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Review

Enzymes in the synthesis of bioactive compounds: the prodigious decades

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Abstract—The growing demand for enantiomerically pure pharmaceuticals has impelled research on enzymes as catalysts for asymmetric synthetic transformations. However, the use of enzymes for this purpose was rather limited until the discovery that enzymes can work in organic solvents. Since the advent of the PCR the number of available enzymes has been growing rapidly and the tailor-made biocatalysts are becoming a reality. Thus, it has been possible the use of enzymes for the synthesis of new innovative medicines such as carbohydrates and their incorporation to modern methods for drug development, such as combinatorial chemistry. Finally, the genomic research is allowing the manipulation of whole genomes opening the door to the combinatorial biosynthesis of compounds. In this review, our intention is to highlight the main landmarks that have led to transfer the chemical efficiency shown by the enzymes in the cell to the synthesis of bioactive molecules in the lab during the last 20 years.

Contents

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'...I can forsee a time in which physiological chemistry will not only make greater use of natural enzymes but will actually resort to creating synthetic ones'.
Emil Fischer, Nobel Lecture, 1902.

1. Introduction

The enzymes advantages and disadvantages as catalysts for organic synthesis, derived from their own nature. Enzymes catalyze reactions with remarkable rate accelerations over background (k_{cat}/k_{uncat} can reach 10^{17}). They are chemoselective and regio- and stereospecific; however, they usually present narrow substrate specificity limiting their application. Enzymes are environmental friendly catalysts since they work in aqueous solutions and at moderate temperatures, but their poor stability under in vitro reaction conditions can become a strong limitation to their use in chemical synthesis. Many enzymes work under similar conditions of pH, temperature, etc., allowing their combination to perform several synthetic steps in one-pot. In this manner, different problems related to the availability of substrates or product inhibitions may be overcome.

Besides the unique properties of enzymes as catalysts, synthetic chemists have been reluctant to employ them for a number of reasons:

(i) most organic compounds are water-insoluble, and the water removal is tedious and expensive;
(ii) limited availability of biocatalysts with the desired activity and substrate specificity;
(iii) poor stability of the enzymes, and
(iv) relatively high cost of the biocatalyst.

These drawbacks have been, at least partially, solved by the advances produced in the biocatalysis field over the last 20 years. Thus, the finding that most enzymes can work in organic solvents,[1] have heightened the use of enzymes in organic synthesis. Recombinant DNA technologies and specially the PCR,[2] have facilitated obtaining new enzymes by a variety of approaches. Protein engineering by rational design[3] or by in vitro evolution[4] permits the modification of the enzyme substrate specificity, stability and other catalytic properties. Using the diversity offered by the immunological system, it has been possible to create tailor-made enzyme active sites into antibodies by immunization with appropriate transition-state analogues.[5] The optimization of recombinant expression systems allows the application of efficient strategies for achieving high-level expression of both natural and engineered enzymes in different host cells, cutting down the cost of the biocatalyst[6] and expanding the array of reactions that can be afforded enzymatically. Finally, the use of immobilized enzymes present two main benefits (i) easy separation of enzyme from the reaction mixture; and (ii) the biocatalyst can be reused several times.[7]

Several excellent comprehensive reviews on enzymes in organic synthesis have been published in recent years.[8] In this review we will summarize the main landmarks that have led to the recognition of enzymes as very useful catalyst in organic synthesis and especially in pharmaceutical processes where chirality is a key factor in the efficacy of many drugs and working with multifunctional molecules is not unusual.

2. General aspects of biocatalysis

2.1. Enzymes in non-aqueous solvents

For a long time enzymes were believed to work efficiently only in aqueous solutions. Consequently, their utilization in organic synthesis was rather scarce since the low water solubility of many substrates represented a serious obstacle. This disadvantage, nevertheless, stimulated the search for systems based on the use of organic solvents in order to increase the solubility of hydrophobic substrates.[9] The methods adopted evolved from the mixtures of water and water-miscible organic solvents, biphasic aqueous-organic systems, reverse micellar systems, and finally in nearly anhydrous organic systems. Because the latter is attractive, it has undergone a rapid expansion among the synthetic chemists during the last two decades.[10]

In principle the replacement of water by an organic solvent should be adverse for the catalytic function of the enzyme, since water molecules are necessary to maintain the catalytically active conformation of the enzyme. However, the real question, as placed by Klibanov who did a leading research in the subject, should not be whether water is required but how much of it is necessary.[1,11] As long as the hydration shell required for retention of enzyme catalytic activity is preserved, the replacement of the rest of the water with an organic solvent should be possible without losing the active conformation. In general, the catalytic activity of enzymes in neat organic solvents is lower than in water. But this decrease in activity could be avoided and effective remedies are emerging.[12] For example, the enzyme activity is usually higher in hydrophobic solvents than in the more hydrophilic ones, since the latter can strip the essential water from the enzyme molecule.[13] The pH is one of the key factors for enzyme activity, but it has not meaning in organic solvents. Instead, in organic solvent media enzymes have 'pH memory'.[14] In practice this 'pH memory' can be achieved by dissolving the enzyme in water of optimal pH followed by freeze-drying or solvent precipitation prior to its use in an organic solvent. In this way the ionization state of the ionogenic groups of the enzyme are retained in the solid state and in organic solvent, which is also necessary for enzyme to function.

However, it has become important to know the effects of the surroundings of the enzyme molecules, in particular counter-ions and the structure of the solid catalyst particles, on the activity and selectivity of enzymes in low-water media.[15]

Ionic liquids are a new class of non-aqueous solvents with non-molecular, ionic character. These solvents are

salts that are liquid at room temperature. Through the choice of cations and anions, the physical and chemical properties can be optimized for each application. The replacement of organic solvents by ionic liquids can lead to remarkable improvements regarding reactivity, selectivity and stability of the enzyme.[16] For lipase catalyzed kinetic resolution of a racemate, it has been shown, that in most cases ionic liquids increase the enantioselectivity of the enzyme and improved conversion compared to organic media.[17]

2.2. Enzyme immobilization

The use of immobilized enzymes presents a number of technological advantages such as the possibility of reusing the biocatalyst and its easy separation from the reaction mixture. There exists a great variety of immobilization methods that can be grouped in several categories:[7,18] (i) immobilization by entrapment; (ii) non-covalent binding by adsorption on inert supports or by affinity immobilization; (iii) enzyme crystallization; (iv) covalent binding onto prefabricated carrier materials.

Sometimes enzyme immobilization is used as synonym of enzyme stabilization. However, not all the immobilization methods improved the stability of the enzyme.[19] Since enzyme immobilization means the restriction of the mobility of the enzyme, protein-protein interactions are greatly retarded which diminishes inactivation caused by aggregation, adsorption on the surface of reaction vessels, dissociation of oligomeric proteins into subunits, and bimolecular processes of proteolysis. For a true stabilization of the enzyme it is necessary stabilize the three-dimensional structure of the protein. This effect is only possible to achieve with those immobilization methods that increase the rigidity of the protein.[20]

2.3. From enzyme over-expression to tailor-made biocatalysts

2.3.1. The PCR revolution and the biocatalysis. Much of the early work on biocatalysis focused on the use of readily available enzymes, such as esterases, proteases and lipases. Although these enzymes are still now largely being used for the asymmetric synthesis of pharmaceuticals, since the advent of PCR and the high-throughput technologies, the advances in the recombinant expression systems, and the genomic research, the array of enzymes available for organic synthesis is greatly expanding. This has made possible to carry out enzymatic reactions that otherwise are difficult to perform by chemical methods, like the synthesis or modification of complex molecules such as carbohydrates, asymmetric carbon–carbon bond formation, kinetic resolution of racemic epoxides or dioxigenation of aromatic compounds.

The natural biodiversity is one of the main sources of new enzymes. Microorganisms living under extreme conditions of temperature, pH, salt concentration or pressure that are difficult or even impossible to be cultured, provide a large number of new enzymes. The new DNA techniques make possible to express enzymes from these organisms in others that can be cultured under controlled conditions such as *Escherichia coli*. Therefore, extremophilic microorganisms are providing biocatalysts able to work under conditions similar to those needed in organic synthesis.[21]

Another source of new enzymes facilitated by the advances in recombinant DNA technologies, is the redesign of pre-existing enzymes. Like the drug discovery process, the redesign of an enzyme can be afforded by *rational* or by *non rational* techniques. Rational redesign is usually restricted to those enzymes, whose structures and mechanisms are well known and understood. The improperly named non-rational methods are of general application since no previous information about the structure or mechanism of the enzyme is necessary. These methods try to reproduce in a lab-time scale the Darwinian scheme of natural evolution, consisting basically in: (i) random genetic mutation; (ii) gene recombination and (iii) selection for higher fitness variants.

