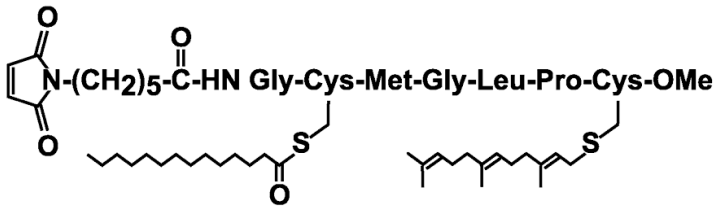
Molecular Biology

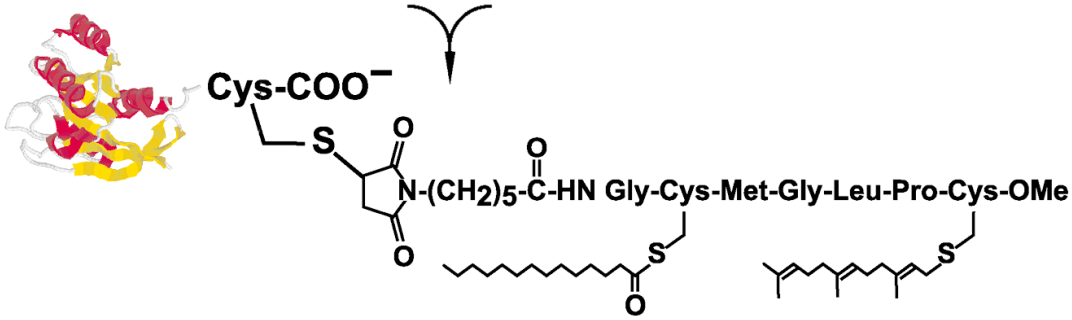
Overexpression of C-Terminally Truncated Ras

Organic Chemistry



Synthesis of Lipidated Ras Peptides

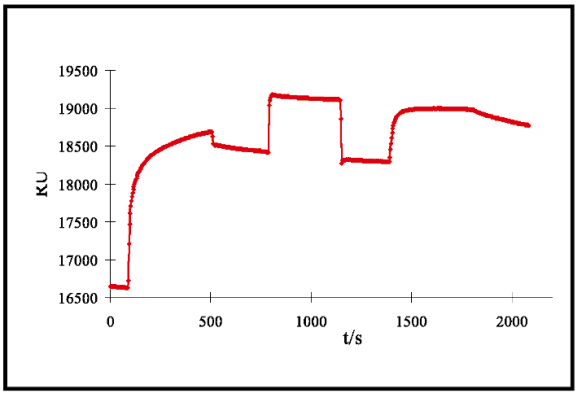
Coupling



Lipidated Ras Proteins

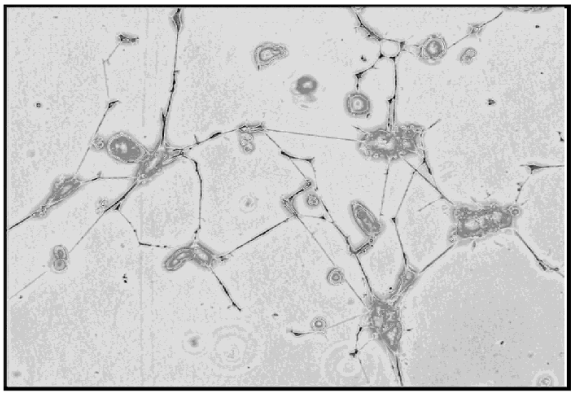
Isolation and Characterization

Biophysics



Surface Plasmon Resonance

Cell Biology



Microinjection (Fluorescence) Microscopy

Biological Result

Dissection of Lipoprotein Interactions in a Membraneous Environment

  1439-4227/00/01/03 $ 17.50+.50/0 CHEMBIOCHEM **2000**, 1, 144 – 169

Linking the Fields—The Interplay of Organic Synthesis, Biophysical Chemistry, and Cell Biology in the Chemical Biology of Protein Lipidation

Dieter Kadereit,[b] Jürgen Kuhlmann,*[a] and Herbert Waldmann*[b]

Dedicated to Professor Horst Kunz on the occasion of his 60th birthday

Research in the biological sciences has undergone a fundamental and dramatic change during the last decades. Whereas biology was more phenomenologically oriented for a long time, today many biological processes are investigated and understood in molecular detail. It has become evident that all biological phenomena have a chemical basis: Biology is based on chemical principles. In the past, this insight had led to the development of biochemistry, molecular biology, and modern pharmacology. Today it increasingly determines the manner in which various biological phenomena are studied. The tools provided by classical biological techniques often are not sufficient to address the prevailing issues in precise molecular detail. Instead, the strengths of both chemical and biological methodology have to be used. Several recent research projects have proven that combining the power of organic synthesis with cell biology may open up entirely new and alternative opportunities for the study of biological problems. In this review we summarize the successful interplay between three disciplines—organic synthesis, biophysics, and cell biology—in the study of protein lipidation and its relevance to targeting of proteins to the plasma membrane of cells in precise molecular detail. This interplay is highlighted by using the Ras protein as a representative example. The development of methods for the synthesis of Ras-derived peptides and fully functional Ras proteins, the determination of their biophysical properties, in particular the ability to bind to model membranes, and finally the use of synthetic Ras peptides and Ras proteins in cell biological experiments are addressed. The successful combination of these three disciplines has led to a better understanding of the factors governing the selective targeting of Ras and related lipid-modified proteins to the plasma membrane.

KEYWORDS:

bioorganic chemistry · biophysics · cell biology · protein lipidation · signal transduction

In its early youth, organic chemistry was so closely connected to biology. I do consider it not only possible but desirable, that the close connection of chemistry with biology […] should be reestablished, as the great chemical secrets of life are only to be unveiled by cooperative work.

Emil Fischer
(Faraday Lecture: "Synthetical Chemistry in Its Relation to Biology": *J. Chem. Soc.* **1907**, 1749)

1. Introduction

During the last decades, the biological sciences have undergone a fundamental and dramatic change. Whereas biology was more phenomenologically oriented for a long time, today many biological processes are investigated and understood in molecular detail. It has become evident that all biological phenomena have a chemical basis: Biology is molecular, it is based on chemical principles. This insight increasingly influences and determines the manner in which research is carried out when studying various biological phenomena. The tools provided by classical biological techniques often are not sufficient to address the prevailing issues in precise molecular detail. Due to the fundamental chemical nature of biological problems, chemical expertise is urgently required, and the strengths of both chemical and biological methodologies have to be used. For instance, several research projects have recently proven that combining the power of organic synthesis with cell biology may

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Dieter Kadereit,

born in 1971 in Bremen (Germany), started studying chemistry at the Universität Hannover in 1991. After investigating rhodium(I)-catalyzed cycloadditions in the group of P. A. Wender at Stanford University in 1994/95, he finished his diploma in Hannover and Aachen with a thesis on manganese-catalyzed asymmetric oxidation reactions in 1996. In 1997 he joined the group of H. Waldmann to work on the synthesis and evaluation of lipidated peptides.

Jürgen Kuhlmann,

born in 1962 in Schwerte a. d. Ruhr (Germany), studied chemistry at the Universität Dortmund and completed his Ph.D. thesis with kinetic and thermodynamic studies on ligand binding of the nicotinic acetylcholine receptor in the group of Alfred Maelicke at the Max Planck Institute for Nutrition Physiology. After three years of work as a supervisor for quality assurance in the environmental analysis laboratories of the Chemische Laboratorien Dr. E. Weßling GmbH in Altenberge (Germany), he returned to Dortmund and became manager of the working group Biophysical Analysis in the department of Structural Biology (Director: Alfred Wittinghofer) in the newly founded Max Planck Institute for Molecular Physiology. The interests of his group are macromolecular interactions in transport processes between nucleus and cytosol, the effect of posttranslational modifications of proteins of the Ras family, and the development of new tests for mutagenity in chemical cancerogenesis.

Editorial Advisory Board Member:[*]
Herbert Waldmann,

born in 1957, received his Dr. rer. nat. degree in 1985 (Universität Mainz, Horst 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 Institute of Molecular Physiology (Department of Chemical Biology), Dortmund, and as Full Professor of Biochemistry at the Universität 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 reasearch 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 his group (further information: www.chemie.uni-karlsruhe.de/OC/akwa/ak.htm).

[*] *Members of the Editorial Advisory Board will be introduced to the readers with their first manuscript.*

open up entirely new and alternative opportunities for the study of biological problems.[1] To use an illustration, chemistry has provided "flashlights" to illuminate the darkness inside cells, for instance in the study of signaling processes through proteins[2] and low molecular weight second messengers.[3]

With the rapidly increasing flow of information emerging from deciphering the genomes of entire organisms, especially the human genome project, synthetic approaches will become more widely applicable. From the nucleotide sequence of a gene, the amino acid sequence of a protein can be deduced, but this does not automatically unravel its biological function. The biological function of a protein is determined by various other factors like specific localization and, in particular, by posttranslational modification. In fact, many proteins are posttranslationally modified, and such information may be obtained from proteomics rather than genomics.[4] To understand the importance of posttranslational modification and the biological role in precise molecular detail, analysis of structure alone, however, is not sufficient. It is necessary to alter the chemical structure and study the different properties resulting. Characteristic partial sequences of modified proteins and, eventually, of entire proteins themselves as well as of analogues with varied structure may provide valuable tools for the study of biological function at the molecular level.

Such modified peptide and protein conjugates whose structure can be rapidly varied at will, are generally not, or only in isolated cases,[5] accessible by biological methods. They therefore have to be synthesized in the laboratory. However, organic synthesis has limitations as well, and the development of new methods of organic synthesis for the construction of multifunctional and sensitive peptide and protein conjugates and their combination with biological techniques may be required. Furthermore, before applying synthetic peptide and protein conjugates in biological studies, their physical properties may have to be determined to precisely interpret the biological data that are subsequently acquired.

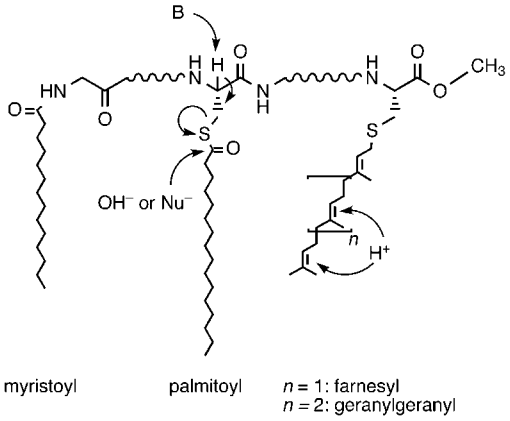
In this review, we summarize the successful interplay between three disciplines—organic synthesis, biophysics, and cell biology—in the study of protein lipidation and its relevance to targeting of proteins to the plasma membrane of cells in precise molecular detail. This interplay is highlighted by using the Ras

protein as a representative example. The development of methods for the synthesis of Ras-derived peptides and fully functional Ras proteins, the determination of their biophysical properties, in particular the ability to bind to model membranes, and finally the use of synthetic Ras peptides and Ras proteins in cell biological experiments are addressed.

This highly interdisciplinary research project may serve as one illustrative example for the multitude of opportunities emerging at the interface between chemistry and biology, which may be best addressed in molecular detail by a combination of both sciences.

2. Protein lipidation

Lipidation of proteins was discovered only two decades ago, and three different types of lipid groups have been found so far (Scheme 1): Myristoylation of the N-terminal amino group of proteins,[6b] *S*-prenylation (farnesyl and geranylgeranyl groups) of cysteine residues at or close to the C terminus, and *S*-palmitoylation[6] of cysteines throughout proteins. In addition, a few *O*-acylated peptides or proteins have been identified.[7, 8]



Scheme 1. *Lipid modifications of proteins. B = base, Nu = nucleophile.*

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. A particularly important example 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_A are palmitoylated at a cysteine close to the C terminus while H-Ras is palmitoylated twice. K-Ras_B is not palmitoylated, but carries a polylysine sequence close to the farnesylated cysteine methyl ester, which enhances binding to the plasma membrane (Figure 1). Lipid modification is essential for both membrane association and biological function of all Ras proteins. Nonlipidated Ras is cytosolic and biologically inactive.

3. Signal transduction through Ras proteins

Ras proteins influence numerous signal transduction processes and function as molecular binary switches that are activated by

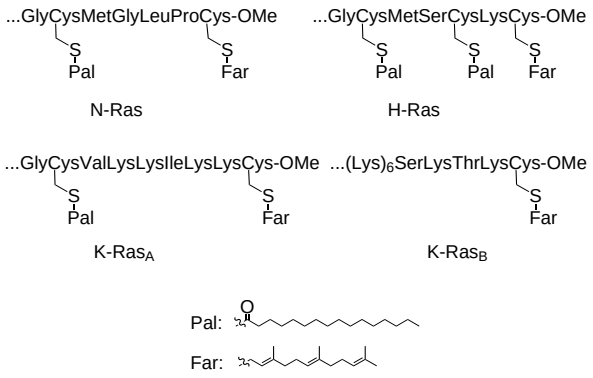


Figure 1. *Structure of lipid-modified C termini of the Ras proteins.*

exchange of bound GDP for GTP and deactivated by hydrolysis of the γ-phosphate group in bound GTP to regenerate GDP.[9] In the Ras signal transduction cascade, monomeric receptor tyrosine kinases dimerize upon binding to an extracellular ligand, for example a growth factor. Then the monomer units activate each other by cross-phosphorylation (Figure 2). The phosphorylated receptors are subsequently recognized by

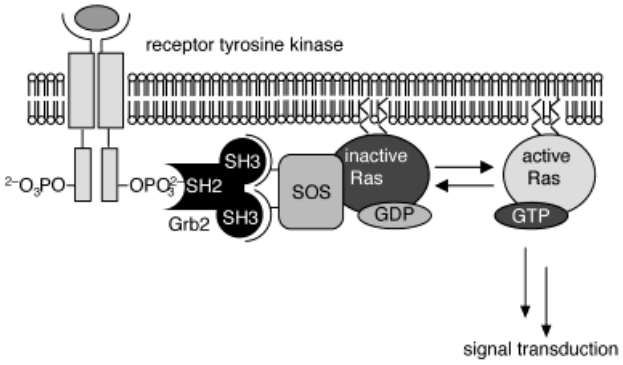


Figure 2. *Ras activation by receptor tyrosine kinases.*

adapter 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 through an SH2 domain to the receptor peptide sequence containing the phosphotyrosine moiety. On the other hand, it recognizes proline-rich sequences in another adapter protein named Sos (son of sevenless, named after a *Drosophila* mutant) 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 exchange of GDP for GTP. The receptor – Grb2 – Sos complex functions as a guanine nucleotide exchange factor (GEF). Activated GTP – Ras then binds to other proteins like Raf, thereby passing the signal on (Figure 3). Association of GTP-bound Ras with a GTPase-activating protein (GAP) leads to hydrolysis of GTP to GDP, resulting in deactivation of the signal transducer.

Activated Ras binds to and activates the cytosolic Raf kinase which then triggers the mitogen-activated protein (MAP) kinase

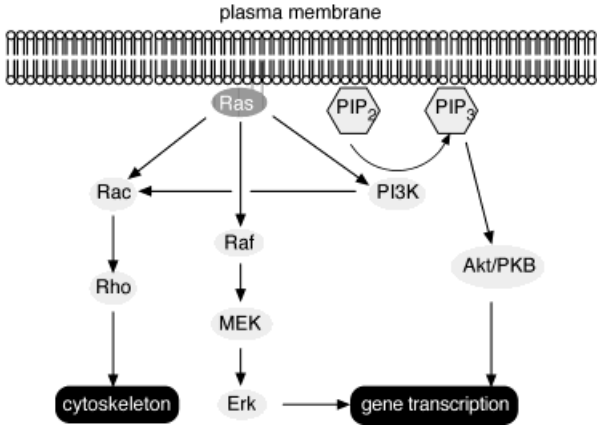


Figure 3. *Signal transduction pathways linked to Ras. PI3K = phosphatidylinositol 3-kinase.*

cascade by phosphorylating the MAP kinase kinase MEK (Figure 3). MEK subsequently phosphorylates Erk, which in turn phosphorylates transcription factors. Upon phosphorylation these proteins gain the ability to enter the nucleus where they influence gene transcription.

