Peptide Synthesis in Water IV. Preparation of *N*-Ethanesulfonylethoxycarbonyl (Esc) Amino Acids and Their Application to Solid Phase Peptide Synthesis

Keiko HOJO,[a] Mitsuko MAEDA,[a] Timothy J. SMITH,[b] Eriko KITA,[a] Fumie YAMAGUCHI,[a] Sachiko YAMAMOTO,[a] and Koichi KAWASAKI*,[a]

[a] *Faculty of Pharmaceutical Sciences, Kobe Gakuin University; Nishi-ku, Kobe 651–2180, Japan: and* [b] *School of Pharmacy, University of Pacific; 3601, Pacific Avenue, Stockton, CA 95211, U.S.A.*
Received November 25, 2003; accepted January 16, 2004

A new *N*-protecting group, ethanesulfonylethoxycarbonyl (Esc), was designed to perform peptide synthesis in both aqueous and organic solvents. Esc-amino acids were prepared by the reaction of Esc-Cl and amino acids. Although Esc-Cl was a highly reactive reagent, it was not stable and decomposed during the purification procedure. A more stable reagent, ethanesulfonylethyl-4-nitrophenyl carbonate (Esc-ONp), was designed for preparation of Esc-amino acids. Esc-ONp was a stable reagent and could be purified by silica gel column chromatography or recrystallization. Esc-amino acids were prepared by the reaction of Esc-ONp and amino acids in good yield. To evaluate Esc-amino acids, Leu-enkephalin amide was synthesized using Esc-amino acids by the solid phase method in water. Removal of the Esc group was performed with 0.025 mol/l NaOH in 50% aqueous ethanol. Leu-enkephalin amide was successfully synthesized on a poly(ethylene glycol)-grafted polystyrene resin. Esc-amino acids have moderate solubility in organic solvents (such as dimethylformamide and acetonitrile). Leu-enkephalin amide was synthesized using Esc-amino acids by the solid phase method in dimethylformamide. Removal of the Esc group was performed with 0.05 mol/l tetrabutylammonium fluoride in dimethylformamide. Synthesis of Leu-enkephalin amide using Esc-amino acids in dimethylformamide was also successful. The yields of synthesis of Leu-enkephalin amide in water and dimethylformamide were 71% and 67%, respectively.

Key words water-soluble protecting group; ethanesulfonylethoxycarbonyl; synthesis in water

Synthesis of peptides was an arduous task before development of the solid phase method. The solid phase method has facilitated automated peptide synthesis and the development of combinatorial chemistry. It has many advantages compared with classical solution peptide synthesis, but requires a large amount of organic solvents. Disposal of organic solvents is one of many important environmental problems, therefore we decided to perform peptide synthesis in water. To perform synthesis in water, protected amino acids must be water-soluble. We have studied water-soluble *N*-protecting groups and we developed 2-[phenyl(methyl)sulfonio]ethoxycarbonyl (Pms)[1,2] as a water-soluble *N*-protecting group. Since the Pms group is an onium salt, it is rather unstable in comparison with *N*-protecting groups which are in general use (such as *t*-butoxycarbonyl group,[3] benzyloxycarbonyl group[4] and 9-fluorenylmethoxycarbonyl (Fmoc) group[5]) for peptide synthesis. Here we report preparation of the ethanesufonylethoxycarbonyl (Esc) group and its application to peptide synthesis in water.

Tesser and Balvert-Geers[6] reported the methanesulfonylethoxycarbonyl (Msc) group as a readily removable *N*-protecting group by treatment with 0.1 or 0.2 N NaOH solution. This protecting group is hydrophilic rather than hydrophobic. We designed the Esc group (Fig. 1), expecting it to display both hydrophilic and hydrophobic character (*i.e.* we expect Esc-amino acids to exhibit reasonable solubility in aqueous and organic solvents). The Esc group was introduced onto amino acids by the reaction of 2-ethanesulfonylethyl chloroformate (Esc-Cl) as shown in Chart 1 (Route 1). 2-Ethanesulfonylethanol was converted to Esc-Cl by treatment with phosgene (prepared from triphosgene)[7] in dichloromethane. Since Esc-Cl was labile and decomposed during purification, it was used without purification for the next acylating reaction. Esc-Cl, H-Phe-OH and triethylamine were allowed to react in aqueous 50% acetonitrile to give Esc-Phe-OH. HPLC analysis of the crude product exhibited impurities and the product could not be purified by silica gel column chromatography and recrystallization. Pure Esc-Phe-OH was obtained by RP-HPLC purification and the yield was 38%. To improve the reaction yield and purification procedure, another synthetic route (Route 2 in Chart 1) to the Esc-amino acid was studied. 2-Ethanesulfonylethanol was reacted with 4-nitrophenyl chloroformate to give ethanesulfonylethyl-4-nitrophenyl carbonate (Esc-ONp). The crude Esc-ONp was stable and could be purified by silica gel column chromatography or recrystallization from a mixture of ether and hexane. The yield was 78%. Other Esc-amino acids (Esc-Leu-OH, Esc-Gly-OH, Esc-Tyr-OH) were also prepared using Esc-ONp and their analytical data are shown in Table 1. Esc-Gly-OH and Esc-Tyr-OH were soluble in water and Esc-Leu-OH and Esc-Phe-OH were not readily soluble in water. Esc-Leu-OH and Esc-Phe-OH were soluble in aqueous 2% Triton X. To evaluate Esc-amino acids, Leu-enkephalin amide was synthesized by the solid phase method in water using Esc-amino acids. For solid phase synthesis in water, a resin which swells appreciably well in water is necessary. Recently, poly(ethylene glycol)-grafted polystyrene resins[8] were developed to increase the swelling ability of the resin in

