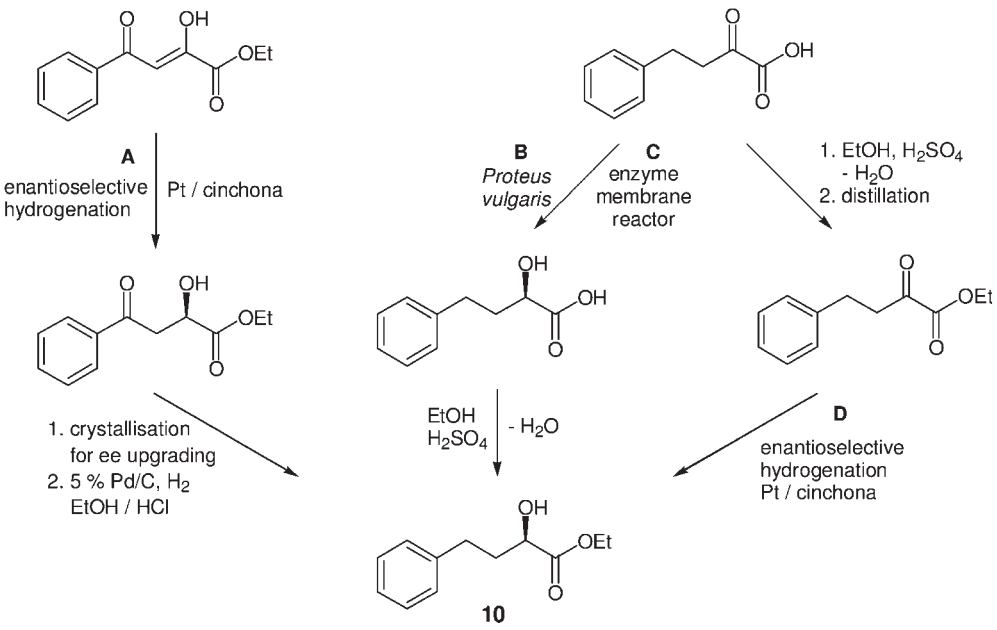
4.2.8 Comparison of Nonenzymatic and Biocatalytic Transformations

The asymmetric syntheses of various chiral compounds have already been compared with regard to their applicability on a technical scale. For example, Blaser and coworkers compared four technically feasible routes (A–D) for the large scale preparation of the chiral building block ethyl (*R*)-2-hydroxy-4-phenylbutyrate (**10**) (Scheme 4.13) [53]. The stereogenic centre is introduced via enantioselective reduction, either via biocatalytic or nonenzymatic methods.

The yields and ee values were significantly higher in the biocatalytic processes, whereas the nonenzymatic reductions needed substantially lower amounts of 'substrates' (cofactor, buffer, cosubstrate) because only hydrogen was used as a



Scheme 4.12 Product scope of HNLs from *Hevea* and *Prunus* (A) and subsequent chemical steps that have been used to synthesize some important bioactive molecules (B).

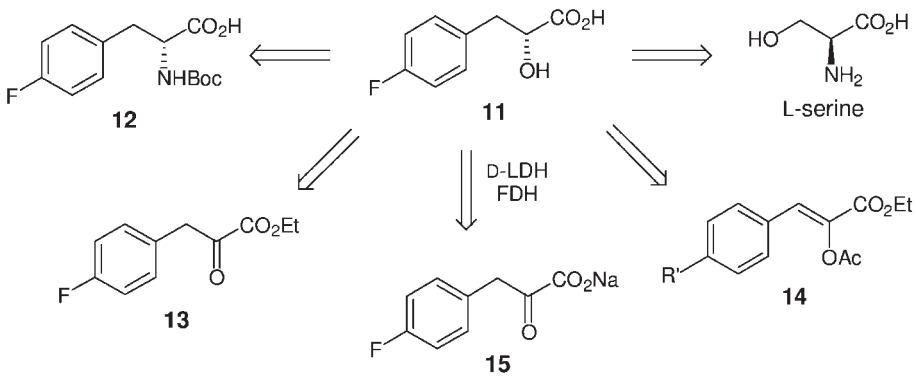


Scheme 4.13 Different nonenzymatic and biocatalytic asymmetric approaches for the synthesis of hydroxy ester **10**.

stoichiometric reducing agent. All enantioselective reduction methods were rather demanding, either because a sensitive catalyst (chemocatalyst!) or a complex biological catalytic system had to be used. Space–time yield was considerably higher and work-up easy for the nonenzymatic process. In the case of the metal-catalyzed hydrogenation, safety concerns are most significant because of the use of flammable hydrogen. In summary, the biocatalytic routes are attractive alternatives to the nonenzymatic processes, provided that space–time yields and the solvent/aqueous buffer consumption can be improved.

The synthesis of (*R*)-2-hydroxy-4-fluorphenyl-propionic acid (**11**), a key building block in the synthesis of the cold drug Ruprintivir, illustrates the parallel approaches usually examined in the search for an ideal synthetic process. The initial approach used a commercially available enantiopure D-phenylalanine derivative **12**, even though the starting material was expensive, prohibiting its use at large scale. L-Serine was also examined as a starting material, however, the synthesis proved to be lengthy, resulting in a poor overall yield. Asymmetric chemocatalysis using **13** or **14** as starting material failed due to poor enantioselectivities or low yields. An enzymatic reduction approach was finally adopted in which D-lactate dehydrogenase (D-LDH) was used to reduce the keto acid **15**. The cofactor was regenerated by formate dehydrogenase (FDH) (Scheme 4.14) [54, 55].

The continuous enzymatic process generates the desired enantiopure product with a high space–time yield (560 g L^{-1} d^{-1}) using a membrane reactor. The synthesis not only reduced the costs significantly at large scale but it can also be used to



Scheme 4.14 Various retrosynthetic strategies for the synthesis of hydroxyacid (**11**).

convert a variety of ketoacid precursors, in contrast to the chemical catalysts mentioned earlier [55].

Several other studies dedicated to the comparison of biocatalytic and nonenzymatic transformations have been published [56–58]. The major aspect can be summarized as follows: if high substrate and product concentration can be reached using low-priced substrates, this will result in an advantage for biocatalytic methods relative to nonenzymatic processes, at least if highly enantioenriched products are needed.

4.3
Some Special Aspects of Biocatalysts, Recent Developments

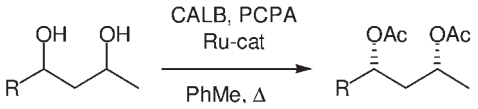
The examples given in Section 4.2 demonstrate that state-of-the-art biocatalytic transformations have reached an extraordinary level making them valuable and competitive methods for use in the pharmaceutical industry. Progress made in the fields of chemoenzymatic synthesis, molecular biology and reaction engineering will improve and enlarge the possible applications of biocatalysts even further.

4.3.1
Dynamic and Parallel Kinetic Resolution

Kinetic racemic resolution, as described in Section 4.2.5, suffers from the fact that the ratio of the substrate enantiomers to be resolved changes during the progress of the transformation. In order to circumvent low enantiomeric excess and low yields of the desired product, strategies like dynamic kinetic resolution (DKR) [59] and parallel kinetic resolution [60] have been introduced. In the first case, racemization, for example, of the substrate is much faster than the undesired transformation of the slower reacting enantiomer, thus keeping an equal ratio of the substrate enantiomers during the whole process. Parallel kinetic resolution makes

use of two different, yet preferably similarly fast reactions, each specific for one of the substrate enantiomers, resulting in the formation of two different products, each containing one enantiomer of the starting material. Still, in this case the maximum yield of the desired enantiomer is restricted to 50%, whereas DKR enables 100% of the theoretical chemical yield of one enantiopure product starting from a racemic mixture.

Both reactions applied can be nonenzymatic or biocatalytic or a combination of both. Bäckvall et al. developed a one-pot synthesis of enantiomerically pure *syn*-1,3-diacetates starting from racemic diastereomeric mixtures of unsymmetrical 1,3-diols by combining an enzymatic transesterification, a ruthenium-catalyzed epimerization and an intermolecular acyl migration reaction in a one pot transformation (Scheme 4.15) [61].



Scheme 4.15 Dynamic kinetic asymmetric transformation with *p*-chlorophenyl acetate (PCPA) as acyl donor (CALB: *Candida antarctica* lipase B).

4.3.2
Different Biocatalytic Approaches to One Building Block

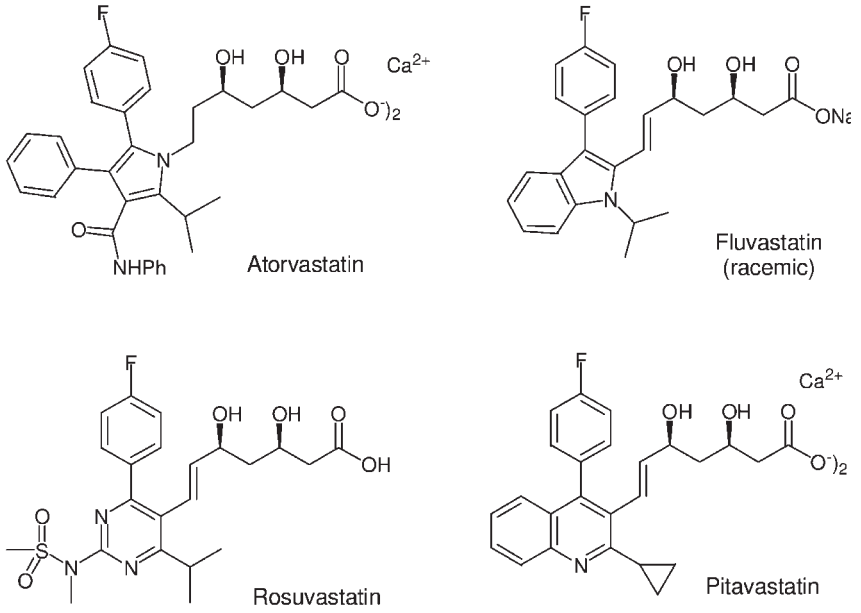
For the synthesis of a specific target molecule various enzymatic transformations are applicable.

Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which catalyzes the reductive conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. This inhibition leads to reduced levels of low-density-lipoprotein (LDL) cholesterol. Synthetic HMG-CoA-reductase inhibitors are depicted in Scheme 4.16, all of which are homochiral with regard to the chiral 3,5-dihydroxyhexanoate sidechain.

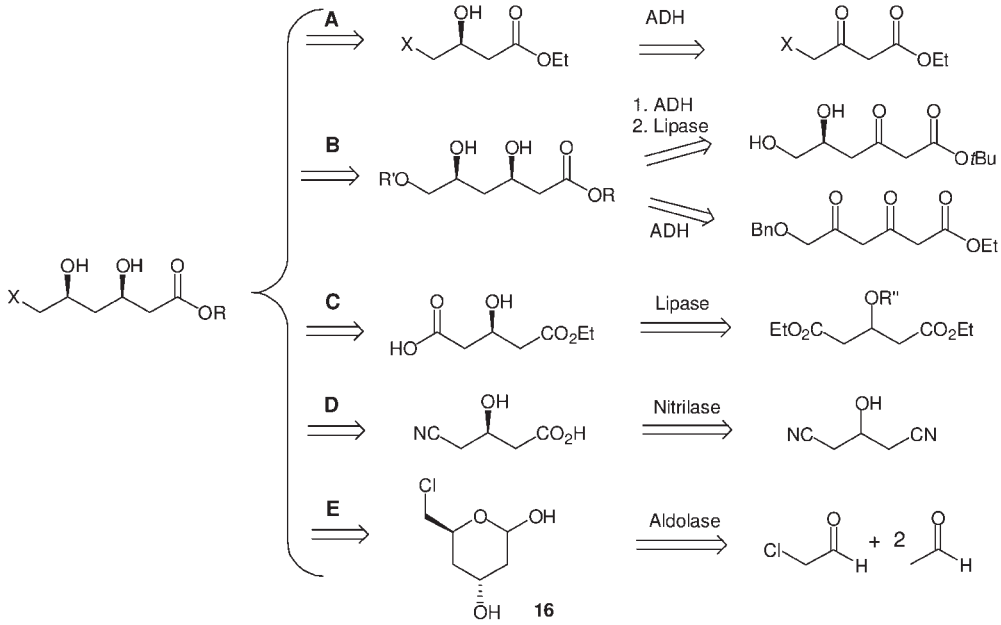
Due to the exceedingly high market value and the requirement for high chemical and stereochemical purity (> 99.5% ee, > 99% de) immense and competitive efforts have been invested in the production of synthetic statins. Pharmaceutical, chemical and biotech companies have recently developed various chemoenzymatic strategies employing different biocatalysts en route to building blocks used for the stereoselective synthesis of the dihydroxyhexanoate side chain (Scheme 4.17) [62].

These examples illustrate that biocatalytic strategies can initiate innovative solutions for problems like cross aldol chemistry, regio- and stereoselective reduction of 1,3-diketones, stereoselective formation of 1,3-diols, or problematic desymmetrization of prochiral compounds. Additionally, several remarkable achievements have been made by optimizing the biocatalytic step for application on the industrial scale.

Product concentrations of 63 g L^{-1} in the organic phase were obtained with an isolated yield of 95% for the ADH-catalyzed transformation (route A) and much higher product concentrations might also be achievable [63].



Scheme 4.16 Synthetic HMG-CoA reductase inhibitors.



Scheme 4.17 Different enzymatic approaches to one stereoisomer of 3,5-dihydroxyhexanoate for application in the synthesis of HMG-CoA reductase inhibitors.

Patel et al. [64] used cell suspensions of *Acinetobacter calcoaceticus* for the stereoselective direduction of ethyl 6-benzyloxy-3,5-dioxohexanoate (route B). The corresponding diol was isolated in 85% yield with an ee of 97%.

The known desymmetrization of prochiral 3-substituted glutarates via enzymatic hydrolysis [65] has been optimized by chemists at Ciba Speciality Chemicals for the synthesis on a large scale [66]. The α-chymotrypsin-catalyzed process is characterized by a high substrate concentration of 285 g L^{-1}, and an isolated yield of 94% product with an ee of 98.2% (route C).

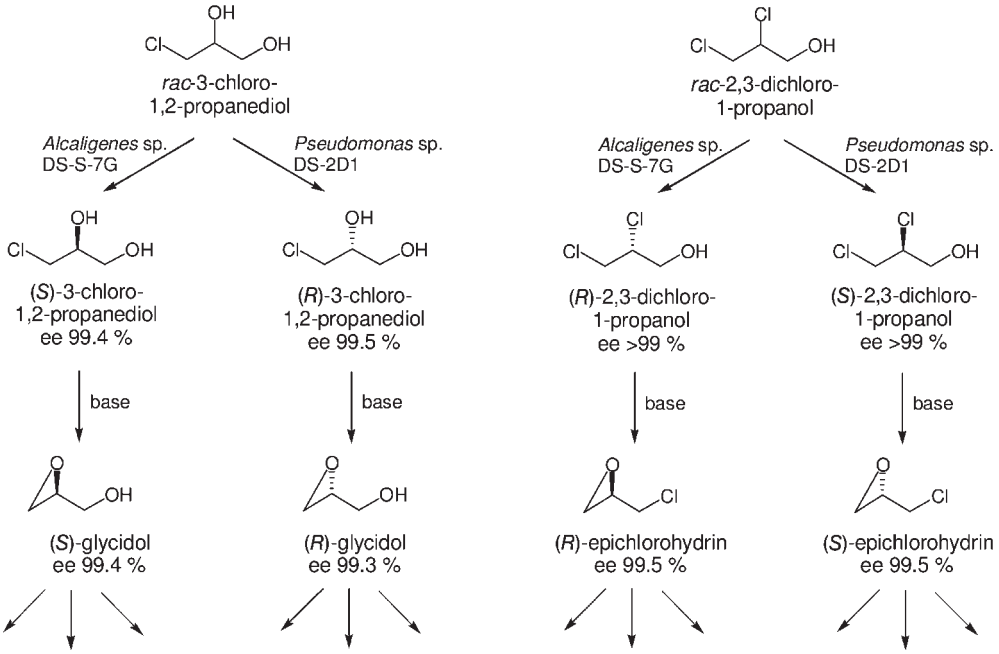
The creation of large genomic libraries by extracting DNA directly from environmental samples has been used by Burk et al. (Diversa) for the identification of more than 200 new nitrilases. Four of the identified enzymes were successfully employed in the desymmetrization of the readily available 3-hydroxyglutaronitrile (route D). Subsequently, a highly enantioselective nitrilase was created through gene site saturation mutagenesis. The efficient enzymatic transformation thus optimized, which was performed on a g-scale with a substrate concentration of 3 M, exhibited high volumetric productivity. After 15 h reaction time the product (ee 98.5%) was isolated in 96% yield, corresponding to a product space–time yield of 619 g L^{-1} d^{-1} [67].

Another highly attractive approach has been developed by scientists at DSM [68] based on a transformation introduced by Wong et al. [69]. The 2-deoxyribose-5-phosphate aldolase (DERA)-catalyzed formation of the pyrane **16** (route E) proceeds through asymmetric C–C bond formation starting from the cheap bulk chemicals acetaldehyde and chloroacetaldehyde. Although the reaction characteristics published in the initial article were not very promising (low substrate concentration, high catalyst loading, 7 d reaction time), this biotransformation is now in operation on an industrial scale. The optimized enzymatic transformation, which is performed at low temperature (2–4 °C), is characterized by a high final product concentration of more than 100 g L^{-1}.

4.3.3 Multipurpose Small Chiral Building Blocks

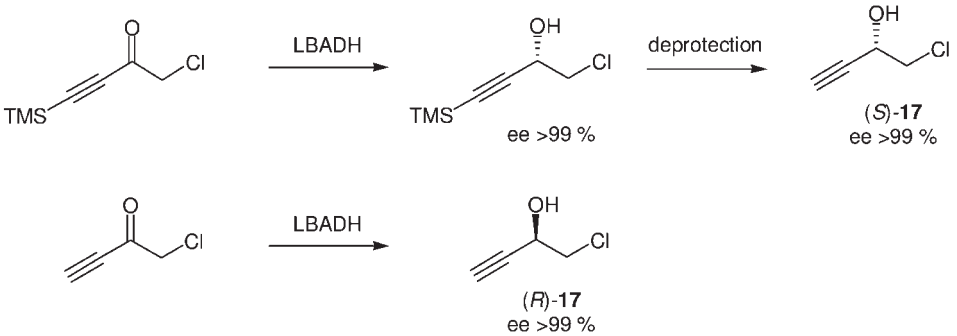
Small chiral molecules like epichlorohydrin, 3-chloro-1,2-propanediol, glycidol, or 4-chloro-3-hydroxybutyrate do have abundant possible applications in the synthesis of pharmaceuticals. Despite this fact, these compounds have not been available in enantiopure form on a large scale although the corresponding racemates are low-priced bulk chemicals. A group at Daiso established whole-cell biocatalytic processes for the racemic resolution of highly functionalized small building blocks (Scheme 4.18) [70].

The microorganisms applied (*Pseudomonas* sp. and *Alcaligenes* sp.) possess dehalogenation activity and are used in a fed-batch fermentation protocol, which has been scaled up to 100–5000 L. The enantiopure products are isolated or purified by extraction, adsorption or distillation at low temperature (because of possible racemization). Although half of the raw material is lost, the processes are economically viable because of the availability of the cheap starting material.



Scheme 4.18 Biocatalytic racemic resolution of small chiral building blocks.

The major aim of producing such highly functionalized molecules is their possible application in the synthesis of many different products. Accordingly, access to both enantiomers of such small chiral compounds is highly desirable for their application as multipurpose building blocks. An interesting new approach to both enantiomers of such a C4 compound has been developed using one enzyme: the LBADH-catalyzed reduction of chloro-butinones both the (*S*)- and the (*R*)-enantiomer of the corresponding alcohol **17** can be obtained selectively, depending on the presence or absence of a trimethylsilyl (TMS)-protecting group (Scheme 4.19) [71].



Scheme 4.19 Enantiodivergent enzymatic reduction of butinones using LBADH.

Similar chiral alkinols have been used manifold in the synthesis of pharmaceuticals and biologically active compounds. Further development in the field of substrate engineering and chemoenzymatic synthesis might enable additional access to general chiral building blocks like compound **17**.

4.3.4 Optimization/New Access Using Molecular Biology Methods

During the past decade, many enzymes have been improved by directed evolution using random mutagenesis, rational protein design, or via a combination of both methods [12, 13]. Early examples were committed to the enhancement of enzyme stability under unusual conditions such as in the presence of organic solvents or towards increased activity. More recently, directed evolution has been used for the creation of enantioselective biocatalysts or, even more, for the reversal of enantioselectivity of an enzyme-catalyzed reaction [72, 73].

New methods like structure-guided recombination, gene or genome shuffling, gene site saturation mutagenesis, or metagenome screening, to name just a few, are developed at rapid pace enabling, together with bioinformatics, the creation of highly specific and efficient biocatalysts [74].

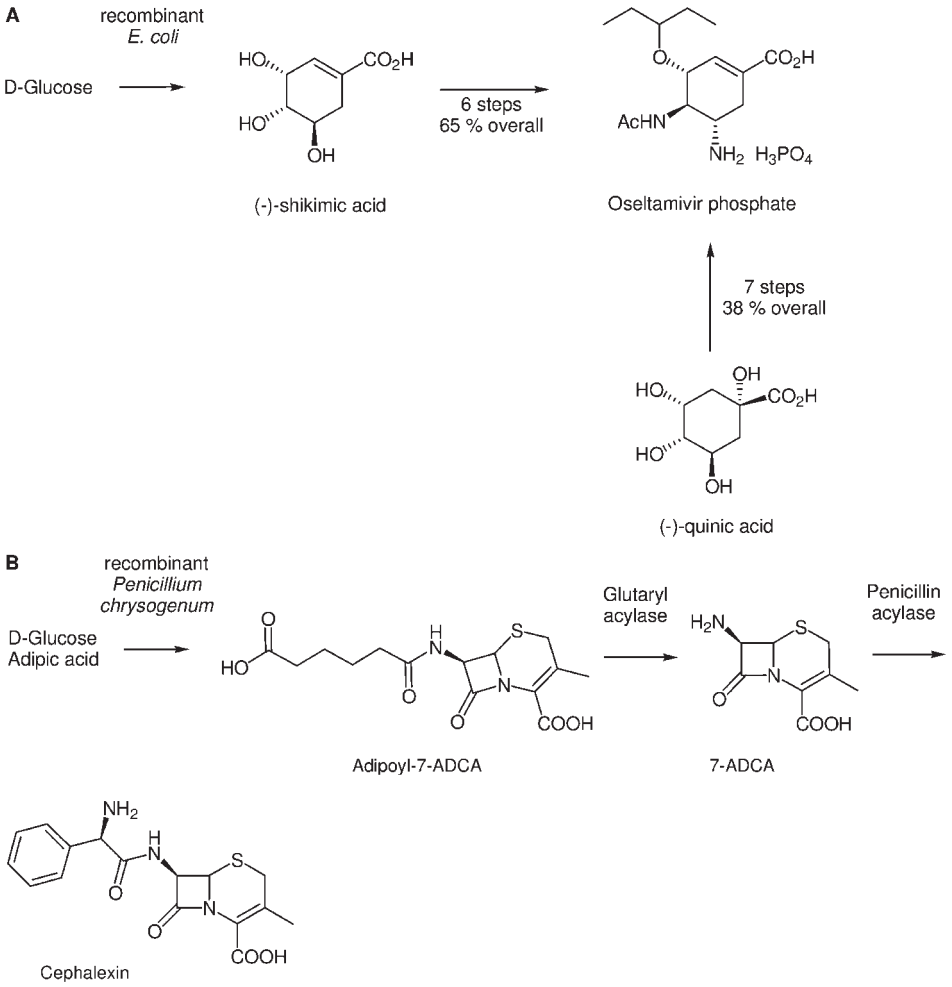
For example, the creation of large genomic libraries by extracting DNA directly from environmental samples (metagenome screening) has been used by Burk et al. for the identification of new nitrilases (Section 4.3.2). Subsequently, a highly enantioselective nitrilase was created through gene site saturation mutagenesis [67].

Most recently, this group applied the metagenome screening strategy for the identification of improved DERA enzymes [75]. More than 15 DERA enzymes were discovered, of which one was used to optimize the process mentioned (Scheme 4.17E) on a 100 g scale. Using a fed-batch strategy, which is necessary because of substrate inhibition, Burk et al. obtained a final product concentration (**16**) of 93 g L^{-1} (>99.5% ee, de 96.6%). Concomitantly, the amount of enzyme was reduced to 2% (wt/wt).

4.3.5 Metabolic Engineering

Metabolic engineering enables the biochemical engineer to steer the metabolism in a living cell in a desired direction. This can be accomplished by silencing certain genes, by overexpression of others but also by introducing genetic information that was not endogeneous to the production organism in the first place. As a result, it is now not only possible to enhance the productivity in an established process – the traditional goal of metabolic engineering – but new products, that are not common to nature, can also become accessible by fermentation.

An example of process optimization by metabolic engineering is the production of intermediates for the synthesis of oseltamivir. This neuraminidase inhibitory drug (Tamiflu®) acts against the common as well as the threatening asian flu. Quinic and shikimic acid can serve as precursors (Scheme 4.20A), but the shiki-



Scheme 4.20 Metabolic engineering route towards the anti-flu drug Oseltamivir (A) and the semi-synthetic cephalosporin Cephalexin (B). Adipoyl-7-ADCAand shikimic acid are produced in recombinant strains of *Penicillium chrysogenum* and *E. coli* respectively.

mic acid acid route is more efficient. Until a few years ago, shikimic acid was in limited supply, since it was harvested from a natural source grown in China. John Frost et al. developed a metabolically engineered *E. coli* strain that was blocked in the common aromatic amino acid pathway at two shikimate kinase genes and which carried various overexpressed and deregulated genes from the aromatic amino acid pathway, resulting in a titer of 71 g L^{-1} shikimic acid and a 27% yield on glucose in a fed-batch fermentation process [76].

As another example of successful metabolic engineering of a fermentation compound that is not common to nature, the adipoyl-7-ADCA process of DSM anti-in-

fectives is depicted in Scheme 4.20B. The genes encoding the expandase enzyme from *Streptomyces clavuligerus* were introduced into an efficient *Penicillium chrysogenum* production host for penicillin. This created a *Penicillium* strain that was able to expand the β-lactam ring of penicillin to a cephalosporin structure. By making additional genetic modifications *Penicillium chrysogenum* was made to accept adipic acid and couple this sidechain to the expanded molecule. The resulting adipoyl-7-ADCA is transported through the cell wall and can thus be isolated. Subsequently, two enzymatic steps are carried out to remove the adipoyl sidechain from adipoyl-7-ADCA (glutaryl acylase) and to couple D-phenylglycine to it (penicillin acylase, see Scheme 4.6D) [77]. This new route is completely water-based and the life cycle analysis of this new process was compared to that of the traditional chemical process as well as the second-generation "green" process (see Section 4.2.4) by the Öko-Institut (Institute for Applied Ecology) in Germany. The results demonstrated that the new fermentation/biotransformation process for cephalexin is not only more economically but also more ecologically sustainable than the traditional chemical process.

4.3.6
Reaction Engineering

During the last decade alternative reaction media like ionic liquids [78], supercritical carbon dioxide ($scCO_2$) [79] or gas phase reactions [80] have attracted much attention to replace conventional solvent systems. For application in the pharmaceutical and food industries these have considerable advantages in comparison to commonly used organic solvents. For example, $scCO_2$ is neither combustible nor explosive and is a GRAS substance acceptable for use in food industry. The low critical temperature (31 °C) makes $scCO_2$ an ideal solvent for heat-sensitive substances and biocatalysis. The ease of recovery of the enzyme, and the possibility of performing reactions which are unfavorable in aqueous solution offers even more advantages. The potential of ionic liquids, $scCO_2$ and gas phases as reaction media has been shown for many different enzymes and even for whole-cell biotransformations.

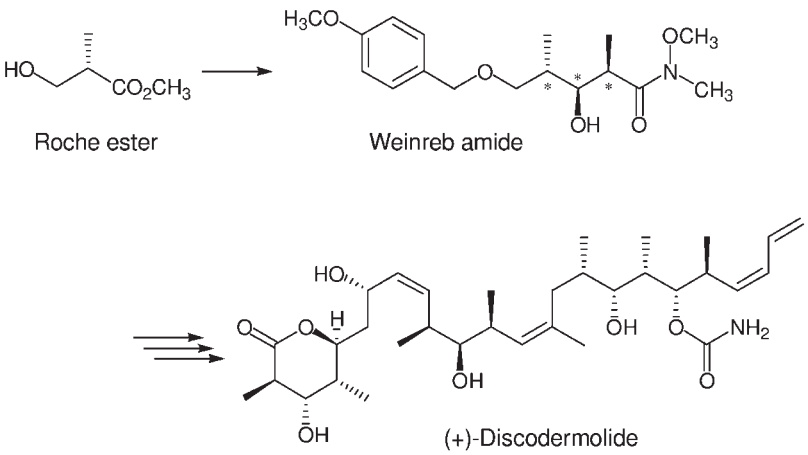
Achievements made within the field of reaction engineering will increase the applicability of biocatalysts even more. For example, the use of microreactors is still in its infancy. Cascade catalysis and multi step conversions [81], a common domain of biocatalysis, will boost the application of biocatalysis for the transformation of highly reactive compounds or intermediates. Moreover, this might diminish operating time and costs as well as consumption of auxiliary chemicals and use of energy. For example, Bacher et al. published the six-step synthesis of labelled riboflavin using eight different enzymes in one reaction vessel [82].

4.4
Conclusions and Outlook

Results discussed in Section 4.2 clearly show that biocatalysis is already an indispensable tool for the synthesis of chiral drugs and drug intermediates. Progress made in the fields of molecular biology, chemistry and reaction engineering, as described in Section 4.3., provides a good basis for further application of biocatalysts on an industrial scale. Nevertheless, we are still in the initial stages of recognizing the great potential of biotransformation methods for generating chiral drugs and drug intermediates.

The last decade has shown that purely chemical strategies like combinatorial chemistry cannot solve the problem of identifying new lead structures possessing high biological activity and selectivity, and at the same time exhibiting low toxicity. On the other hand, virtually all target molecules can be synthesized by chemists, using nonenzymatic and/or biocatalytic methods. However, the scale up is still not an easy task, especially in the case of nonenzymatic methods. Discodermolide, for instance, a potent inhibitor of tumor cell growth, has been synthesized on a 60 g scale. However, the 39-step synthesis is the result of the work of 43 industrial chemists and was build on the preparatory work of two leading academic groups (Scheme 4.21) [83].

It can be assumed that the combination of modern natural product chemistry with molecular biology and biotechnology will promote the launch of and access to new biologically active compounds. Hence, biocatalysis and biotechnology will gain importance, not only for the production of compounds but also for lead structure identification. The generation of lead structure libraries of broad diversity might be a major task, starting e.g. from already known natural products [84] or using so far non-cultured organisms or 'metagenome technology' [85].



Scheme 4.21 Synthesis of Discodermolide performed on a 60 g scale.

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Separations

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5
Resolution of Chiral Drugs and Drug Intermediates by Crystallisation

Kazuhiko Saigo and Kenichi Sakai

5.1
Introduction

The functions of biomolecules, bioactive molecules, ferroelectronic crystalline liquids, organic nonlinear optical molecules and so on often arise from their chirality. This means that the development of methods for obtaining enantiopure compounds is very important. Many methods have been reported for obtaining enantiopure compounds and these can be roughly classified into two categories, enantioseparation and asymmetric synthesis. Each category is further classified into physical, chemical, and biological methods.

Many research groups have been intensively studying physical, chemical, and biological asymmetric syntheses and biological enantioseparation in recent years and have reported a great number of excellent results. In contrast, only a very few research groups have paid attention to the physical and chemical enantioseparations of racemates, and the elucidation of chiral recognition mechanisms during crystallization for these methods. Therefore, if one wants to obtain an enantiopure compound through crystallization one has to carry out the physical or chemical enantioseparation of racemates by trial-and-error, although these enantioseparations are central methods for obtaining enantiopure compounds, not only on the laboratory scale but also on the industrial scale. In order to improve this trial-and-error situation, our group has been carrying out systematic studies on physical and chemical enantioseparations because the elucidation of chiral recognition mechanisms during crystallization is very important for the embodiment of physical and chemical enantioseparations.

5.2
Physical Enantioseparation – Preferential Crystallization

Typical examples of obtaining enantiopure compounds by preferential crystallization are ammonium sodium tartrate, glutamic acid, and methyldopa. In 1848,

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Pasteur reported that the crystals of racemic ammonium sodium tartrate consist of two kinds of enantiomeric crystals and that they can be separated by utilizing tweezers and a magnifying glass [1]. He finally found that the enantiomeric crystals corresponded to the enantiomers of ammonium sodium tartrate; his experiment is the first example of physical enantioseparation. In 1866 Gernez reported the first preferential crystallization. He found that a large amount of highly enantio-enriched ammonium sodium tartrate crystals could be obtained from a supersaturated racemic solution upon contacting with the D- or L-salt crystal [2]. About one century later, preferential crystallization on an industrial scale was performed; in 1960 Ajinomoto started to operate facilities for the enantioseparation of racemic glutamic acid by preferential crystallization to supply sodium L-glutamate in a large quantity [3], and Merck in 1963 also started the preferential crystallization of racemic methyldopa [4].

Figure 5.1 shows the procedure for preferential crystallization. Mutual seedings of small amounts of crystals of both enantiomers of a racemate to a supersaturated solution of the racemate alternatively give large amounts of the corresponding enantiomers. Thus, the preferential crystallization is very simple and therefore a fascinating way to obtain both enantiomers of a racemate. However, preferential crystallization is not always applied to any racemate.

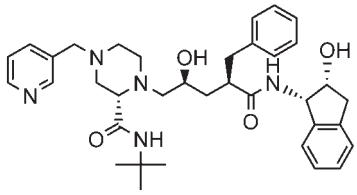


Fig. 5.1 Procedure for preferential crystallization.

There are three crystal modifications of racemic crystals, depending on the difference in strength of the intermolecular interactions between the enantiomerically hetero- and homo-chiral pairs of the racemate; the modifications are racemic compound, conglomerate, and racemic solid solution (Fig. 5.2). Preferential crystallization can only be applied to a conglomerate. However, most racemic crystals are racemic compounds, and conglomerates are very rare (a few percent of racemic crystals in our experience). This means that we have to transform a racemic compound into a conglomerate in order to carry out the enantioseparation of the racemate by preferential crystallization.

Figure 5.3 shows our idea for the transformation of a racemic compound into a conglomerate. In the case of a racemic compound, the intermolecular interactions of the enantiomerically hetero-chiral pair are stronger than those of the homo-chiral pair. However, when we allow the racemic compound to react with an achiral derivatizing agent, which can interact with the racemate molecule by intermolecular interaction(s) obviously stronger than those of the enantiomerically hetero-chiral pair, the interactions for the enantiomerically hetero-chiral pair will be broken, and the possibility of stacking of the enantiomerically homo-chiral pairs will increase.

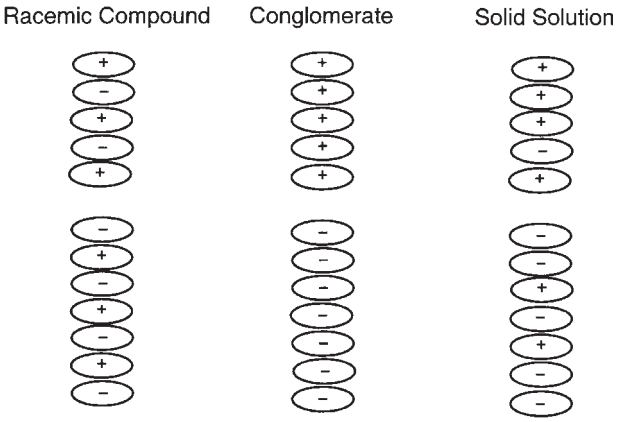


Fig. 5.2 Racemic modification.

As a target racemate, we first selected 1-phenylethylamine, which is a very useful agent in enantioseparations and asymmetric syntheses, and tried to transform it to a conglomerate by salt formation with an achiral derivatizing agent. Among the salts of 1-phenylethylamine with achiral carboxylic acids, the cinnamic acid salt was found to be a conglomerate (Table 5.1) [5]. Mutual seedings of small amounts of the enantiopure salt crystals to a supersaturated solution of the racemic salt gave large amounts of the corresponding enantio-enriched salts (Table 5.2). Recrystallization of each salt, followed by treatment with an aqueous alkaline solution, gave enantiopure 1-phenylethylamine.

In a similar manner, the racemates shown in Fig. 5.4 could be transformed into the corresponding conglomerates very easily by salt formation with achiral derivatizing agents [6]. These results indicate that salt formation with an achiral derivatizing agent is very effective for the transformation of an acidic or basic racemate into a conglomerate.

In the next stage, we examined the crystal structures of the salts derived from 11 kinds of racemic primary amines and 24 kinds of achiral carboxylic acids in order to extract factors governing the formation of conglomerates. Among the combinations, 30 single crystals suitable for X-ray crystallographic analyses were obtained. Their X-ray crystallographic analyses revealed that most of the salt crystals can be classified into two types, 2_1-column crystals and *i*-column crystals, on the basis of

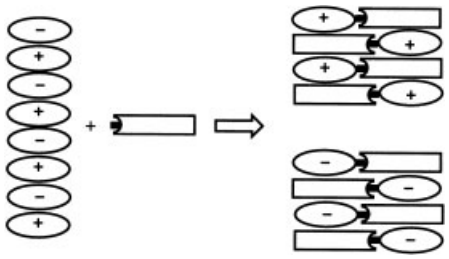
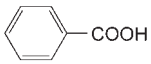
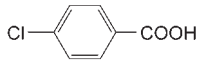
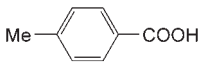
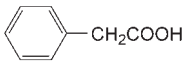
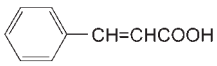
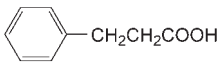


Fig. 5.3 Transformation of a racemic compound into a conglomerate.

Table 5.1 Derivatization of 1-phenylethylamine into a conglomerate upon salt formation with an achiral acid.

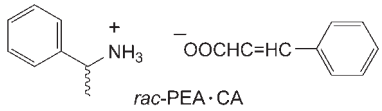
Achiral acid	Conditions for a conglomerate		
	Solubility	Mp	IR
	○	○	×
	×	○	×
	×	△	×
	○	○	×
	○	○	○
	○	○	×

○ Indicating conglomerate formation.
△ Could not be distinct.
× Indicating no conglomerate formation.

the hydrogen-bonding network in the crystals. The 2_1-column crystals are further classified into two crystal types, depending on the packing mode of the 2_1-columns; one type consists of 2_1-columns with homohelicity (homo-chiral 2_1-column crystals), and the other type consists of 2_1-columns with heterohelicity (hetero-chiral 2_1-column crystals). As can be seen from Fig. 5.5, the formation of a homo-chiral 2_1-column crystal is essential for a conglomerate. Therefore, the first way to increase the probability of conglomerate formation would be to increase the possibility of the formation of a homo-chiral 2_1-column crystal [7, 8].

Figure 5.6 shows an example of the crystal structures of the conglomerates we examined [8]. There are clearly 2_1-columns consisting of the units of two amine molecules and two acid molecules. The molecules in the 2_1-columns are packed very closely, and the shape of the 2_1-column is rod-like, which would be favorable for the close packing of the 2_1-columns. Thus, from the viewpoint of intermolecular interactions in conglomerate crystals, the factors governing the formation of conglomerates are hydrogen-bonding interaction to form a 2_1-column and van der Waals interaction between the columns. When a derivatizing agent is flat in shape, the agent molecules effectively partition the molecules of a racemate. Moreover, when the molecular length of the derivatizing agent is similar to that of the racemate, the probability for the formation of a homo-chiral 2_1-column crystal would be almost the same as that for the formation of a hetero-chiral 2_1-column crystal. In contrast,

Table 5.2 The preferential crystallization of racemic 1-phenylethylamine · cinnamic acid salt.



rac-PEA · CA

Supersaturated solution
***rac*-PEA · CA: 15.5 g**
***rac*-PEA · HCl: 15.8 g**
60% MeOH: 100 ml

rac-Salt supplied	Seed (g)	Yield (g)	Optical purity (%)
–	–	3.4	81
3.5	+	5.4	87
5.0	–	7.1	82
6.0	+	5.3	87
6.0	–	6.8	89

Recrystallization from MeOH: 64–82% yield, ~100% optical purity.

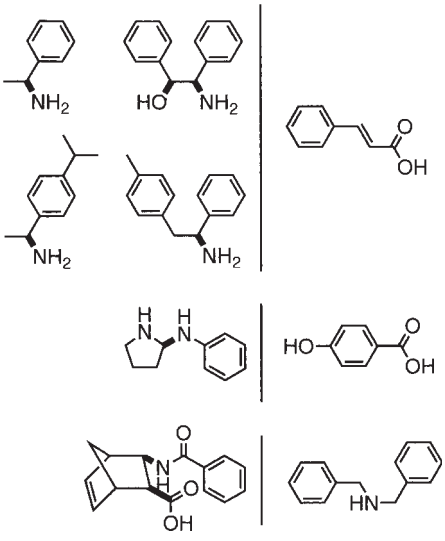


Fig. 5.4 Examples of the transformation of racemates into conglomerates.

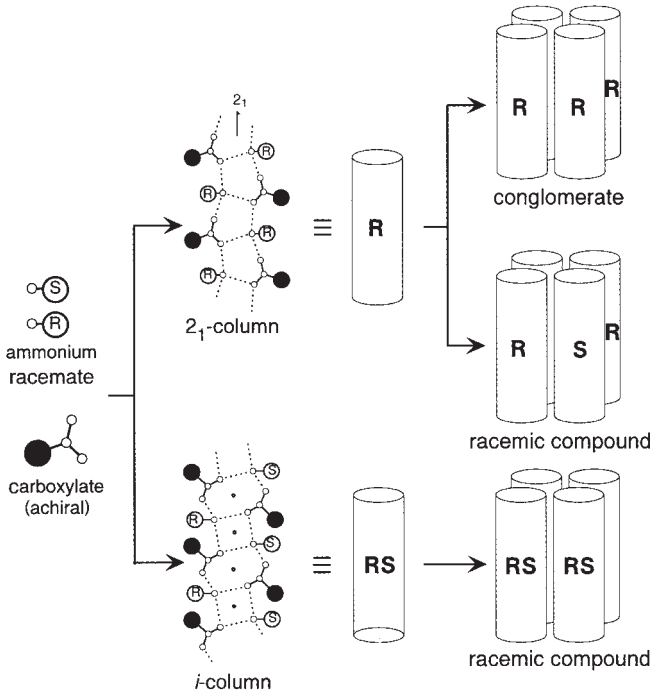


Fig. 5.5 Crystal structures of the salts of racemic amines with achiral carboxylic acids.

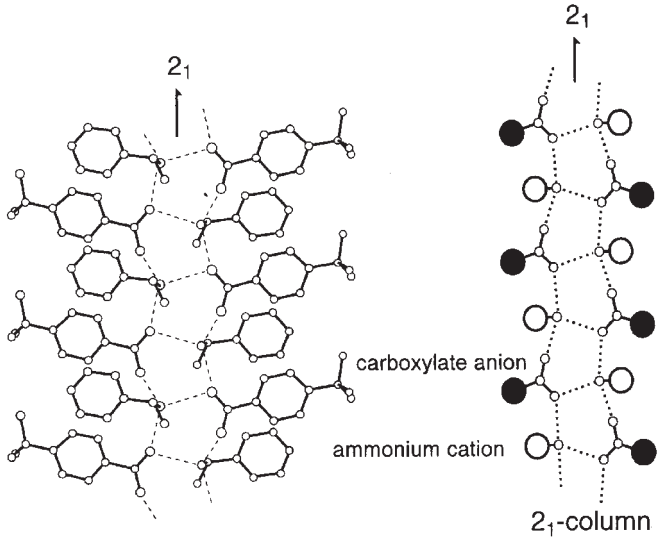


Fig. 5.6 Typical example of the hydrogen network in conglomerates.

when the lengths are very different, 2_1-columns with heterohelicity would aggregate together, as the entanglement of springs of heterohelicity. On the basis of these observations and considerations, a criterion for choosing a suitable derivatizing agent could be proposed; *in order to realize high probability of conglomerate formation, a derivatizing agent is required to satisfy two conditions: that the molecular length is similar to that of a target racemate and that its shape is plate-like.*

5.3
Chemical Enantioseparation – Diastereomeric Salt Formation [9]

The first chemical enantioseparation has been also performed by Pasteur in 1853 [10]. Thereafter, the enantioseparation by the diastereomeric salt formation is most frequently applied to the separation of various racemates in laboratorial and industrial scales.

Figure 5.7 shows the procedure for diastereomeric salt formation. Depending on the difference in solubility of a pair of diastereomeric salts derived from a racemate and a resolving agent, one of the diastereomeric salts can be purified by repeated recrystallization, and the decomposition of the purified diastereomeric salt gives one of the enantiomers of the racemate in a pure form. Thus, the fundamental principle of the diastereomeric salt formation is very simple. However, it is very hard to give a clear answer to the question of how to choose a resolving agent suitable for a given racemate, because the resolving agents have been selected by trial-and-error for the required target racemates.

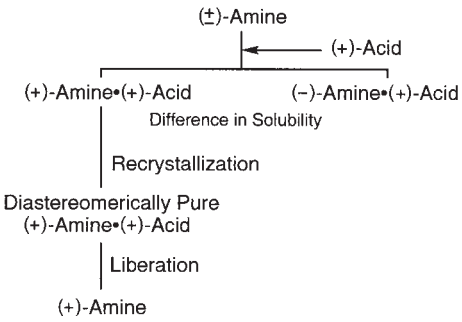
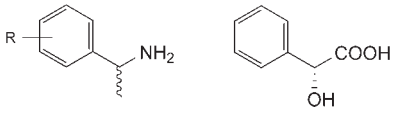


Fig. 5.7 Procedure for diastereomeric salt formation.

In order to overcome this trial-and-error situation, systematic studies on enantioseparations by diastereomeric salt formation for a structurally related series of racemates with a certain resolving agent are highly desirable. As one of such studies, our group determined the resolution efficiency for the enantioseparation of a series of 1-arylethylamine derivatives with mandelic acid [11]. The results in Table 5.3 strongly suggest that there is high correlation between the resolution efficiency and the position of the substituent on the aryl group of the 1-arylethylamine derivatives. High resolution efficiencies could be achieved for the unsubstituted and *o*-substituted derivatives, while the resolution efficiencies were very low

Table 5.3 Enantioseparation of 1-phenylethylamine derivatives with mandelic acid.



Amine (R-)	Yield (%)	Enantiomeric excess (%)	Resolution efficiency
H-	76	87	0.66
o-Me-	71	100	0.71
o-MeO-	69	81	0.56
m-Me-	94	12	0.11
m-MeO-	61	89	0.54
p-Me-	88	4	0.04
p-MeO-	84	4	0.03

Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

for the *p*-substituted derivatives, and the resolution efficiencies of the *m*-substituted derivatives are case-by-case. This phenomenon can be explained by comparing the molecular shapes of the racemates with that of mandelic acid, as shown in Fig. 5.8; there is high correlation between the resolution efficiency and the similarity in molecular length.

Then, in order to clarify this dependence, the difference in crystal structure between pairs of diastereomeric salts was examined [11]. Figure 5.9 shows the typical

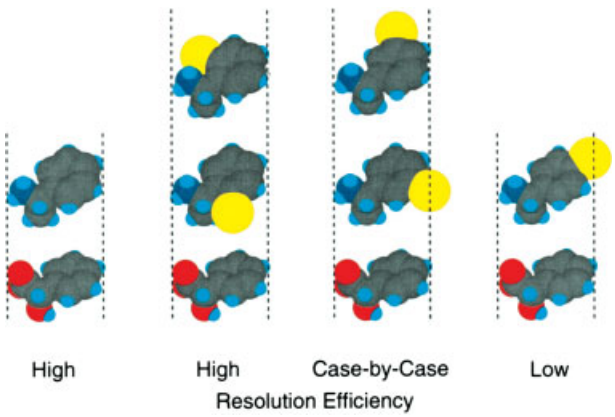


Fig. 5.8 Relation between the resolution efficiency and the relative molecular shape of the amine and mandelic acid.

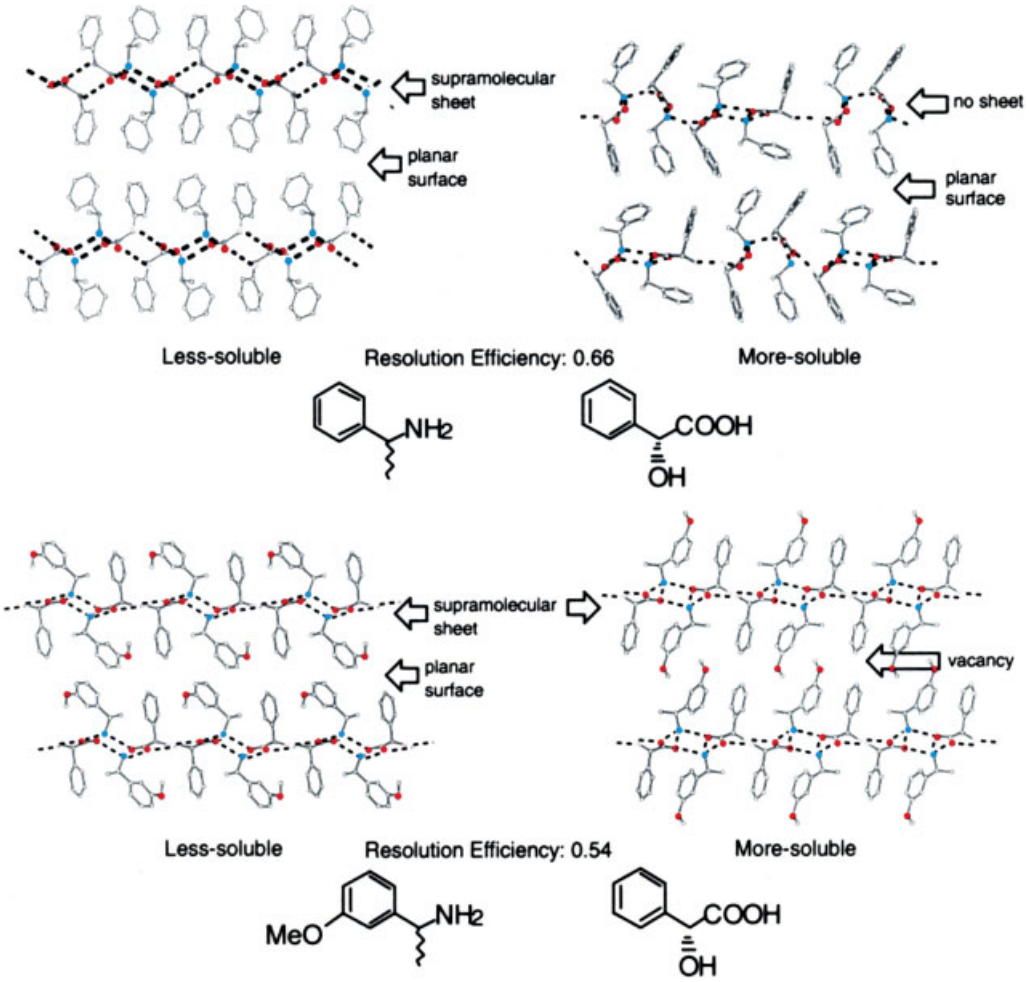


Fig. 5.9 Typical crystal structures of the less- and more-soluble diastereomeric salts in successful enantioseparations.

crystal structures of the less- and more-soluble diastereomeric salts of the 1-phenylethylamine derivatives with mandelic acid, for which the resolution efficiencies are high. In the less-soluble salt crystals there are two common factors for the stabilization of the crystals: the formation of a supramolecular sheet consisting of 2_1-columns, which is favorable from the viewpoint of hydrogen-bonding interaction, and the realization of the planar surfaces of the sheet, which is favorable for the close packing of the sheets by van der Waals interaction. In contrast, the corresponding more-soluble salt crystals satisfy only one of the two factors.

On the other hand, in the cases with low resolution efficiencies, both diastereomeric crystals satisfy only one of the two factors. For example, both diastereomeric salt crystals of 1-(4-methylphenyl)ethylamine with mandelic acid satisfy only one

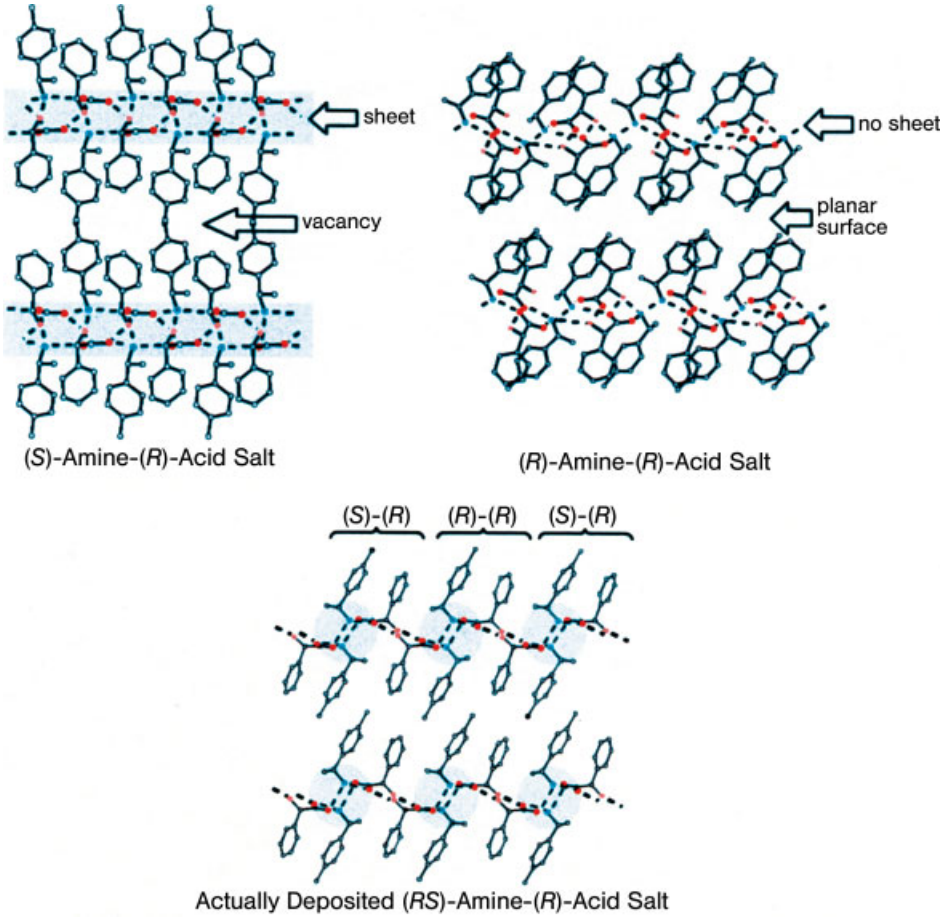
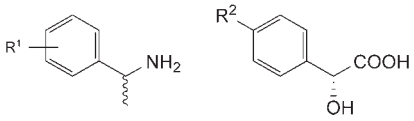


Fig. 5.10 Crystal structures of both diastereomeric salts and the actually deposited salt of racemic 1-(4-methylphenyl)ethylamine with (*R*)-mandelic acid.

of the two factors, the realization of the planar surfaces of an aggregate or the formation of a supramolecular sheet consisting of 2_1-columns. In contrast, the actually deposited crystal can satisfy both of the factors yet contain two kinds of 2_1-columns with heterohelicity, resulting in failure of the enantioseparation (Fig. 5.10).

These results strongly indicate that there are two main factors necessary for the stabilization of the diastereomeric salt crystals with mandelic acid: the formation of a supramolecular sheet consisting of 2_1-columns and the realization of its planar surfaces. When one of a pair of diastereomeric salts can satisfy the two factors for the stabilization of crystals while the other diastereomeric salt satisfies only one of the two factors, the difference in stability between the diastereomeric salt crystals becomes large enough to make the difference in solubility between the diastereomeric salts significantly large, leading to high resolution efficiency.

Table 5.4 Improvement of resolution efficiency upon introduction of a substituent at the *p*-position of mandelic acid.



R^2- / R^1-	MeO-	Me-	H-
p-Me-	0.61 (72% yield, 85% e.e.)	0.54 (85% yield, 63% e.e.)	0.04 (88% yield, 4% e.e.)
p-MeO-	0.29 (62% yield, 46% e.e.)	–	0.03 (84% yield, 4% e.e.)
m-Me-	0.57 (76% yield, 75% e.e.)	–	0.11 (94% yield, 12% e.e.)

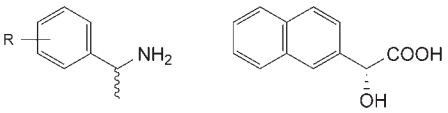
Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

The resolution efficiencies for the enantioseparations of *p*-substituted 1-phenylethylamine derivatives with mandelic acid were very low. This result prompted us to develop new resolving agents more effective than mandelic acid [11]. As mentioned above, one of a pair of diastereomeric salts realizes the formation of a supramolecular sheet consisting of 2_1-columns, although there are large vacancies between the sheets. Then, its planar surfaces are considered to be achieved, if a substituent is introduced at the *p*-position of the phenyl group of mandelic acid or if the phenyl group of mandelic acid is replaced by a large aromatic group to occupy the vacancy. On the basis of this idea, *p*-substituted mandelic acids and 2-naphthylglycolic acid were designed as new resolving agents.

As shown in Table 5.4, the resolution efficiencies for the *p*-substituted 1-phenylethylamine derivatives were greatly improved, as was expected, when *p*-methyl- and *p*-methoxymandelic acids were used in the place of mandelic acid. These results strongly support our explanation that two factors, hydrogen-bonding interaction to form a supramolecular sheet consisting of 2_1-columns and van der Waals interaction between the sheets, govern the stability of diastereomeric salts [11]. The same factors were also found in the diastereomeric salts of racemic arylalkanoic acids with enantiopure amino alcohols [12].

2-Naphthylglycolic acid was also effective for the enantioseparation of a wide variety of *p*-substituted 1-phenylethylamine derivatives (Table 5.5) [13]. Figure 5.11 shows the typical crystal structures of a pair of the diastereomeric salts. The crystal structures are quite similar to each other, suggesting that another factor is contributing to the stabilization of the diastereomeric salt crystals other than hydrogen-bonding and van der Waals interactions. Precise examination of the crystal struc-

Table 5.5 Enantioseparation of 1-phenylethylamine derivatives with 2-naphthylglycolic acid.



Amine (R-)	Yield (%)	Enantiomeric excess (%)	Resolution efficiency
H-	61	96	0.59
o-Me-	76	7	0.05
o-MeO-		Not crystallized	
m-Me-	40	98	0.39
m-MeO-	68	98	0.67
p-Me-	81	95	0.77
p-MeO-	58	87	0.50
p-F-	69	94	0.65
p-Cl-	77	98	0.75
p-Br-	81	93	0.75
p-O_2N-	70	88	0.56
p-Et-	62	>99	0.61
p-^{n}Pr-	80	>99	0.79
p-cyclohexyl-	50	91	0.46

Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

tures revealed that there is a large difference in the CH/π interaction arising from the relative geometries of the aromatic groups in the racemate and 2-naphthylglycolic acid; very effective CH/π interactions contribute to the stabilization of the less-soluble salt crystal, compared with those of the more-soluble salt, although hydrogen-bonding and van der Waals interactions work similarly in both diastereomeric salts. 2-Naphthylglycolic acid can even recognize the chirality of *p*-substituted 1-phenylethylamine derivatives with a large substitutent such as nitro, propyl or cyclohexyl. This suggests that the realization of the planar surfaces of a supramolecular sheet would no longer be impossible in the less-soluble salt crystals, indicating that CH/π interactions play a more significant role than van der Waals interaction for the stabilization of the diastereomeric salt crystals. Such effective CH/π interactions were also observed in the less-soluble salts of racemic acids with *cis*-2-aminobenz[*f*]indan-1-ol, which showed a considerably improved chiral recognition ability compared to the mother compound, *cis*-2-aminoindan-1-ol (Table 5.6) [14].

Thus, there are three factors for the stabilization of diastereomeric salt crystals (Fig. 5.12). The most important factor is hydrogen-bonding interaction, at least to form 2_1-columns and desirably to form a supramolecular sheet consisting of the

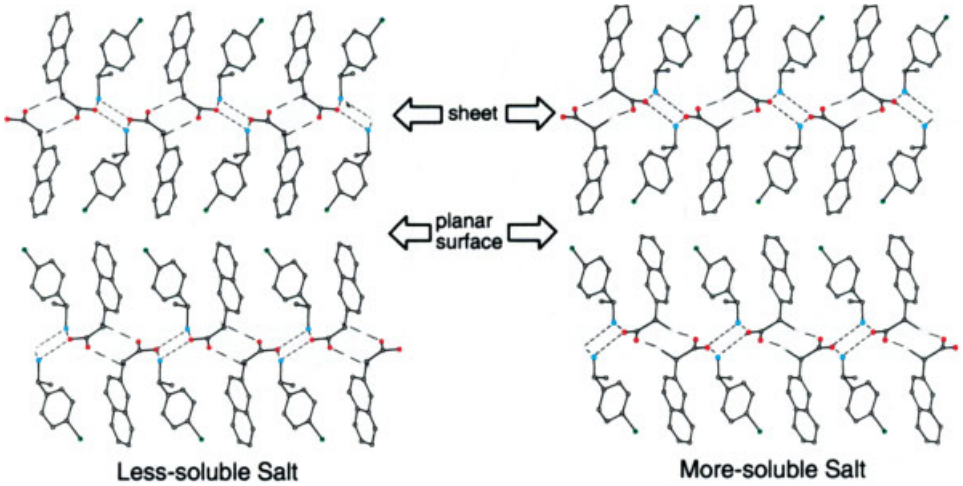


Fig. 5.11 Crystal structures of the less- and more-soluble diastereomeric salts of racemic 1-(4-chlorophenyl)ethylamine with (*R*)-2-naphthylglycolic acid. The short broken lines indicate the hydrogen bonds between the ammonium hydrogens and the carboxylate oxygens. The long broken lines indicate the hydrogen bonds between the hydroxy hydrogens and the carboxylate oxygens at the upper and lower layers.

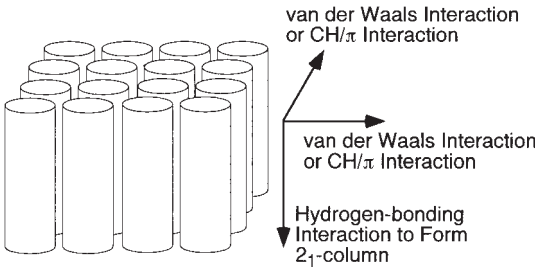
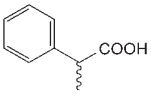
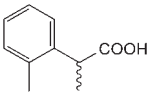
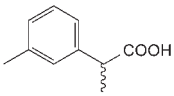
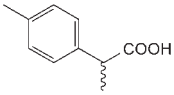
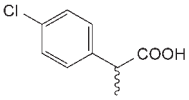
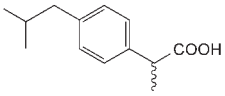
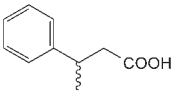
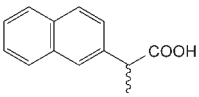
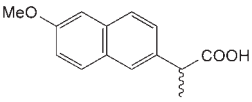


Fig. 5.12 Three-dimensional crystal growth.

2_1-columns. The second factor is CH/π interaction, which plays a significant role for the stabilization of diasteremeric salt crystals. If such a CH/π interaction cannot be expected in crystals, effective van der Waals interaction between the 2_1-columns or supramolecular sheets is important. On the basis of these results, a criterion could be proposed for selecting a suitable resolving agent to achieve successful enantioseparation by the diastereomeric salt formation: *a favorable resolving agent is one in which the molecular length is similar to that of a target racemate or which has aryl groups which can contribute to CH/π interaction.*

Table 5.6 Comparison of resolution efficiency for the enantioseparation by *cis*-2-aminoindan-1-ol and *cis*-2-aminobenz[*f*]indan-1-ol.