GTP – Ras also activates phosphatidylinositol 3-kinase (PI3K) and Rac, which signal through two parallel signal transduction pathways. The GTP-binding proteins Rac and Rho play critical roles in controlling the actin cytoskeleton and in regulation of cell growth.[10] The precise nature of the connection between the Rac/Rho pathway and the MAP kinase cascade is the subject of intense investigation. However, it is likely that activation of members of the Rho family contributes significantly to the Ras-transformed phenotype (see below). PI3-kinase is activated upon direct interaction with active Ras. It then phosphorylates phosphatidylinositol 4,5-diphosphate (PIP_2) to yield phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which acts as a second messenger. PIP_3 is recognized by protein kinase B (PKB, also termed Akt). Binding of Akt/PKB to PIP_3 activates Akt/PKB by localization to the plasma membrane and leads to partial activation of its kinase activity.

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 be one of the critical steps leading to cell transformation.[11] The Ras pathway is highly conserved among different 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 GTPase 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 exemplified by statistical surveys: A mutation in Ras is found in approximately 30 % of all human cancers, and in some of the major malignancies the mutation rate is as high as 80 %.[12] 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.

During the last decades extensive research activities were initiated aiming at the precise understanding of signal transduction processes and, particularly, the biological role and molecular details of Ras-mediated signaling. In general, these investigations were based on genetic and cell biological approaches. They were highly successful in unraveling the general scenario of signal transduction through Ras and in illuminating the molecular details of some of the most important protein – protein interactions, like binding of Ras to Raf.[13] However, the Ras proteins employed in these studies were bacterially synthesized proteins lacking the C-terminal lipid modifications. Completely modified proteins were obtained from baculoviral expression systems in low yields only, and the palmitic thioesters were labile under the isolation conditions.[14] Furthermore, these biological techniques did not allow the introduction of modified lipid groups. Consequently, the function of the lipid groups in signaling through Ras remained 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, a flexible access to differently lipidated and biologically functional Ras proteins (to be used subsequently as molecular probes) was needed. This demand could only be met by an approach that combined techniques of organic synthesis, molecular biology, biophysics, and cell biology.[15] It includes: the development of methods for the synthesis of Ras peptides, the coupling to appropriately designed and expressed Ras mutants, the determination of the biophysical properties of the synthetic neo-Ras proteins, and applications in cell biological experiments. By combination of organic synthesis, biophysics, and cell biology, the precise role of the lipid-modifications of farnesylated and palmitoylated Ras proteins in their selective targeting to the plasma membrane could be better understood.

4. Synthesis of lipid-modified peptides containing one lipid group

Peptides bearing only one type of lipid modification can be synthesized by employing established protecting group techniques. Thioesters are stable under the conditions required for the removal of acid-labile blocking functions, farnesyl and geranylgeranyl thioethers are not attacked at $pH > 7$, and myristoyl amides tolerate both reaction conditions.

Due to their biological relevance, the synthesis of prenylated peptides has been addressed in various studies and the different prenylation procedures were recently reviewed.[16] The synthesis of *N*-acylated compounds follows established procedures of solid-phase chemistry, whereas only a few examples for the synthesis of *S*-palmitoylated peptides have been published.

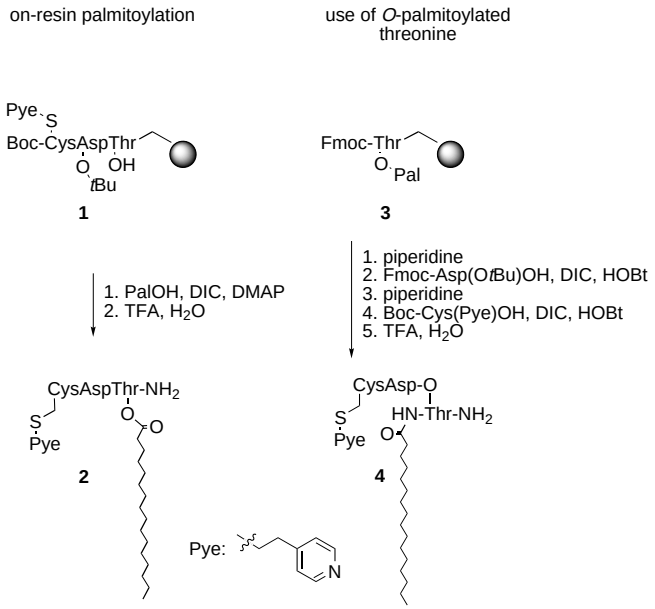
4.1. Synthesis of *N*-myristoylated peptides

N-myristoylation occurs only at the N terminus of signal transducing proteins. In solid-phase peptide synthesis, the lipid modification can be introduced either by using an already modified building block or by acylation of the N-terminal amino

acid. The latter method avoids solution phase chemistry, and the acylation step is completely compatible with solid-phase synthesis.[17]

4.2. Synthesis of *O*-palmitoylated peptides

The C-terminal threonine of the 44-mer peptide PLTX II from the venom of the spider *Plectreurys* is *O*-palmitoylated. The solid-phase synthesis of this compound was attempted by two methods, that is palmitoylation of a Thr-containing peptide resin and incorporation of Fmoc-Thr(Pal) (Scheme 2).[8] To investigate the on-resin palmitoylation, the resin-bound tripeptide Boc-Cys(Pye)-Asp(O*t*Bu)-Thr was treated with a 10-fold excess of palmitic acid, DIC, and DMAP. HPLC analysis showed that the desired product was predominantly formed.

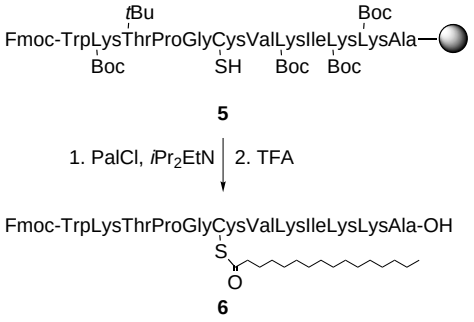


Scheme 2. *Solid-phase synthesis of an* O*-palmitoylated tripeptide. Boc* = tert-*butyloxycarbonyl, DIC* = *diisopropylcarbodiimide, DMAP* = *4-dimethylaminopyridine, HOBT* = *1-hydroxy-1*H*-benzotriazole, Pal* = *palmitoyl, TFA* = *trifluoroacetic acid.*

Incorporation of the Fmoc-Thr(Pal) building block proved to be more complicated. After analysis of the product mixture, Pal-Thr[*O*-Asp-Cys(Pye)]-NH_2 (**4**) was found as the major component indicating that an $O \rightarrow N$ acyl shift had occurred during the first Fmoc deprotection with piperidine. By switching to the Boc strategy, the synthesis of PTLX II was successfully completed.[18] Six fragments were synthesized by solid-phase chemistry and then assembled in solution. Unfortunately, some partial depalmitoylation was observed during the final deprotection procedure with HF/butanedithiol/anisole (90:7.5:2.5).

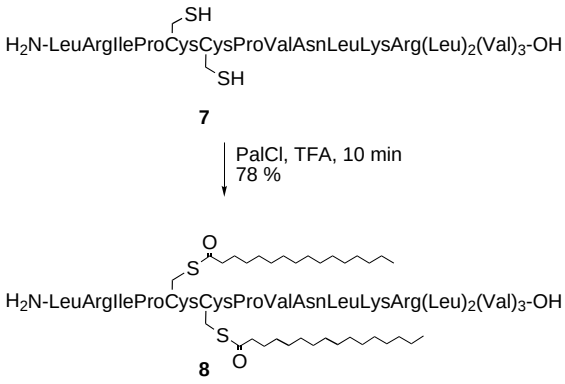
4.3. Synthesis of *S*-palmitoylated peptides

Despite the wide occurrence of *S*-palmitoylated proteins, only few syntheses of *S*-acylated peptides have been reported. A solid-phase procedure similar to the synthesis of the *O*-palmitoylated peptide PTLX II described above was not successful because of a rapid $S \rightarrow N$ acyl shift.[19] An alternative is a final *S*-acylation of a selectively *S*-deprotected peptide in solution[20] or on the solid phase.[21] For the acylation procedure, all other side chain functionalities have to be blocked because often an excess of the acylation reagent is required. For instance, treatment of resin-bound peptide **5** with two equivalents of palmitoyl chloride and two equivalents of diisopropylethylamine resulted in *S*-palmitoylation within twelve hours (Scheme 3). Acylated peptide **6** was then cleaved from the resin and deprotected simultaneously with trifluoroacetic acid giving the crude peptide **6** in 70% overall yield, which was judged to be 90% pure by HPLC analysis.



Scheme 3. *On-resin* S*-palmitoylation of a fully protected peptide. Fmoc* = *9-fluorenylmethoxycarbonyl.*

Recently, the *S*- and *O*-palmitoylation of unprotected peptides in trifluoroacetic acid was described.[22] By treatment with a 20-fold excess of palmitoyl chloride, the peptide **7** was doubly acylated, with a 78% yield of isolated product. The acidic conditions prevented any *N*-acylation which, however, occurred after prolonged reaction times (Scheme 4).



Scheme 4. *Selective* S*-palmitoylation in trifluoroacetic acid.*

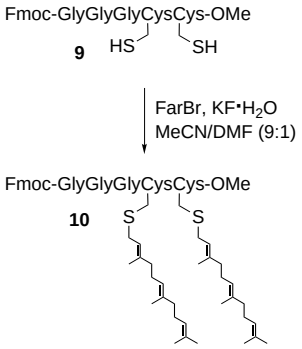
While the *S*-palmitoylation of peptides with acyl chlorides is straightforward, biocatalyzed processes are not yet available for this purpose.

4.4. Synthesis of *S*-prenylated peptides

The synthesis of prenylated peptides[16] is mostly performed by using the solid-phase synthesis of the unmodified peptide followed by *S*-prenylation. A final deprotection step, which should be carried out under basic conditions due to the acid lability of the prenyl group, yields the free modified peptide.

Usually, the prenyl group is only introduced during the final steps of the synthesis, although *S*-alkyl ethers are chemically stable under the conditions of solid-phase peptide synthesis. Prenylation reactions were carried out both under basic and acidic conditions. Several problems were encountered during the *S*-alkylation reaction, that is incomplete conversion due to solubility problems, oxidation of the thiol group to the disulfide, formation of the sulfonium ion when excess alkyl halide was used, alkylation of functional groups other than the cysteine thiol group, and hydrolysis of the prenylation reagent.

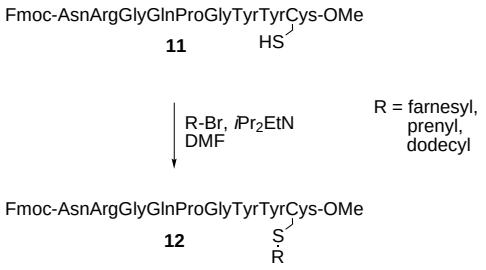
Under basic conditions, oxidation of the thiol group is a major concern, but by working under an inert gas atmosphere, this side reaction is eliminated. If only one equivalent of alkylating agent is used, the thiol groups can be prenylated in the presence of free amino groups. Solubility of the peptide, however, might be too low for a rapid conversion of the starting material. Solvent systems described for this reaction include liquid ammonia, ammonia in methanol, pure DMF, or DMSO/DMF/MeCN mixtures. For instance, peptide **9** was doubly farnesylated in a MeCN/DMF solvent mixture by treatment with an excess of farnesyl bromide in the presence of KF (Scheme 5).[23] This



Scheme 5. *Peptide prenylation under basic conditions. Far = Farnesyl.*

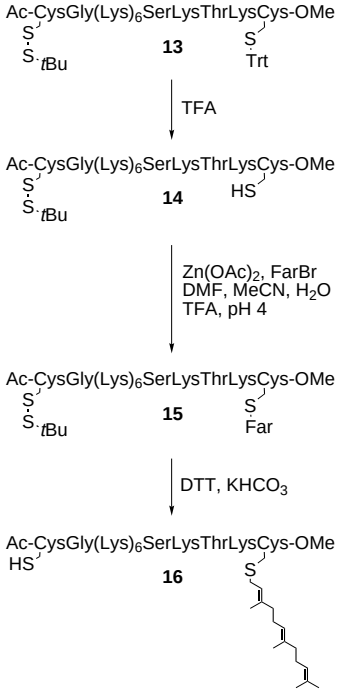
protocol can be generally applied for the *S*-alkylation of cysteine thiol groups.[24] For the synthesis of fungal pheromone analogues, crude peptide **11** was treated with two equivalents of alkyl bromide, for example dodecyl bromide, and eight equivalents of diisopropylethylamine (Scheme 6). After removal of the Fmoc group of **12** with piperidine, the alkylated peptides were isolated by HPLC in 27 – 36 % yield.

The solubility of peptides is often much better in acidic solvents. This property is especially critical for peptides containing several basic functional groups. In the presence of zinc acetate, efficient alkylation was achieved with aqueous/organic solvent mixtures containing TFA or acetic acid. The trityl group of peptide **13**, which corresponds to the K-Ras$_B$ C terminus, was



Scheme 6. *Peptide alkylation under basic conditions.*

cleaved with trifluoroacetic acid (Scheme 7).[25] Farnesylation of **14** under basic conditions (DMF, H_2O, $KHCO_3$) proved to be unsatisfactory, whereas treatment of **14** with farnesyl bromide at pH 4 in the presence of $Zn(OAc)_2$ gave better results. However, *N*-prenylation can occur under these conditions as a side reaction.[26] Finally, reduction of the *tert*-butyl disulfide with

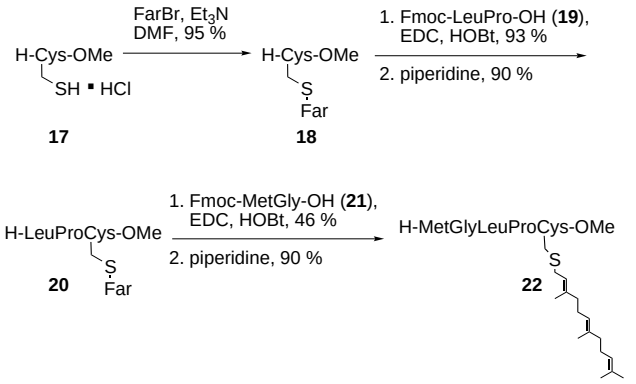


Scheme 7. *Peptide prenylation under acidic conditions. DTT = dithiothreitol, Trt = trityl = triphenylmethyl.*

dithiothreitol led to the formation of the fully modified C-terminal K-Ras$_B$ peptide **16**. An alternative route to prenylated peptides is the enzymatic prenylation catalyzed by either farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase I). For this procedure, a prenyl pyrophosphate is required as a reactant. The enzymatic prenylation is discussed in Section 5.1.

In a completely different approach, the synthesis of *S*-alkylated peptides with previously modified cysteine derivatives takes advantage of the stability of thioethers under basic conditions. By applying the Fmoc strategy, such peptides are

readily available (Scheme 8).[27] For instance, cysteine methyl ester **17** was selectively *S*-farnesylated in high yield under basic conditions. Subsequently, a repeated coupling/Fmoc deprotection made pentapeptide **22** readily accessible. This compound



Scheme 8. *Stepwise synthesis of prenylated peptides, starting with cysteine methylester hydrochloride (**17**). EDC = N′(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride.*

can then be used for further synthetic purposes like the preparation of fluorescently labeled derivatives (see Section 4.6). A major advantage of this methodology is that the critical *S*-alkylation is not performed during the final steps of the preparation procedure, thereby simplifying product separation and isolation. Alternatively, if necessary, the selectivity during the *S*-alkylation of cysteine residues can be easily controlled by using appropriate protecting groups.

4.5. Synthesis of peptides with two different lipid groups

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.[24, 27] 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 because of 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 that can be removed under extremely mild, preferably neutral, conditions.

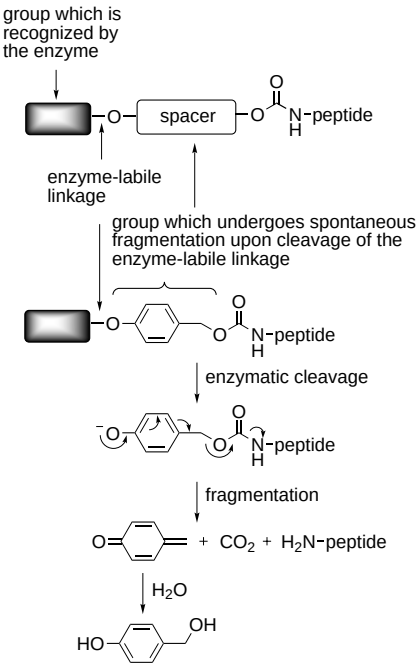
A possible solution is the use of enzyme-labile protecting groups[29] since enzymatic transformations often can be carried out under characteristically mild reaction conditions (pH 6 – 8, room temperature to 40 °C). In addition, enzymes often combine a high specificity for the functional groups they recognize with a large tolerance for substrate structure. Alternatively, transformations employing noble metals offer reaction conditions that are also mild enough to be compatible with sensitive, doubly lipidated peptides.

