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Synthetic peptide conjugates—tailor-made probes for the biology of protein modification and protein processing

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1. Introduction

The development of methods that allow the chemical synthesis of peptides and small proteins is of utmost importance for the advancement of life science-oriented enterprises. Today, peptide synthesis has reached a level of maturity that gives non-chemists the opportunity to readily synthesize well-defined materials suitable for a systematic evaluation of structure–activity relationships. The degree of complexity that can be realized by routine synthesis, however, by no means matches that of naturally occurring proteins. The majority of proteins are post-translationally modified, reflecting the subtle mechanisms by which protein function can be regulated. Of the many types of protein modification possible, this review will focus on the synthesis and use of two very common methods, namely the

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attachment of carbohydrate and lipid groups to amino acid side chains. It will be demonstrated that with the help of these synthetic peptide conjugates, the effect of glycosylation and lipidation on protein structure, localization and function can be studied in molecular detail.

Synthetic peptide conjugates are of high utility not only for analysing but also for influencing biological processes. For example, the attachment of pharmacophoric groups to peptides furnishes conjugates that are invaluable tools for modern cell biology. This feature will be illustrated by describing how these peptide conjugates have helped in unravelling the physiological roles of proteasome-mediated protein degradation and of caspase-mediated proteolysis during apoptosis.

2. Glycopeptides

The most abundant post-translational modification is protein glycosylation, which introduces an enormous structural diversity to proteins. By the attachment of glycans protein structure and activity can be regulated.[1] Glycoproteins are involved in biological recognition events such as cell adhesion, cell differentiation and infection.[2–5] Aberrant glycosylation is associated with various conditions such as autoimmune and infectious diseases and cancer. Since glycoproteins exist in various glycoforms, the isolation of well-defined glycopeptides from natural sources is extremely difficult. In addition, recombinant proteins that are synthesized by cells in a necessary but artificial environment might display an altered glycosylation pattern. Chemical synthesis, however, is able to provide a homogeneous material.

2.1. The glycosidic linkage

Almost all of the naturally occurring glycosidic linkages can be classified into the *N*-glycosides, which are attached to the side chain amide of asparagine, and the more diverse *O*-glycosides, which are linked to the side chains of hydroxyl group-containing amino acids. This review will focus on *O*-glycosylation which is ideally suited to demonstrate that post-translational modifications can increase the chemical complexity by both an altered chemical reactivity and a high structural diversity. The core fragment that is most commonly displayed in *O*-glycopeptides, is the α-D-GalNAc residue attached to serine and threonine. A variety of different tissue-specific glycosyltranferases act upon the α-D-GalNAc bridgehead, leading to a diverse set of so-called mucin-type *O*-glycosides. Mucins are excessively *O*-glycosylated proteins that are expressed on the cell surface of various epithelial cell types.[6] These proteins constitute one important class of tumour-associated antigens and hold much promise as potential targets for tumour therapy (Fig. 1).

The β-*O*-glycosidic attachment of D-*N*-acetylglucosamine (GlcNAc)[7] to serine is found on nuclear pore proteins, transcription factors and cytoskeletal proteins and seems to be involved in transcriptional regulation analogous to phosphorylation.[8,9] Structural proteins such as collagens contain hydroxylysine and frequently are *O*-glycosylated by β-D-

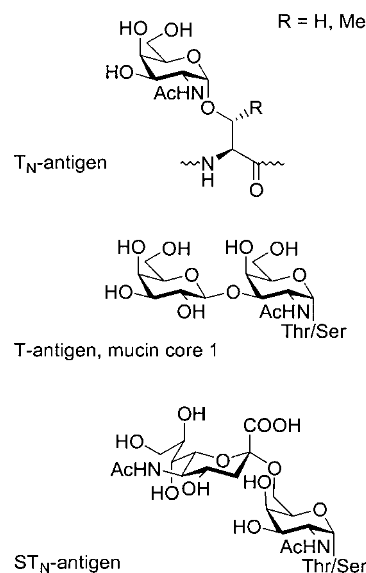


Figure 1. Selected examples of *O*-glycosidically attached oligosaccharides that are found on mucin-type peptides.

galactose or Glcα1→2Galβ1 moieties.[10,11] Interestingly, β-D-Gal-containing peptide fragments of type II collagen seem to induce T-cells mediating rheumatoid arthritis in a mouse model (Fig. 2).

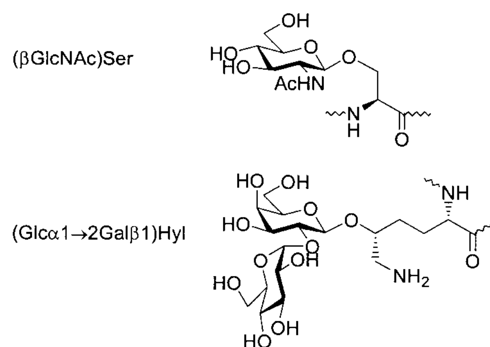
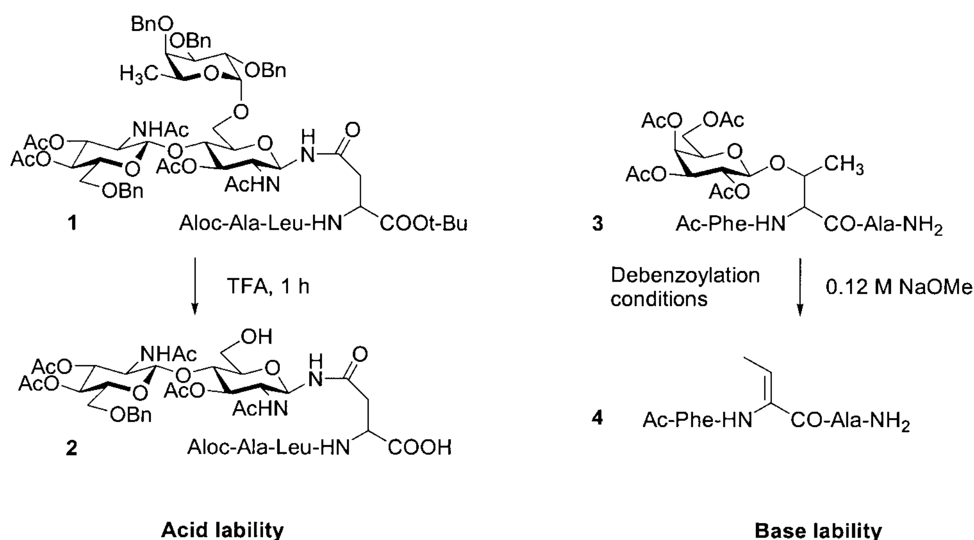


Figure 2. A serine-containing β-*O*-linked GlcNAc moiety is found on nuclear pore proteins, transcription factors and cytoskeletal proteins. The Glcα1→2Galβ disaccharide is found *O*-glycosidically attached to hydroxylysine in collagen and the phenolic hydroxyl group of tyrosine in glycogenine occurs in a glycosylated form.

2.2. Acid and base lability of *O*-glycopeptides

In developing a methodology for glycopeptide synthesis, the additional complexity and lability conferred by the carbohydrate group must be considered. Glycopeptide synthesis therefore presents a synthetic challenge, particularly with respect to protecting group chemistry, which has to allow for selective removal of these groups without harming the acid- and base-labile glycoconjugates. In a well-documented example, acidolysis of the glycopeptide *t*-butyl ester **1** was plagued by undesirable cleavage of the fucosidic linkage (Scheme 1).[12] Fortunately, however, acetylation of the trisaccharide rendered the fucoside less labile. Global acetyl protection of the glycan hydroxyl groups is now a standard technique in solid phase glycopeptide synthesis. The glycosidic linkages of common carbohydrates such as GalNAc, GlcNAc, Gal, Glc and



Scheme 1. Acid lability of the α-fucosidic linkage and β-elimination of *O*-linked glycans under basic conditions.

Man, particularly when they are acylated, survive short treatment with TFA used for removal of side chain protecting groups in solid phase synthesis. The stability of *O*-glycosidic bonds, however, depends on several parameters such as the amount of scavenger used and the structure of the glycopeptide.[13] The number of possible protecting groups is further limited by the base lability of the *O*-glycosidic linkage. Under basic conditions, typically a 0.12M solution of sodium methoxid in methanol, which are normally used for the removal of carbohydrate *O*-benzoyl groups, abstraction of the serine or threonine α-proton, e.g. in **3**, can induce β-elimination of the carbohydrate to furnish the dehydro alanine **4** (Scheme 1).[14] The removal of *O*-acetyl protecting groups proceeds smoothly, however, when a highly diluted solution of sodium methoxide is used (vide infra). Morpholine or piperidine are not basic enough to promote β-elimination and application of the well-established and probably most versatile Fmoc strategy is therefore feasible.[15]

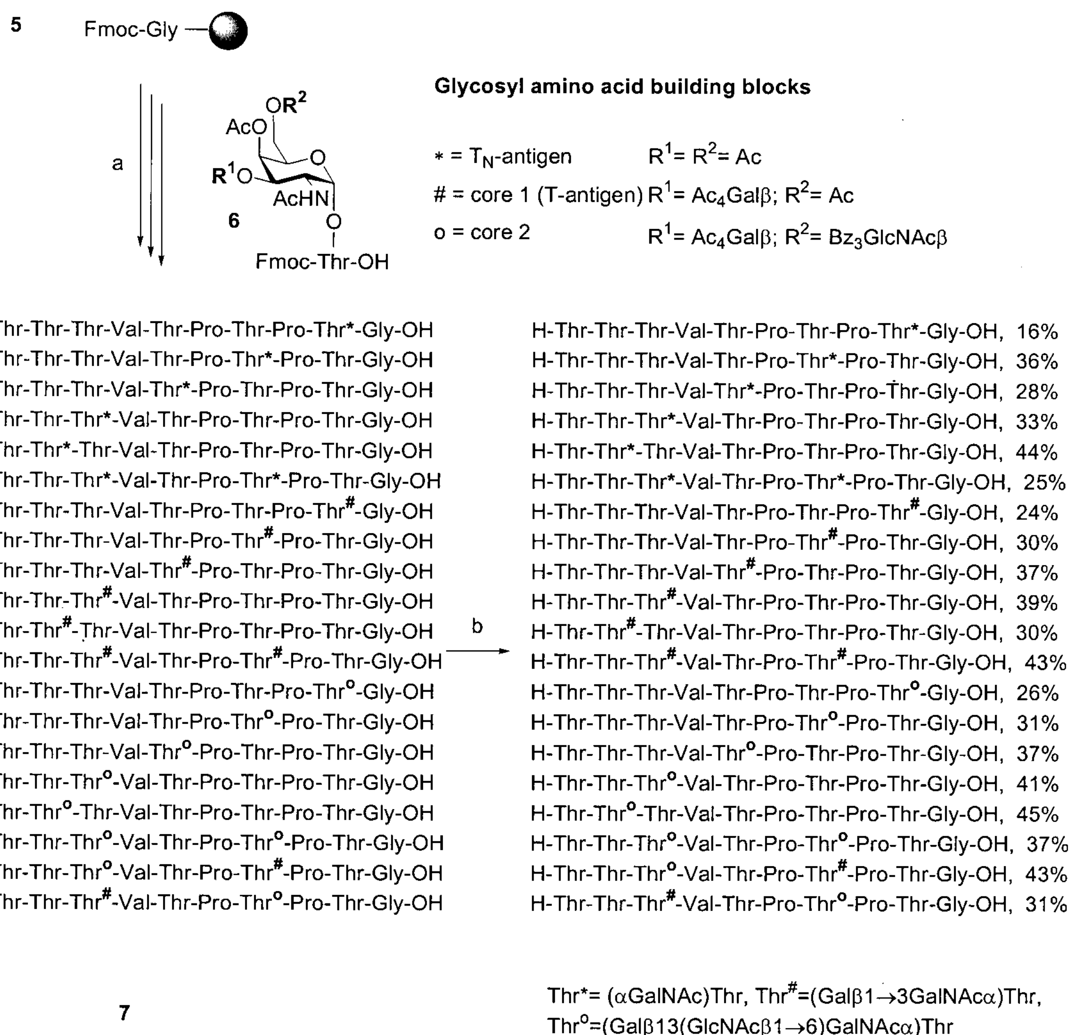
2.3. Solid phase synthesis of *O*-glycopeptides

The synthesis of glycopeptides[16–20] in solution has been successfully carried out, but the repetitive isolation of the intermediates renders this approach rather cumbersome. Solid phase synthesis, however, offers the opportunity to automate the repetitive process.[21–24] In addition, the use of a large excess of the building blocks can drive peptide couplings to completion, which is sometimes difficult to achieve in solution-based approaches. As a result, glycopeptides often can be synthesized in higher yields in the solid phase than in solution. Even more important, however, is the high speed of automated solid phase synthesis and the possibility of readily implementing parallel or combinatorial synthesis formats.[25,26]

The crucial step in any synthesis of a glycopeptide is the introduction of the carbohydrate group. The carbohydrate can be conjugated to a full-length peptide, although stereoselective *O*-glycosylation reactions are difficult to achieve with complex glycosyl donors or acceptors. Preformed glycosyl amino acid building blocks are most commonly employed in the stepwise assembly of the peptide backbone. In this simple yet very efficient approach the correctly protected glycosyl amino acid is coupled in the same way as a simple amino acid. Thus, with a few alterations of the protecting group chemistry, the protocols of modern peptide synthesizers can be used. Access to the desired glycosyl amino acid is, however, required which in most cases is not commercially available. Several recent reviews have focused on the construction of glycosyl amino acid linkages to which the reader is referred.[27,28]

The large abundance of α-*O*-linked GalNAc moieties in *O*-glycopeptides stimulated many research groups to devise solid phase synthesis schemes. The groups of Bock, Meldal and Paulsen have contributed significantly to this field as they recognized the potential of solid phase synthesis and devised parallel and combinatorial synthesis formats. In the synthesis of MUC2 and MUC3 peptides containing oligosaccharides with T_N-antigen, core 1 (T-antigen), core 2, core 3, core 4 and core 6 structure, preformed *O*-acyl protected glycosyl amino acid building blocks were employed in the preparation of 45 differently glycosylated decamers (Scheme 2).[29] For example, the synthesis of the T_N-antigen, core 1 (T-antigen) and core 2 containing MUC2 decamers **8** was performed in parallel on a manual 20-column peptide synthesizer. The Wang resin was chosen as the solid support. TBTU/HOBt activation was performed for the core 1 and the core 2 building blocks (1.5 equiv.) and pentafluorophenyl (Pfp) esters with addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) for the non-glycosylated Fmoc-amino acids **6**. After the TFA cleavage, the glycopeptides **7** were treated with dilute sodium methoxid in methanol to remove the *O*-acetyl and *O*-benzoyl protecting groups.

The conditions required for the removal of *O*-benzoyl protecting groups can give rise to β-elimination and epimerization (Scheme 1). The use of peracetylated building blocks allows the application of milder conditions. Kihlberg and co-workers[30] and Liebe and Kunz[31] independently reported the incorporation of *O*-acetyl protected *O*-sialyl-T_N threonine building blocks. Liebe and Kunz

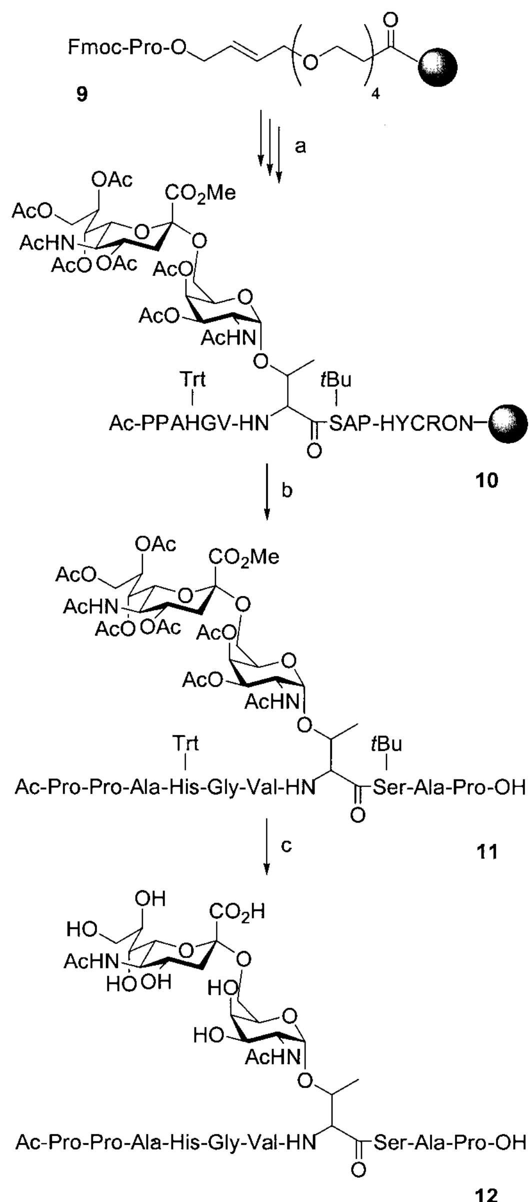


Scheme 2. (a) *i* 20% piperidine, DMF; *ii* 3 equiv. Fmoc-AA-Pfp, Dhbt-OH, DMF (or 1.5 equiv. glycosyl amino acid, TBTU, DIPEA, DMF); cleavage: TFA/H_2O (95:5); (b) NaOMe, MeOH.

employed the HYCRON linker in their synthesis of a sialyl-T_N-containing MUC1 undecamer (Scheme 3). The HYCRON linker had already been reported to enable a highly efficient synthesis of mucine-type *O*-glycopeptides with an overall yield of 95%.[32] The Fmoc-proline derivatized HYCRON-polystyrene resin **9** was extended using TBTU/HOBt activation and the less basic DMF/morpholine for the removal of the Fmoc groups rather than DMF/piperidine. The release of the protected glycopeptide **11** was accomplished by applying a Pd^0-catalysed allyl transfer to the nucleophile morpholine. Subsequent treatment with TFA and aqueous sodium hydroxide removed the protecting groups to give the desired sialyl-T_N-glycopeptide **12**. The high orthogonality of allyl-type linkers is of particular value when protecting group manipulations have to be performed on a solid phase (cf. Scheme 7). It is likewise of high versatility when protected glycopeptide fragments are to be used in solid phase fragment condensations.[33]

Although acetylation is by far the most popular and usually the most versatile means of protecting the carbohydrate hydroxyl groups and stabilizing the glycosidic bonds, there are cases in which side reactions have been reported. On condition that the coupling of the *O*-acetylated glycosyl amino acid to the resin bound peptide is unusually slow an *O*→*N*-acetyl shift might irreversibly block the nucleophile.[30] The basic conditions needed for the *O*-deacetylation can cause cysteine-induced degradations[15] and, if hydrazine is used, hydrazide formation of Asn-residues.[34] β-Elimination as well as epimerization can be problematic if the *O*-deacetylation conditions are not carefully adjusted.

