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# Synthesis of Functionalized Rab GTPases by a Combination of Solution- or Solid-Phase Lipopeptide Synthesis with Expressed Protein Ligation**

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**Abstract:** Prenylated proteins with non-native functionalities are generally very difficult to obtain by recombinant or enzymatic means. The semisynthesis of preparative amounts of prenylated Rab guanosine triphosphatases (GTPases) from recombinant proteins and synthetic prenylated peptides depends largely on the availability of functionalised prenylated peptides corresponding to the proteins' native structure or modifications thereof. Here, we describe and compare solution-phase and solid-phase strategies for the generation of peptides corresponding to the prenylated C terminus of Rab7 GTPase. The solid-phase with utilisation of a hydrazide linker emerges as the more favourable approach. It allows a fast and practical synthesis of pure peptides and gives a high degree of flexibility in their modification. To facilitate the analysis of semisynthetic proteins, the synthesised peptides were equipped with a fluorescent group. Using the described approach, we introduced fluorophores at several different positions of the Rab7 C terminus. The position of the incorporated fluorescent groups in the peptides did not influence the protein-ligation reaction, as the generated peptides could be ligated onto thioester-tagged Rab7. However, it was found that the positioning of the fluorescent group had an influence on the functionality of the Rab7 proteins; analysis of the interaction of the semisynthetic Rab7 proteins with REP (Rab escort protein) and GDI (guanosine diphosphate dissociation inhibitor) molecules revealed that modification of the peptide side chains or of the C-terminal isoprenoid did not significantly interfere with complex formation. However, functionalisation of the C terminus was found to have an adverse effect on complex formation and stability, possibly reflecting low structural flexibility of the Rab GDI/REP molecules in the vicinity of the lipid-binding site.

**Keywords:** lipoproteins · peptides · protein modifications · solid-phase synthesis · solution-phase synthesis

## Introduction

Progress in protein-ligation methods has introduced new possibilities in the field of semisynthetic proteins, by enabling the synthesis of post-translationally modified proteins that were previously not accessible to organic synthesis.[1–4] One of the post-translational modifications that recently became amenable to targeted modification by a combination of organic synthesis and in vitro protein ligation is protein prenylation.[5–7] Prenylation of proteins with farnesyl or geranylgeranyl moieties commonly occurs in eukaryotic cells and was shown to be critical for various cellular processes, such as maintenance of the nuclear envelope, signal transduction and membrane transport. The proteins that bear the most diverse and complex prenyl modifications belong to the Rab guanosine triphosphatase (GTPase) family. These proteins play an important role in regulating membrane transport by acting as molecular switches alternating between active (guanosine triphosphate bound) and inactive (guanosine diphosphate (GDP) bound) conformations. They control vesicular docking, fusion and possibly transport at multiple steps of intracellular vesicular transport.[8,9] More than 60 Rab proteins have been identified in

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[**] GTPase = guanosine triphosphatase.



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mammalian cells, a fact making them the largest family within the Ras-like GTPase superfamily.[10] Rab proteins are post-translationally geranylgeranylated on one or two C-terminal cysteine residues and in some cases are also C-terminally methylated.[11] These modifications allow them to reversibly associate with the cytoplasmic leaflet of intracellular membranes and are critical for their activity. Prenylation of Rab proteins is a complex process requiring the interaction of the GTPase with Rab geranylgeranyltransferase (Rab GGTase) and its accessory protein REP (Rab escort protein). All prenylated Rab proteins are transported between the intracellular membranes and the cytosol by a regulatory protein termed the GDP-dissociation inhibitor (GDI) that associates exclusively with the GDP-bound form of the proteins, thereby maintaining them in the inactive state. Due to the central role of the Rab GTPases in cellular biogenesis, it is not surprising that a number of human diseases were shown to be a consequence of mutations either in Rab GTPases themselves or in their interaction partners, including the Rab GGTase, REP and GDI proteins.[12]

Despite considerable efforts, the elucidation of the biological function of Rab GTPases on the molecular level remains fragmentary. To a large extent this is caused by the difficulties associated with the production and analysis of prenylated proteins and the construction of protein probes for use in in vitro and in vivo assays. This problem, for example, has hampered the elucidation of the mechanism of protein-assisted Rab shuttling between membranes and the cytosol. Such studies require the generation of Rab molecules with functionalised or non-native prenyl structures.

In order to create versatile molecular tools for such studies, we recently developed an intein-mediated synthesis of monogeranylgeranylated Rab7.[7] In this case, a hexapeptide mimicking the last six amino acids of the Rab7 C terminus and containing a geranylgeranylated cysteine methyl ester, a dansyl fluorophore and an N-terminal cysteine for ligation was synthesised and ligated to thioester-tagged Rab7ΔC6. The monoprenylated protein was functionally active and represented a genuine intermediate of the Rab prenylation reaction.

For further studies of the function of Rab, proteins with other functionalities, such as fluorophores displaying environment-dependent spectral characteristics, spin-labels or photoreactive groups, would be highly desirable. Apart from their obvious utility for studies of protein–protein interactions and structure analysis, such proteins are expected to become invaluable tools for in vivo studies of the function of Rab. Such precisely modified proteins, as well as native ones, should be accessible in substantial amounts (that is, on a multimilligram scale) in order to facilitate their application in protein-consuming methods such as protein crystallography, where semisynthetic proteins have already demonstrated their excellent potential.[6] It is therefore crucial that a reliable and efficient method is developed for the fast and flexible synthesis of functionalised prenylated peptides for use in protein-ligation applications.

In our previous reports,[6,7] C-terminal Rab7 peptides were obtained by using solution-phase synthesis. Even though this approach can be used with other peptide modifications, it has major drawbacks, mainly related to speed and flexibility. In order to circumvent these problems, an efficient solid-phase method is needed. Here we describe the development of such a solid-phase method, its comparison with a solution-phase method and its application to the synthesis of fully functional prenylated Rab proteins.

**Considerations for the synthesis of functionalised Rab7 proteins**: The modification of Rab7 proteins can be performed at different positions on the protein. A prerequisite of these modifications is that the functionalised Rab7 should still be biologically active. It is therefore important to preserve its native functional elements as much as possible. In the considerations described below, we discuss the possible positions where the Rab7 C-terminus could be modified.

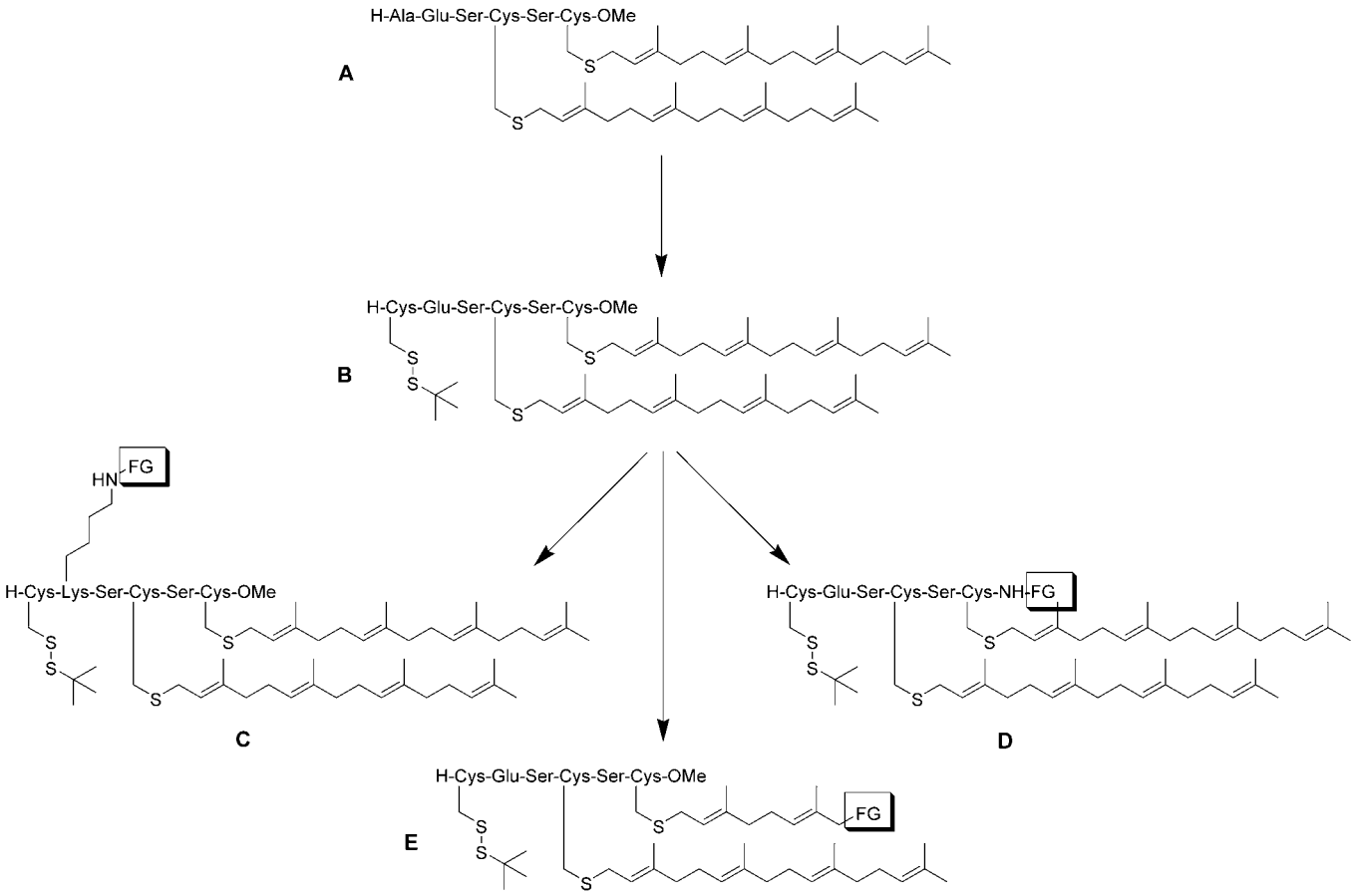
1) The generated peptides should be closely related to the C-terminal amino acid sequence ($Ala^{202}$-Glu-Ser-Cys-Ser-Cys; **A** in Scheme 1) of the original Rab7 protein. Replacement of $Ala^{202}$ with an N-terminal cysteine (**B** in Scheme 1) was chosen to provide a site for ligation because such a replacement is known to preserve Rab function.[13]
2) The C terminus should possibly terminate with a methyl ester since Rab7 is post-translationally modified with a methyl ester that might influence its interaction with other proteins.
3) The nature of the isoprenoid groups should be kept unchanged as much as possible.

In order to identify biologically tolerable positions for incorporation of functional groups, the following sites on the C terminus were selected (Scheme 1):

a) replacement of glutamic acid residue 203 by a fluorescently labelled amino acid (**C**),[7]
b) replacement of a C-terminal methyl group with a fluorescently labelled amide (**D**), and
c) replacement of the geranylgeranyl moiety of the C-terminal cysteine residue with a functionalised prenyl derivative of similar length and size (**E**).

In order to develop the optimal protocol for the synthesis of Rab7 peptides, we decided to synthesise the modified peptides bearing fluorophores at different positions by both solution-phase and solid-phase methods. The fluorophores represent model functional groups that should allow determination of the biological consequences of protein modification at a given position and the generation of biophysical data on their influence on the characterised interactions of Rab GTPases with regulatory proteins.

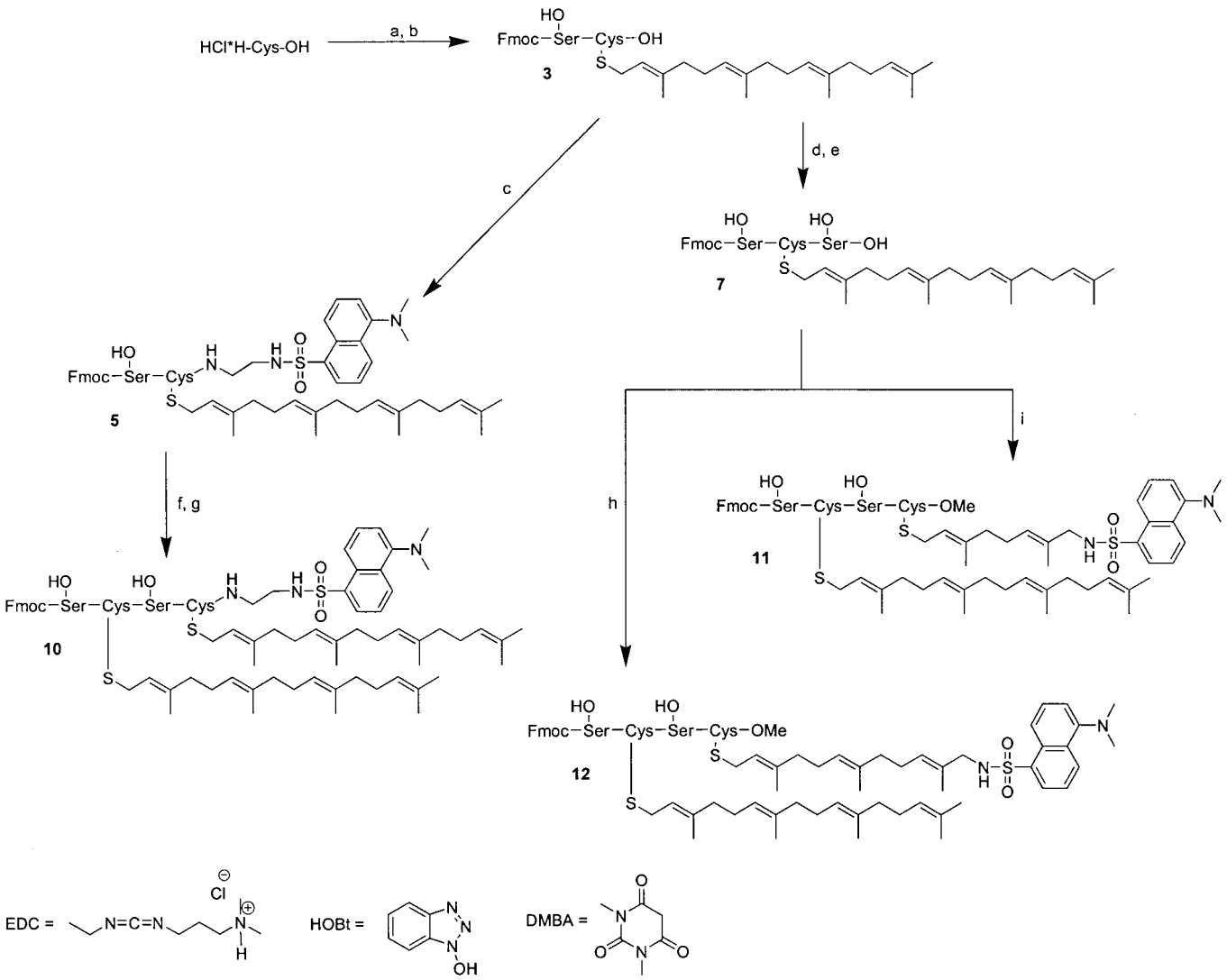



Scheme 1. Planned modifications of Rab peptides as targets for synthesis. **A**: Native C-terminal hexapeptide sequence of Rab 7; **B**: Exchange of alanine for cysteine to enable ligation to a thioester; **C**: Functional group (for example, a fluorophore) incorporated into the peptide sequence at lysine; **D**: Functional group incorporated at the C terminus; **E**: Functional group incorporated into one of the prenyl residues. FG = functional group.

## Results and Discussion

**Solution-phase synthesis**: The synthesis of peptides mimicking the Rab7 C terminus and carrying different functional groups must take into account the pronounced acid lability and intrinsic instability of the geranylgeranyl moieties; these factors limit the number of applicable methods. Upon introduction of the lipid moiety, the number of possible orthogonal protecting groups is dramatically reduced. Furthermore, the bivalent character of the peptides, featuring both highly hydrophobic lipid groups and polar amino acids, makes them difficult to handle. Moreover, transesterification and diketopiperazine side reactions caused by the labile C-terminal methyl ester limit the choice of synthetic strategies. We found that undesired cyclisation does not allow assembly of the hexapeptide by N-terminal linear elongation. Therefore, a block-coupling method was chosen, with dipeptide **3** as a general building block for all the designed peptides (Scheme 2). Coupling of building blocks with C-terminal cysteines in the presence of serines with free side-chain OH groups may lead to racemisation and modification of the unprotected alcohol functionalities of the serines. To circumvent this problem, these couplings were performed by using EDC/HOBt. In most cases the amines were protected with the Fmoc group, although the allyloxycarbonyl (Aloc) group could also be used.