4.5.1. 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 that does not attack other functional groups present in the molecule, especially the palmitoyl thioesters. Finally, they have to embody a functional group which is specifically recognized by the enzyme, and most importantly, an urethane structure to avoid racemization upon amino acid activation. Unfortunately, most enzymes available today do not attack urethanes. This might be due to a diminished reactivity of the urethane carbonyl group. An alternative strategy would be to employ a biocatalyst that attacks a different bond, for example an *O*-alkyl[30] or an ester bond, and to design such a urethane accordingly.

A general strategy for the design of such enzymatically removable urethane protecting groups is to link the urethane to the functional group through a spacer, which is specifically recognized by the enzyme. Upon cleavage of the enzyme-labile 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 9).

The principle of the enzymatic deprotection depicted in Scheme 9 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 biocatalyst's attack. Thus, possible

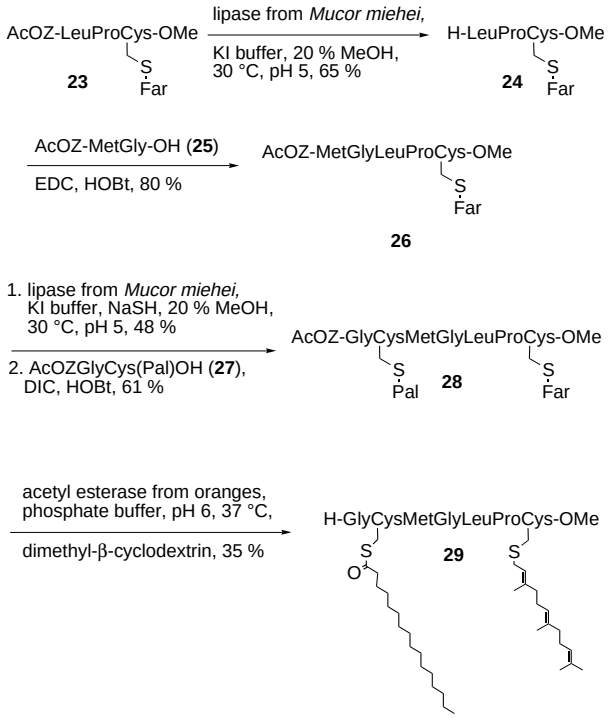


Scheme 9. *Enzymatically removable protecting groups embodying a spacer that can undergo fragmentation.*

unfavorable steric or electronic interactions of the protein with the peptide caused by bulky amino acid side chains are minimized. This enzymatic protecting group technique can therefore be applied for the construction of peptides and analogues thereof containing, for instance, nonnatural amino acids including D-amino acids.

The p-*acetoxybenzyloxycarbonyl (AcOZ) group*

The *p*-acetoxybenzyloxycarbonyl (AcOZ) group, originally introduced as a base-labile blocking function,[31] can be cleaved readily by means of lipase- or esterase-initiated fragmentation under exceptionally mild conditions (pH 5 – 6).[32, 33] AcOZ-protected urethanes can be removed efficiently by a lipase from *Mucor miehei* or an acetyl esterase from the flavedo of oranges.[34] The lipase deprotects even sterically demanding peptides and it tolerates high amounts of methanol as a cosolvent. Acetyl esterase discriminates between acetyl and longer acyl side chains. This feature was especially useful for the removal of AcOZ in the presence of palmitoyl thioester groups (Scheme 10).



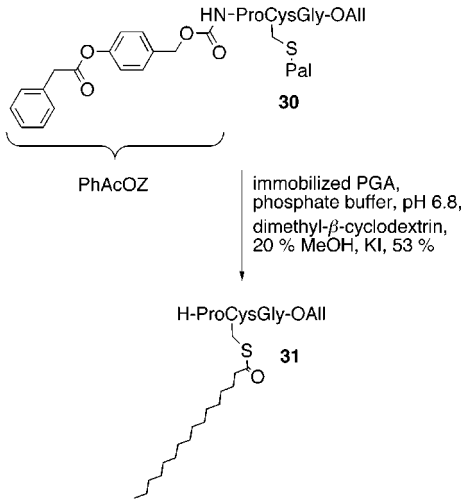
Scheme 10. *AcOZ strategy for the synthesis of palmitoylated and farnesylated N-Ras heptapeptide* ***29****. AcOZ =* p-*acetoxybenzyloxycarbonyl.*

The synthesis of the C-terminal N-Ras heptapeptide **29** proceeded through lipase-catalyzed removal of the AcOZ group from tripeptide **23** in the presence of 20% methanol as solubilizing cosolvent.[33] 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 **25** yielded a pentapeptide that was again deprotected under lipase catalysis. Further elongation resulted in the palmitoylated and farnesylated heptapeptide **28**. Acetyl esterase-catalyzed removal of the AcOZ urethane from **28** was accomplished at pH 6 in the presence of the base-labile thioester group. Dimethyl-β-cyclodextrins were added to improve the solubility of peptide **28**. Cyclodextrins (CDs) are cyclic hexa-, hepta- or octasaccharides (α-, β-, or γ-CDs, respectively) with a hydrophobic cavity. It is assumed that the cyclodextrins can slip over the hydrophobic lipid residues, thereby shielding them from the solvent. Although conversion of **28** was complete, due to its amphiphilic nature part of the desired heptapeptide **29** was lost during the isolation procedure.

The enzyme-initiated fragmentation of *p*-hydroxybenzyl alcohol based linkers has been successfully employed in the development of a prodrug in cancer chemotherapy.[35] After uptake, the therapeutic drug is released by cleavage of the enzyme-labile bond, mediated by intracellular enzymes, and subsequent fragmentation of the spacer.

The p-*phenylacetoxybenzyloxycarbonyl (PhAcOZ) group*

By analogy to the AcOZ group, the PhAcOZ urethane was introduced as an enzyme-labile protecting group for the synthesis of glycosylated and phosphorylated peptides.[36] The phenylacetate is recognized and cleaved by penicillin G acylase (PGA). This technology was used for the synthesis of a 29-mer peptide corresponding to the N terminus of endothelial NO synthase, which embodies two *S*-palmitoyl thioesters and a myristoyl group at the N terminus.[37] By adjusting the pH value between 6.5 to 6.8, enzyme-catalyzed PhAcOZ removal was performed in the presence of these base-labile thioesters. One of the critical deprotection steps in this very demanding synthesis is shown in Scheme 11. The successful completion of a 29-mer peptide, containing three lipid groups, proves the efficiency of the enzyme-cleavable protecting group technology and its applicability to the synthesis of large peptides.



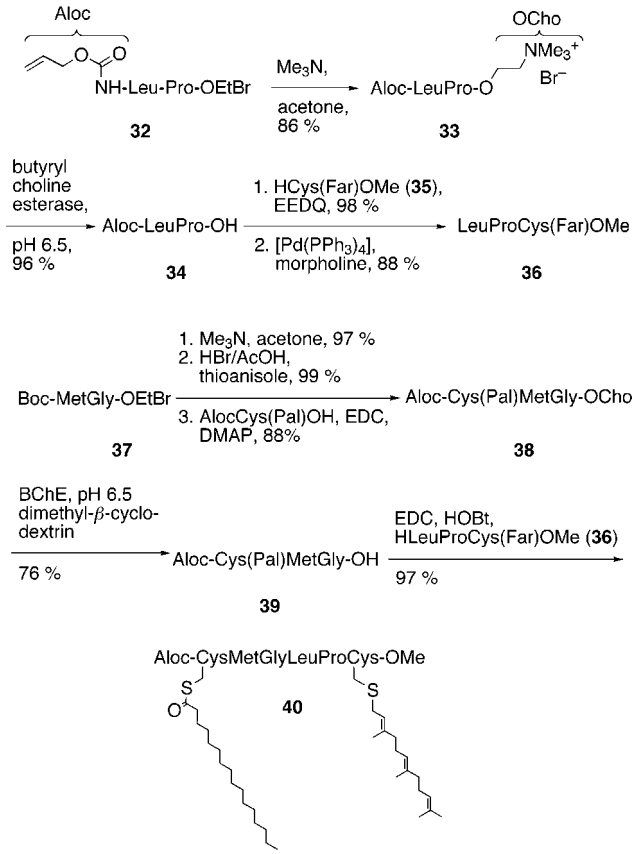
Scheme 11. *Synthesis of a tripeptide employing the PhAcOZ group. Peptide* ***31*** *is one of five building blocks used in the synthesis of a 29-mer peptide from NO synthase. All = allyl, PGA = penicillin G acylase, PhAcOZ =* p-*phenylacetoxybenzyloxycarbonyl.*

4.5.2. C-Terminal enzymatic deprotection

The choline ester (OCho) group

For the selective C-terminal deprotection of acid- and base-sensitive lipidated peptides, the choline ester group was introduced as an enzyme-labile blocking function that can be removed under very mild conditions.[38a] Choline esters of simple peptides, but also of sensitive peptide conjugates like phosphorylated and glycosylated peptides,[39] nucleopeptides,[40] and also lipidated peptides,[38, 41a] 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.

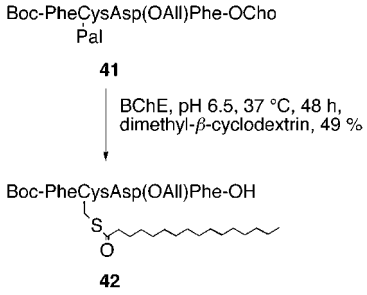
Amino acid choline esters can be synthesized readily by treatment of the corresponding 2-bromoethyl esters with trimethylamine. The charged choline esters have a pronounced solubility in aqueous solvents, a highly desirable property required for the biocatalyzed unmasking of otherwise hydrophobic and poorly soluble lipopeptides. For instance, in a synthesis of N-Ras lipopeptide **40**, the 2-bromoethyl ester **32** was converted into the corresponding choline ester by treatment with NMe_3. It was then removed by cleavage with butyryl choline esterase (BChE) in high yield. C-terminal elongation with *S*-farnesylated cysteine methyl ester by using 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as a condensing reagent followed by palladium-catalyzed cleavage of the allyloxycarbonyl (Aloc) group (see Section 4.5.3) gave access to farnesylated tripeptide **36**. Similarly, 2-bromoethyl ester **37** 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. From the palmitoylated base-labile tripeptide **38**, the choline ester group was removed selectively and in high yield by using the enzyme in the presence of dimethyl-β-cyclodextrin (Scheme 12). Under these conditions, the normally chemically reactive thioester moiety 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 so that 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 **38** is only sparingly soluble in purely aqueous media. Initial experiments with 5% of organic cosolvents resulted in low yields, probably due to denaturation of the enzyme. 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 **38** and in a smooth conversion to the free



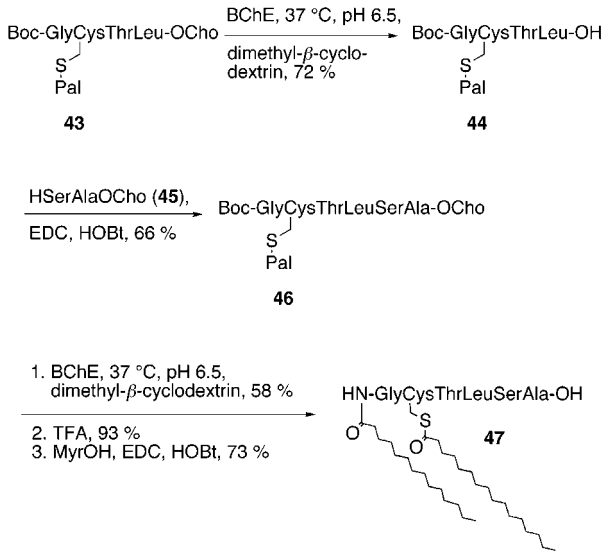
Scheme 12. *Synthesis of an N-Ras peptide employing the choline ester as C-terminal protecting group. Aloc = allyloxycarbonyl, BChE = butyryl choline esterase, EEDQ = 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline, HOCho = choline.*

acid **39**. Efficient coupling of both lipid-modified tripeptides **36** and **39** in high yield completed the synthesis of peptide **40**. Similarly, cyclodextrins were successfully applied in a synthesis of palmitoylated tetrapeptide **42**.[42] Tetrapeptide choline ester **41** was deprotected by BChE in the presence of dimethyl-β-cyclodextrin (Scheme 13). In the absence of the solubility enhancer, this reaction did not proceed.

A similar strategy was applied in the synthesis of the hexapeptide **47** (Scheme 14), which represents the characteristic *N*-myristoylated and *S*-palmitoylated amino terminus of human $G_{\alpha O}$ protein.[41a] The synthesis of the doubly lipidated peptide **47**



Scheme 13. *Enzyme-catalyzed deprotection of a palmitoylated Y_1 receptor peptide.*



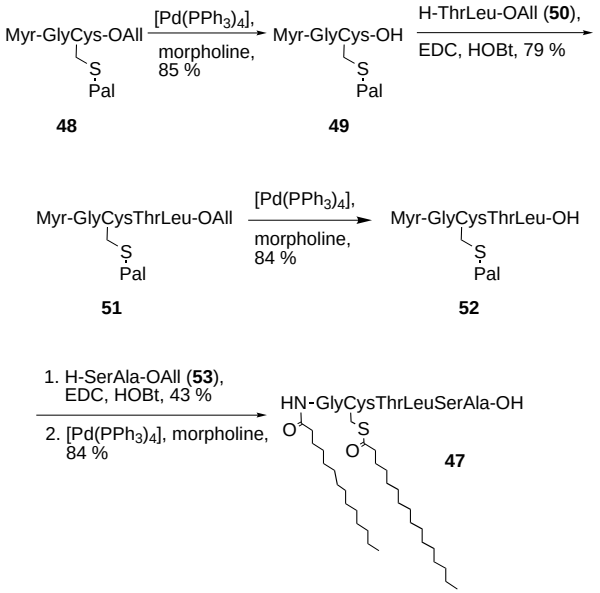
Scheme 14. *Choline ester strategy for the synthesis of a myristoylated and palmitoylated hexapeptide corresponding to the $G_{\alpha O}$ protein N terminus. MyrOH = myristic acid.*

proceeded via enzyme-catalyzed deprotection of the palmitoylated tetrapeptide choline ester **43** in the presence of a cyclodextrin. *C*-terminal elongation with dipeptide choline ester **45** gave the corresponding hexapeptide **46**. Once again, treatment of **46** with BChE in the presence of the solubility-enhancing cyclic heptasaccharide resulted in smooth hydrolysis of the choline ester without affecting the thioester moiety. Finally, treatment with trifluoroacetic acid followed by introduction of the myristoyl group gave the fully modified hexapeptide **47** in high yield. However, in some difficult cases, even the combined use of choline esters and cyclodextrins cannot overcome inherent solubility problems. This observation was particularly made during the unmasking of various *N*-myristoylated lipopeptides. For instance, initially the synthesis of **47** was attempted by starting with a previously myristoylated analogue of **43**. 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.

4.5.3. A noble-metal-sensitive alternative: the allyl ester (All) and the allyloxycarbonyl (Aloc) groups

The Pd^0-sensitive allyl (All) ester and the allyloxycarbonyl (Aloc) urethane groups were widely employed in glycopeptide synthesis[43] and have found widespread application in general organic synthesis, and particularly in peptide conjugate chemistry.[44] The selectivity and mildness of the Pd^0-catalyzed deprotection reaction also allowed for the successful and efficient application of this blocking group technology in the synthesis of acid- and base-labile lipidated peptides. Palladium-catalyzed removal of allyl blocking functions has proven to be a powerful alternative to the enzymatic protecting group methodology described above. The deprotection is usually carried out by the addition of a catalytic amount of $[Pd(PPh_3)_4]$ to a solution of the protected peptide in the presence of a nucleophile. The proper choice of the nucleophile often is crucial and usually depends on the stability of the peptide, as well as on the intended purification method. For instance, morpholine[43a] and dimethylbarbituric acid (DMB)[45] are compatible with base-labile thioesters. Both compounds and their mono-allylated derivatives can be removed by careful extraction with aqueous buffer. In addition, the use of a water-soluble ligand like triphenylphosphanyltrisulfonate sodium salt (TPPTS) and $Pd(OAc)_2$ as the palladium source can result in very pure deprotection products without the need for chromatography.[46] On the other hand, phenylsilane[47] cannot be easily removed by extraction. In particular, its use as an allyl cation scavenger leads to the complete conversion of lipidated peptides, when the use of other nucleophiles results in an incomplete deprotection. An example for the application of the palladium-catalyzed deprotection of allyl esters is the synthesis of the *N*-myristoylated and *S*-palmitoylated hexapeptide **47**, which corresponds to the N terminus of human $G_{\alpha O}$ protein.[41] To compare different protecting-group techniques, this peptide was synthesized employing both the allyl ester (Scheme 14) and alternatively the choline ester (see Section 4.5.2).

