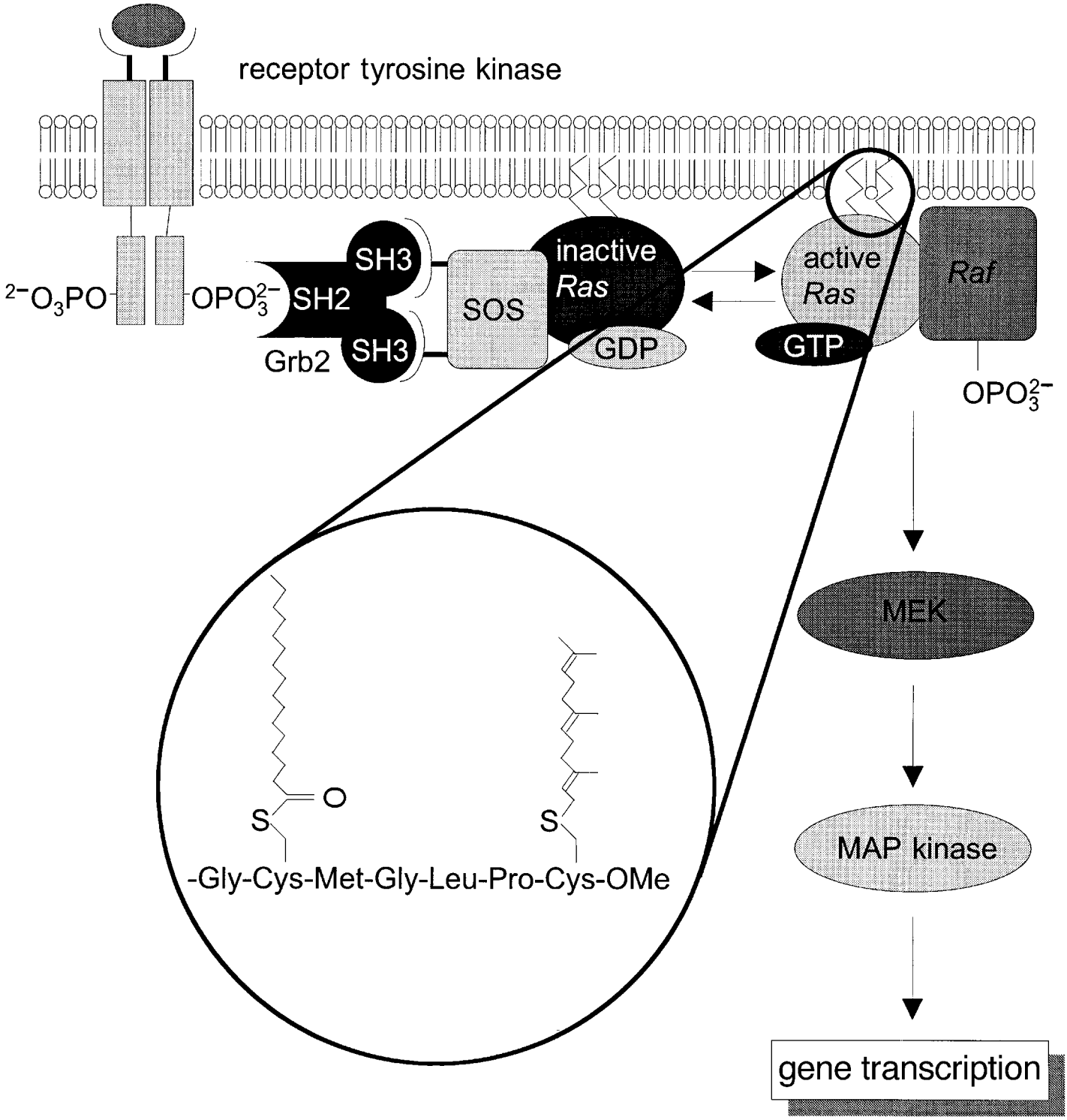

Biological signal transduction regulates important cellular functions such as growth, differentiation, and metabolism. In recent years much progress has been made in the investigation of individual signal pathways. Synthesis of partial structures of proteins involved in signal transduction and other biologically active compounds has helped to elucidate important interactions in molecular detail and to achieve a better understanding of these processes.

Organic Synthesis and Biological Signal Transduction

Klaus Hinterding, Daniel Alonso-Díaz, and Herbert Waldmann*

Dedicated to Professor Dieter Seebach on the occasion of his 60th birthday

Today, many biological phenomena are being investigated and understood in molecular detail, and organic chemistry is increasingly directed towards biological phenomena. Thus, the field of "bioorganic chemistry", which encompasses both biological and chemical research, has developed at the border between these two disciplines. The interplay of organic chemistry and biology will be illustrated in this review with biological signal transduction as an example. For signal transmission through tyrosine kinase receptors and the Ras protein and through G protein coupled receptors, it will be shown how, starting from a biological phenomenon, structural information can be obtained that leads to the development of new chemical methods and syntheses, and subsequently to new reagents for biological studies. In particular, bioorganic research on protein tyrosine kinases, interactions with SH2 and SH3 domains, protein farnesyltransferase, applications of peptide conjugates and inositol phosphate derivatives, protein kinases A and C, protein phosphatases, and ligand-induced dimerization of proteins will be presented.

Keywords: bioorganic chemistry · enzyme inhibitors · peptide conjugates · signal transduction · synthetic methods

What interests us all is finding that deep current—in my case, the most rational understanding of life: its reduction to the molecular details of chemistry.
A. Kornberg[1]

My idea is that chemistry is the central science. Everything that goes on in biology or medicine has a chemical basis.
R. Lerner[2]

1. Introduction

Biological processes are based on chemical reactions and depend on the structure and interactions of the molecules involved. In principle, all biological processes can be reduced to chemical ones: biology is molecular.[3] This insight has influenced current biological research, especially in recent years. Today, many biological phenomena are being investigated and understood in molecular detail, and the increasingly important field of structural biology[4] is becoming more prominent on the horizon of organic chemistry. Just as the dimensions of objects studied in biology have become smaller, organic chemistry has turned increasingly to larger molecules and systems. Owing to the high performance of organic synthesis today, even the most complicated of these natural and active compounds that are recognized as biologically relevant can be synthesized, and noncovalent interactions between large natural molecules that determine processes such as protein–protein and protein–DNA interactions and recognition phenomena at cell surfaces are the focus of "supramolecular chemistry". As the kinds and dimensions of the subjects of biological and organic chemical research have become more similar, these sciences have begun to permeate one another and a growing interdisciplinary field has been established at their interface: "bioorganic chemistry".

Although this term has been used for some time,[5] it still does not have a clear, firm definition (and there is not necessarily a need for this). In our opinion, a "bioorganic chemist" performs active research in chemistry *and* (!) biology[6]: he or she unites the "two cultures of chemistry and biology".[7] However, the emphasis of the research may lie on the chemical or biological side and can, for example, focus on the application of methods and tools of organic chemistry to study biological problems or the use of biological techniques to address chemical problems. These nuances can be more precisely expressed by the terms "chemical biology" and "biological chemistry".

One possible scenario for successful combination of biological and organic chemical research is illustrated in Scheme 1. Here, a well-known biological phenomenon is analyzed for the structures of the molecules involved; these

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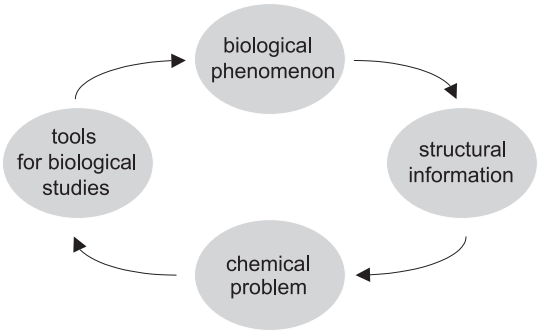


Scheme 1. Possible cooperation between organic synthesis and biology in "bioorganic chemistry".

may be active compounds or biomacromolecules of low molecular weight. Based on this structural information, unsolved synthesis problems are identified and tackled. This is true innovation in the field of organic synthesis. Once these problems have been conquered, the methods and syntheses developed are used to build up new reagents for studying the biological phenomenon under consideration; these are subsequently used in biological experiments. In the ideal case, these reagents are used to glean new knowledge which could not have been obtained (or only with great difficulty) with classical biological techniques. New structural information is derived from this new knowledge foundation, and the cycle of bioorganic research as outlined above starts over again.

In this review the successful interlocking of biological and organic chemical research according to the scenario above will be presented with "biological signal transduction" as an example of current relevance.

2. Biological Signal Transduction[8, 9]

For multicellular organisms to maintain functional and survival capacity, growth, differentiation, and metabolism of a large number of cells must be coordinated by synthesis, secretion, and recognition of signal molecules, sometimes over relatively large distances. Once the signal has been conveyed through the plasma membrane of the target cell, the message is relayed to the cell interior by intracellular signal cascades, and a reaction is triggered. According to their physical characteristics, extracellular signal substances (hormones) are recognized by receptors in the cell interior (lipophilic hormones which can diffuse through the plasma membrane, e.g. steroids and thyroid hormone) or they are bound to receptors on the cell surface (hydrophilic hormones which cannot pass through the cell membrane by diffusion, e.g. peptide hormones and small charged molecules such as adrenaline and histamine).[10] Finally, the signal is switched off and the reaction of the cell to the signal substance ends. The activity of the latter class of hormones and associated signal mediation has been the subject of particularly intensive


H. Waldmann


D. Alonso-Díaz


K. Hinterding

Herbert Waldmann, born in 1957, studied chemistry at the Universität Mainz (Germany) and finished his Ph.D. there in 1985 with Horst Kunz. After a postdoc at Harvard University with George Whitesides, he returned to the Universität Mainz in 1986 and finished his habilitation in 1991. From 1991 to 1993 he was Professor of organic chemistry at the Universität Bonn, and then became Full Professor (Chair) of organic chemistry at the Universität Karlruhe. He has been awarded, among others, the Friedrich-Weygand-Preis, a stipend from the Fonds der Chemischen Industrie, and the Carl-Duisberg-Preis from the Gesellschaft Deutscher Chemiker. His research covers many areas such as diastereo- and enantioselective synthesis, the chemistry of peptides, carbohydrates, and alkaloids, and the use of enzymes in organic chemistry. A main theme of this current work is the combination of methods from organic chemistry with biological techniques, in particular for the synthesis and application of new reagents and tools to study biological questions such as the control of signal transduction pathways.

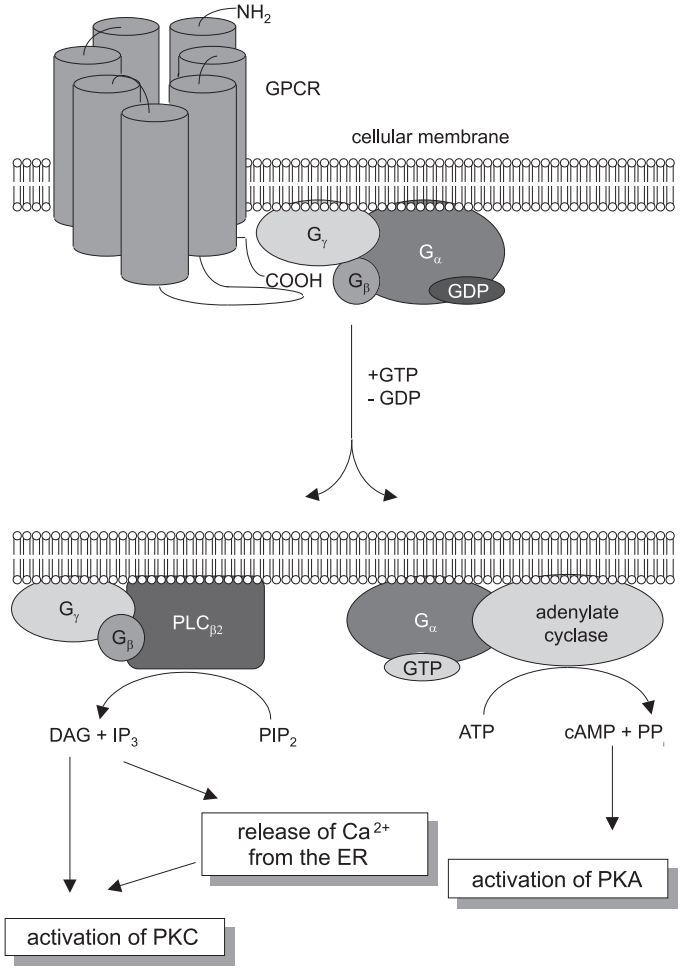
Daniel Alonso-Díaz, born in 1960 in Sabadell (Barcelona, Spain), studied chemistry at the Universidad Autónoma de Barcelona, where he finished his Ph.D. in 1991 on the synthesis of natural products. Afterwards, he conducted research in the pharmaceutical industry and coordinated a Eureka project of the European Union. In 1995 he joined the group of H. Waldmann as a postdoc with a Marie Curie fellowship, where he worked on the total synthesis of biologically active molecules.

Klaus Hinterding, born in 1969 in Neuenkirchen (Germany), studied chemistry between 1989 and 1991 in Freiburg. After a one-year stay at the University of Edinburgh (UK), where he worked on the synthesis of ruthenium and osmium clusters in the group of B. F. G. Johnson, he finished his Diplom in chemistry in 1994 with H. Waldmann in Karlsruhe. He completed his Ph.D. in 1997 on the synthesis of inhibitors for cellular signal transduction processes.

bioorganic research. The knowledge obtained from this work is the central theme of this review.[11] In the following sections the most important intracellular signal cascades relevant to this class of hormones, for which the mode of action has been adequately explained at the cellular and molecular level, are outlined.

2.1. Signal Transduction through G Protein Coupled Receptors and Heterotrimeric G Proteins [8, 9, 12–24]

Many hormonal messengers—for example, adrenaline, noradrenaline, glucagon, corticotropin, luteinizing hormone, and follicle-stimulating hormone—and also signals associated with senses such as taste,[23] smell, and vision[24] are recognized at the cell surface by G protein coupled receptors[12] (GCPRs) made up of seven transmembrane helices (Scheme 2). An

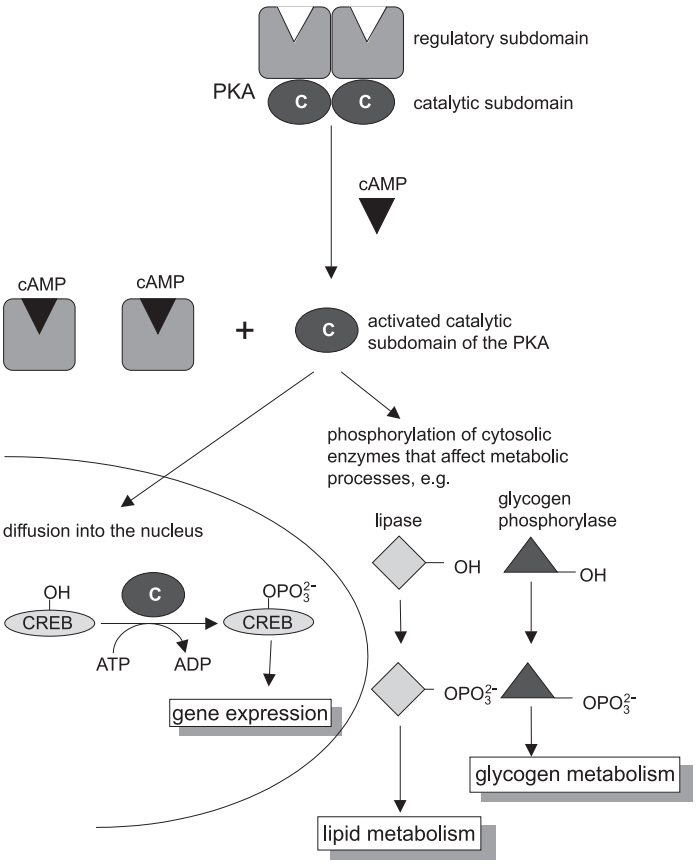


Scheme 2. Formation of "second messengers" in signal transduction through G protein coupled receptors and heterotrimeric G proteins.

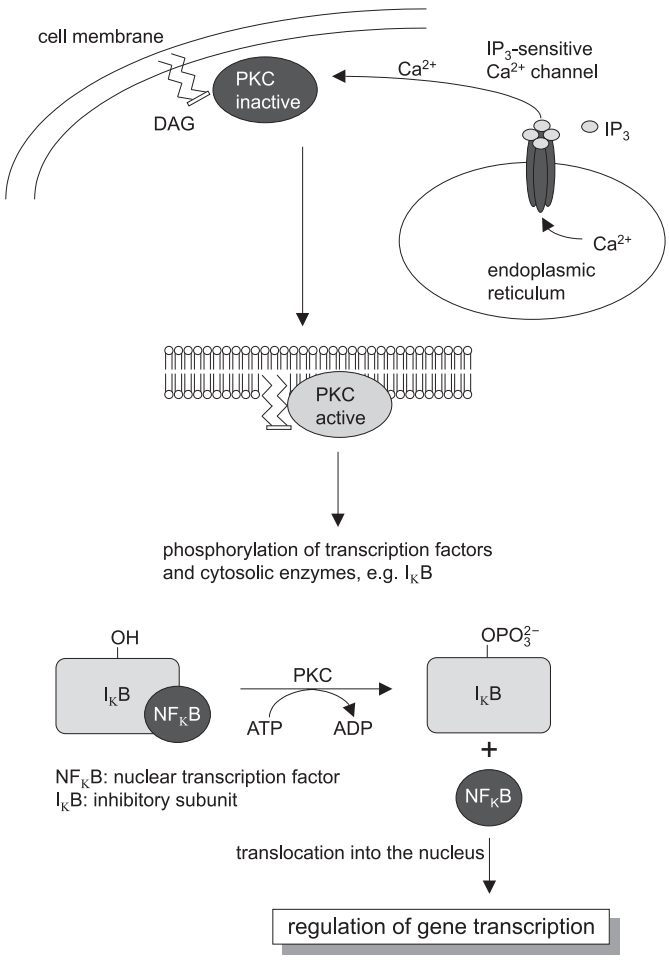
intracellular loop between helices 5 and 6 and the C terminus of the receptor, which is also cytosolic, interact with a heterotrimeric guanine nucleotide binding protein (G protein) located in the membrane. G proteins are made up of three different subunits (G_α, G_β, G_γ),[13–15] of which the α unit has a bound GDP molecule in the inactive form. As a consequence of the interaction with the receptor, GDP is exchanged for GTP on the α unit and the heterotrimeric complex dissociates into G_α/GTP and G_β/G_γ. This activation of the G protein by nucleotide exchange and dissociation is followed by hydrolysis of GTP to GDP and renewed formation of the inactive (but available for reactivation) GDP-binding heterotrimeric complex; thus, the signal is switched off. Before the signal is cancelled, however, G_α/GTP and G_β/G_γ trigger various intracellular signal cascades. If this complicated mechanism is disturbed by external agents, it results in serious complications, both for the cells involved and for the whole organism. For example, cholera toxin brings about a permanent binding of GTP to the α unit of a particular G protein (G_s) and thus continous stimulation of adenylate cyclase, which leads to massive diarrhea. On the other hand, pertussis toxin, which causes whooping cough, inactivates the α unit of the G_0 protein and inhibits stimulation of phospholipase C.

Depending on the heterotrimeric complex from which it is released, G_α/GTP can have a stimulatory ($G_{s\alpha}$) or inhibitory ($G_{i\alpha}$) effect on adenylate cyclase, an enzyme also located in the plasma membrane that catalyzes cyclization of ATP to cyclo-AMP (cAMP) when stimulated (see Scheme 2). This "second messenger" then activates cAMP-dependent protein kinases of Group A (PKA)[16] by releasing their catalytic subunits from an inactive tetrameric complex. According to cell type, PKA then activates various enzymes by phosphorylating them at serine or threonine residues. In liver and muscle cells, for example, activation of PKA takes place when adrenaline binds to the relevant G protein coupled receptors, and leads to degradation of glycogen to glucose 1-phosphate, that is, to mobilization of glucose from the depot form (Scheme 3). In fat-storing adipocytes, an enzyme cascade triggered by adrenaline results in release of fatty acids (Scheme 3). Binding of corticotropin to a G_s-coupled receptor stimulates biosynthesis of steroid hormones, such as cortisol, in the cells of the adrenal cortex. In ovarian cells, the follicle-stimulating hormone triggers increased biosynthesis of estradiol and progesterone, which are needed for expression of female sexual characteristics. In addition, protein kinase A can diffuse into the cell nucleus and stimulate transcription factors, which subsequently activate expression of many genes.

The G_β/G_γ complex (and also G_α) activates the membrane-bound phospholipase $C_{\beta 2}$ ($PLC_{\beta 2}$), which cleaves the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). These substances act as second messengers.[17–19] IP_3 diffuses through the cytosol once it has been released, and is bound by a specific receptor on the endoplasmic reticulum (ER) that opens a channel allowing Ca^{2+} ions to flow from the ER into the cytosol (Scheme 4). Ca^{2+} now functions as a further second messenger that is needed, for example, for activation of protein kinases. DAG remains in the plasma membrane where it binds, together with Ca^{2+} ions and phosphatidylserine, to a class of serine/threonine protein kinases of Group C (PKC; Scheme 4).[20–22] These molecules, which are inactive in



Scheme 3. Activation of protein kinase A and subsequent signal transmission to cytosolic enzymes and transcription factors. CREB = cAMP response element binding protein.



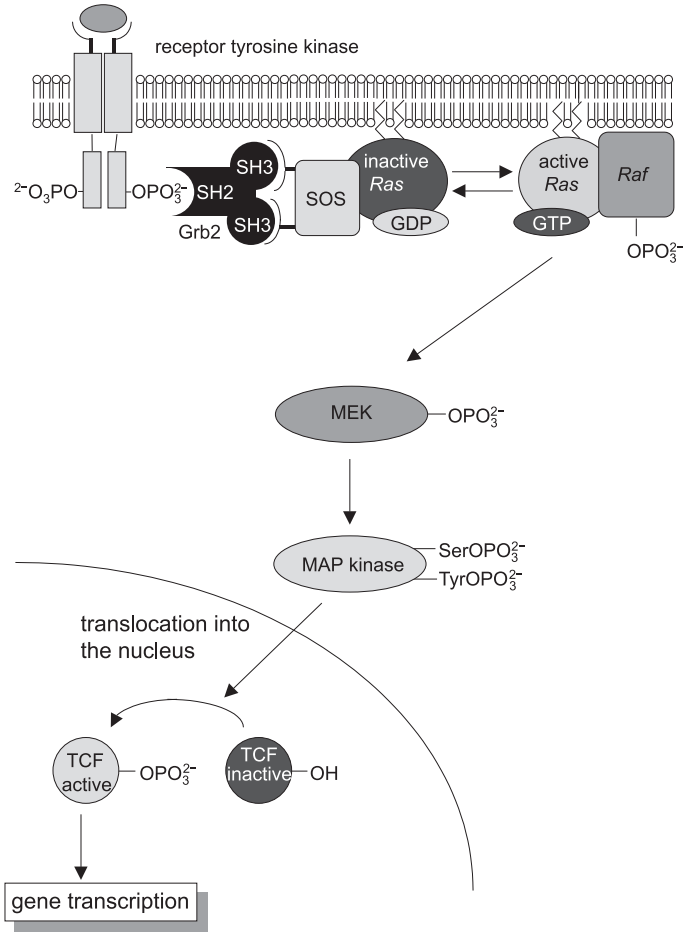
Scheme 4. Activation of protein kinase C by the IP_3/DAG signal pathway and subsequent activation of the transcription factor $NF_\kappa B$.

their cytosolic form, phosphorylate other enzymes after membrane localization, and thus influence a multitude of cell reactions. It is especially relevant that PKC also phosphorylates transcription factors and can thus, according to cell type, induce or suppress the synthesis of particular mRNA molecules. Threrefore, protein kinase C plays an important role in the regulation of cell growth, and it follows that a defective PKC function can lead to misregulated growth and ultimately transformation of the cell (see Section 2.5).

2.2. Signal Transduction through Receptor Tyrosine Kinases and Nonreceptor Tyrosine Kinases—The Ras/MAP Kinase Signal Transduction Cascade[9, 25–33]

Many of the polypeptide hormones that influence cell proliferation and differentiation bind to cell-surface receptors which have tyrosine kinase activity (receptor tyrosine kinases, RTKs). These include the receptors for the epidermal growth factor (EGF), the insulin-like growth factor 1 (IGF-1), the nerve growth factor (NGF) and the platelet-derived growth factor (PDGF), which influence growth, differentiation, and division of epidermal, endothelial, and epithelial cells (EGF), sensory and sympathetic neurons (NGF), and cells of the connective tissue, granulocytes, and the nervous system (PDGF).

The receptor tyrosine kinases, which are monomeric in the active form, are made up of an extracellular binding domain, a single membrane-spanning α helix, and a cytosolic domain with tyrosine kinase activity. On binding an extracellular ligand, the receptors dimerize and the kinase domain of one receptor molecule recognizes and phosphorylates tyrosine residues of the other monomeric unit (Scheme 5). The phosphorylated receptors are subsequently recognized by adaptor molecules, which are not signal molecules themselves but serve to bind the receptor to signal molecules. Of particular importance is the growth factor receptor binding protein 2 (Grb2); this binds, with the help of a SH2 domain (SH2 = sarcoma homology 2), to the peptide sequence of the receptor in which the phosphotyrosine is located. Grb2 also contains two SH3 domains that recognize proline-rich sequences in another adaptor protein, Sos (son of sevenless, named after a Drosophila mutant; Scheme 5). Thus, the cytosolic proteins Grb2 and Sos are localized on the inner side of the cell membrane and correctly aligned. Sos then interacts with the inactive, GDP-binding form of the protein Ras (from rat sarcoma) located in the membrane. Ras exchanges GDP for GTP following interaction with the Grb2/Sos complex, which acts as a kind of "molecular glue"; Grb2/Sos functions as a guanine nucleotide exchange factor (GEF). This activates Ras, which then acts as a molecular switch and diverts the signal arriving from the tyrosine kinase receptor by non-



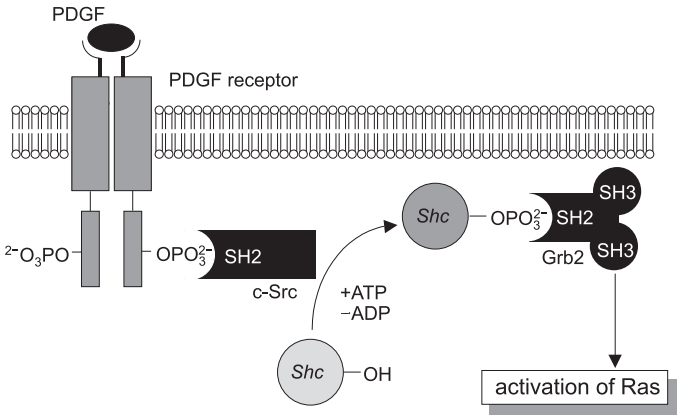
Scheme 5. Signal transduction by the Ras/MAP kinase cascade.

covalent protein – protein interactions to a cascade of protein phosphorylations and into the cell interior (Scheme 5). Ras has weak GTPase activity that hydrolyzes the bound GTP to GDP and thereby terminates the signal. This process is also accelerated by the GAP protein (GTPase-activating protein).

The activated, GTP-binding form of Ras binds to the N terminus of the serine/threonine kinase Raf and localizes Raf (analogous to immobilization of Sos to Grb2) on the plasma membrane. With its C terminus, Raf now binds the protein kinase MAP kinase kinase (MAP = mitogen activated protein)—also known as MEK (from MAP and ERK, extracellular signal regulated kinase)—and phosphorylates it. This activated MEK has tyrosine and serine/threonine kinase activity (dual specificity kinase) and phosphorylates the protein MAP kinase, which is also a serine/threonine-specific kinase. The activated MAP kinase now phosphorylates and activates other kinases, such as the ribosomal S6 kinase; it also diffuses into the cell nucleus and phosphorylates transcription factors there, for example the ternary complex factor (TCF, also known as Elk-1) and the Jun protein, which are thereby activated and stimulate expression of the corresponding genes.

In some cases, the Ras/MAP kinase cascade and the GPCR signal pathway influence the expression of the same gene. In this manner, many genes can be expressed very rapidly by a concerted interlocking of various signal chains. Another way for signal chains to mutually influence each other is the activation of elements of one cascade by members of another (cross talk). PKC and other serine/threonine kinases can phosphorylate MEK and other members of the Ras cascade,[30] and thus establish a cross-link between the GPCR signal pathway and the Ras cascade. Diverse further examples of cross talk are known between various signal cascades (see Section 3.2 and Scheme 12).[30]

Nonreceptor tyrosine kinases (NRTKs)[34]—that is, tyrosine kinases located on the cytosolic side of the plasma membrane without an extracellular domain—can also switch on the Ras cascade.[17, 31] This is how, through a SH2 domain, the NRTK c-Src recognizes the autophosphorylated, and thereby activated, PDGF receptor,[35] which itself has no Grb2 binding site (Scheme 6). c-Src phosphorylates the adaptor protein Shc



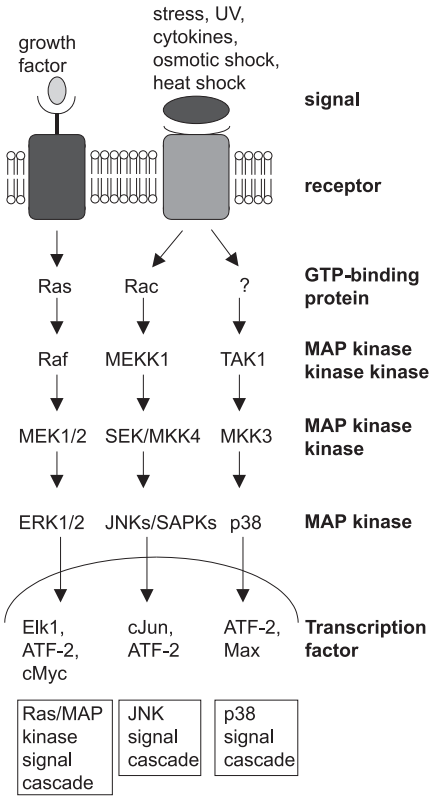
Scheme 6. Activation of the Ras signal cascade by nonreceptor tyrosine kinases (e.g., c-Src).

on a tyrosine residue and thus creates a binding site for the Grb2/Sos complex, which subsequently activates Ras (see above) and passes on the signal.

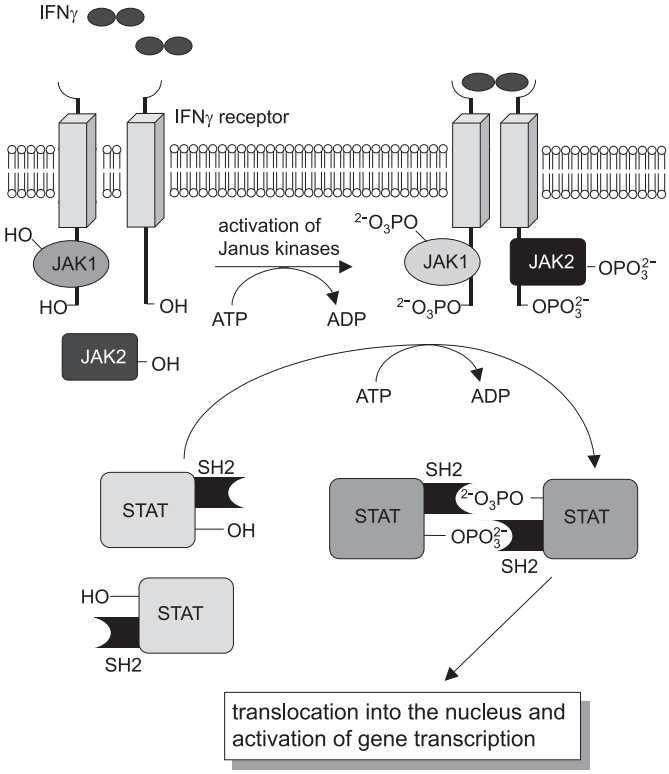
The Ras signal transduction cascade is of extreme physiological importance. It is central to the regulation of cell growth and differentiation, and a mistake in regulating this signal pathway can be one of the critical steps for cell transformation (see Section 2.5). The Ras path is highly conserved in different species,[9, 25–27] and its elements are used in the same way for transmission of growth signals in, for example, yeast, worms, flies, and mammals. Furthermore, it was recently shown that in mammalian cells there are at least three more signal transduction pathways, based on the same scheme as the Ras pathway, which conduct different extracellular signals to the cell nucleus (Scheme 7).[30] This includes, for example, information on stress due to UV light and ionizing radiation, alkylating reagents, osmotic and heat shock, and signals from inflammatory cytokines (see Section 2.3) such as the tumor necrosis factor α (TNF-α) and interleukin 1 (IL – 1).

2.3. Signal Transduction through Janus Kinases and STAT Molecules[9, 36, 37]

A very important pathway for signal transduction in which protein phosphorylation and protein – protein interactions



Scheme 7. Common elements of signal transduction cascades that are activated by growth factors, inflammatory cytokines, genotoxic reagents, and stress factors.



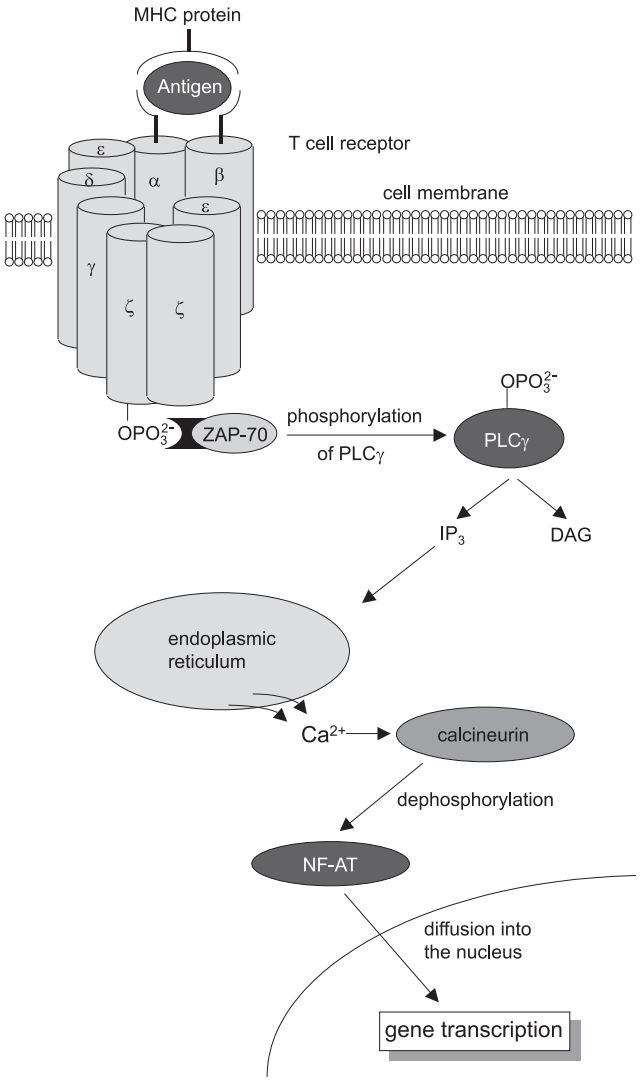
Scheme 8. The JAK/STAT signal transduction cascade. INFγ = interferon γ.

through SH2 domains (see Section 2.2) play a decisive role is for passing on signals triggered by cytokines. Cytokines are soluble proteins that influence growth, differentiation, and function of a variety of cells; they include, for example, interferons α, β, and γ as well as interleukins 2 to 7, 9 to 12, and 15. For example, the interferons are formed in, and secreted by, animal cells following a virus infection. When an interferon binds to plasma-membrane receptors on the target cell, cell growth is inhibited and synthesis is induced of proteins which give the target cell viral resistance.

When a cytokine binds to its receptor, the receptor dimerizes and leads to activation of Janus kinases (JAK or "just another kinase"), a class of tyrosine kinases that are noncovalently associated with the cytosolic domain of the receptor (Scheme 8). The JAK kinases then phosphorylate the receptor itself as well as the STATs (signal transducers and activators of transcription) that subsequently bind to it. On activation, these latent cytosolic transcription factors form homodimeric and heterodimeric complexes that migrate into the cell nucleus and induce transcription of cytokine-dependent genes there.

2.4. Signal Transduction through the T Cell Receptor[38–40]

The signal pathway starting from the T cell receptor (TCR) is essential for maintenance of the T cell dependent cellular immune response; this pathway uses the same transmission mechanism as the signal cascades previously described. On activation of the receptor by an antigen, the T cell first undergoes the transition from the G_0 phase to the G_1 phase; that is, it leaves the resting phase and prepares for cell division.[38] The complex T cell receptors are composed of eight noncovalently bound membrane-spanning subunits (α, β, γ, δ, ε_2, ζ_2). As soon as a macrophage presents an antigen fragment to the receptor through the proteins of the major histocompatibility complex (MHC proteins), the fragment is recognized and bound by the α and β subunits, and the eight individual strands aggregate (Scheme 9). The cytoplasmic domains are brought into spatial proximity and phosphorylated on tyrosine residues by an associated kinase (Lck or Fyn, kinases of the Src family). In particular, phosphorylation of the ζ subunit creates a binding site for the SH2 domain of the protein kinase ZAP-70. This kinase phosphorylates and activates phospholipase C_γ (PLC_γ), which releases inositol trisphosphate (see Section 2.1). This second messenger brings about the release of calcium ion from the endoplasmic reticulum into the cytosol, leading to activation of calcineurin, a Ca^{2+}-dependent Ser/Thr-specific protein phosphatase. Calcineurin now dephosphorylates the cytoplasmic part of the transcription factor NF-AT (nuclear factor of activated T cells), which migrates to the cell nucleus and directs transcription of certain genes responsible for the further correct course of the immune response.[39] As a result, the T cell expresses both the growth factor interleukin 2 (IL-2), which is released into the intracellular space, and also its receptor (IL-2-R), which is anchored in the plasma membrane as a transmembrane protein. This self-stimulation of the T cell



Scheme 9. Signal transduction by the T cell receptor.

(autocrine stimulation) brings about the transition from the G_1 phase to the S phase of the cell cycle and thus leads to division and multiplication of precisely the T cell that recognized the originally presented antigen (clonal selection).

This simple picture of T cell activation can be extended to include the overlap with other signal pathways (cross talk). IP_3 is also released by the GPCR signal pathway (see Section 2.1), and the tyrosine phosphorylated TCR can bind to the SH2 domain of certain adaptor proteins and is thereby linked to the Ras signal path.

2.5. Signal Transduction and Transformation of Cells[41, 42]

The influence of signal transduction cascades on the regulation of cell growth, division, and differentiation is of great importance. In normal cell growth, the interactions of the individual components of the signal cascades are precisely coordinated with one another. However, if a fault occurs in the regulation of an important signal cascade, the cell may be transformed; that is, its growth characteristics are changed and it can develop into a tumor cell. Transformation of a cell can be triggered by various mechanisms (e.g. by DNA-damaging chemical carcinogens), however, one of the most common causes is formation of an oncoprotein (i.e. the product of an oncogene, or cancer gene). Oncogenes are generally derived from normal cell genes responsible for controlling cell growth (protooncogenes), for example, by mutation, or incorrect or misregulated expression. Growth is mostly controlled by growth factors, their receptors, intracellular signal transducers, nuclear transcription factors and cell cycle control proteins. Most oncogenes code for proteins belonging to one of these five classes, whereby representatives of classes 1 – 4 are important elements of the signal transduction cascades described above.

Relatively few oncogenes are derived from genes for growth factors. Genes for growth factor receptors with tyrosine kinase activity can become oncogenes if, on mutation, they code for receptors with kinase activity that stay switched on in the absence of the respective ligand. However, in most cases, oncogenes of this class code for truncated receptors in which the extracellular ligand-binding domain is missing. The oncogene gives a permanent growth signal in both cases.

Most oncogenes derive from genes for proteins involved in intracellular signal transduction cascades. For example, mutations in the $G_{s\alpha}$ subunit of heterotrimeric G proteins (see Scheme 2) bring about loss of GTPase activity of these proteins, which results in continuous stimulation of cAMP synthesis. Therefore, pituitary cells with a mutated $G_{s\alpha}$ protein grow in an uncontrolled fashion owing to the resulting high levels of cAMP, and pituitary gland tumors develop. Similarly, mutations in *ras* genes mean that the Ras protein can no longer be converted from the active, GTP-binding state into the inactive GDP-binding form (see Scheme 5). Consequently, these highly conserved switches emit a permanent growth signal that can lead to tumor formation. Mutations in *ras* genes are found in many human tumors.