Dipeptide **3** was synthesised by initial *S*-alkylation of cysteine with freshly prepared geranylgeranyl chloride **1** in 2 N $NH_3$ in methanol as described before.[14] For solubility reasons, the resulting H-Cys(GerGer)-OH was treated with the preactivated Fmoc-Ser-OSu (**2**) in a 1:1 mixture of methanol and dichloromethane. Dipeptide **3** was treated with freshly prepared dansyl-ethylenediamine **4**[15] to yield dipeptide **5**, the C-terminus for hexapeptide **18** (see Scheme 4 later). Dipeptide **5** was Fmoc-deprotected by using the volatile base diethylamine and the amino acid chain was subsequently elongated with dipeptide **3** to give tetrapeptide **10**. For the synthesis of tetrapeptides **11** and **12**, dipeptide **3** was first elongated to tripeptide **7** by using TFA·H-Ser-OAll (**6**).[16] After deprotection of the allyl ester, tripeptide **7** was obtained with a yield of 50% over two steps. From this tripeptide, the syntheses towards tetrapeptides **11** and **12** diverged, by coupling **7** either to a cysteine methyl ester featuring a geranyl-dansyl thioether **8** or a farnesyl-dansyl thioether **9**. These cysteines were synthesised by following procedures established earlier.[17]

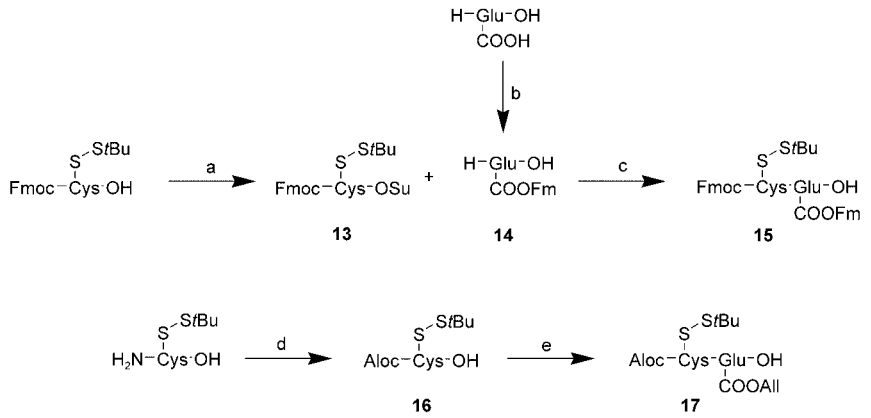



Scheme 2. Synthetic pathway for the solution-phase synthesis of tetrapeptides **10**, **11** and **12**. a) GerGerCl (**1**), 2 N $NH_3$/MeOH, 86%; b) Fmoc-Ser-OSu (**2**), $Et_3N$, $CH_2Cl_2$/MeOH, 44–75%; c) HBTU/HOBt, $Et_3N$, Dansyl-NH-$(CH_2)_2NH_2$ (**4**); d) TFA·H-Ser-OAll (**6**), EDC/HOBt, $Et_3N$, 51%; e) [$Pd(PPh_3)_4$], DMBA, 97%; f) $Et_2NH$, $CH_2Cl_2$; g) Fmoc-Ser-Cys(GerGer)-OH (**3**), HBTU/HOBt, 61%; h) H-Cys(GerDansyl)-OMe (**8**), EDC/HOBt, 40%; i) H-Cys-(FarDansyl)-OMe (**9**), EDC/HOBt, 40%. All = allyl, Dansyl = 5-(dimethylamino)naphthalene-1-sulfonyl, DMBA = *N*,*N*-dimethyl barbituric acid, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, Fmoc = 9-fluorenylmethoxycarbonyl, Ger = geranyl, HBTU = *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxy-1H-benzotriazole, Su = succinimididyl, TFA = trifluoroacetic acid.

For elongation of the tetrapeptides to the desired hexapeptides, dipeptide **15** was devised to feature both the native glutamic acid and a non-native cysteine required for ligation to the protein (Scheme 3). By employment of the Fmoc protecting group for the N-terminal amine and the Fm protecting group for the side-chain carboxylic acid functionality of glutamic acid, a system was generated that allowed simultaneous cleavage of both protecting groups in one final step under basic conditions. First, glutamic acid γ-Fm ester **14** was prepared by following a described procedure.[18] This intermediate was condensed with preactivated Fmoc-Cys(S*t*Bu)-OH to yield the dipeptide **15** in 33% overall yield based on the starting material Fmoc-Cys(S*t*Bu)-OH. In order to explore the use of palladium-labile amine and carboxylic acid protecting groups, we also synthesised dipeptide **17**, which has the amine and acid functionalities protected with an allyl carbamate and an allyl ester, respectively. To this end, the amine functionality of H-Cys-(S*t*Bu)OH was Aloc-protected to yield **16**, and this cysteine was subsequently preactivated and coupled to H-Glu(OAll)-OH to give dipeptide **17** in 47% overall yield.

For the synthesis of hexapeptides **18**–**20** (Scheme 4), the N terminus of the corresponding tetrapeptides **10**–**12** was deprotected by using an $Et_2NH$/dichloromethane mixture (1:3). Each deprotection reaction reached completion within 30 minutes and showed clean conversion. The reaction mixture was co-evaporated with toluene to remove the base. A large excess of toluene with respect to the base had to be

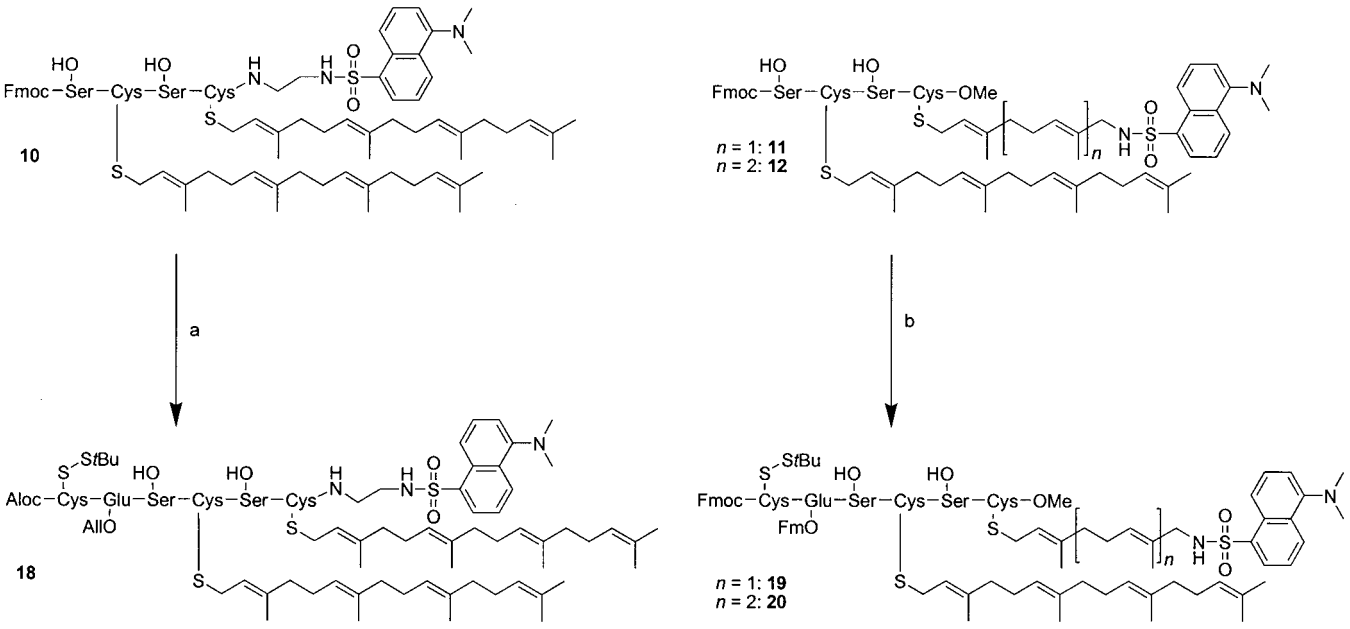
  




Scheme 3. Solution-phase synthesis of N-terminal dipeptides **15** and **17**. a) DCC, SuOH, DME, quantitative; b) FmOH, $HBF_4Et_2O$, $Na_2SO_4$, THF, 58%; c) $ET_3N$, $CH_2Cl_2$, 56%; d) allylchloroformate, EtOAc, 72%; e) 1. DCC, SuOH, DME; 2. $H_2N$-Glu(OAll)-OH·HCl, $Et_3N$, $CH_2Cl_2$, 65%. DCC = *N*,*N*′-dicyclohexylcarbodiimide, DME = 1,2-dimethoxyethane, Fm = 9-fluorenylmethyl, THF = tetrahydrofuran.

used (50 equivalents) to avoid degradation of the peptide. Without dilution with toluene, the dansyl fluorophore appeared to be susceptible to side reactions with the amine. After drying of the amine under high vacuum, the crude product could be used without further purification. Chromatographic purification is possible; however, the tetrapeptides exhibit significant tailing on silica columns and this leads to product loss. The tetrapeptides were treated with dipeptide **15** or **17** to result in the hexapeptides **18–20** in acceptable yields (≈40%, based on **10–12**).

**Solid-phase synthesis**: For the development of a solid-phase synthesis of Rab7 peptides, similar synthetic considerations as for the solution-phase were used. An essential point to be considered was the choice of a linker for connecting the peptide to the solid phase that would allow cleavage of the resin under very mild conditions and with generation of a functional C terminus. In the case of approaches **C** and **E** (Scheme 1), the latter would be a methyl ester as in the native protein. Approach **D** requires the peptide to be cleaved as a functionalised amide. The linker of choice should permit both approaches and should preferably also allow cleavage of the free acid, since Rab proteins are post-translationally modified as methyl esters and also as free acids. All these requirements are fulfilled by the oxidation-sensitive hydrazide linker. 4-Fmoc-hydrazinobenzoyl AM NovaGel (Novabiochem) was therefore employed for the synthesis, based on previous experiences in our group on the synthesis of Ras peptides.[19] We decided not to perform the prenylation of the cysteines on the resin, but instead to rely on a new approach making use of presynthesised Fmoc-protected prenylated cysteines. The reasons for this are threefold. First of all, on-resin prenylation requires large excesses of prenyl halide in order to achieve complete conversion. With regard to geranylgeraniol- or fluorescent-marker-modified prenyl reagents, this

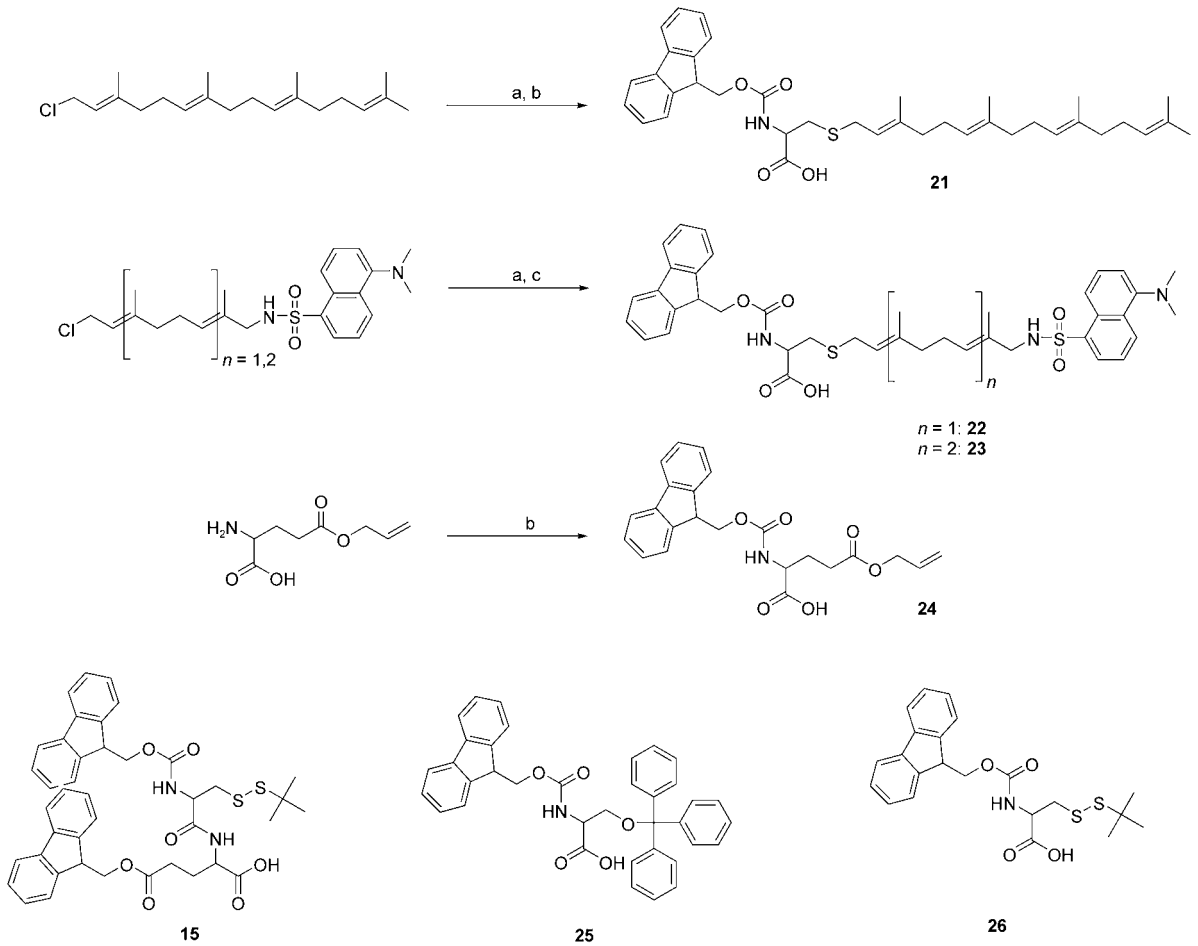



Scheme 4. Solution-phase synthesis of hexapeptides **18**, **19** and **20**. a) 1. $Et_2NH$, $CH_2Cl_2$; 2. Aloc-Cys(S*t*Bu)-Glu(All)OH (**17**), HOBt, HBTU, $Et_3N$, 61%; b) 1. $Et_2NH$, $CH_2Cl_2$; 2. Fmoc-Cys(S*t*Bu)-Glu(Fm)OH (**15**), EDC, HOBt, 42% (based on **11**)/35% (based on **12**). HBTU = *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

approach becomes unpractical because of the challenging and laborious preparation of the expensive building blocks. Secondly, two cysteines need to be consecutively prenylated in the case of Rab7. Performing a stepwise prenylation of the cysteines on the resin would require an extensive protecting-group strategy, thereby rendering the approach unattractive. Finally, the use of preprenylated building blocks allows the insertion of the prenylated cysteines at the position of choice without modification of the protecting-group strategy, thus providing a highly flexible and generic approach applicable to other types of prenylated peptides.

The synthesis of the preprenylated Fmoc-protected cysteines **21**–**23** is displayed in Scheme 5, together with the synthesis of the other required building blocks for the solid-phase peptide synthesis. The synthesis of the prenylated cysteines is based on the *S*-alkylation of cysteine with the appropriate prenyl chloride followed by Fmoc protection of the amine function with Fmoc-OSu. Direct prenylation of Fmoc-Cys-OH is not possible as it results in removal of the Fmoc group, due to the basic conditions required for the prenylation reaction. The prenyl chlorides were synthesised according to established procedures.[14] Optimal conditions for the *S*-alkylation were found with the use of Cys·HCl, since it readily dissolves in methanol, in contrast to Cys or Cys·$H_2O$·HCl. The prenylated cysteines could be purified, but overall yields were usually better when the Fmoc protection was performed directly on the crude reaction mixture, due to the problems associated with chromatography of the amphiphilic intermediates. The Fmoc protection was performed in a mixture of methanol and dichloromethane that solubilises prenyl cysteines. The final products could easily be purified by standard column chromatography, with satisfactory yields.

To investigate the scope of different protecting-group strategies, we not only opted for the use of dipeptide **15** for the N terminus of the hexapeptides, but decided also to examine the use of a palladium-labile protecting group. For this purpose, glutamic acid ester **24** was synthesised; this compound allows its γ-carboxylic acid to be liberated with $[Pd^0(PPh_3)]_4$, while the Fmoc-protected N terminus is left intact. Glutamic acid ester **24** was synthesised by protecting TFA·H-Glu(All)-OH[20] to give Fmoc-Glu(All)-OH in a similar fashion to the prenylated cysteines **21**–**23**. The use of glutamic acid ester **24** requires additional condensation with



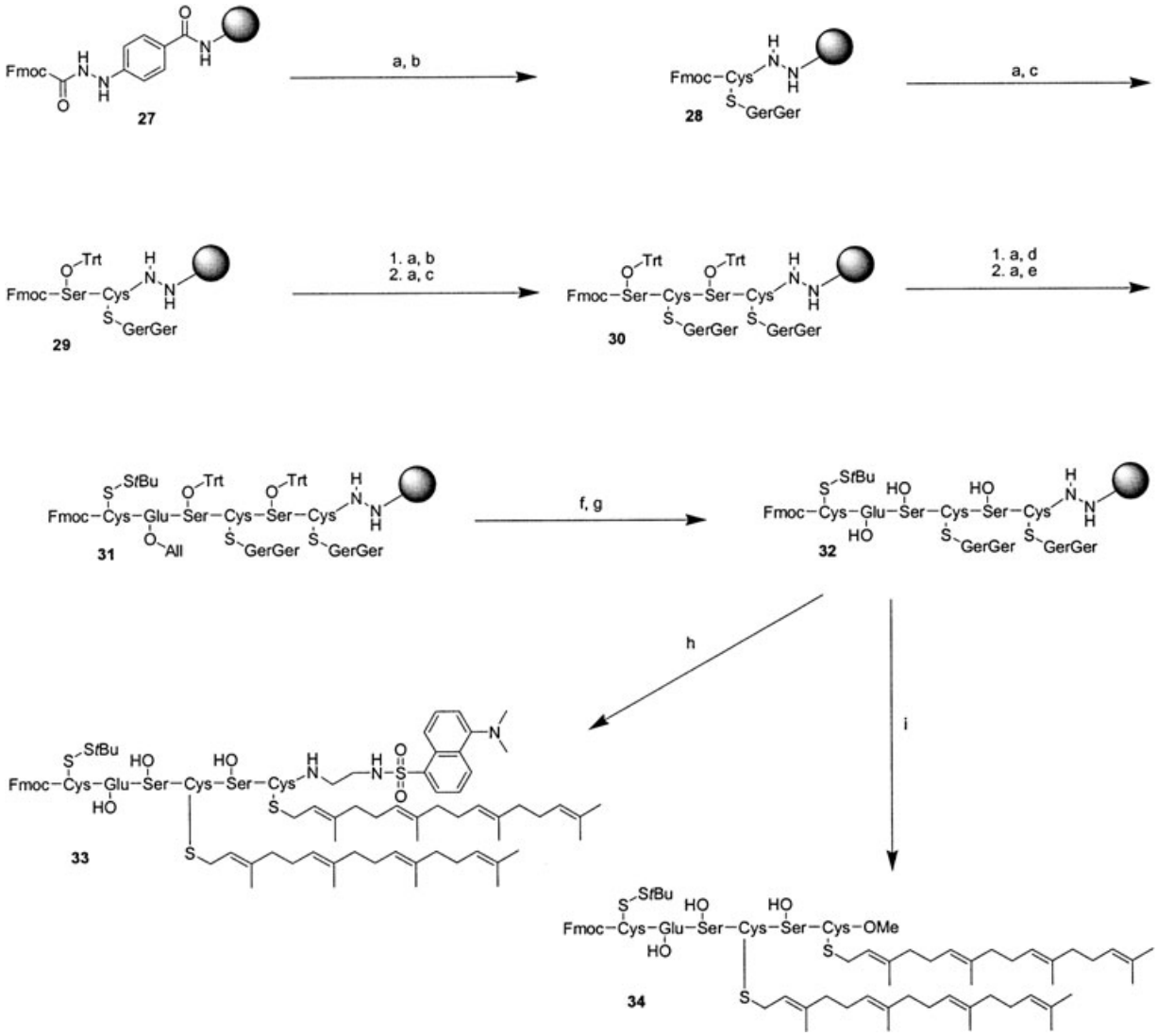

Scheme 5. Building blocks for solid-phase synthesis of Rab7 peptides. The synthetic strategies towards prenylated cysteines **21**–**23** and Fmoc-Glu(All)-OH **24** are displayed. The synthesis of dipeptide **15** is shown in Scheme 3 and building blocks **25** and **26** can be purchased from commercial suppliers. a) H-Cys-OH·HCl, $NH_3$/MeOH, 0 °C; b) Fmoc-Su, $Et_3N$, MeOH/$CH_2Cl_2$, 0 °C, 60 %; c) Fmoc-Su, $Et_3N$, MeOH/$CH_2Cl_2$, 0 °C, $n$ = 1: 75 %; $n$ = 2: 45 %.

cysteine **26** for termination of the sequence. The trityl group was selected as the protecting group for the serines since it can be removed under acidic conditions (1% TFA in dichloromethane) that are mild enough to leave the geranylgeranyl groups intact.

