Application of Pac Ester in Thioester Method for the Synthesis of Cyclopentapeptides†

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Thioester method for the synthesis of cyclopeptides is improved by using Pac (Pac = phenacyl, $CH_2COC_6H_5$) ester as a protecting group of 3-mercaptopropionic acid. The Pac group is easy to be removed from *C*-terminal with zinc in acetic acid. The protected glycine thioester and peptide thioesters synthesized by the improved method, are easy to be purified, so the final linear peptides are pure enough for the following cyclization. Furthermore, this method is flexible for peptide chain elongation, either from *C*-terminal or from *N*-terminal. So it is an efficient and practical method for synthesis of bioactive peptides. Two *N*-protected pentapeptide thioesters, Boc-Pro-Tyr-Leu-Ala-GlySCH$_2$CH$_2$COOPac and Boc-Ala-Tyr-Leu-Ala-Gly-SCH$_2$CH$_2$COOPac were synthesized by the improved thioester method. After deprotecting Pac ester with zinc in aqueous acetic acid and Boc group with trifluoroacetic acid in CH_2Cl_2, two free pentapeptide thioesters were obtained. Ag^+-assisted cyclization in acetate buffered solution afforded two cyclic pentapeptides c(Pro-Tyr-Leu-Ala-Gly) and c(Ala-Tyr-Leu-Ala-Gly). Effects of different buffer pH, different Ag^+ concentrations, *etc*. on the cyclization were studied.

Keywords thioester method, Pac ester, cyclopentapeptide

Introduction

Various methods have been developed for the rapid synthesis of highly pure polypeptides. The solution method for peptide synthesis has been used for 100 years and the solid-phase method developed by Merrifield[1] has been continuously improved over the past 40 years. Among them thioester method is an effective strategy used for synthesis of both linear peptides and cyclic peptides in recent years. It was ascended to 1951 that Wieland found peptide bond could be formed with *N*-protected amino acid *S*-phenylester and amine,[2] but the *S*-phenylester was easy to be hydrolyzed by base. In 1981, Blake developed thiocarboxyl segment condensation strategy.[3] A peptide was prepared with thiocarboxylic acid at the *C*-terminus by solid-phase method and the segment condensation was carried out with an amino component in the presence of silver ions. Similarly, thiocarboxylic acid was unstable and easily decomposed. In 1991, Aimoto improved Blake's thioester method with using 3-mercaptopropionic acid or other similar acids as a thioester group.[4,5] In this method, *N*-protected amino acid thioester was anchored to a resin, then it was elongated to a peptide chain from *C*-terminal by solid-phase method and consequently segment condensation was accomplished in the presence of HOBt, HOOBt, *etc*. and a silver compound such as $AgNO_3$ or AgCl, which facilitated the conversion of thioester moiety to an active ester. After selective removal of the *N*-terminal protecting group, polypeptides were obtained, such as *C*-Myb protein(142-193)-NH_2. The thioester method was further studied by Tam for the synthesis of cyclic peptide.[6-8] Firstly, linear peptide thioester precursors were synthesized by solid-phase method. Then, the linear peptide thioester precursors were cyclized in acetate buffer through silver ion coordination with the linear peptide thioester. Lactones or lactams were obtained by controlling the reaction conditions, such as different acetate buffer pH, or with DMSO as a cosolvent *etc*.

In this paper, an improved thioester method is reported. The carboxyl group of 3-mercaptopropionic acid was protected by Pac (phenacyl, $CH_2COC_6H_5$) group, instead of anchoring to a resin as in Aimoto's method. The coupling procedures were carried out in solution, so the intermediate of each step could be purified. The result was reported briefly elsewhere.[9] Two cyclic pentapeptides, c(Ala-Tyr-Leu-Ala-Gly)[10] (**I**) and c(Pro-Tyr-Leu-Ala-Gly)[11,12](**II**), which were isolated from one kind of Chinese medicinal herbs, *Stellaria yunnanensis* Franch (M) and the roots of *Pseudostellaria heterophylla*, respectively, were synthesized by this thioester method in solution.

Results and discussion

3-Mercaptopropionic acid was used as a thioester group of Boc-glycine. Crystalline Boc-Gly-SCH$_2$CH$_2$COOH was prepared from Boc-Gly-ONp and HSCH$_2$CH$_2$COOH as

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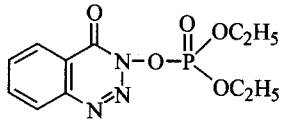
described by Aimoto.[4] Boc-Gly-SCH_2CH_2COOH was esterified easily with 2-bromo-acetophenone (PacBr) in the presence of diisopropyl ethyl amine (DIEA) at room temperature. The resulting Boc-glycine thioester, Boc-Gly-$SCH_2CH_2COOPac$ was then elongated using DEPBT [3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one][13-19] or DCC as a coupling reagent by solution method.