Random and exhaustive mutagenesis can be achieved by several methods the most popular being the error-prone PCR.[22] Nature uses gene recombination to increase the variability produced by mutation. The first method for in vitro recombination of homologous genes was the DNA shuffling described by Stemmer (Fig. 1).[23] Other methods developed for in vitro recombination include incremental truncation,[24] staggered extension process[25] and random-priming recombination.[26] The last step for the in vitro enzyme evolution, and the real bottleneck of this approach, is the identification of the evolved gene with the desired characteristics. The difficulty to find the best mutant in a library of 10^4–10^7 mutants is evident. The identification can be accomplished by screening or by selection.[27] Genetic selection strategies can be applied when enzyme activity is essential for viability and growth. Screening requires to assay individually all the members of the library. Recent improvements in high-throughput screening allow to accomplish this task.[28]

In vitro enzyme evolution has been successfully used to modify enzyme features that are of interest in organic synthesis, such as substrate specificity,[29] optimum pH,[30] stereospecificity[31] or enantioselectivity;[32] this approach has also been applied to improve enzyme stability at high temperature[33] or in organic solvents.[34] A comprehensive list of evolved enzymes can be found in some recent reviews.[35]

2.3.2. Catalytic antibodies: tailor-made biocatalysts. Besides the great diversity of enzymes, there are many important organic transformations for which do not exist any biocatalyst. For this reason, an old longing of the chemist is to create tailored biocatalysts. Basically speaking, enzyme catalysis is based on the stabilization of the transition-state. Thus, if the essential chemical and structural details of enzymatic catalysis are known, one should be able to design and build novel enzymes from scratch. Against the generally accepted concept that proteins with high affinity binding of stable ligands

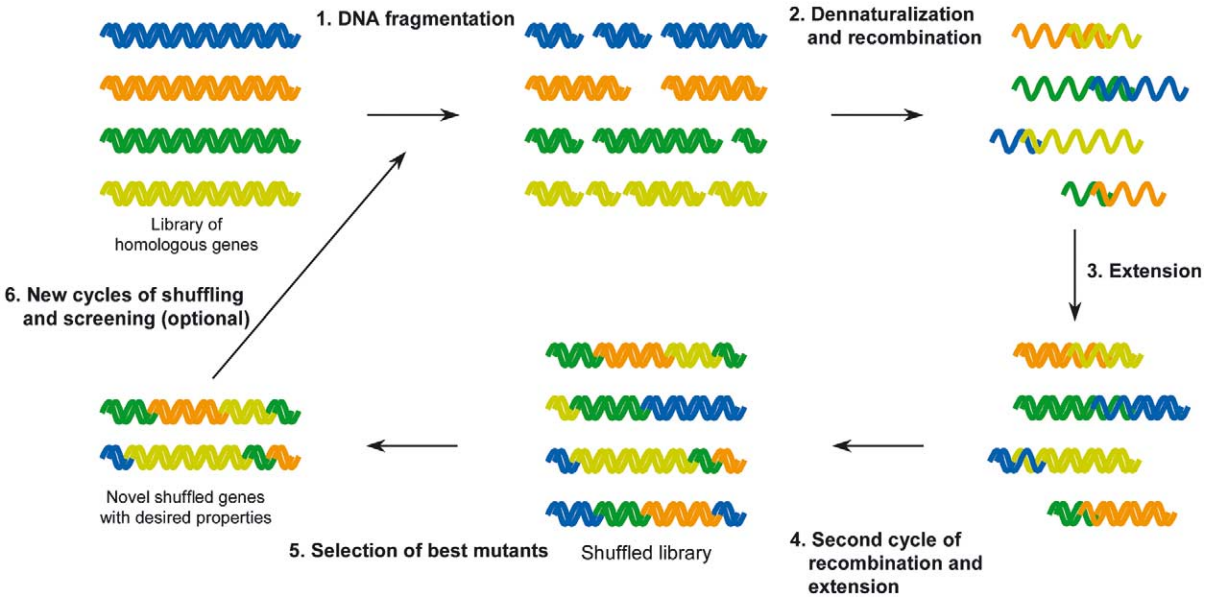

Figure 1. In vitro molecular evolution process by DNA shuffling. This method involves the random fragmentation of homologous DNA sequences. After purification of a pool of small fragments, these are reassembled into a full-length gene in a PCR-like reaction without primers. Homologous sequences from different fragments hybridize and prime each other. Recombination occurs when a fragment from one copy of a gene primes on another copy, causing a template switch. An additional PCR in presence of primers, typically yields a single PCR product of the correct size. Finally, the cloning of this PCR product leads to a combinatorial library of chimeric gene sequences produced by a variable number of crossovers.

do not express catalytic activity, Jencks suggested at the end of the 1960s, that stable molecules resembling the transition state of a reaction might be used as haptens to elicit antibodies with tailored catalytic activities and selectivities.[36] Since the first reports by the groups of Lerner[37] and Schultz[38] in the middle 1980s, more than 100 reactions have now been successfully accelerated using catalytic antibodies (abzymes), including pericyclic processes, group transfer reactions, additions and eliminations, oxidations and reductions, aldol condensations, and miscellaneous cofactor-dependent transformations.[39]

However, the catalytic efficiency of most catalytic antibodies generated to date is lower than that of their enzymatic counterparts. One major advance for the elicitation of antibodies with higher catalytic efficiencies has been the reactive immunization.[40] Usually, haptens are designed to mimic the geometric and electronic features of the reaction transition state (Fig. 2).[41] However, many enzymatic reactions proceed throughout a covalent complex between the enzyme and the substrate. In the reactive immunization, a reactive hapten is designed to promote a specific chemistry, such as the formation of a covalent bond, in the binding pocket of the antibody during its induction. In this way, the selection criterion is shifted from binding to catalysis. Using this approach, highly efficient catalytic antibodies with aldolase activity have been obtained (Fig. 3).[42] The substrate specificity of these antibodies is broader than that observed with any natural aldolase and they are able to catalyze a wide variety of intermolecular aldol reactions between ketone–ketone, ketone–aldehyde,

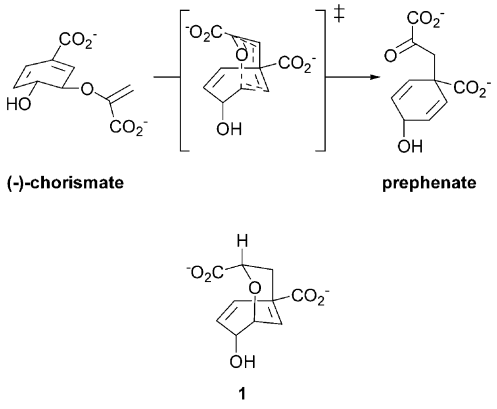

Figure 2. The Claisen rearrangement of (−)-chorismate to form prephenate. The conformationally restricted endo-oxabicyclic dicarboxylic acid **1** mimics the structure of the transition state and was used as the template for generating antibodies with chorismate mutase activity.[41]

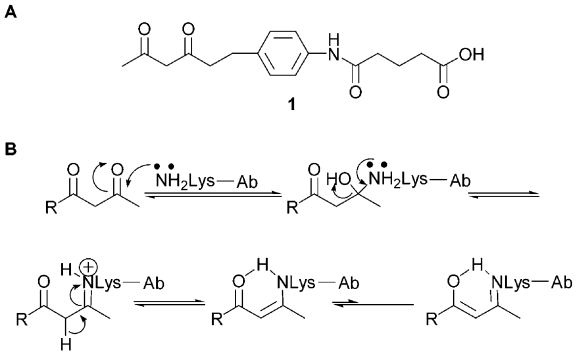

Figure 3. (A) The 1,3 diketone **1** was designed to elicit antibodies with aldolase activity. This hapten can both trap the requisite Lys residue in the antibody binding site (B) to then form the essential enamine intermediate and induce the appropiate binding sites for the two substrates. The aldehyde is represented by the 3-phenylpropiononyl portion of the hapten **1**.[42]

aldehyde–ketone and aldehyde–aldehyde, as well as several intramolecular aldol condensations.In order to find a highly efficient catalytic antibody, the screening of large libraries of antibodies is necessary. Several methods for screening antibody libraries for catalysis instead of binding have been developed. Catalytic enzyme-lined immunosorbent assay (catELISA)[43] is, perhaps, the most widely used. An alternative approach is the chemical selection, in which catalytic antibodies are selected using a mechanism-based screening reagent. This method was demonstrated to select antibodies with glycosidase activity.[44]

3. Enzymes in the synthesis of chiral drugs

Chirality plays a crucial role in nature and it is a key factor in the efficacy of many drugs.[45] During the last two decades, the synthesis of enantiomerically pure compounds has emerged as one of the most important fields of organic synthesis. For this purpose enzymes offer the possibility of performing highly stereoselective transformations under relatively mild reaction conditions avoiding the use of more extreme conditions that could cause problems with isomerization, racemization, epimerization, and rearrangement.