The solid-phase synthesis of hexapeptides **33** and **34** is shown in Scheme 6. The Fmoc group was removed from 4-Fmoc-hydrazinobenzoyl AM NovaGel (**27**) by agitating twice with a solution of 30% piperidine in DMF for 10 min. Fmoc-Cys(GerGer)-OH (**21**) was coupled to the resin after preactivation for 5 min with DIC/HOBt as the coupling reagent in DMF/dichloromethane (1:1), in order to suppress racemisation; this treatment yielded resin **28** after 4 h of agitation. In general, 3 equivalents of cysteine derivative **21** were used, but in cases of poorly accessible amino acids this amount can be reduced to 2 equivalents. The second amino acid, Fmoc-Ser(Trt)-OH, was coupled by using the more reactive HBTU/HOBt combination and with agitation for 2 h. Subsequently, both coupling steps were repeated to yield immobilised tetrapetide **30**. The last two amino acids **24** and **26** were coupled by using a similar technology to that described before to yield the fully protected hexapeptide **31**. The trityl protecting groups and the allyl ester were subsequently removed on the resin to leave only the N-terminal amine and the thiol masked (**32**).

The functionality of the C terminus could be introduced through the cleavage nucleophile. Proper selection of the used nucleophile thus allowed for the combinatorial synthesis of different C termini. Hexapeptide **34** features the native methyl ester at its terminus and was cleaved from the

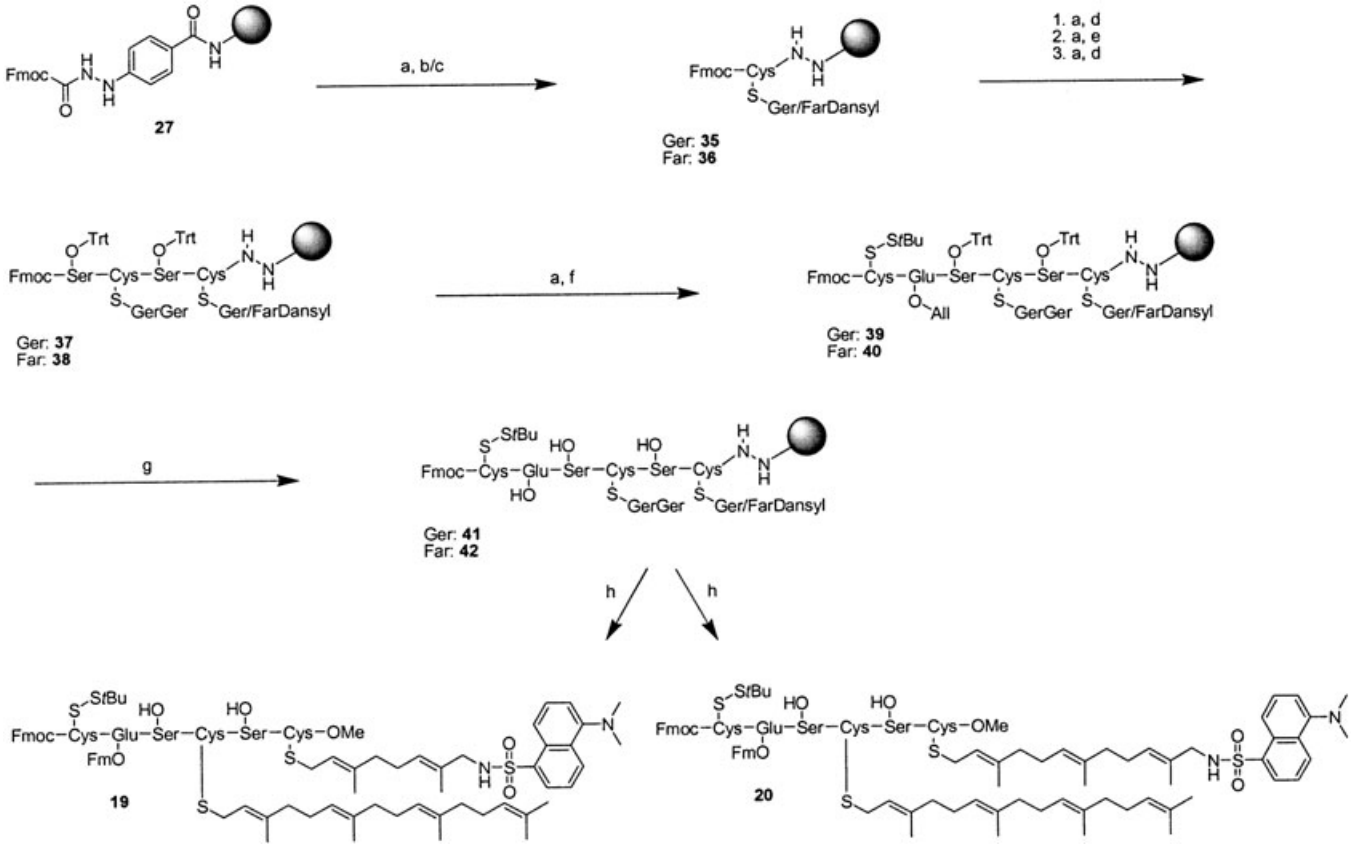



Scheme 6. Solid-phase peptide synthesis of Rab7 peptides featuring a cysteine instead of Ala[202] (**33**) and with an additional fluorescent marker connected at the C terminus (**34**). a) 1. Piperidine/DMF; b) Fmoc-Cys(GerGer)-OH (**21**), DIC, HOBt, $CH_2Cl_2$/DMF (1:1); c) Fmoc-Ser(Trt)-OH (**25**), HBTU, HOBt, DIPEA, $CH_2Cl_2$/DMF (1:1); d) Fmoc-Glu(OAll)-OH (**24**), HBTU, HOBt, DIPEA, $CH_2Cl_2$/DMF (1:1); e) Fmoc-Cys(S*t*Bu)-OH (**26**), DIC, HOBt, $CH_2Cl_2$/DMF (1:1); f) 1% TFA, 2% TES, $CH_2Cl_2$; g) [$Pd(PPh_3)_4$] (0.1 equiv), $PhSiH_3$; h) $Cu(OAc)_2$, pyridine, Dansyl-NH-Et-$NH_2$, THF, $O_2$, 40%; i) $Cu(OAc)_2$, pyridine, $CH_2Cl_2$, $O_2$, MeOH, 50%. DIC = diisopropylcarbodiimide, DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, TES = triethylsilane, Trt = trityl = triphenylmethyl.

resin by using methanol in dichloromethane. Copper acetate and oxygen were used to oxidise the hydrazide to the acyldiazene. After cleavage, the copper salts could be removed by column chromatography, but this was also achieved by simple extraction into a dichloromethane/aqueous acid mixture. Hexapeptide **34** actually constitutes the native sequence of Rab7 with a cysteine replacing the native alanine at position 202. For the synthesis of hexapeptide **33**, dansylated ethylenediamine **4** was used as the nucleophile. In contrast to the usually employed dichloromethane solvent, THF had to be used here; the absence of methanol makes the copper acetate insoluble in dichloromethane. The cleavage proceeds smoothly, although the excess of amine **4** cannot be removed by straightforward evaporation or extraction, in contrast to the case with methanol. However, simple column chromatography sufficed to purify the peptide. Overall yields were in the range of 40–50% based on the original Fmoc loading of the resin. In general, cleavage with methanol gave somewhat higher yields than with amine **4**. Another method commonly applied to oxidise the hydrazine linker relies on the use of *N*-bromosuccinimide (NBS).[21,22] For the peptides required here, however, this oxidation is not suitable, since NBS is not compatible with the geranylgeranyl groups and leads to addition products.

Peptides **33** and **34** are synthesised by a solid-phase approach that diverges at the final cleavage step from the resin. This feature makes this approach to C-terminal functionalisation highly flexible.

For the solid-phase synthesis of hexapeptides **19** and **20** we decided to investigate the use of dipeptide **15** as the N-terminal part (Scheme 7), an approach also used for the solution-phase synthesis. After removal of the Fmoc group from resin **27**, either Fmoc-Cys(Ger-Dansyl)-OH (**22**) or Fmoc-Cys(Far-Dansyl)-OH (**23**) was coupled to the resin after preactivation for 5 min with DIC and HOBt in DMF and dichloromethane. The resulting resins **35** and **36** were next individually subjected to the same synthetic transformations up to the cleavage step. The tetrapeptides **37** and **38** were built up as for tetrapeptide **30**. After cleavage of the Fmoc group, hexapeptides **39** and **40** were formed in one coupling step with dipeptide **15**. After deprotection of the trityl groups, the hexapeptides were cleaved from the resin by using methanol as the nucleophile. This approach leaves the N-terminal cysteine and glutamic acid protected with Fmoc and Fm groups, respectively. We also performed deprotection of the Fmoc and Fm group on the resin, but this approach was less successful. First of all, the free amine of the peptide can undergo cyclisation or oligomerisation with




Scheme 7. Solid-phase peptide synthesis of Rab7 peptides featuring a fluorophore in the membrane-binding geranylgeranyl groups. Hexapeptide **19** contains a geranyl-dansyl prenyl group, while hexapeptide **20** contains a farnesyl-dansyl prenyl group. a) Piperidine/DMF; b) Fmoc-Cys(GerDansyl)-OH (**22**), DIC, HOBt, $CH_2Cl_2$/DMF (1:1); c) Fmoc-Cys(FarDansyl)-OH (**23**), DIC, HOBt, $CH_2Cl_2$/DMF (1:1); d) Fmoc-Ser(Trt)-OH (**25**), HBTU, HOBt, DIPEA, $CH_2Cl_2$/DMF (1:1); e) Fmoc-Cys(GerGer)-OH (**21**), DIC, HOBt, $CH_2Cl_2$/DMF (1:1); f) Fmoc-Cys(SS*t*Bu)-Glu(OFm)-OH (**15**), DIC, HOBt, $CH_2Cl_2$/DMF (1:1); g) 1% TFA, 2% TES, $CH_2Cl_2$; h) $Cu(OAc)_2$, pyridine, THF, $O_2$, MeOH, 50%.

the C termini during cleavage.[22] Secondly, the unprotected peptide was found to be difficult to purify; it showed tailing on both normal- and reversed-phase columns. Together with the side products that occurred upon cleavage of the unprotected peptide, this resulted in a mixture that could not be purified. We therefore chose to perform the final deprotection in solution (see below). The overall yields based on the Fmoc loading of **27** are very high, especially when a somewhat larger batch of resin is used (>50 mg). The peptides could be synthesised and purified within two days. Figure 1 shows the LC/MS trace of the lipidated and fluorescently labelled hexapeptide **19** synthesised by solid-phase peptide chemistry and after cleavage from the resin.

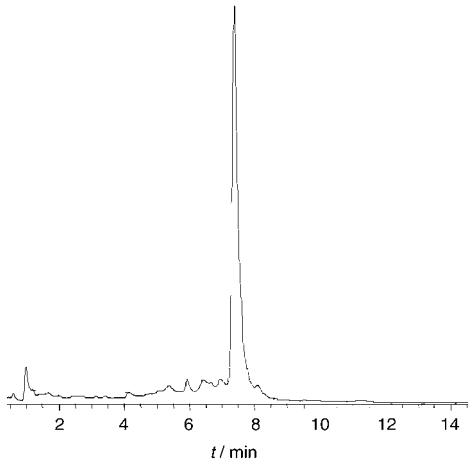



Figure 1. LC/MS trace of hexapeptide **19** after cleavage from the resin.

Divergence in the solid-phase synthesis of hexapeptides **19** and **20** is brought in directly by the coupling of the first amino acid to the resin. This is in contrast to the synthetic approach applied for hexapeptides **33** and **34** and makes the synthesis of peptides modified at the lipophilic geranylgeranyl groups somewhat less flexible for fast structure variation. However, application of parallel synthesis techniques avoids these limitations.

A direct comparison of the solution-phase (Scheme 4) and solid-phase techniques (Scheme 7) for the synthesis of fluorescently labelled peptides **19** and **20** reveals that, in terms of speed, efficiency and flexibility, the solid-phase technique is clearly superior. Thus, by means of the solid-phase method, **19** and **20** are obtained in multimilligram amounts within two days in pure form with overall yields of 50 and 30% respectively, whereas the corresponding synthesis in solution requires several weeks and proceeds with overall yields of 8 and 6%, respectively. In the solid-phase method, only one purification step at the end is needed, while the solution-phase method requires chromatographic separation of the intermediates. Both synthesis strategies allow the flexible introduction of different fluorescent markers and prenyl groups. Notably, the solid-phase method also allows flexible modification of the C terminus upon cleavage from the solid support. If larger amounts (that is, 100 mg) of lipidated peptides are required, application of the solid-phase method will lead to substantial consumption of the lipidated building blocks due to the multiple couplings performed. In such cases, the solution-phase method or a combination of both may be superior. A comparison between the solution- and solid-phase approaches is depicted in Table 1.

**Expressed protein ligation with Rab7ΔC6 thioester**: In order to ascertain that functionalised Rab7 C-terminal peptides could be coupled to thioester-tagged polypeptides by in vitro protein ligation, we performed a series of analytical reactions. The thioester-tagged Rab7ΔC6 protein was mixed with a tenfold molar excess of Fmoc-deprotected peptide **44** or **45** and incubated for 12 h at room temperature under conditions described previously.[23] The reaction mixtures were resolved by sodium dodecylsulfate PAGE and the gels were examined under UV light. As can be seen in Figure 2, in all cases a fluorescent product could be detected at the position corresponding to semisynthetic Rab protein. This indicates that the differently positioned fluorescent groups did not interfere with the ligation reaction. No fluorescent product was observed when the reaction was performed with wild-type Rab protein, a result indicating that the reaction was specific (not shown). As in previously reported cases, the reaction was dependent on the presence of a detergent.[23]

To analyse the ability of the functionalised semisynthetic proteins to form complexes with their interacting proteins, we performed preparative in vitro ligations and purified the ligation products (Figures 2 and 3).[23] At the last stage of purification, the proteins were mixed with Rab GDI or

Table 1. Comparison and characteristics of solid-phase and solution-phase syntheses of lipidated Rab7 C-terminal peptides featuring fluorophores.

| | Solution-phase synthesis | Solid-phase synthesis |
|---|---|---|
| purification | purification of each individual product required, lowers overall yield | only the final product needs to be purified, simple extraction and column chromatography |
| yield | individual reactions have moderate to good coupling yields<br>overall yield: 7–8% | individual reactions go with almost complete conversion<br>overall yield: 40–50% |
| synthesis time | 2–8 weeks | 2 days |
| combinatorial aspects | not suited for library synthesis; new synthetic strategies required for each desired compound and long synthetic pathway | library synthesis possible; depending on point of divergence, synthetic efforts moderate |
| areas of cost | geranylgeranyl group, lower yields | resin and prelipidated cysteine building blocks |

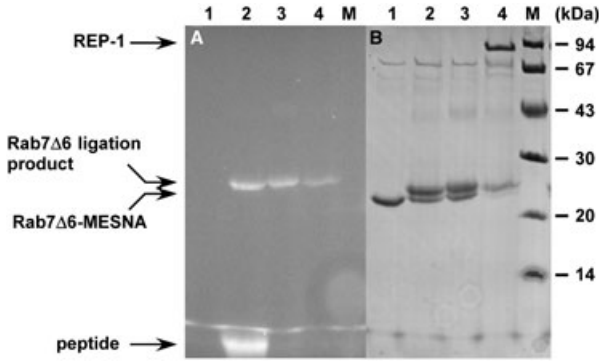



Figure 2. Sodiumdodecyl sulfate PAGE gel of Rab7ΔC6-MESNA thioester before (lane 1) and after (lane 2) ligation to peptide H-Cys-Glu-Ser-Cys(GerGer)-Ser-Cys(Ger-dansysl)-OMe, after removal of unligated peptide (lane 3) and after complex formation with REP-1 and Superdex 200 gel-filtration purification (lane 4). The gel was photographed either in UV light (A) or in visible light after staining with Coomassie blue (B). MESNA = 2-mercaptoethanesulfonic acid.