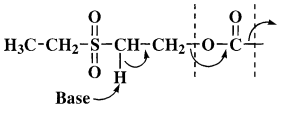

Fig. 1. Structure of the Esc Group

* To whom correspondence should be addressed. e-mail: kawasaki@pharm.kobegakuin.ac.jp

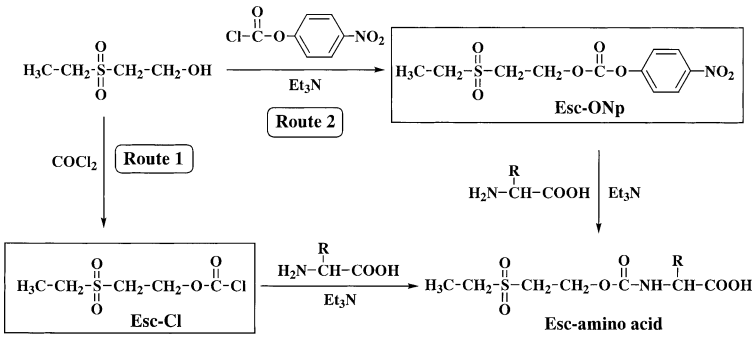

Chart 1. Preparation of Esc-Amino Acids

Table 1. Analytical Data of Synthetic Esc-Amino Acids

Amino acid	MW	TOF-MS (m/z)	$[\alpha]_D^{20}$ (c=1.0, CH_3CN)	Elemental anal. Calcd (Found)			Route 1 yield (%)	Route 2 yield (%)
				C	H	N		
		$(M+Na)^+$		44.73	7.17	4.74		
Leu	281.37	318.26	−9.2	(44.50)	(6.88)	(4.74)	—	68.2
		$(M+Na)^+$		51.05	5.81	4.25		
Phe	329.37	352.12	15.5	(50.80)	(5.67)	(4.22)	39.3	78.3
		$(M+Na)^+$		35.14	5.48	5.85		
Gly	239.25	232.23	—	(35.02)	(5.29)	(5.88)	—	57.0
		$(M+Na)^+$		48.69	5.55	4.06		
Tyr	368.29	368.29	−9.2	(47.98)	(5.23)	(4.06)	—	43.1

various solvents, including water. The present synthesis was performed on the poly(ethylene glycol)-grafted Rink amide polystyrene resin (Rink-PEG amide resin)[9] which was reported to have good swelling ability in water. Coupling reactions were performed with a water-soluble carbodiimide [WSCD, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] in aqueous 2% Triton X. Triton X increases the solubility of Esc-amino acids and increases the swelling ability of the resin. An additive was used to accelerate coupling reactions. Introduction of Esc-Leu-OH on the resin was performed with WSCD in the presence of *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB)[10] for 2 h. The yield of the reaction was satisfactory judging from the Leu-content on the resin. The next coupling reaction (introduction of Esc-Phe-OH on the H-Leu-Rink-PEG amide resin) was performed under the same conditions, but the reaction was slow. Since by-products which did not contain Phe and Gly were obtained after completing the synthesis of Leu-enkephalin amide, we speculated that introduction of Esc-Phe-OH and the reaction for the removal of the Esc group on Phe were slow. We examined the coupling reaction conditions for introduction of Esc-Phe-OH on the H-Leu-Rink-PEG amide resin and the deprotection conditions for the Esc-Phe-Leu-Rink-PEG amide resin. First, various additives [HONB, 1-hydroxybenzotriazole (HOBt),[11] 1-hydroxy-7-azabenzotriazole (HOAt),[12] 1-hydroxysuccimide (HOSu)[13] and imidazole] were examined to stimulate the coupling reaction by the WSCD method. Equi-molar additive to Esc-Phe-OH was used. After the reaction, the Esc-group was removed and the resin was hydrolyzed. Phe and Leu content in an acid hydrolysate was analyzed and the yield was calculated from the amino acid ratio of Phe and Leu. Addition of HOBt, HOAt, imidazole and HOSu did not give better results compared with that of HONB. Fujino *et al.*[10] reported that HONB suppressed a side reaction (*N*-acylurea formation, *C*→*N* rearrangement reaction) of the carbodiimide method. HONB may suppress such side reaction, resulting in a better coupling yield. Addition of HONB gave a satisfactory result and further addition of equi-molar base [diisopropylethylamine (DIEA) or pyridine] to Leu on the resin resulted in the best yield (Table 2).