DEPBT

The synthetic route is shown in Scheme 1.

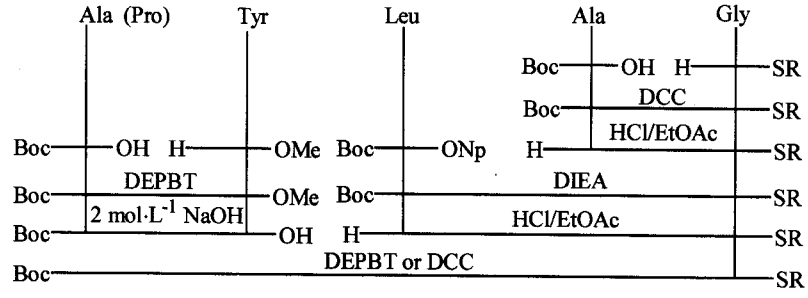
Physical constants of some *N*-protected amino acid and linear peptide thioester derivatives were shown in Table 1.

The phenacyl group can be removed with great facility at room temperature by zinc in 90% aqueous acetic acid.[20] Boc-Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH and Boc-Pro-Tyr-Leu-Ala-Gly-S-CH_2CH_2COOH were obtained in the yields of 97% and 83%, respectively. After deprotecting of Boc group from *N*-terminal with trifluoroacetic acid in CH_2Cl_2 (50%, *V*/*V*), two *N*-free pentapeptide thioesters, TFA·Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**III**) and TFA·Pro-Tyr-Leu-Ala-Gly-S-CH_2CH_2COOH (**IV**) were obtained in almost quantitative yields. The cyclization was carried out in 0.2 mol·L^{-1} sodium acetate buffer from pH 4.6 to 5.8 (Fig. 1). All cyclic peptides were obtained by RP-HPLC and their retention times were exactly the same as those of the standard samples, which were identified by FAB-MS, ^{1}H NMR and amino acid analysis.[17] The effects of different buffer pH, different concentrations of silver ion and substrates **III** and **IV** on the cyclizaton yields were studied (Fig. 2 and Table 2).

The highest yields were 83% and 36% at pH 5.6 and 5.8 for c(Ala-Tyr-Leu-Ala-Gly) (**I**) and c(Pro-Tyr-Leu-Ala-Gly) (**II**), respectively (Fig. 2). It was shown that *N*-terminal amino acid residue affected the cyclization yields greatly. The cyclization yield from peptide thioester **III** with Ala as *N*-terminal residue was much higher than that from peptide thioester **IV** with Pro as *N*-terminal, since Pro has a secondary amino group. As shown in Table 2, at least 3 eq. of silver ion was necessary. Both concentration of substrates **III** or **IV** and concentration of silver ion affected the cyclization yields greatly. At relatively high concentration (5 mmol·L^{-1}) of linear pentapeptide thioester **III** or **IV**, dimerization was not detected by RP-HPLC.

On the other hand, instead of 3-mercaptopropionic acid, benzylmercaptan was used for the preparation of *N*-protected amino acid or peptide thiobenzyl esters. An *N*-protected linear pentapeptide thiobenzyl ester Boc-Pro-Tyr-Leu-Ala-Gly-$SCH_2C_6H_5$ was synthesized by solution method using DEPBT as a coupling reagent (Scheme 2).

Scheme 1 Synthetic route of Boc-Pro-Tyr-Leu-Ala-Gly-$SCH_2CH_2COOPac$ and Boc-Ala-Tyr-Leu-Ala-Gly-$SCH_2CH_2COOPac$



R = $CH_2CH_2COOPac$; Pac $CH_2COC_6H_5$

Boc-GlyOH → Boc-GlyONp → Boc-Gly-SCH_2CH_2COOH → Boc-Gly-$SCH_2CH_2COOPac$

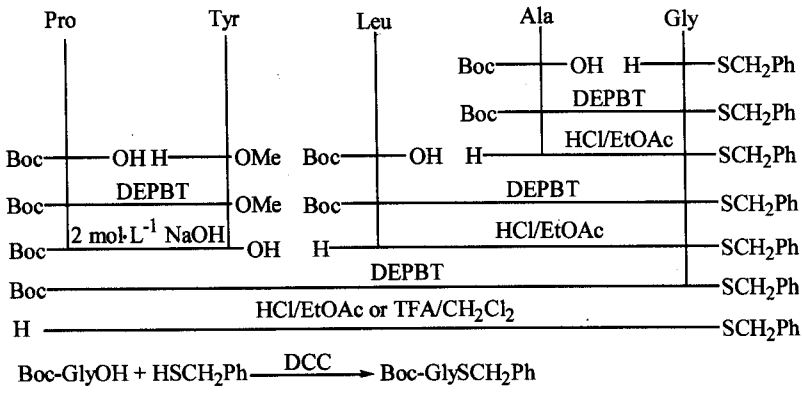
DEPBT: 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one

