Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 6807

www.rsc.org/obc

PAPER

Efficient preparation of Fmoc-aminoacyl-*N*-ethylcysteine unit, a key device for the synthesis of peptide thioesters

Hironobu Hojo,*[a] Hajime Kobayashi,[a] Risa Ubagai,[a] Yuya Asahina,[a] Yuko Nakahara,[a] Hidekazu Katayama,[a] Yukishige Ito[b] and Yoshiaki Nakahara*[a]

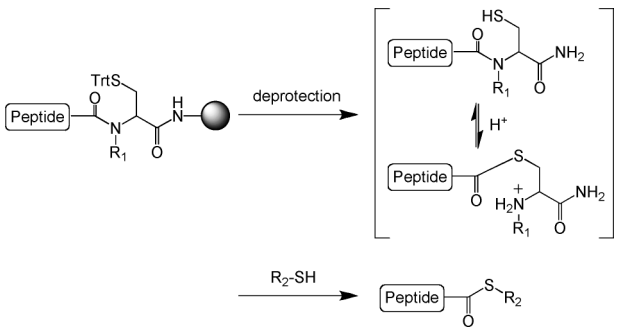
Received 26th May 2011, Accepted 4th July 2011
DOI: 10.1039/c1ob05831b

The synthesis of Fmoc-aminoacyl-*N*-ethyl-*S*-triphenylmethylcysteine, an *N*- to *S*-acyl migratory device for the preparation of peptide thioesters by Fmoc-SPPS (solid-phase peptide synthesis) is described. Condensation of Fmoc-aminoacyl pentafluorophenyl ester and *N*-ethyl-*S*-triphenylmethylcysteine was efficiently performed in the presence of HOOBt (3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine) in DMF. A small amount of diastereomer yielded during the reaction was easily separated by HPLC purification and the highly pure devices were obtained for most of the proteinogenic amino acids.

Introduction

Peptide thioesters have been widely used as key intermediates for the synthesis of (glyco)proteins by ligation methods, such as the thioester method[1] and the native chemical ligation method.[2] This key intermediate was originally synthesized by the Boc (*t*-butoxycarbonyl) method, considering the instability of the thioester linkage to piperidine used for the Fmoc (9-fluorenylmethoxycarbonyl) method.[1] However, peptide thioester preparation by the Fmoc method is attractive for the synthesis of post-translationally modified proteins, such as glycoproteins and phosphorylated proteins, since the linkages that exist in these modifications are generally acid-sensitive. Due to this fact, various methods have been developed to realize the peptide thioester synthesis by the Fmoc method.[3] Recently, we developed a promising strategy for peptide thioester preparation, in which *N*-alkylcysteine (NAC) in the C-terminus of the peptide is used as an *N*–*S* acyl migration device as shown in Scheme 1.[4] This procedure is fully compatible with conventional Fmoc strategy and gives the peptide thioester in excellent yield. In addition, this method provides epimerization-free peptide thioesters. The efficiency of this method has been demonstrated by its application to (glyco)protein syntheses.[5]

The remaining problem in this method was the low yield of peptide thioesters with a chiral amino acid at the C-terminus, which was due to the incomplete coupling of the C-terminal amino acid onto the sterically hindered *N*-alkylcysteine residue. As a solution to this problem, we recently reported loading of C-terminal amino acids as preformed Fmoc-aminoacyl *N*-ethylcysteines to the resin.[4b] Due to this improvement, the yield of peptide thioesters having chiral amino acids at the C-terminus was significantly increased. In the method, the dipeptide unit was obtained by two-step reaction: high-pressure-promoted Fmoc-aminoacylation of *N*-ethyl-*S*-triphenylmethylcysteine allyl ester by Fmoc-amino acid fluoride, followed by deallylation using Pd(0) catalyst. However, the complexity in this method is that the first step requires special apparatus for a high-pressure reaction, which limits the general use of the procedure. Here, we want to report a more general and one-pot procedure for the preparation of Fmoc-aminoacyl *N*-ethylcysteines, which is a significant improvement for the efficient preparation of peptide thioesters by the NAC method.



Scheme 1 Post-SPPS thioesterification using *N*-alkylcysteine device.

Results and discussion

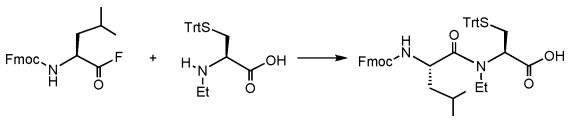
Synthesis of the dipeptide unit using fluoride

In a previous paper, we initially examined the direct coupling of Fmoc-amino acid activated by HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and DIEA (*N*,*N*-diisopropylethylamine) with *N*-ethyl-*S*-triphenylmethylcysteine [H-(*N*-Et)Cys(Trt)-OH].[4b] Although the reaction proceeded quickly, a complex mixture containing the desired dipeptide and the tripeptide by-product, with two *N*-ethylcysteine

[a]*Department of Applied Biochemistry, Institute of Glycoscience, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa, 259-1292, Japan. E-mail: hojo@keyaki.cc.u-tokai.ac.jp*
[b]*RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan. E-mail: yukito@riken.jp*

residues, was obtained. In this reaction, it seemed that the obtained dipeptide further acylated H-(*N*-Et)Cys(Trt)-OH by transesterification from the Fmoc-amino acid OAt ester. Thus, we speculated that carboxy-protection of the H-(*N*-Et)Cys(Trt)-OH is essential to avoid this side reaction. In contrast to our result, however, Brown and Schafmeister succeeded in the acylation of *N*-methylamino acid with free carboxylic acid using Fmoc-amino acid fluorides in HFIP (1,1,1,3,3,3-hexafluoro-2-propanol).[6] According to their report, the reaction proceeds through the formation of a mixed anhydride between *N*-methylamino acid and Fmoc-amino acid fluoride, followed by intramolecular aminolysis by the imino group of the *N*-methylamino acid. The efficiency of the reaction was demonstrated by the syntheses of dipeptides containing *N*-methylamino acids in high yield, typically over 75%. Under their coupling conditions, no base was added to avoid the esterification with the solvent, HFIP. However, we speculated that this base-free condition also prevented the further acylation reaction leading to the tripeptide formation. Thus, we attempted to apply the same method for the synthesis of the dipeptide using Fmoc-Leu fluoride with *N*-ethylcysteine as a model, as shown in Table 1. The application of the exact conditions of Brown's report gave the desired dipeptide in good yield (entry 1), though it took a longer time for the completion of the reaction. We observed some diastereomer formation during the reaction, but this side product was easily separated by RP (reversed-phase) HPLC using an ODS (octadecylsilyl) column. We next tested the coupling in a more common solvent, DMSO. As a result, the product was obtained in an acceptable yield, as shown in entry 2. The increase in temperature accelerated the reaction, but the yield was decreased, as shown in entries 3 to 5.

Table 1 Dipeptide preparation using Fmoc-Leu-F



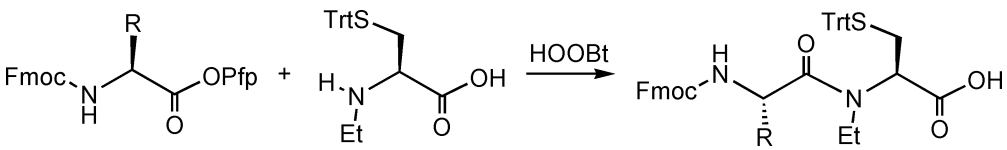
	Solvent	*T* (°C)	Time (h)	Product (%)	D/L (%)[a]
1	HFIP	55	20	69	1.4
2	DMSO	55	24	61	3.3
3	DMSO	65	20	46	8.7
4	DMSO	75	4.5	42	9.5
5	DMSO	85	2	40	12.5

[a] The diastereomer yield was expressed as a ratio of the peak area of the diastereomer and the product on HPLC.