Table 2. Coupling Reaction of Esc-Phe-OH on H-Leu-Rink-PEG Amide Resin in Water

Entry	Coupling reagents	Additive agents	Base	Time	Yield (%)
1	WSCD	HOBt	—	1.5 h	14.6
2	WSCD	HOAt	—	1.5 h	3.5
3	WSCD	HOSu	—	1.5 h	41.6
4	WSCD	HONB	—	1.5 h	78.6
5	WSCD	Imidazole	—	1.5 h	11.7
6	WSCD	HONB	DIEA	30 min	93.4
7	WSCD	HONB	DIEA	1.0 h	100
8	WSCD	HONB	Pyridine	1.5 h	97.2
9	WSCD	HOBt	DIEA	1.5 h	26.1

Coupling reaction was performed with WSCD in the presence of an additive in water. Details of the experiments are described in Experimental.

Removal of the Esc group by various base treatments was examined using Esc-Phe-Leu-Rink-PEG amide resin. Because removal of the Esc group on Phe was slower than that of other synthetic Esc-amino acids, Esc-Phe-Leu-Rink-PEG amide resin was preferentially used as the test compound. The resin was treated with aqueous 0.01 mol/l NaOH (3 times, a few minutes each) and washed repeatedly with water, followed by washing with DMF repeatedly. Then the

resin was swelled in DMF and reacted in DMF with Fmoc-Gly-OH using diisopropylcarbodiimide in the presence of HOBt until the resin gave a negative result in the Kaiser test (ninhydrin test).[14] The resulting resin was treated with 20% piperidine in DMF to remove the Fmoc group, and hydrolyzed. The amino acid ratio of Gly, Phe and Leu in the acid hydrolysate was analyzed and the yield of the deprotection procedure with the Esc-Phe-Leu-Rink-PEG amide resin was calculated. From the results shown in Table 3, complete removal of the Esc group by treatment with 0.01 mol/l NaOH and 0.025 mol/l NaOH was difficult. Even with 0.05 mol/l NaOH treatment (3 times, 3 or 10 min each), complete removal of Esc group could not be observed. Next, the deprotection reaction was examined in a mixture of water and EtOH. Addition of EtOH accelerated the deprotection reaction and gave satisfactory results. The Esc group of the Esc-Phe-Leu-Rink-PEG amide resin was removed completely by treatment with 0.025 mol/l NaOH (3 times, 3 min each) in aqueous 50% EtOH. The Esc group was also removable with 2.5% Na_2CO_3 (3 times, 5 min each) in 50% aqueous EtOH. Considering the results of these coupling and deprotection studies, Leu-enkephalin amide was synthesized in water according to the procedure shown in Table 4. Synthetic Esc-Tyr-Gly-Gly-Phe-Leu-Rink-PEG amide resin was treated with 0.025 mol/l NaOH in 50% aqueous EtOH to remove Esc group, and then treated with a mixture of trifluoroacetic acid (TFA), thioanisole and ethanedithiol (94/3/3) for 2 h. The resulting Leu-enkephalin amide, H-Tyr-Gly-Gly-Phe-Leu-NH_2, was purified by HPLC and the HPLC profile is shown in Fig. 2. The yield of purified Leu-enkephalin amide was 71%.

Since Esc-amino acids also exhibited moderate solubility in organic solvents (such as DMF and acetonitrile), Leu-enkephalin amide was synthesized on Rink amide resin using Esc-amino acids in DMF. The Msc group was reported to be highly base labile and we expected that the Esc group was removable with same basic conditions for deprotection of the Fmoc group. Deprotection of the Esc group was performed by treatment with 20% piperidine/DMF for 30 min. After completion of the synthesis, we purified the product by HPLC, but Leu-enkephalin amide was not obtained. During the synthesis, deprotection procedures were checked by the Kaiser test, but the tests did not give clear positive Kaiser test. To validate a deprotection procedure for removal of the Esc group in DMF, the Esc-Phe-Leu-Rink amide resin was treated with various bases in DMF. As described above, removal of the Esc group on Phe with base was slower than that on other synthetic Esc-amino acids; Esc-Phe-Leu-Rink amide resin was preferentially used as the test compound. To compare with the Msc group, Msc-Phe-Leu-Rink amide resin was treated under the same conditions and the results are shown in Table 5. Fmoc-Leu-Rink amide resin was treated with 20% piperidine/DMF and the resulting resin was coupled with Esc-Phe-OH with diisopropylcarbodiimide/HOBt to give the Esc-Phe-Leu-Rink amide resin. The resin was treated with various bases [20% piperidine/DMF, 2%

Table 3. Deprotection Studies with Esc-Phe-Leu-Rink-PEG Amide Resin and Various Bases in Water

Entry	Reagents	Time (min)	Yield (%)
1	0.025 mol/l NaOH aq.	3, 3, 3	21
2	0.025 mol/l NaOH aq.	10, 10, 10	36
3	0.05 mol/l NaOH aq.	3, 3, 3	96
4	0.05 mol/l NaOH aq.	10, 10, 10	98
5	0.01 mol/l NaOH (EtOH–H_2O)	1, 1, 1	29
6	0.01 mol/l NaOH (EtOH–H_2O)	3, 3, 3	77
7	0.025 mol/l NaOH (EtOH–H_2O)	1, 1, 1	89
8	0.025 mol/l NaOH (EtOH–H_2O)	3, 3, 3	100
9	2.5% $NaHCO_3$ (EtOH–H_2O)	3, 3, 3	16
10	2.5% $NaHCO_3$ (EtOH–H_2O)	5, 5, 5	20
11	2.5% Na_2CO_3 (EtOH–H_2O)	3, 3, 3	87
12	2.5% Na_2CO_3 (EtOH–H_2O)	5, 5, 5	100

Esc-Phe-Leu-Rink-PEG amide resin was treated with various bases in water and the resulting resin was coupled with Fmoc-Gly-OH in DMF. After removal of the Fmoc group, the resin was hydrolyzed. From amino acid analysis of the acid hydrolysate, the deprotection rate of Phe was calculated.