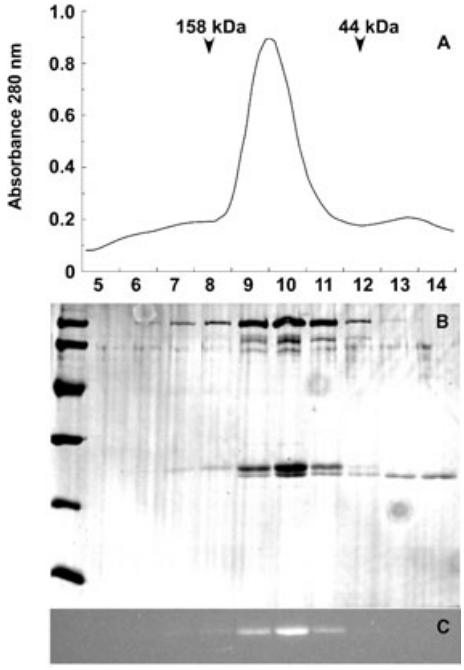



Figure 4. Superdex 200 gel-filtration chromatogram (A) of Rab7ΔC6-CESC(GerGer)SC(Ger-dansyl)-OMe:REP-1 complex and sodiumdodecyl sulfate PAGE gel (B,C) of individual fractions. The gel was photographed either in UV light (C) or in visible light after staining with Coomassie blue (B).

REP-1, concentrated and separated from detergent by size-exclusion chromatography on a Superdex 200 gel-filtration column. At this stage we observed a dramatic difference in the behaviour of the generated proteins. Rab7 proteins containing fluorescent groups on the C terminus failed to form a complex with their molecular chaperones and were retained on the column. The Rab7 protein functionalised with the geranyldansyl group on cysteine residue 207 formed a stoichiometric complex with REP-1 and eluted at the expected position corresponding to approximately 100 kDa (Figure 4).

These results indicate that the introduction of bulky groups directly at the C terminus interferes with complex formation. This finding can be rationalised in the light of recent structure data on the interaction of the lipidated C terminus of Rab GTPases with the lipid-binding site of the GDI molecule.[6,23]

The structure of the Ypt1:GDI complex revealed that the C-terminally attached geranylgeranyl group deeply penetrates the hydrophobic cavity formed by domain II of GDI. In addition, the C-terminal α-carboxyl group of the terminal cysteine residue was found to point towards domain II. The presence of interpretable electron density for this residue suggests that its position is quite fixed. We hypothesise that coordination of the lipid in the cavity largely restricts the conformational freedom of the C-terminal cysteine residue and that bulky C-terminal substituents would clash with residues of domain II; this would explain the observed inability of GDI or REP to interact with Rab proteins modified in such a way (Figure 5). This could help to explain the observed instability of the aforementioned protein complexes.

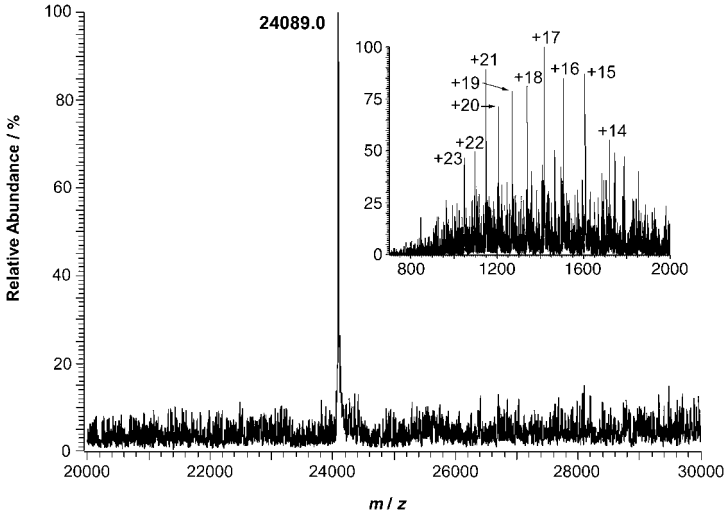



Figure 3. Deconvoluted ESI MS spectra of Rab7ΔC6-CESC(GerGer)SC(Ger-dansyl)-OMe separated from Rep-1 by reversed phase chromatography on a Jupiter C4 reversed-phase column (HPLC–ESI MS). Calculated mass: 24091.69 $[M+H]^+$. Inset: original data.

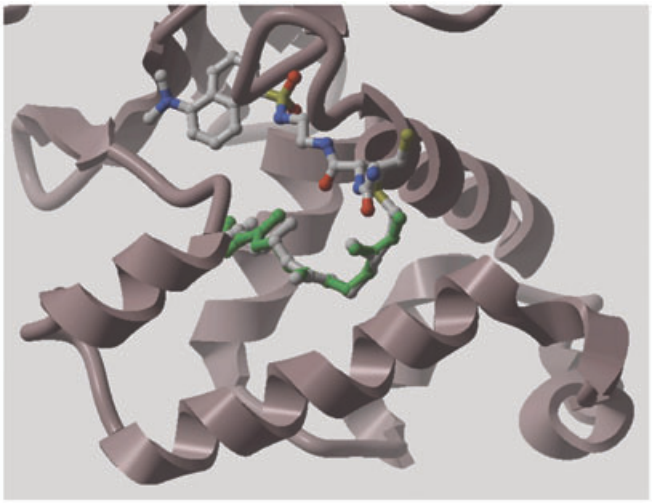


Figure 5. Model showing the potential interaction of the peptide Cys-Cys-(GerGer)-NH-$(CH_2)_2$-NH-Dansyl (ball-and-stick representation) with domain II of Rab GDI (ribbon representation). The lipid (displayed in atomic colours) was superimposed with the geranylgeranyl lipid (green) identified in the crystal structure of Ypt1:GDI complex. Binding of the isoprenoid group in the hydrophobic cavity brings the C-terminal α-carboxyl group into close contact with residues of GDI. This results in steric conflicts between bulky substituents linked to the C terminus of the Rab proteins and the GDI domain II. The model was generated and dispayed by using ICM Pro v3.0–28g (MolSoft LLC).

## Conclusion

We have developed efficient solution-phase and solid-phase methods for the syntheses of lipid-modified peptides representing the characteristic parts of Rab GTPases. Both syntheses employ readily accessible building blocks and allow the flexible and straightforward introduction of reporter groups into both the peptide chain and the lipid residues. In terms of efficiency and flexibility, the solid-phase method is superior to the solution-phase synthesis in the majority of the cases. It gives pure peptides in multimilligram amounts within a much shorter time and with superior overall yields and it allows flexible modification of the C terminus upon release from the solid support.

All generated peptides could be ligated onto thioester-tagged Rab7, a result indicating that the described methods could be used for the production of proteins with a broad array of functionalities incorporated into the C terminus.

Analysis of the interaction of the semisynthetic Rab7 proteins with REP and GDI molecules revealed that modification of various peptide side chains, including the C-terminal isoprenoid moiety, do not significantly interfere with complex formation. This is not entirely unexpected since REP and GDI must have evolved mechanisms to accommodate a number of very heterogeneous Rab C termini. In contrast, derivatisation of the C terminus itself was found to have an adverse effect on complex formation and stability, a fact possibly reflecting low structural flexibility of the Rab GDI/REP molecules in the vicinity of the lipid-binding site.

## Experimental Section

**General**: Chemicals were obtained from Acros, Advanced Chemtech, Aldrich, Biosolve, Fluka or Novabiochem and used without further purification. Analytical chromatography was performed on Merck silica gel 60 $F_{254}$ aluminium sheets and Merck aluminium oxide 60 $F_{254}$ aluminium sheets. Chromatography was performed by using Merck silica gel 60 and Fluka aluminium oxide. Dichloromethane ($CH_2Cl_2$), triethylamine, diisopropylethylamine and piperidine were refluxed under argon over $CaH_2$, THF was refluxed under argon over Na/K and methanol ($CH_3OH$) was refluxed under argon over magnesium; all solvents were freshly distilled prior to usage. $^1H$ and $^{13}C$ NMR spectroscopy data were recorded on a Bruker DRX 500 spectrometer at room temperature. NMR spectra were calibrated to the solvent signals of $CDCl_3$ (7.26 and 77.00 ppm). ESI MS was performed on an Agilent 1100 series binary pump together with a reversed-phase HPLC column (Macherey-Nagel) and a Finnigan Thermoquest LCQ apparatus. FAB MS measurements were taken with a Jeol SX 102 A spectrometer with 3-nitrobenzyl alcohol (3-NBA) as the matrix. MALDI-TOF mass spectra were recorded with a Voyager-DE Pro BioSpectrometer from PerSeptive Biosystems with 2,5-dihydroxybenzoic acid (DHB) as the matrix. Optical rotations were measured by using a Schmidt + Haensch Polartronic HH8 apparatus. The yield and scale of the solid-phase reactions are given with respect to the Fmoc loading as supplied by Novabiochem. All reactions were carried out under an argon atmosphere in dry solvents unless otherwise noted.

**General procedures for the solid-phase synthesis of Rab7 peptides by using the phenylhydrazide linker**: For all peptides, commercially available Fmoc-4-hydrazinobenzoyl NovaGel resin from Novabiochem, with an Fmoc loading of 0.56 mmol $g^{-1}$, was used. All reactions were carried out under an argon atmosphere in polyethylene syringe reactors equipped with a fritted disc. Agitation was achieved by using an orbital shaker.

Fmoc cleavage was achieved by using a degassed solution of 35% piperidine in DMF twice for 7 min. The resin was subsequently washed six times with DMF and three times with dichloromethane.

Amino acids were coupled by using an HBTU/HOBt strategy. Typically, amino acid (4 equiv) was prereacted for 5 min with HBTU (3.6 equiv), HOBt (4 equiv) and (DIPEA; 8 equiv) in DMF/$CH_2Cl_2$ (1:1). The solution was added to the resin and agitated for 2 h at room temperature. Cysteine derivatives and dipeptides were coupled by using a DIC/HOBt strategy in order to suppress racemisation. Typically, amino acid or dipeptide (4 equiv) was prereacted for 5 min with DIC (3 equiv) and HOBt (6 equiv) in DMF/$CH_2Cl_2$ (1:1). Formation of the active ester of the cysteine was confirmed by TLC. The solution was added to the resin and agitated for at least 4 h at room temperature. The resin was subsequently washed six times with dichloromethane and three times with DMF.

Cleavage of the trityl protecting groups from serine was achieved by agitating twice for 30 min with a solution of 1% TFA and 2% TES in dichloromethane. The resin was subsequently washed five times with dichloromethane.

Cleavage of the allyl ester from glutamic acid was achieved by agitating twice for 1 h with a solution of $[Pd(PPh_3)_4]$ (0.1 equiv) and $PhSiH_3$ (10 equiv) in THF. The resin was subsequently washed five times with dichloromethane.

**In vitro protein ligation**: In the ligation reaction, 450 μM Rab7ΔC6-MESNA thioester was mixed with 4 mM peptide in 20 mM $Na_2HPO_4$/$NaH_2PO_4$ (pH 7.5), 0.1 mM $MgCl_2$, 2 μM GDP, 100 mM MESNA and 50 mM cetyltrimethylammonium bromide (CTAB) and the mixture was allowed to react overnight at room temperature with vigorous agitation. The reaction mixture was centrifuged to remove protein and peptide precipitate and the pellet was washed with methanol, dichloromethane (four times), methanol (four times) and distilled water (four times).

**Solubilisation and refolding conditions**: The protein pellet was dissolved in 100 mM tris(hydroxymethyl)aminomethane–HCl (Tris–HCL; pH 8.0), 6 M guanidinium hydrochloride, 1 mM ethylenediaminetetraacetate (EDTA), 100 mM dithioerythritol (DTE) and 1% 3-[3-(cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) to a final concentration of 0.5 mg $mL^{-1}$ for ligated protein. This solution was diluted approxi-

mately 25-fold by adding it dropwise into a buffer containing 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; pH 7.5), 2.5 mM DTE, 2 mM $MgCl_2$, 10 μM GDP, 1% CHAPS, 400 mM arginine·HCl, 400 mM trehalose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA that was being stirred gently at room temperature. After addition of REP-1 or GDI protein (1 equiv), the solution was incubated on ice for 1 h and subsequently dialysed overnight against 25 mM HEPES (pH 7.5) containing 100 mM $(NH_4)_2SO_4$, 10% glycerol, 0.5 mM PMSF, 2 mM $MgCl_2$, 2 μM GDP, 1 mM EDTA and 2.5 mM DTE. The protein complex was concentrated by using a size-exclusion concentrator (Amicon) to a final concentration of approximately 4 mg mL$^{-1}$. Insoluble material was removed by centrifugation and the supernatant was loaded onto a Superdex 200 gel-filtration column (Pharmacia) equilibrated with buffer containing 25 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 10 μM GDP, 2.5 mM DTE, 100 mM $(NH_4)_2SO_4$ and 10% glycerol. Fractions containing both REP and Rab7 were collected, concentrated to approximately 10 mg mL$^{-1}$ and stored in multiple aliquots at −80 °C.

**Synthesis of Fmoc-Ser-Cys(GerGer)-NH-$CH_2$-$CH_2$-NHDansyl (5)**: Dansyl-NH$(CH_2)_2$NH$_2$ (23 mg, 0.156 mmol) was added to an ice-cooled solution of Fmoc-Ser-Cys(GerGer)-OH (50 mg, 0.071 mmol), HOBt (12 mg, 0.171 mmol), HBTU (27 mg, 0.171 mmol) and triethylamine (20 μL, 0.285 mmol) in dichloromethane (5 mL). The solution was stirred for 1 h and was subsequently allowed to warm to RT. After being stirred for 3 h at RT, the solvent was co-evaporated with toluene under reduced pressure. The crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (10:2.5)) to yield the pure title compound **5** as a yellow solid (53 mg, 0.054 mmol, 76%). TLC: $R_f = 0.56$ ($CH_2Cl_2/CH_3OH$ (10:0.5)); $[\alpha]_D^{20} = -9.6$ ($c = 1.2$, $CHCl_3$); m.p. 155–157 °C; $^1$H NMR ($CDCl_3$, 400 MHz, 300 K): $\delta = 8.45$ (d, $J = 8.5$ Hz, 1H; H-4 Dansyl); 8.25 (d, $J = 8.5$ Hz, 1H; H-8 Dansyl); 8.12 (d, $J = 7.3$ Hz, 1H; H-2 Dansyl); 7.70 (d, $J = 7.7$ Hz, 2H; Fmoc); 7.58 (brs, 1H; NH); 7.54 (t, $J = 7.6$ Hz, 1H; Fmoc); 7.52 (t, $J = 7.4$ Hz, 1H; Fmoc); 7.42 (t, $J = 8.0$ Hz, 1H; H-3, Dansyl); 7.32 (t, $J = 8.3$ Hz, 1H; H-7 Dansyl); 7.22 (t, $J = 7.5$ Hz, 2H; Fmoc); 7.20 (d, $J = 7.7$ Hz, 2H; Fmoc); 7.10 (d, $J = 7.5$ Hz, 1H; H-6 Dansyl); 6.51 (t, $J = 7.1$ Hz, 1H; NH); 6.28 (d, $J = 7.0$ Hz, 1H; NH); 5.13 (t, $J = 7.2$ Hz, 1H; $SCH_3CH$=GerGer); 5.05 (m, 3H; C=C*H*, GerGer); 4.56 (m, 1H; α-CH Cys); 4.39 (m, 1H; α-CH Ser); 4.34 (d, $J = 7.2$ Hz, 2H; Fmoc); 4.14 (t, $J = 7.2$ Hz, 1H; Fmoc); 4.01 (m, 1H; $CH_{2a}$ Ser); 3.82 (m, 1H; $CH_{2b}$ Ser); 3.33 (m, 1H; $CH_{2a}$ Cys); 3.29 (m, 1H; $CH_{2b}$ Cys); 3.08 (d, $J = 5.5$ Hz, 2H; S$CH_2$ GerGer); 2.93 (m, 2H; NHC$H_2$C$H_2$NH); 2.80 (m, 8H; NHC$H_2$C$H_2$NH, N(C$H_3$)$_2$ Dansyl); 2.02–1.92 (m, 12H; =C(CH$_3$)C$H_2$C$H_2$, CH$_2$C$H_2$CH=); 1.63 (s, 3H; =C-(CH$_3$)C$H_3$ *trans*); 1.57 (s, 3H; =C(CH$_3$)C$H_3$ *cis*); 1.55 (s, 3H; =C-(CH$_3$)C$H_3$ *cis*); 1.55 (s, 3H; =C(CH$_3$)C$H_3$ *cis*); 1.53 (s, 3H; =C(CH$_3$)C$H_3$ *cis*) ppm; $^{13}$C NMR ($CDCl_3$, 100 MHz, 300 K): $\delta = 171.1$ (CH$_2$NH*C*=O); 170.8 (*C*ONH); 154.4 (CH$_2$O*C*NH); 151.8 (C-5 Dansyl); 143.6, 143.5, 141.1, 140.0 (C-8a, C-9a, C-4a, C-4b Fmoc); 135.2, 134.8 (2×*C*=CH); 134.7, 131.1 (*C*=CH, C-1 Dansyl); 130.3 (C-8 Dansyl); 129.8, 129.4, 129.0 (C-2, C-4a, C-8a Dansyl); 128.4 (*C*=CH, C-3 Dansyl); 128.2 (C-3, C-6 Fmoc); 127.6 (C-2 Fmoc); 127.0 (C-7 Fmoc); 125.1, 125.0 (C-1, C-8 Fmoc); 124.3 (2×C=*C*H); 124.1 (C=*C*H); 123.7 (C-7 Dansyl); 123.0 (C=*C*H); 119.8, 119.1 (C-4, C-5 Fmoc); 118.8 (C-4 Dansyl); 115.1 (C-6 Dansyl); 67.3 (C-10 Fmoc); 62.9 (β-CH$_2$ Ser); 56.2 (α-CH Cys); 53.1 (α-CH Ser); 46.9 (C-9 Fmoc); 45.3 (N(*C*H$_3$)$_2$); 42.2 (NHCH$_2$CH$_2$NH); 39.8, 39.6 (2×=C(CH$_3$)*C*H$_2$CH$_2$); 39.5 (=C(CH$_3$)*C*H$_2$CH$_2$); 32.5 (β-CH$_2$ Cys); 29.6 (S*C*H$_2$C=); 26.6, 26.5 (2×CH$_2$*C*H$_2$CH=); 26.4 (=C(CH$_3$)*C*H$_3$ *trans*); 25.6 (CH$_2$*C*H$_2$CH=); 17.6 (=C(CH$_3$)*C*H$_3$ *cis*); 16.0, 15.9, 15.9 (3×=(CH$_2$)CH$_3$ *cis*) ppm; HR FAB MS (3-NBA): calcd for $C_{55}H_{72}N_5O_7S_2$ [*M*+H]: 978.4864; found: 978.4863.