The doubly lipidated peptide **47** was synthesized from *S*-palmitoylated cysteinyl peptide **48** through a series of selective Pd^0-mediated C-terminal deprotection reactions employing morpholine as the allyl trapping reagent and subsequent chain elongations. As shown in Scheme 15, the noble-metal-catalyzed allyl transfer to morpholine as the accepting nucleophile proceeded in high yields. In the course of these transformations, the base-sensitive thioester was completely stable. In direct comparison to the analogous synthesis employing the enzyme-labile choline ester, the allyl ester group displayed significant



Scheme 15. *Allyl ester strategy for the synthesis of a myristoylated and palmitoylated hexapeptide corresponding to the $G_{\alpha O}$ protein N terminus.*

advantages. Most importantly, the difficulties associated with the limited solubility of the hydrophobic lipidated peptides in aqueous media (see above) were not encountered. The pronounced solubility of lipidated peptides in organic solvents guarantees that the allyl-protected compounds are readily accessible to the catalyst and that deprotection reactions proceed rapidly. Thus, in the case of the $G_{\alpha O}$ peptide syntheses shown in Schemes 18 and 19, the use of the allyl ester group was clearly superior to the choline ester blocking-group strategy.

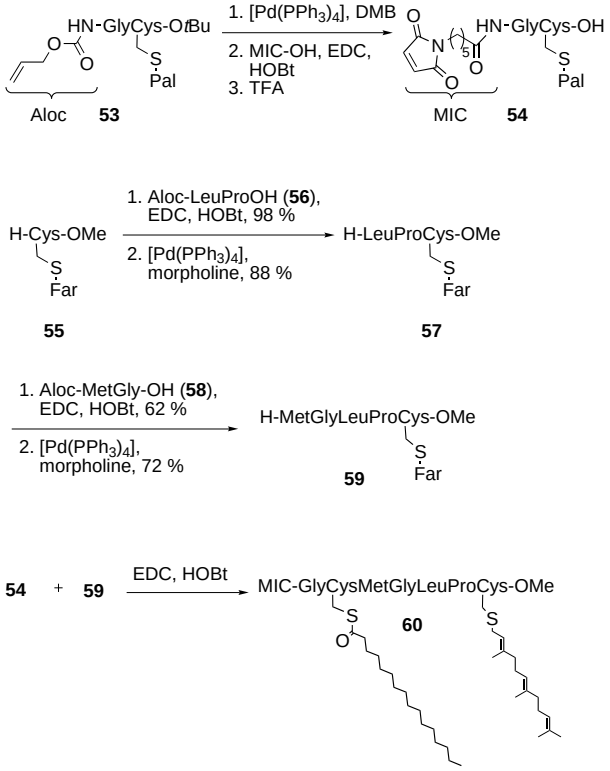
Allyl esters[34, 38b, 48] and the analogous allyloxycarbonyl group[15, 27, 49] have been successfully employed in Ras peptide syntheses. The suitability of the Aloc group for the construction of lipidated peptides is emphasized by the synthesis of the maleimidocaproyl-modified, *S*-palmitoylated and -farnesylated heptapeptide **60**, which corresponds to the N-Ras C terminus (Scheme 15).[49] In contrast to classical urethane-type protecting groups, the Aloc group can be removed in the presence of additional functional groups and under neutral conditions. It is therefore a very convenient protecting group for the synthesis of very hydrophobic lipid-modified peptides, which are not soluble in the aqueous media required for enzyme-catalyzed transformations.

In the synthesis of the maleimidocaproyl-modified peptide **60**, Pd^0-catalyzed deprotection of *S*-palmitoylated dipeptide **54** yielded the corresponding selectively deprotected peptide. After attachment of maleimidocaproic acid (MIC-OH), an acid-mediated removal of the *tert*-butyl ester gave access to the palmitoylated building block **54**. A condensation of **54** with farnesylated pentapeptide **59**, which was readily accessible by using the Aloc methodology[27] as depicted in Scheme 15, or by the Fmoc strategy[27], and alternatively using AcOZ[33] as the protecting group, resulted in the formation of target peptide **60** (Scheme 16).[15, 53]

In a similar fashion, the Aloc group was employed in the synthesis of farnesylated octapeptide **68** (Scheme 17). Pd^0-catalyzed Aloc removal from tripeptide **63** proceeded smoothly in the presence of phenylsilane, which served as the allyl-accepting nucleophile. The resulting peptide **64** was then coupled with tripeptide **62** by treatment with EEDQ as condensation reagent. A repeated C-terminal deprotection/chain elongation procedure resulted in the formation of octapeptide **67**. Subsequent treatment with a palladium(0) catalyst and $PhSiH_3$, followed by DTT, gave farnesylated peptide **68** in high yield.

4.6. Synthesis of lipidated peptides for biological investigations

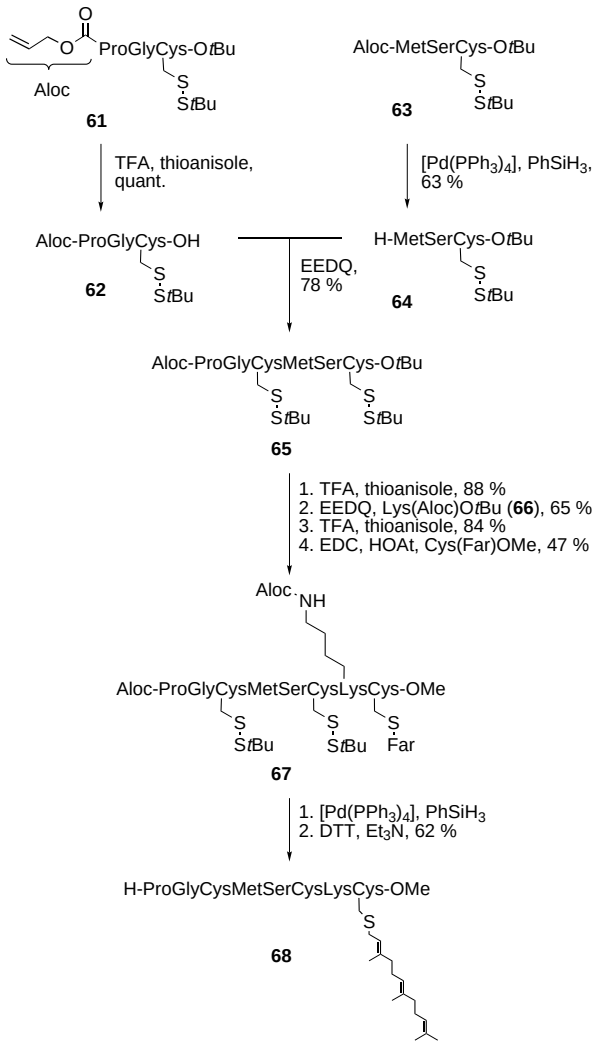
The development of the methodologies discussed above has allowed for the synthesis of a variety of lipid-modified peptides representing characteristic partial structures of the naturally occurring, lipidated parent proteins. However, for the study of biological phenomena, additional analogues with modified lipid or peptide structure may be required. In addition, the introduction of reporter groups, which make monitoring of the intracellular fate of the peptide conjugates possible, may be necessary.



Scheme 16. *Synthesis of maleimidocaproyl(MIC)-modified Ras heptapeptide **60** by using the Aloc strategy. DMB = dimethylbarbituric acid, HOBt = 1-hydroxy-1*H*-benzotriazole.*

Depending on the nature of the lipid group, several problems have been studied. 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 that either cannot be palmitoylated (i.e., Cys →Ser or Cys →Ala mutants) or are irreversibly modified, that is embodying a cysteine hexadecyl thioether instead of the corresponding palmitoyl thioester group. In the case of the N-Ras C terminus and the N-terminal sequence of the human $G_{\alpha O}$ protein such analogues were synthesized (Figure 4). Hexadecyl thioethers were synthesized by alkylation of mercapto groups with hexadecyl bromide according to the protocol of cysteine farnesylation (see Section 4.4).[24, 33, 38b, 41a] 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 that can be removed during the final steps of the synthesis. An example for the preparation of a farnesylated peptide embodying two additional unmodified cysteine residues is described in Section 4.5.3 (Scheme 17).

Similarly, the farnesyl group in proteins may participate in protein-protein interactions,[50, 51] and the existence of farnesyl receptors in membranes was postulated but not proven.[52] On the other hand, only the hydrophobicity of that lipid group might account for its physiological effects. Thus, for the investigation of the biological importance of protein farnesyla-



Scheme 17. *Aloc strategy for the synthesis of a farnesylated octapeptide corresponding to the nonpalmitoylated H-Ras C terminus.*

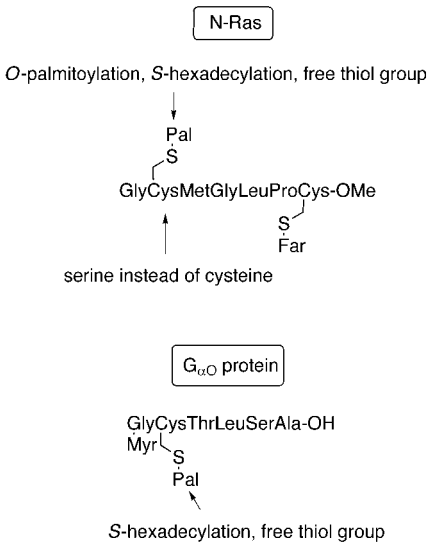


Figure 4. *Variation of S-lipidation or palmitoylatable amino acid residues for the investigation of the biological function of protein palmitoylation.*

tion, analogues are needed that display a similar hydrophobicity but different structure (e.g. *n*-alkyl ethers). Other analogues, especially in the case of palmitoylated Ras proteins, contain a cysteine that has been replaced by a serine (Figure 5).[15, 53] For biological assays, lipidated peptides embodying a fluorescent label like the bimanyl group, the NBD group, or fluoresceine are required for determining membrane binding or subcellular distribution by fluorescence spectroscopy and fluorescence microscopy, respectively. Also, attachment of a biotin group allows to trace modified peptides by means of the protein streptavidine, which may carry a fluorescent label or gold clusters.[54]

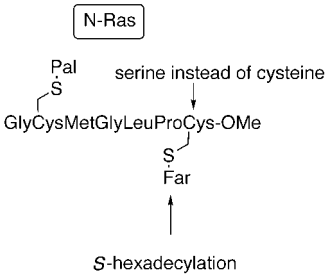
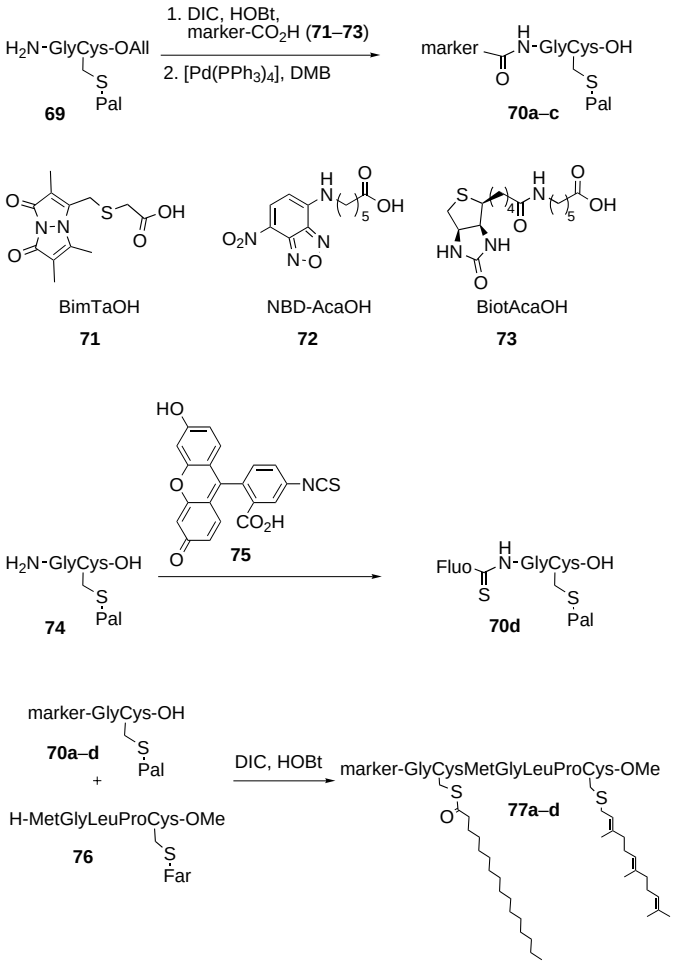


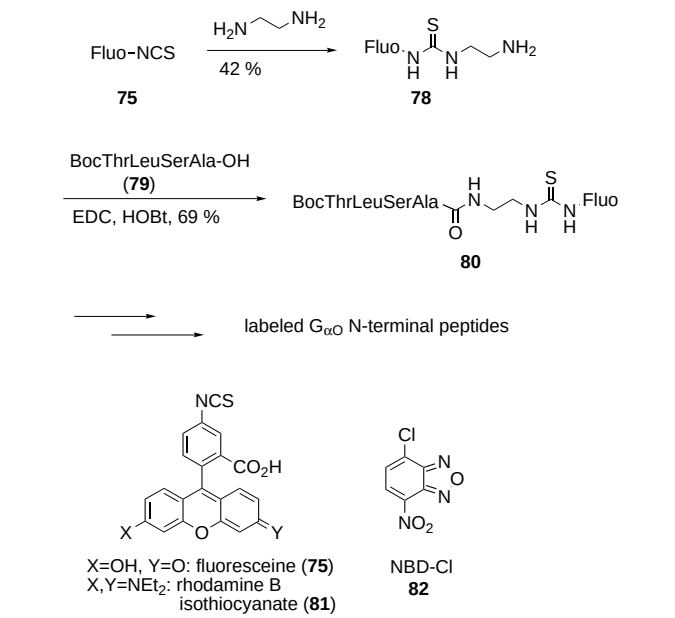
Figure 5. *Peptides for the investigation of farnesyl function.*

In general, such functional groups can be attached to the amino group of selectively N-terminal deprotected peptides like **69** (Scheme 18). Thus, peptide **69** was coupled to *S*-bimanylthioacetic acid (BimTaOH, **71**),[55] 7-nitrobenzofurazene-4-aminocaproic acid (NBD-AcaOH, **72**), or fluoresceine isothiocyanate (**75**) to afford fluorescently labeled peptides **70a**, **b**, **d**, respectively.[33] Accordingly, coupling with biotinylaminocaproic acid (**73**) yielded biotinylated peptides like **70c**.[38b] Condensation of these dipeptides with farnesylated peptide **76** led to the formation of labeled C-terminal N-Ras peptides which were advantageously employed in membrane binding and localization studies (see below).[34, 56]

