Tetrahedron Letters, Vol. 34, No. 7, pp. 1173-1176, 1993
Printed in Great Britain

0040-4039/93 $6.00 + .00
Pergamon Press Ltd

Application of Tetrabutylammonium Salt of N-(9-Fluorenylmethoxycarbonyl)cysteic Acid for Solid Phase Peptide Synthesis: Preparation of Endothelin Antagonistic Cyclic Pentapeptides

Toshio NAGASE,* Uno KUMAGAI, Kenji NIIYAMA, Toshiaki MASE and Kiyofumi ISHIKAWA
New Drug Discovery Research Laboratories, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd.
Tsukuba Techno-Park Oho, 3 Okubo, Tsukuba 300-33, Japan

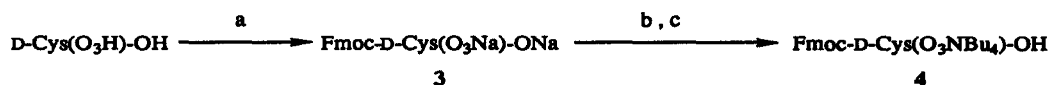
Abstract : According to the Fmoc strategy, highly potent endothelin antagonistic cyclic pentapeptides possessing cysteic acid residue(s) were prepared in solid phase peptide synthesis. Use of a tetrabutylammonium salt overcame low solubility of N-(9-fluorenylmethoxycarbonyl)cysteic acid in a conventional solvent such as DMF.

Endothelin is a potent vasoconstrictor with a sustained and potent pressor activity, which exerts various biological effects on vascular and nonvascular tissues through at least two distinct receptor subtypes (ET_A and ET_B).[1] Substances that antagonize the action of endothelin by specifically inhibiting its binding to receptors may have therapeutic potential in putative endothelin-related diseases. However, such highly potent and selective endothelin antagonists have not been reported previously. In 1989, we isolated ET_A-selective antagonists, BE-18257A (IC_{max50} for ET_A: 3.0 μM, IC_{50} for ET_B: >100 μM) and B (IC_{max50} for ET_A: 1.4 μM, IC_{50} for ET_B: >100 μM) from a microbial fermentation broth.[2,3] Each antagonist is a structurally unique cyclic pentapeptide (CPP) with a DDLDL chirality sequence as shown below, but the low solubility of these substances (Na salt of 2: 210 μg/ml in saline) complicates further pharmacological evaluation. To improve the solubility and potency, we attempted to prepare CPP analogues possessing a more hydrophilic acidic amino acid residue, D-Cys(O_3H), at the position of a D-Glu residue.[4,5] In this communication, we describe the first application of N-(9-fluorenylmethoxycarbonyl) (Fmoc) cysteic acid for conventional solid phase peptide synthesis (SPPS), using its tetrabutylammonium salt as a soluble group protecting the sulfonic acid residue, and preparation of water-soluble endothelin antagonistic CPPs.

cyclo (-D-Trp-D-Glu-Ala-D-Val-Leu-) BE-18257A (1)
cyclo (-D-Trp-D-Glu-Ala-D-*allo*Ile-Leu-) BE-18257B (2)

According to the standard Fmoc strategy, we initially attempted to prepare linear pentapeptide intermediates possessing D-Cys(O_3Na) in SPPS. Fmoc-D-Cys(O_3Na)-OH was, however, not sufficiently soluble in conventional solvents such as CH_2Cl_2, DMF, N-methyl-2-pyrolidone and DMSO (<25 mg/ml) to be introduced to a resin-bound peptide. Generally, 0.1-mmol equiv of a resin-bound amino acid or peptide is treated with a 0.25- a 0.50-mmol/ml solution of Fmoc-amino acid derivatives in SPPS. Therefore, the solubility of Fmoc-D- Cys(O_3X)-OH must be at least 100 mg/ml in the appropriate solvent. To improve the solubility, the counter cation (Na) was changed to ammonium ions. As a result, the tetramethylammonium salt proved not to be sufficiently soluble (<28 mg/ml in DMF) but the tetrabutylammonium and triethylammonium salts were both adequately soluble in DMF (>100 mg/ml) for use in synthesis. During peptide chain elongation or cleavage of a linear pentapeptide from solid support, piperidine or hydrazine was

used as a deprotecting or cleavage agent. Under these conditions, a sulfonic acid residue is preferable to a salt with a strong base because, in this case, the replacement of the counter cation by the above-mentioned basic reagent does not occur as frequently. The tetrabutylammonium salt, Fmoc-D-Cys(O_3NBu_4)-OH (4), was then selected for preparation of linear pentapeptides possessing a D-Cys(O_3H) residue. 4 was prepared as follows (see also Scheme): D-Cys(O_3H)-OH was treated with Fmoc-OSu in the presence of Na_2CO_3 to yield Fmoc-D-Cys(O_3Na)-ONa (3),[6] which was dissolved in water, and the aqueous solution was passed through an ion exchange column (Amberlite IR-120B; H^+-form). The eluate was lyophilized to yield a viscous amorphous Fmoc-D-Cys(O_3H)-OH. The free acid was then treated with 1.0 - 1.1 equiv of a 10% aqueous solution of Bu_4NOH. After the solution was lyophilized, 4 was obtained as a very hygroscopic amorphous solid,[7,8] which was used in SPPS without further purification.



