Chemoenzymatic Synthesis of N-*Ras* Lipopeptides

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Abstract: For the study of biological phenomena influenced by the plasma-membrane-bound *Ras* proteins and other lipidated proteins, characteristic peptides which embody the correct lipid modifications of their parent proteins (palmitoyl thioesters and farnesyl thioethers), as well as analogues thereof, may serve as suitable tools. For the construction of such acid- and base-labile peptide conjugates, the enzyme-labile *p*-acetoxybenzyloxycarbonyl (AcOZ) urethane blocking group was developed. The acetate moiety within the AcOZ group is easily saponified by treatment with acetyl esterase or lipase. After cleavage of the acetate group the resulting quinone methide spontaneously fragments, resulting in the liberation of the desired peptide or peptide conjugates. This enzymatic protecting group technique formed the key step in the synthesis of the characteristic S-palmitoylated and S-farnesylated C-terminus of the human N-*Ras* protein. Deprotections are so mild that no undesired side reactions of the lipid conjugates are observed (i.e., no hydrolysis or β-elimination of the thioester and no acid-mediated attack on the double bonds of the farnesyl group). The combination of enzymatic protecting group techniques with classical chemical methods allowed access to various fluorescent-labeled and differently lipid-modified *Ras* lipopeptides. Their application in biological experiments enabeled the study of the structural requirements for the acylation of *Ras* sequence motifs in vivo and gave insight into the subcellular site at which these modifications occur. The results indicate that the plasma membrane is a major site of cellular S-acylation. This supports a mechanism for the selective subcellular localization of lipidated proteins, including the *Ras* proteins themselves, by kinetic targeting to the plasma membrane.

Introduction

The transduction of stimuli from the extracellular space across the plasma membrane into the cell and ultimately to the cell nucleus is among the most important processes by which cells regulate growth, differentiation, and proliferation. In the signaling cascades[1,2] employed in nature, covalently modified proteins play decisive roles. Among the different types of covalent protein modifications, lipidation is particularly relevant. It can generally arise in three different ways: (1) the co- or posttranslational attachment of myristic acid to an N-terminal glycine, (2) the attachment of palmitic acid to the thiol group of cysteine, and (3) the formation of farnesyl- or geranylgeranyl thioethers of cysteine.[3]

The lipid groups influence the cellular distribution as well as the function of various lipidated proteins important for cellular signaling processes. For instance, the transmembrane G protein coupled receptors are S-palmitoylated; the heterotrimeric G proteins are N-myristoylated, S-palmitoylated, and S-farnesylated; and the *Ras* proteins carry *S*-palmitoyl and *S*-farnesyl groups. In addition, numerous other membrane-associated proteins are lipidated, for example, the enzyme NO_x-synthetase,[4] and various viral envelope proteins[5] are S-palmitoylated.

The biological relevance of lipid-modified proteins is highlighted by the crucial role of the so-called *Ras* proteins in maintaining the regular life cycle of cells. The *Ras* proteins are a class of membrane-bound lipoproteins which are found in organisms as diverse as mammals, flies, worms, and yeast. They serve as central molecular switches[6] and translate the signals given by growth factors via a well-balanced series of noncovalent protein−protein interactions into a cascade of highly specific protein phosphorylations, resulting in the activation of transcription factors (Scheme 1). Thus, *Ras* regulates cell growth and proliferation. If this regulation is disturbed or interrupted, uncontrolled proliferation may occur which may result in the transformation of the cell. Thus, a point mutation in the *Ras* oncogenes coding for the *Ras* proteins is found in ca. 40% of all human cancers, a figure which rises to 80% for some of the major malignancies such as lung, colon, and pancreas cancer.[7]

Due to the important biological roles of lipid-modified proteins, the study of protein lipidation and its biological significance is at the forefront of biological research. Recent reports have revealed that the covalently attached lipids do not only serve to anchor the modified proteins in cytoplasmic or vesicular membranes but rather may be involved directly in the transduction of signals and the regulation of respective effector

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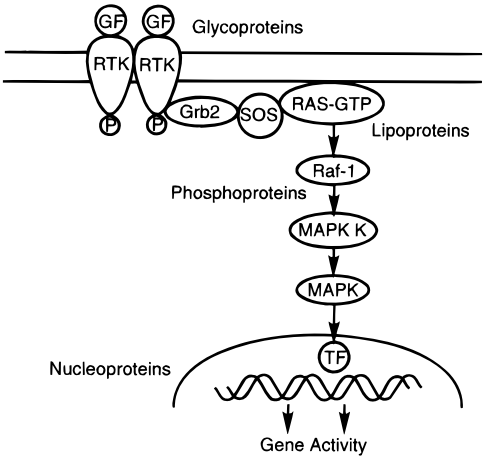
Scheme 1. The *Ras* Signal Transduction Pathway[a]



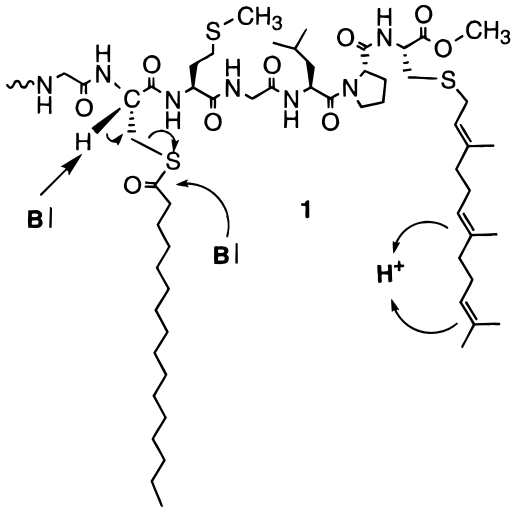
[a] GF = growth factor. RTK = receptor tyrosine kinase. TF = transcription factor.

systems.[8−10] For instance, it has been demonstrated that S-palmitoylation plays a central role in regulating the respective effector systems of the G protein coupled β_2-adrenoceptor[9a] and rhodopsin.[9b] In this system, after stimulation of the β_2-adrenoceptor, the palmitoylation state of the $G_{s\alpha}$ protein coupled to this receptor is altered.[10]

Ras proteins (vide infra) must be lipid-modified and membrane-associated to perform both their normal and oncogenic biological functions. In the nonlipidated forms the *Ras* proteins are cytosolic and cannot transduce signals,[6,11a] and it is believed that the interaction between *Ras* and its downstream effector *Raf* (Scheme 1) is mediated via the farnesyl group present in *Ras*.[11b,c] The finding that lipidation can be crucial to guarantee correct cellular signaling may have far reaching consequences for biological and pharmaceutical research. It may provide important clues for a better understanding of biological signal transduction, as well as for the improvement of the efficiency of drugs which influence signal transduction processes.[7,12]

For the study of biological phenomena which are influenced by lipid-modified proteins, the combination of the techniques of cell biology, organic synthesis, and biophysics opens up new and alternative opportunities.[13−19] For such studies, characteristic lipidated peptides embodying the correct lipid groups and amino acid sequences of their parent lipid-modified proteins and additionally carrying labels by which they can be traced in biological systems (i.e., fluorescent tags which can be detected by fluorescence microscopy and fluorescence spectroscopy) are useful reagents.[15−18]

Scheme 2. Structure of the Characteristic Acid- and Base-Labile C-Terminal Lipoheptapeptide of the Human N-*Ras* Protein



-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe

Thus, fluorescent-labeled lipidated peptides which embody farnesyl thioethers and palmitoyl thioesters have proven to be efficient tools for the study of the membrane binding of lipidated peptides and proteins.[15−17] Such compunds can also shed light on cellular mechanisms by which these covalently modified polypeptides may be targeted to their subcellular destination, that is, the plasma membrane.[17,18] Unfortunately, the synthesis of such peptide conjugates,[13,18−22] for example the characteristic C-terminal lipidated heptapeptide **1** of the human N-*Ras* protein, is severely complicated by their pronounced acid and base lability (Scheme 2). Thus, during acid-mediated removal of the Boc group from S-farnesylated cysteinyl peptides, an attack by the acid on the double bonds of the farnesyl residue always occurs,[19] whereas the thioesters present in S-palmitoylated lipidated peptides hydrolyze spontaneously even at pH 6−7 in aqueous solution.[21] Due to this pronounced acid and base lability, different orthogonally stable blocking functions have to be employed that can be selectively removed under the mildest, preferably neutral conditions. These demands exclude the use of many of the classical chemical protecting groups. However, enzymatic protecting group techniques[13,20] offer viable alternatives to the established classical methods. Enzymatic transformations can be carried out under remarkably mild reaction conditions (pH 6−8, room temperature). In addition, enzymes often combine a high specificity for the structures they recognize and the reactions they catalyze with a broad substrate tolerance. These properties have opened up new and

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advantageous routes to sensitive and multifunctional glyco-,[23] phospho-,[24] glycophospho-,[25] and nucleopeptides.[26] Also, we have recently shown that by means of enzymatic protecting group techniques lipid-modified peptides can be built up.[18,21,22] In this paper we report the application of enzyme-labile protecting groups coupled with classical peptide synthesis to efficiently provide access to lipidated and differently labeled N-*Ras* peptides.

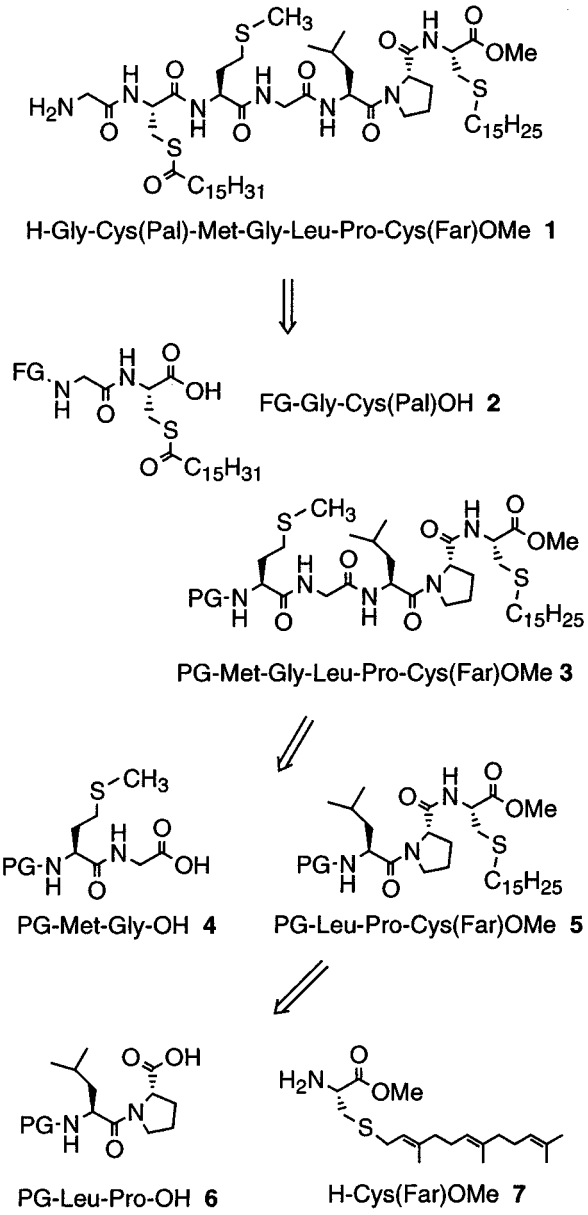
Results and Discussion

Retrosynthetic Analysis of the Lipidated *Ras* Peptide 1. As biologically relevant target compounds, the farnesylated and palmitoylated heptapeptide **1** and fluorescent-labeled analogues of this lipidated peptide were chosen. Compound **1** represents the correctly functionalized C-terminus of the human N-*Ras* protein which in the course of its biosynthesis becomes S-farnesylated, carboxymethylated, and finally S-palmitoylated.[6] In a retrosynthetic sense, **1** could be divided into an N-terminal palmitoylated and protected dipeptide **2** and the pentapeptide **3** (Scheme 3). This strategy was chosen to circumvent problems with intramolecular $S \rightarrow N$ acyl migration, which may arise if peptides carrying an S-acylated cysteine at the N-terminus are deprotected N-terminally.[27] By the avoidance of the deprotection of an N-terminal S-palmitoylated cysteine and the employment of a deblocking of a glycyl-cysteyl peptide instead, this undesired side reaction should be greatly reduced or even excluded entirely. This modular approach also opens up the possibility of introducing differently labeled or modified analogues of **2** at the end of the synthesis. By this convergent strategy, various analogues of the parent N-*Ras* C-terminus should be readily accessible. The constant S-farnesylated pentapeptide **3** was divided into the protected dipeptide **4** and the farnesylated tripeptide ester **5**. Finally, compund **5** was to be built up from the masked dipeptide **6** and S-farnesylated cysteine methyl ester **7**.