Nakahara et al. favoured *O*-benzyl protection for the synthesis of the B-chain of the α2HS-glycoprotein.[35] *O*-Benzyl groups, however, are difficult to remove in the presence of cysteine and methionine residues. In addition, since *O*-benzylation is used in carbohydrate chemistry to prepare armed/activated glycosyl donors and acceptors it inevitably enhances the acid lability of glycosidic bonds. A possible solution to this problem is the use of acid-labile carbohydrate protecting groups. Christiansen-Brams et al. proposed *O*-TMS protection, which is unfortunately too labile to be of general utility.[36] Kihlberg's group has pioneered the use of hindered silyl-type protecting groups



Scheme 3. (a) *i* 50% morpholine, DMF; *ii* Fmoc-AA-OH, TBTU, HOBt, NMM, DMF; (b) $[Pd(PPh_3)_4]$, morpholine, DMF/DMSO (1:1), 42%; (c) *i* TFA, PhOMe, EtSMe; *ii* NaOH, MeOH, 76%.

such as TBDMS- and TBDPS-ether as well as protection through 4-methoxybenzyl ethers.[37] For the solid phase synthesis of glycopeptides derived from type II collagen, the disaccharide-5-hydroxynorvaline conjugate was incorporated into an Fmoc-based protocol (Scheme 4). Starting from the Wang-like hydroxymethylphenoxy-modified TentaGel resin **13**, the Fmoc-amino acids were coupled using DIC/HOBt-activation. The glycosyl amino acid was activated as a hydroxy-azabenzotriazole ester using only 1 equiv. of the building block. After the TFA-cleavage, the desired *O*-disaccharide peptide conjugate **15** was obtained in 45% overall yield. One advantage of this protecting group pattern is that the carbohydrate as well as the peptide side chains are liberated during the TFA-induced cleavage from the resin.

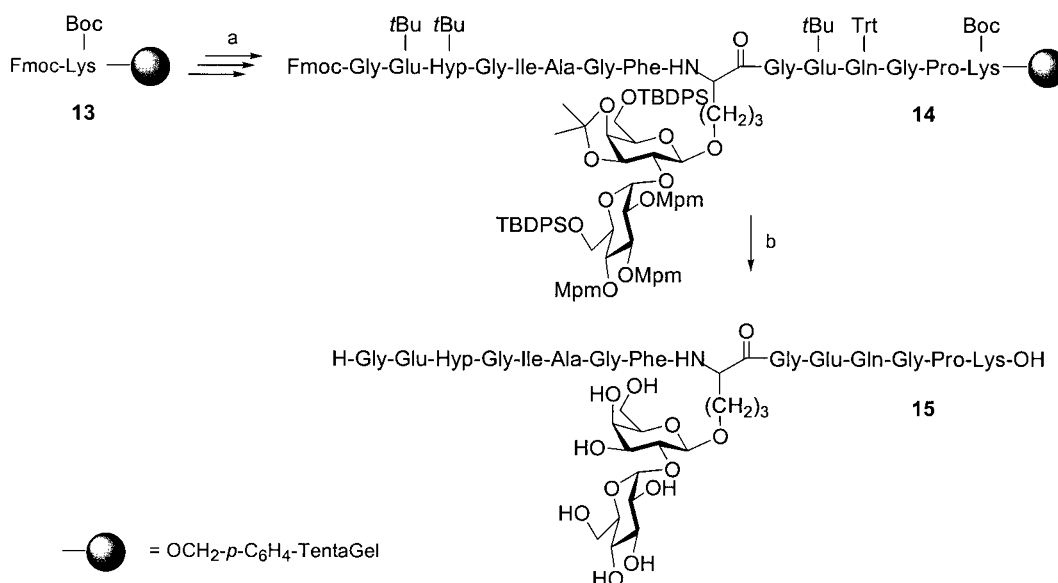
In their quest toward strategies that minimize the number of steps for the building block preparation, Bock, Meldal and Paulsen have synthesized glycopeptides containing *O*-linked 2-azido-2-deoxy-galactosyl residues.[38] The conversion of the 2-azido into a 2-acetamido group was performed on the resin-bound glycopeptides **16** rather than on the building block (Scheme 5).[39] The progress of this conversion was monitored by IR spectroscopy by following the disappearance of the azide stretch. The subsequent TFA-cleavage released the *O*-acetylated glycopeptides **18**. Finally, sodium methoxid-catalysed transacetylation liberated the glycopeptides such as **19** in overall yields ranging from 10 to 57%. Unfortunately, in all syntheses a by-product was formed in 10–15% yield. The reduction of the azide group with thioacetic acid, which was carried out for 2–8 days, led to the formation of thioacetamides, which were sometimes difficult to remove.

A similar strategy has been applied to the solid phase synthesis of glycopeptides containing β-*O*-linked *N*-acetylglucosamine. The 2-acetamido group, which is responsible for the notoriously low yields in glycosylation reactions employing GlcNAc-donors, was replaced by the *N*-dithiasuccinylimido (Dts)[40] group. In a multiple-column synthesis, the use of the building blocks **20** and **21** was compared (Scheme 6).[41] After coupling of the *N*-Dts-protected building block **20**, removal of the Dts group was accomplished by treatment with dithiothreitol in the presence of diisopropylethylamine (DIPEA) followed by *N*-acetylation. Although the synthesis of building block **20** is more straightforward, its incorporation resulted in lower yields compared to the use of the building block **21**.

A convergent route, which would utilize resin-bound glycopeptides as acceptors for on-resin glycosylation reactions, would omit the need to synthesize complex glycosyl amino acid building blocks in solution. Although progress has been made in this area,[42] the necessity of differentiating between numerous protecting groups of the supported glycopeptide complicates the synthesis of complex oligosaccharides.

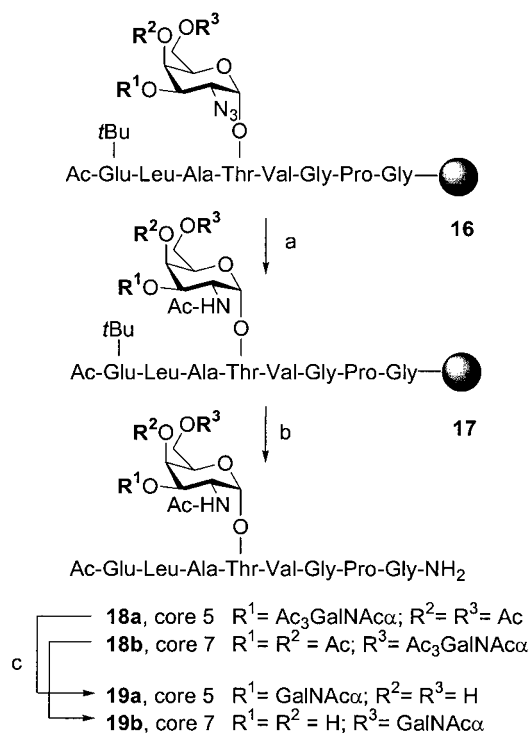
Glycosyltransferases have been shown to produce selective glycosylation reactions in the absence of protecting groups.[43] The application of glycosyltransferases in solid phase synthesis, however, requires a linkage permitting the removal of protecting groups without detaching the supported substrates. The HYCRON linker, which enables both the application of the Boc and the Fmoc strategy, satisfies these demanding properties as demonstrated by the chemoenzymatic solid phase synthesis of glycopeptides containing the SLe^X-tetrasaccharide β-glycosidically linked to threonine residues.[44] In order to allow the use of both aqueous and organic solvents CPG **22** was used as the solid support. The synthesis was performed following the usual Fmoc protocol (Scheme 7). For the removal of all side chain protecting groups, the supported glycopeptide **23** was treated with TFA and the unprotected glycopeptide **24** was then subjected to enzymatic galactosyl and sialyl transfer reactions as pioneered by Schuster et al.[45] The glycoconjugate **26** was released under mild conditions, taking advantage of the Pd^0-catalysed cleavage of the allylic linkage.

A direct glycosylation of peptidic hydroxyl groups would eliminate the bottleneck of glycosyl amino acid synthesis



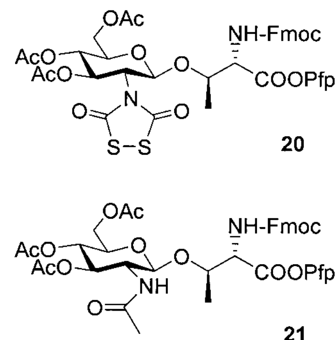
Scheme 4. (a) *i* 20% piperidine, DMF; *ii* Fmoc-AA-OH, DIC, HOBt, DMF (glycosyl amino acid, DIC, HOAt, DMF); (b) TFA/H_2O/PhSMe/ethanedithiol (87.5:5:5:2.5).

and would facilitate access to highly diverse *O*-glycopeptides with variable peptide and carbohydrate structures. Until recently, most efforts were plagued by low yields in the *O*-glycosylation of resin-bound peptide acceptors.[32,46,47] As a recent study shows, the low yields could be due to an effect of the resin.[48] An inert resin, the POEPOP resin, was prepared by anionic polymerization of mono- and bis-epoxypolyethylene glycol, thereby avoiding the presence of amide bonds which could possibly act as scavengers.[49] The supported pentapeptide **28** was subjected to the glycosylation reaction employing five different trichloroacetimidate-activated donors **29a,b**, **32a,b** and **34** (Scheme 8). The yields of the glycopeptides **30a,b**, **33** and **35** obtained after TFA cleavage were reported to be 41–78% relative to the yield determined for the synthesis of the acceptor peptide.

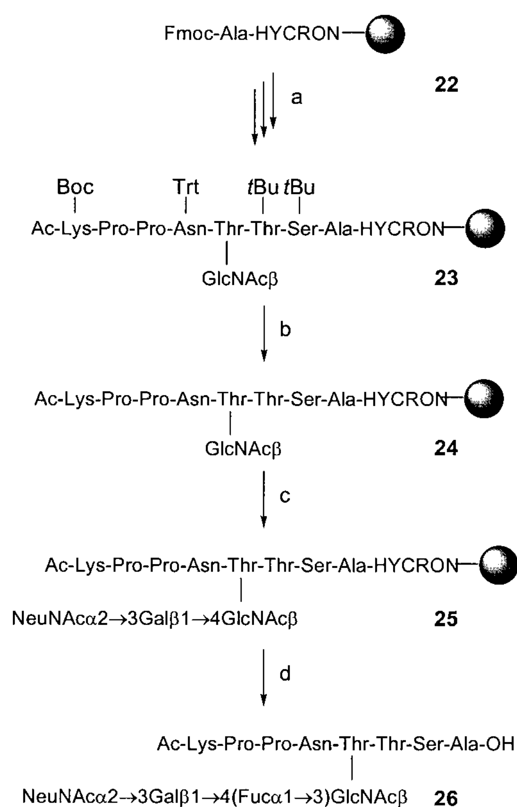


Scheme 5. (a) CH_3COSH; (b) TFA/H_2O (95:5); (c) NaOMe, MeOH, 41% core 5, 42% core 7.

A high degree of convergence with respect to the peptide group is provided by the on-resin condensation of peptide fragments. Fragment condensations can be performed on both the N- and the C-termini when the glycopeptide is connected through the carbohydrate or the peptide side chain to the solid support. Nakahara and co-workers have linked the primary 6-hydroxyl group of an Fmoc/All-protected T-antigen-threonine conjugate to a silyl tether.[50] Removal of a C-terminal allyl group furnished a segment which was first C-terminally and then N-terminally elongated to afford a T-antigen-peptide cluster. Lampe et al. employed an acetal-type linkage to attach a fucosyl threonine via the 3- and 4-hydroxyl groups of fucose.[51] Further elaboration on both the *C*- and the N-terminal end was demonstrated as well as mild acid cleavage to release fucopeptides as SLe^X mimetics.



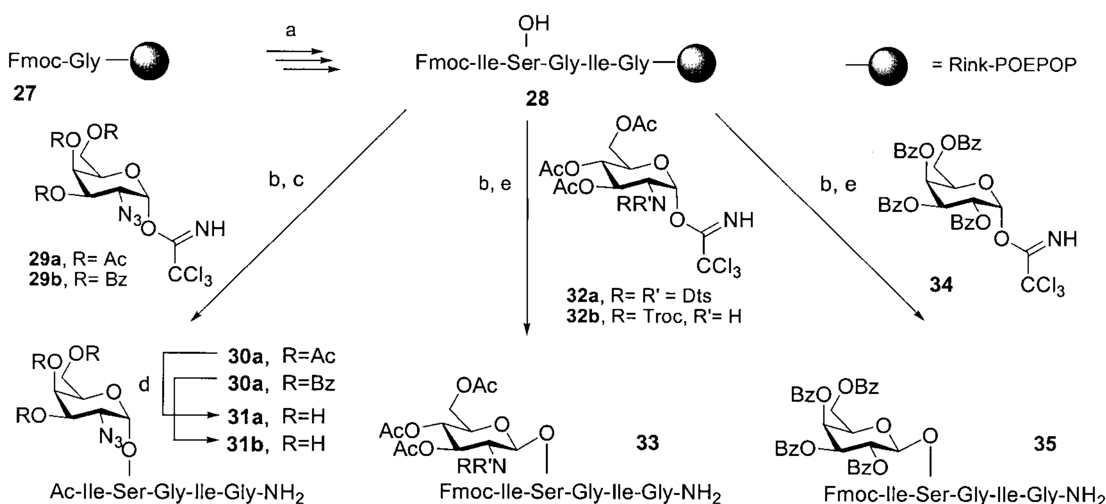
Scheme 6. Building blocks used for the evaluation of on-resin deprotection.



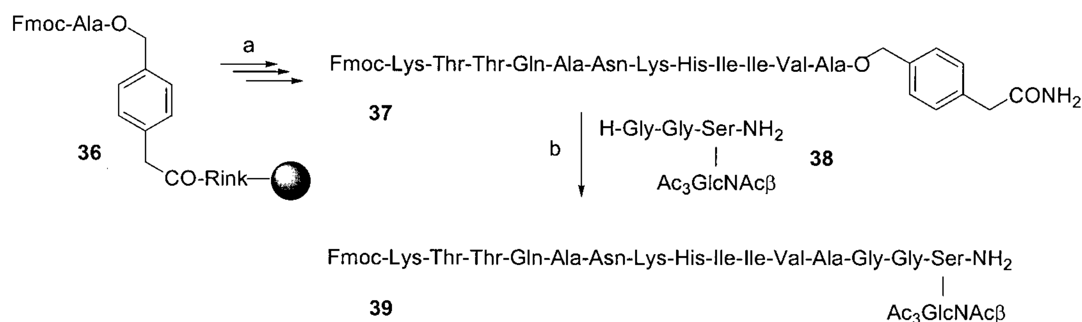
Scheme 7. (a) *i* 50% morpholine, DMF; *ii* Fmoc-AA-OH, HBTU, HOBt, NMM, DMF; *iii* *N*-acetylation: AcOH, HBTU, NMM, HOBt, DMF; (b) TFA/H_2O/ethanedithiol (40:1:1); (c) *i* UDP-Gal, β-1,4-GalTase, 50mM HEPES (pH 7), 5mM $MnCl_2$, 37°C; *ii* CMP-NeuNAc, α-2,3-sialyltransferase, 0.1M HEPES (pH 7), 5mM $MnCl_2$, alk. phosphatase, 37°C; (d) *i* $Pd(PPh_3)_4]$, morpholine, DMF, DMSO, 9%; *ii* α-1,3-FucTase, GDP-Fuc, 0.1M HEPES (pH 7), 37°C, 59%.

Chemical fragment condensations often suffer from the poor solubility of the protected peptide moieties and are prone to racemization at the C-terminus of the acyl donor. In contrast, enzyme-catalysed fragment condensations are free of racemization and use side chain unprotected substrates, increasing the solubility of the peptide fragments. Witte et al. have reported the solid phase synthesis of peptide and glycopeptide esters for subsequent use in enzyme-catalysed fragment condensations (Scheme 9).[52] For the synthesis of a partial sequence of the C-terminal region of ribonuclease B, Rink resin **36** which was loaded with a conjugate of *N*-Fmoc-protected alanine and the acid- and base-stable PAM linker was subjected to a standard Fmoc-based synthesis protocol. Standard TFA-cleavage conditions removed all acid-labile side chain protecting groups and detached the *N*-Fmoc-protected peptide–PAM ester **37** from the solid support. It was essential to use a benzhydrylamine-type linker since PAM amides contrary to PAM acids can serve as acyl donors in enzyme-catalysed peptide couplings. Accordingly, the segment condensation of **37** with the N-terminally unprotected glycotripeptide **38** was achieved by catalysis using the protease subtilisin affording the glycopentadecapeptide **39** in 84% yield.

