Synthesis of Functional *Ras* Lipoproteins and Fluorescent Derivatives

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Abstract: For the study of biological signal transduction, access to correctly lipidated proteins is of utmost importance. Furthermore, access to bioconjugates that embody the correct structure of the protein but that may additionally carry different lipid groups or labels (i.e., fluorescent tags) by which the protein can be traced in biological systems, could provide invaluable reagents. We report here of the development of techniques for the synthesis of a series of modified *Ras* proteins. These modified *Ras* proteins carry a number of different, natural and non-natural lipid residues, and the process was extended to also provide access to a number of fluorescently labeled derivatives. The maleimide group provided the key to link chemically synthesized lipopeptide molecules in a specific and efficient manner to a truncated form of the H-*Ras* protein. Furthermore, a preliminary study on the biological activity of the natural *Ras* protein derivative (containing the normal farnesyl and palmitoyl lipid residues) has shown full biological activity. This result highlights the usefulness of these compounds as invaluable tools for the study of *Ras* signal transduction processes and the plasma membrane localization of the *Ras* proteins.

Introduction

The transduction of stimuli from the extracellular space across the plasma membrane and ultimately into the cell nucleus is one of the most important processes by which cells regulate growth, differentiation, and proliferation. Recent discoveries have shown that the posttranslational modification of certain proteins with lipid units is an essential feature of several critical signal transduction systems in eukaryotic cells.[1,2] Lipidation of protein molecules can arise in three different ways: (1) the co- or posttranslational attachment of myristic acid to a 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] It is believed that the attached lipid groups not only serve to influence the cellular distribution and anchor the modified protein to cytoplasmic or vesicular membranes but may also be involved in the transduction of signals, for instance by facilitating protein−protein and protein−lipid interactions.[4−6]

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 plasma membrane-bound lipoproteins which serve as central molecular switches in cellular signaling cascades.[7] In response to receptor protein tyrosine kinases or other growth factor-dependent stimuli, these proteins become activated, exchanging the normally bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP). In the GTP-bound active state, the *Ras* proteins can then interact with and activate downstream targets, resulting in the activation of transcription factors.[7] The *Ras* cellular signaling pathway is of central importance for the control of cell growth and proliferation and if disturbed uncontrolled proliferation may occur, resulting in transformation of the cell. The importance of the correct functioning of the *Ras* signaling pathway is clearly illustrated by the fact that a point mutation in the ras oncogenes (coding for the *Ras* protein) is found in approximately 30% of human cancers. This figure can increase to a dramatic 80% for some major malignancies such as cancer of the lung, colon, and pancreas.[8] To perform their normal and oncogenic functions, *Ras* proteins must be membrane-associated, and the covalent attachment of lipid residues to their peptide chains is critical to this localization.[9] For the posttranslational processing of *Ras*, a conserved CaaX amino acid sequence (C is cysteine, a is generally an aliphatic amino acid, and X is a serine or methionine residue) is recognized and farnesylated at the

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cysteine residue by the enzyme protein farnesyl transferase (PFTase). After subsequent proteolytic removal of the three C-terminal amino acids and methylation of the resulting farnesylated C-terminal cysteine residue, the peptide chain can then be further modified by introduction of palmitic acid thioesters. The H-, N- and K-*Ras* proteins have been cloned and can be overexpressed and purified from bacterial systems.[10] However, as the protein is not naturally found in prokaryotic cells, such as *Escherichia coli*, the protein is expressed in the unmodified state. Farnesylation, proteolysis and methylation of the isolated protein can be achieved in vitro either via enzymatic reactions, or with cell free extracts.[11] These procedures, however, are inefficient and can only provide very small quantities of partially processed protein. Thus, until this time the only access to fully lipidated *Ras* proteins required lengthy and tedious purification from eukarotic systems.[12]

Previous work from our groups has focused on the synthesis and biological evaluation of correctly lipidated *Ras* peptide fragments.[13] However, access to large quantities of correctly lipidated protein would be even more valuable. Furthermore, access to bioconjugates that embody the correct structure of the protein but may additionally carry different lipid groups or labels by which the protein can be traced in biological systems (i.e. fluorescent tags) could provide invaluable reagents. Recently we described a method to couple synthetically derived lipopeptide fragments to recombinant *Ras* proteins to form modified *Ras* protein derivatives.[14] Building on our preliminary data, in this report we outline the development of the general method for the engineering of protein molecules with the subsequent production of a small library of modified functional *Ras* proteins.

Maleic acid imides (maleimides **1**) are popular constituents of many heterofunctional protein cross-linking agents, and readily undergo alkylation reactions with sulfhydryl groups to form stable thioether bonds **2** (Figure 1).[15] It was realized that this group could provide the key by which chemically synthesized lipopeptide molecules could be linked in a specific manner to thiol residues such as those found in the amino acid cysteine. In particular maleimidocaproic acid (MIC-OH **3**) enabled the specific coupling of chemically synthesized lipopeptides to a modified form of the *Ras* protein.

For the synthesis of the desired lipopeptide molecules, a modular strategy was adopted, whereby a tetrapeptide formed the key intermediate (Figure 2). This tetrapeptide intermediate would allow further elongation at the C-terminus with lipidated or nonlipidated amino acids, as well as the addition of various N-terminal units. For each compound synthesized the MIC-OH **3** group would be positioned at the N-terminus to allow for simple subsequent coupling to the *Ras* proteins. Utilizing this pathway a number of *Ras* derivatives containing natural and

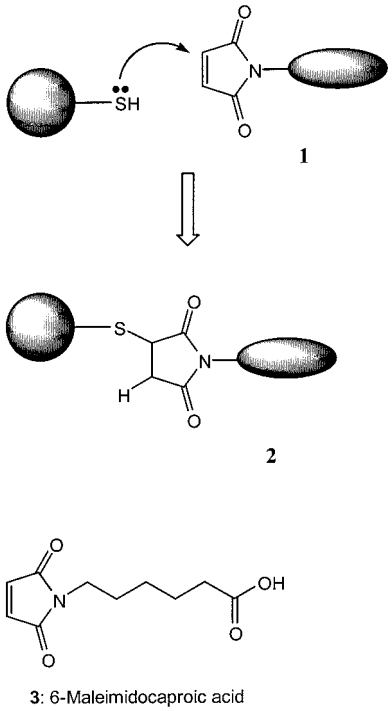

Figure 1. Coupling of the maleimide group **1** to a sulfhydryl group in a protein to form a stable thioether bond **2**.

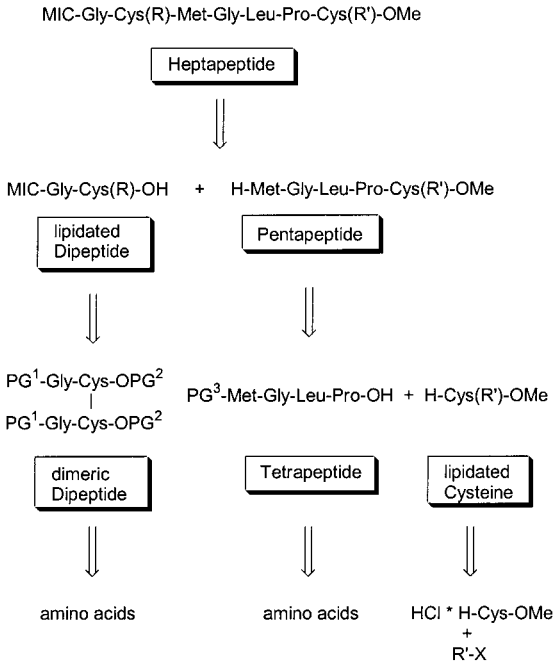

Figure 2. Retrosynthetic scheme for the synthesis of the lipopeptide molecules.

nonnatural lipid residues were produced and the technique was extended to also include a number of fluorescent derivatives. For the synthesis of the fluorescent derivatives the *N*-methylanthranilate group was chosen as it is one of the smallest known fluorescent groups that produces relatively intense fluorescence upon irradiation with UV light.[16]

Preliminary biological assays on the bioconjugates have shown that at least for the natural lipid derivative **43c**, full biological activity is observed. This fact underlines the effectiveness of this general strategy in providing fully active, engineered protein derivatives. It is expected that these proteins

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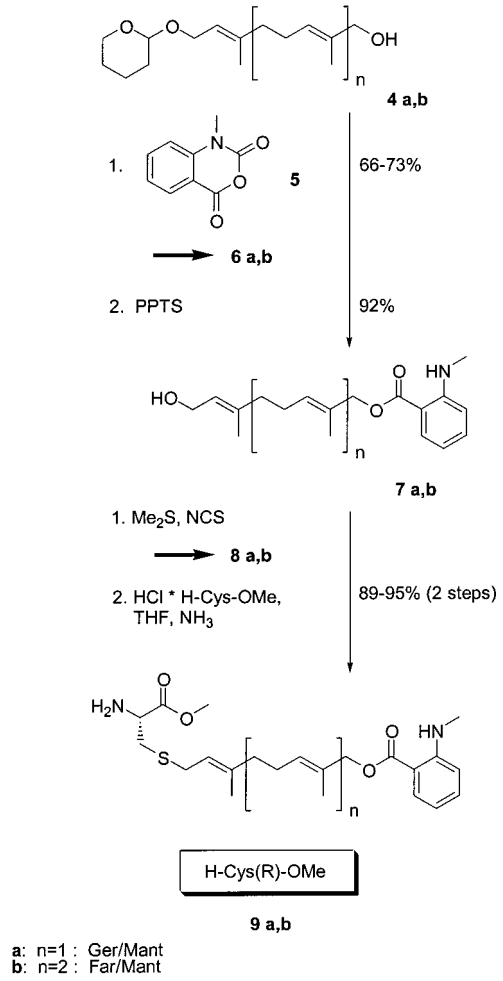
Scheme 1. Synthesis of the Fluorescently Labeled Lipidated Cysteine Molecules **9a**, **9b**

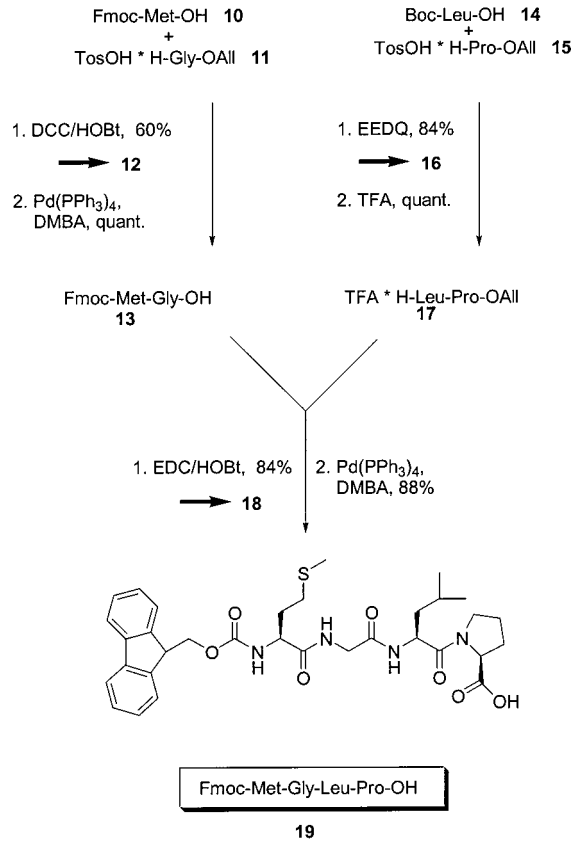


will provide useful tools for further dissecting the biological signal transduction process and the study of selective plasma membrane localization of the *Ras* proteins.

Results

Fluorescent Probes. The synthesis of the novel fluorescently labeled analogues, shown in Scheme 1, starts with the known alcohols **4a** and **4b**,[17,18] both of which are obtained in a two-step sequence starting from commercially available geraniol and farnesol. Attachment of the fluorescent *N*-methylanthranilate group[19] was achieved by acylation of the allylic alcohols with *N*-methylisatoic anhydride **5**, in good yields. Removal of the tetrahydropyran-protecting group from the esters **6a** and **6b**, under acidic conditions furnished the desired fluorescent alcohols **7a** and **7b** in excellent yields. For attachment of the labeled lipids to the cysteine a number of methods were tried. However, by far the best result was obtained utilizing the Corey−Kim chlorination[20] followed by reaction of the allyl chlorides **8a** and **8b** with cysteine methyl ester in THF and liquid ammonia. This procedure, based on a known literature procedure,[21] provided the desired compounds **9a** and **9b** in excellent yields without the need for chromatography.

Scheme 2. Synthesis of the Key Tetrapeptide Intermediate **19**



The Prenylated Pentapeptides. Synthesis of the tetrapeptide intermediate **19** followed standard peptide coupling reactions,[13c,22] and furnished the desired compound in 44% overall yield (Scheme 2). With this compound in hand, condensation with the lipidated cysteine residues **20**, **9a** and **9b**, followed by standard Fmoc deprotection provided the three central pentapeptide intermediates **22a**, **22b**, and **22c** in 60−69% yield (Scheme 3). Access to the first three target molecules was then easily achieved by coupling of the pentapeptides with maleimido caproic acid (MIC-OH **3**), to furnish compounds **41a**, **41b**, and **41c** in excellent overall yields (Scheme 6).