Stereoselective transformations of chiral building blocks in the synthesis of antihypertensive, anticholesterol, anti-Alzheimer, anti-inflammatory, and other pharmaceuticals have been previously reviewed.[46] For achieving these transformations different classes of enzymes have been exploited. The most often used ones are: (i) hydrolases (lipases, esterases proteases/amidases), for the synthesis of esters, acid, peptides/amides; (ii) dehydrogenases, for the oxidation of alcohols and reduction of carbonyls;[47] (iii) mono- and dioxygenases for hydroxylation of unactivated carbon;[48] (iv) aldolases for the stereoselective condensation of carbonyl compounds; and (v) oxynitrile lyase for synthesis of chiral hydroxy nitriles.[49]

Lipases have been the most popular biocatalysts in the synthesis of optically pure drugs. These enzymes are already commercially available and work efficiently in organic solvents. This feature makes possible not only to solubilize substrates which are not soluble in water, also the desired enantioselectivity can be optimized by varying the solvent using the criteria of polarity and hydrophobicity.[50]

However, when only one of the enantiomers is required, the enzymatic resolution of racemic substrates has the limitation that the maximum yield is 50%. There are several ways to overcome this problem: (i) the use of meso compounds or prochiral substrates; (ii) stereoconversion of the remaining enantiomer; and (iii) dynamic kinetic resolution. In the latter case, the substrate is continuously isomerized during the resolution process. Dynamic kinetic resolution will lead to higher enantiomeric ratios of the products, provided that the rate of equilibration of the substrate enantiomers is about the same as, or higher than, the rate of removal of

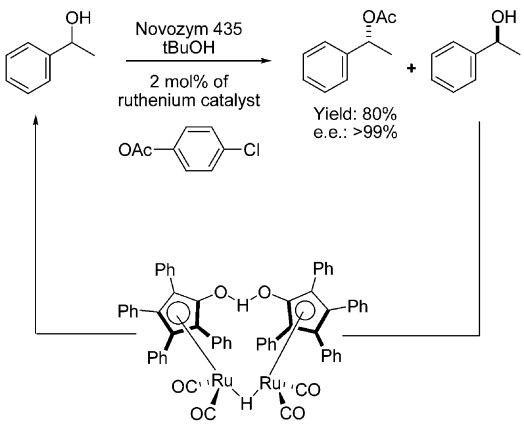

Figure 4. Dynamic kinetic resolution of 1-phenylethyl alcohol.[52]

one enantiomer from the system.[51] One can theoretically obtain 100% yield of one enantiomer. An illustrative example of this approach has been described by Bäckwall et al.[52] They showed that the isomerization of 1-phenylethyl alcohol (Fig. 4) by a ruthenium catalyst and enzymatic acylation with 4-chlorophenyl acetate as acyl donor resulted in transformation of the racemic alcohol to enantiomerically pure acetate. In this example the ruthenium catalyst promotes the racemization of the alcohol substrate. Racemization involves abstraction of the α-proton to give a ketone intermediate and subsequent readdition of hydrogens to the ketone. In most cases reported, the reaction proceeded with >99% ee and in good yield.

Another alternative to kinetic resolution is parallel kinetic resolution of racemic mixtures.[53] In this approach the slower reacting enantiomer is removed by a parallel reaction, ideally at an identical rate. Thus avoiding the decrease in the ee of the product at conversion values close to 50%, due to the continuous increase of the relative concentration (and, therefore, the relative rate of reaction) of the less reactive substrate enantiomer. For instance, Mischitz and Faber studied the asymmetric nucleophilic opening of (±)-3-methyl-2-pentyloxirane (*rac*-**1**, Fig. 5) catalyzed by a crude immobilized enzyme preparation from *Rhodococcus* sp. (NOVO SP 409).[54] The reaction in aqueous buffer gave the diol (*S*)-**2** in only 40% ee. However, when the enzymatic hydrolysis of (±)-**1** was carried out in the pre-

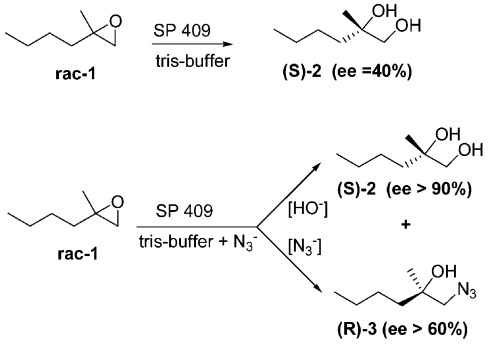

Figure 5. Simultaneous asymmetric hydrolysis and azidolysis of (±)-2-methyl-2-pentyloxirane.[54]

sence of the non-natural nucleophile azide, the (*S*)-diol, **2**, and (*R*)-azidoalcohol, **3**, were obtained in >90% and 60% ee respectively (Fig. 5). Therefore, a simultaneous and opposite enantio-discrimination of two nucleophiles (water and azide) was observed. This parallel kinetic resolution afforded the (*S*)-diol product in higher ee.

4. Enzymatic synthesis of carbohydrates: new tools for new drugs

Traditionally, carbohydrates were associated with structural functions and energy storage. Development of glycobiology and glycochemistry during the past two decades has revealed that carbohydrates are involved in a broad range of biological functions, mainly related to cell recognition events.[55] Many of these events occur at the very early stage of disease development and other signaling processes. Control of such recognition processes has become an important target for new drug development.

Carbohydrates have multiple hydroxyl functions of similar chemical reactivity; for example, five monosaccharides can be linked together to form linear or branched chains that can give rise to 32 million different compounds.[56] Therefore, a special knowledge of selective reactions and protecting groups are needed to achieve successfully their synthesis and modification. Because of that, it is in the field of carbohydrate chemistry where the enzymatic transformations has made the biggest impact during the last years.

4.1. Aldolase enzymes for C–C bond formation

Aldolases have attracted the interest of organic chemists because their ability to catalyze the formation of C–C bonds by an aldol addition reaction between an aldehyde and a ketone, with a high degree of stereochemical control. In general, they show a very strict specificity for the donor substrate (the ketone), but tolerate a broad range of acceptor substrates (the aldehyde).

Dihydroxyacetone phosphate (DHAP) dependent aldolases produce 2-keto-3,4-dihydroxy adducts and, with some exceptions,[57] they control the configuration of the newly formed stereogenic centers. An additional advantage of these enzymes is that they are stereocomplementary, that is, their use allows the synthesis of the four possible diaestereoisomers for a given pair of substrates (Fig. 6). DHAP-dependent aldolases have shown their utility in the synthesis of carbohydrate, carbohydrate-like structures or non-carbohydrate compounds. A comprehensive description can be found in several excellent reviews[58] and references therein.

Pyruvate or phosphoenol pyruvate (PEP) dependent aldolases yield 3-deoxy-2-keto acids. In vivo, pyruvate-dependent aldolases have a catabolic function meanwhile the PEP-dependent aldolases are involved in the biosynthesis of keto acids. These aldolases provided adducts that are not only densely but also differentially functionalized.[59] Thus, four different state of carbon

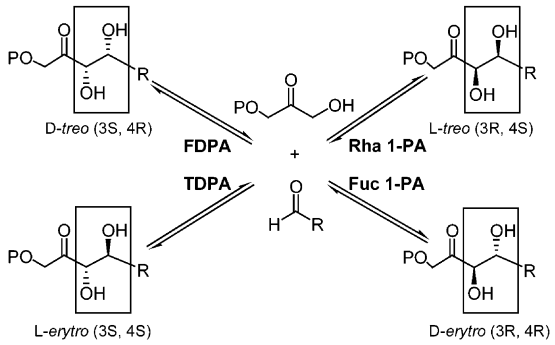

Figure 6. The four DHAP-dependent aldolases are stereocomplementary, allowing the synthesis of the four possible diaestereoisomers for a given pair of substrates.

oxidation can be found in four contiguous carbons. This substitution pattern allows transformation of aldol adducts to a variety of complex natural products (Fig. 7).