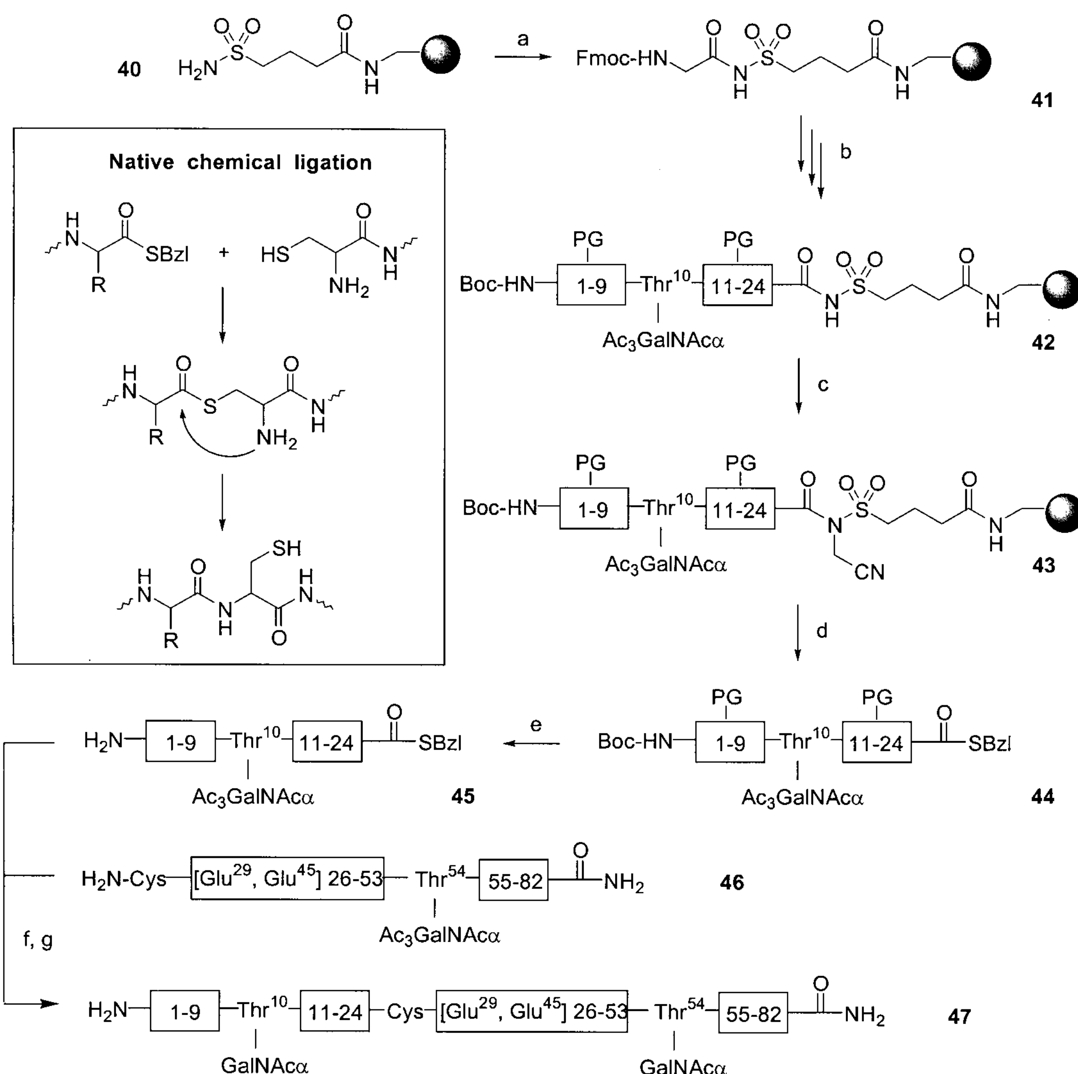
Native Chemical Ligation[53] is a technique which utilizes the selective coupling between a C-terminal peptide thioester and an N-terminal cysteine to enable a chemical condensation of unprotected peptide fragments.[54] The thioester intermediate is subject to a spontaneous intramolecular S→N acyl transfer by which the final ligation product is formed (see inset in Scheme 10). Recently, a Native Chemical Ligation was employed for the total chemical synthesis of the 82-residue glycoprotein **47** (Scheme 10).[55] Crucial for the Native Chemical Ligation is access to peptide thioesters such as **44**. The commonly used standard Fmoc/*t*-Bu strategy is not applicable, since thioesters would not survive the conditions needed for removal of the Fmoc groups. The use of Ellman and co-workers' modification of Kenner's sulfonamide linker,[56] however, allowed the post-assembly formation of the glycopeptide thioester. In this study, the N-terminal 24-mer fragment **42**, which contained one *O*-linked GalNAc moiety, was synthesized on the sulfonamide-modified resin **40** according to Fmoc/*t*-Bu strategy. Thiolytic cleavage of the acid- and base-stable sulfonamide linker commenced with iodoacetonitrile alkylation of the acidic sulfonamide **42**. The resulting tertiary sulfonamide **43** was then cleaved by thiolysis with a large excess of benzyl mercaptan to release the glycopeptide thioester **44**. Subsequent removal of the acid-sensitive protecting groups completed the synthesis of the acyl donor **45**. Using Native



Scheme 8. (a) Standard Fmoc-SPPS; (b) TMSOTf, CH_2Cl_2; (c) *i* piperidine; *ii* Ac_2O; *iii* 95% TFA; (d) NaOMe, MeOH; (e) TFA/H_2O.



Scheme 9. (a) Standard Fmoc-SPPS, cleavage: TFA/Et_3SiH/H_2O (95:2.5:2.5), 89%; (b) subtilisin 8397 K256Y, 50mM triethanolamine/DMF (1:9), 84%.



Scheme 10. (a) Fmoc-Gly, PyBOP, DIPEA, DMF; (b) standard Fmoc-SPPS, N-terminal amino acid coupled as *N*-Boc-protected building block; (c) ICH_2CN, DIPEA, NMP; (d) BnSH, THF; (e) TFA:PhOH:H_2O:PhSMe:EDT (82.5:5:5:5:2.5), 4 h, 21% overall yield (based on resin capacity); (f) 6M Gn·HCl, 100mM NaH_2PO_4, pH 7.5, 4% PhSH, 55%; (g) 5% aq. H_2NNH_2, DTT, 53%.

Chemical Ligation, fragment **45** was coupled to the chemically synthesized C-terminal segment **46** before *O*-deacetylation afforded the fully deprotected glycoprotein 82-mer **47**. It seems reasonable to assume that this technique could provide a basis for the synthesis of a variety of biologically interesting glycoproteins having a degree of complexity that was almost impossible to achieve by previous methods.

2.4. Biological studies with synthetic glycopeptides

These recent improvements in the synthetic methodology and technology make it possible to provide access to homogeneous glycopeptides which are complex enough to study the structural and biological influences of protein glycosylation.[20,57,58] In the following sections, selected examples

will be presented in which synthetic glycopeptides have been employed to unravel the role of carbohydrate attachment in epitope mapping, in MHC binding and T-cell induction and in the immunotherapy of cancer.

2.4.1. Epitope mapping. Monoclonal antibodies directed against tumour-associated glycopeptide antigens are used in in vitro and in vivo tumour diagnostics. Although monoclonal carbohydrate-directed antibodies might display a highly specific binding event, direct elucidation of the recognized structure is complicated by the multivalent nature and the heterogeneity of the non-immunogenic part of the glycoconjugates. In order to study the binding characteristics of these diagnostically relevant carbohydrate-directed antibodies, synthetic glycopeptides are an invaluable tool.

Mucins, heavily *O*-glycosylated proteins expressed on epithelial cells, have been the subject of intense research efforts. The mucin structure is dominated by a variable number of tandem-repeat sequences. In the MUC1 mucin, these span a 20-amino acid sequence, which is highly immunogenic. Upon carcinogenesis certain glycosyl transferases are expressed in lower concentrations leading to aberrant glycosylation of the mucins.[59] Amongst the most important tumour-associated antigens are the T_N- and the sialyl-T_N-antigen, which are found in human colon cancer, ovarian cancer and breast cancer.[60–64] The T-antigen was demonstrated to be tumour specific in breast tissue.[65]

A plethora of monoclonal antibodies directed against tumour-relevant mucin structures exist. The studies of Spencer et al. revealed that, although antibodies might recognize a peptide epitope, increasing glycosylation can confer a significant enhancement of the binding affinity to the peptides.[66] Fluorescence binding quenching studies with the monoclonal antibody C595, which recognizes a PDTR epitope of the MUC1 peptide **48**, showed that the introduction of three GalNAc residues at threonines 9 and 21 and at serine 20 (**49**) increased the MUC1 peptide/antibody association constant. Interestingly, this increase appeared to coincide with a population increase of the PPII helix conformation of **49** as indicated by circular dichroism spectroscopy in cryogenic mixtures. It was therefore proposed that the PPII helix is stabilized by MUC1 glycosylation with GalNAc residues and hence the antibody's binding affinity (Fig. 3).

A similar result was obtained by Karsten et al. who examined the binding of 28 Asp-Thr-Arg (DTR)-specific anti-MUC1 antibodies to 12 synthetic MUC1 20- and 21-mers containing T- and T_N-antigens at various positions.[67] The DTR motif is a preferred target for the majority of the peptide-specific anti-MUC1 antibodies. Although previous studies suggested that this motif represents an effective target for B- and T-cells only if it is non-glycosylated, the binding and inhibition analyses performed by Karsten et al. revealed that glycosylation within this motif can enhance the binding.

^{N}TAPPAHGVT*SAPDTRPAPGS*T*APPA^C

48: S*= Ser, T*= Thr;
49: S*= (αGalNAc)Ser, T*= (αGalNAc)Thr

Figure 3. Attachment of three GalNAc residues to a 25-mer mucin peptide **48** is sufficient to increase both the population of glycopeptides **49** adopting a PPII helix conformation and the binding of the monoclonal antibody C595.[66]

The high specificities of carbohydrate-specific antibodies which were obtained by immunizing mice with human cancer cell line-derived immunogens have been compared with the specificity of antibodies generated by using synthetic clustered sialyl-T_N- and T_N-glycopeptides.[68] Binding and inhibition experiments using the synthetic peptide conjugates revealed that clinically used sialyl-T_N-reactive antibodies cross-reacted with T_N-serine clusters. The only antibody that showed a strong selectivity for the sialyl-T_N-epitope was obtained by immunization with a synthetic immunogen.

2.4.2. Glycopeptides in binding to MHC molecules and T-cell recognition. In the event of a specific immune response towards foreign substances, the immune system follows two principal pathways, namely humoral immunity, in which antibodies specifically bind to the challenging antigens, and cellular immunity, which is mediated by cells rather than molecules and employs cytolytic T-cells for recognition and lysis of antigen-charged cells. The cellular and humoral immune response both depend on the assistance of T-helper cells, the induction of high-affinity antibodies only being possible with the aid of T-helper cells. T-cells recognize antigens on the surface of accessory cells. Every cell constantly converts endogeneous proteins to small peptides by means of its proteasome (4.2). These fragments are transported into the endoplasmatic reticulum, in which the 10–20-mer peptides bind to MHC class I molecules. After transfer through the Golgi network, the peptide–MHC I complex is located on the cell surface and presented to $CD8^+$ cytolytic T-cells provided that certain residues anchor the peptide to the MHC-binding cleft. If the T-cell receptor (TCR) recognizes non-self peptides on the MHC I–peptide binding groove, a cytolytic response towards the antigen-presenting cell can be triggered. Specialized antigen-presenting cells (APC) such as macrophages internalize proteins by endocytosis. After passage through an acidic compartment these proteins are degraded and the resulting peptides are bound to MHC class II molecules. Recognition of MHC II–peptide complexes by T-helper cells is then able to induce the production of cytokines, which are necessary for B-cell activation.

Carbohydrates are known not to bind to MHC molecules, but whether glycopeptide fragments would bind to MHC molecules was not known until early this decade. An excellent review has described the data that have been collected between 1992 and 1997.[24] Recently, Jensen et al. immunized mice with the synthetic glycopeptide **53** and raised MHC class II E^k-restricted T-cell hybridomas that proliferated and secreted interleukin 2 (IL-2) upon activation with **53**.[69] The synthetic glycopeptides that were used for activation of the T-cell hybridomas differed in the glycan moiety, which was attached to a known E^k-binding peptide derived from haemoglobin. Remarkably, 19 of the 22 tested hybridomas responded only to the glycopeptide **53**. The three other clones responded to the unglycosylated peptide

50, H-Val-Ile-Thr-Ala-Phe-Asn-Glu-Gly-Leu-Lys

51, H-Val-Ile-Thr-Ala-Phe-Thr-Glu-Gly-Leu-Lys

52, H-Val-Ile-Thr-Ala-Phe-Ser-Glu-Gly-Leu-Lys

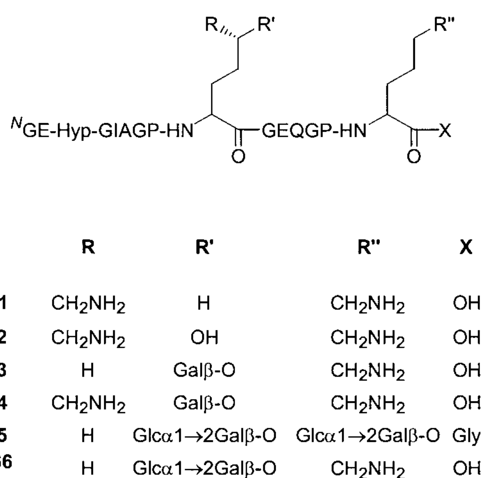
H-Val-Ile-Thr-Ala-Phe-Xxx-Glu-Gly-Leu-Lys

Xxx = (αGalNAc)Thr, **53**; (αGalNAc)Ser, **54**;
(αMan)Thr, **55**; (βGlc)Thr, **56**;
(Galβ1→3GalNAcα)Thr, **57**;
(αGlcNAc)Thr, **58**; (αGalN$_3$)Thr, **59**;
(GlcNAcβ1→6(Galβ1→3)GalNAcα)Thr, **60**

Figure 4. Selected examples of synthetic peptides and glycopeptides that have been used for the evaluation of the carbohydrate specificity of MHC class II-restricted T-cell hybridomas raised against the *O*-glycosylated self peptide **53**.[69]

51, displaying a total lack of cross-reactivity between the glycopeptide and unglycosylated peptide. Most of the hybridomas were equally activatable by the GalNAc-serine- and GalNAc-threonine-containing peptides **54** and **53**, respectively. Seventeen of 19 glycopeptide **53**-responsive hybridomas were able to distinguish between the αGalNAc-containing glycopeptides **53** and **54** and the αGlcNAc-containing glycopeptides **58**, indicating that the glycan is the entity recognized by the T-cell receptor. Since αGalNAc peptides, in which amino acids pointing to the T-cell receptor have been replaced by alanine were unable to activate the hybridomas, it was therefore concluded that both the glycan and the solvent-exposed parts of the glycan-carrying peptide were recognized by the T-cell hybridomas (Fig. 4).

T-cells were shown to recognize a glycopeptide derived from type II collagen.[70] The IL-2-producing hybridomas obtained after immunization of mice with type II collagen were incubated with peptides **61** and **62** together with the glycopeptides **63–66**. The synthetic peptides **61** and **62** activated only a few collagen-reactive T-cell hybridomas although a peptide spanning the same sequence was demonstrated to be immunodominant when obtained through



	R	R'	R''	X
61	CH$_2$NH$_2$	H	CH$_2$NH$_2$	OH
62	CH$_2$NH$_2$	OH	CH$_2$NH$_2$	OH
63	H	Galβ-O	CH$_2$NH$_2$	OH
64	CH$_2$NH$_2$	Galβ-O	CH$_2$NH$_2$	OH
65	H	Glcα1→2Galβ-O	Glcα1→2Galβ-O	Gly
66	H	Glcα1→2Galβ-O	CH$_2$NH$_2$	OH

Figure 5. Synthetic peptides and glycopeptides that were used in order to demonstrate that the majority of T-cells obtained after immunization with native type II collagen specifically recognizes glycopeptide **104** with no cross-reactivity to the unglycosylated peptides **101** and **102**.[70]

proteolytic cleavage of native collagen.[71] It was reasoned that post-translational modification of the lysine residues such as glycosylation was responsible. Indeed, the majority of the hybridomas were stimulated upon incubation with glycopeptide **64**, in which the central hydroxylysine carried the β-galactosyl moiety. The same hybridomas proved unreactive towards glycopeptides **65** and **66**, which contained the Glcα1→2Gal-disaccharide β-linked to hydroxynorvaline, but a few were cross-reactive with glycopeptide **63** containing a β-galactosylated hydroxynorvaline at an identical position. These investigations provided the first example of immunization with a natural glycopeptide eliciting carbohydrate-specific T-cells (Fig. 5).

The molecular basis of the recognition of MHC class I–glycopeptide complexes by the T-cell receptor has been suggested, based on the crystal structures of glycopeptides **68** and **69** with H-2D^b MHC.[72] Both glycopeptides were used to raise H-2D^b-restricted, carbohydrate-specific CTL clones. Interestingly, all clones that were directed against **69** showed a strong cross-reactivity to **68**.[73] In contrast, the CTL clones obtained through immunization with **68** were highly specific for **68**. The crystal structures revealed that the backbone conformations of the two glycopeptides bound by the H-2D^b groove were strikingly different. The highly cross-reactive CTL clone displayed a TCR with a short CDR3 loop to allow access to glycan **69**, which accommodated a large volume. This led to the selection of a highly promiscuous TCR, which also recognized **68**. The TCR directed against the MHC-bound **68** contained a longer CDR3 loop, which was suggested to be able to make additional contacts to the peptide residues (Fig. 6).

67, FAP-Gly-NYPAL

68, FAP-(GlcNAcβ)Ser-NYPAL

69, FAPG-(GlcNAcβ)Ser-YPAL

Figure 6. The crystal structures of the H-2D^b MHC class I molecule in a complex with peptide **67**, glycopeptide **68** or glycopeptide **69** suggested a molecular basis for the non-reciprocal pattern of cross-reactivity of cytotoxic T-cells that were obtained through immunization with **68** and **69**.[72]

2.4.3. Glycopeptides for the immunotherapy of cancer.

During carcinogenesis, the majority of cells experience a dramatic transformation of the glycosylation machinery. As a result, many proteins that are expressed on the surface of the cancer cells display an altered glycosylation pattern.[74] Only a few types of oligosaccharides are tumour-associated and for some tissue, even tumour-specific, structures. These include T$_N$-, sialyl-T$_N$- and T-antigens and Lewis-X and Lewis-A structures, which are strongly increased in expression of cancer cells. Additionally, increased β-1,6-GlcNAc-branching of *N*-linked glycans and a general increase in sialylation are commonly observed. A large body of data obtained in clinical and experimental settings revealed that the increased expression level of certain saccharides is correlated with poor prognosis. These altered glycan structures may therefore be regarded as a means of distinguishing the tumour cell from a normal cell. Vaccination with synthetic tumour antigens with the help of immunostimulatory adjuvants could target the immune system to the cancer cells. Synthetic antigens are usually of low molecular weight and are hence poorly immunogenic.

Conjugation to immunogenic carrier proteins such as KLH or BSA, however, was shown to elicit an immune response that was directed against the synthetic carbohydrate or glycopeptide hapten as well as the tumour cells.[75] Immune responses to synthetic carbohydrate-derived vaccines have until now been largely restricted to the stimulation of antibody production. High antibody titres can prevent metastasis by the eradication of circulating tumour cells. In addition, the antibody response could mediate tumour destruction by targeting the cytolytic complement system to the cancer cells.

Danishefsky's group have synthesized trimeric T_N-peptides as partial structures of mucin-related antigens (Fig. 7).[76] Immunization studies revealed that conjugation to KLH induced high IgM and moderate IgG titres in mice. The sera from mice were able to attack T_N-positive LS-C colon cancer cells as evaluated by flow cytometry assays (detection of surface-bound antibodies) and complement-mediated cytotoxicity assays (^{51}Cr-release upon complement-mediated lysis of target cells). Despite repeated booster immunizations, however, the immune response was dominated by high IgM titres and there was no evidence for an IgM to IgG class switch. Together with the lack of secondary response, this indicates a usually less effective T-cell-independent antibody response, which is characteristic of many carbohydrate antigens. A hexaglycosidic globo H-KLH-conjugate, the most complex vaccine to date that was evaluated in phase I clinical trials,[77] and a sialyl-TN-KLH-conjugate,[78,79] behaved in a similar manner.