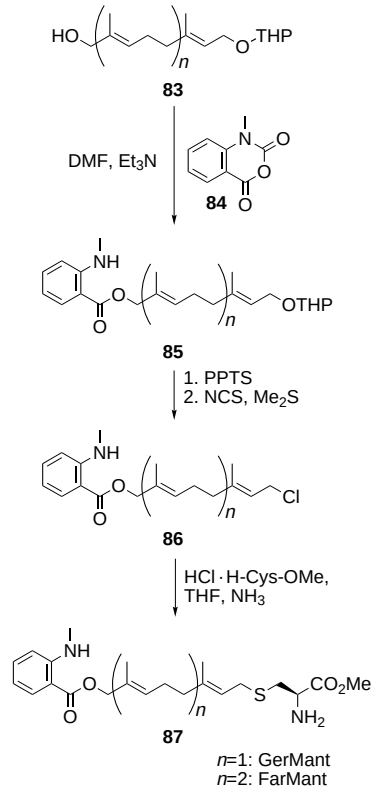
For the introduction of fluorescent markers at the C-terminal carboxy group of peptides, fluoresceine isothiocyanate (**75**) was treated with ethylenediamine (Scheme 19). The amino-functionalized label was then condensed with peptide **79** followed by an N-terminal deprotection/chain elongation process.[41a] Accordingly, rhodamine B isothiocyanate (**81**) and 4-chloro-7-nitrobenzofurazane (NBD-Cl; **82**) were converted into the respective ethylenediamine derivatives and were attached to the peptide carboxy groups. Alternatively, the fluorescent label may be incorporated directly into the lipid residue.[57] Such analogues may embody an *N*-methylanthraniloyl (Mant) group attached to a prenyl moiety (Scheme 20). Prenyl analogues, embodying a fluorescent marker, are accessible starting from the previously described prenyl derivative **83**,[58] which can be synthesized from farnesol or geraniol in a two-step procedure (Scheme 20). Acylation of alcohol **83** with isatoic anhydride (**84**) resulted in the formation of the fluorescent prenyl derivative **85**. Mild acid-mediated removal of the THP protecting group followed by treatment with *N*-chlorosuccinimide furnished the prenyl chloride **86**, which can be used for peptide prenylation as described in Section 4.4 (Scheme 6).[53]



Scheme 18. *Synthesis of lipidated Ras peptides carrying a label at the N terminus. Aca = aminocaproyl, BimTa = S-bimanylthioacetyl, Biot = biotinyl, Fluo = fluoresceinyl, NBD = 7-nitrobenzofurazen-4-yl.*



Scheme 19. *Synthesis of peptides with fluorescent markers at the C terminus.*



Scheme 20. *Synthesis of fluorescently labeled prenylated cysteine methyl ester **87**. Ger = geranyl, NCS = N-chlorosuccinimide, PPTS = pyridinium* p*-toluenesulfonate, THP = tetrahydropyranyl.*

For the synthesis of peptide – protein conjugates (Section 5.2), peptides with a reactive linker group, that is a maleimido group at the N-terminal amino function, were required (Figure 6). The

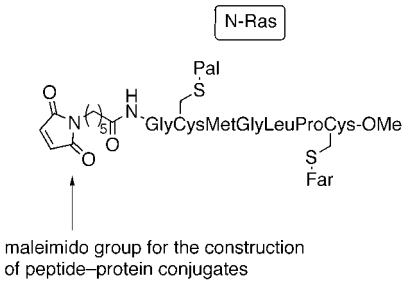


Figure 6. *Maleimido-modified peptides.*

synthesis of these peptides was achieved by selective introduction of the maleimidocaproyl (MIC) linker at the N-terminal peptide amino group as shown in Scheme 16. By appropriate combination of the lipid modifications, linkers, and markers depicted in Schemes 18 – 20 and Figures 4 – 6, a variety of peptides was synthesized and employed as molecular tools in biological investigations, the details of which are outlined in Sections 6 and 7.

5. Synthesis of lipidated proteins

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 experiments to study the binding 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 (see below). However, such model studies only provide a limited amount of information. To approximate the situation in a biological system more precisely, experiments with differently lipidated proteins are required. Clearly, the presence of the entire protein backbone is necessary for particular experiments. Thus, for determining the membrane-binding ability of lipid modifications, studies with lipidated peptides may suffice. However, determining the dependance of the transforming properties of a lipidated protein on its subcellular distribution and function requires the presence of both the membrane-anchoring lipid parts and the domains involved in interaction with further effector proteins responsible for the transforming activity. As mentioned above, the isolation of fully and correctly modified proteins results in low yields with the problem of pronounced lability of thioester bonds throughout the purification process.[14] In addition, such biological techniques are not suitable for the introduction of modified lipid groups into proteins. Thus, methods for the synthesis of differently lipidated proteins were required. For this purpose, two different approaches were developed: 1) the use of biocatalysts for the introduction of (modified) lipid residues and 2) the synthesis of differently lipidated peptides and their coupling with a protein core, which is generated by molecular biology techniques and lacks the lipidation sites.

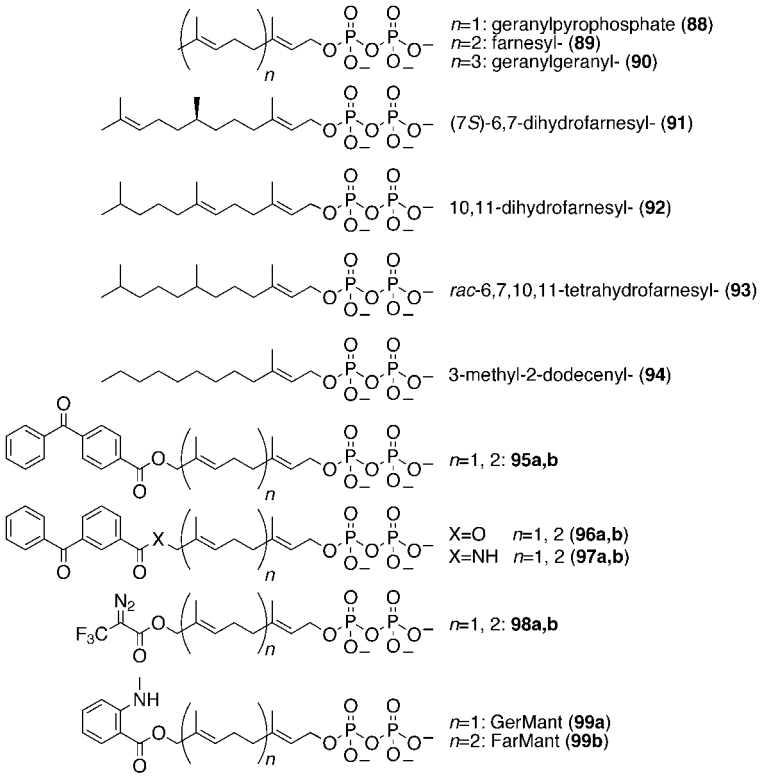


Figure 7. *Prenyl pyrophosphate analogues used as substrates in prenyltransferase-mediated transfer to proteins.*

5.1. Modification of proteins with nonnatural lipid groups

Enzymatic lipidation of entire proteins can be achieved by means of farnesyltransferase (FTase) and geranylgeranyltransferases I and II (GGTase I and II, respectively) together with the corresponding prenyl pyrophosphates, for example farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FTase and GGTase I attach prenyl groups to the cysteine SH group of a C-terminal CaaX sequence of proteins [C is cysteine, a is an aliphatic amino acid, and X is either serine or methionine (required for farnesylation) or leucine (required for geranylgeranylation)]. Both enzymes display a broad substrate tolerance with regard to the protein substrate and the prenyl pyrophosphate.[59]

FTase was successfully used to synthesize a Ras protein and peptides carrying analogues of the farnesyl group in which part of the prenyl moiety was replaced by a photoaffinity label (pyrophosphates **95** – **98**, Figure 7).[58a,b, 60] Such peptides were applied in the study of the mechanism of farnesylation by FTase. FTase was also successfully employed to attach a farnesyl residue and analogues thereof (pyrophosphates **88** – **94**) to H-Ras expressed in bacteria (Figure 7).[51] Furthermore, selective alkylation of H-Ras expressed in bacteria was achieved in *Xenopus* oocytes by inhibiting FPP biosynthesis and injection of FPP analogues.[61]

Although these methods can be used to generate Ras proteins embodying farnesyl groups and analogues thereof, they do not allow for the synthesis of fully processed and correctly lipidated Ras proteins. The biosynthesis of H-, N-, and K_b-Ras proceeds through farnesylation of a precursor protein carrying the C-terminal CaaX sequence, followed by proteolytic removal of the aaX tripeptide and methyl esterification of the resulting C-terminal cysteine residue.[9] Only then are the palmitic acid thioesters introduced. The protease[62] and the methyltransferase[63] required for processing of the C terminus were identified recently. The expression of, for example, the prenylated-protein-specific protease in SF9 cells may be a future tool for the enzymatic generation of modified Ras proteins.[64] Despite numerous and intense attempts the Ras palmitoyltransferase has not yet been identified.[65] Thus, the enzymatic synthesis of fully processed and correctly lipidated Ras proteins is currently not feasible.

Geranylgeranylation by GGTase II was successfully employed for synthesizing lipid-modified and fluorescently labeled Rab proteins.[57] GGTase II recognizes and prenylates C-C and C-X-C sequences at the C termini of proteins. For the covalent modification of a Rab protein, the presence of a Rab escort

protein (REP) is required. The Rab proteins constitute a class of small guanine-nucleotide-binding proteins that are involved in the regulation of vesicular transport and sorting.[66] In the biosynthesis, REP binds to nonprenylated Rab and the binary complex is then recognized and geranylgeranylated by GGTase II. Finally, REP escorts Rab to its destination. Delivery of Rab to a particular vesicle is believed to be mediated by a putative receptor.

To obtain fluorescently labeled Rab proteins as possible tools for the detailed study of the molecular process, nonlipidated Rab was expressed and enzymatically modified. GGPP and FPP analogues **99a** and **b**, respectively, which contain a fluorescent *N*-methylanthraniloyl (Mant) group attached to a prenyl unit, were found to be good substrates for the transferase. They were transferred to the Rab7 protein in the presence of REP in high yield to give fluorescently labeled Rab proteins (Figure 8).[57] Although GGPP is a much better substrate, the enzyme accepted both the Mant-modified FPP analogue and the labeled GGPP analogue. These proteins and related fluorescently labeled Rab derivatives[67] may now be employed, for instance, for identifying the putative Rab receptor and for studying the intracellular distribution of Rab7.

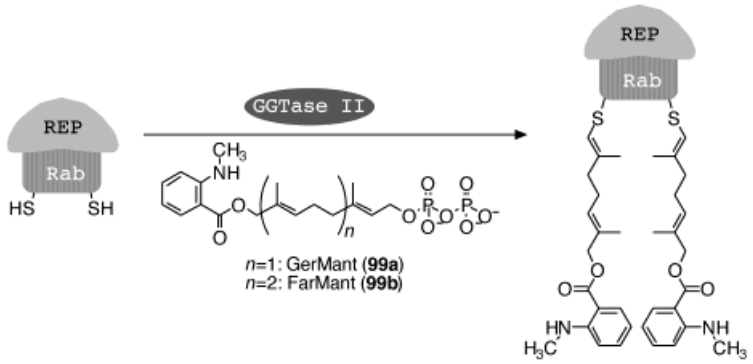


Figure 8. *Geranylgeranyltransferase-II-mediated transfer of fluorescently labeled prenyl analogues onto Rab proteins.*

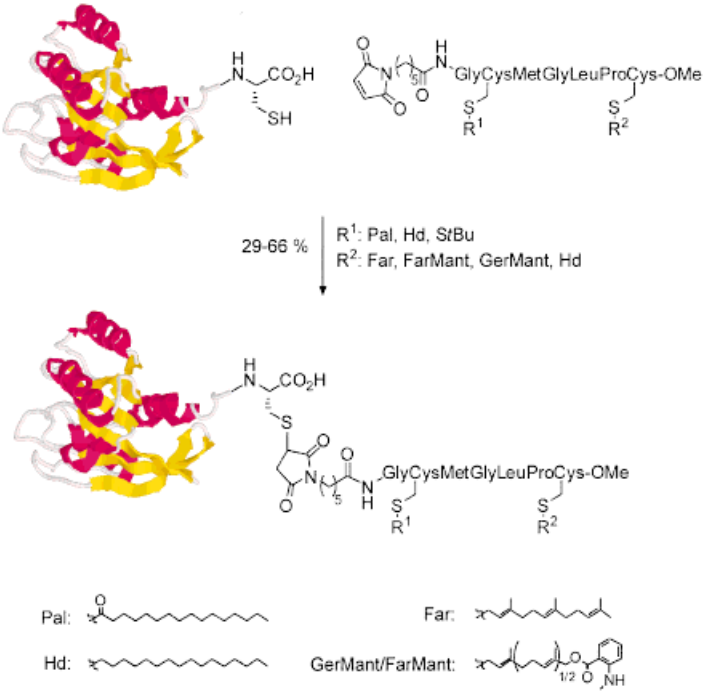
5.2. Synthesis of lipidated proteins by a combination of molecular biology and organic synthesis

The synthesis of differently lipidated proteins by enzyme-catalyzed lipidation is a powerful method. It may, however, be limited by the substrate tolerance of the transferase used. For instance, Mant-modified FPP analogue **99b** was successfully transferred onto Rab7 protein by means of GGTase II, but it was not accepted as a substrate by farnesyltransferase, and Mant-labeled lipidated Ras proteins could not be synthesized by this method.[68] Therefore, generally applicable synthesis methods that are not limited by the substrate tolerance of biocatalysts are highly desirable for providing differently lipidated and biologically active proteins in multi-milligram amounts. Such a method was developed by combining the methods of molecular biology with the techniques of organic synthesis.[15] This approach involves the expression of suitable Ras mutants lacking the lipidatable C terminus and their subsequent coupling with differently lipidated peptides corresponding to the C terminus of Ras by means of maleimido chemistry.

To this end, on the one hand, C-terminally truncated H-Ras proteins were generated by introducing stop codons at position 182 of wild-type human H-Ras and the oncogenic mutant Ras G12V (Ras ΔC and Ras G12VΔC, respectively). These mutants carried a cysteine at the C-terminal position 181, which lies on the surface of the protein and is therefore accessible to external reagents. The proteins have three additional cysteine residues at positions 51, 80, and 118. However, only Cys118 appears to be surface-exposed in the structure of Ras.[69] On the other hand, differently lipidated peptides carrying a maleimidocaproyl (MIC) group at the N terminus were synthesized by means of the enzyme-labile and Pd^0-sensitive protecting groups described in Section 4.5.3.[15]

The Ras proteins were then allowed to react with the MIC-modified peptides in stoichiometric amounts. The maleimido group is a well-established functionality for the covalent modification of proteins.[54] It is known to react specifically with mercapto groups of proteins by conjugate addition of the thiol to the α,β-unsaturated carbonyl compound. The Ras mutants reacted smoothly and in high yield with the MIC-modified peptides. Isolation of the reaction products was extremely straightforward. The hydrophobic coupling products were extracted by Triton X114 (polyethylene glycol *tert*-octylphenylether) phase separation. Triton X114 is soluble in aqueous solution at temperatures below 30°C and shows phase separation at 37°C. Hydrophobic modified proteins remained in the detergent phase after separation while unmodified H-RasΔC was dissolved into the aqueous phase. Mass spectroscopic analysis of the proteins both without and after protease digestion revealed that only one lipopeptide was introduced and that only the C-terminal cysteine had been modified. All semisynthetic Ras proteins retained high solubility in aqueous buffer and could be stored at -70°C without loss of activity.

With this method, a variety of Ras proteins with different lipidation patterns could be synthesized in multi-milligram amounts (Scheme 21). For instance, proteins were generated with the natural lipid combination, which comprises a farnesyl thioether and a palmitoyl thioester. Such proteins are not or only hardly available from biological sources (see above). Furthermore, analogous proteins were synthesized embodying only one lipid residue or in which either the farnesyl or the palmitoyl group was replaced by a stable hexadecyl thioether group. In addition, proteins were built up containing a serine instead of a cysteine residue at the critical sites that normally are lipidated. In a further series of experiments, lipidated Ras proteins were synthesized that carry a fluorescent Mant group incorporated into the farnesyl-type modification (see also above for the Rab proteins).[53] In general, this method proved to be very efficient and extremely straightforward. It yielded the neo-lipoproteins in large amounts and high purity. The biophysical and biological properties of the neo-Ras proteins are discussed in Sections 6 and 7.



Scheme 21. *Synthesis of protein – peptide conjugates using maleimido-modified and differently lipidated Ras peptides.*

The high efficiency of this method notwithstanding, it still introduces nonnatural structural elements, that is the linking MIC group, into the proteins. Thus, a method which creates the natural amide bond would be even more desirable. This goal might be achieved by means of expressed protein ligation.[70] In this technique, a fusion protein, in which the protein of interest is linked to a second domain through a thioester group, is expressed. This thioester intermediate is then trapped with a peptide carrying a cysteine at the N terminus to yield a native peptide bond via rearrangement of the initially formed thioester group. However, although this approach has yielded impressive results, for example in the synthesis of labeled but not posttranslationally modified proteins, it has not yet been successfully applied to the synthesis of lipidated proteins.