2-deoxyribose-5-phosphate aldolase (DERA) catalyzes the aldolic condensation between acetaldehyde and D-glyceraldehyde-3-phospate and determines the *S* configuration of the newly formed stereogenic center.[60] Beside acetaldehyde this enzyme is able to accept propanal, acetone and fluoroacetone as donor substrates.[60b] DERA is the only aldolase able to accept two aldehydes as substrates, allowing sequential bi- or trisubstrate aldol reactions.[61] The unnaturall pyranose obtained after DERA-catalyzed addition of acetaldehyde as donor are useful synthons for the synthesis of natural products as it has been illustrated by the concise total synthesis of epothilones A and C.[62]

Glycine dependent aldolases produce β-hydroxy-α-amino acids that are medicines by themselves or can be used as chiral building blocks for the production of drugs.[63] Their use in synthesis is hampered because the poor stereoselectivity that often leads to mixtures of *erythro* and *threo* compounds.[63] Despite of this limitations, L-threonine aldolase and D-threonine aldolase have been used for the synthesis of several β-hydroxy-α-

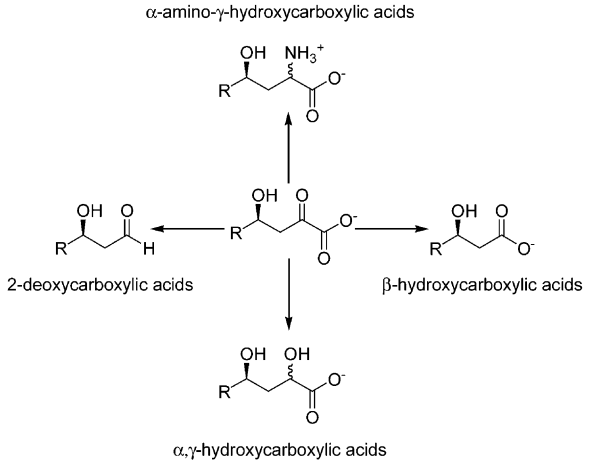

Figure 7. PEP-dependent aldolases provide adducts with four different state of carbon oxidation, allowing their transformation to a variety of complex natural products.[59]

amino acids on a preparative scale.[64] L-Threonine aldolase has been also used for the synthesis of potent sialyl Le^x mimetics[65] and the immunosuppressant mycestericin D.[66]

4.2. From glycosidases to glycosynthases

Glycosidases are readily available enzymes and use simple glycosyl donors, that can be even the free monosacharide. These hydrolytic enzymes are able to catalyze the formation of glycosidic bonds in a stereospecific manner.[67] However, when applied to the synthesis of di- and oligosaccharides the problem of the regioselectivity has to be faced. In contrast to lipase and protease that work in anhydrous organic solvents, glycosidases do not. Therefore, when using for synthesis of glycosides the hydrolysis is always a competitive reaction.

Several tricks have been applied to improve yield and to control the regioselectivity of glycosidase-catalyzed reactions:[68]

(ii) Use of very reactive glycosyl donors, in such a way that it is cleaved more rapidly than the product formed. Under these conditions, the reaction is kinetically controlled.
(ii) Introduction of subtituents at certain positions in the sugar acceptor. For instance, it has been shown that both the nature of the substituent and the anomeric configuration influence the regioselectivity and the yield of the glycosidation.[69]
(iii) Manipulation of the medium, using organic cosolvents to reduce the total volume of water. Also, addition of salts at high concentration has been used in order to reduce the water activity. The use of lipid-coated glycosidases improves the behavior of the enzyme in media with low water activity.[70]

An alternative approach is the use of specifically mutated glycosidase (named glycosynthase), which can efficiently synthesize oligosaccharides, but does not hydrolyze them.[71] For the case of glycosynthases, an active carboxylate nucleophile in the catalytic center of the enzyme is replaced with a nonnucleophilic amino acid side chain, resulting in an enzyme which is catalytically inactive since it cannot form the glycosyl-enzyme intermediate (Fig. 8). However, the mutant enzyme can transfer an activated glycosyl derivative, bound at the active site in the place of the normal glycosyl-enzyme intermediate, to a suitable acceptor bound in the aglycon pocket. Glycosyl-fluorides[72] are appropriate activated glycosyl donors since they are readily synthesized and have a small leaving group. As the disaccharide products are not hydrolyzed this method provide a high-yielding synthetic procedure of oligosaccharides. A similar approach has been used to obtain glycosynthases from endo-glucanases.[73]

4.3. Glycosyltransferases: one enzyme-one linkage

Glycosyltransferases in vivo are responsible for the final 'decoration' of proteins and lipids and contributes to the great variety and complexity of secondary metabolites in plants, bacteria and other organisms. Their strict control over the stereo- and regioselectivity of the newly formed glycosidic bond, have led to the 'one enzyme-one linkage' concept.[74]

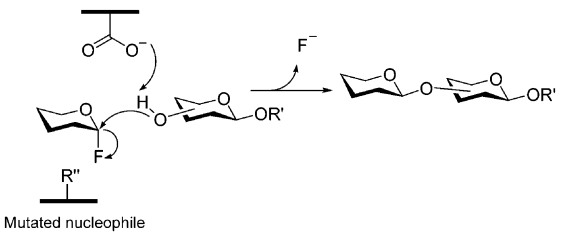

Figure 8. Proposed mechanism of glycosynthases. The enzyme-derived nucleophilic carboxylate (R″) has been mutated to glycine, serine or alanine.[71]

The synthetic utility of glycosyltransferases has been demonstrated in the synthesis of numerous complex oligosaccharides and glycoconjugates.[75]

Enzymes have allowed affording the in vitro synthesis of homogeneus glycoproteins, a difficult task to obtain by in vivo methodologies since glycosylation is a post-transcriptional modification and it is affected by several environmental factors.[76] Several strategies have been developed in the last years for the enzymatic synthesis of glycoproteins (Fig. 9). The combined use of endoglycosidases, glycosyltransferases and proteases has been applied to the synthesis of a homogeneous RNase B glycoform.[77] The use of endoglycosidases A or M allows the exchange of sugar chains to obtain a protein with homogeneous N-linked glycans.[78] Finally, other approach makes use of a natural protein-splicing mechanism mediated by inteins.[79]

In spite of all these impressive examples, the use of glycosyltransferases in synthesis faces two majors drawbacks: (i) their limited availability and (ii) the need of a nucleotide activated sugar as donor.

(i) Historically, most glycosyltransferases studied have been from mammalian sources because these have been the focus of glycobiology and bio-medical studies. Interest is increasing in bacterial glycosyltransferases. Many bacterial glycosyltransferases are able to produce mammalian-like structures and, in addition, in microorganisms it is possible to find transferases with specificities not yet found in mammalian enzymes.[80] An additional advantage of bacterial glycosyltransferases is that they usually show broader substrate specificity than their mammal counterpart.[81] Work in our lab on recombinant α 1,6-fucosyltransferase from *Rhizobium* sp.[82] has shown that this enzyme is able to accommodate a variety of modifications on the acceptor substrate. Thus, it is able to accept as substrate, oligomers and monomer of GlcNAc and it does not seem to present selectivity for the anomeric configuration of the acceptor.

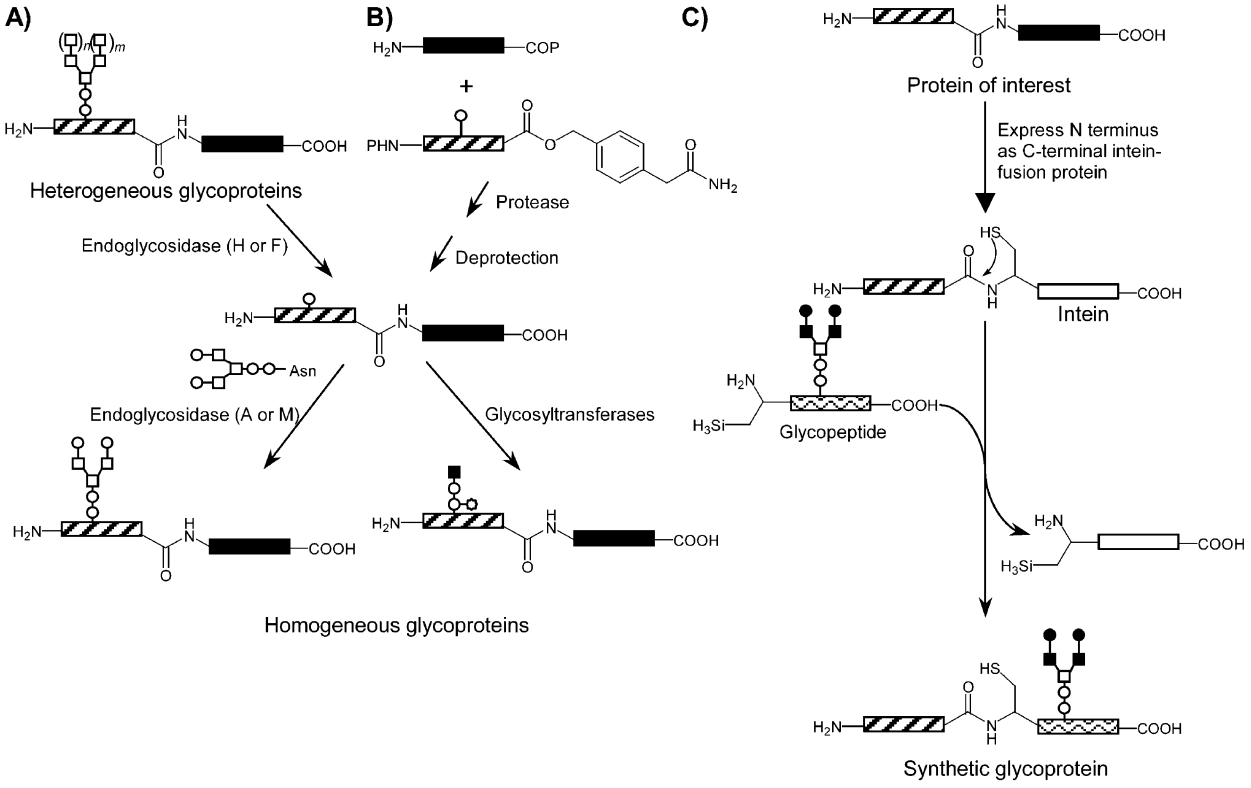

Figure 9. Three strategies for the chemo-enzymatic synthesis of homogeneous glycoproteins. (A) Use of endoglycosidases for the exchange of sugar chains;[78] (B) combined use of endoglycosidases, glycosyltransferases and proteases for the synthesis of a homogeneous glycoforms;[77] (C) glycoprotein synthesis mediated by inteins.[79]