Paramount to the successful execution of this synthesis plan is the availability of an N-terminal blocking group which can be removed under very mild conditions. Due to the acid sensitivity of the farnesylated peptides **3** and **5**, as well as the base-sensitive palmitoyl group in compounds **1** and **2**, classical deprotection techniques were ruled out. The development of an enzyme-labile blocking group was seen as an attractive alternative. This blocking group must have a urethane structure to avoid racemization during activation and be specific for a biocatalyst which does not attack the other funcional groups present, that is the C-terminal methyl ester, the palmitoyl thioester, the farnesyl thioether, and the formed peptide bonds.

Development of the Enzyme-Labile *p*-Acetoxybenzyloxycarbonyl (AcOZ) Urethane Protecting Group. Although in a few cases biocatalyzed hydrolyses of urethane-protected amino acids had previously been observed,[28] the available hydrolases, in general, could not be successfully employed to remove simple urethane protecting groups from peptides. Therefore, a new and general strategy for the development of a class of enzymatically removable urethane blocking groups for peptides and peptide conjugates was developed. This new blocking group should consist of a urethane that embodies a functional group that is specifically recognized by a biocatalyst and that is bound by an enzyme-labile linkage to a functional group that undergoes a spontaneous fragmentation upon cleavage of the enzyme-sensitive bond.[29] In the fragmentation process a carbamic acid derivative is liberated, which decarboxylates to give the desired peptide or peptide conjugate. As a protecting group that might fulfill these criteria, the AcOZ group was chosen[30] (Scheme 4). This group embodies an acetic acid ester which is recognized by the enzyme acetyl esterase from the *flavedo* of oranges.[31] This esterase operates under mild conditions and

Scheme 3. Retrosynthetic Analysis of the C-Terminal N-*Ras* Heptapeptide[a]



[a] FG = protecting group or fluorescent label. PG = protecting group.

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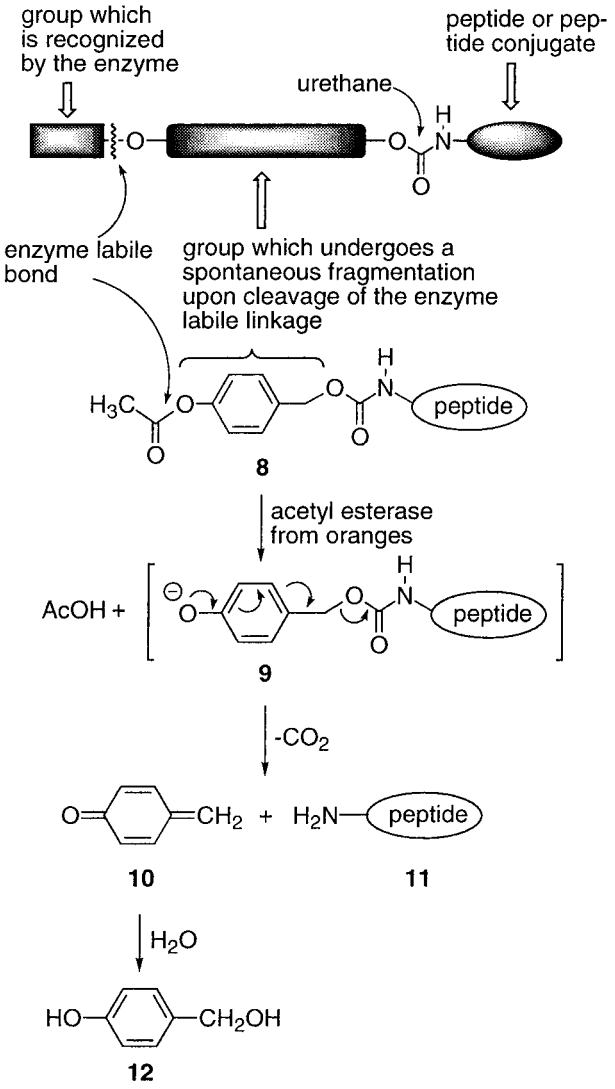
Scheme 4. The AcOZ Protecting Group and Its Removal via Acetyl Esterase-Initiated Fragmentation



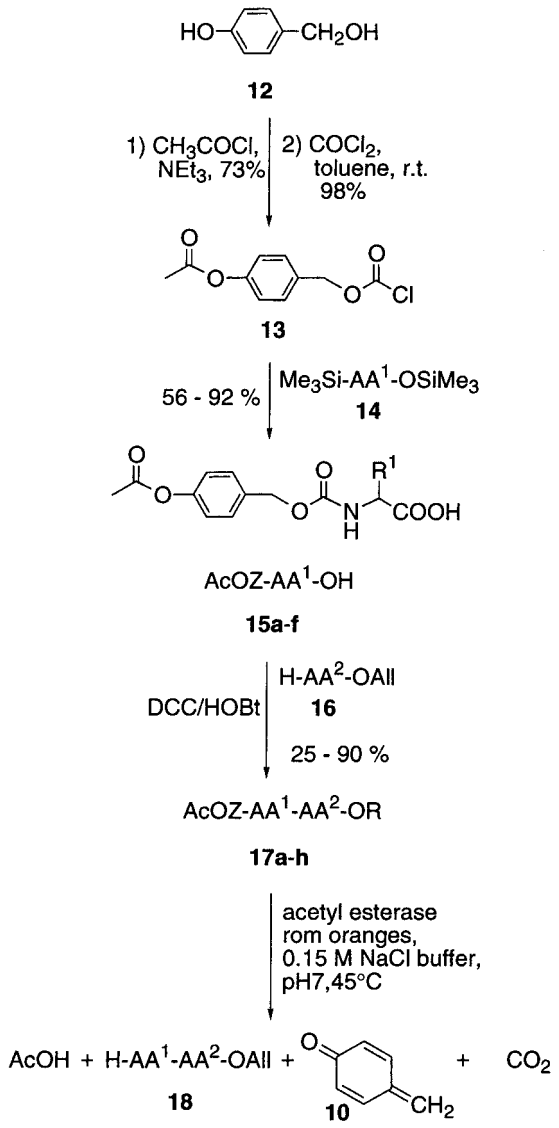
attacks acetates but not longer or branched acyl chains. In addition, it does not display any amidase activity at all. Therefore, acetyl esterase should distinguish between the acetate present in the N-terminal AcOZ group, the C-terminal esters employed in the course of the synthesis of **1**, and the peptide bonds. Furthermore, the AcOZ group contains a *p*-hydroxybenzyl urethane which, after acetyl esterase-mediated hydrolysis of the acetate, may undergo a spontaneous fragmentation reaction to give a quinone methide and the desired amine (Scheme 4).

The principle of the enzymatic deprotection (Scheme 4) is completely general. If a different acyl group is chosen, fragmentation of the resulting *p*-acyloxybenzyl urethane can be initiated with a different hydrolase. This was shown by the use of the *p*-phenylacetoxybenzyloxycarbonyl (PhAcOZ) group, which is cleaved by the enzyme penicillin G acylase, in the recent synthesis of complex, sensitive phosphoglycopeptides.[25] Three further advantages are noted with this general technique. First, the enzyme used recognizes the same element in all cases. Second, the variable peptide or peptide conjugate part of the substrate is remote from the site of the biocatalyst's attack. Thus, possible sterically or electronically unfavorable interactions of the protein with the substrate are ruled out; for example bulky amino acid side chains are guaranteed not to limit the substrate tolerance of the enzyme. Finally, this enzymatic protecting group technique can be used for the construction of peptides and analogues containing unnatural and D-configured amino acids.

The AcOZ-protected peptide allyl esters **17** were synthesized from amino acid allyl esters **16** and AcOZ-protected amino acids **15** by employing established peptide-coupling reagents (Scheme 5). No racemization of the activated amino acid in the formed peptides could be detected by HPLC or NMR techniques. The AcOZ group was introduced into the amino acids by means of *p*-(acetoxy)benzyloxycarbonyl chloride **13** (AcOZCl), which was readily obtained from *p*-hydroxybenzyl alcohol **12** by selective acetylation of the phenolic OH and subsequent formation of the chloroformate (Scheme 5). The acylation of amino acids with **13** was best carried out by treatment of N,O-bissilylated amino acids[32] **14** with the reagent **13**. Under the usual Schotten−Baumann conditions (pH 10−12) presumably the phenyl ester incorporated into the urethane was cleaved and the desired masked amino acids were obtained only in low yield. For the selective enzyme-initiated cleavage of the AcOZ urethane, the fully masked dipeptides **17** were treated with acetyl esterase in NaCl buffer at pH 7 and 45 °C (Scheme 5). In the ensuing reaction the acetates were smoothly hydrolyzed to give the phenolates corresponding to **9** (Scheme 4). These intermediates underwent a spontaneous fragmentation and liberated the deprotected esters **18** in high yields. The quinone methide **10**, which was formed from the phenolate, was trapped by water and converted to *p*-hydroxybenzyl alcohol **12** (see Scheme 4). In this process the enzymatic transformations proceeded with complete chemoselectivity, as no undesired attack of the enzyme on the allyl esters or the peptide bonds was observed. It was not a foregone conclusion that the fragmentation would occur at neutral pH, since in the previously reported nonenzymatic cleavage of the AcOZ group the pH had to be raised to 10−11 to deprotonate the phenol and induce its subsequent conversion into a quinone methide.[30] It is prudent to speculate that the biocatalyst itself might play a role in the deprotonation of the phenol. For instance, some of the basic amino acids might function as bases while the substrate is still close to the biocatalyst after saponification of the acetate.

The scope of this protecting group strategy was shown to be fairly wide (Scheme 5). However, the reaction is limited by substrate solubility in purely aqueous medium. Thus, no reaction was observed for the nonpolar AcOZ-Phe-Phe-OAll, **17a**. Addition of organic cosolvents is neccessarry to solubilize substrates such as **17a** to make them accessible to the esterase. Unfortunately, acetyl esterase is rapidly deactivated in the presence of considerable amounts of solubilizing organic cosolvents.[31] A biocatalyst was sought which would selectively cleave the AcOZ urethane but be less sensitive toward cosolvents. This search revealed that the AcOZ urethanes are also substrates for acetylcholine esterase from the electric eel and, in particular, for the lipases from *Mucor miehei* and *Rhizopus arrhizus*. In the presence of 20 vol % of methanol as cosolvent the *Mucor* lipase readily attacked the sterically demanding phenylalanyl peptide **17a** and removed the N-terminal urethane in high yield and without attack on the C-terminal allyl ester (Scheme 6). Under these conditions, however, the liberated amine competed with the solvent for the electrophile **10** to give an N-*p*-hydroxybenzylated amino acid ester. This undesired side reaction was efficiently suppressed by performing the enzymatic reaction in the presence of iodide as a nucleophile which traps the quinone methide faster than the amine. In addition, protonation of the amine by lowering the pH to 5 was found to be advantageous.

Scheme 5. Synthesis of AcOZ-Protected Dipeptide Allyl Esters and Enzymatic Cleavage of the AcOZ Protecting Group from Dipeptides with Acetyl Esterase from Oranges

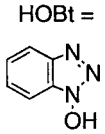


Nr.	AA^1-AA^2	Yield [%]
17 a	Phe-Phe	70
17 b	Phe-Ala	85
17 c	Ala-Ala	85
17 d	Ala-Phe	90
17 e	Ser-Phe	25
17 f	Leu-Pro	74
17 g	Met-Gly	62
17 h	$(Gly)_2$-Cystine	74

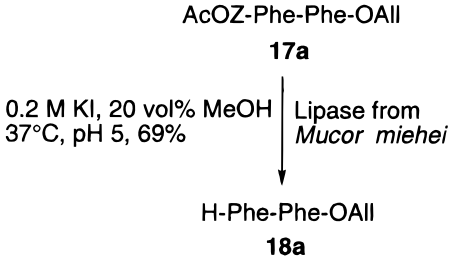
Nr.	AA^1-AA^2	Yield [%]
18 a	Phe-Phe	--
18 b	Phe-Ala	63
18 c	Ala-Ala	57
18 d	Ala-Phe	70
18 e	Ser-Phe	70

DCC = c-Hex−N=C=N-c-Hex

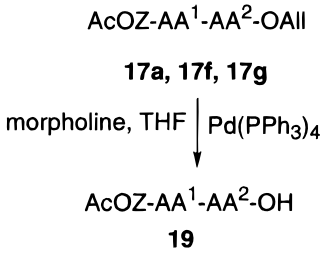
HOBt =



Scheme 6. Removal of the AcOZ Protecting Group with Lipase from *M. miehei*



Scheme 7. Pd-Catalyzed Cleavage of the Allyl Ester Protecting Group from AcOZ Dipeptides



Nr.	AA^1-AA^2	Yield [%]
19 a	Phe-Phe	80
19 b	Leu-Pro	94
19 c	Met-Gly	95

For the projected synthesis of the *Ras* peptides, a selective C-terminal deprotection of the AcOZ-masked peptides was required (see Scheme 3). The AcOZ group proved to be orthogonally stable to the allyl ester blocking function. Thus, upon treatment of the dipeptide esters **17a**, **17f**, and **17g** with $(PPh_3)_4Pd(0)$ in the presence of morpholine as the accepting nucleophile,[33] the C-terminus of the peptides was unmasked without cleavage of the AcOZ urethane to give the carboxylic acids **19** in high yields (Scheme 7).