The first successful attempt to induce a T-cell dependent immune response against carbohydrate epitopes has recently been reported.[80] The synthetic immunogen **69** was designed as a multiple antigenic T_N-glycopeptide containing a well-known T-cell epitope from the type I poliovirus (Fig. 8). Remarkably, an IL-2-secreting T-cell hybridoma cell line specific for the unglycosylated poliovirus peptide (**T** in Fig. 8) showed a strong cross-reactivity to the conjugate **69**. T-cell stimulation was achieved with a 10 000-fold lower dose compared to a construct in which the T_N-antigen was omitted. Clearly, presentation of **69** by MHC was enhanced through either a favourable intra-

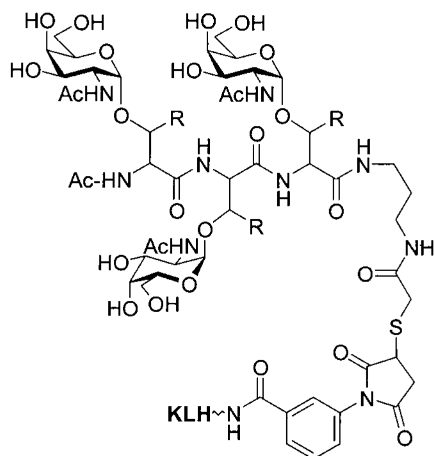
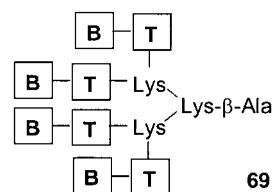


Figure 7. The mucin-derived glycopeptide–KLH conjugate contains three clustered T_N-antigens and was used in clinical trials as a putative synthetic vaccine against prostate cancer.[76]



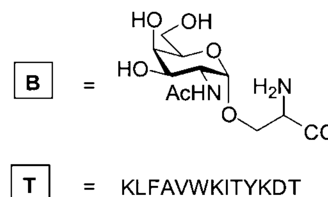


Figure 8. The fully synthetic immunogen **69** that contains a multiply antigenic T_N-glycopeptide as B-cell epitope (**B**) and a well-known T-cell epitope (**T**) was shown to elicit a T-cell-dependent immune response that increased the survival of tumour-bearing mice.[80]

cellular processing or an increased endocytosis of **69** by the antigen-presenting cells. The sera obtained from immunized mice were able to recognize the native T_N-antigen on human Jurkat T-lymphoma and LS180 adenocarcinoma cell lines. Interestingly, the humoral response was dominated by IgG antibodies supporting the notion that a T-cell-dependent response was induced. The survival of tumour-bearing BALB/c mice obtained by grafting the T_N-positive TA3/Ha tumour cell line showed a 2-fold increase after immunization with **69** when compared to the untreated control groups.

3. Lipopeptides

Two decades ago, the first prenylated polypeptide, Rhodotorucine A, the mating factor from the fungus *Rhodospiridium toruloides*, was discovered.[81] The notion that entire proteins could be post-translationally modified, however, has only recently been realized. In one of the key experiments, the use of mevalonate biosynthesis inhibitors revealed that products of mevalonate metabolism other than cholesterol were essential for cell cycle progression.[82,83] It was soon observed that a metabolite of mevalonate was incorporated into proteins.[84] The nuclear membrane-associated protein lamin B was the first of these proteins to be identified.[85,86] The structural analysis exposed a farnesyl group which was attached to a C-terminal cysteine.[87] Interest in protein lipidation then increased rapidly after it was recognized that Ras[88] and Rab[89] proteins are subject to post-translational lipidation. Both proteins belong to the Ras (Rat-adeno-sarcoma) superfamily and are critical for cell signalling regulation and for the regulation of a wide variety of intracellular vesicular trafficking pathways, respectively. The important role of lipidation is to anchor these proteins to the membrane that is primarily essential for the bioactivity. Another role of lipidation is to modulate protein–protein interaction.

Three different types of lipid groups have been found to date (Fig. 9) namely N-terminal myristoylation[90] of a glycine, *S*-prenylation[91,92] of cysteine residues at or close to the

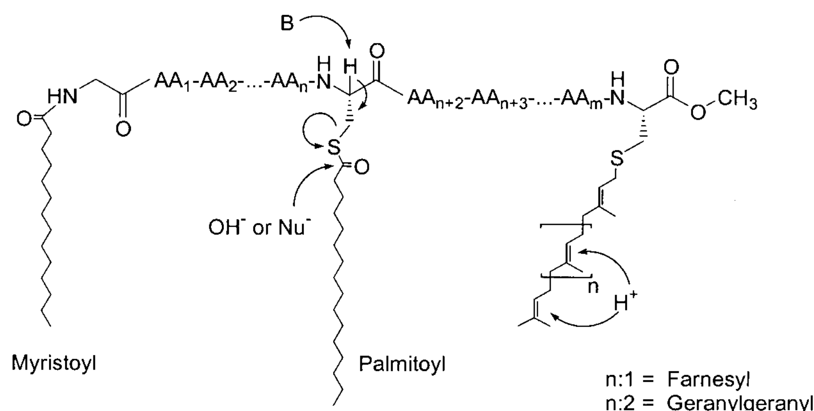


Figure 9. Lipid modifications of proteins and their chemical labilities.

C-terminus (with one of two distinct isoprenoid lipids, the 15-carbon farnesyl or the 20-carbon geranylgeranyl), and *S*-palmitoylation[90,93] of cysteines throughout proteins. In addition, a few *O*-acylated peptides or proteins have been identified.[94,95] Two of the known modifications (prenylation and myristoylation) are metabolically stable, whereas palmitoylation is a dynamic process.[96,97] Prenylated proteins can comprise up to 2% of total cellular protein[98,99] of which geranylgeranylated proteins represent 80%.[100,101]

Particularly in the case of the Ras proteins, fully and correctly lipidated proteins cannot be obtained from yeast or baculoviral expression systems. In addition, such biological techniques are not suitable for the introduction of modified lipid groups into the proteins. It is again chemical synthesis that can provide efficient tools for the further study of these regulation processes.

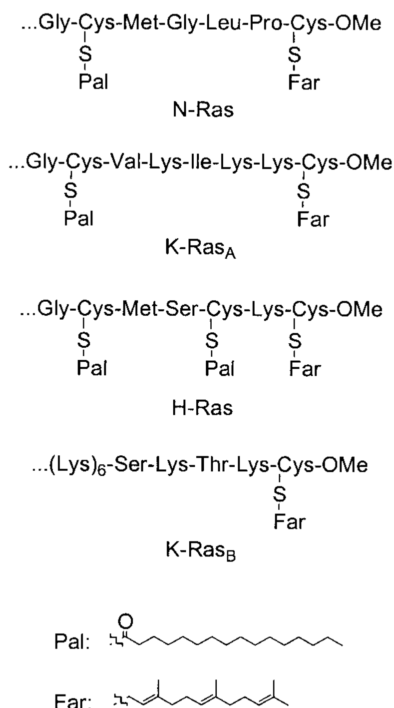


Figure 10. Structures of lipid-modified C-termini of the Ras proteins.

3.1. Ras proteins

Four isoforms of Ras exist, H-Ras, N-Ras, K-Ras$_A$ and K-Ras$_B$. All Ras isoforms terminate in a farnesylated cysteine methyl ester. In addition, fully modified N-Ras and K-Ras$_A$ are palmitoylated at a cysteine residue close to the C-terminus, whereas H-Ras is palmitoylated twice. K-Ras$_B$ is not palmitoylated, but contains a cluster of eight lysine residues near its C-terminus which is thought to confer membrane-anchoring ability by electrostatic contacts with the negatively charged phospholipids (Fig. 10).

Ras proteins act in signal transduction pathways as molecular binary switches. Ras activation involves the exchange of bound GDP for GTP. The reverse reaction, hydrolysis of the bound GTP, regenerates GDP, leading to Ras deactivation.[102–104] Ras proteins are key regulators of cell growth in all eukaryotic cells. Gene expression, apoptosis and remodelling of the actin-cytoskeleton are all controlled by Ras proteins. Mutations of Ras can therefore be highly oncogenic and it comes as no surprise that mutations of Ras are found in approximately 30% of all human cancers, reaching values of 80% for some of the major malignancies.[105–107]

Ras proteins undergo a series of post-translational modifications and Ras trafficking has recently been discussed.[108,109] This involves a cytosolic Ras precursor being farnesylated at a cysteine of a C-terminal recognition motif.[110] A specific protease cleaves a C-terminal tripeptide, leaving a prenylcysteine as the new C-terminus. After C-terminal methylation, one or two further palmitic acids can be attached via a labile thioester linkage to cysteine residues just upstream of the recognition motif. The locus of the palmitoyl transferase is discussed controversially: in the kinetic trapping model,[111,112] only palmitoylation at the plasma membrane results in an irreversible membrane insertion of the farnesylated Ras proteins, whereas in the other models, palmitoylation takes place at the endoplasmic reticulum followed by transfer of the fully lipidated proteins to the plasma membrane via the exocytic pathway.[113,114]

3.2. Rab proteins

Rab proteins belong to the Ras superfamily and play key roles in the secretory and endocytic pathways.[115] In contrast

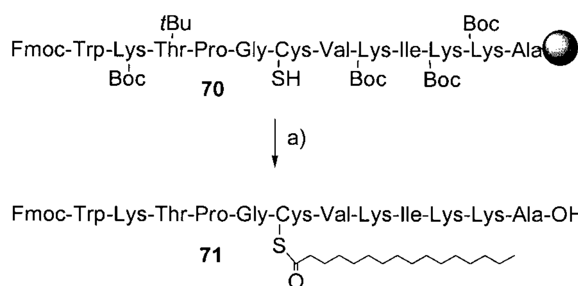
to Ras, most Rabs are doubly geranylgeranylated at a C-terminal motif.[93] Both cysteine residues are modified with geranylgeranyl moieties by geranylgeranyl transferase II (GGTase-II). This enzyme does not recognize a defined C-terminal motif and only acts on a Rab protein if it is in a complex with a special recruiting protein, the Rab escort protein (REP).[116] After prenylation, the Rab protein remains complexed to REP and is delivered to the target membrane.

The signal transduction process and the vesicular trafficking pathway are both still not fully understood. It is shown in the following sections that, for the further study of biological phenomena influenced by these or other proteins, characteristic peptides which embody the correct lipid modifications of their parent proteins, as well as analogues with varied structure or eventually entire proteins, can serve as efficient tools.[117]

3.3. Synthesis of peptides containing one lipid group

Peptides bearing only one type of lipid modification tolerate basic or acidic conditions and can be synthesized by employing established protecting group techniques. The synthesis of *N*- and *O*-acylated compounds, e.g. *N*-myristoylated or *O*-palmitoylated peptides, is possible by following standard solid phase chemistry procedures either by using an already modified building block or by on-resin acylation.[118–121]

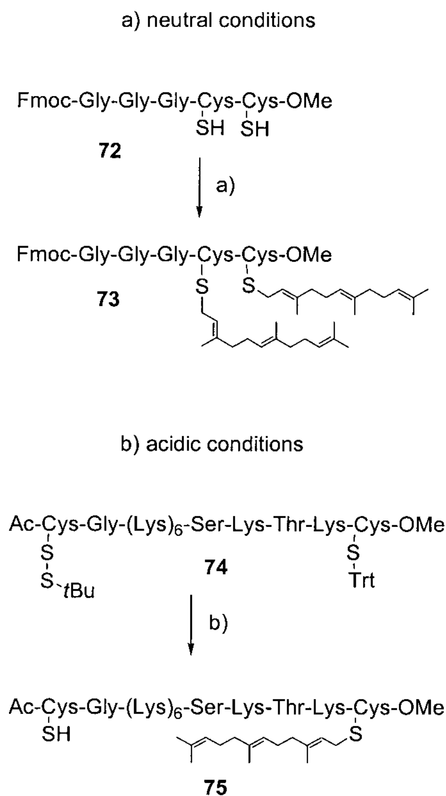
Despite the wide occurrence of *S*-palmitoylated proteins, very few syntheses of *S*-acylated peptides have been reported. *S*-acylation of a selectively *S*-deprotected peptide was demonstrated to be feasible by both solution[122] and solid phase[123] methodology. Treatment of the resin-bound peptide **70** with 2 equiv. of palmitoyl chloride and diisopropylethylamine, for example, resulted in selective *S*-palmitoylation to give the product **71** (Scheme 11). Cleavage from the resin and simultaneous deprotection was achieved by treatment with trifluoroacetic acid.



Scheme 11. On-resin palmitoylation. (a) *i* PalCl, *i*Pr_2EtN; *ii* TFA.

The *S*- and *O*-palmitoylation of unprotected peptides has recently been described.[124] The use of TFA during the acylation reaction prevented the undesired acylation of the amino groups, which, however, did occur under prolonged reaction times.

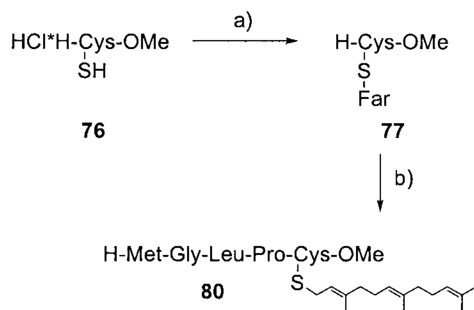
The synthesis of prenylated peptides has been addressed in various studies and the different prenylation procedures have recently been reviewed.[125] The synthesis of the acid-labile prenylated peptides is usually performed by solid phase synthesis of the unmodified peptide followed by *S*-prenylation during the final steps of the synthesis. Depending on the solubility of the peptide, prenylation was accomplished either under neutral, alkaline or acidic conditions adding KF·$2H_2O$,[111] DIPEA[126,127] and $Zn(OAc)_2$,[128,129] respectively (Scheme 12).



Scheme 12. Prenylation under neutral and acidic conditions, respectively. (a) FarBr, KF·$2H_2O$, MeCN/DMF 9:1; (b) *i* TFA; *ii* $Zn(OAc)_2$, FarBr, DMF, MeCN, H_2O, TFA, pH 4; *iii* DTT, $KHCO_3$.

The most common problems that can occur during the *S*-alkylation arise from the limited solubility of the starting materials and oxidation of the thiol group to the disulfide. When an excess of alkyl halide is used, formation of sulfonium ions is possible, which can be accompanied by alkylation of functional groups other than the cysteine thiol. Some of these problems may be avoided by using acidic conditions, since the solubility of peptides is often much higher in acidic solvents and the nucleophilicity of the free amino and carboxyl groups is reduced and disulfide formation thus suppressed. Farnesylation of the peptide **74**, for example, which corresponds to the K-Ras_B C-terminus, under alkaline conditions (DMF, H_2O, $KHCO_3$) proved to be unsatisfactory. Treatment with farnesyl bromide at pH 4 in the presence of $Zn(OAc)_2$, however, furnished the product **75** in a high yield.[128]

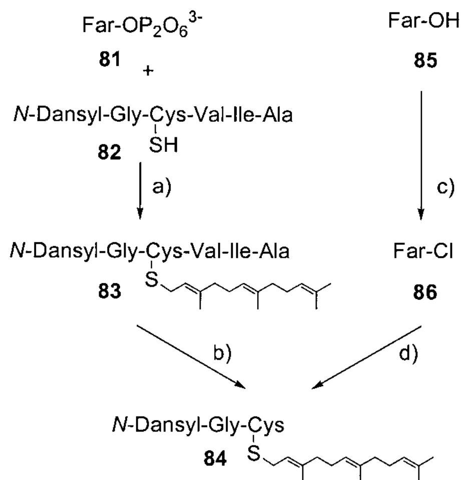
Following a completely different approach, *S*-alkylated peptides have been synthesized according to the Fmoc strategy. Cysteine methyl ester **76** was selectively *S*-farnesylated in high yields and subsequently elaborated to the pentapeptide **80** by repeated coupling and Fmoc deprotection steps (Scheme 13).[130] It should be emphasized that, by introducing the *S*-prenylation through the coupling of a



Scheme 13. Fmoc strategy in the synthesis of alkylated peptides. (a) FarBr, Et_3N, DMF, 95%; (b) *i* Fmoc-Leu-Pro-OH (**78**), EDC, HOBt, 93%; *ii* piperidine, 90%; *iii* Fmoc-Met-Gly-OH (**79**), EDC, HOBt, 46%; *iv* piperidine, 90%.

preformed building block, the product isolation was greatly simplified.

An alternative route to prenylated peptides is enzymatic prenylation which is performed either by a farnesyl transferase (FTase) or a geranylgeranyl transferase I (GGTase-I). For this procedure, a prenyl pyrophosphate is required as a reactant. An elegant approach for analysing the stereochemistry at the C1 farnesyl centre has recently been described.[131] A general route to the dipeptide **84** was either farnesylation of the peptide **82** using FTase followed by enzymatic degradation or alkylation of L-cysteine with farnesyl chloride **86** in NH_3/MeOH[131,132] and DCC coupling of the resulting cysteine adduct with *N*-dansyl-glycine (Scheme 14).



Scheme 14. Enzymatic and chemical synthesis of the farnesylated dipeptide **84**. (a) FTase; (b) carboxypeptidase Y; (c) NCS, Me_2S; (d) *i* Cys, NH_3/MeOH; *ii* *N*-Dansyl-Gly, DCC.