Acid	Resolution efficiency	
	***cis*-2-amino-indan-1-ol**	***cis*-2-amino-benz[*f*]indan-1-ol**
	0.62	0.56
	0.20	0.58
	0.54	0.56
	0.20	0.53
	NC	0.69
	0.06	0.48
	0	0.45
	NC	0.29
	NC	0.15

Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.
NC = Not crystallized.

5.4
The Bridge Between Preferential Crystallization and Diastereomeric Salt Formation

The studies on preferential crystallization and diastereomeric salt formation revealed that the necessary conditions for successful derivatization and enantioseparation are close to each other; the two factors governing the formation of conglomerates are very similar to those governing the stability of diastereomeric salts. Moreover, in order to achieve a high probability of conglomerate formation and in order to realize a high probability of successful enantioseparation by diastereomeric salt formation, the relative molecular length for a racemate and a derivatizing agent or resolving agent is very important. This similarity prompted us to study the combination of racemic acids and racemic amines.

In the case of the salt of a racemic acid and a racemic amine, six crystal modifications are possible (Table 5.7), while there are three crystal modifications for the salt of a racemic acid or amine with an enantiopure amine or acid (Table 5.8). If the successful enantioseparation of an amine with an enantiopure acid by diastereomeric salt formation is assumed, the diastereomers in Table 5.8 should obviously be more stable than the double salt and pseudo-diastereomer. Then, the diastereomers in Table 5.7 should be more stable than the other crystal modifications. On the other hand, this assumption leads to the conclusion that the solubilities of the diastereomers in Table 5.8 are largely different from each other. This

Table 5.7 Crystal modifications for a combination of a racemic acid and a racemic amine.

Acid / Amine	(±) Regular	(+)	(−)	(±) Random
(±) Regular	Racemic compound	Double salt	Double salt	Pseudo-solid solution
(+)	Double salt	Diastereomer	Diastereomer	Pseudo-double salt
(−)	Double salt	Diastereomer	Diastereomer	Pseudo-double salt
(±) Random	Pseudo-solid solution	Pseudo-double salt	Pseudo-double salt	Solid solution

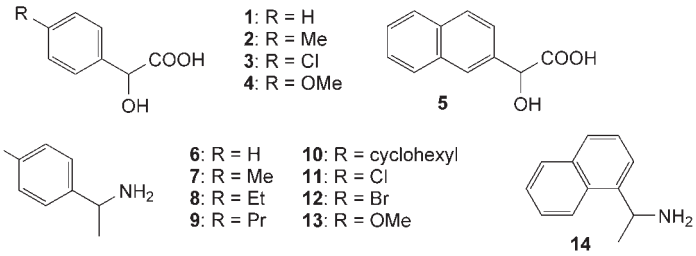
Table 5.8 Crystal modifications for a combination of an enantiopure acid and a racemic amine.

Acid / Amine	(±) Regular	(+)	(−)	(±) Random
(+)	Double salt	Diastereomer	Diastereomer	Pseudo-double salt

means that one pair of the diastereomers in Table 5.7 is less soluble than the other pair; the less-soluble pair is enantiomeric and should be a conglomerate. Thus, the salt of a racemic acid and a racemic amine has a high tendency to crystallize as a conglomerate when the corresponding enantiopure acid and/or amine component of the salt is a suitable resolving agent in the enantioseparation of its counterpart by the diastereomeric salt formation [15].

Table 5.9 shows the crystal modifications of the salts of 1-arylethylamine derivatives with 1-arylglycolic acids that we studied. Among 13 combinations, 7 salts are conglomerates. This means that the proportion of conglomerates is over 50% [15] which is much higher than the normally estimated value of 3–5% for independent racemates. This extremely high probability is also found for the results reported in the literature. For example, Jaques and coworkers examined the crystal modifications of the salts of 16 kinds of acids with 9 kinds of amines [16]. Upon

Table 5.9 Crystal modification for the salts of racemic acids and racemic amines, of which the enantiopure component is efficient for the enantioseparation of the counterpart.



Acid	Amine	Modification	Resolution efficiency with Acid	Amine
1	**6**	R	0.66	0.36
2	**6**	C	0.54	0.41
3	**6**	C	0.59	0.64
4	**6**	R	0.61	0.76
5	**6**	R	0.59	0.54
5	**7**	C	0.77	0.50
5	**8**	R or D	0.61	ND
5	**9**	C	0.79	ND
5	**10**	R	0.46	ND
5	**11**	C	0.75	0.59
5	**12**	C	0.75	ND
5	**13**	R	0.50	ND
5	**14**	C	0.56	0.46

R = racemic compound, C = conglomerate, D = double salt.
Resolution efficiency = yield × e.e.
ND = Not determined.

setting a limit to the combinations, of those for which the resolution efficiencies in the enantioseparations by the diastereomeric salt formation are satisfactory, 11 salts of 24 combinations are conglomerates. This means that the proportion of conglomerates is 46%, which is also very high.

These results strongly suggest that the probability of formation of a conglomerate, which can be enantioseparated by the preferential crystallization, is considerably high for the combinations of racemic acids and racemic amines when the enantiopure component is an efficient resolving agent for its counterpart in the diastereomeric salt formation.

5.5 Process Research on the Enantioseparation of Racemates by Diastereomeric Salt Formation

For the development of enantioseparation processes by diastereomeric salt formation, the selection of a suitable resolving agent is the first concern. As described in Section 5.3, a suitable resolving agent for a target racemate can be selected and/or designed on the basis of the criterion we proposed. However, it is not always easy to obtain a suitable resolving agent because of its availability and/or price. Therefore, we sometimes have to use an easily available and/or cheaper resolving agent and to optimize the operation conditions such as the molar ratio, solvent, temperature, and so on, for the enantioseparation of the target racemate with the resolving agent on an industrial scale; even for such a case, our criterion is helpful.

5.5.1 The Role of Water in the Stabilization of Less-soluble Diastereomeric Salts – A Key Intermediate for the Synthesis of Duloxetine, 3-(Methylamino)-1-(2-thienyl)propan-1-ol [17]

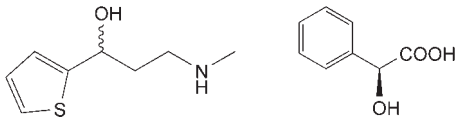
Duloxetine (LY-248686), (*S*)-(+)-*N*-methyl-3-(1-naphthyloxy)-3-(2-thienyl)propylamine, is expected to be not only a new potent antidepressant but also a NE (norepinephrine) reuptake inhibitor, a 5-HT (serotonin) reuptake inhibitor, and a new treatment drug for stress urinary incontinence [18]. In order to produce an enantiopure key intermediate for the synthesis of the (*S*)-amine, the Eli Lilly group proposed various strategies [19]. As a result, they selected the enantioseparation of racemic 3-(dimethylamino)-1-(2-thienyl)propan-1-ol with (*S*)-mandelic acid by diastereomeric salt formation as the most economic and suitable process for industrial-scale production with efficient supporting techniques such as the racemization of the antipode and recycling the recovered materials [20]. However, in the process of demethylation for the preparation of (*S*)-Duloxetine from (S)-3-(dimethylamino)-1-(2-thienyl)propan-1-ol, there are some critical problems, such as low yield and considerable decomposition to give impurities. Thus, a direct synthesis of (*S*)-Duloxetine starting from (*S*)-3-(methylamino)-1-(2-thienyl)propan-1-ol is expected to be a new route for the production of (*S*)-Duloxetine.

In order to find a suitable resolving agent for racemic 3-(methylamino)-1-(2-thienyl)propan-1-ol, typical acidic resolving agents such as (*S*)-mandelic acid, (*R*)-2-methoxy-2-phenylacetic acid, (*R*)-phenylpropionic acid, L-tartaric acid, L-dibenzoyltartaric acid, and L-di-*p*-toluoyltartaric acid were screened using EtOH as solvent. L-di-*p*-toluoyltartaric acid showed the highest resolution efficiency (0.50). However, the enantiomeric excess of the amine recovered from the less-soluble diastereomeric salt was not satisfactory. Moreover, L-di-*p*-toluoyltartaric acid is rather expensive for an industrial-scale application. Thus, we then decided to use (*S*)-mandelic acid, which is commercially available cheaply and in a large quantity, for the enantioseparation of racemic 3-(methylamino)-1-(2-thienyl)propan-1-ol and examined in detail the conditions, especially focusing on the solvent, since no crystal was obtained when EtOH was used as a solvent.

No crystal was obtained from solutions of *tert*-butyl methyl ether (MTBE) and MTBE-EtOH, which were favorable solvents for the enantioseparation of racemic 3-(dimethylamino)-1-(2-thienyl)propan-1-ol with (*S*)-mandelic acid, or from other organic solvents, such as 2-butanol, ethyl acetate, ethyl methyl ketone and diethyl ether. In sharp contrast, fine crystals with acceptable diastereomeric purity (75%) deposited, when water was used as a solvent, although the yield was rather low (20%). The spectral and elemental analyses revealed that the salt crystallized from water was monohydrated; the water molecules stabilizing the less-soluble diastereomeric salt crystal as a result of the close molecular packing with the amine and acid molecules.

In order to improve the resolution efficiency, i.e. to increase the yield of the less-soluble three-component diastereomeric salt without any deterioration in the diastereomeric purity, the effect of water in ethanol was examined for a range of 2–75% (w/w) water contents. Table 5.10 shows that the enantiomeric excess of the amine recovered from the less-soluble diastereomeric salt increased and then decreased with decreasing water content, until finally no crystal was obtained. This result indicates that the presence of water in a solvent is essential for the formation of the less-soluble diastereomeric salt and that the three-component salt could possibly deposit in a larger quantity from a solvent less polar than ethanol. On the basis of this consideration, less polar alcohols were used as solvents in the presence of a small amount of water (Table 5.11). When 2-butanol containing two moles of water was used as a solvent, the highest resolution efficiency was achieved. The diastereomeric salt crystals, obtained in all the systems shown in Table 5.11, contained an equimolar amount of water as a component. These results obviously show that water plays a very important role in the formation of stable diastereomeric salt crystals with satisfactory diastereomeric purity. The recrystallization of the crude salt once from aqueous 2-butanol gave the diastereomeric three-component salt with diastereomeric purity of more than 95%. The final product (*S*)-3-(methylamino)-1-(2-thienyl)propan-1-ol with more than 99.5% ee was obtained upon treatment of the recrystallized salt with aqueous sodium hydroxide, followed by extraction with 2-butanol and crystallization from toluene [21].

Table 5.10 Enantioseparation of 3-(methylamino)-1-(2-thienyl)propan-1-ol with mandelic acid (1).



Solvent water/EtOH	Yield (%)	Enantiomeric excess (%)	Resolution efficiency
100/0	60	68	0.41
75/25	30	72	0.22
50/50	4	86	0.03
25/75	16	85	0.14
5/95	22	70	0.15
2/98		Not crystallized	
0/100		Not crystallized	

Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

Tab 5.11 Enantioseparation of 3-(methylamino)-1-(2-thienyl)propan-1-ol with mandelic acid (2).

Solvent	H_2O/amine molar ratio	Yield (%)	Enantiomeric excess (%)	Resolution efficiency
n-PrOH	1.0	40	71	0.28
2-PrOH	1.0	64	74	0.47
	2.0	94	55	0.52
	3.0	80	71	0.57
n-BuOH	1.0	78	67	0.52
	2.0	72	75	0.54
2-BuOH	1.0	80	63	0.50
	2.0	90	70	0.63
	3.0	78	73	0.57
	4.0	64	76	0.49

Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

5.5.2
Reciprocal Enantioseparation – A Key Intermediate for ACE Inhibitors, 2-Hydroxy-4-phenylbutyric Acid, and 1-(4-Methylphenyl)ethylamine [22]

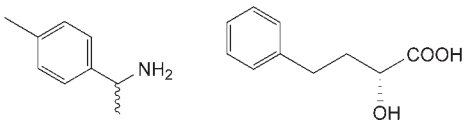
Enantiopure 2-hydroxy-4-phenylbutyric acid, which is a key material for various ACE inhibitors such as Cilazapril, Quinapril, Enalapril, Lisinopril, Indopril, and Ramipril [23], has been obtained through the separation of the diastereomeric methyl ester [24]. However, the process requires multiple recrystallizations in order to obtain the diastereopure ester; this process is comparatively expensive, and the yield is insufficient for industrial-scale production. On the other hand, enantiopure 1-(4-methylphenyl)ethylamine is now known as a useful resolving agent, not only for 2-hydroxy-4-phenylbutyric acid but also for various pharmaceuticals such as Naproxen, Ketoprofen, and chrysanthemum acid [25]. Enantiopure 1-(4-methylphenyl)ethylamine has been obtained by enantioseparation with pyrroglutamic acid, Naproxen, *N*-formylphenylalanine, *threo*-2-hydroxy-3-(2-aminophenylthio)-3-(4-methoxyphenyl)propionic acid, malic acid, camphoric acid, or *N*-acetylleucine [26]. However, both enantiomers of these resolving agents are not always available in the market. Therefore it is difficult to obtain both enantiomers of 2-hydroxy-4-phenylbutyric acid by using these resolving agents. In the course of our studies to find effective resolving agents for racemic 2-hydroxy-4-phenylbutyric acid and 1-(4-methylphenyl)ethylamine, respectively, we found that both can be reciprocally resolved by diastereomeric salt formation.

First, the solvent effect in the enantioseparation of racemic 1-(4-methylphenyl)ethylamine with (*R*)-2-hydroxy-4-phenylbutyric acid was examined. The system using 2-butanone, 2-propanol, or water as a solvent was found to give higher total resolution efficiency, whereas their mixed solvents resulted in lower total resolution efficiency (Table 5.12). For industrial-scale production, the use of a single solvent is better, and water is the best solvent for both economic and environmental reasons. Therefore, water was selected as a solvent, although 2-butanone gave the best total resolution efficiency.

In order to reduce the quantity of the resolving agent and to stabilize the enantioseparation system during crystallization, the molar ratio of (*R*)-2-hydroxy-4-phenylbutyric acid to racemic 1-(4-methylphenyl)ethylamine was examined; HCl corresponding to the deficient amount of the acidic resolving agent was supplemented by applying the Pope and Peachy method [27], and the best result was achieved when the molar ratio of the acidic resolving agent to HCl was 1/1.

When water is used as a solvent, an acidic resolving agent is favorably recycled as an aqueous solution in an industrial-scale operation. However, the presence of NaCl in the aqueous solution cannot be avoided. This arises from the decomposition of the corresponding less-soluble salt with aqueous NaOH to liberate the resolved amine, followed by neutralization with aqueous HCl. Therefore, the enantioseparations were performed in the presence of NaCl to check the possibility of recovering the resolving agent as an aqueous solution. The coexistence of NaCl up to 12% in the aqueous solution did not affect the yield and total resolution efficiency with retention of diastereomeric purity (100%), but when the NaCl concentration

Table 5.12 Enantioseparation of 1-(4-methylphenyl)-ethylamine with 2-hydroxy-4-phenylbutyric acid.



Solvent	Acid/amine molar ratio	Total yield (%)	Enentiomeric excess (%)	Total resolution efficiency
MEK	2/3	71	96	0.69
2-PrOH	2/3	65	95	0.62
MEK/H_2O (90/10)	2/3	58	99	0.58
2-PrOH/H_2O (97/3)	4/5	42	97	0.41
H_2O	2/3	62	99	0.62
	1/1	65	100	0.65

The deficient amount of the acid was supplemented by HCl.
Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

reached 20%, which corresponds to near saturation, the diastereomeric purity was reduced (94%) with decrease in the yield and total resolution efficiency. Thus, in this system, recycling of the resolving agent as an aqueous solution was found to be very difficult and isolation of the resolving agent as a solid by acidification with aqueous HCl might be necessary in order to exclude the influence of NaCl.

In the next stage, the enantioseparation of racemic 2-hydroxy-4-phenylbutyric acid with (*R*)-1-(4-methylphenyl)ethylamine was tried; several solvents (dioxane, 4-methyl-2-pentanone, and water) were tried and a relatively higher resolution efficiency was observed with water which is economically and environmentally favorable. The addition of NaOH was also effective, and the highest resolution efficiency (0.76, 78% yield, 98% enantiomeric excess) was achieved when the molar ratio of the basic resolving agent/NaOH was 1/1 for racemate/water = 1/2.8.

Thus, the reciprocal enantioseparation of 2-hydroxy-4-phenylbutyric acid and 1-(4-methylphenyl)ethylamine was successfully achieved.

5.5.3
Solvent Switch – A Key Intermediate for Lysine Production, α-Amino-ε-caprolactam [28]

Enantiopure α-amino-ε-caprolactam is used not only as a synthetic intermediate for pharmaceuticals [29] but also as a key intermediate for the production of L-lysine [30]. Various enantioseparation procedures for obtaining (*S*)-α-amino-ε-caprolactam have been developed by diastereomeric salt formation using L-amino acid

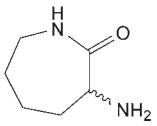
derivatives and L-tartaric acid [31], and by the preferential crystallization of a nickel complex, an amino acid salt, and inorganic acid salts [32]. Enzymatic resolution has also been documented [30, 33]. However, for obtaining a key intermediate of D-lysine, (*R*)-α-amino-ε-caprolactam, the low-yield preferential crystallization or diastereomeric salt formation using an expensive unnatural D-amino acid derivative as a resolving agent had to be employed. Therefore, we re-examined the enantioseparation of racemic α-amino-ε-caprolactam by diastereomeric salt formation to look for a cost-effective procedure.

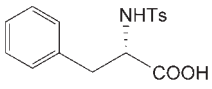
First, in order to find the most suitable resolving agent, commercially available acidic resolving agents, such as L-tartaric acid, di-*p*-toluoyl-D-tartaric acid, dibenzoyl-D-tartaric acid, *N*-tosyl-(*S*)-phenylalanine, *N*-tosyl-(*S*)-alanine, (*S*)-mandelic acid, and (*S*)-2-methoxy-2-phenylacetic acid, were examined, using MeOH as a solvent. Among them, *N*-tosyl-(*S*)-phenylalanine showed the highest resolution efficiency (0.60), whereas the other resolving agents gave very poor results or afforded no crystals at all.

In the next stage, the conditions were optimized using *N*-tosyl-(*S*)-phenylalanine as a resolving agent in various protic solvents (Table 5.13). As a result, the chirality of the enantiomer in excess in the less-soluble diastereomeric salt was found to vary depending on the dielectric constant of the solvent used in the process; by using only one kind of natural resolving agent, both diastereomeric salts containing each enantiomer of α-amino-ε-caprolactam were obtained upon switching the solvent. The (*S*)·(*S*) diastereomeric salt was obtained from solvents having a dielectric constant between 29 and 58, whereas the (*R*)·(*S*) diastereomeric salt was obtained from solvents having a dielectric constant lower than 25 or higher than 63 although the diastereomeric purities were low. Karl Fischer water content determination and elemental and X-ray crystal structure analyses revealed that the (*S*)·(*S*) salt was monohydrated containing a 0.5 equimolar amount of adhered water, whereas no water was detected in the (*R*)·(*S*) salt. These results prompted us to try continuous enantioseparation by simply switching the solvent, because the enantioseparation of an enantio-enriched sample is known usually to give much better result than that of the corresponding racemic sample.

MeOH was the best solvent for the first enantioseparation to give the (*S*)·(*S*)·H_2O salt with 93% diastereomeric purity (30% yield). An equimolar mixture of (*R*)-enriched (40%) α-amino-ε-caprolactam and *N*-tosyl-(*S*)-phenylalanine, which was recovered upon concentrating the mother liquor, was crystallized from 89% aqueous 2-PrOH/H_2O (89/11) to give the (*R*)·(*S*) salt with a high resolution efficiency (41% yield, 93% diastereomeric purity). The processes could be repeated with high reproducibility (the first enantioseparation: 29–31% yield, 91–93% diastereomeric purity; the second enantioseparation: 41–42% yield, 91–94% diastereomeric purity). The diastereomeric purity of the crude salts could be easily improved by recrystallization once from MeOH and 2-PrOH/H_2O (89/11), respectively, to afford the pure salts with a diastereomeric purity of more than 99%. Treatment of the salts with 35% HCl in EtOH, followed by recrystallization gave α-amino-ε-caprolactam·HCl with enantiomeric excess of more than 99.9%.

Table 5.13 Enantioseparation of 1α-amino-ε-caprolactam with *N*-tosyl-L-phenylalanine.





Solvent	Dielectric constant	Yield (%)	Enantiomeric excess (%)	Resolution efficiency
2-PrOH	18	64	32 (*R*)	0.41
EtOH	24	68	7 (*R*)	0.10
2-PrOH/H_2O (89/11)	25	59	29 (*R*)	0.34
EtOH/H_2O (90/10)	29	60	10 (*S*)	0.12
MeOH	33	30	93 (*S*)	0.56
EtOH/H_2O	34	24	99 (*S*)	0.48
(81/19)	34	24	99 (*S*)	0.48
MeOH/H_2O (60/40)	51	9	95 (*S*)	0.17
MeOH/H_2O (45/55)	58	48	3 (*S*)	0.03
MeOH/H_2O (35/65)	63	16	13 (*R*)	0.04
MeOH/H_2O (10/90)	74	37	35 (*R*)	0.26
H_2O	78	30	28 (*R*)	0.17

The deficient amount of the acid was supplemented by HCl.
Yield is based on a half amount of the racemate.
Enantiomeric excess is of the recovered amine.
Resolution efficiency = yield × e.e.

Similar enantioseparation processes applying the solvent switch have been reported for the enantioseparation of racemic 1-phenyl-2-(4-methylphenyl)ethylamine and 2-(4-methoxyphenyl)-3-acetoxy-5-(2-(dimethylamino)ethyl)-2,3-dihydro-1,5-benzothiazepin-4(5H)-one with enantiopure mandelic acid [34].

5.6 Examples of Enantioseparations in the Pharmaceutical Industry

It has been estimated that more than a half of chiral drugs in pharmaceutical markets are produced by diastereomeric salt formation because of its ease of operation and wide applicability [35]. Recent examples for the enantioseparation of chiral drugs and their intermediates are listed in Table 5.14.

Table 5.14 Enantioseparation of drugs and their intermediate by diastereomeric salt formation.

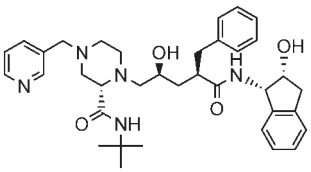
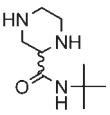
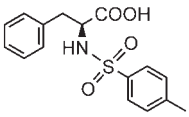
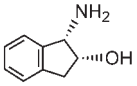
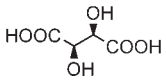
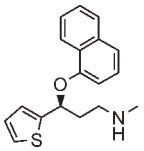
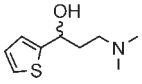
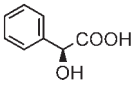
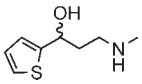
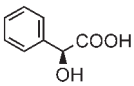
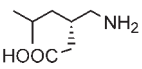
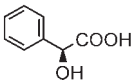
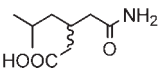
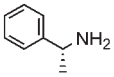
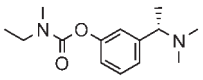
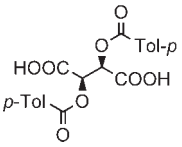
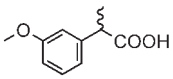
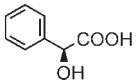
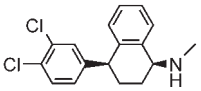
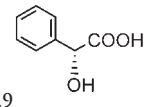
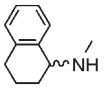
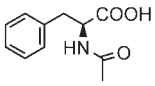
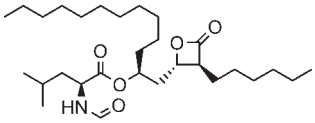
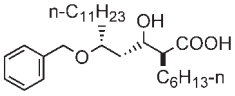
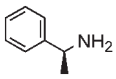
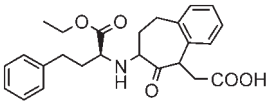
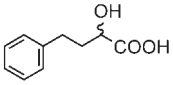
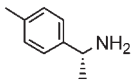
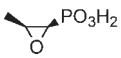
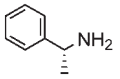
Chiral drug	Intermediate	Resolving agent	Ref.
 Indinavir (HIV drug: protease inhibitor)			
		MeOH: 8 (v/wt) Molar ratio: 0.7 Yield: 96% Enantiomeric excess: 99%	[36]
			
		EtOH/MeOH (1/1): 30 (v/wt) Molar ratio: 1.1 Yield: 86% Enantiomeric excess: 93%	
		$MeOH/H_2O$ (98/2): 9 (v/wt) Molar ratio: 1.2 Yield: 90% Enantiomeric excess: 98%	[37]
 Duloxetine (antidepressant; SNRI)			
		MTBE Molar ratio: 0.45 Yield: 84–86% Enantiomeric excess: 95–98%	[38]
			
		$2\text{-BuOH}/H_2O$ (9/1): 2 (wt/wt) Molar ratio: 1.0 Yield: 90% Enantiomeric excess: 70%	[17]
 Pregabalin (antiepileptic; GABA antagonist)			
		IPA/H_2O (97/3): 8 (wt/wt) Molar ratio: 1.5 Yield: 85% Enantiomeric excess: 82%	[39]
			
		$CHCl_3$/EtOH (99/1): 17 (wt/wt) Molar ratio: 0.7 Yield: 75% Enantiomeric excess: 99%	[39, 40]

Table 5.14 (continued)

Chiral drug	Intermediate	Resolving agent	Refs.
 Rivastigmine (Alzheimer drug, AChE inhibitor			
	MeOH/H_2O: 10 Molar ratio: 1.0 Recryst. 3 times		[41]
			
	MeOH: 2.6 (wt/wt) Molar ratio: 0.6 Yield: 70% Enantiomeric excess: 99%		[42]
 Sertraline (antidepressant; SSRI)			
	EtOH Molar ratio: 0.9 Yield: 80% Enantiomeric excess: 99%		[43]
			
	EeOH: 39 (v/wt) Molar ratio: 1.0 Yield: 81% Enantiomeric excess: not disclosed		[44]
 Orlistat (antiobesity; pancreatic lipase inhibitor)			
	AcOMe: 7 (v/wt) Molar ratio: 0.9 Recryst. 2 times Yield: 53%		[45]
 Benazepril (antihypertensive; ACE inhibitor)			
	H_2O: 2.8 (wt/wt) Molar ratio: 0.6 Yield: 80% Enantiomeric excess: 98%		[22]
 Fosfomycin (antibiotic)			
	n-PrOH/H_2O (87/13): 7 (v/wt) Molar ratio: 0.7 Yield: 82% Enantiomeric excess: 92%		[46]

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6
Isolation and Production of Optically Pure Drugs by Enantioselective Chromatography

Eric Francotte

6.1
Introduction

The systematic investigation of the biological activity of individual stereoisomers has become the rule for all new chiral drugs and chiral considerations are now integral parts of drug research and development and of the regulatory process.

In this context, there has been a considerable development of enantioselective synthetic methodologies, which have now reached a high degree of diversity and complexity. Simultaneously, this trend has created an intensive demand for stereoselective separation techniques and analytical assays for precise determination of the enantiomeric purity of chiral compounds. The development of chiral stationary phases (CSPs) or chiral selectors for gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) rapidly opened a new dimension in the area of separation technologies.

While enantioselective chromatography has become the method of choice for analytical determinations of enantiomeric purity, the significance of the technique on a preparative scale is also gaining increasing recognition as a powerful alternative for the supply of pure enantiomers of bioactive compounds [1–5]. In particular, the concomitant introduction of both, efficient chiral stationary phases, and efficient separation techniques, such as simulated moving-bed (SMB) chromatography, offers new possibilities in the field of chromatographic separations which were not conceivable some years ago.

The successful application of enantioselective chromatography as a valuable approach to the separation of optical isomers on a preparative and even production scale has attracted the attention of most pharmaceutical companies.

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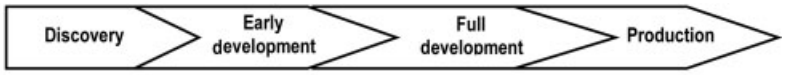
6.2
General Considerations Regarding the Preparation of Single Stereoisomers of Chiral Drugs

6.2.1
The Different Approaches

The requirements for preparing single enantiomers of chiral substances differ considerably with the stage of development of the compound of interest, the goal being to produce a certain amount of pure enantiomers within a certain time frame and under certain cost limitations (Fig. 6.1)



Amount	mg - 50 g	100 g - 10 kg	5 - 100 kg	tons
Needed isomer	both enantiomers	both enantiomers	active enantiomer	active enantiomer
Time frame	days	weeks	months	
Cost importance	minor	minor	middle	major
Scale-up feasibility	minor importance	middle importance	major importance	prerequisite

Options

- Synthesis (chiral pool, auxiliary)
- Catalytic process (ligand, enzyme)
- Diastereomers (crystallization)
- Chromatography

Fig. 6.1 Requirements for the development of chiral drugs.

In the discovery stage, time is the most important factor. The process must be rapid and generally applicable. In early development, the time frame is still relatively short but scale-up feasibility should already be considered. At full development stage, the process must be established, it must be robust and cost becomes a critical factor. At the production scale, cost is a major concern and scale-up feasibility is obviously a prerequisite.

Basically, two major options exist for preparing single enantiomers of chiral compounds. The "chiral approach", consists in designing an enantioselective synthesis of the desired enantiomer. If both enantiomers are needed, it is usually necessary to develop two independent syntheses. The chiral approach includes enantioselective synthesis using substances from the chiral pool and or chiral auxili-

aries, enzymes or stereoselective catalytic processes. In contrast to the chiral approach, the "racemic approach" implies the preparation of the racemate, which is subsequently resolved into the corresponding enantiomers. This preparation is usually achieved by a reaction sequence which generally presents a much lower degree of difficulty than that for the corresponding optically active forms. In the racemic approach, separation methods comprise the widely used technique of crystallisation of diastereoisomers, membrane systems and chromatographic methods. Among the chromatographic methods, the most used approach is undeniably liquid chromatography (LC) separation on chiral stationary phases (CSPs). This successful development has been made possible thanks to the concomitant development of both new efficient chiral stationary phases and powerful separation techniques. In particular, the design of numerous CSPs has provided new tools suitable for preparative separations.

Among the available options, the preferred one will depend on the relative importance of the three mentioned factors (Fig. 6.1), namely time frame, costs and scale-up feasibility.

6.2.2 Enantioselective Chromatography

The enantioselective separation of enantiomers by chromatography on chiral stationary phases has gained increasing recognition over the last 15 years and the technique is now considered as a powerful approach for the preparation of optically pure compounds [1–5]. This trend is clearly demonstrated by the rapidly growing number of applications published in this area. In the field of preparative chromatography it has even been the driving force for the acceptance of the technology as an industrial option, in particular since the introduction of the simulated moving bed technology [6–9].

Chiral stationary phases were applied as a preparative tool for many years before their potential as a powerful technique for the analysis of chiral compounds was recognized.

As early as 1904, Willstätter attempted to separate optical isomers on the optically active natural polymers wool and silk [10]. About 35 years later, the first partial chromatographic resolution of the enantiomers of *p*-phenylene-bis-imino-camphor on lactose was achieved by Henderson and Rule [11], and a few years later by Lecoq for the enantiomers of ephedrine [12], and by Prelog and Wieland for the enantiomers of Troeger's base [13].

However, the real potential of enantioselective chromatography for the preparative separation of optical isomers was definitely established in 1973 by Hesse and Hagel who introduced fully acetylated cellulose (triacetylcellulose) as a new efficient chiral CSP [14]. They successfully achieved the preparative separation of the enantiomers of various chiral compounds. For many years, triacetylcellulose was practically the only chiral stationary phase available for preparative separations and it has been used for the chromatographic resolution of a broad variety of chiral molecules [1–3, 15, 16].

A few years later, Blaschke designed purely synthetic chiral stationary phases obtained by emulsion polymerization of acrylamides prepared from amino acids [17]. These phases had been developed for preparative purposes and proved to be very efficient for the preparative resolution of various chiral drugs for which the enantiomers have been isolated for the first time. These earlier applications include the separation of the enantiomers of the sadly well-known drug thalidomide (Fig. 6.2) [18].

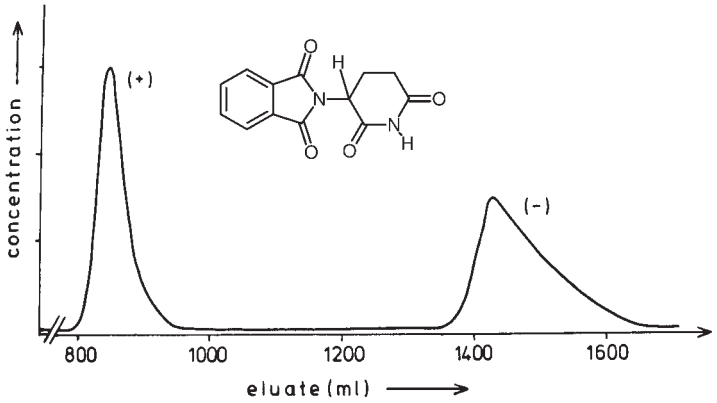


Fig. 6.2 Chromatographic preparative separation of the enantiomers of thalidomide on a chiral polyacrylamide phase (from [18]).

From the early 1980s, a growing number of analytical chiral columns became available and are now routinely used for the determination of the enantiomeric composition of mixtures of optical isomers from enantioselective syntheses, from biological investigations or from pharmacokinetic or toxicology studies. Some of these phases have also become extremely useful for enantioselective preparative separations [1–4, 16].

6.3 Preparative Chiral Stationary Phases

6.3.1 Classification of Chiral Stationary Phases

Basically, one can distinguish three kinds of CSPs, chiral polymers (Type I), achiral matrices (mainly silica gel) modified with chiral moieties (Type II), and imprinted materials (Fig. 6.3).

In the first group (Type I), which comprises most organic polymeric phases, the density of chiral information is generally high and a simultaneous participation of several chiral interaction sites or several polymeric chains has been proved in several instances. Even if it is not always easy to evidence this 'concerted' mechanism,

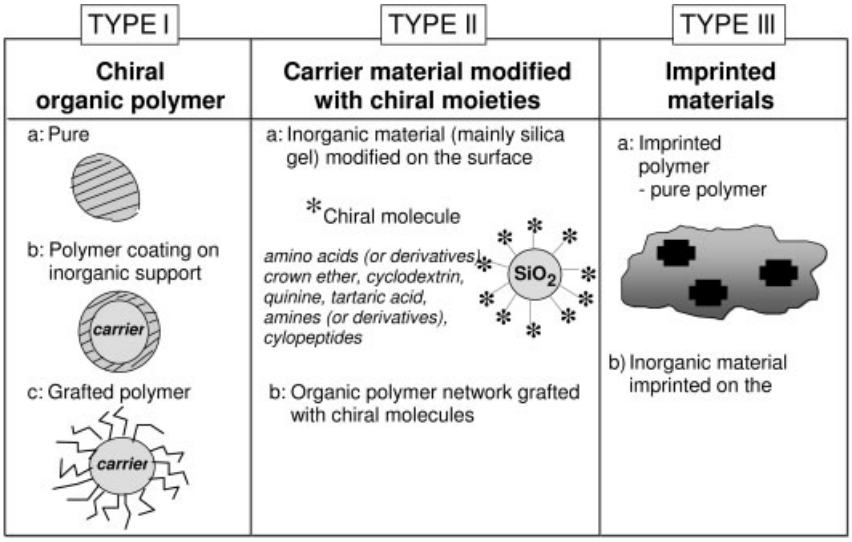


Fig. 6.3 Classification of chiral stationary phases for enantioselective chromatography.

this effect was demonstrated in the case of some cellulose derivatives for which the influence of the supramolecular structure on enantioselectivity was definitely verified [19–21]. This mode of interaction can be defined as "multimolecular or concerted". The polymer can be in pure or diluted form when coated or grafted. Nevertheless, even in the "diluted" form, the possibility of multimolecular interaction is maintained. Oligo- and polysaccharides and their derivatives, polyacrylamides, polyacrylesters and the protein-based phases belong to this type of CSPs.

The second most used type of CSPs (Type II) are chiral sorbents obtained by attaching optically active molecular entities to achiral carriers (mainly silica gel) by means of ionic or covalent bonds. A wide range of optically active moieties have already been applied, including amino acid derivatives, crown ethers, cinchona alkaloids, carbohydrates, amines, tartaric acid derivatives, cyclodextrins, binaphthol etc. Although the silica carrier is also a polymer, in this class of CSPs the chiral interaction sites distributed at the surface or in the network of the achiral support are relatively far away from each other and essentially only a "bimolecular" stereoselective interaction is possible between the chiral solute and the chiral selector. However, in one case Pirkle was able to induce a double bimolecular interaction involving two chiral selectors simultaneously by using a dimeric solute bonded via a long spacer, resulting in very high enantioselectivity [22]. Cyclodextrins as chiral selectors constitute an intermediate case because the inclusion complexation with this macromolecule involves the interaction with several glucose residues but, nevertheless, a simultaneous interaction with two or more cyclodextrin molecules is very rare.

A third group of chiral stationary phases includes those obtained by the imprinting technique initially developed by Wulff [23]. These phases do not contain

Table 6.1 Most used commercially available preparative chiral stationary phases.

Packing name	Chiral selector	Manufacturer
Cellulose and amylose derivatives		
Chiralcel OD™	cellulose 3,5-dimethylphenylcarbamate	Daicel
Chiralcel OF™	cellulose 4-chlorophenylcarbamate	Daicel
Chiralcel OG™	cellulose 4-methylphenylcarbamate	Daicel
Chiralcel OJ™	cellulose *p*-methylbenzoate	Daicel
Chiralcel OK™	cellulose cinnamate	Daicel
Chiralpak IB™	immobilized cellulose 3,5-dimethylphenylcarbamate	Daicel
Chiralpak AD™	amylose 3,5-dimethylphenylcarbamate	Daicel
Chiralpak AS-V™	amylose (*S*)-phenylethylcarbamate	Daicel
CTA-I	cellulose triacetate	Daicel
Synthetic polymers		
ChiraSpher™	poly[(*S*,-*N*-acryloylphenylalanine ethyl ester]	Merck
CHI-DMB™	cross-linked O-3,5-dimethylbenzoyl tartramide	Eka Nobel
CHI-TTB™	cross-linked O-4-*tert*-butylbenzoyl tartramide	Eka Nobel
Cyclodextrin-based CSPs		
ChiraDex™	beta-cyclodextrin	Astec
Cyclobond™	alpha-, beta-, gamma-cyclodextrin	Merck
Chiral Prep CD ST	beta-cyclodextrin	YMC
Chiral Prep CD PM	phenyl-beta-cyclodextrin	YMC
Chirobiotic		
Chirobiotic T™	teicoplanin	Astec
Chirobiotic TAG™	modified teicoplanin	Astec
Chirobiotic V™	vancomycin	Astec
Chirobiotic R™	ristocetin A	Astec
Brush-type CSPs		
DNBLeu	3,5-dinitrobenzoylleucine	Regis
DNBPG-co	3,5-dinitrobenzoylphenylglycine	Regis
DACH-DNB	diaminocyclohexane 3,5-dinitrobenzamide	Regis
Whelk-01™	3,5-dinitrobenzoyl tetrahydrophenanthrene amine	Regis
ULMO™	diphenylethylene diamine 3,5-dinitrobenzamide	Regis
Chiralpak QN-AX™	quinine O-9-(*tert*-butylcarbamoyl)	Daicel
Chiralpak QD-AX™	quinidine O-9-(*tert*-butylcarbamoyl)	Daicel

chiral moieties but they consist of chiral cavities, whose chirality is only due to the three-dimensional imprinting created in the polymer by the reversibly bonded template chiral molecule during the polymerization process. This type of CSP has been intensively investigated by Mossbach [24], Sellergen [25], and Haginaka [26]. However, the real usefulness of this type of phase for preparative applications still needs to be demonstrated.

In all classes of CSPs the classical interaction forces such as ionic, dipolar, hydrophobic, hydrogen bonding, and π–π interactions can be involved.

In practice only a limited number of materials can be reasonably applied for preparative purposes among the broad range of available chiral stationary phases which have been developed for analytical separation of enantiomers. Obviously, the loading capacity of the stationary phase is a major consideration when preparative separations have to be performed. The estimated loading capacity of the most commonly used phases has already been discussed [3, 15, 27, 28].

The next section briefly describes the most important available CSPs for preparative use. These chiral phases are listed in Table 6.1 which also gives the trade name of the respective CSPs.

6.3.2 Polymeric Phases

6.3.2.1 Polysaccharide-based CSPs

A wide range of powerful polysaccharide-based stationary phases have been developed during the last 20 years [29], and several of these CSPs have become available for preparative purposes (Fig. 6.4).

These polymeric materials have been applied as pure polymers in a form adequate for chromatographic purposes or as a coating on an inert achiral support to confer mechanical stability.

For many years, the most widely used cellulose derivative for separations on a preparative scale was cellulose triacetate (CTA-I) introduced in its fully acetylated form by Hesse and Hagel in 1973 [14]. The high versatility and the high loading capacity as well as the low preparation costs have certainly contributed to an extended use of this sorbent, even if there are some practical limitations. For CTA, it must be emphasized that the crystal structure of the polymeric material has a determining influence on the chromatographic properties and the chiral recognition ability [20]. Indeed, cellulose triacetate exists in at least two different crystal polymorphic forms which can confidently be distinguished by X-ray diffraction [20, 21]. Only the so-called CTA I structure (cellulose triacetate, crystal form I) shows a large spectrum of applications.

A broad variety of racemic structures have been resolved on CTA I on a preparative scale (1, 15), but this phase suffers from the fact that it is readly soluble in most organic solvents which are usually applied for chromatographic separations.

However, Okamoto's group in Japan developed a technology consisting of coating macroporous silica gel with about 20 wt% of the polysaccharide derivatives, conferring a much higher mechanical stability to the chiral stationary phase

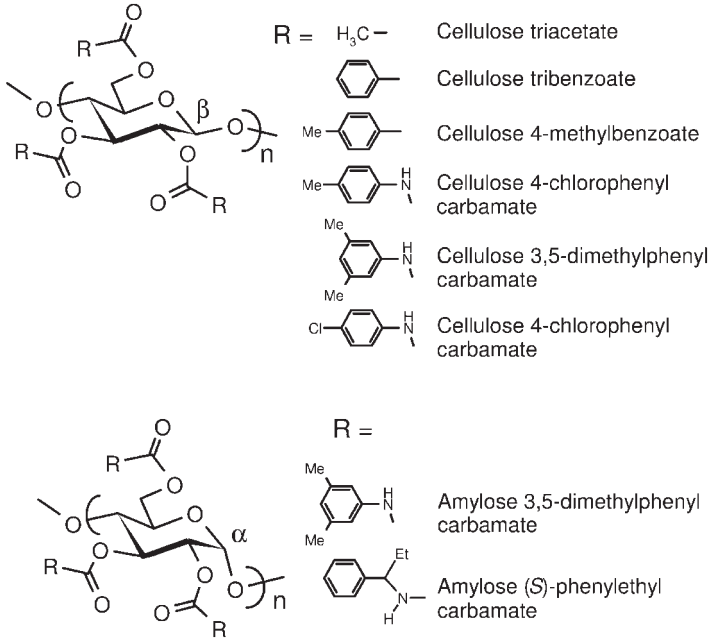


Fig. 6.4 Chemical structures of cellulose- and amylose-based CSPs.

[29–31]. Although a wide range of polysaccharide-based CSPs have been described, only a few derived from cellulose and amylose have been commercialized, according to the list in Fig. 6.4. Among these phases, four have proved to be complementary and are capable together of covering about 90% of all analytical applications [32]. As all analytical separations can potentially be scaled up, one might immediately realize the potential for preparative enantioselective applications. All analytical phases are also available in 20 μm particle size for preparative purposes. Although the inert achiral support confers suitable mechanical stability, it considerably reduces the loading capacity owing to the presence of 75 to 80% of achiral material which does not contribute to chiral discrimination.

The coated polysaccharide-based phases have mostly been used in normal phase conditions, but an increasing number of preparative applications have been reported in supercritical fluid chromatography [33] or reversed phase mode [34]. The broad applicability of the coated polysaccharide-based CSPs has made them very popular and they are now widely used for preparative separation of enantiomers and large-scale applications up to tonnes per year have been reported [35, 36]. The success of these CSPs is documented in numerous papers and these CSPs are the most used phases for analytical and preparative applications.

However, like all the non-immobilized phases, these CSPs have a nonnegligible drawback associated with the relatively good solubility of most polysaccharide derivatives in many common organic solvents like chlorinated alkanes, ethyl acetate, toluene, acetone, tetrahydrofuran, or dioxane. All mobile phases containing these

solvents or mixtures of these solvents must be excluded if one is to avoid irreversible damage of the columns. This is an important limitation for preparative applications, considering that the solubility of the racemates to be resolved is often a major issue when going from the analytical to the preparative scale.

In order to improve the resistance of the polysaccharide-based CSPs to a broader range of solvents, a new generation of this type of phases has recently been designed by immobilizing the chiral polymers on the silica support. Different approaches have been developed to make the polysaccharide-based phases insoluble. Okamoto and his group reported on a process to immobilize cellulose on silica gel through a dicarbamate linkage using diphenyl diisocyanate as a crosslinking agent [37, 38]. However, this approach necessitates additional synthetic steps and appears to negatively affect selectivity as the number of linkage increases. Independently, the groups of Oliveros and Minguillon prepared immobilized polysaccharide CSPs by reacting allyl silica gel with the undecenoyl side chains introduced onto the polysaccharide [39, 40]. Again, this process necessitates additional synthetic steps and has the disadvantage of introducing a structural disturbing factor due to the presence of the undecenoyl ester moieties. The third process, developed in our laboratories, simply consists in exposing the coated phases to a photochemical treatment or in heating in the presence of radical initiators like di-*tert*-butylperoxide [41–44]. By application of one of these processes, immobilization occurs, presumably by crosslinking of the polysaccharide chains. Depending on the derivatizing group and on the polysaccharide type, the thermal or photochemical process is the most effective one (Fig. 6.5). Once immobilized, the CSPs can be used with a large variety of mobile phases without deterioration.

A broad range of immobilized polysaccharide-based CSPs have been prepared according to the latter processes and they show, depending on the applied mobile phase, that (1) significant improvement in selectivity can be achieved; (2) retention time can be better adjusted; (3) considerable increase in solubility of the chiral solute can be obtained. All these features are extremely important for productivity. Practical applications which demonstrate the advantages of these new

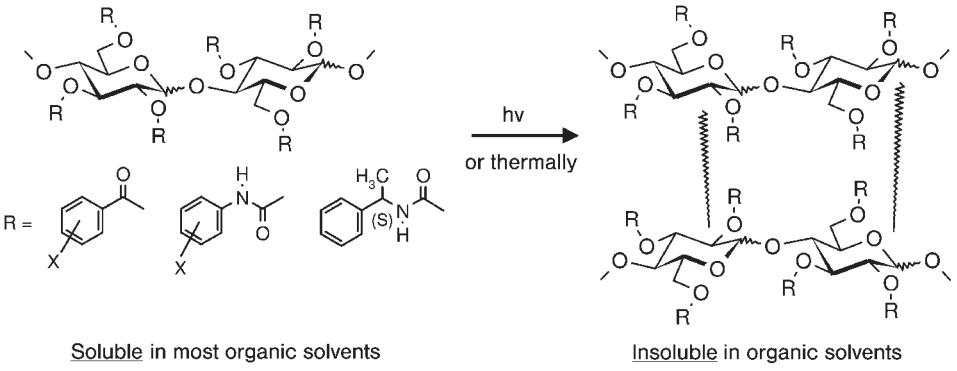


Fig. 6.5 Process of immobilization of polysaccharide-based CSPs according to [42].

phases for preparative purposes, have already been reported [45]. Therefore, it can be anticipated that a rapidly increasing number of applications of these CSPs will be seen in the near future.

6.3.2.2 **Polyacrylamide CSPs**

Cross-linked, optically active polyacrylamides and polymethacrylamides constitute another class of polymeric CSPs. These CSPs were introduced by Blaschke and coworkers about 30 years ago and their usefulness for preparative applications had already been demonstrated before the 1980s [46]. However, the gel structure of these cross-linked polymers prevents utilization at high pressure, and only moderate throughput could be obtained. Improvement in the mechanical performances of these CSPs was achieved by polymerization of the acrylic monomer on the surface of silica gel, yielding a grafted polymer [47, 48].

The preparative separations reported in the literature have been carried out using (*S*)-phenylalanine ethyl ester (a), (*S*)-1-cyclohexylethylamine (b) and menthyl amine (c) [49] as the chiral selector (Fig. 6.6).

Both modes, normal and reversed, have been applied for preparative separations on the silica grafted polyacrylamide CSPs. The feasibility of achieving preparative separations on this last CSP has been demonstrated [48, 50, 51].

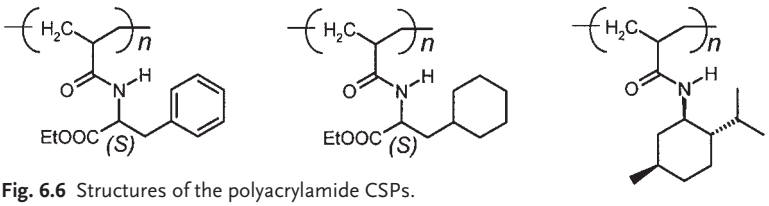


Fig. 6.6 Structures of the polyacrylamide CSPs.

6.3.2.3 **Polymeric CSPs Derived from Tartaric Acid**

Another type of polymeric CSPs (Fig. 6.7) was introduced about 10 years ago by Allenmark et al. and has been commercialized under the trade name of Kromasil CHI-DMB and CHI-TTB [52–54].

These phases have been prepared by polymerization of *N*, *N′*-diallyl derivatives of tartardiamide and grafted onto silica gel. As these phases are crosslinked and bonded to silica gel, they are insoluble in organic solvents and there is no limitation regarding the choice of mobile phase. Normal mode, reversed-phase and supercritical fluid conditions have been applied. They show good mechanical stability, but the relatively high content of achiral silica gel in these CSPs reduces their loading capacity. A few preparative separations have been reported on these two CSPs (Kromasil) [52–54], but so far they have not been widely used for preparative applications.

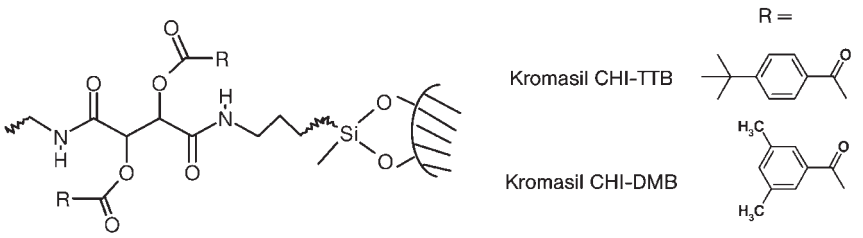


Fig. 6.7 Structure of tartardiamide-based CSPs.

6.3.3 Brush-type CSPs

6.3.3.1 π-Acidic and π-Basic Phases

The brush-type of CSP was introduced by Pirkle who was one of the pioneers of modern enantioselective liquid chromatography [55]. The most frequently used π-acceptor phases are derived from the amino acids phenylglycine (DNBPG) (Fig. 6.8) or leucine (DNBLeu) covalently or ionically bonded to 3-aminopropyl silica gel [56, 57]. These CSPs are commercially available for analytical or preparative separation of enantiomers. Further CSPs based on amino acid or amine chiral selectors such as valine, phenylalanine, tyrosine [58] and 1,2-*trans*-diaminocyclohexane (DACH-DNB phase) [59] and 1,2-*trans*-diphenylethylene diamine (ULMO phase) [60] were also developed (Fig. 6.8). These CSPs have been applied for the preparative separation of the enantiomers of a few racemic compounds, but the number of reported preparative applications has remained very limited over the last 10 years.

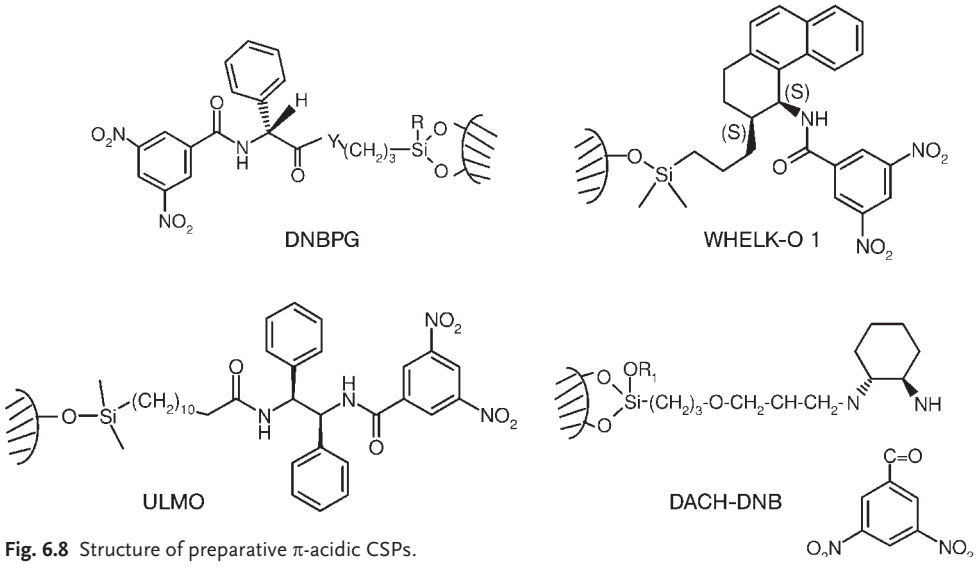


Fig. 6.8 Structure of preparative π-acidic CSPs.

The application of the reciprocality concept has led to the design of various phases of the π-donor/acceptor type [61, 62]. One successful phase is the Whelk-O 1 CSP developed by Pirkle and Welch [63–65].

These chemically modified silica gels are stable at high pressures and exhibit good chromatographic performances. Usually, these CSPs were used under normal-phase conditions, but chiral resolutions under reversed-phase mode [66] or supercritical fluid chromatography [67] have also been performed.

6.3.3.2 **Cyclodextrin-based CSPs**

Cyclodextrins are cyclic olygosaccharides that can form inclusion complexes in their highly hydrophobic chiral cavity with a large variety of molecules (Fig. 6.9).

The size of the cavity, which differs for α-, β-, and γ-cyclodextrins, and the substituent on cyclodextrin play a determining role in the ability to complex a defined molecule. Cyclodextrins CSPs were prepared by immobilizing CD in polymeric structures [68, 69] or on silica gel [70, 71], the latter CSPs showing good performance on an analytical scale. Preparative applications using cyclodextrins as chiral hosts have been reported on polymers obtained by cross-linking of cyclodextrin with ethylene glycol-bis(epoxypropyl) [69] and on the silica modified material Cyclobond I [72], ChiraDex [73]. However, due to their relatively low loading capacity, these phases are not very useful for preparative separation, especially on a larger scale.

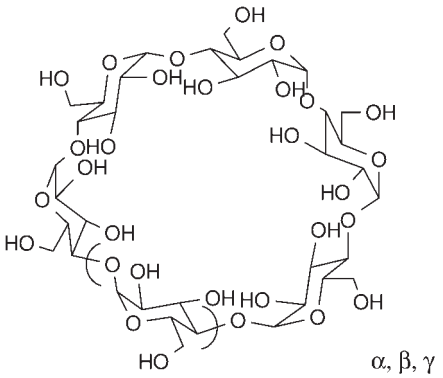


Fig. 6.9 Structures of cyclodextrin selectors.

6.3.3.3 **Chirobiotic CSPs**

A few years ago, Armstrong and his group developed a series of phases based on the macrocyclic glycopeptides vancomycin (Fig. 6.10), teicoplanin, and ristocetin as chiral selectors [74, 75]. These phases have been prepared by covalent immobilization of the glycopeptides to silica gel according to standard procedures. The chiral selectors are characterized by the presence of several chiral cavities provid-

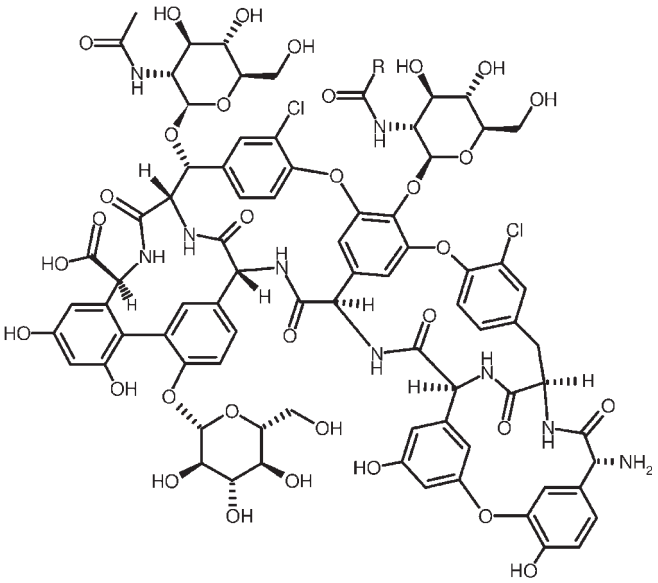


Fig. 6.10 Structure of the antibiotic gycopeptide vancomycin.

ing different environments for enantioselective interactions. The chirobiotic CSPs can be used under multimodal conditions, including SFC [76]. Preparative applications on a scale of 20–40 mg have been reported [77, 78].

6.3.3.4 **Chiral Ion-exchange Stationary Phases**

Recently, Lindner and coworkers developed a series of anion-exchange CSPs based on quinine and quinidine as chiral selectors (Fig. 6.11) [79, 80]. These phases are particularly appropriate for the separation of the enantiomers of chiral acidic compounds. Improvement of the chiral recognition power of these phases by rationally designed structural modifications has led to exceptionally high enantioselectivity which, of course, is of great interest for preparative applications [81]. Screen-

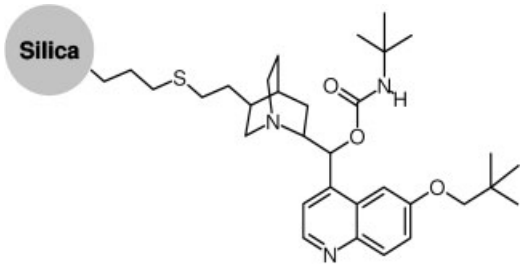


Fig. 6.11 Structure of a quinine-based CSP.

ing these CSPs can also help in the identification of a suitable CSP for large-scale preparative separations.

6.4 Strategies for Performing Enantioselective Separations

For the direct separation of enantiomers by chromatography on chiral stationary phases, two strategies are essentially applicable (Fig. 6.12). The first consists in selecting the best available CSP for the racemic compound of interest, while the second consists in modifying (derivatizing) the racemic solute to accommodate it to a defined CSP until it separates on this particular CSP.

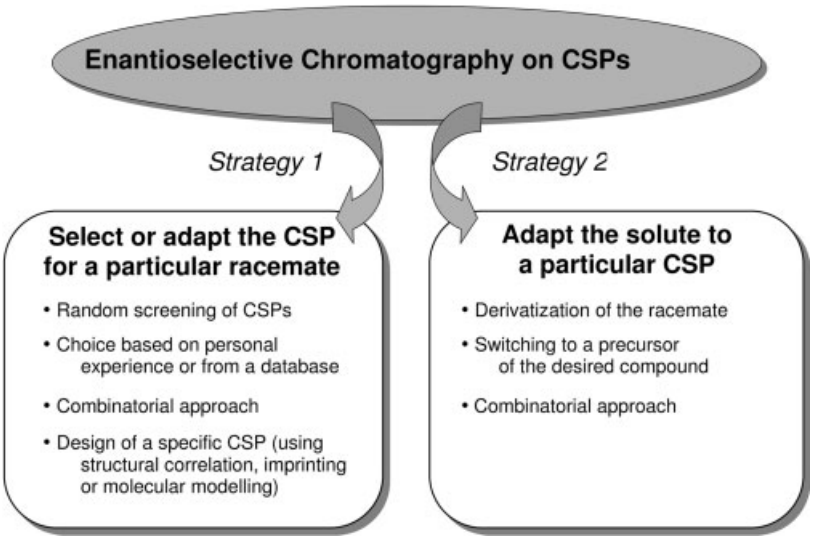


Fig. 6.12 Strategies for enantioselective separations by chromatography.

6.4.1 Selecting the Right CSP

Generally, strategy 1 is applied first and consists in identifying the CSP providing the best selectivity. Numerous preparative applications which follow this approach have been reported. Some tools, such as an electronic data base [82, 83] and user guides are available to help in the choice of the appropriate CSP, but the screening of a few CSPs is still the more commonly used approach in practice. There are only rare cases for which the chromatographic separation could be predicted.

When selecting the CSP, attention must also be paid to the mobile phase, which is often considered as an inert component, but which plays an essential role in the

interaction process. It may not only influence the retention time but can also considerably affect selectivity. This is particularly the case for the polysaccharide-based phases. There are many examples of enantiomers which could not be separated using a mixture of hexane/ethanol but which were well separated on the same CSP with a mixture of hexane/2-propanol [84, 85]. Figure 6.13 illustrates this feature with an example from our laboratory.

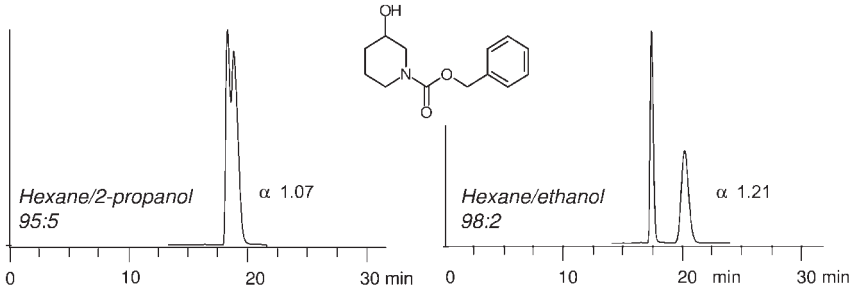


Fig. 6.13 Influence of the mobile phase on enantioselectivity. Separation of the enantiomers of *N*-benzyloxycarbonyl hydroxypiperidine on Chiralcel OD.

There is only one recent report by Blackwell et al. on the investigation of the possible rationalization of such effects for a few chiral stationary phases [86], but the prediction of the influence of the mobile phase on the chiral recognition process clearly remains a challenge. Currently, automated screening devices are usually applied to help to find the optimal mobile phase composition. With the introduction of the new immobilized polysaccharide-based phases, this task has been made even more complicated as they tolerate a much wider range of solvent, which in turn may considerably influence the enantioselectivity of the separation.

In a few cases, the option of preparing tailor-made CSPs for a particular racemic structure has been applied. For example, we prepared on an empirical basis a particular polysaccharide-based CSP for the separation of the enantiomers of the enantiomers of the LTD4 antagonist iralukast and of the antimalaria agent benflumethol [87]. These two racemic drugs were only poorly resolved on the commercially available polysacharide-based phases whereas an excellent separation was obtained on the carbamate derivative of cellulose obtained from cellulose and 3-chloro-4-methylphenylisocyanate. The prepared CSP was also used to perform pharmacokinetic studies.

On a more rational basis (concept of reciprocity), Pirkle and Welch also developed a particular CSP for the separation of the enantiomers of the analgesic agent naproxen and other nonsteroidal anti-inflammatory drugs (NSAID) [88]. It appeared later that this CSP had a relatively broad application range [89].