Mutations in genes for nonreceptor tyrosine kinases can also lead to oncogene formation. The kinase activity and therefore the signal transducing effect of Src (see Scheme 6) can be significantly reduced by phosphorylation of a tyrosine residue at the C terminus. If this phosphorylation site is altered (for example, in Rous sarcoma virus, the last 18 amino acids of the C terminus of Src are missing due to a deletion on the *src* gene), this possibility for regulation is absent and a permanent signal results. Activation of the transforming potential of the Raf serine/threonine kinase (see Scheme 5) also occurs by deletion of regulatory subunits of the normal cellular protein. The C terminus of Raf contains the kinase subunit, whereas the N terminus has two domains that are modulated by the kinase activity. These regulatory domains

are deleted in *raf* oncogenes, and the catalytically active C terminus of the enzyme functions in an uncontrolled manner. Oncogenes coding for nuclear transcription factors often manifest themselves by creating oncoproteins which change the transcription frequency of genes, for example, those coding for growth-stimulating proteins.

A class of oncogenes of great importance for the cell cycle code for tumor suppressors.[43, 44] These proteins monitor the operation of the cell cycle and prevent formation of tumors by arresting cells in the resting phase or even by inducing programmed cell death.[45, 46] If mutations occur in tumor suppressor genes, the cell cycle is no longer properly regulated and mutations accumulate.

The transforming effect of chemical compounds recognized as tumor promoters also arises because of their influence on signal transduction cascades. Whereas normal chemical carcinogens, such as alkylating reagents and polycyclic hydrocarbons, damage the DNA directly (tumor initiators), tumor promoters increase the probability of tumor formation by a tumor initiator. Phorbol esters and teleocidins belong to the tumor promoters. These substances activate protein kinase C by imitating the effect of diacyl glycerol (see Scheme 4).

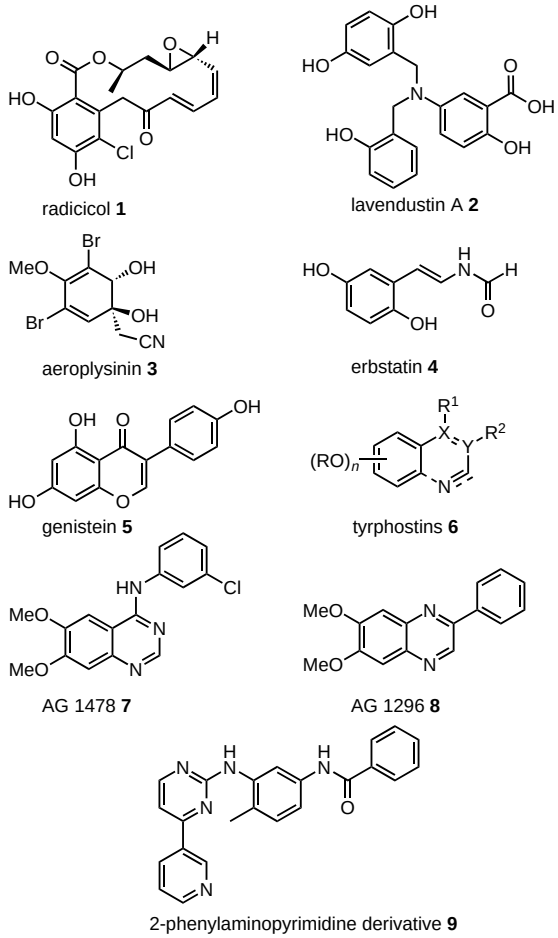
According to our current knowledge, carcinogenesis is generally triggered by several factors in a multistep process; cancer arises due to many effects acting together over a longer period of time. A single oncogene is usually not able to induce malignant growth by itself; different oncogenes must complement each other's activity. Human tumor cells embody several oncogenes, and probably several genetic changes (about four or five) would have to accumulate in a single cell for it to be transformed. Some mutations are particularly prominent because they attack at important points in the regulation of cell growth, division, and differentiation. In about 50% of all human tumors, mutations are found in the gene that codes for the tumor suppressor p53, which is therefore the most commonly occurring oncogene. Of almost equal importance is the *ras* oncogene which is mutated in about 40% of all human tumors. In some of the main types of cancer—such as cancer of the bowel, breast, or pancreas—the mutation rate of the *ras* oncogene is as high as about 80%.

Our understanding of intracellular signal transduction at the molecular level, and selective manipulation of these processes based on such knowledge, opens up fundamental new opportunities for study, and possibly also therapy, of malignant transformations. The rather apt expression "signal transduction therapy" was coined by A. Levitski for this new direction in "molecular medicine".[47]

3. Inhibition of Protein Tyrosine Kinases (PTKs)

PTKs play an important role in cellular signal transduction and the correct course of many genetic programs—for example, in the Ras signal cascade, the Jak/Stat signal pathway, and the TCR signal pathway (see Section 2.2, 2.3, and 2.4, respectively)—and half of all proto-oncogenes discovered so far code for proteins with PTK activity.[48] There is therefore an intensive search underway for selective inhibitors of PTKs.[49]

Such substances would enable selective interruption of the signal transduction cascades and branching of various signal paths to gain a better understanding of the molecular sequence of signal transmission. Furthermore, they could provide ways to treat diseases associated with abnormal PTK activity, such as cancer, psoriasis, restenosis, or septic shock.[49a] Examples of natural products with PTK-inhibiting activity are radicicol (**1**),[50] lavendustin A (**2**),[51] aeroplysinin (**3**),[52] erbstatin (**4**),[53] and genistein (**5**)[54] (Scheme 10).



Scheme 10. Structure of some natural products and synthetic compounds with PTK-inhibitory activity.

Owing to their interesting pharmacological profiles, these substances have already found various uses in biological research. However, the most potent of the currently available inhibitors originate from the synthetic laboratory.

With increasing knowledge of the structure of various PTKs (the primary structures are known for many different PTKs and show high homology in the catalytic domain;[55] furthermore, X-ray structures have been solved for the insulin receptor,[56] c-Src,[57] and some Ser/Thr kinases[58]) and derivation of important structural characteristics of enzyme inhibitors, it has been possible to make significant progress in the development of active and selective inhibitors in recent years. The assumption that it is practically impossible to produce selective ATP-competitive inhibitors (since the ATP binding sites of all PTKs, and even all kinases, share a high structural similarity) has been completely disproved. The

most active and selective inhibitors of the EGF receptor known to date and the PDGF receptor bind reversibly at the ATP binding site.

In the following sections, some examples of natural products with PTK-inhibitory activity and of synthetic inhibitors will be presented.

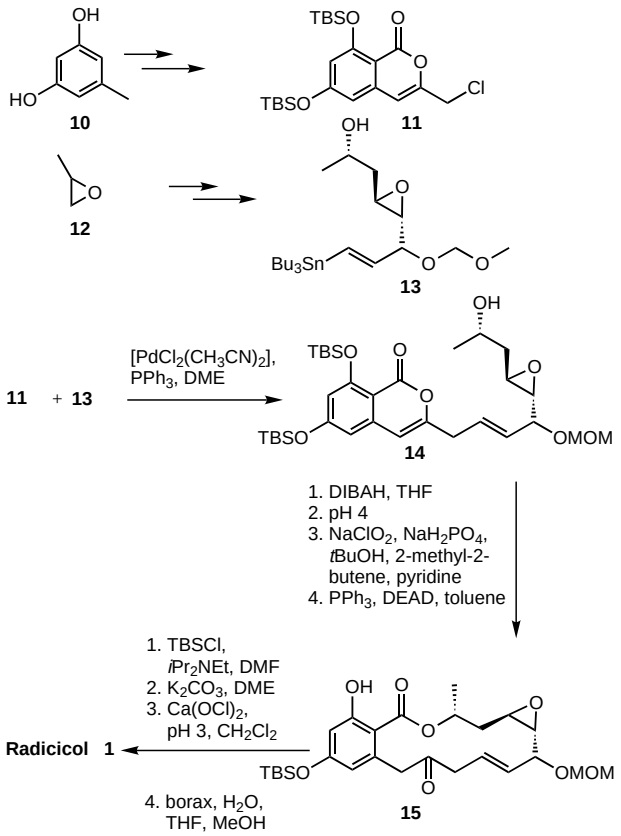
3.1. Radicicol

Radicicol (**1**, Scheme 10), a metabolite of the microparasite *Monocillium nordinii*,[59] shows a variety of biological activities (fungistatic, antitumor activity, PTK inhibitor). Since the natural product is difficult to isolate, the development of an efficient total synthesis provides the opportunity to produce enough material for biological and medical studies and to obtain analogues with altered biological properties.

Particular challenges for the synthesis are construction of the 14-membered macrolactone and a conjugated dienone epoxide unit with an *E* and a *Z* double bond. Based on molecular modeling experiments and the prediction that an established (*E*,*Z*)-dienone epoxide unit would hardly survive the conditions needed for a macrolactonization, the *Z* double bond was created by elimination following successful cyclization of the precursor **15** (Scheme 11) in the only total synthesis[60] published so far. Macrocycle **15** was retrosynthetically converted back into the isocoumarin derivate **14**, which was synthesized by palladium-catalyzed coupling of arene **11** and the vinyl stannane **13**, which contains all the stereogenic centers.

The isocoumarin derivate **11** was synthesized in eleven steps (total yield 31 %) starting from orcinol (**10**). After the free hydroxyl functionality was protected, a further substituent was introduced by a Friedel–Crafts acylation and, following elaboration of the *ortho* methyl group, it was cyclized to **11**.

In the case of the vinyl stannane **13**, particular attention must be paid to the absolute configuration of the three sterocenters: Whereas the two C atoms of the epoxide unit must be *R*-configured, the configuration of the hydroxy-substituted stereocenter must be variable according to whether the acyl functionality (with retention of stereochemistry) or the hydroxyl functionality (with inversion of configuration under S_N2 conditions) should be activated for the macrolactonization. The absolute configuration of the secondary alcohols can be adjusted as needed by nucleophilic opening of enantiomerically pure propylene oxide with lithiated propargyl alcohol that is protected at the hydroxyl functionality. The key steps of the subsequent synthesis were a stereospecific reduction of the acetylene to an (*E*)-allyl alcohol, a Sharpless epoxidation, and the extension of the carbon skeleton with a vinyl stannane. In a palladium-catalyzed Stille reaction, fragments **11** and **13** were coupled to isocoumarin **14**; following release of the ketocarboxylic acid by reduction with DIBAH and reoxidation (the silyl ether group ortho to the carboxyl functionality was selectively cleaved) the macrocycle was closed under Mitsunobu conditions (→**15**). The synthesis was finished with elimination of the MOM acetal, chlorination of the arene under mild conditions, and removal of the silyl protecting group.



Scheme 11. Synthesis of radicicol (**1**). DEAD = diethylazo dicarboxylate, DIBAH = diisobutylammonium hydride, MOM = methoxymethyl, TBS = *tert*-butyldimethylsilyl.

In addition to its activity as an antifungal antibiotic[59] and a tranquilizer,[61] radicicol was examined with particular intensity for its selective and potent PTK-inhibitory activity.[50] In an in vitro assay, the natural product inhibited autophosphorylation of the v-Src tyrosine kinase and phosphorylation of an exogenous substrate with an IC_{50} value of 0.1 $\mu g\,ml^{-1}$. In contrast, the serine/threonine kinases PKA and PKC were only inhibited by much higher concentrations of radicicol. Since the natural product also reduced the degree of phosphorylation of the v-Src kinase in vivo, it was possible to perform experiments on rat fibroblasts transformed by v-Src (SR-3Y1-2 cells); in these cells, radicicol converted the transformed phenotype back into the normal form and arrested the cells in the G_1 and G_2 phase of the cell cycle. When treatment with the macrolide was stopped, the fibroblasts adopted the form of the transformed species again. Since the specificity of activity in vivo was not lost, the morphology of cells transformed by c-*erb-B2* could also be normalized (c-*erb-B2* codes for a mutated, and thus permanently activated, receptor tyrosine kinase), whereas this did not occur with cells transformed by *v-raf-1* (*v-raf-1* codes for a serine/threonine kinase, see Section 2.2). By cultivating Friend leukemia cells in the presence of a low dose of radicicol, it was also demonstrated that phosphorylation and dephosphorylation at tyrosine residues play an important role in the differentation of cells: The fraction of differentiated cells that accumulated hemoglobin increased with increasing concentration of radicicol.

Investigations of structure–activity relationships (SAR), previously only performed for the antifungal activity of the natural product, showed that the biological activity decreased on modification of the macrocyclic ring, particularly of the epoxide function, whereas substitution of the aromatic ring had no influence on activity. Inhibition of the proliferation of HeLa cells, and thus antitumor activity of radicicol, was drastically increased by acylation of the phenolic OH group with stearic and palmitic acid as well as by conversion into an ether.[62] Even though initial investigations show that the PTK-inhibitory activity of radicicol disappears in the presence of reagents that can reduce the epoxide unit, such as dithiothreitol, further research in this area is overdue.

3.2. Tyrphostins

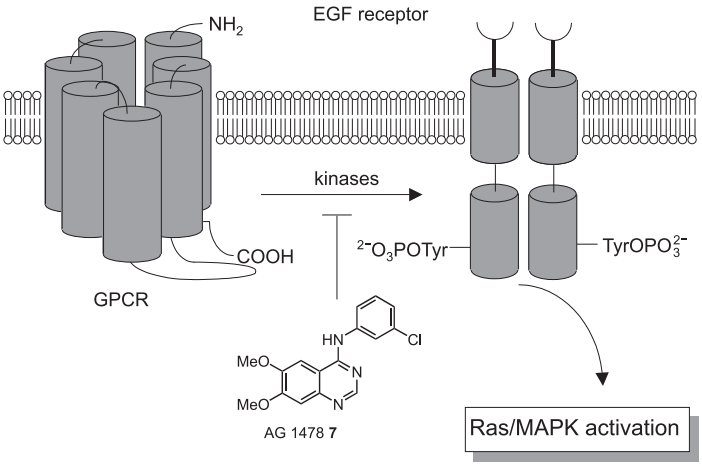
Tyrphostins **6** are particularly well-investigated synthetic PTK inhibitors whose structures are derived from those of erbstatin and tyrosine; they include compounds AG 1478 (**7**) and AG 1296 (**8**, Scheme 10).[49a, b, e] Several hundred representatives of this class of compounds, which are based on a donor-substituted styrene skeleton, have been synthesized and tested for their activity on a large number of kinases.

The majority of the PTK inhibitors found in this way had IC_{50} values in the low micromolar range; however, inhibitors of the newer generation (e.g., **7** and **8**) are often more active by three to four orders of magnitude and are also exceptionally specific for individual receptor subtypes.[49b, e] Tyrphostins are used, for example, to inhibit tumor growth,[63] to reverse transformation of cells,[64] to initiate cell differentiation,[65] to inhibit thrombin-induced platelet aggregation,[66] to inhibit activation of B cells,[67] T cells,[68] and other cell types, to stop growth of psoriatic keratinocytes,[69] and to protect experimental animals from lipopolysaccharide-induced septic shock.[70] In most cases, the molecular target of the inhibitor was precisely determined and, thus, its involvement in the cellular functions described could be demonstrated. These examples show the great potential of synthetic PTK inhibitors as potential medication for diseases such as cancer, restenosis, psoriasis, and sepsis. An interesting finding in these experiments was that, in certain cases, the active compound had to regulate not just a single kinase but rather a broad spectrum of enzymes to achieve the desired pharmaceutical effect.

Beyond the pharmaceutical interest, active and selective PTK inhibitors are also important for research into signal transduction mechanisms. For example, tyrphostin AG 1478 (**7**),[71] which is a highly selective inhibitor of autophosphorylation of the EGF receptor, was used to show that activation of the EGF receptor induced by UV light or strong oxidizing agents, such as H_2O_2, is caused by inhibition of phosphatases that serve to deactivate the receptor again.[72] Similar results were obtained ′with the equally specific tyrphostin AG 1296 (**8**) and the PDGF receptor.[73]

Kinases of the Src family play an important role in the development and progression of tumors, as demonstrated in particular for breast and colon cancer.[74] A 431 cells, which undergo constitutive autophosphorylation of the EGF receptor by autocrine secretion of TGFα (transforming growth factor α), display a tenfold increased activity of the Src kinase in comparison to other cells. It was possible to show that these phenomena are connected, since complete blocking of autophosphorylation of the EGF receptor upon addition of tyrphostin **7** also led to disappearance of the Src activity.[75] Thus, it was demonstrated that increased autophosphorylation of the EGF receptor can be responsible for overactivity of the Src kinase.

Whereas the individual steps of activation of the Ras/MAPK signal path by receptor tyrosine kinases are well understood (see Section 2.2), the activation of this kinase by GPCRs has not yet been explained. It was demonstrated in Rat-1 cells that receptor tyrosine kinases such as the EGF receptor can act as mediators of the mitogenic signals of the G protein coupled receptors; an intracellular crossover of the signal pathways takes place independent of the ligand (in this case, EGF; Scheme 12).[76] If rat fibroblasts are stimulated



Scheme 12. "Cross talk" between G protein coupled receptors and the EGF receptor. MAPK = MAP kinase.

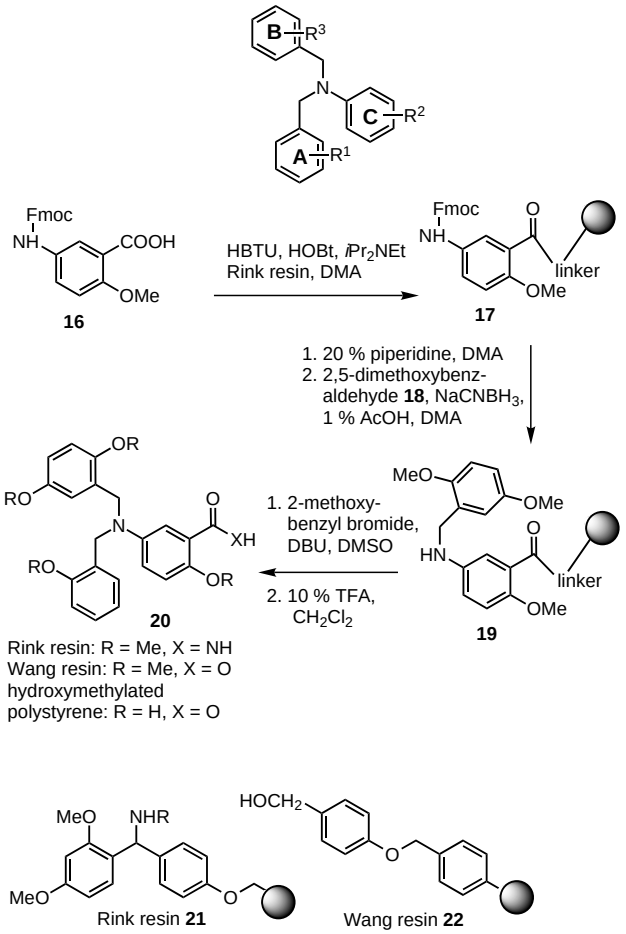
with GPCR agonists such as endothelin 1, lysophosphatidic acid, or thrombin, the receptor for epidermal growth factor is rapidly phosphorylated. If this covalent modification is blocked by addition of the tyrphostin **7** or by transfecting a dominant negative EGF-R mutant into the rat cells, synthesis of the DNA is interrupted and growth is thus stopped. These results give a clear example for crossover of different signal pathways and for the manner in which a membrane receptor, the GPCR, can intracellularly use the activity of another receptor, the EGF-R, without the action of its ligand, EGF. Furthermore, this investigation demonstrates the potential of the use of rapid, effective, and selective in vivo protein tyrosine kinase inhibitors to obtain biological knowledge.

3.3. Lavendustin A and Analogues

An elegant and promising alternative for creation of many potential inhibitors in the shortest time possible is the use of combinatorial chemistry.[77] The natural product lavendustin A (**2**) selectively inhibits the protein tyrosine kinase activity of the EGF receptor, whereas it only has a weak

effect on the activity of the cAMP-dependent kinases PKA or PKC.[78] Detailed kinetic studies show that the substance competes with ATP and peptide substrate, and it reduces the binding affinity of the kinase for both substrates significantly. From these observations, it was concluded that lavendustin A binds to the kinase domain, but outside the binding site for ATP and the peptide. As a target structure for the creation of analogues with modified biological activity by combinatorial synthesis, the substance has ideal properties: The basic skeleton can be assembled from three aromatic units by simple and effective reactions, and it can be bound to a solid phase through the phenolic functionalities or the carboxylic acid.[79]

For the combinatorial synthesis of 60 lavendustin A analogues, four benzyl bromides (**A**), five benzaldehydes (**B**), and three aromatic amino acids (**C**) were used; Rink resin (**21**), Wang resin (**22**), and hydroxymethylated polystyrene were used as polymers (Scheme 13). The Fmoc-protected amino



Scheme 13. Combinatorial synthesis of lavendustin A analogues. DBU = diazabicyclo[5.4.0]undec-7-ene, DMA = *N*,*N*-dimethylacetamide, DMSO = dimethyl sulfoxide, Fmoc = 9-fluorenylmethoxycarbonyl, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxy-1*H*-benzotriazol. The solid support polystyrene is represented by a sphere; for the linker, see **21** and **22**.

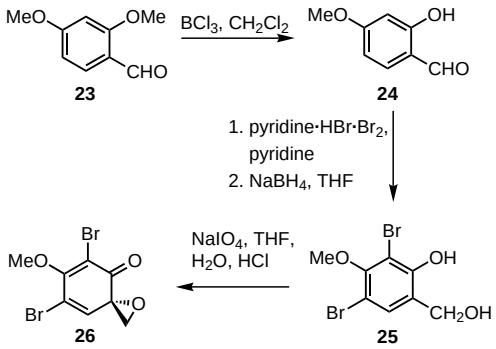
acids **16** (Fmoc = fluorenylmethoxycarbonyl) were first bound to the solid phase through the carboxy functionality (→**17**) and then N-deblocked by treatment with piperidine. Reductive amination with benzaldehydes (e.g. **18**) in the presence of sodium cyanoborohydride led to secondary amines **19**, which were then benzylated in a further step. On cleaving the target molecule from the polymeric phase under acidic (10 % TFA for the Rink resin, 50 % TFA for the Wang resin) or Lewis acid conditions (BBr_3 for hydroxymethylated polystyrene), the individual compounds were obtained protected at the hydroxyl functionality and in the free form. The yields were between 10 and 76 %; the purity was between 30 and 97 %.

3.4. 2-Phenylaminopyrimidine Inhibitors

Recently, 2-phenylaminopyrimidine derivatives were reported to be potent tyrosine kinase inhibitors.[80] By selective variation of the structure and the resulting preferred conformation, it was possible to obtain the highly potent and selective PGDF receptor inhibitor **9** (Scheme 10). Since the substance efficiently penetrates living cells, both autophosphorylation of the receptor and phosphorylation of other substrates were inhibited in vivo. Whereas the inhibitor has no effect on signal transduction stimulated by EGF, insulin, IGF-1, fibroblast growth factor, and phorbol ester, it did block PDGF-induced expression of the c-Fos mRNA in BALB/c 3T3 cells. Based on these findings, it was possible to block proliferation of cells that overexpress the oncogene *v-sis* (v-Sis is a PDGF receptor with a mutation in the transmembrane domain) and that grow due to autocrine PDGF production; the growth of cells transformed by EGF, IL3, and H-Ras was only inhibited by significantly higher concentrations of inhibitor. By feeding **9** to mice in which tumor cells transformed by *v-sis* and *c-sis* had been implanted, it was possible to demonstrate antitumor activity: The degree of tumor reduction was dependent on the dose administered.[81]

3.5. Aeroplysinin

Aeroplysinin (**3**) is a marine natural product which has been isolated in both enantiomeric forms from the sponges *Aplysina aerophoba*[82] and *Ianthella ardis*;[83] it has a highly substituted cyclohexadiene skeleton with two stereocenters. Of its many biological characteristics in addition to its antibiotic activity, its ability to inhibit phosphorylation of exogenous substrates of the EGF receptor in vitro and its antitumor effect on EGF receptor dependent cell lines have attracted attention.[52] However, attempts to investigate the activity of the natural product on autophosphorylation of the EGF receptor in vivo showed that application of the substance did not bring about any change in the phosphotyrosine content of cells.[84] This finding was explained by the high polarity of the *trans* diol unit, which makes it difficult for the substance to penetrate the lipophilic cell membrane. For this reason the more hydrophobic analogue **26** was synthesized (Scheme 14): It has much structural similarity with aeroplysinin, but instead of the diol function it embodies a less hydrophilic epoxyketone unit. This unit also has the advantage that it can be attacked by nucleophiles in the catalytic

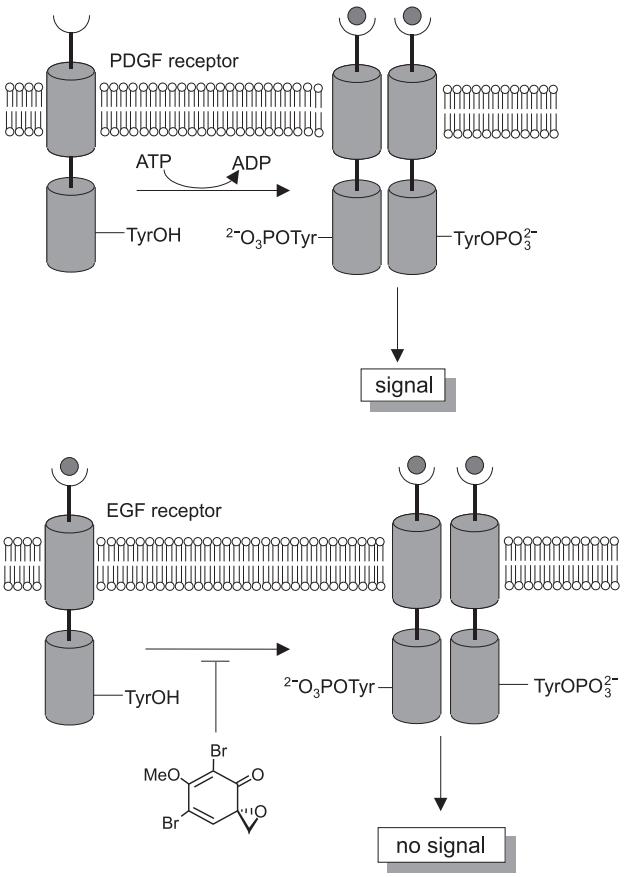


Scheme 14. Synthesis of the biologically active aeroplysinin analogue **26**.

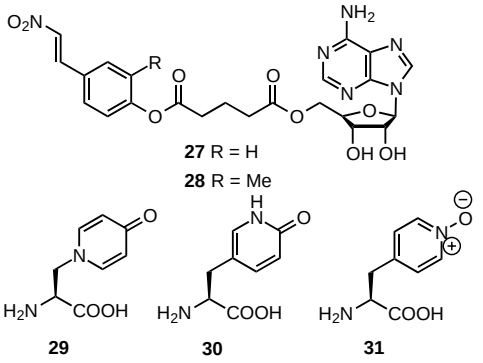
domain of the enzyme, enabling covalent modification, and thus irreversible inhibition, of the tyrosine kinase receptor. Analogue **26** was synthesized in four steps, starting from 2,5-dimethoxybenzaldehyde (**23**), by regioselective cleavage of a methyl ether, bromination, reduction to *ortho*-hydroxybenzyl alcohol, and subsequent Becker-Adler oxidation (total yield 50 %, Scheme 14).

The effect of **26** on autophosphorylation of the EGF receptor was investigated in immortalized rat cells which overexpress the human EGF receptor. The enzyme was inhibited with an IC_{50} value of 10 μM after only five minutes of incubation, whereas autophosphorylation of the PDGF receptor was not blocked, even at a tenfold higher concentration (100 μM, Scheme 15). By synthesis of further structurally related compounds, it was possible to show that the lipophilic bromine atoms and an electrophilic functional group are essential for this rapid in vivo activity. In addition, the epoxide function is opened and an unexpected rearomatization of the system takes place on reaction of **26** with nucleophiles, which supports the above-mentioned hypothesis of a possible covalent modification of the enzyme. The potent and selective activity of this synthetic inhibitor, which occurs almost directly after application of the substance, can now be used to selectively disrupt signals stimulated by EGF.

This is a particularly clear example of how organic synthesis and biological findings can be mutually fruitful: The (biological) observation of the lack of in vivo activity of a natural product leads to rational design and synthesis of an active inhibitor that can now be used in further biological and pharmaceutical experiments.



Scheme 15. Selective blocking of signal transduction by the EGF receptor with the synthetic aeroplysinin analogue **26**, which has no effect on the PDGF receptor (see text for details).



Scheme 16. Bisubstrate analogues and tyrosine mimetics.

3.6. Other Inhibitors

In another interesting experiment, putative bisubstrate analogues were synthesized based on the probable transition state for phosphate transfer from ATP to tyrosine residues.[85] Compounds **27** and **28** contain a nitrostyrene residue as a tyrosine mimetic, which is separated by an alkyl spacer from a glutaryl-adenosine unit that mimicks an ATP molecule (Scheme 16). Compounds **27** and **28** inhibited the EGF receptor with an IC_{50} value of less than 1 mM and also showed significant subtype selectivity for the PTKs v-*Abl* and c-*Src* and also for the Ser/Thr kinase PKC. In an in vivo system with strongly EGF-dependent epidermal murine keratinocytes, these compounds also demonstrated potent antiproliferative activity.

In addition, some publications describe attempts to obtain potent and specific inhibitors of certain tyrosine kinases by incorporating a modified tyrosine derivative in a substrate peptide of the particular kinase. Examples of tyrosine mimetics that should react irreversibly with nucleophilic side chains at the active center of the enzyme are L-3-deoxymimosine (**29**),[86] the pyridone **30**,[87] and the pyridine-*N*-oxide **31**[87], which should react with nucleophiles after O-phosphorylation by the enzyme. However, the observed biological activity was below expectations.

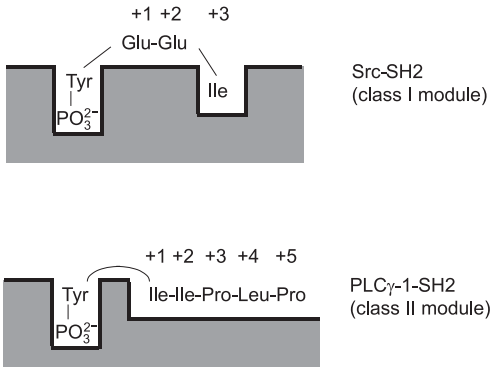
4. Investigation and Inhibition of Interactions with SH2 and SH3 Domains

Noncovalent interactions between proteins play a decisive role in biological phenomena. They are often mediated by modules that are used universally throughout nature and can be found in many proteins such as PTKs, lipid kinases, protein phosphatases, phospholipases, Ras-controlling proteins (see Section 2.2), transcription factors, and adaptor proteins in all eucaryotic cells.[88, 89] These units, which generally have conserved structures independent of the surrounding amino acids and the ligands involved, include the well-investigated SH2 and SH3 domains (see Section 2.2). Combinatorial synthesis of larger peptide libraries was successfully used for investigation of the interactions of SH2 and SH3 domains with their ligands and for discovery of the first modulators of these interactions. Natural products with similar activity are not known at present.

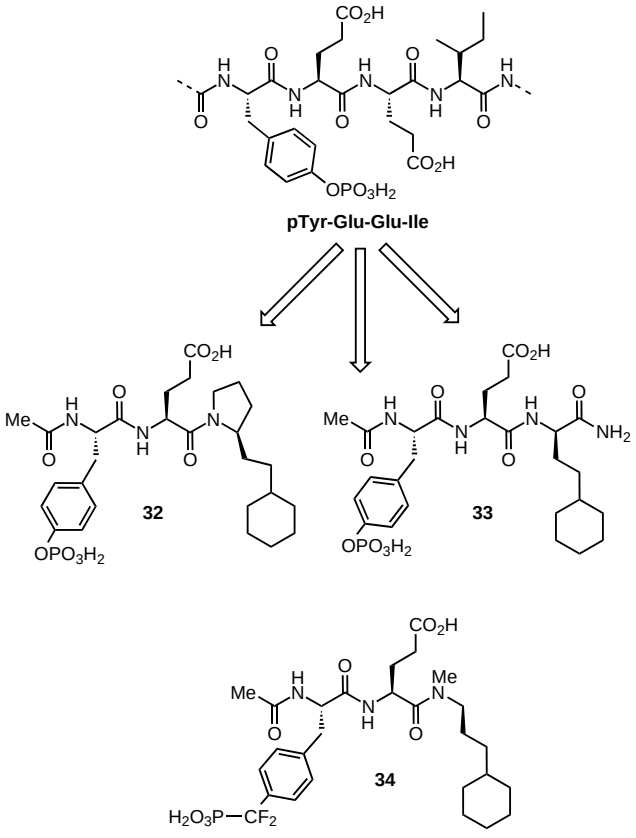
4.1. SH2 Domains

SH2 domains are clusters of about 100 amino acids that recognize and bind peptides phosphorylated on tyrosine residues; the affinity of the ligands is in the region of $K_D = 10-100$ nM.[90] With combinatorial chemistry, it was possible to find a structural basis for the interaction between SH2 domains of certain proteins and their specific binding partners.[91] A library was synthesized of $18^3 = 5832$ phosphopeptides with the general sequence Gly-Asp-Gly-pTyr-Xxx-Xxx-Xxx-Ser-Pro-Leu-Leu-Leu, in which the three Xxx residues were a variety of all natural amino acids except Cys and Trp; the affinity for 14 different SH2 domains was tested. It became apparent that two categories of SH2 domains can be differentiated: class I modules (which include the domains of the proteins Src, Fyn, Lck, Fgr, Abl, Crk, and Nck) bind motifs of the type pTyr-Xxx-Xxx-Ile/Pro (where Xxx is a hydrophilic amino acid), whereas class II modules (which appear in proteins such as p85, PLC$_\gamma$, and SHPTP2) prefer the motif pTyr-Yxx-Zzz-Yyy (where Yyy is a hydrophobic amino acid, and Zzz is any amino acid). With computerized sequence comparisons of the experimentally determined preferred motifs with the sequence of natural proteins, it was possible to identify potential new ligands and target molecules for the different SH2 domains in a straightforward manner. These predictions were confirmed for the binding partners of p85 and PLC$_\gamma$. With the help of NMR and X-ray data, the structures of the domains were determined both in the presence and absence of ligand.[92] Thus, the observed selectivity was explained at the molecular level, and it was shown that the pTyr residue is bound in a deep pocket by ionic (with the phosphate) and hydrophobic interactions (with the aromatic groups), whereas the C-terminal amino acids are either bound by a second pocket (class I domains; a quasi socket is formed here) or by a hydrophobic cleft (Scheme 17).

The high specificity with which the SH2 module of a certain protein recognizes the phosphotyrosine-containing sequences of a different protein makes inhibition or modification of this interaction a rewarding target for bioorganic and medical chemistry research, since it provides the opportunity to disrupt individual signal paths at precisely defined points with suitable substances. In particular, there has been no lack of attempts to find antagonists for the socketlike SH2 domain of the Src kinases which prefer the sequence pTyr-Glu-Glu-Ile (class II). The tetrapeptide analogues **32**, **33**, and **34** (Scheme 18), which all have a hydrophilic (pTyr) and a hydrophobic "plug" (Ile) for the SH2 "socket", have high binding affinities similar to those of longer peptide sequences with the naturally preferred motif (see above).[93, 94] Further



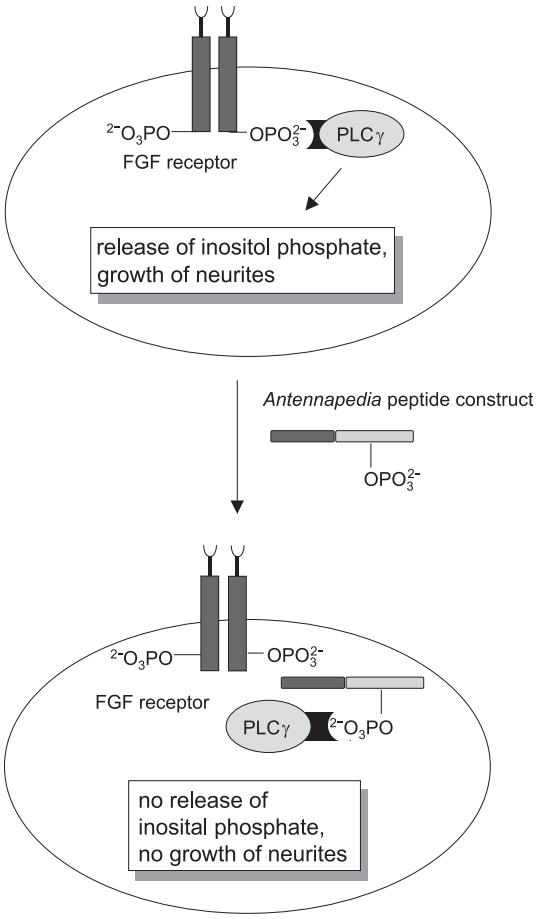
Scheme 17. Schematic representation of the structure of the two classes of SH2 domains.



Scheme 18. Inhibitors, derived from pTyr-Glu-Glu-Ile, of the interaction between the SH2 domain of c-Src and natural ligands.

work on this theme includes combinatorial synthesis of tyrosine-containing peptides[95] as well as the rational design and synthesis of phosphotyrosine peptide mimetics as ligands of c-Src.[96] In addition, conformationally rigid cyclic peptides that have phosphonomethylphenylalanine or phosphotyrosine residues were synthesized as inhibitors of the interaction between PTKs and the SH2 domains of different ligands.[97] The great potential of synthetic inhibitors is demonstrated, for example, by the finding that the pentapeptide acetyl-pTyr-Glu-Glu-Ile-Glu is able to block the interaction between Src and its cellular phosphoprotein partners in cell lysates (i.e. in vitro) even though the peptide has no antiproliferative effect on cells in vivo:[98] It cannot penetrate the cell membrane owing to its high polarity and is degraded by cellular phosphatases and peptidases.

Recently, however, a new and promising possibility for transport of peptide compounds through the membrane was reported. The SH2 domain of phospholipase C (see Section 2.1) recognizes the phosphotyrosine-containing sequences of receptors which are activated on binding their ligands, for example, the receptors for fibroblast growth factor (FGF), EGF, PDGF, or neurotrophin 3 (NT-3). This binding is necessary for hydrolysis of phospholipids and release of inositol phosphates. Phosphorylated peptides corresponding to the phosphotyrosine-containing sequences inhibit this binding in vitro.[99] A sequence of 16 amino acids from the *Antennapedia* protein, which can penetrate biological membranes and thereby transport other peptides and nucleotides,[100] was successfully used to transport these peptides into the cell interior. In an interesting experiment, this sequence was coupled to a nonapeptide corresponding to the sequence recognized by PLC_γ at the FGF receptor. The conjugate was investigated for its activity in cerebral neurons (Scheme 19).[101] Complete inhibition of release of inositol phosphates was achieved with a concentration of 1 $\mu g\,ml^{-1}$ of the phosphorylated peptide, whereas the unphosphorylated peptide showed no activity at all. To demonstrate that the active substance had actually penetrated into the cytoplasm, its uptake of a biotinylated derivative was followed by confocal microscopy. The peptide specifically inhibits FGF-stimulated hydrolysis of phospholipids: Treatment of the cells with PDGF or neurotrophin 3 led to release of inositol phosphates despite the presence of the active substance. Furthermore, it could be shown that activation of PLC_γ is necessary and sufficient for stimulation of FGF-induced growth of neurites, since this growth reaction was blocked in the presence of the peptide inhibitor. These findings document that, with this method, even polar peptides can be used for selective interruption of signal cascades. In the JAK/STAT pathway, the STAT proteins are activated by phosphorylation at tyrosine residues and subsequent dimerization of these transcription factors by interactions between phosphotyrosine and SH2 domains (see Section 2.3). As a result, they migrate into the cell nucleus and bind DNA there. This mechanism was elucidated with phosphotyrosyl peptides which, according to their sequence, were able to induce dissociation of the homodimer and thereby prevent formation of the protein–DNA complex.[102] Non-phosphorylated analogues of the peptide did not trigger this effect.



Scheme 19. In vivo inhibition of the binding of PLC_γ at the FGF receptor by an *Antennapedia* peptide construct.

