Cyclization of *N*-Terminal Fragments of Elcatonin

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Synopsis. It was proved that the cyclization yields of N-terminal fragments of elcatonin were dependent on cyclic positions of the corresponding linear peptides.

Eel calcitonin was isolated by Otani[2] in 1974, which acts to lower Ca^{2+} concentrations in the blood. The [Asu[1,7]] analog (Elcatonin) (Fig. 1), in which L-α-aminosuberic acid is substituted for cystein in sequence positions one and seven of eel calcitonin, has same activities. Synthesis of elcatonin by a conventional solution method has already been reported.[3] In this synthesis, cyclization of N-terminal fragments of elcatonin plays an important role, because the cyclization of peptides is not always achieved in fair yield.[4] In particular, the satisfactory synthetic methods of cyclic peptides are not still established on large scale reactions. We investigated the difference of cyclization efficiency on cyclization positions of cyclic peptides to establish new synthetic procedures of the protected N-terminal fragment of elcatonin.

The cyclic hexapeptide fragment of elcatonin can be cyclized on six kinds of different sites shown in Fig. 2. Usually it is cyclized at position I to give the cyclic peptide, because it is expected that cyclization at this position is free from racemization. We first experimentally studied a comparative demonstration of cyclization of corresponding six kinds of linear protected hexapeptides related to N-terminal fragment of elcatonin, indicated in Fig. 3. And we also investigated the effect of a mixture of alkali metals as an adjunctive cyclization reagent on cyclization[5] to obtain the cyclic hexapeptide in reasonable yields.

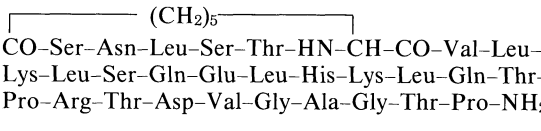


Fig. 1. Primary structure of elcatonin.

Results and Discussion

These hexapeptide derivatives (**1**—**6**) were synthesized by the conventional methods in solution and purified with the conventional procedures. Purity of each synthetic peptide was proved by analytical reversed-phase HPLC, TLC, elemental analysis and amino acid analysis.

Cyclization of each hexapeptide (**1**—**6**) was carried out by two different procedures. One is normal EDC·HCl/HOBt method. Namely, a solution of the respective hexapeptide derivative (0.1 mmol) in DMF (100 ml) was chilled to 0—4 °C, to which was added *N*-methylmorpholine (0.1 mmol), HOBt (0.11 mmol) and EDC·HCl (0.11 mmol), successively. The solution was allowed to stand at room temperature, and stirred for 24 h at room temperature. The other is a mixture of alkali metals additive method. Namely, a suspension of the respective hexapeptide derivative (0.1 mmol) and a mixture of LiCl (0.1 mmol), NaCl (0.1 mmol), KCl (0.1 mmol), and CsCl (0.1 mmol) in DMF (100 ml) was chilled to 0—4 °C, to which was added *N*-methylmorpholine (0.1 mmol), HOBt (0.11 mmol) and EDC·HCl (0.11 mmol), successively. The suspension was allowed to react as similar manners as described

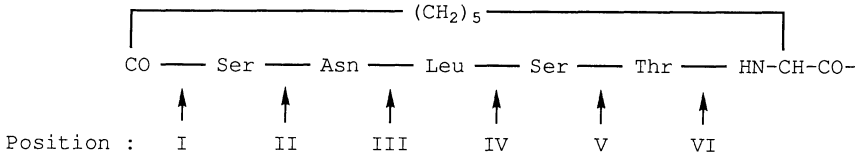


Fig. 2. Reaction sites for cyclization.

1 TFA·H-Ser(Bzl)-Asn-Leu-Ser(Bzl)-Thr(Bzl)-Asu(α-OMe)-OH

2 TFA·H-Asn-Leu-Ser(Bzl)-Thr(Bzl)-Asu(α-OMe)-Ser(Bzl)-OH

3 TFA·H-Leu-Ser(Bzl)-Thr(Bzl)-Asu(α-OMe)-Ser(Bzl)-Asn-OH

4 TFA·H-Ser(Bzl)-Thr(Bzl)-Asu(α-OMe)-Ser(Bzl)-Asn-Leu-OH

5 TFA·H-Thr(Bzl)-Asu(α-OMe)-Ser(Bzl)-Asn-Leu-Ser(Bzl)-OH

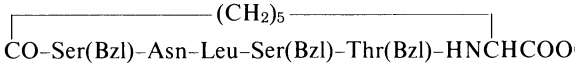
6 TFA·H-Asu(α-OMe)-Ser(Bzl)-Asn-Leu-Ser(Bzl)-Thr(Bzl)-OH

Fig. 3. Amino acid sequences of elcatonin N-terminal fragments for cyclization.