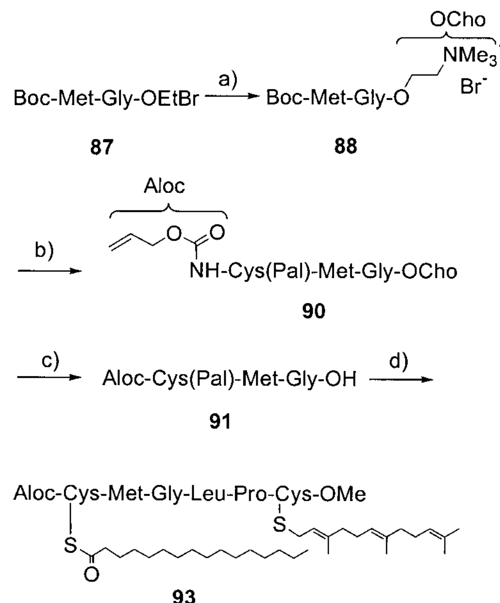
3.4. Synthesis of peptides with two different lipid groups

For the synthesis of peptides incorporating both lipid modifications, namely the acid-labile prenyl group and the base- and nucleophile-labile *S*-acyl group (Fig. 9), the number of suitable protecting groups is dramatically limited. Here, protecting groups are required which can be removed under extremely mild and preferably neutral conditions. The use of enzyme-labile protecting groups is particularly attractive since enzymatic transformations can often be carried out under extremely mild reaction conditions (pH=6–8, rt).[133–135] An alternative strategy is the application of noble metal-mediated transformations which also proceed under virtually neutral conditions.[136,137]

3.4.1. Enzymatic C-terminal deprotection.

The choline ester moiety has been introduced as an enzyme-labile protecting group for the reversible blockage of the C-terminal carboxyl group.[138] Acetylcholine esterase (AChE) and butyrylcholine esterase (BChE) were demonstrated to selectively cleave choline esters of simple peptides and sensitive peptide conjugates such as phosphorylated and glycosylated peptides,[139] nucleopeptides[140] and lipidated peptides.[140–142]

One example of the advantageous use of choline esters is shown in Scheme 15. In the synthesis of the N-Ras lipopeptide **93**, the choline ester **88** was readily prepared by treatment of the corresponding 2-bromoethyl ester **87** with trimethylamine.[143] Removal of the Boc group followed by coupling with palmitoylated cysteine **89** gave access to the tripeptide **90**. The choline ester was selectively removed by means of BChE. The addition of cyclodextrins proved to be a formidable substitute for the standard organic cosolvents, achieving an enhanced solubility of the peptide **90** and a smooth conversion to the free acid. Efficient coupling of the lipid-modified tripeptides **91** and **92** in high yield completed the synthesis of the target hexapeptide **93**. The choline ester strategy was also successfully applied to the synthesis of myristoylated and palmitoylated hexapeptides corresponding to the G_{α_0}-protein N-terminus.[142]

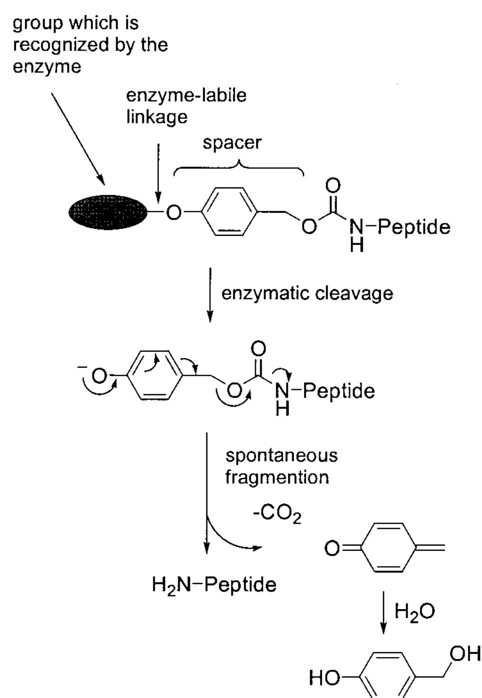


Scheme 15. Chemoenzymatic synthesis of *S*-palmitoylated and *S*-farnesylated hexapeptide **93**. (a) Me_3N, acetone, 97%; (b) *i* HBr/AcOH, thioanisole, 99%; *ii* Aloc-Cys(Pal)-OH (**89**), EDC, DMAP, 88%; (c) BChE, pH 6.5, dimethyl-β-cyclodextrin, 76%; (d) EDC, HOBt, H-Leu-Pro-Cys(Far)-OMe (**92**), 97%.

3.4.2. Enzyme-labile amine protecting groups.

Urethane-type protecting groups are indispensable for avoiding the racemization of activated amino acids. The development of enzyme-labile urethane-type protecting groups is

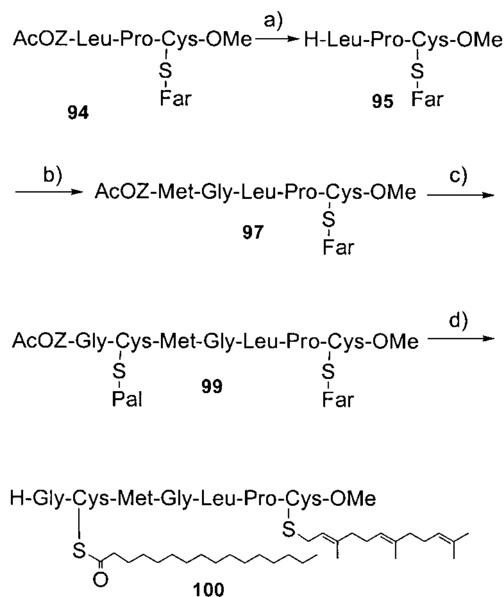
complicated, however, since most available enzymes do not attack urethane structures. The amino protecting group must therefore be designed such that an *O*-alkyl or an *O*-ester bond becomes subject to enzyme-mediated hydrolysis. This can result in the direct release of a carbamate, e.g. BGloc (tetrabenzylglucosylcarbonyl),[144] or in a spontaneous fragmentation of a spacer group which links the urethane and an enzyme-cleavable residue. In both examples, subsequent decarboxylation of the carbamates liberates the amino group, the latter strategy being depicted in Scheme 16. Depending on the acyl group of the *p*-acyloxybenzylurethane, its fragmentation can be initiated by the attack of an appropriate enzyme. It is important to note that the hydrolytic event occurs away from the variable part of the peptide.



Scheme 16. General principle of enzymatic amine deprotection according to the fragmentation strategy.

A frequently used enzyme-labile protecting group is the *p*-acetoxybenzyloxycarbonyl (AcOZ) group which was originally introduced as a base-labile blocking group.[145] AcOZ urethanes can be cleaved efficiently by hydrolytic enzymes such as an acetyl esterase from the flavedo of *Citrus sinensis Pers* and a lipase from *Mucor miehei*.[145–148] The lipase tolerates the large amounts of cosolvent that are often required due to the hydrophobicity of lipidated peptides. In palmitoylated peptides, the *M. miehei* lipase apparently preferentially hydrolysed the palmitic acid thioester rather than the acetate of the blocking function and the acetyl esterase in combination with cyclodextrins proved to be the superior method.

The AcOZ strategy was successfully employed in the synthesis of the C-terminal N-Ras heptapeptide **100**. In this process, the tripeptide **94** was deprotected using the lipase from *M. miehei*[148] (Scheme 17). The presence of 20 vol% of methanol was required to solubilize the lipidated peptide and make it accessible for the biocatalyst. To trap

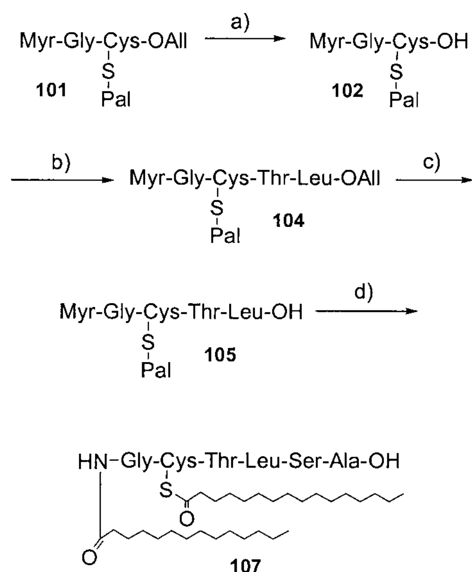


Scheme 17. Synthesis of the C-terminal N-Ras lipoheptapeptide **100**. (a) Lipase from *Mucor miehei*, KI buffer, 20% MeOH, 30°C, pH 5, 65%; (b) AcOZ-Met-Gly-OH (**96**), EDC, HOBt, 80%; (c) *i* lipase from *M. miehei*, KI buffer, NaSH, 20% MeOH, 30°C, pH 5, 48%; *ii* AcOZ-Gly-Cys(Pal)-OH (**98**), DIC, HOBt, 61%; (d) acetyl esterase from oranges, phosphate buffer, pH 6, 37°C, dimethyl-β-cyclodextrin, 35%.

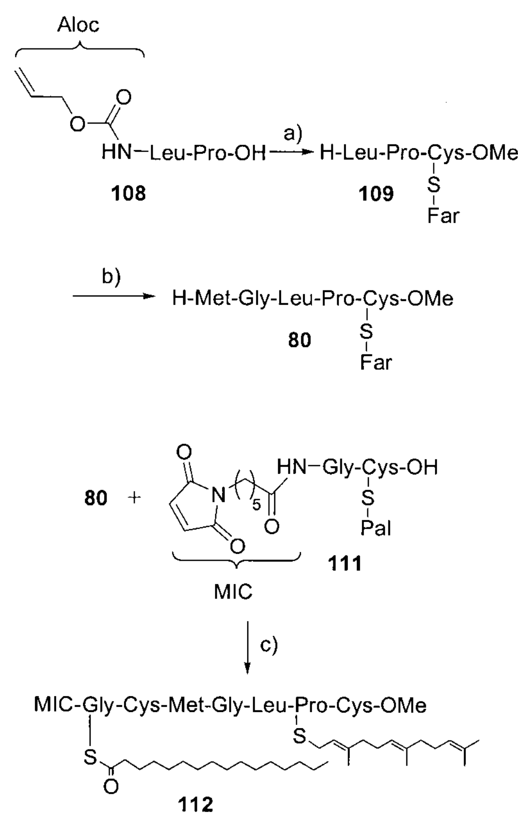
the quinonemethide that is formed upon deprotection, the enzymatic transformation was carried out in the presence of KI and/or NaSH as scavenger nucleophiles. Subsequent elongation and deprotection yielded the farnesylated pentapeptide **97**. Coupling with the AcOZ-protected and *S*-palmitoylated dipeptide carboxylic acid **98** gave access to the doubly lipidated heptapeptide **99**. Finally, the N-terminal blocking group was removed by means of acetyl esterase-mediated fragmentation of the urethane to yield the target peptide **100**.

3.4.3. Allylic esters and the allyloxycarbonyl group: a noble metal-sensitive alternative. Application of the allyl (All)[149,150] and allyloxycarbonyl (Aloc)[130,151] protecting group strategy is of considerable utility, particularly when employed for the protection of carboxyl and amino groups of sensitive peptide conjugates such as lipopeptides. The allyl and the Aloc protecting group are both readily cleaved under very mild and, in particular, neutral conditions in the presence of a Pd^0 catalyst and suitable allyl group scavengers such as morpholine,[151] *N*,*N'*-dimethylbarbituric acid (DMB),[152] phenyltrihydrosilane ($PhSiH_3$) or *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide ($CF_3CON(SiMe_3)CH_3$).[153]

The allyl ester was advantageously employed in the synthesis of the *N*-myristoylated and *S*-palmitoylated N-terminus of human G_{α_0} protein (Scheme 18).[142] Starting with the doubly lipidated dipeptide **101**, the allyl ester was removed in high yield by Pd^0-mediated transfer of the allyl group to morpholine. The gradual elongation with the dipeptide allyl esters **103** and **106** and Pd^0-mediated removal of the allyl group finally delivered the desired lipidated G protein hexapeptide **107**. The three allyl ester cleavages performed in this sequence proceeded with complete selectivity and without any undesired side reactions.

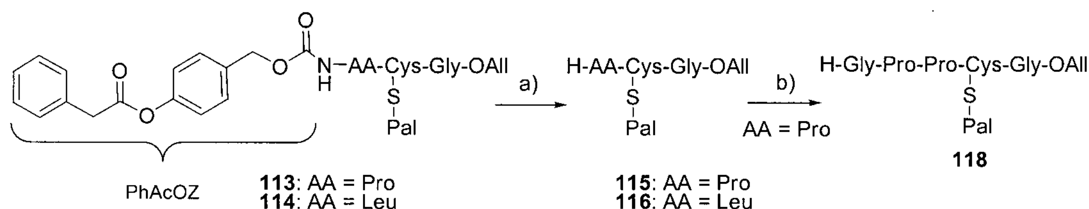


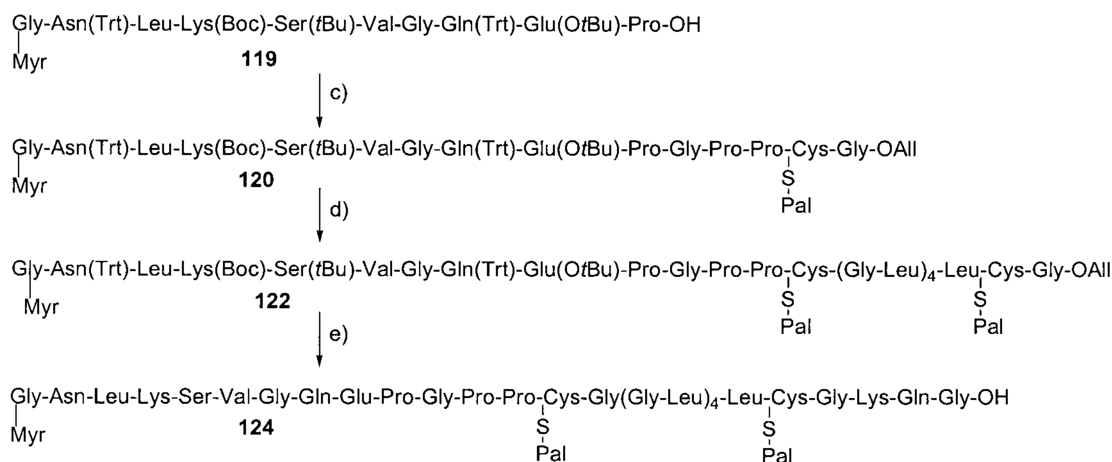
Scheme 18. The allyl ester in the synthesis of the lipidated N-terminus of human G_{α_0} protein. (a) $[Pd(PPh_3)_4]$, morpholine, 85%; (b) H-Thr-Leu-OAll (**103**), EDC, HOBt, 75%; (c) $[Pd(PPh_3)_4]$, morpholine, 84%; (d) *i* H-Ser-Ala-OAll (**106**), EDC, HOBt, 43%; *ii* $[Pd(PPh_3)_4]$, morpholine, 84%.



Scheme 19. Synthesis of the lipidated N-Ras C-terminus via Aloc strategy. (a) *i* Cys(Far)OMe (**77**), EEDQ, 98%; *ii* $[Pd(PPh_3)_4]$, morpholine, 88%; (b) *i* Aloc-Met-Gly-OH (**110**), EDC, HOBt, 62%; *ii* $[Pd(PPh_3)_4]$, morpholine, 72%; (c) EDC, HOBt, 48%.

The allyl ester[141,147,154] and the Aloc group[130,155,156] have been successfully employed in syntheses of various Ras peptides. The suitability of the Aloc group for the construction of lipidated peptides was demonstrated by the synthesis of the maleimidocaproyl (MIC)-modified, *S*-palmitoylated and *S*-farnesylated heptapeptide **112** which corresponds to





Scheme 20. Synthesis of a lipidated 29-mer peptide corresponding to the N-terminus of endothelial NO synthase. (a) Immobilized PGA, phosphate buffer, pH 6.8, dimethyl-β-cyclodextrin, 20% MeOH, KI, 53%; (b) *i* PhAcOZ-Gly-Pro-OH (**117**), HOAt, EDC, CH_2Cl_2, 82%; *ii* CLECS, pH 6.8, 0.1M KI, dimethyl-β-cyclodextrin, 20% MeOH, 39%; (c) H-Gly-Pro-Pro-Cys(Pal)-OAll (**118**), HOOBt, EDC, $CHCl_3/CF_3CH_2OH$ (3:1), 91%; (d) *i* $[Pd(PPh_3)_4]$, DMB, DMSO, 92%; *ii* H-Leu-(Gly-Leu)$_3$-Gly-OAll*CF_3CO_2H (**121**), HOAt, NEt_3, EDC, DMSO, 72%; *iii* $[Pd(PPh_3)_4]$, DMB, DMSO, 87%; *iv* H-Leu-Cys(Pal)-Gly-OAll **116**, HOAt, EDC, NMP, 86%; (e) *i* $[Pd(PPh_3)_4]$, DMB, DMSO, 69%; *ii* H-Lys(Boc)-Gln(Trt)-Gly-O*t*Bu (**123**), HOAt, EDC, NMP, 86%; *iii* CF_3CO_2H/ethandithiol/H_2O (95:2.5:2.5), 31%.

the N-Ras C-terminus (Scheme 19).[155] The pentapeptide **80** is readily accessible via the Aloc methodology.[130] Subsequent condensation with the MIC-modified, *S*-palmitoylated dipeptide **111** resulted in the formation of the target peptide **112**.

The successful interplay of chemical and enzymatic methods for the synthesis of lipidated peptides has recently been demonstrated.[157] In the synthesis of an *N*-myristoylated and doubly *S*-palmitoylated fragment corresponding to the N-terminus of endothelial NO synthase, the allyl ester and the enzyme-removable *p*-phenylacetoxybenzyloxycarbonyl (PhAcOZ) group were employed as key protecting groups.[158] The phenylacetate moiety is recognized and cleaved by penicillin G acylase (PGA).

One of the critical steps was deprotection of the palmitoylated tripeptides **113** and **114** using immobilized penicillin G acylase (Scheme 20). The accessibility of the poorly soluble dipeptides could be enhanced by addition of dimethyl-β-cyclodextrin and 20 vol% of methanol. Coupling of the tripeptide **115** with the dipeptide **117** and deprotection using cross-linked enzyme crystals (CLECs) of penicillin G acylase furnished the palmitoylated pentapeptide **118**. Iterative elongation of the myristoylated decapeptide **119** and removal of the allyl groups afforded the myristoylated and doubly palmitoylated target peptide **124**. The proper choice of solvent and work-up conditions were crucial for the success of the fragment condensations. In addition, lipidated peptides such as **119**, **120** and **122** are highly hydrophobic and tend to form secondary structures. Solubility and isolation problems could be avoided using highly polar solvents, such as DMSO, *N*-methylpyrrolidone (NMP) and $CHCl_3$/trifluoroethanol, for the condensation reactions and DMSO for the allyl ester cleavage.

This successful preparation of a 29-mer peptide, containing three lipid groups, confirms the efficiency of the combined use of the enzymatic deprotection and allyl protecting group technology and its applicability to the synthesis of large peptides.

3.5. Synthetic lipopeptides in biological investigations

The development of the methodologies discussed above has allowed for the synthesis of a variety of lipid-modified peptides representing characteristic partial structures of the parent naturally occurring lipidated proteins. For the study of biological phenomena, however, analogues with a modified lipid or peptide structure may be required. In addition, because of the minute amounts of probes used many biological enterprises depend on the introduction of reporter groups, which also enable the monitoring of the intracellular fate of the peptide conjugates.

Lipidated and biotin-labelled Ras peptides have been successfully used for determining the influence of the lipid residues on the binding of lipid-modified proteins to model membranes (Fig. 11).[141]

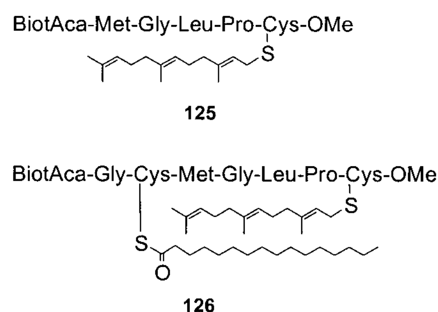


Figure 11. Structures of biotinylated and lipidated peptides used in the BIAcore® system.