**Synthesis of Fmoc-Ser-Cys(GerGer)-Ser-Cys(GerGer)NH-$CH_2$-$CH_2$-NHDansyl (10)**: Diethylamine (200 μL) was added to an ice-cooled solution of Fmoc-Ser-Cys(GerGer)-NH-$(CH_2)_2$-NHDansyl (23 mg, 0.024 mmol) in dichloromethane (800 μL). After the mixture had been stirred for 1 h at 0 °C, the product $H_2$N-Ser-Cys(GerGer)-NH-$(CH_2)_2$-NHDansyl had built up quantitatively (TLC control). The solvent was then co-evaporated with toluene to get the crude product, which was dissolved in dichloromethane (1 mL) and cooled in an ice bath. Fmoc-Ser-Cys(GerGer)-OH (16.5 mg, 0.024 mmol), HOBt (3.8 mg, 0.028 mmol), HBTU (9 mg, 0.028 mmol) and triethylamine (6.5 μL, 0.047 mmol) were added to this ice-cooled solution. The solution was stirred for 1 h and was subsequently allowed to warm to RT. After the mixture was stirred for 3 h at RT, the solvent was co-evaporated with toluene under reduced pressure. The crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (10:0.2)) to yield the pure title compound **10** as a pale-yellow solid (18.7 mg, 0.013 mmol, 54%). TLC: $R_f = 0.73$ ($CH_2Cl_2/CH_3OH$ (10:0.5)); $[\alpha]_D^{20} = -18.5$ ($c = 1.0$, $CHCl_3$); m.p. 160–162 °C; $^1$H NMR ($CDCl_3$, 400 MHz, 300 K): $\delta = 8.53$ (d, $J = 8.5$ Hz, 1H; H-4 Dansyl); 8.28 (d, $J = 8.5$ Hz, 1H; H-8 Dansyl); 8.22 (d, $J = 7.2$ Hz, 1H; H-2 Dansyl); 7.76 (d, $J = 7.5$ Hz, 2H; Fmoc); 7.59 (brs, 1H; NH); 7.58–7.51 (m, 4H; H-3, H-7 Dansyl, 2×H Fmoc); 7.41 (t, $J = 7.5$ Hz, 2H; Fmoc); 7.38 (brs, 1H; NH); 7.34–7.30 (m, 2H; Fmoc); 7.21 (d, $J = 7.5$ Hz, 1H; H-6 Dansyl); 7.28 (brs, 1H; NH); 6.56 (brs, 1H; NH); 6.34 (brs, 1H; NH); 5.69 (brs, 1H; NH); 5.28–5.18 (m, 2H; $SCH_3CH$=GerGer); 5.09 (m, 6H; C=C*H*, GerGer); 4.48–4.35 (m, 4H; 2×α-CH Cys, 2×H Fmoc); 4.25–4.15 (m, 3H; 2×α-CH Ser, 1×H Fmoc); 3.67 (m, 4H; 2×β-CH$_2$ Ser); 3.21–3.08 (m, 8H; 2×β-CH$_2$ Cys, 2×S$CH_2$ GerGer); 3.01 (m, 2H; NHC$H_2$CH$_2$NH); 2.91 (s, 6H; N(C$H_3$)$_2$ Dansyl); 2.84 (m, 2H; NHCH$_2$C$H_2$NH); 2.10–1.85 (m, 24H; =C(CH$_3$)C$H_2$CH$_2$, CH$_2$C$H_2$CH=); 1.67 (s, 6H; =C(CH$_3$)C$H_3$ *trans*); 1.59 (s, 30H; =C-(CH$_3$)C$H_3$ *cis*) ppm; $^{13}$C NMR ($CDCl_3$, 100 MHz, 300 K): $\delta = 170.7$ (CH$_2$NH*C*O); 167.0, 166.8, 165.3 (3×C=O); 157.3 (CH$_2$O*C*NH); 151.8 (C-5 Dansyl); 143.5 (C-8a, C-9a Fmoc); 141.2, 140.3 (C-4a, C-4b Fmoc); 135.4, 135.3 (2×*C*=CH); 134.9 (2×*C*=CH); 134.9 (C-1 Dansyl); 131.3, 131.2 (4×*C*=CH), 130.6 (C-8 Dansyl); 129.8 (C-2, C-4a Dansyl); 129.2 (C-8a Dansyl); 128.3 (C-3 Dansyl); 127.8 (2×C=*C*H); 127.7 (C-3, C-6 Fmoc); 127.1 (C-2 Fmoc); 127.0 (C-7 Fmoc); 126.6, 126.1 (C-1, C-8 Fmoc); 124.4, 124.2 (2×C=*C*H); 124.1 (2×C=*C*H); 123.8 (C-7 Dansyl); 123.6, 123.1 (2×C=*C*H); 119.9 (C-4, C-5 Fmoc); 117.4 (C-4 Dansyl); 111.1 (C-6 Dansyl); 67.7 (C-10 Fmoc); 62.0 (β-CH$_2$ Ser); 46.8 (C-9 Fmoc); 45.3 (N(*C*H$_3$)$_2$); 42.4 (NH*C*H$_2$*C*H$_2$NH); 39.7 (6×=C-(CH$_3$)*C*H$_2$CH$_2$); 39.6 (2×β-CH$_2$ Cys); 29.6 (2×S*C*H$_2$C=); 26.7, 26.6, 26.5 (6×CH$_2$*C*H$_2$CH=); 25.7 (2×=C(CH$_3$)*C*H$_3$ *trans*); 17.6 (2×=C(CH$_3$)*C*H$_3$ *cis*); 16.2, 16.0, 16.0 (6×=(CH$_2$)*C*H$_3$ *cis*) ppm (signals for 4×α-*C*H were not detectable); ESI MS (+ve): *m*/*z* calcd for $C_{81}H_{114}N_7O_{10}S_3$ [*M*+H]: 1440.8; found: 1440.7; MALDI-TOF MS (DHB): calcd for $C_{81}H_{113}N_7O_{10}S_3Na$ [*M*+Na]: 1462.7; found: 1462.4.

**Synthesis of Fmoc-Ser-Cys(GerGer)-Ser-Cys(GerDansyl)-OMe (11)**: HOBt (8.9 mg, 66 μmol), EDC·HCl (9.5 mg, 49 μmol) and triethylamine (7.0 μL, 49 μmol) were added to an ice-cooled solution of Fmoc-Ser-Cys-(GerGer)-Ser-OH (**7**; 26 mg, 33 μmol) and NH$_2$-Cys(Ger-Dansyl)-OMe (**8**; 15.4 mg, 30 μmol) in dichloromethane (5 mL). The reaction was stirred for an additional 1 h at 0 °C and 5 h at RT. Subsequently, the organic layer was diluted with dichloromethane (50 mL) and extracted with aqueous ammonium chloride. The organic layer was dried over $Na_2SO_4$, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (firstly on silica, ethyl acetate → $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (20:1); secondly on alumina(III), $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (17:1)) to yield the pure title compound **11** as an off-white solid (15 mg, 12 μmol, 40%). TLC: $R_f = 0.10$ (alumina, $CH_2Cl_2/CH_3OH$ (25:1)); $[\alpha]_D^{20} = -15.6$ ($c = 0.25$, $CHCl_3$); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta = 8.62$ (brs, 1H; H-4 Dansyl); 8.39 (brs, 1H; H-8 Dansyl); 8.24 (d, $J = 7.2$ Hz, 1H; H-2 Dansyl); 7.8–7.2 (brs, 2H; NH); 7.75 (d, $J = 7.7$ Hz, 2H; Fmoc); 7.58 (m, 4H; H-3 Dansyl, H-7 Dansyl, 2×H Fmoc); 7.39 (t, $J = 7.5$ Hz, 2H; Fmoc); 7.30 (m, 3H; 2×H Fmoc, H-6 Dansyl); 7.1 (brs, 1H; NH); 5.90 (d, $J = 7.0$ Hz, 1H; NH Fmoc); 5.50 (t, $J = 5.5$ Hz, 1H; NH-Dansyl); 5.19 (t, $J = 7.6$ Hz, 1H; C=C*H*); 5.15 (t, $J = 7.3$ Hz, 1H; C=C*H*); 5.11 (m, 4H; C=C*H*); 4.79 (m, 1H; α-C*H* Cys); 4.62 (m, 2H; α-C*H* Cys, α-C*H* Ser); 4.41 (m, 1H; α-C*H* Ser); 4.35 (m, 2H; H-10 Fmoc); 4.17 (m, 1H; H-9 Fmoc); 4.13 (m, 1H; β-C$H_{2a}$ Ser); 4.03 (m, 1H; β-C$H_{2a}$ Ser); 3.76 (m, 2H; 2×β-C$H_{2b}$ Ser); 3.75 (s, 3H; OC$H_3$); 3.39 (d, 2H; C$H_2$NH-Dansyl); 3.17 (m, 4H; S$CH_2$ GerGer, S$CH_2$ Ger-Dansyl); 3.01–2.84 (m, 10H; N(C$H_3$)$_2$, 2×β-C$H_2$ Cys); 2.09–1.84 (m, 16H; =C(CH$_3$)C$H_2$CH$_2$, CH$_2$C$H_2$CH=); 1.67 (s, 3H; =C(CH$_3$)C$H_3$ *trans*); 1.64, 1.60, 1.59, 1.59, 1.58 (5×s, 15H; 5×C$H_3$ *cis*); 1.40 (s, 3H; C$H_3$CCH$_2$-Dansyl) ppm; $^{13}$C NMR ($CDCl_3$, 150 MHz, 300 K): $\delta = 170.4$ (CH$_3$O*C*=O); 152.2 (C-5 Dansyl); 143.6 (C-8a, C-9a Fmoc); 140.8 (C-4a, C-4b Fmoc); 140.2 (*C*=CH); 138.9 (*C*=CH); 136.7 (*C*=CH); 135.2 (C-1 Dansyl); 134.8 (*C*=CH); 130.8 (*C*=CH); 130.7 (*C*=

CH); 130.1 (C-8 Dansyl); 130.0 (C-2 Dansyl); 129.7, 129.3 (C-4a, C-8a Dansyl); 127.6 (C-3 Dansyl); 127.3 (C=*C*H); 127.5 (C-3, C-6 Fmoc); 126.9 (C-2, C-7 Fmoc); 124.8 (C-1, C-8 Fmoc); 124.1, 124.0, 124.0, 123.9 (4×C=*C*H); 124.2 (C-7 Dansyl); 119.7 (C-4, C-5 Fmoc); 120.4 (C-4 Dansyl); 118.9 (C=*C*H); 115.8 (C-6 Dansyl); 67.3 (C-10 Fmoc); 62.9, 62.8 (2×β-*C*H$_2$ Ser); 55.9, 55.0, 54.8, 51.5 (4×α-*C*H); 52.7 (O*C*H$_3$); 50.8 (C=CH*C*H$_2$NH); 47.1 (C-9 Fmoc); 46.4 (N(*C*H$_3$)$_2$); 40.1, 29.9, 29.5, 29.5 (2×β-*C*H$_2$ Cys, 2×S*C*H$_2$CH=); 39.7, 39.4, 39.4, 39.3 (4×=C(CH$_3$)*C*H$_2$CH$_2$); 26.9, 26.7, 26.5, 25.6 (3×CH$_2$*C*H$_2$CH=); 25.6 (=C(CH$_3$)*C*H$_3$ *trans*); 17.8 (=C(CH$_3$)CH$_3$ *cis*), 16.4, 16.0, 15.8, 15.8 (4×=C(CH$_2$)*C*H$_3$ *cis*); 14.2 (=C-(*C*H$_3$)CH$_2$NH) ppm; ESI MS (+ve): *m*/*z* calcd for $C_{70}H_{95}N_6O_{11}S_3$ [*M*+H]: 1291.6; found: 1291.5; calcd for $C_{70}H_{94}N_6NaO_{11}S_3$ [*M*+Na]: 1313.6; found: 1313.6; MALDI-TOF MS (DHB): calcd for [*M*+Na]: 1313.6; found: 1314.3; calcd for [*M*+K]: 1329.6; found: 1330.3.

**Synthesis of Fmoc-Ser-Cys(GerGer)-Ser-Cys(FarDansyl)-OMe (12)**: HOBt (18 mg, 133 μmol), EDC·HCl (26 mg, 133 μmol) and triethylamine (12.5 μL, 89 μmol) were added to an ice-cooled solution of Fmoc-Ser-Cys(GerGer)-Ser-OH (**7**; 70 mg, 89 μmol) and NH$_2$-Cys(Far-Dansyl)-OMe (**9**; 52 mg, 89 μmol) in dichloromethane (5 mL) and DMF (5 mL). The reaction mixture was stirred for an additional 1 h at 0 °C and 5 h at RT. Subsequently, the organic layer was diluted with dichloromethane (100 mL) and extracted with aqueous ammonium chloride. The organic layer was dried over $Na_2SO_4$, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (9:1)), to yield the pure title compound **12** as an off-white solid (15 mg, 12 μmol, 40 %). TLC: 0.35 ($CH_2Cl_2/CH_3OH$ (19:1)); $[\alpha]_D^{20} = -17.7$ ($c = 1.00$, $CHCl_3$); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta = 8.56$ (br s, 1H; H-4 Dansyl); 8.34 (br s, 1H; H-8 Dansyl); 8.23 (d, $J = 7.2$ Hz, 1H; H-2 Dansyl); 7.74 (d, $J = 7.2$ Hz, 2H; Fmoc); 7.55 (m, 4H; H-3 Dansyl, H-7 Dansyl, 2×H Fmoc); 7.4–7.2 (br s, 3H; NH); 7.38 (t, $J = 7.5$ Hz, 2H; Fmoc); 7.29 (t, $J = 6.9$ Hz, 2H; Fmoc) 7.21 (br s, 1H; H-6 Dansyl); 5.90 (d, $J = 6.5$ Hz, 1H; NH Fmoc); 5.20 (br s, 1H; NH-Dansyl); 5.17 (m, 3H; C=*CH*); 5.09 (m, 3H; C=*CH*); 5.00 (t, $J = 6.6$ Hz, 1H; C=*CH*); 4.75 (m, 1H; ∞-*CH* Cys); 4.68 (m, 2H; ∞-*CH* Cys, α-*CH* Ser); 4.43 (m, 1H; α-*CH* Ser); 4.37 (d, $J = 6.7$ Hz, 2H; H-10 Fmoc); 4.19 (t, $J = 6.7$ Hz, 1H; H-9 Fmoc); 4.09 (m, 1H; β-*CH*$_{2a}$ Ser); 4.00 (m, 1H; β-*CH*$_{2a}$ Ser); 3.75 (m, 2H; 2×β-*CH*$_{2b}$ Ser); 3.74 (s, 3H; O*CH*$_3$); 3.39 (d, 2H; *CH*$_2$NH-Dansyl); 3.16 (m, 4H; S*CH*$_2$ GerGer, S*CH*$_2$ Ger-Dansyl,); 2.99–2.84 (m, 4H; 2×β-*CH*$_2$ Cys); 2.90 (br s, 6H; N-(*CH*$_3$)$_2$); 2.07–1.82 (m, 20H; =C(CH$_3$)*CH*$_2$CH$_2$, CH$_2$*CH*$_2$CH=); 1.67 (s, 3H; =C(CH$_3$)*CH*$_3$ *trans*); 1.64, 1.63, 1.59, 1.58, 1.58, 1.52 (6×s, 15H; 5×*CH*$_3$ *cis*); 1.40 (s, 3H; *CH*$_3$CCH$_2$-Dansyl) ppm; $^{13}$C NMR ($CDCl_3$, 125 MHz, 300 K): $\delta = 170.4$ (CH$_3$O*C*=O); 152.4 (C-5 Dansyl); 143.6 (C-8a, C-9a Fmoc); 140.7 (C-4a, C-4b Fmoc); 140.1 (*C*=CH); 138.8 (*C*=CH); 135.7 (*C*=CH); 134.6 (*C*=CH); 131.7 (*C*=CH); 130.7 (*C*=CH); 130.1 (C-8 Dansyl); 130.1 (C-2 Dansyl); 129.5, 129.0 (C-4a, C-8a Dansyl); 127.8 (C-3 Dansyl); 128.7 (C=*C*H); 128.3 (C-3, C-6 Fmoc); 127.6 (C-2, C-7 Fmoc); 125.4 (C-1, C-8 Fmoc); 125.2, 124.8, 124.3, 124.3, 124.2 (5×C=*C*H); 123.8 (C-7 Dansyl); 120.4 (C-4, C-5 Fmoc); 120.3 (C-4 Dansyl); 119.8 (C=*C*H); 115.6 (C-6 Dansyl); 67.7 (C-10 Fmoc); 63.3, 63.2 (2×β-*CH*$_2$ Ser); 56.1, 55.1, 53.7, 52.0 (4×α-*C*H); 53.2 (O*C*H$_3$); 51.8 (C=CH*C*H$_2$NH); 47.4 (C-9 Fmoc); 45.7 (N(*C*H$_3$)$_2$); 33.1, 33.0, (2×β-*C*H$_2$); 29.9, 29.9 (2×S*C*H$_2$CH=); 39.8, 39.8, 39.7, 39.5, 39.4 (5×=C(CH$_3$)*C*H$_2$CH$_2$); 27.2, 26.9, 26.6, 26.6, 26.1 (5×CH$_2$*C*H$_2$CH=); 26.2 (=C(CH$_3$)*C*H$_3$ *trans*); 17.7 (=C(CH$_3$)CH$_3$ *cis*), 16.5, 16.0, 16.0, 15.9, 15.0 (5×=C(CH$_2$)*C*H$_3$ *cis*); 14.3 (=C-(*C*H$_3$)CH$_2$NH) ppm; ESI MS (+ve): *m*/*z* calcd for $C_{75}H_{103}N_6O_{11}S_3$ [*M*+H]: 1359.7; found: 1359.6; calcd for $C_{75}H_{102}N_6NaO_{11}S_3$ [*M*+Na]: 1381.7; found: 1381.7; MALDI-TOF MS (DHB): calcd for [*M*+Na]: 1381.7; found: 1382.5; calcd for [*M*+K]: 1397.6; found: 1398.4.