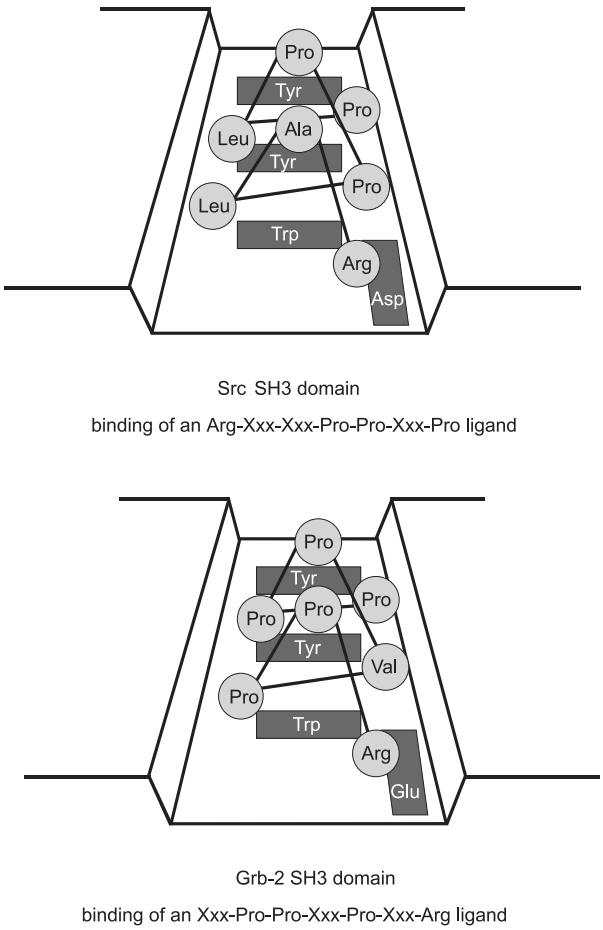
The human estrogen receptor (HER) is a transcription factor that is regulated by binding of a lipophilic hormone; it must also undergo homodimerization to bind to DNA and activate gene transcription. The role of phosphorylation of Tyr 537 of HER in dimerization and activation of the transcription factor was studied with a phosphotyrosine peptide that represents the immediate environment of this phosphorylation site.[103] This dodecapeptide completely inhibits binding of HER to the estrogen response element (ERE) of the DNA and was able, by specific binding of the receptor monomer, to prevent dimerization of the transcription factor. Unphosphorylated peptides and phosphopeptides with other amino acid sequences could not trigger this effect. Based on these findings, a mechanism similar to that for activation of the STAT proteins was postulated, that is, homodimerization by SH2-mediated interactions. A further interesting aspect of this work is that the peptide—in contrast to known antagonists such as clomiphen, tamoxifen and RU 486—inhibits the activity of the estrogen receptor without attacking the hormone binding site.

The protein-tyrosine phosphatase SH-PTP2 has two SH2 domains and, on stimulation by growth factors, it binds to the PDGF receptor, the EGF receptor, and also to the multiple tyrosine phosphorylated insulin receptor substrate 1 (IRS 1). Synthetic phosphotyrosyl peptides with sequence homology to IRS 1 were able to significantly increase dephosphorylation by the enzyme by allosteric activation.[104]

A tyrosyl phosphopeptide derived from EGF receptor, containing the Tyr 1068 of this protein, was used to inhibit interaction of the receptor with the adaptor protein Grb2. Infiltration of this peptide into cells leads to a weaker stimulation of the Ras protein and MAP kinase.[105]

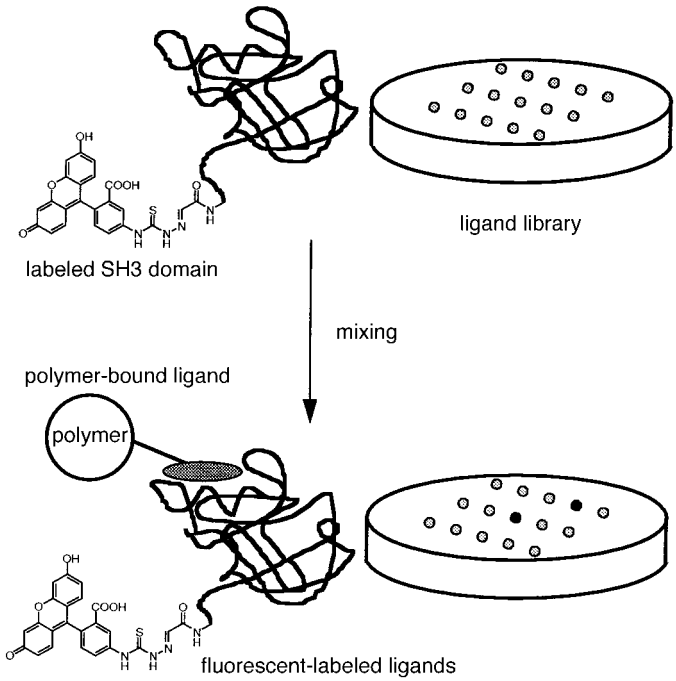
4.2. SH3 Domains

SH3 domains are made up of about 60 amino acids and bind proline-rich sequences that form a left-handed polyproline helix of type II (Scheme 20); the affinity of the peptide is in



Scheme 20. Possible orientation of the Pro-Pro-Xxx-Pro motif in SH3 domains of Src and Grb 2.

the region of $K_D = 5 - 100$ μM.[88] In addition to the determination of the structure of the domains and their ligands,[106] the investigation of the binding specificity and characteristics of this module was also accelerated with combinatorial chemistry.[107] Based on the observation that peptides with the sequence Pro-Pro-Xxx-Pro of the SH3 domain of phosphatidylinositol kinase were bound particularly well, a "biased" library of the general form Xxx-Xxx-Xxx-Pro-Pro-Xxx-Pro-Xxx-Xxx (where Xxx is any amino acid except cysteine) was synthesized which, with the Pro-Pro-X-Pro motif, should have a certain basic affinity to the SH3 domain used.[108] By combinatorial synthesis with the "split and combine" process, about two million different peptides were assembled on poly(dimethylacrylamide) beads, so that exactly one defined compound was attached to each bead. To detect binding of the potential ligands to the SH3 domain, the recombinant SH3 domain of the phosphatidylinositol 3-kinase was coupled to fluorescein-5-thiosemicarbazide following oxidization of the N-terminal serine to glyoxylamide, and the library was treated with this labeled protein (Scheme 21). Of 70 beads that bound

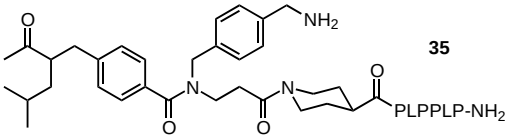


Scheme 21. Combinatorial synthesis of peptide ligands for the SH3 domain of the PI-3 kinase.

to the fluorescent protein, the 17 with the highest fluorescence were selected, and the attached peptides were sequenced by Edman degradation. The binding affinities measured for the ligands thus identified were amongst the highest known ($K_D = 8 - 30$ μM).

Interestingly, two classes of peptides were identified which differ in the location of a basic arginine residue at the C terminus of the common Pro-Pro-Xxx-Pro motif (Xxx-Pro-Pro-Leu-Pro-Xxx-Arg, class I ligands) in one case and at the N terminus (Arg-Xxx-Leu-Pro-Pro-Leu-Pro-Xxx-Xxx, class II ligands) in the other. Determination of the structure from the library of peptides that bind Src SH3 domains with multidimensional NMR techniques gave the surprising result that the helices of the ligand can be bound in two different orientations (Scheme 20). The arginine residue forms a salt bridge with either an aspartate or a glutamate residue in the binding pocket and thus determines the position of the peptide, whereas the side chains of the proline and leucine residues undergo hydrophobic interactions with aromatic amino acids in the protein.[109] Since these noncovalent bonds are common to all domain–ligand systems, the specificity of the SH3 module of a particular protein for its partner is mediated by interactions of the peptide with two of the central loops flanking the binding pocket (Scheme 20).

Identification of peptide-analogous ligands was achieved similarly with a combinatorial method. Four building units from a pool of 64 compounds were linked by amide and urethane bonds to a Pro-Leu-Pro-Pro-Leu-Pro sequence, which should guarantee orientation of the selected analogues in the manner of a class I ligand.[110] The affinity for SH3 domains of the ligands bound to the solid phase was determined with a biotinylated Src SH3 domain that binds to a complex of streptavidin and alkaline phosphatase and thus produces a detectable signal. From a library of 1.1 million compounds, 15 active ligands (e.g. **35**, Scheme 22) with small



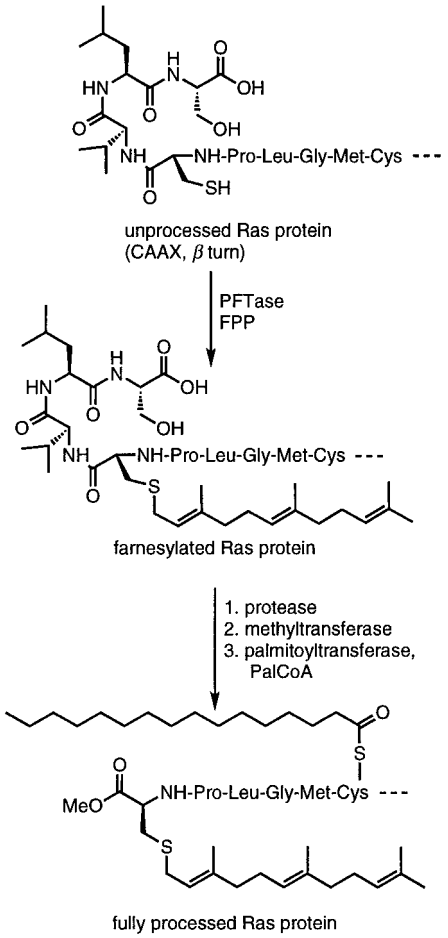
Scheme 22. Non-peptide ligand of the Src SH3 domain. P = Pro, L = Leu.

dissociation constants ($K_D = 3.4 - 30$ μM) were found. With the use of NMR spectroscopy, it was also shown that the binding specificity of peptide-analogous ligands arises from contacts other than those of the peptide binding partner. By designing substances that bind to both contact points, it should be possible to obtain very effective inhibitors for interactions with SH3 domains.[111]

5. Inhibition of the Ras Farnesyltransferase (PFTase)

Ras proteins function as central switches for signals given by growth factors that direct cell growth, cell differentiation, and other genetic programs.[112] The Ras proteins must be associated with the membrane to perform this function. They are anchored in the membrane by co- and post-translational modification of the C terminus of the polypeptide with lipid residues which bind the protein to membranes.[113] To this end, the cysteine of the C-terminal CAAX sequence (C is cysteine, A is generally an aliphatic amino acid, and X is methionine, serine, alanine, or glutamine) of a precursor protein is first enzymatically farnesylated during biosynthesis, the AAX part is then cleaved off by a specific protease, and the free terminal cysteine is finally converted into the methyl ester (Scheme 23). In addition, in H- and N-Ras a further lipid modification of cysteine occurs by formation of palmitoyl thioesters in the immediate vicinity of the CAAX motif. It was shown that farnesylation is essential for the transforming activity of mutated Ras proteins.[114] Inhibition of this lipid modification opens up various possibilities for studying signal transduction processes and for developing alternative medicinal – chemical strategies, for example in tumor therapy.[115]

Since the three-dimensional structure of the heterodimeric Ras farnesyltransferase was only recently obtained,[116] it was necessary to draw on information about substrate specificity as well as the conformation of the substrate in solution and bound to the enzyme (obtained from NMR spectroscopic investigations; the bound ligand Cys-Val-Trp-Met forms a β



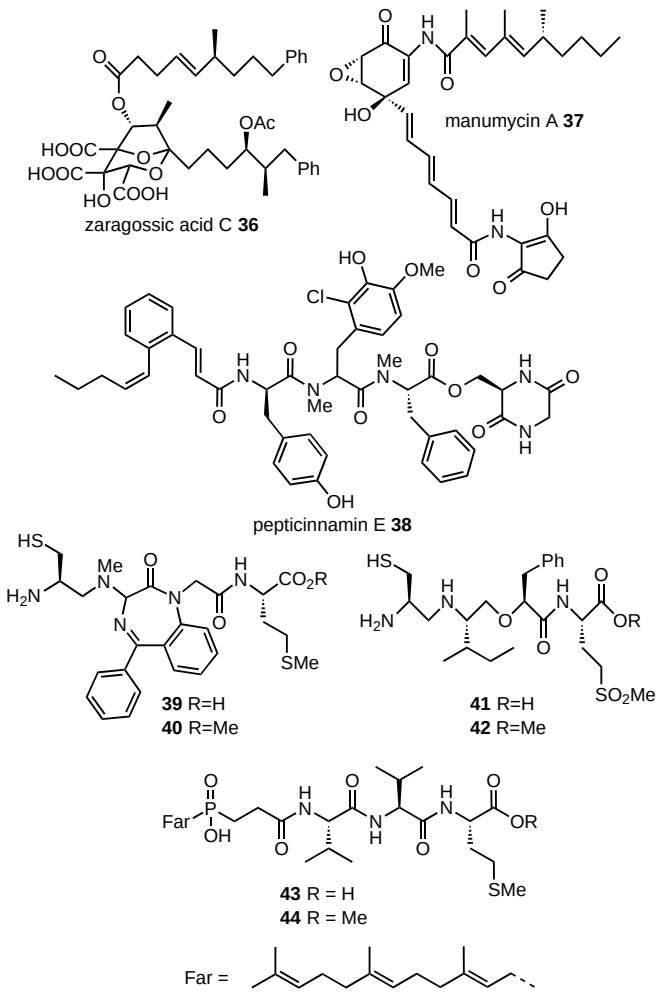
Scheme 23. Post-translational modification of the Ras protein. FPP = farnesyl pyrophosphate, Pal = palmitoyl.

loop of type III (Scheme 23))[117] to develop inhibitors of this enzyme. In addition, natural products with potent PFT-inhibiting activity were found in screening tests including, for example, zaragozic acids (e.g. **36**),[118] manumycin (**37**),[119] and pepticinnamin E (**38**)[120] (Scheme 24).

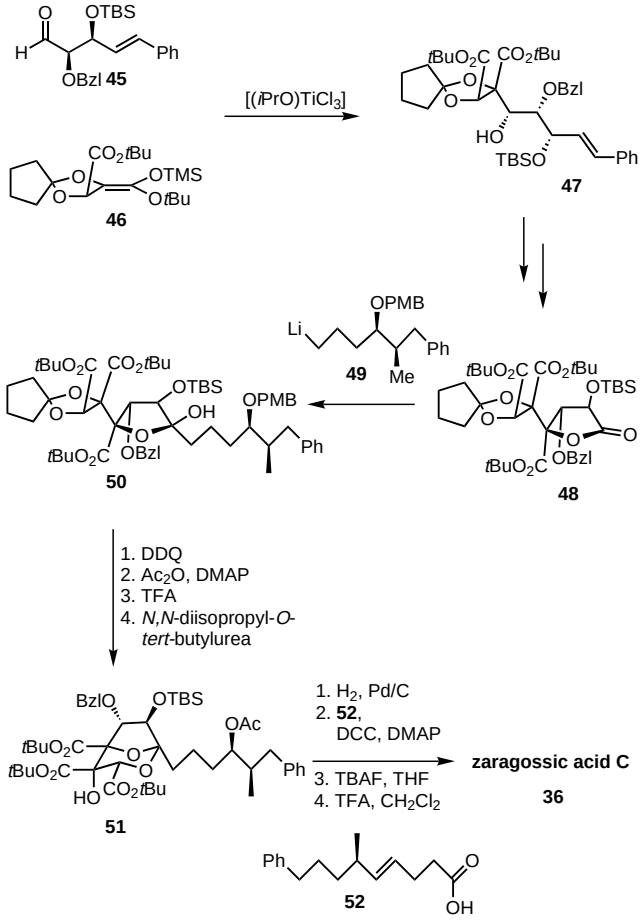
5.1. Naturally Occurring Ras Farnesyltransferase Inhibitors

5.1.1. Zaragozic Acids

The zaragozic acids (e.g. **36**, Scheme 24), first found in fungal cultures in the vicinity of the Spanish town Zaragoza, inhibit squalene synthase in addition to farnesyltransferase.[118] The high therapeutic potential of these complex compounds—which interfere with cholesterol biosynthesis and are therefore of interest for medical indications such as arteriosclerosis, hypertension, and cardiac infarct—was the reason for intensive research leading to the successful total synthesis of this new type of natural product.[121, 122] The biggest challenge was the stereospecific construction of the highly polar 2,8-dioxabicyclo[3.2.1]octane skeleton which carries three carboxy and three hydroxyl groups at five stereocenters. One of these hydroxyl groups must be selectively acylated with a fatty acid side chain. An alkyl side chain must also be attached.



Scheme 24. Inhibitors of the Ras farnesyltransferase.



Scheme 25. Synthesis of the zaragozic acid C. Bzl = benzyl, DCC = *N,N'*-dicyclohexylcarbodiimide, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, DMAP = 4-dimethylaminopyridine, PMB = *para*-methoxybenzyl, TBAF = tetrabutylammonium fluoride.

After removal of the acyl side chain **52**, the resulting alcohol **51** can be retrosynthetically converted back into the lactol **50** (Scheme 25).[122] The latter is obtained by nucleophilic attack of the lithiated alkyl chain in **49** on the lactone **48**, which was obtained from dihydroxysuccinic acid. Assembly of the C_5–C_7 skeleton in **45** was achieved by a highly *syn*-diastereoselective Evans aldol reaction of a boron enolate with phenylacrolein and subsequent removal of the chiral auxiliary. The silyl ketene acetal **46**, which was obtained from the di-*tert*-butyl ester of dihydroxysuccininc acid, underwent a highly diasteroselective aldol reaction with aldehyde **45** to form alcohol **47**. With the help of standard transformations, **47** was converted into lactone **48**, which possesses all eight carbon atoms of the bicyclic skeleton as well as the equivalents of the alcohol and carboxyl groups. The C_1 side chain was introduced by addition of the organolithium compound **49** to **48**. After manipulation of the protecting groups on the side chain, lactol **50** was converted under acid conditions and by transacetalization into the desired bicyclic **51**, in which the carboxyl functionality was protected again as the *tert*-butyl ester. Selective cleavage of the benzyl ether, introduction of the acyl side chain **52**, and deprotection of the hydroxyl and carboxyl functionalities gave zaragozic acid C (**36**). This synthesis should be applicable to the preparation of zaragozic acid A, which only differs from **36** in the alkyl side chain. Noteworthy features of this total synthesis include the high degree of stereocontrol, the low number of steps (21 for the longest linear sequence), the associated high total yield of 15%, and the clever choice of protecting groups.

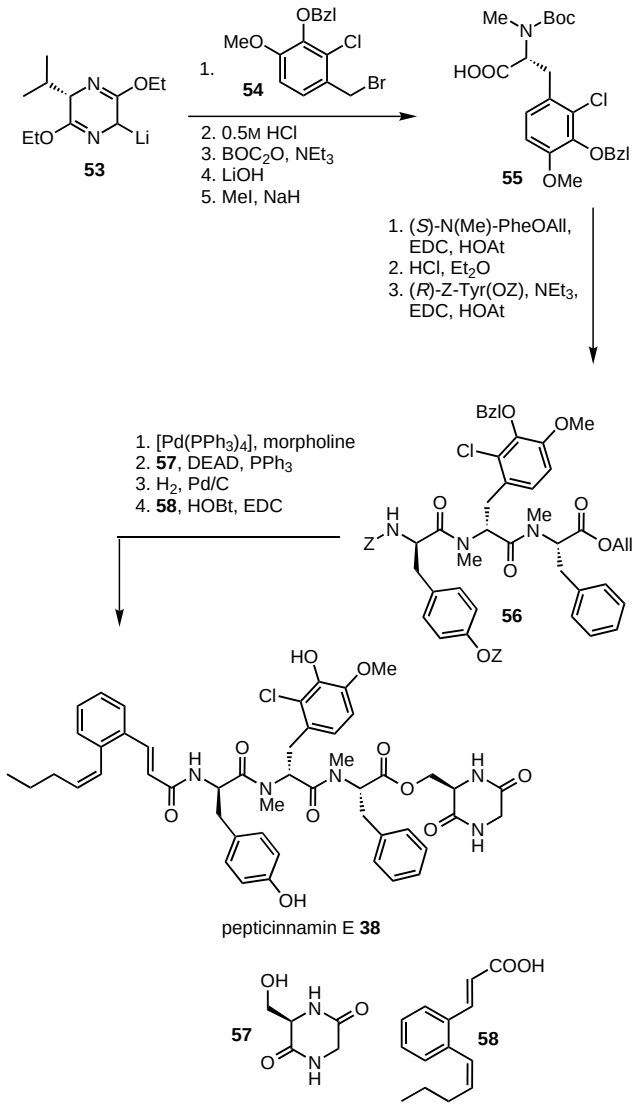
The zaragozic acids show very interesting biological activity: They interfere with the biosynthesis paths of cholesterol by inhibiting the squalene synthase that uses farnesyl pyrophosphate (FPP) as substrate. Zaragozic acid A was also identified as the inhibitor for another enzyme that uses FPP, the Ras farnesyltransferase. It was thereby shown that the natural product acts in competition with FPP rather than with the peptide substrate. Probably, the carboxy and hydroxyl functionalities mimick the bisphosphate, and the hydrophobic side chains imitate the isoprene part of the farnesyl pyrophosphate.[123] If inhibitors of the PFTase or squalene synthase are to be used in pharmaceutical or biological experiments, their selectivity for other prenyl-transferring enzymes—for example, the geranylgeranyl transferases (GGTases)—will be of great importance, since a large number of proteins are modified with the C_{20} diterpenes. By a slight modification of the alkyl side chain of zaragozic acid A, this selectivity was significantly improved; activity against PFTase was raised by a factor of 18, whereas activity against geranylgeranyl trans-

ferase was reduced by a factor of 2.7. These results suggest that other structural modifications may enable further improvement of these characteristics. It is interesting that, in intact cells, both compounds do not influence the function of the Ras protein, even at high concentrations and incubation times of 24 h. Here it can also be assumed that the compounds are too polar, owing to the carboxy and hydroxyl functionalities of the bicyclic core, to be able to penetrate the cell membrane; they would have to be masked to achieve any in vivo effects.

5.1.2. Pepticinnamin E

Pepticinnamin E (**38**, Scheme 24) was isolated from *Streptomyces* species and is a structurally interesting PFTase inhibitor.[120] One can speculate that the natural product is a transition state analogue for farnesyltransferase, in which the hydrophobic alkenyl acrylic acid imitates the prenyl residue and the conformationally rigid N-methylated peptide skeleton imitates the peptide substrate. A total synthesis of the natural product is of considerable interest since the absolute configuration of the unusual central amino acid is unknown. Furthermore, owing to its modular construction, the substance also permits various derivatizations and the combinatorial synthesis of analogues with modified biological activity.

For synthesis of pepticinnamin E[124] (**38**, Scheme 26), acrylic acid **58** was synthesized diastereomerically pure by esterification, *Z*-selective Wittig reaction, reduction to aldehyde, and *E*-selective Perkin reaction, starting from 2-carboxybenzaldehyde (total yield 31 %). The central unusual amino acid in the natural product was obtained in the form of both required enantiomers by a Schöllkopf reaction. For this, lithiated bis(lactim ether) **53** was alkylated with benzyl bromide **54**. Cleavage of the auxiliary, protection of the amino function, ester saponification, and methylation of the urethane gave amino acid **55** and its enantiomer, each in the enantiomerically pure form. The central tripeptide unit **56** was obtained by classical peptide coupling. The allyl ester was cleaved from **56** by Pd^0-mediated allyl transfer with morpholine as accepting nucleophile. Esterification of the carboxylic acid obtained with alcohol **57** by activation of the carboxy function with the help of carbodiimide or trichlorobenzoyl chloride only gave poor yields. Only nucleophilic attack of the carboxylic acid on alcohol **57**, activated according to Mitsunobu, gave the desired ester in satisfactory amounts. Following removal of all benzyl protecting groups, coupling with acrylic acid **58** delivered the natural product **38**.



Scheme 26. Synthesis of pepticinnamin E. All = allyl, Boc = *tert*-butyloxycarbonyl, EDC = *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide, HOAt = 7-aza-1-hydroxy-1*H*-benzotriazole, *Z* = benzyloxycarbonyl.

5.1.3. Manumycin A

Manumycin A (**37**, Scheme 24), a natural product isolated from a *Streptomyces* species,[119] is an example of a class of structurally novel PFTase inhibitors. In addition to the complex parent compound, structurally simpler degradation products and derivatives also block the activity of the PFTase from yeast and rat brain. With the help of manumycin A, a simple test system for in vivo activity of the enzyme inhibitor was established for the development of the thread worm *Caenorabditis elegans*.[125] Both manumycin A and the basic aminoepoxycyclohexenone structure possess antitumor activity. The chemical synthesis[126] of analogues of the natural product should have great potential for providing highly active, selective, and metabolically stable enzyme inhibitors.

5.2. Peptidomimetics

Substances that compete with the peptide, in particular with the CAAX motif, should have particularly advantageous selectivity of inhibition toward other enzymes, since GGTases prefer different sequence motifs (CAAL, where L is leucine) to the PFTases.[115] Benzodiazepines such as **39** and **40** (Scheme 24) are CAAX peptidomimetics, whose central unit imitates a β turn. In addition, they bring the NH_2 terminus of the cysteine analogue and the COOH terminus of the methionine in spatial proximity; these can then complex a Zn^{2+} ion, which is essential for activity of the PFTase and

binding of the peptide substrate.[127] The free acid **39** inhibits the enzyme with an IC_{50} value of less than 1 nM, whereas in intact cells methyl ester **40**, despite its weaker in vitro activity, is significantly more potent; it can penetrate the plasma membrane better owing to its lower polarity. This property can be used to convert the morphology of cells that are transformed by *H-ras* back into the normal form and to inhibit growth of these cells. In contrast, the substance shows no effect on *src*-transformed and untransformed rat fibroblasts. The inhibitor therefore acts selectively on transformed cells and does not influence growth of normal cells. This result is noteworthy because farnesylation of the wild-type H-Ras protein is inhibited by the benzodiazepine, and H-Ras is involved in the growth of normal fibroblasts. Detailed examination of this unexpect result shows that inhibitor **40** reduces the amount of farnesylated H-Ras protein in cells that are both untransformed and transformed by *H-ras*. However, *only* in the transformed cells does this lead to the expected reduction of enzyme activity in the Ras cascade: Both the amount of phosphorylated Raf and the activities of MEK-1, MEK-2, and MAP kinase were reduced in the tumor cells.[128] In addition, stimulation of the Ras signal pathway was not influenced by EGF in normal or *src*-transformed cells. The normal cells apparently have the ability to activate the signal pathway with or without the reduced involvement of Ras. In fact, further experiments have shown that farnesylation of the K-Ras protein by the inhibitors is considerably less influenced than that of H-Ras. K-Ras is also a substrate of the GGTase I and can therefore be geranylgeranylated if activity of the PFTase is reduced.[129] Normal cells and *src*-transformed cells, in contrast to those transformed by *H-ras*, are able to compensate for loss of a Ras protein or an enzyme important for normal cell growth, by activation of alternative pathways.

5.3. Peptide Analogues

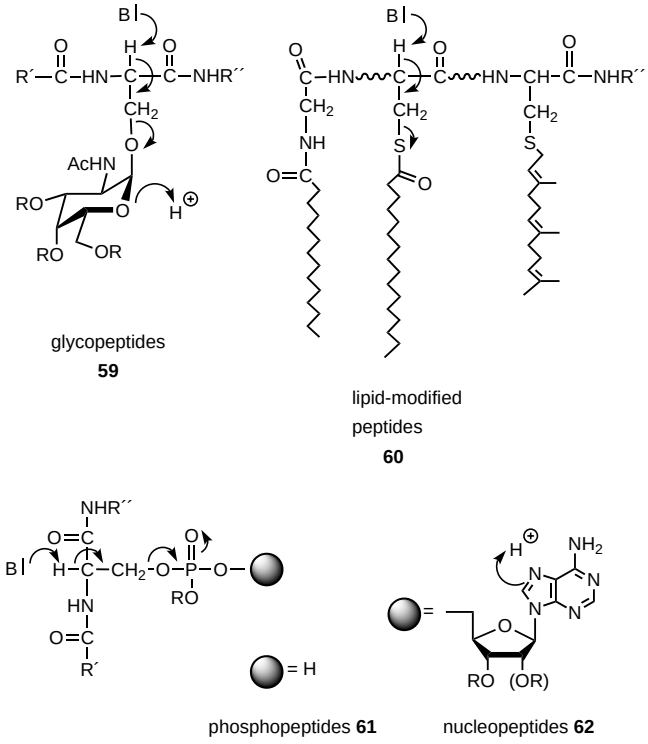
Another inhibitor obtained by rational design is the CAAX β turn mimetic **41** (Scheme 24).[130] It is an analogue of CAAX peptides in which peptide amide bonds are replaced by ether and amine groups. The compound inhibits the PFTase in vitro with an IC_{50} value of 1.8 nM and shows highly specific activity in comparison to inhibition of GGTase I. The less polar methyl ester **42** as prodrug showed better results in vivo and inhibits both farnesylation of the Ras protein and growth of *ras*-transformed cells, whereas proliferation of *raf*- or *mos*-transformed cells was not influenced. Growth of human pancreatic adenocarcinoma cells with mutated *K-ras*, *c-myc*, and p53 genes was inhibited by application of **42**. If the compound is administered over a period of five days to mice with implanted Ras-dependent tumors, tumor growth can be reduced by up to 66% compared to untreated mice, whereas the application of the antitumor antibiotic doxorubicin only resulted in 33% reduction under the same conditions. It is particularly noteworthy that treatment with the β-turn mimetic, in contrast to treatment with doxorubicin, was without any visible side effects such as weight loss.

5.4. Bisubstrate Inhibitors

The phosphinic acids **43** (R = H) and **44** (R = Me; Scheme 24) are examples of bisubstrate analogues for which, a priori, higher activity and selectivity compared to mimetics of the individual substrates alone might be expected.[115] Indeed, **43** is an effective in vitro inhibitor, and the prodrug **44** has activity in cells transformed by *H-ras* and—to a lesser extent—*K-ras*.[131] Prodrug **44** also inhibits growth of malignant cells of the neurofibromatosis type I, which are dependent on overactivation of the wild-type Ras protein.[132] These highly promising results are tempered, however, by the lower bioavailability and in vivo activity in comparison to the peptidomimetics.

6. Peptide Conjugates as Tools for Studying Biological Signal Transduction

The proteins involved in transduction of signals from the plasma membrane into the cell nucleus often have additional covalently linked structural units which are absolutely necessary for fulfillment of their biological function (Scheme 27).



Scheme 27. Structure and lability of peptide conjugates. B = base.

For example, the cell-surface receptors for growth factors (see Scheme 5) and the ligands recognized by them are often glycoproteins in which serine, threonine, and asparagine units are linked to oligosaccharides (**59**). The signal-transmitting proteins located in the membrane, such as the Ras protein, are lipid-modified at cysteine and glycine residues (**60**). In many cases, the signals are passed on by phosphorylating proteins to be switched on at serine, threonine (**61**), and tyrosine

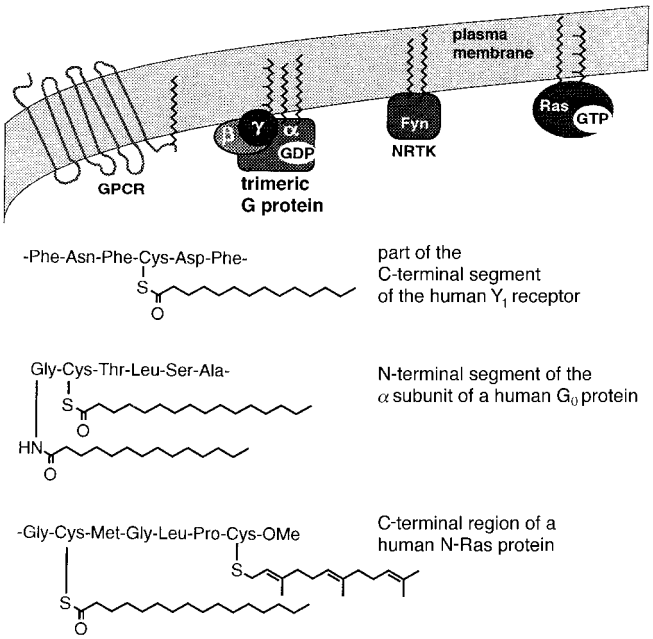
residues; DNA, which is often the target of the signal cascade, exists as a nucleoprotein in which serine, threonine, or tyrosine are linked as phosphoric acid esters to the nucleic acid chain (**62**).

Peptides containing the essential covalently modified structural units of these protein conjugates may be useful tools for studying their biological functions as well as for selectively manipulating the signal cascades in which they are involved (see below). However, synthesis of such peptide conjugates is hindered, by their multifunctionality, which requires the use of many orthogonally stable amino, hydroxyl, mercapto, carboxy, and phosphate protecting groups.[133–136] On the other hand, the peptide conjugates are also particularly acid and base labile. Thus at pH greater than 9, the side chain functionalities are cleaved from the serine glycosides **59**, the lipid-modified peptides **60**, and the phospho- and nucleopeptides **61** and **62** in a β elimination; the thioester in **60** hydrolyzes spontaneously in aqueous solution at pH greater than 7. In acid, there is a danger of anomerization or even cleavage of the *N*- and *O*-glycosidic bonds in **59** and **62**, and the olefins of the farnesyl residue in **60** are easily attacked by acids. In the synthesis of the peptide conjugates of type **59**–**62**, all reactions, particularly the removal of all the diverse protecting groups, must take place under mild (preferentially neutral) conditions, yet with complete preservation of orthogonal stability.

On one hand, peptide conjugates are of great interest as reagents and chemical tools for biological studies. On the other hand, their synthesis is a considerable challenge for organic chemistry. New, alternative synthetic methods must be developed in order to obtain complex peptide conjugates efficiently and with high selectively under very mild conditions. This area of organic synthesis offers an excellent opportunity for initiating bioorganic research as defined in Section 1. The chemistry of the peptide conjugates has received much attention in the last two decades, and has led to the development of high-performance synthetic methods and use of modified peptides in biological studies. In particular, efficient methods are now established for synthesizing glycopeptides[137] and phosphopeptides[138]. The use of enzyme-labile protecting groups[133–136] has been established as a valuable alternative to the classical chemical protecting group techniques, since enzymes often work under mild, neutral conditions, and combine high selectivity for the structural units that they recognize and the reactions they catalyze with broad substrate tolerance. The new synthetic methods resulting from this approach are the focus of the following discussion.

6.1. Lipid-Modified Peptides

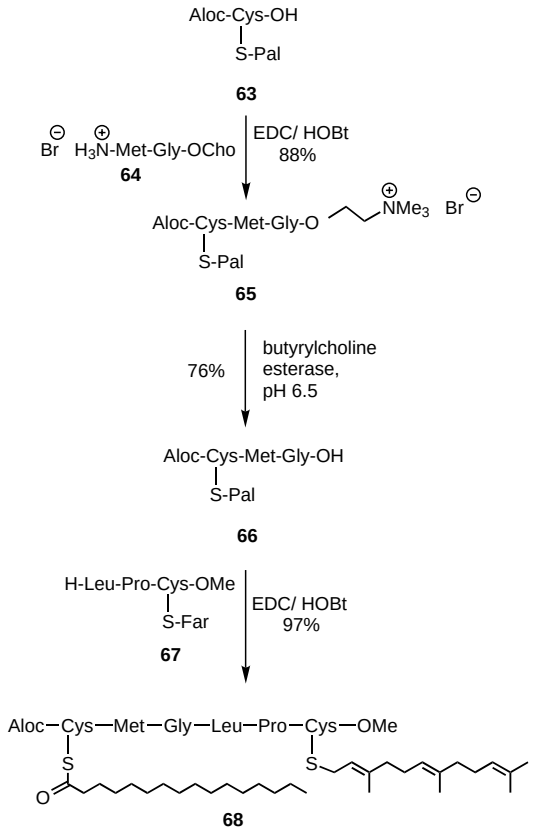
Signal-transducing proteins that are located in the plasma membrane often carry covalently attached lipid residues.[139, 140] Therefore, the C termini of many G protein coupled receptors are *S*-palmitoylated on cysteines; the α subunits of heterotrimeric G proteins and nonreceptor tyrosine kinases are myristoylated at N-terminal glycines and often S-palmitoylated in the immediate vicinity as well. The γ subunits of the G proteins contain an S-farnesylated or S-geranylgeranylated cysteine, and the Ras proteins are S-farnesylated and S-palmitoylated (Scheme 28).



Scheme 28. Structure of lipid-modified proteins bound to a plasma membrane. GPCR = G protein coupled receptor, NRTK = nonreceptor tyrosine kinase.

The lipid residues are necessary to localize the proteins to the site at which they perform their biological function, that is, in the plasma membrane. Therefore, the Ras proteins only fulfill their signal-tranducing function in both normal and transformed cells if they are membrane-bound. This is achieved by lipid modification; non-farnesylated Ras is cytosolic and inactive. Whether the correct lipid modification of proteins fulfills other biological functions and, for example, actively takes part in the process of signal transduction is unclear.[139, 140] There are indications that the interaction of Ras with its substrate Raf occurs through the C terminus of the Ras protein, and the lipid modification seems to play a role[142] (for a similar theory for the interaction of yeast Ras with its target protein, the adenylate cyclase, see ref. [177]). For the G protein coupled β_2 adrenoceptor, it was shown that desensitization following binding of an agonist (i.e. switching the signal off) is accompanied by depalmitoylation of the receptor.[143] Furthermore, addition of the agonist brings about an increase in the degree of palmitoylation of the $G_{s\alpha}$ protein of the β_2 adrenoceptor; its lipid-modified N terminus interacts better with the corresponding β,γ subunit and forms the original heterotrimeric, inactive complex (see Scheme 2, Section 2.1). This process also causes the signal to be switched off.[144] Whether the specific type of lipid modification of individual proteins is a form of subcellular addressing to certain intracellular membranes (e.g. by interaction with a receptor that recognizes lipid residues) is unclear.[141] Lipid-modified peptides have played an important role in the investigation of this last question.

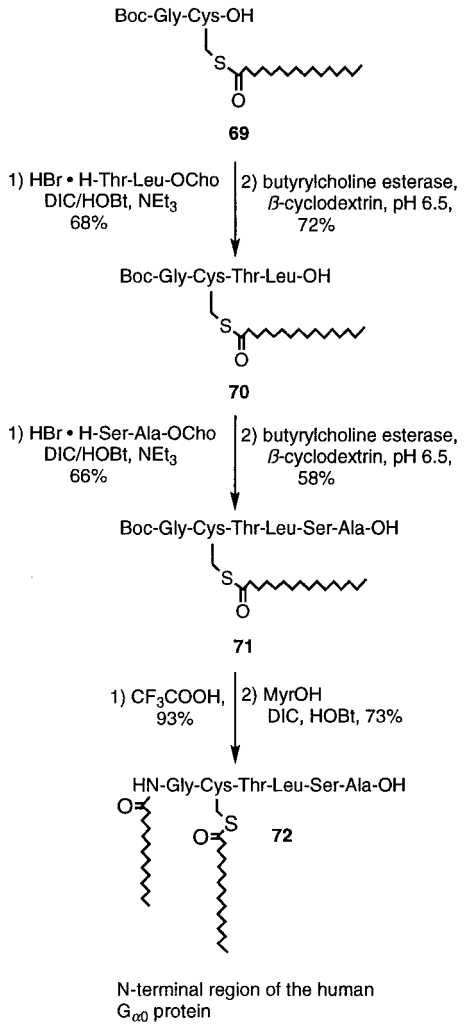
Enzymatic protecting group techniques[133–136] are particularly effective for synthesizing acid- and base-labile lipid-modified peptides. Thus, the choline ester was developed as an enzyme-labile carboxy protecting group that can be released under very mild conditions with butyrylcholine esterase from horse serum; at the same, it ensures better solubility in the aqueous media, which is necessary for biotransformation.[145] An example of its use is in the synthesis of the S-palmitoylated and S-farnesylated lipopeptide **68** of the human N-Ras protein (Scheme 29). The S-palmitoylated tripeptide choline ester **65**



Scheme 29. Chemoenzymatic synthesis of the farnesylated and palmitoylated C terminus of the human N-Ras protein with the enzyme-labile choline ester. Aloc = allyloxycarbonyl, Far = farnesyl, HOCho = choline.

was obtained from the cysteine derivative **63**. In the key step of the synthesis, **65** was completely released without attack at the thioester, which was more reactive under classical conditions, and without detectable β elimination. Extension of the peptide chain of the selectively demasked tripeptide **66** with the S-farnesylated tripeptide methyl ester **67** gave the target compound **68** in high yield.