Synthesis of dipeptides using Pfp ester

If the acylation of *N*-alkylcysteine proceeds through mixed anhydride formation followed by intramolecular aminolysis,[6] a milder acylation reagent would also be used for this synthesis, as the actual acylation step proceeds intramolecularly, irrespective of the acylation reagent. Thus, we next examined the use of more stable Fmoc-amino acid Pfp (pentafluorophenyl) esters supplemented with one equivalent of HOOBt (3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine) as an additive to increase the reactivity.[7] The results are shown in Table 2. In HFIP, the reaction did not proceed

Table 2 Dipeptide preparation using Fmoc-AA-OPfp in the presence of HOOBt



	Fmoc-AA-OPfp	HOOBt (eq)	Solvent	*T* (°C)	Time (h)	Product (%)	D/L (%)[a]
1	Leu	1	HFIP	55	22	0	—
2	Leu	1	DMSO	*rt*	22	36	2.0
3	Leu	1	DMF	*rt*	22	70	0.8
4	Leu	1	DMF	50	3	78	1.6
5	Leu	0	DMF	50	3	47	1.4
6	Ala	1	DMF	50	3	75	1.4
7	Asp(OBu^t)	1	DMF	50	3	77	1.2
8	Glu(OBu^t)	1	DMF	50	5	73	1.4
9	Phe	1	DMF	50	3	74	1.3
10	Gly	1	DMF	50	3	84	—
11	His(Trt)	1	DMF	50	3	66	6.6[b]
12	Ile	1	DMF	50	10	67	0.7
13	Lys(Boc)	1	DMF	50	5	72	1.5
14	Met	1	DMF	50	3	73	1.4
15	Asn(Trt)	1	DMF	50	3	78	*N.D.*
16	Pro	1	DMF	50	10	85	0.9
17	Gln(Trt)	1	DMF	50	5	79	1.2
18	Arg(Pbf)	1	DMF	50	3	54	1.4
19	Ser(Bu^t)	1	DMF	50	3	78	1.2
20	Thr(Bu^t)	1	DMF	50	8	69	0.8
21	Val	1	DMF	50	10	70	1.0
22	Trp(Boc)	1	DMF	50	3	79	2.0
23	Tyr(Bu^t)	1	DMF	50	3	81	1.6

[a] The diastereomer yield was expressed as a ratio of the peak area of the diastereomer and the product on HPLC. [b] Crude Pfp ester was directly used for dipeptide synthesis.

at all. However, in DMSO, the reaction proceeded and the product was obtained in a moderate yield at room temperature. When DMF was used as a solvent, the yield of the product was further improved, even at room temperature, as shown in entry 3. The increase in temperature to 50 °C accelerated the reaction and the product was obtained in 78% isolated yield within 3 h. The addition of HOOBt was essential to increase the yield of the product, as in its absence the yield was significantly decreased, as shown in entry 5. Thus, we decided to use the conditions used for entry 4 for the preparation of other dipeptides. As shown in Table 2 entries 6 to 23, the dipeptide units were obtained in good to moderate yield, except for Arg. Fmoc-Arg(Pbf)-OPfp was easily converted to an inactive lactam form, which resulted in this low yield. In the case of Asn, the separation of the product with its epimer, was not accomplished even with RP-HPLC. Thus, we could not determine epimerization ratio at this stage. However, the ratio was not significant, which is shown in the following section.

In the synthesis of dipeptides with amino acids with bulky side chains (Ile, Pro, Thr and Val), the reaction was not complete within 3 h. In these cases, the elongation of the reaction was effective to increase the yield of the product. Thus, we successfully completed most of the dipeptide units useful for the thioester preparation for segment coupling.

Model peptide thioester synthesis using dipeptide units

In order to demonstrate the usefulness of dipeptide units for peptide thioester synthesis, model pentapeptide thioesters were prepared by the NAC method. As C-terminal amino acids, Asn, Phe and Ser were selected as examples. The synthetic procedure in the case of Asn is shown in Fig. 1. Fmoc-Arg(Pbf)-OH was introduced to Rink amide resin twice. After Fmoc removal, Fmoc-Asn(Trt)-(*N*-Et)Cys(Trt)-OH was introduced using the DIC (*N*,*N*′-diisopropylcarbodiimide)–HOBt (1-hydroxybenzotriazole) method. Then, the chain elongation was carried out using a peptide synthesizer by the Fmoc method. After deprotection by TFA, the crude peptide was dissolved in 50% aq. acetonitrile containing 6 M urea and 5% (v/v) 3-mercaptopropionic acid (MPA), and the solution was kept at 37 °C. As observed in

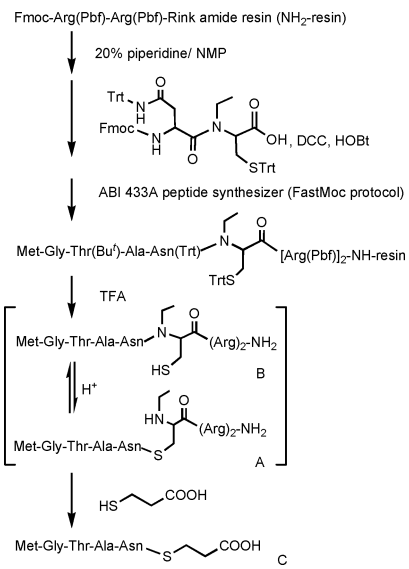


Fig. 1 Synthetic route for peptide thioesters using the dipeptide unit.

Table 3 Synthesis of model peptide thioesters

Peptide thioester	Yield (%)	Epimer/product (%)
QKTEF-SCH_2CH_2COOH	39	< 1
MGTAN-SCH_2CH_2COOH	46	1.8
FKVDS-SCH_2CH_2COOH	32	< 1

previous syntheses, two peaks appeared on the RP-HPLC at T = 0 (Fig. 2), which corresponds to the thioester form (compound A) and the amide form (B). These peaks gradually converted to a new peak (C), which corresponds to the desired thioester. After leaving overnight, the reaction was almost complete, without serious side reactions as shown in Fig. 2. After the reaction mixture was left to stand for another day, the desired peptide thioester was purified by RP-HPLC. As shown in Table 3, the yields were highly increased compared to the previous report, in which the C-terminal chiral amino acids were directly loaded to the resin-bound *N*-ethylcysteine residue by HATU.[4a]

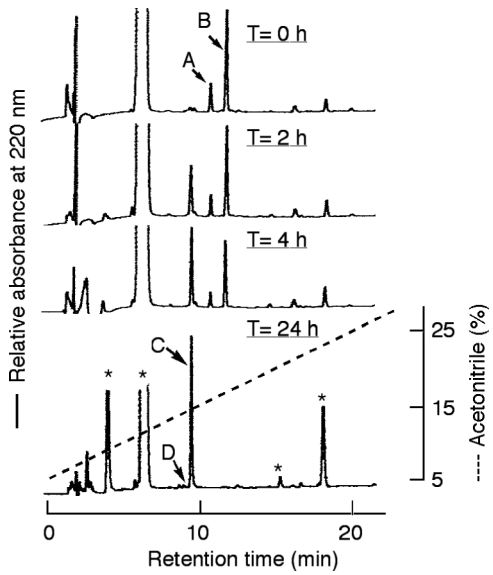


Fig. 2 RP-HPLC profile of the crude peptide thioesterification reaction in Fig. 1. Elution conditions: column, Mightysil RP-18 GP (4.6 × 150 mm, Kanto, Japan) at a flow rate of 1 mL min^{-1}; eluent, A, 0.1% aqueous TFA, B, 0.1% TFA in acetonitrile. Asterisked peaks in the chromatogram (T = 24 h) are non-peptides. Peak D indicates the epimer of compound C.