**Synthesis of Fmoc-Cys(S*t*Bu)-Glu(Fm)-OH (15)**: DCC (376 mg, 1.82 mmol) was added to an ice-cooled solution of Fmoc-Cys(S*t*Bu) (750 mg, 1.74 mmol) and *N*-hydroxysuccinimide (200 mg, 1.74 mmol) in DME (10 mL). The solution was stirred for 1 h and subsequently allowed to warm to RT and stirred for an additional hour. The solution was then cooled in an ice bath and the precipitated dicyclohexylurea (DCU) was removed by filtration. The solvent was evaporated under reduced pressure and the crude product (Fmoc-Cys(S*t*Bu)-Osu (**13**)) was used without further purification. The activated acid and NH$_2$-Glu(Fm)-OH (**14**; 678 mg, 2.09 mmol) were dissolved in dichloromethane (20 mL) and the solution was cooled in an ice bath. Subsequently, triethylamine (293 μL, 2.09 mmol) was added dropwise and the reaction mixture was allowed to warm to RT and stirred overnight. The reaction mixture was diluted with dichloromethane (100 mL) and subsequently extracted twice with 1 M HCl. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2$/formic acid (9:1)) to yield the pure title compound **15** as an off-white solid (720 mg, 0.974 mmol, 56 %). TLC: $R_f = 0.25$ ($CH_2Cl_2$/formic acid (20:1)); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta = 8.50$ (br s, 1H; NH amide); 7.72 (d, 2H; Fmoc); 7.72 (d, 2H; Fm); 7.54 (d, 2H; Fmoc); 7.54 (d, 2H; Fm); 7.37 (t, 2H; Fmoc); 7.37 (t, 2H; Fm); 7.27 (t, 2H; Fmoc); 7.27 (t, 2H; Fm); 5.92 (br d, 1H; NH carbamate); 4.60 (m, 1H; α-*CH* Cys); 4.41–4.31 (m, 5H; H-10 Fmoc, H-10 Fm, α-*CH* Glu); 4.19, 4.17 (2×t, 2H; H-9 Fmoc, H-9 Fm); 3.11 (m, 1H; β-C*H*$_{2a}$ Cys); 3.07 (m, 1H; β-C*H*$_{2b}$ Cys); 2.58 (m, 1H; γ-C*H*$_{2a}$ Glu); 2.53 (m, 1H; γ-C*H*$_{2b}$ Glu); 2.29 (m, 1H; β-C*H*$_{2a}$ Glu); 2.11 (m, 1H; β-C*H*$_{2b}$ Glu); 1.31 (s, 9H; SC(*CH*$_3$)$_3$) ppm; $^{13}$C NMR ($CDCl_3$, 125 MHz, 300 K): $\delta = 174.0$, 173.2, 170.6 (*C*OOH, *C*=O Fm, *C*=O Cys); 156.3 (*C*=O Fmoc); 143.6, 143.5 (C-8a, C-9a Fmoc; C-8a, C-9a Fm); 141.1, 141.1 (C-4a, C-4b Fmoc; C-4a, C-4b Fm); 127.8, 127.7 (C-3, C-6 Fmoc; C-3, C-6 Fm); 127.1, 127.1 (C-2, C-7 Fmoc; C-2, C-7 Fm); 125.1, 125.0 (C-1, C-8 Fmoc; C-1, C-8 Fm); 120.0, 120.0 (C-4, C-5 Fmoc; C-4, C-5 Fm); 67.6, 66.9 (C-10 Fmoc, C-10 Fm); 54.6 (α-*C*H Cys); 52.1 (α-*C*H Glu); 48.5 (S*C*(CH$_3$)$_3$); 46.9, 46.6 (C-9 Fmoc, C-9 Fm); 42.1 (β-*C*H$_2$ Cys); 30.3 (γ-*C*H$_2$ Glu); 29.8 (SC(*CH*$_3$)$_3$); 26.6 (β-*C*H$_2$ Glu) ppm; ESI MS (+ve): *m*/*z* calcd for $C_{41}H_{43}N_2O_7S_2$ [*M*+H]: 739.2; found: 739.1; HR FAB MS (3-NBA): calcd for $C_{41}H_{43}N_2O_7S_2$ [*M*+H]: 739.2433; found: 739.2491.

**Synthesis of Aloc-Cys(S*t*Bu)-OH (16)**: Allyl chloroformate (487 μL, 4.57 mmol) was added dropwise to a solution of H$_2$N-Cys(S*t*Bu)-OH (500 mg, 2.61 mmol) in ethylacetate (50 mL). The reaction mixture was stirred under reflux for 12 h. The solvent was then evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2$/acetic acid (10:1)) to yield the pure title compound **16** as a colourless oil (551 mg, 1.88 mmol, 72 %). TLC: $R_f = 0.44$ ($CH_2Cl_2$/acetic acid (20:1)); $[\alpha]_D^{RT} = +66.0$ ($c = 1.0$, $CHCl_3$); $^1$H NMR ($CDCl_3$, 400 MHz, 300 K): $\delta = 8.60$ (br s, 1H; COO*H*); 7.89 (d, $J = 8.3$ Hz, 1H; NH); 6.30–6.24 (ddd, $J_1 = 17.1$, $J_2 = 11.8$, $J_3 = 5.3$ Hz, 1H; CH$_2$*CH*=); 5.70–5.54 (dd, $J_1 = 13.6$, $J_2 = 1.0$ Hz, 2H; CH=*CH*$_2$); 4.85 (d, $J = 5.0$ Hz, 2H; *CH*$_2$CH=); 4.59–4.54 (m, 1H; α-CH); 3.49–3.45 (dd, $J_1 = 9.0$, $J_2 = 4.1$ Hz, 1H, β-CH$_{2a}$); 3.37–3.31 (dd, $J_1 = 9.8$, $J_2 = 3.3$ Hz, 1H, β-CH$_{2b}$); 1.68 (s, 9H; SC(*CH*$_3$)$_3$) ppm; $^{13}$C NMR ($CDCl_3$, 100 MHz, 300 K): $\delta = 173.1$ (COOH); 156.5 (OCONH); 134.3 (=*C*HCH$_2$); 117.7 (*CH*$_2$=CH); 54.3 (α-CH); 48.5 (O*C*H$_2$CH=, *C*(CH$_3$)$_3$); 42.3 (β-CH$_2$); 29.9 (C(*CH*$_3$)$_3$) ppm; HR FAB MS (3-NBA): calcd for $C_{11}H_{19}NO_4S_2$ [*M*+H]: 294.0834; found: 294.0842.

**Synthesis of Aloc-Cys(S*t*Bu)-Glu(All)-OH (17)**: DCC (348 mg, 1.69 mmol) was added to an ice-cooled solution of Aloc-Cys(S*t*Bu)-OH (**16**; 450 mg, 1.53 mmol) and *N*-hydroxysuccinimide (176 mg, 1.53 mmol) in dimethoxyethane (10 mL). The solution was stirred for 2 h and subsequently allowed to warm to RT and stirred for an additional hour. The solution was then cooled in an ice bath and the precipitated DCU was removed by filtration. The solvent was evaporated under reduced pressure. The crude product Aloc-Cys(S*t*Bu)-OSu and H$_2$N-Glu(All)-OH·HCl (443 mg, 1.53 mmol) were dissolved in dichloromethane (20 mL) and the solution was cooled in an ice bath. Subsequently, triethylamine (425 μL, 3.07 mmol) was added dropwise and the reaction mixture was allowed to come to RT and stirred overnight. The reaction mixture was diluted with dichloromethane (100 mL) and extracted with 1 M HCl and half-saturated sodium carbonate solution. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2$/acetic acid (10:1)) to yield the pure title compound as a white solid (461 mg, 0.997 mmol, 65 %). TLC: $R_f = 0.15$ ($CH_2Cl_2$/acetic acid (20:1)); $[\alpha]_D^{20} = -30.2$ ($c = 1.1$, $CHCl_3$); m.p. 83–85 °C; $^1$H NMR ($CDCl_3$, 400 MHz, 300 K): $\delta = 9.66$ (br s, 1H; COOH); 7.36 (d, $J = 7.8$ Hz, 1H; NH); 6.00 (d, $J = 8.5$ Hz, 1H; NH); 5.99–5.79 (ddd, $J_1 = 17.0$, $J_2 = 10.6$, $J_3 = 5.3$ Hz, 2H; 2×CH$_2$*CH*=); 5.26–5.12 (m, 4H; 2×CH=*CH*$_2$); 4.60–4.40 (m, 2H; α-CH Glu, α-CH Cys); 4.50 (d, $J = 5.5$ Hz, 4H; 2×*CH*$_2$CH=); 3.02 (m, 2H; β-CH$_2$ Cys); 2.41–2.39 (m, 2H; γ-CH$_2$ Glu); 2.21–2.18 (m, 1H; β-CH$_{2a}$

Glu); 2.08–1.99 (m, 1H; β-$CH_{2b}$ Glu); 1.24 (s, 9H; SC($CH_3$)$_3$) ppm; $^{13}$C NMR ($CDCl_3$, 100 MHz, 300 K): $\delta$=173.9 (CHCONH); 172.7 (COOH); 171.3 ($CH_2CO_2$); 156.0 ($CH_2OCO$); 132.4, 131.8 (2×$CH$=$CH_2$); 118.3, 117.7 (2×$CH_2$=CH); 65.3 (α-CH Cys); 51.9 (α-CH Glu); 48.2, 48.1 (2×O$CH_2$CH=, $C$($CH_3$)$_3$); 42.1 (β-$CH_2$ Cys); 30.2 (γ-$CH_2$ Glu); 29.6 (C($CH_3$)$_3$); 26.8 (β-$CH_2$ Glu) ppm; HR FAB MS (3-NBA): calcd for $C_{19}H_{30}N_2O_7S_2Na$ [$M$+Na]: 485.1392; found: 485.1409.

**Synthesis of Aloc-Cys(S*t*Bu)-Glu(All)-Ser-Cys(GerGer)-Ser-Cys-(GerGer)NH-$CH_2$-$CH_2$-NHDansyl (18)**: Diethylamine (400 μL) was added to an ice-cooled solution of Fmoc-Ser-Cys(GerGer)-Ser-Cys-(GerGer)-NH-$(CH_2)_2$-NHDansyl (35 mg, 0.024 mmol) in dichloromethane (1.6 mL). After the mixture had been stirred for 1 h at 0 °C and 1 h at RT, the product $H_2$N-Ser-Cys(GerGer)-Ser-Cys(GerGer)-NH-$(CH_2)_2$-NHDansyl had built up quantitatively (TLC control). The solvent was then co-evaporated with toluene and the crude product was dissolved in dichloromethane (1 mL) and cooled in an ice bath. Aloc-Cys(S*t*Bu)-Glu-(All)-OH (**17**; 11.2 mg, 0.024 mmol), HOBt (3.9 mg, 0.029 mmol), HBTU (9.2 mg, 0.029 mmol) and triethylamine (6.6 μL, 0.048 mmol) were added to this ice-cooled solution. The solution was stirred for 1 h and was subsequently allowed to warm to RT. After the mixture was stirred for 12 h at RT, the solvent was co-evapporated with toluene under reduced pressure. The crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2$→$CH_2Cl_2$/$CH_3OH$ (10:0.2)) to yield the title compound **18** as a pale-yellow oil (24 mg, 0.015 mmol, 61 %). TLC: $R_f$=0.16 ($CH_2Cl_2$/$CH_3OH$ (10:0.5)); $[\alpha]_D^{20}$=−23.7 ($c$=1.0, $CHCl_3$); m.p. 170–173 °C; $^1$H NMR ($CDCl_3$, 400 MHz, 300 K): $\delta$=8.50 (d, $J$=8.3 Hz, 1H; H-4 Dansyl); 8.24 (d, $J$=8.5 Hz, 1H; H-8 Dansyl); 8.15 (d, $J$=7.2 Hz, 1H; H-2 Dansyl); 7.49–7.46 (m, 2H; H-3, H-7 Dansyl); 7.15 (d, $J$=7.5 Hz, 1H; H-6 Dansyl); 5.92–5.82 (m, 2H; 2×O$CH_2$C$H$=); 5.30–5.18 (m, 4H; 2×O$CH_2$CH=C$H_2$); 5.06 (m, 8H; 2×S$CH_3$C$H$=, 6×C=C$H$ GerGer); 4.60–4.30 (m, 11H; 3×α-CH Cys, 2×α-CH Ser, 2×$CH_{2a}$ Ser, 2×O$CH_2$CH=); 3.60 (m, 2H; β-$CH_{2b}$ Ser); 3.35 (m, 3H; 3×β-$CH_{2b}$ Cys); 3.25–2.95 (m, 10H; 3×β-$CH_{2b}$ Cys, α-CH Glu, 2×S$CH_2$ GerGer, NHC$H_2$$CH_2$NH); 2.85 (s, 6H; N($CH_3$)$_2$ Dansyl); 2.78 (m, 2H; NHC$H_2$C$H_2$NH); 2.47 (t, $J$=5.8 Hz, 2H; γ-$CH_2$ Glu); 2.03–1.93 (m, 24H; =C($CH_3$)C$H_2$$CH_2$, $CH_2$C$H_2$CH=); 1.75 (m, 2H; β-$CH_2$ Glu); 1.65 (s, 6H; =C($CH_3$)C$H_3$ *trans*); 1.57 (s, 30H; =C($CH_3$)C$H_3$ *cis*); 1.30 (s, 9H; SC($CH_3$)$_3$) ppm; $^{13}$C NMR ($CDCl_3$, 150 MHz, 300 K): $\delta$=157.2 (C-5 Dansyl); 135.3, 134.9 (2×$CH$=$CH_2$ Aloc, All); 131.2 (C-8 Dansyl); 129.9, 129.8 (2×SC=$CH$); 129.4 (C-2 Dansyl); 128.5 (C-3 Dansyl); 124.3, 124.1 (6×C=$CH$); 123.9 (C-7 Dansyl); 118.4 (C-4 Dansyl); 117.9, 117.8 (2×$CH_2$=CH, Aloc, All); 115.8 (C-6 Dansyl); 55.3 (2×O$CH_2$CH=, 2×β-$CH_2$ Ser); 43.3 (N($CH_3$)$_2$); 42.3 (NHC$H_2$$CH_2$NH); 39.7 (6×=C($CH_3$)$CH_2$$CH_2$); 38.6 (NH$CH_2$$CH_2$NH); 36.6 (3×β-$CH_2$ Cys); 31.4 (γ-$CH_2$ Glu); 29.6 (SC($CH_3$)$_3$); 29.2 (2×S$CH_2$C); 27.1 (β-$CH_2$ Glu); 26.7, 25.6 (6×$CH_2$-$CH_2$CH=); 23.2 (2×=C($CH_3$)$CH_3$ *trans*); 18.4 (2×=C($CH_3$)$CH_3$ *cis*); 16.9, 17.0 (6×=C($CH_2$)$CH_3$ *cis*); ESI MS (+ve): $m/z$ calcd for $C_{85}H_{132}N_9O_{14}S_5$ [$M$+H]: 1662.8; found: 1662.7; MALDI-TOF MS (DHB): calcd for $C_{85}H_{131}N_9O_{14}S_5Na$ [$M$+Na]: 1684.8; found: 1684.5.

**Synthesis of Fmoc-Cys(S*t*Bu)-Glu(Fm)-Ser-Cys(GerGer)-Ser-Cys-(GerDansyl)-OMe (19)**

**Solution-phase synthesis**: Diethylamine (0.5 mL) was added to a solution of **11** (15 mg, 11.0 μmol) in dichloromethane (1.5 mL). The solution was stirred for 45 min and subsequently poured into a flask containing toluene (60 mL). The solvents were removed under reduced pressure, while the temperature was not allowed to exceed 40 °C. The solid was taken up in toluene again (60 mL) and the solvent was again removed under reduced pressure. After drying under high vacuum, the crude product was used without further purification. Deprotected **11** was dissolved in a mixture (2 mL) of DMF and dichloromethane (1:1) and **15** (16.3 mg, 22.0 μmol), HOBt·$H_2O$ (6.7 mg, 44 μmol) and EDC·HCl (4.2 mg, 22.0 μmol) were added at 0 °C. After the mixture had been stirred for 2 h, the reaction was stopped and the solvent was then removed under reduced pressure. The crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2$→$CH_2Cl_2$/$CH_3OH$ (20:1)) to yield the pure title compound **19** as an off-white solid (8.3 mg, 4.6 μmol, 42 %).

**Solid-phase synthesis**: Fmoc-Cys(Ger-Dansyl)-OH (**22**) was coupled by use of DIC/HOBt to deprotected Fmoc-4-hydrazineobenzoyl NovaGel resin **27** (100 mg, 0.56 mmol g$^{-1}$, 56 μmol). After removal of the Fmoc group, Fmoc-Ser(Trt)-OH (**25**), Fmoc-Cys(GerGer)-OH (**21**), Fmoc-Ser-(Trt)-OH (**25**) and Fmoc-Cys(S*t*Bu)-Glu(Fm)-OH (**15**) were consecutively coupled by using either HBTU/HOBt or DIC/HOBt. The Trt groups were removed and the resin was treated with a solution of $Cu(OAc)_2$ (5.4 mg, 29 μmol), pyridine (48 μL, 588 μmol) and methanol (240 μL, 5.9 mmol) in dichloromethane (7 mL) under an oxygen atmosphere for 2 h. The resin was filtered off, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, ethyl acetate/$CH_2Cl_2$ (1:1)→ethyl acetate) to yield the pure title compound **19** as an off-white solid (50 mg, 28 μmol, 50 %).