Scheme. a: Fmoc-OSu, Na_2CO_3, dioxane-H_2O, rt, 19 h, quant.; b: IR-120B (H^+-form), 98%; c: 1.0-1.1 eq. 10% Bu_4NOH, H_2O, quant.

Leu or Nle was selected as a C-terminal amino acid and a resin-bound linear pentapeptide was prepared in SPPS according to the standard Fmoc strategy using a polyamide resin (Ultrosyn B, Pharmacia LKB Biochrom Ltd., Cambridge, England) or an alkoxybenzyl alcohol resin as a solid support. After an N-terminal elongation of a peptide chain and selective deprotection of an N-terminal Fmoc group, a side-chain protected linear pentapeptide was cleaved from the solid support by treatment with hydrazine. The resulting hydrazide was cyclized according to an azide method. A linear pentapeptide without any acid sensitive side-chain protective groups was cleaved by treatment with TFA (in this case, an alkoxybenzyl alcohol resin must be used as a solid support) to afford a linear pentapeptide, which was cyclized according to an HOBT-DCC (or EDCI) method. After deprotection of side-chain protective groups, if necessary, the CPP thus obtained was changed from the tetrabutylammonium salt to the sodium salt by successive treatment of the aqueous solution with strong (IR-120B; H^+-form) and weak (Amberlite IRC-50; Na^+-form) cation exchange columns. Lyophilization of the resulting aqueous eluate yielded the desired CPP as a sodium salt. The use of a solid support to prepare a linear pentapeptide intermediate made it possible to overcome problems due to low solubility of intermediates, and proved to be a rapid, less laborious and highly efficient method for synthesizing CPP analogues. The prepared CPPs and their synthesis conditions are summarized in the Table. As expected, the CPPs possessing a D-Cys(O_3Na) residue (except 8 which was poorly soluble in water, possibly due to the zwitter ionic properties) showed good solubility in water (even in the case of 10, the solubility was more than 10 mg/ml.). Furthermore, all CPPs 5-10 inhibited [^{125}I]-ET-1 binding to ET_A ca. 1000-fold more potently than that to ET_B (10: IC_{50} for ET_B: 15000 nM, also see the Table).[9]

In conclusion, we prepared water-soluble CPPs, which have potent ET_A-selective inhibition activity, in SPPS. The present method of preparation of peptide derivatives possessing a cysteic acid residue(s) in SPPS by means of tetrabutylammonium salt 4, may offer the prospect of converting a hydrophobic peptide derivative to a hydrophilic one, if the modification is permissible with respect to structure-activity relation-

ships.

Table. CPPs Possessing a D-Cys(O_3H) Residue Prepared by SPPS

Compd	Structure[a]	Resin Used[d]	Cyclization Method	Yield (%)[e]	ET_A[f] IC_{max50}
5	*cyclo* (-D-Trp-D-Cys(O_3Na)-Cys(O_3Na)-D-Val-Leu-)	A	azide	24	160
6	*cyclo* (-D-Trp-D-Cys(O_3Na)-Pro-D-*allo*Ile-Leu-)	A	azide	12	17
7	*cyclo* (-D-Trp-D-Cys(O_3Na)-Glu-D-Val-Leu-)	B	azide	50	270
8	*cyclo* (-D-Trp-D-Cys(O_3H)-Lys-D-Val-Leu-)[b]	B	azide	35	400
9	*cyclo* (-D-Trp-D-Cys(O_3Na)-Pro-D-Val-Nle-)	B	HOBT-EDCI	15	60
10	*cyclo* (-D-Trp(CHO)-D-Cys(O_3Na)-Pro-D-Thg-Leu-)[c]	B	HOBT-DCC	42	2.1

[a]All CPPs showed satisfactory 300 MHz ^{1}H NMR, IR and HR FAB-MS spectra supporting the described structures. Purity was more than 95% by HPLC analysis. [b]This compound was obtained as a zwitter ionic form after deprotection of an N^{ϵ}-Boc group of Lys with TFA/ethane dithiol. [c]D-Thg: D-(2-theinyl)glycine. [d]A: Ultrosyn B; B: alkoxybenzyl alcohol resin. [e]Overall isolated yields based on 0.1-0.3 mmol equiv of a Fmoc-Leu- (or -Nle-) resin, not optimized. [f]nM, see ref. 2a on experimental methods.

