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Invited Review

Chemoenzymatic Synthesis of Lipidated Peptides

Dieter Kadereit and **Herbert Waldmann***

Max Planck Institute of Molecular Physiology, Department of Chemical Biology, D-44227 Dortmund, Germany, and University of Dortmund, Department of Organic Chemistry, D-44227 Dortmund, Germany[a]

Summary. This review highlights the use of enzymatic protecting group techniques in the synthesis of lipidated peptides. Lipidated proteins play key roles in signal transduction processes. Moreover, structurally well-defined peptides containing the characteristic linkage region of the peptide backbone with the lipid can provide valuable tools for the study of biological phenomena associated with these protein conjugates. The multifunctionality and pronounced lability towards acids and bases of such compounds render their synthesis a formidable challenge. However, the recent development of enzymatic protection groups provides an efficient access to these sensitive and biologically relevant peptide conjugates under particular mild conditions and with high selectivity.

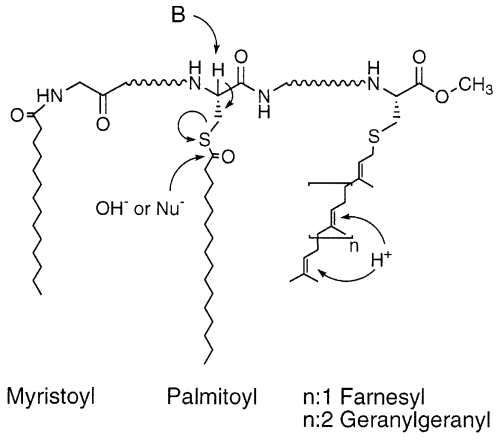
Keywords. Lipidated peptides; Chemoenzymatic Synthesis; Enzyme-labile protecting groups; Ras proteins.

Introduction

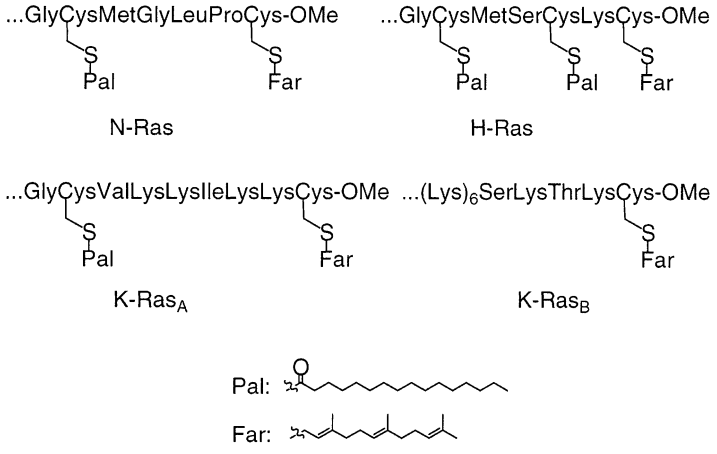
Lipidation of proteins was discovered only two decades ago, and to date only three different types of lipid groups have been found to react with proteins by myristoylation of the N-terminal amino group [1], S-prenylation (farnesyl- and geranylgeranyl groups) of cysteine residues at or close to the C-terminus, and S-palmitoylation [1, 2] of cysteines throughout the chain (Scheme 1).

Lipid modified proteins are often attached to cell membranes. In many cases, they play crucial roles in the transduction of extracellular signals across the plasma membrane and into the nucleus. Particularly important examples are the N-, K-, and H-Ras proteins. All Ras proteins terminate in a farnesylated cysteine methyl ester. In addition, fully modified N-Ras and K-Ras_B are palmitoylated at a cysteine residue close to the C-terminus, whereas H-Ras is palmitoylated twice. K-Ras_A is not palmitoylated, but carries a polylysine sequence close to the farnesylated cysteine methyl ester which enhances binding to the plasma membrane (Scheme 2).

* Corresponding author
[a] Both addresses valid for both authors



Scheme 1. Lipid modifications of proteins



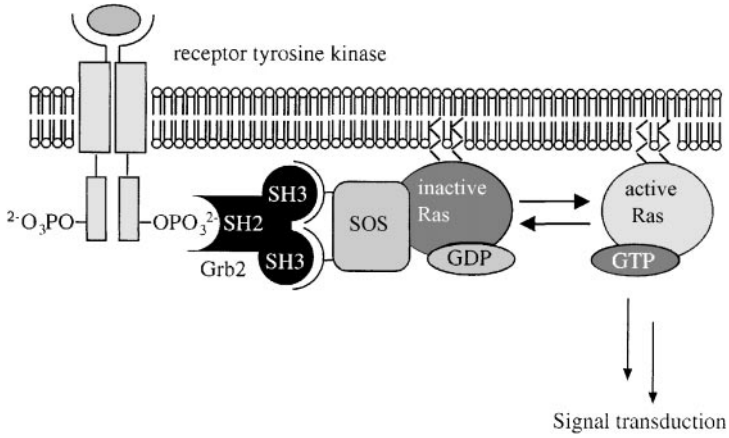
Scheme 2. Structure of lipid-modified C-termini of the Ras proteins

Lipid modification is essential for both membrane association and biological function of all Ras proteins. Non-lipidated Ras is biologically inactive.