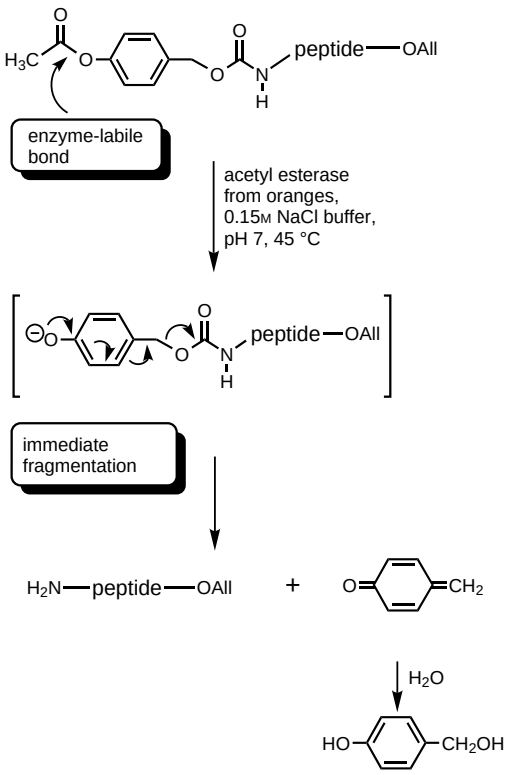
With a second application of this technique, the characteristic N-myristoylated and S-palmitoylated N-terminal hexapeptide of the α_0 subunit of a heterotrimeric $G_{\alpha 0}$ protein was synthesized[146] (Scheme 30). Starting from the S-palmitoylated dipeptide **69**, a tetrapeptide choline ester was synthesized and then enzymatically deblocked at the C terminus under very mild conditions to **70**. Again, there were no undesired side reactions. Successive chain extension and renewed



Scheme 30. Chemoenzymatic synthesis of the myristoylated and palmitoylated N terminus of the α subunit of a heterotrimeric α_0 protein with the enzyme-labile choline ester. DIC = diisopropylcarbodiimide, MyrOH = myristic acid.

choline esterase mediated deprotection delivered the Boc-masked peptide **71**, which was converted into the target compound **72** by cleavage of the urethane group and N-myristoylation.

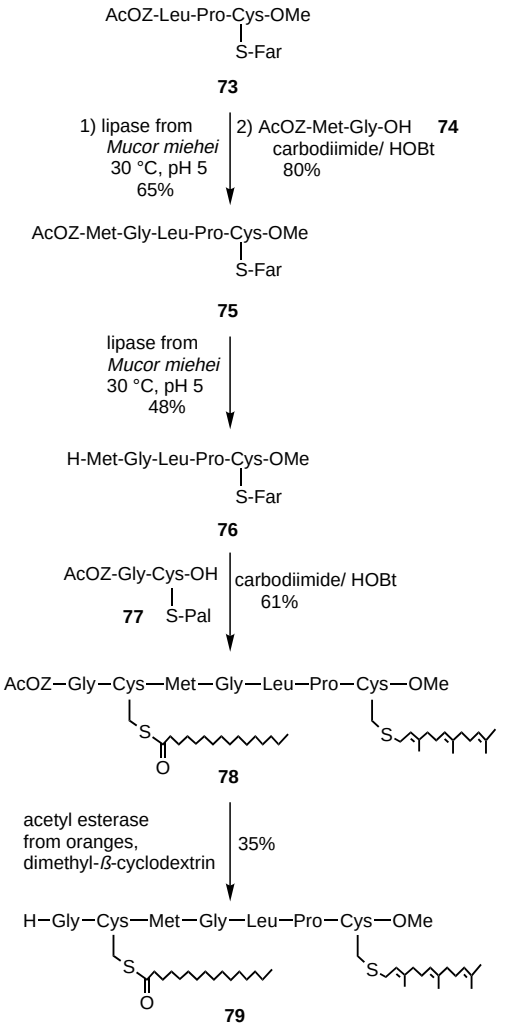
To synthesize acid- and base-labile lipid-modified peptides by N-terminal extension of the peptide chain, it was necessary to develop the first enzyme-labile urethane protecting group for the amino functionality of peptides (Scheme 31).[147] Since no biocatalyst is known for cleaving urethanes from peptides, a strategy was developed in which the enzyme removes a urethane without direct attack on the urethane structure. The p-acetoxybenzyloxycarbonyl (AcOZ) group used for this purpose contains a functional group (acetate) that the biocatalyst (an esterase or lipase) can recognize and that is bound through an enzyme-labile bond (an ester) to a second functional group (a *p*-hydroxybenzyl urethane); following cleavage of the enzyme-labile bond, the urethane undergoes spontaneous fragmentation with release of the desired peptide conjugate (Scheme 31). This is a general principle: Depending on the acyl residue selected (e.g., acetate or phenylacetate), enzymes with different selectivity (e.g., acetyl



Scheme 31. Enzyme-initiated cleavage of the AcOZ urethane protecting group.

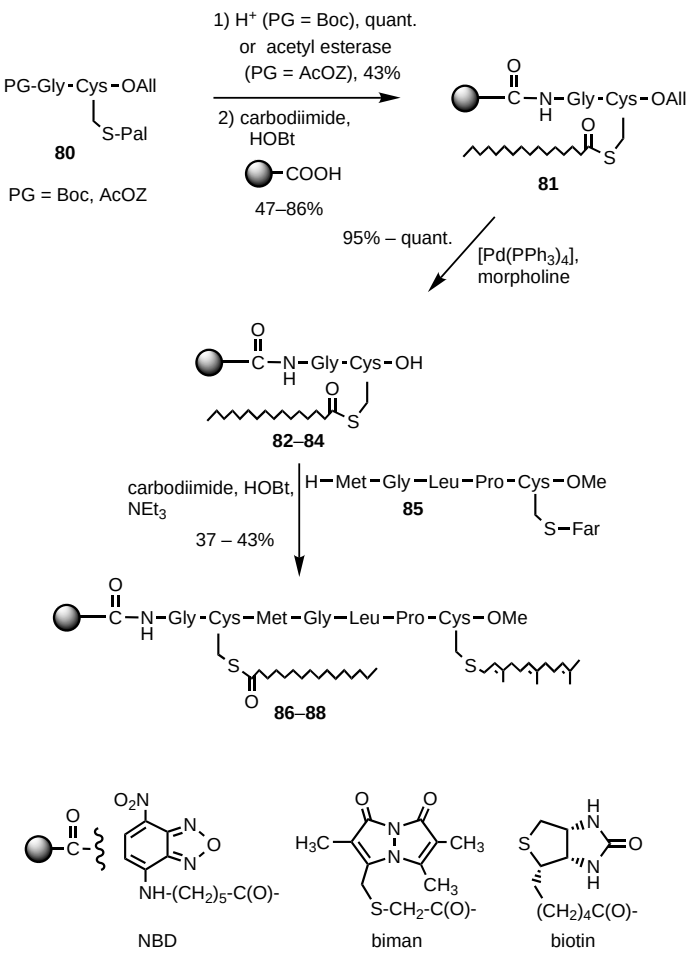
esterase or penicillin G acylase) can be used (see Schemes 32, 51, and 54), and the analogous C-terminal *p*-acyloxybenzyl esters can also be cleaved off by the same mechanism.[148] With the AcOZ urethane, the essential C-terminal lipid-modified heptapeptide ester of the N-Ras protein was constructed (Scheme 32).[147] Deblocking of the urethane-protected farnesylated tripeptide methyl ester **73** by enzyme-initiated fragmentation of the AcOZ group delivered the selectively deprotected peptide. Successive extension of the peptide chain with the AcOZ dipeptide **74** and renewed lipase-mediated release of the urethane protecting group gave the N-Ras pentapeptide **76**; this was condensed with the AcOZ-masked S-palmitoylated dipeptide **77** to give the fully protected lipid-modified heptapeptide **78**. It was subsequently possible to enzymatically release the N-terminal urethane from **78** to give the amino-deblocked Ras heptapeptide **79**, which is available for further chain extensions or linking to additional functional groups.

The enzymatic techniques described here have been proven to be flexible and efficient methods for synthesizing lipid-modified peptides that are both base- and acid-labile. Nevertheless, such conjugates could also be assembled by classical methods, although the different cysteine units to be modified had to be protected with a much more complicated procedure; in general, these were selectively unmasked and functionalized after completion of the peptide chain.[149, 150] If only base-labile palmitic acid thioesters are present in the compound to be demasked, the acid-labile Boc group may be used (see below); acid-labile peptides containing *S*-farnesylcysteine can be deblocked at the N terminus by cleavage of



Scheme 32. Chemoenzymatic synthesis of the C terminus of the human N-Ras protein with use of the enzyme-labile AcOZ urethane protecting group.

the Fmoc urethane.[151] In addition to enzymatic techniques, Pd^0-mediated release of allyl esters has also been demonstrated to be an efficient method for synthesizing sensitive lipid-modified peptides.[152] This protecting group technique demonstrated its potential in the development of a flexible building block system for synthesizing different lipid-modified N-Ras peptides carrying additional fluorophoric groups; owing to the fluorophore properties, these conjugates can be followed in biological and biophysical experiments. The AcOZ- or Boc-protected S-palmitoylated cysteine allyl ester **80** was selectively demasked at the N terminus either enzymatically or under acid conditions, and a fluorescent group was linked to the free amino function (Scheme 33). Without any attack at the palmitic acid thioester, release of the allyl ester was achieved in very high yield by Pd^0-mediated allyl transfer to morpholine as accepting nucleophile. The fluorescently labeled and S-palmitoylated dipeptides **82**–**84** thus obtained were then linked to the farnesylated N-Ras pentapeptide **85** to form Ras heptapeptides **86**–**88** with two lipid modifications; these were used in further biological and biophysical experiments (see below).



Scheme 33. Synthesis of labeled lipopetide derivatives for biological studies. NBD = 4-nitrobenzo-2-oxa-1,3-diazol, PG = protecting group.

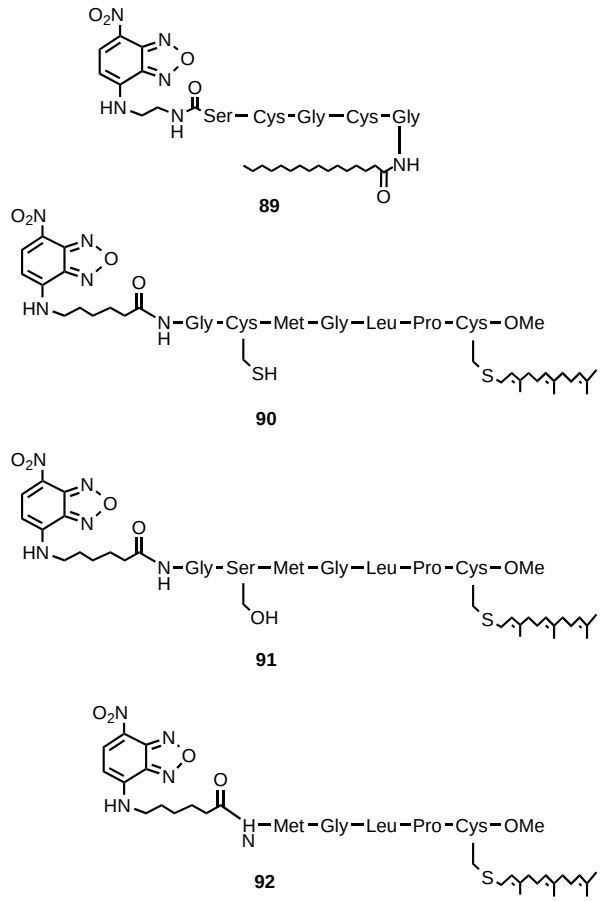
Lipid-modified peptides have been used in many ways as tools, for example, to study enzymes involved in introducing lipid residues into proteins and in processing lipidated proteins. In particular, they have been used to obtain knowledge about the importance of lipid residues in the function of lipid-modified proteins. Issues such as the contribution of different lipid residues to anchoring of proteins in membranes, and whether the type of lipid modification determines the localization of proteins in particular subcellular membranes under certain circumstances, have been addressed by combining biophysical and cell biology techniques for the lipopeptides obtained as described above.[153, 154]

In a series of biophysical investigations on the thermodynamics of the insertion of lipid-modified model peptides in vesicles functioning as model membranes, the contribution of the lipids to lipopeptide membrane affinity was determined.[149, 155–157] This showed that a N-myristoyl or a S-farnesyl group alone cannot contribute enough hydrophobic character to maintain stable membrane insertion of peptides, and therefore also of proteins. Peptides and proteins that carry only a single lipid modification are rapidly inserted into the membrane (within seconds), yet they are also rapidly exchanged between two different membranes (half-time on the order of seconds). A palmitoyl group provides the peptides with about 15-fold higher membrane affinity than a myristoyl residue, and a geranylgeranyl thioether has about the same effect. For a C terminal farnesylated and geranylgeranylated peptide modified as a methyl ester, the membrane affinity is ten times higher than for the analogous S-prenylated carboxylic acid. The C-terminal methylation of a farnesylated protein therefore has a greater effect on its distribution between cytosol and membrane, whereas a geranylgeranylated protein without esterification will be mostly membrane bound. Binding of simple modified peptides to model membranes is therefore strong in the thermodynamic sense but rapidly reversible. A single lipid modification cannot maintain stable insertion of a lipid-modified protein in a membrane.

However, a second lipid modification does achieve stable insertion. Biophysical model experiments on the kinetics of transfer of lipid-modified peptides from one model membrane to another[149, 158] showed that the half-time for transfer of a singly farnesylated or palmitoylated peptide is in the region of seconds. For peptides that are N-myristoylated and S-palmitoylated, or S-palmitoylated and S-farnesylated, the half-times are in the range of hours to days. These values are significantly larger than the half-time for turnover of S-acyl groups of membrane-associated proteins in vivo (≤ 2 h) or recycling of membrane-bound proteins that is mediated by escort proteins. These double lipid modifications give stable membrane binding in a biological context and on a biologically relevant time scale, and can be used as specific structural motifs to localize and anchor proteins in special subcellular membranes. In fact, the N-myristoyl/S-palmitoyl motif is found in nonreceptor tyrosine kinases and the α subunit of heterotrimeric G proteins, and H-Ras and N-Ras are S-palmitoylated and S-farnesylated.

This hypothesis was elegantly tested by in vivo studies with fluorescently labeled N-myristoylased[159] and S-farnesylated peptides.[152, 158, 159] Peptides **89** and **90** (Scheme 34), which represent forms of the N terminus of the human nonreceptor tyrosine kinase Lck and the human N-Ras protein with a single lipid modification, were S-palmitoylated in fibroblast cells; for analogous peptides in which the palmitoylatable cysteine residues were replaced by serine, such as **91**, no incorporation of palmitic acid could be detected. Palmitoylation was also observed for peptides in which the myristoyl residue had been exchanged for other fatty acids (C_{10} to C_{16}) or the farnesyl residue for other alkyl groups (*n*-undecyl, *n*-octyl, *trans*-geranyl).

The site of palmitoylation and the intracellular distribution of peptides with double lipid modification were determined with fluorescent microscopy techniques. This showed that the doubly lipid modified peptides formed from **89** and **90** were concentrated in the plasma membrane of fibroblast cells (Figure 1); this took place under conditions (15 °C) which exclude intracellular transport, for example, by vesicles. For analogous serinyl peptides such as **91** that do not become acylated (see below), such a selective distribution was not observed; the S-*n*-alkylated and geranylated analogues of **90** showed similar behavior. Peptides **89**–**91** were introduced into cells by fusion of the fibroblast membrane with vesicles containing the fluorescently labeled peptide. The results were also confirmed by microinjection of **92** and **86** (Scheme 33;



Scheme 34. Lipid-modified peptides with the NBD marker for biological studies.

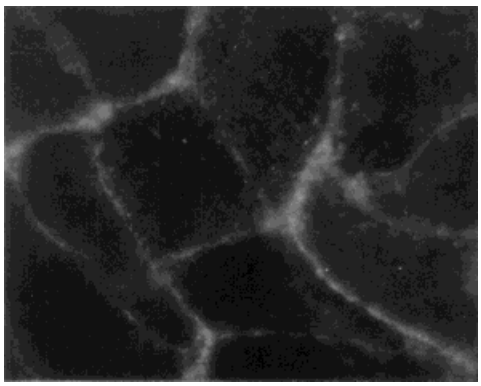


Figure 1. Fluorescence image of CV-1 fibroblasts after treatment with the NBD-labeled and farnesylated lipoheptapeptide **90**. The image shows that the fluorescence is concentrated in the plasma membrane.

NBD-labeled) and subsequent examination by confocal laser fluorescence microscopy.[152] This showed that the farnesylated, but no longer palmitoylatable, pentapeptide **92** was not selectively incorporated into a certain membrane; however, the doubly lipid modified **86** was selectively accumulated in the plasma membrane (Figure 2).

These findings, and those obtained from biophysical experiments, that doubly lipid modified (in contrast to singly

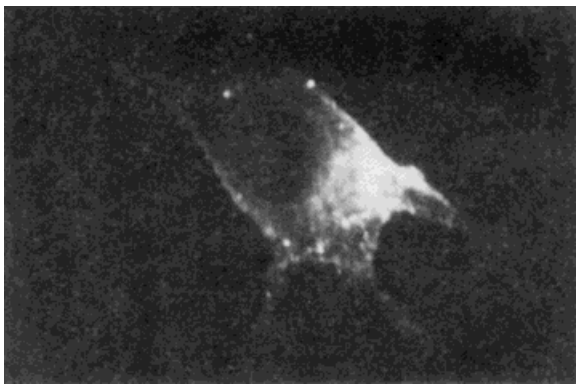
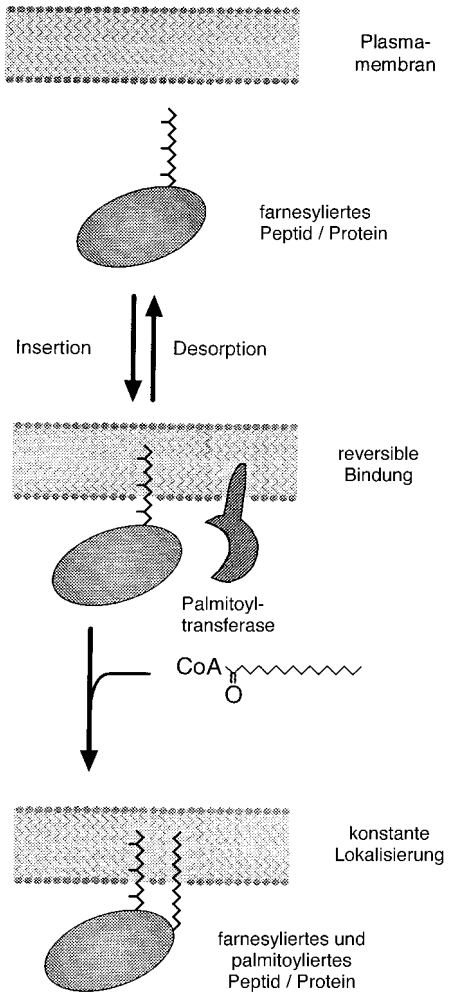


Figure 2. Fluorescence image of NIH-3T3 fibroblasts after microinjection of the NBD-labeled, farnesylated, and palmitoylated Ras heptapeptide **86**. The image shows that the peptide is localized in the plasma membrane.

modified) peptides only exchange very slowly between different membranes when a second lipid residue is introduced (see above) and should therefore concentrate in the cell compartment in which the second lipid modification takes place, led to the conclusion that S-palmitoylation of singly lipidated peptides (and therefore also singly modified proteins) takes place at the plasma membrane.

The results of the biophysical and cell biology investigations support a model[159] for specific localization of proteins by myristoylation/palmitoylation or farnesylation/palmitoylation. According to this model, the specific localization is not only determined by the lipid groups introduced in the course of the biosynthesis (in the case of Ras proteins, the farnesyl residue). Rather, the singly modified proteins can freely diffuse and insert in or desorb from different membranes (Scheme 35). It is only upon S-acylation in a certain membrane compartment that they remain localized in that membrane. In the case of the N-Ras peptide **90** and the $p56^{lck}$ peptide **89**, it is the plasma membrane in which the lipid-modified proteins are actually found. A membrane-bound protein S-acyltransferase that may be responsible for this modification was recently identified; it palmitoylates a farnesylated N-Ras peptide and also the H-Ras protein.[160] If the lipid-modified protein is no longer needed, or the signals transmitted by such proteins must be terminated (regulation of signal chains), the thioester can be cleaved again by a suitable hydrolase, initiating desorption of the protein from the membrane (Scheme 35).

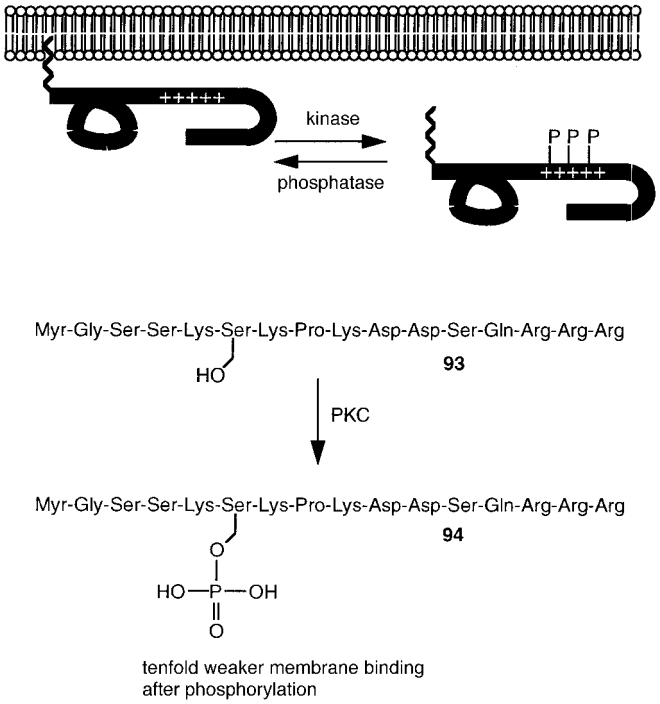
In addition to introduction of a second lipid residue, stable insertion of a protein in a membrane can also be achieved by combining a first lipid residue with a cluster of amino acids that are positively charged under physiological conditions and thus interact with the negatively charged cell membrane (Scheme 36).[153, 154] This cooperative interaction is reduced by phosphorylation of the proteins in the positively charged domains (i.e., by reducing the number of positive charges with negatively charged phosphate), and the protein desorbs from the membrane. The membrane insertion/desorption of proteins can be regulated in this way. A combination of a lipid residue with protonated basic amino acids is found in, for example, the N-myristoylated nonreceptor tyrosine kinase



Scheme 35. Model for insertion of lipid-modified proteins in the plasma membrane by additional S-palmitoylation.

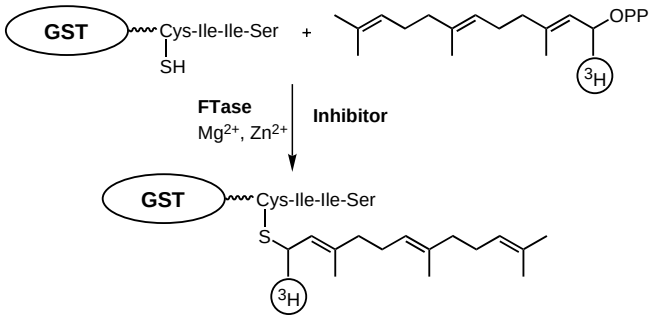
Src, the N-myristoylated alanine-rich C kinase substrate (MARCKS), and the S-farnesylated but not palmitoylated K-Ras protein. For MARCKS, regulation according to the model shown in Scheme 36 was proven by extensive mutation analysis; for the c-Src peptide **93** and the analogous phospholipopeptide **94**, which represent the N terminus of the protein, similar behavior was shown in experiments on a model membrane. The membrane binding of **93** was reduced tenfold on phosphorylation at Ser 12 by protein kinase C.[154] By investigating this and other similar issues with suitable lipid-modified peptides, it might be possible to obtain valuable information leading to development of heterologuous recombinant proteins and their direction to particular locations in the cell where they then influence signal transduction. Furthermore, the development of new active compounds that influence pathological signal mediation processes (e.g. through Ras) might gain a new impetus.

S-Farnesylated cysteinyl peptides have served as tools for studying and inhibiting all enzymes involved in the biosynthesis of S-farnesylated and C-terminal methylated proteins such as the Ras protein (Scheme 5, Scheme 23; see Sections 2.2 and 5). Farnesyltransferase, which transfers the farnesyl residue to the precursor protein, is inhibited with



Scheme 36. Regulation of membrane binding by combination of N-myristoylation with a polybasic amino acid sequence and phosphorylation.

an IC_{50} value in the millimolar range by farnesylated peptides which also have the subsequently cleaved C-terminal AAX sequence (Scheme 37).[161] However, an analogue masked



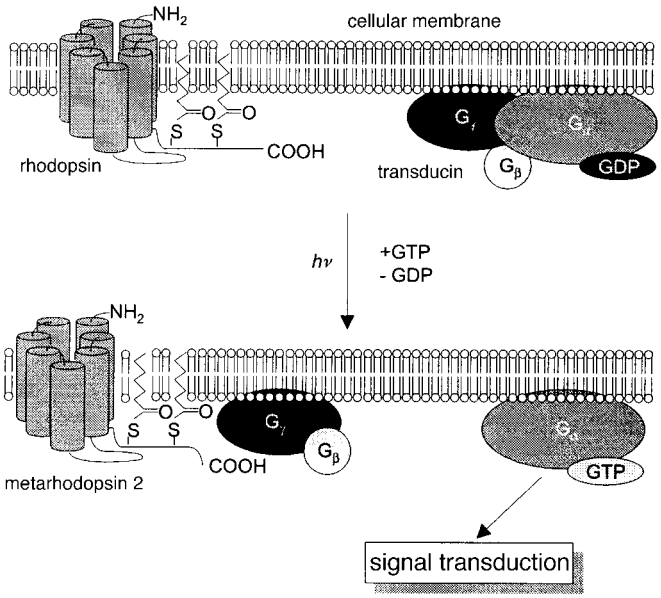
Inhibitor	IC_{50}
CAAX peptide	0.1 – 6 μM
S -farnesyl-CAAX-peptide	1.4 – 8.2 μM
S -farnesyl-Cys-OMe-peptide	no inhibition
Aloc-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe **76**	5.5 mM

Scheme 37. Inhibition of the Ras farnesyltransferase by lipid-modified peptides. GST = glutathion S-transferase.

with a methyl ester which lacks the AAX peptide, corresponding to the processed form of the Ras protein, does not show any inhibitory activity.[151] Nevertheless, weak inhibition is observed if an esterified and also farnesylated and palmitoylated peptide is used as an inhibitor (Scheme 37). This finding indicates a possible feedback inhibition in the biosynthesis of the Ras protein.[151]

Similarly, tetrapeptides with isoprenylated N-terminal cysteine have been used to study proteinases that cleave the C-terminal AAX segment from the precursor protein.[141, 162, 163] It was shown that substrates of the enzyme must have an isoprenylated cysteine residue and that the biocatalyst recognizes the absolute configuration of the amino acids. Based on this knowledge, simple farnesyl peptide analogues were developed as inhibitors of the protease.[164] In addition, S-isoprenylated and simple alkylated peptides were used to track down a methyltransferase that creates the C-terminal methyl ester of the farnesylated protein.[141, 165, 166] This also showed that a farnesylated peptide was able to inhibit the methylation of a natural protein.

With lipid-modified peptides, the unsolved problem was tackled of whether lipid residues, in addition to their importance for membrane anchoring and localization of proteins, also have active roles in the biological function of lipid-modified proteins. The foremost questions here are whether there are specific receptors in the membrane that recognize farnesyl residues and whether the lipid residues are involved in controlling signal transduction processes, for example, through lipid–protein interactions. Signal transduction in photoreceptors through the G protein coupled receptor rhodopsin and the heterotrimeric G protein transducin was chosen as the biological system (see Section 2.1). Upon absorption of light, rhodopsin is activated to metarhodopsin 2, which then interacts with transducin and catalyzes exchange of GDP for GTP at the α subunit; the β,γ subunit is important in this exchange. The α unit with GTP then activates a cGMP-specific phosphodiesterase which transmits the signal to Na channels and thereby passes on the reaction to the light signal (Scheme 38).[141, 167] To test



R-Asp-Lys-Asn-Pro-Phe-Lys-Glu-Leu-Lys-Gly-Gly-Cys-OH
Far

95, R = H
96, R = Y

Scheme 38. Manipulation of signal transmission in the rhodopsin/transducin system by lipid-modified peptides.

whether coupling of the receptor to the G protein is mediated by its γ subunit, the farnesylated peptide **95**, which corresponds to the C terminus of the γ subunit, and *N*-acetyl-*S*-farnesylcysteine were examined as inhibitors of the interaction between rhodopsin and transducin.[168, 169] The peptide **95** stabilizes the active form of the receptor (metarhodopsin 2) and decouples it from transducin. Both the farnesyl residue and the amino acid sequence are important for the effect. It was concluded that the farnesyl residue is of direct importance for the protein–protein interaction and does not just function as a membrane anchor,[168] and that, with the help of S-prenylated cysteine analogues, the interaction of activated receptors with the β,γ subunits of G proteins can be specifically inhibited.[169] Subsequently, analogous peptides such as **96** were used to study the interaction between the α subunit of transducin and the β,γ complex, which can also be inhibited by **96**.[170]

Regardless of any doubt about the conclusions on rhodopsin/transducin drawn from the investigations described above,[141] different farnesyl cysteine analogues and farnesylated peptides show manifold physiological effects suggesting that these compounds interact with signal transduction processes. For example, they interfere with release of superoxide in neutrophiles[171, 172] in which isoprenylated Rac protein plays a role, they block thromboxane- or thrombin-induced platelet aggregation,[173, 174] and they influence the release of insulin from pancreatic cells.[175] These effects do not seem to be due to inhibition of processing of lipid-modified proteins. It seems likely that the isoprenylated peptides influence the function of isoprenylated G proteins, for example, by interaction with proteins in the sense of a receptor-ligand interaction in which they act as their target proteins.

Furthermore, a protein that binds prenylated CAAX peptides with high affinity was identified in microsomal membrane fractions of bovine brain; this protein could be a receptor for prenylated peptides.[176] For yeast Ras, the farnesyl residue of the protein probably serves not only to localize the protein on the plasma membrane but is also involved in the interaction of Ras with its target protein, the adenylate cyclase.[177]

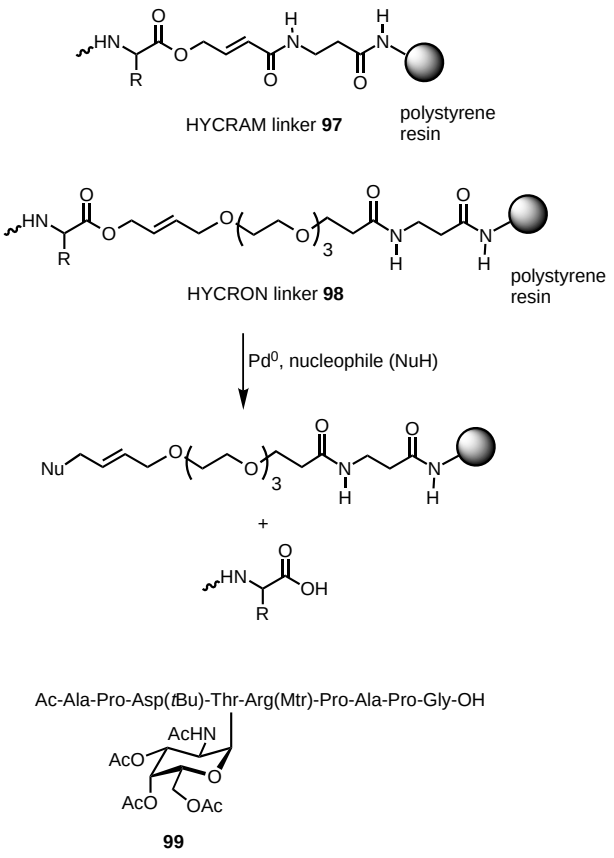
6.2. Glycopeptides

Many of the proteins localized on the extracellular face of the plasma membrane carry complex oligosaccharides. These glycoproteins are intensively involved in regulating communication between cells; they control cell–cell interactions and form cell-surface antigens.[178] Taking this into consideration, it is particularly noteworthy that glycoproteins have also been identified as tumor-associated antigens, that is, as antigens which appear on the surface of tumors but not on normal cells. In addition, many of the cell-surface receptors that take up extracellular signals and pass them through the plasma membrane into the cell interior are glycosylated. The carbohydrate and peptide structures are of importance for biological function of these protein conjugates, as shown, for example, for the proteins of the major histocompatibility

complex (MHC).[179] Whereas proteins with attached complex oligosaccharides almost exclusively fulfill extracellular biological functions on the cell surface, it was recently shown that glycosylation of proteins can play an important intracellular role (see Section 6.3).

Characteristic glycopeptides could be useful tools for investigation of the biological functions of glycoproteins. To synthesize these peptide conjugates, both in solution and on solid phase, powerful techniques for reversible blocking of the amino, carboxy, and alcohol groups have been developed in the last two decades[137] along with methods for gentle release from polymeric carriers.[180–183] Classical chemical synthesis of oligosaccharide units of glycoproteins has also reached a high level of efficiency.[137, 184] The first effective solid-phase techniques were developed to this end.[185]

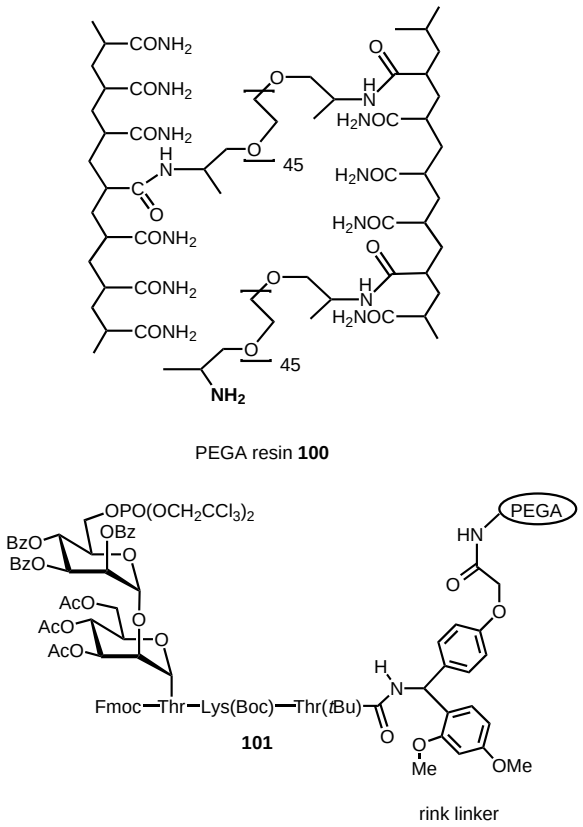
Owing to the performance of established classical chemical techniques, it is possible today to assemble glycosylated peptides on polymeric carriers with a high degree of efficiency. The allylic HYCRAM[180] (**97**) and HYCRON[181] linker groups (**98**, Scheme 39) are particularly advantageous



Scheme 39. Solid-phase glycopeptide synthesis with the HYCRAM and HYCRON linker groups. Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl.

since they permit gentle release of the sensitive glycopeptide from the solid phase in a Pd^0-catalyzed allyl transfer to N- and C-nucleophiles. With HYCRAM linkers, it was possible to synthesize many glycopeptides, with both the acid-labile Boc and base-labile Fmoc groups as protecting functionalities. However, the basic cleavage of the Fmoc group presented problems, for example, nucleophilic attack on the α,β-unsaturated amide group in **97**. Also, the glycopeptides could not be quantitatively released from the solid carrier in some cases. These disadvantages were overcome with the development of **98**. In **98**, a flexible oligoethylene glycol spacer ensures that the Pd^0 complex can progress well to the allylic group during cleavage; this group is no longer present as the α,β-unsaturated carbonyl functionality but rather as the ether and is therefore no longer competitively attacked by nucleophiles. With the HYCRON linker, the glycopeptide **99** was synthesized on a polymeric carrier and subsequently released in a high yield. The method has proven to be equally successful for synthesizing an eicosapeptide.

The PEGA resin **100** was recently developed as an advantageous alternative to the carriers based on polystyrene that are generally used (Scheme 40). In principle, it is made up

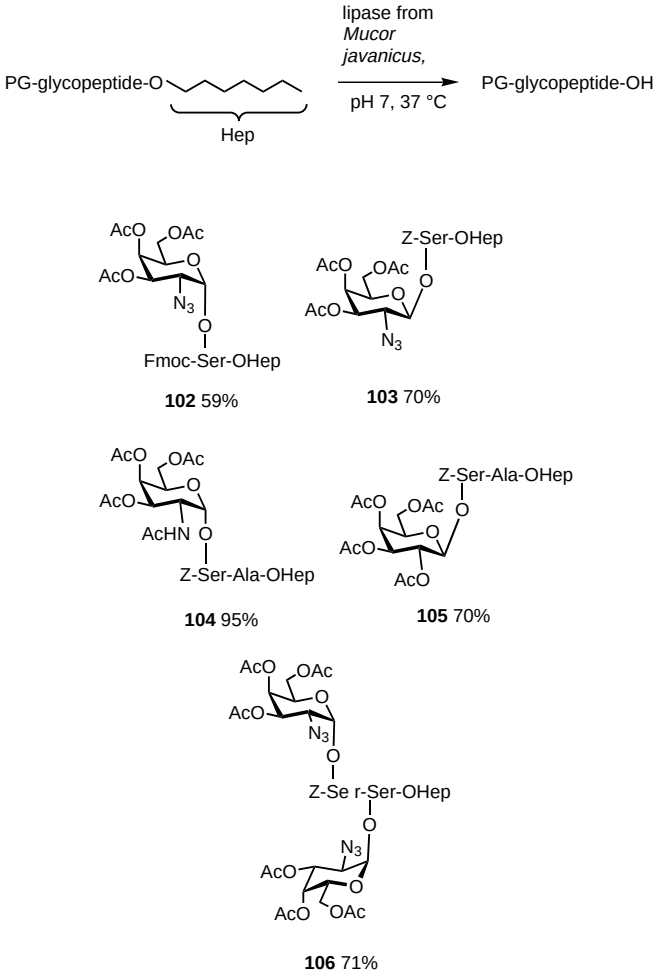


Scheme 40. Solid-phase glycopeptide synthesis on PEGA resin.

of poly(acrylamide) chains which are cross-linked by poly(ethylene glycol) units and carry additional free amino functionalities at which peptides and glycopeptides can be synthesized.[183] The hydrophilic PEGA resin swells very well in different solvents, and its interior is accessible to larger molecules and even enzymes owing to the flexible structure of the matrix. With PEGA resin, numerous glycosylated peptides were synthesized in automated solid-phase syntheses (Scheme 40).[182, 183, 186] Fmoc-urethane was used as N-terminal protecting group, and the glycopeptide to be synthesized was linked to the polymeric carrier through the acid-labile rink linker (see **101**, Scheme 40). Glycopeptides bound on the solid phase were thus synthesized by successive extension of the

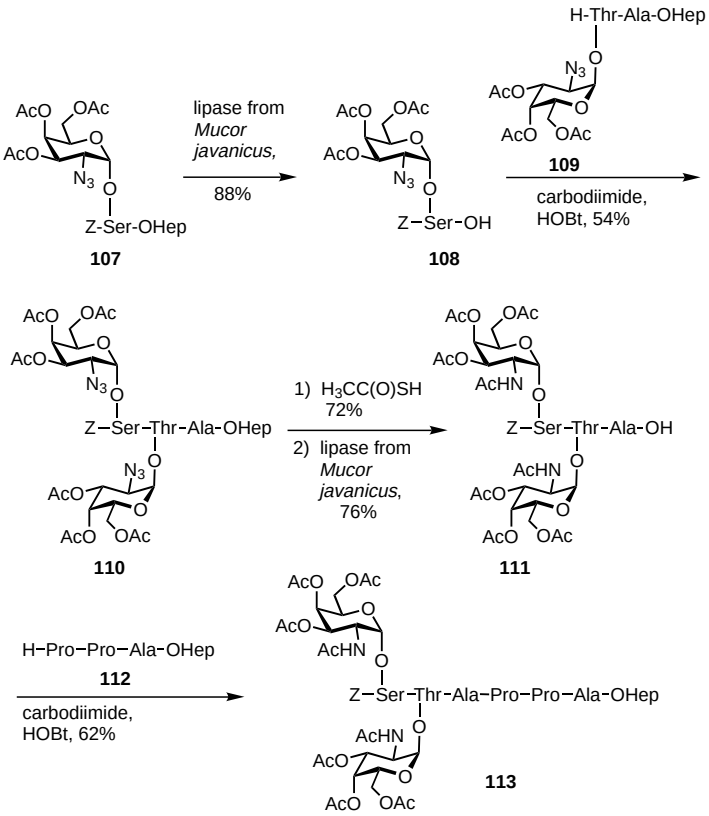
peptide chain. These glycoconjugates were cleaved from the solid phase under slightly acidic conditions and without unwanted side reactions. Glycopeptides can be reliably and efficiently synthesized today by this and other solid-phase techniques with automated methods.