Table 4. Protocol for the Solid Phase Synthesis Using Esc-Amino Acids in Water

Step	Reagents	Time
1	H_2O	3 min×2
2	0.025 mol/l NaOH (H_2O–EtOH)	3 min×3
3	H_2O	3 min×2
4	2.0% Triton X aq.	3 min×3
5	Esc-amino acid WSCD HONB, DIEA in 2.0% Triton X aq.	1 h
6	2.0% Triton X aq.	3 min×3

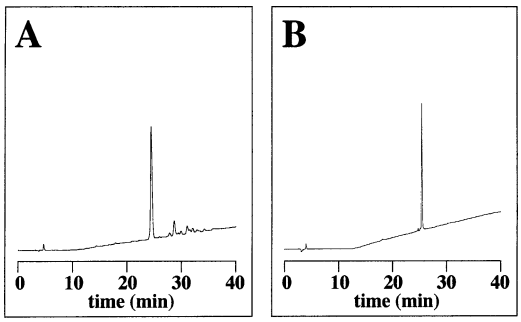

Fig. 2. HPLC Profiles of Synthetic Crude Leu-Enkephalin Amide Synthesized by the Solid Phase Method in Water

A: Crude Leu-enkephalin amide. B: Purified Leu-enkephalin amide. Column, DAISOPAK SP-120-5-ODS-B (4.6×250 mm). Flow rate, 1 ml/min. Eluent, CH_3CN/H_2O containing 0.05% TFA. Gradient: 10/90→50/50 (20 min).

Table 5. Deprotection Studies with Esc- and Msc-Phe-Leu-Rink Amide Resins and Various Bases in DMF

Entry	Protecting group	Reagents	Time (min)	Yield (%)
1	Msc	20% piperidine–DMF	20	<2.0
2	Msc	2.0% DBU–DMF	20	30
3	Msc	0.05 mol/l TBAF–DMF	20	100
4	Esc	20% piperidine–DMF	20	<2.0
5	Esc	2.0% DBU–DMF	20	38
6	Esc	0.05 mol/l TBAF–DMF	20	100
7	Esc	0.05 mol/l TBAF–DMF	5	100

Esc-Phe-Leu-Rink amide resin was treated with various bases in DMF and the resulting resin was coupled with Fmoc-Gly-OH in DMF. After removal of Fmoc group, the resin was hydrolyzed. From amino acid analysis of the acid hydrolysate, the deprotection rate of the Esc group from the Phe residue was calculated.

1,8-diazabicyclo[5,4,0]undec-7-ene (DBU)[15] and 0.05 mol/l tetrabutylammonium fluoride (TBAF)[16]] for 20 min to remove the Esc group and then reacted with Fmoc-Gly-OH and with diisopropylcarbodiimide/HOBt in DMF until the resin gave a negative result with the Kaiser test (ninhydrin test).[14] The resulting resin (Fmoc-Gly-Phe-Leu-Rink amide resin) was treated with 20% piperidine in DMF to remove the Fmoc group, and hydrolyzed. The amino acid ratio of Gly, Phe and Leu in the acid hydrolysate was analyzed and the yield of the deprotection procedure with the Esc-Phe-Leu-Rink amide resin was calculated from the amino acid ratios. Contrary to our expectations, Esc and Msc groups were rather stable to 20% piperidine treatment. Furthermore, Esc and Msc groups could not be removed completely by 2% DBU/DMF treatment for 20 min. Since Wade *et al.*[15] reported that the Fmoc group was removable by 2% DBU/DMF treatment for 5 min, further study regarding the deprotection of Esc and Msc groups was done. Esc-Phe-OH and Msc-Phe-OH were treated with 2% DBU/DMF and the reaction mixture was examined by HPLC. As shown in Fig. 3, complete removal of Msc and Esc groups on Phe could not be achieved by 2% DBU/DMF treatment, even after 220 min. The Esc group tended to be more labile to piperidine and DBU treatment when compared with the Msc group. Esc and Msc groups could be removed completely by 0.05 mol/l TBAF/DMF treatment for 5 min.

