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Peptide Conjugates as Tools for the Study of Biological Signal Transduction

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Abstract—Today, many biological phenomena are being investigated and understood in molecular detail, and organic chemistry is increasingly being directed towards biological phenomena. This review is intended to highlight this interplay of organic chemistry and biology, using biological signal transduction as an example. Lipo-, glyco-, phospho- and nucleoproteins play key roles in the processes whereby chemical signals are passed across cell membranes and further to the cell nucleus. For the study of the biological phenomena associated with these protein conjugates, structurally well-defined peptides containing the characteristic linkage region of the peptide backbone with the lipid, the carbohydrate or the phosphoric acid ester can provide valuable tools. The multi-functionality and pronounced acid- and base-lability of such compounds renders their synthesis a formidable challenge to conventional organic synthesis. However, the recent development of enzymatic protecting groups, provides one of the central techniques which, when coupled with classic chemical synthesis, can provide access to these complex and sensitive biologically relevant peptide conjugates under particularly mild conditions and with high selectivity.

Introduction

Over recent years the dimensions of objects studied in biology have become smaller, while organic chemistry has turned increasingly to larger molecules and systems. This has led to the investigation and understanding of many biological phenomena at the molecular level. Due to the high performance of organic synthesis even the most complicated of those natural and active compounds, which are recognized as biologically relevant, can be synthesized. 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 at their interface, the growing interdisciplinary field of bioorganic chemistry (or chemical biology) has been established. In this review, one possible bioorganic scenario for the successful combination of biological and organic chemical research will be outlined.

In the following chapter we present 'biological signal transduction' as an example of current relevance in biological research. It will then be shown how, based on this structural biological information, new problems in synthetic organic chemistry arise, which have been solved by establishment of new synthetic methods for lipo-, phospho-, glyco- and nucleopeptide compounds. These modified peptide conjugates are subsequently used in biological experiments to glean new knowledge, which could not have been obtained with classical biological techniques or only with much greater effort. According to this scenario, new structural biological information is derived and the cycle of bioorganic research starts over again.[1]

Biological signal transduction[2,3]

In multicellular organisms growth, differentiation, and metabolism of a large number of cells is coordinated via synthesis, secretion and recognition of signal molecules. Extracellular signal substances (hormones), which cannot pass through the cell membrane by diffusion, are bound to receptors on the cell surface. Once the receptor has conveyed the signal through the plasma membrane of the target cell, the message is relayed into the cell interior via intracellular signal cascades, and a reaction is triggered. Finally, the signal is switched off and the reaction of the cell to the signal substance ends. The activity of hormones and associated signal mediation have been the subject of particularly intensive bioorganic research. In the following sections, two of the most important intracellular signal cascades are explained at the cellular and molecular level.

Key words: Bioorganic chemistry; enzyme inhibition; organic synthesis; peptide conjugate; signal transduction.

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Signal transduction via G protein-coupled receptors, and heterotrimeric G proteins[4–16]

Many hormonal messengers are recognized at the cell surface by so-called G protein-coupled receptors (GPCRs).[4] These receptors are made up of seven transmembrane helices (Scheme 1). An intracellular loop between helices 5 and 6, and the C-terminus of the receptor interact with a heterotrimeric G protein (guanine-nucleotide-binding protein) located in the membrane. G proteins contain three different subunits G_{α},G_{β}, G_{γ},[5–7] of which the α-unit has a bound GDP in the inactive form. As a consequence of the interaction of the G protein with the receptor, GDP is exchanged for GTP and the heterotrimeric complex dissociates into G_{α}/GTP and G_{β}/G_{γ}. These two complexes can then trigger various intracellular signal cascades. Subsequent hydrolysis of GTP to GDP results in renewed formation of the inactive GDP-bound heterotrimeric complex, thus switching off the signal. If external agents disturb this complicated mechanism, serious complications result, both for the cells involved and for the whole organism.

Depending on the G protein 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 that is also located in the plasma membrane. Adenylate cyclase catalyzes the cyclization of ATP to cyclo AMP (cAMP), which in turn activates the so-called Group A, cAMP-dependent protein kinases (PKA)[8] by releasing their catalytic subunits from an inactive tetrameric complex (Scheme 1). According to cell type, PKA then activates various enzymes by phosphorylating them at serine/threonine residues. In addition, PKA can diffuse into the cell nucleus and stimulate transcription factors, which subsequently activate expression of many genes.

The G_{β}/G_{γ} complex activates the membrane-bound phospholipase $C_{\beta 2}$ ($PLC_{\beta 2}$) which cleaves the plasma membrane lipid phosphatidyl inositol-4,5-bisphosphate (PIP_2) to the 'secondary messengers' diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3).[9–11] After diffusion through the cytosol, the released IP_3 binds to a specific receptor on the endoplasmatic reticulum, thereby opening a channel to allow Ca^{2+} ions to flow from the endoplasmatic reticulum into the cytosol (Scheme 1). These Ca^{2+} ions are a further 'secondary messenger' needed to activate protein kinases.

DAG remains in the plasma membrane where it binds, together with Ca^{2+} ions and phosphatidyl serine, to a class of Group C serine/threonine protein kinases (PKC).[12–14] After membrane localization these PKA molecules become activated, and phosphorylate other enzymes, thereby influencing a multitude of cell reactions. It is especially relevant that PKC also phosphorylates transcription factors and can thereby control the synthesis of particular mRNA molecules.

Signal transduction via G protein-coupled receptors thus plays an important role in regulation of cell growth, and it follows that defective function can lead to misregulated growth, and ultimately, transformation of the cell.

Signal transduction via receptor-tyrosine kinases and nonreceptor-tyrosine kinases: the *Ras*/MAP kinase signal transduction cascade[3,17–25]

Many of the polypeptide hormones that influence cell proliferation and differentiation bind to cell surface receptors which have tyrosine kinase activity. These are the so-called receptor-tyrosine kinases (RTKs). These enzymes 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 on the other monomeric unit (Scheme 2). The phosphorylated receptors are subsequently recognized by adaptor molecules that have the function of binding the receptor to signal molecules. Of particular importance is the protein *Grb2* (growth factor receptor binding protein 2). The so-called SH2 (sarcoma homology 2) domain of this adaptor molecule consists of ca. 100 amino acids, and binds by non-covalent specific interaction to the peptide sequence of the receptor, in which the phosphotyrosine is located. *Grb2* also contains two SH3 domains that are made up of ca. 60 amino acids, and these bind to the proline-rich sequences in another adaptor protein, *Sos* (son of sevenless, named after a Drosophila mutant) (Scheme 2). By this mechanism, the cytosolic proteins *Grb2* and *Sos* are localized on the inner side of the cell membrane and correctly aligned. This *Grb*/*Sos* complex then functions as a guanine nucleotide exchange factor (GEF) by interacting with the inactive, GDP-binding form of the protein *Ras* (from rat sarcoma) which is located in the membrane. *Ras* is made active by exchanging GDP for GTP. The active GTP bound *Ras* protein then acts as a molecular switch, diverting the signal arriving from the tyrosine kinase receptor (Scheme 2). *Ras* has weak GTPase activity that hydrolyzes the bound GTP to GDP thereby reverting *Ras* to its unactive state and terminating the signal. This process is also accelerated by the GAP protein (GTPase-activating protein).

The activated form of *Ras* binds to the N-terminus of the serine/threonine kinase *Raf* by noncovalent protein–protein interactions and thus localizes *Raf* (analogous to immobilization of *Sos* to *Grb2*) onto the plasma membrane. With its C-terminus, *Raf* now binds the protein kinase known as MEK (from MAP, mitogen activated protein; and ERK, extracellular signal regulated kinase), and activates it by phosphorylation. MEK has tyrosine and serine/threonine kinase activity (dual specificity kinase) and it phosphorylates the protein MAP kinase, which is a serine/threonine-specific kinase.

The activated MAP kinase phosphorylates and activates other kinases, such as the ribosomal S6 kinase. MAP kinase also diffuses into the cell nucleus and phosphorylates transcription factors, such as the ternary complex factor (TCF, also known as *ELK*-1) and the *Jun* protein.

With these transcription factors activated, expression of the corresponding genes begins.

In some cases, the *Ras*/MAP kinase cascade and the GPCR signal pathway influence the expression of the same gene by a concerted interlocking of both signal chains. Another way to establish a connection of two pathways is the activation of elements of one cascade by members of another ('crosstalk'). For example, PKC and other serine/threonine kinases can phosphorylate MEK and other members of the *Ras* cascade.[22]

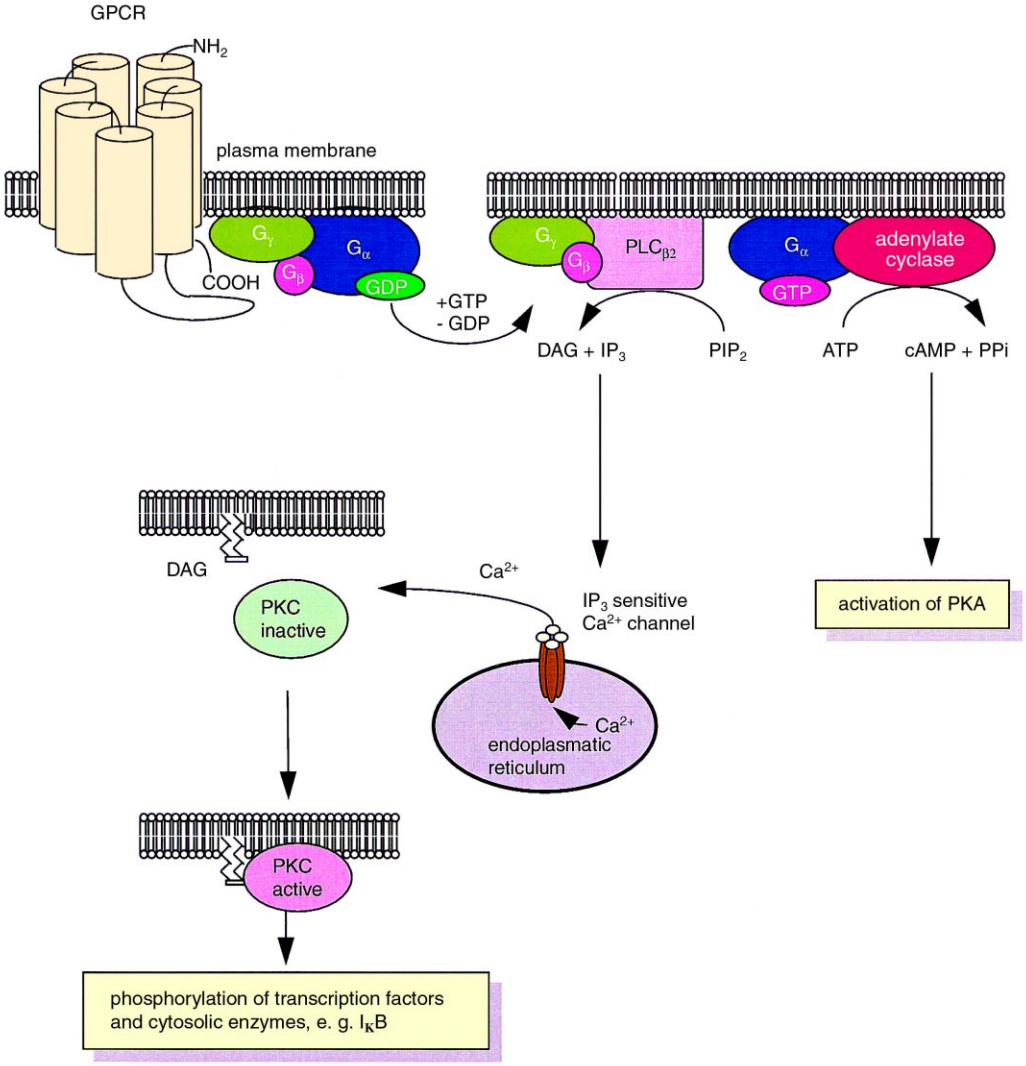
The *Ras* cascade can also be switched on by the so-called nonreceptor tyrosine kinases (NRTKs),[26] that is, tyrosine kinases located on the cytosolic side of the plasma membrane without an extracellular domain.[9,23] By this process the platelet-derived growth factor (PDGF), which itself has no *Grb2* binding site, interacts with the *Grb2*/*Sos* complex via the NRTK *c-Src*, and the adaptor protein *Shc* (Scheme 3).[27] The *Grb2*/*Sos* complex subsequently activates *Ras*, as described above, and passes the signal on.

The *Ras* signal transduction cascade is of paramount physiological importance. It is central to regulation of cell growth and differentiation, and a mistake in regulation of this signal pathway can be one of the critical steps for cell transformation (see next section). The *Ras* path is highly conserved in different species[3,17–19] and its elements are used in the same way for transmission of growth signals in organisms, as diverse as yeast, worms, flies, and mammals.

Other important signal transduction cascades like the one via Janus kinases/STAT molecules,[28,29] or the signalling via the T-cell receptor[30–32] will not be discussed in this review.

Signal transduction and transformation of cells[33,34]

In normal cell growth, the interactions of the individual components of signal transduction cascades are precisely coordinated with one another. However, if a fault occurs in regulation of an important signal cascade, the cell may be transformed (i.e., its growth characteristics are changed and it can develop into a tumor

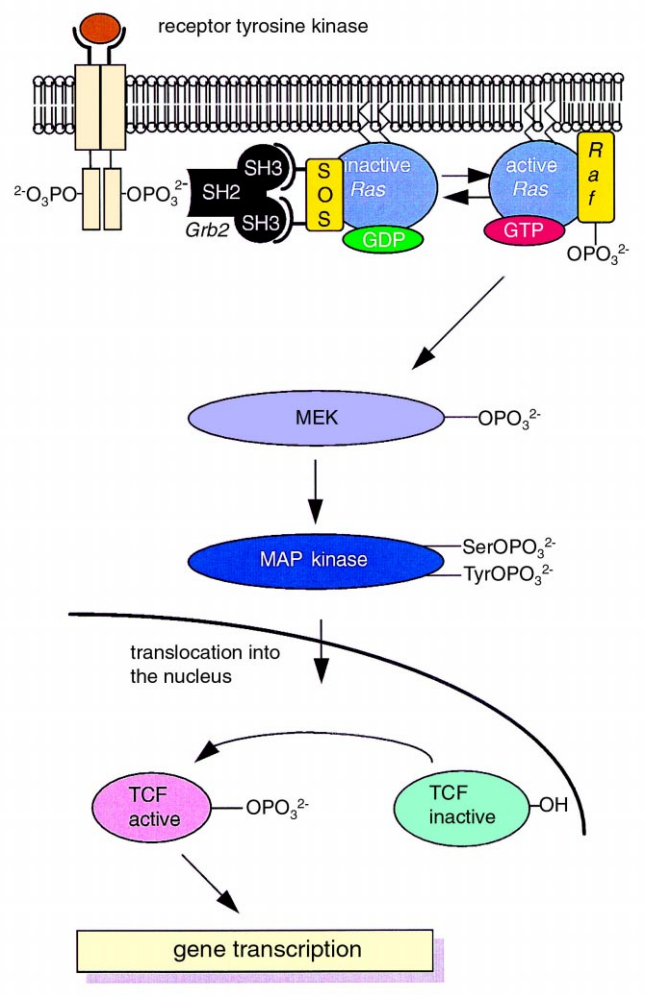


Scheme 1. Formation of 'second messenger' molecules in signal transduction via G-protein-coupled receptors and heterotrimeric G proteins.

cell). Transformation of a cell can be triggered by various mechanisms. One of the most common causes is mutation or wrong expression of cell genes responsible for controlling cell growth. This formation of so-called oncogenes (cancer genes) causes the expression of oncoproteins which lead to the misregulation of signal cascades. Growth is mostly controlled by growth factors, receptors for growth factors, intracellular signal transducers, nuclear transcription factors and cell cycle control proteins. Most oncogenes code for proteins belonging to one of these five classes. 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. However, a number of oncogenes occur for growth factor receptors. Two examples are truncated receptors with the extracellular ligand binding domain missing, or for receptors that stay switched on in the absence of the respective ligand. In both cases, the oncogene gives a permanent growth signal.

Most oncogenes derive from genes for proteins involved in intracellular signal transduction cascades. Thus, mutations in the $G_{s\alpha}$ subunit of heterotrimeric G-proteins (Scheme 1) bring about the loss of GTPase activity, which results in continuous stimulation of cAMP synthesis.



Scheme 2. Signal transduction via the *Ras*/MAP kinase cascade.

Similarly, when mutations in the *ras* gene occur, *Ras* proteins can no longer be converted from the active GTP-binding state, to the inactive GDP-binding form (see Scheme 2). 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 serine/threonine and non-receptor tyrosine kinases can also lead to oncogene formation. The C-terminus of *Raf* contains the serine/threonine kinase subunit whilst 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-terminal enzyme functions in an uncontrolled manner. The kinase activity and thus, the signal transducing effect of *Src* can be significantly reduced by phosphorylation of a tyrosine at the C-terminus (Scheme 3). If this phosphorylation site is altered (for example, in Rous sarcoma virus, the last 18 amino acids of the *Src* C-terminus are missing due to a deletion on the *src* gene), this possibility for regulation is absent and the result is a permanent signal.

Oncogenes coding for nuclear transcription factors often manifest themselves by creating oncoproteins, which change, for example, the transcription frequency of genes coding for growth-stimulating proteins.

According to our current knowledge, carcinogenesis is generally triggered by several causes in a multistep process; cancer arises due to many factors acting together over a long period of time. Human tumor cells embody more than one oncogene and probably several genetic changes (ca. 4–5) would have to accumulate in a single cell for the induction of malignant growth. 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 ca. 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 ca. 80%.