Enzymatic methods have opened up valuable alternatives for selective deblocking of glycopeptides and for synthesis of their oligosaccharide units. For example, selective and gentle enzymatic deblocking of O-glycopeptides was achieved by lipase-mediated hydrolysis of the heptyl ester protecting group. The lipase from *Mucor javanicus* deblocks the C-terminus of the glycosylated amino acids and peptides **102**–**106** without unwanted attack on the N-terminal urethane and the carbohydrate protecting group (Scheme 41).[187] There was



Scheme 41. Chemoenzymatic glycopeptide synthesis with use of the enzyme-labile heptyl (Hep) protecting group (PG).

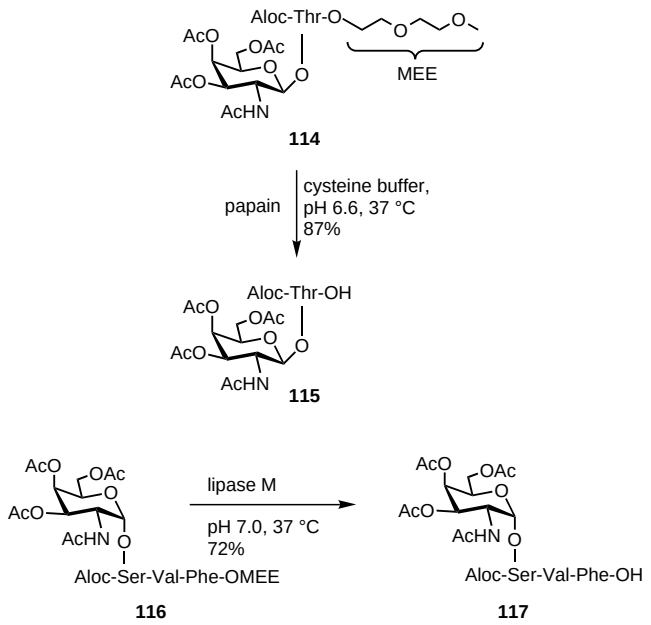
no anomerization or β-elimination under these mild conditions. With this enzymatic protecting group technique, the complex diglycohexapeptide **113** was synthesized; it contains the characteristic linkage region of a tumor-associated antigen found on the surface of human breast carcinomas (Scheme 42). Following selective C-terminal deblocking of the serine glycoside heptyl ester **107**, the carboxylic acid **108** that was released was condensed with the N-terminal deblocked glycopeptide ester **109** to form the diglycotripeptide **110**. The azido group of the latter was converted into the acetamide group, and subsequently the C terminus was selectively released again by enzyme-mediated hydrolysis without any unwanted side reactions (→**111**). The C-terminal extension of the peptide chain gave the desired peptide **113**.



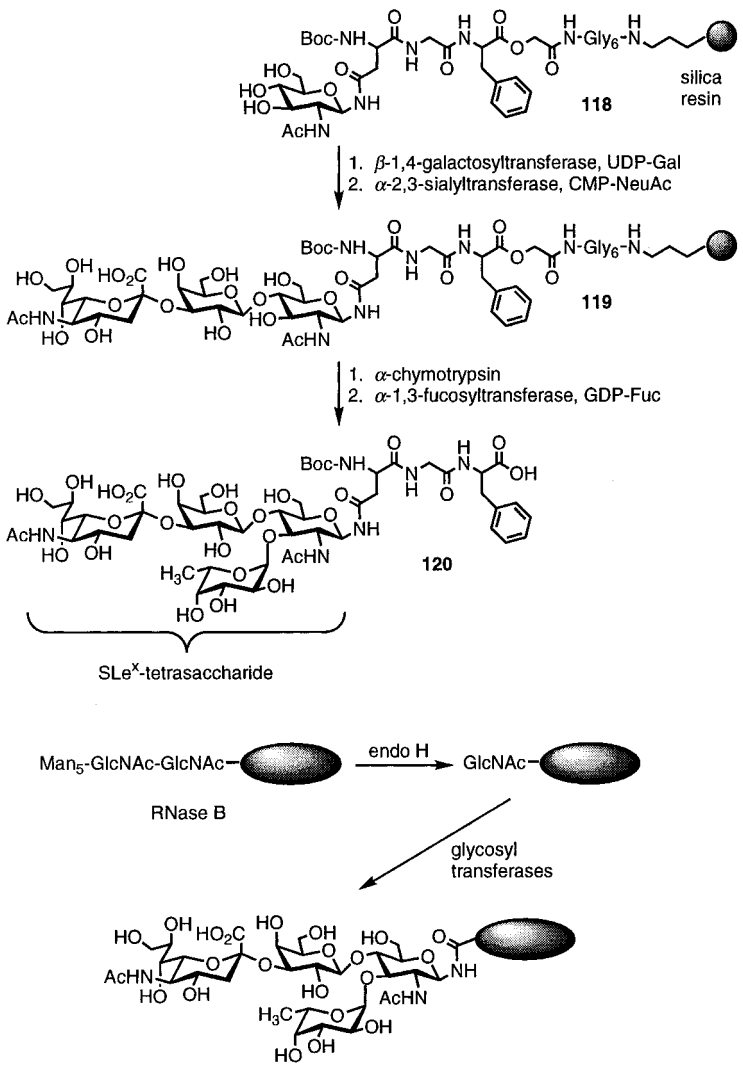
Scheme 42. Synthesis of glycopeptide **113**, which represents a characteristic section of a tumor-associated antigen.

If the very hydrophobic heptyl ester was used, there were problems due to the low solubility of the substrates and, thus, low rate of turnover, especially in the presence of similarly hydrophobic amino acids. These problems were solved by introducing the more hydrophilic 2-(*N*-morpholino)ethyl (MoEt)[188] and, in particular, the methoxyethoxyethyl (MEE) group.[189] The MEE ester was, for example, cleaved from the glycopeptide **116** by lipase-catalyzed hydrolysis (Scheme 43), and the threonine glycoside **114** could be deblocked at the C terminus by papain. The proteases papain, subtilisin, and thermitase were also used for demasking glycopeptide methyl[190] and even *tert*-butyl ester protecting groups.[191]

In the synthesis of glycopeptides, enzymatic transformations were also used to assemble the oligosaccharide. Thus, for example, the glycosylated asparaginyl peptide **118** was synthesized on a polymeric carrier (Scheme 44), and then regio- and stereospecific linking of a galactose and a neuraminic acid unit (→**119**) was achieved in two consecutive enzymatic glycosylations.[192] Following cleavage of the glycopeptide from the solid phase by chymotrypsin-mediated ester hydrolysis at the C terminus of a phenylalanine residue, the characteristic sialyl Lewis^X (SLe^X) tetrasaccharide was completed by fucosylation of the *N*-acetylglucosamine (→**120**).



Scheme 43. Enzymatic glycopeptide synthesis with use of the enzyme-labile methoxyethoxyethyl (MEE) ester.



Scheme 44. Enzymatic synthesis of peptides modified with sialyl Lewis^X and of proteins with glycosyl transferases.

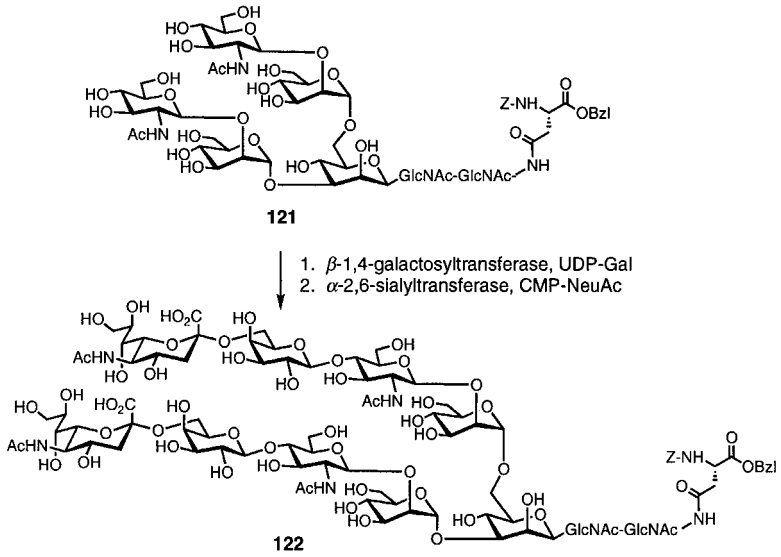
This technique also enabled in vitro synthesis of a glycosylated protein in an impressive manner.[193] For this, an oligosaccharide was cleaved off RNAase B with a glycosidase, and the remaining GlcNAc residue underwent successive enzymatic glycosylations until the protein was attached with the SLe^X tetrasaccharide attached (Scheme 44). With this enzymatic technique, which is based on a principle previously suggested for modifying unwanted glycans of recombinant glycoproteins,[194] it is possible to obtain glycosylated proteins with high homegeneity; this opens up new possibilities for studying the influence of carbohydrates on structure and function of glycoproteins.

A big advantage for the synthesis of complex glycopeptides is offered by combining classical chemical steps with enzymatic glycosylation steps. For example, glycopeptide **121** was synthesized with the help of established carbohydrate chemistry methods, and the oligosaccharide was subsequently extended to the undecasaccharide by enzymatic glycosylations (→**122**, Scheme 45).[195]

Synthetic glycopeptides produced both chemoenzymatically or by classical chemical methods, in solution or on solid phase, have often been used as inhibitors in experiments aimed at blocking the attachment of leucocytes to endothelial cells and the subsequent migration of leucocytes out of blood vessels as a reaction to acute and chronic inflammatory processes. During this process, proteins on the surface of the endothelial cells called selectins recognize complex glycoproteins on the leucocytes carrying the SLe^X carbohydrate epitope;[178, 196] this initiates the migration process.

This interaction was inhibited with glycopeptides with the aim of possibly developing a new therapeutic treatment. Based on data on the conformation of SLe^X in solution, the three-dimensional structure of the human E-selectin, and the structural elements needed for recognizing SLe^X by E-selectin, and supported by molecular modeling, the fucosylated peptides **123**–**125** and further analogues were synthesized and tested as inhibitors (Scheme 46).[197] The peptides each contain the necessary fucose, a structural element carrying a hydroxyl group that imitates the galactose of the SLe^X tetrasaccharide, and a glutaric acid, which functions as the equivalent of the negatively charged group of the sialic acid unit. Compound **123** proved to be as active as the natural SLe^X, whereas **124** and **125** were considerably better inhibitors.

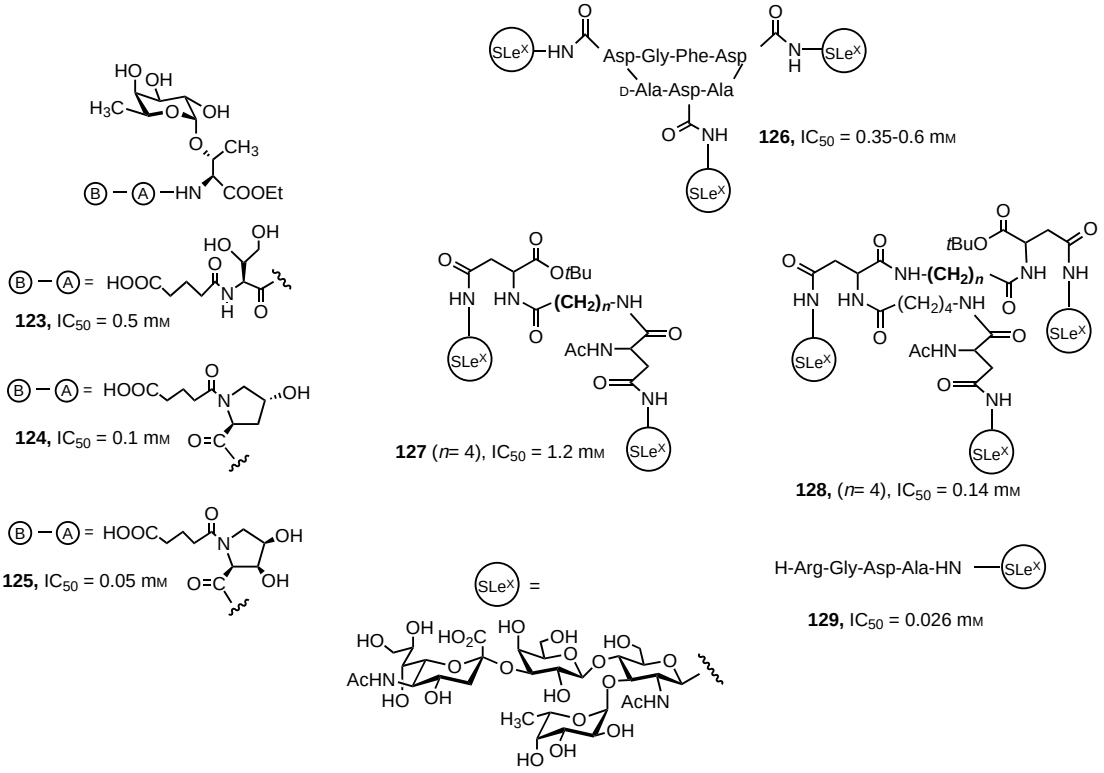
The assumption that the weak interaction of monomeric SLe^X in vivo is increased by "clustering" of ligands



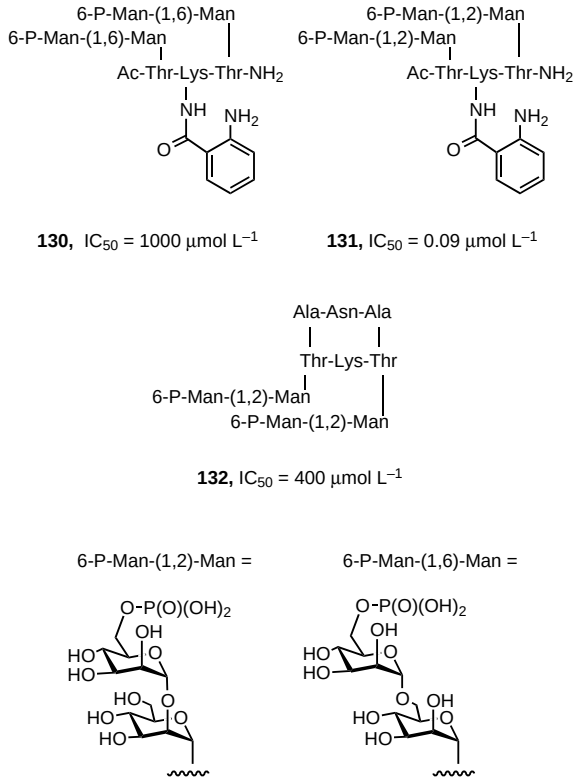
Scheme 45. Synthesis of a complex glycopeptide precursor by successive enzymatic glycosylations.

(multivalence) was examined with the multiple SLe^X glycopeptides **126**–**128** (Scheme 46). In a cell-adhesion test in which binding of **126** to recombinant E-selectin/immunoglobulin fusion proteins was compared to its binding to tumor cells carrying the SLe^X ligand, the trivalent glycopeptide was two to three times more active as an inhibitor than monomeric SLe^X building blocks.[198] In **127** and **128**, the distance of the SLe^X oligosaccharides from one another was varied to obtain information on the size and the spatial orientation of the SLe^X cluster.[199] The divalent ligands **127** were less active than the trivalent analogues **128**, and the inhibition actually depends on the number of methylene groups introduced as spacers. A considerable increase in the cell adhesion inhibitory activity was achieved by linking the SLe^X tetrasaccharide to the tripeptide Arg-Gly-Asp (RGD), which corresponds to the minimum sequence of many adhesion molecules and is, for example, recognized by integrin receptors. The adhesion hybrid **129** thus obtained showed an IC_{50} value of 26 μM in the cell assay and proved to be a very effective inhibitor of adhesion.[200]

Glycopeptides carrying the 6-O-phosphorylated mannose residue were used as reagents for studying the recognition of phosphorylated glycoproteins by the mannose 6-phosphate (Man-6-P) receptor.[186] Lysosomal enzymes carry oligosaccharides with Man-6-P residues. Through these carbohydrates, they are recognized by Man-6-P receptors, then correctly processed, and finally transported to the golgi apparatus. Misregulation of these steps is involved in inflammatory processes in the central nervous system. Based on the suggestion that a second oligosaccharide with two Man-6-P residues is the natural ligand for the receptor, diverse open-chain and cyclic glycopeptides such as **130**–**132** (Scheme 47) were synthesized. A phosphorylated mannose or a phosphorylated disaccharide with α(1,6)- or α(1,2)-linked mannoses was introduced into these compounds, and the length and sequence of the amino acid chains between the saccharides was varied.[186] The glycopeptides were then examined in a



Scheme 46. Synthetic sialyl Lewis^X mimetics.



Scheme 47. Synthetic phosphomannosyl glycopeptides and their inhibitory effect on the phosphomannose receptor.

specially developed ELISA test as inhibitors of binding of the Man-6-P receptor to an immobilized phosphomannan. A high binding affinity was obtained if the ligands are bidental and carry 6′-O-phosphorylated α(1,2)-linked disaccharides; monosaccharides and α(1,6)-linked disaccharides are considerably less active. Three to five amino acids as spacer between the two saccharides were favorable. Cyclic peptides seemed to be conformationally too stiff for good binding.

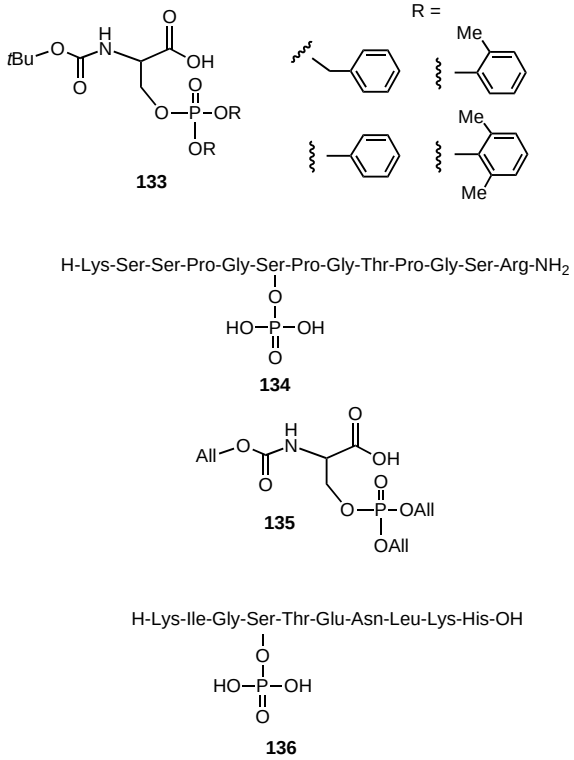
Since glycoproteins could represent the characteristic structures of tumor-associated antigens, corresponding synthetic glycopeptides have also been intensively used as reagents for developing antitumor vaccines.[201]

6.3. Phosphopeptides and Glycophosphopeptides

Protein kinase and phosphatase-mediated phosphorylation of proteins at serine, threonine, and tyrosine residues is used in all organisms to regulte many different intracellular processes, for example, signal transduction from the plasma membrane to the cell nucleus as well as direction of cell growth and division.[202] Characteristic phosphopetides were therefore repeatedly used as reagents for study and selective manipulation of such processes. The synthesis of phosphorylated serine and threonine peptides is impeded to a considerable extent by the extreme base lability of these peptide conjugates (see Scheme 27); protecting groups which can be released under mild conditions are required. However, peptides phosphorylated at tyrosine residues cannot lose the phosphate group by β-elimination, and are therefore much easier to synthesize.[138] The development of methods for their synthesis will not be discussed further here.

Serine/threonine phosphopeptides can in principle be made by post-synthesis phosphorylation of a separately synthesized peptide ("global phosphorylation strategy") or by stepwise synthesis of the target compound in solution or on solid phase with phosphorylated amino acid building units ("building block strategy"). The latter method is generally more flexible.

For the stepwise synthesis of phosphopeptides, Boc-protected building blocks such as **133** initially were used (Scheme 48); the phosphate protecting group had to be

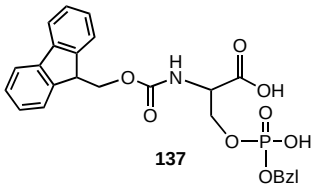


Scheme 48. Phosphopeptides synthesized with fully protected phosphoamino acid building blocks.

carefully chosen to avoid dephosphorylation under the conditions of Boc-group cleavage.[138, 203] Although this technique could be used to synthesize phosphopeptides, such as the partial sequence **134** of the Tau protein, on a polymeric carrier,[204] recently more advantageous synthetic building blocks were developed which were protected in other ways. Use of the N-terminal allyloxycarbonyl (Aloc) protecting group proved to be effective, for example. In a solid-phase synthesis of the Tau protein fragment **136**, the amino acid unit **135** was linked to a hexapeptide assembled on Wang resin with the base-labile N-terminal Fmoc group and acid-labile side-chain protecting groups.[205] By Pd^0-mediated release of the allyl protecting groups in the presence of Me_3SiN_3/Bu_4NF, the N terminus and the phosphate group were simultaneously deblocked without β-elimination. Extension of the peptide chain and subsequent release of the phosphopeptide from the resin, with simultaneous cleavage of the side-chain protecting groups, gave the desired peptide **136**. The disadvantage of this

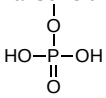
approach, however, is the simultaneous release of the phosphate group and amino functionality, since the latter can itself become activated during the subsequent peptide coupling.

The base-labile Fmoc protecting group, established as a standard method in solid-phase synthesis of nonmodified peptides and glycopeptides, cannot be used without further modification for synthesizing phosphopeptides, since serine and threonine phosphopeptides masked as phosphoric acid esters, unlike the more stable glycopeptides, lose the phosphate group by β-elimination and formation of α,β-dehydroalanyl peptides under the conditions for Fmoc cleavage.[206] This disadvantage was overcome, however, by introduction of the Fmoc-masked unit **137**, which only carries a phosphoric acid diester group (Scheme 49).[207, 208] The phosphate group is deprotonated under basic conditions and, as a poor leaving group, is no longer eliminated. It is stable with respect to treatment with piperidine, which is added to release the Fmoc group, and is apparently not activated upon subsequent extension of the peptide chain. With this building block, the phosphopeptides **138** and **139**, which represent phosphorylated partial sequences of the heat-shock protein 27, were synthesized on solid phase without any occurrence of the unwanted side reactions mentioned above.[208] This is therefore an efficient method for the flexible synthesis of phosphopeptides on polymeric carriers with the established N-terminal Fmoc group.



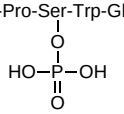
137

H-Ser-Gly-Val-Ser-Glu-Ile-OH



heat-shock protein 27 (87-92) **138**

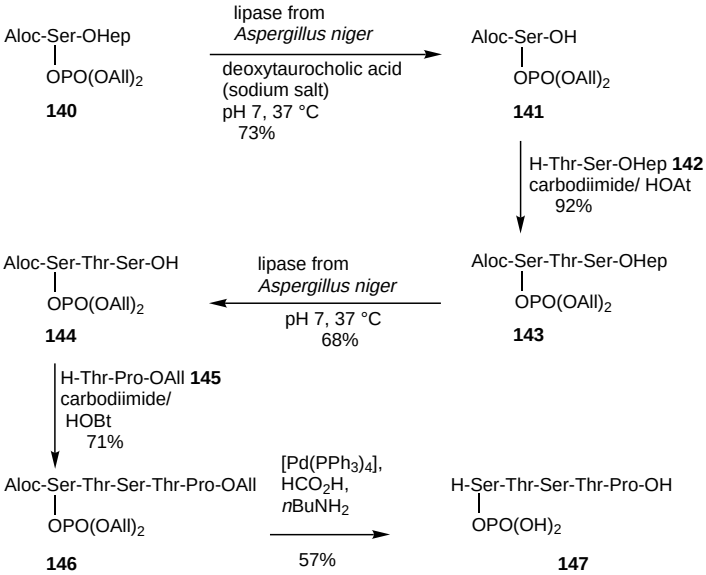
H-Leu-Leu-Arg-Ser-Pro-Ser-Trp-Glu-Pro-Phe-Arg-Cys-NH_2



heat-shock protein 27 (10-20)-Cys-NH_2 **139**

Scheme 49. Phosphopeptides synthesized with phosphoric acid diester **137**. The numbers in parentheses correspond to the sequence numbers in the protein.

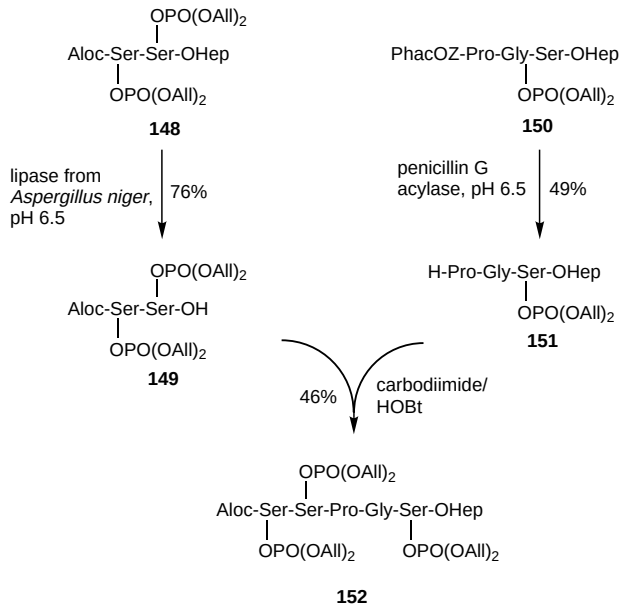
Enzymatic protecting group techniques have also provided interesting and advantageous alternatives to classical chemical methods in phosphopeptide synthesis. Thus, the phosphopentapetide **147** (Scheme 50), which is a characteristic section of the human Raf-1 kinase (see Section 2.2) was synthesized with lipase-mediated hydrolysis of the heptyl ester protecting group (see Section 6.2).[209] The fully protected phosphorylated serine heptyl ester **140** was selectively deblocked at the C terminus with the lipase from *Aspergillus niger*, and, following extension of the peptide chain, the enzyme released only the ester protecting group from the phosphotripeptide ester **143**. Renewed chain extension by a dipeptide and subsequent release of all allyl protecting groups gave the target compound **147**. The conditions for the enzymatic deblocking are so mild that there was no β-elimination of the phosphate residue from the phosphoric acid triester masked peptides.



Scheme 50. Chemoenzymatic phosphopeptide synthesis with the enzyme-labile heptyl ester.

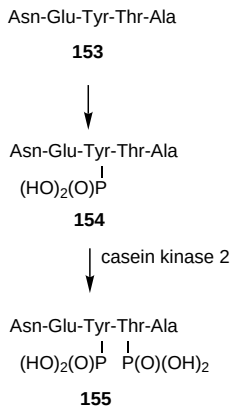
The N-terminal deblocking of phosphorylated peptides was achieved with the phenylacetoxybenzyloxycarbonyl (PhAcOZ) urethane protecting group.[210] This enzyme-labile urethane is an analogue of the AcOZ group described in Section 6.1 and was developed in the course of a phosphoglycopeptide synthesis (see below). On treating the PhAcOZ-masked phosphorylated tripeptide **150** with the enzyme penicillin G acylase, the terminal phenylacetic acid ester group was selectively cleaved off, and the spontaneous fragmentation shown in Scheme 31 was initiated. The phosphopeptide **151**, which is deblocked at the N terminus, that was released was then coupled with the doubly phosphorylated dipeptide **149** to give the pentapeptide **152** (Scheme 51), which is available for further C-terminal enzymatic deblocking. By selective lipase-mediated cleavage of the heptyl ester group, **149** was also obtained from the corresponding fully protected precursor **148**. An alternative possibility for selective N-terminal deblocking of phosphorylated peptides is by direct protection of the amino group with phenylacetic acid and biocatalyzed cleavage of the phenylacetamide with penicillin G acylase.[211]

Phosphorylated peptides have proven to be valuable tools in the study of signal cascades and the phosphoproteins involved therein. Phosphotyrosyl peptides have been repeatedly used in the elucidation of the binding specificity of signal transducing proteins which, for example, recognize phospho-



Scheme 51. Chemoenzymatic phosphopeptide synthesis with use of the enzyme-labile heptyl ester and the PhacOZ protecting group.

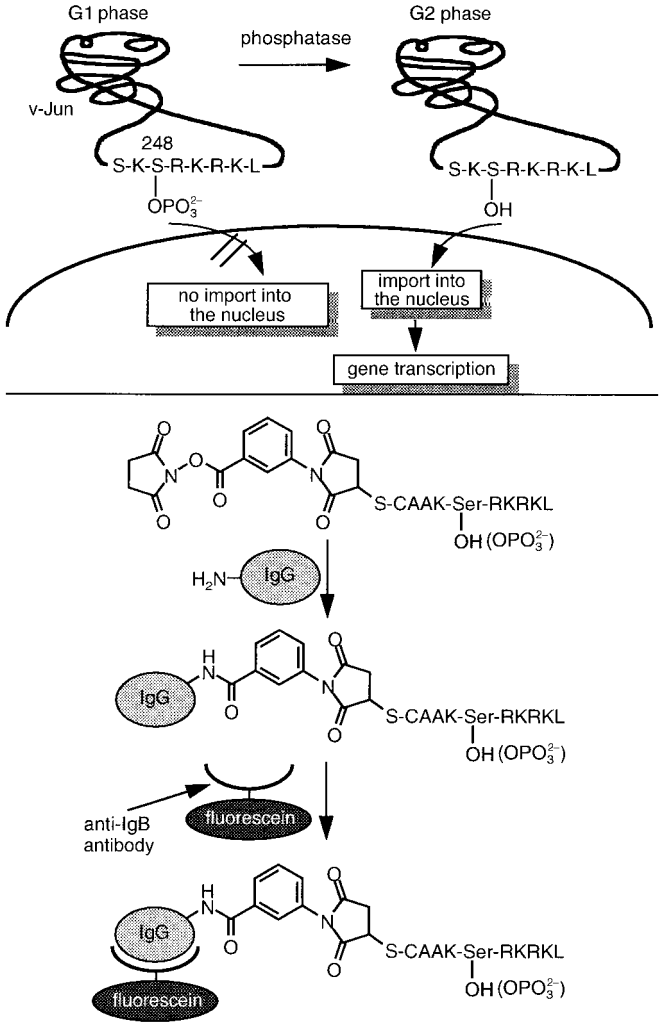
tyrosine residues in Ras and GPCR signal cascades with the help of SH2 domains (see Section 5). Targeted modulation of signal transmission by the JAK/STAT pathway was also achieved with such peptide conjugates (see Section 2.3). Furthermore, peptides modified with tyrosine phosphate which represent autophosphorylation sequences of tyrosine kinase receptors were used for determination of substrate tolerance of protein tyrosine phosphatases.[212] The substrate specificity of the Ser/Thr-specific casein kinase 2 was more closely defined with different phosphorylated peptides of the sequence Ala-Ser/Thr-Tyr-Ser-Ala,[213] which are derived from the phosphorylated form of the autophosphorylation site (Asn-Glu-Tyr-Thr-Ala) of the Src kinase family. Seven phosphopeptides were synthesized with the global phosphorylation strategy (see above) and tested as substrates for casein kinase 2. The enzymatic investigations showed that phosphorylation of the tyrosine residue favors the additional modification of the C-terminal serine; in the natural protein, the tyrosine phosphate group could function as the determinant for a subsequent second phosphorylation by casein kinase 2 at the adjacent C-terminal hydroxyamino acid (Scheme 52). This controlling activity explains why, in the case of the Src kinases, casein kinase 2 deviates from the typical substrate specificity, according to which the amino acid that is C-terminal to the target amino acid should be acidic.



Scheme 52. Enzymatic phosphorylation of a peptide with casein kinase 2.

A peptide in which a serine residue was phosphorylated was very elegantly used to elucidate the structural basis for the cell cycle dependent import of the tumorigenic transcription factor v-Jun in molecular detail.[214] In the G1 phase of the cell cycle, v-Jun is cytosolic. In this form, it is phosphorylated at Ser 248, that is, in the immediate vicinity of its nuclear localization sequence (NLS) which initiates active transport of the protein into the cell nucleus. In the G2 phase, the phosphoric acid ester is removed, however, and the protein concentrates in the cell nucleus. Apparently, the import of the v-Jun protein into the cell nucleus, which is essential for it to function as a transcription factor, is controlled by phosphorylation of the serine in the immediate vicinity of the NLS. That the phosphorylation has this crucial role in regulation of nuclear import was shown with the help of protein kinase and phosphatase inhibitors. In the presence of the serine kinase inhibitor staurosporin (see Section 7), the phosphorylation of v-Jun is suppressed, and the non-phosphorylated protein is transported into the nucleus in the G0, G1, and S phases of the cell cycle. The phosphatase inhibitor ocadaic acid (see Section 9.1) hinders dephosphorylation of the protein, however, and ensures that it also does not get into the nucleus in G2.

To test this hypothesis a phosphorylated peptide and the corresponding nonmodified peptide, both containing the serine residue in question and the NLS, were synthesized and linked to an immunoglobulin through an active ester (Scheme 53). Following microinjection of the protein conju-

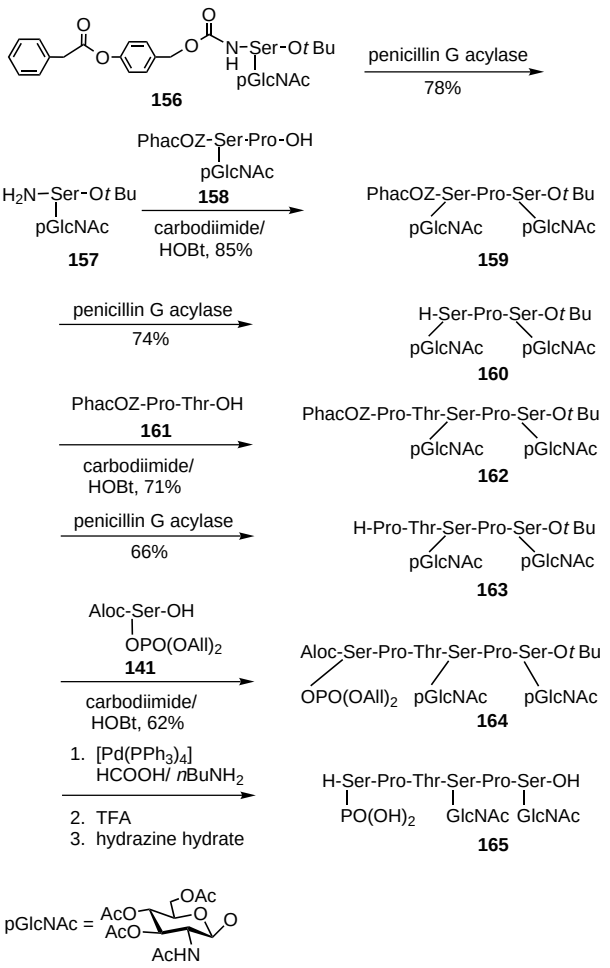


Scheme 53. Regulation of the nuclear import of v-Jun by phosphorylation.

gates into the cytosol of fibroblasts, their intracellular fate could be followed by fluorescence microscopy with a fluorescein-labeled anti-IgG antibody. This showed that the conjugate with the phosphorylated serine residue stayed in the cytosol, whereas the non-phosphorylated analogue was imported into the cell nucleus. Phosphorylated peptides have also often been used as antigenic structural units for creating monoclonal antibodies that recognize particular phosphoproteins.[205, 215] These antibodies serve, for example, as tools for immunodiagnosis of the Tau protein in Alzheimer's disease.

Recently, the glycosidic linkage of *N*-acetylglucosamine (GlcNAc) to the serine and threonine residues of intracellular proteins was reported as a new type of covalent intracellular protein modification.[216] The O-GlcNAc modification is similar to phosphorylation in many ways, especially with respect to its dynamic character and its ubiquitous occurrence, for example, in transcription factors such as Myc, the serum response factor (SRF), Jun, and Fos as well as oncogene products such as the tumor suppressors Rb and p53. Apparently, cells use reversible glycosylation with GlcNAc as a regulatory mechanism. In particular, it appears that phosphorylation and GlcNAc modification have a "yin–yang" relationship to one another, that is, they are not only similar but also often reciprocal to one another. The C-terminal domain of the human RNA polymerase II is multiply glycosylated in the cytosolic enzyme; however, the intranuclear enzyme is highly phosphorylated in the transcription complex and no longer glycosylated, whereby the phosphorylation and glycosylation sites are identical.[216, 217] Peptides containing both modifications could be valuable tools in answering the questions of whether the carbohydrate masks or even marks potential phosphorylation sites, and whether phosphorylation and glycosylation are involved in regulating nuclear import, or even influence transcription of the genetic code. The problems of both phosphopeptide and glycopeptide chemistry must be considered for their synthesis. However, with enzymatic protecting group techniques, the first synthesis of a complex phosphoglycopeptide which represents a characteristic section of the large C-terminal domain of the human RNA polymerase II was achieved.[218] Here, both lipase-mediated cleavage of heptyl ester groups (see Section 6.2) and enzyme-initiated fragmentation of a urethane protecting group (see above and Section 6.1) were employed.

On treating the fully protected glycosylated serine **156**, which includes the enzyme-labile PhAcOZ group at the N terminus (see Scheme 51), with penicillin G acylase, only the phenylacetic acid ester group was cleaved, and the fragmentation of the resulting phenolate shown in Scheme 31 led to release of the desired selectively deblocked amino acid **157** (Scheme 54). After being linked to a glycodipeptide, also PhAcOZ-protected, to form the diglycotripeptide **159**, penicillin G acylase rereleased the N terminus under very gentle conditions. A further cycle of chain extension and enzyme-initiated N-terminal deblocking gave the selectively deprotected diglycopentapeptide **163**. Subsequently, the allyl-masked serine derivative **141**, which was obtained by lipase-mediated cleavage of the heptyl protecting group from the corresponding ester (Scheme 50), was linked to the molecule.



Scheme 54. Chemoenzymatic synthesis of phosphoglycopeptide **165** with use of the enzyme-labile PhAcOZ protecting group.

Finally, successive release of all amino acid and carbohydrate protecting groups gave **165**.

Phosphoglycopeptides such as **146** have not yet been used in biological investigations. However, it was recently shown that a synthetic peptide modified with O-GlcNAc, corresponding to a repeat sequence of the C-terminal domain of the RNA polymerase II, inhibits in vivo gene transcription considerably.[219] The same effect was observed in the presence of an inhibitor of the enzymatic O-GlcNAc cleavage. These findings suggest that the O-GlcNAc modification is important for the transcription process. With synthetic phosphoglycopeptides, it should be possible to obtain valuable information about these and other biological processes.