Signal Transduction *via* Ras Proteins

Ras proteins influence numerous signal transduction processes and function as molecular binary switches which are activated by exchange of *GDP* for *GTP* and deactivated by hydrolysis of the γ-phosphate group in *GTP* to regenerate *GDP* [3].

In the Ras signal transduction cascade, monomeric receptor tyrosine kinases dimerize upon binding to an extracellular ligand, e.g. a growth factor. Then the monomer units activate each other by cross phosphorylation (Scheme 3). The phosphorylated receptors are subsequently recognized by adaptor molecules which link the receptor to signal transducers. Of particular importance is the growth factor receptor binding protein (Grb2). On the one hand, Grb2 binds *via* a SH2 domain to



Scheme 3. Ras activation by receptor tyrosine kinases; SH2: Src (Sarcoma) Homology 2, SH3: Src (Sarcoma) Homology 3, SOS: Son of Sevenless

the receptor peptide sequence containing the phosphotyrosine moiety. On the other hand, it recognizes proline-rich sequences in another adaptor protein named SOS by means of two SH3 domains. Thereby, the cytosolic proteins Grb2 and SOS are localized and correctly aligned on the inner side of the cell membrane. SOS then interacts with the inactive *GDP*-bound form of Ras and activates it by mediating exhange of *GDP* for *GTP*. Activated *GTP*-Ras then binds to further proteins like Raf, thereby passing the signal on.

The Ras signal transduction cascade is of extreme physiological importance. It is central to the regulation of cell growth and differentiation, and false regulation of this signal pathway can affect one of the critical steps leading to cell transformation [4]. The Ras pathway is highly conserved in differen species, and its elements are used in the same way for transmission of growth signals in, for example, yeast, worms, flies, and mammals.

A mutation in Ras genes might cause a high *GDP-GTP* exchange rate or a suppressed *GTP*ase activity. Both kinds of mutations lead to continuously active Ras proteins emitting a permanent growth signal that can result in tumor formation. This malfunction is serious as statistical numbers exemplify: a mutation in Ras is found in approximately 30% of all human cancers, and in some of the major malignancies it reaches 80% [5]. The involvement of Ras genes in human cancer is not limited to their activation by point mutations. It is likely that expression of abnormally high levels of normal Ras products may also contribute to malignancy.

Although the Ras protein and its signalling activity are the subject of intense research activities, the possible functions of the lipid groups in Ras biology remain largely unclear, in particular their role in the selective targeting of Ras to the plasma membrane and the possible involvement in interactions with upstream or downstream effectors. For studying these problems, Ras peptides embodying the correct lipid parts as well as analogs thereof may be efficient tools. Further functionalities may be also required by which the peptides can be traced in biological systems. For their synthesis, enzymatic protecting group techniques have proven to be a key technology. The development of these enzyme-labile blocking functions for the construction of Ras peptides and analogs thereof is the subject of this review.

Enzyme-labile Protecting Groups in the Synthesis of Lipidated Peptides

For the synthesis of lipidated peptides and proteins, the lability of the thioester under basic conditions and towards nucleophiles poses a considerable problem. Furthermore, under acidic conditions addition of the acid to the prenyl group double bonds readily occurs. The combination of both lipid modifications, prenyl and S-acyl groups, dramatically limits the number of usable protecting groups. The acid lability of the prenyl groups (Scheme 1) excludes the application of *tert*-butyl and benzyl-type functions. Similarly, the use of *Fmoc* and related groups is ruled out due to the base lability of thioesters. For the synthesis of peptides embodying an acid-labile farnesyl thioether and a base-labile thioester, new protecting groups are required which can be removed under extremely mild, preferably neutral, conditions.

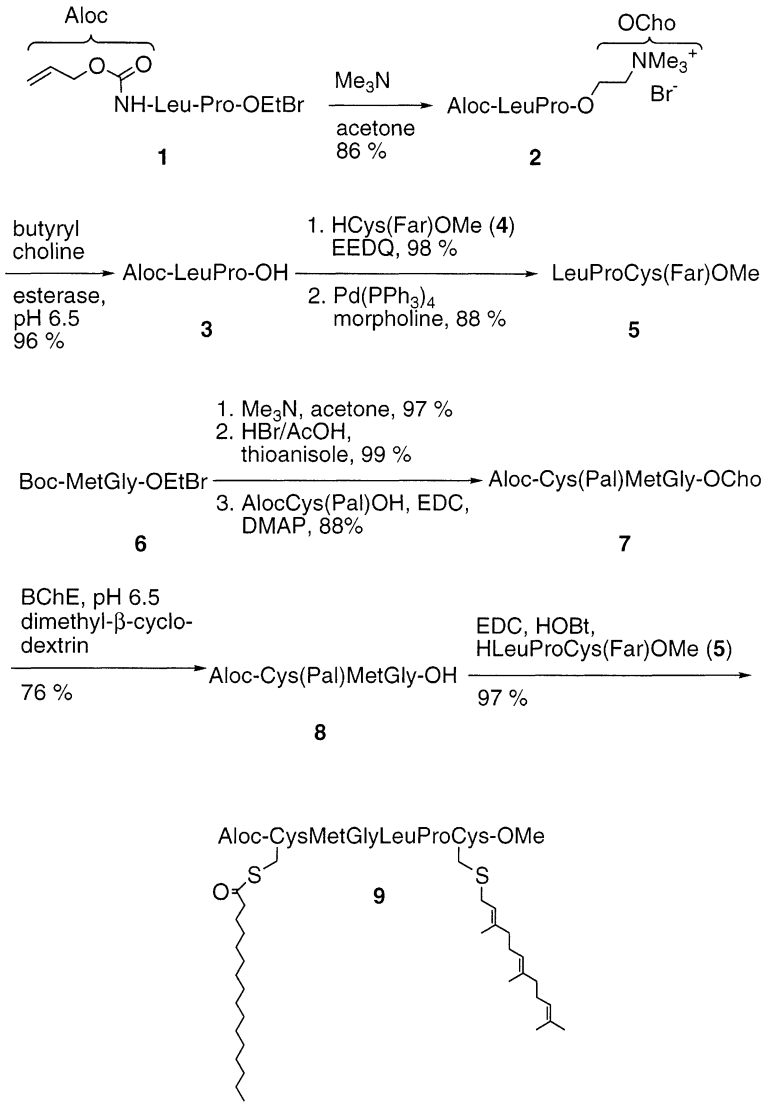
A possible solution is the use of enzyme labile protecting groups [6], since enzymatic transformations often can be carried out under advantageous and characteristically mild reaction conditions ($pH = 6$–8, room temperature to 40°C). In addition, enzymes often combine a high specificity for the recognized substrates with a large tolerance for secondary structures. Alternatively, noble metal transformations offer reaction conditions that are also mild enough to not react with sensitive, doubly lipidated peptides [7].