A monolayer on a BIAcore® chip,[159] which consisted of long-chain alkanethiol molecules, was charged with a second hydrophobic layer by applying small unilamellar vesicles on to the sensor surface. Vesicles were prepared by mixing the carrier lipid DMPC (dimyristoylphosphatidylcholine), a buffer and a solution of the biotinylated peptide or lipid in methanol. Enrichment of streptavidine that binds to the biotinylated peptide leads to a detectable increase of the refractive index. An evaluation of the stability of peptide conjugate insertion revealed that the signal of a surface charged with the monofarnesylated peptide **125** declined by 50% within approximately 3 h (Fig. 12). In contrast, a surface that was loaded with the farnesylated and palmitoylated peptide **126** showed only a moderate loss of the resonance signal, indicating that a double hydrophobic modification of the peptides is necessary to obtain a stable insertion of the peptide–streptavidine complex in the model membrane.

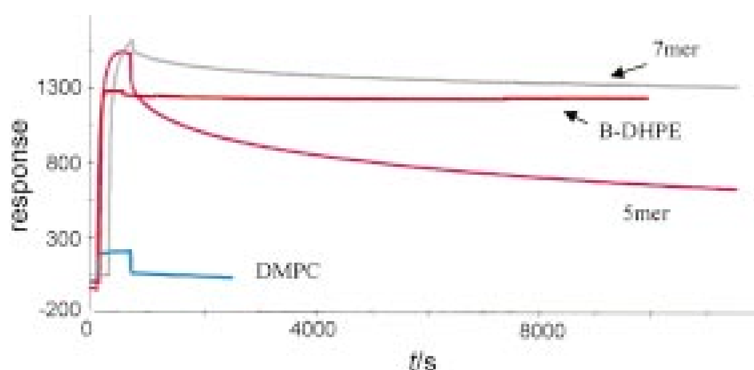
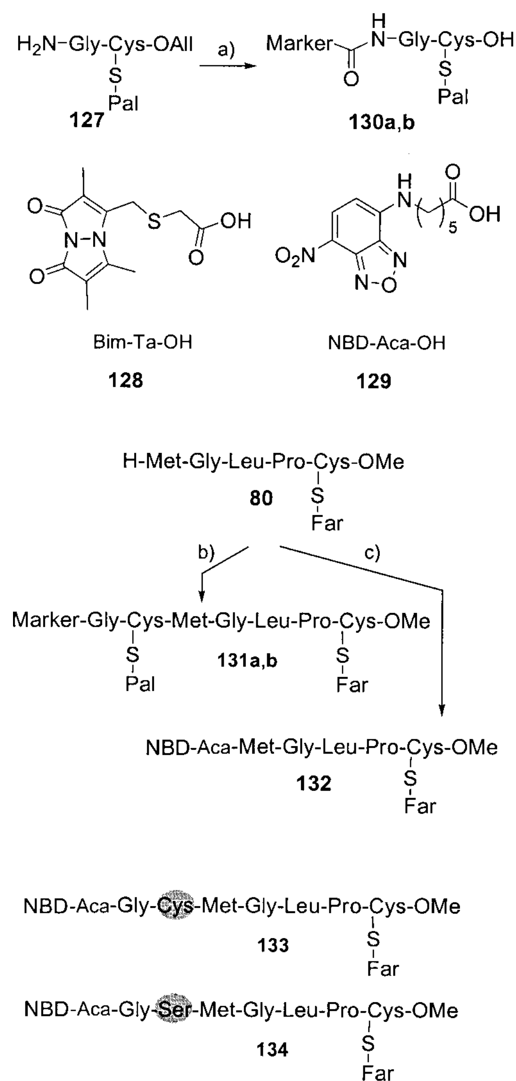


Figure 12. Stability of streptavidin signal after binding to biotinylated lipopeptides or B-DHPE (biotinylated dihexadecanoyl-phosphatidylethanolamine) as a positive control. DMPC vesicles alone show no binding of streptavidin, whereas B-DHPE shows a constant resonance signal, indicating a stable insertion of the DHPE moiety in the DMPC matrix.

For the determination of membrane binding or subcellular distribution by fluorescence microscopy, fully lipid-modified fluorescently labelled peptides were prepared. Fluorescein isothiocyanate, rhodamine B isothiocyanate and 4-chloro-7-nitrobenzofurazan (NBD-Cl) were converted to their respective ethylenediamine conjugates and these were attached to the carboxyl groups of suitably protected peptides.[142] Alternatively, a peptide such as the dipeptide **127** was conjugated via its N-terminus to *S*-bimanylthioacetic acid (Bim-Ta-OH) **128** or 7-nitro-benzofurazene-4-aminocaproic acid (NBC-Aca-OH) **129**. Coupling with the pentapeptide **80** gave access to the fluorescently labelled fully lipid-modified heptapeptides **131a,b** (Scheme 21).[147]

The different NBD-labelled hepta- and pentapeptides corresponded to the C-terminus of human N-Ras and were employed in membrane binding and cell localization studies.[112,147] The peptides **131b** and **132**, for example, were microinjected into NIH3T3 fibroblast cells and the distribution of the fluorophore was monitored by confocal laser fluorescence microscopy.[147] While the farnesylated peptide **132** was distributed in the cytosol, the doubly lipidated heptapeptide **131b** was highly enriched in the plasma membrane.



Scheme 21. Synthesis of N-terminal fluorescently labelled lipid modified peptides **131a,b** and **132**. (a) *i* DIC, HOBt, Marker-CO_2H (**128**, **129**); *ii* $[Pd(PPh_3)_4]$, DMB; (b) **130a,b**, DIC, HOBt; (c) NBD-Aca-OH (**129**), DIC, HOBt.

The heptapeptides **133** and **134** were transferred into CV-1 fibroblasts by incubation with peptide-loaded vesicles.[112] Treatment with [^{3}H]-palmitate resulted in the production of the *S*-[^{3}H]-palmitoylated cysteine-containing peptide **133**. A significant palmitoylation of the peptide **134** which contained a serine instead of a cysteine was not detected. Interestingly, the *S*-acylated peptide was localized at the plasma membrane, even when the incubation was performed in the presence of the vesicular transfer inhibitor, brefeldin A. This indicated that the plasma membrane is a major locus of *S*-acylation of the N-Ras C-terminal peptide. It therefore seems likely that the kinetic trapping model holds true.[111,112] Accordingly, a singly lipid-modified protein which contains an *S*-acylation site in close proximity to the isoprenylated residue is irreversibly inserted into membrane surfaces only when a second hydrophobic modification is attached (e.g. by means of the still putative prenyl protein-specific palmitoyl transferase).[111,112]

With the help of conjugates which link a differently lipidated peptide to a protein, the examination of numerous fully lipid-modified proteins becomes possible. It was therefore required to prepare peptides with a reactive linker such as the maleimido group. The synthesis of these peptides was achieved by selective introduction of the maleimidocaproyl (MIC) linker at the N-terminal peptide amino group (Fig. 13).[155]

Truncated oncogenic H-RasG12V 1–181 proteins with a C-terminal cysteine at position 181 were expressed recombinantly in *E. coli*. These Ras proteins were allowed to react with MIC-modified lipopeptides in stoichiometric amounts (Scheme 22). The biological function of the hybrid proteins was evaluated by microinjection into rat pheochromocytoma PC12 cells. Biologically active oncogenic H-Ras induces cell differentiation as measured by the number of neurites formed.[160] After microinjection of oncogenic truncated H-RasG12V 1–181 lacking the cysteine for farnesylation and for palmitoylation, no neurites were formed (Fig. 14(b)), whereas after microinjection of full-length recombinant oncogenic Ras protein, 79% of injected cells developed neurites (Fig. 14(a)). Microinjection of the

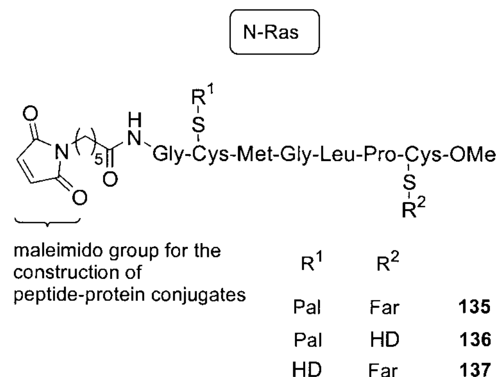
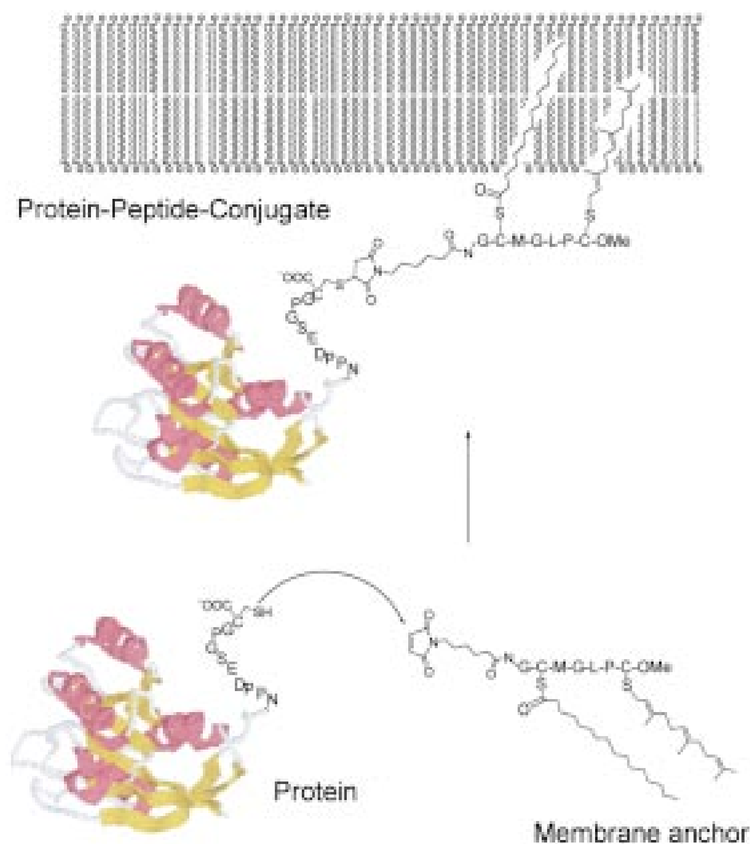


Figure 13. Maleimido-modified peptides.



Scheme 22. Fusion of H-Ras protein to the palmitoylated and farnesylated MIC-modified heptapeptide **135**.

coupling product of H-RasG12V 1–181 heptapeptide **135** (Fig. 14(c)) and H-RasG12V 1–181 with peptide **136** (Fig. 14(d)) resulted in a comparable differentiating phenotype of the PC12 cells. Inter alia it could therefore be shown that the biological activity of Ras is not dependent upon a farnesylated residue and that the hybrid proteins can serve as efficient tools for biochemical, biophysical and biological experiments.

The methodology illustrated in Scheme 22 allows the construction of a widely applicable biological readout system in which any protein can be chemically fused to a membrane-anchoring module. By using an oncogenic Ras protein molecule the ability of different lipopeptide tails to localize the protein to the plasma membrane after microinjection into PC12 cells can be quantified. Conversely, by conjugating a particular lipopeptide tail, which is known to efficiently anchor a protein to the plasma membrane, to various protein molecules, it may be possible to determine the interaction of those protein molecules with or at the plasma membrane or with other membrane-bound proteins.

The development of biological readout systems, such as that described above, should provide invaluable tools for

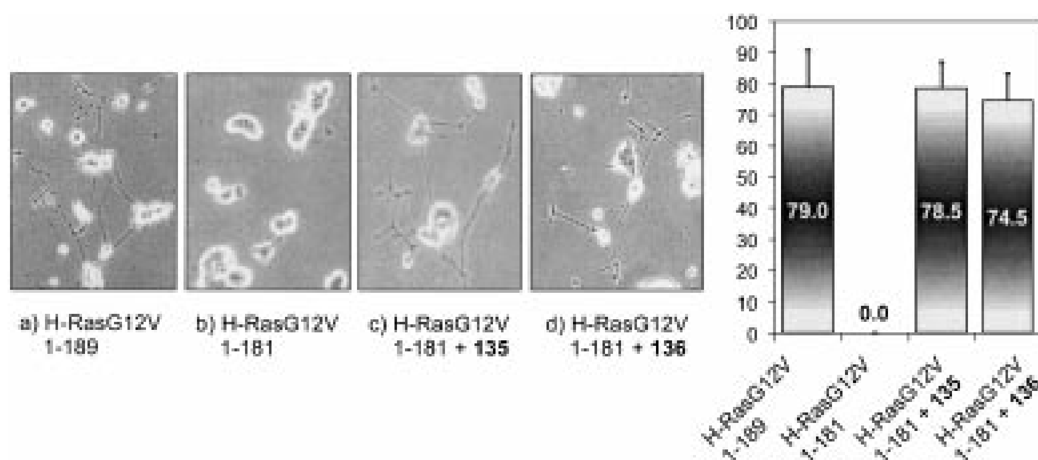


Figure 14. Microinjection of H-Ras proteins and their hybrids (specified below the figure).

determining membrane–protein interactions and may assist in unravelling the molecular details of important biological processes such as cellular signalling cascades.

4. Peptide conjugates as tools for assessing the biological role of protein degradation

4.1. Apoptosis

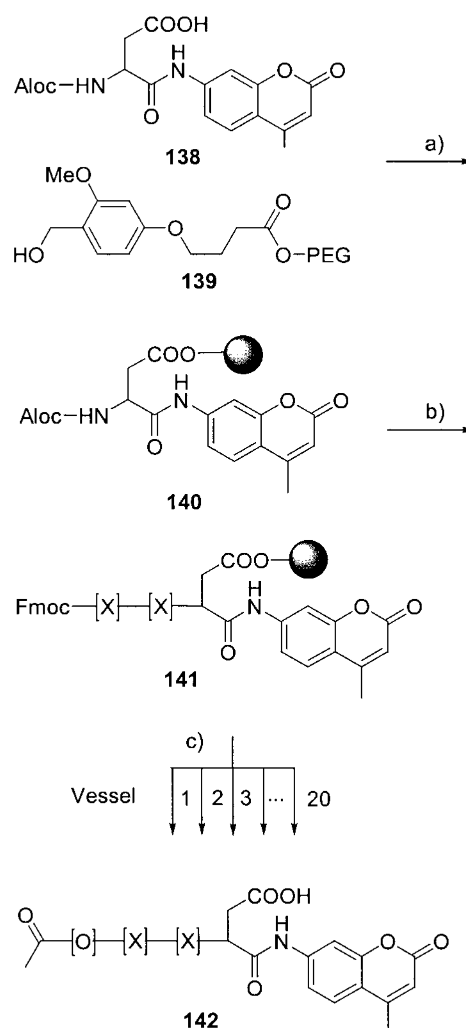
In multicellular organisms, the constancy of cell number (homeostasis) is regulated by the rate of cell proliferation and cell death. Developmental biologists were the first to recognize that the controlled autodigestive process of apoptosis (programmed cell death) is a life-saving event for multicellular organisms.[161,162] Excess and old (erythrocytes, epidermal cells) or potentially dangerous (autoimmune T-cells, mildly injured cells) cells are regularly eliminated by activating their intrinsic cell death machinery. If the homeostasis of the organism, the rate of proliferation and cell death, is out of balance several well-known diseases occur and excessive apoptosis is associated with AIDS, Alzheimer's disease and Parkinson's disease, myocardial infarction, stroke and toxin-induced liver diseases.[163,164] In contrast, the regulatory process of apoptosis is inhibited in cancer, autoimmune disorders and viral infections.

A mechanism of apoptosis has been suggested, based on genetic studies of the nematode *Caenorhabditis elegans*.[161] Sequence-analogy studies of the protein CED-3, one of the two proteins responsible for programmed cell death during *C. elegans* development, revealed a high homology to the human protease, interleukin-1-converting enzyme (ICE). This indicated that proteolysis plays a major role in apoptosis. After less than a decade of frenetic research, a whole family[165] of highly specific proteases could be identified, and these are now called caspases.[166] Once activated (a number of articles have described activating pathways[167–173]) caspases make surgical incisions at a discrete number of special proteins resulting either in activation of a proenzyme or inactivation of a protein but never in its complete degradation. Among the substrates are DNA repair enzymes, cell cycle regulators, transcription proteins, DNA fragmentation factors and also structural proteins such as lamin, actin, Gas2, keratin and spectrin.[165,174,175]

4.1.1. Classification of caspases. The proteolytic mechanism and the substrate specificity of the caspases was investigated using peptide-based substrates and inhibitors.[176] The recognition sequence for the caspases contains at least four amino acids N-terminal from the cleavage site (P1–P4). X-Ray analysis of the two most distinct members, caspase-1 and caspase-3, revealed that proteases of the caspase family contain a cysteine residue at the active site.[177–180] Caspases are very specific proteases, with an unusual and absolute requirement cleavage after aspartic acid.[181]

According to the traditional approach, a large number of different peptide substrates or peptide-based inhibitors has to be individually synthesized and analysed in order to determine the substrate specificity of an unknown protease. A more rapid and efficient approach to analyse the proteolytic specificity of a whole protease family was demonstrated using Positional-Scanning Synthetic Combinatorial Library (PS-SCL).[176,182] This library comprised a fluorogenic tetrapeptide-aminomethylcoumarin (AMC) conjugate with the general structure Ac-X-X-X-Asp-(AMC). If a member of this library is subjected to enzymatic cleavage, a fluorescence signal can be detected that is due to the released AMC reporter. The substrate requirements for the positions P2–P4 were determined by preparing separate sublibraries for every position P2, P3 and P4. In each of the three sublibraries (20 wells, each containing 400 compounds) two positions (X) were occupied by an isokinetic mixture of all proteogenic amino acids whereas the third position was occupied with one of 20 distinct amino acids. A representative example for the preparation of the P4 position library is outlined in Scheme 23.