Table 1 Physical constants of some *N*-protected amino acid and linear peptide thioester derivatives

Compound	Yield (%)	m.p. (℃)	FAB-MS $(M+H)^+$	$[\alpha]_D^{20}$
BocGlySCH_2CH_2COOH[a]	85.0	103—106	264	
BocGly$SCH_2CH_2COOPac$	85.3	73—74	382	
BocAlaGly$SCH_2CH_2COOPac$	75.2	98—99	453	−12.3 (*c*, 1 AcOEt)
BocLeuAlaGly$SCH_2CH_2COOPac$	56.5	108—109	566	−20.8 (*c*, 1 AcOEt)
BocProTyrLeuAlaGly$SCH_2CH_2COOPac$	50.8	132—135	826	−37.5 (*c*, 1 MeOH)
BocAlaTyrLeuAlaGly$SCH_2CH_2COOPac$	44.6	127—130	800	−32.8 (*c*, 1 MeOH)

[a] Total yield from Boc-GlyONp.

Scheme 2 Synthetic route of linear pentapeptide thiobenzyl ester of Pro-Tyr-Leu-Ala-Gly-SCH_2Ph



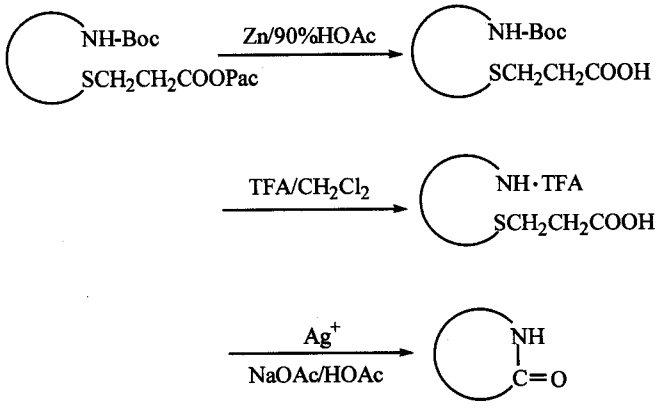


Fig. 1 Scheme of deprotection and cyclization

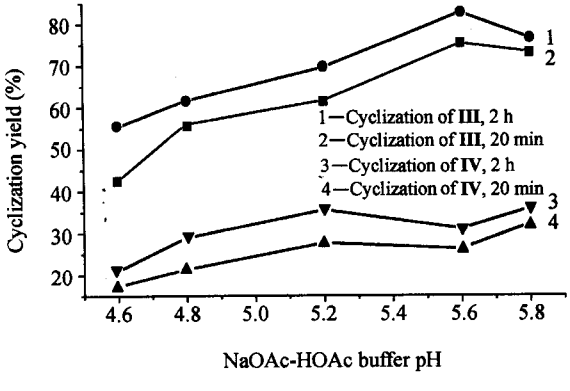


Fig. 2 Effect of different buffer pH on the cyclization yields of TFA·Ala-Tyr-Leu-Ala-Gly-S-CH_2CH_2COOH (**III**) and TFA·Pro-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**IV**).

After deprotection of Boc group of Boc-Pro-Tyr-Leu-Ala-Gly-SCH_2C6H_5 with 50% TFA in CH_2Cl_2 or anhydrous HCl in EtOAc, it was found that thiobenzyl group could not be converted to its corresponding active ester in THF with HOBt, AgCl or $AgNO_3$; or activated by silver ions in acetate buffer with CH_3COOAg. The reason was probably that the thiobenzyl ester group was too stable to release from the linear peptide by the weak interaction between sulfur atom in the thioester and a silver ion in the solution.

Table 2 Cyclization yields (%) of **I** or **II** with different concentrations of Ag^+, **III** or **IV**[a]

Ag^+ (equiv.)	Concentration of **III** or **IV** ($mmol·L^{-1}$)	20 min		2 h	
		I	**II**	**I**	**II**
3	5	73	32	77	36
	1	48	11	62	29
1	5	20	6	36	14
	1	< 5	< 5	9	< 5

[a] RP-HPLC analysis conditions were the same as described in Fig. 2. Ag^+ was provided by CH_3COOAg. c(Ala-Tyr-Leu-Ala-Gly) (**I**); c(Pro-Tyr-Leu-Ala-Gly) (**II**); TFA · Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**III**); TFA·Pro-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**IV**).