For the preparation of 'optimal' CSPs, a new approach based on the combinatorial concept has recently been evaluated by different groups [90–94]. Although this approach is probably not very attractive for the development of specific CSPs for analytical purposes, it could be very valuable for preparative applications as optimized separation conditions may have a determining impact on cost. However,

no practical preparative application of pharmaceutical relevance has been reported so far. The combinatorial approach has also been combined with the reciprocal concept of chromatographic separation [93, 94], leading to the identification of improved CSPs for prostaglandin precursors, aryldihydropyrimidines and profen derivatives.

6.4.2 Selecting the Racemic Solute

The second strategy (Strategy 2 in Fig. 6.12) is also extremely valuable when the separation of the enantiomers of the racemic substance of interest cannot be achieved on available preparative CSPs. This approach consists in selecting a particular CSP and adapting the solute by altering its structure (e. g. derivatization). It can be particularly useful when a limited number of CSPs are available in the working environment or when changing the CSP requires extensive and/or expensive modifications of the hardware (new columns to be packed). We frequently applied this strategy which led to successful resolutions of racemates that otherwise could not be separated on a defined CSP [87].

The strategy of derivatisation is illustrated by the example in Fig. 6.14, which shows the separation of the enantiomers of aliphatic and aromatic alcohols on tribenzoylcellulose as a CSP [95].

In this case, the first choice derivative is the *para*-methoxy benzoate, but for some alcohols, the best selectivity was obtained with the *para*-methyl or *ortho*-methoxy ester [95]. This kind of approach has been exploited to solve practical problems, as shown for the enantiomers of an intermediate used for the synthesis of the chiral anticancer agent edatrexate [2, 87].

6.4.3 Selecting the Right Synthetic Step

In practice, the global strategy very often consists of a combination of the two approaches discussed above.

At an early stage of drug development, it is generally preferred to separate the final active compound, even though a particular intermediate is much better separated. At a larger scale, the situation may be different for cost reasons and it can be more suitable to resolve a synthetic intermediate. In this instance, it is usually preferable to achieve the separation early in the synthetic process to reduce the amount of chemicals to be processed. However, a separation at an early stage of the synthesis can also be disadvantageous because of the risk of racemisation in a later step. All these questions must be carefully answered before choosing the appropriate racemic structure, in particular when a large scale separation is planned.

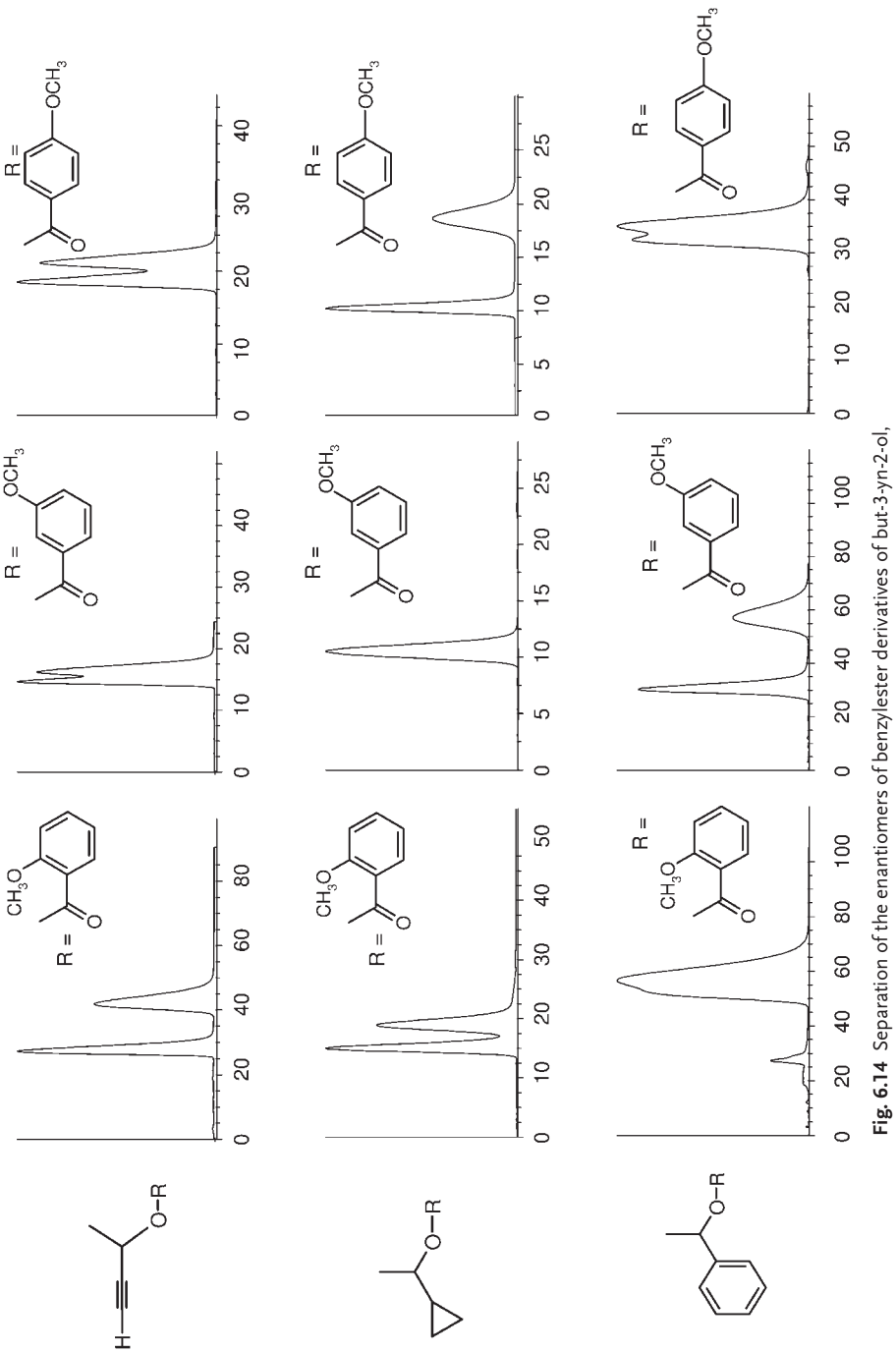


Fig. 6.14 Separation of the enantiomers of benzylester derivatives of but-3-yn-2-ol, cyclopropylethanol, and benzylalcohol on cellulose tribenzoate (beads) [95].

6.5
Preparative Enantioselective Resolution of Chiral Drugs

6.5.1
Laboratory-scale Separations

Considering that most of the enantiomers of chiral molecules can now be separated on commercially available CSPs, chromatography is probably the most general, the most rapid and the most efficient method to prepare the single enantiomers in the amounts required to perform the preliminary biological tests at the discovery stage (a few milligrams to 50 g). In addition, the chromatographic technique usually furnishes, in one operation, both enantiomers obviously needed for comparative testing. At this stage, the development of an enantioselective synthesis for each new chiral entity would be much more time consuming; the development of such a synthesis often requires weeks or months. With the few chiral stationary phases currently available for preparative separations, especially those derived from polysaccharides, it is now possible to resolve almost all racemic compounds by chromatography at a scale of several grams just by scaling up the analytical separation.

This approach has now become an established procedure in many specialized laboratories and does not present any major difficulty. Figure 6.15 shows recent applications from our laboratory for the separation of drug candidates or drug intermediates which have been performed on Chiralpak AD and Chiralcel OJ, respectively [4, 96]. Both the analytical and the corresponding preparative separations are shown.

Most of the small-scale separations published till now are listed in Table 6.2 which summarizes the pharmaceutical applications according to the major therapeutic indications. The most recent applications include those performed on polysaccharide-based CSPs [87, 97–104], on Pirkle and Whelk-O CSPs [104–107], and on Kromasil CHI [98], but the number of published examples probably does not reflect the real degree of utilization of the method which is now routinely applied in many laboratories, especially in all major pharmaceutical companies.The most used technique is the classical elution batch chromatography. Usually, enantioselective separations are performed under isocratic conditions, but when larger separation factors are observed, it may be appropriate to work under gradient conditions. On the other hand, for more difficult separations or to improve throughput, the peak shaving and recycling techniques are often used. We systematically apply this technique in our laboratory for difficult separations and its usefulness has been generally recognized [3, 87, 104].

Apolar normal phase conditions have normally been applied, including supercritical fluid chromatography [3, 108], but the option of using polar mobile phase conditions has also been successfully demonstrated [108, 109].

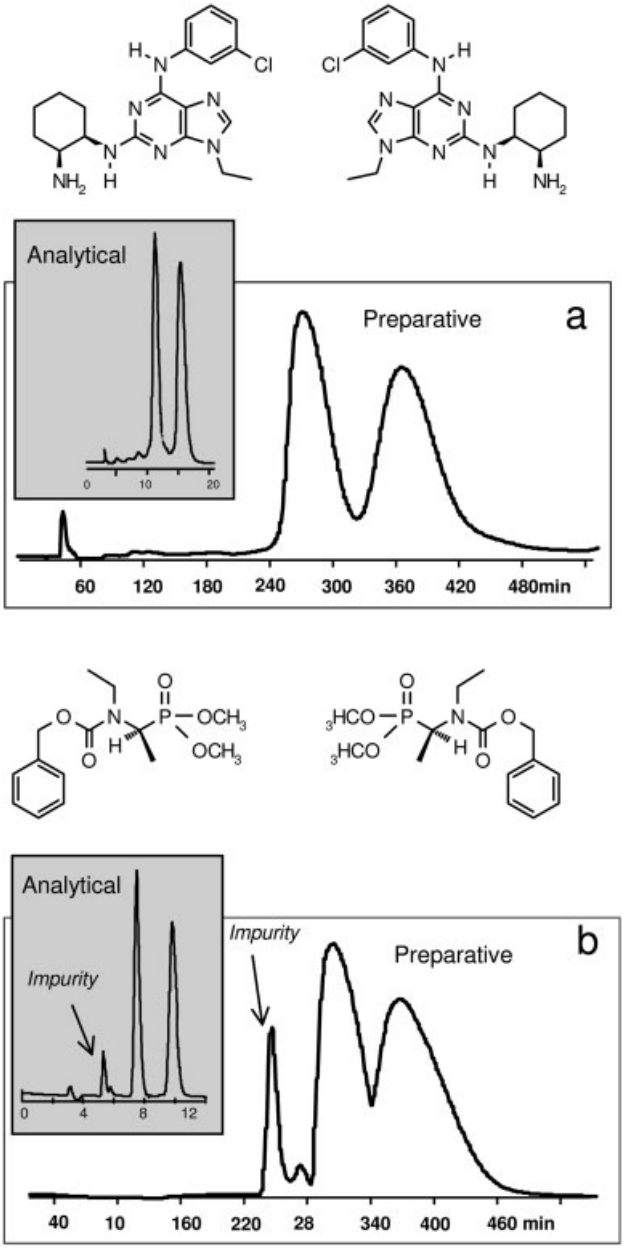


Fig. 6.15 Preparative separation of the enantiomers of (a) a chiral *cis*-diamino-cyclohexane derivative on Chiralpak AD (column 50 × 500 mm, injection 500 mg, flow rate 150 mL min^{-1}), and of (b) *O*,*O*-dimethyl-2-benzyloxy-carbonyl-*N*-ethylamino-phosphate on Chiralcel OJ (column 100 × 500 mm, mobile phase heptane/ethanol 40/60, injection 4 g, flow rate 140 mL min^{-1}).

Table 6.2 Preparative resolutions of racemic drugs and drug intermediates.

Name	Chromatographic technique	CSP	Ref.
Cardiovascular system			
ACE inhibitor (precursor)	MPLC	CTA-I	[1, 2]
Propranolol	HPLC	Chiralcel OD	[134]
Propranolol	SMBC	Chiralcel OD	[135]
Propranolol	SFC	Chiralpak AD	[108]
Atenolol	SMBC	Chiralcel OD	[135]
Pindolol	SMBC	Chiralcel OD	[135]
Alprenolol	HPLC	Chiralcel OD	[134]
Oxyprenolol	HPLC	Chiralcel OD	[134]
Metoprolol derivative	MPLC	CTA-I	[136]
Propranolol derivative	MPLC	CTA-I	[136]
Dihydropyridine	MPLC	CTA-I	[137]
Dihydropyridine	HPLC	Poly-MA	[138]
Verapamil	HPLC	Chiralcel OD	[139]
Norverapamil	HPLC	Chiralcel OD	[139]
Thienopyran	HPLC	Cyclobond I	[72]
Chlortalidone	MPLC	Poly-PEA	[140, 141]
Chlortalidone	MPLC	Poly-MA	[49]
Penflutizide	MPLC	Poly-PEA	[142]
Bendroflumethiazide	MPLC	Poly-PEA	[142]
Buthiazide	MPLC	Poly-PEA	[142]
Warfarin	SFC	Welk-O1	[143]
PAF-antagonist (WEB 2170)	MPLC	CTA-I	[115]
Vasodilator (FK 409)	HPLC	Chiralcel OJ	[144]
Central nervous system			
Vincadifformine	MPLC	CD-Poly	[69]
Quebrachine	MPLC	CD-Poly	[69]
Ketamine	MPLC	CTA-I	[140]
Formoterol	SMBC	Chiralcel OD	[111, 112]
Oxybutynin	HPLC	Chiralpak AD	[145]
Etazepine acetate	MPLC	CTA-I	[133]
Levetiracetam	SMBC	Chiralpak AD	[35, 129]
Mianserin	MPLC	CTA-I	[140]
Citalopram	SMBC	Chiralpak AD	[132]
Guaifenesine	MPLC	CTA-I	[112, 113]
Cetirizine (amide)	HPLC	Chiralpak AD	[117]
Rolipram	MPLC	CTA-I	[140]
Oxazepam	MPLC	Poly-PEA	[141]
Oxazepam	HPLC	DNBPG-io	[146]
Oxazepam acetate	HPLC	DNBPG-co	[147]
Oxazepam analogs	HPLC	DNBPG-co	[147–149]
Oxazepam analog	HPLC	DNBLeu	[148, 150]
Lorazepam	HPLC	DNBPG-io	[146]

Table 6.2 (continued)

Name	Chromatographic technique	CSP	Ref.
RP 59037	HPLC	Chiralcel OC	[151]
RP 60503	HPLC	Chiralcel OC	[151]
RP 60977	HPLC	Chiralcel OC	[151]
CGP 49823	HPLC	Chiralcel OD	[2]
SP-Antagonist	HPLC	Chiralcel OD	[4, 96]
Methaqualone	MPLC	CTA-I	[152, 153]
Barbiturates	MPLC	CTA-I	[18, 154]
Thalidomide	MPLC	Poly-CHMA	[18, 140, 154]
Thalidomide	HPLC	Chiralcel OJ	[103]
Chlormezanone	MPLC	CTA-I	[155, 156]
Thioridazine	SFC	Chiralpak AD	[108]
CPCCOEt (MGlu agonist)	HPLC	Chiralpak AD	[157]
4-phosphonophenyl glycine	HPLC	Chiralcel OJ	[158]
CGS 16920	MPLC	TBC	[1, 87, 124]
Dimiracetam	HPLC	Chiralcel OC	[159]
Infection			
Antibiotic intermediate	MPLC	CTA-I	[160]
Antibiotic intermediate	HPLC	Chiralcel OB	[161]
[^{14}C]-Zileuton	HPLC	ChiralPak AD	[171]
HIV protease inhibitor (Interm.)	SFC	Chiralpak AD	[172]
Anticancer			
Fadrozole	MPLC	TBC	[124, 162]
Fadrozole-D_5	MPLC	Chiralcel OD	[1, 2]
[^{14}C]-Fadrozole	MPLC	TBC	[2, 133]
CGP 27216	MPLC	CTA-I	[163]
Ifosfamide	HPLC	Chiralcel OD	[164]
Ifosfamide	MPLC	Poly-PEA	[165, 166]
Edatrexate intermediate	MPLC	TBC	[87, 124]
Cytotoxic agent	HPLC	DNB-Tyr-A	[173]
Aminoglutethimide	SMBC	Chiralcel OJ	[112]
Antiinflammatory and analgesics			
Oxindanac derivative	MPLC	CTA-I	[2, 169]
Nefopam	MPLC	CTA-I	[170]
Oxapadol	MPLC	CTA-I	[140]
Hydroxyeugenol	MPLC	CTA-I	[171]
Naproxen	HPLC	DNBPG-co	[65]
Gastrointestinal			
Omeprazole	MPLC	CTA I	[173]
ML-1035	HPLC	Chiralcel OD	[174]

Table 6.2 (continued)

Name	Chromatographic technique	CSP	Ref.
Hypocholesterolemic			
Lifibrol metabolite	HPLC	Chiralcel OD	[175]
Dole	SMBC	Chiralcel OF	[122]
Transplantation			
FTY-P precursor	HPLC	Chiralpak AD	[176]
Antihelmintic			
Praziquantel	SMBC	CTA-I	[8]
Miscellaneous drugs/drug intermediates			
Drug intermediate	MPLC	CTA-I	[119]
A-Hydroxyfarnesylphosphonate	HPLC	Chiralcel OD	[177]
SB-553261	SMBC	Chiralpak AD	[178]
Ro 31–8830	HPLC	Chiralcel OD-R	[179]
Melatonin analogs	HPLC	Chiralcel OD	[180]
Melatonin analogs	HPLC	Chiralcel OJ	[180]
sertraline-tetralone	SMBC	amylose 3-chloro-4-Me phenylcarbamate	[132]

For CSP description, see Table 6.1.
CSP = chiral stationary phase; SMBC = simulated moving bed chromatography; HPLC = high-performance liquid chromatography; MPLC = medium pressure liquid chromatography; SFC = super-critical fluid chromatography.

6.5.2 Pilot-scale Separations

In the early stage of development, larger amounts of material are obviously needed. At this stage, chromatography is also increasingly an alternative to the classical approaches of producing optically active compounds, in particular since the introduction of the simulated moving bed (SMB) technology in this application field.

A number of separations of enantiomers at a scale of 100 g and more have been reported in the batch chromatographic mode [3, 104]. For this purpose, larger columns with an internal diameter ranging between 5 and 30 cm have generally been used. The desired amount of enantiomer has been obtained by repetitive injections of small quantities of racemate, mostly under overload conditions. To improve throughput, techniques such as overlapping injections and peak shaving/recycling have been applied. Applications at this scale include the separation of the enantiomers of the benzyl ester of the analgesic agent oxindanac [2], of hetrazepine, of benztriazole

and imidazole derivatives [104], the separation of the enantiomers of rolipram [109], of propranolol [110], of the antiasmathic agent formoterol [111, 112], of the hypolipidaemic agent CGS 26214 [96], of the antitussive agent guaifenesine [112, 113], of the anticancer agent orimeten [112], of the analgesic drug tramadol [114], and of various drug intermediates [115–118] at a scale of 50 to several hundred grams.

Although batch chromatography has been successfully applied for the preparation of up to several kilograms of single enantiomers from the corresponding racemate over the last ten years [1, 2, 104], the SMB technique is increasingly becoming the preferred technology. Hundred gram to several kilogram separations can now be routinely performed. The technical feasibility of scaling-up the technology and its relatively low running costs have rendered this technique very attractive to the pharmaceutical industry. Compared to batch preparative chromatography, SMB technology shows several advantages. In SMB the whole stationary phase is used for the separation, allowing the productivity with respect to the stationary phase to be significantly increased. Moreover, the process is continuous, there is no intermediate fraction to be recycled and the solvent consumption is considerably reduced (up to 80–90%). The increasing interest of the pharmaceutical sector in this technology is emphasised by the increasing number of practical applications recently reported in the literature [111–113, 118–130]. In most of these applications it has been concluded that SMBC is clearly more efficient in terms of productivity and of mobile phase consumption than batch chromatography.

Most of the chiral SMBC separations reported so far have been achieved using polysaccharide-based stationary phases. In particular the high loading capacity of this kind of CSP has been recognized to be a major asset since productivities of 1–10 kg of resolved racemate per kg of CSP per day have been obtained in several instances.

6.5.3
Process-scale Separations

For full development of drugs, cost and scale-up feasibility are determining factors. At this stage, the choice regarding the production process is obviously met and it mainly depends on the cost balance of each process. In the cost calculation, many factors are involved, including equipment and packing investment, running costs (solvent, energy), recycling costs, manpower and environmental impact. Regarding the chromatographic process, it is clear that the possibility of recycling the mobile phase and recycling the 'undesired' enantiomer (after racemisation) have a favorable impact on the cost calculation. Compared to an enantioselective synthetic approach, the chromatographic method may be particularly attractive if the number of reaction steps for preparing the racemate (for chromatographic resolution) is considerably reduced compared to the number of steps needed for the asymmetric synthetic route.

Currently, there is still a limited number of reported applications of chromatographic enantioselective separations at this scale (Table 6.2), but this is probably partly due to the confidential character of these activities. The applications include

the isolation of intermediates for the synthesis of drugs [127] and drug precursors [122, 128].

6.5.4 Production-scale Separations

At the production scale, a limited number of chromatographic applications have been reported so far, but all apply the SMB technology. However, considering the relatively recent introduction of SMB, it is quite remarkable to see that a significant number of production applications have already emerged (Fig. 6.16). All reported applications were performed on polysaccharide-based stationary phases. In the pioneering example of UCB (Belgium), the annual capacity of the system, which comprised 6 columns containing a total of about 50 kg of CSP, was about 12 tonnes of the particular racemate and the desired enantiomer was obtained with a purity of 98.5% and with a recovery of 90% [35, 129, 130]. The drug Levetiracetam is now produced at a scale of several tonnes per year [129]. Other larger units capable of processing annually several tens of tonnes of compound have recently been implemented. These include the production unit at Lundbeck (Denmark) for the isolation of the S-enantiomer of the drug Citalopram [131] and the production unit of the contract company Aerojet Fine Chemicals in Sacramento, USA (Fig. 6.17). Further larger SMB-units are currently built or running in pharmaceutical and contract companies like Bayer (Germany), Daicel (Japan), Finorga (France), Honeywell Speciality Chemicals (Ireland), Novasep (France), UPT/Pharm-Eco (USA). These different examples and the willingness showed by several companies to invest considerably in this new approach for preparing the enantiomers of chiral drugs clearly demonstrates the strong potential of the chromatographic method.

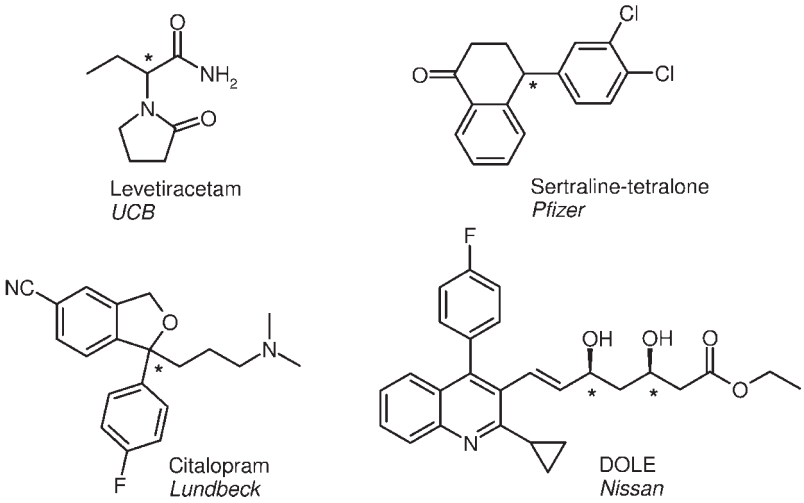


Fig. 6.16 Structures of selected chiral drugs and drug intermediates resolved industrially by simulated moving bed chromatography (SMBC).

Fig. 6.17 Picture of an industrial SMB unit running at Aerojet in Scramento (USA). Courtesy of Aerojet.

6.5 Other Enantioselective Chromatographic Techniques

Further techniques have been investigated as potential methods for the preparation of optically pure compounds. Among these techniques, gas chromatography and supercritical fluid chromatography have already shown some capability in particular cases.

6.5.1 Gas Chromatography

Although gas chromatography (GC) is a well established method for the analytical determination of enantiomeric purity, the number of preparative applications is quite limited. Most of these preparative applications by gas chromatography have been recently reviewed [181] and were performed on a relatively small scale. The method is particularly suited for volatile compounds such as the inhalation anesthetic agents enflurane, isoflurane and desflurane [182] and it has also been recently applied to the resolution of racemic á-ionone [183]. The feasibility of separating the enantiomers by gas phase simulated moving bed chromatography has also been demonstrated for the first time and was applied to the anesthetic enflurane (Fig. 6.18) [184]. However, the productivity of the system was relatively low.

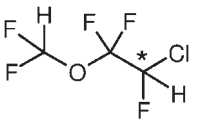


Fig. 6.18 Structure of the chiral anaesthetic agent enflurane.

6.5.2 Membranes

Several attempts to perform enantioselective separations using membranes of a chiral mobile carrier have been reported and have been extensively discussed in a recent review [185]. Various chiral carriers, mainly crown ethers, were used for this purpose but poor enantioselectivity was usually obtained and no preparative application has been described.

Further investigations have led to the development of more efficient systems based on the concept of hollow-fiber membranes. The first applications of this type were reported by Pirkle and Bowen for the separation of the enantiomers of amino acid derivatives using the dioctylamide of (*S*)-*N*-(1-naphthyl)leucine as a chiral selector [186], and by Ding et al. for the enantiomers of leucine using *N*-dodecylhydroxyproline as the chiral carrier [187]. More recently, Nakamura et al. showed the resolution of racemic tryptophan using a bovine serum albumin-multilayered porous hollow-fiber membrane [188]. Okamoto and coworkers also demonstrated the feasibility of separating enantiomers using a cellulose-based membrane for the resolution of racemic oxprenolol [189].

As a continuous process, the hollow-fiber membrane technology shows some potential for preparative use in terms of throughput and cost. However, its relatively poor efficiency currently restricts its applicability to separations exhibiting high enantioselectivity.

6.5.3 Centrifugal Partition Chromatography

As early as 1968, Schloegl et al. used countercurrent partition chromatography to resolve chiral derivatives of ferrocene [190], and several years later, Prelog et al. reported the separation of the enantiomers of norephedrine by partition between two liquid phases, using di-nonyltartrate as the chiral selector [191]. In both instances, only poor effiency was achieved. However, over the last 10 years, the centrifugal partition chromatography (CPC) technique has considerably improved and there has been renewed interest in this technology. It has the advantage of applying liquid solutions of the chiral selectors, avoiding the tedious preparation of chiral stationary phases and making it an attractive alternative approach for preparative applications. The few enantioselective applications (small scale) have recently been reviewed [192], but no practical multi-gram drug application has been reported so far. However, with the development of highly stereoselective chiral selectors, the attractiveness of the CPC technique could reappear. The viability of

this approach for preparative-scale separations was recently demonstrated by Gavioli et al. for the chiral herbicidal agent dichlorprop, which was resolved by CPC using chinchona derivatives as the chiral selectors [193].

6.5.4 Electrophoretic Methods

Electrophoretic methods are widely used alternatives for the analytical determination of the enantiomeric purity of chiral compounds [194]. Due to the high efficiency of capillary electrophoresis, separations can be achieved even when very low selectivities are observed. At a preparative scale, these methods are well established for the purification of proteins and cells [195] but there is very little published on enantioselective separations. Only recently, some interest in chiral preparative applications has been manifested. Separation of the enantiomers of terbutaline [196] and piperoxan [197] have been reported by classical gel electrophoresis using sulfated cyclodextrin as a chiral additive, while the separation of the enantiomers of methadone could be successfully achieved by using free-fluid isotachophoresis [198] and by applying a process called interval-flow electrophoresis [199]. The feasibility of separating the enantiomers of chlopheniramine on a micropreparative scale was also recently shown by applying flow-counterbalanced CE using carboxymethyl β-cyclodextrin as a chiral selector [200]. All these applications were performed at a mg scale or even less and cannot currently compete with the liquid chromatographic methods discussed previously. However, the scope and limitations of the electrophoretic approach still need to be challenged [201].

6.6 Conclusion

There is no doubt that chromatography can be considered as a powerful alternative for the preparation of optically pure compounds. On the laboratory scale it is now the method of choice as it is rapid, easily and generally applicable, and it furnishes both enantiomers. On the pilot and process scale, the chromatographic approach allows a continuous supply of optically pure substances in quantities required to perform the desired biological investigations while other preparative approaches are evaluated. Even on the production scale, and especially since the introduction of the simulated moving bed technology, chromatography must now be considered as one of the possible approaches for obtaining single enantiomers. However, as cost is a major factor at production scale, it will be determining for deciding which approach should be applied, and the choice will remain a case by case decision. Nevertheless, the implementation of the SMB technique at a scale of several tons per year already demonstrates the real potential of this approach.

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7
Stereoselective Chromatographic Methods for Drug Analysis

Norbert M. Maier and Wolfgang Lindner

7.1
Introduction

Chirality is a property inherent to all biological systems, being evident in the nature of not only the basic building blocks of life (amino acids, sugars, lipids), but also in the structure and function of higher order assemblies derived thereof. Thus, biomacromolecules composed from simpler chiral subunits fold into complex three-dimensional architectures exhibiting supramolecular chirality. Subdomains of these sophisticated macromolecular arrangements act as asymmetric binding sites and catalytic centers, capable of recognizing, discriminating and transforming individual stereoisomers of other chiral molecules with extreme levels of selectivity. Stereoselective interactions of biomacromolecules with endogenous chiral entities are essentially involved in all vital biological processes, such as catabolic/metabolic activity, transport, signal transfer, enzyme activity, receptor–ligand interactions, allosteric control, storage, expression and translation of genomic information, and immunochemical response.

The susceptibility of the biological machinery to enantioselective interactions, however, is not limited to chiral compounds of endogenous origin. Frequently, biological systems exert substantial levels of stereoselectivity for exogenous chiral molecules, for example, odorants [1], pheromones [2], agrochemicals [3, 4], environmental pollutants [5–7] and, most importantly, drug compounds [8–11].

The observation of the enantioselective action of chiral drugs dates back to the early days of modern pharmacology [12], but was, in those times, considered a curious, but rather unimportant facet of the global drug activity profiles. This attitude changed in 1961 when the *Thalidomide tragedy* forced the entire scientific community to reconsider enantiomers in context with biological systems as independent entities rather then just different forms of the same drug compound. After administration of racemic thalidomide, a sleep inducing drug, to pregnant women severe teratogenic effects were observed, manifesting themselves in a high number of miscarriages and extreme malformation of surviving newborns. Subsequent toxicological studies of the single enantiomers of thalidomide provided evidence

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that the pronounced teratogenicity was caused by the (*S*)-enantiomer exclusively, and that the tragedy may have been prevented by administration of the corresponding (*R*)-enantiomer possessing the desirable effects [13]. Recent studies, however, question these conclusions as (*R*)-thalidomide was found to undergo fast-rate racemization *in vivo* [13, 14].

Nonetheless, it was this tragic episode that drew major public attention to the potential risks of developing chiral drug compounds as racemic mixtures. In the course of this event, the *racemate-versus-enantiomer issue* became the subject of hot debate among supporting and opposing fundamentalists in drug development circles [15–17]. Confronted with the disastrous consequences of an extreme case of drug enantioselectivity, even less dedicated scientists in academia, pharmaceutical industry and drug regulatory authorities started recognizing drug stereochemistry as a key issue in the development of new pharmaceutically active ingredients. The enhanced awareness of the risk potential associated with the administration of racemic drugs induced a swift paradigm shift within the drug research and development community. Enantiomers of new chiral pharmaceutically active ingredients were no longer looked upon just as different forms of the same compound, but were now considered *a priori* as mixtures of different molecular entities, with potentially distinct pharmacological and toxicological properties. Advancing stereochemically pure drugs was increasingly considered as an attractive strategy to identify and eliminate deleterious by-effects and toxicity residing in inactive stereoisomer(s) early in cost-intensive development campaigns. Reinvestigation of the activity profiles of individual stereoisomers of established chiral drugs provided a deepened understanding of the potential pharmacological benefits of single stereoisomer drugs. The huge body of emerging data provided, for many cases, compelling evidence that the single stereoisomer drugs indeed offer distinct therapeutic advantages over racemic formulations. The most obvious of these merits comprise the reduction in total dosage, the simplification of dose–response relationships, the cancellation of complex drug–drug interactions between the individual enantiomers, and the avoidance of sources of inter-subject variability [9, 10].

In response to the enhanced knowledge on enantioselective drug action, in 1992, the Drug Information Association (DIA) initiated a workshop on chirality, stimulating discussions on the regulatory requirements on chiral drugs [18]. At this forum regulatory authorities and representatives of the pharmaceutical industry in the European Community, USA and Japan debated the regulatory issues concerning chiral drugs. A pragmatic approach to the regulation of chiral drugs was recommended, with the need to justify the development of single stereoisomer drugs with scientific data relating to quality, safety, and efficacy and risk-benefit assessment. Importantly, the advancement of racemic drugs was not objected to, with the final decision as to whether the racemic mixture or a single enantiomer should be developed residing with the applicant for marketing authorization.

Regulatory guidance documents on the development of chiral drugs, mirroring the pragmatic approach advocated by the DIA workshop, were published shortly afterwards in the US and EU [19]. Similar guidance principles were adopted by the local regulatory authorities in Japan and Canada. These documents specified the

nature and quality of the body of information that must be presented in context with new drug applications for chiral active substances, covering chemistry and pharmacy aspects, and providing data on pre-clinical and clinical studies to support drug efficacy claims. The guidelines demand that the applicants recognize the existence of chirality, and separate enantiomers/stereoisomers of drug candidates. Physicochemical properties, pharmacology and toxicity profiles of all enantiomers/stereoisomers have to be established, regardless as to whether the final drug compounds will be launched as pure stereoisomers or as racemates. The final choice of putting on the market single stereoisomers or the racemates has to be fully justified on the basis of the chemical properties, and pre-clinical and clinical trials. In 2000, these guidelines found a common confirmation in the report issued by the International Conference on Harmonization (Guidance on Q6A specification: Test procedures and acceptance criteria for new drug substances and new drug products: Chemical substances).

The recognition of chirality as a new asset in drug development has had an enormous effect on the product pipelines of the major players in the pharmaceutical industry. A most recent analysis of the distribution of worldwide-approved drugs according to their chirality character is given in Fig. 7.1 [20].

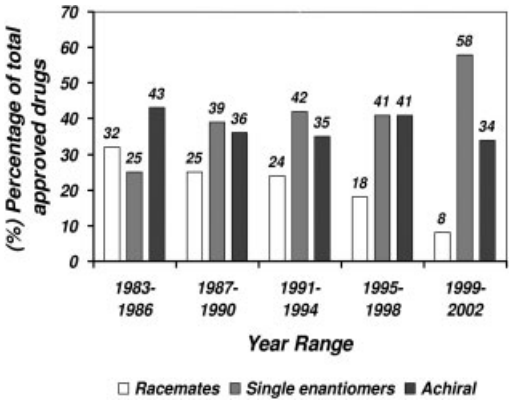


Fig. 7.1 The numbers of world-wide approved drugs within the time period 1983–2002, analyzed according to their chirality character. (Data taken from [20]).

From 1983 to 2002, the percentage of racemates decreased from 32% to 8%, while that of single enantiomer drugs increased from 25% to 58%. Not surprisingly, chiral drugs also make major contributions to the annual revenues of pharmaceutical companies. A recently disclosed ranking of the best selling drugs in 2003 placed nine chiral drugs amongst the top ten performers, with six of them being commercialized as single enantiomers [21].

7.2
The Role of Enantioselective Analysis in Drug Development

The strict regulatory issues concerning chiral drugs have created an urgent demand for efficient technologies for the preparation of enantiomerically pure compounds and dedicated analytical assays for assessing their quality. Enormous research ef-

forts in academic and industrial settings led to significant progress in the areas of asymmetric synthesis [22] and enantioselective biotransformation [23–26] providing an economic basis for large-scale production of enantiomerically pure drug candidates. Simultaneously, major advances have been achieved in the field of analytical chromatographic enantiomer separation methodologies [27–29] establishing a rich repertoire of analytical tools for determination of enantiomer purity.

Enantioselective analytical assays play a key role in the development of chiral drug candidates throughout the entire development cycle. During lead discovery and optimization of active chiral substances enantioselective analytical methods are required to monitor the outcome of asymmetric reactions in which the crucial chiral center(s) are created, and to ensure that the stereochemical integrity is maintained throughout subsequent synthesis steps. In cases where the starting materials already contain the desired chiral centers, initial stereochemical purity must be established by employing validated enantioselective assays [30, 31]. Similar requirements apply to cases in which resolution strategies are deployed to isolate the desired stereoisomer(s) of the active compound(s) [32]. Resolution steps are considered an integral part of the manufacturing process, and the stereochemical purity of the starting materials and intermediates must be assessed for each enrichment cycle. Sensitive enantioselective analytical procedures must also be available to aid the specification of single enantiomer drug candidates. When a chiral drug is presented as a single enantiomer, the unwanted enantiomer is considered an impurity, demanding identification and accurate quantification. In the course of pre-clinical drug assessment, sensitive bioanalytical assays are mandatory to assess the enantiospecificity of toxicological, pharmacokinetic and pharmacodynamic activity profiles, to monitor the formation of chiral metabolites, and to detect potential configurational interconversion [11]. Enantioselective assays must also be implemented in the chemical development issues to guarantee the identity and purity of the active substance before batch release. Finally, suitable enantioselective analytical protocols must be available for quality control of the formulated drugs, demonstrating that the manufacturing process induces no unacceptable changes in enantiomer purity of the final products, and that stereochemical integrity may be maintained for the proposed shelf-lives.

7.3 Separation Techniques in Enantiomer Analysis

Modern drug development campaigns are extremely time- and capital-intensive endeavors, with time-to-market spans up to 12 years and total investment volumes often exceeding 800 million US dollars [33]. The competitive climate in the drug market, the failure of drug development efforts in late stages, and drug prize regulation initiatives all place significant pressure on pharmaceutical companies to enhance the productivity of their pipelines. To achieve this, the pharmaceutical industry has adopted high throughput technologies for synthesis and screening, allowing fast creation and assessment of enormous numbers of drug candidates

within short time periods. For enantioselective analytical assays to be implemented harmonically into this high throughput machinery, they must combine the features of high sensitivity, chemical selectivity and robustness with the added benefits of low sample requirements, high throughput capacity and automation potential. Traditional analytical off-line methods for enantiomer purity determination such as polarimetry or NMR-based assays using chiral solvating agent [34], require often time-consuming sample preparation and mg-amounts of analytes, and therefore are unsuitable for application in modern drug development. In contrast, high resolution separation techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC), capillary chromatography (CE) and capillary electrochromatography (CEC), satisfy many of the crucial demands and are routinely employed methods of choice in drug development settings. HPLC-based enantioselective assays take a dominating role among these methodologies. This privileged position of HPLC-based chiral assays is particularly evident from the number of dedicated publications in the scientific literature. Figure 7.2 shows the results of a literature survey performed in the SciFinder database, using the search statement "chiral separation", and the key words "HPLC", "gas chromatography", "capillary electrophoresis" and "capillary electrochromatography" for subsequent refinement cycles. The comparison of the hit numbers per year identifies HPLC as the most popular enantiomer separation technology, followed by CE-, GC- and,

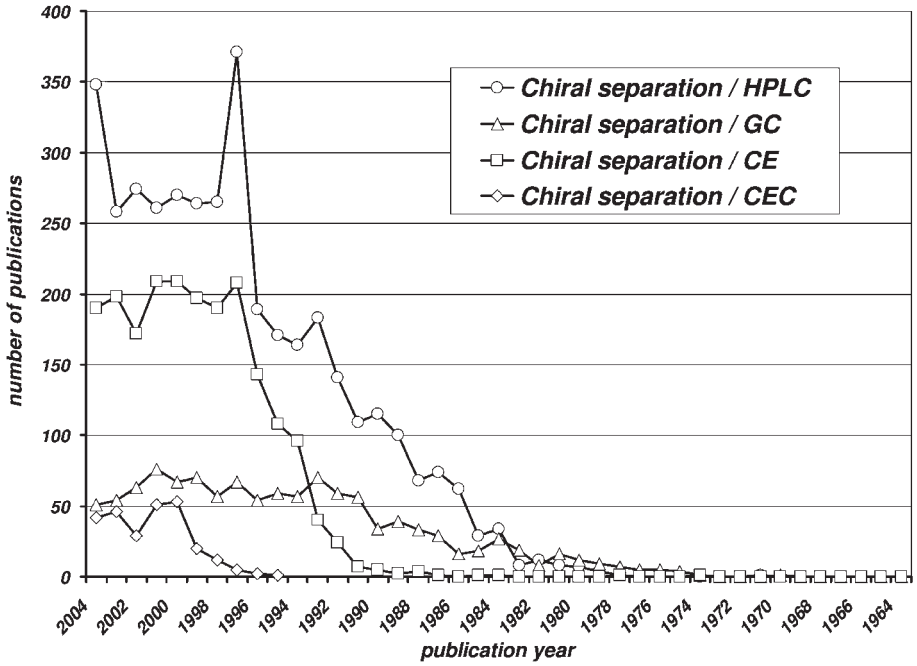


Fig. 7.2 Overview of the number of scientific publications in the field of enantiomer separation employing different separation techniques for the time period 1964–2002. (Data retrieved from the SciFinder Database).

finally, CEC-related techniques. In addition, the interest in HPLC-based enantiomer separation technologies appears to be growing, while the number of publications devoted to GC-, CE- and CEC-based assays is stagnant or even in decline.

The popularity of HPLC-based enantioselective assays in the drug development field is rooted in a number of reasons. One may be a preference of analytical chemists to stick with familiar technologies rather than take chances with new ones. Technical considerations may be another motivation to favor HPLC-based assays over alternative methods. Thus, HPLC instrumentation of high technical standard is readily available at reasonable cost, offers high flexibility through a broad array of detection systems and nowadays has routinely integrated features for automation. A major benefit of HPLC-based assays is their ability to address an immensely broad spectrum of analytes across the entire physicochemical property space. As compared to HPLC, the applicability of GC methodologies is restricted to apolar or medium polar low-molecular-weight analytes showing sufficient levels of volatility and thermal stability [35, 36]. Limitations in applicability are also found for electro-driven CE- and CEC-based enantiomer separation technologies, which are most useful for polar charged or ionizable analytes [37, 38]. No such restrictions, in principle, exist for HPLC-based enantioselective assays. Taking advantage of the immensely rich range of dedicated chiral stationary phases, efficient HPLC assays can be developed for analytes with pronounced differences in physicochemical property profiles, encompassing extremely polar, permanently charged, neutral or highly apolar compounds.

A striking advantage of HPLC-based over GC- and CE/CEC-based protocols is their ready scalability. Generally, chromatographic enantiomer separation protocols developed for analytical applications can conveniently be transformed into preparative procedures, providing expeditious access to enantiomerically pure drug candidates [27]. Particularly in the early development stages, where time constraints prevail over cost considerations, chromatographic enantiomer separation is appreciated as the most efficient way to produce enantiomerically pure drug candidates in gram- to kg-amounts. Recent advances in chromatographic process engineering have adapted simulated moving bed (SMB) technology to enantiomer separation [28, 39]. This continuously operating chromatographic technology allows enantiomer separation at the multi-ton scale, at costs that can compete with traditional manufacturing options. A more detailed account of the role of preparative enantiomer separation in modern drug development can be found in Chapter 6.

7.4 Principle of Enantiomer Separation

Enantiomers are molecular entities that exist in mirror image-like, non-superimposable structures. In isotropic environments enantiomers exhibit identical physicochemical properties, and therefore are indistinguishable under these conditions. On transfer of a pair of enantiomers into asymmetric environments, how-

ever, two diastereomeric species are formed with distinct physicochemical property profiles, on the basis of which physical separation into individual enantiomers may be achieved. For practical purposes, the crucial "asymmetric environment" required to render enantiomers distinguishable is provided in the form of a chiral auxiliary (chiral selector, SO), capable of undergoing covalent (chemically stable) or noncovalent (transient) interactions with the individual enantiomers (selectands, SAs). Depending on the nature of the interactions stabilizing the respective diastereomeric SO–SA species, enantiomer separation strategies are categorized as *indirect* or *direct* enantiomer separation approaches.

7.4.1 Indirect Enantiomer Separation

Indirect enantiomer separation involves the transformation of the SAs of interest into *covalent diastereomers* by conversion with suitably reactive SOs, so-called chiral derivatization agents (CDAs), followed by the separation of the diastereomeric products with achiral chromatographic techniques. Given the longstanding tradition of indirect enantiomer separation methodology, a rich range of CDAs has been established [40–45]. CDAs based on carboxylic acid anhydrides and chlorides, chloroformates, isocyanates, isothiocyanates, activated fluoroaromates and amines are available to convert chiral drugs presenting amino, carboxyl and hydroxy functionalities into the corresponding amide, ester, urea, thiourea or aryl type diastereomers.

The main attraction of the indirect methodology consists in the possibility of using *conventional achiral stationary/mobile phase systems* for separation of the created diastereomers, offering higher chromatographic efficiencies and being generally less expensive than their chiral counterparts. An additional benefit associated with the indirect approach is the opportunity to introduce conveniently CDA-attached chromophoric, fluorophoric or electrochemically active labels into otherwise difficult-to-detect analytes.

From an analytical viewpoint, however, indirect enantiomer separation approaches suffer from several inherent limitations [40]. Principally, indirect methodologies are applicable only to enantiomers presenting a single (or more, but selectively addressable) functional group suitable for derivatization. The chiral derivatization reaction *per se* has to be performed under mild conditions to ensure the stereochemical integrity of the enantiomeric analytes and the CDA. It is essential to drive the derivatization reaction to completion to preclude any "apparent" shifts in the observed enantiomer ratio due to kinetic resolution phenomena. It is also important to recognize that the "resolution" of indirect methods, i. e. the lower limit of detectable enantiomeric excess, is strictly defined by the enantiomeric purity of the employed CDAs. In many commercial CDAs, however, the enantiomeric purities are not specified or are less than 99% [30, 31], precluding the possibility of employing them for the development of accurate enantiomer trace analysis protocols. In context with quantification it must be considered that the analytical species separated are not enantiomers but diastereomeric in nature, and therefore may give rise

to different detector responses. Another limiting aspect of the indirect method is encountered in analytical application within biological matrices; in these environments, the routinely employed excess of CDA can co-derivatize matrix components, creating a forest of background signals which may lead to sample obscuration.

Indirect enantiomer separation protocols have seen intense use in the pioneering days of chiral drug research development, and certainly have helped establish much of the seminal knowledge on enantioselective actions of chiral drugs [40, 41, 45]. Nowadays, however, the popularity of indirect analytical approaches is in decline. The numerous issues that have to be addressed in the course of method validation render the development of indirect enantiomer separation assays a time-consuming and labor-intensive endeavor, hardly compatible with the high throughput requirements of current drug discovery. With the advent of broadly applicable chiral stationary phases, capable of separating the enantiomers of most drugs without the need for prior derivatization, indirect assays may have largely outlived their formerly important role in drug development. The unique opportunity of introducing highly sensitive tags into difficult-to-detect analytes in the presence of complex matrices [42], however, may further ensure a niche for indirect enantiomer separation assays in drug development applications in the future.

7.4.2 Direct Enantiomer Separation

Direct enantiomer separation methodologies circumvent the rather laborious formation of covalent diastereomers, but instead exploit subtle energetic differences of *reversibly formed, noncovalent diastereomeric complexes* for chiral recognition. Direct chromatographic enantiomer separation may be achieved in two different modes, the *chiral mobile phase additive* and the *chiral stationary phase* mode.

7.4.2.1 Chiral Mobile Phase Additives (CMPA)

In chiral mobile phase additive mode enantiomer separation, a *combination of an achiral stationary and a chiral mobile phase* is employed, the latter being created by simply adding a certain amount of an appropriate SO to the eluent. On introduction of a mixture of enantiomers into this system, the individual enantiomers form diastereomeric complexes with the chiral mobile phase additive (CMPA). These transitory diastereomeric complexes may exhibit distinct association/dissociation rates, thermodynamic stabilities and physicochemical properties, and therefore may be separated on an appropriate achiral stationary phase.

CMPA-based enantiomer separation techniques appear attractive from the viewpoints of conceptual simplicity and flexibility as they operate with relatively inexpensive achiral stationary phases and easy-to-prepare chiral mobile phases. In practice, the development of CMPA-based analytical assays may pose a considerable challenge for a number of reasons. In the course of the development and optimization of CMPA-based enantiomer separations a set of conditions must be identified that favor CMPA–analyte interactions and simultaneously maximize

enantioselective distribution into the achiral stationary phase. The underlying association/distribution processes are governed by complex equilibria between the interacting components. The factors governing these multiple interactions are poorly understood, rendering the optimization of CMPA-based enantiomer separation protocols a difficult task. Additional difficulty arises from the fact that these complex systems of interdependent secondary equilibria are very sensitive even to minor interference. Thus, sample injection in a solvent system other than the chiral mobile phase employed, or minor fluctuation in column temperature, may severely affect analyte retention, enantioselectivity and peak shapes. The use of spectroscopically non-transparent CMPAs may compromise detection sensitivity. Moreover, enantiomers separated under CMPA-conditions may eventually elute in the form of diastereomeric SO–SA complexes, and these may exhibit distinct spectroscopic properties and therefore different detector responses. This situation certainly complicates analyte quantification.

Despite these evident drawbacks, a broad variety of SOs have been used in CMPA-based enantiomer separations, including cyclodextrins, proteins, macrocyclic antibiotics, chiral ion-pairing agents, amino acids in combination with transition metal salts, and crown ethers. Recent application for the separation of pharmaceutically relevant chiral compounds utilized β-cyclodextrins [46–48] charged cyclodextrins [49, 50], macrocyclic antibiotics [51, 52] and chiral ion-pairing agents [53, 54]. A more detailed discussion of CMPA-based enantiomer separation is beyond the scope of this chapter. The interested reader is referred to dedicated reviews [55, 56].

7.4.2.2 **Chiral Stationary Phases**

The most convenient and most popular analytical methodology to assess enantiomer purity is the *direct separation of enantiomers on so-called chiral stationary phases (CSPs)*. CSPs consist of an (ideally) inert chromatographic support matrix incorporating chemically or physically immobilized SO species. CSPs may be created by a variety of SO immobilization techniques, including (i) covalent attachment onto the surface of suitably pre-functionalized carrier materials, (ii) physical fixation employing coating techniques, and (iii) incorporation into polymeric networks by copolymerization, or combinations of these procedures.

The permanent confinement of chiral interaction sites to the stationary phase in CSPs provides several operational advantages over CMPA-based enantiomer separation protocols. The anchoring of the SO species onto the stationary phase creates extremely robust tools that can tolerate and successfully perform under a broad range of chromatographic operating conditions. The enhanced stability of CSPs offers considerable flexibility with respect to method optimization parameters, allowing for a wide range of mobile phase solvents and modifiers, but also gradient elution and variable temperature protocols. The possibility of achieving on CSPs enantiomer separation with achiral (i.e. SO-free) mobile phases provides important benefits with respect to analyte detection and quantification. The species eluted after a successful separation from a CSP are *enantiomeric* in nature,

exhibiting *identical spectroscopic properties* with achiral detection systems. Consequently, the recorded detector responses can be used directly to compute enantiomer purity without requiring additional calibration. In addition, the use of mobile phases devoid of SO species diminishes background signal influences and thus improves sensitivity and detection limits as compared to related CMPA-based approaches. This issue is of particular relevance in the context of analytical applications employing mass-sensitive detection systems, for which high concentrations of interfering mobile phase components may induce ion suppression and thus loss in sensitivity.

It also needs to be emphasized that it was the development of robust and broadly applicable CSPs that has laid the foundations for economic chromatographic enantiomer separation on a preparative scale. Although indirect [57–62] and CMPA-based direct [63–65] chromatographic methodologies have seen some use in preparative enantiomer separation, the considerable efforts associated with chemical manipulation and/or recovery of the products render these approaches economically unattractive [66]. Preparative enantiomer separations employing CSPs are not subject to these limitations. With CSPs enantiomers can be processed directly (i.e. without prior derivatization) with readily volatile achiral mobile phases (devoid of SOs), simplifying product recovery to a trivial solvent evaporation step.

7.5 Molecular Requirements for Chiral Recognition

To facilitate the understanding of chiral recognition at the molecular level, a number of attempts have been made to define "minimum criteria" in terms of the required intermolecular SO–SA interactions [67–74]. The currently most widely accepted concept is known as the *three-point rule* [67]. This rule states that for *chiral recognition to occur a minimum of three simultaneous interactions between SO and at least one of the enantiomers is required, with at least one of these interactions being stereochemically dependent*. This concept is graphically illustrated in Fig. 7.3.

On approaching the SO, each of the SA enantiomers can readily establish a set of two interactions with the SO. Due to the different orientations of their functional sites in space, however, only one of the enantiomers is capable of simultaneously undergoing a third interaction with the SO. Thus, chiral recognition between the SA enantiomers and the SO is ultimately achieved by the presence or absence of this crucial *stereochemically dependent* interaction.

Confusion often arises due to misinterpretation of the term *interaction* within the conceptional framework of the three-point rule. It is important to understand that in this particular context *interaction* refers to intermolecular physical forces and their steric implication rather than to specific spatial relationships between substructure elements in the SO and SA entities. This distinction is crucial as intermolecular forces, depending on their physical nature, may be of *single-point* or of *multi-point* quality. For example, forces acting exclusively between specific

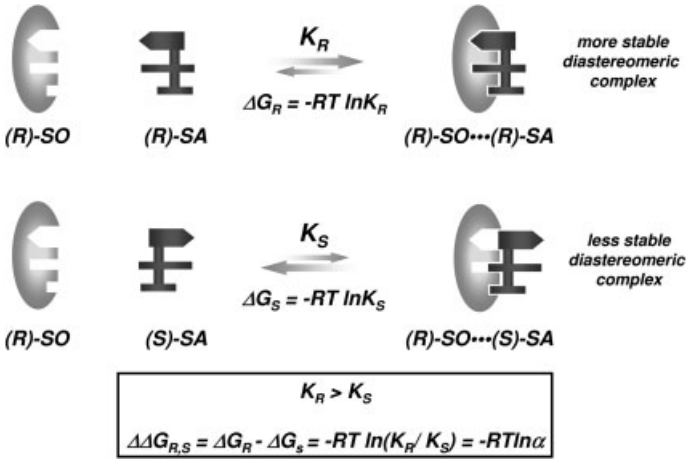


Fig. 7.3 Schematic representation of the thermodynamic principle of chiral recognition.

atoms of SO and SA (hydrogen bonding or end-to-end dipole interactions) may be rightfully classified as *single-point* interactions. Intermolecular forces coupling linear or planar functionality (dipole–dipole stacking and face-to-face π–π-interaction) between SO and SAs impose two-dimensional constraints on the resultant supramolecular assemblies, and therefore must be classified as *multi-point* interactions. Accordingly, the minimum requirements for chiral recognition may be satisfied by two simultaneous intermolecular SO–SA contacts only, provided that they combine the features of a single-point and a two-point interaction (e.g. hydrogen bonding and face-to-face π–π-stacking). It must be emphasized that within the conceptual framework of the three-point rule not all three required interactions need to be attractive; steric repulsion emanating from substructure elements of the SO and/or SAs may effectively contribute to chiral recognition.

7.6 Thermodynamic Principles of Enantiomer Separation

Analytical-scale enantiomer separations on CSPs are usually carried out under linear (non-overloaded) chromatographic conditions, under which the retention of the individual enantiomers can be expressed as

$$k_R = \phi K_{H,R} \tag{7.1}$$

$$k_S = \phi K_{H,S} \tag{7.2}$$

where k_R and k_S represent the corresponding retention factors, and $K_{H,R}$ and $K_{H,S}$ the corresponding equilibrium distribution constants (enantiomer concentration

in the stationary phase divided by the enanationer concentration in the mobile phase) of the (*R*) and (*S*)-enantiomer, respectively, and ϕ represents the volume phase ratio (V_s/V_m).

The chromatographic enantioselectivity $\alpha_{R,S}$ is given by the ratio of the retention factors observed for the individual enantiomers,

$$\alpha_{R,S} = k_R/k_S \tag{7.3}$$

By convention, $\alpha_{R,S}$ is calculated by dividing the retention factor of the more retained enantiomer by that of the less retained enantiomer ($k_R > k_S$).

During the chromatographic process, the reversible binding (and release) of enantiomers from the mobile phase to SO sites in the CSPs is usually fast relative to the chromatographic time scale, and is essentially under thermodynamic control. The magnitude of $\alpha_{R,S}$ can be related to the difference of the free energies ($\Delta\Delta G_{R,S}$) of the diastereomeric association equilibria between chiral SO and the SAs

$$\Delta\Delta G_{R,S} = -RT \ln \alpha_{R,S} \tag{7.4}$$

Equation (7.4) implies that useful levels of enantiomer separation may even be achievable with very small $\Delta\Delta G_{R,S}$-values. For example, at ambient temperature (298 K) a free energy difference as small as 240 J mol^{-1} corresponds to $\alpha_{R,S} = 1.1$, a selectivity that may suffice to achieve baseline separation of enantiomers, even on moderately efficient chromatographic systems.

For a more detailed discussion of the thermodynamic driving forces and the impact of temperature on enantiomer separation it is useful to rewrite Eq. (7.4) in the form of the corresponding Gibbs–Helmholtz equation

$$\Delta\Delta G_{R,S} = -RT \ln \alpha_{R,S} = \Delta\Delta H_{R,S} - T\,\Delta\Delta S_{R,S} \tag{7.5}$$

$$\ln \alpha_{R,S} = -\Delta\Delta H_{R,S}/RT + \Delta\Delta S_{R,S}/R \tag{7.6}$$

where $\Delta\Delta H_{R,S}$ and $\Delta\Delta S_{R,S}$ in Eqs. (7.5) and (7.6) represent the enthalpic and entropic components of enantioselective binding, *R* the general gas constant and *T* the absolute temperature in Kelvin. Experimentally, these quantities for a given SO/SA combination may conveniently be derived by measuring $\alpha_{R,S}$ over a range of temperatures. The emerging data set can then be subjected to van't Hoff analysis, plotting the natural logarithm of the corresponding enantioselectivity data ($\ln\alpha_{R,S}$) versus the reciprocal absolute temperature ($1/T$). This treatment usually provides linear relationships from which the magnitudes of $\Delta\Delta H_{R,S}$ and $\Delta\Delta S_{R,S}$ can be extracted from the slope ($-\Delta\Delta H_{R,S}/R$) and the intercept ($\Delta\Delta S_{R,S}/R$), respectively. For the majority of SO–SA systems employed in CSP-based enantiomer separation, $\Delta\Delta H_{R,S}$ and $\Delta\Delta S_{R,S}$ show opposing effects on $\Delta\Delta G_{R,S}$. The $\Delta\Delta H_{R,S}$ contributions to enantioselective binding tend to be negative, favoring chiral recognition, while the corresponding $\Delta\Delta S_{R,S}$ components generally take positive values,

counteracting chiral recognition. The negative values for $\Delta\Delta H_{R,S}$ emphasize the crucial stabilizing role of short range interactions in SO–SA association; positive $\Delta\Delta S_{R,S}$ values are a manifestation of the energetic costs associated with bimolecular complexation due to losses in rotational and translational freedom. These opposing thermodynamic trends are responsible for the fact that chiral recognition on CSPs is usually *enthalpically controlled*, a situation that becomes evident in a *decrease in enantioselectivity with increasing temperature*. However, the opposite behavior has also been observed on a few occasions, with the level of enantioselectivity being improved on increasing the temperature. These rare cases of *entropically controlled* chiral recognition are preferentially found with macromolecular (polysaccharide- and protein-type) SOs, and are believed to arise from excessive binding-induced desolvation phenomena [75] and/or conformational changes in the backbones of the SOs [76, 77].

It needs to be recognized that deriving thermodynamic information on enantioselective binding directly from chromatographically observed retention data is an oversimplistic approach, assuming that SA retention originates entirely from specific SA interactions with immobilized SO sites. However, the surfaces of most CSPs are strongly heterogeneous in nature. In addition to enantioselective SO sites, CSP surfaces usually harbor a considerable amount of excess functionality, emanating from the supporting matrix, linker groups and spacer units, and this may undergo nonspecific interactions with the SA species. The binding affinity of these nonspecific sites for the SAs is usually lower than that of the enantioselective SO sites. The density of the nonspecific interaction centers, however, is generally much higher than that of the enantioselective ones, and therefore nonspecific interactions may contribute significantly to overall SA retention. Thus, it is important to recognize that experimentally observed retention data reflect *the sum of non-specific and specific binding increments* rather than those of the enantioselective sites alone. Nonspecific increments to overall retention become significant for CSPs derived from low-affinity type SOs or for CSPs with sparse SO surface density. In these cases, a direct van 't Hoff analysis of experimentally observed retention data may produce *apparent* rather than *true thermodynamic quantities*, the mechanistic interpretation of which may give rise to erroneous conclusions [78].

An interesting strategy for deconvoluting the relative contributions of enantioselective (SO-mediated) and nonspecific retention increments has been proposed by Guiochon and coworkers [79–81]. This approach entails (i) the measurement of the adsorption characteristics of SAs on the CSP of interest and (ii) the fitting of these corresponding isotherms to adsorption models accounting for the existence of specific and nonspecific sites. With the fitted coefficients in hand, one can quantify the *densities* and *energies* of both the *selective* and *nonselective binding sites* coexisting on the CSP surface. With this set of information the relative contributions of specific/nonspecific sites to overall SA retention *true enantioselectivity data* can be calculated, and may be used for a thermodynamically meaningful analysis of the underlying chiral recognition process.

Guiochon et al. have also demonstrated that the surface heterogeneity encountered in CSPs presents the primary source of the relatively poor efficiency and

peak tailing phenomena frequently observed with this type of adsorbents [82, 83]. In a series of studies, these workers modeled the consequences of heterogeneous mass transfer and thermodynamics on the quality of peak shapes. These modeling studies were based on the assumption that SA association/dissociation processes at nonspecific (low-affinity) sites should be considerably faster than those at (high-affinity) enantioselective sites [82]. This scenario is a reasonable one within the generally accepted mechanistic view of chiral recognition, which evokes the formation of relatively long-lived SO–SA complexes due to multiple stabilizing, concerted and stereochemically well-defined interactions. Simulation considering linear (i.e. non-overloaded) chromatographic conditions produced band shapes exhibiting a significant level of peak tailing. The authors pointed out that this type of tailing presents an entirely kinetic manifestation of surface heterogeneity, being independent of sample size, and will be seen for all chromatographic processes involving heterogeneous mass transfer. In subsequent work the authors modeled the combined consequences of heterogeneous mass transfer kinetics and sample overload. [83]. In this case the larger sample sizes saturate preferentially the high-affinity enantioselective binding sites, which then no longer exhibit linear isotherm behavior. Modeling of this chromatographically more relevant scenario produced enhanced levels of peak deterioration, consistent with the combined deleterious effects of heterogeneous adsorption thermodynamics and mass transfer kinetics.

7.7 Role of Mobile Phase in Enantiomer Separation

The molecular recognition processes underlying liquid chromatographic enantiomer separation are often discussed considering bimolecular SO–SA interaction only. While this simplistic treatment is convenient for theoretical purposes, this bimolecular view neglects the important role of the mobile phase environment in chiral recognition events. In context with liquid chromatographic enantiomer separation the mobile phase environment is far from being just a passive transporter for the SAs, it is an essential component inherently involved in enantioselective SO–SA association at multiple levels. In fact, the nature of the mobile phase defines the degree of solvation of the immobilized SO and the free SA species, and thus the "energetic barrier" that must be overcome in the course of diastereomeric adsorbate formation. The components of the mobile phase, such as bulk solvents, modifiers, buffer salts and additives, not only dictate the conformational preferences of (flexible) SO and SA molecules but also their ionization status (for charged compounds), and therefore the degree to which their functional group repertoires are capable of complementary intermolecular interactions. Evidently, proper choice of mobile phase media represents a quintessential step in the development and optimization of any chromatographic enantiomer separation protocol. A careful tuning of the mobile phase environment is required to maximize the delicate energetic balance between the diastereomeric SO–SA adsorbates over

the competing background of solvent–SO and solvent–SA interactions. Also, appropriate mobile phase selection is instrumental in suppressing nonspecific enantioselectivity-degrading binding, such as unproductive SA adsorption onto supporting matrices, spacer units and linker functionalities.

