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Solid-Phase Synthesis of Lipidated Peptides

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Abstract: A new flexible and efficient methodology for the solid-phase synthesis of lipidated peptides has been developed. The approach is based on the use of previously synthesized building blocks and overcomes the limitations of previously reported methods, since long doubly lipidated peptides can be synthesized by using this route. Furthermore, it was thus possible to prepare a large number of N- and H-Ras peptides bearing a wide range of reporter and/or linking groups—efficient tools for the investigation of biological processes. In terms of efficiency and flexibility this solid-phase method is superior to the solution-phase synthesis. It gives pure peptides in multi-milligram amounts within a much shorter time and with superior overall yield.

Keywords: building block strategy · lipidated peptides · lipoproteins · solid-phase synthesis

Introduction

The Ras proteins are plasma membrane bound lipoproteins that serve as central molecular switches in biological signal transduction. They cycle between an inactive GDP-bound state and an active GTP-bound state.[1] In the active state, the Ras proteins translate growth-promoting signals into changes in gene expression. These proteins are involved in the regulation of diverse cellular processes such as cell growth and differentiation, the cell cycle, and apoptosis.[2] The importance of the correct functioning of the Ras signal transduction pathway is demonstrated by the fact that a point of mutation in the ras oncogenes is found in approximately 30% of all human cancers.[3] The Ras proteins contain both acid-labile farnesyl thioethers and base-sensitive palmitic acid thioesters and terminate in a cysteine methyl ester (Figure 1). Lipidation of these proteins is essential for the biological function.[4] The lipid groups are believed to serve as anchors of the proteins to the membranes and are possibly involved in protein–protein and protein–lipid interactions.

Tailor-made lipidated peptides representing the characteristic functional parts of their parent proteins are efficient tools for the investigation of biological processes.[5–6] Therefore, the availability of a flexible solid-phase technology for their synthesis is of great importance. To develop such a general and flexible solid-phase method that would be appli-

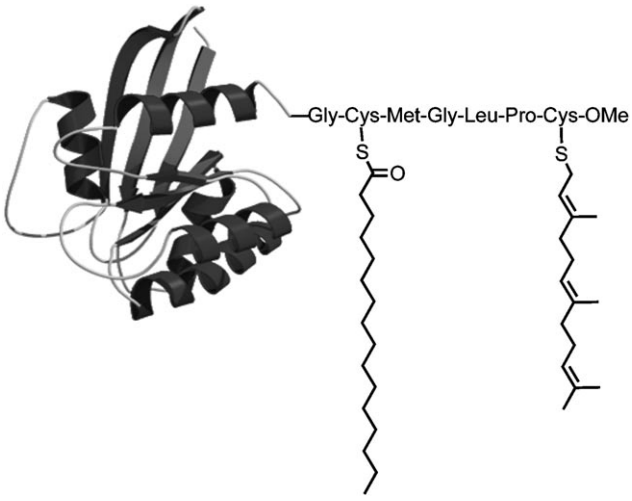

Figure 1. Schematic structure of the human N-Ras protein.

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cable to the synthesis of both acid- and base-labile lipidated peptides several demands must be fulfilled (Figure 2). First, the method requires the use of suitable orthogonally stable protecting groups as well as a linker to the solid support that can be cleaved under mild conditions[6] and which allows release of the peptides as methyl ester, or—if required—equipped with a different functional group, for example, a fluorophore at the C-terminus. Second, the approach should also allow an easy introduction of reporter and/or linking groups needed for further biological investigations.

At the outset of our work only one method for the solid-phase synthesis of double lipidated peptides was available. This employed on-resin lipidation of cysteines after cleavage of suitable protecting groups as the key transformation.[7] However, this method suffers from several drawbacks that hinder its general applicability: 1) the use of a large excess of (sometimes not readily available) lipidation reagents is required, 2) it is not suitable for the synthesis of longer peptides (>10 amino acids), 3) it is not readily automatable. The major challenge to be met by a new more general and flexible approach lies in the elongation of the peptide chain after incorporation of the palmitoylated cysteine, since the thioester rapidly undergoes an intramolecular S,N-acyl shift once the amino group is deprotected (Figure 2).[8]

Herein we describe the development of a technology that fulfils the demands and solves the problems mentioned above. This approach employs the synthesis of lipidated building blocks in solution, which substantially reduces the amount of lipidation reagents required, and subsequent use of these building blocks in the solid-phase synthesis of the target peptides. The use of Fmoc-4-hydrazinobenzoic acid as a linker allows direct access to the methyl esters or free acids after cleavage under mild oxidative conditions.[9] Particularly remarkable is the establishment of coupling conditions that minimize the S,N-acyl shift after incorporation of the palmitoylated cysteine. We demonstrate the efficiency of the new approach in the synthesis of a variety of C-terminal N-Ras and H-Ras peptides that bear a wide range of fluorescent and/or reporter and linking groups. Part of this work has been published in a preliminary communication.[10]

Results and Discussion

Synthesis of the lipidated and labeled building blocks: The building blocks required for the development of the new solid-phase method were synthesized in high overall yields as shown in Scheme 1 using in part transformations described earlier.[11] Only one equivalent of lipidation reagent (farnesyl or palmitoyl chloride) was required, which is notably advantageous in the case of the *N*-methylanthraniloyl (Mant)-labeled cysteine **6**, since this building block requires a laborious multistep synthesis starting from geraniol.[12] Thus a possible use of this reagent in fivefold excess as is required in the on-resin lipidation method[7] is clearly undesired. Not only different lipidated cysteine building blocks were synthesized (**2**, **4**, **5**, and **6**), but also both a NBD (4-nitrobenz-2-oxa-1,3-diazole) fluorescent label (**8**) and a benzophenone group (**10**) were attached to the side chain of lysine.

Solid-phase synthesis of farnesylated and palmitoylated peptides using the pre-lipidated building-blocks: The lipidated cysteines **2** and **4** were used in the solid-phase synthesis of the N- and H-Ras peptides **11** and **12** (Scheme 2). The solid-phase synthesis was performed using the commercially available Fmoc-4-hydrazinobenzoic acid functionalized aminomethyl polystyrene resin (Novabiochem) and standard Fmoc chemistry. The Fmoc group was removed with 20% piperidine in DMF. Standard couplings were performed with five equivalents of amino acid/HBTU/HOBt and 10 equivalents of diisopropylethylamine in DMF for 2 h. Cysteine building blocks were coupled using HBTU/HOBt/trimethylpyridine in CH_2Cl_2/DMF (1:1) to avoid racemization.[13] In these cases extended coupling times were required due to the bulkiness of the lipid residues. The doubly lipidated peptides **11** and **12** were obtained after cleavage from the resin. The hydrazide bond is oxidized by copper acetate. After the nucleophilic attack of methanol, the peptide is released from the resin as a methyl ester (Scheme 2). Filtration of the crude reaction products through a silica gel cartridge allows the peptides to be obtained in high yields (69% for **11** and 60% for **12**) and with high purity (>90%, Figure 3).

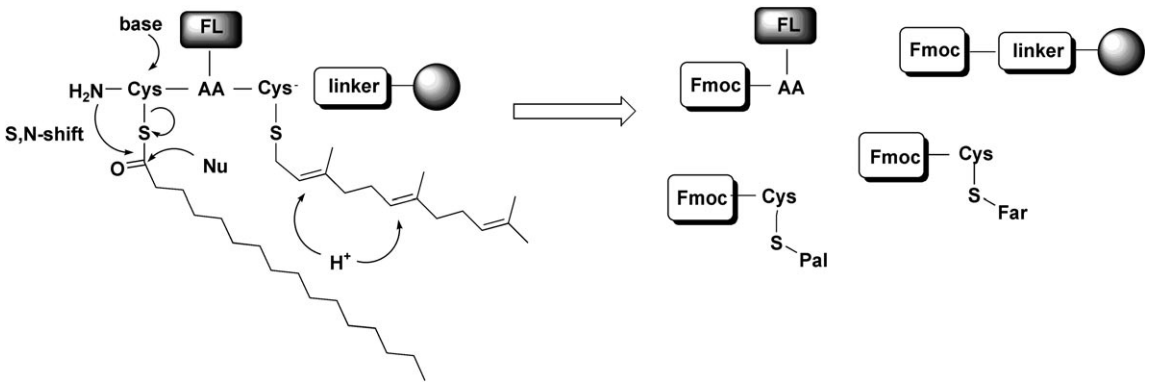
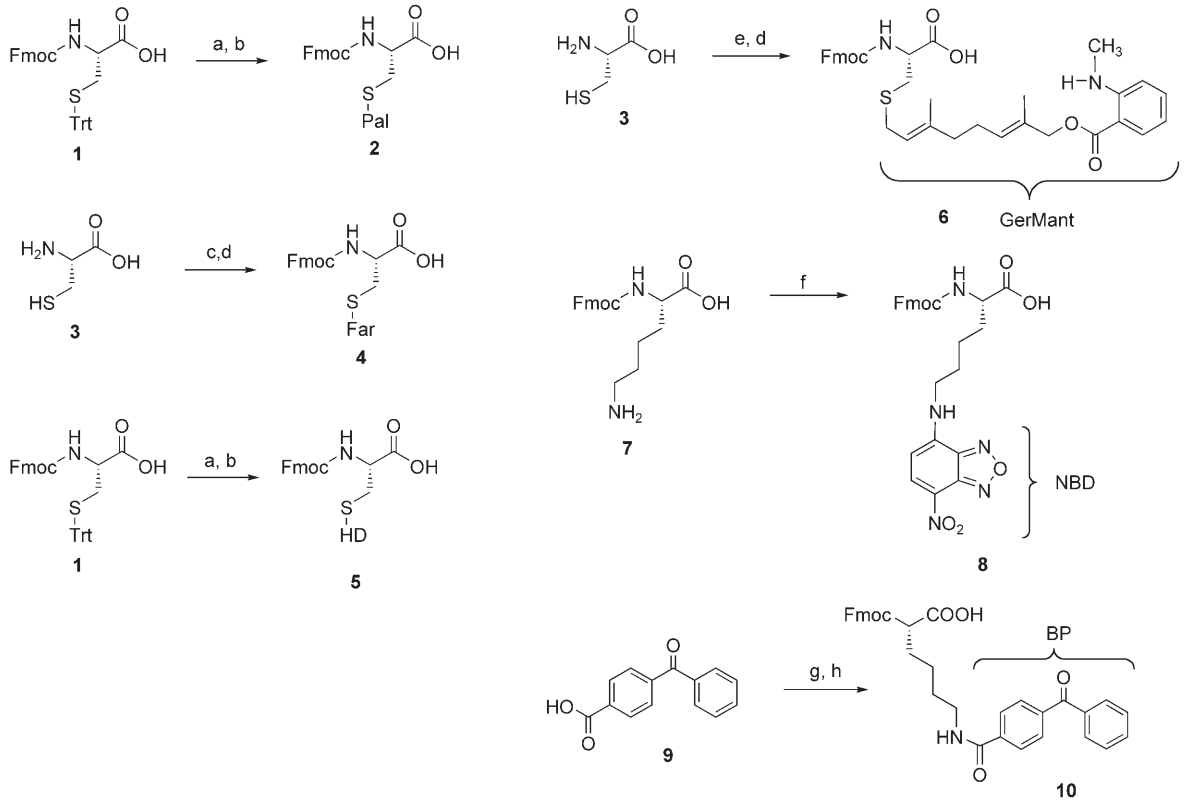