Our understanding of the intracellular signal transduction processes at a molecular level, and selective manipulation of these processes, may open up fundamental new opportunities for study, and possibly also therapy, of malignant transformation.[35]

Peptide conjugates as tools for study of biological signal transduction

The proteins involved in the transduction of signals from outside the cell, through the plasma membrane and finally into the cell nucleus, often have additional covalently linked structural units which are absolutely necessary for

fulfilment of their biological function (Fig. 1). For example, the cell surface receptors for growth factors (see Scheme 2), and the ligands recognized by them, are often glycoproteins in which serine, threonine and asparagine units are linked to oligosaccharides (see **1**, Fig. 1). The signal-transmitting proteins located in the membrane, such as the *Ras* protein, are lipid-modified at cysteine residues and N-terminal glycine (see **2**). In many cases, the signals are passed on by phosphorylating enzymes which switch on the next protein in the cascade by phosphorylating their target proteins at serine, threonine (see **3**) and tyrosine residues. Finally, 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 (see **4**).

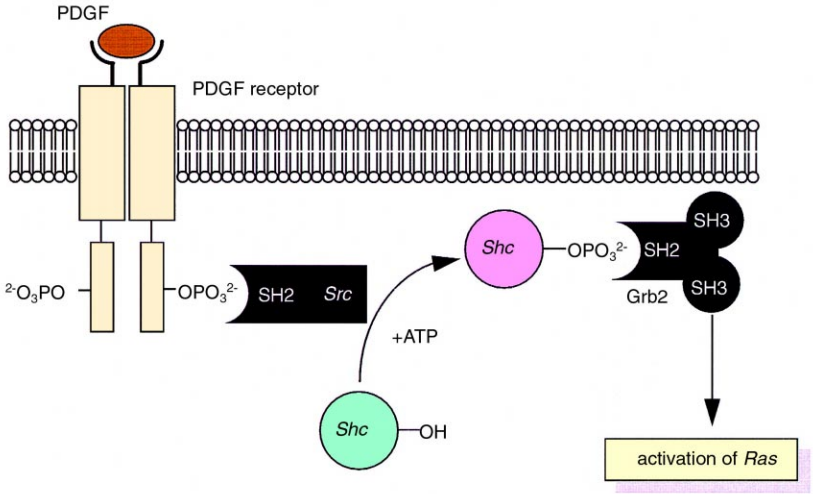
Peptides containing the essential covalently modified structural units of these protein conjugates may be useful tools for the study of their biological functions, and for selective manipulation of the signal cascades in which they are involved. However, synthesis of such peptide conjugates is hindered, on the one hand by their multifunctionality, which requires the use of many orthogonally stable amino, hydroxy, mercapto, carboxy and phosphate protective groups.[36–39] On the other hand, the peptide conjugates are also particularly acid and base labile. Thus at pH > 9, the side chain functions are cleaved from the serine glycosides **1**, the lipid-modified peptides **2** and the phospho- and nucleopeptides **3** and **4** via a β-elimination process; the thioester in **2** hydrolyzes spontaneously in aqueous solution at pH > 7. While in acid, there is a danger of anomerization or even cleavage of the *N*- and *O*-glycosidic bonds in **1** and **4** and the olefins of the farnesyl residue in **2** are easily attacked by acids. In the synthesis of the peptide conjugates of type **1**–**4**, all reactions, and particularly the removal of all the diverse protective groups, must take place under mild, preferably neutral, conditions, yet with complete preservation of orthogonal stability.

New, alternative synthetic methods must be developed in order to obtain these complex peptide conjugates efficiently and highly selectively. Enzyme-labile protective groups[36–39] have been established as valuable alternatives to the classical chemical protective group techniques, since enzymes often work in mild, often neutral conditions and combine high selectivity for the structural units that they recognize and the reactions they catalyze, together with broad substrate tolerance. These new synthetic methods required for the construction of complex peptide conjugates and their application to cellular signal tranduction is the focus of the following discussion.

Lipid-modified peptides

Signal transducing proteins that are located in the plasma membrane often carry covalently attached lipid residues. These lipid functions are necessary for the proteins to perform their biological function.[40–41] Thus, 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 *N*-myristoylated at N-terminal glycines and are often *S*-palmitoylated in the immediate vicinity as well. The γ-subunits of the G proteins contain *S*-farnesylated or *S*-geranylgeranylated cysteine residues, and the *Ras* proteins are *S*-farnesylated and *S*-palmitoylated (Fig. 2). The lipid modification of *Ras* proteins is essential, because they only fulfill their signal tranducing function in both normal and transformed cells if they are lipidated.[40,41]

For synthesis of acid- and base-labile lipid-modified peptides, enzymatic protective group techniques[36–39] form a particularly effective methodology. Thus, the choline ester was developed as an enzyme-labile carboxy protective group that can be released under very mild conditions using butyrylcholine esterase from horse serum; at the same time due to its ionic character, it ensures better solubility in the aqueous media necessary for biotransformation.[42] An example of its use is in the synthesis of the *S*-palmitoylated and *S*-farnesylated characteristic lipopeptide **10** of the human N-*Ras* protein



Scheme 3. Activation of the *Ras* signal cascade by non-receptor tyrosine kinases (e.g., *c-Src*).

(Scheme 4). In the most important step of the synthesis, **8** was completely released without attack at the more reactive thioester, and without detectable β-elimination.

With a second application of this technique, the synthesis of the characteristic *N*-myristoylated and *S*-palmitoylated N-terminal hexapeptide **14** from the α_o subunit of a heterotrimeric G_o protein was achieved (Scheme 5).[43] A tetrapeptide choline ester was synthesized and then enzymatically deblocked at the C-terminus under very mild conditions to **12**. Again, there were no unwanted side reactions. Renewed choline esterase-mediated deprotection delivered the Boc-masked peptide **13**. This was converted to the target compound **14** by cleaving off the urethane and *N*-myristoylation.

To enable synthesis of 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 protective groups for the amino function of peptides. The tetrabenzylglucosyloxycarbonyl (BGloc) group (Scheme 6)[44] and the *p*-acetoxybenzyloxycarbonyl (AcOZ) group (Scheme 7)[45] were developed for this purpose.

The BGloc group is a glycosyloxycarbonyl group with *O*-benzyl protected glucose as the carbohydrate moiety. After removal of the benzyl groups, a mixture of α-glucosidase from baker's yeast and β-glucosidase from almonds, deblocks several BGloc-dipeptide esters by cleavage of the bond between *C*-1 and *O*-1 (Scheme 6). Absolutely no attack at peptide bonds and C-terminal protective groups was observed. In addition, this blocking group enhances the solubility of the compounds in aqueous solutions (i.e., the media in which the enzymatic deprotection has to be carried out). Based on this concept an entire set of enzyme-labile carbohydrate-based blocking groups may be generated, which could be selectively cleaved off by the respective glycosidases.

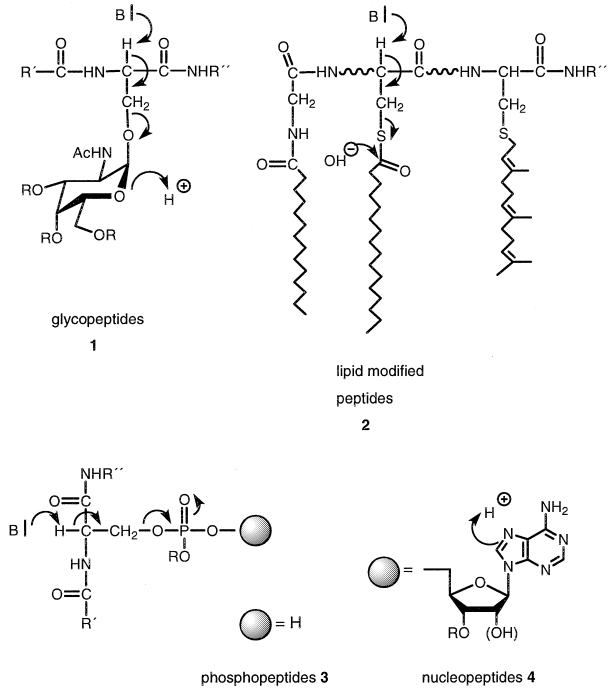


Figure 1. Structure and lability of peptide conjugates.

The AcOZ group contains a functionality (acetate) that the biocatalyst (an esterase or lipase) can recognize and which is bound via 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 7). This is a general principle: depending on the acyl residue selected (e.g. acetate or phenylacetate), enzymes with different selectivity (e.g. acetyl esterase or penicillin G acylase) can be used.

Using the AcOZ urethane, the essential C-terminal lipid-modified heptapeptide ester of the N-*Ras* protein was constructed (Scheme 8).[45] Successive extension of the farnesylated tripeptide methyl ester **15** with the lipase-mediated AcOZ-strategy gave the N-*Ras* pentapeptide **18**. This pentapeptide was condensed with the AcOZ-masked *S*-palmitoylated dipeptide **19** to give the fully protected lipid-modified heptapeptide **20**. It was subsequently possible to enzymatically release the N-terminal urethane from **20** to provide the amino-deblocked *Ras* heptapeptide **21**, which is available for further chain extensions or linking to additional functional groups. The enzymatic techniques described

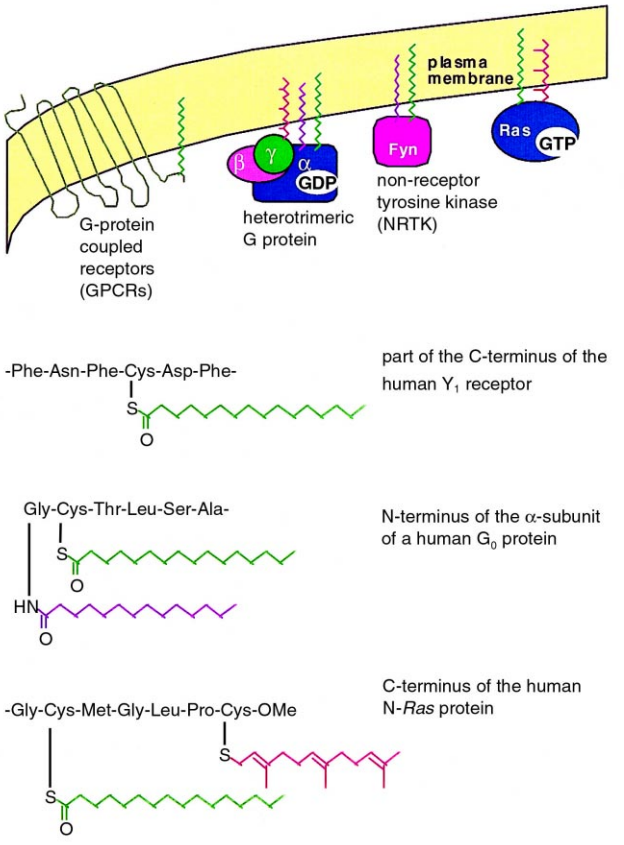
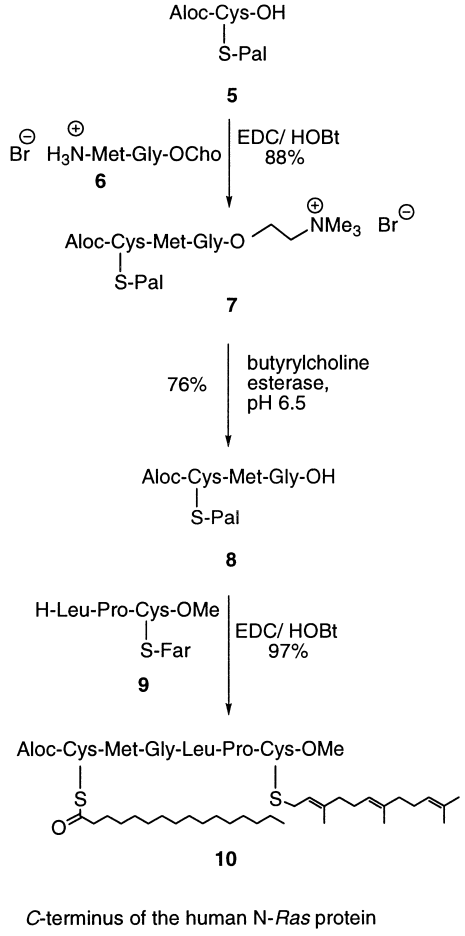


Figure 2. Structure of plasma-membrane-bound lipid-modified proteins; GPCR: G-protein-coupled receptor; NRTK: non-receptor tyrosine kinase.

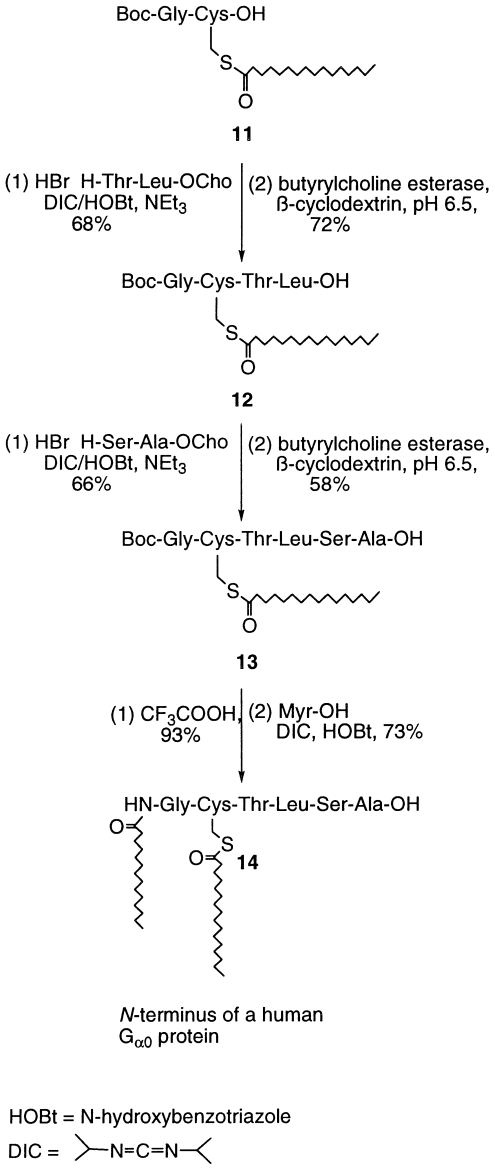
above have been proven to be flexible and efficient methods for the synthesis of lipid-modified peptides, which are both base- and acid-labile. Nevertheless, such conjugates could also be assembled with classical methods although using a much more complicated procedure. The different cysteine units to be modified would have to be protected, generally, selectively unmasked and functionalized after completion of the peptide chain.[46,47] If only base-labile palmitic acid thioesters are present in the compound, the acid-labile Boc group may be used (see below); acid-labile *S*-farnesyl-cysteine-containing peptides can be deblocked at the N-terminus by cleavage of the Fmoc urethane.[48]

In addition to the enzymatic techniques, Pd(0)-mediated release of allyl esters has also been demonstrated to be an efficient method for synthesis of sensitive lipid-modified peptides. This protective group has demonstrated its potential in two additional syntheses of the *S*-palmitoylated and *S*-farnesylated C-terminal sequence of the human N-*Ras* protein and the *N*-myristoylated and *S*-palmitoylated N-terminus of the human $G_{\alpha o}$ protein. Also the *S*-palmitoylated and *S*-geranylgeranylated C-terminal pentapeptide **25** of the R-*Ras* protein has been constructed using this noble-metal catalyzed procedure in the central deprotection steps (Scheme 9).[49] With this technique the C-terminal elongation of the tripeptide **22** could be achieved, with no side reactions occurring at the base labile palmitoyl thioester.

Further application of the allyl ester protective group led to the development of a flexible building block system for synthesis of different lipid-modified N-*Ras* peptides carrying additional fluorophoric groups.[50] The AcOZ- or Boc-protected *S*-palmitoylated cysteine allyl ester **26** was selectively demasked at the N-terminus either enzymatically or under acidic conditions (Scheme 10). Since the acetyl esterase does not tolerate any cosolvents in the enzymatic deprotection step, the low yield is a result of the poor solubility of compound **26** in the aqueous phase. Subsequent coupling of the free amino function to a fluorescent group was easily achieved. Release of the allyl ester was achieved in very high yield by Pd(0)-mediated allyl transfer to morpholine as



Scheme 4. Chemoenzymatic synthesis of the farnesylated and palmitoylated C-terminus of the human N-*Ras* protein using the enzyme-labile choline ester.



Scheme 5. Chemoenzymatic synthesis of the myristoylated and palmitoylated N-terminus of the α-subunit of a heterotrimeric α_O-protein using the enzyme-labile choline ester.

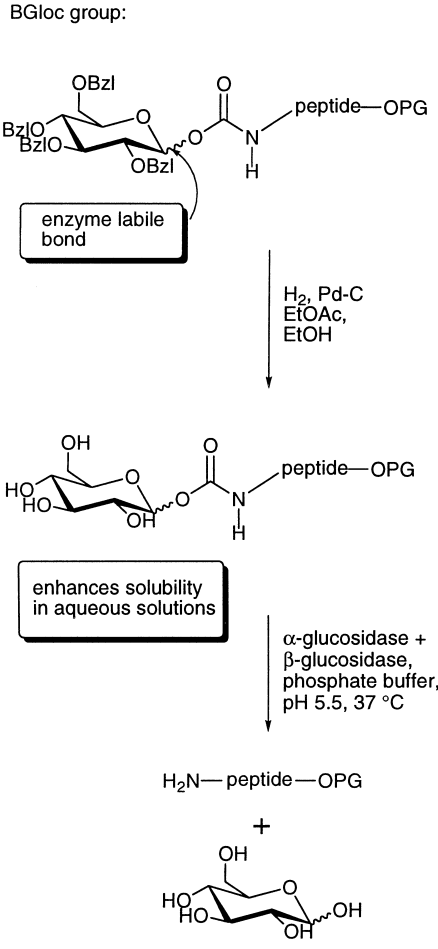
accepting nucleophile, no attack at the palmitic acid thioester was observed. The fluorescently labeled and *S*-palmitoylated dipeptides **28–30** obtained were then linked to the farnesylated N-*Ras* pentapeptide **31** to form *Ras* heptapeptides **32–34** with two lipid modifications. Due to the fluorophore these conjugates can easily be assayed in biophysical and biological experiments.