6. Biophysical properties of synthetic lipidated peptides and proteins

Lipid modifications of proteins and the properties they cause, that is insertion into intracellular membranes, have incising effects upon the kinetic and thermodynamic properties of the corresponding biological interactions. Depending on the partition coefficient for the distribution between aqueous and hydrophobic phase, most of the modified macromolecules will switch from their three-dimensional environment in solution into the two-dimensional system of the fluidic membrane. Although diffusion within membranes is approximately 100-fold slower than in aqueous solution, the probability for two molecules to meet in a lateral matrix can accelerate the overall reaction dramatically (Figure 9).[71] Another striking effect is caused by the local concentrations of the reacting molecules. If two binding partners are translocated to the same membrane their local concentration may exceed the critical value, which is limiting for efficient complex formation. In the case of signal transduction through Ras proteins, the affinity of the Ras nucleotide exchange factor protein Sos towards Ras is too small to allow sufficient binding events for the activation of Ras as long as Sos is in the cytoplasm. In response to an extracellular signal Sos is adressed towards the plasma membrane of the cell by means of an adaptor protein. This membrane adressing generates a local concentration of Ras and Sos molecules in the plasma membrane that is sufficiently above the apparent K_D value of the binding partners and subsequently allows initiation of nucleotide exchange reactions for a biological response (see above).

The membrane itself can contribute to further modifications of the protein – protein interactions. It can provide additional electrostatic and hydrophobic interactions distinct from the lipid anchorage (e.g. electrostatic binding of a negatively charged plasma membrane with a stretch of positive side chains as observed for K-Ras, membrane interaction of hydrophic residues in proteins, or binding of phospholipids by pleckstrin homology

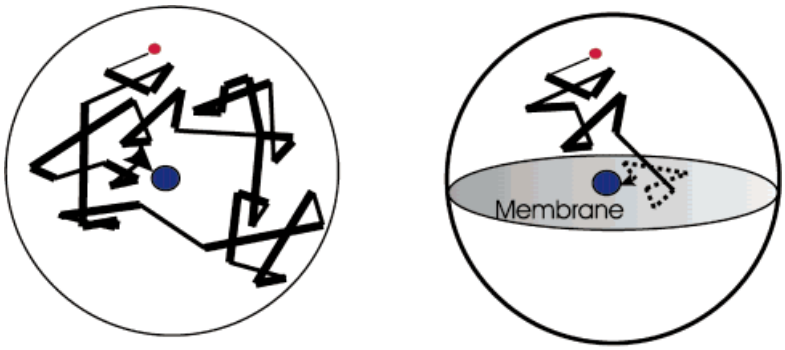


Figure 9. *Acceleration of biochemical reactions by surface-based interactions. See text for details.*

(PH) domains) and thereby affect conformation and/or activity of membrane-associated proteins. In the case of the plasma membrane, the high electric field strength (ca. 10^7 V m^{-1}) cannot be neglected anymore. The conformation of proteins with charged side chains can be influenced by such high fields (e.g. voltage-gated ion channels). Because to these aspects there is high interest in the study of the interactions between membranes and hydrophobically modified proteins (or peptides as model systems), the effects of membranes on protein – protein interactions, and the correlation with biological functions.

The most prominent function of the hydrophobic modification of proteins is the anchorage of the macromolecule in membraneous structures. In biological systems this anchoring is realized by one or more hydrophobic modifications of side groups of the peptide chain and can be supported by additional electrostatic interactions. The contribution of such interactions between positively charged lysine residues of a peptide and

negatively charged lipids as supposed for the plasma membrane of eukaryotic cells has been investigated in binding studies with membranes formed from mixtures of zwitterionic and acidic lipids by filtration, equilibrium dialysis, fluorescence, and microcalorimetry assays.[72] Here, binding does not depend strongly on temperature and pressure but increases sigmoidally with the mole fraction of the acidic lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (PG) as predicted. Decrease in the free energy upon binding to the membrane is due to a positive change in the entropy of the system.

While in the case of electrostatic interactions no lesion of the membrane surface has to occur in the binding step, hydrophobic groups have to penetrate and somehow disturb the intact lipid bilayer of the membrane for anchoring. Fluorescence-based assays have contributed most of the knowledge about kinetics and thermodynamics of such membrane insertions. Based on previous work with bimanyl-labeled lipids[73] the association of isoprenylated di-, tri-, and tetrapeptides representing the C terminus of GTP-binding proteins involved in signal transduction (Ki-Ras, Ral1, Rac2, and RhoC) with phospholipid vesicles has been analyzed with the same fluorophore.[55] The N-terminally coupled bimanyl group shows a large increase in quantum yield when the lipopeptide enters a lipid vesicle (Figure 10 A). Lipopeptides with a single hydrophobic moiety distribute within seconds between the aqueous phase and the outer surfaces of lipid vesicles, which can be described as a simple two-phase partitioning equilibrium. Apparent K_D values for the binding of lipopeptides to the vesicles are in the order of 10^{-5} M for farnesylated and 10^{-7} M for geranylgeranylated peptides if the C-terminal cysteine is carboxymethylated like in the completely processed protein. Removal of the methyl group from the carboxy-terminal cysteine residue decreases the affinity of a given lipopeptide for neutral vesicles by 10- to 20-fold. This effect is even stronger (40-fold reduction) if vesicles that have a negative surface charge under physiological conditions are used (all lipopeptides contain one or two arginines, which give a small electrostatic contribution to the interaction with the membrane). Cysteine-linked farnesyl and geranylgeranyl residues are found to be equivalent to cysteine-linked *n*-alkyl chains of 11 and 14 carbon atoms, respectively, in the strength of their interactions with lipid bilayers. Variations in vesicle lipid composition (cholesterol or aminophospholipid content) only modestly alter the affinity of isoprenylated peptides for the lipid bilayer. Therefore, a single isoprenyl group is sufficient for membrane association only if supported by carboxymethylation of the C-terminal cysteine.

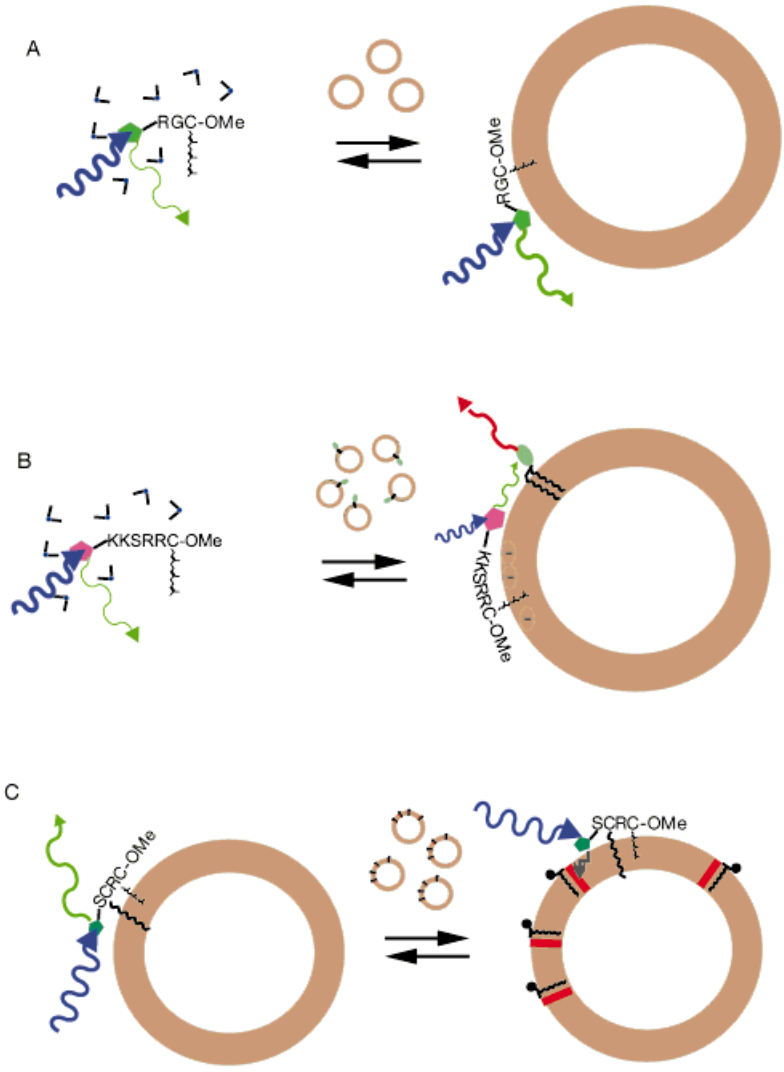


Figure 10. *Experimental setups for fluorescence-based studies upon lipopeptide – vesicle exchange. See text for details.*

To achieve stable membrane binding some proteins utilize hydrophobic and electrostatic interactions in concert. The hydrophobic contribution can be effected by myristoylation (e.g. Src, HIV-1 Gag, MARCKS), farnesylation (e.g. K-Ras4B), or geranylgeranylation (e.g. G25 K). The effect of a combination of positively charged side groups and an isoprenoid modification on membrane binding has been studied with the C termini of K-Ras4B and G25 K.[25] In the G25 K peptide four basic residues (W**KK**S**RR**C) support binding with negatively charged lipid vesicles, while the K-Ras constructs contain eight basic lysine residues (CG**KKKKKK**S**K**T**K**C). Readout for binding was achieved by radiolabelling of the peptide for K-Ras4B or fluorescence resonance energy transfer (FRET) in the case of the G25 K peptide. Here, an N-terminal *N*-acetyltryptophan group in G25 K was excited at 280 nm. If the peptide binds to vesicles containing a trace of dansylated phospholipids (Dansyl-DTPE) the emitted fluorescence light is directly absorbed by the dansyl fluorophore and results in emission of the dansyl group at 510 nm (Figure 10 B).

Hydrophobic and electrostatic properties of the lipopeptides show synergistic effects upon binding to membranes.[74] In the case of G25 K the long isoprenoid chain of the geranylgeranyl

group, carboxymethylation of the C-terminal cysteine and negative charging of the vesicle surface (by phosphoserines) is necessary to achieve nearly irreversible attachment of the lipopeptides. Due to the long stretch of basic amino acids, the electrostatic interaction of the K-Ras4B peptide with negatively charged vesicles results in an approximately 10^3-fold increase in binding compared with a neutral membrane. Further investigations with bimanyl-labeled K-Ras4B peptides demonstrated that relatively small differences in membrane charging (ca. 10 mol%) are sufficient for an electrostatic accumulation in the more negative environment by a factor of 45.[75] With the farnesyl group as a hydrophobic anchor and less strong binding properties, the peptide is still mobile and can swap between vesicles but may find its target membrane by means of the surface-potential-sensing function of its lysine residues. In the case of the *N*-myristoylated, alanine-rich substrate of protein kinase C (MARCKS) the electrostatic component of plasma membrane binding through positively charged side chains can be reduced by phosphorylation of serine residues within the basic cluster. The introduction of one negatively charged phosphate group reduces membrane binding by a factor of ten.[76]

The second class of stable membrane-anchoring motives does not rely on electrostatic interactions but supports the first (often isoprenoid) hydrophobic modification by additional thioester formation with fatty acids (e.g. the H and N isoforms of Ras or in the α subunits of heterotrimeric G proteins) or a second isoprenoid moiety (e.g. Rab proteins). Taking advantage of a bimanyl label, the intervesicle exchange of several lipopeptides with a dual anchor motif was studied in a fluorescence dequenching assay. Here, lipid vesicles were doped with bimanyl-labeled lipopeptides and the fluorescence quencher *N*-oleoyl-1-palmitoyl-2-(12-{[({4-[4-(dimethylamino)phenyl]azo}-benzene)sulfonyl]methylamino}stearoyl)phosphatidylethanol-amine (*N*-oleoyl-DABS-PE). The fluorescence signal of the bimanyl group is quenched by DABS-PE by energy transfer of the excited bimanyl group as long as the distance between both molecules is below a critical range (as within the same vesicle). If these vesicles are diluted with vesicles without quencher, lipopeptides can switch to vesicles free of DABS-PE and the fluorescence signal increases due to the absence of the quencher (Figure 10C).[23]

At physiological temperature (37°C) the dissociation of doubly modified lipopeptides with an isoprenyl thioether and a palmitoyl thioester group is rather slow and characterized by half-times in the order of 50 h. Here, the relative effect of the carboxymethylation is significantly reduced (a free carboxy group at the C-terminal cysteine increases the dissociation rate only fivefold). Due to their length, palmitoyl groups with their C_{16}-carbon chain contribute more efficiently to membrane anchoring than farnesyl or geranylgeranyl modifications (the palmitoyl group has a 5-fold higher apparent affinity for membranes than geranylgeranyl groups and a 100-fold higher affinity than a farnesyl group). This led to the conclusion that the regulation of membrane-anchored proteins has to be achieved by other mechanisms than spontaneous dissociation. In principle, binding to an "escort protein" or *S*-deacylation may induce dissociation of the lipoproteins out of the membrane.

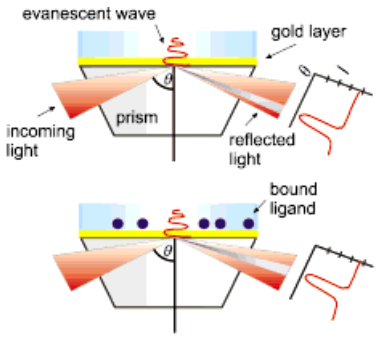


Figure 11. *Readout in surface plasmon resonance. See text for details.*

Another approach to study the interactions of lipopeptides with membranes is the utilization of artificial membranes on the sensor surface of surface plasmon resonance (SPR) systems.[15, 38b] Here, the free electrons (plasmon) of a thin gold layer at the interface between a glass prism and, for example, a buffer solution can interact with the evanescent wave of a light beam, which enters the interface under the condition of total internal reflection. Under defined conditions, the plasmon absorbs energy of the evanescent wave, which results in a gap of reflection intensity for this angle. The resonance angle now reflects the refractive index (RI) of the layer above the gold surface. If the RI changes due to the accumulation of ligands, the resonance angle adopts a new value (Figure 11).[77] In a commercial BIAcore system, the resonance signal is proportional to the mass of macromolecules bound to the membrane and allows analysis with a time resolution of seconds. To test the membrane insertion of lipopeptides, lipids (e.g. dimyristoylphosphatidylcholine, DMPC) were mixed with 5% of biotinylated lipopeptides. Vesicles with defined size distribution of approximately 100 nm diameter were generated by treatment of the lipid–lipopeptide suspensions by sonication and filter extrusion.[38b] These vesicles spontaneously fuse with the alkane thiol surface of the SPR sensor if applied with the volume flow in the SPR system, forming an artificial lipid bilayer with the DMPC–lipopeptide surface orientated towards the solvent (see picture in Figure 12).

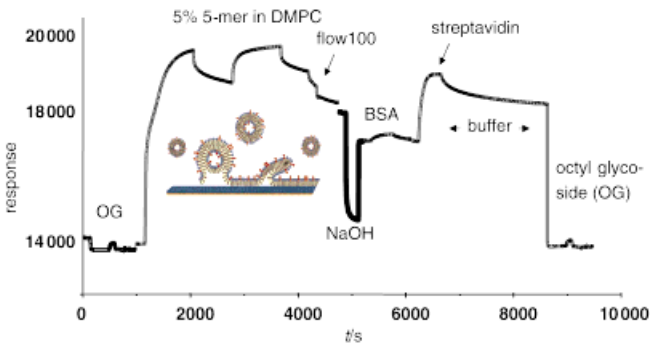


Figure 12. *Generation of artificial membranes on the surface of a hydrophic SPR sensor. See text for details.*

A typical experiment combines treatment of the surface with a non-ionic detergent (e.g. octyl glycoside), two injections with vesicle suspension, conditioning of the surface at high flow rates for buffer washing, and treatment with 10 mM NaOH. Then a solution of bovine serum albumin (BSA) is applied to saturate uncovered spots on the surface. Binding of streptavidin to the biotin headgroups indicates that lipopeptides have been succesfully integrated into the lipid matrix. The cycle is finished by removing all noncovalently bound macromolecules from the sensor surface by an additional injection of octyl glycoside (Figure 12).