Chemoenzymatic Synthesis of the Palmitoylated and Farnesylated C-Terminal Heptapeptide of Human N-*Ras* Protein. The AcOZ group thus proved to be an enzyme-labile urethane blocking function which ensures selective deprotection under the mildest conditions. With this synthetic tool in hand, the construction of the acid- and base-labile *Ras* peptide **1** and fluorescent-labeled analogues thereof was approached. To this end, S-farnesylated cysteine methyl ester **7**[34] was coupled with the C-terminally deblocked, AcOZ-protected peptide **19b** by carbodiimide-mediated activation of the carboxylic acid to give the lipotripeptide **20** in high yield (Scheme 8). From **20** the AcOZ urethane blocking group was removed quantitatively by treatment with lipase from *M. miehei*. The N-terminally deprotected farnesylated peptide **21** was isolated in analytically pure form in 65% yield. Subsequent elongation of the peptide chain with the dipeptide carboxylic acid **19c** yielded the AcOZ-protected lipopentapeptide **22**. From **22** the N-terminal urethane could again be removed by enzyme-initiated fragmentation to give the selectively unmasked farnesylated peptide methyl ester **23**. The enzymatic deprotection of the fairly hydrophobic lipidated peptides **20** and **22** required the presence of 20 vol %

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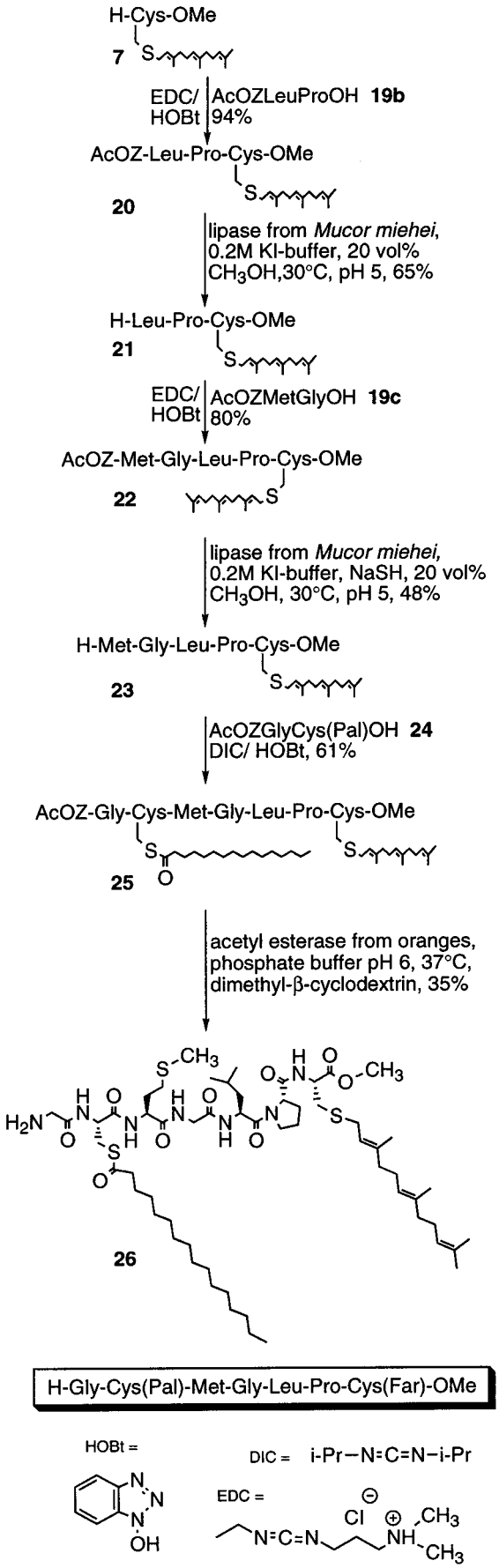
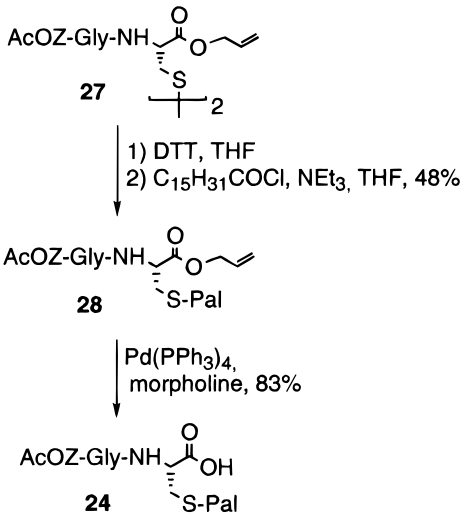
(33) Friedrich-Bochnitschek, S.; Waldmann, H.; Kunz, H. *J. Org. Chem.* **1989**, *54*, 751.
(34) Liakopoulou-Kyriakides, M. *Phytochemistry* **1985**, *24*, 1593.

Scheme 8. Synthesis of the C-Terminal N-*Ras* Lipoheptapeptide



Scheme 9. Synthesis of AcOZGlyCys(Pal)OH



of methanol to solubilize the organic substrates and make them accessible to the biocatalyst. Since acetyl esterase is unstable under these conditions, the removal of the protecting groups from **20** and **22** was carried out with lipase from *M. miehei*. To avoid an undesired attack of the liberated amines on the formed quinone methide, the enzymatic transformations were carried out at pH 5 and in the presence of NaHS and/or KI as trapping nucleophiles as detailed above.

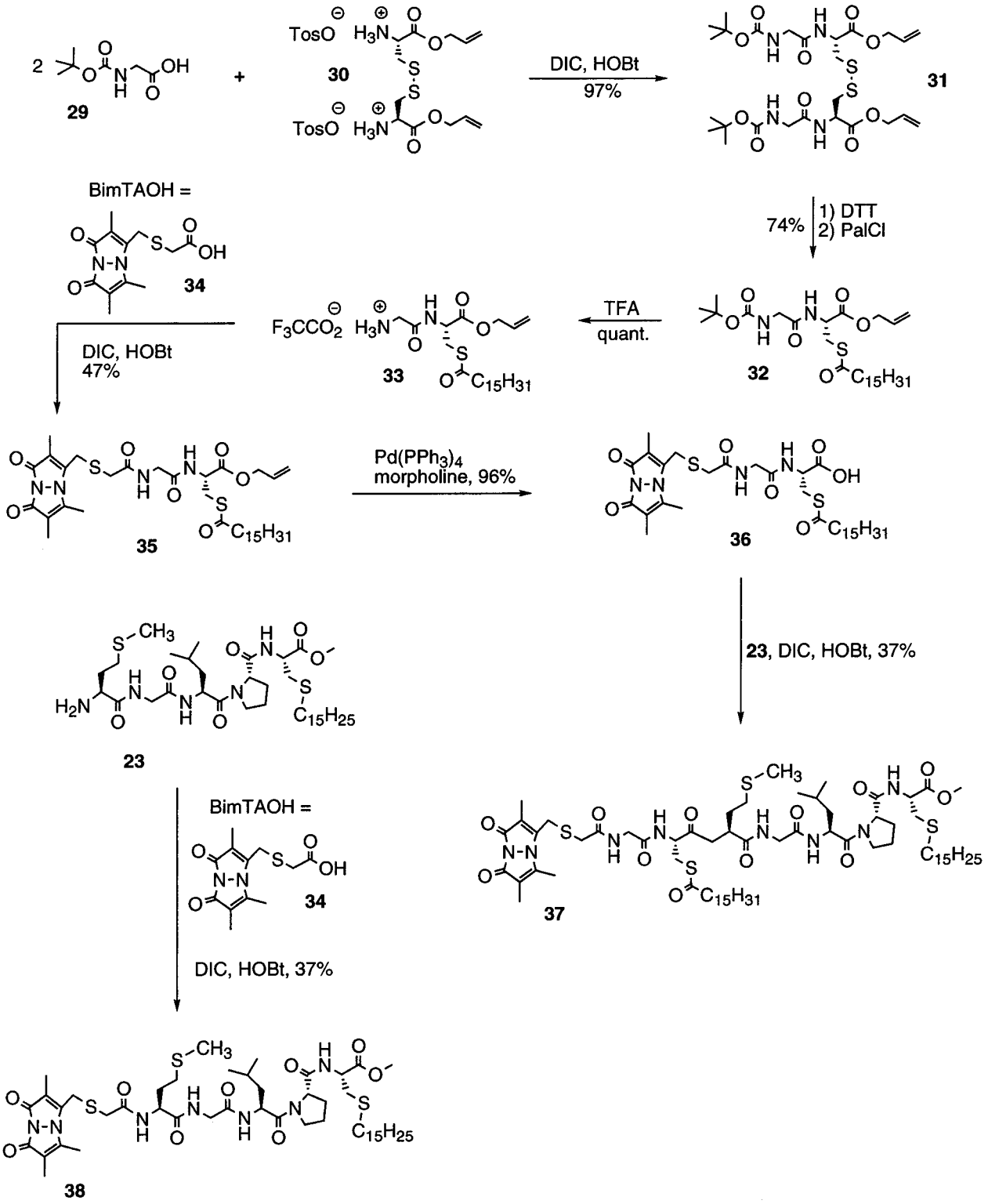
The farnesylated pentapeptide **23** was then coupled with the AcOZ-protected and S-palmitoylated dipeptide carboxylic acid **24** to yield the masked doubly lipidated heptapeptide **25**. Finally, the N-terminal blocking group was removed from compund **25** by means of acetyl esterase-mediated fragmentation of the urethane. In this case the lipase could not be employed, because this biocatalyst preferentially attacks the palmitic acid thioester and not the acetate in the blocking function. Due to the sensitivity of acetyl esterase (vide supra), organic cosolvents could not be introduced. However, the substrate could be rendered accessible to the biocatalyst by the addition of dimethyl-β-cyclodextrin as solubilizing agent.[35] This cyclic heptasaccharide has a hydrophobic cavity that can slip over the palmitoyl or farnesyl residue, thereby solubilizing the peptide. Furthermore, a beneficial effect may be that the palmitoyl thioester becomes protected against enzymatic or nonenzymatic hydrolysis after insertion into the cyclodextrin. Under these conditions, acetyl esterase removed the AcOZ urethane from the palmitoylated and farnesylated heptapeptide **25** with quantitative conversion. Due to its amphiphilic nature, part of the product was lost during workup, and the desired selectively deprotected *Ras* peptide **26** was finally isolated in 35% yield.

In the course of all the enzymatic transformations, no undesired side reaction was observed. The biocatalysts did not attack the C-terminal methyl ester, the acid-sensitive farnesyl thioether, or the base-sensitive thioester. Thus, this enzymatic protecting group technique makes sensitive lipid-modified peptides such as the fully and correctly functionalized C-terminus of the human N-*Ras* protein **26** accessible in an efficient manner.

The AcOZ-masked S-palmitoylated glycyl-cysteine **24** required to complete the synthesis of **26** was built up from the cysteine derivative **27** (Scheme 9). To this end the disulfide bond in **27** was reduced with dithiothreitol (DTT), and with the thiol groups liberated, they were immediately acylated with

(35) Zelinski, T.; Kula, M.-R. *Biocatal. Biotransform.* **1997**, *15*, 57.