The Prenylated Heptapeptides. For the production of the heptapeptide derivatives a modular strategy was adopted, whereby the pentapeptides **22a**, **22b,** and **22c** would provide the central intermediates to which would be coupled the dipeptides **29**, **30**, and **31** (Scheme 6). Access to the protected disulfides **23a** and **23b** was available via literature known procedures.[13c,22] Cleavage of the disulfide bridge by reduction with excess DTT formed the free thiols, which were immediately reacted with either palmitoyl chloride or hexadecyl bromide to provide the desired lipidated dipeptides **24a** and **25a,b** in high yields (Scheme 4). Reaction of the disulfides **23a,b** with *tert*-butanethiol and air also proceeded smoothly to furnish the desired *S*-*tert*-butyl disulfide derivatives **26a,b**.[23]

As an initial approach to the protected dipeptides **29**, **30**, and **31** the N-protected Boc/allyl ester protecting group strategy,[24] found in compound **25a**, was initially chosen. Deprotection of

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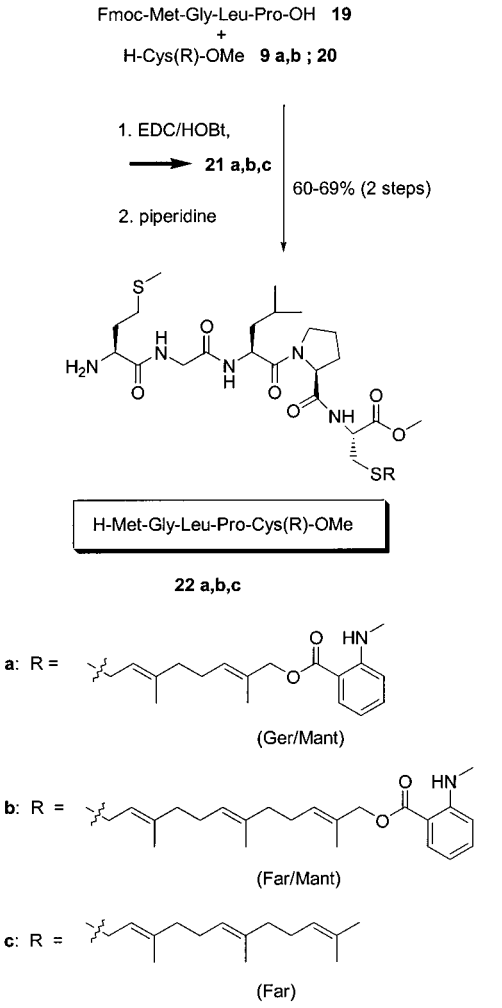
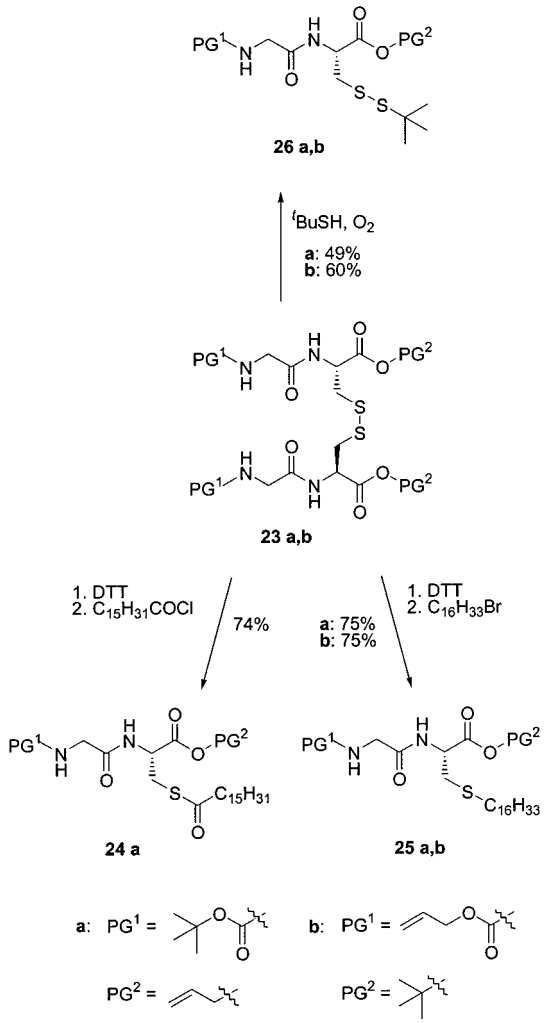
Scheme 3. Coupling of the Lipidated Cysteine Moieties **9a**, **9b**, and **20** to the Tetrapeptide Intermediate **19**, Followed by Deprotection of the Fmoc Group to Produce Pentapeptides **22a**,**b**,**c**



Scheme 4. Formation of the Dipeptide Intermediates **24a**, **25a**,**b**, and **26a**,**b**



the N-terminal Boc group, followed by coupling to MIC-OH **3** proceeded smoothly to furnish **28** (Scheme 5). Unfortunately, deprotection of the allyl ester in compound **28** with tetrakistriphenylphosphine palladium(0) utilizing a number of different allyl scavengers was unsuccessful. In this reaction, the allyl ester was removed cleanly; however, attack also occurred on the olefin moiety within the maleimide group, resulting in the loss of the double bond. Two alternative approaches were then devised to circumvent this problem. The first approach focused on the complete deprotection of the dipeptide to produce compounds **35** and **36**. These compounds were then subsequently coupled to MIC-OH **3** to produce the desired dipeptides **29** and **30**, which could be used for the subsequent coupling reaction. Later studies showed that the maleimide group was quite stable under the conditions of Boc deprotection, with trifluoroacetic acid. Thus, in the second approach to the desired dipeptides, the protecting group arrangement of compound **23a** was reversed. Thereby, removal of the Aloc group from the dipeptides **25b** or **26b**, utilizing tetrakistriphenylphosphine palladium(0) with dimethylbarbituric acid as a scavenger, followed by coupling to the MIC-OH **3**, afforded the protected intermediates **39** and **40**. These two compounds could then be smoothly deprotected by treatment with trifluoroacetic acid in dichloromethane at room temperature for 2 h to provide **30** and **31** in quantitative yields (Scheme 5). This later method, although providing a slightly lower overall yield for the desired compounds, was decided to be superior due to the ease of handling and purification of the intermediates.

With ready access to all three lipidated maleimidocaproyl dipeptides **29**, **30**, and **31**, as well as a sufficient supply of the three major pentapeptide intermediates **22a**, **22b**, and **22c**, the coupling reactions to the target heptapeptides could then be undertaken (Scheme 6). A total of seven heptapeptide derivatives were produced (see Table 1). Each condensation was performed essentially using the same conditions (utilizing EDC and HOBt as coupling reagent), and all reactions provided the desired targets in moderate to good yields. Confirmation of structure for each compound was obtained by ^{1}H and ^{13}C NMR, as well as by FAB and/or MALDI mass spectrometry.

Other Heptapeptide Derivatives. Four more lipid derivatives were also required to undertake a full study of the biological importance of the lipid groups present in the natural protein. In these compounds the effect of the C-terminal cysteine moiety would be examined. Thus, the target compounds for this study would require the C-terminal amino acid to either be lipidated as a hexadecyl thioether, as in compounds **56** and **59** (Scheme 8), or replaced by serine methyl ester as in compounds **62** and

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Scheme 5. Three Approaches to the Desired Dipeptides **29**, **30**, and **31**.

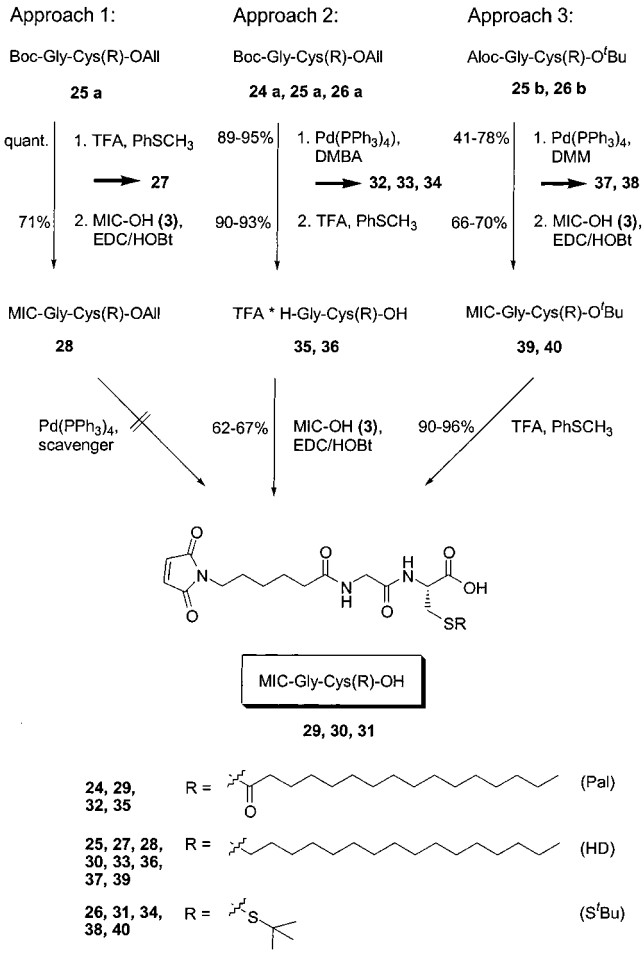

Table 1. Table of All the Target Lipopeptides Synthesized.

type peptide	peptide
MIC-Met-Gly-Leu-Pro-Cys(Far)-OMe	**41c**
MIC-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe	**41a**
MIC-Met-Gly-Leu-Pro-Cys(Far/Mant)-OMe	**41b**
MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe	**43c**
MIC-Gly-Cys(HD)-Met-Gly-Leu-Pro-Cys(Far)-OMe	**44c**
MIC-Gly-Cys(StBu)-Met-Gly-Leu-Pro-Cys(Far)-OMe	**42c**
MIC-Gly-Cys(HD)-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe	**44a**
MIC-Gly-Cys(StBu)-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe	**42a**
MIC-Gly-Cys(HD)-Met-Gly-Leu-Pro-Cys(Far/Mant)-OMe	**44b**
MIC-Gly-Cys(StBu)-Met-Gly-Leu-Pro-Cys(Far/Mant)-OMe	**42b**
MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(HD)-OMe	**56**
MIC-Gly-Cys(StBu)-Met-Gly-Leu-Pro-Cys(HD)-OMe	**59**
MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Ser-OMe	**62**
MIC-Gly-Cys(HD)-Met-Gly-Leu-Pro-Ser-OMe	**65**

65 (Scheme 9). For the synthesis of these compounds, the Fmoc group was thought to be unsatisfactory thus, the Boc-protected tetrapeptide **48** formed the key intermediate (Scheme 7). Synthesis of the tetrapeptide was relatively straightforward based on standard protocols. Condensation of the tetrapeptide with either hexadecylated cysteine methyl ester or serine methyl ester, followed by deprotection of the Boc group provided the desired pentapeptide intermediates **51** and **53** (Scheme 7).

The cysteine hexadecyl pentapeptide was then condensed with both the Boc protected dipeptides **32** and **34** to produce the lipidated heptapeptides **54** and **57**. Deprotection of the Boc groups in both compounds with trifluoroacetic acid/thioanisole, followed by coupling with MIC-OH **3** under standard conditions, provided the targets **56** and **59** (Scheme 8).

The serine-substituted derivative **53** was elaborated in a manner similar to that used for the cysteine hexadecyl compounds. Thus, condensation with the dipeptides **32** and **33**, produced the lipidated heptapeptide derivatives **60** and **63**. Deprotection of the Boc-protecting group with trifluoroacetic acid/thioanisole, followed by coupling of the heptapeptides with MIC-OH **3** under standard conditions furnished the final two target compounds **62** and **65** in excellent overall yields (Scheme 9).