Lipid-modified peptides have been used in many ways as tools 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 in certain circumstances, have been addressed by combination of biophysical and cell biology techniques using the lipopeptides described above.[51–58]

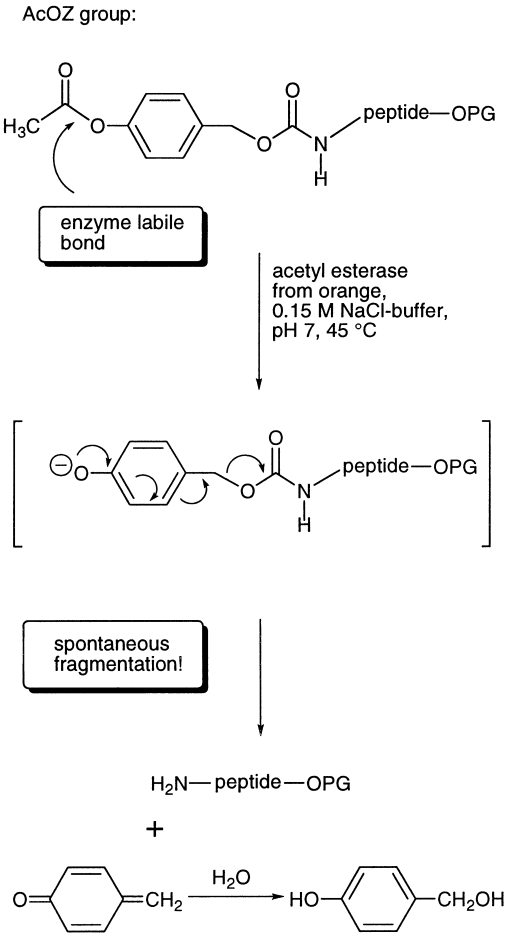
In a series of biophysical investigations the contribution of the lipid portion to the total lipopeptide membrane affinity was determined using vesicles which functioned as model membranes.[46,53–55] This study showed that a single lipid modification cannot contribute enough hydrophobic character to maintain stable membrane insertion of peptides, and therefore also of proteins. The single lipid modified peptides are inserted into the membrane within seconds but rapidly exchanged between two different membranes. Thus, the binding of simple modified peptides to model membranes is strong in the thermodynamic sense but rapidly reversible. Furthermore, it was shown that palmitoylated or geranylgeranylated peptides give higher membrane affinity than the analogous myristoylated or farnesylated peptides. C-terminal methylation can enhance membrane insertion of farnesyl modified compounds.

The introduction of a second lipid modification results in stable insertion. Biophysical model experiments on the kinetics of transfer from one model membrane to another[46,56] showed that the half time for transfer of peptides with *N*-myristoylation and *S*-palmitoylation, or *S*-palmitoylation and *S*-farnesylation was in the region of hours to days. These double lipid modifications might 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 subunits 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 the fluorescently labeled peptides **35** and **36** (Fig. 3).[50,56,57] These peptides represent forms of the



Scheme 6. Enzymatic cleavage of the BGloc urethane protective group.



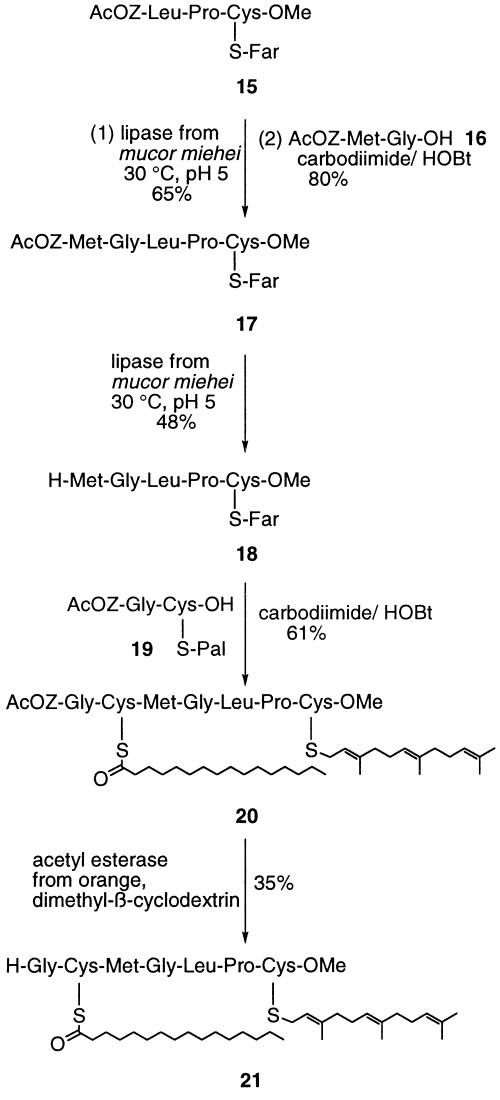
Scheme 7. Enzymatic cleavage of the AcOZ urethane protective group.

N-terminus of the human nonreceptor-tyrosine kinase *Lck* and the human N-*Ras* protein with a single lipid modification. After introduction into fibroblast cells by fusion of the cell membrane with peptide containing vesicles, they were *S*-palmitoylated on cysteine residues. This result is in contrast to analogous peptides, in which cysteine was replaced by serine, such as **37**; in this case no incorporation of palmitic acid could be detected.

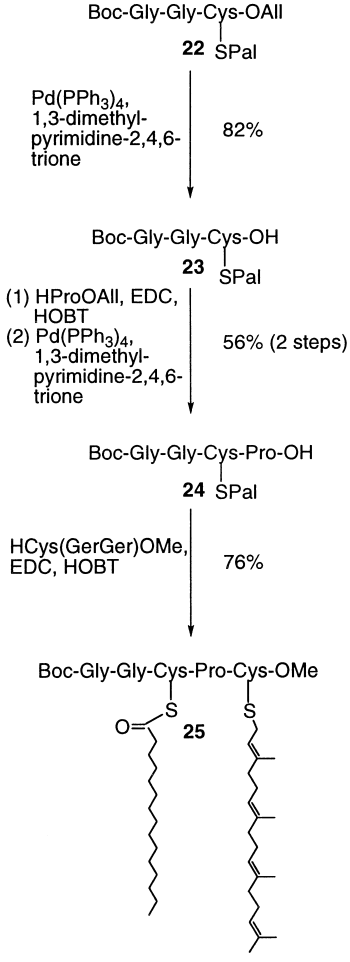
In a series of biological experiments the intracellular distribution of these peptides was determined using fluorescent microscopy techniques. This showed that the doubly lipid-modified peptides formed from **35** and **36** were concentrated in the plasma membrane of fibroblast cells (Fig. 4(a)); this took place under conditions (15 °C) which exclude intracellular transport, for example, by vesicles. For analogous serinyl peptides such as **37** which do not become acylated, such a selective distribution was not observed. The results were also confirmed by microinjection of **38** and **32** (Scheme 10; NBD-labelled) and subsequent examination by confocal laser fluorescence microscopy.[50] This showed that only the doubly lipid-modified **32** was selectively accumulated in the plasma membrane (Fig. 4(b)).

These findings, coupled with those obtained from biophysical experiments, show that doubly lipid-modified peptides (in contrast to singly modified) only exchange very slowly between different membranes (see above) and should therefore concentrate in the cell compartment in which the second lipid modification takes place. This in turn leads 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[57] for specific localization of proteins by myristoylation/palmitoylation or farnesylation/palmitoylation. According to this model, the lipid groups introduced in the course of the biosynthesis (in the case of *Ras* proteins, the farnesyl residue) do not determine the specific localization. Rather, the singularly modified proteins can freely diffuse and insert in different membranes or desorb from these (Scheme 11). It is only upon *S*-acylation in a certain membrane



Scheme 8. Chemoenzymatic synthesis of the C-terminus of the human N-*Ras* protein using the enzyme-labile AcOZ–urethane protective group.



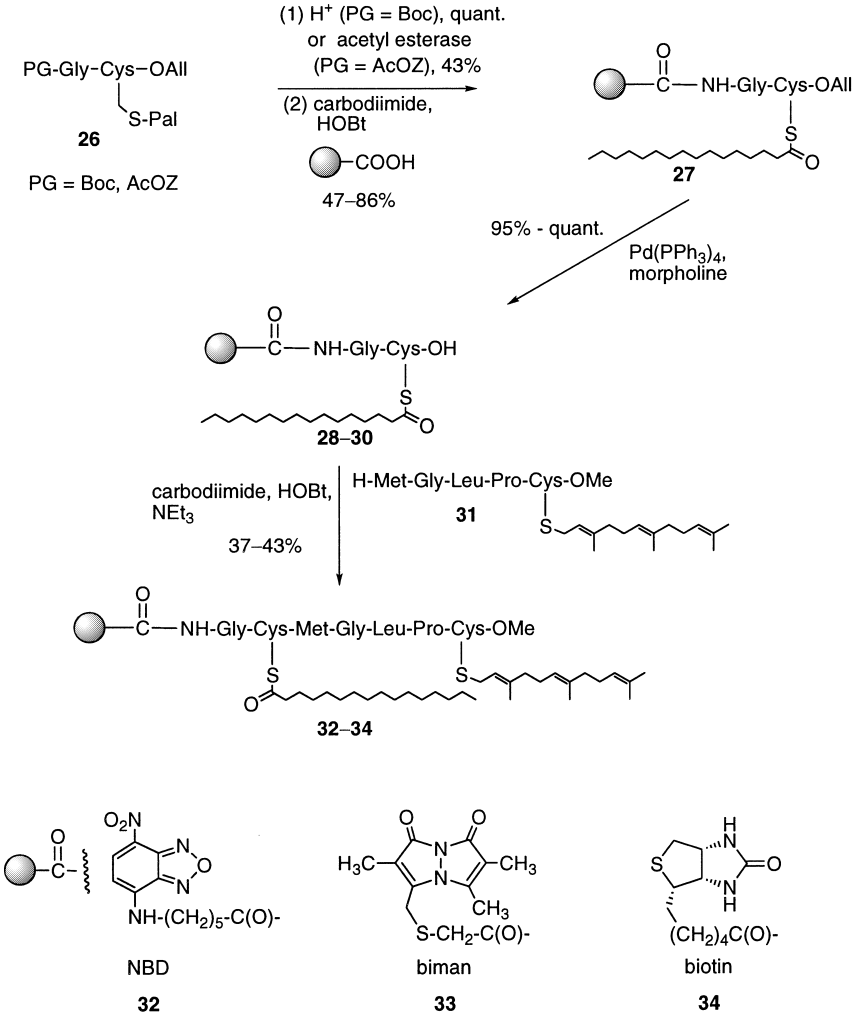
Scheme 9. Synthesis of the *S*-palmitoylated and *S*-geranylgeranylated C-terminus of the human R-*Ras* protein.

compartment, that they remain localized within that membrane. 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 11). A thiolase that may be involved in these processes was recently identified.[58]

In addition to the 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. This positively charged peptide can interact with the negatively charged cell membrane (Scheme 12).[51,52] The membrane insertion/desorption of these proteins can be regulated by phosphorylation in the positively charged domains (i.e., by reducing the number of positive charges using negatively charged phosphate). For *N*-myristoylated alanine-rich C kinase substrate (MARCKS) regulation according to the model was shown by extensive mutation analysis (Scheme 12). Using the N-terminal c-*Src* peptide **39** and the analogous phospholipopeptide **40** in experiments on a model, the reduction of membrane binding by phosphorylation was proven.

Besides their application for the study of membrane localization *S*-farnesylated cysteinyl peptides have served as tools for inhibition of farnesyl transferase. Farnesyl transferase is an enzyme that transfers a farnesyl unit onto a precursor protein, for example that of *Ras* proteins. While the enzyme is inhibited with a millimolar IC_{50} value by farnesylated peptides, which also contain the subsequently cleaved C-terminal AAX sequence,[59] a methyl-ester-masked analogue that lacks the AAX peptide, does not show any inhibitory activity.[48] Nevertheless, weak inhibition is observed if a carboxymethylated, farnesylated and palmitoylated peptide is used as an inhibitor. This finding indicates a possible 'feedback inhibition' in the biosynthesis of the *Ras* protein.[48]

Using lipid-modified peptides, the unsolved problem of whether lipid residues, in addition to their importance for membrane anchoring and localization of proteins, also have active roles in their biological function, was tackled. The foremost question here is whether the lipid



Scheme 10. Synthesis of labeled lipopetide derivatives for biological studies.

residues are involved in the control of signal transduction processes (e.g., via lipid-protein interactions). Signal transduction in photoreceptors via the G protein-coupled receptor rhodopsin and the heterotrimeric G protein transducin was chosen as the biological system (Scheme 13).[60,61] To test whether coupling of the receptor to the G protein is mediated by its γ-subunit, the farnesylated peptide **41**, corresponding to the C-terminus of the γ-subunit, and *N*-acetyl-*S*-farnesyl-cysteine, were examined as inhibitors of the interaction between rhodopsin and transducin.[62,63] It was seen that the peptide **41**, in millimolar concentrations, stabilizes the active form of the receptor (metarhodopsin 2) to a level ca. threefold higher than that of the nonfarnesylated analogue. In addition, peptide **41** (also in ca. 2 mM concentration), was shown to decouple transducin from metarhodopsin 2 to a level of 90% compared against the binding properties of transducin/metarhodopsin 2, in the absence of the peptide. 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.[62] Furthermore, 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.[63] Subsequently, analogous peptides such as **42** can inhibit the interaction between the α subunit of transducin and the β,γ complex.[64]

Although there is some doubt about the conclusions drawn from the rhodopsin/transducin investigation described above,[60] different farnesyl cysteine analogues and farnesylated peptides show manifold physiological effects. These physiological effects clearly show that these compounds interact with signal transduction processes. It seems likely that isoprenylated peptides influence the function of isoprenylated G proteins (e.g., by interaction with proteins in the sense of a receptor-ligand interaction). For yeast *Ras*, probably the farnesyl

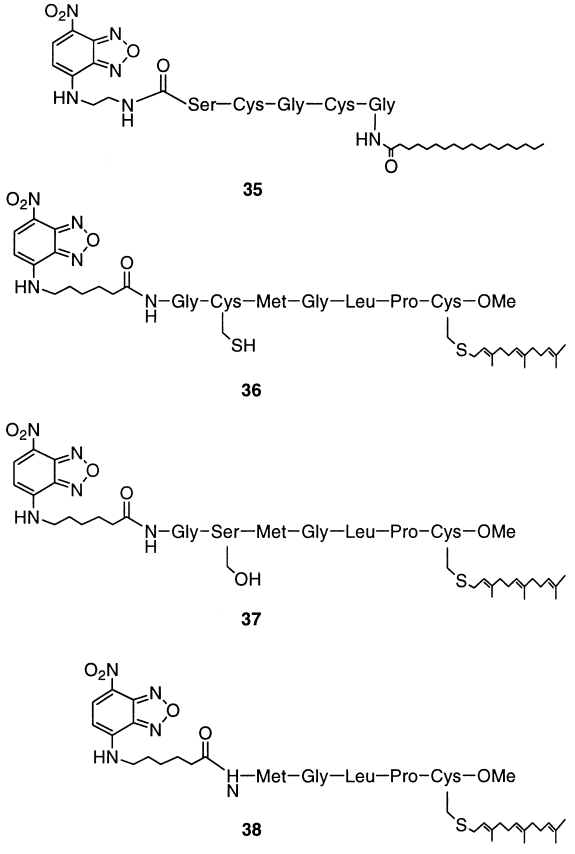


Figure 3. Lipid-modified peptides with the NBD marker, for biological studies.