Leu-enkephalin amide was synthesized using Esc-amino acids on Rink amide resin in DMF according to a procedure shown in Table 6. Removal of the Esc group was accomplished by 2 consecutive 3 min treatments with 0.05 mol/l TBAF/DMF. Synthetic Esc-Tyr-Gly-Gly-Phe-Leu-Rink amide resin was treated with 0.05 mol/l TBAF/DMF and then treated with a mixture of TFA, thioanisole and ethanedithiol (94/3/3). The resulting crude Leu-enkephalin amide was purified by HPLC and the HPLC profile is shown in Fig. 4A. In addition to the desired product (YGGFL), by-products which were deletion peptides of Phe, Gly and Tyr were detected and the yield of the Leu-enkephalin amide was 23%. As shown in Fig. 4A, the major deletion peptide was YGFL and the area ratio of the YGGFL peak and YGFL peak was 1.00 : 0.81. The Esc group of Esc-Phe-OH was completely removable by 2 consecutive 3 min treatments with 0.05 mol/l TBAF/DMF, but the removal of the Esc-group from the Esc-Phe-Leu-Rink amide resin may be incomplete. Since the by-products were derived from incomplete removal of the Esc group, Leu-enkephalin amide was synthesized again employing a longer TBAF treatment step to improve the deprotection procedure. The Esc group was removed by 3 treatments (3 min, 3 min, 10 min) with 0.05 mol/l TBAF/DMF during synthesis and the HPLC profile of synthetic crude Leu-enkephalin is shown in Fig. 4B. The yield of the purified Leu-enkephalin was 67% (calculated from the used resin). The area ratio of the YGGFL peak and YGFL peak was 1.00 : 0.09.

In the preceding papers[1,2] we reported Pms-amino acids as water-soluble *N*-protected amino acids. They are readily soluble in water, but sparingly soluble in organic solvents. Since Esc-amino acids have moderate solubility in both aqueous and organic solvents, they may be used for coupling reactions in various solvents. Pms-amino acids are onium salts and they are rather unstable, but Esc-amino acids are stable compounds. The Esc group is removable with base and removal of the Esc group in water is accelerated with addition of alcohol. The Esc group was removable completely by treatment with 0.025 mol/l NaOH in aqueous 50% EtOH and removable even with 2.5% Na_2CO_3 in aqueous 50% EtOH. Removal of Esc and Msc groups in DMF with piperidine and DBU was rather difficult, but were removable with 0.05 mol/l TBAF/DMF. The Esc group will be a useful protecting group in synthesis of peptides and we were successful in synthesizing Leu-enkephalin amide on a resin in both aqueous and organic solvents.

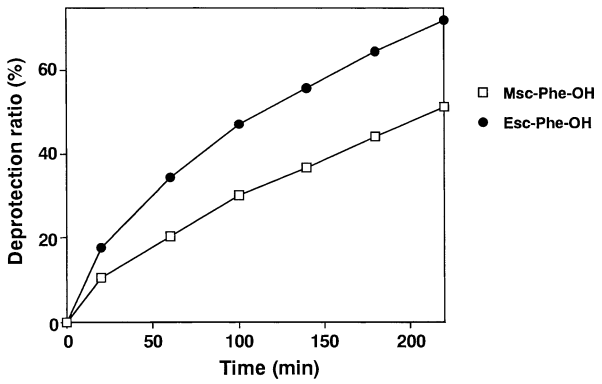

Fig. 3. Deprotection of Esc- and Msc-Groups on Phe by 2% DBU/DMF Treatment

Table 6. Protocol for the Solid Phase Synthesis Using Esc-Amino Acids in DMF

Step	Reagents	Time
1	DMF	3 min×2
2	0.05 mol/l TBAF–DMF	3 min
3	DMF	3 min×2
4	Esc-amino acid DIC, HONB	1 h
5	DMF	3 min×3

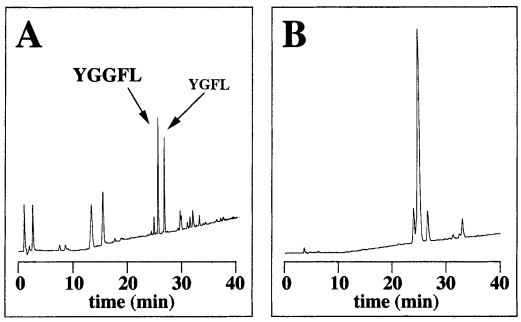

Fig. 4. HPLC Profiles of Synthetic Crude Leu-Enkephalin Amide

Leu-enkephalin amide was prepared by deblocking the Esc group through treatment with 0.05 mol/l TBAF/DMF [A: 3 min (3 times). B: 3 min (2 times) plus 10 min].

Experimental

RP-HPLC was conducted with a Waters 600 on a DAISOPAK column using gradient systems of CH_3CN/H_2O containing 0.05% trifluoroacetic acid. TOF-MS spectra were measured with a Shimadzu/Kratos Kompact MALDI IV mass spectrometer. Synthetic peptides were hydrolyzed with 6 N HCl and amino acid compositions of acid hydrolysates were determined with a Waters Pico·TAG amino acid analyzer. Optical rotations were measured with a JASCO DIP-360 polarimeter.

Melting Points were determined with a Yanagimoto micro-melting point apparatus. Column chromatography was performed on silica gel 60 (BW-

127ZH, Fuji Silica Chemical Co.). ^{1}H-NMR spectra were recorded with a Bruker 400 MHz spectrometer.