In summary, the thioester method for peptide synthesis was improved by using Pac ester as the protecting group of the carboxyl group of 3-mercaptopropionic acid. Two cyclic pentapeptides, c(Ala-Tyr-Leu-Ala-Gly) and c(Pro-Tyr-Leu-Ala-Gly) were synthesized by this thioester method. The protected glycine thioester or protected peptide thioesters were easy to be purified by recrystallization or column separation in each step. Thus the final linear peptide was pure enough for last cyclization. The Pac group was easy to be removed from *C*-terminal with zinc in acetic acid. This method is suitable for peptide chain elongation, from either *C*-terminal or *N*-terminal. Furthermore, it is also an efficient and practical method for synthesis of small bioactive peptides, including cyclic peptides by solution method.

Experimental

^1H NMR spectra were taken on a Bruker ARX200, ARX400 spectrometers. Tetramethylsilane (TMS) was used as an internal standard. Elemental analyses were carried out on an Elementar Vario EL (Germany). Melting points were determined with a Yanaco apparatus and uncorrected. Mass spectra were recorded on a ZAB-HS spectrometer (Micromass, Manchester, UK). And the data of HRMS were recorded by APEX II FT-ICR-MS and BIFLE XIII spectrometers (Bruker Daltonics Inc. Billerica, MA,

USA) using *L*-SIMS ionization. Optical rotation was determined with a Perkin Elmer 341LC. Freeze-drying was carried out on a Flexi-Dry$^{TM}\mu$P, FTS SYSTEMS, INC. (USA). Samples were detected by HPLC on an HP1100 System. Semi-preparation of cyclic pentapeptide was carried out on a Waters 600E LC System, Water 486 monitor. Amino acids used in this paper are all of *L*-configuration. Abbreviations: Standard abbreviations for amino acids and peptide derivatives are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature *Eur. J. Biochem.* **1984**, *138*, 9—37. Other abbreviations: Boc, *tert*-butyloxycarbonyl; Me, methyl; THF, tetrahydrofuran; DMF, *N*, *N*-dimethylformamide; DIEA, diisopropyl ethyl amine; MS, mass spectrometry; NMR, nuclear magnetic resonance. ONp, 4-nitrophenyl ester; Pac, phenacyl; HOBt, 1-hydroxybenzotriazole; HOOBt, 3-hydroxy-1, 2, 3-benzotriazin-4(3*H*)-one; DCC, *N*,*N'*-dicyclohexyl- carbodiimide; DCU, *N*, *N'*-dicyclohexylurea; TLC, thin layer chromatography; RP-HPLC, reversed-phase high performance liquid chromatography. The solvents were dried according to standard methods. Acetonitrile was of chromatographic purity and used in RP-HPLC after ultra filtration.

Boc-Gly-$SCH_2CH_2COOPac$

To a solution of Boc-Gly-SCH_2CH_2COOH (3.16 g, 12 mmol) dissolved in DMF (200 mL), 2-bromoacetophenone (PacBr) (2.39 g, 12 mmol) and DIEA (2.6 g, 20 mmol) were added with stirring at room temperature overnight. After evaporation of the solvent under reduced pressure, the residue was dissolved in ethyl acetate (300 mL). The ethyl acetate layer was washed successively with 5% citric acid (50 mL × 2), water (50 mL), 5% Na_2CO_3 (50 mL) and saturated brine (80 mL × 2), dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The crude product obtained was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (from 20:1 to 2:1, *V*:*V*) as eluent. Recrystallization from petroleum ether/ethyl acetate afforded white powder 3.9 g, yield 85.3%. m.p. 73—74 ℃; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.47 (s, $C(CH_3)_3$, 9H), 2.84 (t, J = 7.4 Hz, 2H, SCH_2CH_2), 3.23 (t, J = 6.4 Hz, 2H, SCH_2CH_2), 4.21 (d, J = 5.4 Hz, 2H, $NHCH_2$), 5.05—5.16 (br, 1H, NH), 5.37 (s, 2H, OCH_2), 7.41—7.58 (m, 2H, Ar(*m*)H), 7.60 (d, J = 6.0 Hz, 1H, Ar(*p*)H), 7.92 (d, J = 7.0 Hz, 2H, Ar(*o*)H,); MS (FAB) *m*/*z*: 382 $(M + H)^+$. Anal. calcd for $C_{18}H_{23}NO_6S$: C 56.69, H 6.04, N 3.68; found C 56.91, H 6.10, N 3.52.