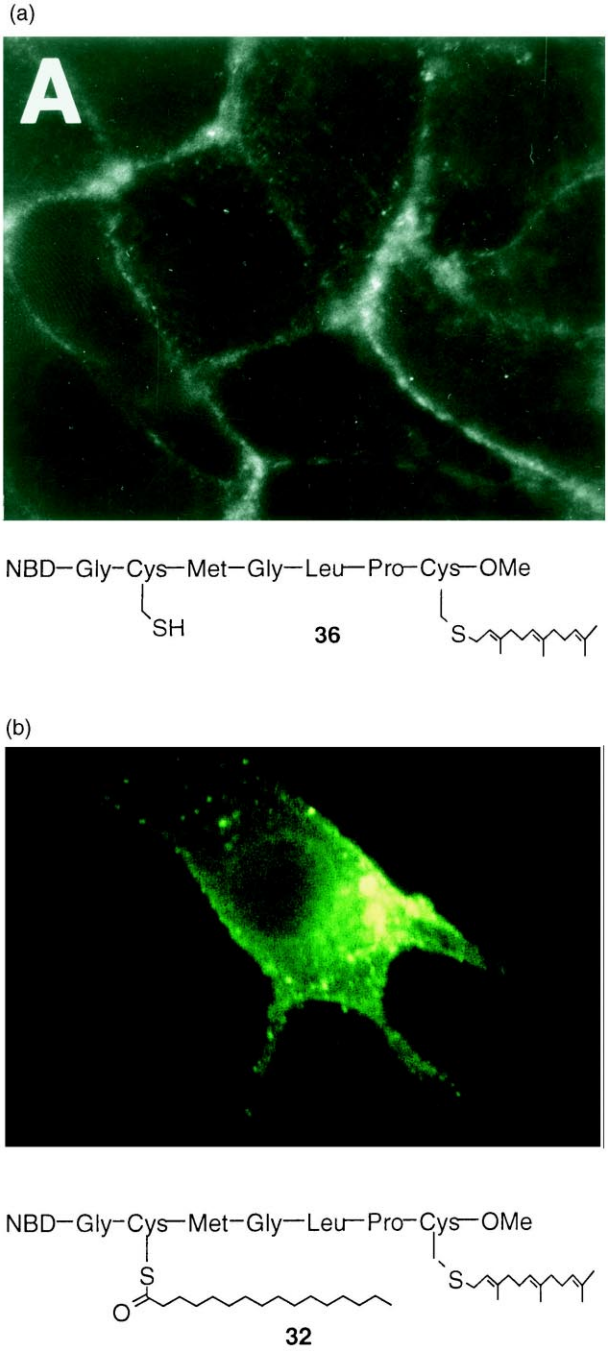
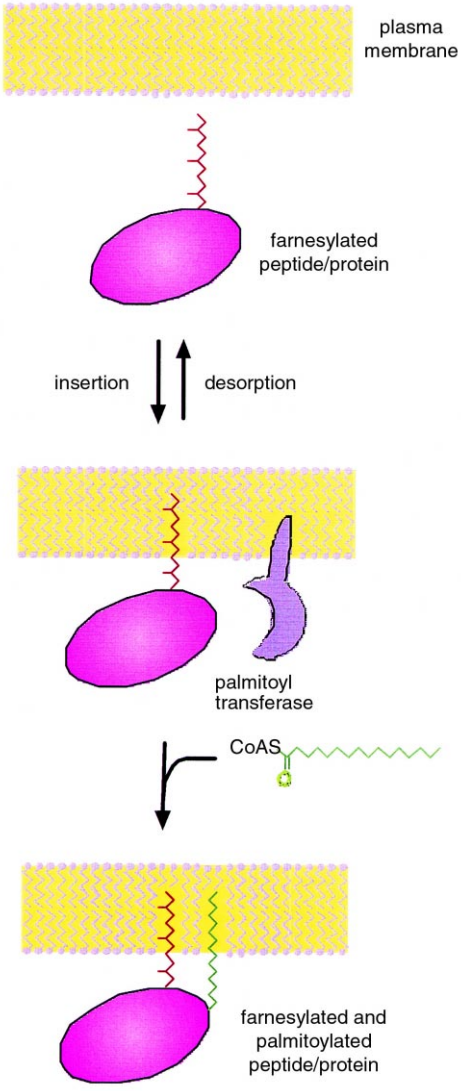


Figure 4. (a) Fluorescence image of CV-1 fibroblasts after inhibition with the NBD-labeled and farnesylated lipoheptapeptide **36**. The image shows that the fluorescence is concentrated in the plasma membrane. (b) Fluorescence image of NIH-3T3 fibroblasts after microinjection of the NBD-labeled, farnsylated and palmitoylated *Ras* heptapeptide **32**. The image shows that the peptide is localized in the plasma membrane.

residue of the protein 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.[65]

Even the specific binding of the *Ras* proteins to the plasma membrane may utilize protein–protein interaction to a high-affinity receptor located in the plasma membrane, that recognizes the isoprenoid substituent.[66] The association of H-*Ras* to the receptor was studied using the natural farnesylated protein, the geranylgeranylated analogue, the non-prenylated protein and *N*-acetyl-*S*-farnesyl-cysteine methyl ester. This study showed a saturable, prenylation dependent binding to the receptor, which also depends on other structural features of the H-*Ras* protein.

This saturation membrane binding to a receptor is consistent with the observation that a series of organic compounds resembling the farnesyl-cysteine of *Ras* proteins, for example *S*-*trans*,*trans* farnesyl thiosalicylic acid



Scheme 11. Model for insertion of lipid-modified proteins in the plasma membrane by additional *S*-palmitoylation.

(FTS), inhibit cell transformation.[67,68] This inhibition is a result of the dislodgement of *Ras* from the membrane of H-*Ras*-transformed cells.[69] FTS and its analogues interfere with the interactions of *Ras* with specific anchorage domains.[70] Dislodged *Ras* becomes unstable and can no longer emit a permanent growth signal.

Glycopeptides

Glycoproteins play important roles in many biological processes, including immune defense, viral replication, cell growth, cell–cell adhesion and inflammation.[71] Many of the proteins localized on the extracellular face of the plasma membrane carry complex oligosaccharides. Some of these glycoproteins are essential for the initial stages of cellular signal transduction, where they act as cell surface receptors that take up extracellular signals, and conduct them through the plasma membrane into the cell interior. Glycoproteins are not only involved in the regulation of communication within cells, but also regulate communication between cells as they control cell–cell interactions and form cell surface antigens.[72] Taking this into consideration, it is particularly noteworthy that glycoproteins have also been identified as tumor-associated antigens i.e., as antigens which appear on the surface of tumors but not on normal cells. It is known that correct carbohydrate and peptide substructures found in such glycoproteins are essential for the correct biological function of these protein conjugates.[73] Whilst proteins with attached complex oligosaccharides almost exclusively fulfill extracellular biological functions on the cell surface, it has recently been shown that glycosylation of proteins can also play an important intracellular role. As will be shown later (in the section on Glycophosphopeptides), new evidence indicates glcosylation/phosphorylation of intracellular proteins may form a switchable mechanism for the final steps of signal transduction, that is, influencing the transcription of the genetic code.

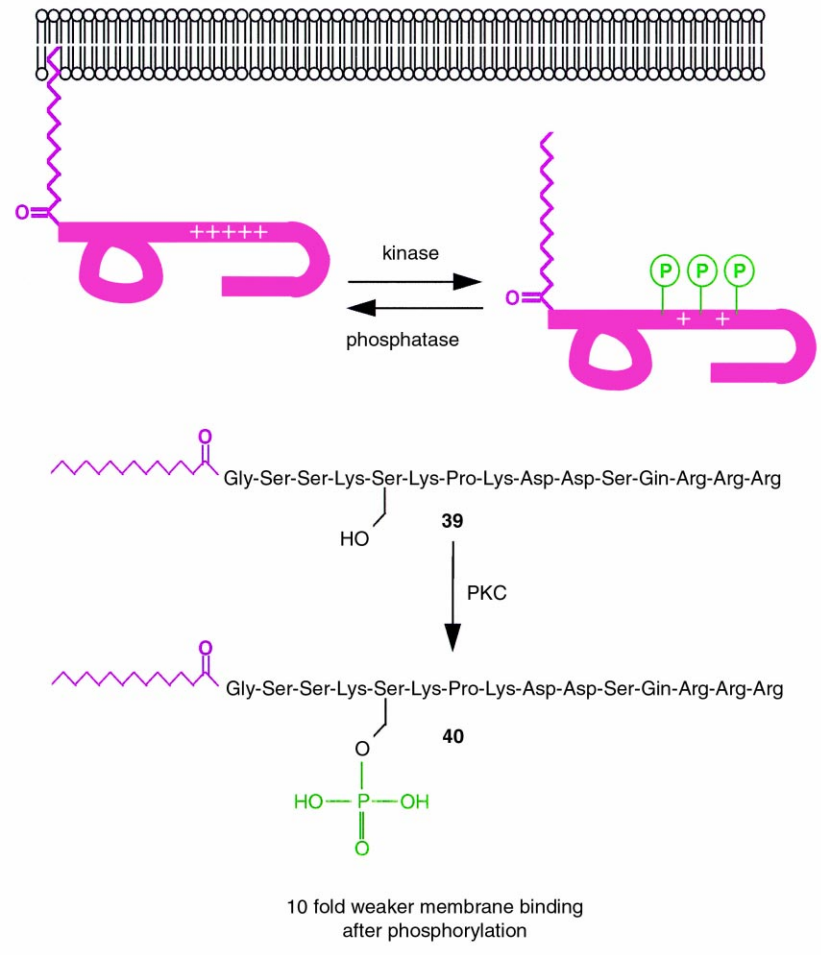
Due to the importance of glycoproteins, access to characteristic glycopeptide units could provide useful tools for investigation of the biological functions of glycoproteins. Classical chemical synthesis of oligosaccharide units of glycoproteins has reached a high level of efficiency.[74] Over the last two decades many powerful techniques for the reversible blocking of the amino, carboxy and alcohol groups have been developed.[74] These techniques can now be utilized, to synthesize glycopeptides both in solution and on solid phase. Advances in methodology, for the gentle release of sensitive molecules from polymeric carriers, also have direct application in solid-phase synthesis of these compounds.[74–79]

The synthesis of glycosylated peptides, on polymeric carrier molecules, can now be realized with a high degree of efficiency. The allylic HYCRAM[74] **43** and HYCRON[75] **44** linker groups (Scheme 14) are particularly advantageous since they permit gentle release of the sensitive glycopeptide from solid-phase carrier, via a Pd(0) catalyzed allyl transfer to *N*- and *C*-nucleophiles. Using HYCRAM linkers, it was possible to synthesize

many glycopeptides, with both the acid-labile Boc, as well as the base-labile Fmoc protecting groups. However, the basic cleavage of the Fmoc group presented problems (e.g., nucleophilic attack on the α,β-unsaturated amide in **43**). Also, the glycopeptides could not, in some cases, be quantitatively released from the solid carrier. These disadvantages were overcome with the development of the HYCRON linker **44**. In compound **44**, a flexible oligoethylene glycol spacer ensures that the Pd(0) complex can progress well to the allylic group during cleavage; this is no longer present as the α,β-unsaturated carbonyl function but rather as the ether and is therefore no longer competitively attacked by nucleophiles. Using the HYCRON linker, the glycopeptide **45** was synthesized on a polymeric carrier and subsequently released with a high yield. This method has proven to be equally successful for the synthesis of an eicosa peptide.[75]

The recent development of the PEGA resin **46** has proven to be an advantageous alternative to carriers based on a polystyrene support (Fig. 5). In principle, it is made up of polyacrylamide chains, which are cross-linked by polyethylene glycol units and carry additional free amino functions at which the synthesis of peptides and glycopeptides can be performed.[77] The hydrophillic PEGA resin swells very well in different solvents, and due to the flexible structure of the matrix, its interior is accessible to larger molecules and even enzymes. Using PEGA resin, numerous glycosylated peptides were synthesized in automated solid-phase syntheses (Fig. 5).[76,77,80] Fmoc-urethane was used as N-terminal protective group and the glycopeptide to be synthesized was linked to the polymeric carrier via the acid-labile Rink anchor (see **47**, Fig. 5). Solid-phase-bound glycopeptides were synthesized by successive extension of the peptide chain. Cleavage of this glycoconjugate from the solid phase was performed under slightly acidic conditions and without unwanted side reactions. Glycopeptides can be reliably and efficiently synthesized today by this and other solid-phase techniques, using automated methods.

The application of enzymatic methods has opened up valuable alternatives for the 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 protective group. The lipase from *Mucor javanicus* deblocks the C-terminus of the glycosylated amino acids and peptides **48–52** without unwanted attack on the N-terminal urethane and the carbohydrate protective group (Scheme 15).[81] The enzymatic deprotection reaction conditions are very

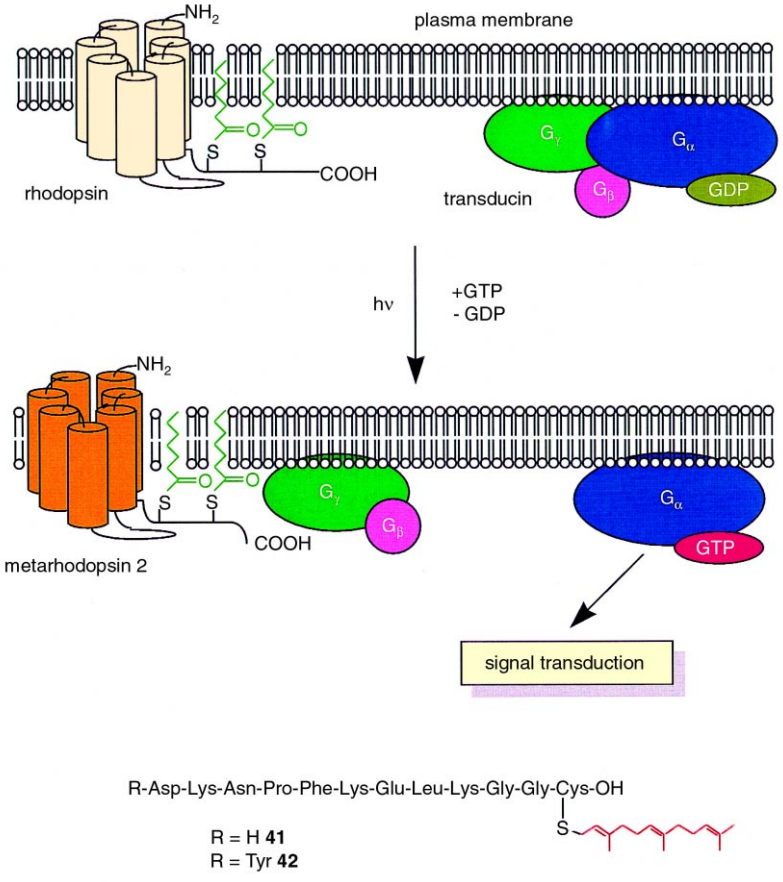


Scheme 12. Regulation of membrane binding by combination of *N*-myristoylation with a polybasic amino acid sequence and phosphorylation.

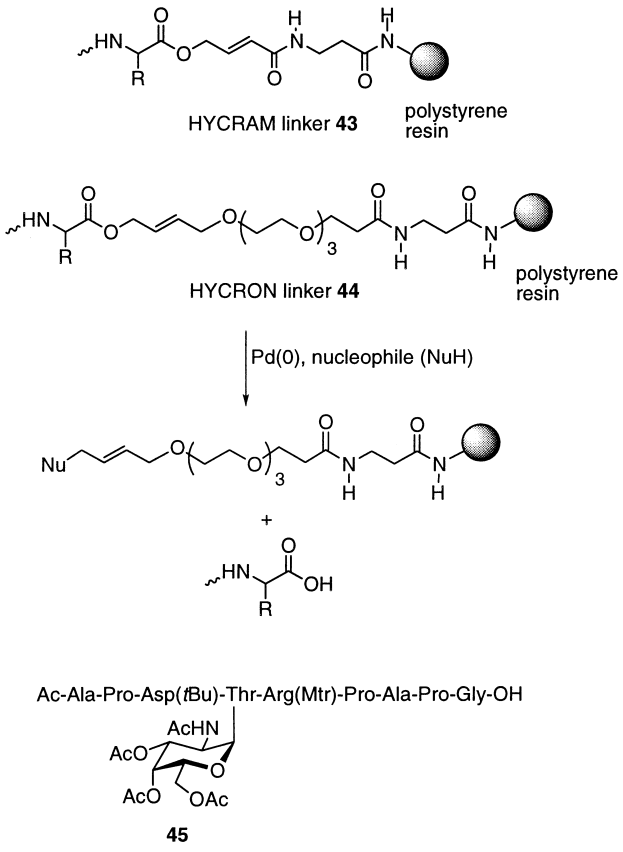
mild and no anomerization or β-elimination could be detected. Using this enzymatic protective group technique, the complex diglycohexapeptide **59** was synthesized; this molecule contains the characteristic linkage region of a tumor-associated antigen found on the surface of human breast carcinomas (Scheme 16). Following selective C-terminal deblocking of the serine glycoside heptyl ester **53**, the carboxylic acid **54** released was condensed with the N-terminal deblocked glycopeptide ester **55** to form the diglycotripeptide **56**. The azido group of the latter was converted to the acetamide and subsequently the C-terminus was selectively released again by enzyme-mediated hydrolysis, without any unwanted side reactions. C-terminal extension of the peptide chain gave the desired peptide **59**. Unfortunately, some problems were associated with the use of the very hydrophobic heptyl ester, this was due to the low solubility of the substrate and thus low rate of turnover, especially in the presence of similarly hydrophobic amino acids. However, these problems were easily solved by introduction of the more hydrophilic 2-(*N*-morpholino)ethyl ester (MoEt)[82] and in particular, the methoxyethoxyethyl (MEE) ester.[83] The MEE ester was, for example, cleaved from the glycopeptide **62** by lipase-catalyzed hydrolysis (Scheme 17) and the threonine glycoside **60** could be deblocked at the C-terminus by papain. The proteases papain, subtilisin and thermitase were also used for demasking of glycopeptidylmethyl[84] and even *tert*-butyl esters.[85]

Enzymatic transformations have also been used successfully for assembly of the oligosaccharide part of glycopeptides. An example of this is the glycosylated asparaginyl peptide **64**. This compound was synthesized on a polymeric carrier (Scheme 18), with regio- and stereospecific linking of a galactose and a neuraminic acid unit to the peptide chain to give **65**, by two consecutive enzymatic glycosylations.[86] Cleavage of the glycopeptide from the solid-phase was achieved by chymotrypsin mediated ester hydrolysis at the C-terminus of a phenylalanine. Completion of the characteristic sialyl Lewis^X tetrasaccharide was achieved by fucosylation of the *N*-acetylglucosamine to give **66**. This technique has also enabled the in vitro synthesis of a glycosylated protein in an impressive manner.[87] For this, an oligosaccharide was cleaved off RNAase B with a glycosidase, and the remaining GlcNAc residue underwent successive enzymatic glycosylations until the protein had the sialyl Lewis^X tetrasaccharide attached (Scheme 18). Using this enzymatic technique, based on a principle previously suggested for the modification of unwanted glycans of recombinant glycoproteins,[88] it is possible to obtain glycosylated proteins with high homogeneity; this opens up new possibilities for study of the influence of carbohydrates on structure and function of glycoproteins.