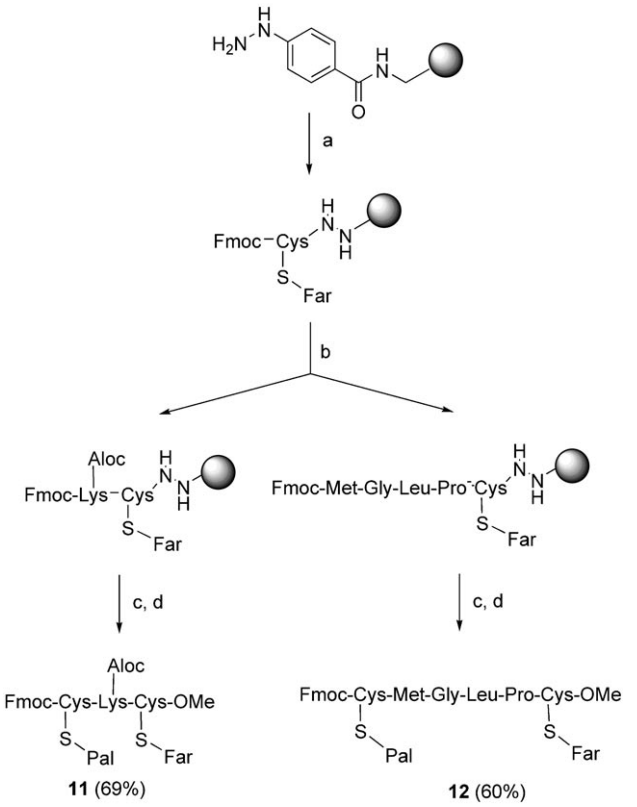
Figure 2. Building block approach to the solid-phase synthesis of doubly lipidated peptides. FL: fluorescent label.



Scheme 1. Synthesis of building blocks **2**, **4**, **5**, **6**, **8**, and **10**. a) 5% TFA, 3% TES, CH_2Cl_2, 1 h, RT; b) TMS-Cl (1.1 equiv), 2 h, reflux, CH_2Cl_2, then Pal-Cl or HD-I (3 equiv), Et_3N (1.5 equiv) dropwise, 3 h, CH_2Cl_2, RT; c) Far-Cl (1 equiv), 4 N NH_3/methanol, 3 h, 0 °C, 1 h, RT; d) Fmoc-OSu (1.1 equiv), Et_3N (1.1 equiv), CH_2Cl_2, 2 h, RT; e) GerMantCl (1 equiv), 4 N NH_3/methanol, 3 h, 0 °C, 1 h, RT; f) NBDCl, THF/methanol, 65 °C, 2 h; g) 1.1 equiv HATU, 2 equiv DIPEA, DMF, 10 min; h) Fmoc-Lys-OH, 5 h, RT. DIPEA: *N,N*-diisopropylethylamine, Far = farnisyl, Fmoc: 9-fluorenylmethyloxycarbonyl, HD-I: hexadecyl iodide, Pal = palmitoyl, TES: triethylsilane, TFA: trifluoroacetic acid, TMS: trimethylsilane, Su: succinimide, HATU: *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate.

Elongation of the peptide chain after incorporation of the palmitoylated cysteine: Further elongation of the peptide chain required finding new conditions to remove the Fmoc protecting group from the N-terminal S-palmitoylated cysteine (Scheme 3), since the thioester is sensitive to nucleophilic attack (Figure 2) and therefore piperidine can not be used. However, the main challenge at this point was to avoid the quick S→N acyl shift once the amino group was free.[8] Gratifyingly, 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU)—a non-nucleophilic hindered base—permitted a fast removal of the Fmoc group and left the thioester intact. The Fmoc group in **13** (Scheme 3) can be cleaved by double treatment with a solution of 1% DBU in DMF each time for 30 s.[14] Fast washing of **14** with DMF (ca. 20 s) and immediate addition of a large excess (10 equiv) of preactivated (10 min) amino acid minimize the S,N-acyl shift of the palmitoyl group. After substantial experimentation we discovered that the polarity of the solvent plays a crucial role in this step (Table 1). In the first attempts we performed the coupling in DMF. The amino acid was activated by using HBTU/HOBt with DIPEA as the base (entry 1, Table 1). In this way we obtained almost exclusively the S,N-shifted N-Ras peptide **17**. By decreasing the polarity of the solvent with different mixtures of CH_2Cl_2/DMF, it was possible to reduce the percentage of shifted peptide to 15% when a 7:1 mixture was used (entry 3, Table 1). The change of the base from DIPEA to TMP did not improve the results (entry 4, Table 1).

Furthermore, the coupling reagent also influenced the extent of the undesired S,N-shift. Thus, the best results were obtained using HATU (5 equiv) as a coupling reagent in CH_2Cl_2/DMF (7:1) (entries 5–7, Table 1). The addition of larger amounts of HATU (10 equiv) did not improve the result (entry 6, Table 1). However, the addition of a fourfold excess of base relative to the reagents gave the best results; less than 5% of the undesired S,N-shifted peptide **17** was formed (entry 7, Table 1). Taking these criteria into consideration, we obtained the desired N-Ras peptide **15** in 98% yield by using HATU (5 equiv) and DIPEA (20 equiv) (Table 1). The development of the coupling conditions initially was performed by using peptide **15** in which the amino acid sequence of the N-Ras C-terminus was incorporated. In subsequent experiments these results were confirmed in the synthesis of peptide **16**, which corresponds to the C-terminus of H-Ras (see below). Notably, in this case the bulky *O*-trityl-protected Fmoc-serine was attached to the N-deprotected S-palmitoylated cysteine. This demonstrates that the results obtained for the N-Ras sequence in which a glycine



Scheme 2. Synthesis of N- and H-Ras peptides **11** and **12** using the prelipidated cysteines. a) 1. 20% piperidine/DMF, 2. Fmoc-Cys(Far)-OH (4 equiv), HBTU/HOBt/TMP (4 equiv), CH_2Cl_2/DMF (1:1), 4 h; b) standard solid-phase synthesis: 1. 20% piperidine/DMF, 2. Fmoc-AA-OH (5 equiv), HBTU/HOBt (5 equiv)/DIPEA (10 equiv), DMF, 2 h; c) 1. 20% piperidine/DMF, 2. Fmoc-Cys(Pal)-OH (4 equiv), HBTU/HOBt/TMP (4 equiv), CH_2Cl_2/DMF (1:1), 4 h; d) $Cu(OAc)_2$ (0.5 equiv), pyridine (30 equiv), acetic acid (50 equiv), methanol (215 equiv), CH_2Cl_2, oxygen, 3 h. HBTU: *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, HOBt: 1-hydroxybenzotriazole, TMP: trimethylpyridine.

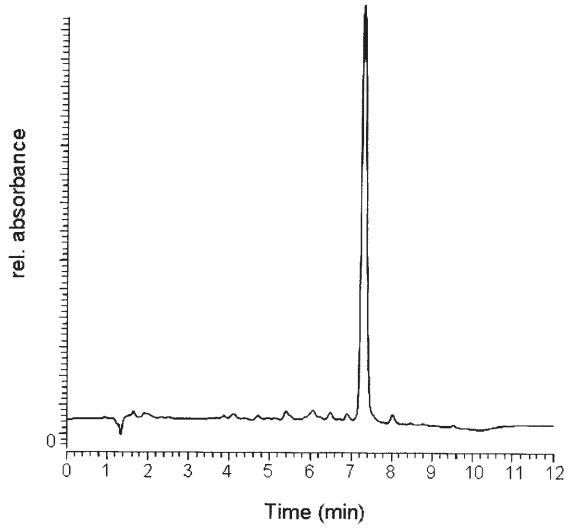
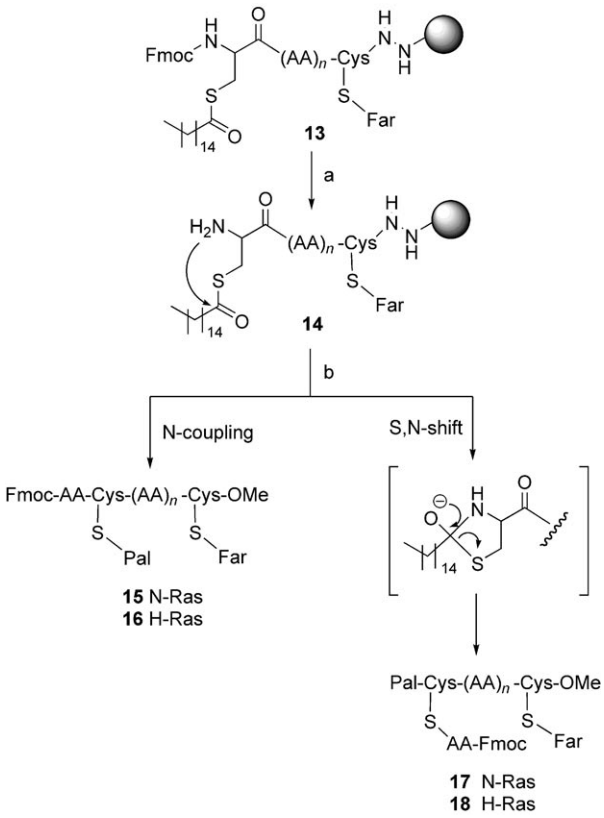


Figure 3. RP-HPLC trace of Fmoc-Cys(Pal)-Lys(Aloc)-Cys(Far)-OMe, **11**.