Enzymatic C-terminal deprotection

For the selective C-terminal deprotection of acid- and base-sensitive lipidated peptides the choline ester moiety was introduced as an enzyme-labile blocking function that can be removed under vey milk conditions [8]. Choline esters of simple peptides, but also of sensitive peptide conjugates like phosphorylated and glycosylated peptides [9], nucleopeptides [10], and also lipidated peptides [8, 11], can be cleaved with choline esterases under virtually neutral conditions. Both acetyl choline esterase (AChE) and butyryl choline esterase (BChE) can be employed for this purpose. As a rule, the butyryl choline esterase catalyzed deprotections proceed faster and result in higher yields. The high specificity of both enzymes for the choline group guarantees that only choline esters are attacked, and complete chemoselectivity is achieved. The conditions for this enzymatic deprotection are so mild that neither acid-labile farnesyl groups nor base-sensitive thioesters are attacked.

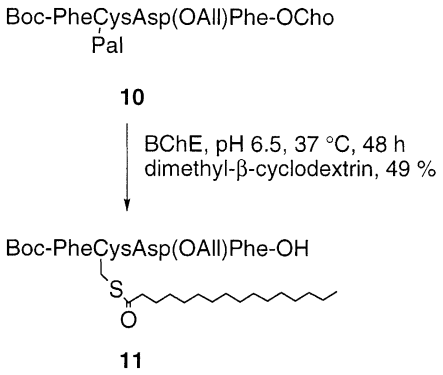
Amino acid choline esters can be synthesized readily by treatment of the corresponding 2-bromoethyl esters with trimethylamine. The charged choline esters have a pronounced solubility in aqueous solvents, a highly desirable property required for the unmasking of otherwise fairly hydrophobic lipopeptides.

For instance, in a synthesis of N-Ras lipopeptide **9**, the 2-bromoethyl ester **1** was converted into the corresponding choline ester by treatment with NMe_3. It was then removed by treatment with butyryl choline esterase (BChE) in high yield. C-Terminal elongation with S-farnesylated cysteine methyl ester using 2-ethoxy-N-ethoxycarbonyl-1,2-dihydroquinoline (*EEDQ*) as a condensing reagent followed by palladium catalyzed *Aloc* cleavage gave access to farnesylated tripeptide **5**. Similarly, 2-bromoethyl ester **6** was converted to the corresponding choline ester. Then the *Boc* group was removed, and the resulting selectively unmasked dipeptide choline ester was coupled to S-palmitoylated allyloxycarbonyl protected cysteine.



Scheme 4. N-Ras synthesis employing the choline ester as C-terminal protecting group; *EEDQ*: 2-ethoxy-N-ethoxycarbonyl-1,2-dihydroquinoline, *DMAP*: dimethylaminopyridine, *EDC*: 1-ethyl-3-(dimethylamino)-propylcarbodiimide hydrochloride, HO*Bt*: 1-hydroxybenzotriazole

From the palmitoylated base-labile tripeptide **7**, the choline ester was removed selectively and in high yield by means of the enzyme in the presence of dimethyl-β-cyclodextrin. Under these conditions, the normally chemically reactive thioester is not attacked. Thus, the biocatalyst reverses the usually observed chemoselectivity. The optimization of this deprotection step proved to be a formidable challenge. Peptide choline esters are usually highly soluble in water; thus, the substrates become readily accessible to the biocatalyst, and the use of additional solubilizing cosolvents that might denature the enzyme may be reduced or rendered unnecessary. However, the S-palmitoylated choline ester **7** is only sparingly soluble in purely aqueous media. Initial experiments using 5% of organic