There was a possibility that Fmoc-Asn(Trt)-(*N*-Et)Cys(Trt)-OH used for the synthesis of compound C was contaminated with a small amount of diastereomer, since it was not separated by RP-HPLC. However, the epimer/product ratio of MGTAN-SCH_2CH_2COOH was only 1.8%, indicating that the epimerization of Asn during the dipeptide synthesis was at most this level, which is comparable to other amino acids. In addition, we confirmed that the post-SPPS thioesterification reaction in the NAC method proceeds essentially epimerization-free.

Conclusion

We successfully established an efficient one-pot procedure for the preparation of Fmoc-aminoacyl *N*-ethylcysteine, a key device for the synthesis of peptide thioesters by the NAC method. This

procedure is mild and simple compared to the previous method using the high pressure promoted method, and thus can be generally applicable for the synthesis of peptide thioesters by the NAC method. The dipeptide units obtained will be efficiently used for the synthesis of (glyco)proteins by ligation methods.

Experimental

General

Specific rotation values were determined with a Jasco P-2200 polarimeter at 20 ± 2 °C for solutions in $CHCl_3$. Column chromatography and flash chromatography were performed on silica gel PSQ 100B (Fuji Silysia) and Wakogel C-500HG (Wako), respectively. TLC and HPTLC were performed on silica gel 60 F_{254} (E. Merck). ^{1}H- and ^{13}C-NMR spectra were recorded with a Jeol AL400 spectrometer. Chemical shifts are expressed in ppm downfield from the signal for internal Me_4Si for solutions in $CDCl_3$. Peptide synthesis was carried out by a Peptide synthesizer 433A (Applied Biosystems) using the FastMoc protocol. HPLC was performed with a recycling preparative HPLC model 9201 (Japan Analytical Industry Co.) on Inertsil ODS-SP using 80% aq acetonitrile containing 0.1% TFA as an eluent. Amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi) after hydrolysis with a 6 M HCl solution at 150 °C for 2 h in an vacuum-sealed tube. Yields of peptides were calculated based on the amino acid analysis data.

Synthesis of Fmoc-aminoacyl *N*-ethyl-*S*-triphenylmethyl-L-cysteine using fluoride

Fmoc-amino acid fluoride (1.2 eq to cysteine) and *N*-ethyl-*S*-triphenylmethyl-L-cysteine were dissolved in HFIP or DMSO at a concentration of 0.1 mol l^{-1} and the solution was stirred at various temperatures and times as shown in Table 1. After the solvent was removed *in vacuo*, the residue was purified by silica gel column chromatography, followed by a recycling HPLC system to obtain the desired dipeptide units.

General procedure for the synthesis of Fmoc-aminoacyl *N*-ethyl-*S*-triphenylmethyl-L-cysteine using Pfp ester in the presence of HOOBt

Equimolar amounts of Fmoc-amino acid Pfp ester, *N*-ethyl-*S*-triphenylmethyl-L-cysteine, and HOOBt were dissolved in DMF at a concentration of 0.25 mol l^{-1} and the solution was stirred at 50 °C. After the solvent was removed *in vacuo*, the residue was purified by silica gel column chromatography, followed by flash chromatography or recycled HPLC to obtain the desired dipeptide units. Usually, the recycling mode in the HPLC system was not necessary, but in the case of Asn, the diastereomer, if any, was not separated even with the recycling mode.

N-(9-Fluorenylmethoxycarbonyl)-L-alanyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.46 (17 : 3 $CHCl_3$–MeOH). $[\alpha]_D$ −28.9 (*c*, 1.3). ^{1}H-NMR: δ 7.74 (d, 2H, *J* = 7.3 Hz, Ar), 7.57 (t, 2H, *J* = 7.8 Hz, Ar), 7.44–7.18 (m, 19H, Ar), 5.83 (d, 1H, *J* = 7.3 Hz, Ala-N*H*), 4.53 (m, 1H, Ala-αH), 4.29 (m, 2H, -CH_2CHAr$_2$), 4.17 (m, 1H, -C*H*Ar$_2$), 3.41 (m, 1H, -CH_2CH$_3$), 3.09 (m, 1H, Cys-βH), 2.91 (m, 2H, -CH_2CH$_3$, Cys-βH), 2.75 (m, 1H, Cys-αH), 1.33 (d, 2H, *J* = 6.8 Hz, Ala-CH_3), 1.02 (brt, 3H, *J* = 7.01 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 60.2 (Cys-αC), 47.0 (Ala-αC), 45.0 (-*C*H$_2$CH$_3$), 30.2 (Cys-βC), 19.4 (Ala-*C*H$_3$), 13.9 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{42}H_{40}N_2O_5S{\cdot}1/2H_2O$: C, 72.70; H, 5.96; N, 4.04; S, 4.62. Found: C, 72.88; H, 6.01; N, 3.92; S, 4.48%. MALDI TOF MS, calcd for $C_{42}H_{40}N_2O_5S$ (M + Na)$^+$: 707.26. Found: *m*/*z* 707.21.

N-(9-Fluorenylmethoxycarbonyl)-O^t-*tert*-butyl-L-aspartyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.55 (9 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −65.8 (*c*, 1.2). ^{1}H-NMR: δ 7.65 (d, 2H, *J* = 7.3 Hz, Ar), 7.45 (t, 2H, *J* = 6.8 Hz, Ar), 7.35–7.09 (m, 19H, Ar), 5.95 (d, 1H, *J* = 9.3 Hz, Asp-N*H*), 4.83 (m, 1H, Asp-αH), 4.18 (m, 2H, -CH_2CHAr$_2$), 4.07 (m, 1H, -C*H*Ar$_2$), 3.47 (m, 1H, -CH_2CH$_3$), 3.05 (dd, 1H, *J* = 5.9, 13.7 Hz, Cys-βH), 2.77 (m, 1H, Cys-βH), 2.71–2.61 (m, 3H, Cys-αH, Asp-βH, -CH_2CH$_3$), 2.44 (dd, 1H, J = 6.3 15.6 Hz, Asp-βH), 1.31 (s, 9H, *t*-Bu), 0.89 (brt, 3H, J 6.8 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 60.8 (Cys-αC), 47.7 (Asp-αC), 44.9 (-*C*H$_2$CH$_3$), 39.1 (Asp-βC), 29.9 (Cys-βC), 13.8 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{47}H_{48}N_2O_7S$: C, 71.92; H, 6.16; N, 3.57; S, 4.08. Found: C, 71.69; H, 6.44; N, 3.37; S, 3.91% MALDI TOF MS, calcd for $C_{47}H_{48}N_2O_7S$ (M + Na)$^+$: 807.31. Found: *m*/*z* 807.29.