Scheme 3. Elongation of the peptide chain after incorporation of the palmitoylated cysteine. a) 1% DBU/DMF; b) coupling of Fmoc-AA-OH.

is introduced, are also valid for elongation of the peptide chain with a sterically demanding bulky amino acid.

N-coupled peptides, **15** and **16**, and S,N-shifted analogues, **17** and **18** could be clearly distinguished in RP-HPLC (Figure 4). These peptides were also identified by means of their ^{1}H NMR spectra, since the CH_2 protons neighboring the carbonyl group of the palmitoyl moiety display different chemical shifts for the N-coupled (ca. 2.50 ppm) and the S,N-shifted (ca. 2.15 ppm) peptides.

Applicability of the method to the synthesis of long lipidated peptides: Several farnesylated and palmitoylated peptides (**15**–**21**) were synthesized to demonstrate the efficiency of the method.(Figure 5) In all cases, pre-lipidated building blocks were used for the solid-phase synthesis. After incorporation of the palmitoylated cysteine, 1% DBU in DMF was used for the Fmoc deprotection and the first coupling was performed under the optimized conditions described above (entry 7, Table 1) to avoid the S,N-shift of the palmitoyl group. Standard conditions (HBTU/HOBt/DIPEA in DMF) were used for the following couplings. It is particularly remarkable that with the new approach it was possible to obtain C-terminal N-Ras decapeptide **19** and tetradecapeptide **20**. Such long farnesylated and palmitoylated peptides were not accessible with the previously reported solid-phase method.[7] The superiority of the new method is also demon-

Table 1. Study of conditions for minimizing S,N-acyl shift in the coupling of the next amino acid after palmitoylated cysteine in the synthesis of N-Ras peptide **15**.[a]

Entry	Coupling method	Base	Solvent [CH_2Cl_2/DMF]	Yield **15** [%]	Yield **17** [%]
1	AA(10 equiv), HBTU (10 equiv), HOBt (10 equiv)	DIPEA(20 equiv)	0:1	<5	>95
2	AA(10 equiv), HBTU (10 equiv), HOBt (10 equiv)	DIPEA (20 equiv)	1:1	55	45
3	AA(10 equiv), HBTU (10 equiv), HOBt (10 equiv)	DIPEA (20 equiv)	7:1	85	15
4	AA(10 equiv), HBTU (10 equiv), HOBt (10 equiv)	TMP (10 equiv)	7:1	75	25
5	AA(5 equiv), HATU (5 equiv)	DIPEA (10 equiv)	7:1	94	6
6	AA(10 equiv), HATU (10 equiv)	DIPEA (20 equiv)	7:1	95	5
7	AA(5 equiv), HATU (5 equiv)	DIPEA (20 equiv)	7:1	98	2

[a] Abbreviations: AA: amino acid, that is Gly in the synthesis of the N-Ras sequence and Ser (Trt) for the H-Ras peptide, TMP: trimethylpyridine.

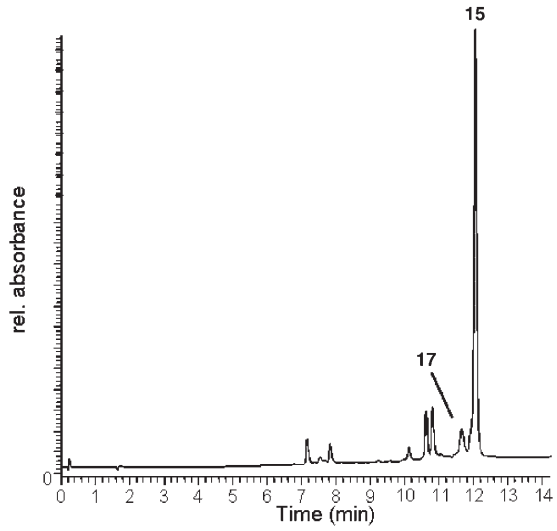


Figure 4. RP-HPLC trace of mixture **15**/**17** (95:5).

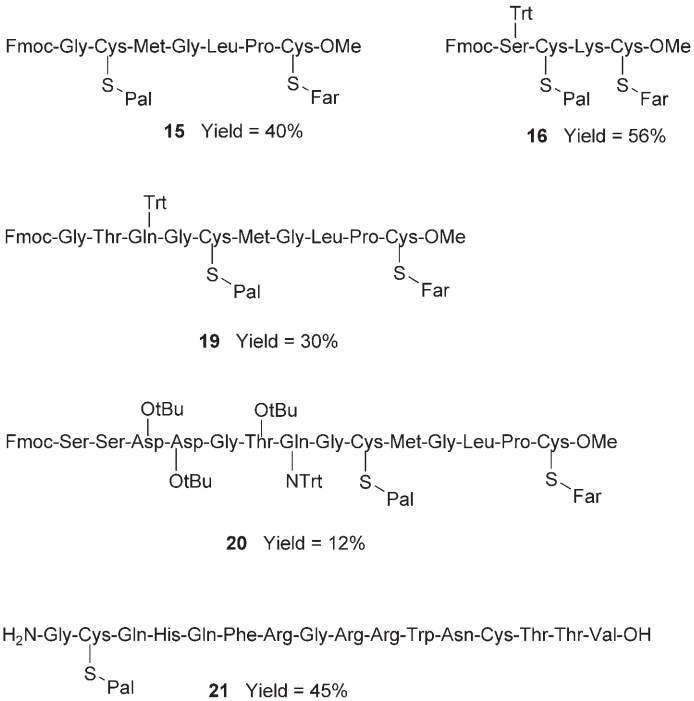


Figure 5. Longer lipidated peptides synthesized by applying the optimized conditions.

strated by means of the synthesis of the palmitoylated hexadecapeptide **21**, which represents a lipidated part of the Wnt protein, a signaling protein involved in numerous events in animal development, such as proliferation and differentiation in several tissues and structures during embryogenesis.[15]

Analysis of the biological activity of Wnt peptide 21: The Wnt proteins are a family of lipid-modified extracellular proteins,[16] which are highly conserved in vertebrates and non-vertebrates. The extracellular Wnt proteins activate the Wnt pathway, which has an impact on cell proliferation, cell transformation, and cell differentiation.[17] The lipid modification is essential for the biological activity of the Wnt protein. The peptide **21** corresponds to the highly conserved sequence of the Wnt proteins. Given this sequence conservation it might be possible that the lipidated core peptide motif is sufficient to activate the downstream signaling pathway. To investigate this possibility, we measured the ability of this peptide to activate the Wnt pathway. The relative level of the intracellular proto-oncoprotein β-catenin served as a parameter for the activity of the Wnt pathway. L cells, which were incubated with a conditioned medium from L Wnt3a cells, served as the positive control. The conditioned medium activated the Wnt pathway in the L cells, as shown by the increase of the relative amount of β-catenin (Figure 6). The β-catenin level did not increase after incubation with the peptide **21**. There was no detectable β-catenin in L cells incubated with the peptide up to a concentration of 5 μM, which is approximately 1000 times higher than the minimal concentration, in which the native protein showed activity.[15a] The lack of activity of the peptide was not due to the solvent. DMSO showed no negative or positive influence on the β-catenin level no matter whether the Wnt3a protein was present or not. Thus, we conclude that the conserved lipidated sequence incorporated into the peptide **21** is not sufficient to activate the Wnt pathway in L cells.

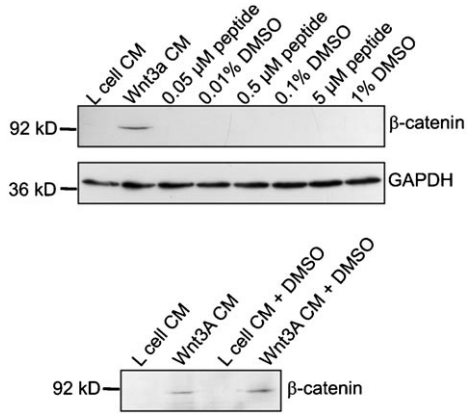


Figure 6. Analysis of the biological activity of peptide **21**. The sizes of marker fragments are indicated on the left in kilodaltons (kDa). Top: Western blot analysis of β-catenin or of GAPD in total lysates of L cells after incubation with conditioned media from L cells (L cell CM), conditioned medium from L Wnt3a cells (Wnt3a CM), peptide **21** or DMSO at the indicated concentrations. Bottom: Control experiment to analyze the influence of the solvent DMSO on the β-catenin level in activated and non-activated L cells. The β-catenin level was analyzed by Western blot in total lysates of L cells after incubation with L cell CM or with Wnt3a CM in the presence or absence of 1% DMSO.

Introduction of fluorescent labels and/or reporter and linking groups into the Ras peptides for further biological investigations: Once the efficiency of the new methodology in the synthesis of long doubly lipidated peptides was demonstrated, the scope of the new approach was explored. For this purpose labeled peptides bearing reporter and/or linking groups required for further biological investigations were synthesized. To demonstrate the versatility of the new method the labels were placed in different positions on the lipidated peptide: 1) within the lipid residue, 2) in the amino acid side chain, 3) at the C-terminus, and 4) at the N-terminus.