(ii) The sugar-nucleotide used by glycosyltransferases as donor, is too expensive to be used in stoichiometric amount in medium or large-scale synthesis and, even more important, the nucleoside phosphate released during the reaction is a natural inhibitor of glycosyltransferases. This problem can be avoided by removing the nucleotide with alkaline phosphatase[83] or, in a more sofisticated way, by in situ regeneration of the glycosil donor. The ability of most of the enzymes to work under similar conditions of pH and temperature together with their high specificity for substrate, allows the combination of several enzymes, each with different catalytic activities, in the same reaction flask. These properties are the basis of the strategy developed by Whitesides et al. in 1982[84] for the synthesis of *N*-acetyllactosamine with in situ regeneration of UDP-Gal. Afterwards, other recycling systems for several sugar-nucleotide have been developed.[85] The use of bifuctional fused enzymes[86] and 'superbeads' containing the necessary enzymes for the regeneration of the sugar-nucleotide co-immobilized on Ni-agarose,[87] are recent improvements to the traditional scheme for in situ sugar-nucleotide regeneration cycle.

In an analogue way, multienzyme systems have been developed for regeneration of other cofactors. Because the interest of bioactive oligosaccharides sulfates, the methods for the recycling of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), are noteworthy.[88]

5. Enzymes enter the field of combinatorial chemistry

In the early 1990s, combinatorial chemistry revolutionized the drug discovery field changing the traditional rational drug design approach for the idea of covering a vast range of chemical diversity where a 'hit' could be found faster.[89] A more recent approach in combinatorial chemistry is to create smaller and focuses libraries. Thus, combinatorial chemistry is used to generate a high degree of structural and chemical diversity on initial lead compounds in order to optimized them for binding, target specificity, bioavailability, etc. Biocatalysts have been recently incorporated to the array of synthetic tools for combinatorial chemistry with special emphasis on focuses libraries. Biocatalysts can be employed in two different combinatorial schemes to generate libraries of compounds: (i) combinatorial biocatalysis, which assembles in vitro new compounds and derivatives using isolated enzymes and (ii) combinatorial biosynthesis, which shuffled or modified the genes of natural biosynthetic pathways to produce in vivo libraries of 'unnatural' natural products.

5.1. Enzymes in polymer-supported synthesis

Combinatorial chemistry often involves the synthesis on polymeric supports. The advantage of this synthetic methodology mainly stems from the ease work up of reactions, which allows the automatization of the process.

In polymer-supported synthesis the substrate is anchored to the polymer through a functional group,

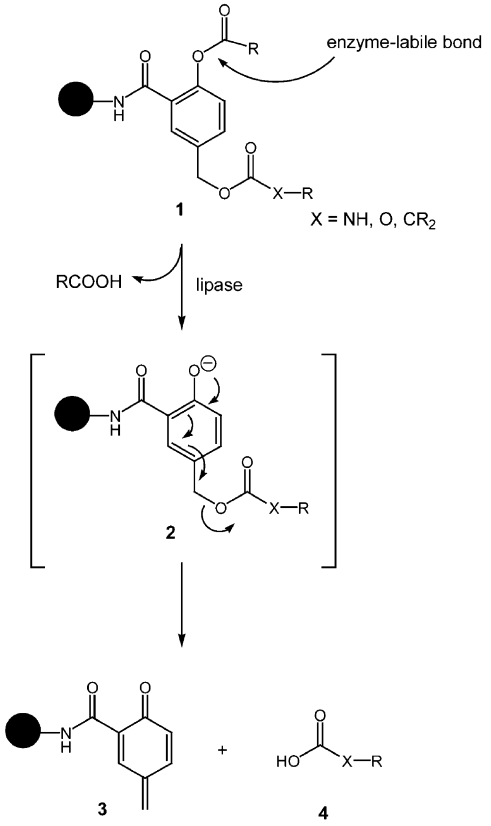

Figure 10. Principle for the development of the enzyme-labile 4-acyloxybenzyloxy linker group.[91]

the so-called linker, which must be stable during the synthesis and has to be cleavable at the end of the synthetic route with high selectivity. Under the mild conditions where enzyme works most of compounds are stable and therefore, the use of enzymes has open up alternative opportunities to release compounds from polymeric supports. Enzyme-labile linkers have been developed, involving the use of hydrolases such as protease, lipase or acylase.[90] For instance, Waldmann et al. described the *exo*-linker **1**, which comprises a 4-acyloxy-3-carboxy-benzyloxy group (Fig. 10).[91] The linker **1** is attached as an amide to the solid phase. It contains an acyl group, for example acetate, which can be cleaved by lipases or estereases. Cleavage of the acyl group by a lipase generated a phenolate **2**, which fragments to give a quinone methide **3** and releases the desired product **4**. The quinone remains attached to the solid phase and is trapped by water or an additional nucleophile.

Enzymes can also be exploited as efficient and selective catalyst in solid-phase synthesis where conventional chemistry is laborious and difficult. Wong et al. reported the synthesis of sialyl Lewis^x glycopeptide on a solid support by the use of glycosyltransferases.[92] In addition, the target product was detached from the solid support through a protease-catalyzed hydrolysis. After this work, several syntheses of oligosacharides on solid support[93] or on soluble polymeric support[94] have been reported. Solid-phase and liquid-phase synthesis of glycopeptides, glycolipids, oligonucleotides and oligosaccharides have been reviewed by Zehavi.[95]

In a different approach using soluble monomethylether of polyethyleneglycol as support, glycosidases have been used for both glycosidation of a sugar acceptor and for removal of the unreacted monosaccharide acceptor.[96] As shown in Figure 11, the glucose anchored to the soluble support is galactosylated using β-galactosidase. Then, the unreacted monosaccharide glucose was removed by the combined use of α- and β-glucosidases to obtained only MPEG-bounded disaccharides. Finally, disaccharides were released from the polymer by cleavage of the linker.[96a]

Recently, Flitsch et al.[97] reported the first example of protease-catalyzed high-yielding peptide synthesis on

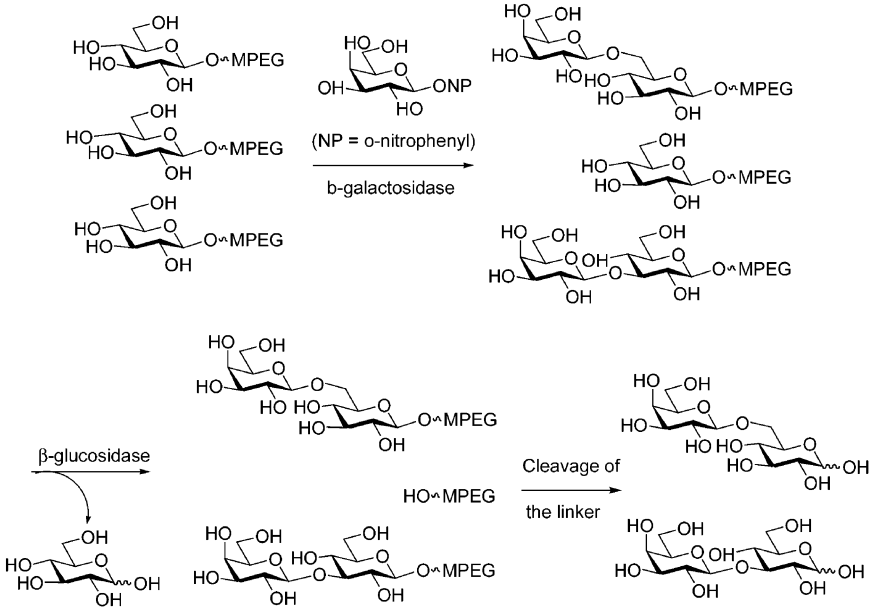

Figure 11. General strategy for the liquid-phase synthesis of disaccharides using glycosidases.[96a]