N-(9-Fluorenylmethoxycarbonyl)-O^5-*tert*-butyl-L-glutamyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.39 (9 : 1 $CHCl_3$–CH_3OH). $[\alpha]_D$ −29.6 (*c*, 1.3). ^{1}H-NMR: δ 7.74 (d, 2H, *J* = 7.3 Hz, Ar), 7.54 (dd, 2H, *J* = 7.8, 13.2 Hz, Ar), 7.44–7.17 (m, 19H, Ar), 5.77 (d, 1H, *J* = 8.8 Hz, Glu-N*H*), 4.57 (m, 1H, Glu-αH), 4.39 (m, 2H, -CH_2CHAr$_2$), 4.17 (t, 1H, *J* = 7.3 Hz, -C*H*Ar$_2$), 3.59 (m, 1H, -CH_2CH$_3$), 3.04 (m, 1H, Cys-βH), 2.88 (m, 2H, -CH_2CH$_3$, Cys-βH), 2.79 (m, 1H, Cys-αH), 2.30 (m, 2H, Glu-γH), 1.99 (m, 1H, Glu-βH), 1.72 (m, 1H, Glu-βH), 1.42 (s, 9H, *t*-Bu), 1.01 (brt, 3H, J 7.1 Hz, -CH$_2$CH_3). ^{13}C NMR: δ 60.2 (Cys-αC), 50.1 (Glu-αC), 47.0 (-*C*H$_2$CH$_3$), 30.1 (Cys-βC), 13.9 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{48}H_{50}N_2O_7S{\cdot}1/2H_2O$: C, 71.35; H, 6.36; N, 3.47; S, 3.97. Found: C, 71.29; H, 6.47; N, 3.39; S, 3.79% MALDI TOF MS, calcd for $C_{48}H_{50}N_2O_7S$ (M + Na)$^+$: 821.32. Found: *m*/*z* 821.21.

N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.52 (9 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −55.7 (*c*, 1.1). ^{1}H-NMR:δ 7.68 (d, 2H, *J* = 7.3 Hz, Ar), 7.50 (t, 2H, *J* = 7.8 Hz, Ar), 7.37–6.98 (m, 24H, Ar), 6.24 (d, 1H, *J* = 8.8 Hz, Phe-N*H*), 4.61 (dd, 1H, *J* = 8.8, 14.6 Hz, Phe-αH), 4.23–4.11 (m, 3H, -CH_2CHAr$_2$, -C*H*Ar$_2$), 3.15 (dd, 1H, *J* = 7.3, 14.2 Hz, Cys-βH), 2.92 (m, 2H, Phe-βH), 2.79 (m, 1H, -CH_2CH$_3$), 2.47 (t, 1H, *J* = 6.8 Hz, Cys-αH), 2.35 (dd, 1H, *J* = 6.3, 14.1 Hz, Cys-βH), 1.96 (m, 1H, -CH_2CH$_3$), 0.73 (t, 3H, *J* = 7.3 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 61.2 (Cys-αC), 51.9 (Phe-αC), 44.3 (-*C*H$_2$CH$_3$), 40.0 (Phe-βC), 29.7 (Cys-βC), 13.6 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{48}H_{44}N_2O_5S$ H_2O: C, 74.01; H, 5.95; N, 3.60; S, 4.12. Found: C, 74.11; H, 6.19; N, 3.35; S, 3.77% MALDI TOF MS, calcd for $C_{48}H_{44}N_2O_5S$ (M + Na)$^+$: 783.29. Found: *m*/*z* 783.25.

N^{α}-(9-Fluorenylmethoxycarbonyl)-N^{τ}-triphenylmethyl-L-histidyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.40 (9 : 1 $CHCl_3$–MeOH, 1% AcOH). $[\alpha]_D$ −19.6 (*c*, 1.0). ^{1}H-NMR: δ 7.88 (d, 2H, J = 7.3 Hz, Ar), 7.79 (d, 0.5 H, J = 8.8 Hz, His-N*H* of conformer 1), 7.65 (t, 2H, J = 7.6 Hz, Ar), 7.43–7.22 (m, 32H, Ar), 7.06 (m, 2H, Ar), 4.73 (m, 0.5H, His-αH of conformer 1), 4.62 (m, 0.5H, His-αH of conformer 2), 4.16 (m, 3H, -CH_2CHAr$_2$), 3.61 (m, 0.5H, -CH_2CH_3), 2.94 (m, 3H, His-βHx2, -CH_2CH_3), 1.03 (m, 3H, -CH_2CH_3). ^{13}C-NMR: δ 59.4 (Cys-αC), 14.2 (-CH_2CH_3). Anal. calcd for $C_{64}H_{56}N_4O_5S$ 2·5H_2O: C, 74.04; H, 5.92; N, 5.40. Found: C, 74.21; H, 5.70; N, 5.28% MALDI TOF MS, calcd for $C_{64}H_{56}N_4O_5S$ (M + Na)$^+$: 1015.39. Found: *m*/*z* 1015.62.

***N*-(9-Fluorenylmethoxycarbonyl)-L-isoleucyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.43 (9 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −23.4 (*c*, 1.1). ^{1}H-NMR:δ 7.74 (d, 2H, J = 7.8 Hz, Ar), 7.56 (brt, 2H, J = 8.5 Hz, Ar), 7.43–7.16 (m, 19H, Ar), 5.79 (d, 1H, J = 9.3, Ile-N*H*), 4.39 (dd, 1H, J = 7.3, 9.3 Hz, Ile-αH), 4.30 (m, 2H, -CH_2CHAr$_2$), 4.17 (t, 1H, J = 7.3 Hz, -CHAr$_2$), 3.48 (m, 1H, -CH_2CH_3), 3.13 (dd, 1H, J = 9.8, 14.6 Hz, Cys-βH), 2.92 (m, 2H, -CH_2CH_3, Cys-βH), 2.59 (dd, 1H, J = 4.9, 9.3 Hz, Cys-αH), 1.73 (m, 1H, Ile-βH), 1.53 (m, 1H, Ile-γH), 1.10 (m, 1H, Ile-γH), 0.99 (t, 3H, J = 6.8 Hz, -CH_2CH_3), 0.90 (m, 6H, Ile-CH_3). ^{13}C-NMR: δ 60.5 (Cys-αC), 55.0 (Ile-αC), 45.6 (-CH_2CH_3), 38.4 (Ile-βC), 31.9 (Cys-βC), 24.0 (Ile-γC), 15.7 (Ile-CH_3), 13.9 (-CH_2CH_3), 11.3 (Ile-CH_3). Anal. calcd for $C_{45}H_{46}N_2O_5S$·1/2H_2O: C, 73.44; H, 6.44; N, 3.81; S, 4.36. Found: C, 73.55; H, 6.54; N, 3.79% MALDI TOF MS, calcd for $C_{45}H_{46}N_2O_5S$ (M + Na)$^+$: 749.30. Found: *m*/*z* 749.71.