Table 1. Yields of Cyclic Hexapeptide

Linear peptide	Yield/%		
	Cyclization at room temperature		Cyclization at low temperature
	Alkali metals		
	Not used	Used	Not used
1	21	25	20
2	37	37	46
3	59	63	86
4	72	67	81
5	57	53	80
6	65	63	85

above. The cyclization yield of each linear peptide was determined with a reversed-phase HPLC analysis in comparison with authentic compound **7** prepared by literature.[3]



CO-Ser(Bzl)-Asn-Leu-Ser(Bzl)-Thr(Bzl)-HNCHCOOCH$_3$ **7**

The HPLC analysis was performed by TSK gel ODS-120T column (4.6ϕ×250 mm), using a linear gradient of 0.1% TFA aqueous solution/MeOH (40/60→0/100, v/v, 1.0 ml min^{-1} of flow rate) in 40 min, and detected by 214 nm UV absorption, and the result was shown in Table 1. In the cyclization reactions, remarkable racemization of Ser in sequence position six of compound **2** (about 30% yield) and Asn in sequence position six of compound **3** (about 10% yield) was observed. But in cyclization of other compounds (**4**—**6**), no significant racemization was observed.

The cyclization yields were dependent on cyclization positions of the cyclic hexapeptide. Namely, cyclization yields of the corresponding linear peptides **3**, **4**, **5**, and **6** were higher than that of the linear peptide **1**, which is expected as a most desirable precursor of the cyclic peptide. These differences are supposed to result from different kind of bond formation; one is peptide bond formation (cyclization positions II—VI) and the other is amide bond formation (cyclization position I). On the other hand, the effect of alkali metals on the cyclization of linear peptide is not always acceptable.

With regard to six kinds of linear hexapeptides, we made an examination of its cyclization in detail. Each hexapeptide derivative was cyclized with EDC·HCl/HOBt method without alkali metals at low temperature to give cyclic peptide **7** in a favorable yield (over 80%) (Table 1), and racemization yields of C-terminal amino acid residues of compounds (**3**—**6**) were also reduced to less than 1%.

These results show cyclization sites of cyclic peptides and condensation conditions for cyclization produce a marked effect on cyclization yield, and the cyclization of the linear peptides **3**, **4**, **5**, and **6** is very useful for the synthesis of the protected elcatonin N-terminal cyclic fragment rather than **1** and **2**.

Experimental

Melting points were determined on a Yanaco micro melting point apparatus model MP-S3. Optical rotations were measured on a JASCO digital polarimeter model DIP-370. R_f^{I} and R_f^{II} values on TLC(Kiesel gel 60F254) refer to solvent systems of $CHCl_3$–MeOH (1:1, v/v) and *n*-BuOH–AcOH–H_2O (4:1:5, v/v, upper phase), respectively. HPLC was performed on a JASCO model 880-UP. Amino acid compositions in acid hydrolyzate (6 M HCl, 110 °C, 24 h, 1 M=1 mol dm^{-3}) were determined with a Hitachi amino acid analyzer model L-8500.

These hexapeptide derivatives (**1**—**6**) were synthesized by the conventional methods in solution using EDC·HCl/HOBt method or *N*-hydroxysuccinimide ester method and purified with the conventional procedures.

Compound 1; mp 172—175 °C; $[\alpha]_D^{24}$ (C 1 DMF) −5.1°; R_f^{I} 0.62; R_f^{II} 0.42; retention time: 19.6 min in HPLC on a TSK gel ODS-120T column (4.6ϕ×250 mm) in 0.1% TFA aqueous solution/MeOH (40/60→0/100, v/v) over 40 min at a flow rate of 1.0 ml min^{-1} at 214 nm; amino acid ratios in acid hydrolyzate: Asp(1) 1.05, Thr(1) 0.99, Ser(2) 1.89, Leu(1) 1.04, Asu(1) 1.03 (recovery 79.4%).
Found: C, 55.56; H, 6.44; N, 8.75%. Calcd for $C_{52}H_{70}N_7O_{15}F_3 \cdot 2H_2O$: C, 55.46; H, 6.62; N, 8.71%.

Compound 2; mp 170—172 °C; $[\alpha]_D^{24}$ (C 1 DMF) 3.7°; R_f^{I} 0.52; R_f^{II} 0.32; retention time: 18.9 min in HPLC under the same conditions as analyzing compound **1**; amino acid ratios in acid hydrolyzate: Asp(1) 1.07, Thr(1) 1.02, Ser(2) 1.85, Leu(1) 1.04, Asu(1) 1.02 (recovery 79.3%).
Found: C, 55.30; H, 6.23; N, 8.74%. Calcd for $C_{52}H_{70}N_7O_{15}F_3 \cdot 2H_2O$: C, 55.46; H, 6.62; N, 8.71%.