Coupling to *Ras*. With a large number of lipopeptide derivatives in place (See Table 1), the compounds could then be coupled to mutated forms of the *Ras* protein. Two truncated versions of the H-*Ras* protein were utilized for this study. The main mutant utilized in this study had the last eight amino acids from the C-terminus removed (including the CaaX box sequence CVLS), leaving a cysteine residue at the C-terminus possibly well accessible for coupling. Truncation of full length H-*Ras* cDNA was achieved with standard PCR-methods. Briefly, a stop codon was introduced into position 182 of the H-*Ras* cDNA, and the resulting PCR product was cloned into an *E. coli* expression vector (ptac expression vector). Protein expression in *E. coli* strain CK600K and purification by ion-exchange chromatography and gel filtration was performed as described.[25] The second mutant utilized in this study was similar to the first but also contained a mutation of a glycine residue at position 12 to a valine residue.[26a] This mutation removes the ability for the *Ras* protein to hydrolyze bound GTP, thus making the protein oncogenic.[26b] Coupling of the peptides to the protein was readily achieved by gently mixing the peptide and protein together in a 1:1 mixture of 11% Triton X-114 detergent and Tris buffer overnight at 4 °C. Purification of the resulting labeled lipoprotein was easily provided by an extraction procedure with Triton X-114 detergent.[27] This detergent has the added property that it undergoes a phase separation from aqueous solutions at 37 °C.[28] Thus, the lipoprotein is extracted into the Triton phase leaving unlabeled *Ras* protein in the aqueous phase, and as can be seen in Figure 3a, yields virtually 95% pure labeled protein. The protein was then separated from the Triton X-114 detergent by simple DEAE ion exchange chromatography. The entire labeling procedure was found to work extremely well, and as can be seen in Table 2, excellent yields for the coupling reaction were obtained. Initial attempts at the coupling reaction utilized a 10-fold excess of the lipopeptide coupling partner. The coupling clearly worked very well; however, it was found in subsequent reactions that the reaction was virtually quantitative and only a 1:1 equivalent of peptide/protein was actually required, with no reduction in the isolated yield.

Characterization of the labeled proteins was obtained by SDS-PAGE gel electrophoresis and electrospray mass spectrometry of the intact labeled proteins (see Figure 3). Full proof for the specific incorporation of the label into the C-terminal cysteine residue was obtained for three representative proteins (proteins coupled with the peptides **41c**, **43c**, and **44c**). In these experiments the labeled proteins were digested with trypsin or chymotrypsin proteases, and the resulting digested samples were then analyzed by electrospray, as well as MALDI mass

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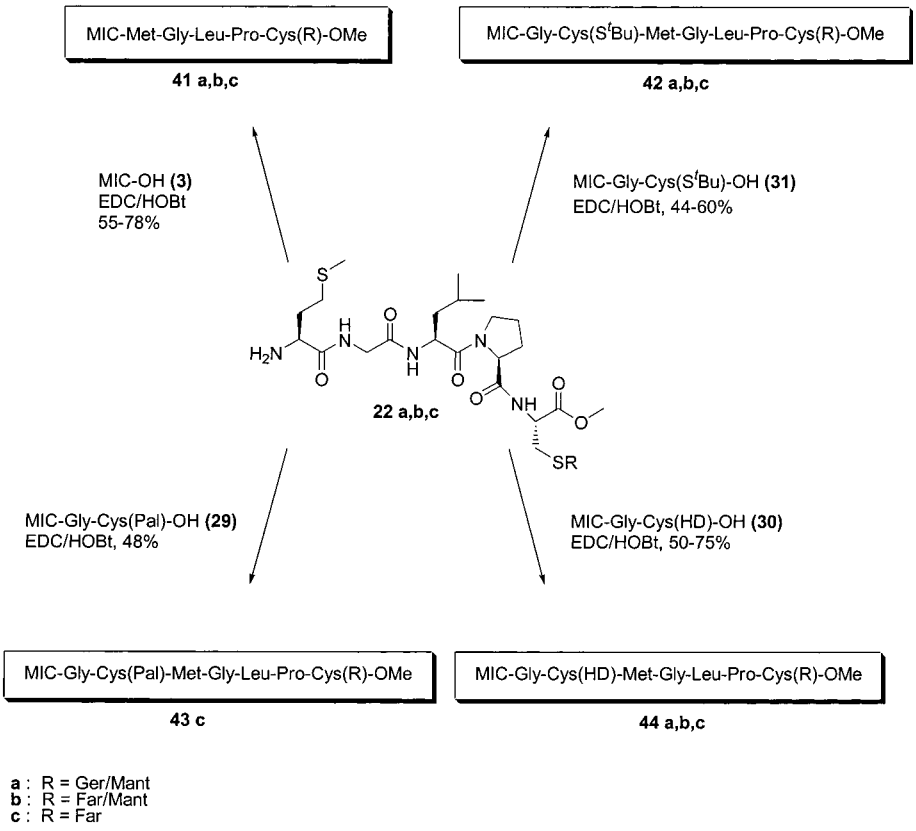
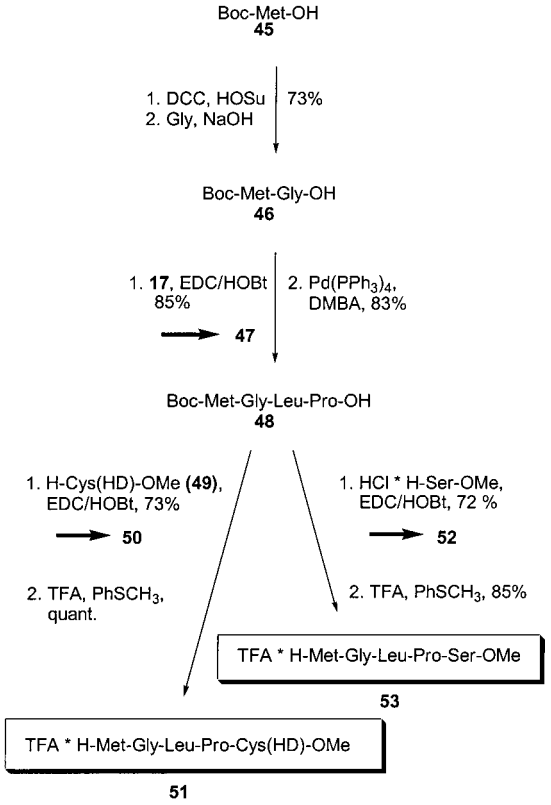
Scheme 6. Formation of the Ten Prenylated Target Molecules, Compounds **41a**,**b**,**c**; **42a**,**b**,**c**; **43c**; **44a**,**b**,**c**



Scheme 7. Synthesis of the N-terminally Deprotected Nonprenylated Pentapeptides **51** and **53**



spectrometry. Through this technique, the peptide fragments from the protease digestion are separated and the molecular weights of individual fragments can be determined. The results from these experiments clearly showed the covalent attachment of the lipopeptides **41c**, **43c**, and **44c** to the C-terminal cysteine, Cys181 of the truncated *Ras* protein, thereby, proving the specificity of the reaction.

Table 2. Table of Results from Coupling Reactions of Lipopeptides to the Truncated *Ras* Protein

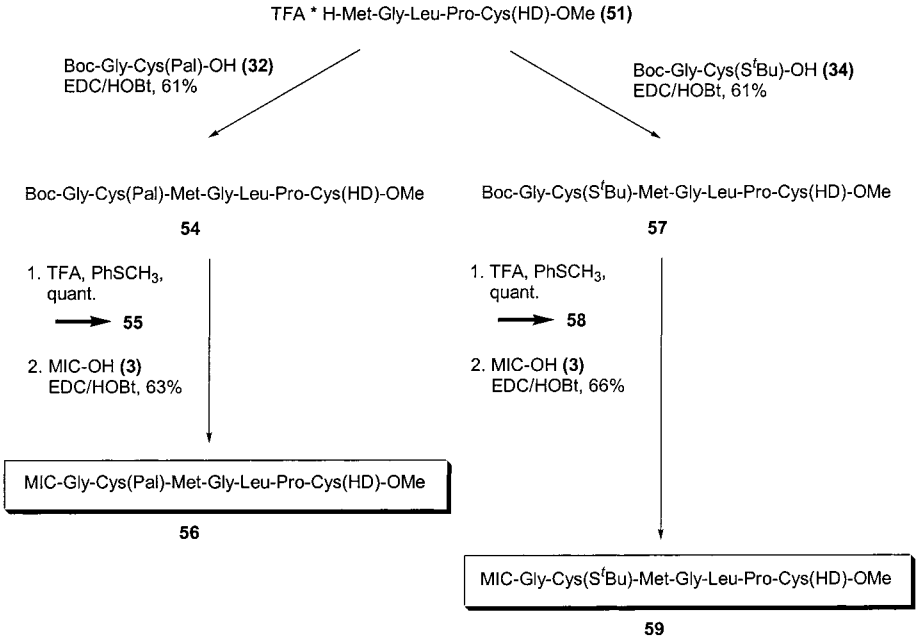
peptide	peptide (mg)	protein (mg)	molar ratio	recovered protein conjugate (mg)	recovered protein conjugate yield (%)
41c	5.03	9.1[a]	12:1	6.24	66
41a	1.50	13.0[a]	2.5:1	7.3	54
41b	0.64	11.5[a]	1:1	6.2	51
43c	6.50	11.6[a]	8.5:1	5.7	46
44c	6.60	11.0[a]	9:1	5.9	50
44a	0.87	10.9[a]	1:1	6.2	53
44b	0.61	10.3[a]	1:1	3.2	29
42c	0.62	10.8[b]	1:1	5.3	49
42a	o.52	8.5[a]	1:1	4.3	50
42b	0.79	12.2[a]	1:1	6.8	56
56	0.33	5.5[b]	1:1	2.2	40
59	0.60	10.2[b]	1:1	6.4	62
62	0.58	9.9[b]	1:1	3.5	35
65	0.73	10.9[a]	1.25:1	5.5	50
	0.55	9.5[b]	1:1	5.6	59

[a] Normal truncated H-*Ras*. [b] Mutated (G12V) truncated H-*Ras*.

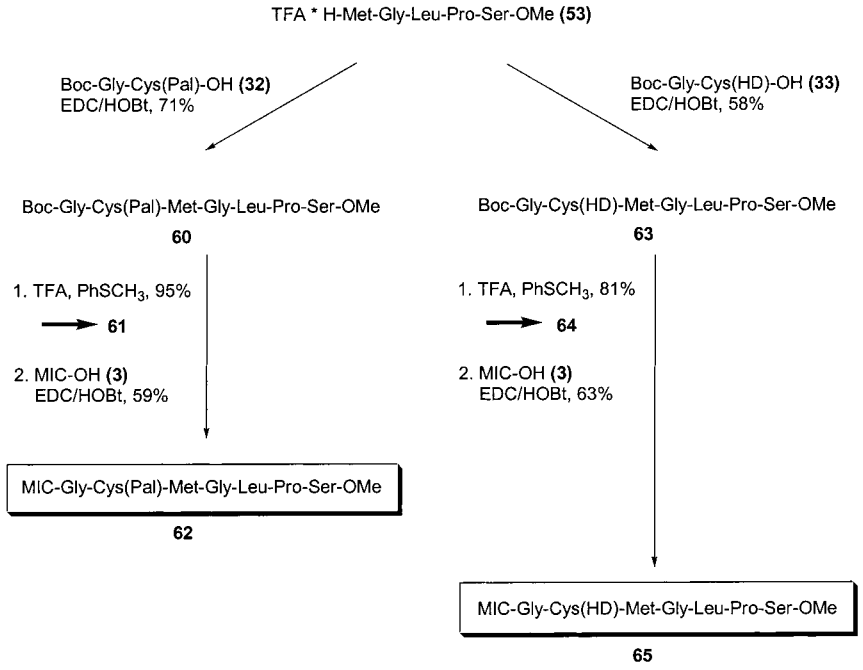
Biological Assays. As a preliminary investigation into the biological activity, the oncogenic protein coupled with the natural lipid derivative **43c** was chosen for microinjection studies in PC12 cells. It had been previously shown that these cells can be induced to differentiate, producing long outgrowths from the cell, after microinjection with functional oncogenic *Ras* protein.[29] In this experiment, two additional protein samples were also microinjected into the PC12 cells as controls, the first sample was the truncated oncogenic H-*Ras* protein (1−181),

(29) Schmidt, G.; Lenzen, C.; Simon, I.; Deuter, R.; Cool, R. H.; Goody, R. S.; Wittinghofer, A. *Oncogene* **1996**, *12*, 87.

Scheme 8. Synthesis of the C-terminal Hexadecylated Heptapeptides **56** and **59**



Scheme 9. Synthesis of the C-terminal Serine Heptapeptides **62** and **65**



while the second was a full length bacterially expressed and therefore unmodified sample of H-*Ras*, that is capable of being modified by the cellular enzymatic machinery. As can be seen in Figure 4, the uncoupled truncated *Ras*, as expected had no effect on the growing cells (Figure 4a), while the full length *Ras* protein sample (Figure 4b) is fully active and produces the expected outgrowths from the cells. Much to our delight, upon microinjection of the coupled oncogenic protein, long outgrowths from the PC12 cells began to form (Figure 4c), thereby proving that this coupled protein derivative was biologically active.