N^2-(9-Fluorenylmethoxycarbonyl)-N^6-*tert*-butoxycarbonyl-L-lysyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.48 (17 : 3 $CHCl_3$–CH_3OH). $[\alpha]_D$ −23.0 (*c*, 1.0).^{1}H-NMR: δ 7.73 (d, 2H, J = 7.3 Hz, Ar), 7.56 (m, 2H, Ar), 7.44–7.16 (m, 19H, Ar), 5.93 (d, 1H, J = 7.8 Hz, Lys-N*H*), 4.54 (m, 1H, Lys-αH), 4.29 (m, 2H, -CH_2CHAr$_2$), 4.16 (m, 1H, -CHAr$_2$), 3.41 (m, 1H, -CH_2CH_3), 2.79 (m, 1H, Cys-αH), 1.40 (s, 9H, *t*-Bu), 1.33 (d, 2H, J = 6.8 Hz, Lys-βH), 1.01 (brt, 3H, J 6.6 Hz, -CH_2CH_3). ^{13}C-NMR: δ 60.1 (Cys-αC), 50.7 (Lys-αC), 30.2 (Cys-βC), 28.4 (C(CH_3)$_3$), 19.4 (Lys-βC), 14.0 (-CH_2CH_3). Anal. calcd for $C_{50}H_{55}N_3O_7S$·2H_2O: C, 68.39; H, 6.77; N, 4.79; S, 3.65. Found: C, 68.51; H, 6.51; N, 4.70; S, 3.58% MALDI TOF MS, calcd for $C_{50}H_{55}N_3O_7S$ (M + Na)$^+$: 864.37. Found: *m*/*z* 864.35.

***N*-(9-Fluorenylmethoxycarbonyl)-L-leucyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.32 (19 : 1 $CHCl_3$–CH_3OH 1% AcOH). $[\alpha]_D$ −33.2 (*c*, 1.6). ^{1}H-NMR: δ 7.74 (d, 2H, J = 7.3 Hz, Ar), 7.56 (t, 2H, J = 7.3 Hz, Ar), 7.46–7.19 (m, 19 H, Ar), 5.58 (d, 1H, J = 9.3 Hz, Leu-N*H*), 4.57 (m, 1H, Leu-αH), 4.31 (m, 2H, -CH_2CHAr$_2$), 4.18 (brt, 1H, J = 7.1 Hz, -CHAr$_2$), 3.40 (m, 1H, -CH_2CH_3), 3.09–2.89 (m, 3H, Cys-βH × 2, -CH_2CH_3), 2.74 (dd, 1H, 4.9, 9.3 Hz, Cys-αH), 1.70 (m, 1H, Leu-γH), 1.52 (m, 1H, Leu-βH), 1.42 (m, 1H, Leu-βH), 1.06 (brt, 3H, J = 7.1 Hz, -CH_2CH_3), 0.96 (d, 3H, J = 6.6 Hz, Leu-δH), 0.94 (d, 3H, J = 6.6 Hz, Leu-δH). ^{13}C-NMR: δ 60.3 (Cys-αC), 24.5 (Leu-γC), 23.4 (Leu-δC), 21.7 (Leu-δC), 14.0 (-CH_2CH_3). Anal. calcd for $C_{45}H_{46}N_2O_5S$·1/3H_2O: C, 73.74; H, 6.42; N, 3.82; S, 4.37. Found: C, 73.78; H, 6.71; N, 3.66; S, 4.10% MALDI TOF MS, calcd for $C_{45}H_{46}N_2O_5S$ (M + Na)$^+$: 749.30. Found: *m*/*z* 749.21.

***N*-(9-Fluorenylmethoxycarbonyl)-L-methionyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.30 (19 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −22.3 (*c*, 1.0). ^{1}H-NMR:δ 7.74 (d, 2H, J = 7.3 Hz, Ar), 7.56 (brt, 2H, J = 8.5 Hz, Ar), 7.43–7.18 (m, 19H, Ar), 5.85 (d, 1H, J = 8.3 Hz, Met-N*H*), 4.70 (m, 1H, Met-αH), 4.30 (d, 2H, -CH_2CHAr$_2$), 4.17 (t, 1H, -CHAr$_2$), 3.51 (m, 1H, -CH_2CH_3), 3.06 (dd, 1H, J = 9.8, 14.1 Hz, Cys-βH), 2.91 (m, 2H, -CH_2CH_3, Cys-βH), 2.78 (m, 1H, Cys-αH), 2.06 (s, 3H, -SCH_3), 1.95 (m, 1H, Met-βH), 1.86 (m, 1H, Met-βH), 1.02 (t, 3H, J = 6.8 Hz, -CH_2CH_3). ^{13}C-NMR: δ 60.4 (Cys-αC), 49.9 (Met-αC), 45.3 (-CH_2CH_3), 30.1 (Cys-βC), 29.9 (Met-γC), 15.6 (Met-CH_3), 14.0 (-CH_2CH_3). Anal. calcd for $C_{44}H_{44}N_2O_5S_2$·1/2H_2O: C, 70.09; H, 6.02; N, 3.72; S, 8.51% Found: C, 70.13; H, 6.19; N, 3.58; S, 8.05. MALDI TOF MS, calcd for $C_{44}H_{44}N_2O_5S_2$ (M + Na)$^+$: 767.26. Found: *m*/*z* 767.33.

N^2-(9-Fluorenylmethoxycarbonyl)-N^4-triphenylmethyl-L-asparaginyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.46 (19 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). ^{1}H-NMR: δ 7.64 (d, 2H, J = 7.3 Hz, Ar), 7.42 (brt, 2H, J = 7.6 Hz, Ar), 7.33–7.08 (m, 34H, Ar), 6.13 (brs, 1H, Asn-N*H*), 4.81 (brd, 1H, J = 6.3 Hz, Asn-αH), 4.18 (m, 1H, -CH_2CHAr), 4.08 (m, 1H, -CH_2CHAr), 4.00 (m, 1H, -CHAr), 3.23 (m, 1H, -CH_2CH_3), 3.05 (m, 1H, Cys-βH), 2.67–2.45 (m, 4H, Cys-α, βH, Asn-βHx2), 0.81 (brs, 3H, -CH_2CH_3). ^{13}C-NMR: δ 59.9 (Cys-αC), 48.7 (Asn-αC), 43.6 (-CH_2CH_3), 39.8 (Asn-βC), 29.8 (Cys-βC), 13.9 (-CH_2CH_3). Anal. calcd for $C_{62}H_{55}N_3O_6S$·2H_2O: C, 74.01; H, 5.91; N, 4.18; S, 3.19. Found: C, 73.95; H, 5.78; N, 4.01; S, 3.05% MALDI TOF MS, calcd for $C_{62}H_{55}N_3O_6S$ (M + Na)$^+$: 992.37. Found: *m*/*z* 992.26.

***N*-(9-Fluorenylmethoxycarbonyl)-L-prolyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.30 (19 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −60.5 (*c*, 1.0). ^{1}H-NMR (DMSO-d_6): δ 7.87 (m, 2H, Ar), 7.67–7.59 (m, 2H, Ar), 7.40–7.21 (m, 19H, Ar), 4.67 (d, 0.6H, Pro-αH of conformer 1), 4.55 (d, 0.4 H, Pro-αH of conformer 2), 4.24–4.01 (m, 3H, -CH_2CHAr), 2.96 (m, 1H, -CH_2CH_3), 2.80 (brt 1H, J = 11.0 Hz, Cys-βH), 2.63 (m, 1H, Cys-βH), 2.30 (m, 0.6 H, Pro-βH of conformer 1), 2.18 (m, 0.4 H, Pro-βH of conformer 2), 1.90–1.71 (3H, Pro-βH, γH x2), 1.08 (t, 3H x0.4, J = 6.6 Hz, -CH_2CH_3 of conformer 2), 0.98 (t, 3H x0.6, J = 6.8 Hz, -CH_2CH_3 of conformer 1). ^{13}C-NMR: δ 59.1, 58.8 (Cys-αC), 43.8, 43.5 (-CH_2CH_3), 30.5 (Cys-βC), 14.3 14.1 (-CH_2CH_3). Anal. calcd for $C_{44}H_{42}N_2O_5S$ 1·5H_2O: C, 71.62; H, 6.15; N, 3.80; S, 4.35. Found: C, 71.97; H, 6.43; N, 3.47; S, 3.69% MALDI TOF MS, calcd for $C_{44}H_{42}N_2O_5S$ (M + Na)$^+$: 733.27. Found: *m*/*z* 733.61.