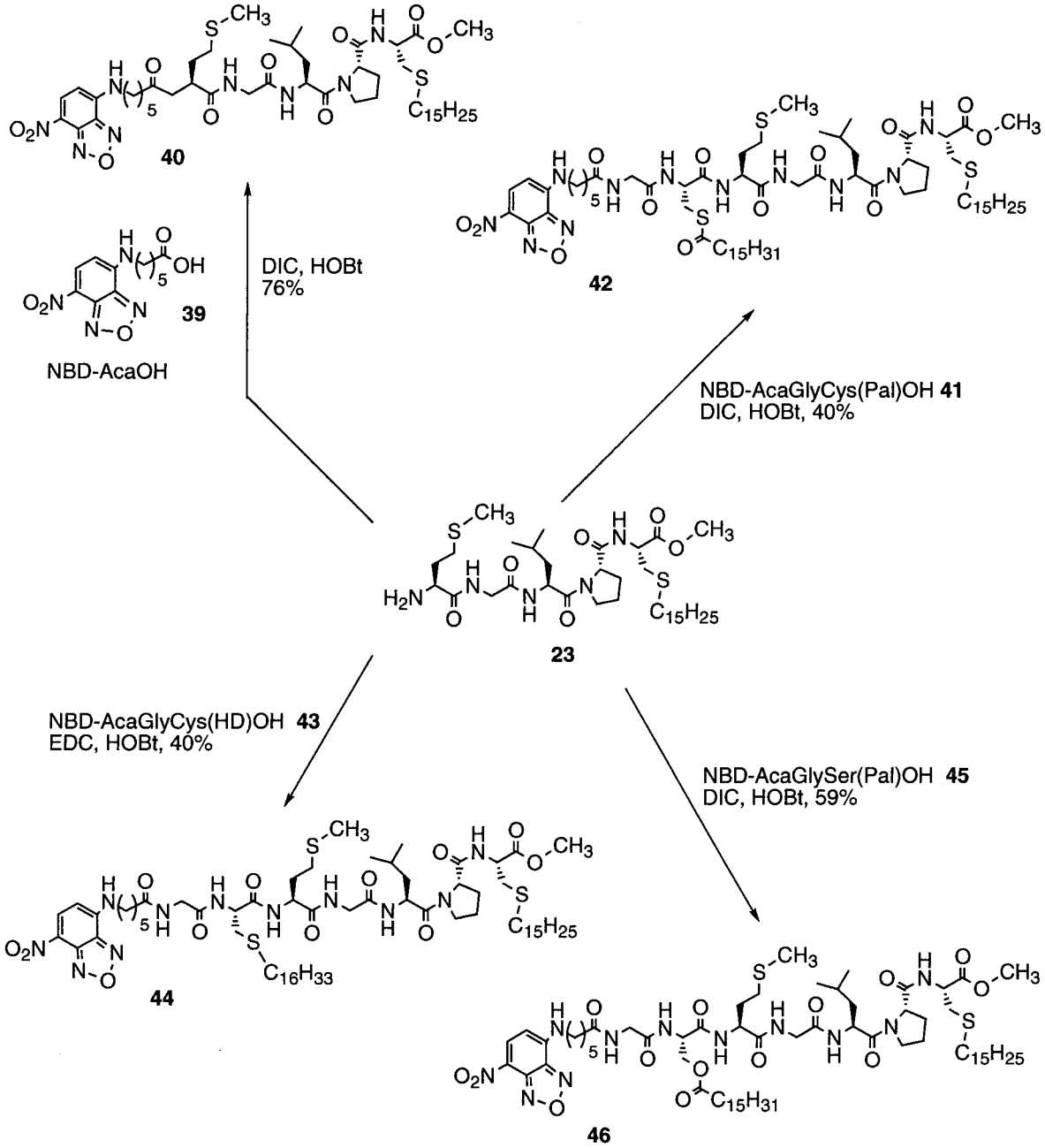
Scheme 10. Synthesis of Palmitoylated and Bimanyl-Labeled Lepoheptapeptides



palmitoyl chloride. The S-palmitoylated dipeptide **28** was then selectively unmasked at the C-terminus by Pd(0)-mediated transfer of the allyl group to morpholine as the accepting nucleophile (vide supra). This finding is remarkable since it indicates that the allyl ester also can be applied advantageously as a mildly removable blocking function in the construction of sensitive lipidated peptides embodying thioesters. Since the corresponding N-terminal allyloxycarbonyl (Aloc) group could also be removed selectively from S-farnesylated peptides,[19] allyl-based blocking functions in general appear to be efficient synthetic tools for the construction of lipid-modified peptides.

Synthesis of Fluorescent-Labeled Analogues of the N-*Ras* Peptide. The *Ras* lipopeptide **26** carries a free N-terminal amino function which may be employed to attach further marker groups, by which it may be traced in biological systems. However, the possibility that the allyl ester could be cleaved selectively from functionalized S-palmitoylated peptide conjugates opened up an opportunity for a more convergent approach. Thus, labeled or modified analogues of the AcOZ-protected and palmitoylated dipeptide carboxylic acid **24** were built up and

Scheme 11. Synthesis of NBD-Labeled and Differently Modified Lipidated Heptapeptides



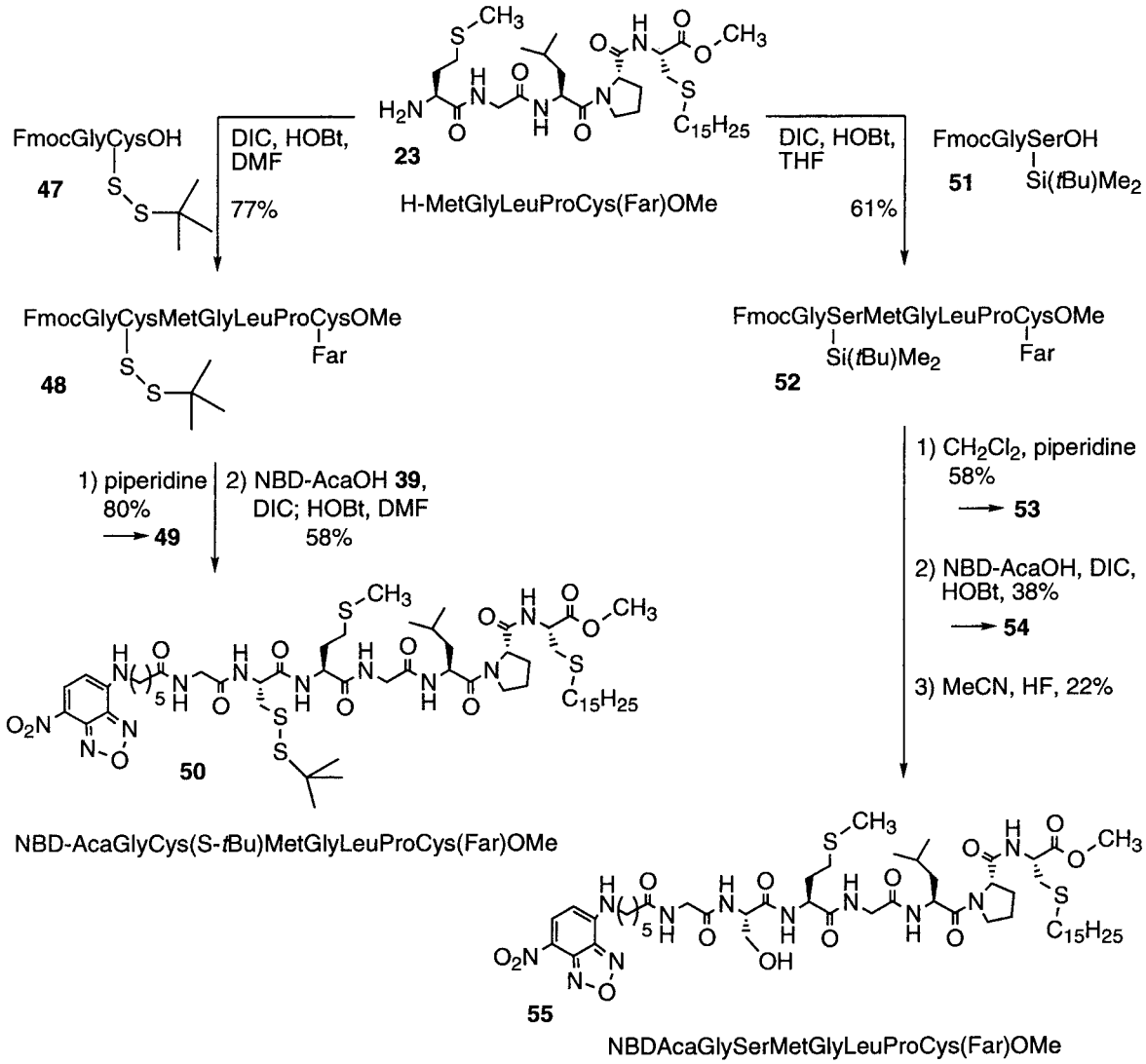
coupled to the N-terminally unmasked S-farnesylated pentapeptide **23**. Three different fluorescent labels were chosen to be introduced into the lipidated peptides, the *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl (NBD) group, the bimanyl group, and the fluoresceinyl (Fluo) moiety. These established fluorescent markers[15,17,18,36] can be detected by means of fluorescence microscopy and fluorescence spectroscopy.

To introduce the bimanyl group, the labeled S-palmitoylated dipeptide **36** was built up as shown in Scheme 10. To this end, bis(Boc)cysteine allyl ester **31** was synthesized and its disulfide bond was cleaved by reduction with DTT. Subsequent palmitoylation yielded the Boc-protected dipeptide ester **32**, from which the Boc group was removed selectively by treatment with trifluoroacetic acid to yield **33** quantitatively. The N-deprotected peptide **33** was then condensed with *S*-bimanylthioacetic acid **34** (BimTaOH) to yield the bimanyl-labeled peptide **35**. From this intermediate the allyl ester was selectively cleaved off to deliver the labeled dipeptide carboxylic acid **36** in excellent yield. Compound **36** was then condensed with the pentapeptide **23** by means of *N*,*N*′-diisopropylcarbodiimide and *N*-hydroxybenzotriazole as condensing reagents to give the bimanyl-labeled palmitoylated and farnesylated N-*Ras* peptide **37** (Scheme 10). In addition, the bimanyl group was directly attached to the pentapeptide **23**, giving rise to the fluorescent-labeled, farnesylated peptide **38** (Scheme 10).

For the introduction of the NBD label, the pentapeptide **23** also served as central intermediate (Scheme 11). Thus **23** was condensed with NBD aminocaproic acid (NBD-Aca-OH **39**), with the S-palmitoylated dipeptide **41**, with the *S*-hexadecyl-modified dipeptide **43**, and with the O-palmitoylated serinyl peptide **45** to give the NBD-labeled and differently modified heptapeptides **40**, **42**, **44**, and **46** (Scheme 11). In addition, **23** was coupled with the Fmoc-masked dipeptide **47** to give the protected heptapeptide **48** (Scheme 12). After removal of the

(36) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, 1996.