All important mobile phase regimes have successfully been employed in liquid chromatographic enantiomer separation, including normal-phase, reversed-phase, polar-organic and supercritical fluid modes. The ideal choice of mobile phase conditions for liquid chromatographic enantiomer separation, however, depends on the nature of the chiral recognition mechanisms of the particular CSP employed. Suitable mobile phase media should support all types of noncovalent interactions involved in enantioselective SO–SA association while keeping the influence of nonspecific interactions to a minimum. Restriction in mobile phase selection may be enforced by CSP stability issues, e. g., precluding conditions that would irreversibly damage the SO molecules, compromise the physical linkage between SO and supporting matrix, or attack the underlying carrier material.

Normal-phase mode mobile phases typically consist of apolar organic bulk solvents (most often hydrocarbons, such as hexane or heptane) and low percentages of polar modifiers (alcohol, ethers, and dioxane). Normal-phase media are characterized by low polarity, and therefore are capable of stabilizing polar and dipolar intermolecular SO–SA interactions, such as hydrogen bonding-, dipole–dipole- and π–π-stacking interactions. On the other hand, intermolecular hydrophobic interactions are effectively disrupted by normal-phase mobile phases. Due to these specific molecular recognition characteristics, normal-phase media represent ideal mobile phase environments for π-donor/acceptor- and polysaccharide-type CSPs, the molecular recognition mechanisms of which largely capitalize on hydrogen bonding-, dipole–dipole- and π–π-stacking SO–SA interactions.

Reversed-phase mode mobile phases are generally composed of mixtures of aqueous buffer systems and water-soluble polar organic solvents (acetonitrile, alcohols, THF). From a molecular recognition viewpoint, reversed-phase mobile phases are highly polar environments, competing strongly with all types of polar SO–SA interactions; thus, solution-exposed intermolecular hydrogen bonds and dipole–dipole stacking motifs are readily disrupted under reversed-phase conditions. Long-range electrostatic interactions between charged SO/SA structural motifs may still persist under these conditions, but can be attenuated by the pH and nature of the buffer salts. Importantly, reversed-phase media strongly favor hydrophobic interactions, i. e. the intermolecular aggregation of apolar SO–SA structure motifs, such as hydrocarbon chains and extended aromatic systems. This particular type of attraction is believed to be driven by an entropically favorable displacement of ordered water molecules from the interfaces of the interacting hydrophobic batches. Under reversed-phase conditions the relative strength of hydrophobic SO–SA interactions can conveniently be tuned by changing the amount of organic modifier, with an increasing modifier content diminishing hydrophobic adhesion. In chromatographic enantiomer separation, reversed-phase mobile phase conditions are preferentially employed with SOs binding enantiomers via a combination of electrostatic and hydrophobic interaction forces, such as bioaffinity- (anti-

bodies, aptamers), glycopeptide-, crown ether-, ion exchange- and ligand exchange-type CSPs.

Polar-organic mode mobile phases are defined as media consisting of polar organic bulk solvents, such as acetonitrile or methanol and small amounts of organic acids and bases. With respect to their molecular recognition properties, polar-organic mobile phases take an intermediate position between normal-mode and reversed-phase media. Polar-organic environments can be expected to suppress effectively hydrophobic SO–SA interactions. The enhanced polarity of the constituting bulk solvents will attenuate, to some extent, all polar interactions, but still preserve strong electrostatic attraction between SO/SA substructure elements, such as hydrogen bonding and ion pairing. Acidic and basic additives in polar-organic environments help to control the ionization status of charged SO/SAs by protonation and/or support the formation of well-defined ion pairs. Polar-organic mobile phases have proved advantageous with a broad assortment of CSPs, including systems based on cyclodextrin- [84, 85], glycopeptide-, ion-exchange- [86] and with polysaccharide-type [87, 88] SOs. An important benefit of polar-organic mode media might be their capacity to simultaneously suppress nonspecific interactions of a hydrophobic as well as polar nature, which are frequently encountered with multifunctional SOs derived from natural compounds.

Supercritical fluid (SFC) media present a highly attractive mobile phase option for liquid chromatographic enantiomer separation [89–93]. Rather than using classical solvent systems, SFC operates with gases kept under supercritical fluid conditions as the bulk mobile phase environment. Currently, carbon dioxide is the most popular SCF medium for chromatographic application, mostly due to its convenient supercritical temperature/pressure characteristics (critical pressure 73.8 bar, critical temperature: 31.1 °C), low toxicity, ready availability and reasonable cost. Inherent to all supercritical fluid media are high diffusivity and low viscosity, features that translate chromatographically into short column equilibration times, low backpressures and fast mass transfer rates. These benefits permit SFC enantiomer separation to be carried out at much higher flow rates than with conventional solvents, resulting in higher analytical (preparative) throughput and vastly enhanced separation efficiency. However, an inherent limitation of supercritical carbon dioxide is its low polarity, which compares to that of pentane. The low polarity of supercritical carbon dioxide can be enhanced to some extent by increasing the temperature and pressure. However, increased pressure results in rather incremental, often insufficient improvements for polar SAs. Addition of polar modifiers, such as methanol or acetonitrile was found to enhance the overall polarity sufficiently to provide chromatographically useful mobile phases. The presence of higher concentrations of polar modifiers often shifts SCF mobile phases from the supercritical to the subcritical regime; fortunately, even at subcritical conditions the advantageous enhanced fluidity characteristics are largely retained. With dedicated instrumentation becoming more readily available, SFC mode enantiomer separation has gained significant popularity. A broad range of chiral SOs has been demonstrated to operate successfully under SFC conditions, including π-donor/acceptor-, cyclodextrin-, polysaccharide-, glycopeptide-type CSPs.

A considerable number of papers dedicated to SFC-based enantiomer separation have been published [89–92], yet little is known on the mode of chiral recognition under these conditions. SFC appears to be capable of reproducing many chiral separations accomplished under normal-phase conditions, but with superior efficiency and in some cases enhanced selectivity. This suggests that the chiral recognition modes operating in SFC may follow mechanisms closely related to those operative under normal-phase conditions.

Many types of CSPs have been demonstrated to express "multimodal" operation capacity, i.e. chiral recognition properties under different mobile phase regimes, although with different levels of success. CSPs based on enantioselective bioaffinity systems (proteins, antibodies and aptamers) present exceptions in that they are restricted to reversed-phase mobile phases. On the contrary, polysaccharide-, glycopeptide- and cyclodextrin-type CSPs have been shown to be fully compatible with all of the discussed mobile phase regimes. For a given CSP/SA combination switching between alternative mobile phases, however, may activate alternative chiral recognition mechanisms, leading to inversion of the enantiomer elution order.

7.8 Chiral Selectors and Chiral Stationary Phases Employed in Liquid Chromatographic Enantiomer Separation

In the course of the development of CSPs, a broad variety of chiral molecules (and materials) has been the subject of scrutiny with respect to chromatographic enantiomer separation capacity. The chiral molecules studied as potential SOs cover virtually the entire chemical and structural diversity space, ranging from low-molecular-weight compounds to polymers of both synthetic and biological origin. So far, the (still ongoing) quest for efficient SOs has resulted in the synthesis of more than 1400 CSPs [94], the properties of which are documented in an almost intractable number of dedicated scientific publications. The outcome of these efforts is manifest in an enormously rich toolbox of more than 200 commercially available CSPs offered by various specialized suppliers.

With this overly rich arsenal of tools in place the development of chromatographic enantiomer separation methods may pose formidable challenges even for well-trained analytical scientists. Specifically, identification of the most suitable CSP/mobile phase combination for a particular analyte and the optimization of the enantiomer separation may present problems beyond routine. In contradistinction to conventional achiral chromatographic materials, for which the selectivity characteristics typically emanate from a singular dominating intermolecular interaction, enantioselective retention on CSPs is governed by mechanisms of enhanced complexity, requiring the concerted establishment of multiple SO–SA interactions. For the majority of SOs the understanding of these delicate "multidimensional" chiral recognition mechanisms is still at an immature level, despite much dedicated research. This general lack of insight into the molecular level re-

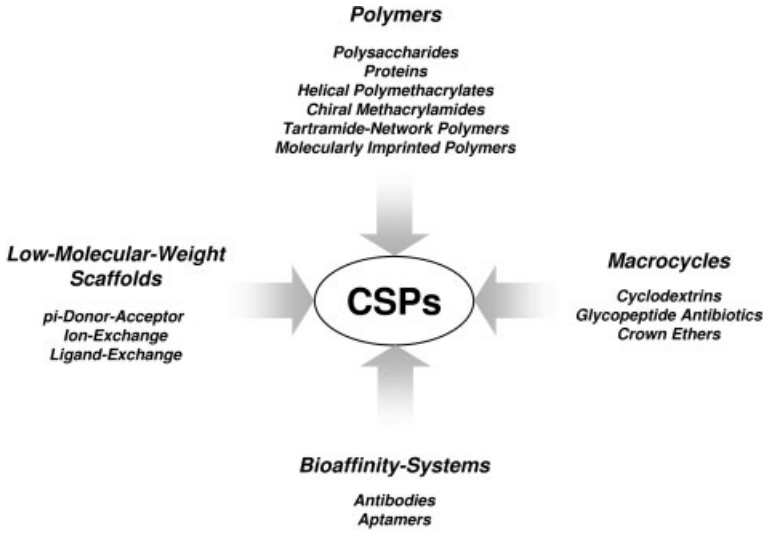


Fig. 7.4 Overview of the major classes of chiral selectors incorporated in chiral stationary phases for liquid chromatographic applications.

quirements of chiral discrimination seriously hampers the advancement of generally applicable rational approaches to CSP selection and reliable predictions of suitable operating conditions.

Therefore, the intent of the following section is to familiarize novices in the field of chromatographic enantiomer separation with the properties of SO systems integrated in the most successful classes of commercial CSPs. In this context important application-relevant aspects of their chiral recognition capabilities will be highlighted, with emphasis being placed on their preferential scope of application and compatibility towards various mobile phase regimes. The primary SO–SA binding mechanisms will be discussed, along with the specific experimental parameters allowing manipulation retention, enantioselectivity and elution order. Advances in the mechanistic understanding of the molecular level requirements of chiral recognition of the individual classes of SOs will also be discussed.

To provide a systematic framework for the subsequent discussion, the CSPs and the underlying SO systems have been categorized into four major groups, using structure-specific features and the molecular weight range as classification criteria (see Fig. 7.4). These groups comprise CSPs derived from polymers of synthetic and biological origin, CSPs integrating macrocyclic scaffolds, CSPs produced from low-molecular-weight molecules and CSPs exploiting bioaffinity as chiral SO.

7.8.1 CSPs Derived from Polymers

7.8.1.1 CSPs Derived from Natural Polymers

7.8.1.1.1 Polysaccharide-type CSPs

The observation of enantioselective binding properties of polysaccharides dates back to the early 50s. At this time Kotake [95] and Dalgliesh [96] achieved thin layer chromatographic separation of amino acid enantiomers on cellulose carriers. However, the poor chiral recognition capacity of native polysaccharides hampered further developments.

Seminal progress was achieved in the early 1970s by Hesse and Hagel, who prepared microcrystalline cellulose triacetate (MCTA) by a heterogeneous reaction procedure. MCTA turned out to be an efficient enantioselective adsorbent, showing favorable preparative loading capacity when operated with water–methanol mixtures. MCTA has been commercialized under the tradenames CTA-I (Merck) and CHIRALCEL CA-1 (Daicel Chemical Industries, Ltd.). A large variety of racemic compounds could be resolved on MCTA, with those containing aromatic substituents showing particularly high enantioselectivities [97, 98]. Dissolution of the microcrystalline material and re-precipitation, however, was found to severely compromise the initial performance characteristics, suggesting that chiral cavities preserved in the microcrystalline structure of MCTA may act as crucial enantioselective binding sites. Addressing the relatively poor mechanical stability of MCTA, Okamoto and coworkers prepared carrier-supported CSP versions by coating MCTA from solutions onto the surface of chemically modified macroporous silica gels [99]. The resulting adsorbents exhibited a radically changed chiral recognition profile, with reversed elution order for some types of analytes. The coated version of MCTA exhibited, however, considerably improved mechanical stability, allowing its use in pressure-driven chromatography.

Expansion of these investigations to other types of polysaccharide derivatives led to the development of silica-coated versions of cellulose and amylose carbamates and benzoates [100–102]. These CSPs showed particularly versatile chiral recognition profiles, resolving an extremely broad assortment of chiral analytes. Several of these CSPs have been commercialized by Daicel Chemical Industries, Ltd., the most important of which are depicted in Fig. 7.5.

A detailed understanding of the chiral recognition principles governing enantioselective analyte binding to polysaccharide derivatives remains to be established. Systematic mechanistic investigations are complicated by the unfavorable physicochemical characteristics of macromolecular polysaccharide derivatives. These challenges include (i) low solubility in solvents favoring chiral recognition, (ii) poorly resolved NMR spectra due to signal broadening by shift averaging and (iii) the complexity associated with the interpretation of spectroscopic data due to the presence of multiple binding site geometries. Okamoto et al. have adopted a multidisciplinary approach to probe important aspects of enantioselective binding, employing chromatographic screening of suitable model analytes, dedicated NMR investi-

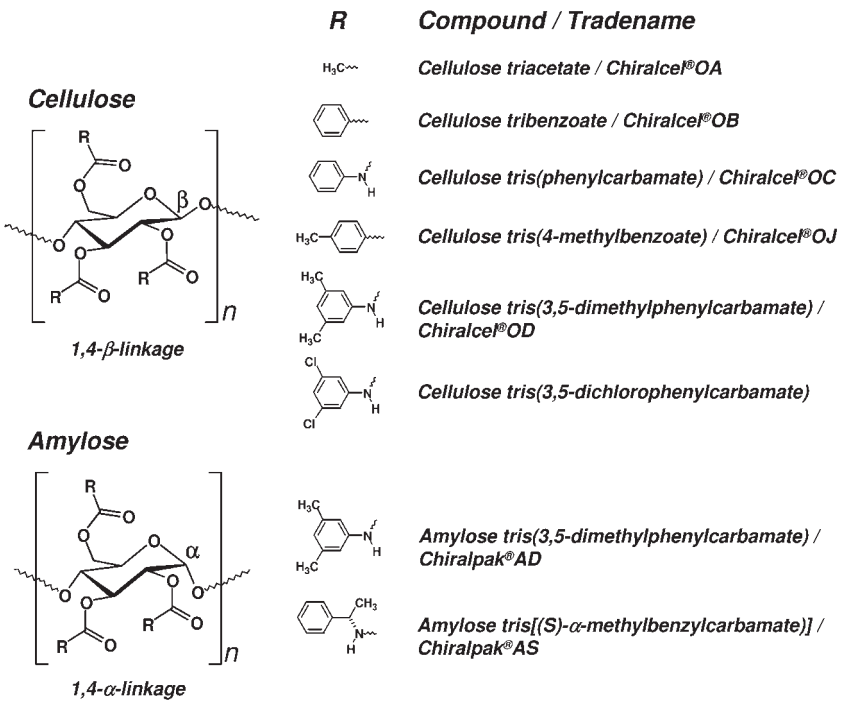


Fig. 7.5 Summary of the chemical structures and tradenames of the most important cellulose and amylose derivatives incorporated in polysaccharide-type chiral stationary phases.

gation using soluble polysaccharide-type SOs and molecular modeling techniques [103, 104]. These studies have revealed important information on the supramolecular structure of cellulose and amylose tris(phenylcarbamate) derivatives. Thus, the authors synthesized an amylose tris(3,5-dimethylphenylcarbamate) derivative with a low degree of polymerization to ensure a sufficient level of solubility for NMR studies [105]. The distance information for proximal 1–4′-protons in adjacent glucose units obtained by NOESY was used as input to probe several possible supramolecular structures by dedicated molecular modeling routines. This procedure revealed that amylose tris(3,5-dimethylphenylcarbamate) adopts a left-handed 4/3 helical superstructure. A similar strategy was employed to explore the supramolecular preferences of cellulose tris(5-fluoro-2-methylphenylcarbamate), for which a left-handed 3/2 helical arrangement was suggested [103]. In both structures, helical grooves lined with polar carbamate groups exist along the main chain. The carbamate groups are located in the interior of these cavities, which are delimited by the hydrophobic aromatic rings. Chiral recognition has been proposed to involve hydrogen bonding and dipole–dipole interactions with the polar carbamate motifs embedded in these chiral cavities, supported by π-stacking and steric interactions contributed by the flanking aromatic groups [102, 104, 105].

The impact of the aromatic substituents on the chromatographic chiral recognition characteristics of cellulose tris(phenylcarbamate) derivatives has been studied in detail [106]. A set of 18 CSPs coated with differently mono- or disubstituted phenylcarbamate derivatives was chromatographically evaluated under identical normal-phase mobile phase conditions with a test set of chiral analytes. The chiral recognition characteristics of the polysaccharide-type CSPs were found to be sensitive to inductive effects originating from the substituents of the incorporated phenyl groups. The 2-substituted derivatives produced poor levels of enantioselectivity, while 4-substituted derivatives carrying methyl-, ethyl-, chloro- and trifluromethyl-groups showed improved chiral recognition capacity. However, 3,4- and 3,5-dimethylphenyl- and dichlorophenylcarbamate derivatives exhibited the best chiral recognition performance among the tested cellulose derivatives.

Cellulose and amylose tris(phenylcarbamates) and tris(benzoates) represent the most broadly applicable class of CSPs currently available. Individual polysaccharide derivatives often show complementary chiral recognition profiles with respect to analyte structure and elution orders [101, 107]. This is particularly true for the cellulose and amolyse tris(3,5-dimethlyphenyl) derivatives (CHIRALCEL®OD and CHIRALPAK®AD), which frequently even display, for a given analyte, opposite enantiomer elution orders. Large-scale screening programs performed in academic and industrial settings have provided evidence that a limited set of polysaccharide-type CSPs are capable of resolving the majority of enantiomer separation challenges encountered [108–113]. For example, Borman et al. reported results of a comprehensive screening campaign of a set of over 100 chemically diverse racemates, employing HPLC, SFC, and CE separation techniques and systems [114]. With only three polysaccharide-type CSPs, CHIRALPAK®AD, the CHIRALCEL®OD and CHIRALCEL®OJ, more than 70% of the racemic analytes could be resolved. Even more favorable figures were disclosed by researchers of Novartis [115], as evident from Fig. 7.6. In this case, 88% of more than 1000 race-

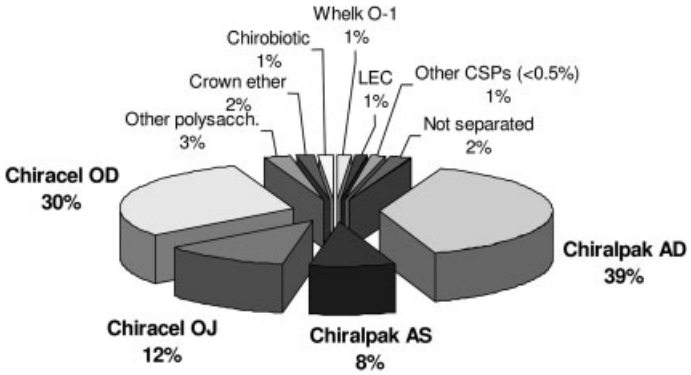


Fig. 7.6 Success rates for liquid chromatographic enantiomer separation of more than 1000 chiral drug discovery compounds on different types of chiral stationary phases. (Data taken from [115]).

mic discovery compounds could be separated into the enantiomers employing only four polysaccharide-type CSPs.

A particular advantage of polysaccharide-type CSPs, the "multimodal" nature of their chiral recognition capacity, makes them compatible with all chromatographically relevant mobile phase regimes. Historically, polysaccharide-type CSPs have been operated under normal-phase conditions, employing mixtures of hexane with polar organic modifiers, such as 2-propanol or ethanol [101, 102]. This mobile phase environment supports chiral recognition mechanisms capitalizing on hydrogen bonding, dipole-stacking, and π-interactions. Additives are generally required for ionizable analytes to reduce peak tailing phenomena. Trifluoroacetic acid is preferentially employed in the case of acidic analytes, while isopropylamine and diethylamine are efficient additives for basic compounds. As an alternative to alcoholic modifiers, a certain type of ether, such as *tert*-butyl methyl ether can be used. However, dichloromethane, chloroform, ethylacetate, tetrahydrofuran, dioxane, toluene and acetone can induce swelling or dissolution of the physically coated derivatives, and are therefore incompatible with polysaccharide type-CSPs, even at low concentration levels.

Polysaccharide-type CSPs have also been shown to be compatible with reserved-phase conditions [116]. However, it should be noted that under reversed-phase conditions the chiral recognition performance of polysaccharide-type CSPs is often diminished compared to normal-phase conditions, due to competing effects of mobile phase components with polar interactions involved in chiral recognition. Dedicated versions of polysaccharide-type CSPs (CHIRALPAK®AD-RH, the CHIRALCEL®OD-RH and CHIRALCEL®OJ-R, CHIRALCEL®AS-RH) have been introduced for reversed-phase applications, with a silica base material being compatible with the more aggressive environments encountered in aqueous solutions. For neutral analytes, methanol/water and acetontrile/water mixtures can be used, with additives of basic or acidic nature having little impact on the enantiomer separation characteristics. Ionizable compounds require additives to improve peak shapes. Phosphoric acid (pH 2) is recommended as an effective mobile phase additive for acidic analytes. Enantiomer separation of basic analytes may be enhanced by addition of chaotropic counter ions, such as hexafluorophosphate salts. Alternatively, basic buffer systems at pH 9 can be employed to efficiently suppress dissociation of basic analytes. The impact of different additives on the reversed-phase enantiomer separation on neutral, acidic and basic analyte is shown in Fig. 7.7.

Polar-organic mobile phases have been demonstrated to be useful for separation of various classes of compounds [87, 88, 117]. This operation mode is of particular interest for the development of preparative applications, and analytical application employing mass-sensitive detection systems showing limited compatibility to reversed-phase/normal-phase media. Recently, Chankvetadze et al. evaluated the chiral recognition performance of four polysaccharide-type CSPs with a set of drugs and drug-like molecules using neat acetonitrile or methanol as mobile phases [88]. With methanol as mobile phase, out of 22 test analytes 16 separated on CHIRALPAK®AD, 12 on CHIRALCEL®OD and 8 on CHIRALCEL®OJ-R. A CSP based on a non-commercial cellulose carbamate derivative, cellulose

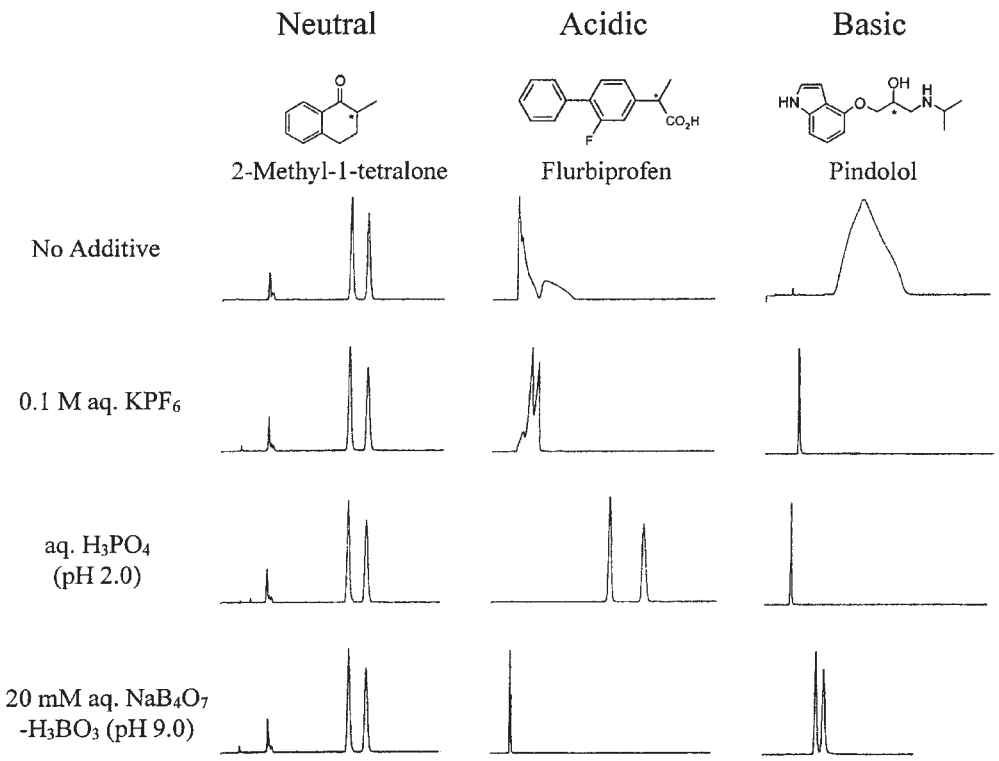


Fig. 7.7 Effect of various types of additives on the reversed-phase enantiomer separation of neutral, acidic and basic analytes on a polysaccharide-type CSP. Column: CHIRALCEL®AD–RH (150 × 4.6 mm i.d.), mobile phase: aqueous mobile phase containing modifier indicated in the figure/acetonitrile (60/40 v/v), flow rate: 0.5 mL min^{-1}, temp.: 25 °C, detection: UV 254 nm. (Reprinted with permission from [116]).

tris(3,5-dichlorophenylcarbamate), gave outstanding performance, separating the complete test set of racemic compounds with acetonitrile as mobile phase. The results of a related, more comprehensive study were reported by Lynam [117]. From a set of more than 80 test compounds, about 30% could be resolved with methanol/ethanol or acetonitrile as mobile phases. The addition of increasing amounts of hexane to the alcohol mixtures enhanced analyte retention, but had little effect on enantioselectivity. From this observation the authors concluded that the chiral recognition mechanisms operating in polar-organic media may be related to those governing enantioselective binding under normal-phase conditions.

Polysaccharides-type CSPs are excellently compatible with SFC conditions [89–93]. The apolar nature of supercritical carbon dioxide requires addition of polar modifier to enhance elution power. The preferred modifiers are alcohols, such as methanol, ethanol and 2-propanol. Methanol is the most frequently used as modifier as it provides the benefits of low viscosity, powerful enhancement of elu-

tion strength, even in low proportions, low toxicity, and reasonable cost. An additional advantage of methanol over other alcohols is ease of evaporation in the case of preparative applications. Admixture of modifiers may shift the mobile phase from the supercritical to subcritical regions, but even under these conditions the favorable characteristics of the mobile phase mixtures are largely retained. These benefits comprise high diffusivity and low viscosity. Chromatographically, these features translate into low back pressure, enabling fast separation at high flow rates. In addition, SFC often produces higher efficiencies and faster column equilibration times than normal-phase media. Enantiomer separation under SFC mode presents a particularly attractive option in industrial settings, where high sample throughput and the possibility to transfer analytical to preparative protocols are frequent requirements [108, 111, 118, 119]. SFC mode enantiomer separation of basic and acidic analytes on polysaccharide-type CSP requires basic and acidic additives to control ionization. Addition of low percentages of isopropylamine and triethylamine were found to improve the peak shapes of basic compounds [120]. Detailed studies suggest that for certain applications different types of amines may produce more favorable effects [121].

The use of additives, however, may induce transient or even persistent conformational changes in the polymeric structure of polysaccharide derivatives, enhancing or attenuating the initial enantiomer separation characteristics. Thus, persistent memory effects were demonstrated to occur with amylose-based CSPs after operation with normal-phase type mobile phase with diethylamine as basic additive [122]. Flushing with 2-propanol was shown to remove most of the memory effect. Acid treatment of the CHIRALPAK®AD-H and CHIRALCEL®OD-H CSPs changed their performance, which was subsequently largely restored by regenerative washing steps with amine-containing mobile phases.

Another unusual thermally induced change in enantiomer separation characteristics was observed for the enantiomers of a dihydropyrimidinone acid derivative and its methyl ester on a CHIRALPAK®AD CSP under normal phase conditions [123]. In the course of variable temperate studies employing ethanol–hexane mixtures as mobile phase, Wang et al. observed nonlinear van 't Hoff behavior for the (*S*)-enantiomer of the acid, but linear behavior for the corresponding (*R*)-enantiomer. The van 't Hoff plot for the α-values was nonlinear, exhibiting a transition at about 30 °C. More importantly, the van 't Hoff plots derived from variable-temperature measurements involving heating from 5 to 50 °C were found to be not superimposable with that obtained by cooling from 50 to 5 °C. The authors interpreted this observation as a thermally induced irreversible conformational change of the polymeric backbone of the amylose derivative incorporated in the CSP. The authors also demonstrated that this phenomenon was sensitive to the polar modifier component of the mobile phase. With 2-propanol the van 't Hoff plots for the acidic analyte were linear and reversible. Equilibration of the CSP with methanol- or water-containing mobile phases, however, led to the thermally irreversible changes observed with ethanol-based mobile phases. Subsequent variable-temperature solid-state ^{13}C-CPMAS and ^{19}F-MAS-NMR experiments of the CSP material performed in the presence and absence of the analytes in ethanol- and trifluor-

oacetic acid-modified hexane revealed that the conformational transition of the amylose derivative was controlled by a single kinetically driven process [124].

Recently, organic sulfonic acids have been suggested as normal-phase and SFC additives to improve peak shapes for basic analytes [125]. These strongly acidic additives proved particularly beneficial for the separation of a broad variety of amines on a CHIRALPAK®AD CSP under normal-phase conditions. The addition of ethanesulfonic and methanesulfonic acid allowed successful separation of a selection of amines which had failed to resolve with less acidic additives.The enantiomer separation of a basic drug compound employing ethanesulfonic acids and trifluoroacetic acid is shown in Fig. 7.8.

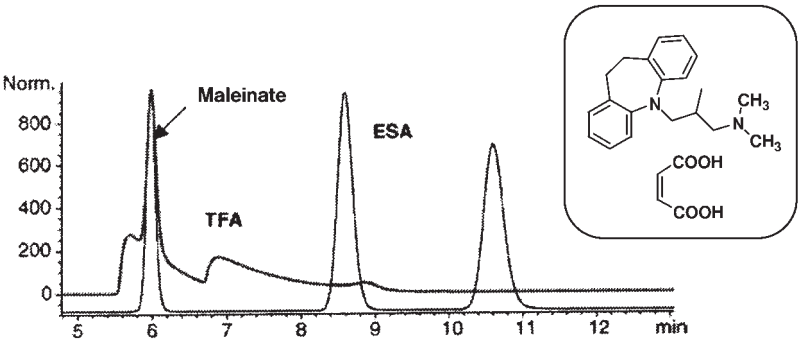


Fig. 7.8 Overlaid chromatograms for trimipramine maleinate obtained on a CHIRALPAK®AD-H CSP in presence of ethanesulfonic acid and trifluoroacetic acid as acidic additives. Column: CHIRALCEL®AD–RH (250 × 4.6 mm i.d.); mobile phase: n-hexane/ethanol/acidic modifier (85/15/0.1 v/v/v) flow rate: 1.0 mL min^{-1}, temp.: 25 °C, detection: UV 210 nm. (Reprinted with permission from [125]).

The origin of the favorable effects of these strongly acidic additives remains to be established. The authors suggested that the strongly acidic additives may play an essential role in the formation of stable ion pairs, or promote a local pH-effect enhancing the enantioselective interaction with the binding sites at the polysaccharide-type CSP.

One of the major limitations of coated polysaccharide-type CSPs is their incompatibility with so-called "non-standard" solvents. Specifically, the exposure of coated CSPs to dichloromethane, chlororform, ethyl acetate, tetrahydrofuran, dioxane, toluene and acetone, induces swelling and/or dissolution of the physically adsorbed polymer films and thus destruction of the columns. To address this serious drawback, a considerable amount of research has focused on the development of immobilized versions with global solvent compatibility. In the last two decades numerous immobilization strategies have been reported [126], and the quest for solvent-stable versions capable of reproducing with fidelity the excellent separation characteristics of their coated congeners is still an active field of study [100, 127–130]. Reported immobilization approaches capitalize on (i) surface at-

tachment of a polysaccharide derivative via carefully engineered bifunctional carbamate/or ester substituents [131], (ii) elegant but demanding enzymatic solid-phase synthesis of polysaccharide segments on silica surfaces followed by dedicated derivatization [132], or (iii) radical cross-linking of polymerizable polysaccharide derivatives after surface coating [133–140]. Probably the conceptually most simple and practical strategy for the immobilization of polysaccharide derivatives has been developed by researchers at Novartis [141]. These workers used a two-step approach, involving the coating of conventional polysaccharide derivatives without performing any additional modifications, followed by a photochemical or thermal cross-linking in the presence of free radical initiators [142]. The resultant CSPs were shown to exhibit excellent stability towards the entire spectrum of organic solvents applied in liquid chromatography combined with excellent chiral recognition capacities.

Most recently, chemically immobilized versions of the most popular amylose and cellulose-type CSPs, CHIRALPAK®AD and CHIRALCEL®OD, have become commercially available under the tradenames CHIRALPAK®IA and CHIRALPAK®IB (Daicel Chemical Industries, Ltd, Japan). This new generation of immobilized

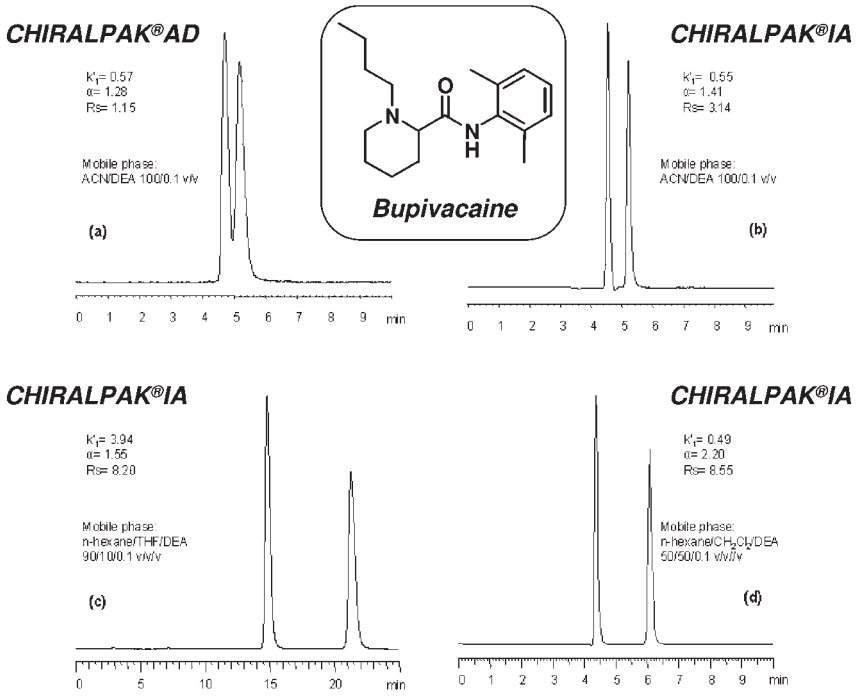
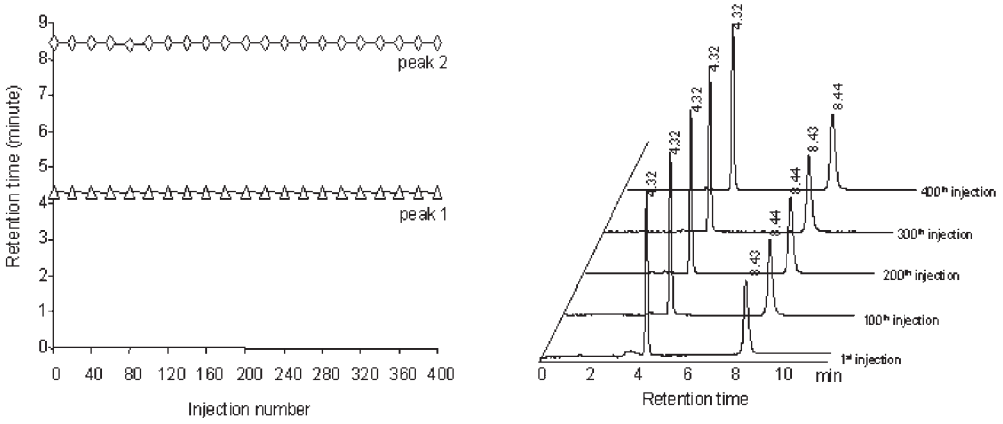


Fig. 7.9 Separation of bupivacaine on the coated (CHIRALPAK®AD) and immobilized (CHIRALPAK®IA) amylose tris(3,5-dimethylphenylcarbamate)-type CSPs. Columns: (250 × 4.6 mm i.d.), mobile phases are indicated in the figure; flow rate: 0.5 mL min^{-1}, temp.: 25 °C, detection: UV 254 nm. (Reprinted with permission from [144]).



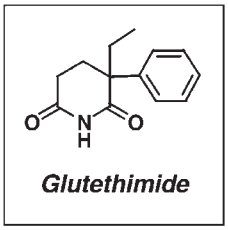


Fig. 7.10 Separation repeatability of glutethimide on CHIRALPAK®IA with 100% ethyl acetate. Column: (250 × 4.6 mm i.d.); flow rate: 1.0 mL min^{-1}, temp.: 25 °C, detection: UV 274 nm. (Reprinted with permission from [144]).

CSPs tolerate ethers, ketones, esters, chlorinated solvents and aromatic solvents, enhancing the scope of application of the coated congeners immensely [143, 144]. Figure 7.9 presents a comparison of the enantiomer separations achieved for bupivacaine on the coated CHIRALPAK®AD and the corresponding immobilized CHIRALPAK®IA employing "standard" and "non-standard" mobile phase mixtures [144]. Excellent durability and inertness of these stabilized CSPs have been demonstrated (see Fig. 7.10), making them an invaluable complement to the established repertoire of coated polysaccharide-type CSPs [144].

However, the (undisclosed) proprietary immobilization process appears to modify the enantiomer separation characteristics as compared to the coated versions [145, 146]. Ghanem et al. compared the chiral recognition profile of a coated CHIRALPAK®AD CSPs with that of the immobilized version, employing hexane/2-propanol containing TFA (0.1%) as mobile phase [145]. They reported superior enantiomer separation for the coated CSP, with some analytes failing to resolve on the immobilized version. These differences in the enantiomer separation capacity of coated and immobilized polysaccharide-type CSPs may complicate attempts at direct method transfer.

7.8.1.1.2 **Protein-type CSPs**

The observation of enantioselective binding of chiral molecules to certain classes of proteins was an early observation in biochemistry. However, it was the emer-

ging interest in drug chirality issues in the late 1970s that triggered, in an academic setting, dedicated research to systematically investigate protein-based chiral recognition phenomena. These efforts led to the development of several protein-type CSPs which proved valuable tools to probe crucial quantitative and qualitative aspects of enantioselective drug action. The most important classes of proteins incorporated in commercially available protein-type CSP are α_1-acid glycoprotein (AGP), human and bovine serum albumin (BSA and HSA), chicken ovomucoid (OMCHI) and the cellulose cleaving enzyme cellobiohydrolase I (CHB I). These protein-type CSPs have seen commercialization under the tradenames CHIRAL-AGP® (ChromTech AB), CHIRAL-HSA® (ChromTech AB), ULTRON ES-OVM® (Shinwa Chemical Industries) and CHIRAL-CBH® (ChromTech AB).

A survey of the recent literature indicates that protein-type CSPs still play a role in the field of bioanalytical monitoring of the stereoselective activity profile of chiral drugs [147–149], despite the large number of alternative enantioselective adsorbents nowadays available. Certainly, the unbroken attraction of protein-type CSPs for bioanalytical applications is rooted in their broad chiral recognition capabilities for drug-like molecules, being fully compatible with biological environments. The scope of applicability of protein-type CSPs, however, is restricted by a number of critical issues. Specifically, the features of low sample capacity, confinement in a choice the mobile phases to (a narrow window of) reversed-phase conditions, poor chromatographic efficiency, risk of irreversible denaturation, susceptibility to biodegradation, and the often quite demanding method development, are limiting factors.

Among the commercial protein-type CSPs, those based on AGP have demonstrated the broadest scope of applications, exhibiting enantioselectivity for numerous basic, acidic and neutral drug compounds [150]. Structurally, AGP consists of a single 181-residue peptide chain supporting 5 heteropolysaccharide grafts. The latter incorporate 14 sialic acid units which render AGP strongly acidic (pI = 2.7) [151]. The polysaccharide content of AGP has been estimated to account for 36 to 40% of the total mass. AGP, covalently attached in a cross-linked fashion onto a chemically modified silica surface, tolerates a wide range of pH (3–7.5), high concentrations of organic solvents (up to 25%) and temperature up to 70 °C. In the course of analytical method development, enantioselectivity and retention on AGP-type CSPs can be controlled by various factors, including mobile phase pH, ionic strength, type and concentration of organic modifier and charged additives, and temperature [150]. The hydrogen bonding characteristics and the hydrophobicity of the organic modifier in the mobile phase can have a significant impact on enantioselectivity. For example, verapamil was not separated with a mobile phase containing 1-propanol, but was completely resolved when 1-propanol was replaced by acetonitrile [150]. Charged modifiers, such as *N*,*N*-dioctylamine and tetrabutylammonium bromide can also be used to control retention and enantioselectivity of basic, neutral and acidic analytes [152]. The effect of these modifiers is believed to result from binding site competition, binding to allosteric sites, or reversibly induced conformational changes. Reversible conformational changes in AGP have also been reported to occur as a consequence of changes in pH (pH 5–7) [153],

temperature [154] or adsorption of certain types of organic modifiers [155, 156]. Yet, little is known on the binding mechanisms or location of the site(s) involving in enantioselective binding of chiral drug molecules to AGP. Experimental evidence indicates that binding may occur in a hydrophobic pocket located in the interior of the protein domains, and that a combination of hydrogen bonding, electrostatic and hydrophobic elements contributes to analyte recognition [151].

CSPs based on OMCHI [157] show a spectrum of application similar to that of AGP, with a capacity to resolve the enantiomers of acidic, neutral and basic drug compounds. Improved long-term stability compared to AGP has been claimed [158, 159]. Recently, it was shown that OMCHI used for preparation of the commercial CSP is heterogeneous, containing about 10% of a second protein termed ovoglycoprotein (OGCHI) [160]. CSPs prepared from the individual purified proteins exhibited excellent enantioselectivity for OGCHI, while OMCHI was practically devoid of chiral recognition properties. The authors concluded that the chiral recognition properties of the commercial CSP originate from the presence of OGCHI.

CSPs incorporating BSA have been successfully employed to separate the enantiomers of acidic and neutral compounds [161–163], such as *N*-acylated amino acids, aromatic amino acids and sulfoxides. Retention and enantioselectivity can be optimized by adjusting pH, ionic strength and organic modifier content of the mobile phase.

HSA bears structural and functional resemblance to BSA, and HSA-type CSPs [164] also show similar enantioselective binding preferences for acidic and neutral drug molecules, such as 2-aryloxypropanoic acids [165], warfarin [166] and benzodiazepines [167]. The chiral recognition mechanism of HSA has been the subject of a number of investigations [168], which revealed that enantioselective binding occurs primarily at two well-defined hydrophobic sites. Acidic drugs have been shown to bind preferentially to the so-called warfarin-azapropazone (site I) and neutral drugs to the indol-benzodiazepine site (site II).

CSPs incorporating CBH I display excellent chiral recognition capacity for β-blocker-type drugs possessing aminoalcohol structure [169, 170]. An unusual temperature dependence of the retention behavior of propranolol was observed on CHB I in buffered aqueous mobile phases at pH 5.5 [80, 81]. Specifically, an increasing in column temperature from 10 to 45 °C induced a decrease in retention of the less retained (*R*)-enantiomer and a simultaneous increase in retention of the more retained (*S*)-enantiomer, leading to a substantial enhancement in enantioselectivity. A detailed thermodynamic study dissecting the nonselective and selective contributions to the global binding energetics revealed an entropically-driven chiral recognition mechanism due to a favorable solvation/desolvation balance. The molecular principles underlying enantioselective recognition of propranolol to CHB I were studied employing rather sophisticated protein fragmentation [171], substrate inhibition [172] experiments and X-ray crystal structure analysis [173]. The combined evidence from these studies established that propranolol binds to the catalytic site of the enzyme [174].

The investigation of chiral recognition of drug molecules by proteins may remain an active field of research in the future as is evidenced by several new types

of promising protein-based CSPs reported in the recent literature. These include chromatographic supports incorporating various types of riboflavin binding proteins [175–177], chicken liver basic fatty acid-binding proteins [178] and penicillin G acylase [179–182].

Apart from their important role in bioanalytical applications in the drug discovery field, various types of protein-type CSPs have been employed successfully in the study of drug–protein binding phenomena. In particular high-performance affinity chromatography techniques on immobilized serum albumins are increasingly used to derive a wealth of qualitative and quantitative information on drug–protein binding, such as equilibrium binding constants, binding kinetics, identification of drug binding sites, demonstration of competitive and allosteric binding phenomena, and drug–drug interactions. A more detailed treatment of these aspects, however, is outside the scope of this work. Interested readers are referred to excellent reviews providing a comprehensive coverage of these topics [168, 183, 184].

7.8.1.2 **CSPs Derived from Synthetic Polymers**

7.8.1.2.1 **Helical Poly(methacrylates)**

In 1981 Okamoto's group reported the successful creation of single-handed helical polymethacrylate polymers from achiral monomers [185]. The synthesis of these polymers was achieved via an anionic polymerization of achiral trityl-methacrylates at low temperatures, triggered by Grignard reagents in presence of catalytic amounts of chiral ligands such as sparteine. The coating of these polymers onto the surface of pre-functionalized macroporous silica gel gave CSPs that showed, in combination with reversed-phase type mobile phases (e.g. water/methanol mixtures), excellent levels of enantioselectivity for certain classes of racemates [186]. Analytes endowed with helical chirality elements and multiple aromatic functionalities were exceptionally well separated, indicating a certain level of SO/analyte complementarity. The CSPs derived from poly(trityl-metharylate) and poly[(diphenyl-3-pyridyl)methyl-methacrylate] have been commercialized by Diacel Chemical Industries Japan Ltd. under the tradenames CHIRALPAK®OT(+) and CHIRALPAK®OP(+). However, the limited scope of application for drug-like targets and a tendency to suffer slow hydrolysis in hydro-organic mobile phase environments render these CSPs of academic rather than pharmaceutical interest.

7.8.1.2.2 **Chiral Poly(methacrylamides)**

In 1974 Blaschke and coworkers introduced new polymer-type CSP materials prepared by suspension polymerization of methacrylamides derived from chiral amines and amino acid esters [187–189]. These CSPs were initially produced in the form of self-supporting beads. However, difficulties arising from extreme polymer swelling and poor mechanical strength prompted later the development of silica-supported composite materials with more favorable chromatographic performance characteristics [190]. These improved versions of the CSPs have been com-

mercialized by Merck under the global tradename ChiraSpher®. Operated under normal-phase conditions, for example with hexane/alcohol or hexane/ether mixtures, these polymeric supports produce useful levels of enantioselectivity for a range of chiral drug molecules [191]. The presence of hydrogen bond donor/acceptor functionalities and/or aromatic substituents in the analytes was reported to improve the changes for successful chiral recognition. A comprehensive review of the chemical and analytical aspects of enantiomer separation with this type of phases has been given by Kinkel [192].

7.8.1.2.3 **Tartramide-network Polymers**

Allenmark and coworkers reported conceptually new polymeric silica-supported network-type CSPs exploiting *O,O′*-disubstituted-*N,N′*-diallyl-L-tartramide as chiral monomers [193, 194]. These CSPs are prepared by a hydrosilylation reaction in the presence of a multifunctional silane and macroporous allyl-functionalized silica gel, leading to composite materials coated with a surface-attached, highly cross-linked polymer film. The authors evaluated the impact of the *O,O′*-substituents attached to the tartramide monomers on the chiral recognition characteristics of the resulting polymeric CSPs. Bis-*O,O′*-dibenzoyl derivatives bearing bulky substituents produced the most versatile enantiomer separation profiles. Polymers incorporating 3,5-dimethylbenzoyl- and 4-*tert*-butylbenzoyl substitution patterns performed especially well and were later introduced into the market under the tradenames CHI-DMB® and CHI-TBB® (Eka Nobel AB). These CSPs show, under normal-phase conditions, favorable chiral recognition characteristics for a broad range of pharmaceutically relevant racemates, including acidic, neutral and basic compounds carrying hydrogen bonding motifs and aromatic functional groups [193]. Peak shapes of ionizable compounds can be significantly improved by the addition of small amounts of basic additives; the addition of strongly acidic additives is discouraged as they may compromise the structural integrity of the polymeric phase.

7.8.1.2.4 **Poly(diaminocyclohexane-*N,N*-diacrylamide)**

A promising concept for the generation of silica-supported polymer-type CSPs with high chromatographic efficiencies was reported recently by Gasparrini et al. [195]. An inherent problem associated with graft-polymerization onto pre-functionalized silica gels is the difficulty in enforcing a homogenous polymer growth from the surface in the confines of the pores. Given the enhanced steric demands of the surface-attached monomers, polymer growth tends to occur preferentially for unconstrained monomers in free solution. This leads to low grafting yields, inhomogeneous polymer coverage and even to pore blocking phenomena, resulting in materials with compromised mass transport kinetics and thus poor chromatographic efficiency. Gasparrini's group addressed this problem by using mesoporous, azo-activated silica particles as starting material for CSP grafting. The concept of this approach is illustrated in Fig. 7.11. The covalently attached radical in-

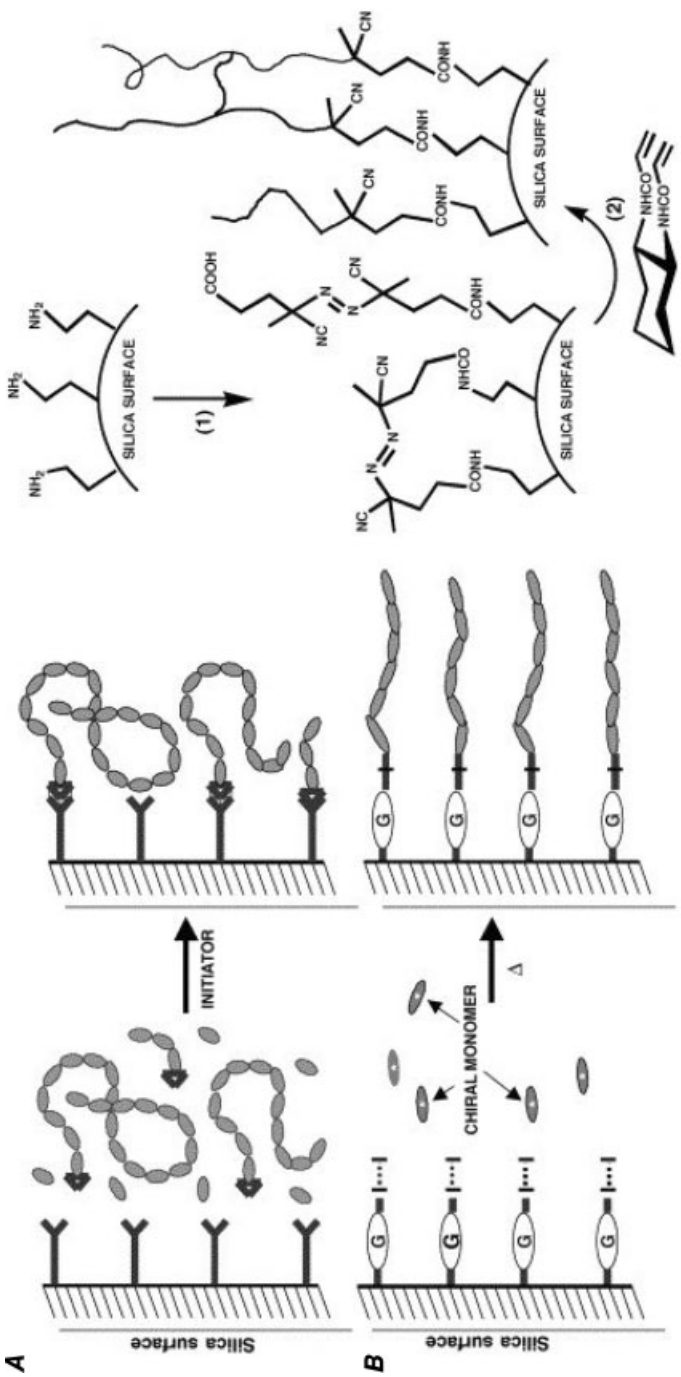


Fig. 7.11 Synthesis of a poly(*trans*-1,2-diaminocyclohexane diacrylamide)-type CSP by surface-initiated graft polymerization. A) conventional protocol by solution-initiated polymerization leading to heterogeneous surfaces; B) polymerization by surface-immobilized azo-initiator allowing for controlled growth, leading to homogeneously grafted surface. (Reprinted with permission from [195]).

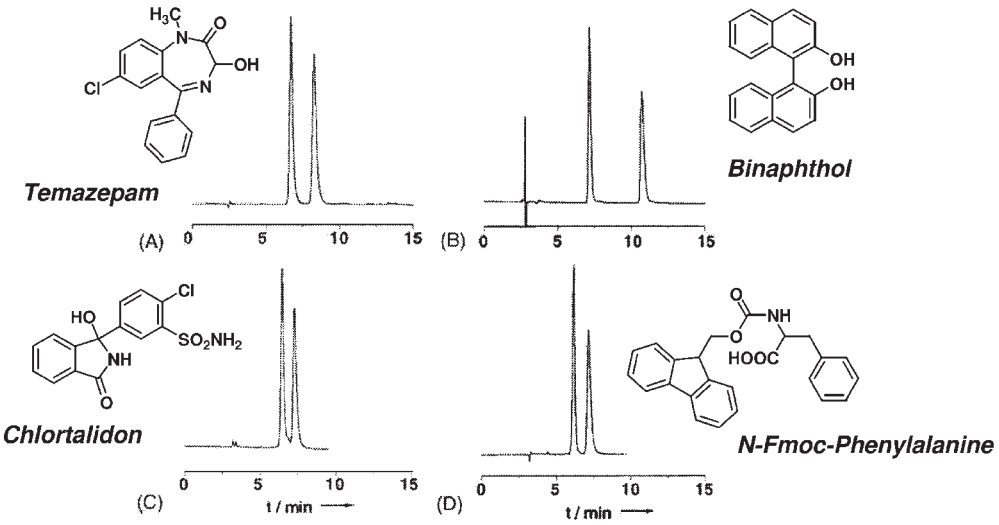


Fig. 7.12 Enantiomer separation of racemic analytes achieved on silica-supported poly(*trans*-1,2-diaminocyclohexane-diacrylamide)-type CSPs prepared by surface-initiated graft polymerization. (A) temazepam, n-hexane/ethanol (50/50 v/v); (B) binaphthol, dichloromethane/methanol (97/3 v/v); (C) chlortalidon, and (D) N-Fmoc-phenylalanine, acetonitrile/methanol 20 mM ammonium acetate (70/30 v/v); column: (250 × 4.6 mm i.d.); flow rate: 1 mL min^{-1}; detection: UV 254 nm; temp.: 25 °C. (Reprinted with permission from [205]).

itiator ensured a highly controlled surface-centered growth of (*trans*-1,2-diaminocyclohexane)-*N,N*-diacrylamide polymer grafts.

A comparison of the resulting CSP with a control material obtained by conventional grafting revealed significant improvement in the chromatographic performance. The CSP obtained by surface-initiated polymerization exhibited excellent chiral recognition performance for a broad range of drug-like compounds, such as benzodiazepines, carboxylic acids and sulfoxides, and proved compatible with both normal-phase and polar-organic mobile phase conditions. Some examples of the achieved separation are shown in Fig. 7.12.

7.8.1.2.5 **Molecularly Imprinted Polymers**

Molecular imprinting technology is probably the conceptually most convenient way to create target-specific polymeric CSPs [196–198]. This approach involves the equilibration of the enantiomer of interest with a large molar excess of a functional monomer, a cross-linking agent and an appropriate porogenic solvent, followed by thermal or photochemical polymerization. During the equilibration stage the functional monomers assemble around the template enantiomer to form noncovalent complexes, which on polymerization become integrated into the marcromolecular network. Elution of the template enantiomer from the resultant bulk polymer leaves behind a polymeric matrix with molecularly imprinted

cavities (molecular imprinted polymer, MIP). These molecularly imprinted cavities are complementary to the template in shape, size and stereochemical features, and therefore can act as target-specific enantioselective binding sites. The conceptual feasibility of the molecular imprinting technology to create enantioselective polymers with target-specific binding capabilities has been amply demonstrated. Numerous enantioselective MIP-type CSPs have been reported for chiral drugs and drug-like molecules, including amino acids, amino acid derivatives, peptides, hormones, antibiotics, profens, various types of amino alcohols and biogenic amines [196].

MIP-type CSPs are appealing in view of their ease of preparation, chemical robustness, low materials costs and convenient control of elution order. Unfortunately, MIP-based CSPs suffer from several serious drawbacks limiting their practical applicability for analytical and preparative enantiomer separation. In particular, MIPs prepared by the classical bulk polymerization approach display low binding site density and heterogeneous binding thermodynamics [199], features which translate chromatographically into poor loading capacity and peak tailing.

While the inherent thermodynamic hurdles associated with MIPs may be difficult to overcome, recent research in molecular imprinting technology has focused on tailoring new MIP formats allowing for improved chromatographic mass transport characteristics [200]. Rather than following the traditional bulk polymerization protocols, MIP-type CSPs are now preferentially prepared in the form of uniformly sized spherical particles and monolithic media. Haginaka's group has established a sophisticated protocol to generate uniformly sized spherical MIPs involving multiple swelling-polymerization technology [201–204]. The generality of this new imprinting approach was recently demonstrated for the preparation of spherical MIP materials for naproxen and nilvadipine. In one of these studies, the authors compared the chromatographic performance characteristic of the spherical nilvadipine MIPs with those of commercially available AGP and OVM protein-type CSPs [201]. Under optimized experimental conditions, the CSP packed with the spherical MIP material showed enantiomer separation characteristics competitive to those observed on the protein-type CSPs, with respect both to efficiency and enantioselectivity.

Yin et al. prepared a monolithic MIP column imprinted against nateglinide, an antidiabetic drug with diastereoselective pharmacological activity, and compared its chromatographic performance with that of a column packed with a MIP produced by conventional bulk polymerization under otherwise identical conditions [205]. The monolithic MIP column showed an appreciable level of selectivity towards related diastereomers and synthetic intermediates and high separation efficiency. Low selectivity and very poor chromatographic efficiency was observed with the MIP prepared by traditional bulk polymerization technology. The corresponding chromatograms and the structures of the diastereomeric analytes are shown in Fig. 7.13.

Despite these promising results further progress in molecular imprinting technology will be needed to make MIP-type materials competitive with the established analytical and preparative CSPs.

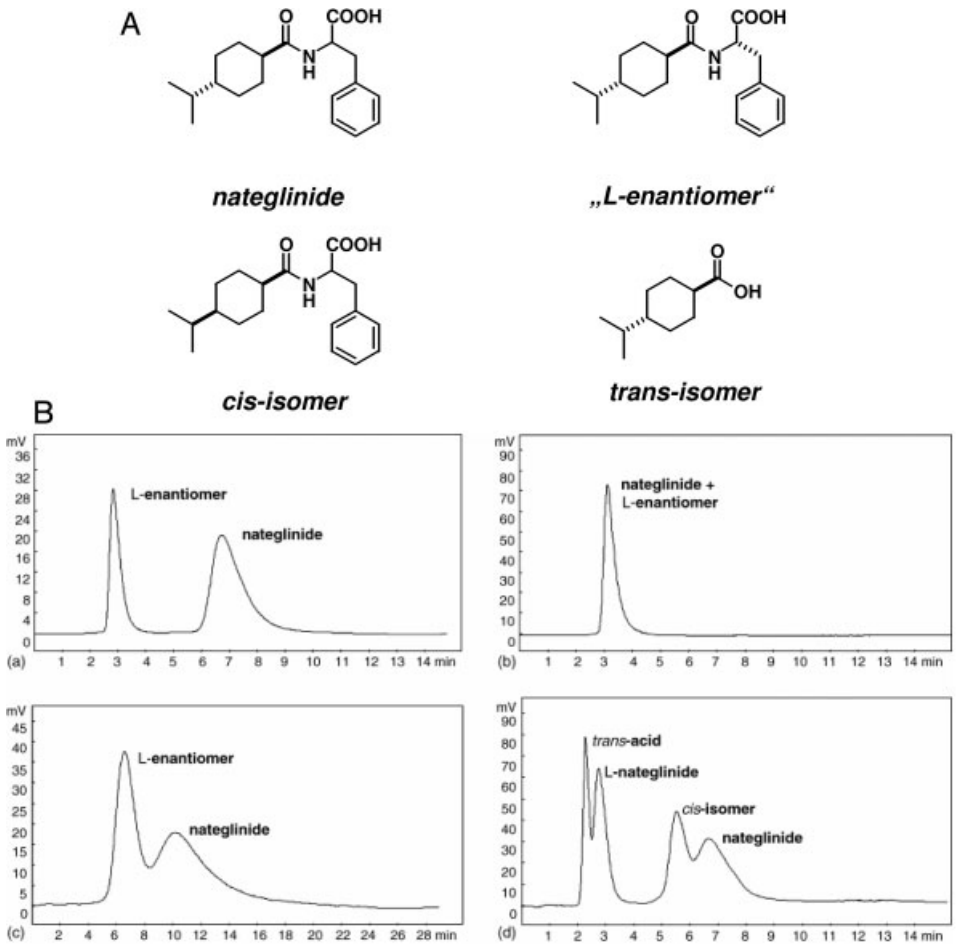


Fig. 7.13 (A) Chemical structures of nateglinide and chemically/diastereomerically related compounds. (B) Separation of nateglinide and related compounds on molecularly imprinted polymers and non-imprinted control polymers. (a) Separation of nateglinide and "L-enantiomer" on the monolithic MIP. (b) Separation of nateglinide and "L-enantiomer" on the non-imprinted control polymers. (c) Separation of nateglinide and "L-enantiomer" on the MIP produced by bulk polymerization. (d) Separation of the analytes on monolithic MIP. Columns: (250 × 4.6 mm i.d.).; mobile phase: acetonitrile; flow rate, 1.0 mL min^{-1}; temp.: 25 °C, detection: UV 210 nm; loaded amount: 50 nmol. (Reprinted with permission from [205]).

7.8.2 Macrocyclic CSPs

7.8.2.1 Cyclodextrin-type CSPs

Cyclodextrins (CDs) represent a class of macrocyclic D-(+)-glucopyranose oligomers interlinked via α-1,4-glycosidic bonds as depicted in Fig. 7.14. The oligomers relevant for liquid chromatographic enantiomer separation consist of 6 (α-CD), 7 (β-CD) and 8 (γ-CD) monosaccharide units, respectively. The global conformations of CDs bear resemblance to truncated cones, possessing an interior lined with hydrophobic methylene groups and glycosidic ether oxygens. The wider and the narrow rims of CDs are decorated with secondary and primary hydroxy groups, respectively, rendering these regions of the macrocycles hydrophilic in nature.

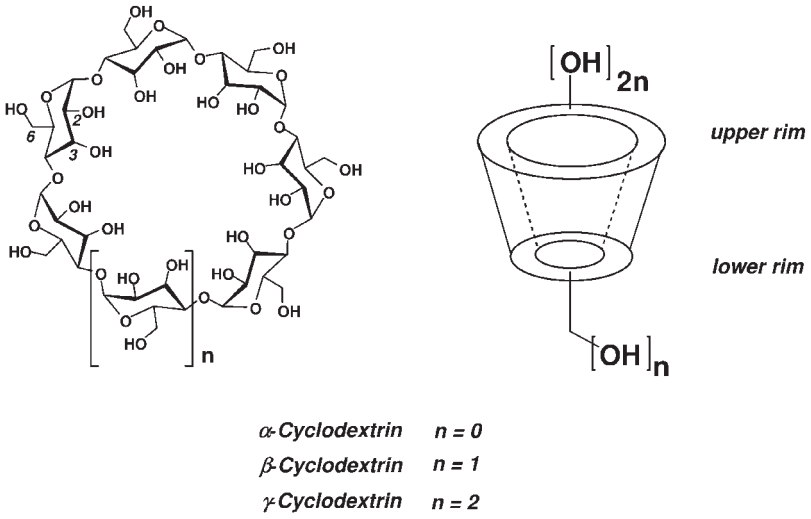


Fig. 7.14 (A) The chemical structure of α-, β, and γ-cyclodextrin. (B) Schematic representation of the cone-like conformation of cyclodextrins, with the upper rim presenting the secondary 2,3-hydroxy and the lower rim the primary 6-hydroxy groups.