**19**: TLC: $R_f$=0.19 ($CH_2Cl_2$/ethyl acetate (1:1)); $[\alpha]_D^{20}$=−25.3 ($c$=0.3, $CHCl_3$); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta$=8.82 (brs, 1H; H-4 Dansyl); 8.59 (brs, 1H; H-8 Dansyl); 8.33 (brs, 1H; NH); 8.28 (d, $J$=7.2 Hz, 1H; H-2 Dansyl); 7.8–7.2 (brs, 4H; NH); 7.74 (d, $J$=7.7 Hz, 2H; Fmoc or Fm); 7.70 (d, $J$=7.5 Hz, 2H; Fmoc or Fm); 7.65 (brm, 2H; H-3 Dansyl, H-7 Dansyl); 7.56 (t, $J$=7.6 Hz, 2H; Fmoc or Fm); 7.50 (t, $J$=7.4 Hz, 2H; Fmoc or Fm); 7.39 (t, $J$=7.5 Hz, 2H; Fmoc or Fm); 7.35 (t, $J$=7.5 Hz, 2H; Fmoc or Fm); 7.27 (m, 4H; 2×H Fmoc, 2×H Fm); 7.22 (brs, 1H; H-6 Dansyl); 5.97 (brs, 1H; NH); 5.55 (brs, 1H; NH-Dansyl); 5.19 (t, $J$=7.2 Hz, 1H; S$CH_2$C$H$=GerGer); 5.13 (t, $J$=7.3 Hz, 1H; C=C$H$); 5.09 (m, 4H; C=C$H$); 4.71 (m, 1H; α-C$H$ Cys); 4.60 (m, 1H; α-C$H$ Cys); 4.55 (m, 1H; α-C$H$ Ser); 4.46 (m, 1H; α-C$H$ Ser); 4.37 (m, 4H; H-10 Fmoc, H-10 Fm); 4.31 (m, 1H; α-C$H$ Cys-SS*t*Bu); 4.27 (m, 1H; α-C$H$ Glu); 4.17 (m, 2H; H-9 Fmoc, H-9 Fm); 4.06 (m, 2H; 2×β-C$H_{2a}$ Ser); 3.82 (m, 2H; 2×β-C$H_{2b}$ Ser); 3.72 (s, 3H; OC$H_3$); 3.41 (d, $J$=5.7 Hz, 2H; C$H_2$NH-Dansyl); 3.25–2.99 (m, 14H; SC$H_2$ GerGer, SC$H_2$ GerDansyl, N(C$H_3$)$_2$, 2×β-C$H_{2a}$ Cys, β-C$H_2$ Cys-SS*t*Bu); 2.93 (m, 1H; β-C$H_{2b}$ Cys); 2.86 (m, 1H; β-C$H_{2b}$ Cys); 2.67 (m, 1H; γ-C$H_{2a}$ Glu); 2.56 (m, 1H; γ-C$H_{2b}$ Glu); 2.12 (brm, 2H; β-C$H_2$ Glu); 2.10–1.85 (m, 16H; =C($CH_3$)C$H_2$$CH_2$, $CH_2$C$H_2$CH=); 1.67 (s, 3H; =C($CH_3$)C$H_3$ *trans*); 1.66 (s, 3H; =C($CH_2$)C$H_3$ *cis*); 1.59 (s, 3H; =C($CH_3$)C$H_3$ *cis*); 1.58–1.57 (3×s, 9H; =C($CH_2$)C$H_3$ *cis*); 1.41 (s, 3H; C$H_3$C$CH_2$-Dansyl); 1.30 (s, 9H; SC($CH_3$)$_3$) ppm; $^{13}$C NMR ($CDCl_3$, 150 MHz, 300 K) $\delta$=171.4 ($CH_3$O$C$=O); 152.5 (C-5 Dansyl); 143.8 (C-8a, C-9a Fmoc; C-8a, C-9a Fm); 141.5 (C-4a, C-4b Fmoc; C-4a, C-4b Fm); 140.6 (*C*=CH); 139.3 (*C*=CH); 135.7 (*C*=CH); 135.4 (C-1 Dansyl); 135.3 (*C*=CH); 131.5 (*C*=CH); 131.0 (*C*=CH); 130.2 (C-8 Dansyl); 129.9 (C-2 Dansyl); 129.9, 129.5 (C-4a, C-8a Dansyl); 128.3 (C-3 Dansyl); 128.1 (C=*C*H); 128.1, 128.0 (C-3, C-6 Fmoc; C-3, C-6 Fm); 127.4, 127.4 (C-2, C-7 Fmoc; C-2, C-7 Fm); 125.4, 125.2 (C-1, C-8 Fmoc; C-1, C-8 Fm); 124.6 (2×C=*C*H); 124.1 (C=*C*H); 123.9 (C-7 Dansyl); 120.7 (C=*C*H); 120.4, 120.3 (C-4, C-5 Fmoc; C-4, C-5 Fm); 120.3 (C-4 Dansyl); 119.7 (C=*C*H); 115.9 (C-6 Dansyl); 68.2, 67.6 (C-10 Fmoc, C-10 Fm); 63.0, 62.2 (2×β-*C*$H_2$ Ser); 57.7, 56.3, 56.1, 55.9, 53.9, 52.4 (6×α-*C*H); 53.0 (O*C*$H_3$); 51.3 (C=CH*C*$H_2$NH); 49.3 (S*C*($CH_3$)$_3$); 47.4, 47.0 (C-9 Fmoc, C-9 Fm); 46.8 (N(*C*$H_3$)$_2$); 41.0, 33.1, 33.3, 33.0, 29.9 (3×β-*C*$H_2$ Cys, 2×S*C*$H_2$CH=); 40.1 (3×=C($CH_3$)*C*$H_2$$CH_2$); 38.9 (=C($CH_3$)$CH_2$*C*$H_2$); 31.5 (γ-*C*$H_2$ Glu); 30.0 (SC(*C*$H_3$)$_3$); 27.1 (3×$CH_2$*C*$H_2$CH=); 26.0 (=C($CH_3$)*C*$H_3$ *trans*); 25.9 ($CH_2$*C*$H_2$CH=); 25.8 (β-*C*$H_2$ Glu); 18.1 (=C($CH_3$)*C*$H_3$ *cis*), 16.5, 16.3, 16.3, 16.3 (4×=C($CH_2$)*C*$H_3$ *cis*); 14.7 (=C(*C*$H_3$)$CH_2$NH) ppm; ESI MS (+ve): $m/z$ calcd for $C_{96}H_{125}N_8O_{15}S_5$ [$M$+H]: 1789.8; found: 1789.4; calcd for $C_{96}H_{125}N_8NaO_{15}S_5$ [$M$+Na]: 1811.8; found: 1811.5; MALDI-TOF MS (DHB): calcd for [$M$+Na]: 1811.8; found: 1811.8; calcd for [$M$+K]: 1827.7; found: 1828.1.

**Synthesis of Fmoc-Cys(S*t*Bu)-Glu(Fm)-Ser-Cys(GerGer)-Ser-Cys-(FarDansyl)-OMe (20)**

**Solution-phase synthesis**: Diethylamine (0.5 mL) was added to a solution of **12** (20 mg, 14.7 μmol) in dichloromethane (1.5 mL). The solution was stirred for 45 min and subsequently poured into a flask containing toluene (60 mL). The solvents were removed under reduced pressure, while the temperature was not allowed to exceed 40 °C. The solid was taken up in toluene again (60 mL) and the solvent was again removed under reduced pressure. After drying under high vacuum, the crude product was used without further purification. Deprotected **12** was dissolved in a mixture (2 mL) of DMF and dichloromethane (1:1), then **15** (22.2 mg, 30.0 μmol), HOBt·$H_2O$ (9.2 mg, 60 μmol) and EDC·HCl (5.7 mg, 30.0 μmol) were added at 0 °C. After the mixture had been stirred for

2 h, the reaction was stopped and the solvent was removed under reduced pressure. The crude mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (20:1)) to yield the pure title compound **20** as an off-white solid (9.6 mg, 5.1 μmol, 35%).

**Solid-phase synthesis**: Fmoc-Cys(Far-Dansyl)-OH (**23**) was coupled by using DIC/HOBt to deprotected Fmoc-4-hydrazineobenzoyl NovaGel resin **27** (25 mg, 0.56 mmol $g^{-1}$, 14 μmol). After removal of the Fmoc group, Fmoc-Ser(Trt)-OH (**25**), Fmoc-Cys(GerGer)-OH (**21**), Fmoc-Ser-(Trt)-OH (**25**) and Fmoc-Cys(S*t*Bu)-Glu(Fm)-OH (**15**) were consecutively coupled by using either HBTU/HOBt or DIC/HOBt. The Trt groups were removed and the resin was treated with a solution of $Cu(OAc)_2$ (1.4 mg, 7.3 μmol), pyridine (12 μL, 147 μmol) and methanol (60 μL, 1.5 mmol) in dichloromethane (2 mL) under an oxygen atmosphere for 2 h. The resin was filtered off, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, ethyl acetate/$CH_2Cl_2$ (1:1)→ethyl acetate) to yield the pure title compound **20** as an off-white solid (9.1 mg, 4.9 μmol, 35%).

**20**: TLC: $R_f$=0.21 ($CH_2Cl_2$/ethyl acetate (1:1)); $[\alpha]_D^{20}=-24.7$ ($c$=0.25, $CHCl_3$); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta$=9.15 (brs, 1H; NH); 8.90 (brs, 1H; H-4 Dansyl); 8.38 (brs, 1H; H-8 Dansyl); 7.85 (d, $J$=7.2 Hz, 1H; H-2 Dansyl); 7.8–7.4 (brs, 4H; NH); 7.75–7.65 (m, 6H; 2×H Fmoc, 2×H Fm, H-3 Dansyl, H-7 Dansyl); 7.58 (m, 4H; 2×H Fmoc, 2×H Fm); 7.37 (m, 4H; 2×H Fmoc, 2×H Fm); 7.27 (m, 5H; 2×H Fmoc, 2×H Fm, H-6 Dansyl); 6.18 (brs, 1H; NH); 5.40 (brs, 1H; NH-Dansyl); 5.22 (m, 2H; C=C*H*); 5.10 (m, 4H; C=C*H*); 4.99 (m, 1H; C=C*H*); 4.75–4.00 (m, 14H; 3×α-C*H* Cys, 2×α-C*H* Ser, H-10 Fmoc, H-10 Fm, α-C*H* Glu, H-9 Fmoc, H-9 Fm, 2×β-C$H_{2a}$ Ser); 3.85–3.50 (m, 7H; 2×β-C$H_{2b}$ Ser, OC$H_3$ C$H_2$NH-Dansyl); 3.45–2.85 (m, 16H; SC$H_2$ GerGer, SC$H_2$ Ger-Dansyl, N(C$H_3$)$_2$, 2×β-C$H_2$ Cys, β-C$H_2$ Cys-S*t*Bu); 2.66–2.10 (m, 4H; γ-C*H* Glu, β-C$H_2$ Glu); 2.15–1.80 (m, 20H; =C(CH$_3$)C$H_2$CH$_2$, CH$_2$C$H_2$CH=); 1.68 (s, 3H; =C(CH$_3$)C$H_3$ *trans*); 1.62 (s, 3H; =C-(CH$_2$)C$H_3$ *cis*); 1.59 (s, 3H; =C(CH$_3$)C$H_3$ *cis*); 1.58–1.57 (3×s, 9H; =C-(CH$_2$)C$H_3$ *cis*); 1.33 (s, 3H; C$H_3$CCH$_2$-Dansyl); 1.28 (s, 9H; SC-(C$H_3$)$_3$) ppm; ESI MS (+ve): $m/z$ calcd for $C_{101}H_{133}N_8O_{15}S_5$ [$M$+H]: 1857.9; found: 1857.6; calcd for $C_{96}H_{132}N_8NaO_{15}S_5$ [$M$+Na]: 1879.8; found: 1879.6; MALDI-TOF MS (DHB): calcd for [$M$+Na]: 1879.8; found: 1879.8.

**Synthesis of Fmoc-Cys(GerGer)-OH (21)**: Triethylamine (206 μL, 1.47 mmol) was added dropwise to an ice-cooled solution of Fmoc-OSu (596 mg, 1.77 mmol) and $NH_2$-Cys(GerGer)-OH (580 mg, 1.47 mmol) in a mixture of methanol (30 mL) and dichloromethane (30 mL). After the reaction mixture had been stirred for an additional 4 h at 0°C, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (9:1)) to yield the pure title compound **21** as a white solid (720 mg, 1.17 mmol, 80%). TLC: $R_f$=0.14 ($CH_2Cl_2/CH_3OH$ (20:1)); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta$=7.74 (d, $J$=7.5 Hz, 2H; Fmoc); 7.60 (d, $J$=7.2 Hz, 2H; Fmoc); 7.38 (t, $J$=7.5 Hz, 2H; Fmoc); 7.29 (t, $J$=7.4 Hz, 2H; Fmoc); 5.80 (brs, 1H; NH carbamate); 5.21 (t, $J$=7.7 Hz, 1H; C=C*H*); 5.08 (m, 3H; C=C*H*); 4.55 (m, 1H; α-C*H* Cys); 4.38 (d, $J$=7.0 Hz, 2H; H-10 Fmoc); 4.22 (t, $J$=7.1 Hz, 1H; H-9 Fmoc); 3.19 (m, 2H; SC$H_2$CH=); 2.99 (m, 1H; β-C$H_{2a}$ Cys); 2.92 (m, 1H; β-C$H_{2b}$ Cys); 2.10–1.95 (m, 12H; =C(CH$_3$)C$H_2$C$H_2$CH=); 1.67, 1.64, 1.59, 1.58, 1.58 (5×s, 15H; C$H_3$) ppm; $^{13}$C NMR ($CDCl_3$, 125 MHz, 300 K): $\delta$=173.2 (*C*OOH); 156.1 (*C*=O Fmoc); 143.8 (C-8a, C-9a Fmoc); 141.1 (C-4a, C-4b Fmoc); 139.6 (*C*=CH); 135.0 (*C*=CH); 134.6 (*C*=CH); 130.9 (*C*=CH); 127.4 (C-3, C-6 Fmoc); 126.8 (C-2, C-7 Fmoc); 124.8 (C-1, C-8 Fmoc); 124.1 (C=*C*H); 123.9 (C=*C*H); 123.5 (C=*C*H); 119.6 (C-4, C-5 Fmoc); 119.4 (C=*C*H); 66.8 (C-10 Fmoc); 53.5 (α-*C*H); 46.8 (C-9 Fmoc); 39.4, 39.4, 39.3 (3×=C(CH$_3$)*C*H$_2$CH$_2$); 33.1 (β-*C*H$_2$ Cys); 29.7 (S*C*H$_2$CH=); 26.4, 26.3, 26.2 (3×CH$_2$*C*H$_2$CH=); 25.3 (=C(CH$_2$)*C*H$_3$ *trans*); 17.2, 15.7, 15.6, 15.6 (4×*C*H$_3$) ppm; HR-FAB MS (3-NBA): calcd for $C_{38}H_{49}NO_4SNa$ [$M$]: 638.3280; found: 638.3302.

**Synthesis of Fmoc-Cys(Ger-Dansyl)-OH (22)**: Triethylamine (51 μL, 0.366 mmol) was added dropwise to an ice-cooled solution of Fmoc-OSu (148 mg, 0.439 mmol) and $NH_2$-Cys(Ger-Dansyl)-OH (185 mg, 0.366 mmol) in a mixture of methanol (8 mL) and dichloromethane (8 mL). After the reaction mixture had been stirred for an additional 2 h at 0°C and 1 h at RT, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (12.5:1)) to yield the pure title compound **22** as a yellow oil (260 mg, 0.357 mmol, 97%). TLC: $R_f$=0.20 ($CH_2Cl_2/CH_3OH$ (17:1)); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta$=8.47 (d, $J$=8.2 Hz, 1H; H-4 Dansyl); 8.31 (d, $J$=8.5 Hz, 1H; H-8 Dansyl); 8.19 (d, $J$=7.2 Hz, 1H; H-2 Dansyl); 7.70 (brd, 2H; Fmoc); 7.57 (brd, 2H; Fmoc); 7.52–7.44 (2×t, $J$=8.0, $J$=7.7 Hz, 2H; H-3 Dansyl, H-7 Dansyl); 7.33 (brt, 2H; Fmoc); 7.23 (brt, 2H; Fmoc); 7.12 (d, $J$=7.2 Hz, 1H; H-6 Dansyl); 5.95 (brs, 1H; NH carbamate); 5.37 (brs, 1H; NH-Dansyl); 5.06 (m, 2H; C=C*H*); 4.50 (m, 1H; α-C*H* Cys); 4.32 (m, 2H; H-10 Fmoc); 4.17 (m, 1H; H-9 Fmoc); 3.36 (d, $J$=5.6 Hz, 2H; C$H_2$NH-Dansyl); 3.19–3.01 (m, 3H; SC$H_2$CH=, β-C$H_{2a}$ Cys); 2.92–2.78 (m, 1H; β-C$H_{2b}$ Cys); 2.83 (s, 6H; N(C$H_3$)$_2$) 1.84 (m, 4H; =C(CH$_3$)C$H_2$C$H_2$CH=); 1.50 (s, 3H; =C(CH$_2$)C$H_3$ *cis*); 1.33 (s, 3H; C$H_3$CCH$_2$-Dansyl) ppm; $^{13}$C NMR ($CDCl_3$, 125 MHz, 300 K): $\delta$=174.3 (*C*OOH); 153.3 (*C*=O carbamate) 151.7 (C-5 Dansyl); 143.8 (C-8a, C-9a Fmoc); 141.1 (C-4a, C-4b Fmoc); 139.4 (*C*=CH); 135.2 (C-1 Dansyl); 130.5 (*C*=CH); 130.1 (C-8 Dansyl); 129.9, 129.5 (C-4a, C-8a Dansyl); 129.4 (C-2 Dansyl); 128.2 (C-3 Dansyl); 127.6 (C-3, C-6 Fmoc); 127.0 (C=*C*H); 127.0 (C-2, C-7 Fmoc); 125.2 (C-1, C-8 Fmoc); 123.1 (C-7 Dansyl); 120.4, 119.8 (C-4 Dansyl, C-4, C-5 Fmoc); 119.0 (C=*C*H); 115.1 (C-6 Dansyl); 67.0 (C-10 Fmoc); 52.8 (α-*C*H); 50.9 (C=CH*C*H$_2$NH); 47.0 (C-9 Fmoc); 45.3 (N(*C*H$_3$)$_2$); 39.7, 38.6 (β-*C*H$_2$ Cys, =C(CH$_3$)*C*H$_2$CH$_2$); 29.8 (S*C*H$_2$CH=); 25.7 (CH$_2$*C*H$_2$CH=); 15.9 (=C(CH$_2$)*C*H$_3$ *cis*); 14.1 (=C(*C*H$_3$)CH$_2$NH) ppm; HR FAB MS (3-NBA): calcd for $C_{40}H_{45}N_3O_6S_2$ [$M$]: 727.2750; found: 727.2762.