Discussion

Previous studies from our group have focused on the production of lipopeptides as tools for studying cellular signaling cascades and selective membrane targeting of lipidated proteins.[13,30] Efficient methods for the synthesis of these sensitive compounds have been developed and problems associated with the acid and base sensitivity of these compounds can be alleviated by the correct protecting group strategy.[22a,24,31] As useful as the lipopeptides have been fully lipid modified proteins would provide the ultimate tools. Such molecules would naturally contain the correct protein and lipid structures or could alternatively carry different lipid groups or labels by which the proteins could be traced in biological systems (i.e., fluorescent tags which can be detected by fluorescence microscopy and

(30) For reviews see (a) Hinterding, K.; Alonso-Díaz, D.; Waldmann, H. *Angew. Chem.* **1998**, *110*, 716 and *Angew. Chem., Int. Ed.* **1998**, *37*, 688. (b) Kappes, T.; Waldmann, H. *Liebigs Ann./ Recl.* **1997**, 803. (c) Eisele, F.; Owen, D. J.; Waldmann, H. *Bioorg. Med. Chem.* **1999**, *7*, 193.

(31) Schelhaas, M.; Waldmann, H. *Angew. Chem.* **1996**, *108*, 2192 and *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2056.

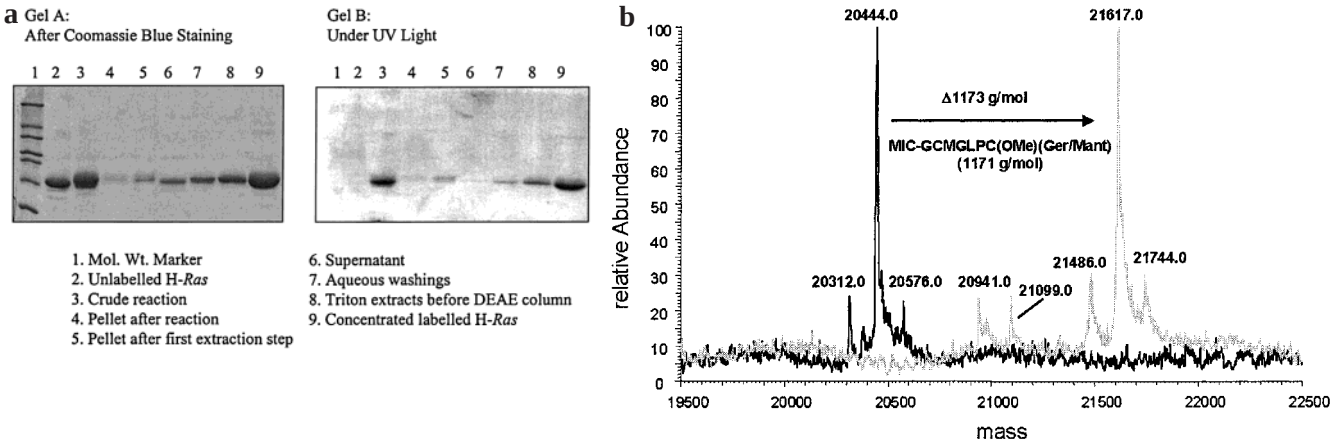


Figure 3. An example of characterization of the product from the coupling reaction of H-*Ras* 1−181 with lipopeptide **41b**. (a) Picture of SDS-PAGE gels for coupling reaction. The differences in electrophoretic mobility between labeled and unlabeled H-*Ras* 1−181 are very small, unlabeled H-*Ras* migrated slightly faster. Lanes 6 and 8 of both gels show the good separation of labeled and unlabeled H-*Ras* employing the Triton X-114 method. Gel A shows the slight difference in electophoretic mobility, gel B shows under UV-excitation virtually no fluorescent protein in lane 6. A small amount of labeled protein is lost during washing the detergent phases with fresh buffer (lane 7). Lanes 8 and 9 show the high purity of isolated labeled H-*Ras*. (b) Electrospray mass spectra of H-*Ras* 1-181 (black) and of the protein coupled with a lipopeptide **42a** (gray). Nanoelectrospray mass spectrometry of starting material and product in a LCQ ion-trap instrument from Finnigan shows the expected shift from RasΔC (calculated MW: 20446 g·mol^{-1}, black trace) to *Ras*-7merGer/Mant (21617 g·mol^{-1}, gray trace). Starting material and product show minor peaks due to small amounts of protein without the first N-terminal cycteine (20312/21486 g·mol^{-1}). Precision of the instrument ±4 Da.

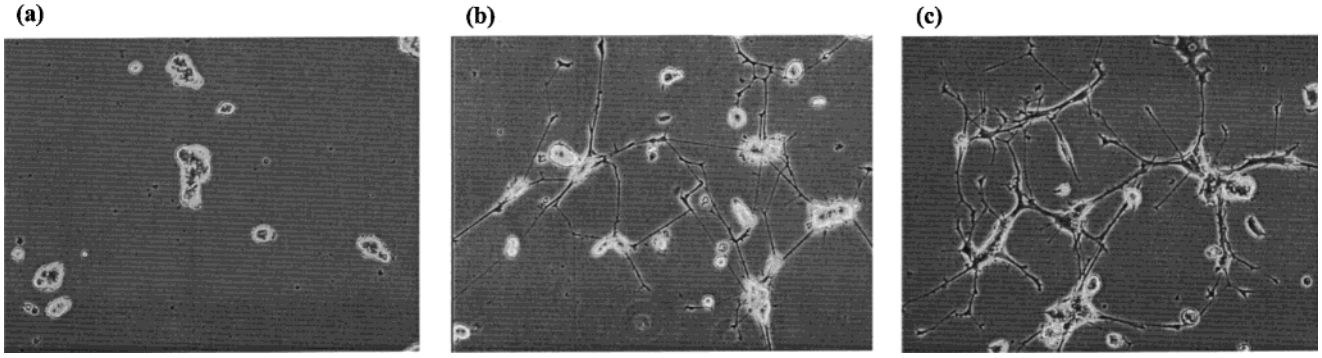


Figure 4. Pictures from PC 12 cells after microinjection of three different oncogenic protein samples of H-*Ras*. Picture (a) shows cells in which the truncated form of the oncogenic H-*Ras* (1−181) was injected. Because of the absence of modification sites, this protein can't be modified by the cellular enzymatic machinery. As expected, there is no effect on the cell growth. As shown in the picture (b), when the full-length oncogenic H-*Ras*, which can be modified, is injected into the cells, long outgrowths are formed. In picture (c) cells are shown which formed such outgrowths, too, after injection of the truncated oncogenic H-*Ras* (1−181) coupled with the lipid derivative **43c**. This result shows the biological activity of those protein conjugates.

fluorescence spectroscopy). We recently reported a general method by which synthetically derived lipopeptide chains could be selectively coupled to a recombinant *Ras* protein.[14] Here we describe the production of a small library of engineered *Ras* protein derivatives. These engineered proteins were easily synthesized, purified and characterized and may provide the foundation for a wide arsenal of derivatives which would be of use for further biological studies. It is expected that this coupling procedure should also be completely general and thus could be further utilized for the production of other important bioconjugates.

The maleimide group has been widely used as a popular constituent of many heterobifunctional cross-linking agents.[15] The double bond of the maleimide group **1** can undergo alkylation reactions with sulfhydryl groups to form stable thioether bonds 2 (Figure 1). Although maleimides can also react with amines and other nucleophiles, the reaction is specific with sulfhydryl groups in the pH range of 6.5−7.5. Thus, at pH 7 the maleimide group will react a 1000 times faster with the sulfhydryl of cysteine than with the ϵ amine of lysine.[32] Furthermore, the derivative maleimidocaproic acid (MIC-OH **3**) is a stable commercially available compound, which can easily be incorporated into peptide molecules via standard peptide coupling procedures. It was for these reasons that the maleimide group was thought to provide the key to linking the lipopeptide molecules specifically to the *Ras* protein.

For the synthesis of the desired target molecules we initially saw that the tetrapeptide **19** would provide the major intermediate to all targets (Figure 2). This compound would allow further elongation at the C-terminus with differently labeled cysteine amino acids or other amino acids, as well as allow the addition of various *N*-terminal pieces. For each compound the maleimido caproic acid group would be placed at the *N*-terminus for subsequent attachment onto the mutated *Ras* protein. As noted above, synthesis of the desired lipopeptides followed established protocols developed within our group,[13c,22a,24] and access to eight mono- and dilipidated peptide derivatives, as well as six mono- and dilipidated fluorescent derivatives was achieved (see Table 1).

(32) (a) Smyth, D. G.; Nagamatsu, A.; Fruton, J. S. *J. Am. Chem. Soc.* **1960**, *82*, 4600. (b) Smyth, D. G.; Blumenfeld, O. O.; Konigsberg, W. *Biochem. J.* **1964**, *91*, 589.

The synthesis of a range of lipid derivatives was thought to be useful to probe the biological activity of the newly synthesized *Ras* proteins, in particular, the influence of the different membrane anchors on distribution and signaling ability of the new biopolymers. A number of biological assays for monitoring the in vivo activity of *Ras* already exist,[29,33] and so by producing a series of differently lipidated *Ras* proteins, the specific effect of the different lipid groups could be studied. Thus, in one set of compounds the *S*-palmitoyl thioester, which occurs in the natural protein, was replaced by either; (1) no lipidation site at all (compounds **41a**,**b**,**c**), (2) by a metabolically stable thioether of similar lipophilicity (compounds **44a**,**b**,**c**), or (3) by a free thiol group (compounds **42a**,**b**,**c**). The free thiol group can be generated in situ from the S-*tert*-butyl disulfide by treatment of the coupled protein with dithiothreitol.[13b] In one case (compound **43c**), the S-palmitoyl thioester was introduced into the peptide to form a *Ras* derivative with both natural lipid residues. It has previously been shown that *S*-palmitoylation/deacylation of both *Ras* proteins and peptide fragments is a rapidly reversible process within a cell.[13b] For this reason we believe that for microinjection studies, proteins coupled with either the palmitoyl derivative **43c** or the free thiol derivatives **42a**,**b**,**c** are essentially equivalent.

To undertake a full study of the biological importance of the lipid groups present in the natural protein, four more compounds were synthesized. In these compounds the effect of the C-terminal cysteine moiety would be examined. Thus, the second set of compounds contained the normal native palmitoylation site (either as the palmitoylated thioester derivative or as the free thiol derivative) combined with changes in the *S*-farnesyl thioether moiety. In these molecules, replacement of the farnesylated cysteine methyl ester with a hexadecylated cysteine methyl ester (compounds **56** and **59**) is expected to show if the farnesyl group itself is important or if the presence of any lipid group is sufficient for biological activity of the *Ras* proteins. Finally with the removal of a C-terminal lipidation site, by replacement with a nonlipidizable serine residue (compounds **62** and **65**), the monolipidated derivatives were completed.

The Fluorescent Derivatives. It was believed at the onset of the project that fluorescent groups attached to the peptide could provide invaluable compounds for further in vivo biological studies utilizing the technique of microinjection followed by fluorescence microscopy. Earlier studies from our groups had shown that problems could be encountered with loss of fluorescence from fluorescently labeled lipopeptides.[34] It was believed that by placing the fluorescent marker within the lipid group, no interference with the attachment of the peptide to the protein, should occur. Furthermore, with the fluorescent group in the lipid moiety, once the labeled protein is injected into cells, the lipid group would insert into intracellular membranes and thus, would (1) protect the fluorescent group from hydrolysis, and (2) is expected to increase the groups quantum yield. There were some initial reservations that the presence of a bulky fluorescent group within the lipid portion of the protein molecules may effect the binding of the lipid molecule to the biological lipid bilayer. It was for this reason that the fluorescent *N*-methylanthranilate group was chosen, as it is one of the smallest known compounds that produces relatively intense fluorescence upon irradiation with UV light.[16] It was decided that two different lipid chain lengths would also be advantageous as the effect of the chain length on protein binding could also be observed.

Coupling to H-*Ras*. With a large number of lipopeptide derivatives in place the compounds could then be coupled to the mutated forms of the *Ras* protein (see Table 2). The main mutant used in this study, H-*Ras* 1−181, was a mutant of the H-*Ras* protein in which the last eight amino acid residues had been removed from its sequence leaving a single surface available cysteine residue at the C-terminus. This protein is expressed in high level from an *E. coli* expression vector, and is easily purified.[25] The coupling reaction between the peptide and the protein was extremely straightforward, resulting in high yields of coupled protein after a relatively simple extraction procedure followed by anion exchange chromatography. Furthermore, it was found that the coupling reaction required only one equivalent of each of the coupling partners, thus highlighting the efficiency of the maleimide-sulfhydryl reaction. The H-*Ras* protein used in this study does in fact have three more cysteine residues within its sequence. It could thus not be excluded, that the maleimide group would react with one of these cysteine residues, although the crystal structure shows that they are not easily accessible.[35] Proof for the specific incorporation of the lipopeptide tail to the C-terminal cysteine residue (and not to other amino acid residues) was obtained for three representative proteins. Thus, proteolysis of the labeled proteins, followed by mass spetrometry of the fragments clearly showed the presence of a single lipopeptide covalently attached to the C-terminal cysteine 181 residue.