Boc-Ala-Gly-$SCH_2CH_2COOPac$

Boc-Gly-$SCH_2CH_2COOPac$ (3.04 g, 8 mmol) was dissolved in a solution of saturated HCl in 10 mL of EtOAc and stirred at room temperature for 15 min. The solution was evaporated to dryness *in vacuo*. The excess HCl was removed by evaporation with anhydrous methanol (20 mL × 2) and anhydrous benzene (20 mL × 2) *in vacuo*, and the residue was suspended in 80 mL of THF. Boc-Ala-OH (1.51 g, 8 mmol) and HOBt (1.19 g, 8.8 mmol) were added. The pH of solution was adjusted to 7 with DIEA. The solution was stirred and cooled in an ice-water bath while DCC (1.81 g, 8.8 mmol) was added. Stirring was continued for one hour at 0 ℃ and overnight at room temperature. The DCU separated was removed by filtration and the solvent evaporated *in vacuo*. The residue was dissolved in ethyl acetate (250 mL). The ethyl acetate layer was washed successively with 5% citric acid (30 mL × 2), water (30 mL), 5% Na_2CO_3(30 mL) and saturated brine (50 mL × 2), dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The crude product obtained was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (from 20:1 to 1:1, *V*:*V*) as eluent. Recrystallization from petroleum ether/ethyl acetate afforded white powder 2.72 g, yield 75.2%. m.p. 98—99 ℃; $[\alpha]_D^{20}$ − 12.3 (*c* 1, AcOEt); ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.41 (d, J = 7.2 Hz, 3H, CH_3), 1.46 (s, 9H, $C(CH_3)_3$), 2.82 (t, J = 7.4 Hz, 2H, SCH_2CH_2), 3.22 (t, J = 6.4 Hz, 2H, SCH_2CH_2), 4.19—4.25 (m, 3H, $CH(CH_3)NH$, CH_2NH), 4.92—5.08 (br, 1H, NH(Gly)), 5.37 (s, 2H, OCH_2CO), 6.80—6.95 (br, 1H, NH(Ala)), 7.42—7.58 (m, 2H, Ar(*m*)H), 7.61 (d, J = 6.0 Hz, 1H, Ar(*p*)H), 7.92 (d, J = 7.0 Hz, 2H, Ar(*o*)H,); MS (FAB) *m*/*z*: 453 $(M + H)^+$. Anal. calcd for $C_{21}H_{28}N_2O_7S$: C 55.75, H 6.19, N 6.19; found C 55.65, H 5.83, N 6.06.

Boc-Leu-Ala-Gly-$SCH_2CH_2COOPac$

Boc-Ala-Gly-$SCH_2CH_2COOPac$ (2.4 g, 5.31 mmol) was dissolved in a solution of saturated HCl in 10 mL of EtOAc and stirred at room temperature for 20 min. The solution was evaporated to dryness *in vacuo*. The excess HCl was removed by evaporation with anhydrous methanol (30 mL × 2) and anhydrous benzene (30 mL × 2) *in vacuo*, and the residue was suspended in 10 mL of DMF. Boc-LeuONp (1.93 mg, 5.31 mmol) was added and the pH of solution was adjusted to 8—9 with DIEA. The solution monitored by TLC was stirred at room temperature for 3 h. Saturated brine (20 mL) was added. The solution was extracted by ethyl acetate (50 mL × 4). The organic layer was washed successively to colorless with 5% citric acid (30 mL × 2), water (30 mL), 5% Na_2CO_3(30 mL) and saturated brine (30 mL × 2), dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (from 5:1 to 1:2, *V*:*V*) as eluent. Recrystallization from petroleum ether/ethyl acetate afforded white powder 1.39 g, yield 56.5%. m.p. 108—109 ℃; $[\alpha]_D^{20}$ − 20.8 (*c* 1, AcOEt); ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 0.82—1.04 (d, J = 6.0

Hz, 6H, $CH(CH_3)_2$), 1.43 (d, J = 4.4 Hz, 3H, CH_3), 1.45 (s, 9H, $C(CH_3)_3$), 1.58—1.86 (m, 3H, $CH_2CH(CH_3)_2$), 2.82 (t, J = 7.2 Hz, 2H, CH_2CH_2), 3.21 (t, J = 6.8 Hz, 2H, CH_2CH_2), 3.94—4.24 (m, 3H, $CH(CH_3)NH$, CH_2NH), 4.55 (t, J = 7.0 Hz, 1H, $NHCH(CO)CH_2$), 4.93 (br, 1H, NH(Gly)), 5.37 (s, 2H, OCH_2CO), 6.60—6.86 (br, 1H, NH(Ala)), 7.18—7.38 (br, 1H, NH(Leu)), 7.38—7.58 (m, 2H, Ar(m)H), 7.61 (d, J = 6.0 Hz, 1H, Ar(p)H), 7.91 (d, J = 7.2 Hz, 2H, Ar(o)H); MS (FAB) m/z: 566 $(M+H)^+$. Anal. calcd for $C_{27}H_{39}N_3O_8S$: C 57.34, H 6.90, N 7.43; found C 57.32, H 6.88, N 7.28.