Figure 13 shows the incubation of 500 nM streptavidin with surfaces generated from DMPC vesicles containing 5% of biotinylated heptapeptide Biot-Cys(Pal)MetGlyLeuProCys(Far)-OMe or pentapeptide Biot-MetGlyLeuProCys(Far)-OMe. Due to the high affinity of streptavidin towards biotin (K_D ca. 10^{-15} M) and the very slow dissociation rate of this system, dissociation of the

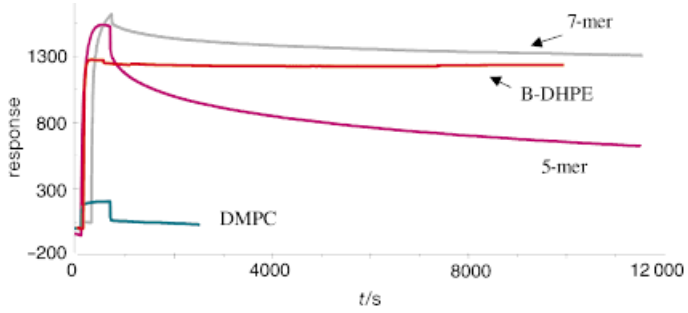


Figure 13. *Binding and dissociation of streptavidin to biotinylated lipopeptides inserted into an SPR membrane. See text for details.*

streptavidin – biotin complex can be neglected at 20°C. The decrease in signal strength occuring during washing the surface with buffer therefore reflects the dissociation of the lipopeptide out of the artificial membrane. An artificial surface formed from DMPC alone did not bind streptavidin, while DMPC vesicles containing biotinylated dihexadecanoyl phosphatidylethanolamine (B-DHPE) exhibit a similar binding pattern for streptavidin and no significant reduction in resonance signal strength during the observed dissociation phase.

Again, there is a clear difference between peptides bearing one or two hydrophobic modifications in their ability to persist in the lipid layer. A farnesylated and palmitoylated heptapeptide dissociates rather slowly, whereas a pentapeptide that is only farnesylated has an observed half-time in the matrix of less than two hours. While these findings agree qualitatively with the results from vesicle experiments in solution,[55] their values differ by two orders of magnitude compared to data derived from intervesicle transfer.[56] Despite of other experimental parameters (e.g. temperature) in the SPR system, dissociated lipopeptides have a high probability of binding back to the sensor surface because they are not trapped by vesicles in solution.

A new quality in the analysis of hydrophobically posttranslationally modified proteins could be achieved by the construction

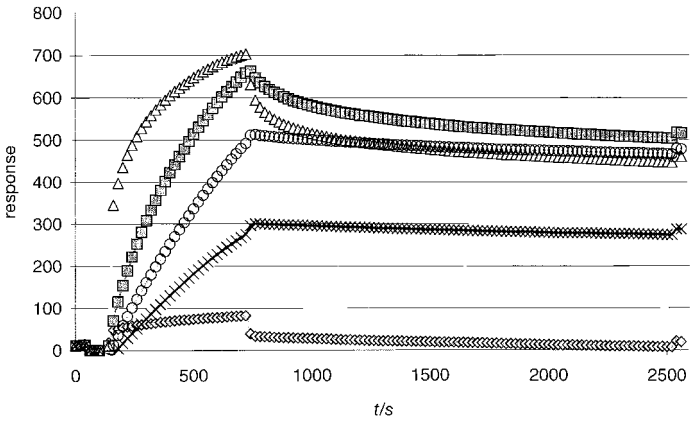


Figure 14. *Dissociation of protein – lipopeptide constructs out of the artificial membrane of a BIAcore sensor. See text for details. Symbols: soluble H-RasΔC (◆), H-RasΔC coupled to farnesylated pentapeptide (5Far, ▲), a palmitoylated and farnesylated heptapeptide (7PalFar, ○), a hexadecylated and farnesylated heptapeptide (7HDFar, ×), and in vitro farnesylated, full-length H-Ras (■).*

of lipidated proteins through a combination of bioorganic synthesis of activated lipopeptides and expression of the protein backbone in bacteria, as described in Section 5.2. The physicochemical properties of such artificial lipoproteins differ substantially from those of the corresponding lipopeptides. The pronounced dominance of the hydrophilic protein moiety (e.g. 181 amino acids for the Ras protein) over a short lipopeptide with one or two hydrophobic modifications keeps the construct soluble up to 10^{-4} M, while the biotinylated or fluorescently labeled lipopeptides exhibit low solubility in aqueous solutions and can be applied in the biophysical experiments only in vesicle-integrated form or dissolved in organic solvents. Thus, lipoproteins could be injected over the surface of a lipid-covered SPR sensor in a detergent-free buffer solution and showed spontaneous insertion into the artificial membrane.[15] Again, two hydrophobic modifications are necessary for stable insertion into the lipid layer, whereas lipoproteins with a farnesyl group only dissociate significantly faster out of the membrane (Figure 14). Therefore the isoprenylation of a protein is sufficient to allow interaction with membraneous structures, while trapping of the molecule at a particular location requires a second hydrophobic anchor. Interaction between the Ras protein and its effector Raf kinase depends on complex formation of Ras with GTP (instead of the Ras·GDP complex which is present in the resting cell). If a synthetically modified Ras protein with a palmitoylated and farnesylated lipopeptide at its C terminus is inserted into an artificial membrane of a BIAcore sensor, a GST fusion construct with the Ras-binding domain (RBD) of Raf kinase shows only weak nonspecific binding (mostly due to the GST domain). This binding increases specifically if the Ras-complexed GDP is exchanged on the surface for the nonhydrolyzable GTP analogue GppNHp by treatment with EDTA and this compound (Figure 15).[78] The SPR setup can now be used for the study of interactions between membrane-associated proteins and their effectors and regulators in a membrane environment mimicking the situation in the living cell.

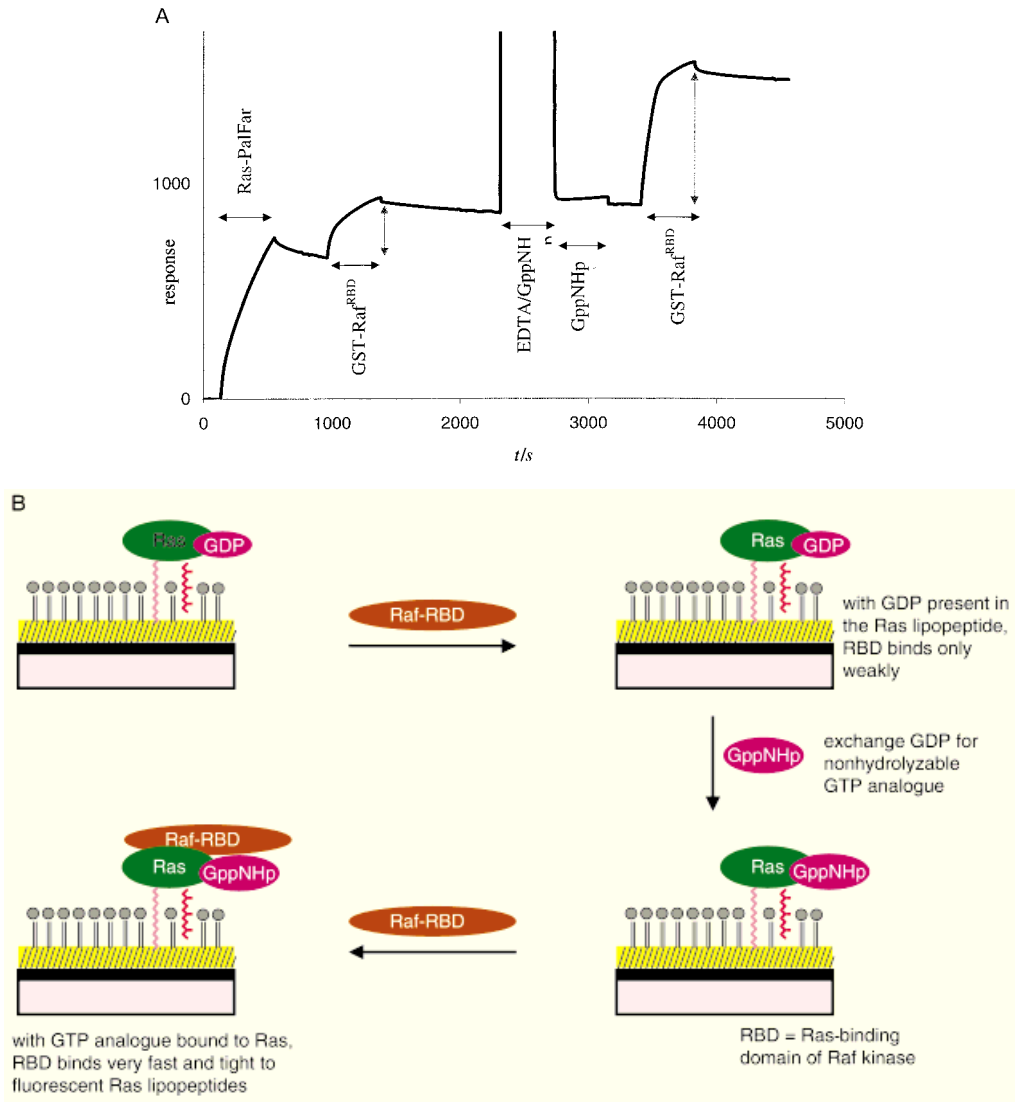


Figure 15. *Specific binding of the Ras-binding domain of the Ras effector Raf kinase to Ras – lipopeptide constructs inserted into a membraneous SPR sensor surface. Figure A shows the response curve of the process, the individual steps are schematically represented in Figure B. See text for further details.*

7. Biological properties of synthetically lipidated peptides and proteins

Biophysical analysis follows a reductionistic approach: Restriction to a small number of parameters and interaction partners allows high reproducibility and normally straightforward interpretation of the results. Despite these advantages several properties of a living cell elude themselves from being emulated in a biophysical setup. So far, distribution in subcellular structures, interactions with membrane-spanning proteins, intracelluar transport, and modifications by several enzymes could be addressed more or less exclusively in biological experiments.

Two kinds of applications of enzymes are described with regard to lipopeptides. In a first approach, enzymes are applied to introduce, for example, isoprenoid structures into peptides or proteins. Here, the incorporation of radiolabeled prenyl alcohols and their analogues into mammalian cellular proteins[79] has been shown, as well as the replacement of the farnesyl group of H-Ras by lipid analogues in vitro with farnesyltransferase and the analysis of their biological functions by microinjection into *Xenopus* oocytes.[61] On the other hand, lipopeptides have been used to analyze function and specificity of enzymes. Synthetic peptides bearing the sequence of the doubly *S*-geranylgeranylated C termini of Rab proteins were applied to test substrate recognition by the membrane-bound prenyl-protein-specific methyltransferase,[80] N-terminally acetylated, C-terminally *S*-farnesylated peptides with two additional C-terminal amino acids were synthesized to analyze the specificity of a prenyl-protein-specific endoprotease (PPEP) purified from rat liver microsomes in competition experiments with the natural CaaX motif,[81] and isoprenoids with the fluorophore *N*-methylanthraniloylic acid were used to study the isoprenylation of Rab proteins (and to thereby generate fluorescently labeled proteins for further approaches).[57]

Eukaryotic cells utilize an efficient transport system that delivers macromolecules fast and securely to their destination. In the case of the small GTP-binding proteins of the Ras family, the modified C terminus seems to be sufficient for addressing the polypeptide to its target membrane (which is, in the case of Ras itself, the plasma membrane). Lipopeptides having the C-terminal structure of N-Ras (either a pentamer with a C-terminal carboxymethylation and farnesylation or a heptapeptide with an additional palmitoyl thioester group) and an N-terminal 7-nitrobenz-2-oxa-1,3-diazolyl (NBD) fluorophore were microinjected into NIH3T3 fibroblast cells, and the distribution of the fluorophore was monitored by confocal laser fluorescence microscopy. Accumulation of the protein in the plasma membrane was efficient only for peptides with two hydrophobic modification sites, whereas the farnesylated but not palmitoylated peptide was distributed in the cytosol (Figure 16).[34] In a related experiment, CV-1 fibroblasts were incubated with fluorescent N-Ras lipopeptides bearing a free palmitoylation site. These peptides cause staining of the CV-1 plasma membrane (Figure 17) and efficient *S*-

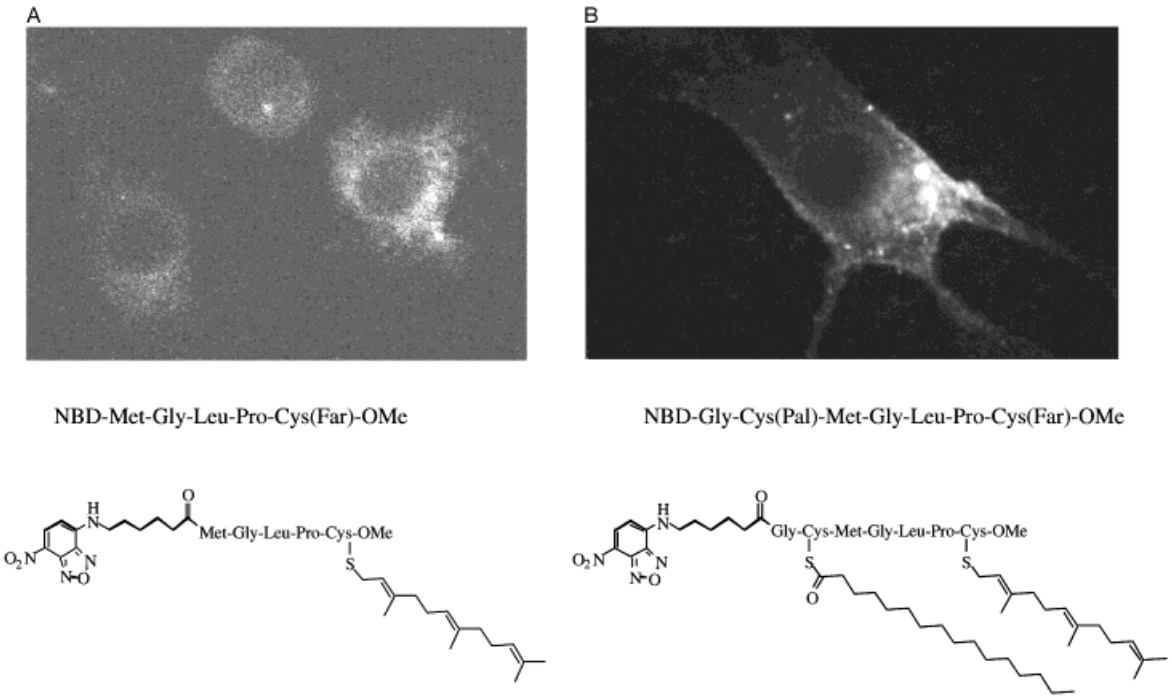


Figure 16. *Confocal laser scanning microscopy of NIH3T3 cells after microinjection with either a farnesylated pentameric (A) or a farnesylated and palmitoylated heptameric peptide (B) corresponding to the C-terminal sequence of N-Ras.*

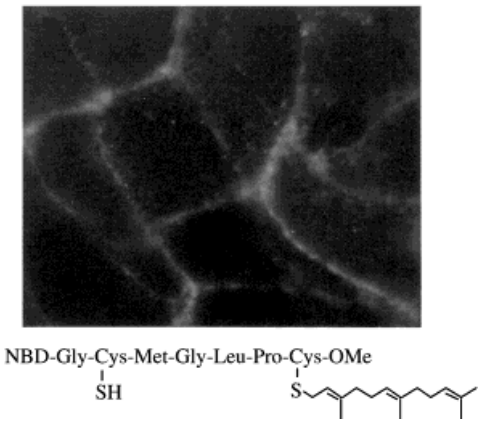


Figure 17. *Accumulation of lipopeptide NBD-GCMGLPC(Far)-OMe in the plasma membrane of CV-1 fibroblast cells visualized by fluorescence microscopy.*

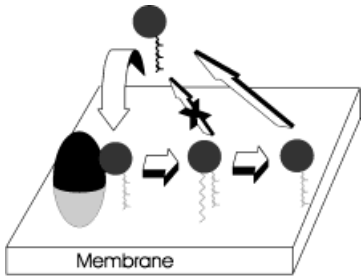


Figure 18. *Kinetic trapping model for the accumulation of isoprenylated proteins with a free acylation site in specific membranes.*

acylation even if the farnesyl group was replaced by an *n*-octyl group.[56] The association of the N-Ras lipopeptides with the plasma membrane was not affected by brefeldin A (which blocks endosomal transport) or reduced temperatures, which inhibit vesicular transport by a different mechanism. These findings support the kinetic targeting model, where a singly lipid-modified protein bearing an *S*-acylation site near the isoprenylated residue can switch between different membrane surfaces inside a cell. If the protein enters a membrane with an acylating enzyme (e.g. the still putative prenyl-protein-specific palmitoyltransferase), the protein acquires its second hydrophobic modification and is now trapped in the distinct membrane as long as its thioester group is not hydrolyzed (Figure 18).[23] However, the findings with CV-1 cell transfection studies particularly contradict results obtained with GFP constructs of N-Ras or N-Ras proteins.[82] In these experiments a contribution of endosomal transport to trafficking of N- and H-Ras could be observed. Again, only constructs with a free palmitoylation site were accumulated in the plasma membrane.