The side chain carboxylic acid of *N*-Aloc-L-aspartic acid-α-AMC **138** was first attached to the resin **139** functionalized with the HMPB linker. After removal of the Aloc protecting



Scheme 23. Preparation of the P4 position library **142**. X represents the isokinetic mixture of proteogenic amino acids and O represents the spatially addressed amino acid. (a) DIAD/PPh_3; (b) *i* $Pd(Ph_3)_4$/DMB; *ii* isokinetic equiv. Fmoc-X-OH/HOBt/EDC; *iii* 25% pip/DMF; *iv* isokinetic equiv. Fmoc-X-OH/HOBt/EDC; (c) *i* transfer to 20 vessels; *ii* 25% pip/DMF; *iii* Fmoc-O-OH/HOBt/EDC; *iv* 25% pip/DMF; *v* Ac_2O/py/DMF; *vi* TFA/H_2O/PhOH/TIS.[182]

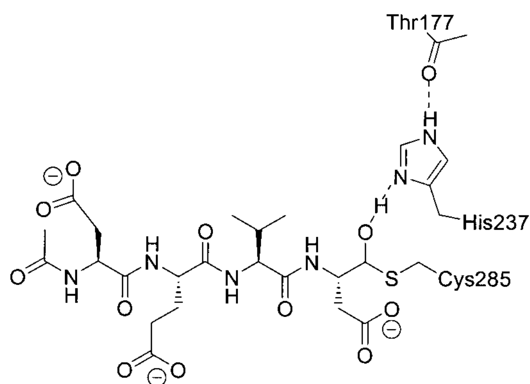
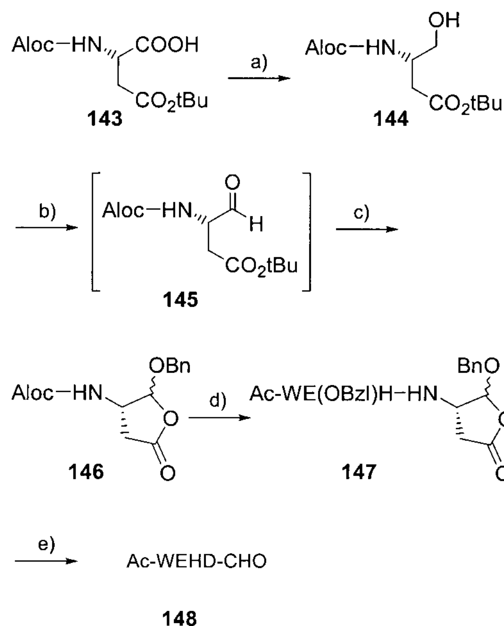


Figure 15. Complex of caspase-3 active site and the peptide aldehyde inhibitor, Ac-DEVD-CHO.[171]

group, an isokinetic mixture of Fmoc-protected amino acids (X) was coupled. In an isokinetic mixture, the proportion of each amino acid in the reaction mixture is varied inversely to its reaction rate, guaranteeing the synthesis of an equimolar mixture of each peptide.[183] The Fmoc group was removed before a second coupling with the isokinetic mixture of Fmoc-amino acids was performed. The polymer **141** was then transferred into 20 individual reaction vessels. The Fmoc groups were removed and one single amino acid (O) was added to each vessel, which now had a distinct amino acid residue at the P4 position. After the final Fmoc removal and terminal *N*-acetylation, the tetrapeptide-AMC conjugate was detached from the resin by TFA treatment.

Thornberry and co-workers were able to distinguish three subfamilies. The nature of the P4 position proved to be the most important for the subclassification. Addition of group I caspases (-1, -4, -5), for example, resulted only in a strong fluorescence when peptide conjugates were used that were synthesized in the vessels that contained hydrophobic amino acids such as tryptophan as single amino acids. Group II caspases (-2, -3, -7, CED-3) require aspartic acid at the P4 position. Group III caspases (-6, -8, -9, -10) are less specific but prefer branched, aliphatic side chains (Val, Leu).



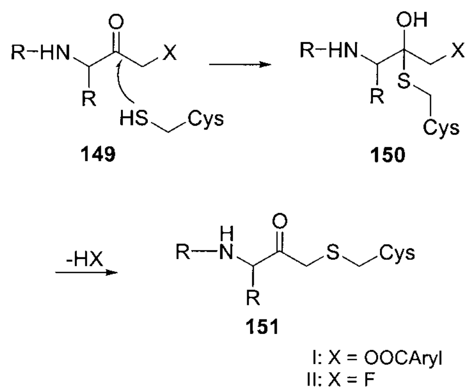
Scheme 24. Preparation of the caspase-1-specific tetrapeptide aldehyde inhibitor, Ac-WEHD-CHO (**148**). (a) *i* Butylchloroformiate/NMM; *ii* $NaBH_4$; (b) DMSO/$(COCl)_2$/Et_3N; (c) *i* BnOH/TsOH/Sieves; *ii* TFA; (d) Ac-WE(OBzl)H-OH/$(PPh_3)_2PdCl_2$/Bu_3SnH/EDC/HOBt; (e) H_2/$Pd(OH)_2$/C.

4.1.2. Caspase inhibitors. Immense efforts have been undertaken to develop potent tools which allow an intervention into programmed cell death. Specific inhibitors were synthesized firstly to block specific caspases, in order to study their physiological role in apoptosis. Specific caspase inhibitors might additionally become therapeutic tools for curing diseases caused by irregulated apoptosis.

A suitable motif for the design of caspase inhibitors proved to be the linkage of a recognition moiety, usually a tri- or tetrapeptide, with an electrophile that readily reacts with the thiol group of the active site cysteine residue.[184,185] Peptide aldehydes are amongst the most potent and widely used caspase inhibitors.[181,186] Based on the results of PS-SCL, the optimal peptide aldehyde inhibitor for caspase-1, Ac-WEHD-CHO, displays a dissociation constant K_i of 56pM[182] and the caspase-3 inhibitor, Ac-DEVD-CHO, has a K_i value of 230pM.[179] Cocrystallization of caspase-1 and caspase-3 with their corresponding aldehyde inhibitors enabled access to the crystal structures. The inhibitor formed a covalent but easily hydrolyzable adduct by generating a thiohemiacetal with the cysteine residue. Surprisingly, the carbonyl group of the aldehydes was not involved in binding to a putative oxyanion hole. Instead, the oxyanion appeared to participate in the hydrogen-bond network which involved the catalytic histidine residue (Fig. 15).[181]

A common feature of all caspases is that an aspartic acid residue is essentially required at the P1 position and hence, only one amino acid aldehyde precursor **146** needed to be prepared, which in a second step could be conjugated to different peptides (Scheme 24).[182,187] The selectively protected aspartic acid building block **143**, for example, was converted to the mixed anhydride and then reduced to the corresponding alcohol **144**. After a Swern oxidation, the generated aldehyde **145** was treated with benzyl alcohol. Subsequent TFA treatment furnished the *O*-benzylacylal **146** as a masked aspartal. For the synthesis of a caspase inhibitor, the removal of the Aloc group and the attachment of a tripeptide can be performed simultaneously, the caspase-1-specific inhibitor, Ac-WEHD-CHO, **148** being generated by in situ coupling of the tripeptide, Ac-WE(OBzl)H-OH and final hydrogenolysis.

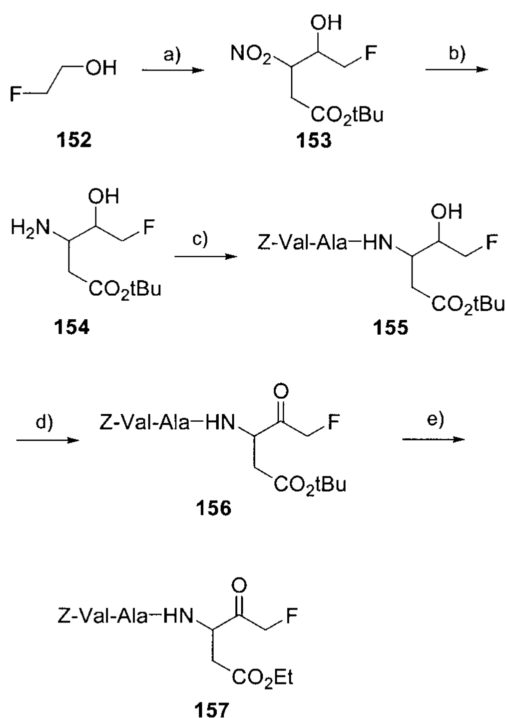
Unfortunately, peptide aldehydes do not only inhibit cysteine proteases such as caspases but also serine and threonine proteases (see Section 4.2.2). In addition, peptide aldehydes readily react with all types of nucleophiles and their cellular uptake is insufficient. In the quest for high affinity inhibitors showing a high selectivity and membrane permeability, two classes of irreversible cysteinase inhibitors were developed, namely the fluoromethyl and the acyloxymethyl ketones. In a first reversible step, the nucleophilic thiol of the enzyme attacks the keto group forming a



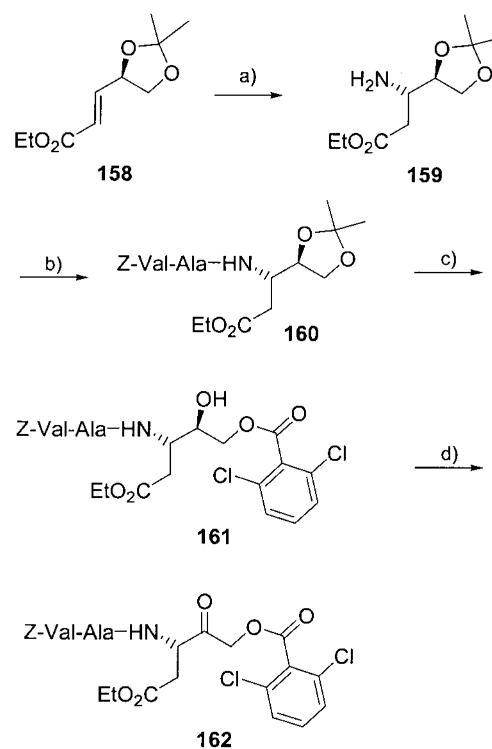
Scheme 25. Irreversible caspase inhibition by acyloxymethyl (I) and fluoromethyl (II) ketones. In a first step, the nucleophilic thiol group of the cysteine residue attacks the keto group forming a thiohemiketal (**150**) and subsequently replaces the α-substituent, generating a thiomethyl ketone (**151**).

thiohemiketal and irreversible inactivation of the enzyme then proceeds through displacement of the leaving group, X, to form a thiomethyl ketone with the active Cys site of the enzyme (Scheme 25).

Among the α-substituted ketones, the fluoromethyl ketones were found to fulfil the criteria for a selective cysteinase inhibitor.[184] The rate of alkylation of the model thiol, glutathione, was reduced to 0.2% compared to the first generation chloromethyl ketone. Fluoromethyl ketones still display a high degree of reactivity towards cysteine proteases, whilst serine proteases, as desired, show a considerably reduced susceptibility. The most promising fluoromethyl ketone-based caspase inhibitor is Z-VAD-fmk **157**. The increased lipophilicity of Z-VAD-fmk **157** confers a high membrane permeability which has helped in elucidating the role of caspases in apoptosis, particularly when living cells were used.[165,188]



Scheme 26. Preparation of the fluoromethyl ketone **157**. (a) *i* $(COCl)_2$/DMSO/Et_3N; *ii* 3-nitropropyl acid *t*-butyl ester; (b) H_2/Raney-Nickel; (c) Z-Val-Ala-OH/HOBt/DMAP/EDC; (d) Dess–Martin; (e) *i* TFA; *ii* EtOH/HCl.



Scheme 27. Preparation of the acyloxymethyl ketone **162**. (a) *i* $BnNH_2$; *ii* H_2, Pd/C; (b) Z-Val-Ala-OH/HOBt/EDC; (c) *i* EtOH/H_2O K10 Montmorillonite; *ii* 2,6-Dichlorobenzoyl chloride/py/DMAP/DMPU; (d) Dess–Martin.

Acyloxymethyl ketones were originally developed as irreversible inhibitors for the lysosomal cysteinase, cathepsin B.[189] In this class of α-substituted ketones, the displaced group is an arylcarboxylate which is known to be a weak leaving group in S_N2 reactions. By varying the substitution pattern of the arylcarboxylate a fine tuning is possible. The 2,4,6-trimethylbenzoyloxymethyl ketone, for example, was 12-fold less reactive against displacement by other thiols than the corresponding fluoromethyl ketone.[189] The most notable feature of caspase-1 inhibition by tetrapeptide acyloxymethyl ketones is the extraordinarily rapid rate of inactivation observed, which is independent of the leaving group pK_a.[190] It was therefore concluded that the rate-determining step was the binding and not the substitution reaction.

Revesz et al. have developed methods suitable for large-scale preparation of aspartate-based acyloxymethyl and fluoromethyl ketones.[191] The synthesis of the fluoromethyl ketone **157** commenced by subjecting 2-fluoroethanol **152** to a Swern oxidation followed by 1,2-addition of 3-nitropropanoic acid *t*-butyl ester. Hydrogenation of the nitroalcohol **153**, which was formed furnished the amine **154** which was coupled with the dipeptide, Z-Val-Ala-OH, using carbodiimide activation. A subsequent Dess–Martin oxidation delivered the fluoromethyl ketone **156** which,

after transesterification, yielded the desired fluoromethylketone **157** (Scheme 26).

For the synthesis of the acyloxymethylketone **162**, pentenecarboxylic acid ethyl ester **158** was treated with benzylamine and subsequently hydrogenated. The released amine **159** was coupled to the dipeptide, Z-Val-Ala-OH, as described above. The ketal **160** was subsequently converted to the diol which was selectively esterified at the primary hydroxyl group to yield the benzoate **161**. A subsequent Dess–Martin oxidation completed the synthesis of the acyloxymethylketone **162** (Scheme 27).

4.2. The proteasome

In a living cell, the majority of proteins is constantly turned over by a dynamic process of degradation and synthesis. When out of balance, cells can start to proliferate in an uncontrolled manner, a feature characteristic of tumour cells. Eukaryotic cells evolved two major systems for the degradation of proteins, one of which involves a lysosomal pathway, in which, preferably, membrane-associated and extracellular proteins are endocytosed and degraded by lysosomal proteases (e.g. cathepsins). The majority of cytosolic proteins are hydrolysed by an alternative proteolytic system, proteasome or multicatalytic protease complex.

The 26 S proteasome is located in both the cytosol and the nucleus of all eukaryotic cells.[192–194] It consists of the 20 S core particle as the proteolytic centre and the 19 S regulatory complex at the entry of the 20 S core particle. The 20 S core particle is a cylinder of 28 subunits arranged in four stacked rings.[195] In *T. acidophilum*, the 20 S proteasome is comprised exclusively of α- and β-subunits, which build an $\alpha_7\beta_7\beta_7\alpha_7$ structure (Fig. 16).[196]

Proteolysis occurs in the central chamber which is assembled by the β-subunits. The α-subunits form narrow gates (13 Å diameter) and therefore prevent unspecific degradation of folded proteins. In eukaryotic proteasomes, the α-ring allows the binding of the regulatory 19 S complex. The 19 S complex consists of ca. 20 different subunits with a chaperone- and transporter-like function. Other subunits are required for the binding and cleavage of ubiquitinated protein substrates.[197,198]

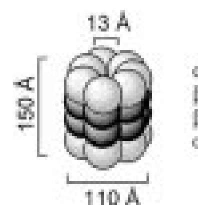
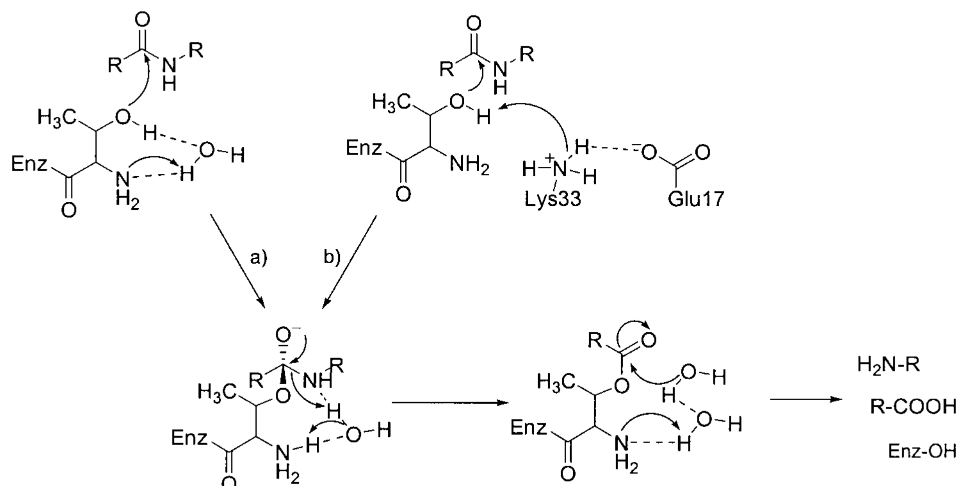


Figure 16. Schematic representation of the 20 S proteasome of *Thermoplasma acidophilum*. The catalytic activity is centred inside the β-subunit.

4.2.1. Peptidase activity of the proteasome.

In the mammalian proteasome, multicatalytic protease, three distinct protease activities have been identified.[199] One activity is described 'trypsin-like', because it preferentially hydrolyses peptide substrates after basic residues, one 'chymotrypsin-like', hydrolysing after large hydrophobic residues and one 'post-glutamyl-peptide hydrolytic' (PGPH) activity, cleaving after acidic residues. For the latter activity, recent results indicated a higher specificity for Asp than for Glu, so that 'caspase-like' seems to be a more appropriate description.[200] Although this classification is useful for model peptides, it should be noted, however, that natural protein substrates are hydrolysed at almost every position.[195]

Mutational analysis of the β-subunit of *T. acidophilum* indicated that the N-terminal threonine residue was essential for proteolytic activity.[201] The X-ray structure of the 20 S proteasome, in which the aldehyde inhibitor, Ac-Leu-Leu-Norleucinal, was covalently bound via a hemiacetal to the side chain hydroxyl group of the N-terminal Thr1, revealed a particular type of protease.[196] In contrast to classical serine and cysteine proteases, there was no catalytic triad. The side chain hydroxyl group of Thr1 is probably activated by either the free amino terminus of Thr1 or the ε-amino group of a proximal lysine 33 (Scheme 28).[202]



Scheme 28. Two possible mechanisms for proteolytic activity of the proteasome. The essential base for acceptance of the hydroxyl proton in the transition state resides in (a) the N-terminal amino group of Thr1, or in (b) a lysine amino group found near the active site.

4.2.2. Proteasome inhibitors. Essential in understanding the physiological role of the proteasome in mammalian cells is the knowledge of how to modulate its proteolytic activity in vitro and in vivo. In the last few years, several novel proteasome inhibitors have been developed.[202,203] A recently developed class of inhibitors is based on a combination of two inhibitory functions. These bivalent inhibitors display an increased selectivity for the distinct proteolytic activities of the proteasome. A classical peptide aldehyde, for example, was equipped with a maleimide function as a thiol-reactive handle, leading to a high selectivity for the trypsin-like proteolytic activity of the proteasome (Fig. 17).[204] Within a few minutes of incubation a complete inactivation of the β2-subunit was achieved. However, one limitation for the use of this inhibitor in more complex systems is the fact that the maleimide moiety is reactive towards all types of thiols.