Boc-Pro-Tyr-Leu-Ala-Gly-$SCH_2CH_2COOPac$

Boc-Pro-Tyr-Leu-Ala-Gly-$SCH_2CH_2COOPac$ was synthesized from Boc-Pro-Tyr-OH and HCl·Leu-Ala-Gly-$SCH_2CH_2COOPac$, which was obtained from Boc-Leu-Ala-Gly-$SCH_2CH_2COOPac$ (566 mg, 0.1 mmol) with saturated HCl in 5 mL of EtOAc, employing the same procedure described for protected dipeptide Boc-Ala-Gly-S-$CH_2CH_2COOPac$. The crude product was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (from 10:1 to 1:2, $V:V$) as eluent. Recrystallization from ethyl acetate afforded white powder 419.0 mg, yield 50.8%. m.p. 132—135 ℃; $[\alpha]_D^{20}$ − 37.5 (c 1, AcOEt); ^1H NMR ($CDCl_3$, 400 MHz) δ: 0.91 (t, J = 5.8 Hz, 6H), 1.39 (s, 9H), 1.47 (d, J = 7.2 Hz, 3H), 1.58 (d, J = 11.2Hz, 3H), 1.73 (br, 2H), 1.88 (br, 3H), 2.17 (br, 2H), 2.80 (t, J = 7.3 Hz, 2H), 3.06 (d, J = 6.2 Hz, 2H), 3.17 (t, J = 6.9 Hz, 2H), 3.43 (br, 2H), 4.02 (q, J = 5.3 Hz, 1H), 4.08 (br, 1H), 4.32 (q, J = 5.3 Hz, 1H), 4.38—4.62 (m, 3H), 5.36 (d, J = 3.8 Hz, 2H), 6.44 (br, 1H), 6.82 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 8.0 Hz, 2H), 7.19 (d, J = 6.4Hz, 1H), 7.49 (t, J = 7.6 Hz, 3H), 7.62 (t, J = 7.3 Hz, 1H), 7.90 (d, J = 7.6 Hz, 1H); MS (FAB) m/z: 826 $(M+H)^+$. Anal. calcd for $C_{41}H_{55}N_5O_{11}S$: C 59.61, H 6.72, N 8.48. found C 59.45, H 6.60, N 8.40.

Boc-Ala-Tyr-Leu-Ala-Gly-$SCH_2CH_2COOPac$

After deprotection Boc-group of Boc-Leu-Ala-Gly-$SCH_2CH_2COOPac$ (344.7 mg, 0.61 mmol) with saturated HCl in 5 mL of AcOEt, the residue was dissolved in DMF (4 mL). Boc-Ala-Tyr-OH (235.4 mg, 0.67 mmol) and DEPBT (200.6 mg, 0.67 mmol) were added to the solution. The pH of solution was adjusted to 8—9 by DIEA. The solution was stirred for 8 h at room temperature. The washing procedure was the same as that of Boc-Ala-Gly-$SCH_2CH_2COOPac$. The residue was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (from 10:1 to 1:2, $V:V$) as eluent and afforded 217.4 mg, yield 44.6%. m.p. 127—130 ℃; $[\alpha]_D^{20}$ − 32.8 (c 1, MeOH); ^1H NMR ($CDCl_3$, 400 MHz) δ: 0.91 (t, J = 5.5 Hz, 6H), 1.33 (d, J = 6.9 Hz, 3H), 1.36 (s, 9H), 1.48 (d, J = 7.3 Hz, 3H), 1.53—1.80 (m, 4H), 2.80 (t, J = 7.2 Hz, 2H), 2.93—3.13 (m, 2H), 3.17 (t, J = 7.0 Hz, 2H), 3.92 (br, 1H), 4.06 (q, J = 5.5 Hz, 1H), 4.24 (q, J = 6.4 Hz, 1H), 4.36—4.64 (m, 3H), 5.18—5.35 (br, 1H), 5.36 (s, 2H), 6.74 (br, 1H), 6.80 (d, J = 7.6 Hz, 2H), 6.98 (d, J = 8.1 Hz, 2H), 7.19 (br, 1H), 7.36 (br, 1H), 7.49 (t, J = 7.8 Hz, 2H), 7.62 (t, J = 7.8 Hz, 2H), 7.90 (d, J = 7.4 Hz, 2H); HRMS calcd for $C_{39}H_{53}N_5O_{11}S$ 800.3540 $(M+H)^+$, found 800.3522.