6.4. Nucleopeptides

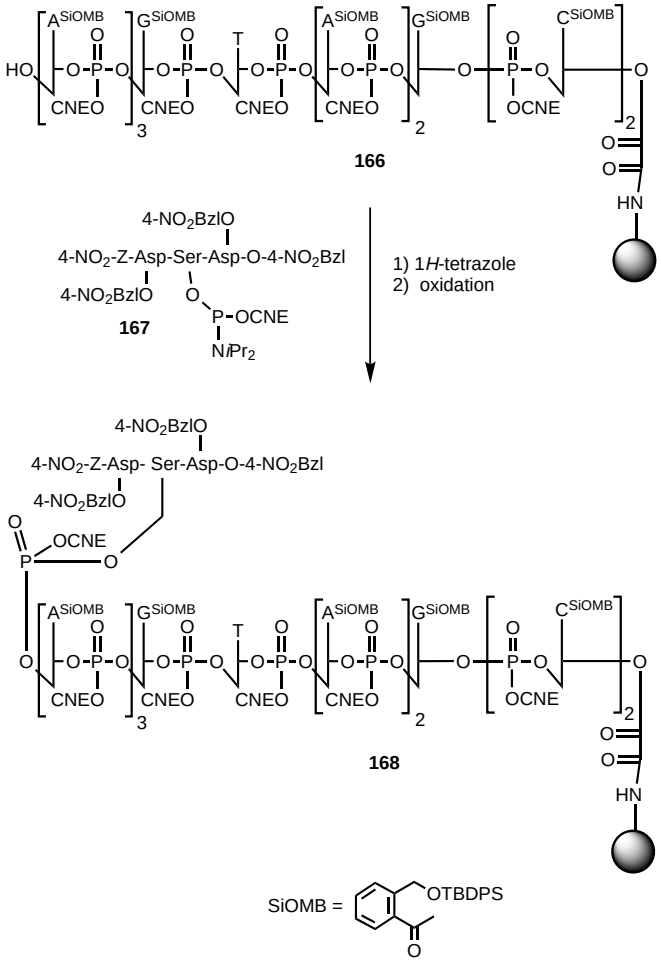
Nucleopeptides are involved in transcription of the genetic code in many different organisms. For example, formation of nucleopeptides plays a crucial role in priming replication of viruses,[220] DNA topoisomerases create nucleopeptides as part of their function,[221] and DNA exists as a nucleoprotein covalently linked to the matrix of the cell nucleus. Thus, characteristic nucleopeptides could open up many new

possibilities for studying gene transcription and particularly for developing new and alternative antiviral agents.

The synthesis of nucleopeptides presents in principle the same problems as that of phosphopeptides (see Section 6.3). However, the number of orthogonally stable protecting groups to be used increases considerably owing to the additional functional groups of the carbohydrates and nucleobases; also the acid lability of the purine N-glycoside forbids use of acid-labile protecting groups.

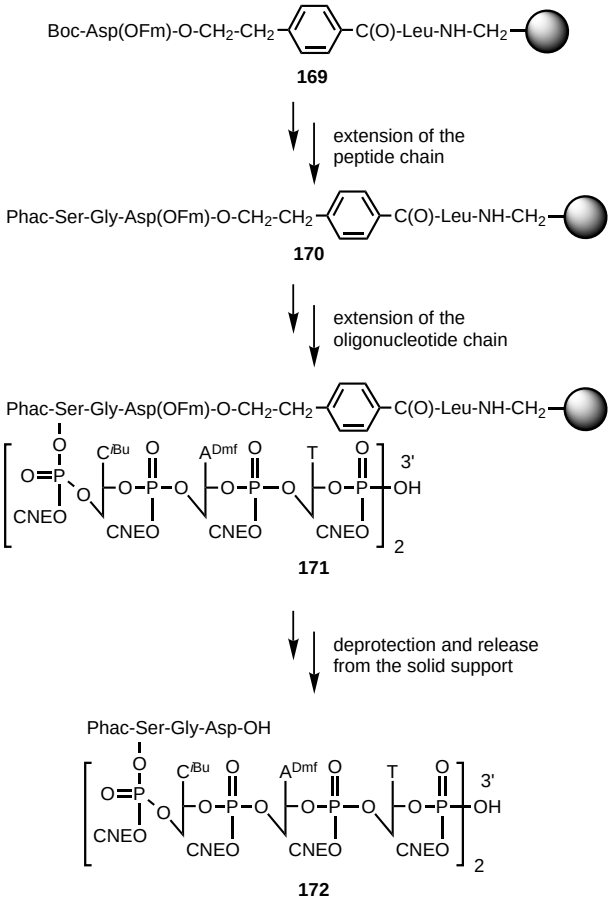
Initial work[222] showed that protecting groups established in peptide chemistry, such as allyl esters, often do not provide a suitable solution for this problem;[223] however, the first protecting group combinations were recently developed for synthesizing tyrosine nucleopeptides[223, 224] and serine/threonine nucleopeptides[223, 225–228] in solution and on polymeric carriers. Since the tyrosine derivatives—as in the case of phosphopeptides—are not destroyed by base-induced β-elimination, only synthesis of serine/threonine peptide conjugates are considered in this review.

Two general strategies were developed for the solid-phase synthesis of serine nucleopeptides. In a convergent method[225] the immobilized oligonucleotide **166** was first created and coupled to the separately synthesized peptide phosphoramidite **167** (Scheme 55). The protecting groups in the peptide



Scheme 55. Solid-phase synthesis of nucleopeptides by linking of a preformed peptide to an oligonucleotide. CNE = cyanethyl, TBDPS = *tert*-butyldiphenylsilyl.

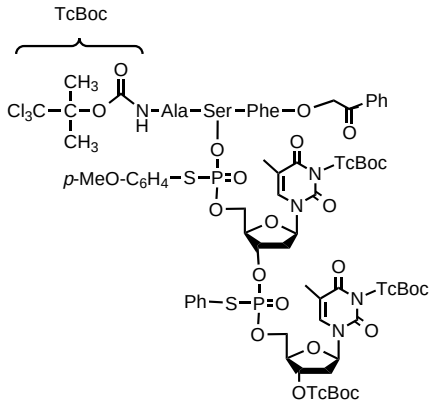
and oligonucleotide portions as well as the linker to the solid carrier were chosen so that the desired nucleopeptide was obtained in two concluding steps. Deblocking remained incomplete, however. An alternative procedure was the stepwise synthesis first of peptide **170** on a polymeric carrier by the Boc method followed by successive synthesis of the oligonucleotide chain[226] (Scheme 56). Subsequently, the linker to the polymeric carrier was cleaved by fluoride, and the base protecting groups were removed by treatment with concentrated ammonia. The desired nucleopeptide **172** was isolated in a total yield of 33 %. In neither case was it reported whether β-elimination occurred under the conditions used for cleavage.



Scheme 56. Solid-phase synthesis of nucleopeptides by successive extension of the peptide and nucleotide chains. Dmf = dimethylformamidinyl, Fm = fluorenylmethyl, Phac = phenylacetyl.

For the synthesis of nucleopeptides in solution, a strategy was successfully used in which the amino functionalities of the nucleobases and the N-terminal amino acids were protected with the trichloro-Boc group (TcBoc) and the C-terminus was protected as the phenacyl ester (Pac, Scheme 57).[227] In a convergent synthesis, a tripeptide was linked with a dinucleotide to give the nucleopeptide **173**; the protective functionalities mentioned were removed in one step without β-elimination.

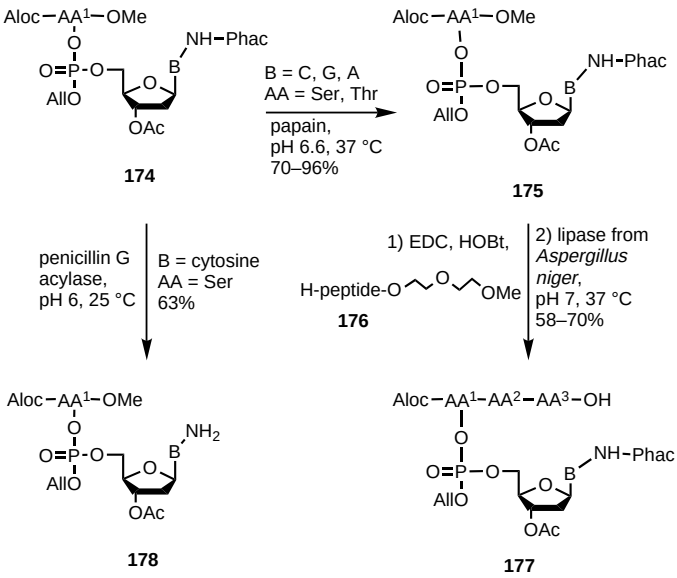
A stepwise flexible synthesis of nucleopeptides in solution, which, according to the previously described procedure, is not



173

Scheme 57. Combinations of protecting groups for the synthesis of nucleopeptides in solution.

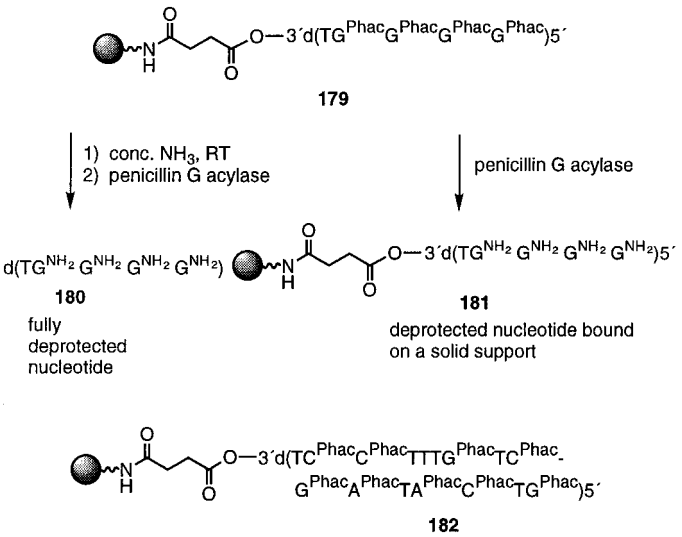
possible due to lack of orthogonality of the protecting groups, was achieved by combining classical chemical methods with enzymatic protecting group techniques,[228] however (Scheme 58). The nucleoamino acid **174** was synthesized for



Scheme 58. Nucleopeptide synthesis with use of enzyme-labile protecting groups.

this purpose, and the C-terminal methyl ester was cleaved off in high yield. Following extension of the amino acid chain with dipeptide diethylene glycol monomethyl ether (Dem) esters,[189] the selective C-terminal deblocking was performed again; the lipase from *Aspergillus niger* was the biocatalyst of choice. There was no β-elimination or attack on the other diverse protecting groups during the course of the enzymatic deblocking. On the other hand, the allyl protecting groups used could be selectively removed from **174** (B = cytosine) in a Pd^0-mediated transfer of the allyl groups, and the targeted deblocking of the amino functionality of the nuceobases of **174** was achieved by release of the phenylacetamide with penicillin G acylase.

This possibility for enzymatic deblocking of nucleobases is not limited to mononucleotides. For example, the PhAc-protected oligonucleotides **179** and **182** were synthesized on a solid carrier and cleaved off without deblocking the nucleobases[229] (Scheme 59). Release of all PhAc groups from the



Scheme 59. Enzymatic deblocking of the amino groups of oligoneucleotides in solution and on solid phase (controlled pore glass (CPG) beads, which are represented by spheres).

penta- and hexadecanucleotides was achieved by treatment with penicillin G acylase at pH 7 and room temperature. Furthermore, the enzyme even deblocked oligonucleotides such as **179** that were solid-phase anchored on the carrier (Scheme 59) without attacking the linker groups. This finding is of particular interest considering the possible application of biocatalysts in combinatorial synthesis on polymeric carriers.[230] Synthetic nucleopeptides have not yet been used for biological studies.

7. Modulation of the Activity of Protein Kinases C and A

Protein kinase C (PKC)[231] is a serine/threonine kinase that performs an important function in signal cascades in various cell types:[232] Cellular processes such as proliferation and differentiation are regulated by this function (see Section 2.1).[233–235] At present, at least 12 classes of the isoenzymes of PKC have been identified in mammalian cells,[236–238] which, according to the conditions required for activation, can be divided into three or four subfamilies.[239] The conventional PKC (cPKC) includes the isoenzymes α, β_1, β_2, β_3, and γ and requires calcium and diacyl glycerol (DAG) or phorbol ester for activation. The subfamily of the novel PKC (nPKC) includes the ε, δ, η, and o enzymes; their activity is independent of calcium but requires DAG or phorbol ester. The atypical PKC (aPKC) is represented by the subtypes ζ, δ, and τ, which function independent of calcium, diacyl glycerol, and phorbol esters, whereas the isoenzyme PKCμ has not been further classified. To perform its function, the latter

subtype requires membrane-bound phospholipids such as phosphatidylserine[233, 240] to localize the PKC at the plasma membrane. This illustrates that glycerolipids, sphingolipids and their metabolic products as well as phospholipases and phosphatidylinositol kinases can influence signal transduction processes (see Section 2.1).

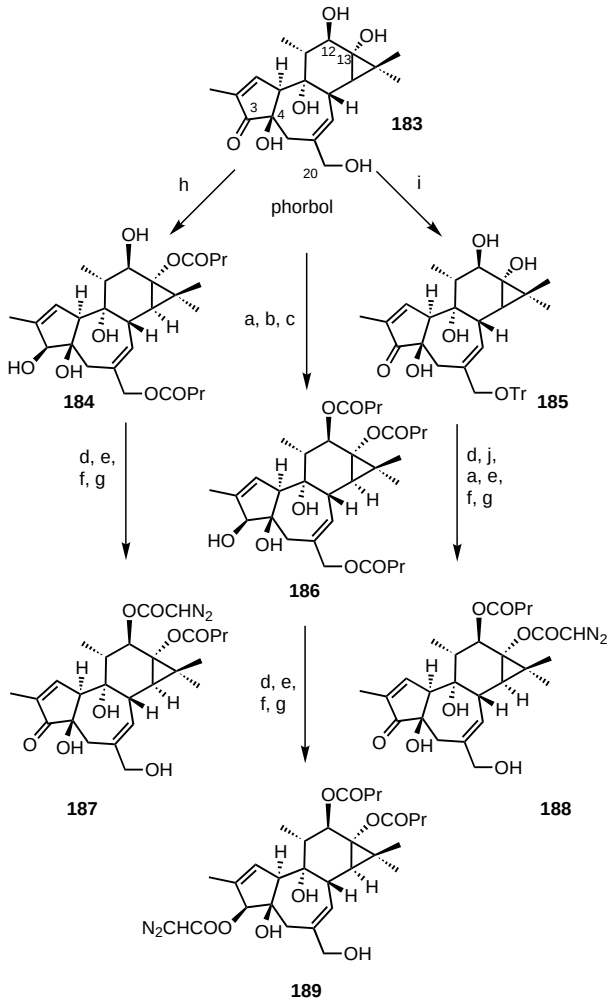
7.1. Modulation of the Activity of PKC by Binding to the Regulatory Domain

The activation of PKC by diacyl glycerol (see Section 2.1, Scheme 2) can also be achieved by various natural products such as the phorbol esters, teleocidins, bryostatins, and aplysia toxins.[241–245] With molecular modeling, an attempt was made to find a common pharmacophore for these structurally very different compounds.[246–251] This showed that a double cysteine rich motif in the C1 region of the PKC is necessary and sufficient for binding of diacyl glycerol;[244, 252–255] this finding was supported by an X-ray structural analysis of the complex of PKCδ and phorbol-13 acetate. Binding of the natural product to the protein through five hydrogen bridges creates a common hydrophobic surface and enables binding of the whole complex to the plasma membrane.[256] The structural requirements for formation of the PKC/activator/phospholipid aggregate are, however, for the most part unknown.

7.1.1. Phorbol Esters

Phorbol esters promote tumor growth by binding to the PKC and activating the enzyme over a long period, since, in contrast to DAG, they are metabolically stable. Two total syntheses of the structurally complex and synthetically very demanding phorbol ester have been published,[257a b] and diverse derivatives have been assembled starting from the natural product itself. For example, photoactivable substances carrying carbene precursors in the form of diazoacetyls on various hydroxyl groups of the molecule have been synthesized.[257c] Of six hydroxyl groups (on C3, C4, C9, C12, C13, and C20) those at C4 and C20 are probably needed for binding to the PKC, whereas that at C9 is sterically too well protected for a chemical derivatization. Starting from 12,13-phorbol dibutyrate, analogues with photolabile markers at C3, C12, and C13 were synthesized and tested for biological activity (Scheme 60). First, the relevant hydroxyl functionalities were esterified with Boc-glycine, and following cleavage of the Boc protecting group the free amine was diazotized. Selective hydrolysis of the butyl ester with $Ba(OH)_2$ in methanol gave the desired product.

To synthesize **189**, the three hydroxyl groups were acylated at C12, C13, and C20 with butyric acid anhydride in the presence of triethylamine and DMAP. Subsequently, reduction of the keto group with sodium borohydride/cerium trichloride and treatment with tetrabutylammonium fluoride (TBAF) selectively produced the biologically relevant β-alcohol **186**, which could be converted to the diazoacetic acid ester **189** with the procedure described above. If phorbol was acylated without addition of DMAP, a selective derivatization took place at C13 and C20 to provide the phorbol analogue



Scheme 60. Synthesis of photoactivable phorbol esters starting from phorbol. a) $[CH_3(CH_2)_2CO]_2O$, DMAP, NEt_3, CH_2Cl_2; b) $NaBH_4$, $CeCl_3$, MeOH; c) TBAF, THF; d) Boc-Gly, DMAP, NEt_3, DCC, THF; e) 1N HCl, AcOH; f) $NaNO_2$, H_2O/CH_2Cl_2, H_2SO_4; g) $Ba(OH)_2$, MeOH; h) $[CH_3(CH_2)_2CO]_2O$, NEt_3, THF/CH_2Cl_2; i) $(C_6H_5)_3CCl$, pyridine; j) $HClO_4$, MeOH.

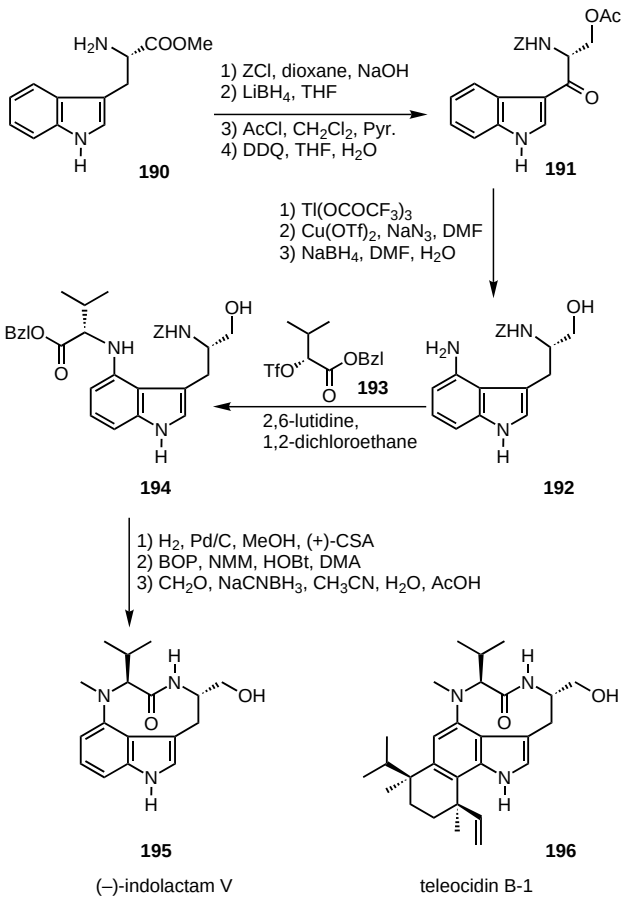
187, which is selectively labeled at C12. The various reactivities of the hydroxyl functionalities could also be used for labeling the C13 position (**188**). First, the most reactive primary alcohol was tritylated at C20, then the C13 position was selectively esterified with Boc-glycine. Following deprotonation of the primary hydroxyl groups, the C12,C20-dibutyl ester was obtained and selectively hydrolyzed to **188**.

The compounds synthesized were used in an investigation of the binding affinity and photoaffinity labeling of peptide C;[258] this represents a cysteine-rich subunit of the regulatory domain of PKCγ, and binds 12,13-[^{3}H]phorbol dibutyrate with high affinity in the presence of phosphatidylserine. Furthermore, like the PKC itself, it recognizes activators such as dioctanoyl glycerol and teleocidin B-4. Peptide C was not photolabeled by **187** and **188**, yet it was labeled by **189**. This indicates that the C3-hydroxyl group of the phorbol is close to the polar region of the phosphatidylserine in the bound complex.

Phorbol esters have been used in many different biological studies. For example, phorbol-12-myristate-13 acetate (PMA) triggers differentiation of the leukemia cell line HL60 into macrophage-like cells, which prove to be cytostatic and cytotoxic for malignant human cells in vitro.[259] Use of PMA also delivered information about the role of ceramide in inducing apoptosis through the Fas/Apo-1 cell-surface receptor. The effect triggered by ceramide was reduced by activation of PKC through PMA, which indicates that PKC modulates initiation of the ceramide-induced apoptosis.[260] The observation that different membrane transport processes are stimulated by PMA led to the conclusion that activation of PKC is involved in these processes and plays an important role in regulation of exocytosis, transport of receptors, and constitutive transport of proteins through the golgi complex.[261, 262] PMA was also successfully employed in studies of glucose uptake by translocation of the glucose transporter Glut 4 (see also Section 9.1). This showed that Glut 4 can be stimulated by insulin through PI3 kinase, through GTP (possibly through GTP-binding proteins), and through PKC (on activation by PMA).[263] Indications that PKC is also involved in signal mediation through nitric oxide formed in endothelial cells[264] were obtained with the phorbol derivative phorbol-12,13-dibutyrate (PDBu).[265] Thus incubation of rat hepatocytes led to release of the messenger NO. Finally, it has been shown with the help of PMA that a PMA-resistent isoform of PKC is directly involved in secretion of exocrine glands induced by the muscarinic agonist carbachol.[266]

7.1.2. Teleocidin and Indolactam V

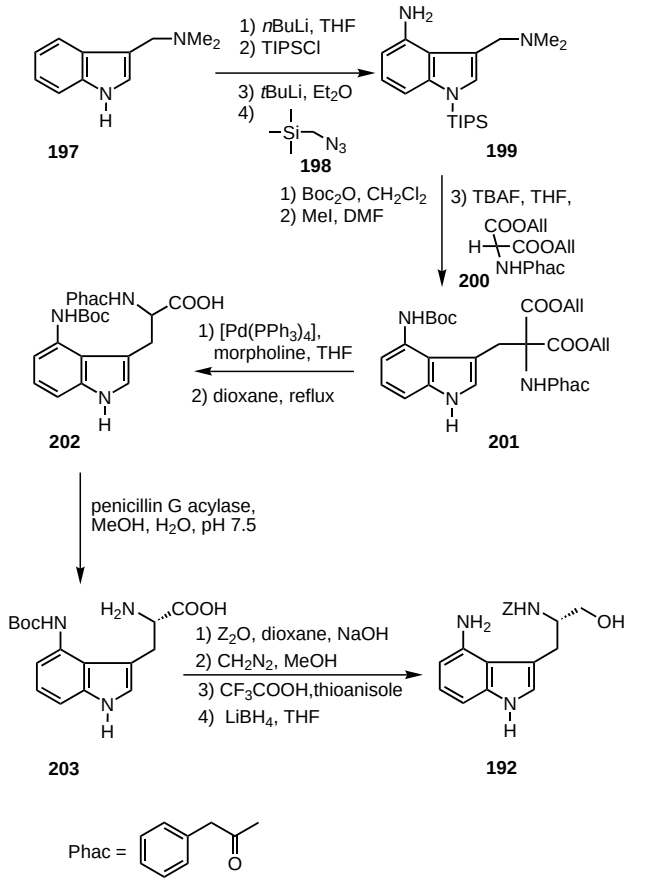
Teleocidin and indolactams are a family of indole alkaloids that promote tumor growth and, like phorbol ester, activate PKC. They were therefore used in studies of the biological relevance of this enzyme. The main problems in the total synthesis of these natural products,[267–273] all of which have the (−)-indolactam backbone (Scheme 61), were the regioselective substitution of the indole at C4 and C7, the ring closure to the nine-membered lactam ring, and control of the stereochemistry at C9 and C12 (see, for example, **195** and **196**, Scheme 61). Starting from L-tryptophan methyl ester, the ketone **191** was first obtained by protection of the amine, reduction of the ester with lithium borohydride, and subsequent acylation of the alcohol as well as a DDQ oxidation (Scheme 61).[270a] This ketone was metalated at the 4-position of the indole backbone with thallium(III) trifluoroacetate and converted to the 4-amino derivative **192** by treatment with sodium azide/copper triflate and subsequent reduction of the azido and keto functionalities. By a stereospecific reaction of the aminoindole **192** with the triflate **193** derived from D-valine, the missing stereocenter of the indolactam was assembled at C12. Hydrogenolysis released both the carboxyl and the amino functionalities so that lactam formation could be achieved with the help of 1-benzotriazolyloxytris(dimethylamino)phosphonium (BOP) hexafluorophosphate. The missing N-methyl group was introduced by reductive amination with formaldehyde in the presence of sodium cyanoborohydride.



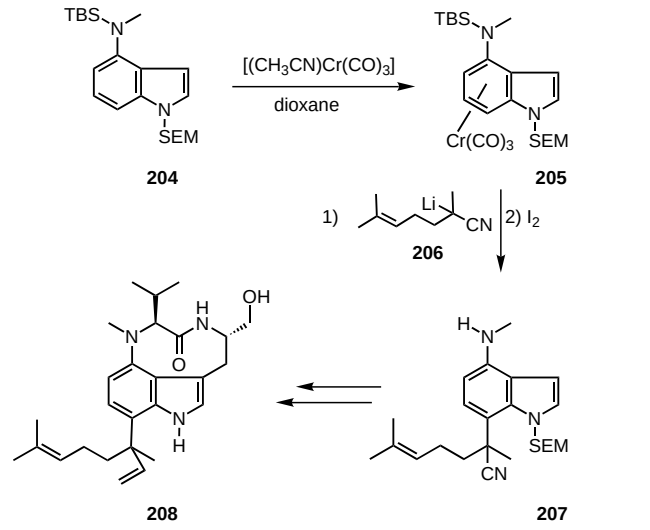
Scheme 61. Synthesis of (−)-indolactam V and teleocidin B-1. (+)-CSA = (+)camphorsulfonic acid, NMM = *N*-methylmorpholine, Tf = trifluoromethanesulfonyl.

In an alternative synthesis,[270b] the thallium reagent was avoided by selective lithiation at the 4-position of the TIPS-protected gramine **197** (TIPS = triisopropylsilyl) and introduction of the amino functionaliaty with trimethylsilylmethyl azide (Scheme 62). Following protection of the primary amine group, the side chain was assembled on an intermediary α,β-unsaturated imine by quaternization of the amine group and conjugated addition of phenylacetylaminodiallyl malonate. Cleavage of the allyl ester and decarboxylation of the dicarboxylic acid gave the racemic phenylacetamidotryptophan derivative, which was resolved into the enantiomers with penicillin G acylase. Amino acid **203**, which was selectively derivatized at the C4 position of the indole ring, could be converted into alcohol **192**, a precursor for indolactam V (see above).

The teleocidins have a greater PKC-activating effect than indolactam V owing to their higher lipophilicity, and, therefore, their synthesis[274, 275] and particularly the regioselective substitution at the 7-position has been given a great deal of attention. This derivatization was achieved by nucleophilic addition at an indole ring activated by a tricarbonylchromium fragment (Scheme 63). Furthermore, the C7 position of the indolactam V can be functionalized by a Vilsmeier formylation[276] to obtain a photolabile indolactam V derivative. For this, the indolactam V was protected as the acetate and then formylated at C7 of the indole backbone (→**209**). Condensation of the aldehyde with nitrohexane (hydrophobic residues

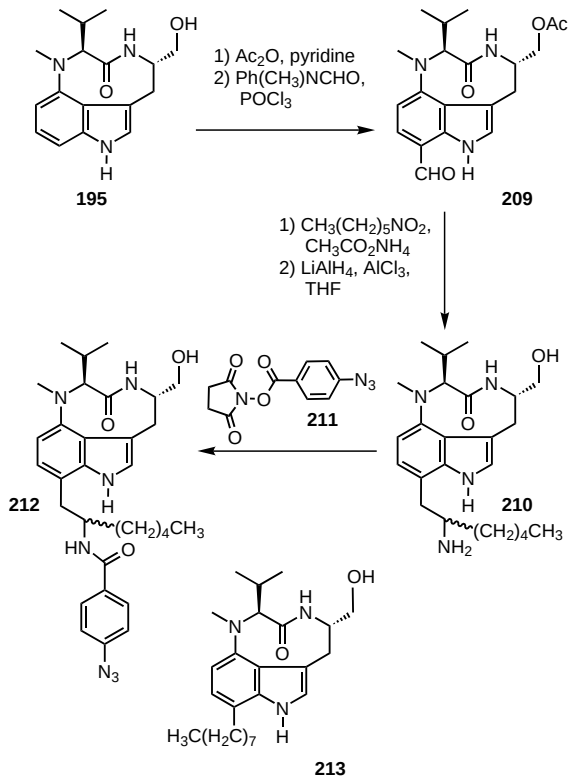


Scheme 62. Synthesis of the indolactam V precursor **192** with enzymatic racemate resolution.



Scheme 63. Synthesis of teleocidin **208**. SEM = trimethylsilylethoxymethyl.

at the C7 position of teleocidine seem to increase the tumor-promoting activity of this substance)[277] and subsequent reduction delivered amine **210**, to which ^{3}H-labeled succinimido 4-azidobenzoate (**211**) was added (Scheme 64). With these compounds in many different in vitro assay systems, attempts were made to identify the binding partners and



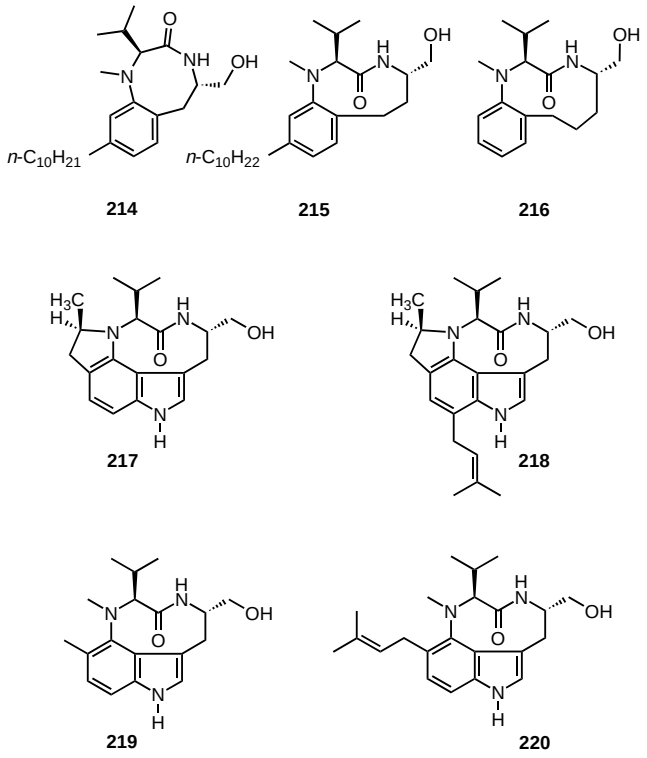
Scheme 64. Synthesis of the photolabile teleocidin derivative **212** and the biologically active (−)-7-octylindolactam (**213**).

binding sites of these natural-product analogues by photoaffinity labeling. These experiments were unsuccessful, however.

Indolactam V was used in mechanistic studies of the regulation of muscle contraction triggered by calcium/calmodulin-mediated activation of myosin kinase.[278, 279] Direct activation of PKC leads to contraction of arterioles, and indolactam V also proved to be a potent constrictor of small arteries. It was suggested that the alkaloid modulates the influence of Ca^{2+} on the contractile processes and brings about an increase in phosphorylation of myosin kinase.[280] Indolactam V was also used to develop a rapid and generally applicable phenotype assay of PKC activity for identification of PKC-activating or -inhibiting substances.[281] The (−)-7-octylindolactam **213** was successfully used in studies of the receptor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA). GABA is recognized by two receptors ($GABA_A$ and $GABA_B$), which become desensitized on binding the ligand. Activation of PKC also leads to desensitization of $GABA_A$; use of **213** showed that the receptor is phosphorylated in this process. It is assumed that the purpose of the PKC signal system is to switch off receptors that were not completely desensitized by GABA. Finally, **213** was used in a study of phosphoinositide conversion in glioma cells. Conversion of this messenger substance is initiated by binding of an agonist to the ATP receptor, and it is assumed that PKC is involved in control of this process, by negative feedback. (−)-7-Octylindolactam and also PMA thus bring about desensitization by activation of PKC.[283]

7.1.3. Indolactam Analogues

Teleocidins and indolactams can adopt two stable conformations in solution, a twist conformation with a *cis* amide that is preferred by five- to eight-membered rings, and a sofa conformation with *trans* amide stucture that dominates for compounds with rings with ten or more members. To find out which of these two conformations is relevant for biological activity, different conformationally restricted analogues were designed, synthesized, and tested for biological activity.[273, 284–288] For example, the indole ring was exchanged for a benzene ring, and lactams were synthesized with different ring sizes that should adopt the twist (e.g. **214**) or the boat (**215** or **216**) conformation.[284] To ensure that the compounds were also sufficiently hydrophobic, a *n*-decyl chain was added to the arene. The activity of the compounds synthesized was examined in assays for growth inhibition, cell adhesion, and differentiation of human promyelocytic leukemia cells (HL-60) to monocytes. The twist conformation is the biologically active conformation of teleocidins. It was also possible to determine the optimal length of the alkyl side chains as 10 – 14 carbon atoms (Scheme 65).[285]



Scheme 65. Conformationally restricted indolactam analogues.

By replacing the indole backbone with a benzofuran ring, it was shown that the indole nitrogen atom influences the isoenzyme selectivity, but only plays a supporting role in the interaction with the PKC.[287] With an aza-Claisen *trans*-formation as the key step, analogues **217** and **218** with a twist conformation were synthesized. Examination of the biological activity of these and other derivatives as well as of the analogues **219** and **220**, which imitate the sofa conformation, also showed that it is the twist form that is biologically relevant.[288]

7.1.4 Other PKC Activators

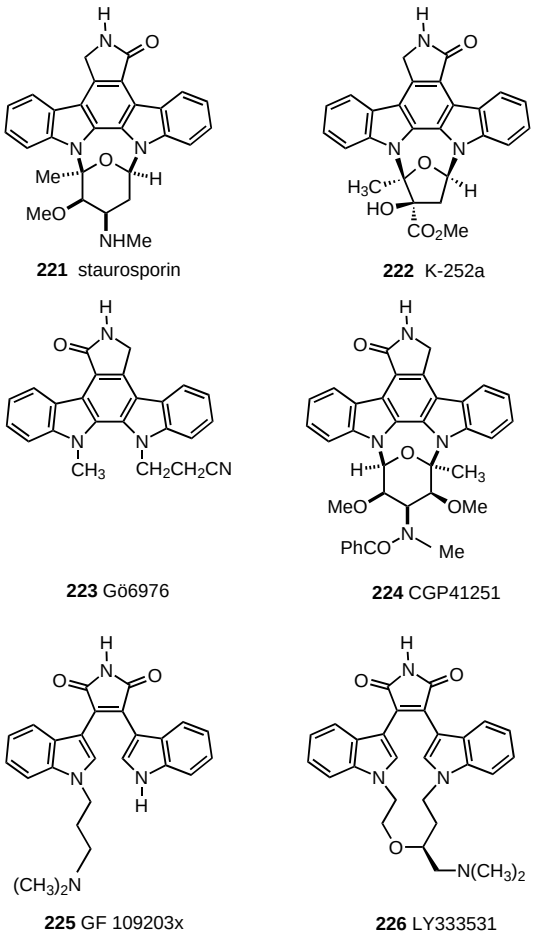
Diacyl glycerol (DAG) analogues were examined in detail as activators of PKC. This showed that the stereochemistry of the glycerol derivative is important for binding to the enzyme. *sn*-1,2-DAG analogues are recognized, *sn*-2,3- and 1,3-DAG derivatives are inactive. Two ester carbonyl groups and an alcohol were needed for activation.[289] The length of the acyl group may vary; *sn*-1,2-dibutyroyl glycerol is inactive, however, since it is taken up by membranes and forms micelles. Cyclic analogues were also synthesized but proved to be inactive. Of the diverse analogues examined, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and 1,2-dioctanoyl-*sn* glycerol (DOG) turned out to be particularly valuable reagents for biological studies.[290] The high selectivity with which the DAG analogues recognize PKC indicates that at least three interactions with the enzyme are important; these are probably hydrogen bonds with the two ester carbonyl groups and the OH functionality.[291] Other PKC activators which will not be discussed here are mezerine,[292] 6-(*N*-decylamino)-4-hydroxymethylindole (DHI),[293] and ingenol.[294, 295]

7.2. Inhibition of PKC

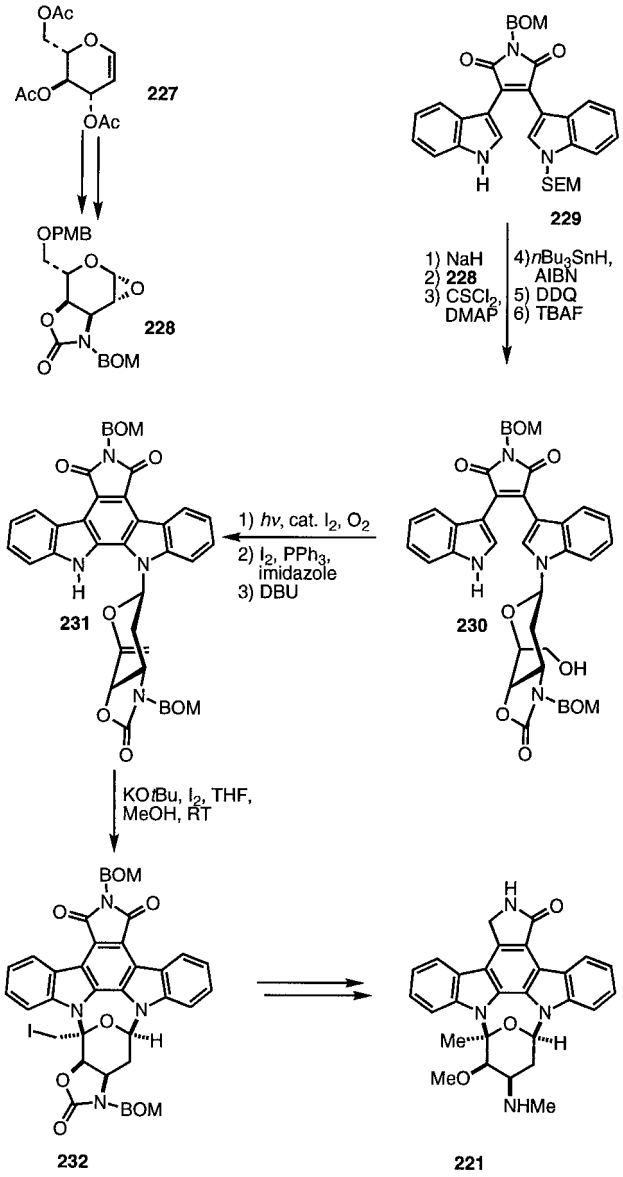
Since PKC has a decisive role in many different processes, discovery of selective inhibitors of this kinase is of great importance. Of particular interest is the development of inhibitors that show selectivity for the different isoenzymes (see Section 7). Staurosporin[296] and K-252a[297] are microbial products from the indole carbazole alkaloid family (Scheme 66) with potential antitumor activity. Staurosporin is one of the most potent PKC inhibitors known with activity in the nanomolar region,[298] and it interacts with the ATP binding site in the catalytic domain.[299, 300] The total synthesis of this natural product was recently reported;[301–304] one of the biggest synthetic challenges was to link two glycosidic bonds to the only weakly nuceolophilic indole nitrogen atoms.

The monosaccharide epoxide **228** used for this synthesis was obtained from tri-*O*-acetyl-L-glucal (**227**, Scheme 67).[301, 302] It proved to be a suitable glycosyl donor for linkage with the aglycon precursor **229**, which was obtained from N-BOM-masked dibromomaleinimide in a three-step sequence. The heterocycle was N-deprotonated and subsequently coupled with the epoxide **228** formed in situ. Following deoxygenation at the C2 position of the glycoside under Barton conditions and deprotection of the primary hydroxl group in the carbohydrate as well as of the second indole nitrogen atom, **230** was obtained. A photocyclization followed by conversion of the primary alcohol into the corresponding iodide and elimination of hydrogen iodide gave *exo*-glycal **231**. The key step in the synthesis was activation of the double bond with iodine accompanied by cyclization to **232**. Following dehalogenation, opening of the oxazolidinone, methylation, cleavage of the BOM group, and reduction of the imide to the amide, staurosporin and its enantiomer were produced.

(+)-Staurosporin and the closely related natural products (+)-K-252a, (+)-RK-286c, and (+)-MLR-52 were also ob-



Scheme 66. Structures of staurosporin, K-252-a, and analogues of these natural products.