Scheme 5. Enzyme catalyzed deprotection of a palmitoylated Y_1 receptor peptide

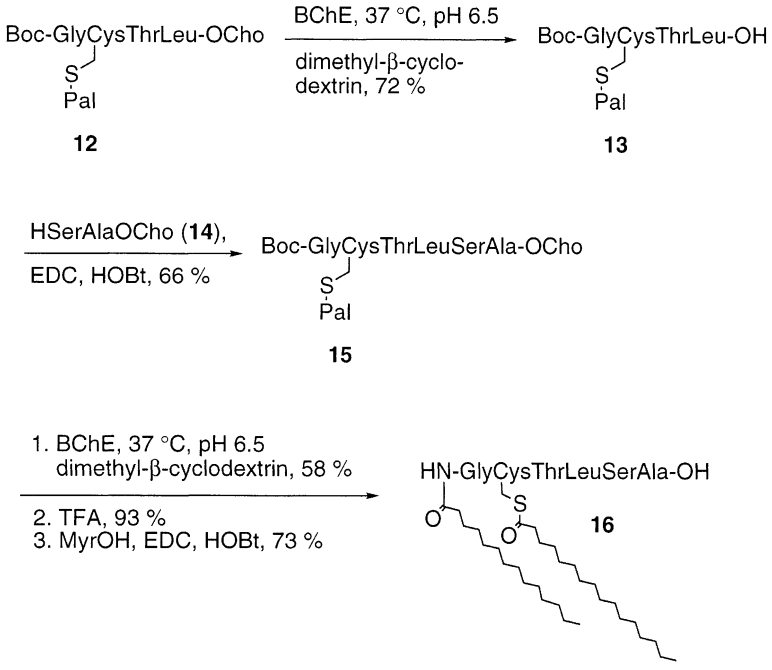
cosolvents resulted in low yields, probably due to denaturation. Furthermore, in the presence of methanol, the biocatalyst catalyzes a transesterification to yield the undesired methyl ester. The addition of dimethyl-β-cyclodextrin instead of standard organic cosolvents, however, resulted in an enhanced solubility of peptide **7** and in a smooth conversion to the free acid **8**. Cyclodextrins are cyclic heptasaccharides with a hydrophobic cavity. It is assumed that the cyclodextrines can slip over the hydrophobic lipid residues, thereby shielding them from the solvent. In addition, this shielding may also prevent non-enzymatic hydrolysis of the thioesters. Efficient coupling of both lipidmodified tripeptides **5** and **8** in high yield completed the synthesis of the target peptide **9**.

Similarly, cyclodextrins were successfully applied in a synthesis of palmitoylated tetrapeptide **11** [12]. Tetrapeptide choline ester **10** was deprotected by BChE in the presence of dimethyl-β-cyclodextrin. In the absence of the solubility enhancer this reaction did not proceed.

A similar strategy was applied in the synthesis of the hexapeptide **16** which represents the characteristic N-myristoylated and S-palmitoylated amino terminus of the $G_{\alpha 0}$-protein [11].

The synthesis of the doubly lipidated peptide **16** proceeded *via* enzyme catalyzed deprotection of the palmitoylated tetrapeptide choline ester **12** in the presence of cyclodextrin. C-Terminal elongation with dipeptide choline ester **14** gave the corresponding hexapeptide **15**. Once again treatment of **15** with BChE in the presence of the solubility enhancing cyclic heptasaccharide resulted in smooth hydrolysis of the choline ester without affecting the thioester. Finally, treatment with trifluoroacetic acid (*TFA*) followed by introduction of the myristoyl group gave the fully modified hexapeptide **16** in good yield.

However, in severe cases, even the combined use of choline esters and cyclodextrins can not overcome inherent solubility problems. This observation was particularly present in various N-myristoylated lipopeptides. For instance, initially the synthesis of **16** was attempted by starting with a previously myristoylated analogue of **12**. The doubly lipidated peptides were only slightly soluble in the aqueous buffer even in the presence of cyclodextrins, and no enzymatic deprotection could be observed. Addition of organic cosolvents did not improve the deprotection but rather resulted in denaturation of the enzyme.



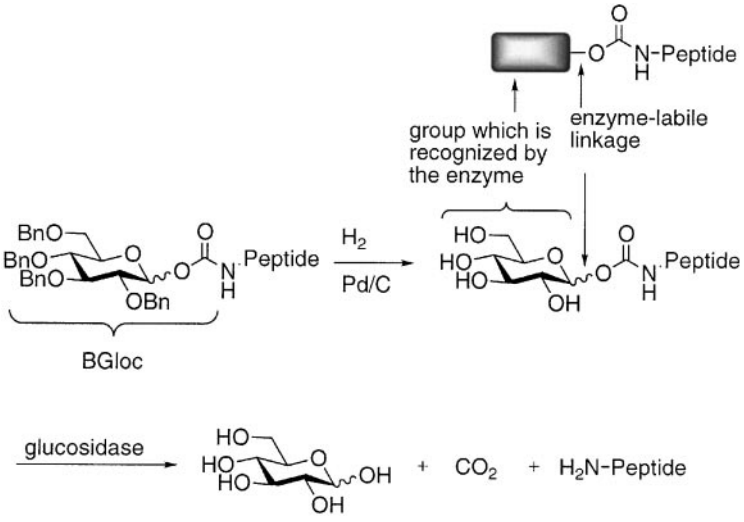
Scheme 6. Choline ester strategy for the synthesis of a myristoylated and palmitoylated hexapeptide corresponding to the $G_{\alpha 0}$-protein N-terminus; *TFA*: trifluoroacetic acid, *EDC*: 1-ethyl-3-(dimethylamino)-propylcarbodiimide hydrochloride, HO*Bt*: 1-hydroxybenzotriazole