In the synthesis of complex glycopeptides, it is often advantageous to combine classical chemical steps with



Scheme 13. Manipulation of signal transmission in the rhodopsin/transducin system by lipid-modified peptides.



Scheme 14. Solid-phase glycopeptide synthesis using the HYCRAM and the HYCRON linker groups.

enzymatic glycosylation steps. For example, the glycopeptide **67** was synthesized with the help of established carbohydrate chemistry methods and the oligosaccharide was subsequently extended to the undecasaccharide **68** by enzymatic glycosylations (Scheme 19).[89]

The methods illustrated above clearly show that ready access to synthetic glycopeptides can be reached either by solution- or solid-phase syntheses, utilizing both classical chemical methods coupled with chemoenzymatic procedures. These synthetic glycopeptides find many important uses in biological studies, one such study is the mechanism of acute and chronic inflammation processes.

In this process, proteins on the surface of endothelial cells recognize the so-called selectins which are complex glycoproteins on the leucocytes carrying multiple copies of the sialyl Lewis^X (SLe^X) carbohydrate epitope.[72,90] Once the endothelial cell and leucocytes meet and are attached, the leucocytes subsequently migrate out of blood vessels and may cause acute and chronic inflammation. This migration of the leucocytes is an example of the process known as protein shedding.[135]

With the aim of developing new therapeutic treatments, glycopeptides were produced that were expected to block the attachment of leucocytes to the endothelial cells, and halt their subsequent migration. Based on data of the conformation of SLe^X in solution, the three-dimensional structure of the human E-selectin, the structural elements needed for recognition of SLe^X by E-selectin and supported by molecular modeling, the fucosylated peptides **69–71** and further analogues were synthesized and tested as inhibitors (Fig. 6).[91] The peptides each contain the necessary fucose, a hydroxy group carrying a structural element that imitates the galactose of the SLe^X tetrasaccharide, and a glutaric acid that functions as the equivalent of the negatively charged group of the sialic acid unit. It was found that compound **69** proved to be as active as the natural sialyl Lewis^X, whilst derivatives **70** and **71** were considerably better inhibitors.

The assumption that the weak interaction of monomeric SLe^X in vivo is increased by 'clustering' of ligands (multivalence) was examined using the multiple SLe^X glycopeptides **72, 73**, and **74** (Fig. 6). In a cell adhesion test in which binding of **72** to recombinant E-selectin-immunoglobulin fusion proteins was compared against its binding to SLe^X ligand-carrying tumor cells, it was found that the trivalent glycopeptide was two- to three-fold more active as an inhibitor than the monomeric SLe^X building blocks.[92] In compounds **73** and **74** the distance of the SLe^X oligosaccharides from one another

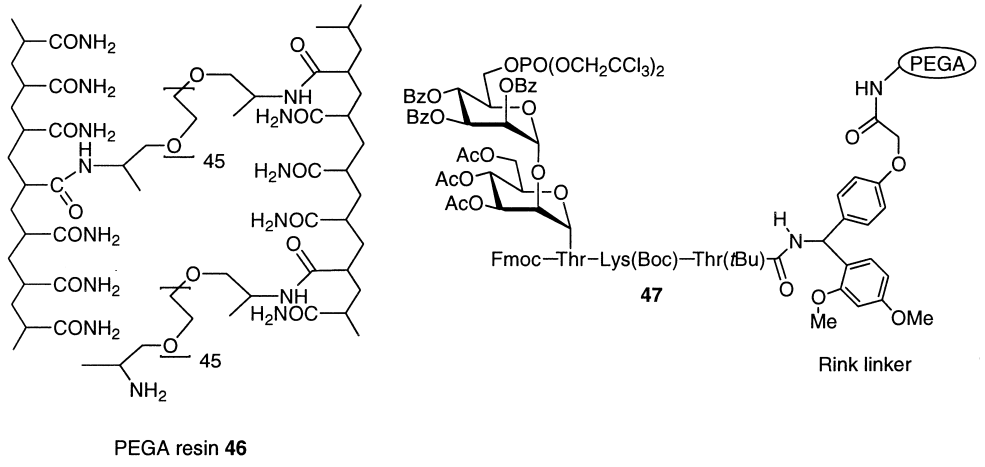
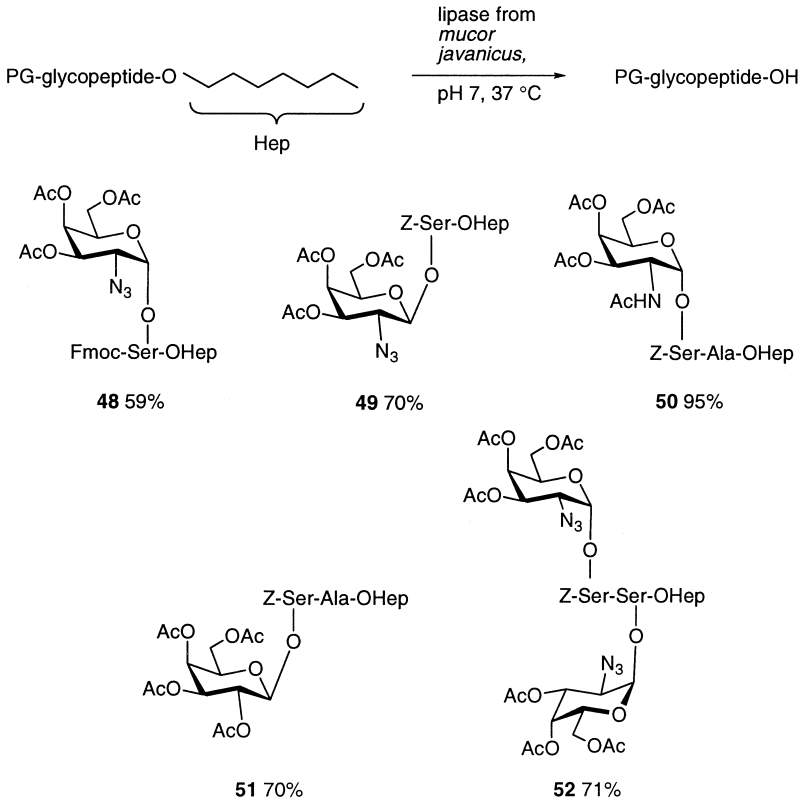
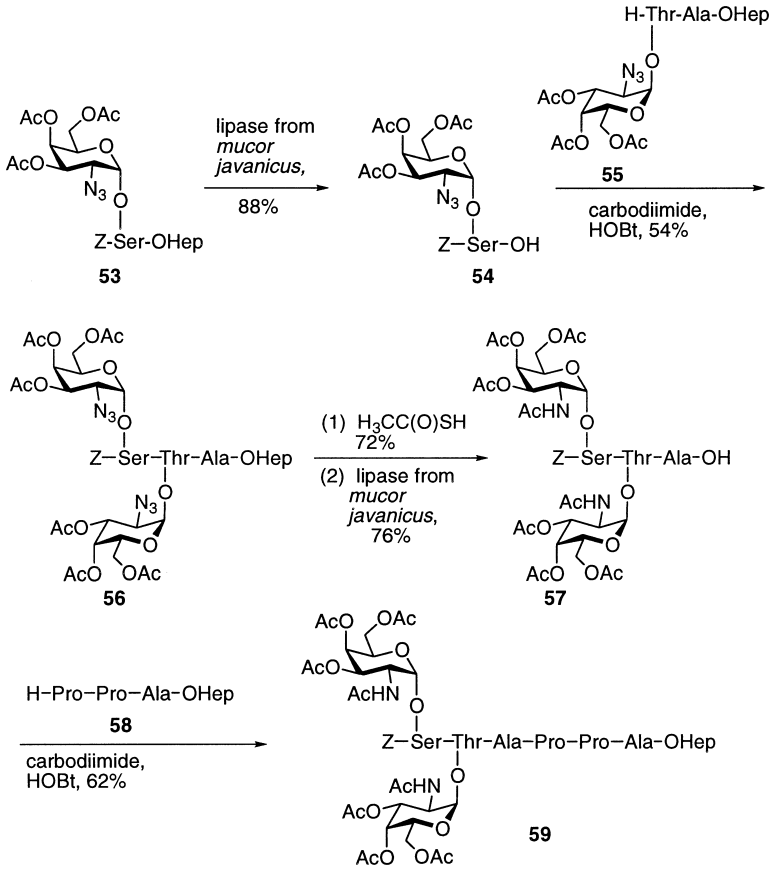


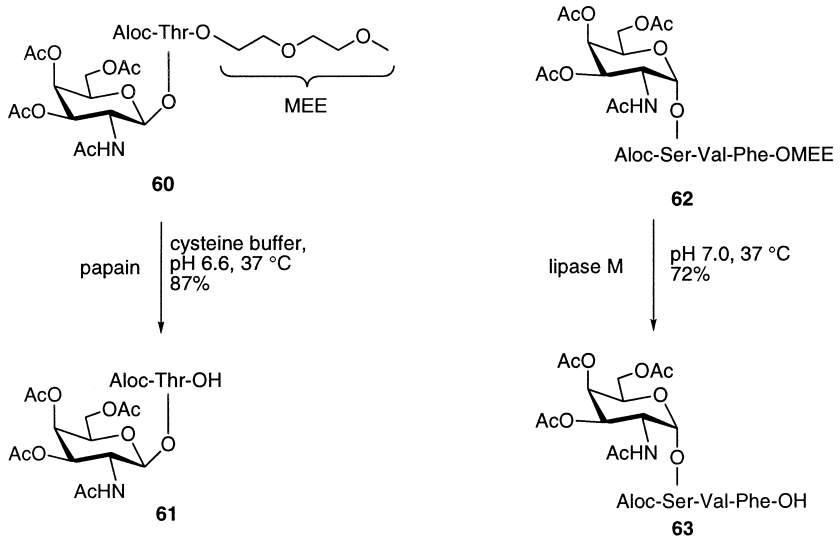
Figure 5. Solid-phase glycopeptide synthesis on PEGA resin.



Scheme 15. Chemoenzymatic glycopeptide synthesis using the enzyme-labile heptyl (Hep) protective group.



Scheme 16. Synthesis of the glycopeptide **59** that represents a characteristic section of a tumor-associated antigen.



Scheme 17. Enzymatic glycopeptide synthesis using the enzyme-labile methoxyethoxyethyl (MEE) ester.

was varied to obtain information about the size and the spatial orientation of the SLe^X cluster.[93] It was shown that the divalent ligands found in **73** were less active than the trivalent analogues **74**, and that 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). This tripeptide corresponds to the minimum sequence of many adhesion molecules and is, for example, recognized by integrin receptors. The adhesion hybrid **75** thus obtained showed an IC_{50} value of 26 μM in the cell assay and proved to be a very effective inhibitor of adhesion.[94]

Recent work aimed at understanding the relationship between binding, clustering and proteolytic release of L-selectin, utilized the effects of L-selectin ligands on the cell surface presentation of the protein (Fig. 7).[136] These compounds termed neoglycopolymers are based on the L-selectin glycoprotein structure and display multiple copies of the SLe^X cluster on their surface. It was shown that monovalent derivatives of the trisaccharide $Lewis^X$, which are inhibitors of L-selectin (IC_{50} values of 1–3 mM) did not cause L-selectin shedding. In contrast the neoglycopolymers induced L-selectin shedding from human leukocytes in a dose dependent manner. Furthermore, it was shown that the neoglycopolymers showed no signs of activation, and selectively initiated L-selectin shedding, as opposed to activation promoted shedding processes, which results in the cleavage of a number of surface molecules coupled with an increase in the surface concentration of the β2-integrin Mac-1. These results show that the glycoprotein like neoglycopolymers, may provide useful compounds that can minimize cell-surface levels of L-selectin without simultaneously initiating other signaling pathways.

For the study of the recognition of phosphorylated glycoproteins by the mannose-6-phosphate (Man-6-P) receptor, glycopeptides carrying the 6-*O*-phosphorylated mannose residue were used as reagents.[80] It is known that lysosomal enzymes carry oligosaccharides with Man-6-P residues. Recognition of the carbohydrate residue by Man-6-P receptors is required before the enzyme is processed and finally transported to the Golgi apparatus. Misregulation of these steps results in an inflammatory process within 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 **76–78** (Fig. 8) were synthesized. Introduction of a phosphorylated mannose or a phosphorylated disaccharide with α-(1,6)- or α-(1,2) linked mannoses into these compounds provided a base set of derivatives. Further derivatives were made by variation of the length and sequence of the amino acid chains between the saccharides.[80] These glycopeptides were then examined, in a specially developed ELISA test, as inhibitors of binding of the mannose-6-P receptor to an immobilized phospho-mannan. It was found that 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. Favorable results were also obtained with a three to five amino acid separator between the two saccharides, whereas 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 development of antitumor vaccines.[95]

Phosphopeptides and glycophosphopeptides

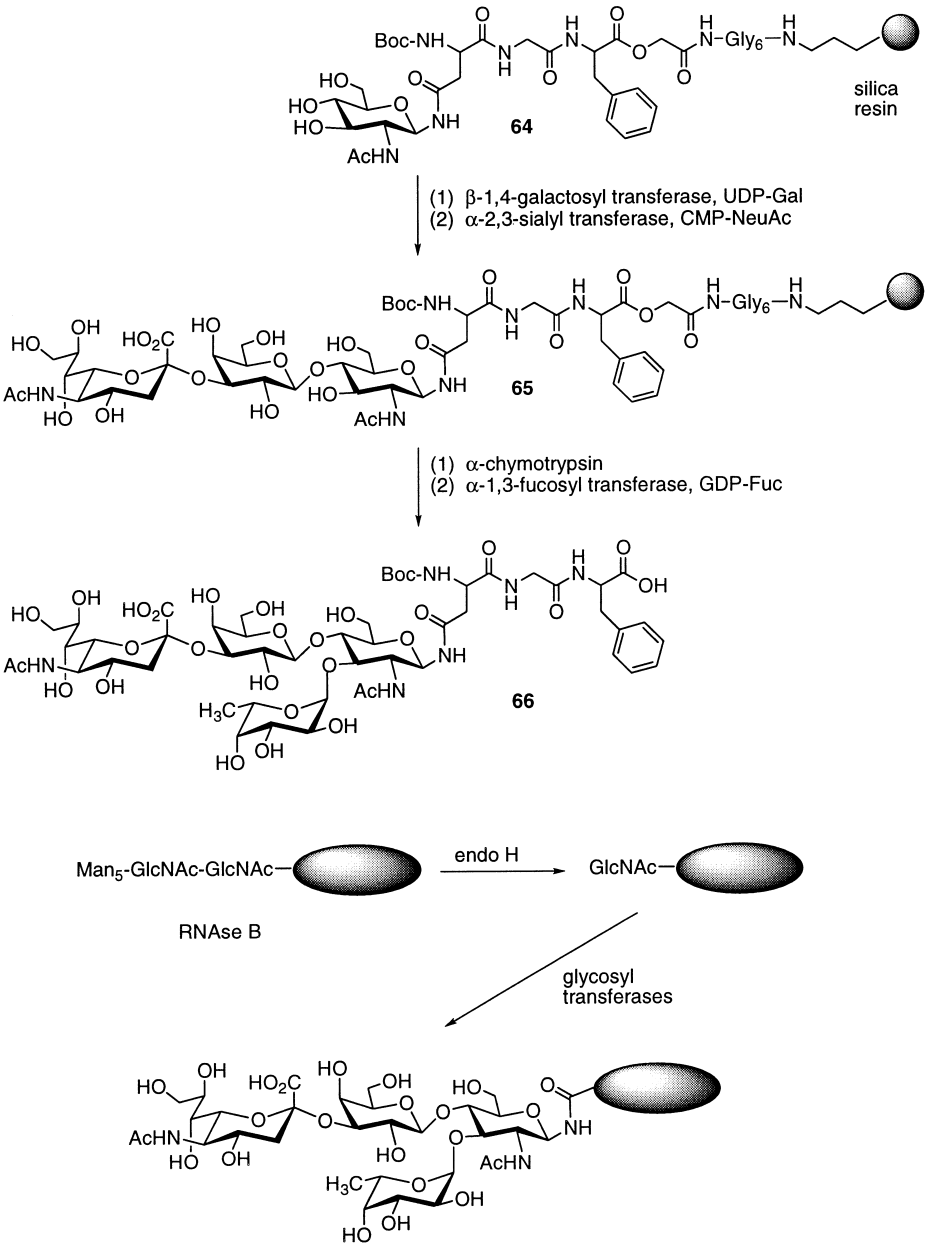
Phosphopeptides. Reversible protein phosphorylation is widely recognized as an integral mechanism for the regulation of many cellular processes. These covalent protein modifications are employed by all organisms for numerous purposes (e.g., the control of extracellular

chemical signals to the cell nucleus, regulation of the transcription of the genetic code by transcription factors, the control of the cell cycle and the regulation of cell growth, and proliferation). Specific protein kinase and phosphatase enzymes achieve phosphorylation and dephosphorylation of these proteins, at serine, threonine, and tyrosine residues. Due to the importance of such phosphopeptides, characteristic peptides incorporating the correctly phosphorylated residues of parent proteins serve as invaluable tools and reagents.[96]

Peptides phosphorylated at tyrosine residues are generally very stable and fairly easy to synthesize by relatively standard chemistry.[96] Thus, the development of methods for their synthesis will not be discussed further here. However, the synthesis of phosphorylated serine and threonine peptides is impeded to a considerable extent by the extreme base lability of these peptide conjugates (Fig. 1); for access to these compounds, protective groups that can be released under mild conditions are required.

Synthesis of serine/threonine phosphopeptides can be achieved in principle 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, using phosphorylated amino acids building units ('building block strategy'). The latter method is generally more flexible.