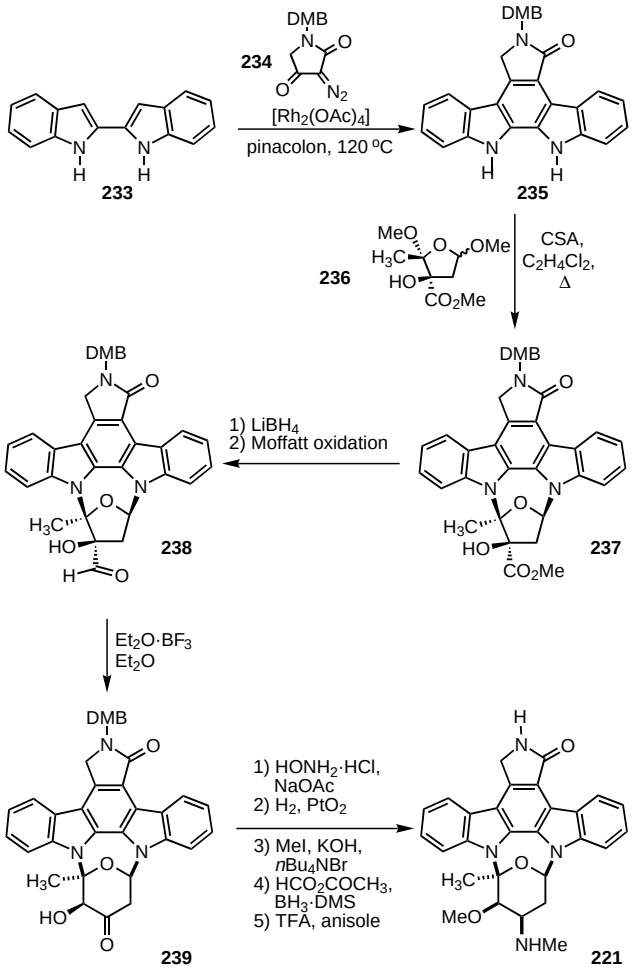
Scheme 67. Synthesis of staurosporin by successive glycosylations. AIBN = azobisisobutyronitrile, BOM = benzyloxymethyl.

tained starting from the common intermediate **239** (Scheme 68).[303, 304] For their synthesis, the carbene precursor **234** was allowed to react with the 2,2′-bis(indole) **233** in the presence of dirhodium tetraacetate to the 3,4-dimethoxybenzyl-protected aglycon **235**. In a cycloglycosylation reaction with the furanose derivative (−)-**236** in the presence of camphorsulfonic acid, the bis(indole) gave **237**, which underwent a ring extension to the pyran derivative **239** following reduction of the ester to the aldehyde upon treatment with boron trifluoride etherate. Reductive amination of the ketone via the oxime and alkylation delivered staurosporin in a total of 19 steps.

Investigation of staurosporin and isomeric compounds in two cell lines, on three human PKC isoenzymes, and on topoisomerase I showed that the spatial conformation of the lactam C5 carbonyl group and the pyranosyl C4′ methylamino functionality are more important for biological activity than the conformation of the pyranosyl C3′ methoxy functionality and the pyranosyl C2′ methyl group.[302]

Owing to the highly conserved structure of the catalytic domain of different protein kinases, staurosporin (**221**) and K-252a (**222**) are only unselective inhibitors, however,[305] so further analogues were synthesized to try to find more selective enzyme inhibitors. Examples are the analogues **223**–**225** (Scheme 66). Compound **223** belongs to a class of analogues in which the amino sugar was replaced by different alkyl substituents,[306] and it represents one of the most active and selective compounds of this type. The second class of inhibitors, of which **224** is an example, are semisynthetic derivatives of the natural product, whereby **224** inhibits PKC much more effectively than, for example, PKA and tyrosine kinases.[307] The third class are bis(indolyl) maleimides.[308–311]

An increased level of diacyl glycerol and the resulting long-lasting activation of PKC in macrovascular tissue is responsible for development of chronic vascular complications in diabetes.[312] The observation that the PKC isoenzyme β_2 is strongly expressed in the retina, heart, and aorta is particularly relevant to this problem. To determine whether this isoenzyme is responsible for the complications, inhibitors that specifically block this PKC subtype were needed.[313] The staurosporin analogue **226** proved to be a potent compound which was nontoxic and could be orally administered in a rat model. With **226**, the vascular misfunction could be



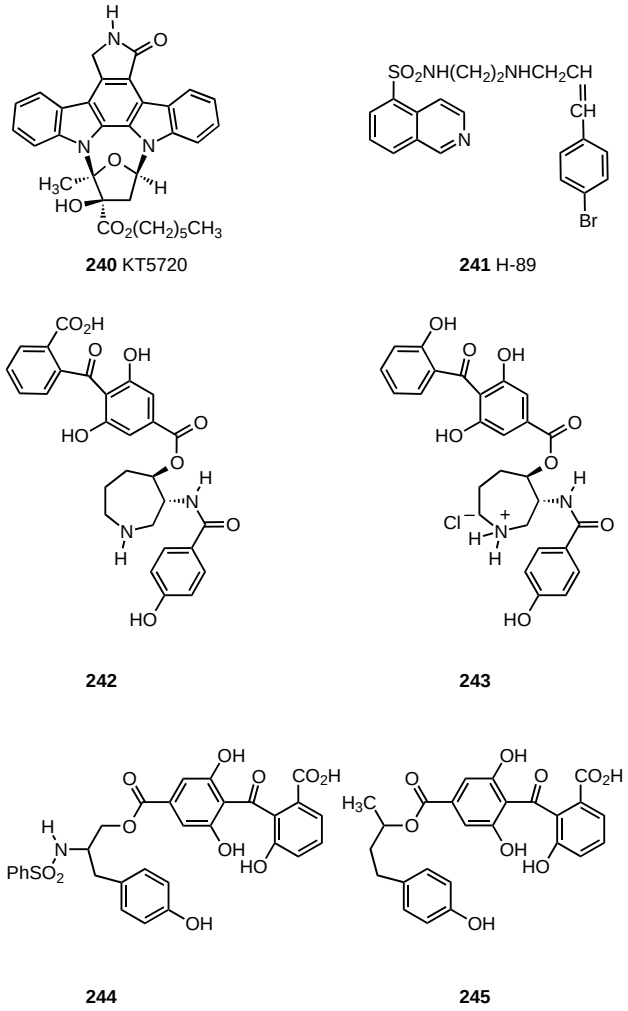
Scheme 68. Synthesis of staurosporin by cycloglycosylation and rearrangement.

corrected, and it was shown that the β_2 PKC isoenzyme is responsible for the corresponding complications in diabetes.

7.3. Inhibition of Protein Kinase A

Protein kinase A (PKA) is a serine/threonine kinase that is activated by cAMP upon stimulation of G protein coupled receptors; it transmits the incoming signal into the cell interior by phosphorylation of other target proteins (see Section 2.1). Since PKA is involved in many important processes, there is a lot of interest in developing selective inhibitors for this enzyme. PKA is made up of a heterotetrameric complex of two catalytic and two regulatory subunits. When cAMP binds to the regulatory subunit, the conformation of the enzyme changes and leads to release of the catalytic subunit, which then phosphorylates the target proteins.

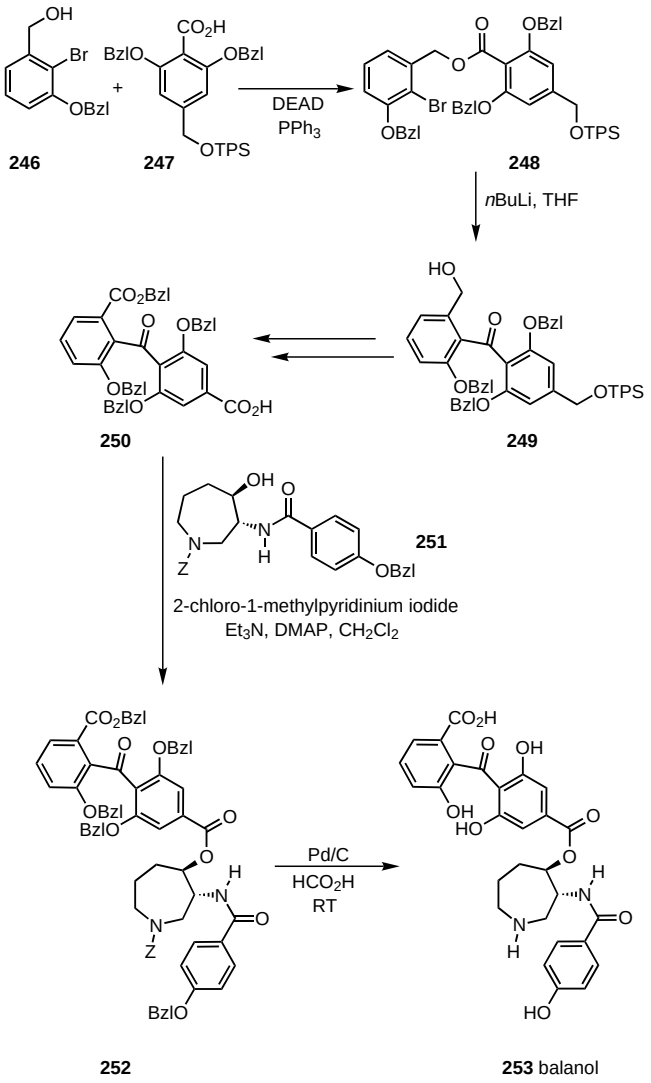
Some oligopeptides such as Ala-kemptide (kemptide = H-Leu-Arg-Arg-Ala-Ser-Leu-Gly-OH) and Asn-Ala-kemptide recognize and bind the PKA catalytic subunit, and inhibit enzyme activity by imitating the influence of the regulatory domain. Interest has been focused on non-peptide natural products and their derivatives, however, which has led to the discovery of two of the most selective nonpeptide inhibitors: KT 5720 (**240**), which is structurally related to staurosporin, and the isoquinolone sulfonamide derivative H-89 (**241**, Scheme 69).



Scheme 69. Inhibitors of protein kinase A.

The fungal metabolite balanol (**253**)[314–316] is also a PKA inhibitor ($IC_{50} = 4$ nM) which, however, inhibits PKC with equal efficiency. Several total syntheses of the natural product have been performed in order to develop analogues with higher selectivity.[317–321] In a convergent strategy to this end, two similarly complex partial structures of the molecule (**250** and **251**) were linked together, and the natural product was obtained by simple removal of the benzyl protecting groups (Scheme 70). Starting from D-serine, hexahydroazepine **251** was obtained in 15 steps (total yield 36%). To synthesize benzophenone **250**, the brominated benzyl alcohol **246** was linked to the aromatic carboxylic acid **247** in a Mitsunobu reaction to give ester **248**. Metalation with *n*-butyl lithium converted **248** into benzophenone **249**, from which the intermediate **250** was obtained by standard transformations. Esterification of **250** and **251** and subsequent deblocking produced balanol (**253**).

To elucidate which structural characteristics of the natural product are responsible for kinase inhibition, further balanol



Scheme 70. Convergent total synthesis of balanol. TPS = triphenylsilyl.

analogues were synthesized by the procedure described above.[318] Two of the compounds obtained, 10″-deoxybalanol (**242**) and 14″-decarboxybalanol hydrochloride (**243**), inhibited PKA more effectively than PKC and were more potent enzyme inhibitors than KT 5720 (**240**) and H-89 (**241**).[322] Balanol analogues in which the perhydroazepine ring and the *p*-hydroxybenzamide group are replaced by an acyclic unit, such as **244** and **245** (Scheme 69), show inhibitory activity. They inhibit PKC much more effectively than PKA and other serine/threonine kinases.[323]

8. Inositol Phosphate Analogues

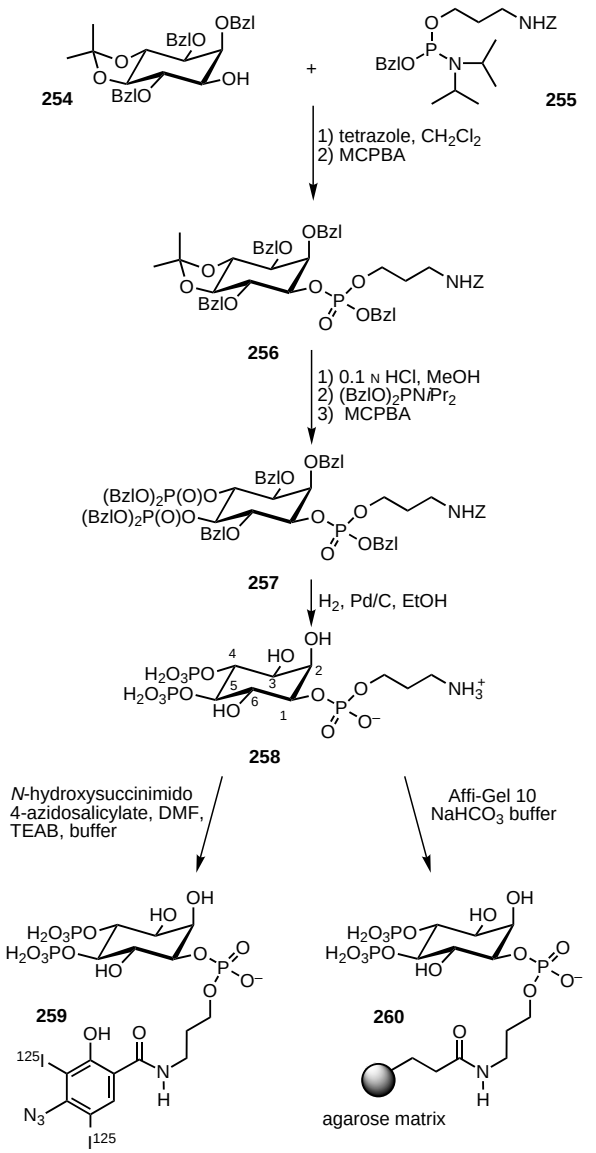
Inositol derivatives play a crucial role in signal transduction through heterotrimeric G proteins (see Section 2.1). Furthermore, they are also involved in other signal cascades. Activation of receptor tyrosine kinases can therefore lead to stimulation of the phosphoinositol-3 kinase (PI-3K),[324] which produces phosphatidylinositol 3,4,5-trisphosphate.[325] This is then converted into the corresponding 3,4-bisphosphate, which serves as a membrane anchor recognized by protein kinase B (PKB, *Akt*) through pleckstrin homology domains and thus brings about membrane localization of this protein. The PKB is subsequently activated by phosphorylation and switches on a further cascade.[326] Owing to the high biological relevance of inositol phosphate derivatives and the possibilities opened up for selective manipulation of physiological processes such as insulin secretion,[327, 328] a multitude of analogues have been synthesized and used as tools in biological studies and as lead substances for the development of new pharmaceuticals. As a result, the chemistry of inositol phosphates is highly developed and has been extensively reported in several reviews.[18, 19] Only a few newer developments and applications in biological studies will be presented here.

8.1. Inositol Phosphate Derivatives

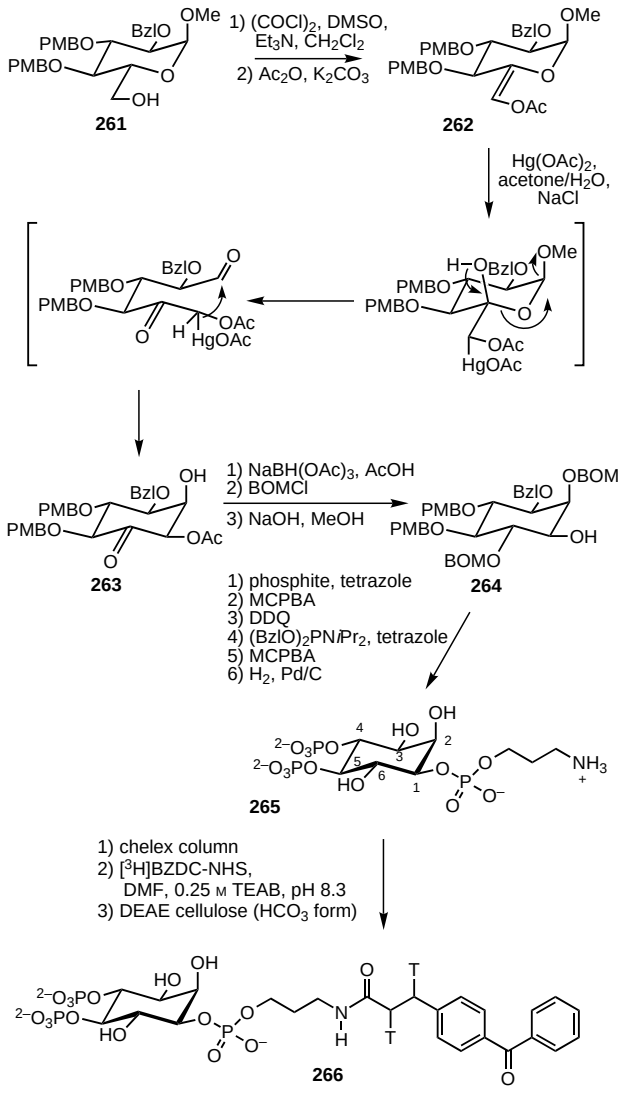
The focus of interest in inositol phosphate chemistry was particularly on the 1,4,5-trisphosphate and the investigation of the structural basis for the recognition of this mediator by the 1,4,5-IP_3 kinase, by the 1,4,5-IP_3 5-phosphatase, and by its receptor leading to calcium mobilization from intracellular reserves. By synthesizing a range of derivatives, it was possible to elucidate the molecular causes of these interactions in detail. According to these findings, the presence of the 4,5-bisphosphate motif seems to be essential for recognition by the receptor; furthermore, an additional phosphate group at the C1 position leads to increased release of Ca^{2+}. The presence of sterically demanding substituents at the C1 phosphate and the 2-OH group is tolerated. The phosphate group at C4 seems to be of particular importance for phosphorylation of the 1,4,5-IP_3 by the 1,4,5-IP_3 kinase. Modifications of the C5 phosphate are tolerated by the kinase; likewise, the C1 phosphate is important but not essential. For dephosphorylation of 1,4,5-IP_3 by the 5-phosphatase, the C1 phosphate group is probably of particular importance.

Based on this knowledge, derivatives suitable for photoaffinity labeling (**259**) were synthesized and an affinity matrix (**260**) was produced for further study of the 1,4,5-IP_3 receptor (Scheme 71).[329] For this purpose, the partially protected inositol **254** was converted into phosphate **256**, which could be selectively deblocked and phosphorylated to the 1,4,5-trisphosphate **257**. Subsequent removal of all benzyl protecting groups gave the inositol derivative **258**, from which the photoactivable and radioactively labeled 4-azidosalicylamide (**259**) and the affinity matrix **260** based on agarose could be synthesized. In an in vitro test system, trisphosphates **258** and **259** displaced [^{3}H]-myo-1,4,5-IP_3 from purified rat-brain receptors and released calcium from receptor preparations reconstituted in liposomes loaded with Ca^{2+}.[331] Both compounds were neither substrates nor inhibitors for the 1,4,5-IP_3 5-phosphatase and the 1,4,5-IP_3 kinase. With the immobilized derivative **260**, it was possible to isolate and purify receptors with good activity.

Recently, an improved general method was developed for the assembly of different enantiomerically pure IP_n derivatives and their functionalization to affinity matrices and



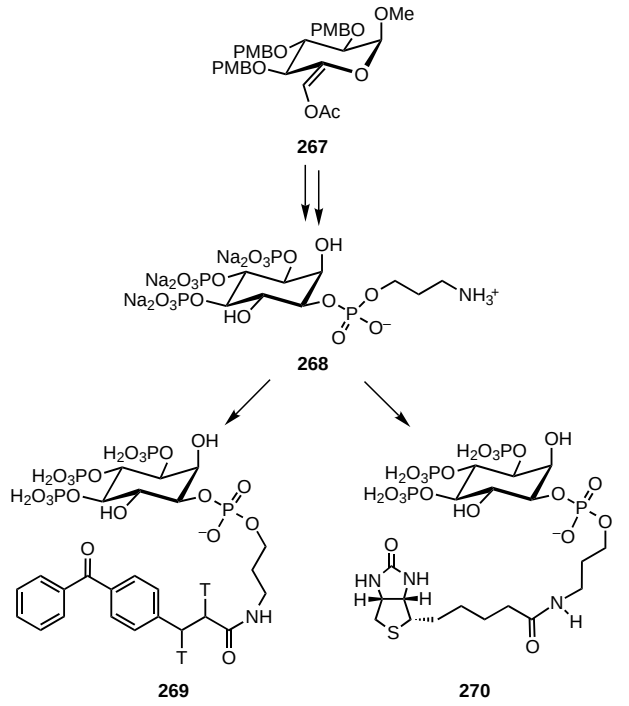
Scheme 71. Synthesis of inositol trisphosphates for photoaffinity labeling and production of an affinity matrix. MCPBA = *meta*-chloroperbenzoic acid, TEAB = tetraethylammonium bromide.



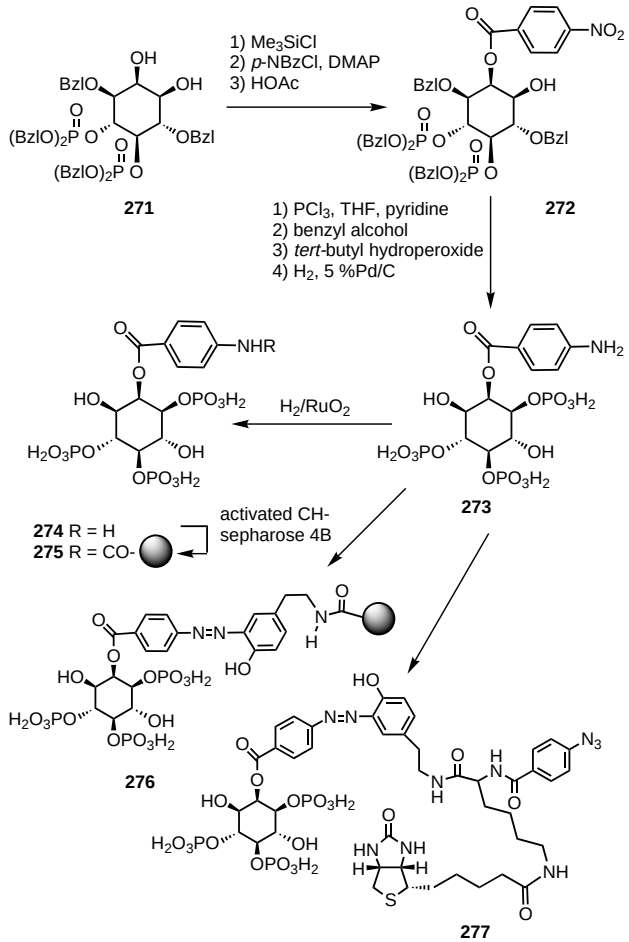
Scheme 72. Synthesis of an inositol phosphate analogue for biological studies. See Scheme 79 for the structure of BZDC-NHS (**308**), DEAE = diethylaminoethyl, T = ^{3}H.

photolabile analogues.[332] Starting from D-glucose, the differently protected glycoside **261**, which is selectively deblocked at the primary OH group, was synthesized, oxidized to the aldehyde, and converted into enol acetate **262** (Scheme 72). On treatment with mercury(II) acetate in aqueous solution, **262** reacted by ring opening and alternative ring closure to ionose **263**, which was stereoselectively reduced and then masked at 2-OH and 6-OH as the benzyloxymethyl (BOM) ether. Selective cleavage of the acetyl group enabled introduction of an aminoalkyl-modified phosphate group and following selective removal of the PMB groups, phosphorylation, and release of the remaining hydroxyl functionalities, derivative **265** was obtained. A radioactively labeled and photoactivable side chain was subsequently linked to the aminopropyl group (→**266**). Amide **266** was used, for example, to mark the PIP_2-binding pleckstrin homology (PH) domain of phospholipase C δ_1.[333, 334] By varying the pattern of protecting groups, 1,3,4,5-IP_4-tetrakisphosphates such as **269** and **270** were assembled with the same procedure (Scheme 73). Also IP_4 derivatives were used for producing affinity matrices and photoaffinity markers. With IP_4 affinity chromatography, different tetrakisphosphate receptors were purified. In addition, an IP_6 receptor was isolated and labeled.[335–337]

2-Acyl derivatives were also used as photoaffinity markers and for affinity chromatography (Scheme 74).[338] The synthesis started from the 4,5-bisphosphate **271**, which was obtained in five steps from D-*myo*-inositol. Manipulation of the protecting groups delivered the monohydroxyl derivative **272**, which—following phosphorylation, oxidation, and hydrogenolysis—gave trisphosphate **273**. Reduction of the aromatic ring gave the carboxylic acid derivative **274**. Compounds **273** and **274** were examined for interaction with the 1,4,5-IP_3 5-phosphatase and the 1,4,5-IP_3 3-kinase, exchange with D-[^{3}H]-1,4,5-IP_3, and ability to mobilize calcium ions. This showed that both analogues are substrates of the phosphatase and could release calcium from saponin-permeabilized[339–340]



Scheme 73. Synthesis of the inositol tetrakisphosphates **269** and **270**.

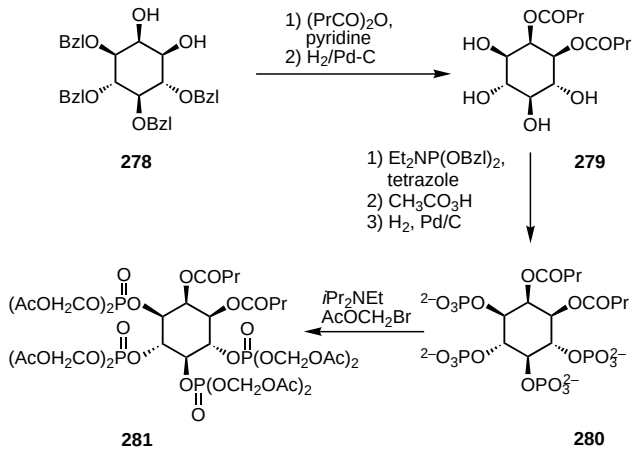


Scheme 74. Synthesis of the affinity matrices **275** and **276** as well as the biotinylated derivative **277**. *p*-NBzCl = *para*-nitrobenzyl chloride.

macrophages; only the D isomer of **274** was a substrate of the kinase, however. Likewise, displacement of D-[^{3}H]-1,4,5-IP_3 from its binding site in microsomal fractions of rat brain was observed. This led to the conclusion that the 2-OH functionality of the D-1,4,5-IP_3 is probably not involved in recognition by the receptor. Starting from derivatives **273** and **274**, affinity matrices such as **275** and **276** (Scheme 74) were produced for isolating corresponding cellular binding partners of 1,4,5-IP_3. To that end, **274** was coupled to an activated CH-sepharose 4B (→**275**). Amine **273**, following formation of the diazonium salt, opened up a route to matrix **276**. Similarly, the biotinylated analogue **277** was constructed, which could be detected without radioactive labeling with the biotin/avidin system. Labeling of a 1,4,5-IP_3 5-phosphatase from erythrocyte ghosts was achieved through the again activated amino functionality.[341]

The labeled analogues **266** and **269** were used as tools for isolating the cellular binding partner of the corresponding natural compounds and for labeling the exact sequences of the binding domains of these proteins. For example, the binding domain of the IP_3 receptor and of the PIP_2-binding protein were examined with [^{3}H]-BZDC-1,4,5-IP_3 (**266**).[333] In addition to the well-known role of the 4,5-bisphosphate in receptor recognition, it was also shown that the hydrophobic interaction is important for PLC isoenzymes for binding through the PH domain. Furthermore, four IP_4-binding proteins were isolated from rat brain, one of which was efficiently labeled by [^{3}H]-BZDC-1,4,5-IP_4 (**269**). This protein, called centaurin, has a PKC zinc finger and other domains in addition to the IP_4 binding domain; it also seems to be the first protein that binds PIP_3 more effectively than IP_4.[342]

Deblocked inositol phosphates cannot penetrate membranes at all, or only with great difficulty, owing to their high polarity; therefore, their use in biological studies is often limited. This problem was elegantly solved with the help of protected precursors such as **281** (Scheme 75).[343] In **281**, four



Scheme 75. Synthesis of a membrane-accessible inositol tetrakisphosphate analogue.

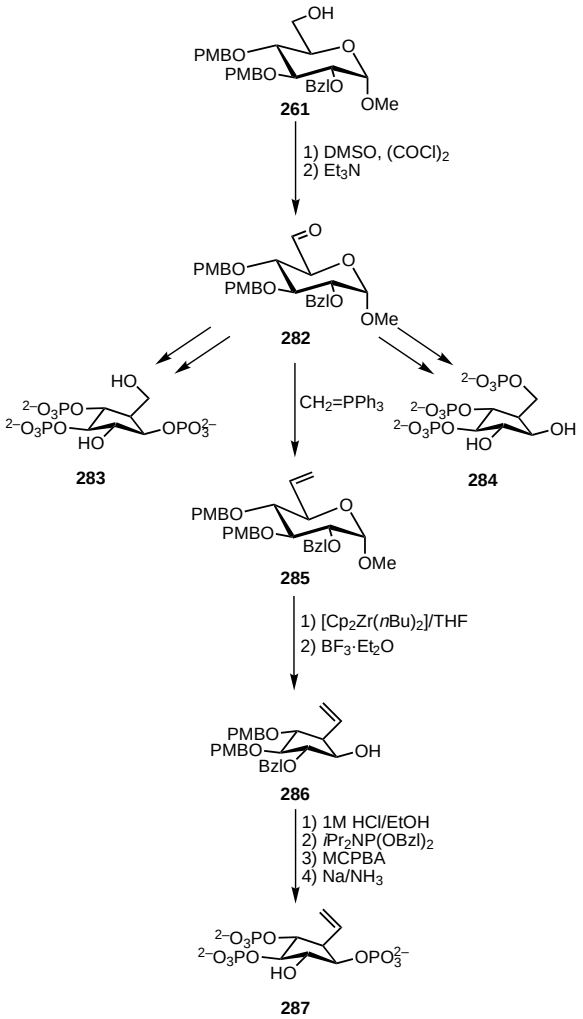
phosphate groups are protected as acetoxymethyl esters, and the OH groups of the inositol units are masked as butyrates. This lipophilic prodrug form of the inositol 3,4,5,6-tetrakisphosphate can penetrate membranes well and is intracellu-

larly deblocked by esterases and lipases to 3,4,5,6-IP_4 rapidly. With this reagent, it was possible to study the role of 3,4,5,6-IP_4 in the regulation of chloride secretion.[343] Chloride channels, which are regulated by cyclic nucleotides and the intracellular Ca^{2+} concentration, control the secretion of salt and liquid in intestinal epithelial cells; osmoregulation, pH balance, and neurotransmission also depend on the regulation of the chloride concentration. With **281**, it was shown that an increased flow of chloride ions brought about by raised Ca^{2+} concentration (e.g., triggered by histamine) is inhibited by 3,4,5,6-IP_4. The study also showed that, in the presence of a high 3,4,5,6-IP_4 concentration, chloride secretion is independent of Ca^{2+} concentration.

8.2. New Inositol Phosphate Analogues

The 1,4,5-IP_3 derivatives **287**, **283**, and **284** were developed as new inositol phosphate analogues with a modified ring; they have a five-membered ring instead of the cyclohexane structure (Scheme 76).[344] In **287** the positions 1, 4, 5, and 6 are still in the correct spatial orientation, but a C atom in the contracted ring is deleted. Several studies have shown that this C2 position is not needed for recognition by the 1,4,5-IP_3 receptor. To synthesize analogue **287**, the selectively deblocked glucose derivative **261** was synthesized and converted into the vinyl carbohydrate **285** by Swern oxidation and subsequent Wittig methylenation. Upon treatment with $[Cp_2Zr(n\mathrm{Bu})_2]$ and then with boron trifluoride etherate, **285** underwent a ring contraction to the vinyl cyclopentane **286**. Standard manipulations delivered the 1,4,5-IP_3 analogue **287**. With **287**, it was possible to mobilize calcium ions from 1,4,5-IP_3 receptors of platelets, although the concentration needed was higher than for 1,4,5-IP_3 itself.

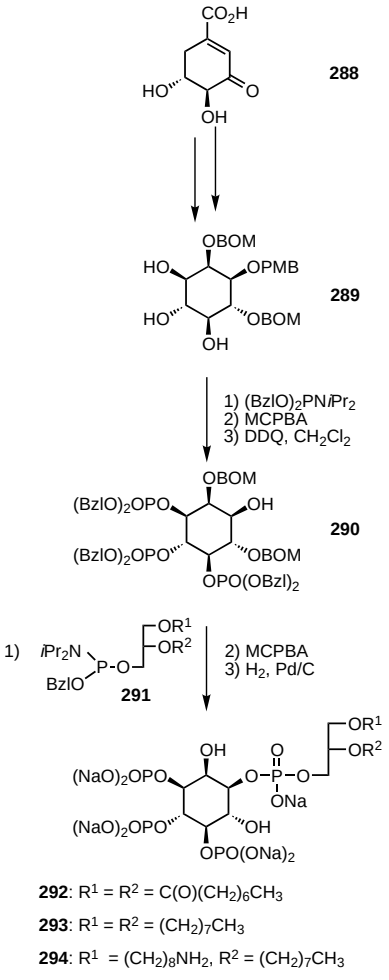
Starting from the intermediate **282**, analogues **283** and **284** were synthesized with the help of a ring contraction mediated by samarium(II) iodide. Compound **283** proved to be superior to the vinyl derivative **287** in mobilizing Ca^{2+}, whereas **284** hardly mobilized Ca^{2+} at all. These observations illustrate that a six-membered ring is not necessary for the development of 1,4,5-IP_3 agonists, and thus open up new perspectives for the investigation and disruption of the inositol signal pathways.



Scheme 76. Synthesis of ring-modified inositol phosphate analogues.

8.3. Analogues of Phosphatidylinositol Phosphates

Enantiomerically pure diester and diether analogues of PIP_2 and PIP_3 were constructed from compounds from the chiral pool, for example, dehydroshikimic acid (**288**, Scheme 77).

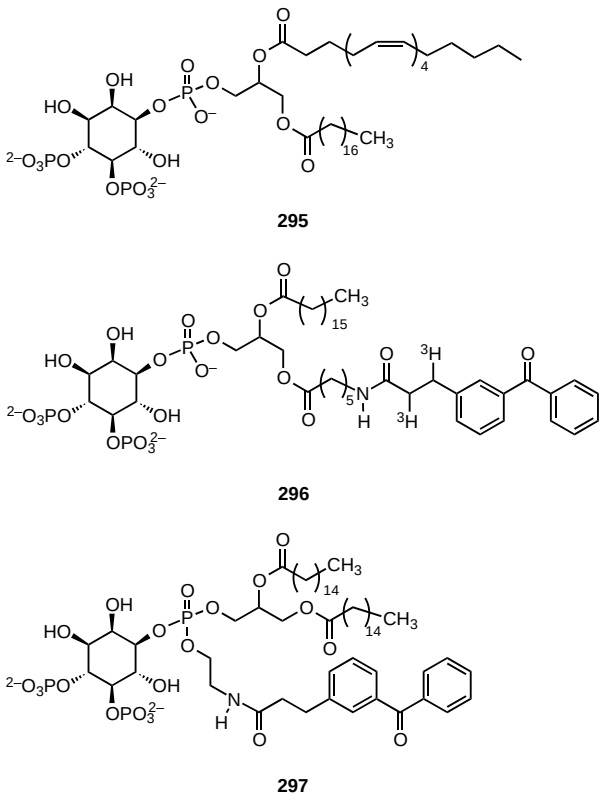


Scheme 77. Synthesis of phosphatidylinositol phosphates starting from dehydroshikimic acid.

To synthesize the ester or ether derivatives **292**–**294**, the natural product was first converted into triol **289**, which gave trisphosphate **290** on phosphorylation and removal of the PMB group. Condensation with the phosphoramidates **291**, oxidation with perchloric acid and deprotection gave the PIP_3 analogues **292**–**294**.[345] The water-soluble derivatives **292** and **293** stimulated the calcium-independent PKCδ, PKCε, and PKCη isoenzymes in vitro,[345, 346] whereas **294** can be used for coupling to antigens and for producing affinity matrices.

Based on the structure of PIP_3 **295** and the IP_3 analogues described, derivatives of PIP_3 with reporter groups, glycerol **296** and phosphotriester **297** were synthesized (Scheme 78).[322, 347, 348] Their synthesis started from the inositol derivatives **298** and **299** and used phosphoramidates **306** or **307** (Scheme 79). In the end product, the hydroxyl functionalities previously protected as PMB ethers are now phosphorylated; the alcohols previously protected as benzyl or benzyloxymethyl esters are now free. To incorporate reporter groups in the glycerol side chain, fragments **298** or **299** and **307** were coupled to one another and further elaborated; thus, PIP_2 and PIP_3 analogues were obtained. The PIP_2 and PIP_3 analogues modified at the phosphotriester (**304** and **305**) were similarly obtained following reaction of phosphoramidate **306** with inositol derivatives **298** and **299** and further elaboration.

These PIP_2 analogues and related compounds were used in photolabeling and fluorescence spectrometry tests. It was shown that the myristoylated alanine-rich C kinase substrate (MARCKS) brings about an encapsulation of PIP_2 and phosphatidylserine in lateral domains of the cell membrane through electrostatic interactions, and thus initiates a reversible inhibition of hydrolysis of PIP_2.[349]



295

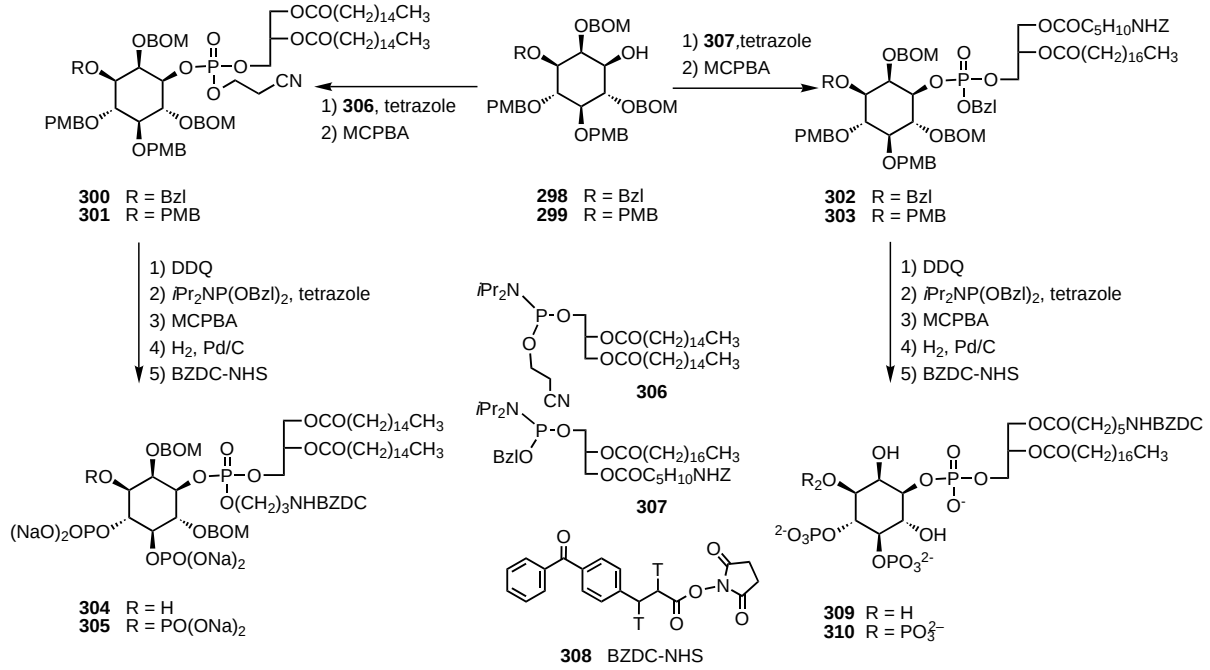
296

297

Scheme 78. Structures of phosphatidylinositol phosphate **295** and labeled analogues.