Enzyme labile amine protecting groups

The development of enzyme-labile protecting groups for the N-terminus of peptides and peptide conjugates poses a major challenge. First, these blocking functions have to be removable under mild conditions to prevent base mediated thioester hydrolysis as well as acid catalyzed addition to the prenyl double bonds. In addition, they should be removable with a biocatalyst which does not attack other functional groups present in the molecule, especially the palmitoyl thioesters. Finally, they have to embody a function group which is specifically recognized by the enzyme, preferably an urethane structure to avoid racemization upon amino acid activation. Unfortunately, most enzymes available today do not attack urethanes which might be due to diminished reactivity of the urethane carbonyl group. An alternative strategy would be to employ a biocatalyst which attacks a different bond, *e.g.* an O-alkyl or an ester bond, and to design such a urethane accordingly.

For instance, the design of the carbohydrate based tetrabenzylglucosyloxy-carbonyl (*BGloc*) protecting group is based on the ability of glycosidases to hydrolyze the glycosidic bond of the corresponding sugar, the bond between C-1 and O-1 [13]. In this case, the enzyme (here: a glucosidase) initially cleaves the glycosidic C-O bond to the carbohydrate thereby rendering an attack on the less reactive urethane unnecessary (Scheme 7).

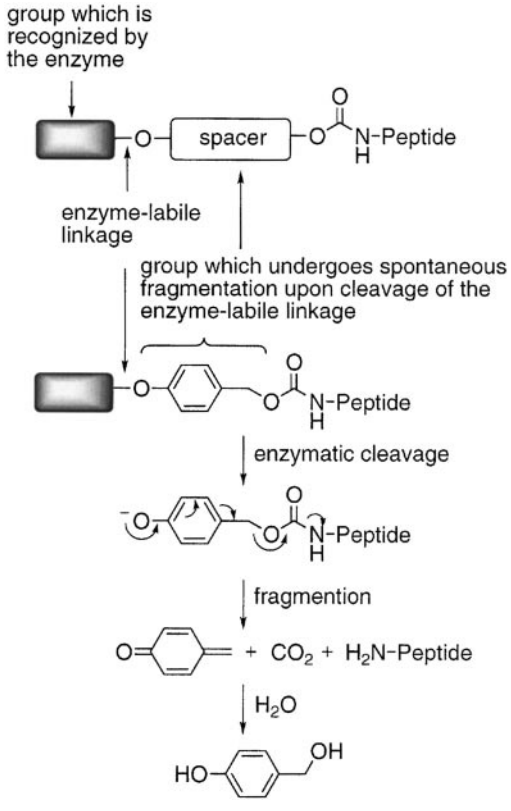
The removal of *BGloc* proceeds *via* a two step chemoenzymatic protocol. First, in the chemical step, the benzyl ethers are cleaved by hydrogenolysis. Second, enzaymatic treatment with a glucosidase to yield glucose, CO_2, and the desired free



Scheme 7. The *BGloc* protecting group

amine was achieved. However, such carbohydrate based protecting group have not yet been used in the synthesis of lipidated peptides.

Another general strategy for the design of enzymatically removable protecting groups is to link the urethane *via* a spacer to the functional group which is



Scheme 8. Enzymatically removable protecting groups embodying a fragmenting spacer unit

specifically recognized by the enzyme. Upon cleavage of the enzyme sensitive bond, the spacer undergoes spontaneous fragmentation. In this fragmentation process, a carbamic acid derivative is liberated which decarboxylates to finally yield the desired peptide or peptide conjugate (Scheme 8).

The principle of the enzymatic deprotection depicted in Scheme 8 is general. Depending on the acyl group chosen, the fragmentation of the resulting *p*-acyloxybenzyl urethane can be initiated with an appropriate enzyme. An additional advantageous feature is that the variable peptide part of the substrate is remote from the site of the biocatalysis. Thus, possible unfavourably steric or electronic interactions of the protein with the peptide caused by bulky amino acid side are minimized. Therefore, this enzymatic protecting group technique can be applied for the construction of peptides and analogues thereof containing, for instance, unnatural amino acids including the *D* amino acids.