CDs have a longstanding tradition as versatile host systems in the molecular recognition field, and have seen extensive use in various enantiomer separation techniques [206, 207], including GC, LC, CE and CEC. The widespread applications of CDs for enantiomer separation of chiral drug compounds have been detailed in recent reviews [55, 208].

A broad variety of bonded CD-type CSPs have been developed and commercialized for liquid chromatographic application. Table 7.1 summarizes the tradenames, the types of incorporated CD derivative and the suppliers of these CSPs. Typically, the CD molecules in these CSPs are covalently attached to functiona-

lized silica surfaces via one or more of the primary hydroxy groups, aligning the CD molecule in such a way that the wider rim faces the solution [209]. Apart from CSPs incorporating native CDs, enantioselective adsorbents with various derivatives have been developed. These additional functionalities may have supportive effects on chiral recognition by enhancing the size of the chiral cavity and providing additional supportive interaction sites.

Table 7.1 Overview of commercial cyclodextrin (CD)-type CSPs.

CSP tradename	Incorporated cyclodextrin derivative	Supplier
Cyclobond I 2000	Native β-CD	Astec
Cyclobond I 2000 AC	Acetylated β-CD	Astec
Cyclobond I 2000 SP	(S)-hydroxypropyl β-CD	Astec
Cyclobond I 2000 RSP	(R,S)-hydroxypropyl-β-CD	Astec
Cyclobond I 2000 SN	(S)-1-(1-naphthyl)ethyl carbamoyl-β-CD	Astec
Cyclobond I 2000 RN	(R)-1-(1-naphthyl)ethyl carbamoyl-β-CD	Astec
Cyclobond I 2000 DMP	3,5-dimethylphenyl carbamoyl-β-CD	Astec
Cyclobond II	Native γ-CD	Astec
Cyclobond III	Native α-CD	Astec
Cyclobond IIAC	Acetylated γ-CD	Astec
Cyclobond IIIAC	Acetylated α-CD	Astec
ChiraDex	Native α-CD	Merck
ChiraDex Gamma	Native γ-CD	Merck
Ultron ES-CD	Native β-CD	Shinwa Chemical Industries, Ltd
Ultron ES-PhCD	Phenylcarbamoyl β-CD	Shinwa Chemical Industries, Ltd

CD-based CSPs are multi-modal with respect to their molecular recognition capacities, and may be operated in normal-phase mode, polar organic mode or reversed-phase mode. Operation of CD-type CSPs in reversed-phase conditions is known to trigger chiral inclusion-type recognition mechanisms, with binding modes involving the (partial) transfer of hydrophobic analyte from the polar mobile phase into the apolar interior of the macrocycle. The degree of incorporation generally depends on the size of the CD cavity. Substituted phenyl, naphthyl and hetereoaromatic rings can conveniently be accommodated in a β-CD cavity, while larger analytes are expected to fit preferentially γ-CD and smaller α-CD cavities. Polar functionalities in the analytes may preferentially give rise to interaction with the secondary hydroxy groups located at the wide rim of the CD cavities. The relative strength of inclusion-type CD–analyte interactions can effectively be tuned via the mobile phase composition. Organic solvents with the capacity to competitively

replace analytes from the interior of CD cavities can be used to attenuate CD–analyte binding. The efficiency of this replacement effect increases in the order water < methanol < propanol ~ acetonitrile < tetrahydrofuran. However, increasing the solvent hydrophobicity also has been found to compromise enantioselectivity, making the weekly displacing methanol the modifier of choice. Polar analyte–CD interactions may be attenuated in a number of ways by adjusting the mobile phase pH, buffer type and buffer concentration, and column temperature.

When CD-type CSPs are operated under normal-phase conditions, e. g. with hexane/alcohol mixtures, inclusion phenomena are no longer major driving forces for CD–analyte interactions [210]. Instead, chiral recognition mechanisms capitalizing on specific polar interaction with the exterior of the CD-cavity prevail. In normal-phase mode, CDs comprising additional functional groups, such as chiral 1-(1-naphthyl)ethyl carbamates, have been demonstrated to perform particularly well. Inversion of the enantiomer elution order for a given analyte have been observed depending on the absolute configuration of the attached 1-(1-naphthyl)ethyl carbamates. This observation suggests that enantioselective analyte binding is primarily controlled by the chirality of the attached 1-(1-naphthyl)ethyl carbamate groups rather than by the supporting CD cavity.

Organic polar mode conditions have proved particularly useful with CD-type CSPs for the separation of polar chiral drugs which failed to resolve with reversed-phase and normal-mode mobile phases [84, 85]. These "magic" organic-polar mobile phases are typically composed of 85–100% acetonitrile, 0.001–1.2% glacial acetic acid and 0.001–1.2% triethylamine. With these mixtures enantiomer separation could be achieved for a broad range of analytes possessing amine functionality (β-blockers), but also for compounds with phenolic and carboxylic groups (profens, warfarin). A mechanistic rationale advanced for chiral recognition with CDs in polar-organic mobile phases suggests that the solvent molecule may preferentially occupy the CD cavity, forcing the analytes to interact enantioselectively with the polar hydroxy groups located at the rims.

7.8.2.2 **Glycopeptide-type CSPs**

CSPs based on glycopeptide antibiotics have been introduced by Armstrong and proved highly versatile tools to address a broad range of liquid chromatographic analytical enantiomer separation challenges [211]. Currently, CSPs based on vancomycin [212], teicoplanin [213], ristocetin A [214] and the aglycon of teicoplanin [215] are marketed by ASTEC under the tradnemes Chirobiotic V, Chirobiotic T, Chirobiotic R and Chirobiotic TAG.

The antibiotics of the glycopeptide class are molecules of considerable structural complexity as is evident from Fig. 7.15. As a common structural feature they share a basket-shaped aglycone framework, composed of three or four interconnected cyclo-tripeptides rich in aromatic elements. The individual antibiotics show different degrees of *O*-glycosylation, with one to three saccharide units attached at the periphery of the peptide backbone. The relatively rigid peptidic scaffolds provide a stereochemically well-defined positioning of multiple functionalities within glyco-

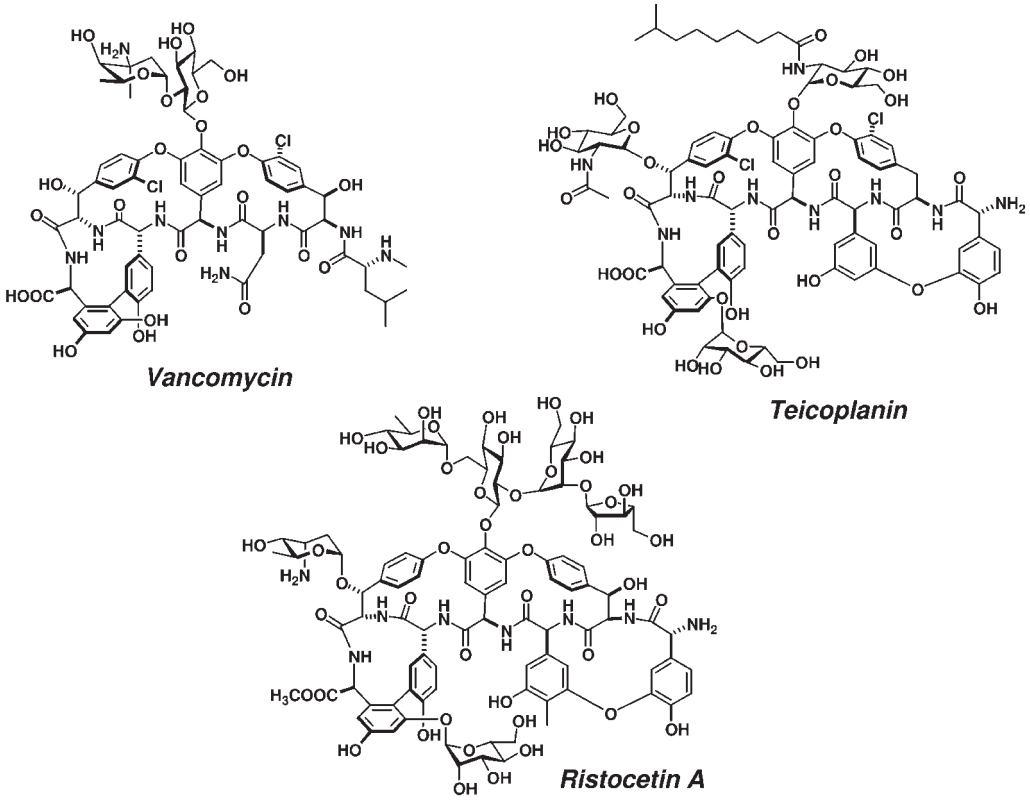


Fig. 7.15 Chemical structures of the glycopeptide-type antibiotics vancomycin, teicoplanin and ristocetin A. For teicoplanin the prevalent derivative (A_2-2, 85 %) of the teicoplanin complex is shown.

peptide antibiotics. The repertoire of these functional groups includes ionizable carboxylic, phenols and amino groups, electron rich aromatic rings and amides, offering ample opportunities for enantioselective interactions with analytes.

The inherent structural complexity of glycopeptide antibiotics has proved a major obstacle in the development of a molecular-level understanding of the underlying chiral recognition mechanisms. However, several studies have been reported addressing selected mechanistic aspects of glycopeptide-type CSPs. Nair et al. have investigated changes in the chiral recognition performance of vancomycin in the presence of copper ions [216], which are known to form a strong complex with functionalities located at the *N*-terminus of the peptide. The presence of copper ions was found to compromise enantioselectivity severely, indicating that functional groups in proximity to the *N*-terminus are crucial contributors to chiral recognition. Armstrong et al. studied the impact of chemical modification on the chiral recognition capabilities of vancomycin [212]. Silica-attached vancomycin was treated with a

large excess of 3,5-dimethylphenyl isocyanate to transform the reactive amino and hydroxy groups into the corresponding urea and carbamate derivatives, and the enantiomer separation performance of the chemically modified CSP was compared with that of the parent CSP. Only minor changes in enantioselectivity profile for some heterocyclic analytes were observed, suggesting that chiral discrimination is predominately controlled by the peptide core structure. Similar experiments were performed by Berthod et al., employing as derivatization agents the sterically demanding enantiomers of 1-(1-naphthylethyl)-isocyanate. The resultant diastereomeric CSPs showed somewhat enhanced enantiomer separation characteristics as compared to the unmodified CSP, resolving a range of *N*-blocked amino acids derivatives that could not be separated on the parent CSP. However, for some analytes enantioselectivity was lost, indicating that the introduction of the sterically demanding carbamate groups may block some of the essential binding sites at the vancomycin framework. Changes in elution order were not observed on derivatization, pointing to chiral recognition processes preferentially capitalizing on vancomycin-based structure elements. Another important mechanistic study addressed the potential role of the carbohydrate grafts in chiral recognition on teicoplanin-based CSP [215]. For this purpose a CSP incorporating the aglycone of teicoplanin was elaborated, and chromatographically evaluated versus a control CSP harboring native teicoplanin. The aglycon-type CSP exhibited substantially enhanced levels of enantioselectivity for all tested α-amino acids, with improvements in enantioselective free energy ($\Delta\Delta G$) in the range of 1.2 to 4.2 kJ mol^{-1}. Figure 7.16 shows the separation of DOPA on the teicoplanin-type and on the respective aglycon-type CSP.

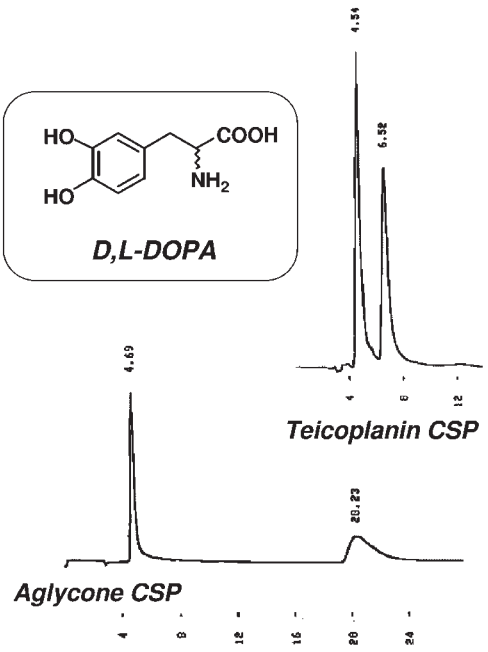


Fig. 7.16 Enantiomer separation of DOPA achieved on a teicoplanin-type and the corresponding aclycon-type CSP. Columns: (250 × 4.6 mm i.d.); mobile phase: methanol/water (60/40 v/v) pH 4.1 TEA + acetic acid; flow rate: 1 mL min^{-1}; detection: UV 254 nm; temp.: 22 °C. (Reprinted with permission from [215]).

From these observations the authors concluded that the enantioselective amino acid binding site must be located at the aglycon backbone, and the carbohydrate grafts in proximity to this site may interfere with efficient enantioselective binding due to steric congestion. For other classes of analytes, however, the carbohydrate deletion had deleterious effects. For example, the teicoplanin-type CSP gave significantly superior enantiomer separation performance for heterocyclic drugs. For a few analytes, enantioselectivity was even lost completely on the aglycone-type CSP, emphasizing for these cases the essential contributions of carbohydrate motifs to chiral recognition.

Evidently, glycopeptide antibiotics harbor multiple functional group assemblies that may act as potential enantioselective binding sites for a wide range of analytes. However, appropriate adjustment of the mobile phase environment is required to exploit the "multimodal" chiral recognition mechanisms [217]. Reversed-phase mobile phase conditions have been reported to activate the chiral recognition, mechanism benefiting from a combination of hydrophobic inclusion and ionic interactions and strong hydrogen bonding. Polar-organic mobile phase environments are recommended to enforce enantioselective interactions based on hydrogen bonding and electrostatic binding increments. Normal-phase conditions were proposed to promote the resolution of analytes capable of undergoing π–π-stacking, dipole–dipole and hydrogen bonding interactions.

Statistical evidence disclosed by the manufacturer [217] indicates that successful enantiomer separation with glycopeptide-type CSPs is preferentially achieved with reversed-phase (40 %) and polar-organic mobile phases (40 %), while normal-phase conditions appear to be less useful (5 %). An independent large-scale screen carried out in industrial settings on the three major glycopeptide-type CSPs with a test set of 55 pharmaceutically relevant analytes (starting materials, intermediates, drugs) confirmed this picture [218]. In this case, the combined success rate with reversed-phase and polar-organic mobile phases was 67 %, with the vancomycin- and the teicoplanin-type CSPs showing superior performance over the ristocetin-type CSP. Similar separation scores for drugs and drug-like compounds were reported by others [114], employing gradient reversed-phase and polar organic screens.

Glycopeptide-type CSPs have been reported to show chiral recognition capabilities for amides, acids, esters, aminoalcohols, cyclic amines, underivatized amino acids, their *N*-blocked amino acids, oligopeptides, hydroxcarboxylic acids, aryloxycarboxylic acids and phenols [212, 214, 215, 219, 220]. In general, glycopeptide-type CSPs show overlapping substrate specificities, a fact that is not surprising considering their close structural resemblance. However, this feature of closely related chiral recognition capabilities has been claimed to offer advantages in method development and optimization [217]. Thus, poor quality enantiomer separation obtained on one glycopeptide-type CSP may be improved by simply switching to a congener under the same mobile phase conditions. This "principle of complementary separations" is demonstrated in Fig. 7.17.

Another attractive feature of glycopeptide-type CSPs is a certain level of predictability concerning the separability of structurally related racemates. Thus, if for a given compound enantiomer separation can be achieved on a glycopeptide-type

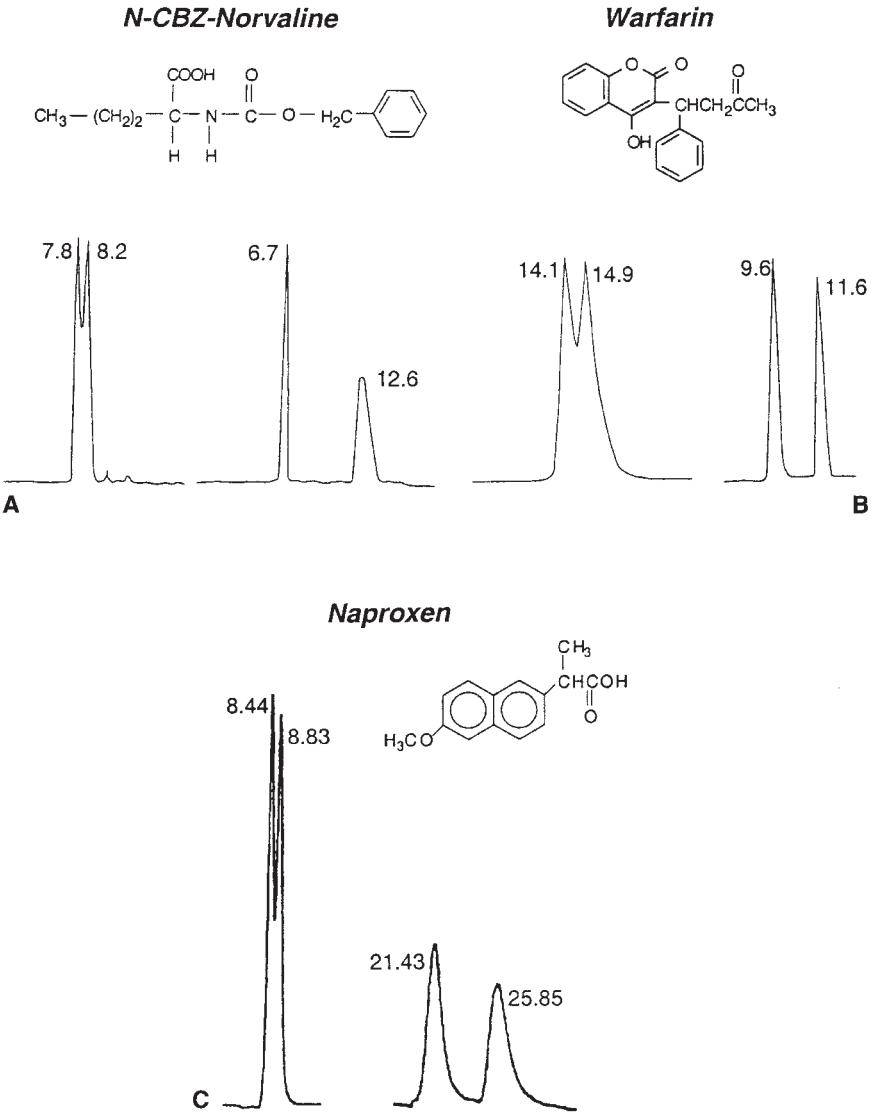


Fig. 7.17 Complementary chiral recognition profiles of glycopeptide-type CSPs. (A) *N*-CBZ-norvaline on vancomycin (left) and teicoplanin (right). Mobile phase: methanol/1% triethylammonium acetate (20/80 v/v) pH 4.1. (B) Warfarin on teicoplanin (left) and vancomycin (right). Mobile phase: acetonitrile/1% triethylammonium acetate (10/90 v/v) pH 4.1. (C) Naproxen on teicoplanin (left) and ristocetin A (right). Mobile phase: methanol/0.1% triethylammonium acetate (30/70 v/v) pH 4.1. Columns (250 × 4.6 mm i.d.); flow rate: 1 mL min^{-1}; temp.: 23 °C. (Reprinted with permission from [217]).

CSP, then there is a good chance that compounds with similar stereogenic environments may also resolve on this CSP under the same or similar chromatographic conditions [217].

A recent observation is that glycopeptide-type CSPs show promising enantiomer separation capabilities under SFC conditions. In the course of a comprehensive screening study, Lui et al. evaluated a set of 111 chiral test compounds, including heterocycles, profens, β-blockers, sulfoxides, *N*-blocked amino acids and natural amino acids, on commercial glycopeptide-type CSPs [221]. More than 90% of the test compounds could be resolved employing carbon dioxide–methanol as the mobile phase, containing low amounts of acidic and basic additives. Among the tested CSPs, Chirobiotic TAG proved most efficient, followed by Chirobiotic T and Chirobiotic R.

7.8.2.3 **Crown Ether-type CSP**

CSPs based on chiral crown ethers have been pioneered by Cram et al., who achieved resolution of series of amino acids esters on silica- and polystyrene-supported bis-(1,1′-binaphthyl)crown ether derivatives [222, 223]. The primary molecular recognition principle underlying crown ether-triggered enantiomer separation is based on the formation of multiple hydrogen bonds between the protonated primary amino group of the analyte and the ether oxygens of the crown framework [224, 225]. This crucial requirement restricts the scope of applications of crown ether-type CSPs to chiral compounds presenting primary amino groups in close proximity to stereogenic centers, such as amino acids, amino acid derivatives, primary amines and amino alcohols. Advances in the field of crown ether-type CSPs have been summarized in a recent review [226].

Shinbo's group introduced CSPs comprising (3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 (for chemical structure see Fig. 7.18A) dynamically coated on octadecyl silica gel [227], which proved efficient tools for resolving a broad range of racemic amino acids and related compounds. A related CSP has been commercialized under the tradename CROWNPAK CR by Daicel Chemical Industries, Tokyo, Japan. Coated crown ether-type CSPs have been successfully employed for enantiomer

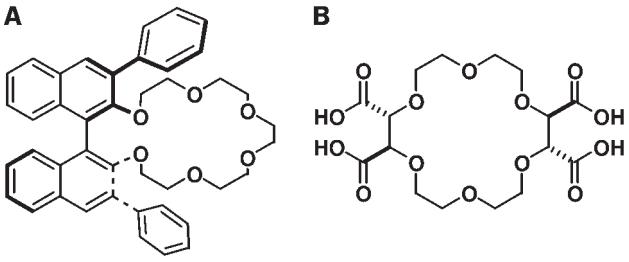


Fig. 7.18 Chiral crown ethers employed as chiral selectors in CSPs for liquid chromatographic enantiomer separation. (A) (3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 employed in coated CSPs. (B) (18-crown-6-2,3,11,12)-tetracarboxylic acid used for the preparation of immobilized CSPs.

separation of various basic drug compounds, such as aminoglutethimide, baclofen and primaquine [228].

These CSPs are preferentially operated under reversed-phase conditions in the presence of acidic additives to ensure full protonation of the analyte's amino function. The proportion of organic modifier (preferentially methanol), however, is restricted to an upper maximum of 15% (v/v) to avoid column deterioration due to crown ether bleeding. Different types of acids may be used as suitable acidic additives, such as formic, trifluoroacetic, sulfuric and perchloric acid, with the latter often providing the best chromatographic performance. Analyte retention, enantioselectivity and resolution can be controlled by the modifier content. Generally, increasing the proportion of organic modifier in the mobile phase diminishes analyte retention, and tends to improve enantioselectivity and resolution. Additional factors influencing the chromatographic behavior on crown ether-type CSP are the concentration of acidic additive and column temperature. Increasing additive concentration enhances retention and enantioselectivity, and the same trend is seen on lowering the column temperature.

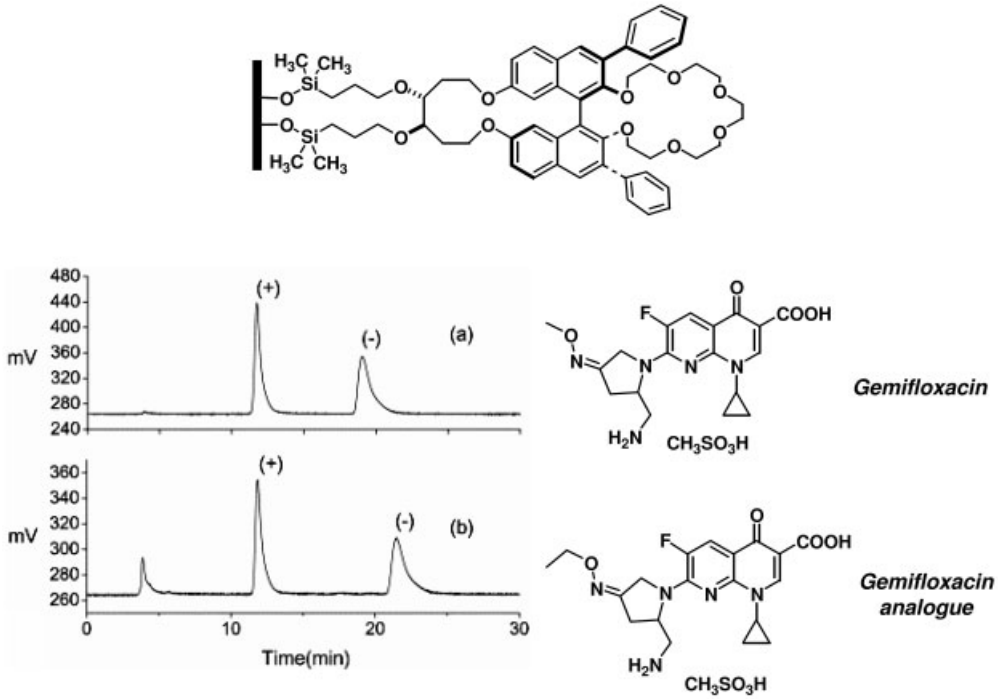


Fig. 7.19 Representative chromatograms for the resolution of (a) gemifloxacin (methanesulfonate form) and the (b) *O*-ethyloxime analogue of gemifloxacin on an immobilized crown ether-type CSP. Column: (250 × 4.6 mm i.d.); mobile phase: 80% acetonitrile in H_2O+H_2SO_4 (10 mM) + ammonium acetate (10 mM); flow rate: 0.8 mL min^{-1}. detection: 254 nm UV; temp.: 20 °C. The sign of optical rotation of the two enantiomers was determined by an in-line Shodex OR-2 polarimetric detector set at 589 nm. (Reprinted with permission from [231]).

The restrictions imposed by the sensitive nature of coated crown ether-type CSPs have recently been addressed by the introduction of CSPs incorporating covalently immobilized crown ether derivatives [229, 230]. This new generation of CSPs can be operated with mobile phases containing high concentrations of organic modifiers. An example is given in Fig. 7.19, showing the separation of the enantiomers of the antibacterial drug gemifloxacin and a related compound on an immobilized crown ether-type CSP employing a mobile phase containing 80% acetonitrile [231].

A CSP comprising silica-attached (18-crown-6–2,3,11,12)-tetracarboxylic acid (for chemical structure see Fig. 7.18B) has been commercialized as CHIRALHYUN-CR-1 by K-MAC Corp., Daejon, Korea. This chemically robust CSP holds promise to facilitate method development by offering improved long term stability and full compatibility with a broad range of organic solvents. Recent experimental evidence demonstrated that the multifunctional crown ether incorporated in this CSP also expresses chiral recognition capacity for analytes devoid of primary amino groups. Thus, Steffeck et al. could achieve with this CSP enantiomer separation for several β-blocker-type analytes comprising secondary amino functions [232, 233]. Similar unexpected results were reported by Hyun, who observed with this CSP the resolution of *N*-acylated amino acids [234].

7.8.3 CSPs Based on Low-Molecular-Weight Molecules

7.8.3.1 Donor–Acceptor-type CSPs

Donor–acceptor-type CSPs capitalize on synthetic or semi-synthetic chiral low-molecular-weight SOs capable of enantioselectively recognizing analytes by complementary arrays of nonionic attractive interactions [67]. The repertoire of these nonionic interactions generally comprises hydrogen bonding, π–π-stacking, dipole–dipole-stacking and steric interactions.

The majority of donor–acceptor-type SOs has been designed to exploit π–π-stacking interactions between electron-rich and electron-deficient aromatic systems as the primary attractive interaction force. The chemical structures of several popular π-donor–acceptor-type CSPs are given in Fig. 7.20.

Major contributions in this field have been made by Pirkle and coworkers, who can claim credit for the development of the first commercial CSP based on 3,5-dinitrobenzoyl-phenylglycine (DNBPG®, Regis Technologies). Another achievement of this group is the intelligent exploitation of the "reciprocity principle of chiral recognition" in support of the development of highly efficient target-specific π-donor–acceptor type SOs [235]. This principle states that the roles of SO and analytes are interchangeable; thus, if a single enantiomer of a SO has different affinities for a pair of analyte enantiomers, then a single enantiomer of this analyte will express different affinities for the enantiomers of the SO. The practical consequence of this fundamental thermodynamic concept is that CSPs incorporating a single enantiomer of the target analyte can be employed to screen libraries of racemic SO candidates for promising target-specific SO candidates.

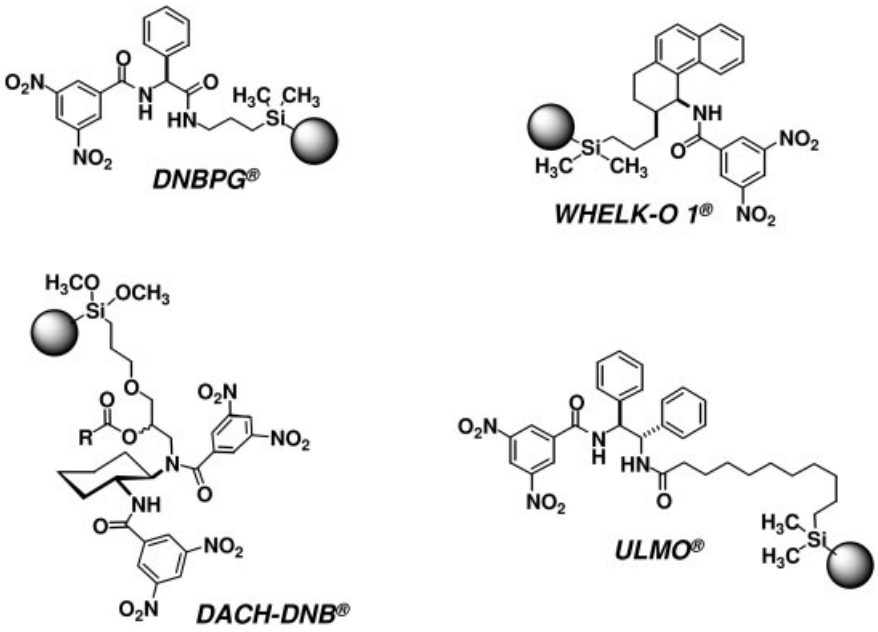


Fig. 7.20 Chemical structures of selected commercial π-donor–acceptor-type CSPs.

An impressive demonstration of the efficiency of this approach was given by Pirkle's group with the successful development of a π-donor–acceptor CSP for the nonsteroidal antiinflammatory drug (NSAID) naproxen [236]. To gain knowledge on the crucial structural requirements for enantioselective recognition of this drug compound, they attached (*S*)-naproxen via an ester-linkage to silica gel, and used this analyte-type CSP to screen a selection of racemic DNB-derivatives. The chromatographic results provided information (and inspiration) on the crucial motifs required for enantioselective naproxen binding, namely face-to-edge and face-to-face π–π-stacking and hydrogen bonding interactions. In addition, the knowledge derived from the screen allowed the proposal of a chiral recognition rationale, which provided guidance in the structure-based design of a dedicated SO for naproxen. Synthesis and immobilization of this SO candidate, 4-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene, indeed provided a CSP with excellent enantioselective binding properties for naproxen. Serendipitously, this particular CSP demonstrated an enhanced chiral recognition profile, being capable of resolving the enantiomers of a broad array of structurally diverse classes of analytes, including many structures of pharmaceutical interest. This rationally designed CSP, commercialized under the tradename WHELK-O 1® (Regis Technologies), represents probably the most versatile π-donor–acceptor-type CSP currently available.

Other useful π-donor–acceptor-type CSPs have been advanced from C_2-symmetric diamine scaffolds. Gasparrini and coworkers developed a series of CSPs

based on silica-supported bis-*N*,*N*′-(3,5-dinitrobenzoyl)-1,2-diamino cyclohexane (DNB-DACH, Regis Technologies) with a relatively broad chiral recognition profile for sulfoxide, phosphinoxides, selenoxides, β-amino acid esters and a variety of drug compounds [237–239]. A similar type of CSP was advanced by Uray et al. based on *N*-3,5-dinitrobenzoyl-*N*′-undecanyl-1,2-diphenyl-1,2-diamine (ULMO, Regis Technologies), showing useful levels of enantioselectivity for a variety of heterocyclic drugs [240, 241], pharmaceutically relevant carboxylic acids [242], arylarylcarbinols [243] and, in particular, for a broad range of arylcarbinols [244]. As an example, the chromatographic separation of four pairs of racemic arylcarbinols on (R,R)-UMLO CSP is given in Fig. 7.21.

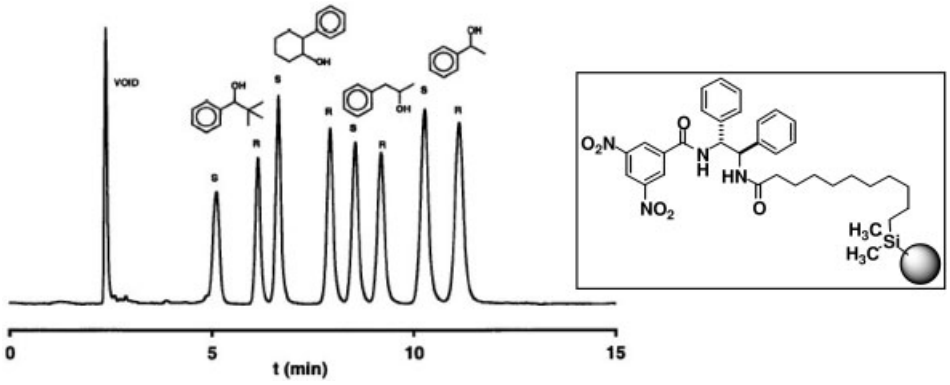


Fig. 7.21 Single run separation of the enantiomers of four arylcarbinols on a (*R*,*R*)-ULMO CSP. Column: (250 × 4.6 mm i.d.); mobile phase, n-heptane/isopropanol/(99.5/0.5 v/v); flow rate: 1 mL min^{-1}; detection: UV 254 nm; temp.: 25 °C. (Reprinted with permission from [244]).

Donor–acceptor-type CSPs derived from synthetic low-molecular-weight chiral scaffolds offer several advantages over those derived from more complex natural compounds [67]. In general, the building blocks for these SOs are available in both enantiomeric forms, from which enantiomeric CSPs with identical chiral recognition profiles, but opposite enantiomer elution characteristics, may be prepared. The robustness of low-molecular-weight SOs make them compatible with a wide range of immobilization chemistries, and their modest spatial demands guarantee high surface coverage, and thus favorable loading capacity. The features of limited structural complexity, ready solubility and ease of structure modification make low-molecular-weight SOs amenable to mechanistic scrutiny by spectroscopic techniques [245–248] and facilitate insightful chemical "site-directed mutagenesis" experiments. The insights gained in studies with soluble versions with low-molecular-weight SOs have significantly enhanced the understanding of chiral recognition processes in general. The derived mechanistic rationales helped to establish reliable rules for the elution order-based assignment of absolute con-

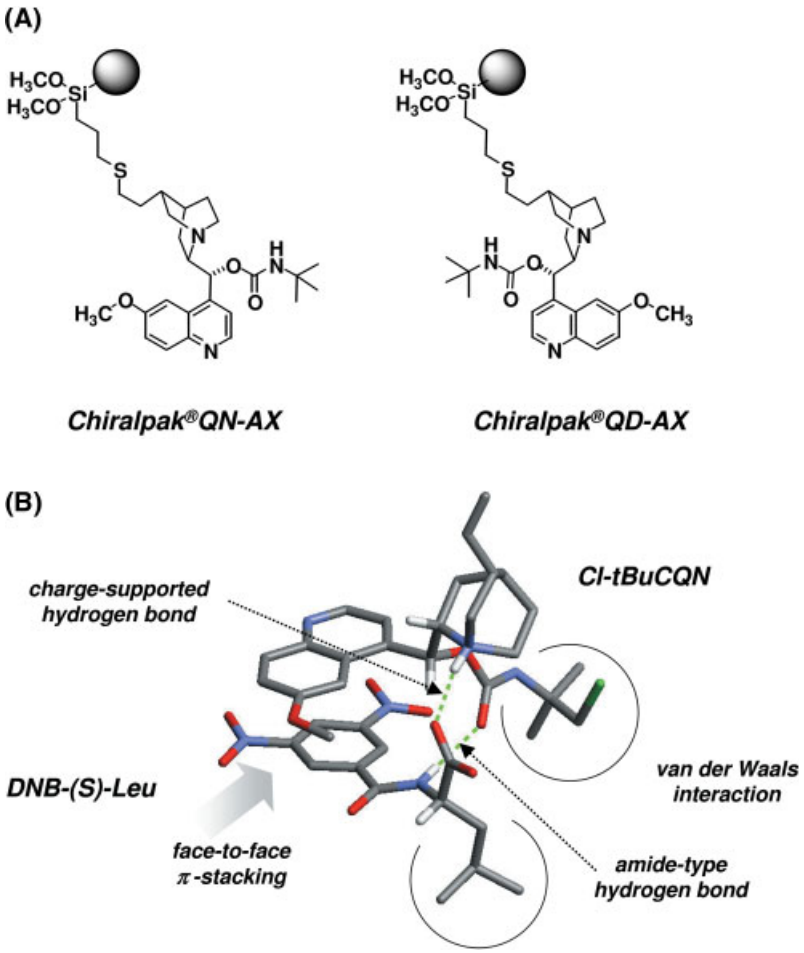


Fig. 7.22 (A) Chemical structures of the quinine and quinidine *tert*-butylcarbamate-based CHIRALPAK®QN-AX and CHIRALPAK®QD-AX anion exchange-type CSPs. These CSPs show pseudo-enantiomeric chiral recognition profiles. (B) X-ray crystal structure of the more stable β-chloro-*tert*-butylcarbamoyl quinine/3,5-*N*-dinitrobenzoyl-(*S*)-leucine ion pair, reflecting the crucial interactions involved in chiral recognition.

figuration [249–251], and enabled, in certain cases, the knowledge-based design of SOs displaying receptorial levels of enantioselectivity [235, 252, 253].

Disadvantages of donor–acceptor type CSPs are their rather limited scope of application and incompatibility with polar-organic and reversed-phase mobile phase conditions. Thus, the spatially well-defined, but restricted functional group repertoires of donor–acceptor-type SOs can satisfy the chiral recognition requirements of a few classes of analytes only. In addition, to produce useful levels of enantioselectivity with donor–acceptor-type CSPs, analytes may frequently require dedicated (achiral) derivatization to attenuate basicity/acidity and/or to complement

functionality essential for chiral recognition. The noncovalent interaction forces effecting chiral recognition on donor–acceptor-type CSPs are easily disrupted by high-polarity and hydrogen-bonding solvents, confining the use of these CSPs to normal-phase environments.

7.8.3.2 **Ion-exchange-type CSPs**

Ion exchange-type CSPs rely on enantioselective ion-pairing interactions between complementarily charged SO and analyte species. Enantioselective ion-pairing as a molecular recognition principle may be encountered with all types of CSPs incorporating ionizable SO systems, including those based on glycopeptide and proteins. In the following, however, the discussion will focus on CSPs for which the enantioselective ion-pairing between charged SO and analyte species represent the dominating interaction force.

In 1996 Lämmerhofer et al. demonstrated that CSPs based on silica-immobilized quinine and quinidine 9*O*-carbamates exhibit, under buffered reversed-phase operation conditions, excellent chiral recognition capabilities for a wide range of chiral acids [254]. Analysis of the underlying retention behavior established a mixed mode mechanism involving ion-pairing as the primary interaction force and minor contributions originating from hydrophobic adsorption. Structure-based optimization of the first generation of cinchona carbamate-type SOs led to the development of CSPs incorporating the sterically demanding 9*O*-*tert*-butylcarbamate derivatives of quinine (tBuCQN) and quinidine (tBuCQD) [255]. These improved CSPs, offering enhanced chiral recognition performance and an extended scope of application, have recently been commercialized under the tradenames CHIRALPAK®QN-AX and CHIRALPAK®QD-AX by Chiral Technology Europe (Strasbourg, France). The chemical structures of these CSPs are depicted in Fig. 7.22A.

Comprehensive chromatographic [255, 256], spectroscopic [257–259], molecular modeling [257, 259] and X-ray crystal structure [255, 256, 259] studies have been carried out to elucidate the crucial intermolecular forces governing enantioselective binding of acidic analytes to these SOs. The huge body of experimental data emerging from these interdisciplinary efforts advocates a chiral recognition mode involving intermolecular ion-pairing as the primary attractive interaction force, supported by an array of simultaneously acting secondary binding increments. These stabilizing intermolecular contacts are evident from the X-ray crystal structure of the more stable SO/analyte ion pair complex between β-chloro-*tert*-butylcarbamoyl quinine (Cl-tBuCQN) and 3,5-*N*-dinitrobenzoyl-(*S*)-leucine (DNB-(*S*)-Leu) depicted in Fig. 7.22B.

Hence, DNB-(*S*)-Leu is intercalated into a cleft-like binding pocket located between the quinoline and the carbamate segments of Cl-tBuCQN, and forms, via its carboxylate group, a charged-supported hydrogen bond to the protonated quinuclidine nitrogen in Cl-tBuCQN. The aromatic portions of DNB-(*S*)-Leu and Cl-tBuCQN are engaged in stabilizing face-to-face π–π-stacking interactions. Further stabilization of the ion pair complex is achieved by an amide-type hydrogen bond existing between the carbamate carbonyl of Cl-tBuCQN and the amide-NH group

of DNB-(*S*)-Leu. Close contacts between the hydrophobic batches of the Cl-tBuCQN carbamate group and DNB-(*S*)-Leu side chain indicate additional stabilizing arising from van der Waals attraction. The results of solution-phase NMR studies carried out with the individual diastereomeric tBuCQN/DNB-Leu complexes in CD_3OD at chromatographically relevant concentrations confirmed this interaction mode [259]. Chiral recognition was shown to originate from the inability of DNB-(*R*)-Leu to access the enantioselective tBuCQN binding site and benefit from the embedded secondary interaction elements. Recently disclosed solid-phase MAS NMR spectroscopic binding studies of DNB-Leu with silica-bound tBuCQN provided consistent information, adding confidence that the proposed chiral recognition mechanism has physical relevance for the chromatographic processes occurring in the stationary/mobile phase interface [258].

Enantiomer separation of acidic analytes on cinchona carbamate anion exchange type-CSP generally requires buffered reversed-phase or polar organic mobile phase conditions [86]. Retention and enantioselectivity can be controlled by the mobile phase pH, the concentration and type of the buffer salts, the nature and content of the organic modifiers, and temperature. For the majority of acidic analytes, optimum levels of enantioselectivity are observed in a range of pH 4 to 6. Analyte retention can most conveniently be adjusted over a wide range without compromising enantioselectivity by increasing the concentration of buffer salts, such as ammonium acetate or ammonium formate. Excessively strong retention occasionally observed with hydrophobic acids can be attenuated by increasing the amount of organic modifiers, or, preferably, by switching to polar organic mobile phase conditions. Nonaqueous phases consisting of methanol and low concentrations of ammonium acetate and acetic acid generally reproduce the enantioselectivity found under reversed-phase conditions, with the added advantages of reduced retention and enhanced efficiency. Temperature can also be exploited to tune the chromatographic performance. Chiral recognition of acidic analytes on cinchona carbamate anion exchange-type CSPs are enthalpically controlled processes [260], with decreasing operation temperature enhancing both analyte retention and enantioselectivity. Alternatively, performing enantiomer separation at elevated temperatures is a convenient option to improve mass transfer kinetics and increase column efficiency.

Cinchona carbamate anion exchange-type CSPs cover an extremely broad scope of application for different classes of chiral acidic analytes, comprising carboxylic, sulfonic, phosphonic and phosphoric acids [86]. The enantiomers of *N*-blocked α- and β- and γ-amino acids are particularly well-resolved and well-established interaction models allow, in many cases, elution order-based assignments of absolute analyte configuration [261, 262]. Cinchona carbamate-type CSPs also resolve a broad range of chiral acidic compounds of biological and pharmaceutical relevance, such as 2-arylpropionic acids [86], phyrethroic acids [263], phosphinic acid analogues of dipeptides [261], heptelidic acid and atrolactic acid [86], 1,4-dihydropyridine monocarboxylic acid [264], thyroxine and related compounds [265]. The enantiomer separation of thyroxine and triiodothyronine from a pharmaceutical formulation on the CHIRALPAK®QN-AX CSP is shown in Fig. 7.23.

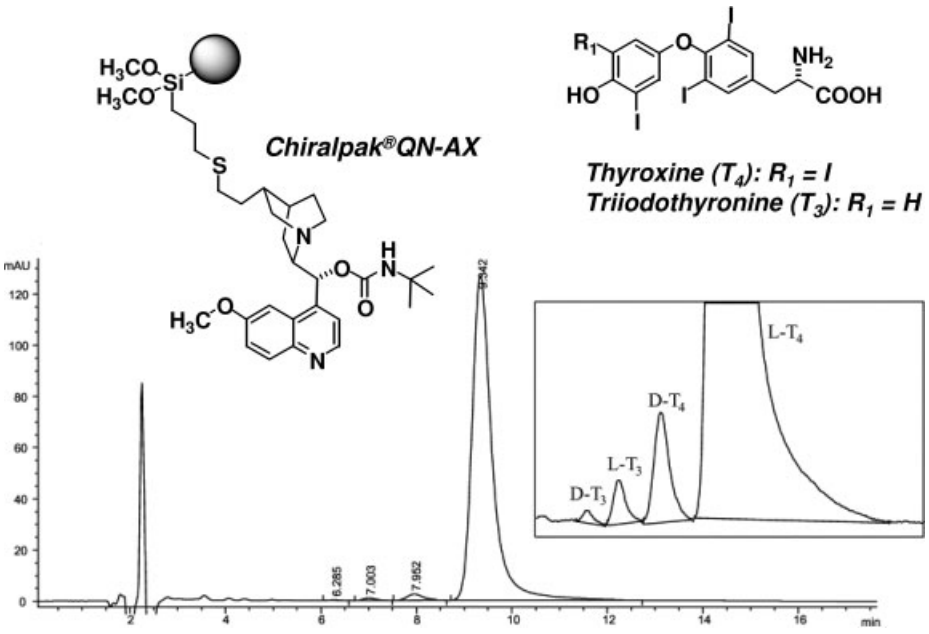


Fig. 7.23 Identification of impurities in pharmaceutical formulation of L-thyroxine sodium. Column: CHIRALPAK®QN-AX (150 × 4.0 mm i.d.); acetonitrile/0.05 M aqueous ammonium acetate (60/40 v/v) pH 4.5; flow rate: 0.7 mL min^{-1}; detection: UV 240 nm; temp.: 25 °C. (Reprinted with permission from [265]).

Importantly, the well-known pseudo-enantiomeric behavior of quinine and quinidine is also reflected in the chiral recognition characteristics of the corresponding carbamate derivatives. Under identical chromatographic conditions, CSPs incorporating quinine carbamates produce, for a given analyte, very similar levels of enantioselectivity to the corresponding quinidine congeners, but opposite enantiomer elution orders. This complementary chiral recognition profile may facilitate analytical method development. Cinchona carbamate-type CSPs also show considerable promise for preparative enantiomer separation applications. Loading capacities up to 1 g racemate per kg CSP have been achieved under touching band conditions for well-resolved (alpha > 2) acidic analytes.

Recent studies indicate that cinchona carbamate-type CSPs may also present an interesting option to address enantiomer separation problems involving nonacidic analytes. Thus, the enantiomers of a set of quinazolone-based drug candidates have been successfully resolved [266], employing acetonitrile and methanol-containing hydro-organic mobile phases. The enantiomer separation of various types of arlyalcohol derivatives under normal-phase conditions has also been achieved on cinchona-type carbamate CSPs [267–269].

7.8.3.2 Ligand-exchange-type CSPs

Ligand-exchange-type CSPs capitalize on the reversible enantioselective coordination of immobilized SOs and analyte species from the mobile phase around suitable metal ions. Depending on the steric and functional properties of the analytes these diastereomeric ternary chelate complexes show different rates of formation and/or thermodynamic stabilities, giving rise to different column residence times. This particular type of chromatographic enantiomer separation technique, developed and popularized by Davankov and coworkers [270], was the first to achieve the complete enantiomer separation of racemic compounds [271]. An essential requirement for the applicability of ligand-exchange chromatography is the presence of metal-chelating functionalities in both SO and the analytes. Suitable structure motifs should offer bidentate and (tridentate) groups, such as hydroxy, amino and carboxylic functionalities as found in α- and β-amino acids, amino alcohols, α-hydroxy acids and dansyl and dabsyl derivatives of α- and β-amino acids. As chelating metal ions Cu(II) and Ni(II) have been preferentially employed, but Zn(II), Cd(II) and Hg(II) have been shown to be suitable alternatives. Concerning the SO ligands, rigid cyclic amino acids, such as proline and hydroxyproline, have been demonstrated to give the best results in combination with Cu(II). The nature of the immobilization chemistry and the type of chromatographic support employed for CSP preparation has been shown to have an important impact on the general chiral recognition performance. The repertoire of parameters exploitable for method optimization includes mobile phase pH, type and concentration of buffer salts, nature and content of organic modifier and column temperature.

Due to the rather restrictive requirements in terms of analyte structure, the applicability of ligand-exchange-type CSPs is limited to a rather narrow range. In the early years of chromatographic enantiomer separation, ligand-exchange-type CSPs were the first choice for the assessment enantiomer purity of underivatized amino acids. However, method development with ligand exchange-type CSPs can experimentally be quite demanding [272], and column efficiencies tend to be rather poor [273]. With the advent of easier-to-operate CSPs with competing chiral recognition profiles, such as glycopeptide- and crown ether-type CSPs, ligand-exchange-type CSPs have lost their privileged position. However, their excellent chiral recognition performance for aliphatic α-hydroxy acids is still appreciated.

A variety of CSPs operating on ligand-exchange-type principles are commercially available from Daicel Chemical Industries, Ltd (Japan). Among these, CHIRALPAK®MA(+), based on *N,N*-dioctyl-L-alanine, and the proline-type CHIRALPAK®WH appear to be the most broadly applicable CSPs.

7.8.4 CSPs Based on Target-specific Bioaffinity Systems

Tracing analytes at low concentration levels in biological environments is a frequently encountered bioanalytical challenge in the course of drug development. Unarguably, the availability of target-specific affinity systems allowing the direct

quantification of the analyte(s) of interest in the presence of complex biological matrices would greatly facilitate this task. Conventional SOs show relatively broad substrate specificity and modest levels of binding affinity and hardly qualify for this task. Recent advances in the fields of microbiology and protein engineering have established a rich toolbox of techniques for the *in vitro* evolution of biomolecules with unprecedented target-specific binding properties. Among these enantioselective antibodies [274] and small-molecule-binding oligonucleotides [275], so-called aptamers, hold considerable promise as new analytical tools for isolation and quantification of chiral drugs.

7.8.4.1 **Antibody-type CSPs**

Nevanen et al. reported the generation of stereoselective recombinant antibody fragments for finrozole, a diarylalkyltriazole-type drug, incorporating two stereogenic centers, and evaluated their utility for the chromatographic enantiomer separation [276]. The antibody fragments were engineered to carry a histidine-tag at the *C*-terminus, which allowed immobilization to a chelating sepharose support by copper-mediated affinity binding. Loading of the racemic mixture of the drug and washing with neutral aqueous buffer led to the elution of the less strongly bound (*R*,*S*)-enantiomer in the flow through. Recovery of the specifically bound (*S*,*R*)-enantiomer, however, required rather harsh conditions to disrupt the high affinity interaction between the immobilized antibody and its target. Release of the specifically bound enantiomer was achieved by addition of 40% (v/v) methanol to the elution buffer. Some loss in binding capacity became evident after 20 loading/elution cycles, indicating antibody denaturation and/or compromised immobilization. In subsequent work the authors re-engineered the binding sites of these antibody fragments to attenuate binding affinity and thus allow for less stringent elution conditions [277]. Site-directed mutagenesis targeting amino acid residues located at the substrate binding domain produced a number of enantioselective mutants with up to ten-fold decreased binding affinities. These affinity-attenuated mutants could be used for multiple rounds of enantiomer separation without significant decay in capacity and selectivity.

In related work Hofstetter and coworkers evaluated immobilized monoclonal anti-D- and anti-L-phenylalanine antibodies as target-specific CSPs [278]. For this purpose the antibodies were covalently attached onto a polystyrene-based macroporous perfusion resin, allowing high-flow-rate operation at low back pressures. To avoid antibody denaturation enantiomer separations were carried out with phosphate buffered saline at pH 7.4, mimicking physiological conditions. Excellent levels of enantioselectivity were observed for the target analyte, phenylalanine ($\alpha > 90$), but also for structurally related amino acids, such as histidine ($\alpha = 70$), tryptophan ($\alpha = 61$) and kynurenine ($\alpha = 59$). The observed elution order was in all cases consistent with that observed for the target analyte. These observations indicate that the employed antibodies preferentially sense functionality located at the amino acid's stereogenic center, while exhibiting relaxed specificity towards side chain motifs. In a follow-up paper these workers studied the influence of a num-

ber of operational parameters on the chromatographic chiral recognition performance of these antibody-type CSPs, including mobile phase pH, ionic strength, effect of modifiers and flow rate [279]. Increasing the flow rate was found to diminish the retention of the specifically bound enantiomer. This unexpected behavior was rationalized as a consequence of enhanced dissociation rates due to free ligand depletion in the proximity of the binding site. The effect of flow rate on the enantiomer separation of D,L-phenylalanine on the anti-L-amino acid antibody-type CSP is shown in Fig. 7.24.

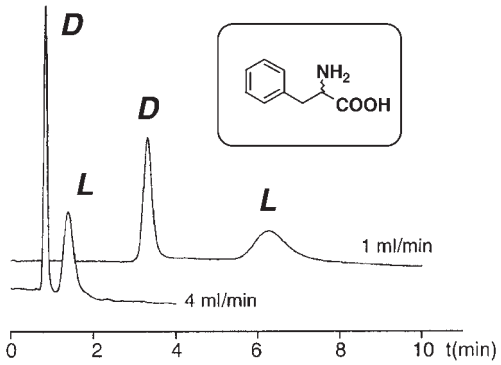


Fig. 7.24 Enantiomer separation of D,L-phenylalanine on the anti-L-amino acid antibody-type CSP at 1 mL min^{-1} and 4 mL min^{-1}. Separations were carried out at 25 °C using phosphate buffered saline as mobile phase. (Reprinted with permission from [279]).

Increasing temperature led to losses in analyte retention and enantioselectivity, consistent with an enthalpically controlled chiral recognition process. Mobile phase pH-values higher or lower than pH 7.4 induced significant losses in retention and enantioselectivity, reflecting the fact that the molecular recognition capacity of antibodies is confined to physiological conditions by Nature. Mixed effects were seen concerning the impact of ionic strength. While the CSP based on the anti-(L)-amino acid antibody suffered losses in selectivity and retention with increasing ionic strength, the anti-(D)-amino acid antibody column was found to be insensitive to increasing ionic strength. The presence of organic modifiers, such as methanol, ethanol, propanol, 2-propanol or acetonitrile, in the mobile phase generally diminished retention and enantioselectivity.

Poor long term stability may present one of the major obstacles for future analytical application of CSPs based on enantioselective antibodies. Hofstetter and coworkers, however, reported that in their hands anti-amino acid antibody-type CSPs, when exclusively operated with phosphate buffered saline pH 7.4, could be reused for several thousand analyses over a period of three years without any significant loss in performance [279].

7.8.4.2 **Aptamer-type CSPs**

In the last decade, DNA and RNA aptamers have emerged as powerful target-specific affinity probes for many analytical applications. Aptamers with antibody-like affinity and target-specificity can be extracted from easily accessible combinatorial

oligonucleotide libraries using an evolutionary *in vitro* process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [280, 281]. This process involves the synthesis of a huge pool of oligonucleotides flanked by primer sequences, making them amenable to amplification by polymerase chain reaction (PCR) technology. Then, employing affinity chromatography on supports with immobilized targets, sequences showing target-specific binding are extracted. These binders are recovered and amplified by PCR to generate a new pool enriched in cognate sequences. These oligonucleotides are subjected to additional rounds of affinity selection of increasing stringency (evolutionary pressure), leading ultimately to the retrieval of the sequences with the most potent binding characteristics.

SELEX has been demonstrated to be a powerful tool for the evolution of enantioselective binders expressing extreme levels of selectivity. For example, Geiger et al. have employed SELEX to extract from RNA pools comprising 10^{15} sequences aptamers specific for L-arginine, with dissociation constants in the low μM-range and an unprecedented level of enantioselectivity $\alpha > 12000$ [282]. This outstanding result was achieved by employing a sophisticated selection procedure of high stringency. The selection strategy included heat denaturation to suppress misfolding, counter-selection with compounds structurally related to the target to enhance specificity, and pre-selection with the target enantiomer to remove low-affinity binders.

The utility of immobilized aptamers for chromatographic enantiomer separation was explored by the group of Peyrin [283–286]. In a proof-of-concept study these workers immobilized a biotin-tagged 55-base DNA-aptamer selected for the all-D-enantiomer of arginine-vassopressin onto an avidine-functionalized perfusion-type support [285]. They could achieve an excellent level of enantioselectivity for a racemic mixture of the all-D-peptide and all-L-peptide, with a mobile phase consisting of 5 mM phosphate buffer, 3 mM $MgCl_2$ at pH 6. Study of the chiral recognition mechanism of the all-D-peptide binding revealed desolvation, adaptive conformational transition and charge–charge interactions as crucial contributions. In an extension of this work, these authors demonstrated the general applicability of aptamer-based enantiomer separation for small biologically active molecules, such as D-adenosine and L-tyrosinamide [286]. The corresponding aptamer-type CSPs gave modest enantioselectivity for adenosine ($\alpha = 3.72$), but excellent enantioselectivity was observed for tyrosinamide ($\alpha = 79$). However, these figures should be considered with care as very little retention was obtained for the less strongly retained enantiomers. Also, the authors noted a significant loss in binding capacity for the L-tyrosinamide column, indicating oligonucleotide degradation. This stability issue was addressed in a subsequent contribution using the so-called "spiegelmer" approach. Specifically, the authors compared the long term stability of a "natural" D-RNA aptamer selected against L-arginine with that of the corresponding "non-natural" L-RNA aptamer [284]. It needs to be emphasized that these aptamers are enantiomers, expected to display identical levels of enantioselectivity but opposite preference of enantiomer binding. Within a period of one week the CSP based on the natural D-RNA-aptamer lost more than 65% of its

initial retention for L-arginine, while the corresponding L-RNA-aptamer showed no decay in binding capacity for D-arginine, even after more than one month. These findings suggest that the non-natural L-RNA-aptamer may present an excellent alternative to biodegradation-prone natural D-RNA-aptamers.

7.9 Conclusions

Over the last two decades, chromatographic enantiomer separation on chiral stationary phases has developed to be an indispensable tool in chiral drug discovery. Nowadays, enantioselective chromatography is involved in virtually all stages of drug development, providing fast access to pure stereoisomers, establishing crucial information on stereoselective activity/toxicity profiles, guiding process development efforts, and ensuring the quality and safety of the finished drug product.

Currently ongoing research in the field of chromatographic enantiomer separation may help to further advance its utility for drug research applications. In particular, recent progress in the fields of innovative chromatographic supports, automation and miniaturization holds promise of enhanced ease of operation, sensitivity and throughput capacity. The implementation of chiral stationary phases based on monolithic media will allow enantioselective assays to be carried out at high flow rates with minimal loss in efficiency. In enantioselective bioanalysis, the coupling of chromatographic assays with mass-sensitive detection systems has been shown to dramatically enhance the information content, facilitating the simultaneous monitoring of drugs and their metabolites with unprecedented sensitivity. The time-consuming trial-and-error associated with method development of enantioselective assays is increasingly replaced by fully automated chromatographic screening technologies.

In the not too distant future, when genomics, proteomics and related "ics" disciplines will finally start fulfilling their great promises, a host of new targets may emerge. Although the "druggability" of these targets and the chemical nature of ideal drugs directed towards these targets remain subjects of debate, it is safe to assume that the drugs of the future will feature molecular or supramolecular chirality. In this context, chirality issues can be expected to remain a central topic in drug development in the future.

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8
Capillary Electrophoresis Coupled to Mass Spectrometry for Chiral Drugs Analysis*

Serge Rudaz and Jean-Luc Veuthey

8.1
Introduction

As Louis Pasteur proposed in his time, enantiomers can be separated as diastereomeric derivatives after a chemical reaction with a chiral selector using fractional recrystallization (see Chapter 5). However, separation techniques such as chromatography and electrophoresis are now recognized for the separation of enantiomers. Even if chromatography is considered as the method of choice for chiral analytical purposes (see Chapter 7), capillary electrophoresis (CE) has recently gained importance in the field of stereoselective analysis.

Since its introduction in the 80s, CE has rapidly become a powerful separation technique and has found numerous applications. High efficiency, short analysis time, rapid method development, simple instrumentation, low cost per analysis and low sample requirement are the main reasons for this success. In the case of chiral analysis, the separation is generally performed by adding a chiral selector to the background electrolyte solution (BGE). The best resolution can easily be obtained by changing the chiral selector, its concentration or other analytical conditions, including simultaneous addition of several stereoselective agents. Therefore, simple instrumentation and method development allow, without difficulty, optimal separation conditions. UV–VIS spectrophotometry is often chosen for the on-line detection of compounds separated by CE. However, sensitivity is low, due to the short optical pathlength afforded by the small internal diameter of capillaries and requires relatively high analyte concentrations. It follows that the major evolution in recent methodologies is improvement of the detection systems.

Due to its high sensitivity and specificity, mass spectrometry (MS) is a detector of choice in micro-separation techniques such as CE. This selective detector provides additional advantages by allowing high speed analysis, giving information about the mass, and potentially, the structure of the separated compounds. This

* Please find a list of abbreviations at the end of this chapter.

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information is highly desirable for an unambiguous identification and confirmation of components in complex mixtures.

In this context, the on-line coupling of CE with MS has evolved into an efficient, sensitive and selective technique for the chiral analysis of drugs. This chapter does not intend to cover all the recent developments in the application of electromigration approaches coupled with conventional detection techniques such as UV, electrochemical, fluorescence or LIF [1, 2] for the enantioselective analysis of drugs. Moreover, achiral CE-MS applications have been summarised in recent reviews and these can be consulted for a more systematic coverage of the field [3–5]. This chapter will therefore focus on the electrophoresis-based enantioseparation technique coupled with mass spectrometry.

8.2 Capillary Electrophoresis (CE)

Separation by electrophoresis is obtained by differential migration of solutes in an electrical field. In CE, separation is performed in capillaries which are usually filled only with a buffer solution, namely background electrolyte (BGE). The high electrical resistance of the capillary enables the application of very high electrical fields (100 to 500 V cm^{-1}) with only minimal heat generation. Moreover, the large surface-to-volume ratio of the capillary efficiently dissipates the generated heat. Application of high electrical fields results in short analysis time as well as high efficiency and resolution. In addition, minimal sample requirements (1 to 10 nL) and the overall simplicity of the instrumentation give CE numerous advantages vis-à-vis conventional separation techniques.