9. Inhibition of Phosphatases

Phosphorylation and dephosphorylation of proteins are critical components of signal transduction mechanisms that regulate cell differentiation and growth (see Sections 3, 6, and



300 R = Bzl
301 R = PMB

298 R = Bzl
299 R = PMB

302 R = Bzl
303 R = PMB

306

307

308 BZDC-NHS

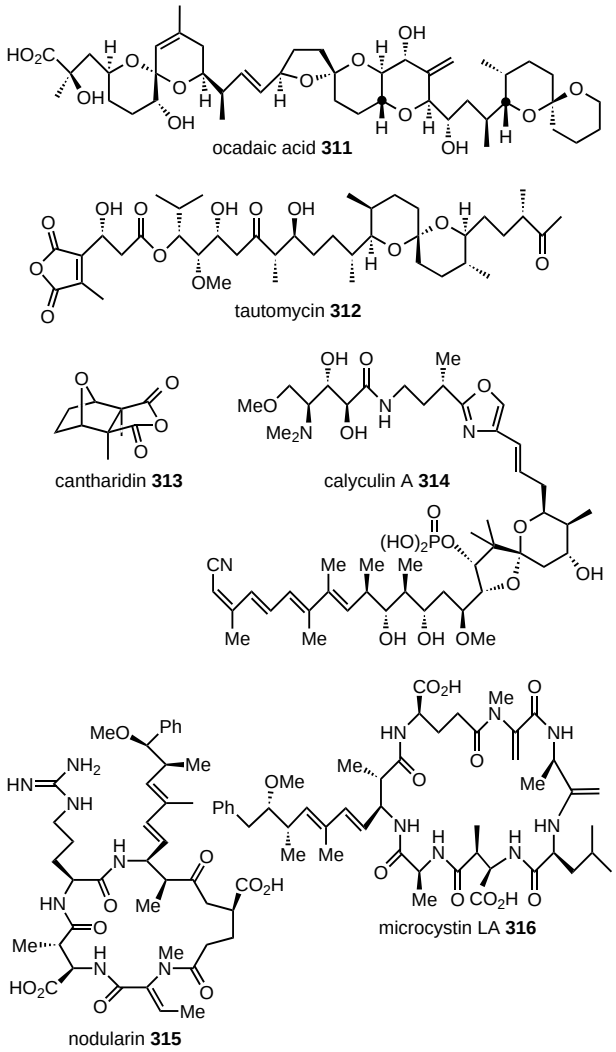
304 R = H
305 R = $PO(ONa)_2$

309 R = H
310 R = PO_3^{2-}

Scheme 79. Synthesis of labeled phosphatidylinositol phosphate analogues.

7). The position of this modulatable equilibrium is determined by the activity and concentration of protein kinases and phosphatases,[350] which reinforce or weaken one another according to the cellular context.[351] A disturbance of this balance can lead to disease and to transformation of the cell. In Sections 3 and 7, tyrosine- and serine/threonine-specific kinases were presented; in the following, their cellular opponents will be described.

Eucaryotic phosphatases[350] are structurally and functionally varied enzymes that are divided into several families. The Ser/Thr-specific phosphatases differ considerably in their catalytic domains from the "dual-specificity" phosphatases and from the tyrosine-specific phosphatases, which both have a high sequence homology. The structure of several serine/threonine and tyrosine phosphatases was obtained by crystal structure analysis. The catalytic mechanisms are also known.[352] The importance of these enzymes for cell physiology is underlined by the fact that they are often the target of microbial or viral attack. For example, λ-bacteriophage codes for a Ser/Thr phosphatase, the virulence plasmid from *Yersinia* for a Tyr phosphatase, and the *Vaccinia* virus for a phosphatase that dephosphorylates both Ser/Thr and Tyr residues.[350b] Inhibitors of these biocatalysts are important tools for in vivo investigations and have led to the discovery of a range of physiological functions of protein dephosphorylation. In particular, natural products such as ocadaic acid (**311**),[353] tautomycin (**312**),[354] calyculin A (**314**),[355] cantharidin (**313**),[356] nodularins such as (**315**),[357] and microcystins such as **316**[358] (Scheme 80) have been used. The special role of the phosphatase inhibitor FK 506 and cyclosporin A will be described in Section 10.



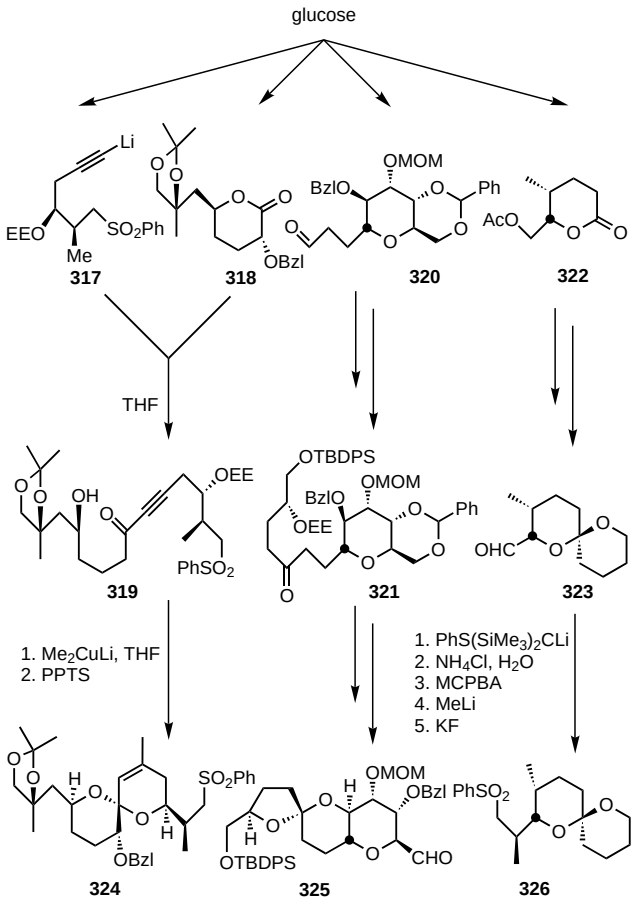
Scheme 80. Natural products that inhibit phosphatases.

9.1. Ocadaic Acid

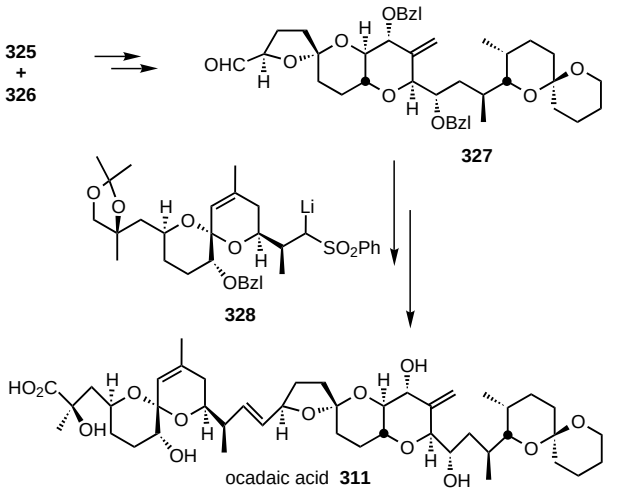
Ocadaic acid is a polyether derivative of a C_{38} fatty acid isolated from two different marine sponges.[353] In addition to its activity as a diarrheogenic shell-fish toxin, it has also been reported as a tumor promoter[359] and a specific Ser/Thr-phosphatase inhibitor.[360] Unlike the PKC-activating phorbol esters (see Section 7), it promotes tumor growth by inhibiting the dephosphorylation of the PKC's target proteins. Owing to its manifold biological activities and the interesting polyether structure with 17 steroecenters, the natural product is an exciting goal for a total synthesis. The correct assembly of the stereocenters is the biggest challenge. The complex molecule was retrosynthetically converted into fragments **324**–**326** (Scheme 81),[361] which were each assembled starting from glucose derivatives.

The key step in the synthesis of fragment **324**[362] (Scheme 81) was nucleophilic attack of acetylide **317** on lactone **318**, which are both obtained from glucose. The only carbon atom still missing was introduced by a stereoselective conjugate addition of a methyl cuprate to the acetylenic ketone **319**, and the ring was then closed to the spiroketal by acid catalysis. Fragment **325** (Scheme 81) was also synthesized from glucose.[363] Standard reactions gave aldehyde **320**, in which the benzylidene group fixed the conformation of the pyranosyl ring. The carbon backbone was extended by adding a lithiated sulfone with formation of ketone **321**. Selective manipulation of the protecting group enabled formation of the ketal and subsequently completion of fragment **325**. In the synthesis of the third fragment (Scheme 81), an enantiomerically pure glucose derivative also functioned as a starting material.[364] Following functionalization of the pyranose backbone, lactone **322** was opened with a lithiated sulfone and then converted into aldehyde **323**, which was converted into the alkene in a Petersen olefination. Stereoselective introduction of the missing methyl group was achieved by chelate-controlled heteroconjugate addition at the vinyl sulfone produced. This key step in the synthesis sequence was also used in the production of fragment **324**.

To complete the synthesis of the natural product **311** (Scheme 82),[365] phenylsulfone **326** was deprotonated, coupled with fragment **325**, and the phenylsulfone group subsequently removed. The correct stereochemistry of the newly created alcohol was set by oxidation and stereoselective reduction; further transformations then led to fragment **327**. This was coupled to the lithiated sulfone **328** with formation of the necessary olefin. Manipulation of the protecting groups and oxidation to the acid gave the natural product. The



Scheme 81. Synthesis of the individual fragments of ocadaic acid. EE = ethoxyethyl, PPTS = pyridinium-*p*-toluene sulfonate.



Scheme 82. Synthesis of ocadaic acid.

multitude of latent hydroxyl groups in the molecule called for a careful protecting group strategy. In the course of the synthesis, silyl (TBDPS), acetal (MOM, THP, benzylidene, ethoxyethyl) and acetyl protecting groups were used and were subsequently replaced by benzyl ethers, so that in the last step, complete deprotection of the molecule was achieved with lithium in ammonia.

The importance of ocadaic acid for investigating biological and medical phenomena is illustrated by the fact that in 1995 and 1996 this natural product was used in biological experiments described in about 450 and 400 publications, respectively. In particular, the ability of the natural product to selectively inhibit the Ser/Thr phosphatase PP2A (IC_{50} = 1 nM) without influencing the Ser/Thr phosphatase PP1 (IC_{50} = 330 nM) was put to use.[366] Fundamental knowledge was obtained in the investigation of programmed cell death, or apoptosis. By treating different cancer cell lines with ocadaic acid, it was shown that the cells already have the whole protein repertoire necessary for apoptosis, regardless of their position in the cell cycle.[367, 368] A de novo synthesis of additional proteins is not necessary. Phosphatases are thus involved in the signal cascade leading to apoptosis. However, further investigations are needed for better understanding of the whole process, since ocadaic acid is also able to protect lymphocytes from apoptosis induced by other reagents.[369]

In further experiments, the influence of ocadaic acid on K 562 leukemia cells was investigated.[370] Treatment with the natural product reduced expression of the genes *c-myc* and *max*. These code for proteins that play a key role in controlling cell growth and cell differentiation and which must heterodimerize in the cell nucleus for this function.[371] Reduction of expression of these genes induces apoptosis in the treated cells. It was shown with several reporter genes that the natural product does not cause general reduction of gene expression. In addition, PP2A is the only phosphatase that is inhibited. Treatment of the cells with the phosphatase inhibitor calyculin A (**314**, Scheme 80) gave similar results, whereas the ocadaic acid tetraacetate, which is inactive towards the enzymes, did not bring about any change in gene expression. One hypothesis that explains these observations is that the phosphatase PP2A dephosphorylates and activates transcription factors that are needed for transcription of *c-myc* and *max* (see Section 6.3).

The role of the phosphatase PP2A in resistance to insulin mediated by the tumor necrosis factor (TNF-α) was shown by treatment of skeletal muscle cells from rat with ocadaic acid.[372] The insulin resistance is caused because TNF-α blocks the insulin-stimulated phosphorylation of MEK triggered by Ras and Raf, and thus the subsequent activation of the MAP kinase (see Section 2.2). Cancellation of this effect by addition of ocadaic acid, and observation of increased PP2A activity on stimulation by TNF-α, indicate a role for this enzyme, which apparently dephosphorylates MEK and thereby inactivates it. In contrast, other phosphatase inhibitors such as orthovanadate or FK 506 (see Section 10) do not lead to removal of the TNF-α-mediated insulin resistance.

In further work on insulin-stimulated transport of glucose and translocation of a glucose transporter (Glut 4) from intracellular vesicles to the cell membrane, it was shown that ocadaic acid imitates the effect of insulin on human adipocytes: The rate of glucose transport is increased, and migration of Glut 4 to the cell membrane is stimulated.[373] In contrast to the insulin-stimulated signal path, the effect of ocadaic acid is independent of the activity of the PI-3 kinase, a lipid kinase. These findings suggest that several signal paths regulate glucose transport; this opens up new avenues for

treatment of diabetes. The exact cause of the effect of ocadaic acid has not been investigated further.

With ocadaic acid as a selective inhibitor of the PP2A phosphatase, at low concentrations of about 10 nM, this enzyme is involved in EGF-induced regulation of MAP kinase in A431 cells[374] and in hyperphosphorylation of the Tau protein, which is important in Alzheimer's disease.[375]

9.2. Calyculin A

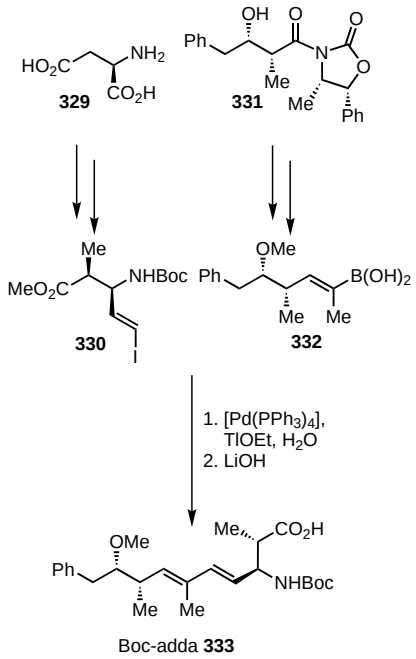
The secondary metabolite calyculin A (**314**, Scheme 80) was isolated in 1986 from a marine sponge[355] and was reported a bit later as a phosphatase inhibitor.[376] In contrast to ocadaic acid, which inhibits PP2A by a factor of 10–100 better than PP1, calyculin A blocks both enzymes with similar IC_{50} values. The relative stereochemistry of the natural product was obtained by a crystal structure analysis;[355] the absolute configuration was elucidated by a first total synthesis in which particular attention had to be paid to the hydrolysis-labile phosphate unit and the conjugated tetraene unit.[377] Calcyculin A has a broader enzyme specificity than ocadaic acid but otherwise has very similar biological activity.

If human tumor cells are exposed to γ-radiation or heat, intensive DNA fragmentation and morphological changes take place which are characteristic of apoptosis. In the presence of 20 nM calyculin A or 500 nM ocadaic acid, the programmed cell death is hindered, however.[378] Calyculin A has the same effect at low concentrations in photodynamic therapy (PDT). In this procedure for tumor treatment, apoptosis is triggered by laser radiation in cells which have been treated with photosensitive reagents. Alignment of the light source enables targeted destruction of malignant tissue. In leukemia cells from mice, the characteristic DNA fragmentation takes place very rapidly (within 30 min) after radiation.[379] Treatment of the cells with calyculin A prevents programmed cell death, whereas the PKC inhibitor staurosporin accelerates the process. From the very rapid occurrence of apoptosis, the authors concluded that photodynamic therapy initiates a late step in the whole process (this is probably the reason for the broad applicability of PDT; in contrast, chemotherapeutical agents influence earlier steps in programmed cell death which are no longer present or suppressed in tumor cells) and that a Ser/Thr phosphorylation/dephosphorylation is involved in a late stage of the signal transduction processes leading to cell death.

Calyculin A and ocadaic acid function as strong tumor promoters (see above and Section 9.1) in that they prevent dephosphorylation of target proteins of PKC; they have the same effect as activation of the Ser/Thr kinase by phorbol esters (see Section 7).[350b] Both natural products bring about translocation of PKC from the cytosol to the cell membrane in different cell types; calyculin A does this at lower concentrations (10 nM) than ocadaic acid (1 μM).[380] This membrane association is also initiated by phorbol esters and leads to activation of the kinase.[381] Despite differences in the structure and the cellular target proteins, the different natural products promote tumor growth in a similar way, by membrane localization of the PKC. Further applications of calyculin A include examination of the TNF-α-mediated insulin resistance,[382] modulation of the activity of the Ras farnesyltransferase by phosphorylation/dephosphorylation,[383] and the role of the phosphatases PP1 and PP2A in glucose-induced regulation of glycogen synthase.[384]

9.3. Nodularins and Microcystins

Nodularins such as **315**[357] and microcystins such as **316**[358] (Scheme 80) are cyclic penta- and heptapeptides, respectively, produced by cyanobacteria. These compounds are hepatotoxic and responsible for the poisonousness of water in which blue-green algae bloom. In addition, they function as potent inhibitors of the Ser/Thr phosphatases PP1 and PP2A and as tumor promoters. Both natural products are structurally related and have a common unusual β-amino acid, adda (**333**, Scheme 83), as well as D-*erythro*-β-methylaspartic acid.



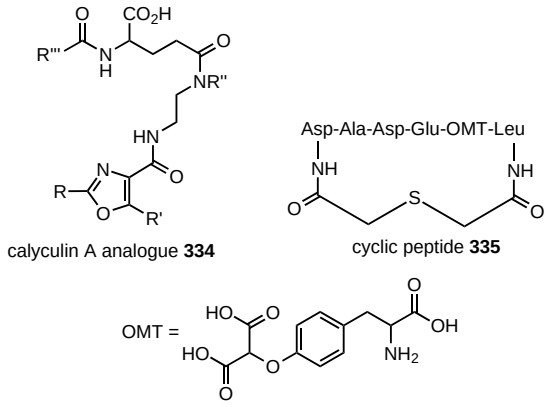
Scheme 83. Synthesis of Boc-adda.

Owing to their many biological properties, there is much interest in the synthesis of these natural products and of structurally related nonnatural analogues.[385] For this purpose, an efficient and stereospecific synthesis of **333** is required.[386] In a recently published initial total synthesis of microcystin LA,[385] **333** was obtained by a Suzuki coupling of the vinyl iodide **330**, which is obtained from a stereoselectively β-methylated aspartic acid, and boronic acid **332**, which was synthesized from the *syn*-aldol **331** (Scheme 83).

9.4. Other Inhibitors

An example for the development of new synthetic phosphatase inhibitors was recently reported.[387] Based on the few

available SAR[388] and molecular modeling investigations[389] concerning the activity of the naturally occurring inhibitors, the highly simplified calyculin A analogue **334** (Scheme 84)



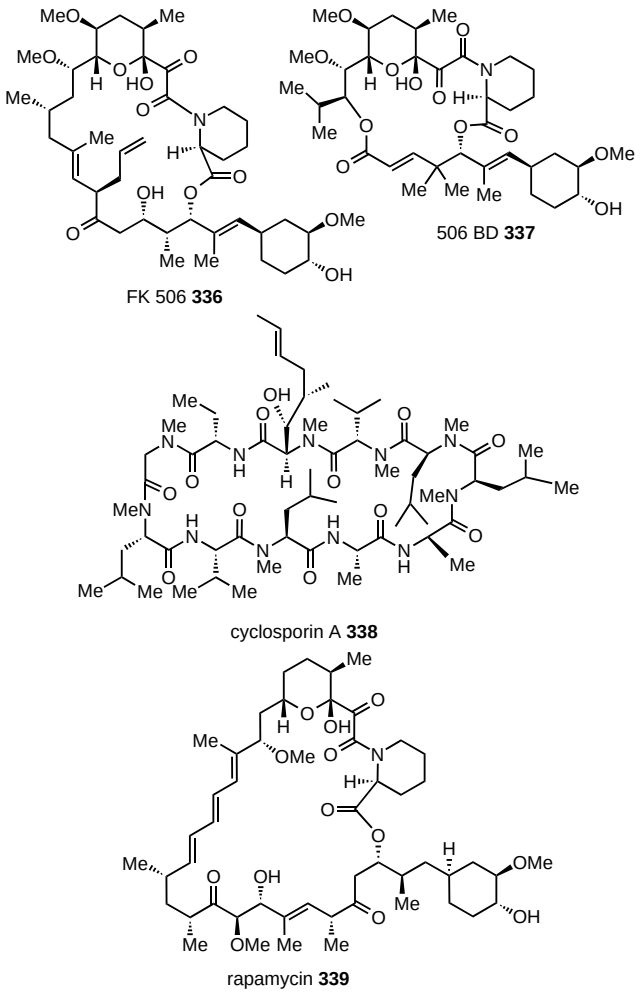
Scheme 84. Synthetic phosphatase inhibitors.

was designed, which contains an essential carboxylate functionality, a hydrophobic residue, and hydrogen donors and acceptors. With a combinatorial strategy, 18 derivatives of **334** were synthesized and tested for activity against the phosphatases PP1 and PP2A as well as in human breast cancer cells. Some compounds in the library blocked the enzyme activity (although at considerably higher concentrations than calyculin A), and in whole cell systems an antiproliferative activity developed, so that the planned further experiments promise interesting results.

Starting from the amino acids sequences of preferred substrates of individual phosphatases, it was also possible to synthesize selective peptide enzyme inhibitors. It is advantageous to replace phosphotyrosine with hydrolysis-labile derivatives such as phosphonomethylphenylalanine, difluorophosphonomethylphenylalanine, or *O*-malonyltyrosine (OMT). In this way, cyclic peptides derived from an autophosphorylation sequence of the EGF receptor were synthesized which were stable with respect to proteolysis. Those compounds that contained the OMT mimetic (e.g. **335**, Fig. 24) were potent in vitro inhibitors of the phosphatase PTP1.[390] It would be interesting to investigate whether there are structural similarities between this class of enzyme inhibitors and the microcystins and nodularins mentioned above.

10. Induced Dimerization of Proteins by Synthetic Ligands[391]

One of the most illustrative examples for the cross-fertilization of organic synthesis and biology is the elucidation of the mechanism of the immunosuppressants FK 506 (**336**) and cyclosporin (CsA, **338**; Scheme 85) as well as T cell activation (see Sections 2 and 4 and Scheme 9). Since this fascinating area of research has already been extensively described elsewhere,[392] it will only be mentioned briefly here. FK 506 and cyclosporin are natural products that block Ca^{2+}-dependent signal paths, and, due to their inhibitory effect on



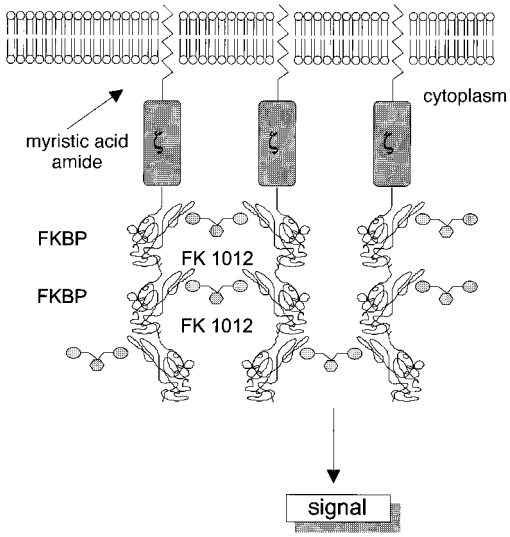
Scheme 85. Immunosuppressants and the synthetic ligand 506 BD.

the transcription of a range of genes in T cells, they have been used as immunosuppressants in organ transplantation. With immobilized derivatives of these ligands, their physiological receptor proteins, the immunophilins cyclophilin (Cyp) and FK 506 binding protein (FKBP) were identified. At first it was assumed that the activity of the natural products immuno suppressiol was due to inhibition of the rotamase activity of these proteins, which is essential for the activation of T cells. However, this hypothesis had to be abandoned when it was discovered that both rapamycin (**339**) and the fully synthetic FK 506 binding domain (506 BD, **337**; Scheme 85) bind to FKBP and inhibit its rotamase activity but that rapamycin inhibits a signal path other than that of FK 506 and CsA, and 506 BD does not inhibit any signal transmission. Building on the hypothesis that the ligand–receptor complex influences another molecule of the transduction cascade, the cellular binding partner of the "activated complex" of FK 506 and FKBP was found, again with the help of affinity chromatography. This turned out to be the calmodulin-dependent phosphatase calcineurin; its enzymatic activity is inhibited by binding to FK 506/FKBP but is not influenced by FK 506, FKBP, Cyp, rapamycin, 506 BD, or rapamycin/FKBP. This enzyme inhibition hinders phosphorylation of the transcription factor NF-AT (see Scheme 9) leading to arrest of the cell

cycle in the G_0 phase and thus to the observed immunosuppression. It is interesting that the phosphatase calcineurin is also inhibited by the complex of CsA and cyclophilin. The natural products have two protein-binding domains; they bind to the immunophilins with one and to calcineurin with the other, and can therefore be described as "chemical inducers of dimerization".[391] Stimulated by this work, quite new possibilities and concepts have grown from the investigation of signal transduction processes.

Ligand-mediated signal transduction processes are often initiated by di- or oligomerization of receptors.[393] However, the extracellular and transmembrane domains are only needed to a limited extent: Their function is primarily to bring the enyzmatically active intracellular domains into the immediate vicinity of one another so that, for example, a transphosphorylation can take place. Based on this assumption, a dimer was synthesized starting from FK 506 in which two FK 506 monomers are linked to one another through a functional group within the calcineurin binding domain.[394] This dimeric ligand, FK 1012 (**340**, Scheme 86), should still be able to bind FKBP, but inhibition of the TCR signal path by essential binding to the phosphatase calcineurin is no longer possible.

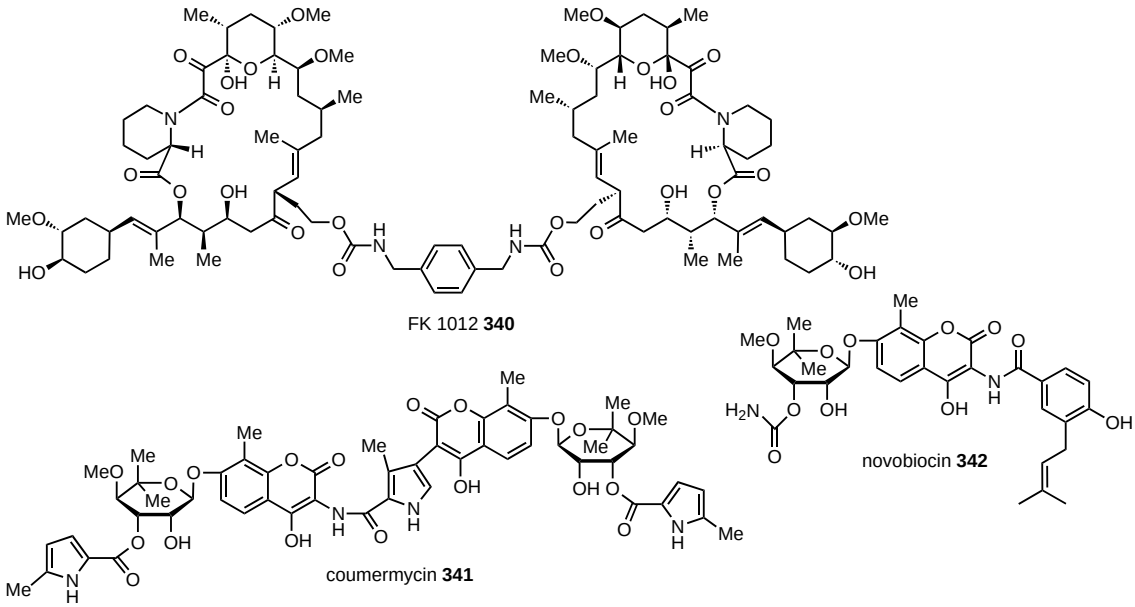
To show the applicability of this property in biological studies, Tag–Jurkat T cells were transfected with chimeric Src/ζ-FKBP receptors constructed from the N terminus of Src (this protein is myristoylated and thus brings about a membrane localization), the ζ-chain of the TCR (to induce a kinase cascade upon dimerization of the receptors, see Scheme 9), and several copies of the FKBP. On treating these cells with the synthetic dimer **340**, the receptors oligomerized in that the ligand bound them to one another through its FKBP binding site (Scheme 87). As a consequence of this artificial aggregation and the kinase cascade thereby initiated, activation of reporter genes was observed similar to that elicited by antibody cross-linking. If the myristoylation, and thus the membrane association of the receptor, was prevented by exchanging the N-terminal glycine of the Src protein for



Scheme 87. Activation of chimeric receptors by FK 1012.

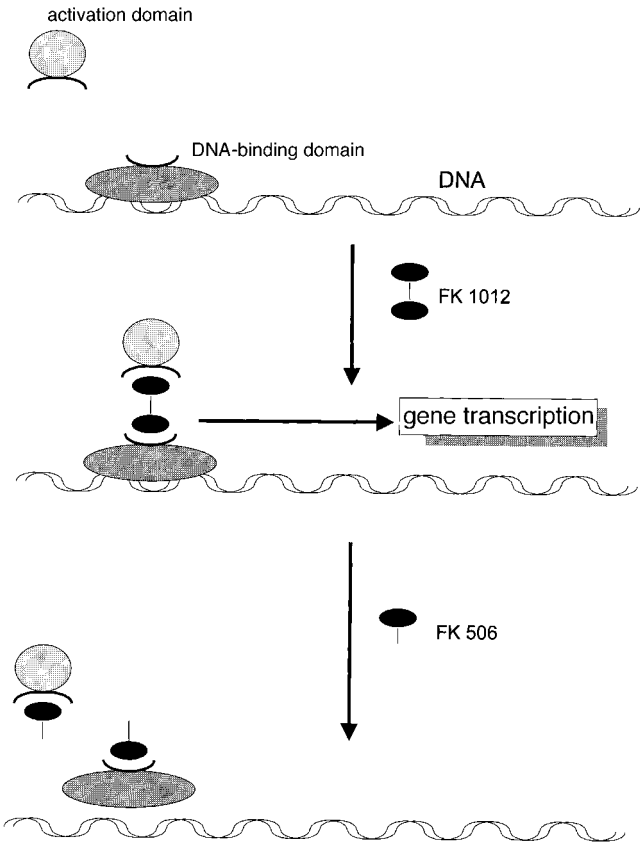
alanine, no activation of the signal path was detected. The same finding was observed if the receptor only contained one copy of FKBP, whereas three copies gave maximal activity. It was thus concluded that it is not the extracellular and the transmembrane domains of the TCR that are necessary for activating the TCR signal path; however, the receptor must be localized on the cytosolic side of the plasma membrane. Ligand-induced conformational changes in the T cell receptor apparently do not play an essential role. In addition, an oligomerization of the receptor seems to be necessary, but a simple dimerization by the synthesized ligands does not switch the signal cascade on. The artificial signal can be switched off by addition of the monomeric FK 506 or CsA; therefore, the transduction cascade can be activated or terminated on command.

This method was also used to selectively switch on transcription of genes. Transcription factors are made up of



Scheme 86. Ligands that induce protein dimerization and the monomeric novobiocin.

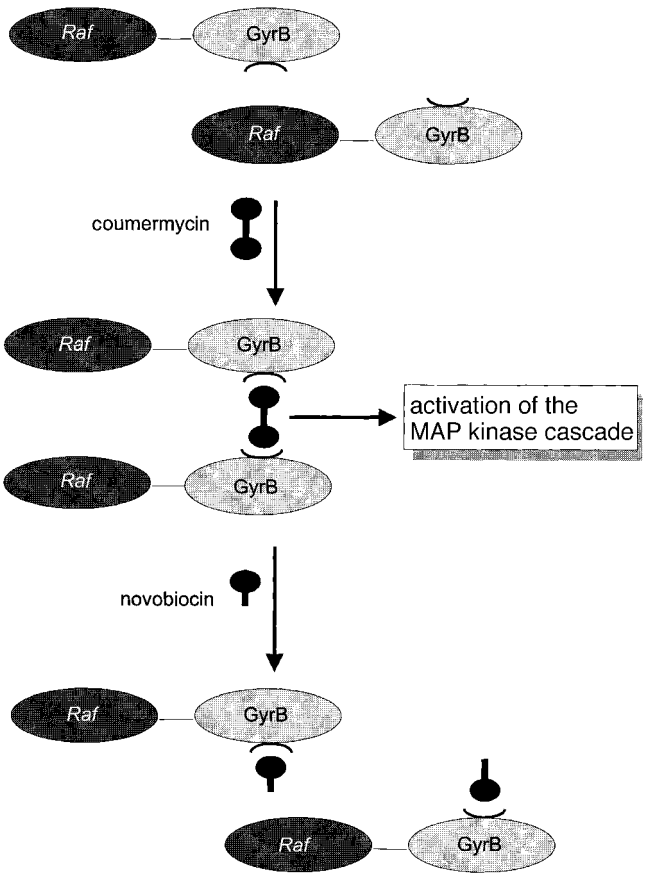
functionally different domains which mediate binding to DNA and activation of transcription independent of one another.[395] Since no covalent bonds are needed here, it is possible to bring such domains close to each other with the help of dimeric ligands and thus induce transcription. For this purpose, chimeric proteins were used, each containing FKBP and, in one case, a DNA-binding domain (GF3 or HF3), and in another, an activation domain (NF3V1); these were expressed in cells (Scheme 88).[396] Treatment of these cells



Scheme 88. Induction of gene transcription by a synthetic ligand.

with the ligand FK 1012 (**340**) led to the activation of transcription, which could be stopped by addition of the monomer FK 506. In addition, the observations suggest that the activation domain must be permanently bound to the DNA for maintenance of transcription and that a solitary occurrence of complex formation is not sufficient.

In a further investigation, signal transduction through the Raf kinase was examined with the help of induced dimerization.[397] Coumermycin (**341**, Scheme 86) is an antibiotic that binds to two molecules of the B subunit of a bacterial DNA gyrase (GyrB) and induces a dimerization. To investigate the mechanism that leads to activation of the Raf-1 kinase (see Section 2.2), chimeric Raf fusion proteins were created in which the GyrB subunit was linked to the C terminus of Raf. If these proteins were expressed in Cos cells, their catalytic activity (i.e., phosphorylation of MEK) could be induced by addition of the antibiotic **341** (Scheme 89). High doses of coumermycin or novobiocin (**342**, a monomeric analogue of coumermycin, Scheme 86) blocked the MEK activation; this



Scheme 89. Activation of the MAP kinase cascade by coumermycin-induced dimerization of the Raf kinase.

is consistent with the hypothesis of activation by dimerization. If a mutant of Raf (R89L), which does not interact with Ras and therefore should not be localized in the plasma membrane, was used for these experiments, the same coumermycin-dependent reaction was observed as for the wild-type protein. Dimerization of Raf kinase seems to be sufficient for activation, and a membrane localization is not necessary. The role of the Ras protein in the activation of Raf, according to these results, may be to induce aggregation of the kinase. Investigations in which dimerization of chimeric FKBP-Raf fusion proteins were induced with the help of FK 1012 led to similar results.[398]

With a dimer of the cyclosporin A, a Fas-mediated apoptosis was induced in cells in which the intracellular domain of the Fas receptor was expressed linked to cyclophilin.[391] A synthetic construct of FK 506 and cyclosporin A, FKCsA, was also successfully used for the aggregation of two different proteins.[399]

The potential of this conceptionally new class of active compounds is increased by the fact that they are generally orally applicable and are easily taken up by cells. For example, a therapeutic protein, human growth hormone (hGH), was produced with the help of the natural product rapamycin by controlling transcription in mice implanted with a cell construct.[400] The cell construct contained the hGH gene and two regulatory proteins which were equipped with recognition sequences for rapamycin.

11. Conclusions and Outlook

The examples described in this review for the successful interlocking of organic chemical and biological search to investigate and influence biological signal transduction are an impressive demonstration of the capabilities of interdisciplinary research in the field of bioorganic chemistry, according to the definition given in the introduction. Today, more and more biological phenomena are being investigated, elucidated, and understood in molecular detail at a rapidly increasing speed. For organic chemistry, this opens up a multitude of new spheres of activity, in which its capabilities can be used to the full extent and in which new, great, and important challenges are presented which must be taken up: In mastering them, organic chemistry can rise to a key role. For biology, bioorganic research presents new alternative possibilities to obtain results faster, more directly, and often with a greater degree of precision and clarity; in most cases, these results could hardly be achieved with methods established today, or only with much greater effort. Establishing bioorganic chemistry at the border between organic chemistry and biology as a capable and widely applicable field, such as physical chemistry between physics and chemistry, requires the readiness and will of organic chemists and biologists to step over the boundary to the neighboring discipline. The language and methods of the other must be learnt, and there must be mutual respect (and one must not just regard the neighboring science as a source of themes or substances) and dedication to the issues to be addressed with real cooperation. The future will show whether we can rise to this challenge.

Motivation to write this review came from our own work in linking organic chemical and biological research in the field of signal transduction at the Universität Mainz, Universität Bonn, and Universität Karlsruhe. Our work is mainly mentioned in Sections 3 and 5–7. The following persons (in alphabetical order) have been involved in the past and at present: D. Alonso-Díaz, D. Brom, A. Cotté, F. Eisele, S. Gabold, S. Glomsda, P. Hagenbuch, A. Heuser, K. Hinterding, V. Jungmann, D. Kadereit, T. Kappes, B. Klaholz, V. Klein, N. Kuder, K. Kuhn, Y. Li, T. Lipps, R. Machauer, B. Meseguer-Vidagany, E. Nägele, D. Owen, T. Pathak, T. Pohl, A. Reidel, J. Sander, M. Schelhaas, U. Schlede, D. Sebastian, T. Schmid, T. Schmittberger, S. Schulze, P. Stahl, F. Stieber, P. Stöber, T. Zelinski. For generous support of our work, we would like to thank the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung, the Fonds der Chemischen Industrie, the European Union, the Alexander-von-Humboldt-Stiftung, the Krupp Stiftung, Boehringer Mannheim GmbH, Bayer AG, BASF AG, Degussa AG, and Boehringer Ingelheim KG.

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[1] A. Kornberg, *The Golden Helix—Inside Biotech Ventures*, University Science Books, Sausalito, CA (USA), **1995**, p. 4.
[2] *Chem. Eng. News* **1996**, *74*(20), 39.
[3] See also G. M. Whitesides, *Angew. Chem.* **1990**, *102*, 1247–1257; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 1209–1219.
[4] This situation is also demonstrated by the founding of diverse new journals on the subject, which have been met with great interest and respect, for example, *Nature Structural Biology*, *Structure*, *Current Opinion in Structural Biology*.
[5] See, for example, H. Dugas, *Bioorganic Chemistry*, 3rd ed., Springer, New York, **1995**.
[6] Recently, the emphasis on biological aspects seems to have gained importance. Therefore, the Chemistry Department of Harvard University was recently renamed Department of Chemistry and Chemical Biology (and not Biological Chemistry!); Harvard University is also going to open an Institute for Chemistry and Cell Biology (*Chem. Eng. News* **1997**, *75*(24), 31) and at the Scripps Research Institute, the Skaggs Department of Chemical Biology has been opened.[2]
[7] See a) ref. [1], p. 13, b) A. Kornberg, *Chem. Biol.* **1996**, *3*, 3–5. In this essay, the author identifies one of the main differences between chemical and biological research as the different way that chemists and biologists tackle problems: "Chemists seek precise answers to well-defined problems, whereas biologists are content with approximate answers to complex problems" (ref. [7a], p. 14); "Chemists seem more conservative, analytical and clanish, […] They obtain precise data with relatively few and elegant techniques. […] Biologists on the other hand seem more artistic, eclectic and right-brain dominated. They focus on complex phenomena in cells and organisms using a wider range of techniques with less presicion." (ref [7b], p. 3–4). We are furthermore of the opinion that communication problems arise between biologists and chemists due to their education and the ways of thinking thereby impressed upon them. Therefore, for an organic chemist, the expression "glucose 6-phosphate" evokes a structural association and the biological function remains in the background, whereas a biologist will first think of the role of this carbohydrate derivative for instance in glycolysis, and the structure is secondary for him. Chemists and biologists look at different, yet inseparable, sides of the same coin.
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[9] For a comprehensive presentation, see a) H. Lodish, D. Baltimore, A. Berk, S. L. Zipursky, P. Matsudeira, J. Darnell, *Molekulare Zellbiologie*, 2nd ed, de Gruyter, Berlin, **1996**, chap. 20; b) G. Krauss, *Biochemie der Regulation und Signaltransduktion*, WILEY-VCH, Weinheim, **1997**.
[10] A third class is formed by lipophilic hormones with membrane receptors, such as the arachidonic acid derivatives, which are continuously produced and secreted by many cell types and are often degraded again in the immediate vicinity of the site at which they were biosynthesized.
[11] The influence on behaviour of other organisms of the same type by pheromones will not be discussed here.
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