The incorporation of a maleimidocaproyl group (MIC) at the N-terminus of the peptide allows the ligation to proteins through conjugate addition of cysteine thiol groups.[5a,b] We have synthesized several N-Ras peptides carrying the maleimido group with different combinations of lipid patterns (**22**–**24**), such as the farnesylated and palmitoylated peptide **22** or the double farnesylated compound **24** (Figure 7a). Ras proteins bearing a photoactivatable benzophenone group are efficient tools to study the interaction and cross-linking with possible Ras-binding proteins.[18] The interest in such proteins led us to synthesize lipidated peptides **25** and **26** carrying the benzophenone group (Figure 7b). This reporter group was placed in the side chain of the lysine using the previously synthesized building block **10** (Scheme 1). The introduction of the biotin marker should facilitate both the detection as well as the purification of the cross-linked products.

Furthermore, we synthesized a wide range of N- and H-Ras peptides fluorescently labeled with, for example, the NBD group (Table 2). Several of these peptides bear the NBD marker at the N-terminus, whereas in other cases an Fmoc-Lys(NBD)-OH **8** building block was used for the synthesis. Most of these peptides also incorporate the MIC group which can be employed for ligation to proteins.[5a–b] The presence of an N-terminal cysteine in **27** and **32** also permits the coupling to proteins by way of expressed protein ligation.[19] Moreover, the peptide **35** with the D-configuration was synthesized for further stereochemical studies.

Finally, Mant and Bodipy-FL (4,4-difluoro-4-borata-3a-azonia-4a-aza-*s*-indacene)[20] were also used as fluorescent labels (Table 2). Peptide **37** incorporates the GerMant-analogue of the farnesyl group. The introduction of the fluorescent label into the lipid moiety leaves the C-terminus free for possible ligation to proteins.

We did not synthesize a previously labeled lysine building block for the synthesis of the peptide **38**, since Bodipy-FL is not stable under typical Fmoc deprotection conditions (20% piperidine). Fmoc-Lys(Aloc)-OH was used instead as a building block for the peptide synthesis. After coupling of Fmoc-Gly-OH, the Aloc group was removed, and the Bodipy-FL was coupled to the lysine on the resin. Then, the Fmoc group of Gly was cleaved with 1% DBU in DMF, and the MIC group was attached to the N-terminus.

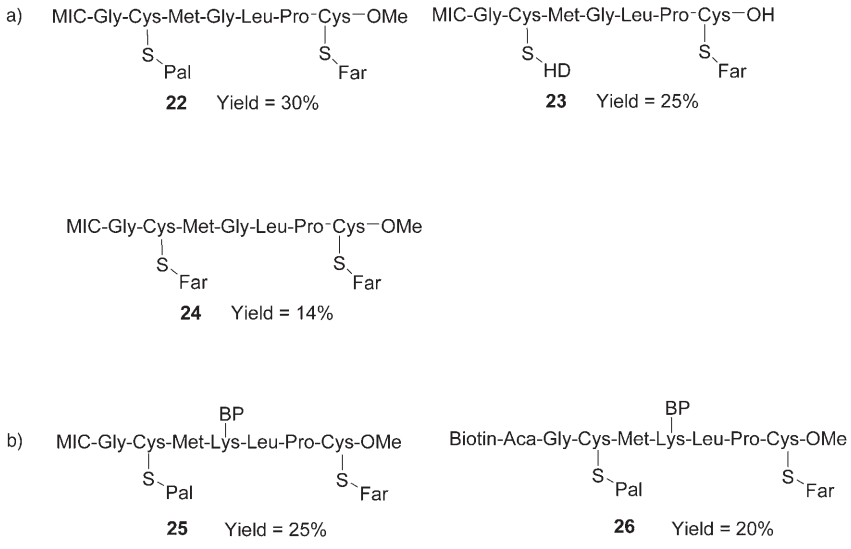
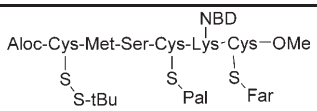
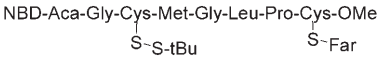
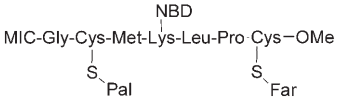
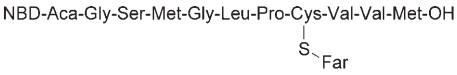
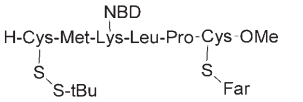
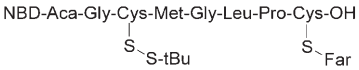
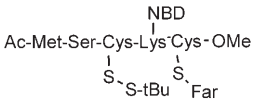
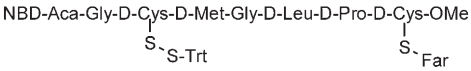
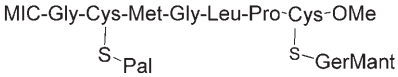
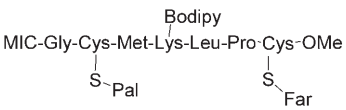


Figure 7. Peptides incorporating reporter and linking groups.

Table 2. Results of the syntheses of fluorescent labeled peptides by means of the new method.

Entry	Compound	Peptide	Yield [%]
1	**27**		28
2	**28**		22
3	**29**		28
4	**30**		32
5	**31**		25
6	**32**		38
7	**33**		37
8	**34**		12
9	**35**		40
10	**36**		22
11	**37**		31
12	**38**		30

Conclusion

We have developed a flexible and efficient methodology for the solid-phase synthesis of lipidated peptides based on the use of pre-synthesized building blocks. This approach overcomes the limitations of the previously reported method, since it gives access to long doubly lipidated peptides and furthermore it meets the demands for a widely applicable potentially automatable methodology. Its efficiency is demonstrated by the synthesis of a series of N- and H-Ras peptides carrying not only different combinations of lipid patterns, but also a wide range of reporter and/or linking groups needed for further biological, biophysical, and biochemical investigations. In terms of efficiency and flexibility this solid-phase method is superior to the solution-phase synthesis. It gives pure peptides in multimilligram amounts within much shorter time and with superior overall yield.

Experimental Section

^{1}H and ^{13}C NMR data were recorded on a 400 MHz spectrometer. Electrospray mass spectrometry (ESI-MS) was performed in the positive mode on an Agilent 1100 series instrument. Analytical reverse-phase HPLC separation was performed by using a C_4 column with a flow rate of 1 mL min^{-1}. A solvent system was used that consisted of a isocratic elution with 80% acetonitrile/water, 0.1% formic acid for 1 min followed by a linear gradient to 100% acetonitrile for 7 min and then again isocratic elution with 100% acetonitrile for 4 min. FAB measurements were taken with a Jeol SX 102 A apparatus by using a 3-nitrobenzyl alcohol (3-NBA) matrix. Optical rotations were measured with a Perkin-Elmer 241 Polarimeter. Melting points were measured with a Büchi B-540 apparatus. MALDI MS spectra was recorded by using 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxy cinnamic acid (CHCA) as matrix.

General procedure for the synthesis of lipidated and labeled peptides: Commercially available Fmoc-4-hydrazinobenzoyl NovaGel resin was used for all solid-phase reactions. The 50-mL reactor flask had a frit at the bottom, a stopcock, which permitted rapid filtration and washing of the resin, and a side arm, which allowed the supply of nitrogen or argon. The yield and scale of the solid-phase reactions are given with respect to the amount of the first amino acid coupled onto the resin. The resin loading was determined by measuring the Fmoc groups remaining on the resin by the established UV method. To this end, a small amount of resin (~3 mg) was treated with 20% piperidine/DMF solution (7.5 mL) for 30 min, and the UV absorption of the solution at 301 nm ($\varepsilon = 7800\,\text{M}^{-1}\,\text{cm}^{-1}$) was determined.

All amino acids and NBD-labeled Lys or benzophenone-labeled Lys were coupled by using HBTU/HOBt chemistry. Typically, the amino acid (AA) (5 equiv) was treated for 2 min with HBTU (5 equiv), HOBt (5 equiv), and DIPEA (10 equiv) in DMF. The solution was added to the resin, which was then agitated for 2 h at room temperature. The resin was washed after coupling and Fmoc-deprotection with DMF (5×2 min). Every step was carried out under an argon atmosphere except the washings. The trityl group was cleaved by shaking the resin-bound peptides in a solution of 1% TFA and 2% triethylsilane in dichloromethane (2.5 mL/0.1 g of resin) for 2 h.

Cysteine building blocks were coupled by using HBTU/HOBt/TMP in CH_2Cl_2/DMF (1:1). After optimization of the coupling times for the lipidated building blocks, the following conditions were used: Fmoc-Cys-(Far)-OH was coupled by using four equivalents for 5 h, Fmoc-Cys(Pal)-OH using four equivalents overnight, and Fmoc-Cys(GerMant)-OH using 1.5 equivalents for 24 h. Standard Fmoc chemistry and piperidine deprotection (20% in DMF 4×2 min) were used until palmitoylated Cys was incorporated in the peptide. After incorporation of Fmoc-Cys(Pal)-OH, 1% DBU/DMF (1 mL/0.1 g of resin, 2×30 s) was used for Fmoc de-

protection. To avoid an S,N-shift, HATU in CH_2Cl_2/DMF (7:1) was used for the subsequent coupling of amino acids.

Oxidative cleavage of peptides from the solid support: The resin was treated with a solution of $Cu(OAc)_2$ (0.5 equiv with respect to the initial resin loading), pyridine (30 equiv), acetic acid (50 equiv), and nucleophile (215 equiv)—methanol in dichloromethane or water in THF (5 mL/0.1 g of resin)—for 3 h under an oxygen atmosphere. The resin was filtered, and the solvent was evaporated under reduced pressure. The crude residue was washed three times with toluene and after each washing it was evaporated to dryness. The oily residue was purified by using a short silica gel column (2 or 5 mL) with ethyl acetate and methanol (0–5% depending on the peptide) as the eluent.