A second truncated form of the *Ras* protein was also used in the coupling reactions. This protein carries basically the same truncation of the tail as the other "natural" protein. However, this protein in addition carries a mutation at the twelve position whereby, the natural glycine is replaced by a valine residue. This mutation renders the protein unable to hydrolyze bound GTP, thereby, keeping the protein in a continuously active state.[26] This particular oncogenic protein, once derivatized with the various lipopeptides, provides a useful tool for assessing the biological activity of the protein−peptide bioconjugates, as the continuous "ON" signal enables the quick detection of active and membrane-bound *Ras* proteins. As expected there was no detectable difference between the handling and coupling of the two different *Ras* proteins with the various peptide fragments.

Biological Activity. As a preliminary investigation into the biological activity of these derivatives, the product from the coupling reaction of the oncogenic H-*Ras* protein with the natural lipopeptide N-*Ras* fragment **43c** was tested by microinjection into PC12 cells. Microinjection into these cells forms a typical standard assay to assess the biological activity of *Ras* proteins, as once injected with oncogenic *Ras* proteins they quickly begin to differentiate, producing long outgrowths. In our experiment after microinjection of the protein sample into the PC12 cells a formation of long outgrowths was observed. This result proves the biological activity of the engineered protein, and also proves that the maleimide group has little, if any effect on protein activity.

Conclusions

The study of *Ras* signal transduction is an important field especially when it is considered that many useful drugs to combat diseases such as cancer, may develop from full understanding of this important pathway.[8] The development of

(33) Biological assays were adopted for microinjection by Schmidt, G. in her Ph.D. thesis, based on different cell lines: NIH3T3 cells: Stacey, D. W.; Kung, H. F. *Nature* **1984**, *310*, 508. Fos-LacZ cells: Lloyd, A. C.; Paterson, H. F.; Morris, J. D.; Hall, A, Marshall; C. J. *EMBO J.* **1989**, *8*, 1099. PC 12 cells: Greene, L. A.; Tischler, A. S. *Proc. Nat. Acad. Sci. U.S.A.* **1976**, *73*, 2424.

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tools to study such processes is an integral part of this goal. Here through the successful combination of organic chemistry, biochemistry and cell biology we hope to better understand the molecular details of complex processes such as those involved in signal transduction and membrane targeting. In this study, access to the rather sensitive lipopeptide molecules was efficiently achieved utilizing a range of powerful synthetic protocols, previously developed within our group,[13,22a,24] while the maleimide group provided the key reagent for the efficient and specific coupling of the synthetic lipopeptide derivatives to truncated forms of the H-*Ras* protein. The natural and nonnatural engineered protein derivatives produced in this work are expected to provide important tools for further studying the *Ras* signaling pathway. Microinjection experiments on one of the lipopeptide−protein conjugates has shown the bioconjugate to possess full biological activity.

It is important to note that the methodology developed here forms the basis for a general biological readout system, comprised of a protein head and a lipopeptide tail. It is expected that the methodology should be completely general and will allow for the specific engineering of many different protein bioconjugates. As shown in this study, by using an oncogenic *Ras* protein molecule, coupled to different lipopeptide tails, we can obtain a quantifiable biological readout of the ability of different lipopeptide tails to localize the protein to the plasma membrane after microinjection into PC12 cells. Conversely, taking a lipopeptide tail that can efficiently anchor a protein to the plasma membrane (such as compounds **42**, **43**, or **44**) and coupling it to various protein molecules we may be able to determine the possible interaction of those particular protein molecules with or at the plasma membrane. Such a system may also prove useful for studying the interactions of these various protein molecules with other membrane bound proteins. The development of general biological readout systems, such as the one outlined here, should provide invaluable tools for determining membrane−protein interactions and may assist in determining the molecular details of important biological processes such as cellular signaling cascades.

Experimental Section

General Methods. Proton and carbon NMR spectra were recorded on Bruker AC-250, Bruker AM-400 and Bruker DRX-500 spectrometers. NMR spectra were obtained in either deuteriochloroform, deuterio methanol, or deuteriochloroform/deuterio methanol mixture. Proton and carbon spectra are reported in parts per million downfield from an internal standard of Me4Si. EI-MS and FAB-MS were recorded on a Finnigan MAT 90 machine, MALDI-MS were recorded on a Voyager machine by Applied Biosystems, applied matrices are given for each particular compound. ESI-MS were recorded on a LCQ Ion Trap instrument by Finnigan. Infrared Spectra were recorded on a Bruker IFS88 FTIR spectrometer as a KBr disk. UV spectra were recorded on a Perkin-Elmer UV/vis spectrometer in methanol. Combustion analysis was carried out on a Heraeus CHN-Rapid analyzer. Flash chromatography was carried out on columns packaged with Baker silica gel (30−60 μm). TLC was carried out on Kieselgel 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.[36] All peptide couplings were performed utilizing standard synthetic methods (see Supporting Information).

Standard Peptide Coupling Conditions. Unless otherwise stated, all peptide coupling reactions were performed based on the following standard procedure: To a 1:1 (molar) solution of each coupling partner dissolved in CH_2Cl_2 (5−10 mL) at 0 °C was added 1-hydroxybenzotriazole (HOBt) (1.5 equiv), followed by ethyl-(dimethylamino)-propylcarbodiimide (EDC) (1.2 equiv). The reaction was warmed to room temperature and stirred for 18 h. The reaction mixture was then diluted with ethyl acetate (50−100 mL) and was extracted with 0.5 M HCl (2 × 10 mL), 1 M NaHCO3 (2 × 10 mL), and finally with brine solution (2 × 10 mL). The organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The product could then be isolated from the remaining residue by flash chromatography on silica gel using the solvent systems noted for each particular compound.

Standard Fmoc-Protecting Group Removal. To a solution of the protected peptide in CH_2Cl_2 (5−10 mL) at room temperature was added piperidine (1−2 mL). The reaction was warmed to room temperature and stirred for 18 h. After removing the solvent by azeotropic distillation with toluene (2 × 30 mL) and chloroform (30 mL), the product was isolated from the remaining residues by flash chromatography on silica gel using the solvent systems noted for each particular compound.

Standard Procedure for the Removal of Aloc Groups or Allyl Esters. To a solution of the protected peptide in dry THF (5−10 mL) was added dimethylbarbituric acid (0.55 equiv), followed by a catalytic amount of tetrakis(triphenylphosphine)palladium(0). The reaction was monitored by TLC, and was judged complete with the disappearance of starting material (generally 2−3 h). The solvent was then removed under reduced pressure. The product could be isolated from the remaining residue by the methods noted for each particular compound.

Standard Procedure of the Removal of Boc Groups or *tert*-Butyl Esters. To a solution of the protected peptide in CH_2Cl_2 (5 mL) and thioanisole (0.5 mL) was added trifluoroacetic acid (2.5−5 mL). The reaction was then left to stir for 1−2 h at room temperature. The solvent was removed by azeotropic distillation with toluene (2 × 50 mL) and chloroform (50 mL). The desired product was isolated from the remaining residues by the methods noted for each particular compound.

(*E*,*E*)-8-*O*-(2-*N*-Methyl-aminobenzoyl)-1-(tetrahydropyran)-3,7-dimethyl-2,6-octandiene (6a). A mixture of the anhydride **5** (440 mg, 2.4 mmol) and DMAP (26 mg, 0.24 mmol), dissolved in dry DMF (2 mL), was heated to 65 °C with stirring under an atmosphere of dry argon. A mixture of the alcohol **4a** (620 mg, 2 mmol) and dry triethylamine (340 μL, 2.4 mmol), dissolved in dry DMF (2 mL), was then slowly added to the anhydride solution over a period of 40 min. The reaction was left stirring at this temperature for 3.5 h, after which time TLC indicated the reaction was virtually complete. The solution was poured into a separatory funnel containing ethyl acetate (100 mL) and was washed once with 1:1 water/brine solution (10 mL) followed by brine (10 mL). The combined aqueous phases were back-extracted three times with ethyl acetate (10 mL). The combined organic phases were dried over $MgSO_4$ and filtered, and the solvent was removed under reduced pressure. Purification of the resulting oily residue by flash chromatography on silica gel with hexane/ethyl acetate (8:1), yielded 711 mg (75%) of the desired product **6a** as a colorless oil, followed by 60 mg (10%) of starting material: $R_f = 0.65$ (hexane/ethyl acetate 3:1). UV (MeOH) λ_{max} 223 nm (ϵ 29 958), 256 nm (ϵ 9960), 355 nm (ϵ 7130). IR (KBr) ν_{max} 3378, 2940 br s, 1682, 1607, 1581, 1520, 1239, 1128, 1085, 1023, 751 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 (dd, J = 1.6, 8.0 Hz, 1H, arom CH); 7.67 (br s, 1H, NH); 7.34 (ddd, J = 1.6, 7.1, 8.4 Hz, 1H, arom CH); 6.62 (dd, J = 0.8, 8.4 Hz, 1H, arom CH); 6.56 (ddd, J = 0.8, 7.1, 8.0 Hz, 1H, arom CH); 5.51 (dt, J = 1.0, 6.4 Hz, 1H, CH Ger); 5.38 (dt, J = 1.1, 7.4 Hz, 1H, CH Ger); 4.62 (s, 2H, CO_2CH_2); 4.61 (t, J = 3.6 Hz, 1H, OCHO); 4.23 (dd, J = 6.4, 11.7 Hz, 1H, CH_{2a}O Ger); 4.01 (dd, J = 7.4, 11.7 Hz, 1H, CH_{2b}O Ger); 3.87 (m, 1H, CH_{2a}O THP); 3.49 (m, 1H, CH_{2b}O THP); 2.87 (s, 3H, NHCH_3); 2.17−2.22 (m, 2H, CH_2 Ger); 2.07−2.10 (m, 2H, CH_2 Ger); 1.79−1.85 (m, 1H, CH_{2a} THP); 1.71 (s, 3H, CH_3 Ger); 1.69 (m, 1H partially obscured, CH_{2b} THP); 1.68 (s, 3H, CH_3 Ger); 1.47−1.60 (m, 4H, 2 * CH_2 THP). ^{13}C NMR (125.6 MHz, $CDCl_3$): δ 168.3 (CO_2);

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152.0 (arom quart. CNH); 139.4 (quart. C Ger); 134.5; 131.5 (2 * arom CH); 130.6 (quart. C Ger); 128.6; 121.1 (2 * CH Ger); 114.3; 110.6 (2 * arom CH); 110.0 (arom quart. *C*CO_2); 97.7 (CH THP); 69.6 (CO_2*C*H2); 63.5 (CH_2O THP); 62.1 (CH_2O Ger); 39.0 (CH_2 Ger); 30.7 (CH_2 THP); 29.4 ($NHCH_3$); 26.0; 25.5; 19.6 (3 * CH_2 THP); 16.4; 14.0 (2 * CH_3 Ger). MS (EI) *m*/*z* (rel intensity) 387 (M^+, 8.2), 302 (26), 151 (100), 134 (24), 105 (14), 85 (88). HRMS (EI) *m*/*z*: calcd for (M^+) $C_{23}H_{33}NO_4$ 387.2409, found 387.2397. Anal. Calcd: C, 71.29; H, 8.58; N, 3.61. Found: C, 71.54; H, 8.47; N, 3.90.

(*E*,*E*)-8-O−(2-*N*-Methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandien-1-ol (7a). Compound **6a** (500 mg, 1.3 mmol) was dissolved in ethanol (5 mL). Solid PPTS (36 mg, 0.14 mmol) was added and the reaction was heated at 60 °C for 3 h. After this time, the solvent was removed under reduced pressure, and the oily residue was purified by flash chromatography on silica gel using hexane/ethyl acetate (3:1) as eluent, and yielded 362 mg (92%) of compound **7a** as a colorless oil: R_f = 0.34 (hexane/ethyl acetate 3:1). UV (MeOH) λ_{max} 223 nm (ϵ 36 325), 256 nm (ϵ 11 857), 355 nm (ϵ 8497). IR (KBr) ν_{max} 3379 br s, 2914 br s, 1680, 1607, 1581, 1520, 1242, 1128, 1087, 751 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.92 (dd, *J* = 1.6, 8.0 Hz, 1H, arom CH); 7.60 (br s, 1H, NH); 7.37 (ddd, *J* = 1.6, 7.1, 8.5 Hz, 1H, arom CH); 6.65 (dd, *J* = 1.0, 8.5 Hz, 1H, arom CH); 6.58 (ddd, *J* = 1.0, 7.1, 8.5 Hz, 1H, arom CH); 5.50 (dt, *J* = 1.3, 7.0 Hz, 1H, CH Ger); 5.40 (dt, *J* = 1.3, 6.8 Hz, 1H, CH Ger); 4.63 (s, 2H, CO_2CH_2); 4.12 (d, *J* = 6.8 Hz, 2H, CH_2O Ger); 2.89 (s, 3H, NHC*H*$_3$); 2.12−2.21 (m, 2H, CH_2 Ger); 2.06−2.09 (m, 2H, CH_2 Ger); 1.71 (s, 3H, CH_3 Ger); 1.67 (s, 3H, CH_3 Ger). ^{13}C NMR (125.6 MHz, $CDCl_3$): δ 168.4 (CO_2); 152.0 (arom quart. CNH); 138.8 (quart. C Ger); 134.6; 131.5 (2 * arom CH); 130.6 (quart. C Ger); 128.4; 123.9 (2 * CH Ger); 114.3; 110.7 (2 * arom CH); 110.0 (arom quart. $\underline{C}CO_2$); 69.6 ($CO_2\underline{C}H_2$); 59.2 (CH_2O Ger); 38.9 (CH_2 Ger); 29.5 ($NH\underline{C}H_3$); 25.9 (CH_2 Ger); 16.2; 14.0 (2 * CH_3 Ger). MS (EI) *m*/*z* (rel intensity) 303 (M^+, 12), 151 (100), 134 (21), 105 (23). HRMS (EI) *m*/*z*: calcd for (M^+) $C_{18}H_{25}NO_3$ 303.1834, found 303.1823. Anal. Calcd: C, 71.24; H, 8.31; N, 4.62. Found: C, 71.09; H, 8.31; N, 4.91.