Scheme 12. Synthesis of NBD-Labeled Heptapeptides



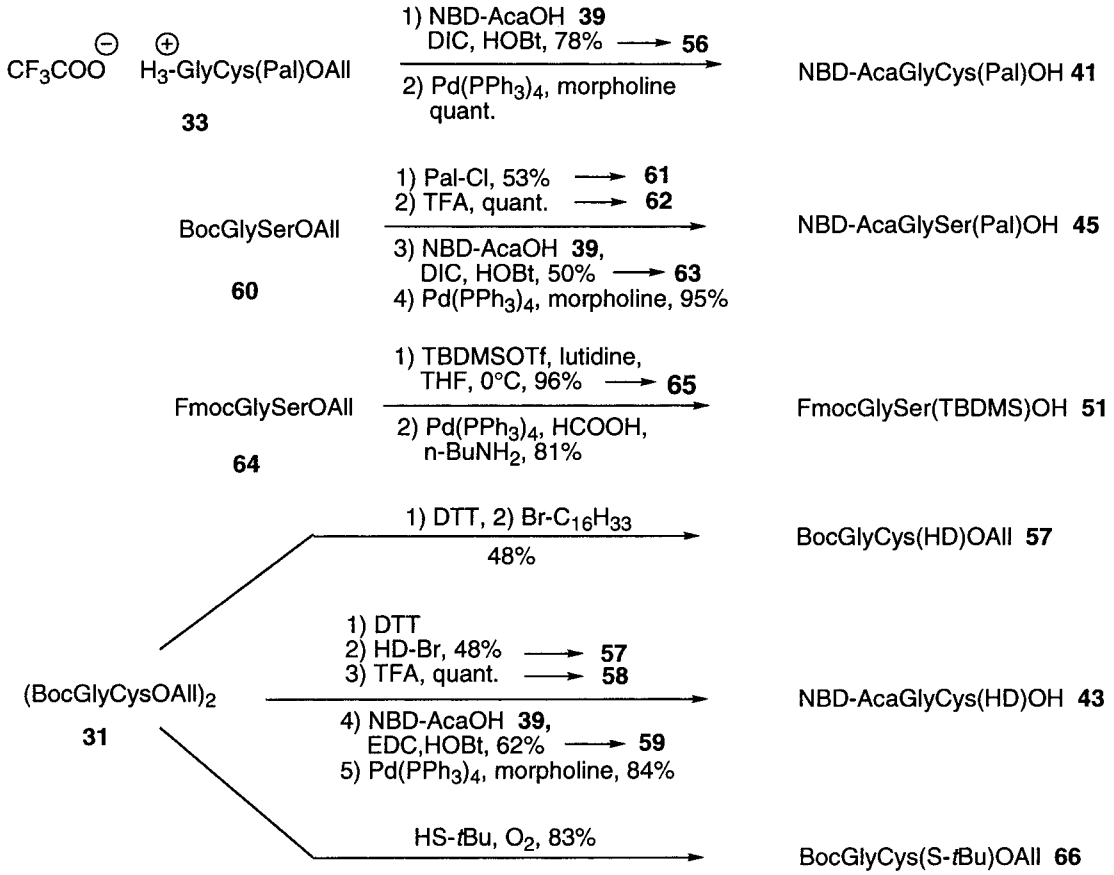
Fmoc group, the NBD label was introduced to yield the heptapeptide **50**, which embodies a cysteine protected as a disulfide. Furthermore, condensation of **23** with the O-silylated serinyl peptide **51** gave the masked heptapetide **52**, which was converted to the NBD-labeled heptapeptide **55** via removal of the Fmoc group, introduction of the NBD label, and liberation of the serine OH. The peptides **40**, **42**, **44**, **46**, **50**, and **55** all carry the same C-terminus, but the N-terminus was varied. Thus, in one set of compounds the *S*-palmitoyl thioester which occurs in the natural proteins was replaced by a metabolically stable thioether of similar lipophilicity and an SH group (which can be generated in situ from the *S*-*tert*-butyl disulfide by treatment with dithiothreitol). In the second set of compounds the cysteine was replaced by serine which is either O-palmitoylated or has an unmasked OH group.

The building blocks **41**, **43**, **45**, and **51** required for the syntheses described in Schemes 11 and 12 were built up as shown in Scheme 13 (the construction of **47** followed an established route[37]). The S-palmitoylated dipeptide **41** was obtained in high yield from the N-terminally deprotected dipeptide **33** by coupling with NBD aminocaproic acid and subsequent selective removal of the C-terminal allyl ester (see also Scheme 9). The analogous dipeptide **43** which carries a hexadexcyl thioether instead of a palmitic acid thioester was synthesized by reduction of the disulfide of the fully protected cysteine derivative **31** and subsequent alkylation of the liberated mercapto group with hexadecyl bromide. After removal of the N-terminal Boc group and introduction of the NBD label, the C-terminal allyl ester was cleaved by Pd-mediated allyl transfer to morpholine. For the construction of the O-palmitoylated NBD-labeled building block **45**, the dipeptide **60** was O-palmitoylated, the Boc group was removed, and the amine was coupled to NBD aminocaproic acid. Finally, the C-terminus was selectively unmasked by Pd-mediated allyl ester cleavage. The O-protected serine peptide **51** was obtained from the dipeptide **64** by O-silylation and subsequent liberation of the carboxylic acid.

Fluoresceine-labeled *Ras* peptides were synthesized as depicted in Scheme 14. Treatment of the Boc-protected cystine allyl ester **31** with *tert*-butyl thiol under aerobic conditions yielded the mixed disulfide **66** (Scheme 13) which was C- and N-terminally deprotected in high yield and then linked to fluoresceine by treatment with fluoresceine isothiocyanate (FITC) to give the labeled peptide **69**. In addition, **31** was converted to **32** as described above (Scheme 10) and then the allyl ester and the Boc group were split off. Treatment of the unmasked peptide **72** thus obtained with fluoresceine isothiocyanate delivered the fluorescent and S-palmitoylated glycyl-

(37) Wünsch, E. In *Houben-Weyl, Methoden der Organischen Chemie*; Georg Thieme Verlag: Stuttgart, 1974; Vol. 15/1, p 794.

Scheme 13. Synthesis of Lipid-Modified Dipeptides **41**, **43**, **45**, **51**, **57**, and **66**



cysteine **73** in high yield. Finally, from the *S*-hexadecyl-modified dipeptide ester **57** (obtained from **31** as described in Scheme 13) the N- and C-terminal blocking functions were removed and fluoresceine was introduced by treatment with the isothiocyanate to give the desired labeled dipeptide **77**. Compounds **69**, **73**, and **77** were then condensed with the farnesylated pentapeptide **23** (Scheme 14). Thereby the N-*Ras* heptapeptides **70**, **74**, and **78** were obtained. They once more embody an S-palmitoylated cysteine (**74**), a cysteine which carries a thioether instead of a thioester (**78**), or a precursor to an unmodified cysteine (**70**).

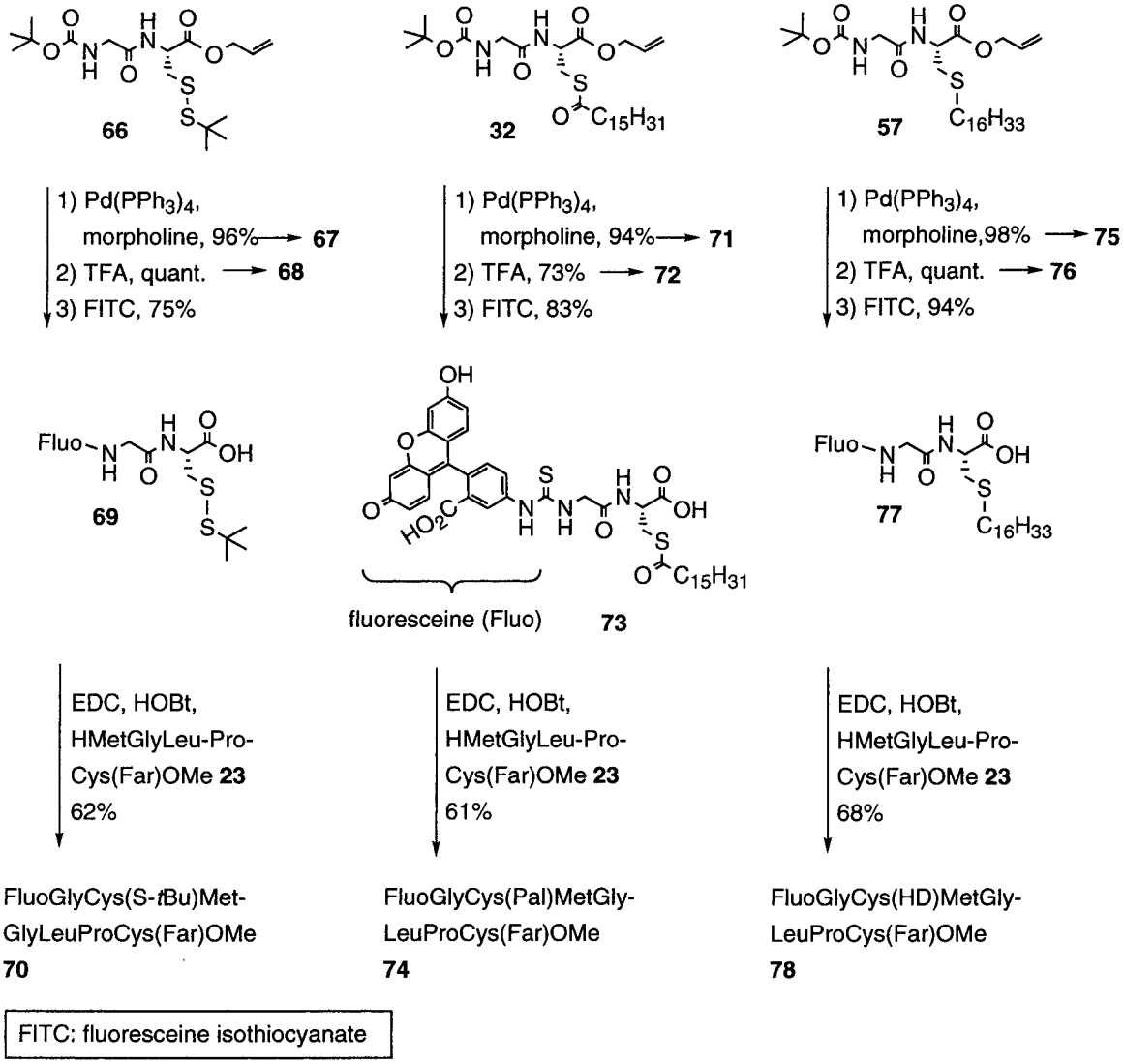
A Model for the Targeting of Lipidated Peptides and Proteins to the Plasma Membrane by S-palmitoylation. By means of the syntheses depicted in Schemes 10−14, a set of fluorescent-labeled N-*Ras*-derived lipidated peptides for further biological and bioorganic investigations is available. In addition to the different fluorescent groups, either the *S*-palmitoyl/*S*-farnesyl motif found in the N-*Ras* protein is incorporated by these peptides, or the S-palmitoylated cysteine is replaced by an *O*-palmitoylated serine, a serine, a cysteine, or an *S*-alkylated cysteine. These modifications of the underlying structure of the C-terminus of the human N-*Ras* protein open up interesting opportunities to address several biological problems by means of suitable in vivo and in vitro experiments. For instance, the structural requirements for the acylation of *Ras* sequence motifs in vivo and the subcellular site at which this modification occurs could be studied. Thus, initial in vivo cell biology experiments were carried out which addressed these problems by a combination of membrane fusion and fluorescence microscopy techniques[17b] or by means of microinjection and confocal laser fluorescence microscopy.[18] In addition, biophysical experiments were performed which yielded characteristic data describing the strength and the kinetics of membrane binding and spontaneous intermembrane transfer of the differently lipidated peptides.[17b] Taken together, the results obtained in these experiments revealed that peptides which are S-farnesylated and S-palmitoylated are specifically localized to the plasma membranes whereas peptides which are only farnesylated do not accumulate at this subcellular site. The observations suggested that the plasma membrane is itself a major site of cellular S-acylation. They support a mechanism for the selective subcellular localization of lipidated proteins, including the *Ras* proteins themselves, by kinetic targeting to the plasma membrane via S-acylation of farnesylated proteins at this membrane.[15,17]

According to this mechanism which is schematically described in Scheme 15, the proteins are farnesylated first, thereby gaining the ability to bind reversibly to different membranes. Only after attachment of a second lipid residue, for instance, S-acylation of a cysteine by an *S*-palmitoyltransferase bound to the plasma membrane, are the lipidated proteins localized to the particular membrane at which this second lipid group is introduced. In addition to fluorescence spectroscopic and fluorescence microscopic techniques, the fluoresceine-labeled peptides may be traced by means of antibodies which specifically recognize this dye. Such antibodies are, for instance, available in fluoresceine-modified form or labeled with colloidal gold.[36] Thereby the possibility to perform experiments with labeled *neolipoproteins* (i.e., the antibody bound to the lipidated peptide) and to employ electron microscopy (if the gold-labeled antibody is used) is opened up.

Conclusion

By means of the enzymatic protecting group techniques described in this paper, sensitive N-*Ras*-related peptides are available in an efficient manner. The chosen strategy allows

Scheme 14. Synthesis of Differently Modified, Fluoresceine-Labeled *Ras* Peptides



the flexible introduction of different modifications of the peptide chain and of various fluorescent labels. The peptide conjugates accessible by this strategy can now be employed as valuable tools for biological applications and should open up new opportunities for interdisciplinary research enterprises at the interface between chemistry and biology.

Experimental Section

^{1}H and ^{13}C NMR spectra were recorded on Bruker AM 400 and DRX 500 spectrometers. High-resolution mass spectra and FAB mass spectra were measured on a Finnigan MAT 90 spectrometer. Specific rotations were measured with a Perkin-Elmer 241 Polarimeter. Flash chromatography was performed using Baker silica gel (230−400 mesh ASTM). Reaction progress was monitored by TLC using Merck silica gel $60F_{254}$ aluminum sheets (Merck, Darmstadt, Germany).