Fmoc-Cys(Pal)-OH (2): Trifluoroacetic acid (2.5 mL) and triethylsilane (1.5 mL) were added to a solution of Fmoc-Cys(Trt)-OH (**1**) (2 g, 3.4 mmol) in CH_2Cl_2 (50 mL), and the mixture was stirred at room temperature for 2 h under an argon atmosphere. The solvent was evaporated under reduced pressure, and the crude residue was washed three times with toluene and after each washing it was evaporated to dryness. The solid residue was transferred to a sinter funnel and washed twice with pentane to remove the released triphenylmethane. The remaining solid (1.2 g) was dissolved in CH_2Cl_2 (25 mL), trimethylsilyl chloride (0.48 mL, 3.7 mmol) was added, and the reaction mixture was heated to reflux for 2 h. After the mixture was cooled to room temperature, palmitoyl chloride (3.1 mL, 10.2 mmol) was added, followed by dropwise addition of a solution of triethylamine (0.78 mL, 5.6 mmol) in CH_2Cl_2 (15 mL) over 3 h under an argon atmosphere. After the mixture had been stirred for one hour, the solvent was evaporated, and the crude mixture was purified by flash chromatography on silica gel using a gradient of 0–20% ethyl acetate in cyclohexane. The eluent furnished a white solid (1.6 g, 82%). R_f= 0.6 (ethyl acetate/cyclohexane (2:1), 1% acetic acid). m.p. 85–86°C. $[\alpha]_D^{20}=-7.2$ ($c=1.69$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.76$ (d, $J=7.5$ Hz, 2H), 7.62–7.55 (m, 2H), 7.40 (t, $J=7.5$ Hz, 2H), 7.29 (t, $J=$ 7.5 Hz, 2H), 5.64 (d, $J=7.8$ Hz, 1H), 4.62 (m, 1H), 4.39 (d, $J=7.2$ Hz, 2H), 4.24 (t, $J=7.2$ Hz, 1H), 3.50–3.34 (m, 2H), 2.59 (d, $J=7.6$ Hz, 2H), 1.70–1.60 (m, 2H), 1.23 (s, 24H), 0.89 ppm (t, $J=6.9$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=199.5$, 174.5, 156.2, 143.8, 141.4, 127.9, 127.3, 125.4, 120.2, 67.8, 62.8, 54.3, 47.4, 44.5, 32.4, 30.8, 30.12, 30.11, 30.10, 30.08, 30.03, 29.9, 29.8, 29.7, 29.4, 26.0, 23.1, 14.6 ppm; MS (FAB, 3-NBA): calcd for $C_{34}H_{47}NO_5S$ $[M+Na]^+$ 604.3073, found 604.3085.

Fmoc-Cys(Far)-OH (4): A solution of cysteine hydrochloride monohydrate (**3**) (1.0 g, 5.7 mmol) in MeOH (11 mL) was cooled to 0°C, and a 7N solution of ammonia in MeOH (15 mL) was slowly added. After 5 min farnesyl chloride (1.38 mL, 5.7 mmol) was added. The reaction mixture was stirred at 0°C for 3 h and then at room temperature for 1 h, the solvent was evaporated, the solid residue was washed with pentane (3×10 mL), and CH_2Cl_2 (50 mL) was added. The resulting suspension was cooled to 0°C and triethylamine (0.88 mL, 6.28 mmol) and Fmoc-succinimide (2.12 g, 6.28 mmol) were added. The reaction mixture was stirred overnight at room temperature and concentrated in vacuum. The product was obtained by flash chromatography on silica gel using a gradient of 0–4% methanol in dicholoromethane as the eluent to give a pale yellow oil that can be triturated with pentane to yield a sticky solid (2.3 g, 65%). $R_f=0.7$ (dichloromethane/methanol (9:1)). $[\alpha]_D^{20}=-4.3$ ($c=$ 0.94 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.76$ (d, $J=7.4$ Hz, 2H), 7.61 (d, $J=7.5$ Hz, 2H), 7.40 (t, $J=7.4$ Hz, 2H), 7.31 (t, $J=7.4$ Hz, 2H), 5.60 (d, $J=8.0$ Hz, 1H), 5.22 (t, $J=7.4$ Hz, 1H), 5.12–5.06 (m, 2H), 4.63 (m, 1H), 4.47–4.39 (m, 2H), 4.24 (t, $J=7.0$ Hz, 1H), 3.27–3.15 (m, 2H), 3.04–2.90 (m, 2H), 2.14–1.93 (m, 8H), 1.68 (s, 3H), 1.65 (s, 3H), 1.59 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=175.7$, 156.1, 143.8, 141.4, 140.5, 135.6, 131.5, 127.9, 127.3, 125.3, 124.5, 123.9, 120.2, 119.6, 67.7, 53.9, 47.5, 40.1, 40.0, 33.5, 30.5, 27.2, 26.9, 26.2, 18.2, 16.6, 16.5 pm; MS (FAB, 3-NBA): calcd for $C_{33}H_{41}NO_4S$ $[M+Na]^+$ 570.2654, found 570.2760.

Fmoc-D-Cys(Far)-OH: The synthesis was performed as described for the L-enantiomer. $[\alpha]_D^{20}=+$ 4.2 ($c=0.94$ in $CHCl_3$).

Fmoc-Cys(HD)-OH (5): Trifluoroacetic acid (1 mL) and triethlysilane (0.6 mL) were added to a solution of Fmoc-Cys(Trt)-OH (**1**) (0.5 g, 3.4 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred at room temperature for 2 h under an argon atmosphere. The solvent was evaporated under reduced pressure and the crude product was washed three times with toluene and after each washing it was evaporated to dryness. The solid residue was transferred to a sinter funnel and washed twice with pentane to remove the released triphenylmethane. The remaining solid (0.3 g) was dissolved in DMF and cooled to 0°C, then triethylamine (5 equiv) and a solution of hexadecyl iodide (3 equiv) in DMF was added. The mixture was stirred at 0°C and then at room temperature overnight. The solvent was evaporated and the crude mixture was purified by flash chromatography on silica gel using a gradient of 0–20% ethyl acetate in cyclohexane as the eluent. The eluent furnished a white solid (0.14 g, 30%). $[\alpha]_D^{20}=-5$ ($c=1.00$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.77$ (d, $J=7.4$ Hz, 2H), 7.61 (m, 2H), 7.41 (t, $J=7.4$ Hz, 2H), 7.32 (t, $J=7.4$ Hz, 2H), 4.65 (m, 1H, Fmoc), 4.42 (m, 2H), 4.25 (t, $J=7.0$ Hz, 1H), 3.05 (m, 2H), 2.56 (m, 2H), 1.57 (t, $J=7.2$ Hz, 2H), 1.26 (m, 28H), 0.89 ppm (t, $J=7.0$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=$ 174.9, 156.6, 143.7, 141.3, 127.7, 127.1, 125.1, 120.0, 67.4, 53.6, 47.1, 34.2, 32.9, 31.9, 29.7, 29.4, 29.2, 28.8, 22.7, 14.1 ppm; (FAB, 3-NBA): calcd for $C_{34}H_{49}NO_4S$ $[M+Na]^+$ 590.3280, found 590.3300.

Fmoc-Cys(GerMant)-OH (6): A solution of cysteine hydrochloride monohydrate (0.39 g, 2.25 mmol) in MeOH (8 mL) was cooled to 0°C, and a 7N solution of ammonia in MeOH (10 mL) was added slowly. After 5 min, GerMant-chloride[12] ((*E*,*E*)-8-*O*-(2-*N*-methyl-aminobenzoyl)-3,7-dimethyl-2,6-octandiene-1-chloride) (0.47 g, 1.5 mmol) was added and the mixture was stirred at 0°C for 3 h and then at room temperature for 1 h. The solvent was evaporated, the solid residue was washed with pentane (3×5 mL), and CH_2Cl_2 (20 mL) was added. The resulting suspension was cooled to 0°C and triethylamine (0.31 mL, 2.25 mmol) and Fmoc-succinimide (0.76 g, 2.25 mmol) were added. The reaction mixture was stirred overnight at room temperature and then concentrated in vacuum. The oily residue was purified by flash chromatography on silica gel using a gradient of 0–4% methanol in dicholoromethane as eluent to afford a colorless oil (0.53 g, 57%). $R_f=0.7$ (dichloromethane/methanol 9:1). $[\alpha]_D^{20}=-1.0$ ($c=0.93$ in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.93$ (d, $J=8.0$ Hz, 1H), 7.75 (d, $J=7.5$ Hz, 2H), 7.62–7.58 (m, 2H), 7.42–7.34 (m, 3H), 7.28 (t, $J=7.5$ Hz, 2H), 6.66 (d, $J=8.0$ Hz, 1H), 6.59 (t, $J=8.0$ Hz, 1H), 5.66 (d, $J=6.9$ Hz, 1H), 5.46 (t, $J=6.9$ Hz, 1H), 5.21 (t, $J=7.5$ Hz, 1H), 4.63–4.58 (m, 3H, distinguished singlet at 4.63 ppm), 4.45–4.38 (m, 2H), 4.22 (t, $J=7.0$ Hz, 1H), 3.25–3.13 (m, 2H), 3.04–2.88 (m, 5H, distinguished singlet at 2.89 ppm), 2.20–2.15 (m, 2H), 2.10–2.05 (m, 2H), 1.71 (s, 3H), 1.65 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=175.1$, 168.6, 156.1, 152.2, 143.8, 141.4, 139.7, 134.8, 131.8, 130.8, 128.6, 127.9, 127.3, 125.3, 120.3, 120.2, 114.6, 111.1, 110.2, 70.1, 67.7, 53.8, 47.5, 39.3, 33.6, 30.4, 30.0, 26.4, 16.6, 14.6 ppm; MS (FAB, 3-NBA): calcd for $C_{36}H_{40}N_2O_6S$ $[M+H]^+$ 629.2685, found 629.2653, $[M+Na]^+$ 651.2505, found 651.2496.