N^2-(9-Fluorenylmethoxycarbonyl)-N^5-triphenylmethyl-L-glutaminyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.45 (19 : 1 $CHCl_3$–CH_3OH 1% AcOH). $[\alpha]_D$ −20.9 (*c*, 1.0). ^{1}H-NMR: δ 7.73 (d, 2H, *J* = 7.3 Hz, Ar), 7.55 (dd, 2H, J = 7.8 15.6 Hz, Ar), 7.40–7.15 (m, 34H, Ar), 5.85 (d, 1H, *J* = 7.8 Hz, Gln-N*H*), 4.37 (m, 1H, Gln-αH), 4.32 (d, 2H, *J* = 7.3 Hz, -CH_2CHAr), 4.16 (t, 1H, *J* = 6.8 Hz, -C*H*Ar), 3.39 (m, 1H, -CH_2CH$_3$), 2.95 (dd, 1H, J = 10.7 14.1 Hz, Cys-βH), 2.87 (m, 2H, Cys-α, βH), 2.69 (m, 1H, -CH_2CH$_3$), 2.32 (m, 2H, Gln-γH), 2.09 (m, 1H, Gln-βH), 1.64 (m, 1H, Gln-βH), 0.87 (t, 3H, -CH$_2$CH_3). ^{13}C-NMR: δ 59.8 (Cys-αC), 50.4 (Gln-αC), 44.5 (-*C*H$_2$CH$_3$), 32.6 (Gln-γC), 30.1 (Cys-βC), 29.5 (Gln-βC), 14.0 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{63}H_{57}N_3O_6S{\cdot}1.5H_2O$: C, 74.83; H, 5.98; N, 4.16; S, 3.17. Found: C, 74.76; H, 6.14; N, 3.91; S, 2.96% MALDI TOF MS, calcd for $C_{63}H_{57}N_3O_6S$ (M + Na)$^+$: 1006.39. Found: *m*/*z* 1006.29.

***N*-(9-Fluorenylmethoxycarbonyl)-N^g-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)**

R_f 0.38 (9 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −22.9 (*c*, 1.1). ^{1}H-NMR (DMSO-d$_6$): δ 7.89 (d, 2H, *J* = 7.8 Hz, Ar), 7.73 (dd, 2H, *J* = 7.3, 12.7 Hz, Ar), 7.59 (d, 1H, *J* = 8.3 Hz, Arg-N*H*), 7.43–7.25 (m, 19 H, Ar), 4.34 (m, 1H, Arg-αH), 4.22 (m, 3H, -CH_2C*H*Ar$_2$), 3.31 (m, Cys-αH), 3.05 (m, 3H, -CH_2CH$_3$, Arg-δH × 2), 2.92 (s, 2H, Pbf: -CH_2-), 2.52 (s, 3H, Pbf: -CH_3), 2.44 (s, 3H, Pbf: -CH_3), 1.99 (s, 3H, Pbf: -CH_3), 1.54 (1H, Arg-βH), 1.48 (1H, Arg-βH), 1.39 (s, 6H, -CH_3 x2), 1.03 (brt, 3H, *J* = 6.6 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 59.3 (Cys-αC), 42.5 (Pbf: -*C*H$_2$-), 28.3 (Pbf: -*C*H$_3$), 19.0 (Pbf: -*C*H$_3$), 17.6 (Pbf: -*C*H$_3$), 14.3 (-CH$_2$*C*H$_3$) 12.3 (Pbf: -*C*H$_3$). Anal. calcd for $C_{58}H_{63}N_5O_8S_2{\cdot}1.5H_2O$: C, 66.39; H, 6.34; N, 6.67. Found: C, 66.33; H, 6.37; N, 6.31% MALDI TOF MS, calcd for $C_{58}H_{63}N_5O_8S_2$ (M + Na)$^+$: 1044.4. Found: *m*/*z* 1044.40.

***N*-(9-Fluorenylmethoxycarbonyl)-O^3-*tert*-butyl-L-seryl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.43 (9 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −55.7 (*c*, 1.0). ^{1}H-NMR:δ 7.74 (d, 2H, *J* = 7.8 Hz, Ar), 7.58 (dd, 2H, *J* = 7.3, 15.1 Hz, Ar), 7.42–7.16 (m, 19H, Ar), 5.88 (d, 1H, *J* = 8.8 Hz, Ser-N*H*), 4.66 (m, 1H, Ser-αH), 4.29 (m, 2H, -CH_2CHAr$_2$), 4.18 (m, 1H, -C*H*Ar$_2$), 3.66 (m, 1H, -CH_2CH$_3$), 3.51 (m, 1H, Ser-βH), 3.38 (brt, 1H, *J* = 8.5 Hz, Ser-βH), 3.21 (dd, 1H, *J* = 6.3, 14.1 Hz, Cys-βH), 2.83–2.74 (m, 2H, Cys-α, βH), 2.52 (m, 1H, -CH_2CH$_3$), 1.07 (s, 9H, *t*-Bu), 0.96 (t, 3H, *J* = 7.1 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 63.2 (Ser-βC), 61.0 (Cys-αC), 50.5 (Ser-αC), 44.5 (-*C*H$_2$CH$_3$), 29.9 (Cys-βC), 27.2 (C(*C*H$_3$)$_3$), 13.7 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{46}H_{48}N_2O_6S$: C, 72.99; H, 6.39; N, 3.70; S, 4.24. Found: C, 72.65; H, 6.54; N, 3.68; S, 4.11% MALDI TOF MS, calcd for $C_{46}H_{48}N_2O_6S$ (M + Na)$^+$: 779.31. Found: *m*/*z* 779.37.

***N*-(9-Fluorenylmethoxycarbonyl)-O^3-*tert*-butyl-L-threonyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.43 (19 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −27.8 (*c*, 1.0). ^{1}H-NMR:δ 7.74 (d, 2H, *J* = 7.3 Hz, Ar), 7.57 (dd, 2H, *J* = 7.3, 13.2 Hz, Ar), 7.44 (d, 4H, *J* = 7.3 Hz, Ar), 7.36 (t, 2H, *J* = 7.3 Hz, Ar), 7.29–7.17 (m, 13H, Ar), 5.76 (d, 1H, *J* = 8.3 Hz, Thr-N*H*), 4.48 (dd, 1H, *J* = 5.4, 8.3 Hz, Thr-αH), 4.36–4.26 (m, 2H, -CH_2CHAr$_2$), 4.19 (m, 1H, -C*H*Ar$_2$), 3.84 (m, 2H, Thr-βH, -CH_2CH$_3$), 3.04 (dd, 1H, *J* = 5,4, 14.1 Hz, Cys-αH), 2.91 (dd, 1H, *J* = 8.3, 14.1 Hz, Cys-βH), 2.78 (m, 2H, Cys-βH, -CH_2CH$_3$), 1.16 (s, 9H, *t*-Bu), 1.08 (d, 3H, *J* = 6.3 Hz, Thr-γH), 0.98 (t, 3H, *J* = 7.3 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 68.7 (Thr-βC), 60.7 (Cys-αC), 55.1 (Thr-αC), 45.3 (-*C*H$_2$CH$_3$), 30.3 (Cys-βC), 28.2 (C(*C*H$_3$)$_3$), 18.8 (Thr-*C*H$_3$), 13.7 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{47}H_{50}N_2O_6S{\cdot}H_2O$: C, 71.55; H, 6.64; N, 3.55; O, 14.19; S, 4.06. Found: C, 71.37; H, 6.57; N, 3.41; S, 3.90% MALDI TOF MS, calcd for $C_{47}H_{50}N_2O_6S$ (M + Na)$^+$: 793.33. Found: *m*/*z* 793.07.