The p-acetoxybenzyloxycarbonyl group (AcOZ)

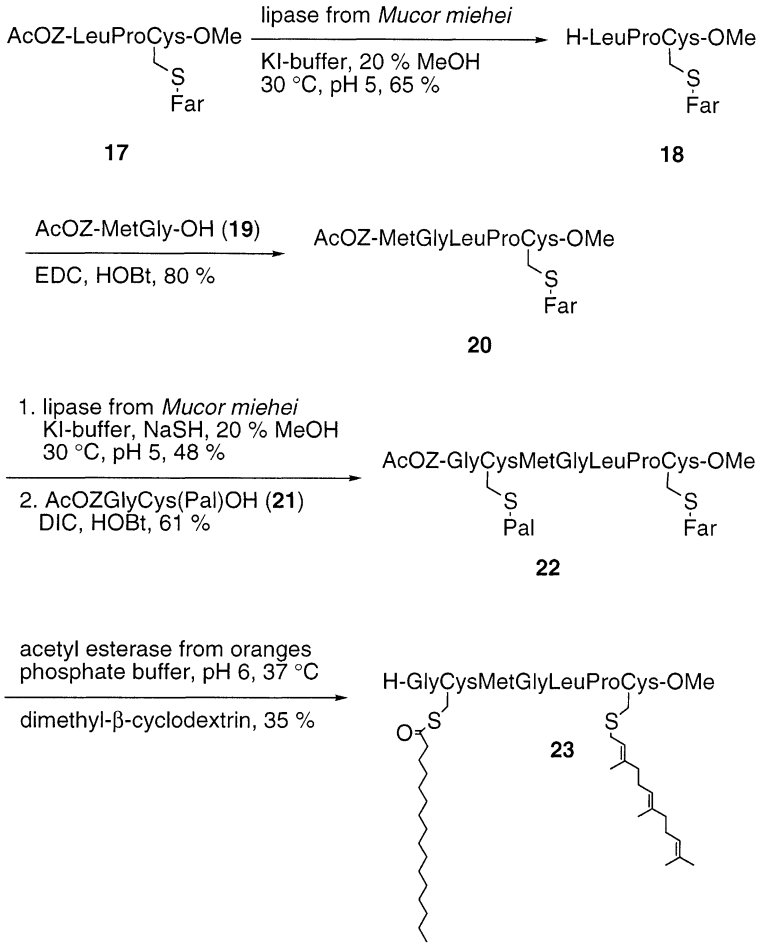
The *p*-acetoxybenzyloxycarbonyl (AcOZ) group, originally introduced as base-labile blocking function [14], can be cleaved readily by means of lipase- or esterase- initiated fragmentation under exceptionally mild conditions (*pH* 5–6) [15, 16].

AcOZ urethanes can be removed efficiently by a lipase from *Mucor miehei* and an acetyl esterase from the *flavedo* of oranges [17]. The lipase deprotected even sterically demanding amino acids. In addition, it tolerated high amounts of methanol as a cosolvent. On the other hand, the acetyl esterase discriminated between acetyl and longer acyl side chains. This feature was useful especially for the removal of AcOZ in the presence of palmitoyl thioesters (Scheme 9).

The synthesis of the C-terminal N-Ras heptapeptide **23** proceeded *via* lipase catalyzed removal of the AcOZ group from tripeptide **17** in the presence of 20% methanol as solubilizing cosolvent [16]. During the course of this reaction, one equivalent of quinone methide was formed by fragmentation of the linker. To trap this reactive intermediate, an excess of potassium iodide was added. It should be noted that the cysteine methyl ester was not affected under these conditions. N-terminal elongation with dipeptide **19** yielded a pentapeptide that was again deprotected under lipase catalysis. Further elongation resulted in the palmitoylated and farnesylated heptapeptide **22**. Acetyl esterase catalyzed removal of the AcOZ urethane from **22** was accomplished at *pH* 6 in the presence of the base labile thioester. Dimethyl-β-cyclodextrins were added to improve solubility of peptide **22**. Although conversion of **22** was complete, due to its amphiphilic nature part of the desired heptapeptide **23** was lost during the isolation procedure.

Synthesis of Lipidated Peptides for Biological Investigations

The development of the methodologies discussed thus far has allowed for the synthesis of a variety of lipid modified peptides representing characteristic partial structures of naturally occuring lipidated proteins. However, for the study of biological phenomena, additional analogues with modified lipid or peptide structure may be required. In addition, the introduction of reporter groups, which allow for monitoring the intracellular fate of the peptide conjugates, may be necessary. Depending on the nature of the lipid group, several problems have been studied.

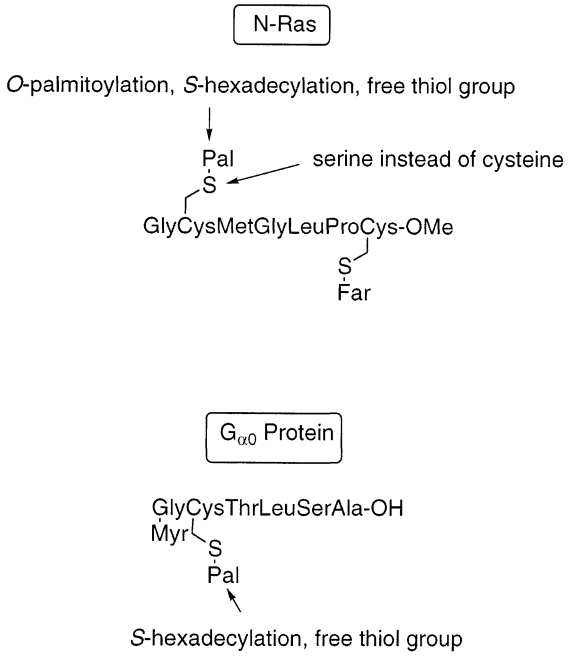


Scheme 9. Use of the AcOZ urethane in the synthesis of palmitoylated and farnesylated N-Ras heptapeptide **23**

Under physiological conditions, S-palmitoylation is a reversible process. Thus, the regulation of palmitoylation/depalmitoylation processes may be involved in the steering of biological phenomena like regulated membrane trapping mechanisms. To investigate such mechanisms, peptides are needed which either can not be palmitoylated (*i.e. Cys→Ser* or *Cys→Ala* mutants) or which are irreversibly modified, i.e. embodying a cysteine hexadecyl thioether instead of the corresponding palmitoyl thioester. In the case of the N-Ras C-terminus and the N-terminal sequence of the human $G_{\alpha 0}$-protein (Scheme 10), such analogues were synthesized.