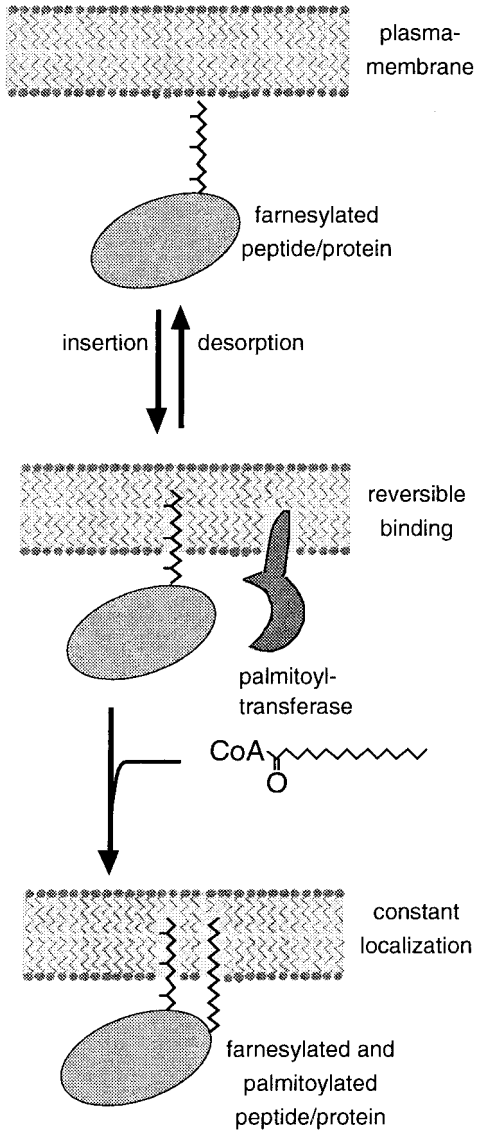
All reagents were obtained from Fluka (Buchs, Switzerland), Aldrich (Steinheim, Germany), or Sigma (Deisenhofen, Germany). All solvents were dried and distilled using standard procedures [Perrin, D.D.; Armarego, W.L.F. *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon: Oxford, 1988].

4-(Acetoxy)benzyl Alcohol (12). To a suspension of 4-hydroxybenzyl alcohol (**12**) (7.5 g, 60 mmol) in ethyl acetate (100 mL) at 0 °C under argon was added triethylamine (8.4 mL, 60 mmol) followed by acetyl chloride (4.2 g, 4.7 mL, 60 mmol), dissolved in ethyl acetate (50 mL). The mixture was stirred for 5 h, after which time the precipitate was filtered off and the solvent was evaporated in vacuo. The product 4-(acetoxy)benzyl alcohol was isolated from the residue by flash chromatography (silica gel; hexane/ethyl acetate 3:2) to yield 7.3 g (73%) of **12** as a yellow oil. $R_f = 0.43$ (hexane/ethyl acetate 3:2). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.3 (s, 3H, CH_3); 2.6 (s, 1H, OH); 4.55 (s, 2H, CH_2); 7.05 (d, $J = 8$ Hz, 2H, C*H*CCH$_2$ aromatic); 7.3 (d, $J = 8$ Hz, 2H, OCCH aromatic). MS *m*/*e* calcd for (M^+) $C_9H_{10}O_3$ 166.062, found 166.063.

4-(Acetoxy)benzyl Chloroformate (AcOZ-Cl) (13). To a solution of phosgene (0.1 mol) in toluene (50 mL) at 0 °C under argon was added a solution of 4-(acetoxy)benzyl alcohol (7 g, 42 mmol) in toluene (50 mL). The solution was stirred overnight, and the excess of phosgene and the solvent were evaporated in vacuo. The product AcOZ-Cl (**13**) was isolated as yellow oil. Yield: 9.4 g (98%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.3 (s, 3H, CH_3); 5.3 (s, 2H, CH_2); 7.1 (d, $J = 8$ Hz, 2H, C*H*CCH$_2$ aromatic); 7.4 (d, $J = 8$ Hz, 2H, OCCH aromatic). MS *m*/*e* calcd for (M^+) $C_{10}H_9O_4Cl$ 228.018, found 228.017.

Synthesis of *N*-(4-(Acetoxy)benzyloxycarbonyl) Amino Acids (15). To a suspension of the amino acid (6 mmol) in THF (50 mL) was added trimethylsilyl chloride (12 mmol) (for serine 18 mmol), and the mixture was warmed to 50 °C for 30 min. After the mixture cooled to 0 °C, triethylamine (12 mmol) was added (for serine 18 mmol) followed by 4-(acetoxy)benzyl chloroformate (**13**) (15 mmol). The solution was stirred for 1 h at 0 °C and for 12 h at room temperature. The solvent was evaporated in vacuo, and the residue was dissolved in 20 mL of 1 M $NaHCO_3$/ether 1:1. The pH of the aqueous phase was adjusted to pH 2 with 1 M HCl. After being extracted three times with ethyl

Scheme 15. Proposed Model for the Targeting of Lipidated Proteins to the Plama Membrane



acetate, the combined organic layers were dried over $MgSO_4$ and the solvent was distilled off in vacuo.

***N*-(4-(Acetoxy)benzyloxycarbonyl)-L-alanine (AcOZ-AlaOH) (15a).** Data for **15a**: white crystals. Yield: 1.1 g (68%). Mp 90 °C. $[\alpha]^{20}_D = -14.5°$ ($c = 0.5$, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$): δ 1.5 (d, $J = 7$ Hz, 3H, CH_3 Ala); 2.3 (s, 3H, CH_3CO); 4.4 (m, 1H, α-CH Ala); 5.1 (s, 2H, CH_2O); 5.4 (d, $J = 8$ Hz, 1H, NH urethane); 7.1 (d, $J = 8$ Hz, 2H, C*H*CCH_2 aromatic); 7.4 (d, $J = 8$ Hz, 2H, OCCH aromatic); 9.8 (s, 1H, COOH). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 18.4 (CH_3 Ala); 21.2 (*C*H_3CO); 49.5 (α-CH Ala); 66.4 (CH_2O); 129.4 (CH aromatic); 121.7 (CH aromatic); 150.5 (C_q aromatic); 133.8 (C_q aromatic); 155.8 (OCONH); 169.6 (CO); 177.5 (COOH). MS *m*/*e*: calcd for (M^+) $C_{13}H_{15}O_6N$ 281.089, found 281.089. Anal. Calcd: C, 55.50; H, 5.38; N, 4.98. Found: C, 55.31; H, 5.36; N, 4.64.

Synthesis of *N*-(4-(Acetoxy)benzyloxycarbonyl)-dipeptide Allyl Esters (17). To a solution of *N*-(4-(acetoxy)benzyloxycarbonyl) amino acid (**15**) (6 mmol) and amino acid allyl ester hydrotosylate (**16**) (6 mmol) in THF (100 mL) under argon was added triethylamine (6 mmol). The solution was cooled to 0 °C, and HOBt (12 mmol) and a solution of DCC (7.2 mmol) in THF (50 mL) were added slowly. The solution was stirred overnight, the precipitate was filtered off, and the solvent was evaporated in vacuo. The residue was dissolved in dichloromethane and extracted with 1 M HCl, 1 M $NaHCO_3$, and distilled water. The organic layer was dried over $MgSO_4$, and the solvent was evaporated in vacuo. The remaining residue was purified by flash chromatography on silica gel.

***N*-(4-(Acetoxy)benzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine Allyl Ester (AcOZPhePheOAll) (17a).** Data for **17a**: white crystals. Yield: 2.3 g (70%). $R_f = 0.48$ (hexane/ethyl acetate 3:2). mp 143 °C. $[\alpha]^{20}_D = +23°$ ($c = 0.5$, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 2.3 (s, 3H, CH_3CO); 2.9−3.1 (m, 4H, 2 × β-CH_2 Phe); 4.4 (m, 1H, α-CH Phe); 4.6 (d, $J = 7$ Hz, 2H, OCH_2); 4.8 (m, 1H, α-CH Phe); 5.1 (s, 2H, CH_2O); 5.2 (m, 2H, CH_2 allyl); 5.3 (d, $J = 8$ Hz, 1H, NH urethane); 5.8−6.0 (m, 1H, CH allyl); 6.9−7.4 (m, 14H, 2 × OCCH aromatic, 2 × C*H*CCH_2 aromatic, 2 × C_6H_5), 6.2 (s, 1H, NH amide). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 21.1 (*C*H_3CO); 37.9 (β-CH_2 Phe); 38.3 (β-CH_2 Phe); 53.3 (α-CH Phe); 56.0 (α-CH Phe); 66.1, 66.4 (OCH_2, CH_2O); 119.1 (CH_2 allyl); 121.4; 127.1; 128.5; 128.8; 129.3; 129.8; 130.1 (14 × CH aromatic); 133.8 (CH allyl); 135.5; 136.2; 150.5 (4 × C_q aromatic); 155.7 (OCONH); 169.3 (CO); 170.3 (CO); 170.6 (CO). MS *m*/*e*: calcd for (M^+) $C_{31}H_{32}O_7N_2$ 544.220, found 544.218. Anal. Calcd: C, 68.37; H, 5.92; N, 5.14. Found: C, 68.38; H, 6.03; N, 5.49.

Bis[*N*-(4-(Acetoxy)benzyloxycarbonyl)-glycyl]-L-Cystine Bis-Allyl Ester [$(AcOZGly)_2Cys_2(OAll)_2$] (17h). To a solution of AcOZ-GlyOH (**15f**) (3.3 g, 12 mmol) in THF (100 mL) at 0 °C under argon was added $H_2Cys_2(OAll)_2$·2 TosOH (**55**) (4.17 g, 6 mmol), NEt_3 (1.31 g, 1.75 mL, 13 mmol), N-hydroxybenzotriazole (HOBt) (3.4 g, 26 mmol), and diisopropylcarbodiimide (DIC) (1.89 g, 2.4 mL, 15 mmol) in THF (50 mL). The mixture was stirred for 24 h at room temperature, the precipitate was filtered off, and the solvent was evaporated in vacuo. The residue was dissolved in dichloromethane (100 mL) and extracted with 1 M HCl (30 mL), 1 M $NaHCO_3$ (30 mL), and water (30 mL). The organic layer was dried over $MgSO_4$, the solvent was distilled off in vacuo, and the product $(AcOZGly)_2Cys_2(OAll)_2$ (**17h**) was isolated from the residue by flash chromatography (silica gel, ethyl acetate/*n*-hexane 4:1) to yield 7.3 g (74%) of a white amorphous solid. $R_f = 0.35$ (ethyl acetate/*n*-hexane 4:1). $[\alpha]^{20}_D = -28°$ ($c = 0.9$, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$): δ 7.4 (d, $J = 8$ Hz, 4H, 4 aromatic OCCH); 7.1 (d, $J = 8$ Hz, 4H, 4 aromatic C*H*CCH_2); 5.8−6.0 (m, 2H, 2 CH allyl); 5.2−5.5 (m, 6H, 2 allyl CH_2, 2 NH urethane); 5.1 (s, 4H, 2 CH_2O); 4.8 (m, 2H, 2 Cys α-CH); 4.6 (d, $J = 7$ Hz, 4H, 2 OCH_2); 3.9 (d, $J = 5.6$ Hz, 4H, 2 Gly CH_2); 3.3 (m, 4H, 2 Cys β-CH_2); 2.3 (s, 6H, 2 CH_3CO). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 21.1 (*C*H_3CO); 40.7 (β-CH_2 Cys); 44.3 (CH_2 Gly); 52.1 (α-CH Cys); 66.5, 66.4 (CH_2O AcOZ, OCH_2 allyl); 119.2 (CH_2 allyl); 121.7 (2 × CH aromatic); 129.4 (2 × CH aromatic); 131.2 (CH allyl); 133.9 (C_q aromatic); 150.4 (C_q aromatic); 156.1 (OCONH); 169.5 (CO); 169.6 (CO); 169.7 (CO). MS *m*/*e*: calcd for (M^+) $C_{36}H_{43}O_{14}N_4S_2$ 818.217, found 819.233. Anal. Calcd: C, 52.80; H, 5.17; N, 6.84. Found: C, 52.82; H, 5.48; N, 7.25.