***N*-(9-Fluorenylmethoxycarbonyl)-valyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.31 (19 : 1 $CHCl_3$–CH_3OH 0.5% Ac OH). $[\alpha]_D$ −31.1 (*c*, 1.0). ^{1}H-NMR:δ 7.74 (d, 2H, *J* = 7.8 Hz, Ar), 7.56 (dd, 2H, *J* = 7.3, 10.2 Hz, Ar), 7.43–7.17 (m, 19H, Ar), 5.77 (d, 1H, *J* = 9.3 Hz, Val-N*H*), 4.37 (dd, 1H, 6.3, 9.3 Hz, Val-αH), 4.29 (d, 2H, -CH_2CHAr$_2$), 4.18 (brt, 1H, *J* = 7.3 Hz, -C*H*Ar$_2$), 3.49 (m, 1H, -CH_2CH$_3$), 3.09 (dd, 1H, *J* = 9.8, 14.6 Hz, Cys-βH), 2.94 (m, 2H, -CH_2CH$_3$, Cys-βH), 2.61 (dd, 1H, *J* = 4.9, 8.8 Hz, Cys-αH), 1.98 (m, 1H, Val-βH), 1.00 (brt, 1H, *J* = 7.1 Hz, -CH$_2$CH_3), 0.96–0.86 (m, 6H, Val-CH_3). ^{13}C-NMR: δ 60.4 (Cys-αC), 55.6 (Val-αC), 45.6 (-*C*H$_2$CH$_3$), 30.3 (Cys-βC), 19.6 (Val-*C*H$_3$), 17.4 (Val-*C*H$_3$), 14.0 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{44}H_{44}N_2O_5S{\cdot}1/2\ H_2O$: C, 73.21; H, 6.28; N, 3.88; O, 12.19; S, 4.44. Found: C, 73.50; H, 6.44; N, 3.85; S, 4.27% MALDI TOF MS, calcd for $C_{44}H_{44}N_2O_5S$ (M + Na)$^+$: 735.29. Found: *m*/*z* 735.52.

N^a-(9-Fluorenylmethoxycarbonyl)-N^1-*tert*-butoxycarbonyl-tryptophyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine

R_f 0.39 (19 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −23.0 (*c*, 1.2). ^{1}H-NMR:δ 7.76 (t, 2H, *J* = 7.1 Hz, Ar), 7.61–7.03 (m, 26H, Ar), 6.60 (d, 1H, *J* = 8.8 Hz, Trp-N*H*), 4.92 (m, 1H, Trp-αH), 4.38 (m, 1H, -CH_2CHAr$_2$), 4.23 (m, 2H, -CH_2C*H*Ar$_2$), 3.23 (dd, 1H, *J* = 8.8, 13.7 Hz, Trp-βH), 3.11–3.03 (m, 2H, Trp-βH, -CH_2CH$_3$), 2.93 (m, 1H, Cys-αH), 2.70 (m, 1H, Cys-βH), 2.36 (m, 1H, -CH_2CH$_3$), 2.30 (m 1H, Cys-αH), 1.57 (s, 9H, Bu^t), 0.82 (t, 3H, *J* = 6.8 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 60.7 (Cys-αC), 50.9 (Trp-αC), 29.8 (Cys-βC), 28.2 (C(*C*H$_3$)$_3$), 13.7 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{55}H_{53}N_3O_7S{\cdot}2H_2O$: C, 70.57; H, 6.14; N, 4.49; O, 15.38; S, 3.43. Found: C, 70.63; H, 5.92; N, 4.59; S, 3.38% MALDI TOF MS, calcd for $C_{55}H_{53}N_3O_7S$ (M + Na)$^+$: 922.35. Found: *m*/*z* 922.39.

***N*-(9-Fluorenylmethoxycarbonyl)-O^4-*tert*-butyl-tyrosyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine**

R_f 0.38 (19 : 1 $CHCl_3$–CH_3OH 0.5% AcOH). $[\alpha]_D$ −64.0 (*c*, 1.0). ^{1}H-NMR: δ 7.76 (d, 2H, *J* = 7.3 Hz, Ar), 7.59 (dd, 2H, *J* = 7.3, 13.7 Hz, Ar), 7.41–7.15 (m, 19H, Ar), 7.03 (d, 2H, *J* = 8.3 Hz, Ar), 6.67 (m, 2H, Ar), 6.19 (d, 1H, *J* = 8.3 Hz, Tyr-N*H*), 4.60 (m, 1H, Tyr-αH), 4.34–4.20 (m, 3H, -CH_2CHAr$_2$, -C*H*Ar$_2$), 3.29 (m, 1H, Cys-βH), 2.97 (m, 2H, Tyr-βH), 2.71 (m, 1H, -CH_2CH$_3$), 2.50 (m, 2H, Cys-α, βH), 1.94 (m, 1H, -CH_2CH$_3$), 1.19 (m, 9H, *t*-Bu), 0.76 (brt, 3H, *J* = 7.1 Hz, -CH$_2$CH_3). ^{13}C-NMR: δ 61.4 (Cys-αC), 52.3 (Tyr-αC), 44.1 (-*C*H$_2$CH$_3$), 39.6 (Tyr-βC), 29.7 (Cys-βC), 28.7 (C(*C*H$_3$)$_3$), 13.6 (-CH$_2$*C*H$_3$). Anal. calcd for $C_{52}H_{52}N_2O_6S{\cdot}0.7H_2O$: C, 73.85; H, 6.36; N, 3.31; S, 3.79. Found: C, 73.81; H, 6.47; N, 3.25; S, 3.66% MALDI TOF MS, calcd for $C_{52}H_{52}N_2O_6S$ (M + Na)$^+$: 855.34. Found: *m*/*z* 855.38.

Synthesis of peptide thioesters

Fmoc-Rink amide MBHA resin (74 mg, 25 μmol) was subjected to automated synthesis by the FastMoc protocol to give Arg(Pbf)-Arg(Pbf)-NH-resin. To this resin, Fmoc-amino acyl *N*-ethyl-*S*-tritylcysteine (50 μmol), activated by DIC (11.6 μl, 75 μmol) and HOBt (10.1 mg, 75 μmol) in dichloromethane for 30 min at room temperature, was added. After the mixture was shaken overnight, the peptide chain was elongated by the FastMoc protocol. A part of the resin obtained (30 mg) was treated with a TFA cocktail (TFA : H_2O : triisopropylsilane 90 : 5 : 5, 400 μl) at room temperature for 1 h. After removing TFA under a nitrogen stream, the peptide was precipitated with ether. The precipitate was washed twice with ether, dried *in vacuo*, and dissolved in 3 ml of 50% aqueous acetonitrile containing 6 M urea and 5% (v/v) 3-mercaptopropionic acid (MPA). After the mixture was filtered, the filtrate was kept at 37 °C for 2 d. The solution was loaded on a RP-HPLC column (Mightysil 5C18, 10 × 250 mm) and the fraction containing the product was isolated and lyophilized to give the desired peptide thioesters.