Initial studies on the stepwise synthesis of phosphopeptides focused on the use of Boc-protected building blocks, such as **79**, (Fig. 9). In this work the phosphate protective group had to be carefully chosen to avoid

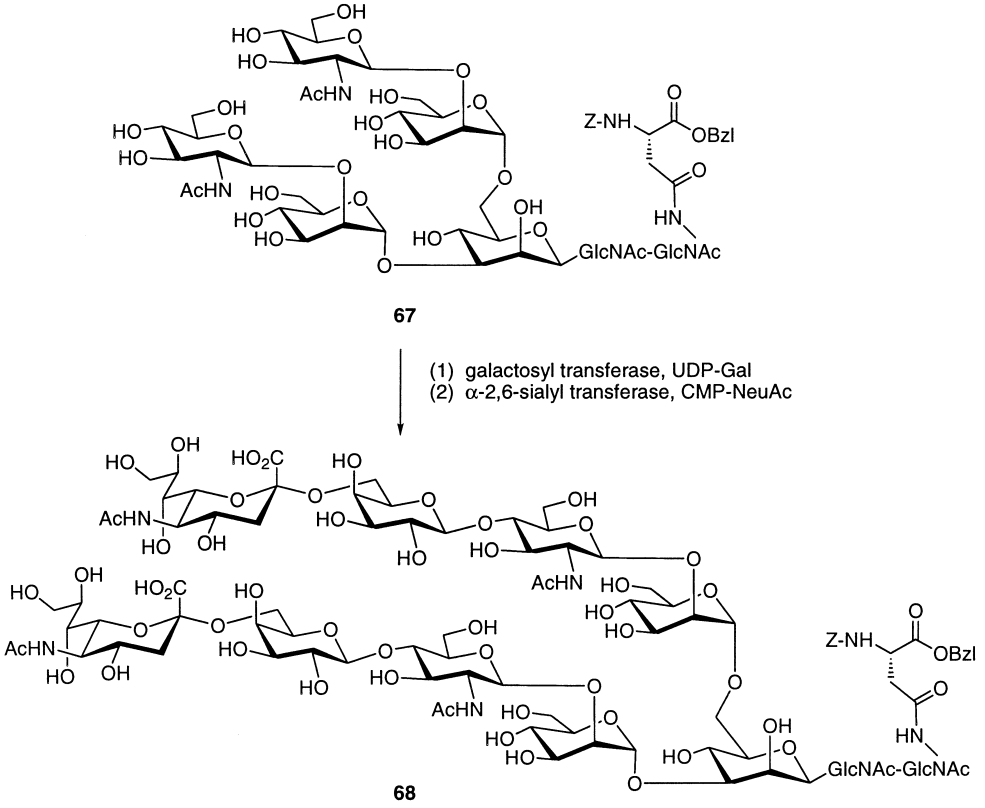


Scheme 18. Enzymatic synthesis of sialyl Lewis^X-modified peptides and proteins with glycosyl transferases.

dephosphorylation under the conditions of Boc group cleavage.[96e,97] This technique, although successfully used to synthesize the partial sequence **80** of the Tau protein on a polymeric carrier, has been superceded by improved protecting group strategies.[98] The use of the N-terminal allyloxycarbonyl (Aloc) protective group has proven to be quite effective. For example, in a solid-phase synthesis of the Tau protein fragment **82**, the amino acid unit **81** (Fig. 9) was linked to a hexapeptide assembled on Wang resin using the base labile N-terminal fluorenylmethoxycarbonyl (Fmoc) group and acid-labile side chain protective functions.[99] By palladium(0)-mediated release of the allyl protective groups in the presence of Me_3SiN_3/Bu_4NF, the N-terminus and the phosphate 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 protective groups, gave the desired peptide **82**. One disadvantage of this approach however, is the simultaneous release of the amino function and the phosphate group, which can itself become activated during the subsequent peptide coupling.

Unfortunately, the base-labile Fmoc protective group cannot be used for synthesis of phosphopeptides without further modification. The reason for this is that under the conditions of Fmoc cleavage, serine and threonine phosphopeptides masked as phosphoric acid esters (unlike the more stable glycopeptides), lose the phosphate by β-elimination and formation of α,β-dehydroalanyl peptides.[100] Introduction of the Fmoc-masked unit **83**, which only carries a phosphoric acid diester (Fig. 10), has recently been shown to be an effective alternative.[101,102] The phosphate is deprotonated under basic conditions and is no longer eliminated as it is now a poor leaving group. This group is stable under treatment with piperidine (required to release the Fmoc group), and is not apparently activated during the subsequent extension of the peptide chain. Using this building block, the phosphopeptides **84** and **85**, which represent phosphorylated partial sequences of the heat shock protein, were synthesized on solid phase without any occurrence of the unwanted side reaction mentioned above.[102]

The use of enzymatic protective group techniques has also provided interesting and advantageous alternatives to classical chemical methods in phosphopeptide synthesis. An example of this is the recent synthesis of the phosphopentapeptide **93** (Scheme 20), which is a characteristic section of the human *Raf*-1 kinase (see the earlier second sub-section of the section on Biological signal transduction). This synthesis was achieved using lipase-mediated hydrolysis of the heptyl ester protective group.[103,104] The fully protected phosphorylated serine heptyl ester **86** was selectively deblocked at the C-terminus using the lipase from *Aspergillus niger*, and following extension of the peptide chain, the enzyme treatment released only the ester protective group from the phosphotripeptide ester. Renewed chain extension by a dipeptide and subsequent release of all allyl protective groups gave the target compound **93**. The conditions for the enzymatic deblocking are so mild that no β-elimination of the phosphate residue from



Scheme 19. synthesis of a complex glycopeptide precursor by successive enzymatic glycosylations.

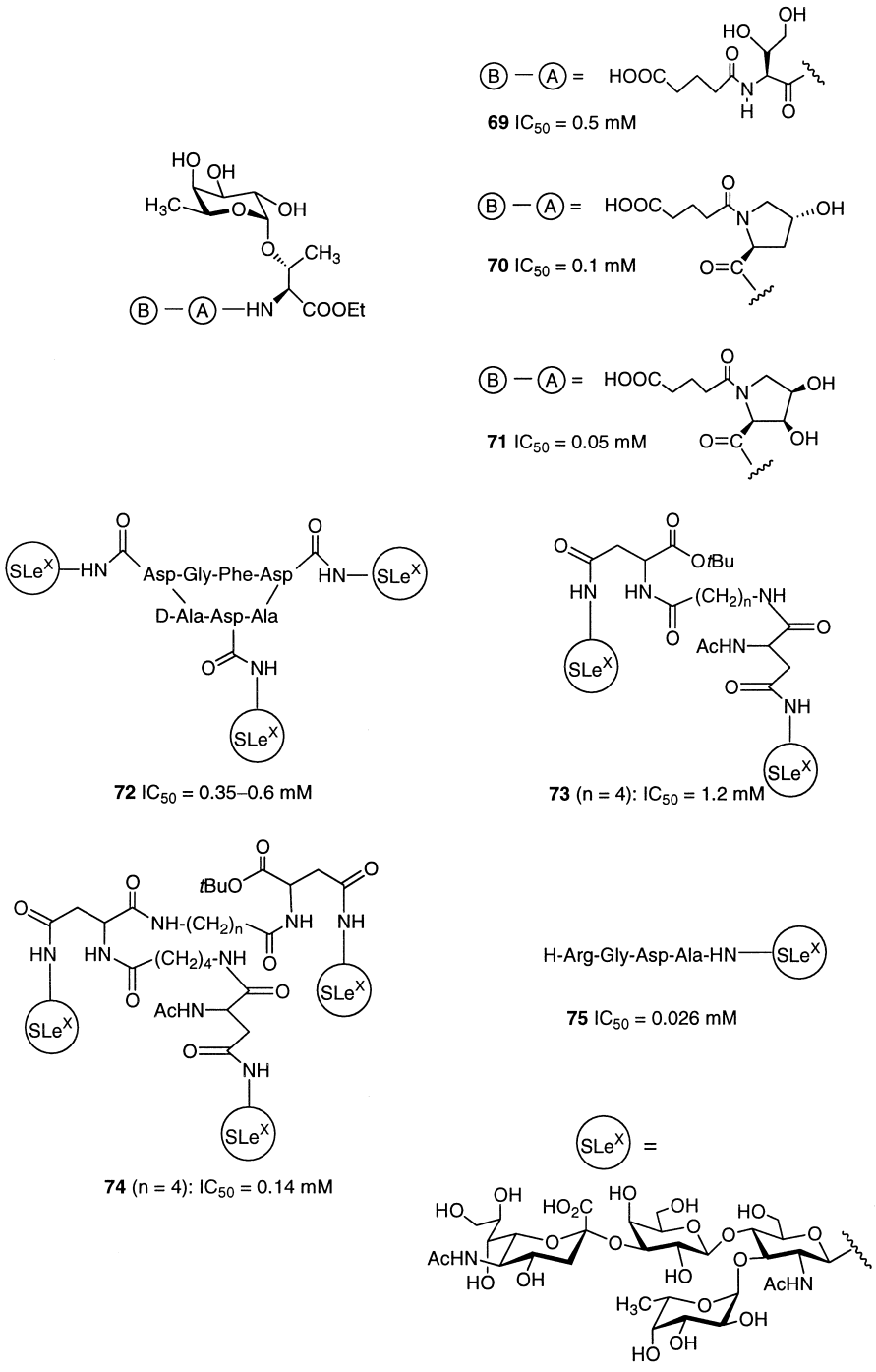


Figure 6. Synthetic sialyl Lewis^X mimetics.

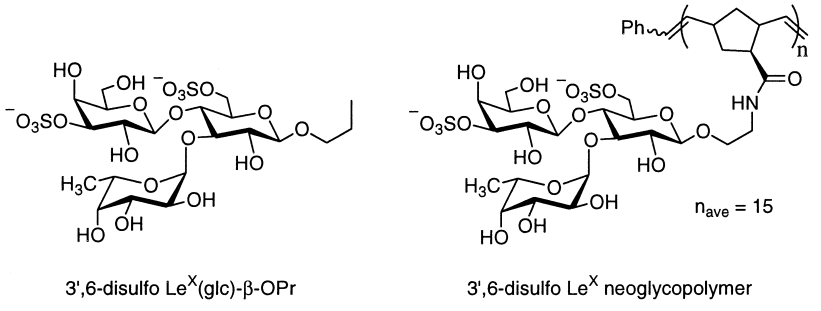


Figure 7. Synthetic siayl Lewis^X mimetic and neoglycopolymer for studies on L-selectin shedding.

the phosphoric acid ester-masked peptides could be observed.

N-terminal deblocking of phosphorylated peptides was achieved using the phenylacetoxybenzyloxycarbonyl (PhAcOZ) urethane protective group.[105] This enzyme-labile urethane is an analogue of the AcOZ group described in the earlier sub-section on lipid-modified peptides above, and was developed in the course of a phosphoglycopeptide synthesis (see below). On treating the PhAcOZ-masked phosphorylated tripeptide **96** with the enzyme penicillin G acylase, the terminal phenylacetic acid ester was selectively cleaved off and the spontaneous fragmentation was initiated (Scheme 21). With the release of the N-terminal deblocked phosphopeptide **97**, it was then coupled with the doubly phosphorylated dipeptide **95** to give the pentapeptide **98**, which is available for further C-terminal enzymatic deblocking. By selective lipase-mediated heptyl ester cleavage, compound **95** was also obtained from the corresponding fully protected precursor **94**. An alternative possibility for selective N-terminal deblocking of phosphorylated

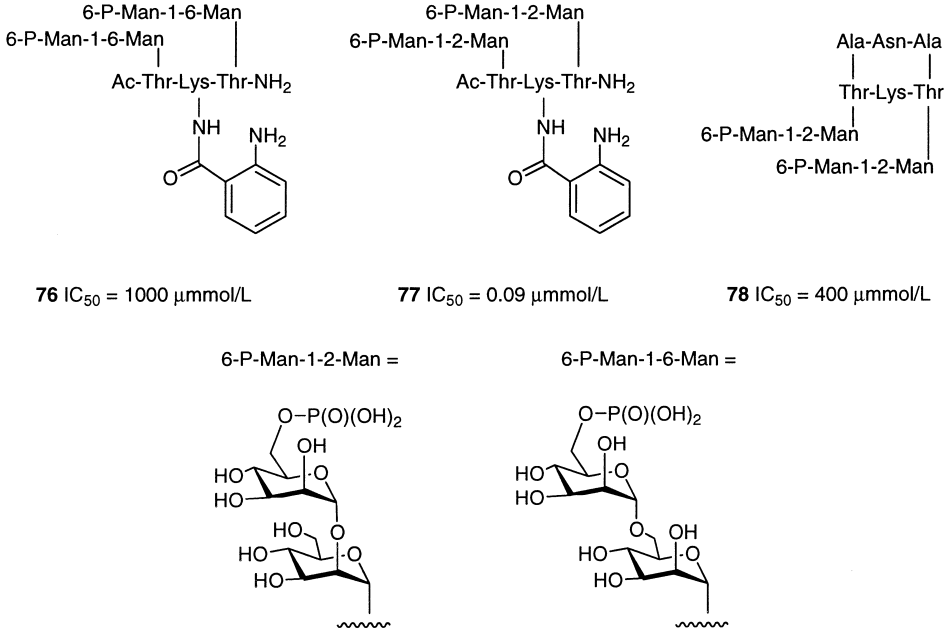


Figure 8. Synthetic phosphomannosyl glycopeptides and their inhibitory effect on the phosphomannose receptor.

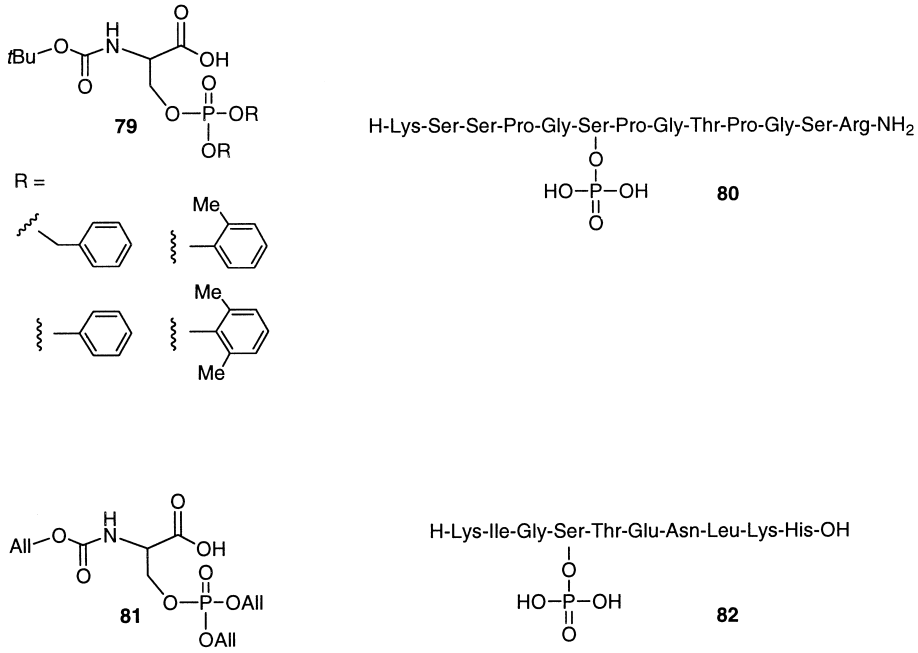
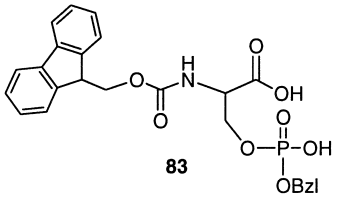


Figure 9. Phosphopeptides synthesized with fully protected phosphoamino acid building blocks.

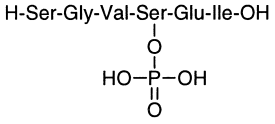
peptides is by direct protection of the amino group with phenylacetic acid and biocatalyzed cleavage of the phenylacetamide with penicillin G acylase.[103,106]

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 that, for example, recognize phosphotyrosine residues in *Ras* and GPCR signal cascades using SH2 domains.[107–109] Targeted modulation of signal transmission via the JAK/STAT pathway was also achieved using such peptide conjugates.[110] Furthermore, tyrosine–phosphate-modified peptides, which represent autophosphorylation sequences of tyrosine kinase receptors, were used for the determination of substrate tolerance of protein tyrosine phosphatases.[111] The substrate specificity of the Ser/Thr-specific casein kinase 2 was more closely defined using different phosphorylated peptides of the sequence Ala-Ser/Thr-Tyr-Ser-Ala,[112] derived from the phosphorylated form of the autophosphorylation site (Asn-Glu-Tyr-Thr-Ala) of the *Src* kinase family. Seven phosphopeptides were synthesized using the 'global phosphorylation' strategy (see above) and these were tested as substrates for casein kinase 2. The enzymatic investigations showed that phosphorylation of the tyrosine favors the additional modification of the C-terminal serine. In the natural protein, the tyrosine phosphate could function as the determinant for a subsequent second phosphorylation by casein kinase 2 at the C-terminal neighbouring hydroxyamino acid (Scheme 22). This controlling activity explains why casein kinase 2, in the case of the *Src* kinases, shows such substrate specificity, according to which amino acid C-terminal to the target amino acid should be acidic.