Compound 3; mp 85—88 °C; $[\alpha]_D^{24}$ (C 1 DMF) 14.7°; R_f^{I} 0.46; R_f^{II} 0.28; retention time: 16.3 min in HPLC under the same conditions as analyzing compound **1**; amino acid ratios in acid hydrolyzate: Asp(1) 1.08, Thr(1) 1.01, Ser(2) 1.86, Leu(1) 1.01, Asu(1) 1.04 (recovery 75.8%).
Found: C, 55.61; H, 6.57; N, 8.35%. Calcd for $C_{52}H_{70}N_7O_{15}F_3 \cdot 2H_2O$: C, 55.46; H, 6.62; N, 8.71%.

Compound 4; mp 94—97 °C; $[\alpha]_D^{24}$ (C 1 DMF) 13.3°; R_f^{I} 0.52; R_f^{II} 0.39; retention time: 18.0 min in HPLC under the same conditions as analyzing compound **1**; amino acid ratios in acid hydrolyzate: Asp(1) 1.09, Thr(1) 0.98, Ser(2) 1.87, Leu(1) 1.04, Asu(1) 1.02 (recovery 75.9%).
Found: C, 55.39; H, 6.39; N, 8.73%. Calcd for $C_{52}H_{70}N_7O_{15}F_3 \cdot 2H_2O$: C, 55.46; H, 6.62; N, 8.71%.

Compound 5; mp 157—160 °C; $[\alpha]_D^{24}$ (C 1 DMF) −7.5°; R_f^{I} 0.63; R_f^{II} 0.35; retention time: 17.5 min in HPLC under the same conditions as analyzing compound **1**; amino acid ratios in acid hydrolyzate: Asp(1) 1.12, Thr(1) 0.90, Ser(2) 1.97, Leu(1) 1.11, Asu(1) 0.89 (recovery 78.4%).
Found: C, 55.91; H, 6.59; N, 8.78%. Calcd for $C_{52}H_{70}N_7O_{15}F_3 \cdot 3/2H_2O$: C, 55.91; H, 6.59; N, 8.78%.

Compound 6; mp 165—167 °C; $[\alpha]_D^{24}$ (C 1 DMF) 3.6°; R_f^{I} 0.60; R_f^{II} 0.28; retention time: 18.0 min in HPLC under the same conditions as analyzing compound **1**; amino acid ratios in acid hydrolyzate: Asp(1) 1.07, Thr(1) 0.98, Ser(2) 1.89, Leu(1) 1.08, Asu(1) 0.98 (recovery 78.1%).
Found: C, 55.41; H, 6.34; N, 8.87%. Calcd for $C_{52}H_{70}N_7O_{15}F_3 \cdot 2H_2O$: C, 55.46; H, 6.62; N, 8.71%.

Cyclization under Low Temperature Conditions: A solution of the respective hexapeptide derivative (108 mg, 0.1 mmol) in DMF (100 ml) was chilled to 0—4 °C, to which was added *N*-methylmorpholine (10.2 µl, 0.1 mmol), HOBt (16.8 mg, 0.11 mmol), and EDC·HCl (21.1 mg, 0.11 mmol), successively. After the reaction mixture was stirred for 24 h at 2—4 °C, to which EDC·HCl (5.8 mg, 0.03 mmol) was added and stirred for further 48 h at 2—4 °C. The cyclization yield was determined by HPLC analysis as described in the text.

References

1) Abbreviations used as follows: Asu(α-OMe), L-α-

aminosuberic acid α-methyl ester residue; Bzl, benzyl; DMF, *N,N*-dimethylformamide; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid.

2) M. Otani, H. Yamauchi, T. Meguro, S. Kitazawa, S. Watanabe, and H. Orino, *J. Biochem.*, **79**, 345 (1976).

3) T. Morikawa, E. Munekata, S. Sakakibara, T. Noda, and M. Otani, *Experientia,* **32**, 1104 (1976); S. Sakakibara, T. Morikawa, and E. Munekata, Jpn Tokkyo Koho, JP 53041677 [78041677].

4) T. Katagi and H. Kataoka, "The Second Series of Pharmaceutical Research and Development," ed by I. Suzuki, Hirokawa Publishing Company, Tokyo (1991), Vol. 14, p. 259.

5) H. Kataoka and T. Katagi, *Chem. Pharm. Bull.*, **37**, 3166 (1989).