A fundamental constituent of CE operation is the so-called electro-osmotic flow (EOF) which is a consequence of the surface charge on the interior capillary wall. Under particular conditions, surface ionization creates a mobile double-layer at the capillary wall to maintain the charge balance. When the voltage is applied across the capillary, the cations forming the double-layer are attracted toward the cathode. Because the cations are solvated, their movement drags the bulk solution in the same direction. A unique feature of EOF in the capillary is a flat flow profile. Since the driving force of the flow is uniformly distributed along the capillary, there is no pressure drop within the capillary and the flow remains nearly constant throughout. The flat flow profile is beneficial since it avoids contributing directly to the dispersion of the solute zone. This contrasts with the laminar flow profile observed in pressure-driven techniques such as liquid chromatography. Therefore, in CE, transport of analytes is due to two phenomena: electrophoretic migration and electro-osmosis. As a result of the EOF and as a function of electrophoretic and electro-osmotic mobilities, all sample components, be they cations, neutrals or anions, are drawn towards the cathode and the detector, although with different velocities.

8.3
CE-MS Coupling

UV–VIS spectrophotometry is considered as the detector of choice in capillary electrophoresis. However, beside its relatively low sensitivity, many pharmaceutical and biological substances do not possess a chromophore and, therefore, require a derivatization procedure. Other detection techniques have been coupled to CE such as electrochemical detection (EC) and fluorescence, especially laser-induced fluorescence (LIF), but with several limitations. EC is limited to electroactive substances and the interfacing with capillary electrophoresis is not trivial since it requires a decoupling and the physical alignment of the electrode with the end of the capillary. With LIF, only a limited number of substances are fluorescent at the appropriate wavelength. Therefore, a derivatization procedure is required which compromises the time gain and the small sample volume capabilities of CE. Finally, with all these detection methods, peak identity is generally confirmed using migration times only which is often insufficient to identify unequivocally compounds of interest, since real samples may contain unknown interferences and the parameters influencing migration time are not easily controlled in EC.

Mass spectrometry (MS) possesses the unique ability to identify separated compounds and is now used routinely with gas and liquid chromatography. This selective and highly sensitive detection mode is also compatible with capillary electrophoresis and provides a powerful combination for performing rapid, efficient and sensitive analysis.

8.3.1
CE-ESI-MS

Several ionization methods have been applied for CE-MS coupling. Matrix-assisted laser desorption ionization (MALDI), continuous flow fast atom bombardment (FAB), laser vaporization ionization with UV laser, sonic spray ionization and electrospray ionization (ESI) have all been used for coupling CE to MS. However, ESI is now undoubtedly the most widely used ionization technique, employing numerous analyzers including quadrupoles, magnetic sector, Fourier transform ion cyclotron resonance, time-of-flight and trapping devices. However, quadrupole detectors have predominantly been applied in CE-MS [6–8].

ESI is a mild ionization technique which is compatible with a large flow rate from nL min^{-1} up to several mL min^{-1}. It allows the detection of singly and multiply charged compounds and is especially suited for the analysis of polar and moderately polar compounds in the mass range from 10^2 to 10^5 Da.

Unlike spectroscopic detectors which are performed on capillary, the outlet end of the CE must be removed from the vial and positioned in front of the mass spectrometer inlet. This process must be performed without sacrificing separation efficiency and ensuring electrical continuity. Therefore, the interface between CE and ESI-MS is one of the keys to the success of this technique. Three modes have been reported, including liquid–liquid junction, sheath–liquid and sheathless interfaces

[9–12]. However, because of its instrumental simplicity and versatility, together with the possibility of enhancing the ionization process by chemical reaction, the co-axial sheath–flow interface represents the most common approach when connecting CE with ESI-MS. In this arrangement (Fig. 8.1), the fused silica capillary is located in the centre of the sprayer while the middle capillary (usually in stainless steel) provides a coaxial sheath liquid make-up flow to ensure stable electrospray as well as electrical contact at the capillary outlet. Generally, the sheath liquid is introduced at a flow rate of a few μL min^{-1} and is significantly higher than the CE flow rate, typically in the range of 100 nL min^{-1}. Finally, the outer capillary, also in stainless steel, supplies the nebulizing gas to assist spray formation.

CE-ESI-MS coupling induces some limitations concerning the choice of the background electrolyte solution. Indeed, nonvolatile buffers commonly used in CE, such as phosphate, borate and citrate, are not compatible with MS, although a make-up flow containing 50 to 80% organic solvent may encompass this incompatibility. Therefore, volatile buffers such as formic acid, trifluoroacetic acid, ammonium acetate, acetic acid and so on are often recommended. Furthermore, CE separations require the presence of additives in the BGE such as micelles, microemulsions, ion-pairing agents and chiral selectors to improve selectivity but they are not directly compatible with CE-ESI-MS coupling. These substances may have detrimental effects on ESI-MS performance, since they can enhance the contamination risk of the ionization chamber and can suppress the analyte signal. However, different strategies have been reported to address this incompatibility such as the partial filling technique (see Sections 8.5 and 8.6) or capillary electrochromatography (see Section 8.9).

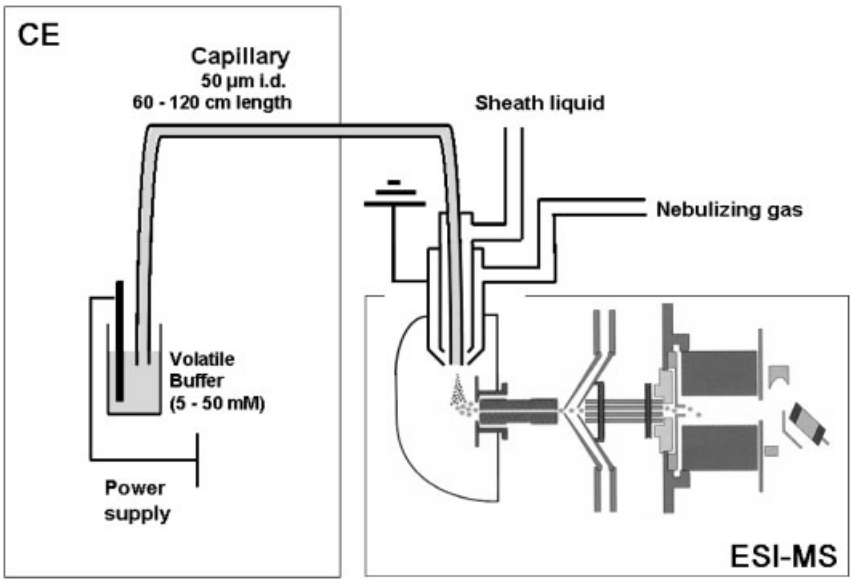

Fig. 8.1 Instrumental set-up of CE-ESI-MS with a sheath–liquid interface.

It can be noted that optimizing a CE-ESI-MS procedure involves a compromise since better electrophoretic efficiencies are obtained in the presence of high ionic strength buffers, whereas the ionization process in electrospray is impaired under these conditions. Finally, even if ESI-MS is a concentration sensitive detector, the dilution of BGE by the sheath liquid does not significantly affect sensitivity since the ionization process occurs in the gas phase and both sheath liquid and buffer electrolyte are completely evaporated.

8.3.2 Other CE-MS Coupling

As already mentioned, almost all CE-MS analyses have been reported with an ESI interface and a single quadrupole mass spectrometer. The performance with this kind of arrangement is largely sufficient in several cases. However, with the need for more sensitive and informative analytical techniques, other combinations will certainly emerge in the near future. Tandem MS and ion trapping devices are already frequently used in gas and liquid chromatography and will also find their application in capillary electrophoresis in the proteomics and metabolomics domains. A new generation of Fourier transform ion cyclotron resonance and time of flight MS, which allow high resolution, high mass accuracy and fast acquisition rates, open new avenues for CE-MS.

Moreover, besides ESI, which will remain the preferred ionization technique, other atmospheric pressure ionization modes such as atmospheric pressure chemical ionization (APCI) and, more recently, atmospheric pressure photoionization (APPI) can be used as interesting alternatives. These interfaces might be of great interest in CE-MS, since they allow different selectivities and are more tolerant than ESI with respect to nonvolatile electrolytes. However, the lack of commercially available interfaces appears to be one of the limiting factors of these ionization techniques for CE analysis.

8.4 Chiral Separation Strategies

The most frequently applied CE techniques for chiral separations are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and chiral capillary electrochromatography (CEC). Two strategies are generally adopted for the enantiomer resolution in these electrophoretic techniques :

- The direct resolution of enantiomers in a chiral stationary phase (CSP) which contains an immobilized selector. In this case, labile diastereomers are formed on the support (CEC).
- The dynamic mode where resolution of enantiomers is carried out by adding a chiral selector directly in the BGE for *in situ* formation of diastereomeric derivatives (CZE, MECK).

In all cases, the chiral selector creates a stereoselective environment which enables the separation of the two enantiomers. One of the most attractive advantages of CZE and MEKC for enantiomer separation is that the separation media can easily be modified during method development to screen the most appropriate stereoselective environment (chiral selector type and concentration). Furthermore, one can rapidly find the optimum operating conditions at low cost according to the minimal amount of additives and solvents required. Another relevant advantage of CE over other separative techniques such as LC or CEC, is the possibility of a reversal of the enantiomeric migration order by simple procedures such as changing the chiral selector nature or system polarity [13, 14]. This important feature allows the minor enantiomer in the sample to migrate in front of the major one.
Besides the coupling of CEC and MS which appears effortless, only a few applications exist and most of the published chiral separations in CE-MS concern the dynamic mode. However, as previously mentioned, hyphenation of chiral CZE or MECK with MS is severely hampered by the presence of nonvolatile additives, which often leads to a significant loss of ionization efficiency and/or contamination of the ionization chamber. Considerable progress has been made in the last few years to circumvent these limitations and therefore to increase the range of application for chiral CE-MS.

Various chiral selector additives, such as chiral crown ethers, proteins, antibiotics, bile salts, chiral micelles and ergot alkaloids, have been reported in the literature [15–17]. An extensive review of the numerous selectors of CE is outside the scope of the present chapter. Nevertheless, CDs are by far the most widely used selectors in chiral CE. CD are nonionic cyclic oligosaccharides consisting of six, seven or eight glucose units and are called α-, β-, and γ-CD, respectively.

Neutral and charged CD derivatives, with various functional groups, have been developed to induce different stereoselective interactions and enhance enantioselectivity and solubility. Their use in CE was first reported by Terabe for the separation of positional isomers [18]. Later, Fanali obtained a chiral separation in CZE by simply adding CD to the BGE [19].

The pioneering work on chiral CE-ESI-MS was that of Hennion et al. [20] in 1995 that demonstrated the selectivity and sensitivity advantages of MS as a CE detector when using a stereoselective agent in the BGE. The enantiomers of both terbutaline and ephedrine were separated with a BGE containing an appropriate concentration of heptakis(2,6-di-O-methyl)-β-CD (DM-β-CD) at acidic pH. In SIM mode, a 10^3 sensitivity gain was achieved compared to UV detection for urine samples spiked with terbutaline. Both the free drug enantiomers and the noncovalent enantiomer–CD inclusion complexes were detected by MS. However, the ion current abundance of the free analyte was found to be eight times higher than the enantiomer–CD inclusion complexes, demonstrating for the first time the interference problem afforded by the simultaneous presence of analytes and chiral selectors on the ionization side. Lu and Cole studied in detail the CD concentration effect in BGE on the relative abundance of several basic compounds including terbutaline, ketamine and propranolol [21]. In this study, DM-β-CD and (2-hydoxypropyl)-β-CD (HP-β-CD) were used. The addition of minor quantities of organic

modifiers to BGE was found to improve ESI stability but, for all the studied compounds, there was a significant decrease in ionic current abundance while the chiral selector concentration increased in BGE. Otsuka et al. [22] successfully analysed phenoxy acid herbicide enantiomers by negative ionization mode with heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) as chiral selector. The latter migrated directly into the ESI interface and, in order to reduce the noise, a nonaqueous BGE was recommended.

In the chiral CE-MS field, an important application concerns the analysis of amphetamines and their derivatives. These compounds are powerful stimulants of the central nervous system and are often misused by recreational users. A chronic abuse of so-called "designer drugs" often leads to hallucinations and psychosis, as well as dysphoria and depression upon withdrawal. All amphetamines possess an asymmetric carbon and it is well known that the activities are different for both enantiomers. Therefore, stereoselective analysis is of great importance in clinical and forensic studies. Another chiral selector, the negatively charged highly sulfated-γ-CD (HS-γ-CD) (see Section 8.6.2), was used by Iwata et al. [23] for the simultaneous stereoselective separation of amphetamine-type stimulants. Investigations were performed in the negative polarity mode with detection at the anode. The cationic analyte migrated towards the anodic detection as a negatively charged complex with the anionic CD derivatives. The migrated amphetamines–CD complex was dissociated at the ESI interface, and only basic compounds went into the MS detector set in cationic detection mode. All 18 individual enantiomers were identified by their mass spectra and their specific fragmentation pattern. The detection limit of MS/MS was 10 times more sensitive than that for single MS. However, the elevated monitored electrophoretic current, afforded by the relatively high concentration of this polyanionic charged chiral selector, forced the use of water instead of buffering BGE. Analysis times were rather long (ca. 55–min for the complete analysis) and the complete dissociation of the migrated amphetamines–CD complex was not investigated.

Hence, as previously mentioned, interfacing chiral CE with ESI/MS is severely hampered by the presence of nonvolatile additives, such as CD, which often leads to a significant loss of electrospray efficiency and ion source contamination. In order to circumvent these limitations, several approaches have been investigated, such as the combined column coupling with voltage switching and the partial-filling technique.

In the first approach, Lamoree et al. chose a coupled capillary system in which the chiral separation occurred in the first capillary containing both chiral selectors and analytes. This technique was used to achieve CE-MS analysis with DM-β-CD for the stereoselective separation of ropivacaine. The latter was transferred via a PTFE union located in a plexiglass connection vial to a second capillary hyphenated to ESI-MS. A relatively complex sequence of timing events was necessary to ensure the complete analyte transfer and prevent the chiral selectors from entering the electrospray chamber [24]. On the other hand, the partial-filling technique has emerged as a simpler, straightforward and efficient valuable alternative to avoid potential interference of the chiral selector with MS detection.

8.5 Partial Filling

The partial filling technique (PFT), first introduced by Valtcheva et al. [25] and modified by Terabe [26], was originally developed to improve UV sensitivity in enantioseparation systems which involve chiral selectors eliciting a strong detector response, such as proteins or macrocyclic antibiotics [27–29]. This technique involves filling a discrete portion of the capillary with a background electrolyte containing a suitable amount of chiral selector for enantiomeric separation (partial filling). Another important advantage of PFT is the low consumption of chiral selector because CE vials do not contain any chiral selector [30]. More recently, this method was applied in chiral CE-MS determination to avoid the presence of a chiral selector in the MS detector ion source.

8.5.1 Partial Filling with Crown Ethers

Crown ethers are macrocyclic polyethers, first synthesised in 1967, which form stable inclusion complexes with alkali, alkaline-earth and primary ammonium cations. A more advanced generation of crown ethers derivatized with carboxylic groups (18-crown-6-tetracarboxylic acid) was introduced by Cram et al. [31]. The cyclic polyether forms a cavity where the analyte can penetrate with its hydrophilic part and interact with oxygen atoms. To date, even though more than 100 different chiral amines have been separated with crown ethers, only a few applications have demonstrated the possibility of MS detection. Tanaka et al. successfully analysed several racemic analytes with this chiral selector [32]. The separation of racemic 3-aminopyrrolidine and racemic α-amino-ε-caprolactam offered a higher sensitivity by MS detection because of the lack of UV absorption of these analytes. The chiral selector concentration and the separation zone length were carefully investigated. Stereoselective resolutions were found to increase with a high concentration of crown ethers and a long separation zone.

8.5.2 Partial Filling with Neutral Derivatized CD

The main problem encountered with PFT with a neutral selector remains the prevention of the chiral selector from entering the ionization source. This problem becomes particularly important at a high pH where EOF is important. Therefore, an acidic buffer pH or a coated capillary to minimize EOF is of the utmost importance. In addition, it has to be noted that the electrospray ionization process is pneumatically assisted when a sheath–liquid interface is used. The coaxial sheath gas induces an aspirating phenomenon in the capillary which may considerably affect the separation quality. This can be due to the decrease in interaction between analytes and the chiral selector and to a hydrodynamic flow induced by the Venturi effect at the capillary end [33, 34].

Javerfalk et al. [35] first applied PFT with a neutral CD, methyl-β-CD, for the separation of racemic bupivacaine and ropivacaine. To avoid introducing the selector into the MS chamber, the capillary was coated with polyacrylamide to minimize EOF. Prior to sample injection, the capillary was partially filled with methyl-β-CD dissolved in 50 mM acetate buffer at acidic pH (pH 3). After voltage application, the analytes migrated towards the detector through the zone containing the neutral selector, which acted as a pseudo-stationary phase. Under these conditions, an impurity of 0.25% of (*R*)-ropivacaine was detected in the presence of (*S*)-ropivacaine. For the separation of amphetamines and other related derivatives, Cherkaoui et al. employed a PVA-coated capillary to decrease EOF [36]. To prevent a detrimental aspiration effect, no nebulisation pressure was used with neutral HP-β-CD as chiral recognition agent. Filling 90% of the separation capillary with 40 mM ammonium formate buffer at pH 3 with an appropriate amount of HP-β-CD allowed a rapid and baseline enantioseparation of five amphetamines. With the same chiral selector, Grard et al. [37] demonstrated that detection sensitivity can be drastically improved by using tandem mass spectrometry (MS-MS) detection with PFT rather than with UV detection. The capillary was partially filled with HP-β-CD dissolved in a volatile 50 mM ammonium formate buffer at pH 4. The effects of chiral selector concentration and separation zone length were investigated for the stereoselective separation of adrenoreceptor antagonist drugs. Because enantioseparation decreases on lowering the selector zone length, a partial capillary filling at 80% with an appropriate HP-β-CD concentration was selected as a compromise between chiral resolution and MS sensitivity. The signal to noise ratio and peak efficiencies were found to increase with a low concentration of chiral selector. LOD were found to be 5 ng mL^{-1} with tandem MS detection (CE-MS/MS), 75 ng mL^{-1} with single MS and 150 ng mL^{-1} in CE-UV, with the advantage of higher selectivity and sensitivity detection. Toussaint et al. [38] demonstrated the possibility of resolving clenbuterol enantiomers with DM-β-CD as chiral selector using PFT after a solid-phase extraction (SPE) of plasma samples. With a fused silica capillary, EOF was almost completely suppressed at acidic pH to prevent DM-β-CD interference at the MS interface. Iio et al. [39] demonstrated the possibility of a CD dual system comprised of a mixture of β-CD and DM-β-CD for the simultaneous chiral determination of methamphetamine (MA), amphetamine (AP), dimethylamphetamine (DMA) and *p*-hydroxymethamphetamine (*p*OHMA) in urine. With an acidic BGE (formic acid), the detection limits were 30 ng mL^{-1} for the enantiomers of MA and AP and 50 ng mL^{-1} for the enantiomers of *p*OHMA in the SIM mode. This detection level was sufficient to analyse urine samples of MA addicts and DMA addicts after SPE. Excellent migration time repeatability (<0.05%) was obtained after correction with an internal standard.

8.6
Partial Filling – Counter Current Technique

In order to further improve PFT, a charged chiral selector offers the best approach for the stereoselective determination of chiral compounds when CE is hyphenated with MS. There are several relevant advantages with a selector presenting self-mobility under an electric field. Firstly, ionic derivatives often lead to better solubility and display additional electrostatic interactions. Secondly, and as already demonstrated by several authors [40–43], a chiral selector with a mobility opposite to that of the analyte shows a relevant resolving power even at very low concentrations. In fact, and according to the theoretical approach of Wren and Rowe [44], the mobility difference between the free and complexed analytes is increased with oppositely charged chiral selectors, often leading to a higher resolution than with a neutral chiral selector. Therefore, enhanced enantioselectivities can be achieved when the electromigrations of the chiral selector and the analytes are opposite (counter current process) [45]. According to this, the self-mobility of analytes must be sufficient to reach the detector while the charged chiral selector should migrate in the opposite direction (see Fig. 8.2). Furthermore, a low concentration of the stereoselective agent induces lower electrophoretic currents [46]. Finally, because these chiral selectors migrate in the ionised state with their own electrophoretic mobility on the opposite side of the MS detection, the partial-filling counter cur-

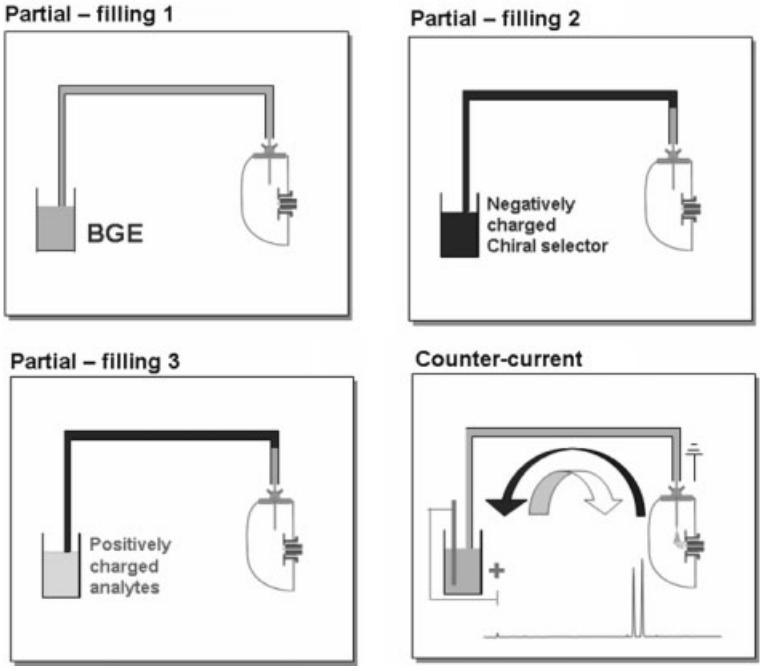

Fig. 8.2 Schematic presentation of the partial filling counter-current method.

rent technique (PFT-CC) prevents the contamination of the ion source with the chiral selector which, in turn, improves the sensitivity and stability of the mass spectrometer. Therefore, both positively and negatively charged chiral selectors were explored for chiral CE-MS of anionic and cationic analytes, respectively.

8.6.1
Anionic Analytes – Positively Charged Chiral Selectors

For the separation of anionic analytes, the positively charged macrocyclic antibiotic vancomycin is one of the most commonly used chiral selectors. It was introduced by Armstrong and coworkers as a new class of chiral selector in CE, HPLC and thin-layer chromatography for the separation of a wide range of enantiomers [29, 47–52]. It contains multiple stereogenic centers and a variety of functional groups. It is a glycopeptide antibiotic which forms a characteristic "basket" shape, and presents several carbohydrate moieties. The PFT-CC was historically developed according to the strongly absorbing nature of these selectors in the UV region of the spectrum. Fanali et al. [53] demonstrated the usefulness of the PFT-CC technique for the chiral CE-ESI-MS analysis of several well-established anionic pharmaceutical drugs (chiral anionic arylpropionic acids), such as ibuprofen and its metabolites, as well as etodolac and its metabolites. The advantages of MS peak purity testing and selective monitoring of unresolved peaks were demonstrated for the determination of these compounds in biological matrices. For ibuprofen, MS detection was necessary to achieve peak identification of the parent drug in the presence of its co-migrating phase I metabolite, 2-hydroxyibuprofen. The enantiomer separation of racemic isocitric acid lactone was achieved by Tanaka et al. [54] using vancomycin as chiral selector. The same author explored other positively charged antibiotics such as avidin which provided a stereoselective resolution of anionic compounds such as ibuprofen, ketoprofen, warfarin and camphor sulfonic acid [55]. In this study, quaternary ammonium β-CD (QA) was also investigated. While the separation of tropic acid was achieved with a polyacrylamide-coated capillary, some disturbances at the MS detection were observed because of the significant presence of sodium chloride as counter-ion and impurity of this cationic CD.

8.6.2
Cationic Analytes – Negatively Charged Chiral Selectors

Basic compounds are important in different areas, such as environmental, food and pharmaceutical industries. In the pharmaceutical area in particular, it is estimated that over 85% of ionisable drugs possess a basic function. Therefore, it is of crucial importance to develop efficient methods for the analysis of these compounds in procedures such as quality control, forensic and clinical analysis, therapeutic drug monitoring, metabolism studies, etc. The first work concerning negatively charged CDs for the separation of basic compounds was published in 1998 by the Blaschke group at Munster University [56]. Several pharmaceutical cationic analytes (etilefrine, mianserine, dimethindene and chloropheniramine) used as

model compounds were successfully resolved with carboxymethyl β-CD (CM-β-CD), a negatively charged chiral selector at pH > 3. In this work another charged CD was investigated, the sulfobutylether-β-CD (SBE-β-CD), for the separation of mianserin enantiomers, opening the way for sulfated CD as an important class of chiral selector. CM-β-CD was also selected for the analysis of methadone (Mtd), a synthetic opiate used in drug addict maintenance programs and in the management of severe pain. The enantiomeric separation of Mtd was achieved in a PVA-coated capillary as well as in a conventional fused-silica capillary. With the latter, in the presence of CM-β-CD, migration times were shorter but resolution and sensitivity were impaired, probably because of CD in the MS source. Mtd enantiomers were separated with a 40 mM ammonium acetate buffer, pH 4.0, containing a low concentration of CM-β-CD. By working in SIM mode, Mtd enantiomer detection was performed without ambiguity in the presence of its two major metabolites. In comparison to UV detection, working in SIM mode increased the sensitivity by a factor of 10, allowing the monitoring of Mtd in serum samples of patients undergoing therapy. Almost the same analytical conditions were successfully applied for the chiral resolution of venlafaxine, a second generation antidepressant drug and its major active metabolite, *O*-desmethyl venlafaxine [36].

An extensive chemometric study was performed by Rudaz et al. [34] to evaluate the main electrophoretic parameters involved in the enantioseparation of cationic compounds by CE-ESI-MS. With a volatile buffer consisting of 20 mM ammonium acetate at pH 4.0, the stereoselective analysis of Mtd as a model compound was compared using three different CD, including CM-β-CD and SBE-β-CD. In order to study the counter current process, the simultaneous evaluation of the main experimental factors involved with the PFT was carried out by means of experimental designs. A full factorial design was applied to determine the effects and significance of three relevant factors, namely the chiral selector concentration, the separation zone length and the nebulisation gas pressure. Results revealed that the nebulization pressure involved in the electrospray process and the CD concentration had a significant effect on enantiomeric resolution, while the effect of the separation zone length was less pronounced. As previously mentioned, the pseudo-EOF induced by the sheath–liquid interface, which represents the most relevant difference from the CE-UV set-up, and particularly the nebulisation gas pressure, affected dramatically the quality of the separation. However, this effect was less pronounced in the case of negatively charged CD, probably due to the counter-current migration process. SBE-β-CD proved to be the most appropriate, leading to the highest enantiomeric resolution. The potential of the PFT-CC in CE-ESI-MS combined with SBE-β-CD was further demonstrated for the stereoselective analysis of other chiral drugs in biological matrices, such as tramadol and its phase I metabolites [57]. In spite of the peak overlapping observed in the reconstructed ion electropherogram (RIC), the recording of selected masses allowed unambiguous determination of each analyte, which demonstrates the high selectivity of MS compared to conventional detectors. Acquisition in the SIM mode allowed one to enhance both sensitivity and selectivity. All compounds were baseline-resolved within a reasonable analysis time.

Similar conclusions were reached in another application of Cherkaoui et al. [58] concerning the potential of negatively charged CD for the chiral separation of anesthetic drug substances. Among the three investigated CDs, the sulfated-β-CD was found to be the most effective for the simultaneous enantioseparation of bupivacaine, mepivacaine, prilocaine and ketamine. Under optimized CE-ESI-MS conditions, the same chiral selector allowed a chiral separation of atropine enantiomers. The recording of extracted masses in SIM mode led to an unambiguous determination of each alkaloid enantiomer. The method performances were evaluated with homatropine as internal standard. The relative standard deviations of migration time and peak area ratio were 0.2 and 5.5%, respectively. The limit of detection (LOD) was of the order of ppb. Compared to UV detection, this corresponded to a 10^3 factor sensitivity improvement attributed to the high proton affinity of tropane alkaloids as well as their low molar absorptivity. As a result, this method was directly applied to the enantiomeric determination of atropine in plant extracts without any additional pre-concentration step [59].

Highly sulfated cyclodextrins (HS-CD) of different cavity size with a defined substitution degree have recently been introduced by Vigh et al. [60–66] as single isomers and from commercial sources as mixtures of randomly sulfated CD [67, 68]. These chiral selectors perform a very efficient stereoselective separation of various compounds (acidic, neutral and basic) and appear as first choice when a rapid screening of chiral molecules needs to be carried out [69]. Stereoselective resolutions were obtained at a low concentration of HS-γ-CD, demonstrating the high resolution power of this chiral selector towards basic selected compounds such as tramadol and its phase I metabolites as presented in Fig. 8.3 as well as other cationic pharmaceutical compounds including Mtd, venlafaxine and fluoxetine [70]. According to previous investigations, the sheath liquid composition consisted of isopranol–water (50/50, v/v) in the presence of 0.1% formic acid and the flow rate was maintained at 3 μL min^{-1}. These conditions allowed a high signal-to-noise ratio as well as a stable electrospray current [59, 71]. With the same percentage of filled capillary (i.e. 83%), a higher concentration of HS-γ-CD was necessary with the MS set-up compared to UV detection to reach the maximum enantioresolution for the four selected analytes. The significant increase in HS-γ-CD concentration was necessary to counterbalance the negative influence of the nebulisation gas pressure. It is noteworthy that with the increase in the chiral selector concentration, only one enantiomer was detected, achieving an infinite enantioselectivity. In fact, a CD concentration was determined for each analyte where one enantiomer still migrated cationically while the other migrated anionically. This particular behavior is a spectacular example of the infinite enantiomeric resolution that can be attained when the analyte charge is opposite to that of the chiral resolving agent. When the bonding constant of the two enantiomers and/or the ionic mobilities of the two diastereomeric analyte–chiral selector complexes are different from each other, the chiral selector concentration, where the enantiomeric mobilities change from cationic to anionic, is different for the two enantiomers. Consequently, as first discussed by Vigh et al. [72], within the "Charged resolving agent migration" (Charm) model, in some cases a resolving agent concentration

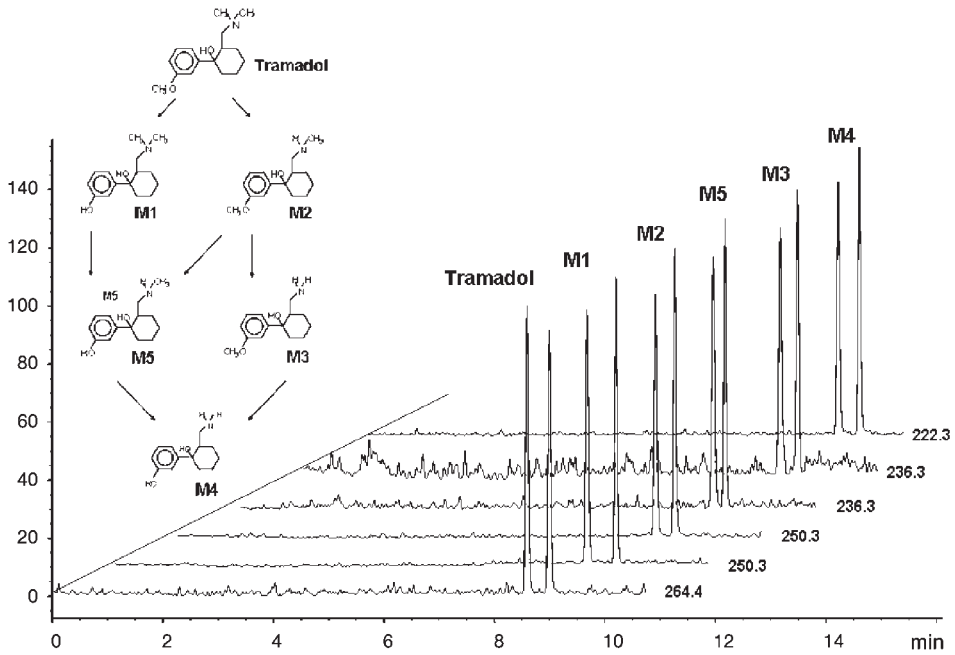

Fig. 8.3 CE-ESI-MS enantioseparation of tramadol and its phase I metabolites (M1-M5) in the presence of a negatively charged chiral selector. Experimental conditions; CE conditions: running buffer, 20 mM ammonium formate at pH 2.5 in the presence of HS-γ-CD (0.2%); fused silica capillary 60 cm 50 μm I.D.; partial filling of the capillary (52%); sample concentration, 1 μg mL^{-1}; pressure injection, 50 mbar for 10 s; applied voltage, 30 kV; temperature, 20 °C. MS conditions: SIM positive ion mode (4 ions); capillary voltage, 3 kV; fragmentor, 70 V; drying gas N_2, flow and temperature, 6 L min^{-1} and 150 °C; nebulizer pressure 4 psi; sheath liquid, 0.5% formic acid in water–isopropanol (50/50, v/v); sheath flow, 3 μL min^{-1}.

exists where one enantiomer still migrates cationically while the other migrates anionically and never reaches the MS source, as presented in Fig. 8.4. As soon as the effective mobility of the stronger binding enantiomer becomes negative, the separation selectivity attains an infinitely large value [73]. Further experiments demonstrated that this phenomenon was independent of the zone length.

8.7
Chiral Micellar Electrokinetic Chromatography

In addition to CZE, micellar electrokinetic chromatography (MEKC) is one of the most widely used CE modes. Electrokinetic chromatography separations involving micelles were first described to extend CZE application to electrically neutral substances. Today, MEKC is an accepted method for the simultaneous separation of both neutral and ionic solutes. The method requires the addition of a surfactant

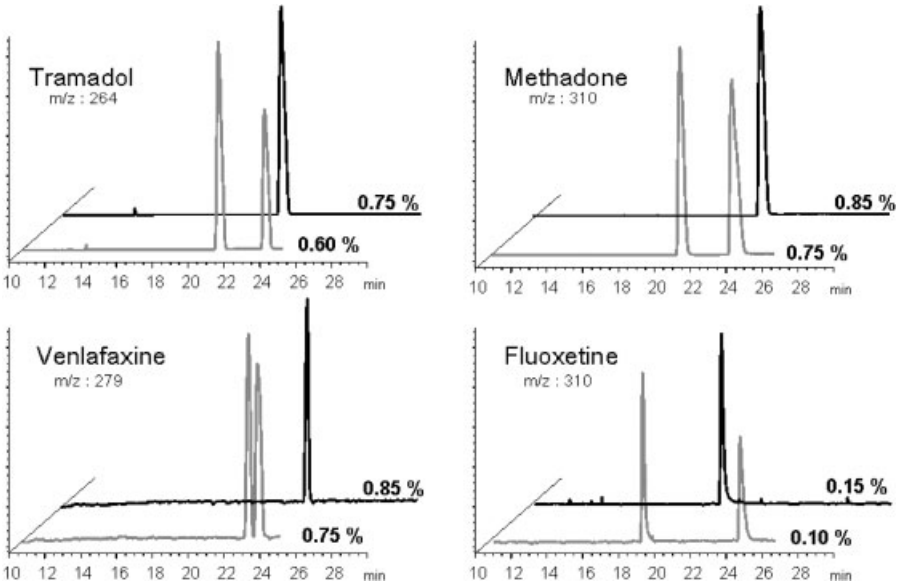

Fig. 8.4 CE-ESI-MS enantioseparation under optimal conditions (lower gray traces) and infinite enantioselectivity (upper black traces) of tramadol (*m/z* 264), methadone (*m/z* 310), venlafaxine (*m/z* 279) and fluoxetine (*m/z* 310) with HS-γ-CD as chiral selector. BGE 30 mM ammonium formate at pH 2.5, 83% capillary filled, HS-γ-CD concentration (0.10–0.85 %). Uncoated fused silica capillary: *L* = 75.0 cm, I.D. 50 ¿m. Applied voltage, 25 kV (ramp voltage 0.5 min). Pressure injection; 50 mbar for 20 s. Temperature, 20 °C. Analyte concentrations, methadone (Mtd) 10 μg mL^{-1}, tramadol (T) 10 μg mL^{-1}, venlafaxine (Vx) 10 μg mL^{-1} and fluoxetine (Flx) 5 μg mL^{-1}. MS conditions: SIM positive ion mode; capillary voltage, 4.5 kV; fragmentor, 40 V; drying gas N_2 flow and temperature , 4 L min^{-1} and 200 °C; nebulizer pressure 4 psi; sheath liquid, 0.1% formic acid in water–isopropanol (50/50, v/v) ; sheath flow, 3 μL min^{-1}. (Adapted from [70]).

such as sodium dodecyl sulfate (SDS) to the BGE. Above the critical micelle concentration (CMC), aggregates of individual surfactant molecules, micelles, are formed. Micelles are essentially spherical with the hydrophobic tails of the surfactant molecules orientated towards the center, to avoid interactions with the hydrophilic buffer, and the charged heads oriented towards the BGE. Selectivity is based on the differential partitioning of solutes between micellar and aqueous phases and differential electrokinetic migration, including electro-osmotic and electrophoretic mobilities. Many surfactants are commercially available but only some are chiral. Amino acid-derived synthetic surfactants, bile salts, an abundant source of chiral surfactants, and a number of others have been successfully used for enantiomer separation, including electrically neutral compounds. Another possibility is to employ CD together with ionic micelles, such as sodium dodecyl sulfate (SDS). This feature was originally developed to separate highly hydrophobic compounds, which are almost totally incorporated into the micelles and cannot be separated [74]. Addition of CD to a MECK solution changes the apparent distribu-

tion coefficients between micellar and nonmicellar phases, because CD can include hydrophobic compounds in its cavity and thus increases the apparent solubility of the aqueous phase. Nevertheless, the coupling of MEKC with MS appears to be very difficult as high SDS concentration produces a strong interfering signal whatever the ionization mode. Furthermore, SDS–analyte adduct formation suppresses ionization and results in high detection limits for cationic analytes. The possibility of using a new polymeric material such as a molecular micelle or micelle polymer is an attractive approach when coupling with MS is projected. These molecular micelles are difficult to ionize due to the covalent bond formed between the surfactant monomer and, therefore, they can be used at a low concentration and produce a MS signal increase by a low surface activity. Shamsi et al. was the first to report on chiral surfactant in MEKC-MS [75]. Recently, a baseline chiral resolution of (±)-1,1′-bi-2-naphthol (BOH) was reported with a molecular micelle at a concentration lower than the CMC of a similar conventional surfactant [30].

8.8 Quantitative Aspects in CE-MS and Parameters for CE-ESI-MS Coupling

Despite the widespread use of CE-MS for qualitative analysis, few quantitative applications have been published for routine analysis, and the validation of CE-MS methods according to generally accepted criteria is very uncommon. To our knowledge, only a few validation procedures are reported in the literature. Although CE methods can be validated like chromatographic techniques, there are some specific characteristics to be discussed when quantitative determinations are expected.

Capillary cassettes designed for CE-MS allow capillaries to exit the instrument. They generally present a UV detection window at a short distance from the inlet position. The total length of the capillary varies from 120 to 60 cm if the UV detection is by-passed. In this case, maximum field strength is available but does not allow simultaneous UV and MS detection.

In order to expand the CE potential for quantification, the selected ion monitoring (SIM) mode is to be preferred for its high selectivity and sensitivity. It can be noted that sensitivity improvement over UV–VIS spectrometry is closely related to the nature of the compound (molar absorptivity or protonation or deprotonation capacity); the awaited gain in sensitivity can diverge from about 10^1 to 10^4. Tandem mass spectrometry (MS^2) appears to be relevantly advantageous for quantitative purposes [23, 37]. In fact, the selectivity issue is of crucial importance in chiral CE due to the complex composition of the BGE.

Some electrospray parameters are known to be critical for achieving stable conditions and thereby good quantitative results. These parameters are the sheath liquid composition and flow rate, the nebulizing gas pressure, the applied electrospray voltage and the capillary outlet position. On the other hand, in previous studies, the impact of drying gas flow rate and temperature on stability and sensitivity were demonstrated to be moderate [3, 76]. Most of the quoted parameters are well described in the literature, apart from the capillary position which is disregarded for CE-ESI-

MS applications, probably because it is strongly dependent on the instrument used and on the interface geometry. The electric current created by ions reaching the MS capillary and the electric current due to ions striking the end plate ESI parameters must be carefully controlled. These electric currents are of the utmost importance to prevent unwanted corona discharge and for a good system sensitivity and stability. Their instrumental measurements were described by Bruins [7] and depended on different parameters, including the positioning of the sprayer inside the source. As a result, their intensity can be adjusted by modifying either the applied electrospray voltage or the position of the CE capillary outlet. Prior to starting a sequence, analyses have to be performed to ensure that these electric currents remain stable, otherwise, the capillary position has to be slightly adjusted to achieve good quantitative results. Furthermore, the capillary current monitoring can be a diagnostic tool of the chiral selector's entrance into the nebulisation chamber.

The choice of an appropriate sheath liquid and its flow rate is essential to achieve good performance. This choice is a compromise between separation (to maintain an efficient electrophoretic separation) and ionization performances (to assist droplet formation and spray stability). Most CE-ESI-MS applications described in the literature for the analysis of protonated compounds use a sheath liquid containing a mixture of organic solvent, water and formic or acetic acid. In method development, the impact of the nature of the sheath liquid on the expected chiral separation can be evaluated by placing it in the outlet vial. The solubility of the chiral resolving agent in the sheath liquid has to be carefully investigated to avoid its precipitation at the spray needle.

The applied nebulizing gas pressure (NGP) influences migration times, resolution and sensitivity [33, 36, 59]. For example, increasing the NGP from 2 to 30 psi produced a suction effect inside the capillary, which was detrimental for CE performance (decrease in efficiency and resolution). Moreover, because of this suction effect, air may enter during the injection process, provoking unstable CE currents and impeding separation performance. In contrast, increasing the NGP seemed beneficial for the electrospray process, since better sensitivity was achieved. The generated flow was found to decrease significantly when increasing the capillary length, suggesting that high NGP is better tolerated in a longer capillary.

Choosing an internal standard to correct errors due to sample preparation and injection reduced the impact of variability on the final trueness and precision of the developed method. Peak area can be corrected (peak area/migration time) to avoid the migration time drift influence, because of the temperature affecting both electro-osmosis and electrophoretic mobilities as well as buffer electrolysis, adsorption into the capillary wall and so on.

8.9
Capillary Electrochromatography Coupled to Mass Spectrometry

Capillary electrochromatography (CEC) is a hybrid between electrophoresis and high-performance liquid chromatography. Silica-based particles are packed into

capillaries, and CEC separation results from both the effect of electrophoresis and partitioning [2, 77, 78]. This technique, which combines the advantages of CE (i.e. separation efficiency) with those of LC (i.e. well characterised retention), has recently generated great interest. Brush-type, proteins, macrocyclic antibiotic, CDs, natural and molecular imprint-based polymer have already been immobilised as chiral selectors in CEC. One important advantage of CEC over CE is the possibility of using chiral selectors insoluble in common BGE or which possess a detector response. The main operating problem with CEC is the preparation of frit, required to prevent the packed bed movement under the effect of the EOF or pressure. Applications of CEC appear to be similar to LC and CE, including the determination of impurities, main components assay and, of course, chiral separation. For the latter, this technique can be realised in two basic modes: CEC in wall-coated open tubular capillaries [79–82] and CEC packed with chiral stationary phases [83]. Using the former approach, Schurig et al. [84] reported the chiral separation of hexobarbital in urine samples with a modified permethylated β-CD capillary. Zheng and Shamsi [85] demonstrated the possibility of CEC-MS with a commercially packed column for the determination of warfarin and coumachlor enantiomers in human plasma. Benefits of the presented method are its minimal sample and mobile phase consumption and the ability to differentiate nonracemic ratio with 1% of the minor enantiomer in the presence of 99% of the major one. Moreover, CEC presents several advantages over other electrophoretic modes for the separation of chiral compounds. Especially in the choice of ionization approach, the absence of properly designed devices appears to be a major task for a much broader application of this promising technique.

8.10 Discussion and Conclusion

The increased need for stereoselective analyses has induced a tremendous development of analytical techniques resolving enantiomers. Among these techniques, liquid chromatography, and more recently capillary electrophoresis (CE), are recognized as methods of choice for the chiral separation of pharmaceutical compounds. Chiral discrimination by CE is generally achieved with the direct separation method where the chiral selector is simply added to the background electrolyte (BGE).

It can be noted that the lack of sensitivity of CE for biological fluid concentrations remains the major bottleneck. However, coupling CE with mass spectrometry (MS) can overcome this drawback. These two analytical techniques are orthogonal and therefore their mutual complementarity is important. Hyphenation of CE with mass spectrometry appears appropriate for the stereoselective analysis of numerous compounds in various matrices. Moreover, the use of mass spectrometry in the selected ion monitoring mode results in very high selectivity, as well as improved sensitivity compared to conventional UV detection.

To achieve chiral separation, various chiral selectors have been applied, including neutral and ionized compounds such as negatively charged cyclodextrins

(CD). The partial-filling technique (PFT) proved to be a suitable and efficient approach to avoid MS source contamination, as well as signal suppression due to nonvolatile additives. Therefore, the PFT technique is particularly adapted with chiral selectors added into the electrolyte solution. Because of the counter-current contribution, charged chiral selectors were found to be more suitable for the on-line MS detection of separated enantiomers. Capillary electrochromatography with chiral stationary phases has also been developed, but to a lesser extent.

Abbreviations

BGE	Background electrolyte
β-CD	Native β-cyclodextrin
CD	Cyclodextrin
CD-MEKC	Cyclodextrin-modified electrokinetic micellar chromatography
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CM-β-CD	Carboxymethyl-β-cyclodextrin
CMC	Critical micelle concentration
CZE	Capillary zone electrophoresis
DM-β-CD	Heptakis(2,6-di-O-methyl)-β-cyclodextrin
EOF	Electro-osmotic flow
ESI	Electrospray ionization
HPLC	High-performance liquid chromatography
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HS-γ-CD	Highly sulfated-γ-CD
MEKC	Micellar electrokinetic chromatography
NGP	Nebulizing gas pressure
RIC	Reconstructed ion monitoring
SBE-β-CD	Sulfobutylether-β-cyclodextrin
SDS	Sodium dodecyl sulfate
SEE-β-CD	Sulfoethyl-β-cyclodextrin
SIM	Single ion monitoring
Succ-β-CD	Succinyl-β-cyclodextrin
TM-β-CD	Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin

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9
Powerful Chiral Molecular Tools for Preparation of Enantiopure Alcohols and Simultaneous Determination of Their Absolute Configurations by X-Ray Crystallography and/or ^{1}H NMR Anisotropy Methods

Nobuyuki Harada

9.1
Introduction

It is well recognized that molecular chirality is essential to life processes, and that most biologically active compounds controlling physiological functions of living organisms are chiral. Hence, in the structural study of biologically active compounds, including natural and synthetic drugs, determination of the absolute configuration becomes the first major issue. The second issue is the chiral synthesis of biologically active compounds that become pharmaceutical targets and how efficiently the desired enantiomers can be synthesized with 100% enantiopurity or enantiomeric excess (% ee). Furthermore, studies on chiral functional molecules and molecular machines, such as the light-powered chiral molecular motor developed in our laboratory, has been rapidly progressing in recent years. Therefore, the unambiguous determination of the absolute configuration of chiral compounds as well as their chiral syntheses are of vital importance in the field of pharmaceutical and material sciences.

We have recently developed chiral carboxylic acids as novel molecular tools useful for enantioresolution and simultaneous determination of the absolute configuration of various alcohols. These chiral molecular tools are very powerful for the facile preparation of enantiomers with 100% ee and also for the absolute configurational assignment. Methods using these chiral tools have been successfully applied to various compounds, and their methodologies and applications are explained throughout this chapter.

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9.2
Methodologies for Determining Absolute Configuration

9.2.1
Nonempirical Methods for Determining Absolute Configurations of Chiral Compounds

The methods for determining the absolute configurations of chiral compounds are summarized in Table 9.1 with the nonempirical methods in the top half of the table.

It is well known that the absolute configuration of chiral compounds was first determined by the Bijvoet using X-ray crystallography [1]. In X-ray crystallography, since the anomalous dispersion effect of heavy atoms can be measured accurately under proper conditions, the absolute stereostructure obtained is unambiguous and reliable. In addition, the molecule can be projected as a three-dimensional structure, and, therefore, the method has been employed extensively. However,

Table 9.1 Methods for determining the absolute configurations of chiral compounds.

Method	Phenomena key points and/or reagents	Ref.
Non-empirical methods		
X-ray crystallography	Heavy atom effect	[1]
CD spectroscopy	Exciton coupling	[2(a)]
		[2(b)]
	Twisted π-electron system	[2(c)]
VCD and/or OR	*ab initio* calculation	[3(a)]
		[3(b)]
		[3(c)]
		[3(d)]
Relative and/or empirical methods		
X-ray crystallography	Internal reference of absolute configuration	
	CSDP acid	[5–8, 11, 59]
	inclusion complex	[9, 10]
^{1}H NMR spectroscopy	Anisotropy effect	
	MTPA acid	[13]
	MPA acid	[15]
	MTPA, 1NMA, 2NMA, 9AMA, 2ATMA acids	[12]
	MTPA, 1NMA, 2NMA, 9AMA acids	[14]
	Enantioresolution and anisotropy effect MαNP acid	[50, 59, 60]
Chemical correlation		
Comparison of CD spectra		

the X-ray method needs single crystals of suitable size good for X-ray diffraction, and so the critical problem is how to obtain such single crystals.

The CD exciton chirality method [2] is also useful because the absolute configuration can be determined in a nonempirical manner, and it does not require crystallization. Furthermore, chiral chemical and biological reactions are traceable by CD, and even the absolute configurations and conformations of unstable compounds can be obtained by this method. However, since some compounds are not ideal targets for this method, the results must be interpreted carefully.

Recently the *ab initio* calculation method of vibrational circular dichroism (VCD), optical rotatory dispersion (ORD), and electronic circular dichroism (ECD) has been developed as the third nonempirical method [3, 4]. The method is applicable to compounds having no chromophore, and so should be widely used in future.

9.2.2
Relative and/or Empirical Methods for Determining Absolute Configuration Using an Internal Reference of Absolute Configuration

These methods are summarized in the bottom half of Table 9.1.

Absolute configuration can be obtained by determining the relative configuration at the position of interest against a reference compound or substituent with known absolute configuration. A typical example is the X-ray crystallography taken after the introduction of a chiral auxiliary with known absolute configuration (Fig. 9.1) [5–8]. In this case, the absolute configuration of the point in question can be automatically determined using the chirality of the auxiliary introduced as an internal reference. Consequently, the samples do not need to contain heavy atoms for the anomalous dispersion effect. The result obtained is very clear, even when the final *R*-value is not small enough due to the poor quality of the single crystal. A variety of methods to link an internal reference to the target molecule have been developed. For example, there are ionic bonding as in conventional acid–base salts, covalent bonding as in esters or amides, and the recently developed use of inclusion complexes [9, 10]. These relative X-ray crystallographic methods are expected to find widespread application, as exemplified by the recent determination of the absolute configurations of chiral C_{60} fullerene *cis*-3 bisadducts by X-ray crystallography [11].

Recently, the proton nuclear magnetic resonance (^{1}H NMR) anisotropy method has often been employed as the relative method, and it is useful for the study of the absolute configuration of natural products [12–15]. In particular, the absolute configurations of secondary alcohols are frequently determined using the advanced Mosher method developed by Kusumi et al. [12]. In this case, the absolute configurations of chiral auxiliaries, such as Mosher's reagent [α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA)] [12–14] and Trost's reagent [α-methoxyphenylacetic acid (MPA)] [15] are known, and the preferred conformation of the esters formed with chiral secondary alcohols and MTPA or MPA acid is rationalized. In addition, the aromatic substituent (phenyl group) generates a magnetic

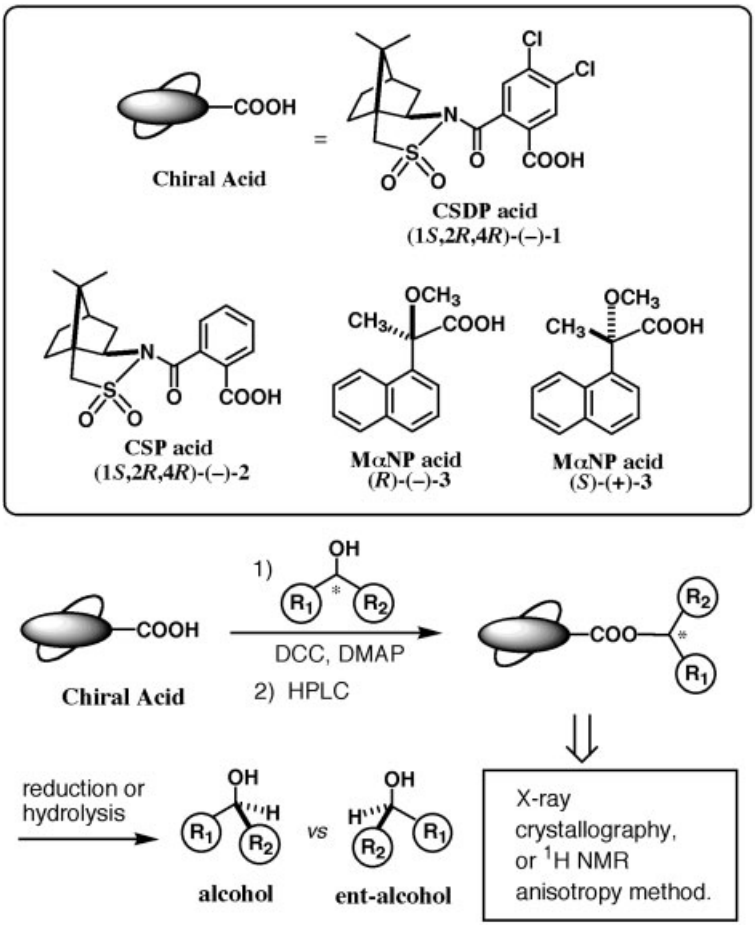


Fig. 9.1 Enantioresolution and determination of absolute configuration of alcohols using chiral carboxylic acids [5–8].

anisotropy effect due to the ring current induced under the external magnetic field, and so the proton NMR signals of the alcohol moiety facing the phenyl group in the preferred conformation are moved to a higher magnetic field (high field shift). By observing the ^{1}H NMR anisotropy effect, the absolute configuration of the alcohol part can be determined. This method is very convenient, since it does not require crystallization of compounds. One problem with this method is that it is based on the assumption of preferred conformation of molecules in solution. However, it is highly reliable since the method itself has a self-diagnostic function; in some exceptional cases, to which the NMR anisotropy method is not applicable, the observed $\Delta\delta$ values reflecting the anisotropy effect distribute randomly. On the other hand, in the majority of cases leading to the correct assignment, the $\Delta\delta$ values show a reasonable distribution pattern. Therefore, the applic-

ability of the NMR anisotropy method can be judged from the distribution pattern of the observed $\Delta\delta$ values. Although the method has been widely applied to secondary alcohols, the method could be extended to other kinds of compounds.

9.3 CSDP Acid, Camphorsultam Dichlorophthalic Acid (–)-1, Useful for the Enantioresolution of Alcohols by HPLC and Simultaneous Determination of Their Absolute Configurations by X-ray Crystallography

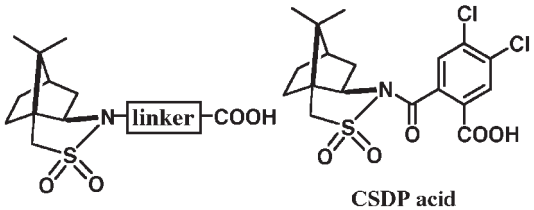
We consider that the most reliable and powerful method for determining the absolute configuration is the X-ray crystallography of compounds containing a chiral auxiliary with known absolute configuration as the internal reference, as described above. Namely, the absolute configuration of the point in question can be unambiguously determined from the ORTEP drawing showing a relative stereochemistry, because the absolute configuration of the chiral auxiliary is already known. Therefore, it is very easy to determine the absolute configuration, and there is no possibility of making a mistake in the assignment.

We also consider that a highly efficient method for preparing an appropriate amount of various chiral compounds with 100% enantiopurity on the laboratory scale is the enantioresolution method, as illustrated in Fig. 9.1. In this method, a chiral auxiliary is covalently bonded to racemates, and the obtained diastereomeric mixture can be separated by conventional HPLC on silica gel. If the chromatogram shows a base-line separation, the diastereomers obtained are enantiopure.

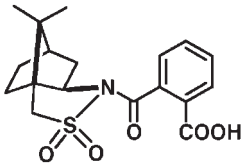
As chiral auxiliaries useful for preparation of enantiopure alcohols and simultaneous determination of their absolute configurations, we have developed a chiral molecular tool, camphorsultam dichlorophthalic acid (CSDP acid) (–)-**1** connecting (1*S*,2*R*,4*R*)-2,10-camphorsultam and 4,5-dichlorophthalic acid (Fig. 9.2), and have applied this chiral tool to various compounds [16–40]. The 2,10-camphorsultam was selected because of its good affinity with silica gel used in HPLC, allowing good separation of the two diastereomers. In addition, the sultam amide moiety is effective for providing prismatic single crystals suitable for X-ray diffraction. Furthermore, the (1*S*,2*R*,4*R*) established absolute stereochemistry of 2,10-camphorsultam is useful as the internal reference of absolute configuration. To connect alcohols, an ester bond was chosen, because it could be readily formed and cleaved off. Accordingly, phthalic acid or 4,5-dichlorophthalic acid was selected as a linker (Fig. 9.2) [16, 17].

The desired molecular tool, CSDP acid (–)-**1**, was synthesized by reacting (1*S*,2*R*,4*R*)-(–)-2,10-camphorsultam anion with 4,5-dichlorophthalic anhydride [(–)-**1**, mp 221 °C from EtOH; $[\alpha]_D^{20}$ –101.1 (*c* 1.375, MeOH); Fig. 9.2]. This carboxylic acid was condensed with alcohol in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) [17].

Various chiral drugs with a diphenylmethanol skeleton have been developed, as shown in Fig. 9.3 [18–24]. Therefore it is clear that the biological activity of those



CSDP acid
(1*S*,2*R*,4*R*)-(−)-1



CSP acid
(1*S*,2*R*,4*R*)-(−)-2

Fig. 9.2 Design of a chiral molecular tool, CSDP and CSP acids containing the 2,10-camphorsultam moiety.

drugs depends on their absolute configurations. Although the absolute configurations of some drugs have been determined, those of others have remained undetermined. In addition, those drugs were prepared mostly by means of asymmetric syntheses and/or enzymatic reactions. Therefore, it is hard to obtain enantiopure drugs without purification by recrystallization. How can we determine the absolute configuration of these chiral drugs and also obtain enantiopure compounds? To solve these problems, we have applied the CSDP acid method to various diphenylmethanols as follows.

To exemplify a general procedure of the CSDP acid method, we show here the results for chiral (2,6-dimethylphenyl)phenylmethanol **10**. The CSP acid (–)-**1** was allowed to react with (±)-**10** using DCC and DMAP in CH_2Cl_2 yielding diastereomeric esters, which were effectively separated by HPLC on silica gel: hexane/EtOAc = 6:1; α = 1.25, R_s = 1.94 (Figs. 9.4 and 9.5) [25]. The first-eluted ester (–)-**11a** obtained was recrystallized from EtOH to give prism-shaped crystals. A single crystal of **11a** was subjected to X-ray analysis affording the ORTEP drawing as shown in Fig. 9.6, from which the absolute configuration of the alcohol part was clearly determined as *R* based on the absolute configuration of the camphorsultam moiety used as an internal reference. The *R* absolute configuration of **11a** was also confirmed by the heavy atom effect of its two chlorine and sulfur atoms. The reduction of the first-eluted ester (*R*)-(–)-**11a** with $LiAlH_4$ in THF yielded enantiopure alcohol (*R*)-(+)-**10** [25]. Although reduction with $LiAlH_4$ was used here to recover the alcohol, it was found later that solvolysis with K_2CO_3 in MeOH, a milder reaction, is also applicable for most CSDP esters.

As shown in Table 9.2 (see p. 292), the CSDP acid method has been successfully applied to various substituted diphenylmethanols **12–27** and **36–41** (entries 2–14

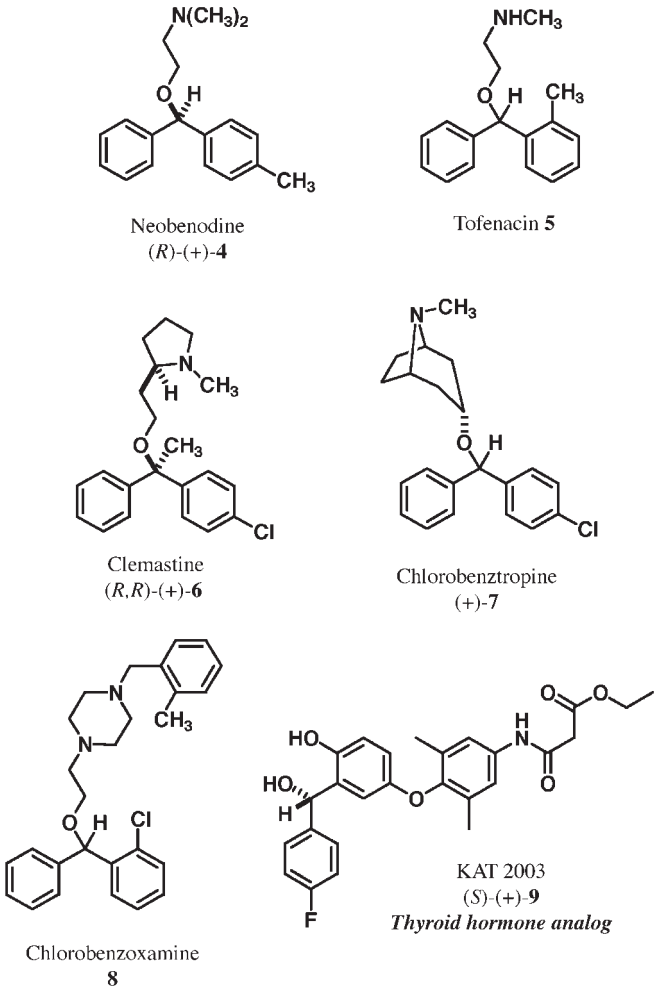


Fig. 9.3 Chiral drugs with a diarylmethanol skeleton [18–24].

and 23–28). Namely the diastereomeric esters prepared from racemic alcohols and CSDP acid (1*S*,2*R*,4*R*)-(–)-**1** were effectively separated by HPLC on silica gel with a separation factor $\alpha = 1.10–1.34$. It is known that if the separation factor α is larger than 1.10, the two components are base-line separable, yielding pure compounds. For alcohols **17**, **23**, **25**, **36**, **37**, and **39–41**, the CSDP acid method was applied in a straightforward manner; the separated CSDP esters were recrystallized giving single crystals, which were subjected to X-ray crystallography (entries 7, 11, 12, 23, 24, and 26–28). The absolute configurations of the alcohol parts were thus explicitly determined.