Hexadecyl thioethers were synthesized by alkylation of mercapto groups with hexadecyl bromide [8b,11,16,18]. If free thiol groups in peptides are generated for or during a biological experiment, a suitable protecting group (e.g. *tert*-butyl disulfide) must be introduced which can be removed during the final steps of the synthesis.

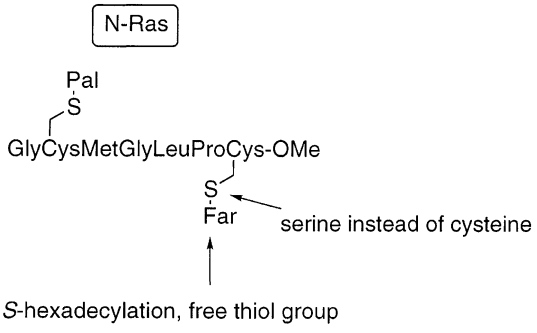
Similarly, the farnesyl group in proteins may participate in protein-protein interactions. Even more so, the existence of farnesyl receptors in membranes is believed to be possible. On the other hand, only the hydrophobicity of that lipid group might account for its physiological consequences. Thus, for the investigation



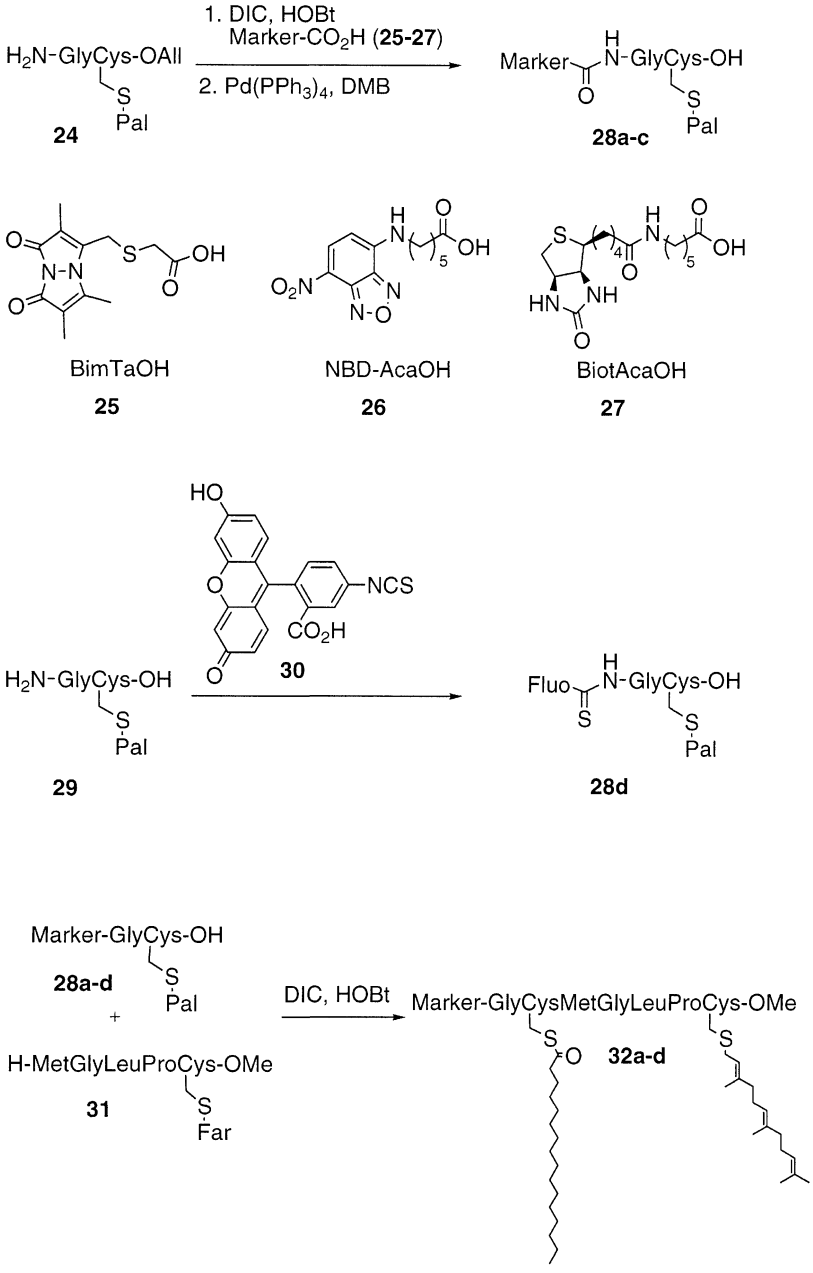
Scheme 10. Variation of S-lipidation and amino acid residues for the investigation of the biological function of protein palmitoylation

of the biological importance of protein farnesylation, analogues are needed which display a similar hydrophobicity but different structure (*e.g. n*-alkyl ethers). Other analogues, especially in the case of palmitoylated Ras proteins, contain either an unmodified cysteine or a cysteine which has been replaced for a serine [19].