Fmoc-Lys(BP)-OH (10): Fmoc-Lys-OH (0.40 g, 1.10 mmol) was added was added to a solution of 4-benzoylbenzoic acid (0.25 g, 1.10 mmol), HATU (0.42 g, 1.10 mmol), and DIPEA (0.20 mL, 2.20 mmol) in DMF (20 mL). The solution was stirred at room temperature for 5 h. After evaporation of the solvent, the residue was dissolved in AcOEt and washed with H_2O, dried over $MgSO_4$, and concentrated in vacuum to afford a white solid (0.48 g, 75%). $[\alpha]_D^{20}=-$ 2.1 ($c=0.92$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta=7.85$ (d, $J=8.4$ Hz, 2H), 7.74–7.69 (m, 6H), 7.57–7.52 (m, 3H), 7.44 (t, $J=7.4$ Hz, 2H), 7.34 (t, $J=7.4$ Hz, 2H), 7.24 (t, $J=7.4$ Hz, 2H), 4.40–4.30 (m, 2H), 44.17–4.09 (m, 2H), 3.46 (m, 2H), 1.95–1.47 ppm (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=196.5$, 167.6, 156.6, 150.5, 143.8, 141.5, 140.3, 137.1, 133.2, 130.3, 128.7, 127.3, 120.2, 67.3, 60.7, 47.3, 40.1, 32.0, 29.0, 22.6, 14.2 ppm; MS (FAB, 3-NBA): calcd for $C_{35}H_{33}N_2O_6S$ $[M+H]^+$ 577.2260, found 577.2365.

Fmoc-Cys(Pal)-Lys(Aloc)-Cys(Far)-OMe (11): Yield: 0.051 mmol; 69%. $[\alpha]_D^{20}=-21.2$ ($c=0.20$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.68$ (d, $J=7.6$ Hz, 2H, Fmoc), 7.52 (d, $J=9.6$ Hz, 2H; Fmoc), 7.32 (t, $J=$ 7.4 Hz, 2H, Fmoc), 7.23 (t, $J=7.4$ Hz, 2H; Fmoc), 6.94 (s, 1H; NH), 6.82 (s, 1H), 5.83–5.75 (m, 2H), 5.19 (d, $J=18$ Hz, 1H), 5.15–4.97 (m, 5H), 4.66 (m, 1H), 4.50–4.24 (m, 6H, Fmoc, Aloc), 4.14 (m, 1H), 3.67 (s, 3H, OMe), 3.30–2.75 (m, 8H), 2.51 (t, $J=7.4$ Hz, 2H, Pal), 2.1–1.81 (m, 8H, Far), 1.68–1.59 (m, 2H, Pal), 1.60 (s, 3H, Far), 1.58 (s, 3H; CH_3 Far), 1.52

(s, 6H, Far), 1.46–1.43 (m, 2H), 1.36–1.33 (m, 2H), 1.18 (s, 24H, Pal), 0.81 ppm (t, $J=7.0$ Hz, 3H, Pal); MS (ESI +): calcd for $C_{63}H_{94}N_4O_9S_2$ $[M+H]^+$: 1115.7; found: 1115.3.

Fmoc-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe (12): Yield: 0.044 mmol; 60%. $[\alpha]_D^{20}=-17.0$ ($c=0.40$ in CH_2Cl_2). ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=7.69$ (d, $J=7.6$ Hz, 2H, Fmoc), 7.53 (m, 2H, Fmoc), 7.33 (t, $J=7.4$ Hz, 2H, Fmoc), 7.24 (t, $J=7.2$ Hz, 2H, Fmoc), 5.10 (t, $J=$ 7.2 Hz, 1H, Far), 5.01 (m, 2H, Far), 4.70 (m, 1H), 4.49 (m, 1H), 4.30 (m, 2H), 4.15 m (1H), 3,65–3.48 (m, 5H, distinguished singlet at 3.60, OMe), 2.51 (t, $J=7.3$ Hz, 2H, Pal), 2.15–1.81 (m, 17H), 1.65–1.40 (m, 14H, distinguished singlets at 1.60 and 1.52, Far), 1.35–1.17 (m, 30H), 0.92–0.85 (m, 4H), 0.80 ppm (t, $J=7.0$ Hz, 6H, Leu, Pal); MS (ESI): calcd for $C_{71}H_{108}N_6O_{10}S_3$ $[M+H]^+$ 1301.7; found 1301.4.

Fmoc-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe (15): Yield: 0.031 mmol; 40%. $[\alpha]_D^{20}=-27.0$ ($c=0.22$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.74$ (d, $J=7.3$ Hz, 2H, Fmoc), 7.60 (m, 2H, Fmoc), 7.37 (t, $J=7.3$ Hz, 2H, Fmoc), 7,28 (t, $J=7.3$ Hz, 2H, Fmoc), 5.13 (t, $J=7.2$ Hz, 1H, Far), 5.07 (m, 2H, Far), 4.82 (m, 1H), 4.65 (m, 2H), 4.51 (m, 1H), 4.33 (t, $J=7.0$ Hz, 1H), 4.20 (t, $J=7.0$ Hz, 1H), 3.99 (m, 2H), 3.77–3.45 (m, 5H, distinguished singlet at 3.68, OMe), 3.12–3.23 (m, 3H), 2.89–3.04 (m, 2H), 2.65–2.70 (m, 1H), 2.52 (t, $J=7.2$ Hz, 2H, Pal), 2.46 (t, $J=$ 7.2 Hz, 2H), 2.19–1.95 (m, 17H), 1.66–1.53 (m, 17H, distinguished singlets at 1.66, 1.62 and 1.58, Far), 1.35–1.15 (m, 24H), 0.95–0.75 ppm (m, 9H, Leu, Pal); MS (ESI): calcd for $C_{73}H_{111}N_7O_{11}S_3$ $[M+H]^+$ 1358.8; found 1358.6.

Fmoc-Ser(Trt)-Cys(Pal)-Lys(Aloc)-Cys(Far)-OMe (16): Yield: 0.041 mmol; 56%. $[\alpha]_D^{20}=-20.3$ ($c=0.21$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.69$ (d, $J=7.5$ Hz, 2H, Pal), 7.57–7.52 (m, 2H, Pal), 7.37–7.20 (m, 19H, Pal, Trt), 5.87–5.75 (m, 2H), 5.21 (d, $J=17.2$ Hz, 1H, Far) 5.15–4.93 (m, 5H, Far), 4.68–3.92 (m, 10H), 3.78–3.65 (m, 5H, distinguished singlet at 3.68 ppm, OMe), 3.35–2.72 (m, 8H), 2.52 (t, $J=7.6$ Hz, 2H, Pal), 2.05–1.85 (m, 10H), 1.65–1.25 (m, 18H, distinguished singlets at 1.60, 1.59, and 1.52, Far), 1.18 and 1.16 (2×s, 24H), 0.81 ppm (t, $J=$ 6.9 Hz, 3H, Pal); MS (ESI): calcd for $C_{85}H_{113}N_5O_{11}S_2$ $[M+Na]^+$ 1466.8, found 1446.8.

Fmoc-Gly-Thr-Gln(Trt)-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe (19): Yield: 0.022 mmol; 30%. $[\alpha]_D^{20}=+$ 1.3 ($c=0.74$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ(characteristic signals)=7.67 (m, Fmoc), 7.50 (m, Fmoc), 7.33 (m, Fmoc), 7.00–7.22 (m, Fmoc), 5.01 (m, Far), 3.60–3.70 (m, distinguished singlet at 3.65, OMe), 1.42–1.70 (m, distinguished singlets at 1.62 and 1.53, 4 CH_3 Far), 0.90–1,40 (m, 12 CH_2 Pal), 0.75–0.85 ppm (m, CH_3 Pal, 2 CH_3 Leu); MS (ESI): calcd for $C_{103}H_{143}N_{11}O_{16}S_3$ $[M+H]^+$ 1887.0; found 1887.3.

Fmoc-Ser-Ser-Asp(O*t*Bu)-Asp(O*t*Bu)-Gly-Thr(O*t*Bu)-Gln(Trt)-Gly-Cys-(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe (20): Yield: 0.010 mmol; 12%. $[\alpha]_D^{20}=-20.4$ ($c=0.21$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ(characteristic signals)=7.69 (m, Fmoc), 7.54 (m, Fmoc), 7.33 (m, Fmoc), 7.04–7.23 (m, Fmoc, Trt), 5.01 (m, Far), 3.60–3.70 (m, distinguished singlet at 3.66, OMe), 1.42–1.70 (m, distinguished singlets at 1.60 and 1.53, 4 CH_3 Far), 0.90–1,40 (m, 12 CH_2 Pal, *t*Bu), 0.76–0.85 ppm (m, CH_3 Pal, 2 CH_3 Leu); MS (MALDI, DHB): calcd for $C_{129}H_{187}N_{15}O_{26}S_3$ $[M+Na]^+$ 2481.3; found 2481.8; $[M+K]^+$ 2497.3; found 2497.9.

NH_2-Gly-Cys(Pal)-Gln-His-Gln-Phe-Arg-Gly-Arg-Arg-Trp-Asn-Cys-Thr-Thr-Val-OH (21): The Pbf, Boc, Trt, and *t*Bu groups were cleaved from the amino acid residues on resin with 95% TFA and 5% water for 2 h. The resin was treated with a solution of NBS (20 equiv) and pyridine (20 equiv) in CH_2Cl_2 (3 mL) for 5 min, washed with DCM (3×5 mL), and then with THF (3×5 mL). A solution of water (50 equiv) in THF (3 mL) was added and the mixture was stirred for 4 h at room temperature. The resin was filtered off and washed four times with THF and the solvent was evaporated under reduced pressure. Yield: 0.023 mmol; 45%. $[\alpha]_D^{20}=$ +3.9 ($c=0.43$ in MeOH). ^{1}H NMR (400 MHz, CD_3CN): $\delta=$(characteristic signals) 8.77 (m, 2H, NH Trp, NH His), 7.98–7.93 (m, 19H, C=NH Arg, NH), 7.73–7.63 (m, 6H, *m*-Phe, Trp), 7.34–7.27 (m, 3H, *o*-Phe, *p*-Phe), 7.00 (s, 2H, His, Trp), 2.67 (t, $J=7.0$ Hz, 2H, Pal), 1.35–1.29 (m, 28H, Pal), 1.21 (d, $J=4.3$ Hz, 6H, Thr), 0.96–0.89 ppm (m, 9H, Pal, Val); MS (ESI): calcd for $C_{97}H_{155}N_{31}O_{23}S_2$ $[M+2Na]^{2+}$ 1116.1; found 1115.7.

MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe (22): Yield: 0.022 mmol; 30%. $[\alpha]_D^{20}=-22.0$ ($c=0.42$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=6.62$ (s, 2H, MIC), 5.12 (t, $J=7.2$ Hz, 1H, Far), 5.02 (m, 2H, Far), 4.52 (m, 1H) 4.33 (t, $J=7.0$ Hz, 1H), 4.20 (t, $J=7.0$ Hz, 1H), 3.99 (m, 2H), 3.78–3.44 (m, 7H, distinguished singlet at 3.68, OMe), 3.12–3.23 (m, 3H), 2.89–3.04 (m, 2H), 2.65–2.70 (m, 1H), 2.54 (t, $J=7.2$ Hz, 2H, Pal), 2.46 (t, $J=7.2$ Hz, 2H), 2.19–1.95 (m, 19H), 1.66–1.53 (m, 17H, distinguished singlets at 1.66, 1.62 and 1.58, Far), 1.35–1.15 (m, 30H), 0.92–0.79 ppm (m, 9H, Leu, Pal); MS (ESI): calcd for $C_{68}H_{112}N_8O_{12}S_3$ $[M+H]^+$ 1329.8; found 1329.6.

MIC-Gly-Cys(HD)-Met-Gly-Leu-Pro-Cys(Far)-OH (23): Yield: 0.012 mmol; 25%. $[\alpha]_D^{20}=-20.4$ ($c=0.30$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=6.65$ (s, 2H, MIC), 5.10 (t, $J=7.2$ Hz, 1H, Far), 5.00 (m, 2H, Far), 4.50 (m, 1H), 4.30 (t, $J=7.0$ Hz, 1H), 4.20 (t, $J=7.0$ Hz, 1H), 3.99 (m, 2H), 3.78–3.44 (m, 4H), 3.12–3.23 (m, 3H), 2.89–3.04 (m, 2H), 2.65–2.70 (m, 1H), 2.45 (t, $J=7.2$ Hz, 2H), 2.19–1.95 (m, 21H), 1.64–1.53 (m, 17H, distinguished singlets at 1.64, 1.61 and 1.57, Far), 1.30–1.10 (m, 32H), 0.92–0.79 ppm (m, 9H, Leu, HD); MS (ESI): calcd for $C_{67}H_{112}N_8O_{11}S_3$ $[M+H]^+$ 1300.8 found 1300.9.

MIC-Gly-Cys(Far)-Met-Gly-Leu-Pro-Cys(Far)-OMe (24): Yield: 0.048 mmol; 14%. $[\alpha]_D^{20}=-15.5$ ($c=1.28$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=6.62$ (s, 2H, MIC), 5.02–5.12 (m, 6H, Far), 4.7–4.2 (m, 8H), 3.6–3.1 (m, 7H), 2.6–2.15 (m, 10H), 2.1–1.3 (m, 27H), 1.3–0.99 (m, 24H, Far), 0.6–0.95 ppm (6H, Leu); MS (ESI): calcd for $C_{67}H_{106}N_8O_{11}S_3$ $[M+H]^+$ 1295.7; found 1295.7.

MIC-Gly-Cys(Pal)-Met-Lys(BP)-Leu-Pro-Cys(Far)-OMe (25): Yield: 0.018 mmol; 25%. $[\alpha]_D^{20}=-25.0$ (c=0.20 in MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.81$ (d, $J=8.4$ Hz, 2H, BP), 7.74 (d, $J=7.3$ Hz, 2H, BP), 7,65 (d, $J=7.3$ Hz, 2H, BP), 7.55 (t, $J=7.4$ Hz, 1H, BP), 7,46 (2H, $J=$ 8.4 Hz, 2H, BP), 6.60 (s, 2H, MIC), 5.13 (t, $J=7.2$ Hz, 1H, Far), 5.02 (m, 2H, Far), 4.52 (m, 1H), 4.30 (t, $J=7.0$ Hz, 1H), 4.20 (t, $J=7.0$ Hz, 1H), 3.96 (m, 2H), 3.75–3.44 (m, 7H, distinguished singlet at 3.65, OMe), 3.12–3.20 (m, 3H), 2.89–3.04 (m, 4H), 2.65–2.70 (m, 1H), 2.51 (t, $J=$ 7.3 Hz, 2H, Pal), 2.15–1.90 (m, 25H), 1.62–1.47 (m, 17H, distinguished singlets at 1.65, 1.62 and 1.57, Far), 1.35–1.15 (m, 30H), 0.92–0.79 ppm (m, 9H, Leu, Pal); MS(ESI): calcd for $C_{86}H_{129}N_9O_{14}S_3$ $[M+H]^+$ 1608.9; found 1608.6.

Biotin-Aca-Gly-Cys(Pal)-Met-Lys(BP)-Leu-Pro-Cys(Far)-OMe (26): Yield: 0.015 mmol, 20%. $[\alpha]_D^{20}=-24.0$ ($c=0.25$ in MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ(characteristic signals)=7.85 (d, $J=8.2$ Hz, 2H, BP), 7.78 (d, $J=8$,2 Hz, 2H, BP), 7.72 (d, $J=7.0$ Hz, 2H, BP), 7.55 (t, $J=$ 7.4 Hz, 1H, BP), 7.43 (t, $J=7.8$ Hz, 2H, BP), 5.29 (m, 1H), 5.14 (m, 1H, Far), 5.02 (m, 2H, Far), 3.69–3.60 (m, 7H, distinguished singlet at 3.69, OMe), 2.02–1.89 (m, 14H), 1.65–1.45 (m, 12H, distinguished singlets at 1.61 and 1.53, Far), 1.35–1.10 (m, 30H, Pal), 0.83–0.77 ppm (m, 9H, Leu, Pal); MS(ESI): calcd for $C_{92}H_{143}N_{11}O_{14}S_4$ $[M+H]^+$ 1754.0; found 1754.7.

Aloc-Cys(S-*t*Bu)-Met-Ser-Cys(Pal)-Lys(NBD)-Cys(Far)-OMe (27): Yield: 0.020 mmol; 28%. $[\alpha]_D^{20}=-18.4$ ($c=0.30$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ(characteristic signals)=8.46 (m, 1H, NBD), 6.15 (m, 1H, NBD), 5.88 (m, 1H, Aloc), 3.72 (s, 3H, OCH_3), 2.55 (t, $J=$ 7.1 Hz, 2H, Pal), 1.99 (s, 3H), 1.65 (s, 3H, Far), 1.57 (s, 6H, Far), 1.31 (s, 9H, *t*Bu), 1.22 (s, 24H, Pal), 0.85 ppm (t, $J=7.1$ Hz, 3H, Pal); MS (MALDI, DHB): calcd for $C_{69}H_{112}N_{10}O_{14}S_5$ $[M+Na]^+$ 1487.7, found 1490.0.

NBD-Aca-Gly-Cys(SBu^t)-Met-Gly-Leu-Pro-Cys(Far)-OMe (28): Yield: 0.013 mmol, 22%. $[\alpha]_D^{20}=-$ 32.3 ($c=0.6$ in CH_2Cl_2). ^{1}H NMR (400 MHz.$CDCl_3$): $\delta=8.42$ (d, $J=8.2$, 1H, NBD), 6.1 (m,1H, NBD), 5.2–4.95 (m, 3H, Far), 4.78–4.1 (m, 8H), 3.7–3.3 (m, 9H), 3.2–2.5 (m, 6H), 2.31–2.15 (m, 2H), 2.1–1.6 (m, 19H), 1.6–1.3 (m, 2H), 1.3–0.99 (m, 27H), 0.6–0.95 ppm (m, 6H); MS (MALDI): calcd for $C_{58}H_{91}N_{11}O_{12}S_4$ $[M+Na]$ 1285.67 found 1286.28.

MIC-Gly-Cys(Pal)-Met-Lys(NBD)-Leu-Pro-Cys(Far)-OMe (29): Yield: 0.020 mmol; 28%. $[\alpha]_D^{20}=-28.3$ ($c=0.60$ in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.36$ (d, $J=8.4$ Hz, 1H, NBD), 6.62 (s, 2H, MIC), 6.09 (m, 1H), 5.12 (t, $J=7.2$ Hz, 1H, Far), 5.02 (m, 2H, Far), 4.52 (m, 1H) 4.33 (t, $J=7.0$ Hz, 1H), 4.20 (t, $J=7.0$ Hz, 1H), 3.99 (m, 2H), 3.78–3.44 (m, 7H, distinguished singlet at 3.68, OMe), 3.12–3.23 (m, 3H), 2.89–3.04 (m, 4H), 2.65–2.70 (m, 1H), 2.54 (t, $J=7.2$ Hz, 2H, Pal), 2.19–1.95 (m, 25H),

1.66–1.53 (m, 17H, distinguished singlets at 1.66, 1.62 and 1.58, Far), 1.35–1.15 (m, 30H), 0.92–0.79 ppm (m, 9H, Leu, Pal). MS (ESI): calcd for $C_{78}H_{122}N_{12}O_{15}S_3$ $[M+H]^+$ 1563.8; found 1563.6.

NBD-Aca-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe (30): Yield: 0.019 mmol; 32%. $[\alpha]_D^{20}=-18.4$ ($c=0.30$ in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): $\delta=8.1$ (d, $J=8.2$, 1H, NBD), 6.5 (m, 1H, NBD), 5.4–5.2 (m, 3H, Far), 4.8–4.1 (m, 8H), 3.7–3.3 (m, 9H), 3.3–2.7 (m, 6H), 2.6–2.4 (m, 4H), 2.1–1.6 (m, 19H, CH_2), 1.7–13 (m, 16H, Far), 1.3–1.0 (m, 30H), 0.7–0.95 (6H, CH_3 Leu, Pal) ppm. MS (ESI): calcd for $C_{70}H_{113}N_{11}O_{13}S_3$ $[M+H]^+$ 1412.7 found 1412.9.