Table 1. Enzyme catalyzed reactions with application to combinatorial biocatalysis[98a]

Introduction of new functional groups	Modification of existing functional groups	Addition onto functional groups
Carbon–carbon bond formation	Oxidation of alcohols to aldehydes and ketones	Acylation
Hydroxylation	Reduction of aldehydes and ketones to alcohols	vynil esters
Hydrogenation	Oxidation of sulfides to sulfoxides	trihaloethyl esters
Halogenation	Oxidation of amino groups to nitro groups	vinyl carbonates
Peroxidation	Oxidation of thiols to thioaldehydes	vinyl carbamates
Epoxidation	Hydrolysis of nitriles to amides and carboxilic acids	oxime esters
Cycloadditions	Replacements of amino groups with hydroxyl groups	oxime carbonates
Halohydrin formation	Lactonization	bifunctional esters
Addition of amines	Isomerization	Glycosylation
	Epimerization	glycosides
	Dealkylation	aminoglycosides
	Methyl transfer	glycosilic acids
		Amidation
		amides
		peptides
		hydrazides
		Phosphorylation
		phosphates
		phospholipids

solid support in bulk aqueous buffer, with no need for organic cosolvent or activated carboxylic acid.

These and other many examples of solid and liquid-phase biocatalytic synthesis show that the biocatalytic synthetic machinery is ready to be used for the preparation of compound libraries.

5.2. Combinatorial biocatalysis

The creation of focused libraries is based on derivatization of existing molecules. This approach mimics the chemistry that occurs in biological systems where precursors are modified by the action of biocatalysts. For modification of existing lead compounds, the special properties of enzyme as catalysts report some advantages over synthetic chemical reactions:[98]

(i) the high chemo-selectivity of enzymatic reactions allows modifying only one kind of functional groups in a scaffold molecule that typically contains several different functional groups.
(ii) the enzyme regioselectivity provide the opportunity for specific combinatorial modification of lead molecules with multiple copies of the same functional group, for example, glycosylation catalyzed by glycosyltransferases with different regioselectivity.
(iii) the enzyme stereocontrol over the reaction permit a combinatorial approach to the three-dimensional structure of the compound, for example, using the four DHAP-dependent aldolases.[58b]

A myriad of biocatalytic reactions are available for combinatorial biocatalysis (Table 1). These reactions can be grouped in three mayor categories:[98a] (i) introduction of new functional groups; (ii) modification of existing functionalities; and (iii) addition onto functional groups.

An important issue regarding combinatorial bicatalysis is that of orthogonality.[99] Modification of a substrate by one enzyme, 'A', may prevent it from being substrate for another enzyme, 'B', while the modification of the initial substrate by 'B', may not preclude it from being a substrate for the first enzyme, 'A' (Fig. 12). The reactions are performed iteratively. A first generation of derivatives is modified by another round of biocatalytic reactions at additional reactive sites to produce a second generation of derivatives. After several iterations is possible to create a great number of derivatives from the original lead compound (Fig. 13).

Although combinatorial biocatalysis is an emerging technology in the field of drug discovery, published application have expanded at a growing rate. New advances including iterative derivatization of small molecules and complex natural products, regioselectively controlled libraries, novel one-pot library synthesis, etc., have been recently reviewed.[100]

5.3. Let the cell make the chemistry: from metabolic engineering to combinatorial biosynthesis

Fermentation processes is a traditional approach of the pharmaceutical industry for obtaining natural-bioactive products. The possibility to manipulate metabolic pathways at genetic level has opened the door to use the cell like a chemical factory for the production of new 'unnatural' natural products. The whole-cell approach

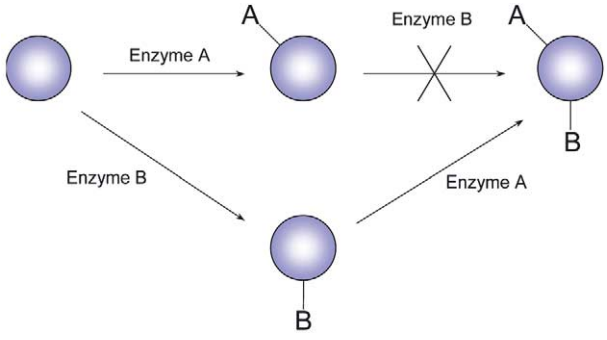

Figure 12. Orthogonality of biocatalysts.[99]

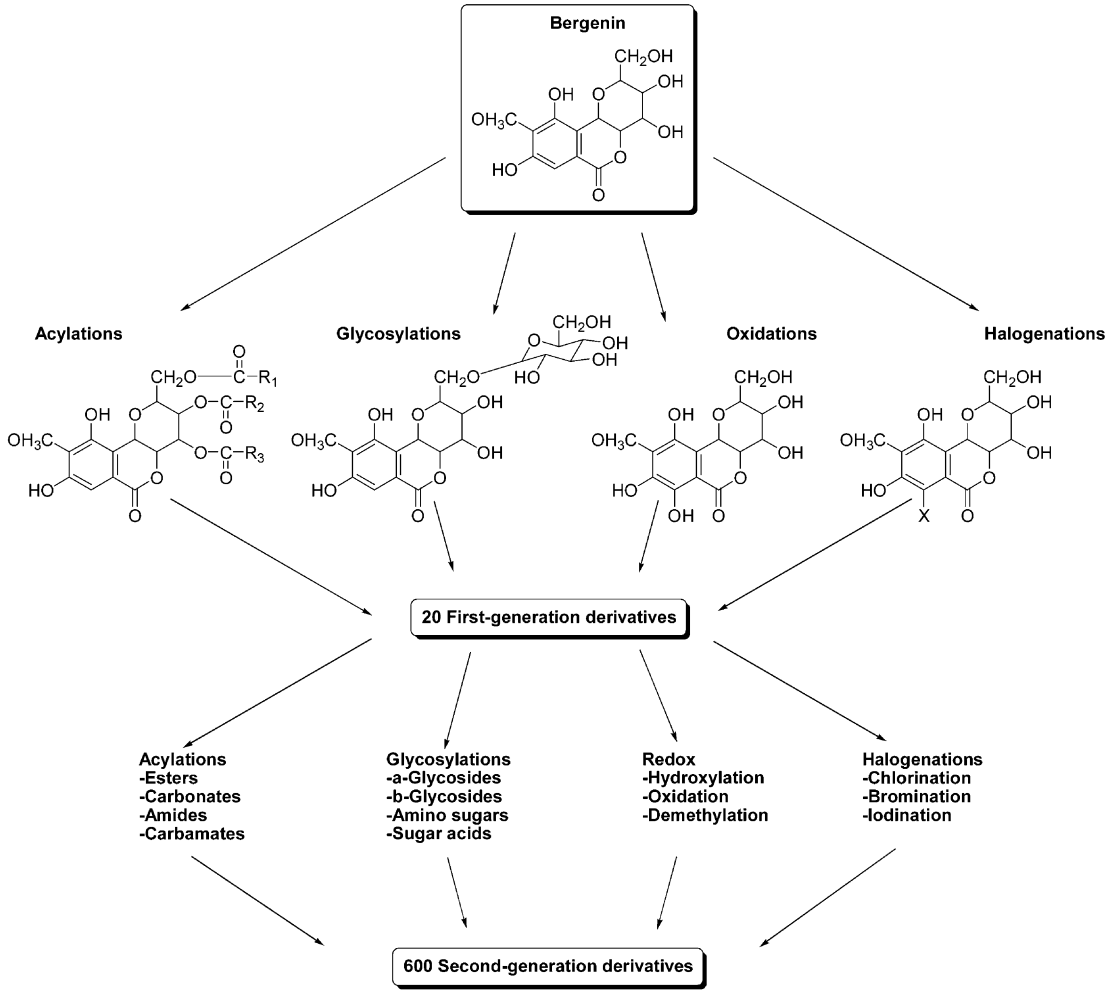

Figure 13. Iterative synthesis of a 600-member library from the flavonoid bergenin.[98b]

is especially appealing to obtain compounds produced by the consecutive action of several enzymes or when co-factor regeneration is required.

5.3.1. The cell factory: an alternative strategy for the synthesis of oligosaccharides. Recombinant whole-cells over-expressing glycosyltransferases, have been used as biocatalyst for the synthesis of oligosaccharides[101] in a similar way that is done with the isolated enzymes. That is, the sugar-nucleotide donor and the corresponding acceptor are incubated in the presence of the recombinant cells. The concept of cell factory implies the use of the cell metabolic machinery — natural or engineered — for the production of the desired compound from simple and inexpensive substrates.