For biological assays, lipidated peptides embodying a fluorescent label like the bimanyl-, the *NBD*-group, or fluoresceine are required for determining cell uptake, membrane binding or subcellular distribution by fluorescence spectroscopy and fluorescence microscopy. Also, attachment of a biotin group allows researches to trace such modified peptides by means of the protein streptavidine, which may carry a fluorescent lable or gold clusters [30]. In general, such functional groups can be attached to the amino group of selectively N-terminal deprotected

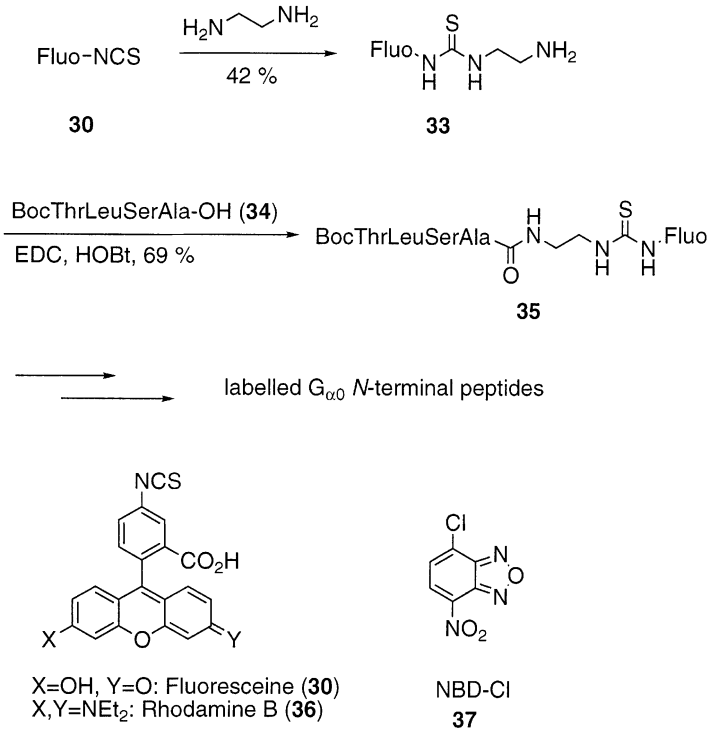


Scheme 11. Peptides for the investigation of the biological function of the farnesyl thioether



Scheme 12. Synthesis of lipidated peptides carrying a fluorescent label at the N-terminus; *DIC*: diisopropylcarbodiimide, HO*Bt*: N-hydroxybenzotriazole, *DMB*: dimethylbarbituric acid

peptides like **24** (Scheme 12). Thus, lipidated peptide **24** was treated with S-bimanylthioacetic acid (*BimTa*OH, **25**), 7-nitrobenzofurazene-4-aminocaproic acid (*NBD-Aca*OH, **26**), or fluoresceine isothiocyanate (**30**) to afford fluorescent-labelled peptides **28** [16]. Accordingly, coupling with biotinylaminocaproic acid (**27**) yielded biotinylated peptides like **28c** [8b]. Condensation of these dipeptides with farnesylated peptide **31**, which is available *via* AcOZ-technology (Scheme 9), led to the formation of labelled C-terminal N-Ras peptides.



Scheme 13. Synthesis of lipidated peptides with a fluorescent marker at the C-terminus

For the introduction of fluorescent markers at the C-terminal carboxy group in peptides, fluoresceine isothiocyanate (**30**) was treated with ethylenediamine (Scheme 11). The amino-functionalized label was then condensed with peptide **34** followed by an N-terminal deprotection/chain elongation process [11]. Accordingly, rhodamine B isothiocyanate (**36**) and 4-chloro-7-nitrobenzofurazene (*NBD*-Cl, **37**) were converted into the respective ethylenediamine derivative and were attached to the peptide carboxyl groups.

Lipidated peptides embodying the characteristic linkage region found in the parent lipoproteins and bearing additional functional groups which could be traced in biological systems or which allowed for their use in biophysical experiments were used successfully in model studies. The application of lipid modified conjugates in binding experiments to vesicles and model membranes, in membrane fusion experiments, and in microinjection studies has led to the proposal of a mechanism for the targeting of Ras proteins to the plasma membrane [17, 21].

For discussion of these experiments and the consequences for targeting of Ras and related proteins to the plasma membrane the reader is referred to more comprehensive review [22].

Conclusions and outlook

The enzyme-mediated syntheses of lipidated peptides described in the previous sections clearly demonstrate the capabilities of enzymatic transformations, in particular enzymatic protecting group techniques. Today, an increasing number of

biological phenomena is being investigated, elucidated, and understood in molecular detail at a rapidly increasing speed. For organic chemistry, this opens up a multitude of new spheres of activity in which its power and strength can be used to the full extent and in which new, great, and important challenges are presented. In mastering them, organic chemistry can rise to a key role.

Acknowledgements

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