Alcohols **12** and **13** were previously enantioresolved by means of CSP acid (1*S*,2*R*,4*R*)-(–)-**2**, a similar chiral auxiliary developed by us as shown in Figs. 9.1

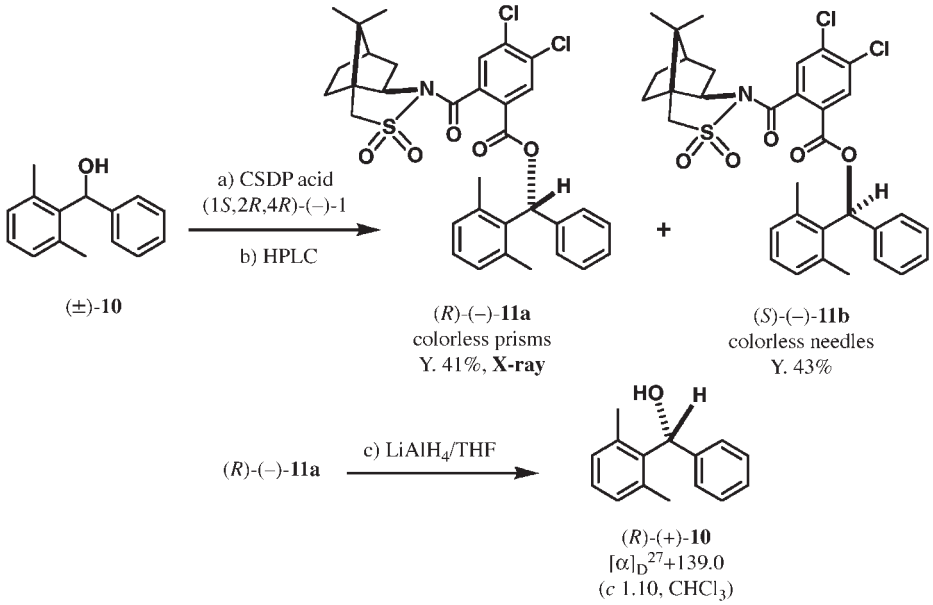


Fig. 9.4 (a) Preparation of CSDP esters: DCC, DMAP in CH_2Cl_2. (b) HPLC on silica gel. (c) Recovery of enantiopure alcohol (*R*)-(+)-**10** [25].

and 9.2, and the absolute configurations of their CSP esters were determined by X-ray crystallography (entries 2′ and 3′). So, by comparison with the data, the absolute configurations of CSDP acid esters of alcohols **12** and **13** were established by chemical correlation. It should be noted that the CSDP acid esters of **12** and **13** were more effectively separated by HPLC on silica gel than the corresponding CSP acid esters: separation factor $\alpha = 1.20–1.26$ vs. 1.1 (entries 2, 2′, 3, and 3′). In general, CSP acid esters have low solubility, possibly due to their better crystallinity, resulting in a longer elution time and smaller α value in HPLC on silica gel. In addition, CSP esters were often obtained as fine needles, which were unsuitable for X-ray crystallography. Therefore CSDP acid **1** is more useful in most cases than CSP acid **2**.

In the case of the halogenated alcohols **14** and **15**, their diastereomeric CSDP esters were obtained as fine crystals, which were unsuitable for X-ray crystallography, so the enantiopure alcohol recovered (–)-**14** was converted to camphanate ester, the absolute configuration of which was determined by X-ray crystallography as *R* (entry 4). Alcohol (–)-**15** was treated in the same way, but its caphanate ester was not suitable for X-ray analysis (entry 5). The absolute configuration of (–)-**15** was determined as *R* by comparison of its CD spectrum with that of (*R*)-(–)-**14**.

Methyl-substituted alcohol **16** could not be enantioresolved by the CSDP acid method, because of the small difference in substituent effects: Me vs. H (entry 6). Therefore, we adopted the chemical conversion method as follows; racemic alcohol **17** with 4-Me and 4′-Br groups was effectively enantioresolved as CSDP esters,

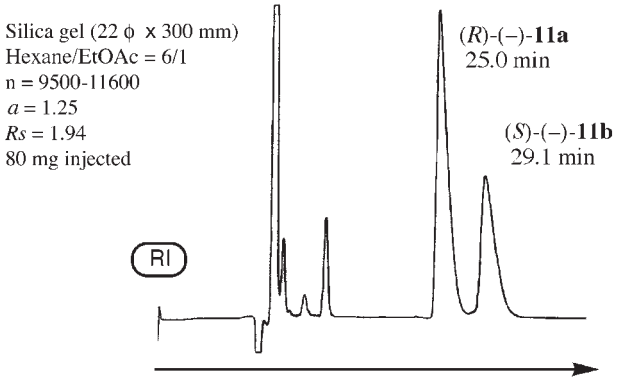


Fig. 9.5 HPLC separation of CSDP esters **11a** and **11b** [25].

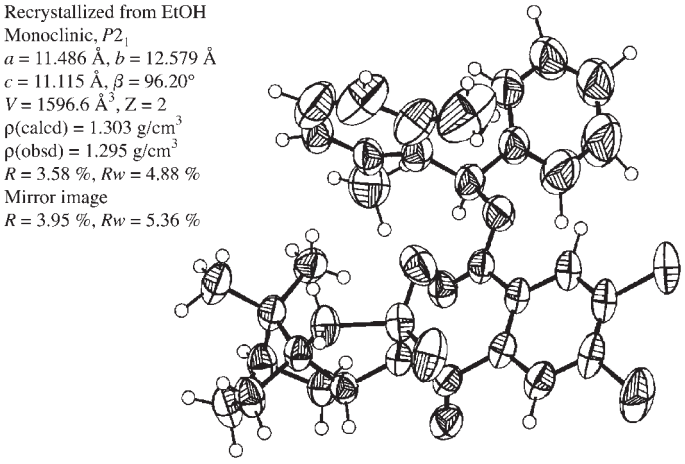


Fig. 9.6 ORTEP drawing of CSDP ester (*R*)-(–)-**11a** [25].

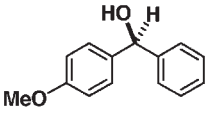
the absolute configuration of which was determined by X-ray crystallography (entry 7). The enantiopure alcohol (*R*)-(–)-**17** obtained was reduced to remove the Br atom yielding (*S*)-(–)-**16**.

Alcohols **19** and **21** are very unique chiral compounds, the chirality of which is generated by the substitution of isotopes: in the case of **19**, H vs. D; in the case of **21**, ^{12}C vs. ^{13}C, so it is very difficult to recognize such an extremely small chirality directly. To synthesize enantiopure alcohols **19** and **21**, and to determine their absolute configurations, the indirect chemical conversion method was employed as follows. For example, deuterium-substituted/4-Br alcohol **20** was similarly enantioresolved as in the case of compound **14** (entry 9). The enantiopure alcohol (*S*)-(–)-**20** obtained was reduced to remove the Br atom yielding (*S*)-(–)-**19**, which exhibits a negative CD Cotton effect at 270.4 nm. In a similar way, ^{13}C-substituted

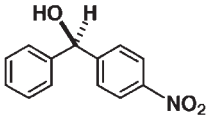
Table 9.2 Enantioresolution of alcohols by HPLC on silica gel using (1*S*,2*R*,4*R*)-(–)-CSDP acid **1**, and determination of their absolute configurations by X-ray crystallography.]

Entry	Alcohol	Solvent[b]	α[c]	*Rs*[d]	X-ray[e]	Abs. config. First Fr.	Ref.
1	**10**	H/EA = 6/1	1.25	1.94	y (1st Fr.)	*R*	[25]
2	**12**	H/EA = 4/1	1.20	0.91	–	*S*	[26]
2′[a]	**12**	H/EA = 4/1	1.1	1.3	y (1st Fr.)	*S*	[27]
3	**13**	H/EA = 5/1	1.26	1.37	–	*R*	[26]
3′[a]	**13**	H/EA = 5/1	1.1	1.6	y (1st Fr.)	*R*	[27]
4	**14**	H/EA = 8/1	1.1	1.3	y[f]	*R*	[28]
5	**15**	H/EA = 6/1	1.17	0.95	–	*R*	[26]
6	**16**	H/EA = 7/1	–	–	–	–	[27]
7	**17**	H/EA = 8/1	1.18	0.83	y (1st Fr.)	*R*	[27]
8	**18**	H/EA = 4/1	1.1	1.0	–	*R*	[26]
9	**20**	H/EA = 8/1	1.21	1.07	y[f]	*S*	[28]
10	**22**	H/EA = 4/1	1.27	1.20	y[f]	*S*	[29]
11	**23**	H/EA = 5/1	1.12	1.01	y (1st Fr.)	*S*	[30]
12	**25**	H/EA = 4/1	1.14	0.91	y (2nd Fr.)[g]	*R*	[30, 31]
13	**26**	H/EA = 10/1	1.26	1.03	–	*R*	[30]
14	**27**	H/EA = 6/1	1.26	1.29	–	*R*	[32]
15	**28**	H/EA = 5/1	1.16	1.11	y (1st Fr.)	*S*	[33]
16	**29**	H/EA = 5/1	1.12	0.87	y (1st Fr.)	*S*	[33]
17	**30**	H/EA = 2/1	1.11	0.88	–	*R*	[33]
18	**31**	H/EA = 2/1	1.38	1.19	y (1st Fr.)	*R*	[33]
19	**32**	H/EA = 7/1	1.18	1.06	y (2nd Fr.)	3*R*,4*R*	[17, 34]
20	**33**	H/EA = 7/1	1.23	1.27	y (1st Fr.) y (2nd Fr.)]	1*R*,2*S*	[35]
21	**34**	H/EA = 10/1	1.30	1.74	y (1st Fr.)	1*S*,4*R*	[17]
22	**35**	H/EA = 3/1	1.2	1.6	y (2nd Fr.)	a*R*,a*R*	[36, 37]
23	**36**	H/EA = 5/1	1.34	2.37	y (1st Fr.)	*R*	[38]
24	**37**	H/EA = 5/1	1.16	1.22	y (1st Fr.) y (2nd Fr.)]	*R*	[38]
25	**38**	H/EA = 5/1	1.11	1.33	–	*R*	[38]
26	**39**	H/EA = 4/1	1.21	2.50	y (1st Fr.)	*S*	[38]
27	**40**	H/EA = 5/1	1.16	1.42	y (1st Fr.)	*S*	[39]
28	**41**	H/EA = 4/1	1.15	1.34	y (1st Fr.)	*S*	[39]
29	**42**	H/EA = 4/1	1.27	1.49	y[h]	*S*	[40]

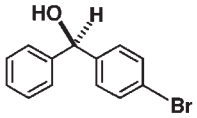
a) Esters with (1*S*,2*R*,4*R*)-(–)-CSP acid **2**. **b)** H = n-hexane, EA = ethyl acetate. **c)** Separation factor $\alpha = (t_2 - t_0)/(t_1 - t_0)$ where t_1 and t_2 are the retention times of the first- and second-eluted fractions, respectively, and t_0 is the retention time of an unretained compound (void volume marker). **d)** Resolution factor $Rs = 2\,(t_2 - t_1)/(W_1 + W_2)$ where W_1 and W_2 are the band-widths of the first- and second-eluted fractions at the base-line level, respectively. **e)** y = yes. **f)** X-ray analysis of camphanate ester. **g)** CSDP ester of the primary alcohol moiety. **h)** X-ray analysis of 4-bromobenzoate.



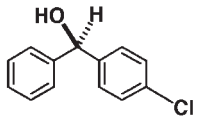
(*S*)-(–)-**12**



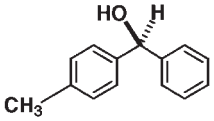
(*R*)-(–)-**13**



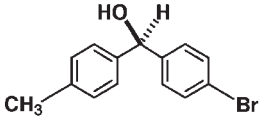
(*R*)-(–)-**14**



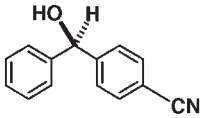
(*R*)-(–)-**15**



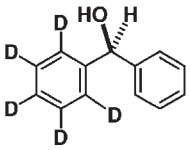
(*S*)-(–)-**16**



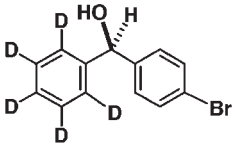
(*R*)-(–)-**17**



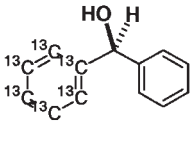
(*R*)-(–)-**18**



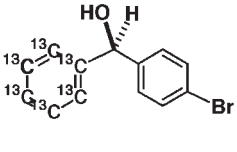
[CD(–)270.4]-(*S*)-**19**



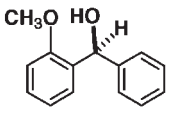
(*S*)-(–)-**20**



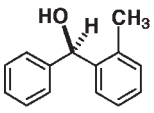
[CD(–)270]-(*S*)-**21**



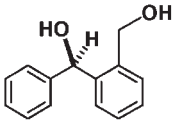
(*S*)-(–)-**22**



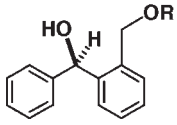
(*S*)-(–)-**23**



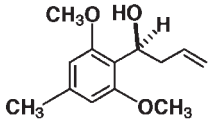
(*R*)-(–)-**24**



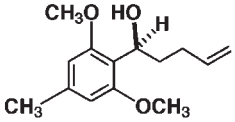
(*R*)-(+)-**25**



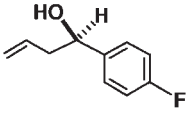
(*R*)-**26**: R = TBDMS
(*R*)-**27**: R = TBDPS



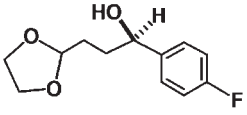
(*S*)-(–)-**28**



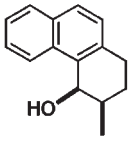
(*S*)-(–)-**29**



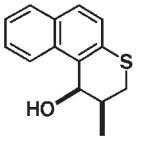
(*R*)-(+)-**30**



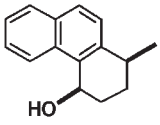
(*R*)-(+)-**31**



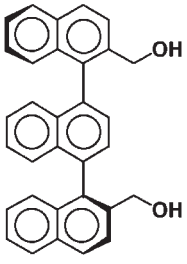
(3*R*,4*R*)-(+)-**32**



(1*R*,2*S*)-(+)-**33**

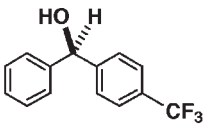


(1*S*,4*R*)-**34**

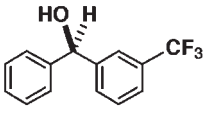


(a*R*,a*R*)-(–)-**35**

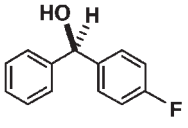
Structures to Table 9.2



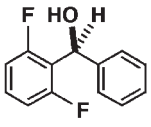
(*R*)-(–)-**36**



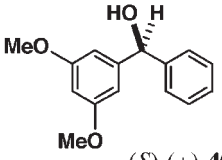
(*R*)-(–)-**37**



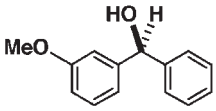
(*R*)-(–)-**38**



(*S*)-(–)-**39**



(*S*)-(+)-**40**



(*S*)-(+)-**41**

Structures to Table 9.2

diphenylmethanol [CD(–)270]-**21** was synthesized in an enantiopure form and its absolute configuration was determined as *S* (entry 10).

Although the CSDP acid method was directly applicable to *o*-methoxy-substituted alcohol **23** (entry 11), *o*-methyl-substituted alcohol **24** could not be enantioresolved as the CSDP acid esters, so the indirect method was adopted as follows; *o*-hydroxymethyl-substituted alcohol **25** was enantioresolved as CSDP esters, where the primary alcohol moiety was esterified (entry 12). Enantiopure alcohol (*R*)-(+)-**25** was then converted to the target compound (*R*)-(–)-**24**. It should be noted that the absolute configuration of alcohol **24** was once estimated on the basis of an asymmetric reaction mechanism, but it was revised by this study. The data of alcohols **26** and **27** indicate that the HPLC separation as CSDP esters is easier for silyl ethers (entries 13 and 14).

The CSDP acid method was applicable to benzyl alcohols **28–31** and naphthalene alcohols **32–34**, the CSDP esters of which were effectively separated by HPLC on silica gel with α = 1.11–1.38 (entries 15–21). In addition, except only for the one case of **30**, the absolute configurations of their CSDP esters were determined by X-ray crystallography. Ternaphthalene-diol **35** is a unique compound having three naphthalene chromophores in chiral positions. Therefore, it was expected that it would show intense exciton-coupled CD, from which its absolute configuration could be determined. The CSDP esters of **35** were separable with α = 1.2 (entry 22), and the absolute configuration of the second-eluted fraction was determined by X-ray crystallography. The (a*R*,a*R*) configuration of (–)-**35** agreed with the assignment by the CD exciton chirality method.

Various fluorinated diphenylmethanols **36–39** were also enantioresolved as CSDP esters (entries 23–26). In the case of alcohols **36**, **37**, and **39**, their absolute configurations were determined by X-ray crystallography. To those fluorinated alcohols, the method of MαNP acid **3** could be applied for enantioresolution and also for determination of their absolute configurations by the ^{1}H NMR anisotropy method, as discussed below. *Meta*-substituted diphenylmethanols **40** and **41** were enantioresolved by the CSDP acid method yielding enantiopure alcohols, the abso-

lute configurations of which were unambiguously determined by X-ray crystallography (entries 27 and 28).

A very important example is the case of 2-(1-naphthyl)propane-1,2-diol **42**, which was isolated as a chiral metabolite of 1-isopropylnaphthalene in rabbits. The metabolite, however, was not enantiopure and its absolute configuration had only been estimated empirically, based on the reaction mechanism. To obtain the enantiopure diol **42** and to determine its absolute configuration unambiguously, the method of CSDP acid was applied to (±)-**42** (Fig. 9.7) [40]. In this case, only the primary alcohol part was esterified, and the diastereomeric mixture obtained was clearly separated by HPLC on silica gel: hexane/EtOAc = 4 : 1, $\alpha = 1.27$, $R_s = 1.14$ (entry 29 and Fig. 9.8). In this HPLC, the presence of a free tertiary hydroxy group is important, because the protection of the tertiary alcohol group led to poor separation.

Despite repeated recrystallizations, both diastereomers **43a** and **43b** were obtained only as amorphous solids. Therefore, the first-eluted fraction (–)-**43a** was reduced with $LiAlH_4$ to yield enantiopure glycol (–)-**42**, which was further converted to 4-bromobenzoate (–)-**44** (Fig. 9.7a). By recrystallization from EtOH, (–)-**44** gave good single crystals suitable for X-ray analysis, and consequently its absolute configuration was explicitly determined as *S* by the Bijvoet pair measurement of the anomalous dispersion effect of the bromine atom contained (Fig. 9.7b) [40].

Furthermore, we have obtained enantiopure 2-methoxy-2-(1-naphthyl)propionic acid (MαNP acid) (*S*)-(+)-(**3**) via several reactions from diol (*S*)-(–)-**42** (Fig. 9.7c) [40]. We have discovered that this novel carboxylic acid, MαNP acid **3**, is also effective for enantioresolution and simultaneous determination of the absolute configuration of various secondary alcohols by the ^{1}H NMR anisotropy method [41–52, 56–58]. The results obtained by the ^{1}H NMR anisotropy method are, of course, consistent with those by the X-ray method. Therefore, the methods of CSDP and MαNP acids are useful as complementary molecular tools, as discussed in this chapter.

9.4
A Novel Chiral Molecular Tool, 2-Methoxy-2-(1-naphthyl)propionic Acid (MαNP Acid (*S*)-(+)-3), Useful for Enantioresolution of Alcohols and Simultaneous Determination of Their Absolute Configurations by the ^{1}H NMR Anisotropy Method

We have discussed above the design and applications of CSDP acid useful for both the synthesis of enantiopure compounds and the unambiguous determination of their absolute configurations by X-ray analysis. The X-ray crystallographic method using the internal reference of absolute configuration thus leads to the unambiguous and reliable determination of absolute configuration. However, the drawback of X-ray crystallography is that the method needs single crystals and, therefore, it is not applicable to noncrystalline materials. However, in daily experiments, prismatic single crystals suitable for X-ray analysis are not always obtainable. So is

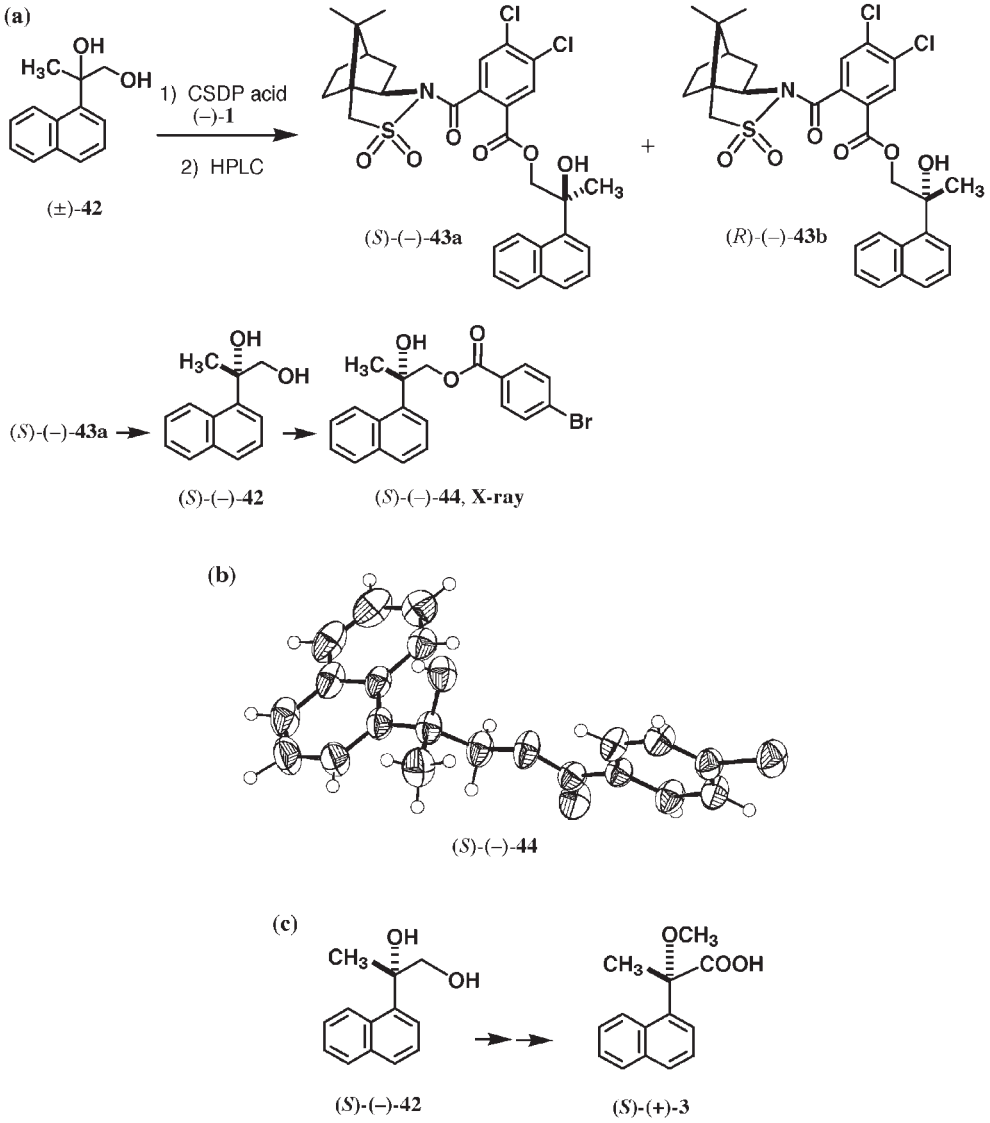


Fig. 9.7 (a) Enantioresolution and determination of the absolute configuration of 2-(1-naphthyl)propane-1,2-diol (**42**). (b) ORTEP drawing of 4-bromobenzoate (*S*)-(–)-**44**, whose absolute configuration was determined by the heavy atom effect of the bromine atom. (c) Preparation of (*S*)-(+)-MαNP acid (**3**) from glycol (*S*)-(–)-**42** [40].

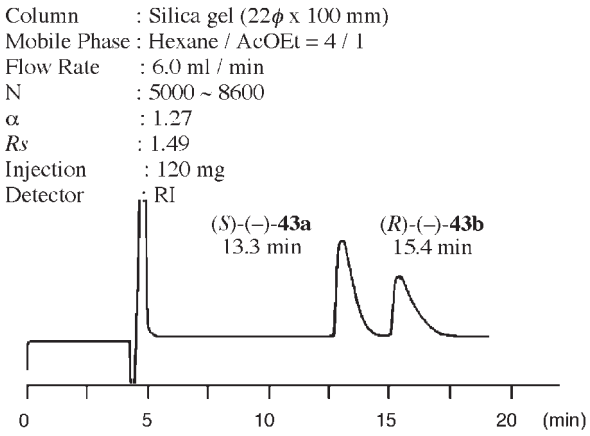


Fig. 9.8 HPLC separation of esters (*S*)-(–)-**43a** and (*R*)-(–)-**43b**.

there any other method applicable to noncrystalline materials? In addition, the applications of the CSDP acid method have been mostly applied to aromatic compounds as shown in Table 9.2. So, a powerful method applicable to aliphatic compounds was required.

We have discovered that 2-methoxy-2-(1-naphthyl)propionic acid (MαNP acid (**3**), Figs. 9.1 and 9.7 c), is remarkably effective in the enantioresolution of aliphatic alcohols, especially acyclic aliphatic alcohols [41–52, 56–58]. In the ^{1}H NMR spectra of the esters formed from MαNP acid **3** and alcohols, the chemical shifts of the protons in the alcohol moiety are strongly affected by the magnetic anisotropy effect induced by the naphthyl group. Therefore, this MαNP acid **3** can be used as the chiral auxiliary of the advanced Mosher method [12–15] useful for determining the absolute configuration of secondary alcohols. Another advantage of the MαNP acid **3** is that it does not racemize, because the α-position of **3** is fully substituted, and therefore, it is easy to prepare the enantiopure acid **3**. As discussed below, MαNP acid **3** is a very powerful chiral derivatizing agent, which simultaneously enables both enantioresolution of secondary alcohols and determination of their absolute configurations. Namely, the MαNP acid method explained here is very useful for enantioresolution of racemic alcohols and also for determination of the absolute configurations of natural products and biologically active synthetic chiral compounds, e. g., chiral drugs. In this sense, the chiral MαNP acid **3** is superior to the conventional chiral acids: Mosher's MTPA acid [13], Trost's MPA acid [15], 1- and 2-NMA acids developed by the Riguera [14] and Kusumi [12] groups.

In the following sections, the principle and applications of this chiral MαNP acid method are described: (1) the synthesis of chiral MαNP acid **3**, (2) the enantioresolution of racemic acid **3** with chiral alcohols, (3) the absolute configurational and conformational analyses of MαNP acid esters by NMR and CD spectroscopic methods, (4) the enantioresolution of racemic alcohols and determination

of their absolute configuration using chiral MαNP acid **3**, (5) the recovery of chiral alcohols with 100% enantiopurity from the separated diastereomeric esters.

9.4.1
Facile Synthesis of MαNP Acid (3) and Its Enantioresolution with Natural (–)-Menthol [40, 42, 50]

To synthesize a large amount of enantiopure chiral MαNP acid (**3**), the facile synthesis and enantioresolution of racemic acid **3** were carried out as shown in Fig. 9.9. In general, for enantioresolution of carboxylic acids, chiral synthetic amines or alkaloids have been used. However, we have adopted the following novel strategy using chiral alcohols; chiral alcohols are condensed with racemic acid **3** and the diastereomeric esters formed are separated by HPLC on silica gel.

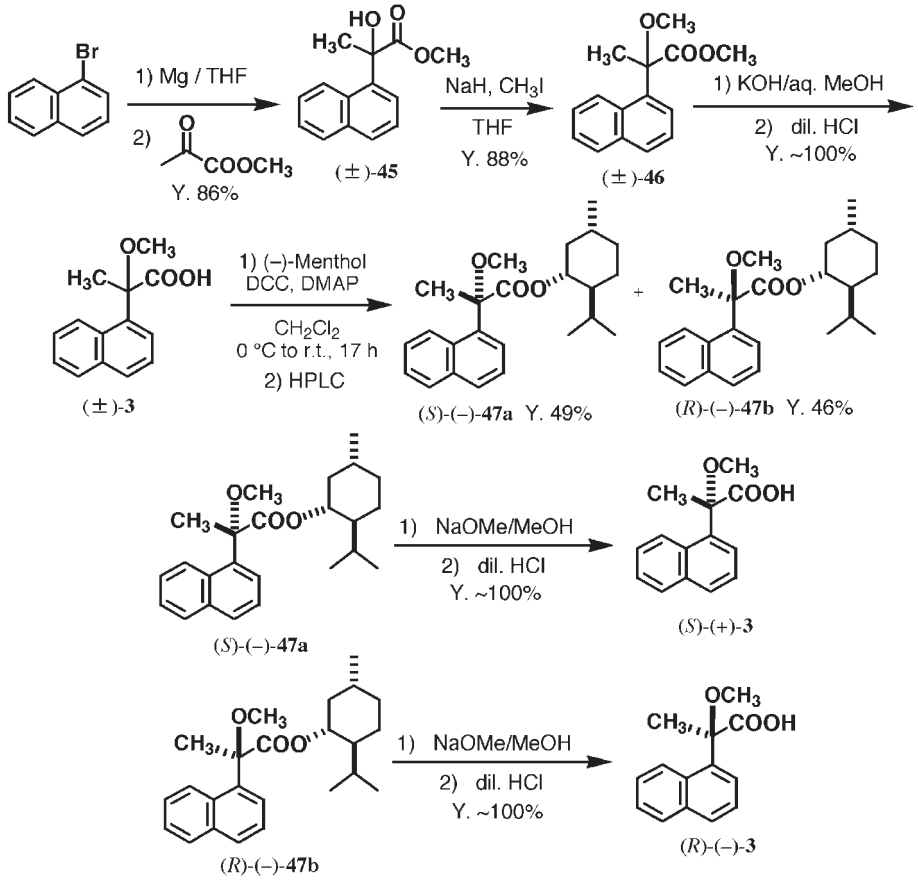


Fig. 9.9 Preparation of enantiopure MαNP acids (*S*)-(+)-**3** and (*R*)-(–)-**3** [50].

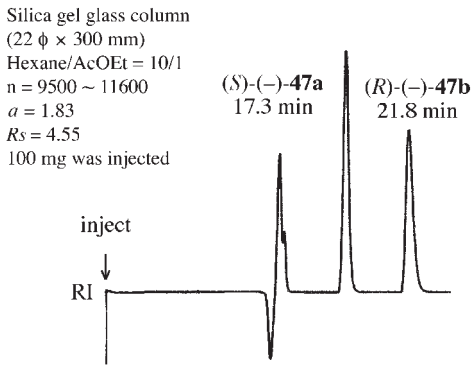


Fig. 9.10 HPLC separation of MαNP acid menthol esters (*S*)-(–)-**47a** and (*R*)-(–)-**47b** [50].

The separated esters are then hydrolyzed to yield both enantiomers of the desired carboxylic acids **3**.

As a chiral alcohol, naturally occurring (–)-menthol was selected and esterified with racemic acid **3**. It was surprising that the diastereomeric esters **47a** and **47b** formed were very easily separated by HPLC on silica gel (hexane/EtOAc =10 : 1) as illustrated in Fig. 9.10. The separation and resolution factors were extraordinarily high (α = 1.83, R_s = 4.55), indicating that acid **3** has great ability to recognize the chirality of the alcohols. The efficiency in separation enabled the HPLC on a preparative scale: esters **47a**/**47b** (1.0–1.8 g) were separable in one run using a glass column of silica gel (25 ϕ × 400 mm). The first-eluted ester **47a** was subjected to solvolysis to yield chiral acid (+)-**3**, while the second-eluted ester **47b** gave acid (–)-**3**. To determine the absolute configurations of chiral acids **3** obtained, they were converted to methyl esters and their CD spectra measured. By comparison of these CD spectra with that of the authentic sample with known absolute configuration established by X-ray analysis and chemical correlation (Fig. 9.7), the absolute configurations of chiral acids **3** were determined as (*S*)-(+) and (*R*)-(–), respectively, leading to the assignment of (*S*)-(–)-**47a** and (*R*)-(–)-**47b** (Fig. 9.9).

9.4.2
The ^{1}H NMR Anisotropy Method for Determining the Absolute Configuration of Secondary Alcohols: the Sector Rule and Applications [42, 50]

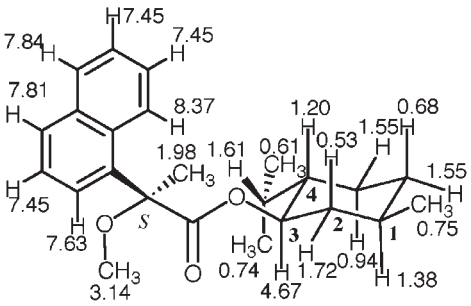
As described above, the ^{1}H NMR anisotropy method has been frequently used as a relative and empirical method for determining the absolute configurations of chiral organic compounds [10–13]. In particular, the advanced Mosher method for chiral secondary alcohols has been successfully employed in the field of natural products. In the cases of Mosher's MTPA and Trost's MPA acids, the phenyl group exhibits the magnetic anisotropy effect induced by the aromatic ring current, affecting the chemical shift (δ) of protons in the alcohol part. Therefore, the absolute configuration of chiral alcohol can be determined by the difference ($\Delta\delta$) in

the chemical shifts of the esters formed with (*R*) and (*S*) carboxylic acids: $\Delta\delta = \delta(R) - \delta(S)$ or $\Delta\delta = \delta(S) - \delta(R)$. We have found that MαNP acid **3** is superior to Mosher's MTPA and Trost's MPA acids, because the magnetic anisotropy effect of the naphthyl group is much larger than that of a phenyl group and, therefore, larger $\Delta\delta$ values are obtained. So, the absolute configuration of chiral alcohols can be unambiguously determined, when using MαNP acid **3** as a chiral NMR anisotropy reagent. Moreover, MαNP acid has the further advantage of not racemizing, because the α-position of **3** is fully substituted. For these reasons, it is advisable to use MαNP acid **3**, rather than other conventional chiral acids, for determining the absolute configuration of chiral alcohols including natural products.

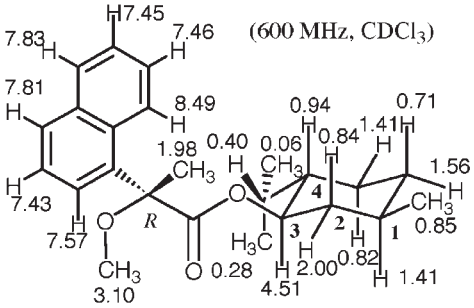
All NMR proton peaks of diastereomeric menthol MαNP esters **47a** and **47b** were fully assigned by various methods including two-dimensional ones (^{1}H, ^{1}H-^{1}H COSY, ^{13}C, ^{1}H-^{13}C COSY, HMBC; Fig. 9.11 a). The protons of the isopropyl group in ester **47b** appeared at much higher fields than in ester **47a**. On the other hand, the protons in the 2-position in **47a** appeared at higher fields than in ester **47b**. Those high field shifts are obviously due to the magnetic anisotropy effect induced by the naphthyl group of the MαNP acid moiety.

To determine the absolute configuration from the ^{1}H NMR anisotropy effect, it is necessary to determine the preferred conformation of each diastereomer. In esters **47a** and **47b**, the absolute configurations of MαNP acid and menthol moieties are established as described above, and so the following stable conformations are proposed to satisfy the anisotropy effects observed in the NMR spectra (Fig. 9.11). Namely, the two oxygen atoms of the methoxyl and ester carbonyl groups are synperiplanar (*syn*) to each other in their stable conformations. In addition, the ester carbonyl oxygen atom is also *syn* to the alcohol methine proton. Therefore, the methoxyl group, ester group, and alcohol methine proton lie in the same plane, which is called the MαNP plane (Figs. 9.11 and 9.12). These *syn* conformations are similar to those proposed for MPA esters. In ester **47a**, the naphthyl group and H-2 protons are on the same front side of the MαNP plane, and the H-2 protons are located above the naphthyl plane. Therefore, the H-2 protons feel the magnetic anisotropy effect of high field shift, and so appear at higher field. In ester **47b**, the naphthyl group is close to the isopropyl group, and the high field shifts of isopropyl protons are observable.

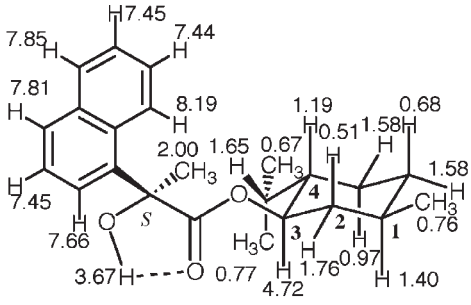
The predominance of the *syn* conformations in esters **47a** and **47b** is also supported by the comparison of the NMR data with those of 2-hydroxy-2-(1-naphthyl)-propionic acid (HαNP) menthol esters **48a** and **48b** shown in Fig. 9.11(b). From the NMR chemical shift and IR data, it is obvious that the tertiary hydroxy group of HαNP esters is hydrogen-bonded to the oxygen atom of the ester carbonyl group, i.e. the hydroxy group and the ester carbonyl oxygen atom take a *syn* conformation. We have found a very interesting fact that the NMR chemical shift data of MαNP acid menthol ester (*S*;1*R*,3*R*,4*S*)-(–)-**47a**, especially those of the menthol part, are very similar to those of HαNP acid menthol ester (*S*;1*R*,3*R*,4*S*)-(–)-**48a** as shown in Fig. 9.11(a) and (b). The same is true for the pairs of other diastereomers, (*R*;1*R*,3*R*,4*S*)-(–)-**47b** and HαNP acid menthol ester (*R*;1*R*,3*R*,4*S*)-(–)-**48b** (Fig. 9.11). These facts indicate that MαNP acid menthol esters take the *syn* con-



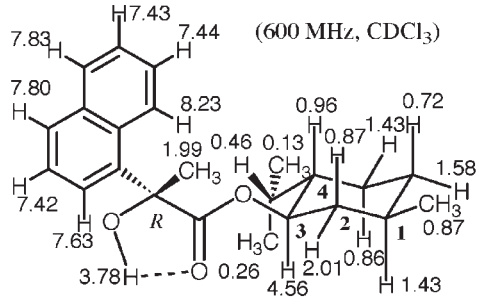
(a) **(*S*;1*R*,3*R*,4*S*)-(–)-47a**



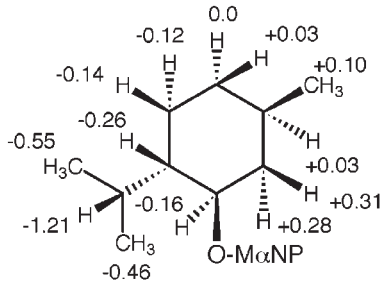
(*R*;1*R*,3*R*,4*S*)-(–)-47b



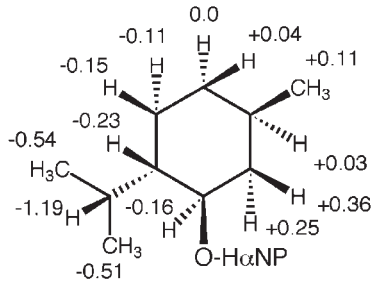
(b) **(*S*;1*R*,3*R*,4*S*)-(–)-48a**



(*R*;1*R*,3*R*,4*S*)-(+)-48b



$\Delta\delta = \delta(R) - \delta(S)$
$= \delta(\mathbf{47b}) - \delta(\mathbf{47a})$



$\Delta\delta = \delta(R) - \delta(S)$
$= \delta(\mathbf{48b}) - \delta(\mathbf{48a})$

Fig. 9.11 NMR data of MαNP and HαNP acid menthol esters [42, 50].

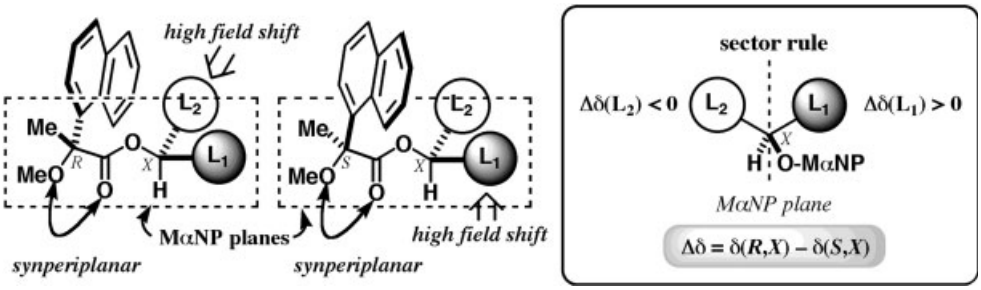


Fig. 9.12 The preferred conformation of MαNP esters, and the sector rule for determining the absolute configuration of chiral alcohols by using NMR $\Delta\delta$ values [39].

formation, as HαNP acid menthol esters usually do. This fully explains the observed magnetic anisotropy effects.

By using the NMR anisotropy effect of MαNP esters, the sector rule for determining the absolute configuration of secondary alcohols can be deduced (Fig. 9.12). The basic procedure is as follows; (*R*)-MαNP and (*S*)-MαNP acids are separately allowed to react with a chiral alcohol, the absolute configuration of which is defined as *X*. So, the ester prepared from (*R*)-MαNP acid has the (*R*,*X*) absolute configuration, while the other ester prepared from (*S*)-MαNP acid has the (*S*,*X*) absolute configuration. All NMR proton signals of (*R*,*X*)- and (*S*,*X*)-esters are fully assigned by careful analysis. If necessary, the use of two-dimensional spectra is suggested. The $\Delta\delta$ values ($\Delta\delta = \delta(R,X) - \delta(S,X)$) are calculated for all protons in the alcohol moiety. Figure 9.12 shows the sector rule for the MαNP ester, where the MαNP group is placed on the down and front side, while the methine proton of the secondary alcohol is on the down and rear side. The group L_1 with protons exhibiting positive $\Delta\delta$ values is placed on the right side, while the group L_2 with protons showing negative $\Delta\delta$ values on the left side. From this projection, the absolute configuration X of the chiral alcohol can be determined.

The magnetic anisotropy effect of chiral MαNP acid is much stronger than that of conventional chiral carboxylic acid (Fig. 9.13). For instance, the $\Delta\delta$ values of the MαNP-menthol ester are ca. four times larger than those of Mosher's MTPA ester [13] (Fig. 9.13 b): twice the value for Trost's MPA ester [15] (Fig. 9.13 c); comparable to 1-NMA and 2-NMA esters reported by Riguera [14] and Kusumi et al. [12]. MαNP acid is thus effective for determining the absolute configuration of natural products.

Some examples of the application of this MαNP acid method to chiral alcohols are shown in Fig. 9.14.

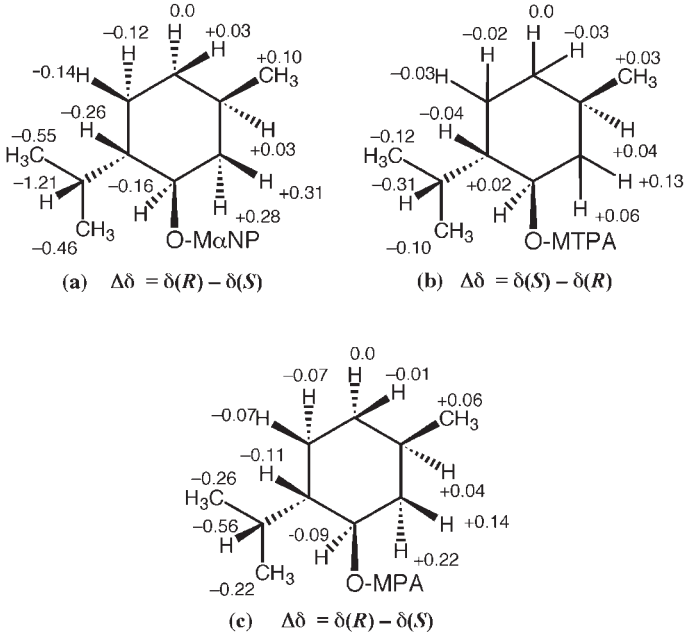


Fig. 9.13 Comparison of the NMR $\Delta\delta$ values of menthol esters formed with chiral carboxylic acids [42].

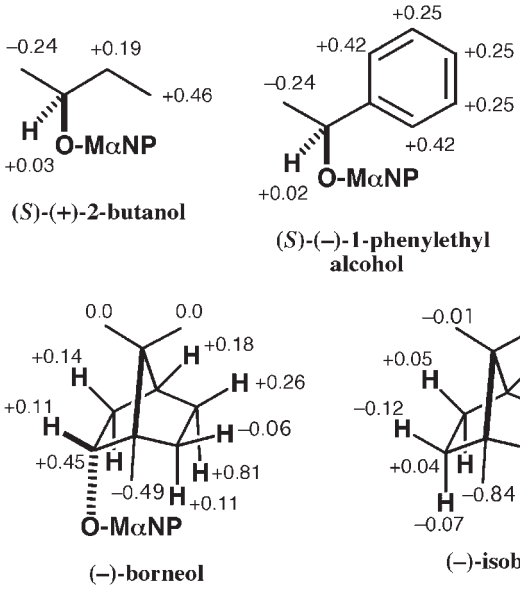


Fig. 9.14 The NMR $\Delta\delta$ values and absolute configurations determined by the MαNP acid method [42].

9.4.3
Enantioresolution of Various Alcohols Using MαNP Acid and Simultaneous Determination of Their Absolute Configurations [44, 50]

Another extraordinary quality of MαNP acid is its excellent ability in chiral recognition. For example, as discussed above, racemic MαNP acid could be successfully enantioresolved as the esters of natural (–)-menthol; the diastereomeric esters formed were clearly separated by HPLC on silica gel. MαNP acid could also be enantioresolved with other chiral alcohols. These facts logically indicate that if enantiopure MαNP acid is used, racemic alcohols can be enantioresolved. In fact, we have succeeded in the enantioresolution of various alcohols using enantiopure MαNP acid (*S*)-(+)-**3** as exemplified in Fig. 9.15.

This novel chiral MαNP acid (*S*)-(+)-**3** has thus a remarkable enantioresolving power for alcohols, especially for aliphatic alcohols. For instance, in the case of

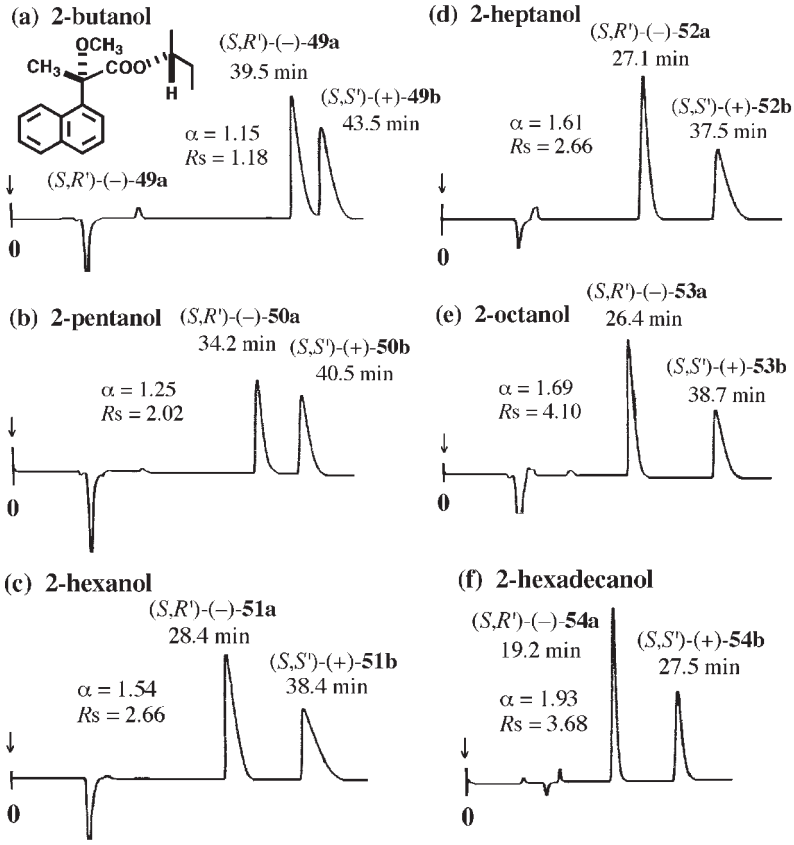


Fig. 9.15 HPLC separation of diastereomeric esters formed from aliphatic alcohols and (*S*)-(+)-MαNP acid **3** (silica gel, 22 ϕ × 300 mm, hexane/EtOAc = 20 : 1) [44].

2-butanol, the diastereomeric esters can be baseline separated with the separation factor $\alpha = 1.15$ and resolution factor $R_s = 1.18$. In this case, it is obvious that the chiral carboxylic acid **3** recognizes well the slight difference between methyl and ethyl groups. This is an excellent and practical method since the chiral acid **3** exhibits a high resolving power to aliphatic alcohols, to which in general asymmetric syntheses are hardly applicable.

The next question is how is the absolute configuration of the alcohol moiety determined.The absolute configurations of separated diastereomers can be determined by applying the ^{1}H NMR anisotropy method using chiral MαNP acid as described above. A general scheme is illustrated in Fig. 9.16. Racemic alcohol is esterified with MαNP acid (*S*)-(+)-**3** yielding a mixture of diastereomeric esters, which are separated by HPLC on silica gel. The absolute configuration of the first-eluted ester is defined as (*S,X*), where *S* denotes the absolute configuration of the MαNP acid part, while *X* denotes that of the alcohol part. So, the absolute configuration of the second-eluted ester is expressed as (*S,–X*), where *–X* indicates the opposite absolute configuration of *X*. The original definition of the $\Delta\delta$ value is $\Delta\delta = \delta(R,X) - \delta(S,X)$, and so the value of $\delta(R,X)$ is required to calculate the $\Delta\delta$ value. However, the enantiomer (*R,X*) does not exist in this scheme, and so the original equation for $\Delta\delta$ is not useful here.

To solve the above problem, the following conversion of the equation was performed. Since the ester (*S,–X*) is the enantiomer of ester (*R,X*), their NMR data should be identical: $\delta(R,X) = \delta(S,-X)$. Therefore, $\Delta\delta = \delta(R,X) - \delta(S,X) = \delta(S,-X) - \delta(S,X) = \delta(\text{2nd fr.}) - \delta(\text{1st fr.})$. So, the absolute configuration *X* of the first-eluted fraction can be determined from the $\Delta\delta$ value which is obtained by subtracting the chemical shift of the first-eluted fraction from that of the second-eluted frac-

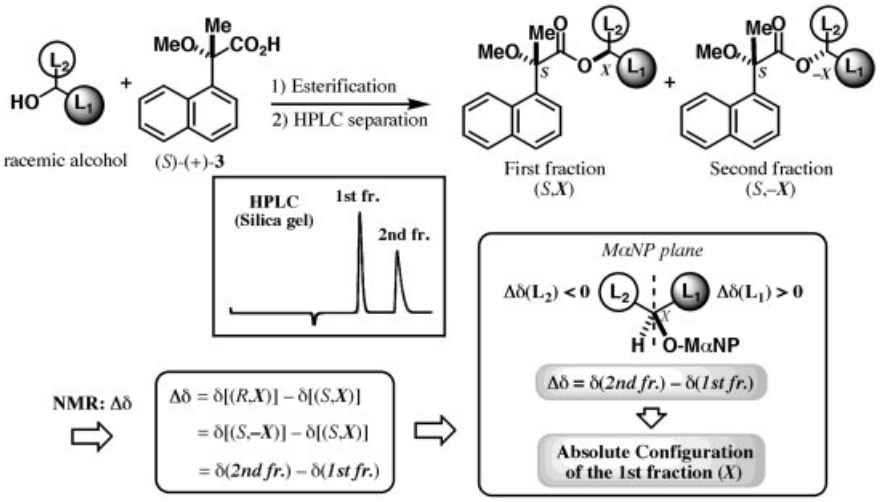


Fig. 9.16 Enantioresolution of racemic alcohol as (*S*)-MαNP esters, and determination of the absolute configuration of the first-eluted fraction by the NMR anisotropy method [38, 45, 50].

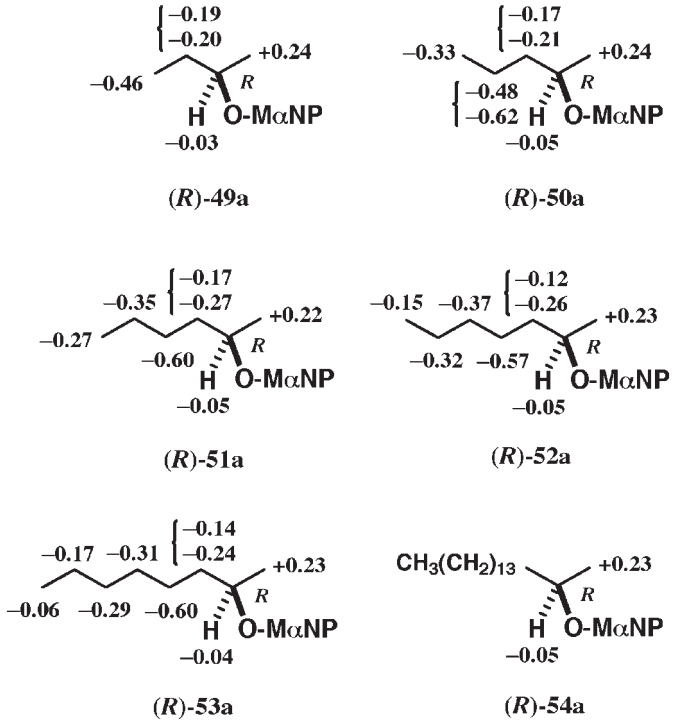


Fig. 9.17 Determination of the absolute configurations of the alcoholic part of the first-eluted esters by the NMR anisotropy method using (*S*)-(+)-MαNP acid, **3** and the observed $\Delta\delta$ values [44].

tion (Fig. 9.16). This method has been applied to the esters shown in Fig. 9.15, giving $\Delta\delta$ values and the absolute configurations of the first-eluted esters (Fig. 9.17). The $\Delta\delta$ values are reasonably distributed: positive values on the right, and negative values on the left. Thus the absolute configuration of the first-eluted ester can be determined, and the opposite absolute configuration is, of course, assigned to the second-eluted ester. It should be noted that when MαNP acid (*R*)-(–)-**3** is used, the $\Delta\delta$ value is defined as $\Delta\delta = \delta(R,X) - \delta(S,X) = \delta(R,X) - \delta(R,-X) = \delta$(1st fr.) – δ(2nd fr.).

The next step is the recovery of enantiopure alcohol and chiral MαNP acid **3**. As exemplified in Fig. 9.18, both enantiopure alcohols were readily obtained by the solvolysis of the separated esters [47, 50]. The chiral MαNP acid **3** was also recovered and could be recycled.

How good is the enantiopurity of the recovered alcohols? In our method, both diastereomeric esters obtained are enantiopure, if the MαNP acid **3** used is enantiopure, because they are fully separated in HPLC. The MαNP acid **3** was enantioresolved with natural (–)-menthol, the enantiopurity of which was confirmed as 100% by gas chromatography using a chiral stationary phase [50].

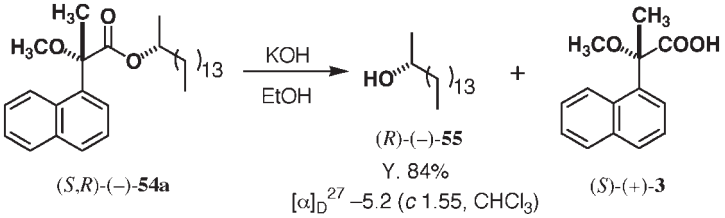


Fig. 9.18 Recovery of enantiopure alcohol and MαNP acid.

As described here, MαNP acid has excellent enantioresolving power despite its simple molecular structure and the absence of so-called hetero atoms. Furthermore, the chiral acid **3** is superior to Mosher's MTPA and Trost's MPA acids in the magnetic anisotropy effect.

9.4.4
Recent Applications of the MαNP Acid Method to Various Alcohols [38, 44, 50]

The MαNP acid method has been successfully applied to various racemic alcohols listed in Table 9.3 for preparation of enantiopure secondary alcohols and the simultaneous determination of their absolute configurations. If the separation factor α is as large as in the case of 1-octyn-3-ol **56** (entry 2 in Table 9.3, α = 1.88), a large-scale HPLC separation of diastereomeric MαNP esters is feasible. For example, in the case of esters **64a** and **64b** derived from alcohol **56**, ca. 0.85–1.0 g of the mixture was separable in one run by the HPLC (hexane/EtOAc = 20 : 1) using a silica gel glass column (22 Φ × 300 mm) (Figs. 9.19 and 9.20).

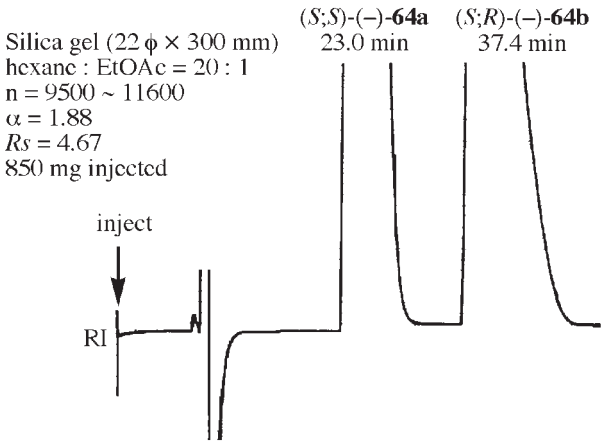
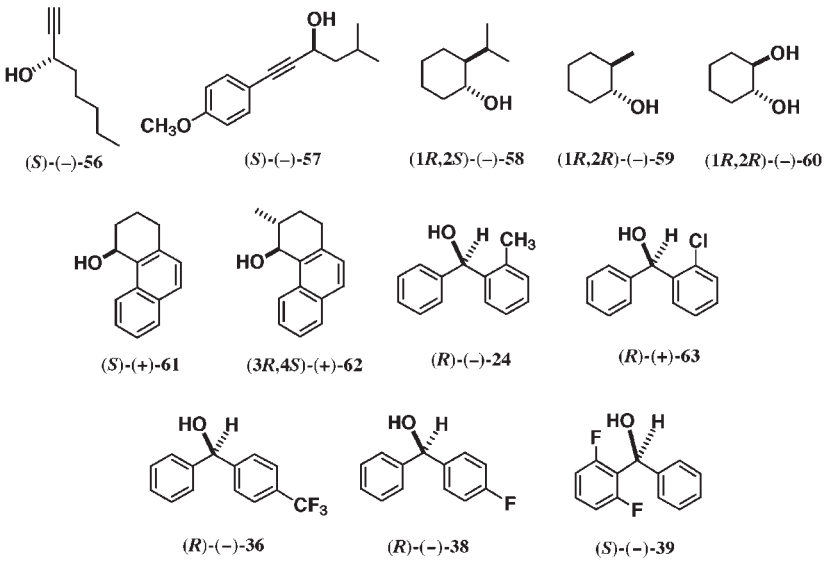


Fig. 9.19 A large-scale HPLC separation of diastereomeric esters (*S*;*S*)-(−)-**64a** and (*S*;*R*)-(−)-**64b** [50].

Table 9.3 HPLC[a] separation of diastereomeric esters formed from alcohols with MαNP acid (*S*)-(+)-**3**, determination of their absolute configurations by the ^{1}H NMR anisotropy method, and absolute configurations of recovered chiral alcohols.

Entry	Alcohol	Solvent[b]	α[c]	*Rs*[d]	Ester (1st Fr.)	Chiral alcohol (1st Fr)	Ref.
1	(±)-**55**	H/EA = 20/1	1.93	3.68	(*S*;*R*)-(−)-**54a**	(*R*)-(−)-**55**	[44, 50]
2	(±)-**56**	H/EA = 20/1	1.88	4.67	(*S*;*S*)-(−)-**64a**	(*S*)-(−)-**56**	[44, 50]
3	(±)-**57**	H/EA = 10/1	1.30	2.38	(*S*;*S*)-(−)-**65a**	(*S*)-(−)-**57**	[50]
4	(±)-**58**	H/EA = 20/1	1.88	4.97	(*S*;1*R*,2*S*)-(−)-**66a**	(1*R*,2*S*)-(−)-**58**	[50]
5	(±)-**59**	H/EA = 20/1	1.21	1.54	(*S*;1*R*,2*R*)-(−)-**67a**	(1*R*,2*R*)-(−)-**59**	[50]
6	(±)-**60**	H/EA = 2/1	1.35	1.82	(*S*;1*R*,2*R*)-(−)-**68a**[e]	(1*R*,2*R*)-(−)-**60**	[50]
7	(±)-**61**	H/EA = 10/1	1.22	1.54	(*S*;*S*)-(+)-**69a**	(*S*)-(+)-**61**	[50]
8	(±)-**62**	H/EA = 15/1	1.46	2.77	(*S*;3*R*,4*S*)-(+)-**70a**	(3*R*,4*S*)-(+)-**62**	[50]
9	(±)-**24**	H/EA = 15/1	1.12	1.45	(*S*;*R*)-(−)-**71a**	(*R*)-(−)-**24**	[50]
10	(±)-**63**	H/EA = 15/1	1.10	1.40	(*S*;*R*)-(−)-**72a**	(*R*)-(+)-**63**	[50]
11	(±)-**36**	H/EA = 8/1	1.39	4.84	(*S*;*R*)-(−)-**73a**	(*R*)-(−)-**36**	[38]
12	(±)-**38**	H/EA = 10/1	1.18	2.55	(*S*;*R*)-(−)-**74a**	(*R*)-(−)-**38**	[38]
13	(±)-**39**	H/EA = 8/1	1.08	1.28	(*S*;*S*)-(−)-**75a**	(*S*)-(−)-**39**	[38]

a) Glass column (22 ϕ × 300 mm, or 25 ϕ × 400 mm) of silica gel (particle size 5–10 μm). **b)** H = *n*-hexane, EA = ethyl acetate. **c)** Separation factor $\alpha = (t_2 - t_0)/(t_1 - t_0)$ where t_1 and t_2 are the retention times of the first- and second-eluted fractions, respectively, and t_0 is the retention time of an unretained compound (void volume marker). **d)** Resolution factor $Rs = 2(t_2 - t_1)/(W_1 + W_2)$ where W_1 and W_2 are the band-widths of the first- and second-eluted fractions at the base-line level, respectively. **e)** Mono-MαNP ester.



(*S*)-(−)-**56** (*S*)-(−)-**57** (1*R*,2*S*)-(−)-**58** (1*R*,2*R*)-(−)-**59** (1*R*,2*R*)-(−)-**60**

(*S*)-(+)-**61** (3*R*,4*S*)-(+)-**62** (*R*)-(−)-**24** (*R*)-(+)-**63**

(*R*)-(−)-**36** (*R*)-(−)-**38** (*S*)-(−)-**39**

Structures to Table 9.3

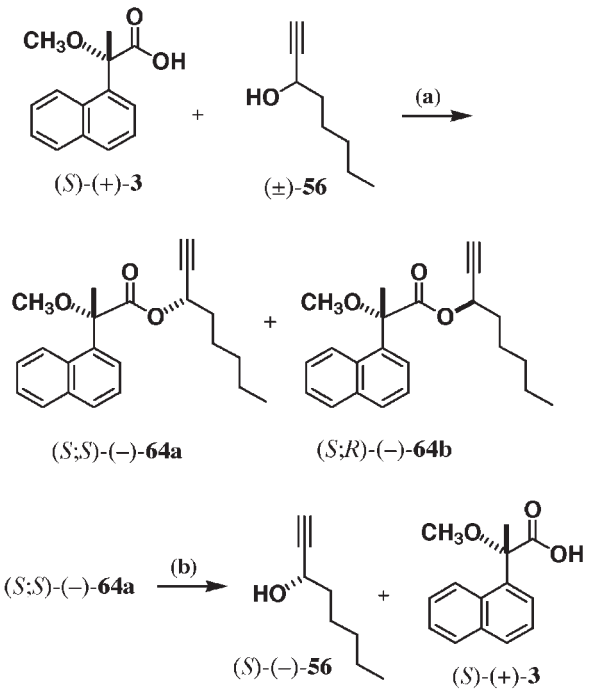


Fig. 9.20 Preparation of enantiopure 1-octyn-3-ol (*S*)-(−)-**56** [50].