Esc-Phe-OH Prepared through Route 1 in Chart 1 To a solution of 2-ethansulfonylethanol (276 mg, 2.0 mmol) and triphosgen (394 mg, 1.33 mmol) in dichloromethane (30 ml), Et_3N (0.56 ml, 4.0 mmol) in dichloromethane (20 ml) was added at 0 °C and the mixture was stirred at 0 °C for 1.5 h. After removal of the solvent *in vacuo* at 10 °C, the residue was dissolved in acetonitrile (15 ml). The solution was added to a solution of Phe (330 mg, 2.0 mmol) and Et_3N (0.35 ml, 2.5 mmol) in 50% aqueous acetonitrile (30 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h. After evaporation *in vacuo*, the residue was purified by HPLC. Yield: 125 mg, 38%. ^{1}H-NMR (CD_3CN) δ: 7.30—7.22 (5H, m), 5.97 (1H, br d), 4.40 (1H, m), 4.32 (2H, t, J=5.3 Hz), 3.21 (2H, t, J=5.3 Hz), 3.18 (1H, dd, J=14.0, 5.0 Hz), 3.06 (2H, q, J=7.4 Hz), 2.92 (1H, dd, J=14.0, 9.2 Hz), 1.25 (3H, t, J=7.4 Hz). $[\alpha]_D^{23}$ −15.54° (c=1.0, CH_3CN). mp 78 °C. TOF-MS m/z: 352.12 $[M+Na]^+$ (Calcd for $C_{14}H_{19}NO_6SNa$: 352.36). *Anal.* Calcd for $C_{14}H_{19}NO_6$: C, 51.05; H, 5.81; N, 4.25. Found: C, 50.80; H, 5.67; N,4.22.

Esc-ONp To a solution of ethylsulfonylethanol (249 mg, 1.8 mmol) and Et_3N (278 μl, 2.0 mmol) in dichloromethane (30 ml), 4-nitrophenylchloroformate (402 mg, 2.0 mmol) in dichloromethane (5 ml) was added, and the mixture was refluxed at 40 °C for 4 h. After filtration, the solvent was removed *in vacuo* and the residue was purified by column chromatography on silica gel using hexane–ethyl acetate (2/1) as an eluent. Yield: 69%, colorless crystal, mp 49 °C, ^{1}H-NMR (CD_3CN) δ: 8.30 (2H, d, J=9.3 Hz), 7.40 (2H, d, J=9.3 Hz), 4.74 (2H, t, J=5.8 Hz), 3.44 (2H, t, J=5.8 Hz), 3.14 (3H, q, J=7.5 Hz), 1.46 (3H, t, J=7.5 Hz). TOF-MS m/z: 326.61 $[M+Na]^+$ (Calcd for $C_{10}H_{11}NO_7SNa$: 326.29). *Anal.* Calcd for $C_{11}H_{13}NO_7S$: C, 43.56; H, 4.32; N, 4.62. Found: C, 43.34; H, 4.28; N, 4.62.

Esc-Phe-OH Prepared through Route 2 in Chart 1 To a solution of Phe (82.5 mg, 0.5 mmol) and Et_3N (70 μl, 0.5 mmol) in 50% aqueous acetonitrile (30 ml), Esc-ONp (181 mg, 0.6 mmol) in acetonitrile (5 ml) was added and the mixture was stirred overnight at room temperature. After removal of the solvent *in vacuo*, the residue was dissolved in ethyl acetate. The solution was extracted with 5% $NaHCO_3$ and the aqueous layer was acidified to pH 3 with 1 N HCl. The mixture was extracted with ethyl acetate and the extract was washed with saturated sodium chloride solution and evaporated *in vacuo*. The residue was crystallized from ether–hexane. Yield: 128 mg, 78%. The product was identified with Esc-Phe-OH prepared through Route 1 by NMR and MS.

The following Esc-amino acids were prepared according to the procedure described above: Esc-Gly-OH: Yield: 57.0% (colorless solid). ^{1}H-NMR (CD_3CN) δ: 5.98 (1H, br s) 4.40 (2H, t, J=5.3 Hz), 3.81 (2H, s), 3.29 (2H, t, J=5.3 Hz), 3.06 (3H, s), 1.31 (3H, t, J=7.5 Hz). mp 73 °C, TOF-MS m/z: 262.36 $[M+Na]^+$ (Calcd for $C_7H_{13}NO_6SNa$: 262.24). *Anal.* Calcd for $C_6H_{11}NO_6S$: C, 35.14; H, 5.48; N, 5.85. Found: C, 35.02; H, 5.29; N, 5.88.

Esc-Leu-OH: Yield: 68.2% (colorless solid). ^{1}H-NMR (CD_3CN) δ: 5.98 (1H, br d), 4.38 (2H, t, J=5.3 Hz), 4.13 (H, q, J=7.1 Hz), 3.27 (2H, t, J=5.3 Hz), 3.06 (3H, q, J=7.4 Hz), 1.70 (1H, m), 1.57 (2H, t, J=7.1 Hz), 1.29 (3H, t, J=7.4 Hz), 0.93 (3H, d, J=7.1 Hz), 0.90 (3H, d, J=7.1 Hz). $[\alpha]_D^{24}$ −14.73° (c=1.0, CH_3CN). mp 50 °C. TOF-MS m/z: 318.26 $[M+Na]^+$ (Calcd for $C_{14}H_{20}NO_6SNa$: 318.34). *Anal.* Calcd for $C_{14}H_{20}NO_6S$: C, 44.73; H, 7.17; N, 4.74. Found: C, 44.50; H, 6.88; N, 4.74.