(*E*,*E*)-8-*O*-(2-*N*-Methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene-1-chloride (8a). To a solution of *N*-chlorosuccinimide (30.5 mg, 0.23 mmol) in CH_2Cl_2 (1 mL), stirring at −40 °C under an atmosphere of argon, was added dimethyl sulfide (18.5 uL, 0.28 mmol) dropwise. The reaction was then warmed to 0 °C with an ice bath and kept at this temperature for 5 min. The reaction was then cooled back to −40 °C, and a solution of the alcohol **7a** (63 mg, 0.21 mmol) in CH_2Cl_2 (2 mL), was added dropwise. The resulting milky white solution was then left stirring to warm to 0 °C over 1 h. The reaction was maintained at 0 °C for another hour, after which time it was then warmed to room temperature for a further 15 min. The resulting clear yellow solution was poured into a separating funnel containing pentane (100 mL) and ice cold brine solution (20 mL). The layers were separated, and the organic phase was washed once more with ice cold brine solution. The combined aqueous phases were back-extracted with pentane (2 × 20 mL). The combined organic phase was dried over Na_2SO_4 and filtered, and the solvent was removed under reduced pressure, to yield the allylic chloride **8a** as a pale yellow oil (62 mg, 92%): R_f = 0.63 (hexane/ethyl acetate 6:1). UV (MeOH) λ_{max} 219 nm (ϵ 44 908), 253 nm (ϵ 11 954), 353 nm (ϵ 8432). IR (KBr) ν_{max} 3379, 2933 br s, 1682, 1607, 1581, 1520, 1241, 1174, 1128, 1087, 844, 751 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.92 (dd, *J* = 1.6, 8.0 Hz, 1H, arom CH); 7.66 (br s, 1H, NH); 7.38 (ddd, *J* = 1.6, 7.1, 8.4 Hz, 1H, arom CH); 6.66 (dd, *J* = 1.0, 8.4 Hz, 1H, arom CH); 6.58 (ddd, *J* = 1.0, 7.1, 8.0 Hz, 1H, arom CH); 5.43−5.49 (m, 2H, 2 * CH Ger); 4.63 (s, 2H, CO_2CH_2); 4.08 (d, *J* = 8.0 Hz, 2H, CH_2Cl Ger); 2.90 (s, 3H, $NHC\underline{H}_3$); 2.18−2.21 (m, 2H, CH_2 Ger); 2.09−2.13 (m, 2H, CH_2 Ger); 1.73 (s, 3H, CH_3 Ger); 1.72 (s, 3H, CH_3 Ger). ^{13}C NMR (125.6 MHz, $CDCl_3$): δ 168.4 (CO_2); 152.1 (arom quart. CNH); 142.1 (quart. C Ger); 134.6; 131.5 (2 * arom CH); 130.9 (quart. C Ger); 128.0; 120.7 (2 * CH Ger); 114.3; 110.7 (2 * arom CH); 110.0 (arom quart. *C*CO_2); 69.6 (CO_2-CH_2); 41.0 (CH_2Cl Ger); 38.8 (CH_2 Ger); 29.6 ($NHCH_3$); 25.8 (CH_2 Ger); 16.1; 14.1 (2 * CH_3 Ger). MS (EI) *m*/*z* (rel intensity) 321 (M^+, 19), 151 (100), 134 (30), 133 (17), 105 (32). HRMS (EI) *m*/*z*: calcd for (M^+) $C_{18}H_{24}ClNO_2$ 321.1495, found 321.1511. Anal. Calcd: C, 67.26; H, 7.53; N, 4.36. Found: C, 67.07; H, 7.61; N, 4.30.

***S*-[(*E*,*E*)-8-*O*-(2-*N*-Methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene]-L-cysteine Methyl Ester (H-Cys(Ger/Mant)-OMe) (9a).** To a solution L-cysteine methyl ester hydrochloride (73.0 mg, 0.42 mmol) in dry liquid ammonia (10 mL), stirring vigorously at −40 °C under an atmosphere of argon, was added a solution of the chloride **8a** dissolved in dry THF (10 mL). The resulting clear solution was then left to slowly warm overnight. After evaporation of the remaining solution the resulting white solid was dissolved in water (50 mL) and extracted with Et_2O (5 × 20 mL). The combined organic phase was dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield compound **9a** as a pale yellow oil (142 mg, 89%). R_f = 0.53 (hexane/ethyl acetate 1:2). $[\alpha]^{22}_D$ = +11.2 (*c* = 1.0, CH_3OH). UV (MeOH) λ_{max} 199 nm (ϵ 29 653), 220 nm (ϵ 31 033), 253 nm (ϵ 9106), 354 nm (ϵ 6220). IR (KBr) ν_{max} 3379, 2925 br s, 1742, 1681, 1607, 1580, 1520, 1240 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.89 (dd, *J* = 1.6, 8.0 Hz, 1H, arom CH); 7.62 (br d, *J* = 4.7 Hz, 1H, NH); 7.34 (ddd, *J* = 1.6, 7.0, 8.4 Hz, 1H, arom CH); 6.62 (dd, *J* = 1.0, 8.4 Hz, 1H, arom CH); 6.55 (ddd, *J* = 1.0, 7.0, 8.0 Hz, 1H, arom CH); 5.47 (t, *J* = 7.0 Hz, 1H, CH Ger); 5.21 (t, *J* = 7.8 Hz, 1H, CH Ger); 4.60 (s, 2H, CO_2CH_2); 3.70 (s, 3H, OCH_3); 3.59 (dd, *J* = 4.7, 7.7 Hz, 1H, α-CH Cys); 3.09−3.20 (m, 2H, α-CH_2 Ger); 2.86 (d, *J* = 4.7 Hz, 3H, $NHC\underline{H}_3$); 2.83 (dd, *J* = 4.7, 13.5 Hz, 1H, *β*-CH_{2a} Cys); 2.64 (dd, *J* = 7.7, 13.5 Hz, 1H, *β*-CH_{2b} Cys); 2.00−2.20 (m, 4H, 2 * CH_2 Ger); 1.76 (s, 2H, NH_2); 1.69 (s, 3H, CH_3 Ger); 1.64 (s, 3H, CH_3 Ger). ^{13}C NMR (125.6 MHz, $CDCl_3$): δ 174.4 (CO_2Me); 168.3 (CO_2); 151.9 (arom quart. CNH); 138.8 (quart. C Ger); 134.5; 131.4 (2 * arom CH); 130.6 (quart. C Ger); 128.3; 120.3 (2 * CH Ger); 114.2; 110.6 (2 * arom CH); 110.0 (arom quart. $\underline{C}CO_2$); 69.5 ($CO_2\underline{C}H_2$); 54.1 (α-CH Cys); 52.1 (OCH_3); 38.9 (α-CH_2 Ger); 36.3 (*β*-CH_2 Cys); 29.6 (CH_2 Ger); 29.4 ($NHCH_3$); 26.0 (CH_2 Ger); 16.0; 14.0 (2 * CH_3 Ger). MS (EI) *m*/*z* (rel intensity) 420 (M^+, 27), 151 (100), 134 (68), 105 (20), 88 (22). HRMS (EI) *m*/*z*: calcd for (M^+) $C_{22}H_{32}N_2O_4S$ 420.2070, found 420.2083.

***N*-(9-Fluorenylmethyloxycarbonyl)-L-methionylglycyl-L-leucyl-L-prolyl-*S*-[(*E*,*E*)-8-*O*-(2-*N*-methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene]-L-cysteine Methyl Ester (Fmoc-Met-Gly-Leu-Pro-Cys-(Ger/Mant)-OMe) (21a).** This compound was prepared utilizing the standard peptide coupling procedure with the tetrapeptide **19** (250 mg, 0.39 mmol) and the lipidated cysteine **9a** (164 mg, 0.39 mmol). Purification by flash chromatography on silica gel starting with ethyl acetate, followed by ethyl acetate/methanol (95:5) as eluent, yielded 272 mg (68%) of the desired product **21a** as a colorless oil. R_f = 0.6 (ethyl acetate). $[\alpha]^{22}_D$ = −52.9 (*c* = 1.0, CH_3OH). ^1H NMR (500 MHz, $CDCl_3$): δ 7.91 (dd, *J* = 1.6, 8.0 Hz, 1H, arom CH Mant); 7.75 (d, *J* = 7.5 Hz, 2H, arom CH Fmoc); 7.58 (d, *J* = 7.4 Hz, 2H, arom CH Fmoc); 7.35−7.47 (m, 4H, 2 * arom CH Fmoc, arom CH Mant, CONH); 7.30 (m, 2H, arom CH Fmoc); 7.03 (br s, 1H, CONH); 6.92 (br s, 1H, CONH); 6.67 (d, *J* = 8.4 Hz, 1H, arom CH Mant); 6.59 (t, *J* = 8.0 Hz, 1H, arom CH Mant); 5.62 (d, *J* = 7.7 Hz, 1H, OCONH); 5.48 (t, *J* = 6.9 Hz, 1H, CH Ger); 5.15 (t, *J* = 7.9 Hz, 1H, CH Ger); 4.81−4.84 (m, 1H, α-CH); 4.60−4.68 (m, 2H, 2 * α-CH), 4.63 (s, 2H, CO_2CH_2); 4.30−4.47 (m, 3H, OCH_2 Fmoc, α-CH); 4.21 (t, *J* = 6.6 Hz, 1H, CH Fmoc); 3.90−4.13 (m, 2H, α-CH_2 Gly); 3.75−3.79 (m, 1H, *δ*-CH_{2a} Pro); 3.72 (s, 3H, OCH_3); 3.61−3.64 (m, 1H, *δ*-CH_{2b} Pro); 3.16 (dd, *J* = 8.1, 13.3 Hz, 1H, α-CH_{2a} Ger); 3.06 (dd, *J* = 7.4, 13.3 Hz, 1H, α-CH_{2b} Ger); 2.92 (dd, *J* = 4.7, 13.8 Hz, 1H, *β*-CH_{2a} Cys); 2.90 (s, 3H, NHC*H*$_3$); 2.72 (dd, *J* = 6.9, 13.8 Hz, 1H, *β*-CH_{2b} Cys); 2.54 (br s, 2H, *γ*-CH_2 Met); 2.09 (s, 3H, SCH_3); 1.90−2.30 (m, 8H, 2 * CH_2 Ger, *β*-CH_2 Met, *β*-CH_2 Pro); 1.71 (s, 3H, CH_3 Ger); 1.65 (s, 3H, CH_3 Ger); 1.50−1.71 (m, 5H, *γ*-CH_2 Pro, *β*-CH_2 Leu, *γ*-CH Leu); 0.94 (d, *J* = 6.5 Hz, 3H, 1 * *ω*-CH_3 Leu); 0.91 (d, *J* = 6.5 Hz, 3H, 1 * *ω*-CH_3 Leu). ^{13}C NMR (125.6 MHz, $CDCl_3$): δ 171.8; 171.6; 171.1; 168.3; 168.2 (5 * C═O); 156.1 (OCONH); 151.9 (arom quart. CNH); 143.7; 143.5; 141.1 (3 * quart. C Fmoc); 139.1 (quart. C Ger); 134.5; 131.4 (2 * arom CH); 130.5 (quart. C Ger); 128.3 (CH Ger); 127.6; 126.9; 125.0 (3 * arom CH, Fmoc); 120.0 (CH Ger); 119.8 (arom CH, Fmoc), 114.1; 110.6 (2 * arom CH); 109.8 (arom $\underline{C}q$-CO_2); 69.5 ($CO_2-\underline{C}H_2$); 66.9 (OCH_2 Fmoc); 59.6 (α-CH Pro); 53.9 (α-CH Met); 52.2 ($\underline{O}CH_3$); 52.1 (α-CH Cys); 48.6 (α-CH Leu); 47.4 (*δ*-CH_2 Pro); 47.0 (CH Fmoc); 42.9 (α-CH_2 Gly); 41.6 (*β*-CH_2 Leu); 38.9 (α-CH_2 Ger); 32.5 (*β*-CH_2 Cys); 32.0 (CH_2 Met); 29.9 (CH_2 Ger); 29.4

($NHCH_3$); 29.3 (CH_2 Met); 28.6 (β-CH_2 Pro); 26.7; 26.0 (2 * CH_2 Ger); 24.7 (γ-CH_2 Pro); 24.4 (γ-CH Leu); 23.1; 21.9 (2 * ω-CH_3 Leu); 15.9 (CH_3 Ger); 15.1 (SCH_3); 13.9 (CH_3 Ger). HRMS (FAB; 3-NBA) *m/z*: calcd for $(M + H)^+$ $C_{55}H_{73}N_6O_{10}S_2$ 1041.452, found 1041.483. Anal. Calcd: C, 63.44; H, 6.97; N, 8.07. Found: C, 63.05; H, 6.82; N, 7.96.