TFA·Pro-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**IV**)

Boc-Pro-Tyr-Leu-Ala-Gly-$SCH_2CH_2COOPac$ (124.8 mg, 0.15 mmol) was dissolved in 10 mL of 90% aqueous acetic acid. Zinc dust (0.5 g) prepared freshly was added to the solution under stirring at room temperature for 1 h monitored by TLC. The zinc residue was filtered and washed with methanol (10 mL × 2). The filtrate was evaporated to remove methanol *in vacuo* and extracted with ethyl acetate (15 mL × 3). The organic layer was washed successively with saturated brine (10 mL), water (5 mL × 2) and saturated brine (10 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was purified by preparative-TLC on silica gel with chloroform:methanol:acetic acid (90:5:0.5, $V:V:V$) as eluent and afforded white compound 88.8 mg, yield 83.0%. The white compound was dissolved in TFA/CH_2Cl_2 (V/V, 50%) with stirring at room temperature monitored by TLC. The solution was evaporated to dryness. The residue was dissolved in water (1—2 mL) and lyophilized to afford white compound 84.6 mg, yield 93.4%. m.p. 135—139 ℃. HRMS calcd for $C_{28}H_{41}N_5O_8S$ 608.2748 $(M+H)^+$, found 608.2730.

TFA·Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**III**)

Boc-Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH [125.8 mg, yield 97.2%, MS (FAB) m/z: 704 $(M+Na)^+$] was obtained from Boc-Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2-COOPac (150 mg, 0.19 mmol), and TFA·Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (90.7 mg) was obtained in the same procedure described above. Yield 68.7%. HRMS calcd for $C_{26}H_{39}N_5O_8S$ 582.2592 $(M+H)^+$, found 582.2581.

Cyclization of TFA·Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**III**) *and* TFA·Pro-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (**IV**)

TFA·Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH and TFA·Pro-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH were dissolved in 0.2 mol·L^{-1} acetate buffer (5 and 1 mmol·L^{-1}, respectively) at pH 5.8, 5.6, 5.2, 4.8 and 4.6, respectively with stirring at room temperature. Then, CH_3COOAg (15

and 5 mmol/L, respectively) was added to the solution. The cyclization was monitored and analyzed by RP-HPLC, which was performed on a Microsorb column (250 × 4.6 mm) at a flow rate of 1 mL/min with a 30 min linear gradient of 5%—50% CH_3CN in H_2O and a 10 min linear gradient of 50%—5% CH_3CN in H_2O containing 0.1% TFA at 260 nm.

Semi-preparative HPLC *of* c(Ala-Tyr-Leu-Ala-Gly) (**I**) *and* c (Pro-Tyr-Ile-Ala-Gly) (**II**)

TFA·Ala-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (14 mg, 0.02 mol·L^{-1}) was dissolved in 4 mL of 0.2 mol·L^{-1} acetate buffer at pH 5.6. Then, CH_3COOAg (10 mg, 0.06 mmol) was added to the solution with stirring at room temperature. After filtration, the solution was condensed to about 1 mL *in vacuo*. Semi-preparation by RP-HPLC and followed by freeze-drying afforded the cyclic pentapeptide c(Ala-Tyr-Leu-Ala-Gly) (6.4 mg, 13.5 μmol), yield 67%. MS (FAB) *m/z*: 476 $(M+H)^+$. TFA·Pro-Tyr-Leu-Ala-Gly-SCH_2CH_2COOH (28 mg, 0.038 mmol) was dissolved in 7.6 mL of 0.2 mol·L^{-1} acetate buffer at pH 5.8. Then, CH_3COOAg (20 mg, 0.12 mmol) was added to the solution with stirring at room temperature for 2 h. After filtration, the solution was condensed to about 1 mL *in vacuo*. Semi-preparation by RP-HPLC and the following freeze-drying afforded the cyclic pentapeptide c(Pro-Tyr-Ile-Ala-Gly) (5.0 mg, , 9.98 μmol), yield 26%. MS (FAB) *m/z*: 502 $(M+H)^+$.

HPLC semi-preparation condition: Waters 600E LC System, Vydac™ column, C_{18} (250 × 10 mm) with a 30 min linear gradient of 5%—50% CH_3CN in H_2O and a 10 min linear gradient of 50%—5% CH_3CN in H_2O containing 0.1% TFA at a flow rate of 2 mL/min at 220 nm.