Replacement of the farnesyl group by lipid analogues could be performed for full-length Ras proteins in vitro by means of the enzyme farnesyltransferase. When such partially modified Ras constructs were applied into *Xenopus* oocytes, the cellular machinery completed modification (endoprotease activity, carboxymethylation, and palmitoylation). In these cases the H-Ras farnesyl group could be stripped off of most of the isoprenoid features that distinguish it from a fatty acid without any apparent effect on its ability to induce oocyte maturation and activation of mitogen-activated protein kinase. In contrast, replacement by the less hydrophobic isoprenoid geranyl causes severely delayed oocyte activation. Analysis of posttranslational processing suggested that the isoprenoid modification could

influence the kinetics of C-terminal proteolysis and/or methylation.[51, 61]

The powerful tool of molecular genetics allows the modification of each single amino acid in the peptide chain of a protein, for example deletion of side chain residues necessary for isoprenylation or palmitoylation[83] or introduction of additional charged amino acids for electrostatic interaction with the plasma membrane.[84] Even some artificial modifications can be introduced by means of recombinant enzymes as shown above. The limitations of molecular biology become obvious if hydrophobic modifications do not match the specificity of the enzymes available or if the nature of the chemical bond should be changed. For example, this is necessary if the labile palmitoyl thioester group of membrane-anchored proteins should be replaced by stable thioether moieties having the corresponding alkane chains. These restrictions could be overcome with the coupling of C-terminally truncated protein and activated lipopeptides as described in Section 5.2. These neo-lipoproteins could be synthesized in large amounts and proved to be efficient tools for biochemical, biophysical, and biological experiments. Their biological activity has been demonstrated in experiments with the rat pheochromocytoma cell line PC12. This cell line can be induced to differentiate by oncogenic Ras proteins[85] and this effect can be correlated to the transforming potential of these mutants. If oncogenic Ras protein (substitution of glycine by valine at codon 12, RasG12V) from bacterial synthesis is microinjected into PC12 cells the enzymatic machinery of the cell performs all modification steps (as for endogenous Ras) to generate active oncogenic protein. As a consequence, most of the cells develop neurite-like outgrowths (Figure 19).[86]

Protein – lipopeptide constructs carrying the oncogenic mutation and the natural C-terminal modification introduced by the lipopeptide also induce neurite outgrowth in the same manner as full-length nonmodified RasG12V (Figure 20).[15] Cells microinjected with the truncated RasG12VΔ181 do not respond to the oncogenic protein since the protein can no longer be modified

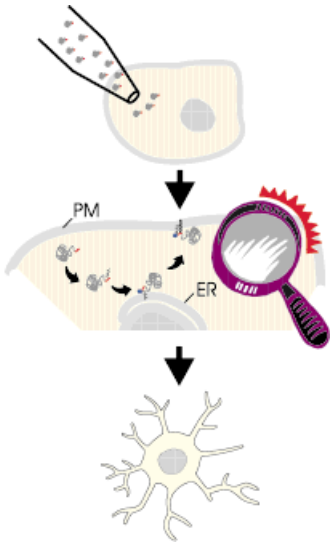


Figure 19. Schematic representation of a transformation experiment with PC-12 cells. ER = endoplasmic reticulum; PM = plasma membrane.

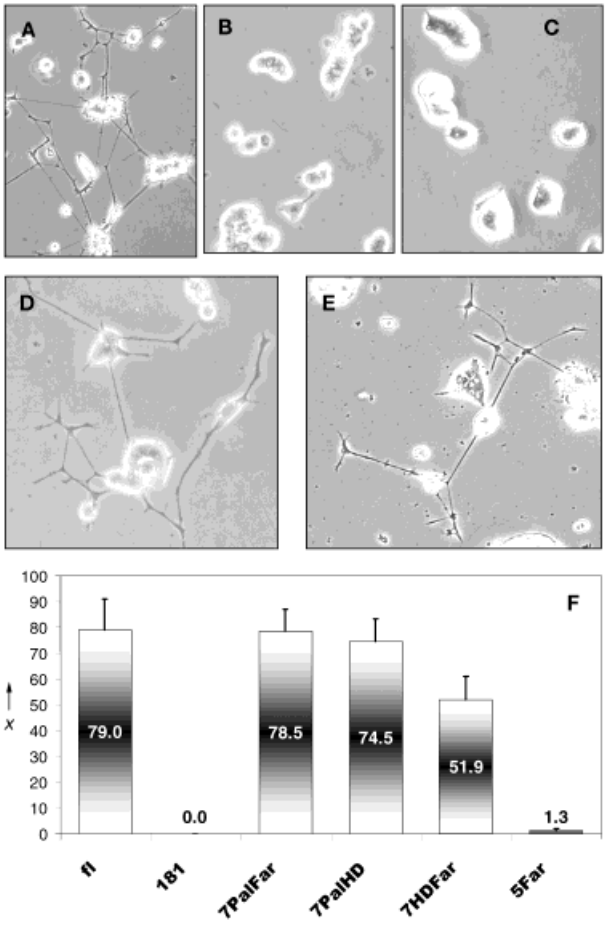


Figure 20. Biological activity of Ras – lipopeptide constructs in microinjection experiments: A) H-RasG12V (fl); B) C-terminally truncated H-RasG12VΔC (181); C – E) H-RasG12VΔC chemically coupled to (C) the farnesylated pentapeptide MIC-MGLPC(Far)-OMe (5Far), (D) the farnesylated and palmitoylated heptapeptide MIC-GC(Pal)MGLPC(Far)-OMe (7PalFar), (E) the hexadecylated and palmitoylated heptapeptide MIC-GC(Pal)MGLPC(Hd)-OMe (7PalHD); F) quantification of the microinjection experiments. X = efficiency of transformation.

posttranslationally in the cell. A RasG12V construct with a C-terminal farnesyl thioether group and carboxymethylation but without palmitoylation is nearly inactive. This is in agreement with transfection experiments in which Ras constructs with mutations in the palmitoylatable cysteine residues were used. These constructs had no effect on farnesylation but dramatically reduced transforming activity and plasma membrane localization,[83] indicating that one hydrophobic modification is not sufficient for the biological activity of H-Ras.

If the farnesyl moiety of the lipopeptide is replaced by a linear unbranched alkane chain, the corresponding coupling product displays the same biological activity as the farnesylated analogue. This finding is in line with experiments utilizing structural analogues of the farnesyl group that were enzymatically incorporated into the H-Ras protein. These proteins showed biological activity even if the modification had a reduced isoprenoid character.[61] Therefore, no specific isoprenylation receptor seems to be involved in the localization of Ras to the plasma membrane—a hydropobic alkane chain and a palmitate group are sufficient.

One of the major advantages of the strategy for the synthesis of hybrid proteins is the ability to design well-chosen analogues of C-terminal modifications relying on the efficiency of chemical lipopeptide synthesis. If the labile palmitoyl thioester is replaced by a stable hexadecyl thioether the resulting hexadecylated (HD) and farnesylated (Far) hybrid protein Ras(G12V) – HDFar is also biologically active but the efficiency of neurite formation is significantly reduced. The reduced biological readout of the 7HDFar hybrid additionally indicates that the palmitoylation step of H-Ras occurs in the plasma membrane. The farnesylated and hexadecylated protein will insert into any cellular membrane with a low probability of detaching from it. In contrast, the reversibility of thioester formation may allow palmitoylated constructs to switch between several membrane structures after induced or spontaneous hydrolysis of the thioester bond. If a corresponding protein-specific palmitoyltransferase was located exclusively in the plasma membrane, Ras constructs, which may become palmitoylated, would automatically accumulate there.

By combining bacterial expression and chemical synthesis Ras constructs with the properties of the posttranslationally modified protein can be generated. These hybrid proteins can insert into artifical and biological membranes, have been proven to be efficient tools for biochemical, biophysical, and biological experiments, and can be synthesized in large amounts. In principle, the same method is applicable to many of the Ras-related GTP-binding proteins or the γ subunit of heterotrimeric G proteins. As an outlook, protein – lipopeptide constructs with a natural peptide bond connection should be accessible by using the chemistry presented and a protein ligation system based on intein activity.[84]

In the case of Ras, questions concerning its posttranslational modification remain to be answered. It is ambiguous whether it is the localization of a palmitoyltransferase that traps H-Ras specifically in the plasma membrane or whether a specific receptor recognizes the hydrophobic modification and/or peptide sequences. It would also be desirable to investigate the influence of the chemical nature of the lipid modification on the strength and specificity of membrane insertion and whether the lipid needs to be connected through a dynamic and labile thioester bond. It is also still controversial whether the posttranslational modification of Ras modulates its interaction with guanine nucleotide exchange factors and GTPase-activating proteins or effectors, or whether the lipid environment of the membrane contributes to these interactions. The role of an acylprotein thioesterase in the regulation of Ras activity needs to be addressed as well.[23]

There is great potential in the synthesis of natural and artificial lipidated proteins, which cannot be achieved by classical methods of molecular biology. The power of organic synthesis allows to modify the structur of the hydrophobic C terminus at will. The approach also facilitates quantitative analysis with recently established biosensors, classical biochemistry, or cellular readouts. Contributions of isoprenyl and thioester groups or amino acid composition to interactions with partner proteins may be dissected independently. The availability of lipoproteins modified with fluorescent reporter groups will allow analysis of two-dimensional reaction kinetics on artificial membrane surfaces to more closely mimic signal transduction processes, many of which take place on the cytoplasmic side of membranes. Hybrid proteins may also be useful to screen for compounds that inhibit the membrane insertion of Ras by using the protein palmitoyltransferase as a target or other, as yet unidentified proteins necessary for the biological function of Ras. In this way, new routes leading to the development of anticancer drugs may be unravelled.

8. Conclusion and outlook

In this review we have summarized the successful interplay between organic synthesis, biophysics, and cell biology in the study of protein lipidation and its role in the selective targeting of proteins like Ras to the plasma membrane. The development of new methods for the synthesis of sensitive Ras peptides and entire Ras proteins, the analysis of their membrane-binding properties by means of vesicle-based assays and surface plasmon resonance techniques, and the use of synthetic Ras peptides and Ras proteins in microinjection experiments led to a better understanding of the molecular details that govern plasma membrane binding of lipidated proteins and the mechanisms by which selective plasma membrane trapping is achieved (Figure 21)

This highly interdisciplinary research provides an illustrative and representative example of what we define as "Bioorganic Chemistry" or "Chemical Biology".[87] In this field, research has to be carried out in both chemistry and biology. The researcher has to cross the barrier and bridge the undoubtedly existing gap in research culture between these two disciplines.[88] The researcher will be rewarded by experiencing the excitement that is created in both disciplines, by the ability to describe a biological phenomenon in the precise molecular language of chemistry, and by gaining insights that could not have been obtained by employing either discipline alone.

As demonstrated above for the Ras proteins, the logic of chemical biology/bioorganic chemistry may follow a cycle of investigation that begins with the analysis of a biological phenomenon, in particular the structural information available for the individual biomacromolecules (i.e. proteins or protein conjugates) or low-molecular-weight compounds (i.e. natural products or drugs) influencing it (Scheme 22). Based on these structural data, unsolved chemical problems are identified and solved by developing new synthetic methods and techniques or by devising new pathways to a desired product. If required, the biophysical properties of the synthesized compounds are determined and used for designing new syntheses and planning subsequent biological experiments. Finally, these compounds are used in biological studies aimed at gaining new insights into the biological phenomenon of interest. The results emerging from such experiments may then be used as the basis for further rounds of investigation following the same scenario. Thus, the cycle of investigations sketched in Scheme 22 actually is not closed, rather, it is the beginning of a spiral winding forward towards the future. This general scenario is applicable to the study of many biological problems in molecular detail. It may serve as a guideline for the planning of interdisciplinary research

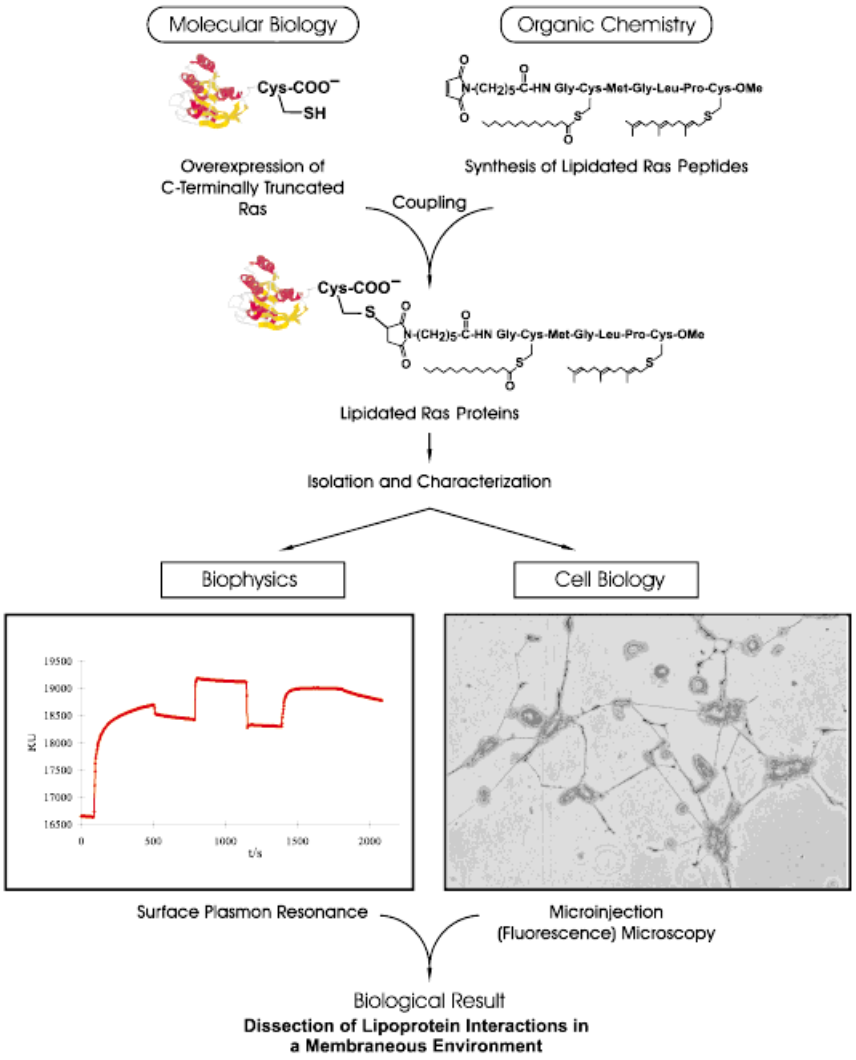
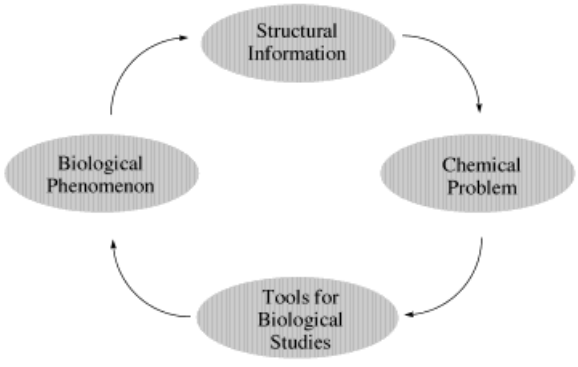


Figure 21. *An interdisciplinary approach toward the investigation of biological phenomena.*

enterprises at the interface between chemistry and biology, but various other approaches are imaginable as well. "Synthesize—measure—microinject!" and similar abbreviating imperatives may become the guidelines for recognizing and understanding the molecular basis of biological phenomena in the decades to come.



Scheme 22. *Interplay between organic chemistry and biology.*

Our research at the interface between chemistry and biology was carried out by motivated researchers. Their names are found in the references to our work. We are grateful to the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, the Bundesministerium für Bildung und Forschung, BASF AG, Bayer AG, Roche Diagnostics, and Degussa-Hüls AG for funding our research.

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