L-Methionylglycyl-L-leucyl-L-prolyl-*S*-[(*E*,*E*)-8-*O*-(2-*N*-methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene]-L-cysteine Methyl Ester (H-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe) (22a). This compound was prepared utilizing the standard Fmoc deprotection procedure using the pentapeptide **21a** (130 mg, 0.125 mmol). Purification by flash chromatography on silica gel starting with ethyl acetate, followed by ethyl acetate/methanol (80:20), yielded 95 mg (93%) of the desired product **22a** as a colorless oil. $R_f = 0.2$ (ethyl acetate/methanol 95:5). $[\alpha]^{22}_D = -43.1$ ($c = 1.0$, CH_3OH). ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ 5:1): δ 7.83 (dd, $J = 1.5$, 8.0 Hz, 1H, arom CH); 7.70 (d, $J = 7.7$ Hz, 1H, CONH); 7.56 (d, $J = 7.5$ Hz, 1H, CONH); 7.29−7.32 (m, 2H, arom CH, CONH); 6.60 (d, $J = 8.4$ Hz, 1H, arom CH); 6.51 (t, $J = 7.5$ Hz, 1H, arom CH); 5.43 (t, $J = 6.6$ Hz, 1H, CH Ger); 5.13 (t, $J = 7.2$ Hz, 1H, CH Ger); 4.61 (m, 1H, α-CH); 4.55 (s, 3H, α-CH, CO_2CH_2); 4.46−4.49 (m, 1H, α-CH); 4.04 (obscured by MeOH, 2H, α-CH_2 Gly); 3.72−3.76 (m, 1H, δ-CH_{2a} Pro); 3.68 (s, 3H, OCH_3); 3.51−3.55 (m, 1H, δ-CH_{2b} Pro); 3.28 (br, 1H, α-CH Met); 3.12 (dd, $J = 8.3$, 13.6 Hz, 1H, α-CH_{2a} Ger); 3.04 (m, 1H, α-CH_{2b} Ger); 2.88 (dd, $J = 5.0$, 13.7 Hz, 1H, β-CH_{2a} Cys); 2.83 (s, 3H, $NHCH_3$); 2.72 (dd, $J = 7.0$, 13.8 Hz, 1H, β-CH_{2b} Cys); 2.55 (t, $J = 7.4$ Hz, 2H, γ-CH_2 Met); 1.91−2.12 (m, 10H, 2 * CH_2 Ger, β-CH_2 Met, β-CH_2 Pro, γ-CH_2 Pro); 2.06 (s, 3H, SCH_3); 1.76 (m, 1H, γ-CH Leu); 1.65 (s, 3H, CH_3 Ger); 1.60 (s, 3H, CH_3 Ger); 1.40−1.65 (m, 2H, β-CH_2 Leu); 0.88 (d, $J = 5.2$ Hz, 6H, 2 *ω-CH_3 Leu). ^{13}C NMR (125.6 MHz, $CDCl_3$):δ 172.9; 171.9; 171.5; 169.5; 169.4; 168.7 (6 * C═O); 152.2 (arom quart. CNH); 139.7 (quart. C Ger); 134.9; 131.7 (2 * arom CH); 130.8 (quart. C Ger); 128.6; 120.1 (2 * CH Ger); 114.6; 111.0 (2 * arom CH); 110.1 (arom quart. *C*CO_2); 69.9 (CO_2CH_2); 60.2 (α-CH Pro); 52.8 (α-CH Met); 52.7 (OCH_3); 52.3 (α-CH Cys); 49.8 (α-CH Leu); 47.5 (δ-CH_2 Pro); 42.7 (α-CH_2 Gly); 40.6 (β-CH_2 Leu); 39.2 (α-CH_2 Ger); 32.9 (β-CH_2 Cys); 30.4 (CH_2 Met); 29.8 (CH_2 Ger); 29.5 ($NHCH_3$); 29.2 (CH_2 Met); 28.4 (β-CH_2 Pro); 26.3 (CH_2 Ger); 25.0 (γ-CH_2 Pro); 24.8 (γ-CH Leu); 23.3; 21.5 (2 *ω-CH_3 Leu); 16.1 (CH_3 Ger); 15.0 (SCH_3); 14.1 (CH_3 Ger). HRMS (FAB; 3-NBA) *m/z*: calcd for $(M + H)^+$ $C_{40}H_{62}N_6O_8S_2$ 819.4218, found 819.4149.

***N*-(6-Maleimidocaproyl)-L-methionylglycyl-L-leucyl-L-prolyl-*S*-[(*E*,*E*)-8-*O*-(2-*N*-methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene]-L-cysteine Methyl Ester (MIC-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe) (41a).** This compound was prepared following the standard coupling procedure using the pentapeptide **22a** (64 mg, 0.08 mmol) and maleimidocaproic acid **3** (18 mg, 0.09 mmol, 1.1 eq.). The compound was purified by flash chromatography on silica gel starting with ethyl acetate, followed by ethyl acetate/methanol (98:2), and furnished 44 mg (55%) of the desired target molecule **41a** as a colorless oil. $R_f = 0.33$ (ethyl acetate/methanol 98:2). $[\alpha]^{22}_D = -41.6$ ($c = 1.0$, CH_2Cl_2). ^1H NMR (500 MHz, $CDCl_3$): δ 7.90 (dd, $J = 1.6$, 8.0 Hz, 1H, arom CH); 7.58−7.62 (m, 2H, CONH, NH); 7.57 (d, $J = 7.6$ Hz, 1H, arom CH); 7.36 (m, 1H, arom CH); 7.24 (br s, 1H, CONH); 6.67 (s, 2H, CH═CH MIC); 6.64 (d, $J = 8.5$ Hz, 1H, arom CH); 6.55−6.57 (m, 2H, arom CH, CONH); 5.46−5.49 (m, 1H, CH Ger); 5.17 (t, $J = 8.2$ Hz, 1H, CH Ger); 4.83 (dt, $J = 4.4$, 9.1 Hz, 1H, α-CH); 4.59−4.66 (m, 3H, 3 *α-CH); 4.61 (s, 2H, CO_2CH_2); 4.00−4.06 (m, 2H, α-CH_2 Gly); 3.80−3.84 (m, 1H, δ-CH_{2a} Pro); 3.71 (s, 3H, OCH_3); 3.62−3.66 (m, 1H, δ-CH_{2b} Pro); 3.48 (t, $J = 7.2$ Hz, 2H, NCH_2 MIC); 3.16 (dd, $J = 8.3$, 13.2 Hz, 1H, α-CH_{2a} Ger); 3.04 (dd, $J = 7.2$, 13.2 Hz, 1H, α-CH_{2b} Ger); 2.90 (partially obscured, 1H, β-CH_{2a} Cys); 2.89 (d, $J = 4.8$ Hz, 3H, NHC*H*$_3$); 2.70 (dd, $J = 7.3$, 13.8 Hz, 1H, β-CH_{2b} Cys); 2.50−2.55 (m, 2H, γ-CH_2 Met); 2.12−2.25 (m, 3H, β-CH_{2a} Met, α-CH_2 MIC); 2.08 (s, 3H, SCH_3); 1.72−2.11 (m, 9H, 2 * CH_2 Ger, β-CH_{2b} Met, β-CH_2 Pro, γ-CH_2 Pro); 1.70 (s, 3H, CH_3 Ger); 1.65 (s, 3H, CH_3 Ger); 1.50−1.70 (m, 6H, 2 * CH_2 MIC, γ-CH Leu, β-CH_{2a} Leu); 1.23−1.31 (m, 3H, CH_2 MIC, β-CH_{2b} Leu); 0.93 (d, $J = 6.5$ Hz, 3H, 1 *ω-CH_3 Leu); 0.91 (d, $J = 6.5$ Hz, 3H, 1 *ω-CH_3 Leu). 13C NMR (125.6 MHz, $CDCl_3$):δ 173.1; 172.3; 171.7; 171.3; 171.2; 170.9 (2 signals overlapped); 168.4; 168.4 (9 * C═O); 152.1 (arom quart. CNH); 139.4 (quart. C Ger); 134.6 (arom CH); 134.1 (CH═CH MIC); 131.5 (arom CH); 130.7 (quart. C Ger); 128.4; 120.0 (2 * CH Ger); 114.3; 110.7 (2 * arom CH); 110.0 (arom quart. *C*CO_2); 69.7 (CO_2-CH_2); 59.8 (α-CH Pro); 52.5; 52.2 (α-CH Met, α-CH Cys); 52.0 (OCH_3); 48.9 (α-CH Leu); 47.5 (δ-CH_2 Pro); 42.9 (α-CH_2 Gly); 41.7 (β-CH_2 Leu); 39.1 (α-CH_2 Ger); 37.6 (NCH_2 MIC); 36.1 (α-CH_2 MIC); 32.9 (β-CH_2 Cys); 31.5; 30.1 (3 signals 2 * CH_2 Met, CH_2 Ger); 29.5 ($NHCH_3$); 28.2 (2 signals overlapped, CH_2 MIC, β-CH_2 Pro); 26.3 (CH_2 Ger); 26.2 (CH_2 MIC); 24.9; 24.8 (γ-CH_2 Pro, CH_2 MIC); 24.6 (γ-CH Leu); 23.3; 21.9 (2 *ω-CH_3 Leu); 16.1 (CH_3 Ger); 15.3 (SCH_3); 14.0 (CH_3 Ger). HRMS (FAB; 3-NBA) *m/z*: calcd for $(M + H)^+$ $C_{50}H_{74}N_7O_{11}S_2$ 1012.489, found 1012.465.

***N*-(6-Maleimidocaproyl)-glycyl-*tert*-butylthio-L-cysteyl-L-methionylglycyl-L-leucyl-L-prolyl-*S*-[(*E*,*E*)-8-*O*-(2-*N*-methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene]-L-cysteine Methyl Ester (MIC-Gly-Cys(StBu)-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe) (42a).** This compound was prepared following the standard coupling procedure using the pentapeptide 22a (29.5 mg, 0.036 mmol) and MIC-Gly-Cys-(S^{t}Bu)-OH **31** (18.2 mg, 0.039 mmol, 1.1 eq.). The compound was purified by flash chromatography on silica gel using gradient elution starting with ethyl acetate, followed by ethyl acetate/methanol (98:2) and finally with ethyl acetate/methanol (95:5). After purification 20.0 mg (44%) of the desired target molecule **42a** was obtained as a colorless oil. $R_f = 0.3$ (ethyl acetate/methanol 95:5). $[\alpha]^{22}_D = -51.8$ ($c = 1.0$, CH_2Cl_2). ^1H NMR (500 MHz, $CDCl_3/CD_3OD$, 3:1): δ 8.30 (d, $J = 7.2$ Hz, 1H, CONH); 8.22 (d, $J = 6.9$ Hz, 1H, CONH); 7.87 (dd, $J = 1.6$, 8.0 Hz, 1H, arom CH); 7.76 (br s, 1H, NH); 7.67 (d, $J = 7.6$ Hz, 1H, CONH); 7.50 (d, $J = 8.0$ Hz, 1H, CONH); 7.44 (d, $J = 5.3$ Hz, 1H, CONH); 7.34−7.36 (m, 1H, arom CH); 6.96 (s, 2H, CH═CH MIC); 6.65 (d, $J = 8.5$ Hz, 1H, arom CH); 6.56 (dt, $J = 1.0$, 8.0 Hz, 1H, arom CH); 5.47 (t, $J = 6.6$ Hz, 1H, CH Ger); 5.16 (t, $J = 7.1$ Hz, 1H, CH Ger); 4.52−4.70 (m, 4H, 4 *α-CH, α-CH Cys_{Ger}, α-CH Cys_{HD}, α-CH Pro, α-CH Leu); 4.59 (s, 2H, CO_2CH_2); 4.13−4.22 (obscured by MeOH, 2H, α-CH_{2a} Gly, α-CH Met); 3.85−3.90 (m, 2H, α-CH_2 Gly′); 3.81−3.85 (m, 1H, δ-CH_{2a} Pro); 3.71 (s, 3H, OCH_3); 3.56−3.71 (m, 2H, δ-CH_{2b} Pro, α-CH_{2b} Gly); 3.47 (t, $J = 7.2$ Hz, 2H, NCH_2 MIC); 3.15 (dd, $J = 8.3$, 13.2 Hz, 1H, α-CH_{2a} Ger); 3.01−3.07 (m, 2H, α-CH_{2b} Ger, β-CH_{2a} Cys_{StBu}); 2.94 (dd, $J = 6.5$, 13.5 Hz, 1H, β-CH_{2b} Cys_{StBu}); 2.90 (dd, $J = 4.9$, 13.8 Hz, 1H, β-CH2a CysGer); 2.87 (s, 3H, $NHCH_3$); 2.70 (dd, $J = 7.2$, 13.8 Hz, 1H, β-CH_{2b} CysGer); 2.58−2.62 (m, 1H, γ-CH_{2a} Met); 2.50−2.54 (m, 1H, γ-CH_{2b} Met); 2.00−2.29 (m, 12H, 2 * CH_2 Ger, α-CH_2 MIC, β-CH_2 Met, β-CH_2 Pro, γ-CH_2 Pro); 2.06 (s, 3H, SCH_3); 1.69 (s, 3H, CH_3 Ger); 1.64 (s, 3H, CH_3 Ger); 1.44−1.78 (m, 7H, 2 * CH_2 MIC, γ-CH Leu, β-CH_2 Leu); 1.30 (br s, 11H, CH_2 MIC, $SC(CH_3)_3$); 0.92 (d, $J = 6.7$ Hz, 3H, 1 *ω-CH_3 Leu); 0.90 (d, $J = 6.7$ Hz, 3H, 1 *ω-CH_3 Leu). MS (FAB; 3-NBA) *m/z*: found 1260 $(M + H)^+$.