Boc-Gly-SCH_2Ph

Boc-Gly-OH (1.314 g, 7.5 mmol), $PhCH_2SH$ (0.93 g, 7.5 mmol) and DMAP (91.5 mg, 0.75 mmol) were dissolved in CH_2Cl_2 (30 mL). The solution was stirred and cooled in an ice-water bath while DCC (1.7 g, 8.25 mmol) was added. Stirring was continued for one hour at 0 ℃ and overnight at room temperature. The DCU separated was removed by filtration and the solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (250 mL). The ethyl acetate layer was washed successively with 5% citric acid (30 mL × 2), water (30 mL), 5% Na_2CO_3 (30 mL × 2) and saturated brine (50 mL × 2), dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. Recrystallization from petroleum ether/ethyl acetate obtained 1.584 g, yield 75%. m.p. 85—86 ℃; R_f: 0.67 (AcOEt : petroleum ether = 1:2, *V*:*V*). MS (FAB) *m/z*: 282 $(M+H)^+$, 304 $(M+Na)^+$. Anal. calcd for $C_{14}H_{19}NO_3S$: C 59.76, H 6.81, N 4.98; found C 59.82, H 6.78, N 5.05.

Boc-Ala-Gly-SCH_2Ph

Boc-Gly-SCH_2Ph (1.12 g, 4 mmol) was deprotected as described above and dissolved in THF (50 mL). Boc-Ala-OH (0.76 g, 4 mmol), HOBt (0.57 g, 4.2 mmol) and Et_3N (0.445 g, 4.4 mmol) were added to the solution under stirring. DCC (0.865 g, 4.2 mmol) was added at room temperature. Stirring was continued overnight. The crude product was purified by column chromatography on silica gel with petroleum ether/ethyl acetate to afford white powder 0.592 g, yield 42%. m.p. 82—83 ℃; R_f: 0.24 (AcOEt:petroleum ether = 1:2, *V*:*V*); $[\alpha]_D^{20}$ + 7.6 (*c* 0.25, AcOEt); MS (FAB) *m/z*: 353 $(M+H)^+$.

Boc-Leu-Ala-Gly-SCH_2Ph

Boc-Ala-Gly-SCH_2Ph (140.8 mg, 0.4 mmol) was deprotected as described above and dissolved in THF (10 mL). Boc-Leu-OH (120 mg, 0.48 mmol), DEPBT (131.6 mg, 0.44 mmol) and Et_3N (84.8 mg, 0.84 mmol) were added to the solution under stirring. Stirring was continued for 24 h. The crude product was purified by column chromatography on silica gel with petroleum ether/ethyl acetate as eluent to give white powder 131.2 mg, yield 71%. m.p. 123.5—124.5 ℃; R_f: 0.54 (AcOEt: petroleum ether = 2 : 1, *V* : *V*); $[\alpha]_D^{20}$ − 28.9 (*c* 1, AcOEt); MS (FAB) *m/z*: 466 $(M+H)^+$, 488 $(M+Na)^+$. Anal. calcd for $C_{23}H_{35}N_3O_5S$: C 59.33, H 7.58, N 9.03; found C 59.63, H 7.58, N 8.90.

HCl·Pro-Tyr-Leu-Ala-Gly-SCH_2Ph

Boc-Leu-Ala-Gly-SCH_2Ph (130 mg, 0.28 mmol) was deprotected as described above and dissolved in THF (20 mL). Boc-Pro-Tyr-OH[19] (116.5 mg, 0.31 mmol), DEPBT (92 mg, 0.31 mmol) and Et_3N (60 mg, 0.59 mmol) were added to the solution under stirring. Stirring was continued overnight. The crude product was purified by column chromatography on silica gel with petroleum ether/ethyl acetate as eluent to afford white powder 142.6 mg, yield 70%. m.p. 163—169 ℃; R_f: 0.44 (AcOEt: petroleum ether = 9 : 1, *V* : *V*). MS (FAB) *m/z*: 726 $(M+H)^+$, 748 $(M+Na)^+$. Boc-Pro-Tyr-Leu-Ala-Gly-SCH_2Ph (50 mg) was dissolved in a solution of saturated HCl in 5 mL of EtOAc to afford HCl·Pro-Tyr-Leu-Ala-Gly-SCH_2Ph. MS (FAB) *m/z*: 626 $(M+H)^+$. HRMS calcd for $C_{32}H_{43}N_5O_6S$ 626.3007 $(M+H)^+$, found 626.3000.

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