**Synthesis of Fmoc-Cys(Far-Dansyl)-OH (23)**: Triethylamine (51 μL, 0.366 mmol) was added dropwise to an ice-cooled solution of Fmoc-OSu (8.2 mg, 24 μmol) and $NH_2$-Cys(Far-Dansyl)-OH (11.5 mg, 20 μmol) in a mixture of methanol (1 mL) and dichloromethane (1 mL). After the reaction mixture had been stirred for an additional 2 h at 0°C and 1 h at RT, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (20:1)) to yield the pure title compound **23** as a yellow oil (12 mg, 15 μmol, 75%). TLC: $R_f$=0.24 ($CH_2Cl_2/CH_3OH$ (17:1)); $^1$H NMR ($CDCl_3$+$CD_3OD$, 500 MHz, 300 K): $\delta$=8.52 (brs, 1H; H-4 Dansyl); 8.30 (brs, 1H; H-8 Dansyl); 8.15 (d, $J$=6.8 Hz, 1H; H-2 Dansyl); 7.70 (d, $J$=7.5 Hz, 2H; Fmoc); 7.55–7.46 (m, 4H; 2×Fmoc, H-3 Dansyl, H-7 Dansyl); 7.33 (t, $J$=7.2 Hz, 2H; Fmoc); 7.24 (t, $J$=7.5 Hz, 2H; Fmoc); 7.18 (brs, 1H; H-6 Dansyl); 5.10 (m, 2H; C=C*H*); 4.95 (m, 1H; C=C*H*); 4.46 (m, 1H; α-C*H* Cys); 4.32 (d, $J$=7.2 Hz, 2H; H-10 Fmoc); 4.17 (t, $J$=6.7 Hz, 1H; H-9 Fmoc); 3.32 (brd, 2H; C$H_2$NH-Dansyl); 3.28–3.06 (m, 3H; SC$H_2$CH=, β-C$H_{2a}$ Cys); 2.92–2.78 (m, 1H; β-C$H_{2b}$ Cys); 2.87 (s, 6H; N(C$H_3$)$_2$); 2.05–1.72 (m, 8H; =C-(CH$_3$)C$H_2$C$H_2$CH=); 1.57 (s, 3H; =C(CH$_2$)C$H_3$ *cis*); 1.46 (s, 3H; =C-(CH$_2$)C$H_3$ *cis*); 1.33 (s, 3H; C$H_3$CCH$_2$-Dansyl) ppm; MALDI-TOF MS (DHB): calcd for [$M$+H]: 796.3; found: 796.3; calcd for [$M$+Na]: 818.3; found: 818.4; HR FAB MS (3-NBA): calcd for $C_{45}H_{53}N_3O_6S_2$ [$M$+H]: 796.3454; found: 796.3480.

**Synthesis of Fmoc-Glu(OAll)-OH (24)**: Triethylamine (414 μL, 2.99 mmol) was added dropwise to an ice-cooled solution of Fmoc-OSu (500 mg, 1.49 mmol) and $H_2$N-Glu(All)-OH·HCl (334 mg, 2.99 mmol) in a mixture of methanol (1 mL) and dichloromethane (3 mL). After the reaction mixture had been stirred for an additional 2 h at 0°C and 1 h at RT, the solvent was evaporated under reduced pressure and the crude reaction mixture was purified by column chromatography (silica, $CH_2Cl_2 \rightarrow CH_2Cl_2/CH_3OH$ (10:1)) to yield the pure title compound **24** as a white oil (318 mg, 0.775 mmol, 52%). TLC: $R_f$=0.2 ($CH_2Cl_2/CH_3OH$ (10:1)); $[\alpha]_D^{20}=+66.0$ ($c$=1.0, $CHCl_3$); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): $\delta$=7.75 (d, $J$=7.2 Hz, 2H; Fmoc); 7.57 (t, $J$=7.5 Hz, 2H; Fmoc); 7.38 (t, $J$=7.3 Hz, 2H; Fmoc); 7.30 (t, $J$=7.5 Hz, 2H; Fmoc); 5.89–5.86 (ddd, $J_1$=17.0, $J_2$=11.5, $J_3$=5.2 Hz, 1H; CH$_2$C*H*=); 5.63 (d, $J$=7.5 Hz, 1H; NH); 5.31–5.21 (dd, $J_1$=13.6, $J_2$=1.0 Hz, 2H; CH=C$H_2$); 4.56 (d, $J$=5.2 Hz, 2H; H-10 Fmoc); 4.40–4.39 (m, 3H; H-9 Fmoc, OC$H_2$CH=); 4.20 (t, $J$=7.0 Hz, 1H; α-CH); 2.51–2.44 (m, 2H; γ-CH$_2$); 2.29–2.26 (m, 1H; β-CH$_{2a}$); 2.06–2.04 (m, 1H; β-CH$_{2b}$) ppm; $^{13}$C NMR ($CDCl_3$, 125 MHz, 300 K): $\delta$=170.4 (COOH); 160.8 (CH$_2$O*C*NH); 143.6, 141.3 (C-8a, C-9a, C-4a, C-4b Fmoc); 131.8 (=*C*HCH$_2$); 127.7 (C-2 Fmoc); 127.1 (C-7

Fmoc); 125.1 (C-1, C-8 Fmoc); 120.0 (C-4, C-5 Fmoc); 118.5 ($CH_2$=CH); 67.1 (C-10 Fmoc); 65.5 (α-CH); 50.7 ($COCH_2C$=); 47.1 (C-9 Fmoc), 30.9 (β-$CH_2$); 27.2 (γ-$CH_2$) ppm; HR FAB MS (3-NBA): calcd for $C_{23}H_{232}NO_6$ [*M*+H]: 410.1603; found: 410.1613.

**Synthesis of Fmoc-Cys(S*t*Bu)-Glu-Ser-Cys(GerGer)-Ser-Cys(GerGer)-NHCH$_2$-CH$_2$NH-Dansyl (33)**: The resin-bound hexapeptide **31** was synthesised as described for the resin-bound hexapeptides **41** and **42** (see Scheme 7) Cleavage from the resin: The resin was treated with 0.005 M $Cu(OAc)_2$ solution in THF and $H_2N$-$CH_2$-$CH_2$-NH-Dansyl (3 equiv) for 3 h under an oxygen atmosphere at room temperature. The solution was co-evaporated with toluene and the residue was dissolved in DCM. The organic phase was washed with 1 N HCl and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (silica, $CH_2Cl_2$→$CH_2Cl_2$/$CH_3OH$ (10:1)) to yield the title compound **33** as a pale-yellow solid (overall yield referring to the loading of the resin: 40%). TLC: $R_f$=0.46 ($CH_2Cl_2$/$CH_3OH$ (10:1)); $[\alpha]_D^{20}$=−23.7 (*c*=1.0, $CHCl_3$); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): δ=8.45 (br d, *J*=10.5 Hz, 1H; H-4 Dansyl); 8.23 (d, *J*=10.5 Hz, 1H; H-8 Dansyl); 8.11 (d, *J*=7.5 Hz, 1H; H-2 Dansyl); 7.71 (t, *J*=6.4 Hz, 2H; Fmoc); 7.57–7.45 (m, 4H; 2×H Fmoc, H-3 Dansyl, H-7 Dansyl); 7.34 (m, 4H; Fmoc); 7.11 (m, 2H; H-6 Dansyl, NH); 6.76 (br d, 1H; NH); 5.26 (m, 2H; 2×$SCH_2$=C*H*); 5.15 (m, 6H; C=C*H*); 4.63–4.18 (m, 9H; 3×α-C*H* Cys, 2×α-C*H* Ser, α-C*H* Glu, 3H Fmoc); 3.64–3.50 (m, 4H; 2×β-$CH_2$ Ser); 3.50 (m, 10H; S$CH_2$ GerGer, 3×β-$CH_2$ Cys); 2.80 (m, 10H; $CH_2CH_2$NHDansyl, N($CH_3$)$_2$); 2.26 (m, 4H; γ-$CH_2$ Glu, β-$CH_2$ Glu); 2.05–1.85 (m, 24H; 6×=C($CH_3$)$CH_2CH_2$, 6×$CH_2CH_2$CH=); 1.60 (s, 6H; 2×=C($CH_3$)$CH_3$ *trans*); 1.52 (s, 6H; 2×=C($CH_2$)$CH_3$ *cis*); 1.24–1.18 (m, 27H; 6×=C($CH_2$)$CH_3$ *cis*, SC($CH_3$)$_3$) ppm; ESI MS (+ve): *m*/*z* calcd for $C_{93}H_{134}N_9O_{14}S_5$ [*M*+H]: 1760.9; found: 1761.5; calcd for $C_{93}H_{133}N_9NaO_{14}S_5$ [*M*+Na]: 1782.8; found: 1783.9.

**Synthesis of Fmoc-Cys(S*t*Bu)-Glu-Ser-Cys(GerGer)-Ser-Cys(GerGer)-OMe (34)**: The resin-bound hexapeptide **31** was synthesised as described for the resin-bound hexapeptides **41** and **42** (see scheme 7) Cleavage from the resin: The resin was treated with MeOH (100 equiv) in 0.005 M $Cu(OAc)_2$/0.01 M pyridine solution in THF twice for 3 h under an oxygen atmosphere at room temperature. The solution was co-evaporated with toluene and the residue was dissolved in DCM. The organic phase was washed with 1 N HCl and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (silica, $CH_2Cl_2$→$CH_2Cl_2$/$CH_3OH$ (10:1)) to yield the title compound **34** as a pale-yellow solid (overall yield referring to the loading of the resin: 50%). TLC: $R_f$=0.48 ($CH_2Cl_2$/$CH_3OH$ (10:1)); $[\alpha]_D^{20}$=−58.0 (*c*=1.0, $CHCl_3$); m.p. 169–171 °C; $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): δ=7.67 (d, *J*=10.0 Hz, 2H; Fmoc); 7.57–7.54 (m, 4H; Fmoc); 7.35 (m, 2H; Fmoc); 5.25 (m, 1H; NH); 5.13 (m, 2H; $SCH_2CH$=GerGer); 5.02 (m, 6H; C=C*H*); 4.63–4.58 (m, 3H; 3×α-C*H* Cys); 4.35–4.45 (m, 5H; 2×α-C*H* Ser, 3×H Fmoc); 4.14 (m, 1H; α-C*H* Glu); 3.65 (m, 4H; 2×β-$CH_2$ Ser); 3.57 (s, 3H; O$CH_3$); 3.50 (m, 6H; 3×β-$CH_2$ Cys); 3.32 (m, 4H; 2×S$CH_2$ GerGer); 3.09 (m, 2H; γ-$CH_2$ Glu); 2.00–1.87 (br m, 26H; β-$CH_2$ Glu, 6×=C($CH_3$)$CH_2CH_2$, 6×$CH_2CH_2$CH=); 1.60 (s, 6H; =C($CH_3$)$CH_3$ *trans*); 1.55 (s, 6H; =C($CH_3$)$CH_3$ *cis*); 1.24–1.18 (2×s, 27H; 6×=C($CH_2$)$CH_3$ *cis*, SC($CH_3$)$_3$) ppm; ESI MS: (+ve): *m*/*z* calcd for $C_{80}H_{119}N_6O_{13}S_4$ [*M*+H]: 1499.7; found: 1499.5; MALDI-TOF MS (DHB): calcd for $C_{80}H_{118}N_6O_{13}S_4Na$ [*M*+Na]: 1521.8; found: 1521.6.

**Synthesis of H-Cys(S*t*Bu)-Glu-Ser-Cys(GerGer)-Ser-Cys(GerDansyl)-OMe (44)**: Diethylamine (0.5 mL) was added to a solution of **19** (12 mg, 6.7 μmol) in dichloromethane (1.5 mL). The solution was stirred for 45 min and subsequently poured into a flask containing toluene (60 mL). The solvents were removed under reduced pressure, while the temperature was not allowed to exceed 40 °C. The solid was taken up in toluene again (60 mL) and the solvent was again removed under reduced pressure. After drying of the crude product under high vacuum, the compound was purified by column chromatography (silica, ethyl acetate→$CH_3OH$/ethyl acetate (1:1)) to yield the product as a light-yellow waxy solid (7.8 mg, 5.6 μmol, 84%). TLC: $R_f$=0.48 ($CH_3OH$/ethyl acetate (1:4)); $^1$H NMR ($CDCl_3$, 500 MHz, 300 K): δ=8.51 (br s, 1H; H-4 Dansyl); 8.32 (br s, 1H; H-8 Dansyl); 8.19 (br s, 1H; H-2 Dansyl); 7.54, 7.49 (2×br s, 2H; H-3 Dansyl, H-7 Dansyl); 7.18 (br s, 1H; H-6 Dansyl); 5.09 (m, 6H; C=C*H*); 4.75–4.45 (m, 1H; 2×α-C*H* Cys, 2×α-C*H* Ser); 4.28 (m, 1H; α-C*H* Cys-SS*t*Bu); 4.25 (m, 1H; α-C*H* Glu); 4.05 (m, 2H; 2×β-$CH_{2a}$ Ser); 3.83 (m, 2H; 2×β-$CH_{2b}$ Ser); 3.72 (br s, 3H; O$CH_3$); 3.38 (br s, 2H; $CH_2$NH-Dansyl); 3.18–2.80 (m, 14H; 2×S$CH_2$, N($CH_3$)$_2$, 3×β-$CH_2$); 2.65–1.85 (m, 20H; γ-$CH_2$ Glu, β-$CH_2$ Glu, =C($CH_3$)$CH_2CH_2$, $CH_2CH_2$CH=); 1.70 (s, 3H; =C($CH_3$)$CH_3$ *trans*); 1.65 (s, 3H; =C($CH_2$)$CH_3$ *cis*); 1.60 (br s, 12H; 4×=C($CH_3$)$CH_3$ *cis*); 1.41 (br s, 3H; $CH_3CCH_2$-Dansyl); 1.30 (br s, 9H; SC($CH_3$)$_3$) ppm; ESI MS (+ve): *m*/*z* calcd for $C_{67}H_{104}N_8O_{13}S_5$ [*M*]: 1388.6; found: 1388.5; calcd for $C_{67}H_{103}N_8NaO_{13}S_5$ [*M*+Na−H]: 1410.6; found: 1410.5; MALDI-TOF MS (DHB): calcd for [*M*+Na−H]: 1410.6; found: 1410.6; calcd for [*M*+K−H]: 1426.6; found: 1426.5.

**Synthesis of H-Cys(S*t*Bu)-Glu-Ser-Cys(GerGer)-Ser-Cys(GerGer)NH-CH$_2$-CH$_2$-NHDansyl (45)**: Hexapeptide **18** (4 mg, $2.41\times10^{-3}$ mmol) was dissolved in THF (1 mL). Morpholine (2.4 equiv, 0.5 μL, $5.78\times10^{-3}$ mmol) and [Pd($Ph_3$)$_4$] (0.1 equiv, 0.28 mg, $0.24\times10^{-3}$ mmol) were added to the solution under an argon atmosphere. The solution was stirred for two hours in the dark at room temperature. After evaporation of the solvent under reduced pressure, the residue was purified by column chromatography (silica, $CH_2Cl_2$→$CH_2Cl_2$/$CH_3OH$ (10:1)) to yield the title compound as a white solid (3 mg, $1.95\times10^{-3}$ mmol, 81%). TLC: $R_f$=0.48 ($CH_2Cl_2$/$CH_3OH$ (10:1)); $^1$H NMR ($CDCl_3$+1% MeOD, 500 MHz, 300 K): δ=8.56 (br s, 1H; H-4 Dansyl); 8.24 (br s, 1H; H-8 Dansyl); 7.67 (m, 1H; H-2 Dansyl); 7.55, 7.48 (2×m, 2H; H-3 Dansyl, H-7 Dansyl); 7.19 (br s, 1H; H-6 Dansyl); 5.35 (t, *J*=4.9 Hz, 2H; $SCH_2CH$ GerGer); 5.30 (br s, 6H; C=C*H*); 5.06 (m, 3H; 3×α-C*H* Cys); 4.69 (weak signal, 2H; 2×α-C*H* Ser); 4.08 (weak signal, 1H; α-C*H* Glu); 3.65 (m, 4H; 2×β-$CH_2$ Ser); 3.65, 3.57–3.49 (2×m, 6H; 3×β-$CH_2$ Cys); 3.40–3.35 (m, 4H; 2×S$CH_2$ GerGer); 3.04 (m, 2H; γ-$CH_2$ Glu); 2.89 (br s, 10H; N($CH_3$)$_2$, $CH_2CH_2$NHDansyl); 2.35 (t, *J*=9.5 Hz, 2H; β-$CH_2$ Glu); 2.03–1.99 (br m, 12H; 6×=C($CH_3$)$CH_2CH_2$); 1.67–1.59 (m, 24H; 2×=C($CH_3$)$CH_3$ *trans*, 2×=C($CH_2$)$CH_3$ *cis*, 6×$CH_2CH_2$CH=); 1.30–1.25 (br s, 27H; 6×=C($CH_3$)$CH_3$ *cis*, SC($CH_3$)$_3$) ppm; ESI MS (+ve): *m*/*z* calcd for $C_{78}H_{124}N_9O_{12}S_2$ [*M*+H]: 1538.8; found: 1538.7; MALDI-TOF MS (DHB): calcd for $C_{78}H_{124}N_9NaO_{12}S_2$ [*M*+Na+H]: 1561.8; found: 1561.9.

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