The HPLC separation data of diastereomeric esters prepared from other racemic alcohols **24**, **36**, **38**, **39**, and **57**-**63** with MαNP acid (*S*)-(+)-**3** are listed in Table 9.3. It should be emphasized that for most alcohols, their diastereomeric MαNP esters are clearly separated with α values of 1.10–1.88. Phenylacetylene alcohol **57** was separable as the MαNP esters **65a**/**65b** (α = 1.30, entry 3). Substituted cyclohexanols **58** and **59** were also effectively separated as MαNP esters (entries 4 and 5). Especially, the α value of *trans*-2-isopropylcyclohexanol MαNP esters **66a**/**66b** is as large as 1.88, which is comparable to that of the menthol case. On the other hand, in the case of *trans*-2-methylcyclohexanol MαNP esters **67a**/**67b**, the α value is relatively small, α = 1.21. These results indicate that the combination of a longer and larger alkyl group on one side and a smaller alkyl group on the other side leads to better separation of two diastereomers, as seen in 2-hexadecanol esters **54a**/**54b** (see Fig. 9.15) and *trans*-2-isopropylcyclohexanol MαNP esters **66a**/**66b**.

Entry 6 is an interesting case, where mono-MαNP esters **68a**/**68b** of *trans*-1,2-cyclohexanediol **60** were sufficiently separated, despite the existence of a polar hydroxy group (α = 1.35). In the cases of cyclic naphthalene alcohols **61** and **62**, their MαNP esters were separated well, but the values depend on the neighboring substituent. Namely, the MαNP esters **70a**/**70b** of *trans*-alcohol **62** were more effi-

ciently separated (α = 1.46) than those of unsubstituted alcohol **61** (esters **69a**/**69b**, α = 1.22) (entries 7 and 8). The enantioresolution of *ortho*-substituted diphenylmethanols **24** and **63** was one of the most difficult cases. We have previously applied the CSDP acid method to these alcohols as described above [30, 31]. However, the diastereomeric CSDP esters of **24** appeared as a single peak in HPLC, indicating no separation at all. However, the MαNP esters **71a**/**71b** of alcohol **24** were base-line separated (α = 1.12, entry 9). In a similar way, alcohol **63** was also enantioresolved as MαNP esters **72a**/**72b** (α = 1.10, entry 10).

The MαNP acid method was also applied to racemic fluorinated diphenylmethanols **36**, **38**, and **39** (Table 9.3). A diastereomeric mixture of esters **73a** and **73b** prepared from (4-trifluoromethylphenyl)phenylmethanol **36** was separated well by HPLC on silica gel: α = 1.39; *R*s = 4.84 (entry 11). Other fluorinated diphenylmethanols were similarly esterified with (*S*)-(+)-**3**, and the diastereomeric mixtures obtained were subjected to HPLC on silica gel. Diastereomeric MαNP esters of (4-fluorophenyl)phenylmethanol **38** were separated well with α-values of 1.18 (entry 12). On the other hand, it was a little difficult to separate the diastereomeric MαNP esters of (2,6-difluorophenyl)phenylmethanol **39**, because of its small α-value: α = 1.08.

By applying the ^{1}H NMR anisotropy method, the absolute configurations of the first-eluted MαNP esters **64a**–**75a** were determined, as illustrated in Fig. 9.21. The observed $\Delta\delta$ values are distributed in a reasonable manner; protons near the MαNP group show larger $\Delta\delta$ values than remote ones. In the 1-octyn-3-ol MαNP esters **64a**/**64b**, the acetylene proton showing a positive $\Delta\delta$ value (+0.11) is placed on the right side, while the pentyl group having negative $\Delta\delta$ values is on the left side. So, the absolute configuration of (–)-**64a** was assigned as *S*. In the phenyl acetylene alcohol esters **65a**/**65b**, the phenyl protons show clearly positive $\Delta\delta$ values, despite the long distance from the MαNP group, while the iso-butyl group shows large negative $\Delta\delta$ values. Therefore, the *S* absolute configuration was assigned to (–)-**65a**.

In the cyclic alcohol esters **66a**/**66b** and **67a**/**67b**, the observed $\Delta\delta$ values are similar at the corresponding positions, leading to the (1*R*,2*S*)-absolute configuration of (–)-**66a** and the (1*R*,2*R*)-absolute configuration of (–)-**67a**. The case of vicinal diol mono-MαNP esters **68a**/**68b** is a unique example; there was some concern that the conformation of the MαNP group might be deviated from the ideal *syn*-conformation by the effect of the adjacent polar hydroxy group. However, the distribution pattern of observed $\Delta\delta$ values is similar to that of esters **66a**/**66b**, although their absolute values are different. So, the (1*R*,2*R*) absolute configuration was assigned to (–)-**68a**.

The naphthalene-cyclic alcohol esters **69a**/**69b** are also interesting cases; the naphthalene moiety contained in the alcohol skeleton also works as a strong ^{1}H NMR anisotropy-inducing group. Therefore, it was considered that the ^{1}H NMR anisotropy effect of MαNP esters **69a**/**69b** might become complex because of the two naphthalene groups. However, the $\Delta\delta$ values observed are reasonably distributed, even in the naphthalene region. Furthermore it should be emphasized that the 2-axial proton exhibits a very large negative $\Delta\delta$ value (–1.36 ~ –1.29) as shown

in Fig. 9.21. This phenomenon is interpreted as follows; the MαNP ester group takes an axial orientation in both **69a** and **69b** because of the *peri*-position of the naphthalene group, and, therefore, in the *syn*-conformation of **69b**, the 2-axial proton is located just below the naphthalene ring of the MαNP moiety, falling in the area of high field shift. From the observed $\Delta\delta$ data, the *S* absolute configuration was unambiguously assigned to the first-eluted ester (+)-**69a**.

It should also be noted that the boundary line of $\Delta\delta$ values is tilted to the right side, as indicated by the dotted line in **69a** (Fig. 9.21), i.e. the MαNP plane dividing the space into two sectors of $\Delta\delta$ is moved from the regular position of the C4–C1 line to that of C4–C10. This phenomenon implies that the MαNP ester moiety declines toward the aliphatic side of C-3, not toward the aromatic side of C-4a, because of steric hindrance. This conformation was in fact proved by the X-ray crystallographic analysis of ester **69a**, which indicated that the ester plane was tilted from the alcohol methine proton plane by 41.7° to the methylene side at the C-3 position [45]. The $\Delta\delta$ data of *trans*-methyl alcohol esters **70a**/**70b** are similar to those of esters **69a**/**69b** except for that of the methyl group, leading to the (3*R*,4*S*) absolute configuration of (+)-**70a**.

In the case of (2-methylphenyl)phenylmethanol MαNP esters **71a**/**71b**, it was easy to assign the proton signals of two phenyl groups, leading to the *R* absolute configuration of (–)-**71a**, which was corroborated by X-ray crystallography. In the 2-methylphenyl group on the left side, the $\Delta\delta$ value of the H-5 proton (–0.45) is larger than that of the H-3 proton (–0.22), indicating that esters **71a**/**71b** take a preferred conformation where the H-5 proton is more shielded by the naphthyl group than the H-3 proton. A similar phenomenon was also observed in esters **72a**/**72b**; the $\Delta\delta$ value of the H-5 proton (–0.46) is larger than that of the H-3 proton (–0.14). The absolute configuration of **72a** was unambiguously determined as *R*.

Similarly, the absolute configurations of fluorinated diphenylmethanol MαNP esters **73a**–**75a** were assigned as shown in Fig. 9.21. In the case of **73a** and **74a**, the phenyl groups have large positive $\Delta\delta$ values (+0.11 ~ +0.45 ppm), while the fluorinated phenyl groups show large negative $\Delta\delta$ values (–0.58 ~ –0.33 ppm), and therefore *R* absolute configurations were assigned to the first-eluted esters. On the other hand, in the case of (2,6-difluorophenyl)phenylmethanol **39**, the first-eluted MαNP ester **75a** takes the *S* absolute configuration (Fig. 9.21). The observed $\Delta\delta$ values distribute with regularity; *ortho*-protons have the largest $\Delta\delta$ values (0.49 ~ 0.58 ppm), *meta*-protons middle $\Delta\delta$ values (0.20 ~ 0.37 ppm), and *para*-protons the smallest $\Delta\delta$ values (0.11 ~ 0.20 ppm). These data indicate that the absolute configurational assignments performed by the ^{1}H NMR anisotropy method using MαNP acid are reliable. In the case of alcohols **36** and **39**, the absolute configurations determined by the MαNP acid method naturally agreed with those obtained by X-ray crystallography.

Enantiopure alcohols were recovered from the corresponding diastereomeric MαNP esters, (1) by hydrolysis with KOH in EtOH, or (2) by solvolysis with $NaOCH_3$ in MeOH followed by treatment with water, or (3) by reduction with $LiAlH_4$, or (4) by hydrolysis with K_2CO_3 in MeOH (Table 9.3). The MαNP acid method is thus very useful for the preparation of enantiopure alcohols and also

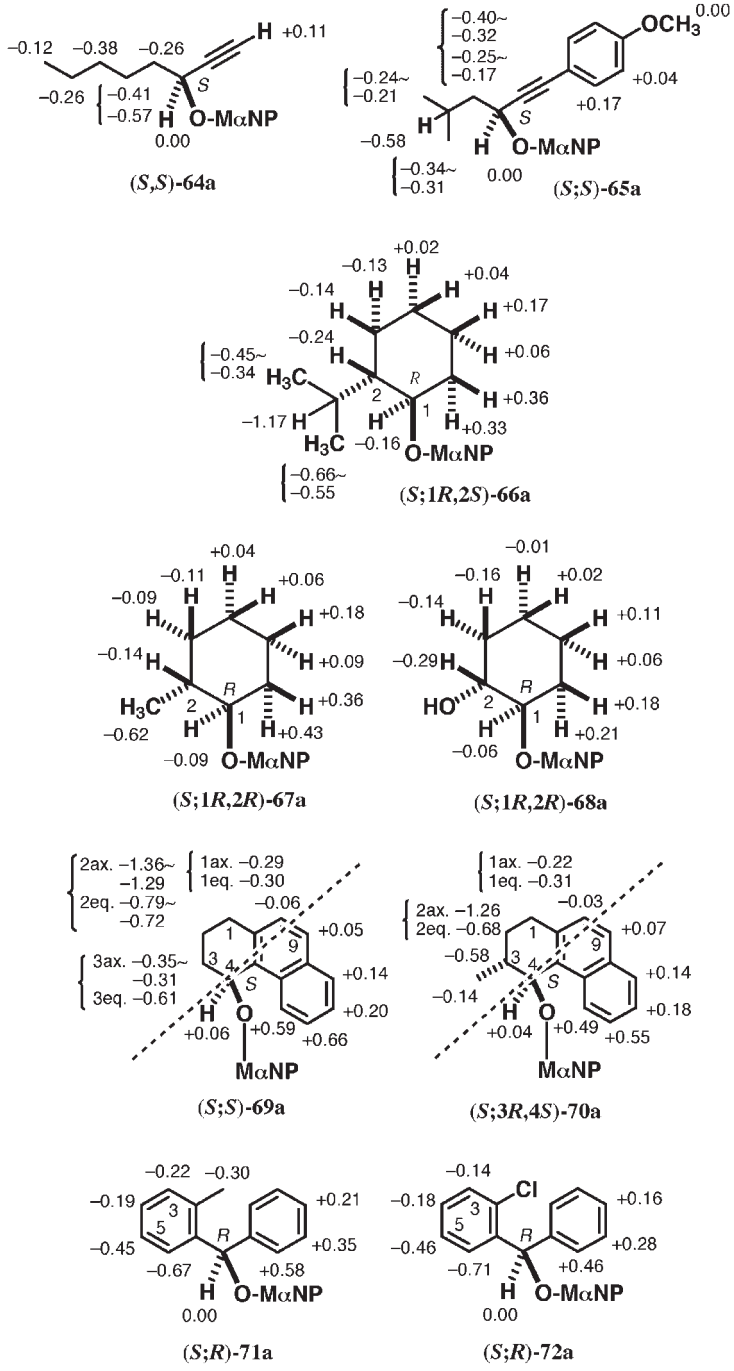


Fig. 9.21 (legend see p. 313)

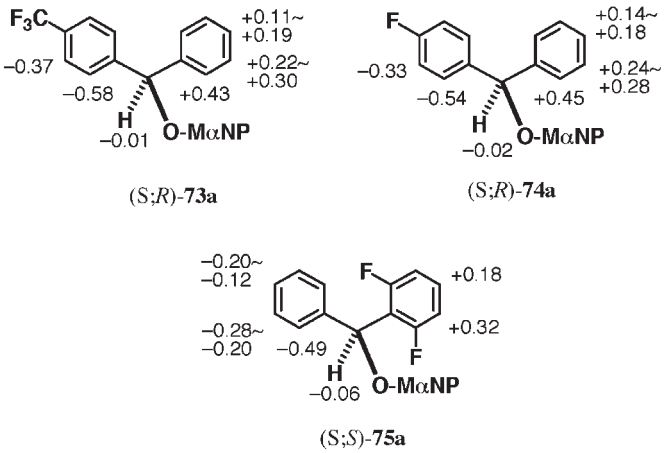


Fig. 9.21 Determination of absolute configurations by the ^{1}H NMR anisotropy method using (*S*)-(+)-MαNP acid **3**: observed $\Delta\delta$ values and the absolute configuration of the first-eluted esters [38, 50].

for the simultaneous determination of their absolute configurations by the ^{1}H NMR anisotropy method.

9.4.5
Application of the MαNP Acid Method to Chiral *meta*-Substituted Diphenylmethanols [39]

If the purpose is only to determine the absolute configuration of chiral alcohols, the MαNP acid method is applicable in the following manner. For example, alcohol (+)-**40** was esterified with (*R*)-(–)-MαNP acids **3**, yielding ester (*R,X*)-**76**, where *X* denotes the absolute configuration of alcohol moiety (Fig. 9.22). A similar reaction was repeated using (*S*)-(+)-MαNP acid **3** affording ester (*S,X*)-**76**. The ^{1}H NMR spectra of both products were fully assigned using the ^{1}H, ^{1}H-^{1}H COSY, ^{13}C, HMQC, and HMBC methods. The $\Delta\delta$ values ($\Delta\delta = \delta(R,X) - \delta(S,X)$) were calculated as shown in Fig. 9.22. The 3,5-dimethoxyphenyl group has positive $\Delta\delta$ values, while the phenyl has negative ones. By applying the sector rule shown in Fig. 9.12, the absolute configuration of the alcohol part was determined as $X = S$. This result agrees with the *S* absolute configuration as previously determined by the X-ray method (Table 9.3). It was again established that the ^{1}H NMR anisotropy method using MαNP acid is safely applicable to chiral alcohols.

The same MαNP method was next applied to enantiopure alcohol (+)-**77**, which was obtained by the CSDP acid method. Its absolute configuration, however, had remained undetermined, because its CSDP esters did not crystallize as ideal single crystals suitable for X-ray analysis. To determine the absolute configuration of (+)-**77**, it was subjected to the esterification with MαNP acids yielding (*R,X*)-**78** and (*S,X*)-**78** (Fig. 9.22). The $\Delta\delta$ values were similarly calculated as shown in

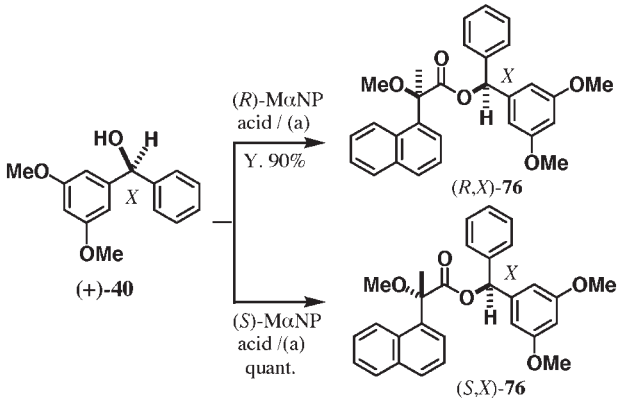


Fig. 9.22 The *S* absolute configuration of alcohol (+)-**40** as determined by the ^{1}H NMR anisotropy method using MαNP acid: (a) DCC, DMAP, CSA/CH_2Cl_2, r.t. [39].

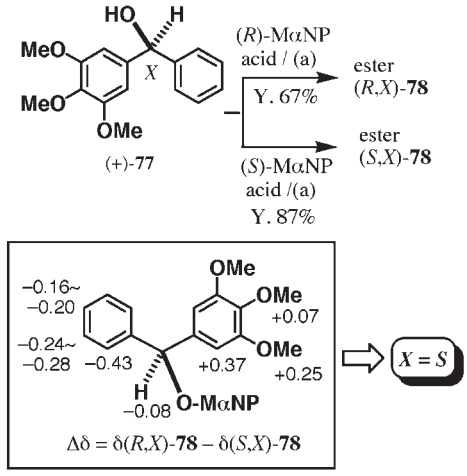


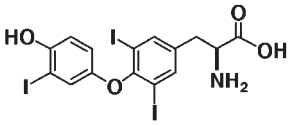
Fig. 9.23 The *S* absolute configuration of alcohol (+)-**77** as determined by the ^{1}H NMR anisotropy method using MαNP acid: (a) DCC, DMAP, CSA/CH_2Cl_2, r.t. [39].

Fig. 9.22. The 3,4,5-trimethoxyphenyl group has positive $\Delta\delta$ values, while the phenyl has negative ones. The magnitude and distribution pattern of the $\Delta\delta$ values are very similar to those of the MαNP esters **76** except for the methoxy group at the 4-position. Therefore the *S* absolute configuration was unambiguously assigned to alcohol (+)-**77**.

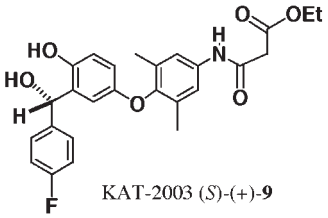
As described above, the methods of CSDP acid and MαNP acid are very powerful for the preparation of enantiopure alcohols and also for simultaneous determination of their absolute configurations. If single crystals of CSDP esters are not available, the ^{1}H NMR anisotropy method using "MαNP acid" is powerful as the complementary tool.

9.5
Absolute Configuration of the Thyroid Hormone Analog KAT-2003 as Determined by the ^{1}H NMR Anisotropy Method with MαNP Acid [52]

Triiodothyronine (L-T_3, **79**) and related analogs have the effect of lowering cholesterol levels in animal models [53] and humans [54] (Fig. 9.24). However, these agents are not used therapeutically because of adverse cardiac side effects, which arise either directly by acting on cardiac receptors or indirectly through an increase in the metabolic rate [55]. It was reported that the chiral thyroid hormone analogue KAT-2003 (+)-**9** showed hypocholesterolemic activity and decrease in the hepatic triglyceride content with reduced cardiac side effects [23]. In addition, it was also found that the dextrorotatory enantiomer of KAT-2003 (+)-**9** had significant inhibitory effect on the second primary hepatocellular carcinoma in rats [23, 24]. On the other hand, the opposite enantiomer (–)-**9** was less active than (+)-**9** [23]. So the chiral thyroid hormone analogue KAT-2003 (+)-**9** has been developed as a potent medicine. However, its absolute configuration has remained undetermined.



Triiodothyronine (L-T_3) **79**



KAT-2003 (*S*)-(+)-**9**

Fig. 9.24 Structures of triiodothyronine **79** and KAT-2003 (*S*)-(+)-**9** [52].

KAT-2003 (+)-**9**, $[\alpha]_D^{28}$ +32.2 (*c* 1.00, MeOH), having a chiral diphenylmethanol moiety has been prepared by the asymmetric reduction of the corresponding ketone with chiral reagent, (+)-*B*-chlorodiisopinocamphenylborane (DIP-chloride™). As discussed above, we have successfully applied the method of CSDP acid **1** to various substituted diphenylmethanols for enantioresolving racemic alcohols and also for determining their absolute configurations by X-ray crystallography. So we first applied the CSDP acid method to KAT-2003 **9** and its methyl ether derivative. However, it was unsuccessful in separating diastereomeric CSDP esters by HPLC.

We next applied the method of MαNP acid **3**, which is very effective for enantioresolving racemic alcohols and also for determining the absolute configuration of chiral alcohols by the ^{1}H NMR anisotropy method as described above. We explain here the application of the MαNP acid method to the chiral thyroid hormone analogue KAT-2003 (+)-**9** to determine its absolute configuration.

To apply the MαNP acid method, the phenol group of KAT-2003 has to be protected, e.g., as methyl ether. So, racemic KAT-2003 (±)-**9** was methylated with CH_3I and Cs_2CO_3 in acetonitrile yielding trimethylated alcohol (±)-**80** in a good yield (Fig. 9.25). Esterification of (±)-**80** with (*S*)-(+)-MαNP acid **3** gave a diastereomeric mixture of esters, which was easily separated by HPLC on silica gel

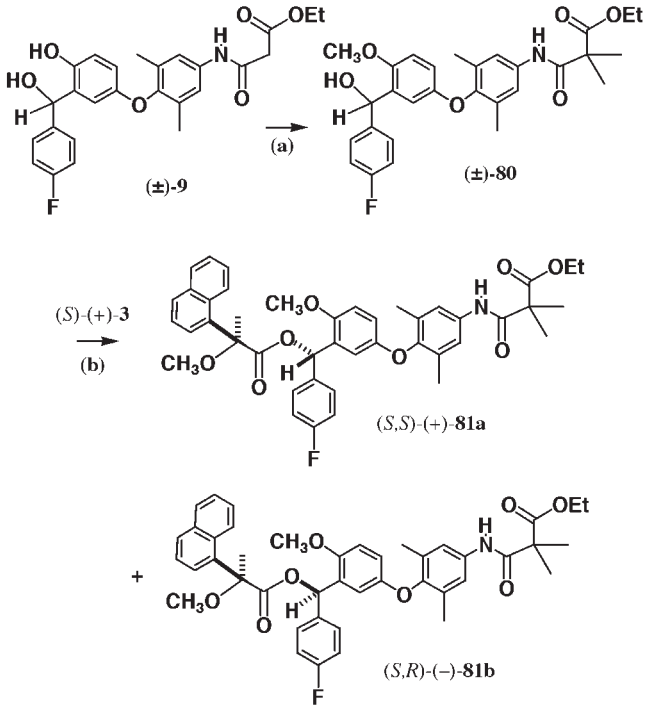


Fig. 9.25 (a) CH_3I, Cs_2CO_3/CH_3CN: (±)-**80**, 75%.
(b) DCC, DMAP, CSA/CH_2Cl_2, r.t.: (+)-**81a**, 45%; (–)-**81b**, 50% [52].

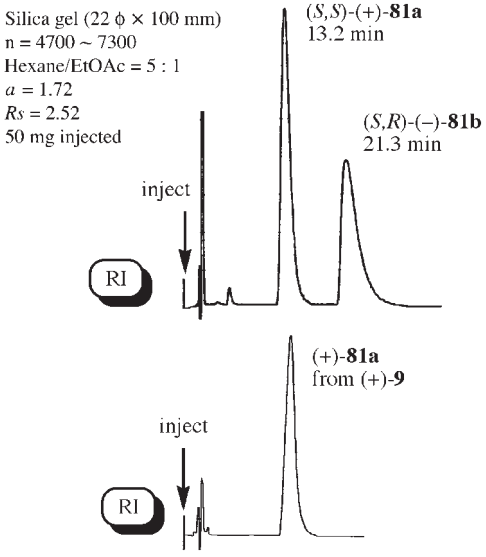


Fig. 9.26 HPLC separation of diastereomeric esters (*S*,*S*)-(+)-**81a** and (*S*,*R*)-(–)-**81b** [52].

(Fig. 9.26). In general, the MαNP esters of less polar aliphatic alcohols, such as 2-hexadecanol, are more effectively separated by HPLC on silica gel than the MαNP esters of polar alcohols, as discussed above. Therefore it was very surprising to us to find that the diastereomeric mixture of MαNP esters **81a**/**81b** composed of polar groups was clearly separated by HPLC on silica gel (hexane/EtOAc = 5 : 1) as shown in Fig. 9.26: separation factor α = 1.72; resolution factor *R*s = 2.52. It should be emphasized that a mixture (50 mg) of these two esters was separable even with a HPLC silica gel column of 10 cm length. The first-eluted ester (+)-**81a** (45%, $[\alpha]_D^{27}$ +23.0 (*c* 1.215, $CHCl_3$)) and the second one (–)-**81b** (50%, $[\alpha]_D^{27}$ –77.3 (*c* 1.255, $CHCl_3$)) were obtained.

To determine the absolute configuration of the first-eluted ester (+)-**81a** by the ^{1}H NMR anisotropy method, all NMR signals of both diastereomeric esters (+)-**81a** and (–)-**81b** were fully assigned by the ^{1}H, ^{1}H-^{1}H COSY, ^{13}C, HMQC, and HMBC methods (Fig. 9.27). The anisotropy value, $\Delta\delta = \delta(R,X) - \delta(S,X) = \delta$(second-eluted ester) – δ(first-eluted ester) = δ(**81b**) – δ(**81a**), was calculated for all protons as shown in Fig. 9.27. According to the sector rule, the 4-fluorophenyl group with negative $\Delta\delta$ values was placed on the left side, while the remaining group with positive $\Delta\delta$ values was placed on the right side. From the projection, the absolute configuration of the first-eluted ester was determined as *X* = *S*, leading to the absolute configurations (*S*,*S*)-(+)-**81a** and (*S*,*R*)-(–)-**81b**.

To assign the absolute configuration of chiral KAT-2003 (+)-**9**, the compound was treated in the same way giving trimethyl derivative (–)-**80** (82%, $[\alpha]_D^{25}$ –3.2 (*c* 1.825, $CHCl_3$)) (Fig. 9.28), the spectroscopic data of which agreed with those of racemate (±)-**80** except for optical rotation (Fig. 9.26). Alcohol (–)-**80** was esterified with (*S*)-(+)-MαNP acid **3** to give ester (+)-**81a** which was completely identical with

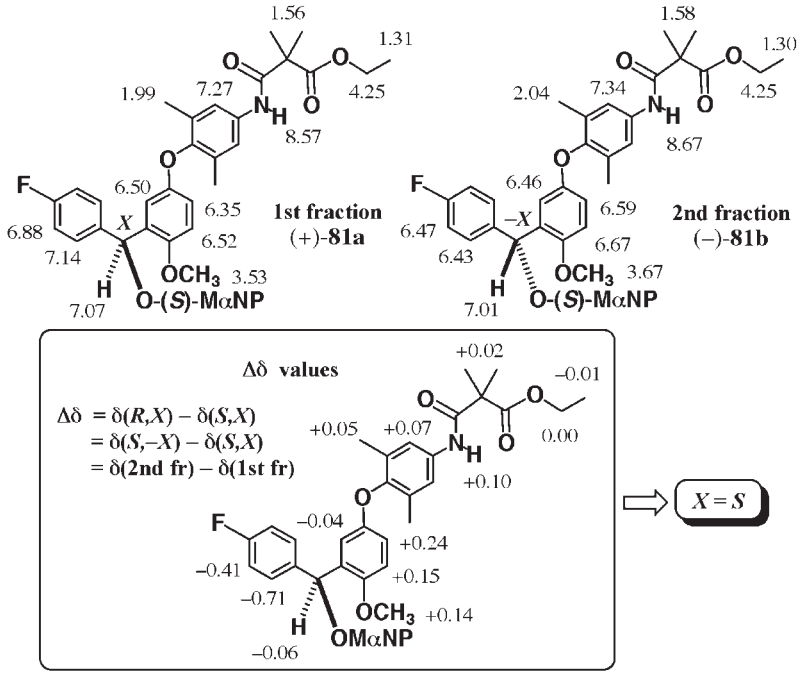


Fig. 9.27 The ^{1}H NMR chemical shift data (600 MHz, $CDCl_3$) of esters (+)-**81a** and (−)-**81b** and the absolute configuration of the first-eluted ester (+)-**81a** as determined by the ^{1}H NMR anisotropy method using (*S*)-MαNP acid [52].

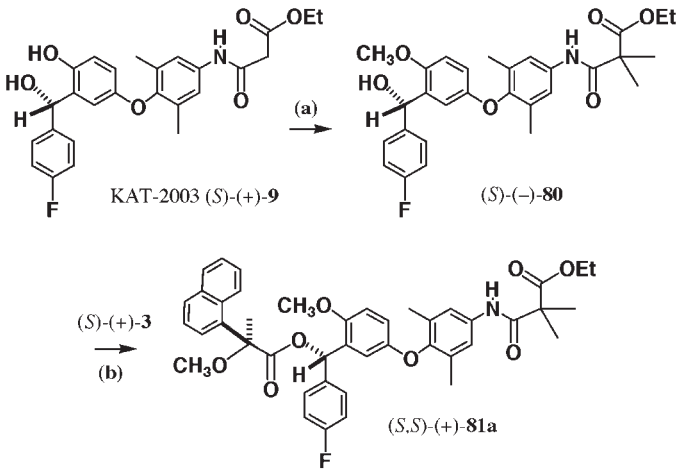


Fig. 9.28 (a) CH_3I, Cs_2CO_3/CH_3CN: (−)-**80**, 82%. (b) DCC, DMAP, CSA/CH_2Cl_2, r.t.: (+)-**81a**, 87% [52].

the first-eluted ester (*S*,*S*)-(+)-**81a** obtained by HPLC in Fig. 9.26. Therefore the absolute configuration of KAT-2003 (+)-**9** was established as *S*.

As described above, the absolute configuration of the chiral thyroid hormone analog KAT-2003 (+)-**9** was unambiguously determined as *S* by the ^{1}H NMR anisotropy method using novel MαNP acid (*S*)-(+)-**3**. This result indicates that MαNP method is very powerful for determining the absolute configurations of chiral synthetic drugs.

9.6 Conclusion

We have developed chiral molecular tools and *successfully applied these CDAs (chiral derivatizing agents) to the preparation of enantiopure alcohols by HPLC separation, and simultaneous determination of their absolute configurations by X-ray crystallography and/or by the ^{1}H NMR anisotropy method*. The X-ray crystallographic method using an internal reference is, of course, the best for determining absolute configuration. However, ideal single crystals are not always obtainable. In such a case, the ^{1}H NMR method using MαNP acid, which requires no crystallization, is effective. In enantioresolution, chiral CSDP acid and MαNP acid are thus useful as complementary molecular tools. If the resolution with one CDA is unsuccessful, the use of the other is suggested. The methods described above are very powerful for the preparation of enantiomeric alcohols with 100% enantiopurity and also for simultaneous determination of their absolute configurations. Further studies of these CDAs are in progress [56–58].

Acknowledgements

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10
Keywords in Chirality Modeling: Molecular Modeling of Chirality – Software and Literature Research on Chirality in Modeling, Chirality in Docking, Chiral Ligand–Receptor Interaction and Symmetry

Gerd Folkers, Mine Yarim, and Pavel Pospisil

10.1
Introduction

A substantial step towards the understanding of the physical, chemical, or biological properties of a molecule is to study and analyze its spatial shape. Besides the constitution, a major shape-determining feature is the configuration of a molecule, i. e. the stereochemistry. Furthermore, molecular chirality plays a major role in many areas of chemistry. Enantiomers often exhibit quite different physical, chemical, and biological properties. The exploration of the configurational space of a molecule and the analysis of the various isomers a molecule can adopt is therefore of great importance.

This protocol presents the application of modern computer-aided modeling tools dealing with chirality – stereo drawing, chiral centers annotation, chiral ligand docking and analysis of complexes with the receptor, chiral descriptors in QSAR, simulation of protein–DNA or DNA–DNA complexes with respect to their chirality, chiral catalysis.

At the end of the chapter are presented popular chemical softwares and their capabilities to "calculate" with the chirality. Chemical 2D structural editors – ChemDraw (CambridgeSoft), ACD/Labs ChemSketch (ACD), ISISDraw (MDL) – permit the stereo centers and E/Z bonds registration and therefore the subsequent transfer to the correct 3D form. Chirality drawing has to be allowed in order to determine the correct chirality. Similarly, large software packages allowing expert molecular modeling (Sybyl Tripos, Accelrys, Schrödinger, MOE...) also permit basic chirality recognition and annotation. Finally, more complex software allows the chiral induction, protein–ligand recognition, biological activity, 3D-QSAR chiral descriptors to be studied.

One has to recognize that tremendous progress in stereochemistry and chirality viewing brought "ray tracing", which can be explained as a rendering of the 3D object by rays of light. Light direction and path is calculated for every point at the screen of our computer thus enabling three-dimensional realistic visualization of the molecule. Such representation does not need any schematic annotation of the stereocenter.

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10.2 Chirality in QSAR

Because of the stereospecifity of biological effects, QSAR (quantitative structure–activity relationships) methods must be capable of taking into account atomic chiralities. Indeed, one of the most popular 3D-QSAR methods, CoMFA and other CoMFA-like methods take into account chirality by default, since the molecular fields of chiral isomers are different. If compounds are highly flexible and no experimental structural information about the receptor–ligand complexes is available, CoMFA (and CoMFA-like) methods are not always applicable. Several shortcomings and problems have motivated researchers to consider improvements to these techniques. The first idea for improvement was to modify the conventional 2D descriptors to make them chirality-sensitive [1].

Chiral information is related to symmetry, it must be supplied in addition to geometric data. It is conceivable that a pre-geometric molecular paradigm such as a topological model could incorporate chiral information [1, 2].

The first attempt to consider chirality descriptors in QSAR studies was made by de Julián-Ortiz et al., when several chirality-sensitive molecular and charge topological indices were introduced [3]. Both chirality descriptors and conventional MolconnZ [4] descriptors have been implemented in QSAR studies of a series of D_2 dopamine and σ receptor ligands. It was shown that the resulting QSAR models had better statistics and predictive power for the IC_{50} values of the ligands than those obtained with conventional descriptors alone. In addition, using these chirality descriptors, a set of chiral barbiturates were correctly classified as sedatives or stimulants [3].

Several series of novel chirality descriptors of chemical organic molecules were introduced by Golbraikh et al. [5, 6]. These descriptors have been implemented in a QSAR study with a high content of chiral and enantiomeric compounds. It was shown that for all data sets 2D-QSAR models that use a combination of chirality descriptors with conventional topological descriptors afford better or similar predictive ability when compared to models generated with 3D-QSAR approaches. 2D-QSAR methods enhanced by chirality descriptors present a powerful alternative to popular 3D-QSAR approaches.

Chirality molecular topological descriptors (CMTD) defined in Refs. [5, 6] included modified overall Zagreb indices, molecular connectivity indices, extended connectivity indices and overall connectivity indices. All of the indices make use of the so-called chirality correction, which can be a real or imaginary number added to or subtracted from vertex degrees of a hydrogen-depleted molecular graph corresponding to atoms in *R*- and *S*-configurations, respectively. For example, the conventional index ${}^1\chi$ is defined as ${}^1\chi = \sum_{\text{All edges } ij} (a_i a_j)^{-0.5}$, where a_i and a_j are the vertex degrees of adjacent atoms i and j. The chirality index ${}^1\chi$ is defined as ${}^1\chi = \sum_{\text{All edges } ij} (a_i \pm c_i)^{-0.5}(a_j \pm c_j)^{-0.5}$, where c_i is the chirality correction for atom i. The plus sign is used, if the atom is in the *R*-configuration, and the minus sign is used, if the atom is in the *S*-configuration. For achiral atoms, the chirality correction is zero [5, 6].

Keinan et al. found that the quantitative degree of chirality of substrates correlates with their efficiency of reaction with active sites [7]. The degree of chirality, a global shape descriptor, was determined by the use of the continuous chirality measure (CCM) methodology developed previously, which treats chirality as a continuous structural property and not as a binary quality (chiral/not chiral) [8]. The generality of this new type of shape–activity correlation is demonstrated for five receptor/substrate systems: trypsin/arylammonium inhibitors; the D2-dopamine receptor/dopamine derivative agonists; trypsin/organophosphate inhibitors; acetylcholinesterase/organophosphates; and butyrylcholinesterase/organophosphates. The correlations were obtained both for active-site induced chiral conformers and for inherently chiral inhibitors. Interestingly, for some of these cases the correlation of activity with structure is hidden when classical parameters, such as chain length, are taken, but is revealed with this shape descriptor. For two cases they show that the CCM approach is capable of corroborating the assignment of the pharmacophore moiety. Keinan et al. define and make a distinction between the quantitative enantioselectivity ratio, which is the ratio of the slopes of the correlation lines for two enantiomeric series and which serves as a measure of enantioselectivity; and the quantitative chirality-sensitivity ratio, which compares the sensitivity to chirality changes of different enzymes towards the same set of inhibitors. The findings of this study are quite nontrivial because symmetry and chirality are global shape parameters and not specific descriptors of the intricate geometry of the drug or of the active site. Keinan et al. propose tentatively that these results may indicate two different recognition mechanisms: shape recognition and chemical recognition: The first is the classical exact key-locking and the second is, interestingly!, the low resolution determination of the overall shape of the substrate [8].

10.3 Molecular Modeling in Chiral Chromatography

Modeling the interactions between an analyte and a chiral stationary phase (CSP) can be achieved by combining several computational tools: from quantum chemical to molecular mechanics calculations of analyte–receptor interactions to Monte Carlo (MC) and classical molecular dynamic simulations (MDS) [9, 10]. An example of the computation of an analyte–CSP interaction can be found in the studies of Lipkowitz et al. who investigated the chiral recognition modes of Pirkle-type CSPs [9, 11] or of Yashima et al. who calculated the interaction energies between chiral selector and enantiomers to gain an insight into the chiral recognition mechanism of the chiral selector [12]. Attempts to determine possible interaction modes between analytes and modeled strands of cellulose-based CSPs were also undertaken by Azzolina et al. [13] and Camilleri et al. [14].

The computational simulation of the interaction between analytes in a reversed phase liquid chromatography (RP-LC) system by means of molecular mechanics and dynamics simulations was reported by Lukulay and McGuffin [15]. As in

RP-LC, multivariate analysis methods such as principle component analysis (PCA) and nonlinear mapping (NML), or comparative molecular field analysis (CoMFA) approaches and linear free energy-related (LFER) equations have been used to derive structure–retention relationships in chiral chromatography [16–18].

Chiral discrimination has been a subject of great interest in the development, use and action of pharmaceutical agents. Kim et al. investigated by molecular modeling the chiral discrimination by β-CD (cyclodextrin) through the differences in the interaction energies and configuration of inclusion complexes. The calculated results are in agreement with experimental observation in predicting the correct elution order in propranolol separation. Their results have shown that molecular modeling methods such as Monte Carlo docking and molecular dynamics simulations successfully explain the experimental result of the chiral recognition process of propranolol by β-CD [19].

The group of Lipkowitz et al. also performed computational studies of chiral catalysts using COMFA of an asymmetric Diels-Alder reaction with catalysts containing bisoxazoline or phospinooxazoline ligands that are known to induce asymmetry [20]. Approximately 70% of the variance in the observed enantiomeric excess can be attributed to the sterical field and the remainder of the variance to the electrostatic field.

10.4
Chirality of Protein Residues, Homology Modeling

The 3D models are generally evaluated by relying on geometrical preferences of the amino acid residues or atoms that are derived from known protein structures. Essential for interpreting 3D protein models is the estimation of their accuracy, both the overall accuracy and the accuracy in the individual regions of a model. The errors in models arise from two main sources, the failure of the conformational search to find the optimal conformation and the failure of the scoring function to identify the optimal conformation.

A basic requirement for a model is to have good stereochemistry. The most useful programs for evaluating stereochemistry are PROCHECK [21], PROCHECK-NMR [22], AQUA [22], SQUID [23], and WHATCHECK [24]. The features of a model that are checked by these programs include bond lengths, bond angles, peptide bond and sidechain ring planarities, chirality, mainchain and sidechain torsion angles, and clashes between nonbonded pairs of atoms. In addition to good stereochemistry, a model also has to have low energy according to a molecular mechanics force field, such as that of CHARMM22 [25].

10.5
Chiral Selective Binding, MDS Methods

10.5.1
DNA

Enantiomers of chiral metal complexes have attracted considerable attention as potential structural probes of DNA conformation. Norden and Tjerneld [26] first reported the preference of the Δ enantiomer of Tris(dipyridyl)Fe(II) for the right-handed B-form DNA. The Barton laboratory subsequently developed an elaborate series of chiral metal complexes, some of which were reported to recognize specific DNA conformational features [27–29]. A comprehensive review of the interaction of chiral metal complexes with DNA [30] indicated, however, that the structural selectivity of these agents is ambiguous in many cases.

Qu et al. [31] reported biophysical studies of the binding interactions of daunorubicin and its (–) enantiomer (WP900) with DNA. Binding of the enantiomeric pair to DNA is clearly chirally selective, and each of the enantiomers was found to act as an allosteric effector of DNA conformation. The results provide a striking example of chiral-selective recognition of DNA. (–) Daunorubicin (WP900) binds to DNA with unique structural selectivity and strongly prefers left-handed Z-DNA to the normal right handed B-form, in contrast to daunorubicin, for which the

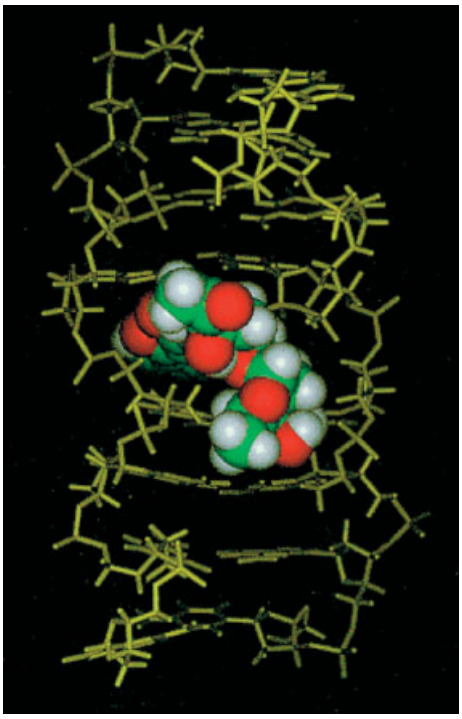


Fig. 10.1 Energy-minimized average model of the left-handed Z-DNA–WP900 intercalation complex formed with the $d(CGCGCGCG)_2$ duplex.

pattern of selectivity is reversed. Molecular dynamics simulations show that intercalation into left-handed Z-DNA represents a plausible mode of WP900 binding (Fig. 10.1).

10.5.2
Topoisomerase II-DNA Crossover Recognition

Timsit et al. reported that for forming symmetric ternary complexes between topoisomerase II and tight DNA crossovers, two geometric solutions were possible, depending on the chirality of the crosses. In the first, the two DNA segments are symmetrically recognized by the enzyme while each single double helix binds asymmetrically the protein dimer. In the second, each double helix is symmetrically recognized by the protein around its dyad axis, while the two DNA segments have their own binding modes. Each solution, which was consistent with different sets of experimental data from the literature, could correspond to different functions of the enzyme. This work provided structural insights for a better understanding of the role of chirality and symmetry in topoisomerase II-DNA crossover recognition, suggested testable experiments to further elucidate the structure of ternary complexes, and raised new questions about the relationships between the mechanism of strand passage and strand exchange catalyzed by the enzyme [32].

10.5.3
Chiral Catalysis

An interesting example of a computational tool in chirality is stereo-cartography. Lipkowitz et al. presented the stereo-cartography as a computational mapping that can predict the location of maximal stereo induction around the chiral catalyst [33]. A hypothesis concerns asymmetric induction by chiral catalysts (catalyst of the chemical reaction, e. g. during synthesis) stating that chiral catalysts (efficient at inducing asymmetry) will have their region of maximum stereo induction spatially congruent (matching) with the site of the chemistry but inefficient catalysts will not. Stereo-cartography is a simple mapping strategy used to assess where the region of maximum stereo induction is located around a given catalyst. This is calculated using a grid of points, with the chiral ligand being placed in this grid. At each grid point, a Boltzmann-weighted energy is determined between the probe (transition-state probe) and the catalyst for a large number of probe orientations. Both antipodes probes *R* and *S* are considered in the calculation. The intermolecular energy was calculated using a suitable force field with electrostatically fitted point charges. The authors used the software Spartan, force field AMBER and catalysts structures were taken from the Cambridge Structural Database. The key point about this mapping procedure is that it is based on energy calculations using well-known potential energy functions. The protocol compares interaction energies between mirror image probes at each point in space around the catalyst being considered. The probes are models of the actual transition states of the reac-

tion being catalyzed by a particular catalyst. The hypothesis was tested on three Diels-Alder reactions. 17 of the 18 catalysts conformed to the hypothesis.

10.5.4
Chiral Ligand–Receptor Interactions – Proteins

Several studies have been carried out in order to study chiral ligand recognition by the receptor target. The methods of modeling used are quantum-chemical and semi-empirical binding energy calculations, mainly taking into account the conformational restraints of the ligand and receptor residues, and the minimization of the complex. The following examples represent steric and energetic modeling of the chiral ligand–protein complexes, docking and the screening application.

Schmidt et al. [34] calculated $\delta G_{\text{binding}}$ (given as the difference of the free energies of solvation δG_{solv} of the complex partners (chiral ligand – protein)) in the bound and unbound states. They developed a microscopic model for the numerical calculation of free energies of solvation that could be used for the evaluation of differences in the free energies of binding $\delta\delta G$ binding of chiral complex partners. The establishment of a solvation energy surface density function led to the propagation of an interaction site point charge representation of solute atoms. Chirality aspects were studied. The solvent is modeled by means of integral equation theory which accounts for the structure and hydrogen bonding of the solvent phase.

Manual docking by Anzini et al. [35] of tifluadom-related structural derivatives correlates with the previously reported SAR of these derivatives [36]. Because of the previous results with *S* and *R* tifluadom, a different specificity of action of new enantiomer derivatives was explored. Derivatives were docked to the homology model of the κ-opioid receptor. The ligands in this study were docked manually into the minimized average structure of the receptor, the main criteria were the formation of charge-reinforced hydrogen bonds between the protonated nitrogen atom of the ligands with aspartate and between the carbonyl oxygen and the histidin. The binding energies of the minimized ligand–receptor complexes were calculated as the sum of the total interaction energy between the receptor and the ligand, the distortion energy of the ligand calculated with respect to the optimized energy of the free molecule, and the conformational energy change in the receptor induced by ligand binding. Distortion energies were obtained by minimizing the ligand and the receptor separately, resulting in the same geometry in each minimized complex. The binding studies showed that some derivatives bind to the opioid receptor subtypes as eutomers, in the same stereoselective manner. (A eutomer is an active isomer of a drug with the desired biological effect.) On the other hand the 7-chloro derivative binds the subtypes apparently without stereoselectivity.

Rotticci et al. [37] investigated the interaction between the substrates and the lipase of *Candida Antarctica*. Lipase-catalyzed asymmetric transformation is one of the best methods of preparing optically active compounds. The rational design of enenatioselectivity is possible if based on thorough models. They built such a

model based on two different binding modes for alcohol enantiomers in the active site of the enzyme. Unfavorable interactions were found between the halogen moiety of the fast-reacting *S*-enantiomers and a region situated at the bottom of the active site (stereoselectivity pocket). The binding site of the wild-type and mutant lipases was investigated with the methyl and organic bromine probes of the GRID program. Mutations of the crucial residues were refined with MDS. Single point mutations gave rise to one variant with doubled enantioselectivity as well as one variant with annihilated (removed) enantioselectivity towards the target halohydrins. An increase in the volume of the stereoselectivity pocket caused a decrease in enantioselectivity, while changes in electrostatic potential increased enantioselectivity. The modeled mutations suggested in enantioselectivity were well in agreement with the proposed model concerning the chiral recognition of alcohol enantiomers by this lipase.

Gallagher et al. [38] used a sequence-alignment model of the component of epoxygenase that catalyzes the stereoselective epoxygenation of alkenes to chiral epoxides. A model of the binding site, supported by chemical modification and fluorescence probing, revealed the hydrophobic oxygen/substrate active site. Molecular docking of substrates suggested that two alanin residues are responsible for the stereoselectivity of the enzyme, clamping the intermediate and/or product of the reaction and thereby controlling the configuration of the epoxide produced. If the two residues are glycines (as in methane monooxygenase), these residues do not provide sufficient steric hindrance to prevent rotation of the intermediate and, therefore, the product of the reaction catalyzed by this enzyme is achiral. From the simulated docking of both propene enantiomers, it appears that the methyl functional group is essential for the free rotation of the ligand in the active site. The selectivity may be determined by the specific orientation of either an intermediate in the reaction or the product before its release from the active site. Based on modeling studies and experimental studies it was concluded that the epoxygenase binds specifically to the *R*-enantiomer and is hence stereoselective.

Shin and Kim [39] used the accessible surface area of essential amino acid residues of the amine pyruvate aminotransferase and various amino donors and acceptors to explore the active site structure. Their results suggested a model consisting of two pockets, one large and the other small. The size difference between the binding pockets and the strong repulsion for a carboxylate in the small pocket were key determinants of the substrate specificity and stereoselectivity.

Ott et al. [40] docked manually, and refined with a subsequent molecular dynamics protocol, *R*- and *S*-enantiomers of antagonist derivatives to the metabotropic glutamate 1 receptor. To suggest a plausible binding mode for both enantiomers, they built a molecular mechanics model of the putative seven TM domain of hmGlu1 based on the α-carbon template of the TM helices of rhodopsin. A receptor docking hypothesis suggests that the OH of Thr815 comes in close contact with the oxime OH of the (–)-enantiomer, whereas no such close interactions could be demonstrated by docking of the (+) form.

Venketachalam et al. [41] in studies of the effect of stereo and regiochemistry on HIV-1 reverse transcripase modeled the potency of chiral halopyridyl and tiazolyl

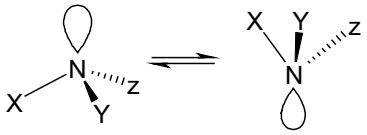
PETT derivatives. Modeling studies indicated that because of the asymmetric geometry of the non-nucleoside inhibitors of the reverse transcriptase (NNRTI) binding pocket, the *R*-stereoisomers would fit the binding pocket much better than the corresponding *S*-stereoisomers, as reflected by their 10^4-fold lower *K*i values. All the *R*-isomers showed potent anti-HIV activity and inhibited the replication of the resistant HIV-1 strains at nanomolar concentrations (compared to their less potent S-enantiomers). Docking and the structure–activity relationship among the derivatives showed preference for the pyridyl unit with halo-substitutions primarily at the 5-position, demonstrating the importance of both the stereochemistry as well as regioselectivity.

The importance of including *R*- and *S*-enantiomers in the chemical database designated for the *virtual screening* is illustrated by the discovery by Wang et al., using chiral docking, of an apoptosis inducer [42]. They screened almost 200 000 compounds from the MDL/ACD 3D database into the BakBH3 peptide binding pocket of the Bcl-2 protein, using DOCK 3.5. In the process, the interaction of ligands with the Bcl-2 surface pocket of each molecule in different orientations was scored by a shape complementarity scoring function. The docking orientations of the 1000 best scored molecules were optimized using their own SPL script in Sybyl 6.2 (Tripos) but with the addition of manual and individual examination of every ligand analysis. They created and analyzed enantiomers to ligands with chiral atoms and one of the nonpeptidic and chiral ligands of Bcl-2 was discovered to be active.

10.6 Docking of Chiral Compounds

Docking of chiral compounds does not differ basically from any standard automated or manual docking procedures; the 3D compound is docked flexibly into the rigid binding site. As the compound is in 3D, chirality included in the 3D structure is taken by default. However, docking algorithms with incremental construction of the ligand in the active site (FlexX) attach the fragments of the ligand *in situ*, based on the stereo information of chiral atoms. In FlexX, it is possible to decide upon the function *R* or *S* to investigate whether the ligand may undergo favorable binding in both isomers. If the other isomer fits the target site, such a hit differs from the original input in the database (that can be the opposite isomer or, in reality, the racemic mixture). This is a bit of a problem since modelers that use this feature find the hit with inverse chirality that may not be stored in stock or may have to be regioselectively synthesized.

FlexX also considers the pseudochirality of the pyramidal sp3 amine nitrogen (sybyl atom type N.3) inversion. If three of the groups attached to the N atom are different and the lone pair is considered as the fourth group then FlexX is able to invert this amine to the pseudoinverted position. This is not available in other docking programs and the possibility of inverting the sp3 N atom considerably increases the conformational space of the ligand.



The chemical databases used in docking are usually stereochemically defined within their 3D coordinates. Enantiomers are not stored. If it is necessary to invert the chiral centers for compound entry in the database, specific scripts using the function "invert" chiral centers should be applied. This enlarges the database significantly.

The potential compounds that become chiral during enzymatic catalysis remain problematic in docking. So-called "induced chirality" when binding to the active site can be modeled if the product is known. This is an argument for implementing the chirality generator to entries in the chemical database prior to docking. Achiral compounds that are docked can be found to be inactive, in contrast to their chiral analogs. For example, we can imagine an amine derivative that during the catalysis becomes protonated and, therefore chiral, and can bind tightly to the enzyme. Induced chirality may be classified as the problem of protonation/ionization and tautomerisation in virtual docking. Vice versa, there may also be cases where a chiral compound becomes achiral.

10.7 Molecular Modeling Software Dealing with Chirality and Some References to Its Successful Application

Basically, all computational chemical software, small and large software packages, deal with the basic stereochemical annotations of molecules. The basic capabilities of these programs are the identification of chiral centers (some even prochiral centers), determination of the stereochemical type or the chirality, chiral center inversion and storing of this information in a chemical database. More advanced features in chirality are: elucidation of the stereochemistry of complexed molecules, identification of wrong stereo drawings, generation of stereocenters for use in combinatorial libraries, registry and stereochemistry annotation in databases and use of that annotation for programs converting 2D structures to 3D.

Both the chirality of an atom and the cis–trans isomerism about a double bond can be determined by using a set of rules developed by and adopted by IUPAC [43]. These rules govern the sequencing of substituents about the chiral atom or double bond. Once the substituents are assigned a priority, simple geometric algorithms determine which type of isomerism is present. These same rules are used to determine the prochirality of an atom.

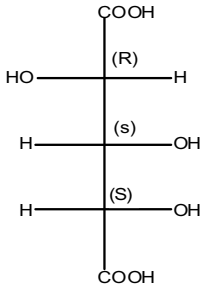
R–*S* isomerism of an atom is determined by positioning a three-dimensional representation of the atom so that the lowest group in the sequence is oriented away from the viewer. If the remaining substituents are arranged in a clockwise manner by priority, the center is designated *R*, otherwise it is *S*.

Z–*E* isomerism about a double bond is a more general designation than cis–trans and is determined by examining the bond's substituents and sequencing them in the manner prescribed by IUPAC. The special relationship between the higher priority substituent on each end of the double bond is examined relative to a reference plane including the two double bonded atoms and drawn perpendicular to the plane of the four substituents. If the two higher priority substituents lie on the same side of this reference plane, the isomerism is denoted as *Z*; if they lie on opposite sides, it is *E*.

Other, more specific annotations are available depending on the program (see below). A special case is the program Chirano which assigns chirality to oligonucleotidic chains.

10.7.1
ChemDraw 6.0 (CambridgeSoft) –an Example of the Classical Program

Supported stereochemical descriptors in computational software are, according to the Cahn–Ingold–Prelog, (CIP) system *E*/*Z* for double bonds and *R*/*S* for asymmetric centers. Pseudoasymmetric centers are also recognized as descriptors *r*/*s* (e. g. 2,3,4-trihydroxyglutaric acid, see Fischer projection below) in ChemDraw 6.0.



10.7.2
Chirano – Chirality of Nucleic Acid Chains

The program Chirano (*chira*lity analysis for oligonucleotides) reads the chirality from the PDB format of oligonucleotides (such as RNAs, DNAs). As the author Nikolai Ulyanov (UCSF) told us, Chirano checks that the stereochemistry of sugars corresponds to what is expected of beta-D-nucleosides or their 2′-deoxy derivatives, as defined by the iUPAC-IUB commission [44] and it complains if it does not. Chirano also checks the naming convention for the geminal protons; e. g., it will complain if H2′ (pro-S) and H2″ (pro-R) are switched in deoxyriboses. The author wrote this program when nothing of the sort was available to the computational community. Recently, there is available a nice validation tool with RCSB – AutoDep, which does a similar thing. (www.rcsb.org).

10.7.3
Corina – Chirality in 3D Structure Generator

Corina provides access to the configurational space of molecules. As mentioned above, Corina generates by default one single stereoisomer by taking into consideration the stereo information given in the input connection table and by making reasonable assumptions for missing stereo information. The driver option "–d stergen" forces Corina to automatically identify stereocenters and to generate all. Furthermore, the option "-d preserve" allows one to retain the configuration at atoms which have a defined stereochemistry (i. e., a stereo descriptor is given in the input file).

Corina as a leader in the generation of high-quality, low-energy 3D coordinates (conformations) to the 2D structures (drawn in various formats – SD/RD files, SMILES, MOL/MOL2 etc.) handles stereochemical information properly. This means that *R*- and *S*-chiral centers specified in 2D structures are kept. If such information is not available, Corina creates centers with lower conformational energy, or both enantiomers when requested. Stergen determines all stereocenters in a given input structure and generates the 3D structures of all possible, but unique, stereoisomers. Chiral tetrahedral centers as well as cis/trans double bonds are considered. Maximally 16 stereoisomeric compounds are generated, and a maximum number of 4 stereocenters are processed. The user can define which centers should be processed. For molecules with defined stereochemistry, a stereodescriptor is given in the input structure. Stereoisomers are named uniquely.

By default, Corina considers atom stereodescriptors and wedge symbols for chiral centers as well as bond descriptors indicating cis or trans double bonds. If the input option -i sdfict (SDF ignore cis/trans) is set Corina ignores all bond descriptors which define cis- or trans-configurated double bonds in order to convert also those structures with unreasonably defined descriptors, e. g., if a trans double bond is specified in a small ring system, or with ambiguous definitions, e. g., contradictory definitions in conjugated systems.

For pyramidal ring nitrogen atoms having one exocyclic substituent, Corina can produce conformations having both possible configurations at the nitrogen atoms (option -d rc,flapn). Corina would generate for 1,4-dimethyl-piperidine (SMILES: CN1CCC(C)CC1) (when no stereochemistry is specified), four chair conformations with all combinations of the two substituents in equatorial and axial positions.

10.7.4
Omicron from OpenEye Software – Chirality from 1D Formulas

This program respects chirality and cis–trans specifications when creating 3D from their "1D" SMILES representations.

10.7.5
Cache Software BioMedCache – Chirality Convention in Semiempirical Calculations

See the work published in Ref [45].

In BioMedCache, as it is the expert software with various MOPAC calculation methods, the MOPAC convention for chirality can be mentioned. So-called torsion or dihedral angle coherency exists because MOPAC calculations do not distinguish between enantiomers, consequently the sign of the dihedrals can be multiplied by –1 and the calculations will be unaffected. However, if chirality is important, a user should be aware of the sign convention used. The dihedral angle convention used in MOPAC is that defined by Klyne and Prelog. In this convention, four atoms, AXYB, with a dihedral angle of 90°, will have atom B rotated by 90° clockwise relative to A when X and Y are lined up in the direction of sight, X being nearer to the eye. In their words, "To distinguish between enantiomeric types the angle τ is considered as positive when it is measured clockwise from the front substituent A to the rear substituent B, and negative when it is measured anticlockwise." The alternative convention was used in programs which preceded MOPAC.

10.7.6
Accelrys

Accelrys contains several special types of chirality annotations; among those are annotations – no-chirality, – chirality *R* or *S*, – pseudo atoms PSEUDO_*R* and PSEUDO_*S*. Accelrys as an expert software package can offer multiple methods to model the chiral recognition, see Ref. [46].

The *S*-isomer interacts more strongly than the *R*-isomer because the orientation of the phenyl group attached to the chiral carbon allows closer contact with the host. Monte Carlo docking simulations would be useful for the prediction of the chiral recognition ability of β-cyclodextrins.

10.7.7
Schrödinger – Generation of Stereoisomers

LigPrep of Schrödinger can generate stereoisomers consistent with specified stereochemical information (e.g. parities in SD files) by varying the chiralities of the atoms for which chiralities are missing. Alternatively, the chiralities of all chiral atoms may be varied.

LigPrep is a robust package for preparing high-quality 3D molecular models of drug-like molecules from 2D or 3D structures. Useful variations on ionization state, stereochemistry and ring conformations can be requested, with the benefit of producing potentially multiple output structures for each input structure. A single command can be used to process thousands of input structures.

10.7.8
Tripos – Stereochemistry Module StereoPlex

As any basic package, Tripos determines, fix, annotates chirality *R* and *S*, also D and L of amino acids, inverts chirality on user demand, searches for stereoisomers. Tripos Sybyl also differentiates and annotates chiral atoms as in chiral ring fusion atoms, chiral bridgehead atoms, chiral ring atoms, chiral acyclic atoms, and chiral acyclic double bonds. In the docking program FlexX (BioSolvelT), during the docking procedure *R*/*S*, *E*/*Z* and N.3 pseudo-stereo nitrogen centers can change the chirality.

Tripos mainly contains StereoPlex™ that is designed specifically to address the need for additional stereomers in your 3D databases. StereoPlex functions as a 3D *database-extender* and can be used either by itself or as a CONCORD pre-processor. What is more, when compounds have more than a single stereocenter, StereoPlex actually increases the 3D shape diversity of the databases, thus becoming a *database-diversifier*. While diastereomers and enantiomers often "look" the same to distance-based pharmacophores or database queries, to biological receptors they are as different as night and day. StereoPlex has several features:

- Select which of the 2^n possible stereomers are to be generated
- Sterically and topologically impossible stereomers automatically eliminated
- Specify unique naming conventions for new stereomers
- Prioritize stereocenters such as acyclic double bonds, ring fusion atoms, ring bridgehead atoms, etc.

StereoPlex connects UNITY and CombiLibMaker often using CONCORD to rapidly generate high quality 3D coordinate sets for the compounds entered into the databases or combinatorial libraries. With StereoPlex new combinatorial libraries containing chiral structures can be validated, enhanced and expanded by using a combination of StereoPlex and CONCORD.

Tripos also contains ConGen that determines the relative configurations of a chiral molecule from NMR data. ConGen uses estimates of inter-proton distances from experimental NOE data as constraints during SYBYL molecular dynamics. The main feature is a period of dynamics at 8000 K, during which most chiral sites are frequently inverted. This allows the distance constraints to guide the molecule into a configuration consistent with the NOE data. Configurations returned by ConGen are tested for agreement with the experimental inter-proton coupling constants as well as with the NOE data. ConGen test ran successfully on organic molecules of known stereochemistry, with 5–17 chiral centers. The procedure is tolerant of large errors in the estimated inter-proton distances and is reasonably fast. ConGen is expected to work well for molecules that occur in solution in one main conformation. ConGen requires 2 or 3 NOE-derived distance constraints for each chiral center to be determined.

10.7.9 MOE – DAPPER

Wildman et al. have described improvements to their recent 3D-QSAR program DAPPER and presented further validation of the technique [48]. DAPPER was developed in the Molecular Operating Environment (MOE) [49] which is equipped to determine chirality using the Cahn–Ingold–Prelog (CIP) [50] chirality rules. MOE assigns a value of 1 or –1 for chiral molecules and zero for achiral molecules. DAPPER, was developed with this in mind and therefore can easily incorporate different conformation search techniques and chirality metrics. A new chirality measure provides a quantitative description of the overall chirality of a given conformation of a molecule without assigning values to individual atoms. In this article, they discuss the analysis of several implementations of various conformation search techniques, a novel chirality metric, and their incorporation into DAPPER with validation on three standard medicinal chemistry data sets (dihydrofolate reductase, angiotensin converting enzyme, acetylcholin-esterase). They have demonstrated clear predictive power in the case of 20 DHFR inhibitors [48].

Software Websites

ChemDraw: http://products.cambridgesoft.com

Chirano: http://picasso.ucsf.edu/~ulyanov/chirano.html

Corina: http://www2.chemie.uni-erlangen.de/software/corina/free_struct.html

Omicron (OpenEye): http://www.eyesopen.com/products/toolkits/omicron.html

BioMedCache (Cache Fujitsu): http://www.cachesoftware.com/biomedcache/

Accelrys: http://www.accelrys.com

Schrödinger: http://www.schrodinger.com

Tripos – StereoPlex: http://www.tripos.com/sciTech/inSilicoDisc/chemInfo/stereoplex.html

MOE: http://www.chemcomp.com

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Subject Index

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