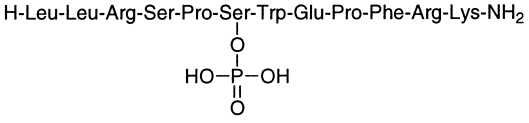
Recent work has shown the elegant use of a serine phosphorylated peptide to elucidate the molecular



83

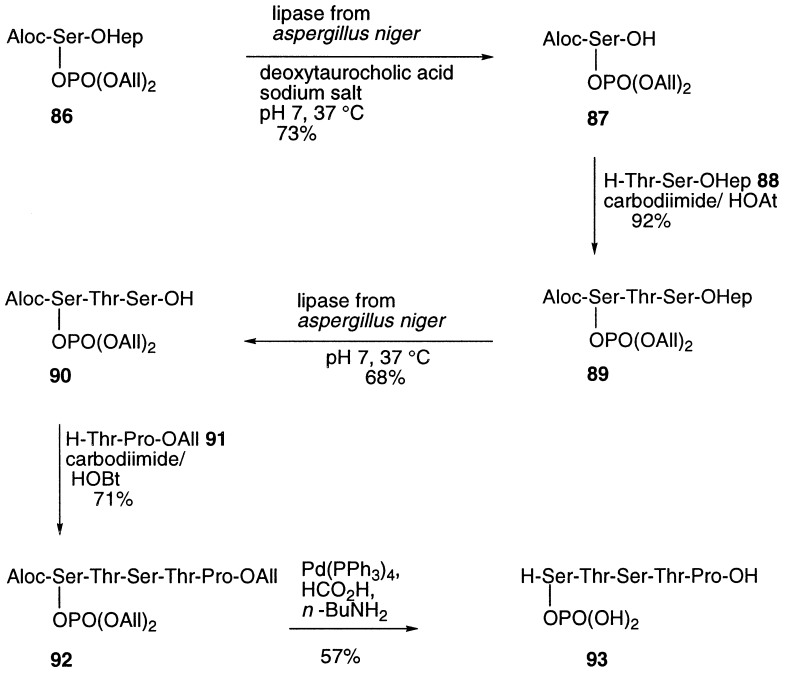


heat shock protein 27 (87-92) **84**



heat shock protein 27 (10-20)-Cys-NH_2 **85**

Figure 10. Phosphopeptides synthesized using the phosphoric acid diester **83**.



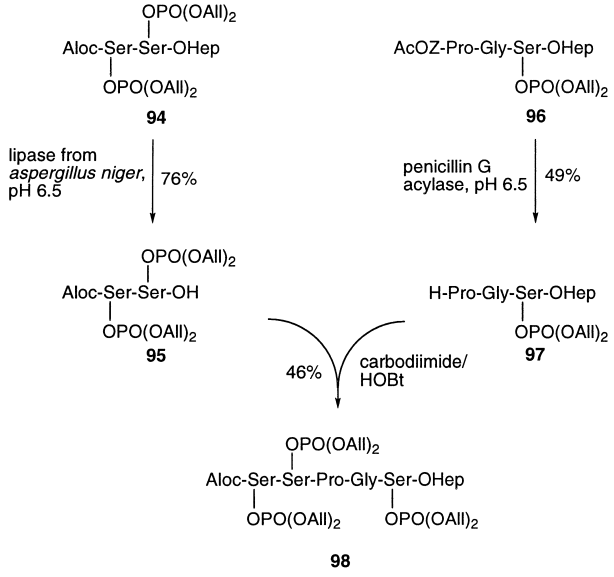
Scheme 20. Chemoenzymatic phosphopeptide synthesis using the enzyme-labile heptyl ester.

details, and the structural basis for the cell cycle-dependent import of the tumorigenic transcription factor v-*Jun*.[113] In the G1 phase of the cell cycle, v-*Jun* is cytosolic. In this form, it is phosphorylated at serine 248, that is, in the immediate vicinity of its nuclear-localizing amino acid sequence (NLS) that initiates active transport of the protein into the cell nucleus. However, in the G2 phase, the phosphoric acid ester is removed, and the protein concentrates in the cell nucleus. It was thought that 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 a serine residue in the immediate vicinity of the NLS. Support for the idea that the phosphorylation has this crucial role in regulation of nuclear import came initially with the help of protein kinase- and phosphatase-inhibitors. A more in depth study to test this hypothesis utilized a phosphorylated peptide and the corresponding non-modified peptide; both peptides contained the serine in question and the NLS. Both these compounds, once synthesized, were then linked to an immunoglobulin via an active ester (Scheme 23). Following microinjection of the protein conjugates into the cytosol of fibroblasts, their intracellular fate could be followed by fluorescence microscopy using a fluorescein-labeled anti-IgG antibody. The result from this experiment showed that the conjugate with phosphorylated serine stayed in the cytosol whereas the nonphosphorylated analogue was imported into the cell nucleus.[113]

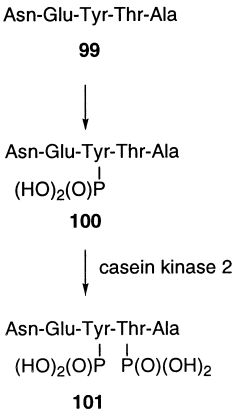
Phosphopeptide libraries have also recently been used to determine the sequence specificity and phosphorylation dependence of the peptidyl–prolyl isomerase, Pin1.[114] Pin1 is a highly conserved enzyme, which posseses both a WW domain and PPIase activity.[115] PPIases catalyze the relatively slow peptidyl–prolyl isomerization of proteins, allowing relaxation of local energetically unfavourable conformational states.[116] Pin1 both negatively regulates entry into mitosis and is required for normal progression through mitosis in human and yeast cells. In this work, a degenerate series of peptides was utilized to probe the specificity of Pin1. It was found that Pin1 preferentially isomerised proline residues preceded by a phosphorylated serine or phosphorylated threonine residue, with up to 1300-fold selectivity compared with unphosphorylated peptides. The results from this study suggest a two step mechanism for mitotic regulation. Phosphorylation at specific serine/threonine–proline sites by mitotic kinases creates a binding site for Pin1, which in turn induces conformational changes by catalyzing prolyl isomerization. These local conformational changes thereby alter the activity of mitotic phosphoproteins, their ability to interact with other proteins or trigger their degradation.

Phosphorylated peptides have also often been used as antigenic structural units for creation of monoclonal antibodies that recognize particular phosphoproteins.[99,117] These antibodies serve, for example, as tools for immunodiagnosis of the Tau protein in Alzheimer's disease.

Glycophosphopeptides. Recently, a new type of covalent intracellular protein modification was reported. In this process a single glycosidic linkage of *N*-acetylglucosamine to serine and threonine residues of intracellular proteins is formed.[118] This *O*-GlcNAc modification is similar to phosphorylation in many ways, especially with respect to its dynamic character and its ubiquitous occurrence (e.g., on transcription factors such as *Myc*, the serum response factor (SRF), *Jun* and *Fos* and oncogene products such as the tumor suppressors Rb and p53). The limited data available to date suggest that cells use reversible glycosylation with *O*-GlcNAc as a regulatory mechanism. Also there are indications that phosphorylation and glycosylation by *O*-GlcNAc may have a so-called 'yin yang' relationship to one another (i.e., they are not only similar but are often reciprocal to one another). An illustrative example of this process is found within the C-terminal domain of the human RNA polymerase II. Whilst this enzyme is present in the cytosolic form, the C-terminus is heavily glycosylated, however the intranuclear elongating transcription complex is no longer glycosylated and instead is highly phosphorylated. In both forms of the enzyme the



Scheme 21. Chemoenzymatic phophopeptide synthesis using the enzyme-labile heptyl ester and the AcOZ protective group.

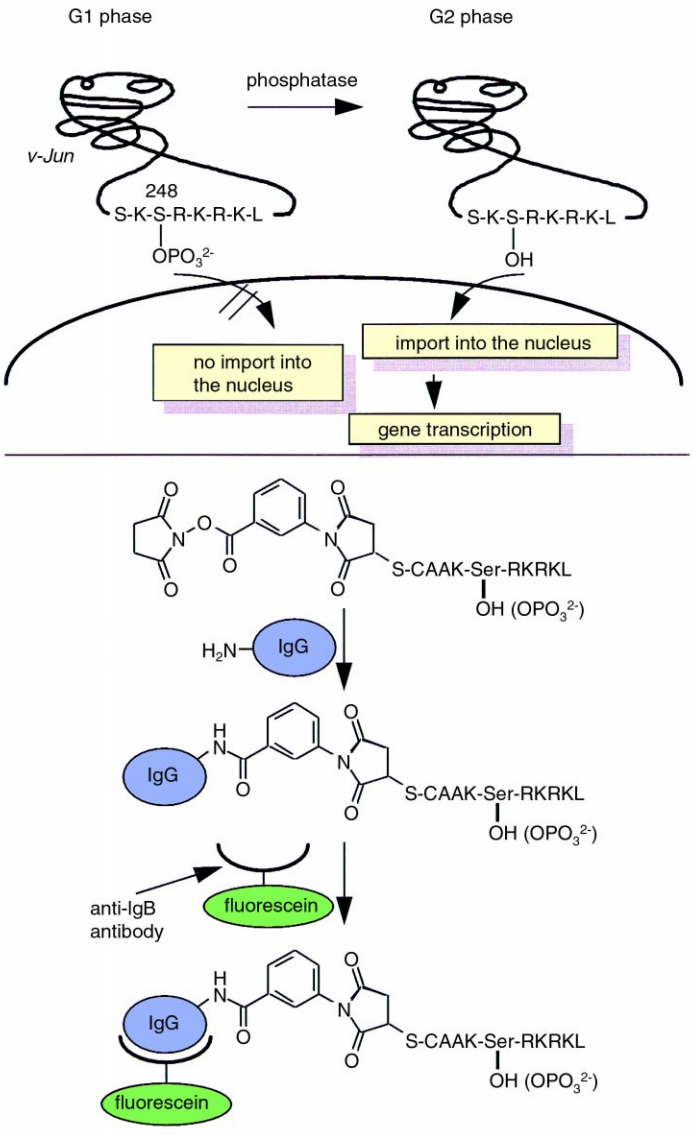


Scheme 22. Enzymatic phosphorylation of a peptide with casein kinase 2.

phosphorylation and glycosylation sites are identical.,[118,119] Peptides containing both modifications could provide valuable tools in answering such questions as whether the carbohydrate masks or even marks potential phosphorylation sites, as well as whether phosphorylation/glycosylation are involved in regulation of nuclear import, or even influence transcription of the genetic code.

In designing the syntheses of such glycophosphopeptides the problems of both phosphopeptide and glycopeptide chemistry must be considered. The combination of multifunctionality, as well as the pronounced acid and base sensitivity of the conjugates provides a formidable challenge to conventional organic synthesis. To this end, application of enzymatic protecting group techniques has shown to be extremely powerful in providing access to such complex molecules. Recently, the first synthesis of a phosphoglycopeptide **111**, which represents a characteristic section of the large C-terminal domain of the human RNA polymerase II, was achieved.[120] In this synthesis, both lipase-mediated cleavage of heptyl esters (see the earlier section on Glycopeptides) and also enzyme-initiated fragmentation of a urethane protective group (see the earlier section on Lipid-modified peptides) were employed.

Utilizing the enzymatic protecting group strategy outlined previously compound **111** was synthesized (Scheme 24). The fully protected glycosylated serine **102**, which includes the enzyme-labile PhAcOZ group at the N-terminus (see also Scheme 21), was treated with penicillin G acylase. Only the phenylacetic acid ester was cleaved and the fragmentation of the resulting phenolate, shown in Scheme 7, led to release of the desired selectively deblocked amino acid **103** (Scheme 24). After linking to a glycodipeptide, also PhAcOZ-protected, to form the diglycotripeptide **105**, penicillin G acylase re-released the N-terminus under very gentle conditions. A further cycle of chain extension and enzyme-initiated N-terminal deblocking gave the selectively deprotected



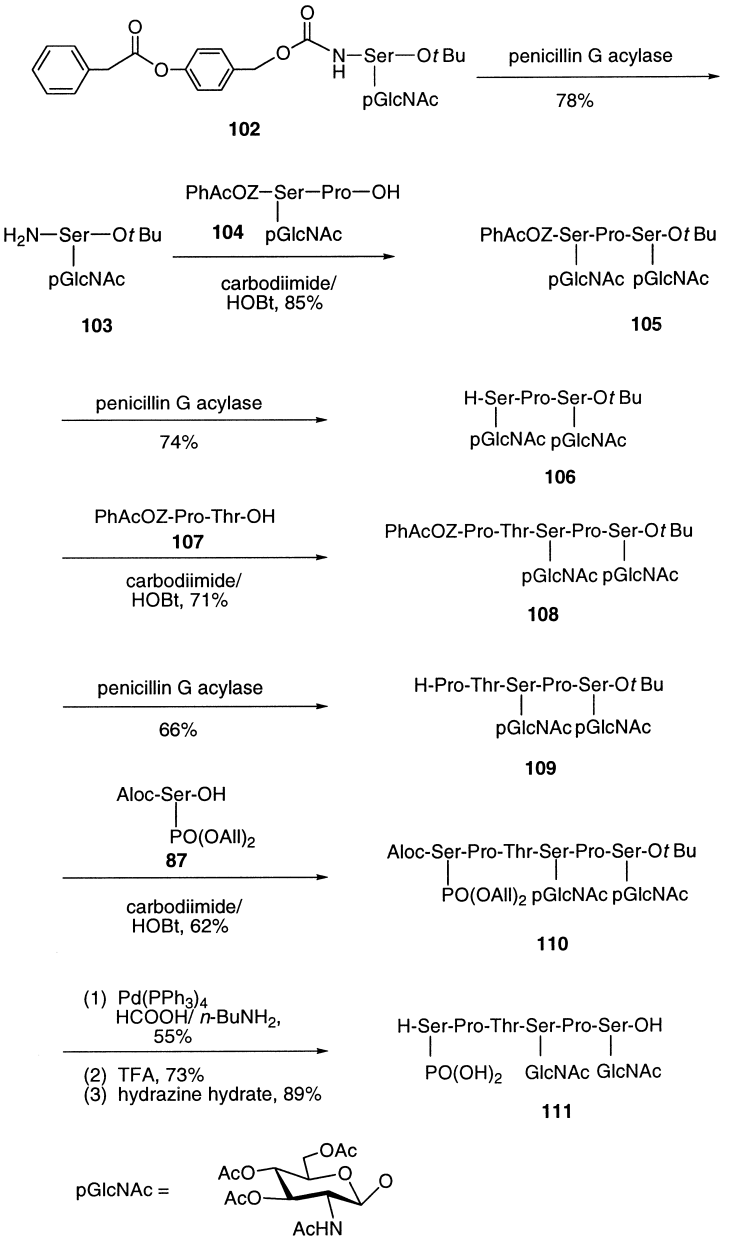
Scheme 23. Regulation of the nuclear import of v-*Jun* by phosphorylation.

diglycopentapeptide **109**. Subsequently, the allyl-masked serine derivative **87** was linked to the molecule; as shown in Scheme 20, **87** was obtained by lipase-mediated cleavage of the heptyl protective group from the corresponding ester. Finally, successive removal of all amino acid and carbohydrate protective groups gave the desired target molecule **111**.

Phosphoglycopeptides such as **111** have not yet been used in biological investigations. However, it was recently shown that a synthetic *O*-GlcNAc-modified peptide, corresponding to a repeat sequence of the C-terminal domain of the RNA polymerase II, inhibits in vivo gene transcription considerably.[121] 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. Using synthetic phosphoglycopeptides, it should be possible to obtain valuable information about these and other biological processes.

Nucleopeptides

The final stage of cellular signal transduction is the receipt of the message to the final target, followed by the desired response. This response in most cases is the transcription of DNA. Nucleopeptides in which the hydroxy group of a serine, a threonine or a tyrosine residue is linked via a phosphodiester group to the 3′ or 5′ end of DNA or RNA, play decisive roles in many important biological processes. In particular, nucleopeptides are involved in the transcription of the genetic code in many different organisms. For example, formation of nucleopeptide compounds plays a crucial role in priming replication of



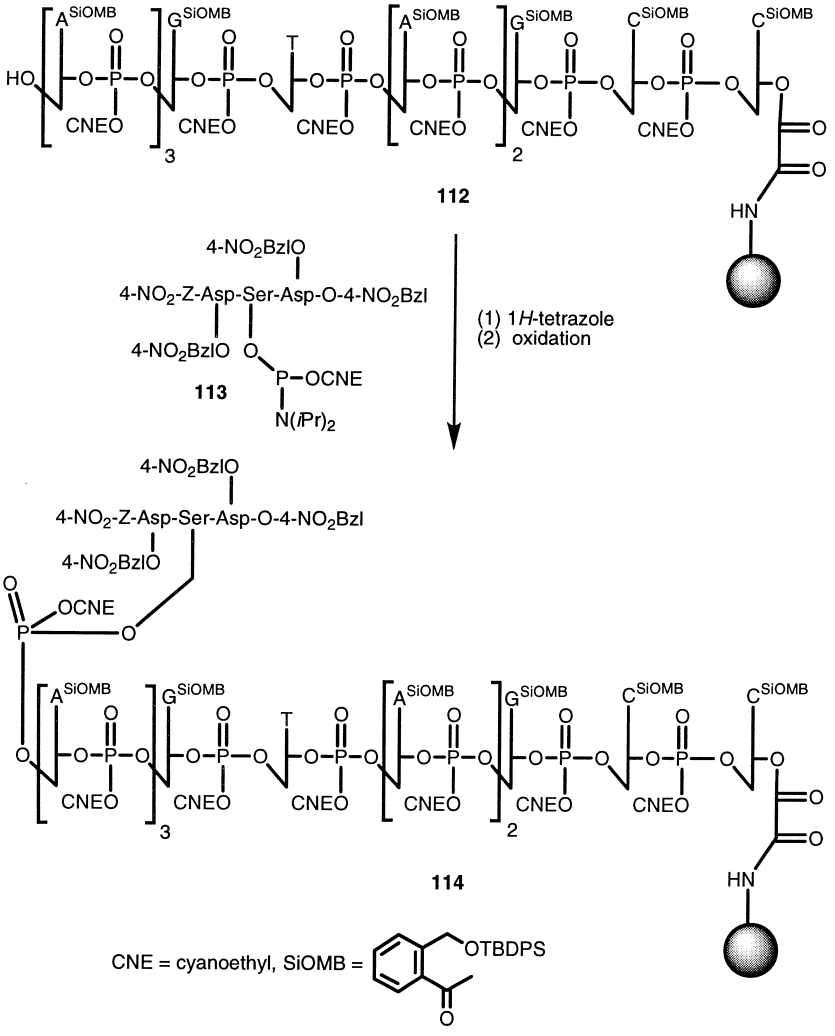
Scheme 24. Chemoenzymatic synthesis of the phosphoglycopeptide **111** using the enzyme-labile PhAcOZ protective group.

viruses,[122] DNA topoisomerases create nucleopeptides as part of their function[123] and DNA exists as a nucleoprotein covalently linked to the matrix of the cell nucleus. For this reason characteristic nucleopeptides could open up many new possibilities, for example, for study of gene transcription and particularly for development of new and alternative antiviral agents.