Esc-Tyr-OH: Yield: 56% (colorless solid). ^{1}H-NMR (CD_3CN) δ: 7.07 (2H, d, J=8.6 Hz), 6.74 (2H, d, J=8.6 Hz, ArH 3, 5), 5.97 (1H, br d), 4.39 (2H, t, J=5.3 Hz), 4.32 (H, m), 3.30 (2H, t, J=5.3 Hz), 3.13 (1H, dd, J=14.0, 5.0 Hz), 3.06 (2H, q, J=7.4 Hz), 2.83 (1H, dd, J=14.0, 9.2 Hz), 1.25 (3H, t, J=7.4 Hz), $[\alpha]_D^{24}$ −9.2 (c=1.0, CH_3CN). mp 75 °C. TOF-MS m/z: 368.29 $[M+Na]^+$ (Calcd for $C_{14}H_{19}NO_7SNa$: 368.36). *Anal.* Calcd for $C_{14}H_{19}NO_7S$: C, 48.69; H, 5.55; N, 4.06. Found: C, 47.98; H, 5.23; N, 4.06.

Coupling Reactions of Esc-Phe-OH on H-Leu-Rink-PEG Amide Resin in Water Esc-Phe-OH (16 mg, 50 μmol) was coupled with H-Leu-Rink-PEG amide resin (48 mg, 12.5 μmol. Prepared by Fmoc strategy in DMF)) in aqueous 2% Triton X. WSCD (9.5 mg, 50 μmol) and an additive were used for the coupling reaction. Various additives [HOBt (6.8 mg, 50 μmol), HOAt (6.8 mg, 50 μmol), HOSu (5.8 mg, 50 μmol), HONB (9.0 mg, 50 μmol) and imidazole (3.4 mg, 50 μmol)] were tested to increase the coupling yield. DIEA (8.7 μl, 50 μmol) or pyridine (4.0 μl, 50 μmol) was added to keep reaction mixture at pH 8. After the coupling reaction, the Esc group was removed with aqueous 0.025 mol/l NaOH and the resin was hydrolyzed. Phe and Leu content in a hydrolysate was analyzed and each coupling yield was calculated from the amino acid ratio of Phe and Leu. Results are summarized in Table 2.

Deprotection Studies Involving the Esc Group on Esc-Phe-Leu-Rink-PEG Amide Resin with Various Bases in Water Esc-Phe-OH (32.9 mg, 0.1 mmol) and the H-Leu-Rink-PEG amide resin (192 mg, 50 μmol) was reacted with diisopropylcarbodiimide (15.6 μl, 0.1 mmol) in the presence of HOBt (13.5 mg, 0.1 mmol) in DMF until the resin gave a negative Kaiser test. The resulting Esc-Phe-Leu-Rink-PEG-resin was washed with DMF and dichloromethane and dried *in vacuo*. Yield 216 mg (99%). Esc-Phe-Leu-Rink-PEG-resin (10 mg each) was treated with various base solutions (0.01 mol/l NaOH in aqueous 50% EtOH, 0.025 mol/l NaOH in 50% aqueous EtOH, aqueous 0.05 mol/l NaOH solution, 2.5% Na_2CO_3 in aqueous 50% EtOH, 2.5% $NaHCO_3$ in aqueous 50% EtOH). After base treatment, the resin was washed with H_2O and DMF. Then Fmoc-Gly-OH (3.0 mg, 10 μmol) was coupled on the resin with diisopropylcarbodiimide (1.6 μl, 10 μmol) and HOBt (1.4 mg, 10 μmol) in DMF. The resulting resin was washed with DMF and dichloromethane, and dried *in vacuo*. The resin was hydrolyzed and Gly, Phe and Leu content in the hydrolysate were analyzed. Each deprotection yield was calculated from the amino acid ratio of Gly and Leu. Results are summarized in Table 3.

Deprotection Studies Involving Esc and Msc Groups of Esc- and Msc-Phe-Leu-Rink Amide Resins with Various Bases in DMF Esc-Phe-Leu-Rink amide resin was prepared from Fmoc-Rink amide resin (100 mg, 62 μmol) *via* Fmoc-Leu-Rink amide resin using diisopropylcarbodiimide/HOBt as a coupling reagent in DMF. Yield 104 mg (98%). The rseins (10 mg each) were swelled with DMF and treated with 20% piperideine, 2% DBU/DMF and 0.05 mol/l TBAF/DMF respectively. After base treatment, the each resin was washed with DMF and coupled with Fmoc-Gly-OH (9.0 mg, 30 μmol) using diisopropylcarbodiimide/HOBt in DMF until the reaction mixture showed negative Kaiser test. The resulting resins were washed with DMF and dichloromethane, and dried *in vacuo*. The resins were hydrolyzed and Gly, Phe and Leu content in hydrolysates were analyzed. Each deprotection yield was calculated from the amino acid ratio of Gly and Leu.

Deprotection studies involving the Msc group of the Msc-Phe-Leu-Rink amide resin with various bases in DMF were performed in a similar manner as described above.

2% DBU/DMF Treatment of Esc-Phe-OH Esc-Phe-OH (2.0 mg, 6.1 μmol) was dissolved in 2% DBU/DMF (400 μl) and the solution was stirred at room temperature. An aliquot (10 μl) was taken periodically for analysis by HPLC and the rate of deprotection was calculated by measurement of the peak area of Esc-Phe-OH. Results are shown in Fig. 3. Complete removal of the Esc group was not observed even after 220 min.