Thus, *E. coli* cells transformed with a plasmid codifying for the enzymes to recycle UDP-Gal — sucrose synthase and UDP-galactose-4-epimerase[102] — and different galactosyltransferases have been used as a galactoside-producing factory.[103]

Endo et al. have developed an alternative approach for the large-scale production of sugar-nucleotides and oligosaccharides through coupling of engineered bacteria.[104] This system is based in the combined use of *Corynebacterium ammoniagenes*, a bacterium able to produce UTP from inexpensive orotic acid, with *E. coli* strains metabolically engineered with the different sugar-nucleotide biosynthetic genes. Therefore, *E. coli* cells expressing galactose-1-phosphate uridyltransferase, galactokinase, glucose-1-phosphate uridyltransferase and pyrophosphatase, coupled with *C. ammoniages* cells were able to accumulate 44 g/L of UDP-Gal in the culture supernatant after a 21 h reaction (Fig. 14A).[105] In a similar way, system for the production of other sugar-nucleotides like UDP-glucose, UDP-*N*-acetylglucosamine,[106] GDP-mannose, GDP-fucose[107] and CMP-neuraminic acid[108] have been developed. For this last system, two recombinant *E. coli* strains were used; one expressing the CTP synthase and the other expresses the CMP-NeuAc synthase (Fig. 14B).

When these sugar-nucleotide production systems are coupled with a new strain of *E. coli* over-expressing a glycosyltransferase, different oligosaccharides can be efficiently produced (Fig. 14).[105,107–109]

5.3.2. Combinatorial biosynthesis: creating new polyketides. Polyketides belongs to a very important family of compound for the pharmaceutical industry. Nowadays, more than 40 polyketide drugs are in the market

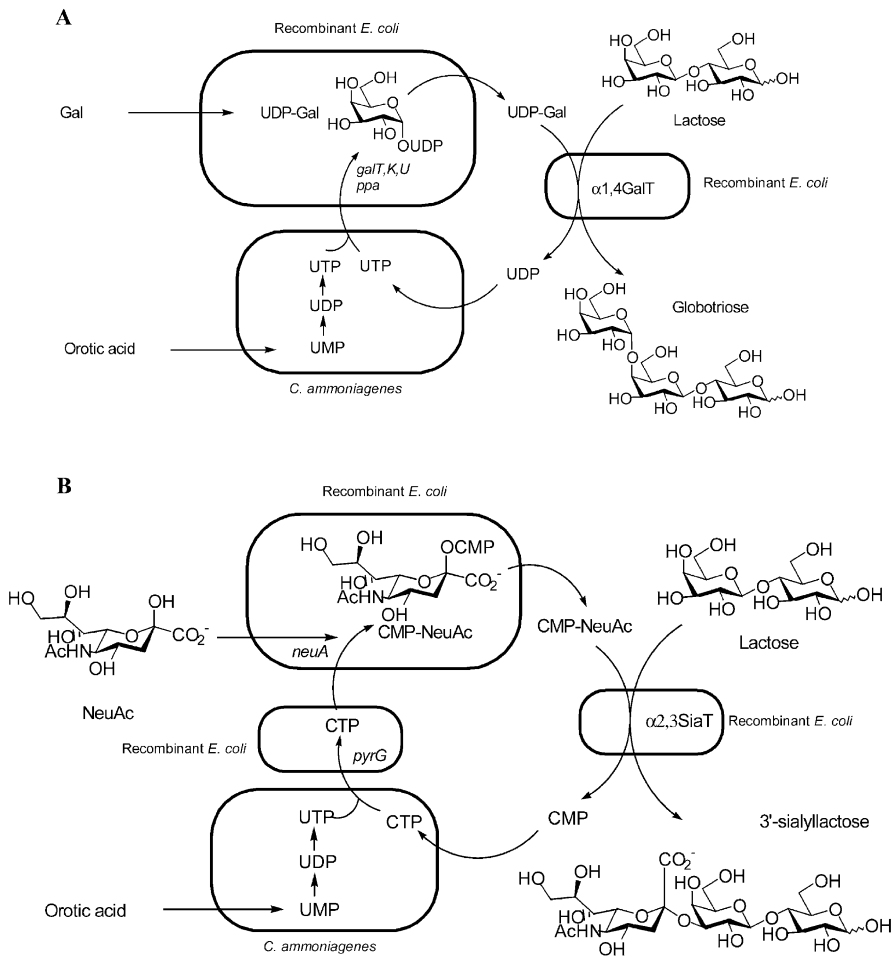

Figure 14. Production of sugar-nucleotides and oligosaccharides by combined use of *C. ammoniagenes* with *E. coli* strains metabolically engineered. (A) Production system for UDP-Gal and globotriose.[105] (B) Production system for CMP-NeuAc and 3′-sialyllactose.[108]

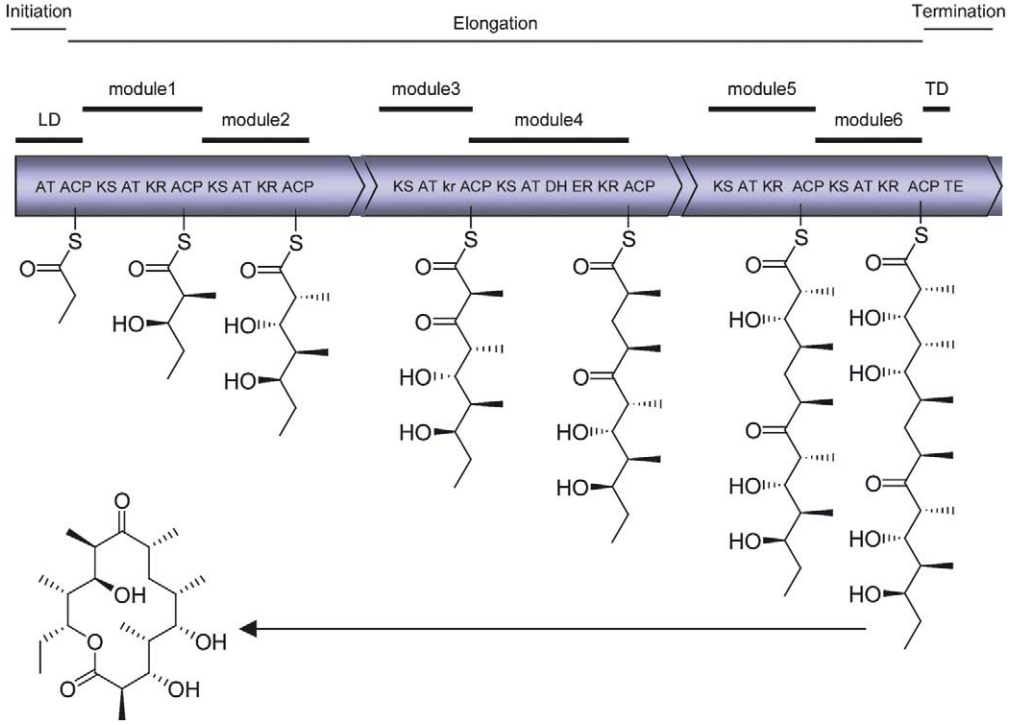

Figure 15. The erythromycin PKS system. The pathway has three PKS polypeptides containing six modules and loading and releasing domains. The cyclized structure shown needs to be further modified to bring erythromycin to its final structure.

including antibiotics, immunosuppresants, antifungals and cancer chemotherapeutics.[110] Production of new polyketides is probably the best example showing the advanced application of combinatorial biosynthesis, which has been facilitated by the modular structure of polyketide synthases (PKSs). PKSs complex catalyzes the assembly of specific types of polyketides from simple carboxylic acids.[111] The synthesis begins when the starter substrate is loaded in the acyl carrier protein (ACP) as a thiol ester. Then, the acyl moiety is transferred to β-ketoacyl:ACP synthase (KS) domain leading to a two-carbon atoms increase of the chain length. This is followed by successive two carbon extensions in a new ACP. Eventually, the polyketide is completely synthesized and, then, released by the activity of a specific thioester (TE) domain (Fig. 15).