NBD-Aca-Gly-Ser-Met-Gly-Leu-Pro-Cys(Far)-Val-Val-Met-OH (31): Yield: 0.018 mmol, 25%. $[\alpha]_D^{20}=-27.3$ ($c=0.36$ in CH_2Cl_2). ^1H NMR (400 MHz.$CDCl_3$): $\delta=8.42$ (m, 1H, NBD), 6.9 (m,1H, NBD), 5.3 (m, 3H, Far), 4.0–3.2 (m, 20H), 3.0–2.5 (m, 8H), 2.4–2.2 (m, 4H), 2.3–1.5 (m, 23H), 1.5–1.0 (m,18H), 0.5–0.96 ppm (m, 18H); MS (ESI): calcd for $C_{68}H_{108}N_{14}O_{16}S_3$ $[M+H]^+$ 1473.7, found 1473.3.

H-Cys(S-*t*Bu)-Met-Lys(NBD)-Leu-Pro-Cys(Far)-OMe (32): (0.028 mmol,38%). $[\alpha]_D^{20}=-$ 15.4 ($c=0.40$ in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): $\delta=8.40$ (d, $J=8.4$ Hz, 1H, NBD), 6.09 (m, 1H), 5.13 (m, 1H, Far), 5.01 (m, 2H, Far), 4.55–4.65 (m, 2H), 3.66–3.72 (m, 5H, OMe), 3.41–3.53 (m, 2H), 2.95–3.15 (m, 2H), 1.90–2.05 (m, 17H), 1.53–1.61 (m, 21H), 1.19 (s, 9H, Leu), 0.81–0.87 ppm (m, 6H); MS (ESI): calcd for $C_{54}H_{86}N_{10}O_{10}S_4$ $[M+H]^+$ 1163.5; found 1163.4.

NBD-Aca-Gly-Cys(S-*t*Bu)-Met-Gly-Leu-Pro-Cys(Far)-OH (33): Yield: 0.011 mmol, 37%. $[\alpha]_D^{20}=-6.2$ ($c=0.93$ in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): $\delta=8.2$ (d, $J=8.3$, 1H, NBD), 6.08 (m, 1H, NBD), 5.4–5.2 (m, 3H, Far), 4.7–4.1 (m, 8H), 3.7–3.38 (m, 6H), 3.1–2.5 (m, 6H), 2.15 (m, 2H), 2.00–1.8 (m, 19H), 1.8–1.4 (m, 2H), 1.3–1.0 (m, 27H), 1.0–0.7 ppm (m, 6H, Leu); MS-MALDI (DBH): calcd for $C_{57}H_{89}N_{11}O_{12}S_4$ $[M+Na]$ 1271.7; found 1271.9.

Ac-Met-Ser-Cys(SBut)-Lys(NBD)-Cys(Far)-OMe (34): Acetylation of N-terminus: after Fmoc-cleavage from the Met residue, the N-terminus was acetylated using a cocktail of five equivalents of acetic acid/HBTU/HOBt/DIPEA in CH_2Cl_2/DMF (1:1) for 4 h. Yield: 0.009 mmol; 12%. $[\alpha]_D^{20}=95.2$ ($c=0.70$ in CH_2Cl_2). ^1H NMR (400 MHz.$CDCl_3$): $\delta=8.42$ (m,1H, NBD), 6.10 (m, 1H, NBD), 5.30 (m,1H, Far), 5.00 (m, 2H, Far), 4.78–4.10 (m, 4H), 3.70–3.00 (m, 10H, OMe), 2.80 (m, 2H), 3.21–2.50 (m, 6H), 2.10–1.81 (m, 15H), 1.80–1.20 (m, 6H) 1.20–1.02 ppm (m, 21H, Far); MS-(ESI): calcd for $C_{48}H_{75}N_9O_{11}S_4$ $[M+H]^+$ 1083.43; found 1083.50.

NBD-Aca-Gly-D-Cys(Trt)-D-Met-Gly-D-Leu-D-Pro-D-Cys(Far)-OMe (35): Yield: 0.035 mmol; 40%. $[\alpha]_D^{20}=+3.0$ ($c=0.71$ in CH_2Cl_2). ^1H NMR (400 MHz.$CDCl_3$): $\delta=8.42$ (m ,1H, NBD), 7.06–7.45 (m,15H, Trt), 6.9 (m,1H, NBD),5.2–4.95 (m, 3H, Far), 4.78–4.1 (m, 8H), 3.7–3.3 (m, 9H), 3.2–2.5 (m, 6H), 2.31–2.15 (m, 2H), 2.1–1.3 (m, 21H, Far), 1.3–0.99 (m, 18H, Far, Aca), 0.6–0.95 ppm (m, 6H, Leu); MS (ESI): calcd for $C_{73}H_{97}N_{11}O_{12}S_3$ $[M+Na]^+$ 1438.65; found 1438.71.

NBD-Aca-Gly-Ser-Met-Gly-Leu-Pro-Cys(Far)-OMe (36): Yield: 0.013 mmol; 22%. $[\alpha]_D^{20}=-2.4$ ($c=0.70$ in CH_3CN). ^1H NMR (400 MHz.$CDCl_3$): $\delta=8.42$ (m,1H; CH NBD), 6.9 (m, 1H; CH NBD),5.35 (m, 3H; 3× C=CH Far), 4.2–4.0 (m, 8H), 3.8–3.2 (m,9H),3.0–2.5 (m, 6H), 2.4–2.2 (m, 2H), 2.2–1.5 (m, 21H, Far, Aca), 1.5–1.0 (m,18H; Far, Aca),0.6–0.95 ppm (6H, Leu); MS-MALDI (DBH): calcd for $C_{54}H_{83}N_{11}O_{13}S_2$ $[M+K]^+$ 1197.44; found 1197.79.

MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(GerMant)-OMe (37): Yield: 0.011 mmol; 31%. $[\alpha]_D^{20}=-15.4$ ($c=0.30$ in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): δ(characteristic signals)=7.91 (d, $J=8.0$ Hz, 1H, Mant), 7.36 (m, 1H, Mant), 6.81 (m, 1H, Mant), 6.69 (m, 1H, Mant), 6.65 (s, 2H, MIC), 5.46 (m, 1H, Ger), 5.15 (m, 1H, Ger), 3.70 (s, 3H, OCH_3), 2.89 (s, 3H, NCH_3), 2.52 (t, $J=7.6$ Hz, 2H, α-CH_2 Pal), 1.98 (s, 3H; SCH_3), 1.68 (s, 3H, Ger), 1.63 (s, 3H, Ger), 1.21 (s, 28H, Pal + CH_2 MIC), 0.93–0.88 (m, 6H, CH_3 Leu), 0.84 ppm (t, $J=6.9$ Hz, 3H, CH_3 Pal); MS (MALDI, CHCA): calcd for $C_{72}H_{113}N_9O_{13}S_3$ $[M+Na]^+$ 1430.8, found 1434.2; calcd for $[M+K]^+$ 1446.7, found 1450.0.

MIC-Gly-Cys(Pal)-Met-Lys(Bodipy)-Leu-Pro-Cys(Far)-OMe (38): Yield: 0.037 mmol; 30%. $[\alpha]_D^{20}=-11.6$ ($c=0.30$ in CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): $\delta=7.63$–7.58 (m, 1H, Bodipy), 7.41–7.38 (m, 1H, Bodipy), 7.11–6.95 (m, 1H, Bodipy), 6.82–6.87 (m, 1H, Bodipy), 6.61 (s, 2H, MIC), 5.28 (m, 1H), 5.02 (m,1H), 3.70–3.59 (m, 5H), 2.49 (m, 2H), 2.18–2.16 (m, 2H), 1.98–1.92 (m, 6H), 1.70–1.40 (m, 12H, distinguished singlets at 1.60 and 1.53), 1.35–1.10 (m, 28H, Pal), 0.83–0.77 ppm (m, 12H, Leu, Pal); MS (ESI): calcd for $C_{86}H_{134}BF_2N_{11}O_{13}S_3$ $[M+H]^+$ 1674.94, found 1674.78; $[M-F+H]^+$ 1655.94, found 1655.14.

Biological assay: The peptide **21** was solubilized in DMSO at 5 mM concentration. L-M(TK-) cells (L cells) from murine subcutaneous connective tissue and Wnt3a transfected L cells (L Wnt3a cells) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C and 10% CO_2.[21] L Wnt3a cells express and secrete biologically active Wnt-3a.[22] Conditioned media from L cells or from L Wnt3a cells were prepared as described.[16] Assays to test the activity of the Wnt peptide were performed as described for the purified protein.[15a] L cells at approximately 75% confluence were incubated for 4.5 h with conditioned media from L cells or from L Wnt3a cells. In parallel, L cells were incubated with different concentrations of the peptide **21** and of DMSO as control. Assays were performed with the peptide or DMSO in a full medium containing 10% fetal calf serum. After incubation, the cells were washed with PBS and lysed in cell lysis buffer (25 mM Tris/HCl, 2 mM EDTA, 10% glycerol, 1% Triton, 2 mM DTE, pH 7.8) by incubation for 5 min on ice. Total lysates were equilibrated to equal protein concentrations with cell lysis buffer. Samples were electrophoresed on 12.5% SDS polyacrylamide gels and electro-blotted on a PVDF membrane (Amersham Biosciences). Membranes were blocked in TPBS (PBS, 0.1% Tween) with 5% powdered milk. The membrane was probed with a monoclonal anti-β-catenin antibody (Transduction Laboratories) at 1:1000 dilution or with a monoclonal anti-GAPDH antibody (abcam) at 1:30000 dilution in TPBS for 1 h at room temperature. After the membrane had been washed three times in TPBS, it was probed with a horseraddish peroxidase coupled goat-anti-mouse antibody (Amersham Biosciences) as secondary antibody. Luminescence detection was performed with the commercial ECL Plus Western blot detection system (Amersham Biosciences).

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