Solid Phase Synthesis of Leu-Enkephalin Amide in Water Fmoc-Rink-PEG amide resin (100 mg, amino content 25 μmol) was treated with 20% piperidine/DMF to remove the Fmoc group. After washing with DMF and aqueous 2.0% Triton X, synthesis was performed according to a protocol shown in Table 4. Esc-Leu-OH (29.5 mg, 0.1 mmol), Esc-Phe-OH (32.9 mg, 0.1 mmol), Esc-Gly-OH (24.7 mg, 0.1 mmol), and Esc-Tyr-OH (34.5 mg, 0.1 mmol) was coupled in turn with WSCD (19.1 mg, 1.0 mmol)/HONB 17.9 mg (1.0 mmol) in a presence of DIEA (17.4 μl, 0.1 mmol). Removal of the Esc group was carried out with 0.025 mol/l NaOH in 50% aqueous EtOH. Synthetic H-Tyr-Gly-Gly-Phe-Leu-Rink-PEG amide resin (109 mg) was treated with TFA-thioanisole-ethandithiol (94-3-3, 5 ml) for 2 h at room temperature. After filtration, TFA was removed *in vacuo* and the residue was dissolved in water. The solution was washed with ether and lyophilized. The crude product was purified by HPLC. Yield (based on amino group content of the resin) 12 mg (71%. Fluffy powder). $[\alpha]_D^{24}$ +9.0° (c=0.2, H_2O), TOF-MS m/z: 555.3 $[M+1]^+$ (Calcd for $C_{28}H_{39}N_6O_6$: 555.64). Amino acid ratios in an acid hydrolysate: Tyr 0.93; Gly 1.88; Phe 1.01; Leu 1.00. (average recovery: 97%).

Solid Phase Synthesis of Leu-Enkephalin Amide in DMF Synthesis was carried out using Esc-amino acids on Fmoc-Rink amide resin (50 mg, amino group content 33 μmol) in DMF according to the protocol shown in Table 6. Coupling reactions of each Esc-amino acid (130 μmol) were carried out with diisopropylcarbodiimide/HONB and the Esc group was removed with 0.05 mol/l TBAF/DMF. Synthetic H-Tyr-Gly-Gly-Phe-Leu-Rink amide resin was treated with TFA–thioanisole–ethanedithiol (94–3–3) and the resulting peptide was purified by HPLC. Yield) 14.8 mg (67% based on the amino group content of the starting resin. fluffy powder). $[\alpha]_D^{24}$ +9.0° (c=0.2, H_2O), TOF-MS m/z: 555.3 $[M+1]^+$ (Calcd for $C_{28}H_{39}N_6O_6$: 555.64). Amino acid ratios in an acid hydrolysate: Tyr 1.01; Gly 2.00, Phe 1.01; Leu 0.89. (average recovery: 98%).

References

1) Hojo K., Maeda M., Kawasaki K., *J. Peptide Sci.*, **7**, 615—618 (2001).
2) Hojo K., Maeda M., Takahara Y., Yamamoto S., Kawasaki K., *Tetrahedron Lett.*, **44**, 2849—2851 (2003).

3) McKay F. C., Albertson W. F., *J. Am. Chem. Soc.*, **79**, 4686—4690 (1957).
4) Bergmann M., Zervas L., *Chem. Ber.*, **65**, 1192—1201 (1932).
5) Carpino L. A., Han G. Y., *J. Am. Chem. Soc.*, **92**, 5748—5749 (1970).
6) Tesser G. I., Balvert-Beers I. C., *Int. J. Peptide Protein Res.*, **7**, 295—305 (1975).
7) Eckert H., Forster B., *Angew. Chem. Int. Ed. Engl.*, **26**, 894—895 (1987).
8) Bayer E., Rapp W., "Chemistry of Peptides and Proteins," ed. by Volter E., Bayer E., Ovchinikov Y. A., Ivanov V. T., Walter de Gryyter, Berlin, 1986, pp. 3—8.
9) Sheehan J. C., Cruickshnk P. A., Boshrt G. L., *J. Org. Chem.*, **26**, 2525—2528 (1983).
10) Fujino M., Kobayashi S., Obayashi M., Fukuda T., Shinagawa S., Nishimura O., *Chem. Pharm. Bull.*, **22**, 1857—1863 (1974).
11) Konig W., Geiger R., *Chem. Ber.*, **103**, 788—798 (1970).
12) Carpino L. A., EI-Faham A., Minor C. A., Albericio F., *J. Chem. Soc. Commun.*, **1994**, 201—203 (1994).
13) Fields G. B., Noble R. L., *Int. J. Peptide Protein Res.*, **35**, 161—214 (1990).
14) Kaiser E., Colescotto R. L., Bossinger C. D., Cook P. I., *Anal. Biochem.*, **34**, 595—598 (1970).
15) Wade J. D., Bedford J., Sheppard R. C., Tregear G. W., *Peptide Res.*, **4**, 194—199 (1991).
16) Ueki M., Amemiya M., *Tetrahedron Lett.*, **52**, 6617—6620 (1987).