Enzymatic Removal of the *N*-(4-(Acetoxy)benzyloxycarbonyl) Protecting Group from Dipeptide Allyl Esters with Acetyl Esterase. To a suspension of *N*-(4-(acetoxy)benzyloxycarbonyl)-dipeptide allyl ester (**17**) (0.18 mmol) in 0.15 M NaCl (200 mL) solution at pH 7 was added 5 units of acetyl esterase, and the mixture was stirred at 40 °C for 12 h. The solution was extracted five times with ethyl acetate, the combined organic layers were dried over $MgSO_4$, and the solvent was evaporated in vacuo. The remaining residue was purified by flash chromatography on silica gel.

L-Phenylalanyl-L-alanine Allyl Ester (HPheAlaOAll) (18b). Data for **18b**: yellow oil. Yield 8.7 mg (63%). $R_f = 0.55$ (ethyl acetate/methanol 4:1). $[\alpha]^{20}_D = -14°$ ($c = 0.3$, $CHCl_3$). ^1H NMR (250 MHz, $CDCl_3$): δ 1.4 (d, $J = 7$ Hz, 3H, CH_3 Ala); 2.1 (s, 2H, NH_2); 2.7 (m, 1H, β-CH_2 Phe); 3.0 (m, 1H, β-CH_2 Phe); 3.7 (m, 1H, α-CH Phe); 4.5 (m, 1H, α-CH Ala); 4.6 (m, 2H, OCH_2); 5.2−5.3 (m, 2H, CH_2 allyl); 5.8−6.0 (m, 1H, CH allyl); 7.1−7.3 (m, 5H, C_6H_5); 7.6 (d, $J = 8$ Hz, 1H, NH amide). MS *m*/*e* calcd for (M^+) $C_{15}H_{20}O_3N_2$ 276.147, found 276.146.

l-Phenylalanyl-l-phenylalanine Allyl Ester (HPhePheOAll) (18a) via Enzymatic Deprotection of AcOZPhePheOAll (17a) with Lipase from *M. miehei*. AcOZPhePheOAll (**17a**) (0.2 mmol) was dissolved with ultrasonication in a mixture of a 0.2 M KI solution (160 mL) (pH 5) and methanol (40 mL). Fifty units lipase from *M. miehei* was added, and the mixture was stirred at 37 °C and at pH 5 for 12 h. The solution was extracted three times with dichloromethane, the combined organic

layers were dried over $MgSO_4$, and the solvent was evaporated in vacuo. The product HPhePheOAll (**18a**) was isolated from the residue by flash chromatography (silica gel, ethyl acetate/methanol 4:1) to yield 12 mg (69%) of **18a** as a yellow oil. $R_f = 0.9$ (ethyl acetate/methanol 4:1). $[\alpha]^{20}_D = -45°$ ($c = 0.3$, $CHCl_3$). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.6 (m, 1H, β-CH_2 Phe); 3.0 (m, 3H, β-CH_2 Phe); 3,6 (m, 1H, α-CH Phe); 4.5 (d, $J = 7$ Hz, 2H, OCH_2); 4.8 (m, 1H, α-CH Phe); 5.2 (m, 2H, CH_2 allyl); 5.7−5.9 (m, 1H, CH allyl); 6.9−7.3 (m, 10H, 2 × C_6H_5); 7.6 (d, $J = 10$ Hz, 1H, NH amide). MS *m/e*: calcd for (M^+) $C_{21}H_{24}O_3N_2$ 352.178, found 352.177.

Cleavage of the Allyl Ester Protecting Group from *N*-(4-(Acetoxy)benzyloxycarbonyl)-dipeptide Allyl Esters. To a solution of *N*-(4-(acetoxy)benzyloxycarbonyl)-dipeptide allyl ester (0.6 mmol) (**17**) in THF (50 mL) under argon were added $Pd(PPh_3)_4$ (0.06 mmol) and morpholine (0.72 mmol). The solution was stirred at room temperature for 30 min, the solvent was evaporated in vacuo, and the product was isolated from the residue by flash chromatography on silica gel.

***N*-(4-(Acetoxy)benzyloxycarbonyl)-L-phenylalanyl-phenylalanine (AcOZPhePheOH) (19a).** Data for **19a**: clear oil. Yield 40 mg (80%). $R_f = 0.5$ (ethyl acetate/hexane 7:3 + 5% acetic acid). $[\alpha]^{20}_D = +28°$ ($c = 0.5$, $CHCl_3$). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.3 (s, 3H, CH_3CO); 2.9−3.2 (m, 4H, 2 × β-CH_2 Phe); 4.4 (m, 1H, α-CH Phe); 4.5 (m, 1H, α-CH Phe); 5.0 (s, 2H, $C_6H_4CH_2$); 5.5 (d, $J = 8$ Hz, 1H, NH urethane); 6.5 (d, $J = 8.5$ Hz, 1H, NH amide); 6.9−7.3 (m, 14H, CH aromatic). MS *m/e* calcd for (M^+) $C_{28}H_{28}O_7N_2$ 504.189, found 504.188.

***N*-(4-(Acetoxy)benzyloxycarbonyl)-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteine Methyl Ester (AcOZLeuProCys(Far)OMe) (20).** To a solution of AcOZ-LeuProOH (**19b**) (0.63 g, 1.5 mmol) and of HCys-(Far)OMe[34] (**7**) (0.5 g, 1.5 mmol) in dichloromethane (10 mL) under argon was added a solution of HOBt (3 mmol, 0.4 g) and EDC (0.34 g, 1.8 mmol) in dichloromethane (10 mL). The solution was stirred for 24 h at room temperature; and was extracted with 1 M HCl, 1 M $NaHCO_3$, and distilled water and dried over $MgSO_4$. The solvent was removed in vacuo, and the product AcOZLeuProCys(Far)OMe (**20**) was isolated from the residue by flash chromatography (silica gel, *n*-hexane/ethyl acetate 1:1) to yield 1 g (94%) of **20** as a yellow oil. $R_f = 0.3$ (*n*-hexane/ethyl acetate 1:1). $[\alpha]^{20}_D = -58°$ ($c = 0.5$, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.9 (d, $J = 7$ Hz, 3H, CH_3 Leu); 1.0 (d, $J = 7$ Hz, 3H, CH_3 Leu); 1.5 (m, 2H, Leu γ-CH); 1.6 (s, 6H, 2 × CH_3 Far); 1.65 (s, 6H, 2 × Far CH_3); 1.7 (m, 1H, Leu β-CH_2); 1.8−2.1 (m, 11H, 4 × CH_2 Far, CH_2 Pro (3H)); 2.3 (s, 3H CH_3CO); 2.35 (m, 1H, CH_2 Pro); 2.75 (dd, $J_1 = 6$ Hz, $J_2 = 13.8$ Hz, 1H, β-CH_2 Cys); 2.95 (dd, $J_1 = 6.7$ Hz, $J_2 = 13.7$ Hz, 1H, Cys β-CH_2); 3.0−3.2 (m, 2H, CH_2 Far); 3.6 (m, 1H, δ-CH_{2a} Pro); 3.7 (m, 1H, δ-CH_{2b} Pro); 3.75 (s, 3H, CH_3CO); 4.55 (m, 1H, α-CH); 4.6 (m, 1H, α-CH); 4.65 (m, 1H, α-CH); 5.0 (s, 2H, $C_6H_4CH_2O$); 5.1 (m, 2H, 2 × CH Far); 5.2 (t, $J = 7$ Hz, CH Far); 5.7 (d, $J = 8.5$ Hz, 1H, NH urethane); 7.1 (d, $J = 8$ Hz, 2H, 2 × C*H*CCH$_2$ aromatic); 7.3 (d, $J = 8$ Hz, 2H, 2 × OCCH aromatic); 7.4 (d, $J = 9$ Hz, 1H, NH amide). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 15.95 (C(*C*H_3)$_2$ Far); 16.07 (C(*C*H_3)$_2$ Far); 17.6 (Far CH_3); 21.0 (*C*H_3CO); 21.58 (CH_3 Leu); 23.3 (CH_3 Leu); 24.5 (Leu γ-CH); 24.9 (CH_3 Far); 25.6 (CH_2 Pro); 26.4 (CH_2 Far); 26.4 (CH_2 Far); 26.6 (CH_2 Far); 27.2 (CH_2 Pro); 29.6 (CH_2 Far); 33.1 (β-CH_2 Cys); 39.6 (2 × CH_2 Far); 42.2 (Leu β-CH_2); 47.1 (Pro δ-CH_2); 50.8 (OCH_3); 51.7 (α-CH Leu); 52.1 (α-CH Cys); 59.64 (α-CH Pro); 66.0 ($C_6H_5CH_2O$); 119.6 (CH Far); 121.6 (2 × CH aromatic); 123.7 (CH Far); 124.3 (CH Far); 129.1 (2 × CH aromatic); 131.1(C_q Far); 133.9 (C_q aromatic); 135.8 (C_q Far); 139.6 (C_q Far); 150.3 (C_q aromatic); 156.1 (OCONH); 169.3 (CO); 170.9 (CO); 171.06 (CO); 172.7 (CO). MS *m/e*: calcd for (M^+) $C_{40}H_{59}O_8N_3S$ 741.402, found 741.401. Anal. Calcd: C, 64.75; H, 8.01; N, 5.66. Found: C, 64.30; H, 7.83; N, 5.48.

L-Leucyl-L-prolyl-(*S*-farnesyl)-L-cysteine Methyl Ester (HLeuProCys(Far)OMe) (21). AcOZLeuProCys(Far)OMe (**20**) (20 mg, 27 μmol) was dissolved with ultrasonication in a mixture of 0.2 M KJ solution (160 mL) (pH 5) and methanol (40 mL). Next, 25 units of lipase from *M. miehei* was added, and the mixture was stirred at 37 °C and at pH 5 for 12 h. The solution was extracted three times with dichloromethane, and the organic layer was dried over $MgSO_4$. The solvent was evaporated in vacuo, and the product HLeuProCys(Far)-OMe (**21**) was isolated from the residue by flash chromatography (silica gel, ethyl acetate/methanol 4:1) to yield 9.6 mg (65%) of **21** as a yellow oil. $R_f = 0.16$ (ethyl acetate/methanol 4:1). $[\alpha]^{20}_D = -50°$ ($c = 1$, $CHCl_3$). ^{1}H NMR (400 MHz; $CDCl_3$) δ 0.9 (m, 6H, 2 × CH_3 Leu); 1.4 (m, 2H, γ-CH Leu); 1.6 (s, 6H, 2 × CH_3 Far); 1.65 (s, 6H, 2 × CH_3 Far); 1.7 (m, 1H, β-CH_2 Leu); 1.8−2.2 (m, 13H, 4 CH_2 Far, CH_2 Pro (3H), NH_2); 2.3 (m, 1H, CH_2 Pro); 2.75 (dd, $J_1 = 7$ Hz, $J_2 = 15$ Hz, 1H, β-CH_{2a} Cys); 2.95 (dd, $J_1 = 5$ Hz, $J_2 = 14$ Hz, 1H, β-CH_{2b} Cys); 3.0−3.2 (m, 2H, CH_2 Far); 3.5−3.7 (m, 3H, δ-CH_2 Pro, α-CH Leu); 3.75 (s, 3H, CH_3CO); 4.6−4.7 (m, 3H, 2 × α-CH); 5.1 (m, 2H, 2 × CH Far); 5.2 (t, $J = 7$ Hz, CH Far); 7.5 (d, $J = 9$ Hz, 1H, NH amide). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 16.0 (CH_3 Far); 16.1 (CH_3 Far); 17.7 (CH_3 Far); 21.4 (CH_3 Leu); 23.6 (CH_3 Leu); 24.6 (CH_3 Far); 25.0 (γ-CH Leu); 25.7 (CH_2 Pro); 26.4 (CH_2 Far); 26.7 (CH_2 Far); 27.0 (CH_2 Pro); 29.6 (CH_2 Far); 33.2 (CH_2 Far); 39.6 (α-CH_2 Far); 39.7 (β-CH_2 Cys); 44.5 (β-CH_2 Leu); 46.8 (CH_2 Pro); 51.2 (α-CH Leu); 51.7 (α-CH Cys); 52.4 (OCH_3); 59.7 (α-CH Pro); 119.6 (CH Far); 123.7 CH Far); 124.3 CH Far); 131.2 (C_q Far); 135.3 (C_q Far); 139.8 (C_q Far); 170.8 (C═O); 171.0 (C═O); 176.2 (C═O). MS *m/e*: calcd for (M^+) $C_{30}H_{51}N_3O_4S$ 549.360, found 549.357.