Synthesis of nucleopeptides presents the same problems, in principle, as phosphopeptide synthesis (see above). However, the number of protective groups to be used, which must also be orthogonally stable, increases considerably due to the additional functional groups of the carbohydrates and nucleobases; also the acid lability of the purine *N*-glycoside forbids the use of acid-labile protective groups. Due to this combination of sensitive functionalities, only very few reports on the successful construction of nucleopeptides have appeared. Initial work[124] showed that protective groups that are established for use in peptide chemistry, such as allyl esters, often do not provide a suitable solution for this problem.[125] However, the first protective group combinations were recently developed for synthesis of tyrosine nucleopeptides,[125,126] and of serine/threonine nucleopeptides,[125,127–130] in solution and on polymeric carriers. Since the tyrosine derivatives are not destroyed by base-induced β-elimination, only synthesis of serine/threonine peptide conjugates will be considered in this review.

For the development of a solid-phase synthesis of serine nucleopeptides, two general strategies have been developed. In the first strategy, a convergent method,[127] the immobilized oligonucleotide **112** was created and then coupled to a separately synthesized peptide amidophosphite **113** (Scheme 25). The protective groups in the peptide and oligonucleotide parts and the linker to the solid carrier were chosen so that the desired nucleopeptide was obtained in two concluding steps. Unfortunately, deblocking of compound **114** remained incomplete.

In the second strategy a peptide **116** was synthesized, stepwise on a polymeric carrier by the Boc method. This was followed by the successive synthesis of the oligonucleotide chain[128] (Scheme 26). Subsequent cleavage of the linker from the polymeric carrier with fluoride, followed by removal of the base protective groups by treatment with concentrated ammonia resulted in formation of the desired nucleopeptide **118** isolated in a total yield of

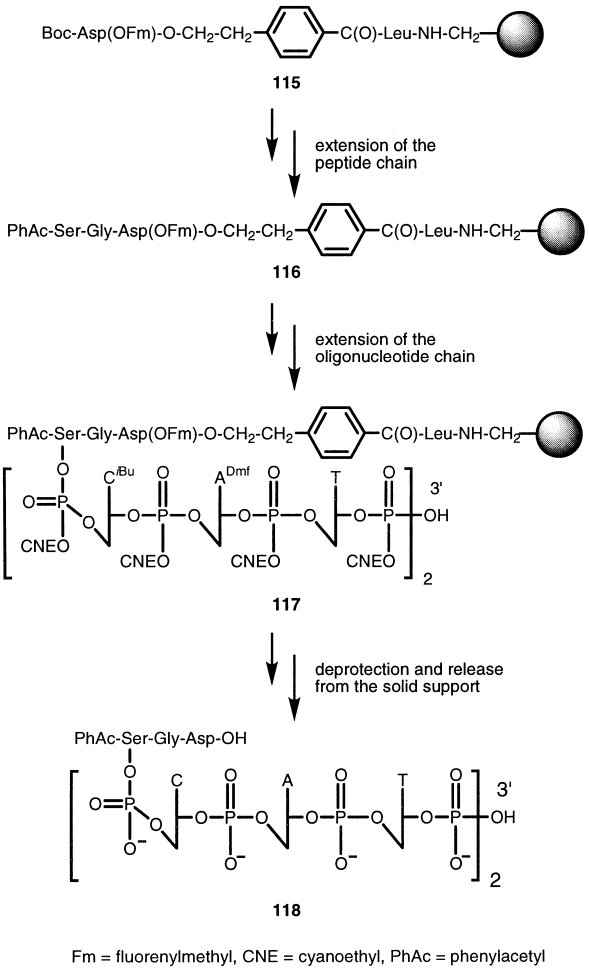


Scheme 25. Solid-phase synthesis of nucleopeptides by linking a preformed peptide to an oligonucleotide.

33%. Unfortunately, in neither case was it reported whether β-elimination occurred under the conditions used for cleavage.

For the synthesis of nucleopeptides in solution, an effective strategy has been realized whereby the trichloro-Boc group (TcBoc) was utilized for the protection of the amino functions of the nucleobases and for the N-terminal amino acids, and the C-terminus was protected as the phenacyl ester (Pac) (Fig. 11).[129] In a convergent synthesis, a tripeptide was linked with a dinucleotide to give the nucleopeptide **119**; the protective functions mentioned above were removed in one step without β-elimination.

Unlike the convergent syntheses outlined above, the stepwise flexible synthesis of nucleopeptides in solution was considered to be particularly challenging, due to lack of orthogonality of the protective groups. However, by a combination of classical chemical methods coupled with enzymatic protective group techniques, access to the desired target compounds can be achieved (Scheme 27).[130–132] A representative example of this strategy applied to the synthesis of nucleopeptides is illustrated in Scheme 27. The nucleoamino acid **120** contains several orthogonally stable blocking groups



Scheme 26. Solid-phase synthesis of nucleopeptides by successive extension of the peptide and nucleotide chains.

that can on the one hand tolerate the conditions of peptide and nucleotide synthesis, and on the other hand can be removed under very mild conditions selectively, without attack on the peptide–oligonucleotide linkage. Compound **120** was recently applied in the synthesis of a series of nucleotripeptides **125** (Scheme 27). In this work the C-terminal function was removed selectively by saponification with the enzyme papain from *Carica papaya*, at pH 6.6 and 37°C. After elongation of the peptide chain with amino acid or peptide methoxy (ME), methoxyethoxyethyl (MEE) **122**[83] or choline (Cho) esters **123**, once more the C-terminal ester blocking function could be removed smoothly. Thus, the methyl and methoxyethoxy esters could be cleaved off by treatment with the lipase from *Aspergillus niger*, whereas the choline ester group is selectively attacked by butyrylcholine esterase from horse serum (Scheme 27). The nucleoamino acids and peptides once built up could then be fully deprotected. To this end, the enzyme labile *N*-phenylacetyl (PhAc) group, which was employed to mask the amino functions of the nucleobases, was removed by treatment with penicillin G acylase from *Escherichia coli,* the *O*-acetate present in the deoxyribose was saponified by means of lipase from wheat germ or hydrazine and finally the allyl protecting groups present were cleaved by Pd(0)-mediated allyl transfer to phenyl silane as an allyl accepting nucleophile, thereby yielding compounds **127**.[130–132] This possibility for enzymatic deblocking of nucleobases is not limited to mononucleotides. For example, the PhAc-protected oligonucleotides **128** and **131** were synthesized on a solid carrier and cleaved off without deblocking the nucleobases (Scheme 28).[133] Release of all PhAc groups from the penta- and hexadecanucleotides was achieved by penicillin G acylase treatment at pH 7 and room temperature. Furthermore, the enzyme even deblocked solid phase-anchored oligonucleotides, such as **128**, on the carrier (Scheme 28) without attacking the linker groups. This finding is of particular interest considering the possible application of biocatalysts in combinatorial synthesis on polymeric carriers.[134] Synthetic nucleopeptides have not yet been used for biological studies.

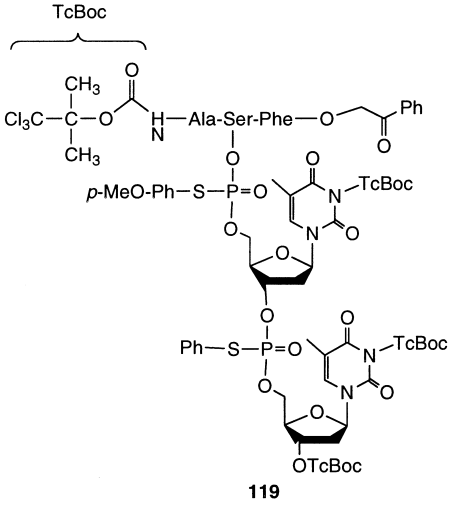
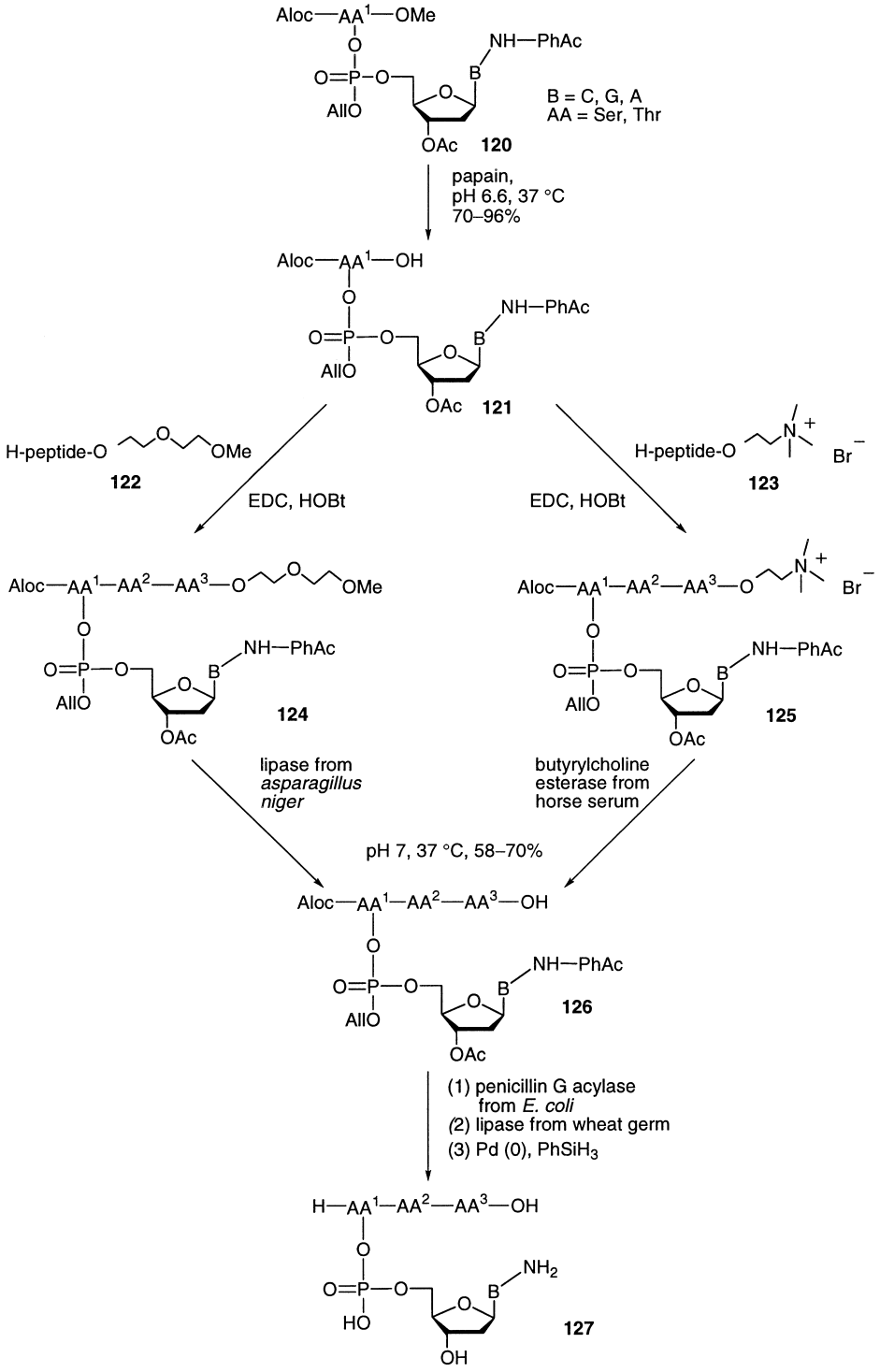


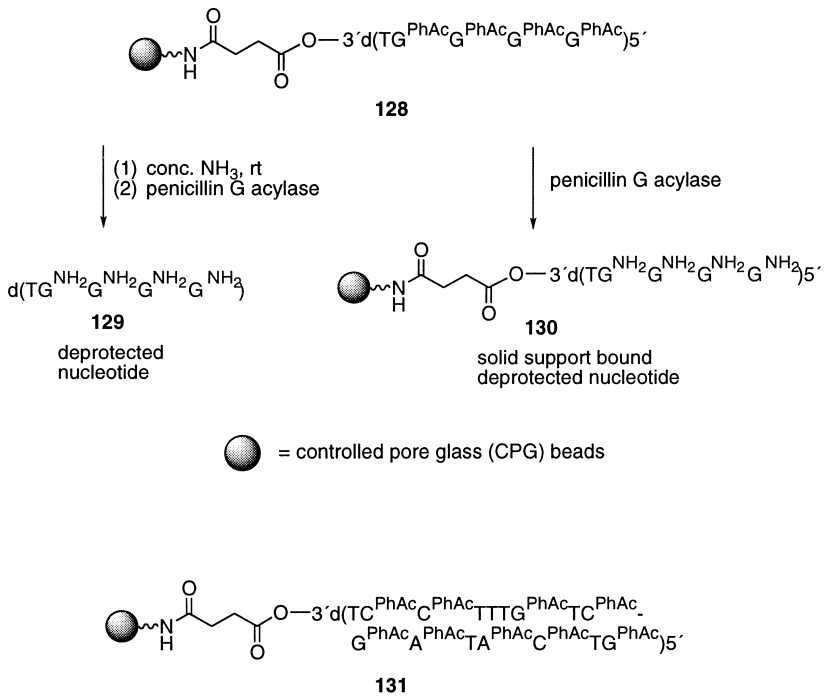
Figure 11. Protective group combinations for synthesis of nucleopeptides in solution.

Conclusions and Outlook

The examples described in the previous sections of successful interlocking of organic chemical and biological work in investigation and influence of biological signal transduction are an impressive demonstration of the capabilities of interdisciplinary research in the field of bioorganic chemistry. 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 that 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.



Scheme 27. Nucleopeptide synthesis using enzyme-labile protective groups.



Scheme 28. Enzymatic deblocking of the amino groups of oligneucleotides in solution and on solid phase.

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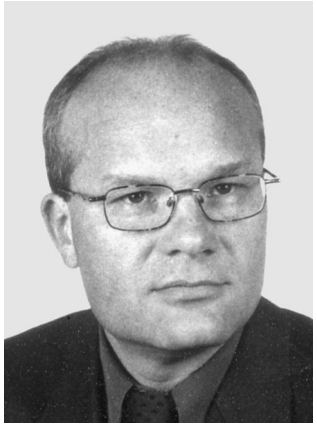
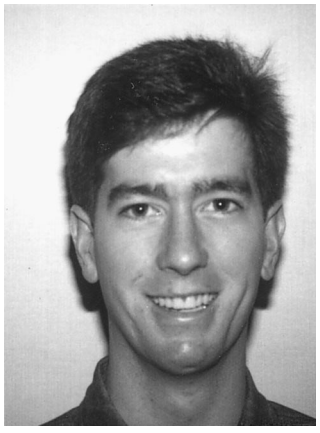
Bibliographical Sketch

Frank Eisele was born in Karlsruhe in 1970. He studied chemistry at the Universität Karlsruhe and received his diploma degree in 1996. He joined the research group of Professor H. Waldmann in 1996 and in his graduate studies he is concentrating on the development of chemoenzymatic techniques for the synthesis of peptide conjugates, in particular complex lipopeptides, which are relevant in studies of signal transduction processes and viral infection of cells.



Herbert Waldmann was born in Neuwied in 1957. He studied chemistry at the Universität Mainz and received his Dr. rer. nat. in 1985 under the guidance of Horst Kunz. After postdoctoral studies from 1985 to 1986 with George Whitesides at Harvard University he returned to the University of Mainz and received his habilitation in 1991. After holding a professorship at the Rheinische Friedrich-Wilhelms-Universität Bonn from 1991 to 1993, he moved to the Universität Karlsruhe where he is now Full Professor of Organic Chemistry. Herbert Waldmann has been the recipient of the Friedrich Weygand award for the advancement of peptide chemistry and the Carl Duisberg award from the Gesellschaft Deutscher Chemiker. His current research interests include bioorganic chemistry, in particular the synthesis and biological evaluation of peptide conjugates and natural products that are involved in biological signal transduction processes as well as biocatalysis, stereoselective synthesis, peptide-, carbohydrate- and alkaloid chemistry and combinatorial chemistry.



David J. Owen was born in 1970 in Brisbane, Australia. After completing his Bachelor of Science degree with first class honors, at the University of Queensland in 1991, he then moved to the Australian National University, and undertook a Ph. D. under the guidance of Professor Lewis Mander on the partial synthesis of gibberellins. With the completion of his Ph. D. in 1995, he then undertook postdoctoral studies with Professor C. Dale Poulter at the University of Utah from 1995 to 1997. In 1997 he received a Humboldt fellowship and is presently working within the research group of Professor H. Waldmann, on the synthesis of fluorescently labeled lipopeptides and their application in studies on biological signal transduction.