For exploiting the unique topography of the six active sites, two peptide aldehydes were conjugated to a polyethylene glycol (PEG) spacer.[205] The highly solvated and unstructured PEG was chosen to mimic a random coiled polypeptide substrate. The coupling of well-established peptide aldehyde inhibitors to the PEG dicarboxylic acid (Scheme 29) furnished homobivalent and heterobivalent peptide conjugates. The former conjugate, which contained either Leu-Leu-Nle-H or Arg-Val-Arg-H as the head groups for chymotrypsin-like or trypsin-like activity, respectively, improved the selective inhibition by 2-fold compared to the single peptide aldehyde inhibitor. Interestingly, a heterobivalent inhibitor **172**, which consisted of one Leu-Leu-Nle-H and one Arg-Val-Arg-H moiety, blocked both the chymotrypsin- and the trypsin-like activity at nanomolar concentrations. Unfortunately, peptide aldehydes also inhibit cysteine proteases such as lysosomal cathepsins and calpains. The reactive aldehydes can, additionally, bind unspecifically to free amino groups of any protein to form Schiff bases. Novel inhibitors were therefore developed with the aim of achieving a higher selectivity in targeting the Thr protease.

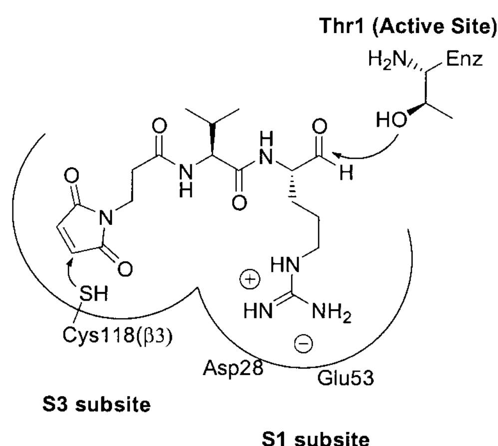
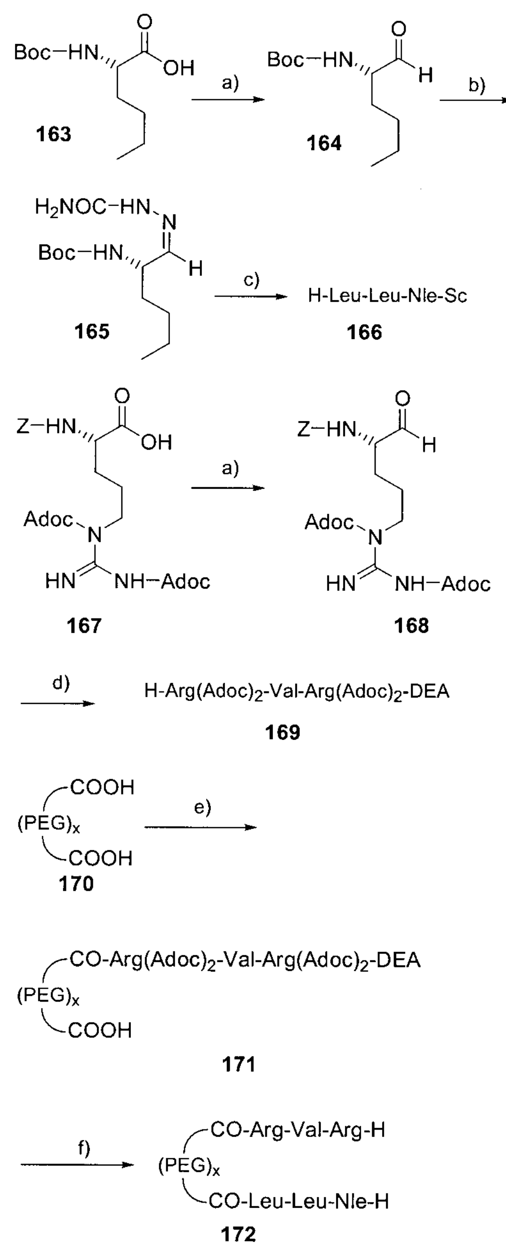


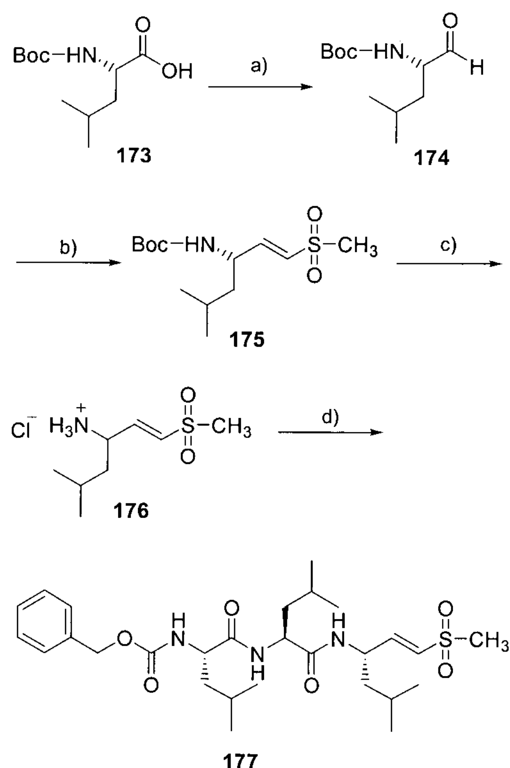
Figure 17. A schematic representation of the bifunctional inhibitor, maleoyl-β-Ala-Val-Arginal in a complex with the β2-subunit of the yeast proteasome, containing exclusively a Cys residue in close proximity. According to the proposed mechanism, the inhibitor first reversibly forms a hemiacetal with the active site Thr. Subsequently, the thio group adds to the maleimide leading to an irreversible blocking of the trypsin-like proteolytic activity of the proteasome.[195]



Scheme 29. Preparation of bivalent peptide aldehyde inhibitors. (a) *i* CH_3NHOCH_3/TBTU/HOBt/DIPEA; *ii* $LiAlH_4$; (b) NaOAc/semicarbazide HCl; (c) *i* 25% TFA/DCM; *ii* Boc-Leu-Leu-OSu; *iii* 25% TFA/DCM; (d) *i* EtOH/HCl; *ii* H_2/Pd–C; (e) 1 equiv. **169**/TBTU/HOBt/DIPEA; (f) *i* 2 equiv. **166**/TBTU/HOBt/DIPEA; *ii* AcOH/37% HCHO/MeOH; *iii* 95% TFA.

A second class of peptide-based proteasome inhibitors, the peptide–vinyl sulfones, proved to be very effective.[206,207] These peptides contain a vinyl sulfone moiety at the C-terminus acting as a Michael acceptor for the nucleophilic hydroxyl group of the catalytic Thr1. For the preparation of the vinyl sulfones, the amino acid **173** is converted to the Weinreb amide and subsequently reduced to the aminoaldehyde **174** (Scheme 30). The vinyl sulfone **175** is then generated by a Wittig reaction. After removal of the Boc-protecting group, Z-Leu-Leu-OH is coupled under standard conditions to furnish **177**.

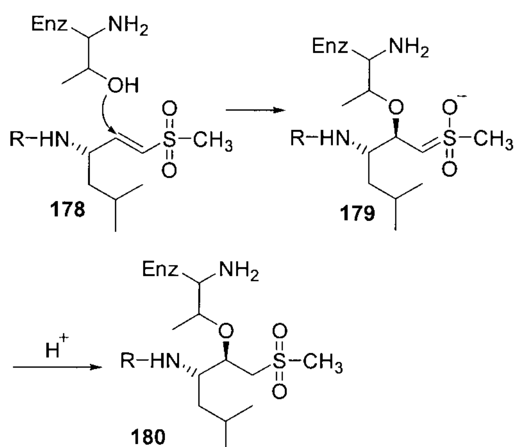
In contrast to the peptide aldehydes, the vinyl sulfones act as



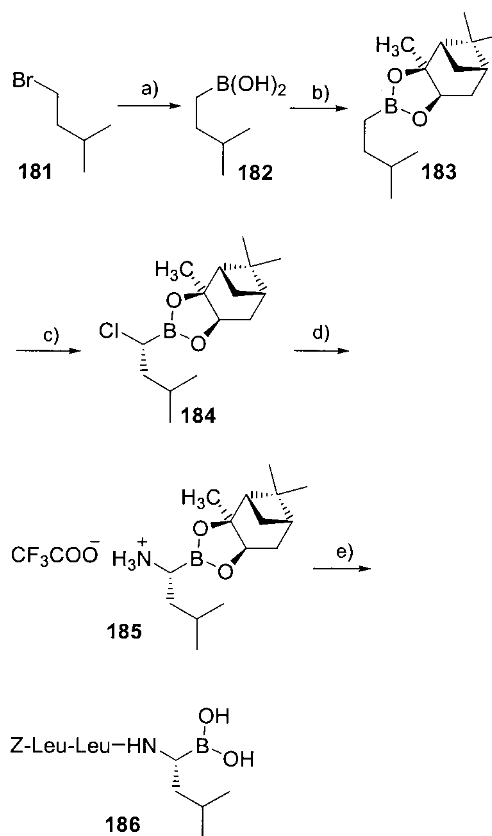
Scheme 30. Preparation of the vinyl sulfone **177**. (a) *i* CH_3NHOCH_3/PyBOP/DIPEA; *ii* $LiAlH_4$; (b) $(EtO)_2P(O)CH_2S(O)_2CH_3$/NaH/TsOH; (c) 4M HCl/1,4-dioxane; (d) Z-Leu-Leu-OH/PyBOP/DIPEA.

suicide substrates leading to an irreversible linkage between the inhibitor and the substrate (Scheme 31). Originally, the vinyl sulfones were developed as inhibitors against the softly nucleophilic cysteine proteases,[208] but they were surprisingly less reactive against lysosomal cysteine proteases than the peptide aldehydes. In addition, Z-tri-leucine vinyl sulfones were demonstrated to covalently block all of the active β-subunits of the proteasome whereas the corresponding aldehydes only blocked the chymotrypsin and post-acidic subunits.

Peptidyl boronic acids are a promising class of novel highly



Scheme 31. Mechanism of inactivation of proteasome by vinyl sulfone. The vinyl sulfone acts as a Michael acceptor for the nucleophilic hydroxyl group of the active site Thr1, blocking its proteolytic activity irreversibly.



Scheme 32. Preparation of peptidyl boronic acids. (a) *i* $MgB(OEt)_3$; *ii* H_2O/H_2SO_4; (b) pinanediol; (c) $LiCHCl_2$; (d) *i* $LiN(SiMe_3)_2$; *ii* TFA; (e) *i* Z-Leu-Leu-OH/TBTU/DIPEA; *ii* *i*-$BuB(OH)_2$/aq. HCl.

selective peptide-based proteasome inhibitors.[209] Compared to the corresponding aldehyde, peptidyl boronic acids display an inhibitory potency that is increased by 100-fold (e.g. Z-Leu-Leu-Leucinal K_i=4nM and Z-Leu-Leu-Leu-$B(OH)_2$ K_i=0.03nM). The boronic acid reversibly binds to the active site of the proteasome, but due to the very low S—B bond strength, cysteine proteases such as lysosomal cathepsins are not subject to inhibition by peptidyl boronic acids at low picomolar concentrations. For the synthesis of peptidyl boronic acids, an initial Grignard reaction formed the *i*-butylboronic acid **182** (Scheme 32).[210,211] Esterification was performed by treating the boronic acid with pinanediol. The boronic ester **183** was chlorinated using (dichloromethyl)lithium to afford **184**. Subsequent treatment with lithiohexamethyldisilazane and acid desilylation yielded the leucine analogue amino boronate **185**. Under standard conditions the peptide moiety was coupled and the free peptidyl boronic acid **186** was liberated.[209]

4.2.3. Inhibitors as tools for cell biologists. Due to the availability of potent inhibitors in the past few years, several unexpected results have demonstrated the physiological importance of proteasome-mediated protein degradation.[203] The blocking of the proteasome pathway enabled the stabilization of short-lived proteins and made it possible to isolate and identify the physiological role of several regulatory proteins (e.g. NF-κB, p53 and CDK).[198] The degradation of these proteins was believed for a long time to take place in lysosomes. Proteasome inhibitors, however, were able to block 90% of the degradation of abnormal and

regulatory proteins.[212,213] In contrast, lysosomal inhibitors accounted for a minority of 10–20% of total protein degradation.

Proteolytic degradation by the proteasome plays an important role in the immune response to foreign pathogens (cf. Section 2.4.2).[193] The short peptides that are presented by cell surface MHC molecules are generated during breakdown of intracellular proteins by the proteasome. By blocking the protein breakdown, peptide aldehydes and lactacystin[214] also completely prevented the MHC presentation on the cell surface.[212,213] Further evidence for the important role of the proteasome in the antigen presentation was the discovery that the cytokine γ-interferon (IFN-γ) which stimulates antigen presentation induces a reorganization of the proteasome.[215] After treatment of the cell with IFN-γ in the newly built proteasomes, the proteolytic β-subunits β1, β2 and β5 are replaced by new subunits LMP2, LMP7 and MECL1. This novel 'immunoproteasome' has enabled the processing and the presentation of antigens to be improved in order to ensure a highly efficient immune response.

5. Conclusion and outlook

The examples described in this review demonstrate that the recent improvements in the synthetic methodology have enabled the synthesis of a variety of modified peptides such as glycopeptides, lipopeptides and peptide pharmacophore conjugates. It is particularly encouraging that the degree of peptide conjugate complexity that can be accessed by current techniques meets the many needs of biological and medicinal research. The use of synthetic glycopeptides revealed that the attachment of carbohydrates can create and mask B- and T-cell epitopes, which will have an impact on tumour diagnostics and tumour therapy. It now seems possible to direct proteins to plasma membranes in order to study the biological role of membrane insertion. Only through the design of highly specific caspase and proteasome inhibitors has the unravelling of the physiological importance of protein degradation and processing at the molecular level been achieved. The synthesis of very complex target molecules, however, is still a research project in its own right and remains an area for highly specialized chemists. One of the main goals will therefore be to match the different time scales of chemical and biological research. Solid phase-based synthesis appears to be a well-suited and automatable synthetic tool that allows a rapid and, if desired, combinatorial, access to peptide modifications. The combined use of chemical and enzymatic methods with orthogonal ligation strategies such as Native Chemical Ligation holds much promise and can enable the synthesis of entire proteins and protein modifications within a reasonable time.

The human genome has been sequenced and is expected to facilitate the identification of disease-related proteins. As the age of proteomics commences, it will become of utmost importance to analyse and influence post-translational protein modifications. Routine procedures of protein over-expression do not allow a complete control of protein modification and processing. A promising approach is the intein-mediated Expressed Protein Ligation, a splicing event, which might provide a general biocatalysis of protein fragment condensation within a living cell.

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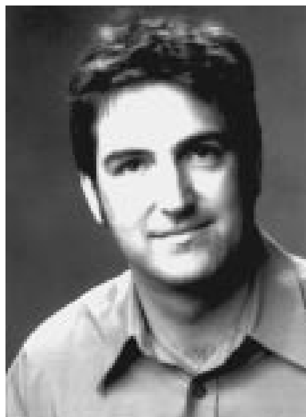
Biographical sketch



Oliver Seitz was born in Frankfurt, Germany, in 1966 and received his Diploma in chemistry from the University of Mainz in 1992. He obtained his Ph.D. there in 1995 under the supervision of Professor Horst Kunz developing a new linker for solid phase glycopeptide synthesis. In 1996 he worked as a postdoctoral fellow in the laboratories of Professor Chi-Huey Wong at the Scripps Research Institute in La Jolla, California. He returned to Germany in 1997 and started his work towards the Habilitation under the chair of Professor Herbert Waldmann at the University of Karlsruhe. In 2000, he moved to the Max-Planck-Institut of Molecular Physiology in Dortmund where he leads a group in the Department of Chemical Biology and the Institute of Organic Chemistry at the University of Dortmund. His research interests include the synthesis and functionalization of biopolymers such as peptides, glycopeptides, nucleic acids and analogues for the further usage as biomolecular tools. Most recently he has been working on the development of new strategies for the functionalization of peptide nucleic acids.

Amos Mattes, born in 1972 in Strümpfelbach, Germany, studied chemistry at the universities of Kaiserslautern and Würzburg. At the Ecole Supérieure de Chimie Physique Electronique (CPE) in Lyon, France, he developed analogues of cyclophosphamide in 1996. The following year he finished his diploma in bioinorganic chemistry with a thesis on the C/Si bioisosterism of potential antimuscarinics in the group of Professor R. Tacke. In 1998 he commenced work on his Ph.D. thesis which he performs in the group of Oliver Seitz. His current research interest is focused on the synthesis of oligonucleotides and peptide nucleic acids and their use in template-directed reactions.

Ines Heinemann, born in Eckernförde, Germany, in 1971, studied chemistry and biology at the University of Kiel. In 1997 she received her Diploma in chemistry after working on the synthesis of macrocyclic musk odorants under the supervision of Professor W. Tochtermann. She obtained the Diploma in biology in 1998 with a thesis on proteins and protein complexes at the outer chloroplastic envelope membrane which she carried out in the group of Professor J. Soll. In 1998, she joined the group of Professor H. Waldmann in Karlsruhe to work on the synthesis and evaluation of lipidated peptides and proteins.

Herbert Waldmann, born in 1957, received his Dr. rer. nat. in 1985 (Universität Mainz, H. Kunz). After postdoctoral studies (1985–1986, Harvard University, George Whitesides) and habilitation (1991, Universität Mainz) he accepted a professorship at the Universität Bonn in 1991. In 1993 he moved to the Universität Karlsruhe as Full Professor of Organic Chemistry. In 1999 he was appointed as Director at the Max-Planck-Institut of Molecular Physiology, Dortmund (Department of Chemical Biology) and as Full Professor of Biochemistry at the University of Dortmund. Herbert Waldmann has been the recipient of the Friedrich Weygand Award for the advancement of peptide chemistry, of the Carl Duisberg Award of the Gesellschaft Deutscher Chemiker and the Steinhofer Award of the Steinhofer Foundation. His current research interests include bioorganic chemistry and natural product synthesis as well as biocatalysis, stereoselective synthesis and combinatorial chemistry. A major focus of his research activities is on the combination of organic chemistry, biophysics and biology for the synthesis and biological evaluation of peptide and protein conjugates that are involved in biological signal transduction processes. Most recently syntheses of natural products and natural product derived compound libraries on polymeric supports have been investigated by the Waldmann group (see the home page for further information: www.mpi-dortmund.mpg.de).