***N*-(4-(Acetoxy)benzyloxycarbonyl)-L-methionyl-glycyl-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteine Methyl Ester (AcOZMetGlyLeu-ProCys(Far)OMe) (22).** To a solution of HLeuProCys(Far)OMe (**21**) (27 mg, 50 μmol) and AcOZ-MetGlyOH (**19c**) (20 mg, 50 μmol) in dichloromethane (10 mL) at 0 °C under argon was added a solution of HOBt (13.6 mg, 100 μmol) and of EDC (11.5 mg, 60 μmol) in dichloromethane (5 mL) and the mixture was stirred for 24 h at room temperature. The solution was extracted with 1 M HCl, 1 M $NaHCO_3$, and distilled water and dried over $MgSO_4$. The solvent was evaporated in vacuo, and the product AcOZMetGlyLeuProCys(Far)OMe (**22**) was isolated from the residue by flash chromatography (silica gel, ethyl acetate/methanol 9:1) to yield 37 mg (80%) of **22** as a yellow oil. $R_f = 0.58$ (ethyl acetate/methanol 9:1). $[\alpha]^{20}_D = -56°$ ($c = 0.6$, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.9 (m, 6H, 2 × CH_3 Leu); 1.2 (m, 2H, γ-CH_2 Leu); 1.6 (s, 6H, 2 × CH_3 Far); 1.65 (s, 6H, 2 CH_3 Far); 1.7 (m, 1H, β-CH_2 Leu); 1.8−2.2 (m, 17H, 4 × CH_2 Far, 2 × CH_2 Pro, γ-CH_2 Met, SCH_3); 2.3 (s, 3H, CH_3CO); 2.6 (m, 2H, β-CH_2 Met); 2.75 (m, 1H, β-CH_2 Cys); 2.95 (m, 1H, β-CH_2 Cys); 3.0−3.2 (m, 2H, CH_2 Far); 3.5−3.7 (m, 2H, CH_2 Pro); 3.75 (s, 3H, OCH_3); 4.0 (m, 2H, CH_2 Gly); 4.4 (m, 1H, α-CH); 4.8 (m, 1H, α-CH); 5.1−5.2 (m, 4H, CH_2O, 2 × CH Far); 5.2 (t, $J = 7$ Hz, 1H, CH Far); 5.8 (d, $J = 8$ Hz, 1H, NH urethane); 6.8 (d, $J = 8$ Hz, 1H, NH amide); 7.1 (d, $J = 8$ Hz, 2H, 2 aromatic C*H*CCH$_2$); 7.15 (d, $J = 8$ Hz, 1H, NH amide); 7.2 (d, $J = 8$ Hz, 2H, 2 × OCCH aromatic); 7.5 (d, $J = 8$ Hz, 1H, NH amide). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 15.2 (SCH_3); 16.0 (Far C(*C*H_3)$_2$); 16.1 (Far C(*C*H_3)$_2$); 17.7 (Far CH_3); 21.1 (*C*H_3CO); 21.8 (CH_3 Leu); 21.9 (CH_3 Leu); 24.7 (γ-CH Leu); 24.9 (CH_3 Far); 25.7 (CH_2 Pro); 26.5 (CH_2 Far); 26.7 (CH_2 Far); 27.7 (CH_2 Pro); 29.6 (CH_2 Met); 30.0 (CH_2 Met); 32.6 (β-CH_2 Cys); 39.7 (CH_2 Far); 41.9 (CH_2 Gly); 42.9 (β-CH_2 Leu); 47.3 (δ-CH_2 Pro); 51.9 (α-CH Leu); 52.5 (α-CH Cys); 53.9 (α-CH Met); 59.8 (α-CH Pro); 66.4 (CH_2O); 119.6 (CH Far); 121.7 (2 × CH aromatic); 123.7 (CH Far); 124.3 (CH Far); 129.4 (2 × CH aromatic); 131.3 (C_q Far); 133.8 (C_q aromatic); 135.3 (C_q Far); 139.9 (C_q Far); 150.5 (C_q aromatic); 156.9 (OCONH); 171.1 (CO); 171.5 (CO); 171.6 (CO); 171.9 (CO); 172.0 (CO); 172.8 (CO). MS *m/e*: calcd for (M^+) $C_{47}H_{71}O_{10}N_5S_2$ 929.464, found 929.4510. Anal. Calcd (dihydrate): C, 58.42; H, 7.82; N, 7.25. Found: C, 58.47; H, 7.39; N, 7.19.

L-Methionyl-glycyl-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteine Methyl Ester (HMetGlyLeuProCys(Far)OMe) (23). AcOZMetGlyLeu-ProCys(Far)OMe (**22**) (20 mg, 27 μmol) was dissolved with ultrasonication in a mixture of 0.2 M KJ solution (160 mL) (pH5) and methanol (40 mL). Next, 25 units of lipase from *M. miehei* was added, and the mixture was stirred at 37 °C and at pH 5 for 12 h. The solution was extracted three times with dichloromethane and the organic layer was dried over $MgSO_4$. The solvent was evaporated in vacuo, and the product HMetGlyLeuProCys(Far)OMe (**23**) was isolated from the residue by flash chromatography (silica gel, ethyl acetate/methanol 4:1) to yield 9.5 mg (48%) of **23** as a yellow oil. $R_f = 0.25$ (ethyl acetate/methanol 4:1); $[\alpha]^{20}_D = -78°$ ($c = 0.5$, $CHCL_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.9 (m, 6H, 2 × CH_3 Leu); 1.4 (m, 2H, γ-CH Leu); 1.55

(s, 6H, 2 × CH_3 Far); 1.65 (s, 6H, 2 × CH_3 Far); 1.7 (m, 1H, β-CH_2 Leu); 1.8−2.2 (m, 17H, 4 × CH_2 Far, 2 × CH_2 Pro, γ-CH_2 Met, SCH_3); 2.6 (m, 2H, β-CH_2 Met); 2.7 (m, 1H, β-CH_2 Cys); 3.0−3.2 (m, 2H, CH_2 Far); 2.9 (m, 1H, β-CH_2 Cys); 3.5 (m, 1H, δ-CH_2 Pro); 3.6 (m, 1H, δ-CH_2 Pro); 3.7 (s, 3H, OCH_3); 3.8 (m, 1H α-CH Met); 4.1 (m, 2H, CH_2 Gly); 4.6 (m, 1H, α-CH); 4.7 (m, 1H, α-CH); 4.9 (m, 1H, α-CH); 5.1−5.2 (m, 2H, 2 × CH Far); 5.2 (t, J = 7 Hz, 1H, CH Far); 6.8 (d, J = 8 Hz, 1H, NH amide); 7.15 (d, J = 8 Hz, 1H, NH amide); 7.5 (d, J = 8 Hz, 1H, NH amide). MS *m*/*e*: calcd for (M^+) $C_{37}H_{63}N_5O_6S_2$ 737.429, found 737.419.

***N*-(4-(Acetoxy)benzyloxycarbonyl)-glycyl-(*S*-palmitoyl)-L-cysteyl-L-methionyl-glycyl-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteine Methyl Ester (AcOZGlyCys(Pal)MetGlyLeuProCys(Far)OMe) (25).** To a solution of HMetGlyLeuProCys(Far)OMe (**23**) (50 mg, 67 μmol) in DMF (5 mL) at 0 °C under argon was added AcOZGlyCys(Pal)OH (**24**) (41 mg, 67 μmol), EDC (19 mg, 100 μmol), and HOBt (18 mg, 130 μmol). The mixture was stirred for 12 h at room temperature, the solvent was evaporated in vacuo, and the residue was dissolved in dichloromethane. The solution was extracted with 0.5 M HCl, the organic layer was dried over $MgSO_4$, and the solvent was distilled off in vacuo. The product AcOZGlyCys(Pal)MetGlyLeuProCys(Far)OMe (**25**) was isolated from the residue by flash chromatography (silica gel, *n*-hexane/ethyl acetate/methanol 5:5:1) to yield 27 mg (61%) of **25** as colorless oil. R_f = 0.33 (*n*-hexane/ethyl acetate/methanol 5:5:1). $[\alpha]^{20}_D$ = −40° (c = 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.9 (m, 9H, 2 × CH_3 Leu, ω-CH_3 Pal); 1.3−1.35 (m, 25H, γ-CH Leu, 12 × CH_2 Pal); 1.7−1.55 (m, 16H, β-CH_2 Leu, 2 × CH_3 Far, β-CH_2 Pal, 2 × CH_3 Far); 1.7−2.2 (m, 17H, 4 × CH_2 Far, 2 × CH_2 Pro, γ-CH_2 Met, SCH_3); 2.3 (s, 3H, CH_3CO); 2.5 (m, α-CH_2 Pal); 2.7 (m, 2H, β-CH_2 Met); 2.9 (m, 2H, β-CH_2 Cys); 3.0−3.4 (m, 4H, β-CH_2 Cys, CH_2 Far); 3.6 (m, 2H, δ-CH_2 Pro); 3.7 (s, 3H, OCH_3); 3.9 (m, 2H, CH_2 Gly); 4.1 (m, 2H, CH_2 Gly); 4.5−4.7 (m, 4H, 4 × α-CH); 4.8 (m, 1H, α-CH); 5.0 (s, 2H, CH_2CCH_2O); 5.1−5.2 (m, 2H 2 × CH Far); 5.2 (m, 1H, CH Far); 6.0 (m, 1H, NH urethane); 7.1 (d, J = 8 Hz, 2H, 2 × CH_2-CCH_2O aromatic); 7.4 (d, J = 8 Hz, 2H, 2 × OCCH aromatic); 7.0−8.1 (m, 5H, 5 NH amide). MS *m*/*e*: calcd for (M^+) $C_{68}H_{109}O_{13}N_7S_3$ 1328.8, found: 239 [$C_{15}H_{31}CO$](40); 107 [$HOC_6H_4CH_2$](100).

Glycyl-(*S*-palmitoyl)-L-cysteyl-L-methionyl-glycyl-L-leucyl-L-prolyl-(*S*-farnesyl)-L-cysteine Methyl Ester (HGlyCys(Pal)MetGlyLeuProCys(Far)OMe) (26). AcOZGlyCys(Pal)MetGlyLeuProCys(Far)-OMe (**25**) (8 mg, 6 μmol) and dimethyl-β-cyclodextrin (720 mg, 600 μmol) were dissolved in methanol (5 mL). The solution was poured into phosphate buffer (100 mL) (20 mM, pH 6). Next, 8 units of acetyl esterase was added, and the mixture was stirred at 37 °C and at pH 6 for 24 h. The solution was extracted four times with chloroform (50 mL), and the organic layer was dried over $MgSO_4$. The solvent was evaporated in vacuo, and the product HGlyCys(Pal)MetGlyLeuProCys-(Far)OMe (**26**) was isolated from the residue by flash chromatography (silica gel, ethyl acetate/methanol 9:1) to yield 2.5 mg (35%) of **26** as a white waxy solid. R_f = 0.8 (ethyl acetate/methanol 9:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.9 (m, 9H, 2 × CH_3 Leu, ω-CH_3 Pal); 1.3−1.35 (m, 25H, γ-CH Leu, 12 × CH_2 Pal); 1.55−1.7 (m, 17H, β-CH_2 Leu, 2 × CH_3 Far, β-CH_2 Pal, 2 × CH_3 Far); 1.7−2.2 (m, 17H, 4 × CH_2 Far, 2 × CH_2 Pro, γ-CH_2 Met, SCH_3); 2.5 (m, α-CH_2 Pal); 2.7 (m, 2H, β-CH_2 Met); 2.9 (m, 2H, β-CH_2 Cys); 3.0−3.4 (m, 4H, β-CH_2 Cys, CH_2 Far); 3.6 (m, 2H, δ-CH_2 Pro); 3.7 (s, 3H, OCH_3); 3.8 (m, 2H, CH_2 Gly); 4.1 (m, 2H, CH_2 Gly); 4.5−4.8 (m, 4H, 4 × α-CH); 5.1−5.2 (m, 3H, 3 CH Far); 7.0−8.1 (m, 5H, 5 × NH amide). FAB-MS (3-NBA) *m*/*e*: calcd for (M + H) $C_{58}H_{100}O_9N_7S_3$ 1135.7, found 1135 (<1).

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Supporting Information Available: Experimental procedures and ^{1}H and ^{13}C NMR data for compounds **15b**−**15f**, **17b**−**17g**, **18c**−**18e**, **19b**−**19c**, **24**, **28**, **31**−**38**, **40**−**46**, **48**−**63**, **65**−**78** as well as copies of the ^{1}H and ^{13}C NMR spectra of compounds **18a**−**18e**, **19a**, **21**, **23**−**26**, **34**−**38**, **40**−**46**, **48**−**56**, **63**, **65**, **73**, **74** (85 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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