Gln-Lys-Thr-Glu-Phe-SCH_2CH_2COOH

39% based on the amino group in the initial resin. MALDI-TOF mass, found: *m/z* 740.5, calcd for $(M + H)^+$: 740.3. Amino acid analysis: $Thr_{0.91}Glu_{2.05}Phe_1Lys_{1.02}$.

Met-Gly-Thr-Ala-Asn-SCH_2CH_2COOH

46% based on the amino group in the initial resin. MALDI-TOF mass, found: *m/z* 581.0, calcd for $(M + H)^+$: 581.2. Amino acid analysis: $Asp_{1.11}Thr_{1.03}Gly_1Ala_{0.99}Met_{1.00}$.

Phe-Lys-Val-Asp-Ser-SCH_2CH_2COOH

32% based on the amino group in the initial resin. MALDI-TOF mass, found: *m/z* 683.1, calcd for $(M + H)^+$: 683.3. Amino acid analysis: $Asp_{1.04}Ser_{0.85}Val_{1.01}Phe_1Lys_{1.02}$.

Acknowledgements

This work was supported by a grant-in-aid for creative scientific research (17GS0420) from the Japan Society for the Promotion of Science, and by a grant-in-aid for scientific research from the Ministry of Education, Sports, Science and Technology of Japan (20380069). We thank Dr H. Koshino for HRNMR, Dr T. Nakamura for HRMS, and staff of the analysis laboratory for elemental analysis at Riken. We also thank Tokai University for their support with a grant-in-aid for high technology research.

Notes and references

1 (*a*) H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, 1991, **64**, 111–117; (*b*) S. Aimoto, *Biopolymers*, 1999, **51**, 247–265.
2 (*a*) P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776–779; (*b*) P. E. Dawson and S. B. H. Kent, *Annu. Rev. Biochem.*, 2000, **69**, 923–960.
3 (*a*) S. Futaki, K. Sogawa, J. Maruyama, T. Asahara, M. Niwa and H. Hojo, *Tetrahedron Lett.*, 1997, **38**, 6237–6240; (*b*) X. Li, T. Kawakami and S. Aimoto, *Tetrahedron Lett.*, 1998, **39**, 8669–8672; (*c*) Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman and C. R. Bertozzi, *J. Am. Chem. Soc.*, 1999, **121**, 11684–11689; (*d*) R. Ingenito, E. Bianchi, D. Fattori and A. Pessi, *J. Am. Chem. Soc.*, 1999, **121**, 11369–11374; (*e*) J. Alsina, T. S. Yokumu and G. Barany, *J. Org. Chem.*, 1999, **64**, 8761–8769; (*f*) A. B. Clippingdale, C. J. Barrow and J. D. Wade, *J. Pept. Sci.*, 2000, **6**, 225–234; (*g*) D. Swinnen and D. Hilvert, *Org. Lett.*, 2000, **2**, 2439–2442; (*h*) A. R. Mezo, R. P. Cheng and B. Imperiali, *J. Am. Chem. Soc.*, 2001, **123**, 3885–3891; (*i*) A. Ishii, H. Hojo, Y. Nakahara, Y. Ito and Y. Nakahara, *Biosci., Biotechnol., Biochem.*, 2002, **66**, 225–232; (*j*) J. Brask, F. Albericio and K. Jensen, *Org. Lett.*, 2003, **5**, 2951–2953; (*k*) J. A. Cararero, B. J. Hackel, J. J. de Yoreo and A. R. Mitchell, *J. Org. Chem.*, 2004, **69**, 4145–4151; (*l*) J. D. Warren, A. R. Miller, S. J. Keding and S. J. Danishefsky, *J. Am. Chem. Soc.*, 2004, **126**, 6576–6578; (*m*) P. Botti, M. Villain, S. Manganiello and H. Gaertner, *Org. Lett.*, 2004, **6**, 4861–4864; (*n*) T. Kawakami, M. Sumida, K. Nakamura, T. Vorherr and S. Aimoto, *Tetrahedron Lett.*, 2005, **46**, 8805–8807; (*o*) N. Ollivier, J.-B. Behr, O. El-Mahdi, A. Blanpain and O. Melnyk, *Org. Lett.*, 2005, **7**, 2647–2650; (*p*) Y. Ohta, S. Itoh, A. Shigenaga, S. Shintaku, N. Fujii and A. Otaka, *Org. Lett.*, 2006, **8**, 467–470; (*q*) F. Nagaike, Y. Onuma, C. Kanazawa, H. Hojo, A. Ueki, Y. Nakahara and Y. Nakahara, *Org. Lett.*, 2006, **8**, 4465–4468; (*r*) S. Manabe, T. Sugioka and Y. Ito, *Tetrahedron Lett.*, 2007, **48**, 849–853; (*s*) T. Kawakami and S. Aimoto, *Tetrahedron Lett.*, 2007, **48**, 1903–1905; (*t*) S. Ficht, R. J. Payne, R. T. Guy and C.-H. Wong, *Chem.–Eur. J.*, 2008, **14**, 3620–3629; (*u*) D. Lelièvre, P. Barta, V. Aucagne and A. F. Delmas, *Tetrahedron Lett.*, 2008, **49**, 4016–4019; (*v*) J. Kang, J. P. Richardson and D. Macmillan, *Chem. Commun.*, 2009, 407–409; (*w*) S. Tsuda, A. Shigenaga, K. Bando and A. Otaka, *Org. Lett.*, 2009, **11**, 823–826; (*x*) T. Kawakami and S. Aimoto, *Tetrahedron*, 2009, **65**, 3871–3877; (*y*) A. P. Tofteng, K. K. Sørensen, K. W. Conde-Frieboes, T. Hoeg-Jensen and K. J. Jensen, *Angew. Chem., Int. Ed.*, 2009, **48**, 7411–7414.
4 (*a*) H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.*, 2007, **48**, 25–28 2007, **48**, 1299; (*b*) Y. Nakahara, I. Matsuo, Y. Ito, R. Ubagai, H. Hojo and Y. Nakahara, *Tetrahedron Lett.*, 2010, **51**, 407–410.
5 (*a*) H. Hojo, Y. Murasawa, H. Katayama, T. Ohira, Y. Nakahara and Y. Nakahara, *Org. Biomol. Chem.*, 2008, **6**, 1808–1813; (*b*) H. Katayama, H. Hojo, T. Ohira and Y. Nakahara, *Tetrahedron Lett.*, 2008, **49**, 5492–5494; (*c*) C. Ozawa, H. Katayama, H. Hojo, Y. Nakahara and Y. Nakahara, *Org. Lett.*, 2008, **10**, 3531–3533; (*d*) H. Katayama, T. Utsumi, C. Ozawa, Y. Nakahara, H. Hojo and Y. Nakahara, *Tetrahedron Lett.*, 2009, **50**, 818–821; (*e*) H. Katayama, H. Hojo, I. Shimizu, Y. Nakahara and Y. Nakahara, *Org. Biomol. Chem.*, 2010, **8**, 1966–1972.
6 Z. Z. Brown and C. E. Schafmeister, *J. Am. Chem. Soc.*, 2008, **130**, 14382–14383.
7 E. Atherton, J. L. Holder, M. Meldal, R. C. Sheppard and R. M. Valerio, *J. Chem. Soc., Perkin Trans. 1*, 1988, 2887–2894.