***N*-(6-Maleimidocaproyl)-glycyl-*S*-hexadecyl-L-cysteyl-L-methionylglycyl-L-leucyl-L-prolyl-*S*-[(*E*,*E*)-8-*O*-(2-*N*-methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene]-L-cysteine Methyl Ester (MIC-Gly-Cys-(HD)-Met-Gly-Leu-Pro-Cys(Ger/Mant)-OMe) (44a).** This compound was prepared utilizing the standard coupling procedure with the pentapeptide **22a** (27 mg, 0.033 mmol) and MIC-Gly-Cys(HD)-OH **30** (20 mg, 0.033 mmol). The compound was purified by flash chromatography on silica gel using gradient elution starting with ethyl acetate, followed by ethyl acetate/methanol (98:2) and furnished 25.5 mg (52%) of the desired target molecule **44a** as a colorless oil. $R_f = 0.63$ (ethyl acetate/methanol 98:2). $[\alpha]^{22}_D = -56.3$ ($c = 1.0$, CH_2Cl_2). ^1H NMR (500 MHz, $CDCl_3$): δ 7.91 (dd, $J = 1.5$, 7.9 Hz, 2H, arom CH, CONH); 7.63 (br s, 1H, NH); 7.55 (d, $J = 7.5$ Hz, 1H, CONH); 7.37 (t, $J = 6.9$ Hz, 2H, arom CH, CONH); 7.20 (d, $J = 7.9$ Hz, 1H, CONH); 6.68 (s, 2H, CH═CH MIC); 6.66−6.68 (m, 1H, arom CH); 6.58 (t, $J = 7.9$ Hz, 1H, arom CH); 5.49 (t, $J = 6.5$ Hz, 1H, CH Ger); 5.17 (t, $J = 7.7$ Hz, 1H, CH Ger); 4.74−4.78 (m, 1H, α-CH Leu); 4.69−4.72 (m, 1H, α-CH CysGer); 4.62 (s, 2H, CO_2CH_2); 4.51−4.59 (m, 2H, α-CH Cys_{HD} α-CH Pro); 4.37 (dd, $J = 7.7$, 17.0 Hz, 1H, α-CH_{2a} Gly); 4.27 (br, 1H, α-CH Met); 3.91−3.96 (m, 2H, α-CH_2 Gly′); 3.85−3.88 (m, 1H, δ-CH_{2a} Pro); 3.74 (s, 3H, OCH_3); 3.54−3.73 (m, 2H, δ-CH_{2b} Pro, α-CH_{2b} Gly); 3.50 (t, $J = 7.1$ Hz, 2H, NCH_2 MIC); 3.16 (dd, $J = 8.2$, 13.3 Hz, 1H, α-CH_{2a} Ger); 3.05 (dd, $J = 4.5$, 13.3 Hz, 1H, α-CH_{2b} Ger); 2.90 (d, $J = 4.9$ Hz, 3H, $NHCH_3$); 2.81−2.96 (m, 3H, β-CH_{2a} Cys_{Ger}, β-CH_2 CysHD); 2.71 (dd, $J = 6.6$, 13.7

Hz, 1H, β-CH_{2b} Cys_{Ger}); 2.55−2.67 (m, 2H, γ-CH_2 Met); 2.52 (t, J = 7.4 Hz, 2H, α-CH_2 HD); 1.98−2.41 (m, 12H, 2 * CH_2 Ger, α-CH_2 MIC, β-CH_2 Met, β-CH_2 Pro, γ-CH_2 Pro); 2.08 (s, 3H, SCH_3); 1.72 (s, 3H, CH_3 Ger); 1.66 (s, 3H, CH_3 Ger); 1.42−1.80 (m, 9H, 2 * CH_2 MIC, β-CH_2 HD, γ-CH Leu, β-CH_2 Leu); 1.24 (br s, 28H, CH_2 MIC, $(CH_2)_{13}$); 0.93 (t, J = 6.4 Hz, 6H, 2 *ω-CH_3 Leu); 0.87 (t, J = 6.9 Hz, 3H, ω-CH_3 HD). ^{13}C NMR (125.6 MHz, $CDCl_3$):δ 174.9; 173.4; 171.4; 171.2; 171.1; 170.9 (2 signals overlapped); 170.8; 170.6; 169.7; 168.5 (11 * C═O); 152.1 (arom quart. CNH); 139.5 (quart. C Ger); 134.7 (arom CH); 134.1 (CH═CH MIC); 131.5 (arom CH); 130.8 (quart. C Ger); 128.4; 120.0 (2 * CH Ger); 114.3; 110.7 (2 * arom CH); 110.0 (arom quart. $\underline{C}CO_2$); 69.7 ($CO_2\underline{C}H_2$); 60.1 (α-CH Pro); 53.4; 53.1 (α-CH Met, α-C$\underline{H}$ Cys); 52.6 (OCH_3); 51.9 (α-CH Cys); 49.1(α-CH Leu); 47.4 (δ-CH_2 Pro); 44.0; 43.0 (2 *α-CH_2 Gly); 40.6 (β-CH_2 Leu); 39.1 (α-CH_2 Ger); 37.6 (NCH_2 MIC); 35.9 (α-CH_2 MIC); 33.2; 32.7; 32.7; 31.9; 30.6; 29.7 (2 *β-CH_2 Cys, 2 * CH_2 Met, CH_2 Ger, 9 * CH_2 HD); 29.6 ($NHCH_3$); 29.6; 29.4; 29.3; 28.9; 28.5; 28.2; 27.8; 26.3; 26.2; 25.0; 24.9 (3 * CH_2 MIC, 5 * CH_2 HD, CH_2 Ger, β-CH_2 Pro, γ-CH_2 Pro); 24.6 (γ-CH Leu); 23.3 (1 *ω-CH_3 Leu); 22.7 (CH_2 HD); 21.7 (1 *ω-CH_3 Leu); 16.1 (CH_3 Ger); 15.0 (SCH_3); 14.2 (CH_3 Ger); 14.1 (ω-CH_3 HD). MS (FAB; 3-NBA) *m*/*z*: found 1396.0 $(M + H)^+$.

***S*-Hexadecyl-L-cysteine Methyl Ester (H-Cys(HD)-OMe) (49).** To a solution of 1.0 g (5.81 mmol) HCl * H−Cys-OMe (1.0 g, 5.81 mmol), dissolved in dry DMF (10 mL), was added at 0 °C diisopropylethylamine (2.5 mL, 14.50 mmol) followed by hexadecylbromide (3.6 mL, 11.62 mmol). After stirring for 3 h at room temperature, the solvent was removed under reduced pressure and the residue was taken up in 1 M HCl (80 mL). The product was extracted by washing with CH_2Cl_2 (3 × 60 mL). The organic layer was washed with 1 M $NaHCO_3$ (100 mL) and water (100 mL) and dried over $MgSO_4$. After removing the solvent, the product was purified by flash chromatography on silica gel using hexane/ethyl acetate (1:1) as eluent and furnished 541 mg (26%) of the desired material as a white solid. R_f = 0.3 (hexane/ethyl acetate 2:3). $[\alpha]^{22}_D$ = −3.1 (c = 1.0, $CHCl_3$). ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.74 (s, 3H, OCH_3); 3.65 (dd, J = 4.7, 7.3 Hz, 1H, α-CH Cys); 2.91 (dd, J = 4.6, 13.5 Hz, 1H, β-CH_{2a} Cys); 2.76 (dd, J = 6.3, 13.5 Hz, 1H, β-CH_{2b} Cys); 2.51 (t, J = 7.4 Hz, 2H, α-CH_2 HD); 2.08 (s, br, 2H, NH_2); 1.50−1.59 (m, 2H, β-CH_2 HD); 1.24 (s, br, 26H, $(CH_2)_{13}$ HD); 0.86 (t, J = 7.0 Hz, 3H, ω-CH_3 HD). HRMS (EI) *m*/*z*: calcd for (M^+) $C_{20}H_{41}NO_2S$ 359.2858, found 359.2842. Anal. Calcd: C, 66.80; H, 11.49; N, 3.89. Found: C, 67.20; H, 11.25; N, 3.41.

Coupling to *Ras*. Prior to the coupling reaction, a sample of the mutant *Ras* protein (1−181) was passed through a desalting column to remove any excess salts and DTE required for storage of the protein. At all stages of the coupling reaction, all samples containing protein, unless otherwise stated, were kept at or below 4 °C. To a solution of the desired peptide dissolved in methanol (20 μL) in a 2 mL Eppendorf tube was added a solution of 11% Triton X-114 in water (1 mL). The solution was sonicated for approximately 10 min until a slightly cloudy, homogeneous solution was obtained. The detergent solution was cooled to 0 °C, and an aqueous solution (buffer: 20 mM Tris/HCl, 5 mM $MgCl_2$, pH 7.4) containing the *Ras* protein was added to the peptide solution (approximately a 1:1 solution of the peptide- and protein-containing solutions). The reaction tube was sealed under argon and placed on a rotary mixer and left to react at 4 °C. After 15 h, the solution was then centrifuged to remove any denatured protein. The supernatant was transferred into a fresh 15 mL centrifuge tube, diluted with 3 mL buffer (containing 2mM DTE), and heated to 37 °C upon which the solution turned milky white. Centrifugation at room-temperature resulted in a phase separation of an 11% Triton X-114 detergent phase from the aqueous phase. The aqueous phase was removed and extracted two more times with a 11% Triton X-114 detergent solution (2 × 1 mL). The detergent phases were combined and washed three times with fresh buffer (3 × 7 mL). The resulting extracted solution was diluted to 30 mL with fresh buffer, and the sample was purified by DEAE ion exchange chromatography following a standard protocol: After removal of the Triton X-114 by purging the column with buffer, the labeled protein was eluted in 2 mL portions using a NaCl-gradient (0 M to 1 M NaCl within 25 min with a flow rate of of 1 mL/min). Fractions containing the desired protein were combined, concentrated, and desalted using a Vivaspin protein concentrator with a 10,000 Da exclusion membrane. The protein was then snap frozen and stored at −80 °C. Yields for each coupling reaction are given in Table 2.

Proof for the specific coupling of the lipopeptides to the proteins was obtained by SDS-PAGE gel electrophoresis, as well as by electrospray mass spectrometry of the intact labeled protein. Further proof for the specific incorporation of the lipopeptide onto cysteine residue 181 was obtained by digestion of three of the protein samples (H-*Ras* (1−181) + peptide **41c**, H-*Ras* (1−181) + peptide **43c**, H-*Ras* (1−181) + peptide **44c**) with trypsin and chymotrypsin proteases followed by mass spectrometry of the individual fragments. This final result clearly showed incorporation of the lipopeptide tail to the last cysteine Cys181, thereby proving the specific incorporation of the peptide group.

Microinjection Experiments. PC12 cell culture and microinjections were performed as described.[29] Briefly, PC12 cells were prestimulated with NGF (100 ng/mL) for 3 days and kept in NGF-free medium for another 2 days. *Ras* proteins were diluted to 150 μM in PBS containing 10 μM fluorescein dextran as a marker for identification. 40 h after injection the transformed cells showed a typical differentiated phenotype with neurite outgrowth.

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Supporting Information Available: Experimental procedures, spectroscopic and analytical data for all compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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