PKSs control the selection of the starter substrate, the length of the chain, the reduction degree and the stereochemistry of the newly formed chiral centres.[112] Thus, inactivating, altering the order of the genes in the PKS biosynthesis pathway or exchanging genes from different organisms, novel hybrid polyketides can be obtained (Fig. 16).[113] However, the design of new poly-

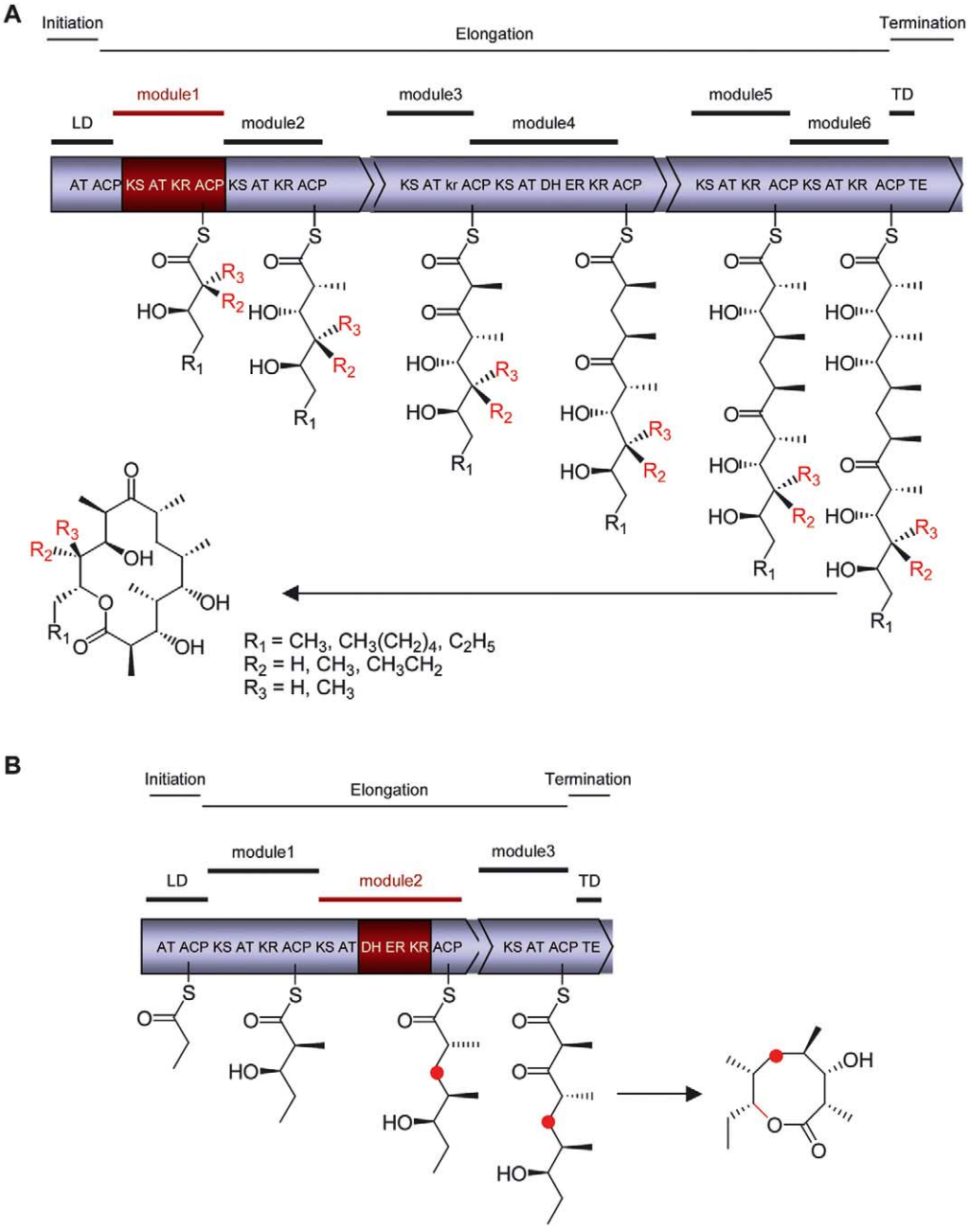

Figure 16. Two different strategies for the synthesis of 'unnatural' natural products by combinatorial biosynthesis. (A) Precursor-directed polyketide biosynthesis; inactivation of the first ketosynthase (KS) prevent the production of the second-step precursor, allowing to prime the second module with synthetic analogues of the product of the first module.[113a] (B) Introduction of auxiliary catalytic domains; by replacing the ketoreductase (KR) domain of module 2 with a KR-DH(dehydratase)-ER(enoylreductase) tridomain from the rapamycin synthase ( ) new chemistry is introduced into the reaction sequence catalysed by the module.[113c]

ketides must face some dificulties. After being released from the PKSs, the polyketide must be cycled by non-PKS enzymes and heterologous cyclases may not work properly on the 'unnatural' linear intermediate, which results in the formation of incorrectly cyclized products.[114] Also, the ACPs are not always interchangeable.[114a] Nevertheless, this approach has provided a large number of novel compounds.[115]

The final enzymatic trimming with carbohydrates, methyl groups, etc., gives even more structural diversity to the resulting aromatic compounds than the PKS. The importance of the sugar moiety in the biological activity of these compounds has led to the idea of using combinatorial biosynthesis to modify the glycosylation pattern of the aglycon.[116] The richest group of sugars present on many polyketides is the 6-deoxyhexoses. A great number of genes involved on the biosynthesis of these carbohydrates have been already identified and isolated.[117] Many of the glycosyltransferases involved in the biosynthesis of secondary metabolites are known to possess relaxed specificity for the sugar moiety and the aglycon.[118]

Valuable hybrid pharmacological compounds such as antibiotics can be made by combinatorial biosynthesis with bacterial deoxysugar biosynthesis genes. One approach is to introduce heterologous genes into a mutant strain blocked in the biosynthesis of its own deoxysugar.[119] These recombinant strains can synthesize novel sugar derivatives through the combined action of the host genes and the incorporated genes. Another approach to incorporate different sugars in the aglycon is to exchange glycosyltransferase genes between species producing structurally related bioactive compounds.[120]

6. Conclusions

Medicinal chemistry has decisively contributed to the humankind welfare during the last century. Nowadays, the appearance of new diseases, the rising of antibiotic-resistant pathogenic bacteria, the growing consciousness of the variable activity of different optical isomers, the search for higher selectivity and less toxicity, the individualization of the medicine in the post-genomic era, etc., are new challenges that pharmaceutical research must face. In this new landscape, the exciting advances in the biocatalysis field during the past years have placed this technology in position to play an important role in the drug discovery process.

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Biographies

Dr. Eduardo García-Junceda was born in Madrid, Spain, in 1961. He studied Biology at the Complutense University of Madrid (Spain). He completed his doctoral studies in 1990 at the Plant Physiology Department, Faculty of Biology (UCM) under the supervision of Prof. Dr. C. Vicente Córdoba. From 1990 to 1992, he worked as a postdoctoral fellow at the CSIC first in the Department of Instrumental Analysis and Enviromental Chemistry (group of Dr. J. Sanz) of the Institute of Organic Chemistry and later in the Department of Biocatalysis (group of Dr. J. M. Guisán) of the Institute of Catalysis and Petrochemistry. In 1992 Dr. García-Junceda joined as postdoctoral fellow the Dr. Chi-Huey Wong's Group in The Scripps Research Institute (La Jolla, California), where he began to work on the obtainment and use of enzymes for carbohydrate synthesis. After his return to Spain in 1995, he was appointed Research Staff Member in the Institute of Organic Chemistry of CSIC in 1997. Dr. García-Junceda's research interest include development of new biocatalysts and their application in the chemo-enzymatic synthesis of bioactive compounds, with particular emphasis on carbohydrates and glycoconjugates.

Juan F. García-García was born in Madrid, Spain in 1975. He studied for his B. A. in Biochemistry at the Autónoma University of Madrid, graduating in 2001. In 2001 he joined the Institute of Organic Chemistry belonging to the Spanish Council for Scientific Research (CSIC), where he started his D. Phil. Studies under the supervision of Dr. Eduardo García-Junceda, investigating the use of several carbohydrate-related enzymes in the synthesis of different carbohydrate compounds.

Dr. Agatha Bastida was born in Madrid, Spain, in 1964. She received her degree in Chemistry at the Autónoma University of Madrid. In 1992, she obtained her PhD in the Laboratory of Dr. J. M Guisán at the CSIC working on synthesis of peptides with immobilized enzymes. From 1993 to 1994, as Postdoctoral Fellow of the European Union, she worked at the Research Department of Unilever (United Kingdom) on synthesis of polyols esters with enzymes. She returned to Spain, to the Institute of Catalisis (CSIC), where she worked on the resolution of racemic carboxylic acid esters and regioselective hydrolysis of peracetylated sugars with lipases. In 1998 she joined Dr. Eduardo García-Junceda's group where she is working on the cloning and expression of glycosyltransferases and their use in synthesis.

Dr. Alfonso Fernández-Mayoralas was born in Madrid, Spain, in 1960. He studied Chemistry at the Autónoma University of Madrid and received his PhD degree from this university in 1987, under the supervision of Professor Manuel Martín-Lomas. Postdoctoral periods with Professors Sinaÿ (ENS, Paris), Gigg (NIMR, Mill Hill) and Klibanov (MIT) followed. In 1990 he joined the Spanish Council for Scientific Research (CSIC) where he currently holds the position of Scientific Researcher. His research interests include enzymatic synthesis, carbohydrate chemistry, bioorganic and medicinal chemistry.