***cyclo* (-D-Trp-D-Cys(O_3Na)-Cys(O_3Na)-D-Val-Leu-) (5)**: Using Fmoc-Leu-resin (1.00 g, 0.096 mmol equiv), H-D-Trp-D-Cys(O_3NBu_4)-Cys(O_3NBu_4)-D-Val-Leu-resin was prepared with a manual peptide synthesizer (Biolynx 4175, Pharmacia LKB Biochrom. Ltd., Cambridge, England). According to the instructions for standard operation of the synthesizer, 2.5 equiv of Fmoc-amino acid, HOBT and DIPC were used in each condensation step. The resin bound linear pentapeptide was then treated with 3 ml of 10% hydrazine hydrate in dioxane/MeOH (9:1) at room temperature for 2h. The resin was filtered and washed with dioxane/MeOH (9:1). The combined filtrate and washings were neutralized with dry ice and concentrated under reduced pressure. The residue was dissolved in water and purified by reverse-phase column chromatography (Cosmosil $75C_{18}$-OPN, Nacalai Tesque, Kyoto, Japan), using H_2O-MeOH (1:1) as the eluent, to yield the pentapeptide hydrazide (22.2 mg, 25%) as a colorless powder. The hydrazide (20.0 mg, 0.021 mmol) was dissolved in DMF (0.4 ml) and treated with isoamyl nitrite (4.0 µl, 0.030 mmol) at pH 2-3 at -30 to -20 °C. Cyclization of the resulting azide was effected at -20 °C by making the solution dilute with DMF (4.0 ml) and making it basic (pH 8-9) with triethylamine. The reaction mixture was concentrated in vacuo and the residue was dissolved in water. The aqueous solution was successively passed through cation exchange columns, Amberlite IR-120B (H^+-form) and Amberlite IRC-50 (Na^+-form). The eluate was concentrated under reduced pressure to yield **5** (14.9 mg, 94%) as a colorless powder; mp: 286 °C (dec.); High Resolution FAB-MS: Found: 701.2283; Calcd for $C_{28}H_{40}N_6O_{11}S_2$ + H: 701.2275.

***cyclo* (-D-Trp-D-Cys(O_3Na)-Pro-D-Val-Nle-) (9)**: Fmoc-Nle-resin (200 mg, 0.476 mmol/g) was packed in a polypropylene column (10 mm Φ x 60 mm) and SPPS was performed manually as follows: deprotection of an Fmoc group with 20% piperidine/DMF (3 ml x 5 min x 3); washing of the resin with DMF

(3 ml x 1 min x 6); condensation with Fmoc-amino acid (2.5 equiv) in the presence of HOBT (2.5 equiv) and DIPC (2.5 equiv) in DMF (1 ml) (rt, 2 h); washing of the resin with DMF (3 ml x 1 min x 4). The resin-bound linear pentapeptide was treated with TFA/phenol (95/5) (3 ml x 20 min x 2). The resin was filtered and washed with TFA (3 ml x 5 min x 2). Filtrate and washings were combined and concentrated under reduced pressure to yield a linear pentapeptide (39 mg, 43%) as a pale yellow powder. To a DMF (2 ml) solution of EDCI (8 mg, 1.5 equiv) and HOBT (7 mg, 1.5 equiv), was added a DMF (2 ml) solution of the linear pentapeptide (26 mg) at 0 °C over a period of 1 h, and the resulting mixture was stirred at room temperature for 16 h. After removal of the solvent, the residue was treated using the procedure described above to yield 9 (6.8 mg, 35%) as a pale yellow powder; mp: 217-221 °C; High Resolution FAB-MS: Found: 647.2825; Calcd for $C_{30}H_{42}N_6O_8S$ + H: 647.2863.

Acknowledgment: It is a pleasure to acknowledge the contributions of Mr. S. Nakajima (NMR spectra), Mr. S. Abe (MS spectra) and Mr. R. Nishioka (HPLC analysis). We are also grateful to Ms. A. Thomas and Ms. D. LeBlanc, Merck & Co., for their critical reading of this manuscript.

References and Notes

1. Review for endothelin: A. M. Doherty, *J. Med. Chem.*, **1992**, *35*, 1493.
2. a) isolation: K. Kojiri, M. Ihara, S. Nakajima, K. Kawamura, K. Funaishi, M. Yano and H. Suda, *J. Antibiot.*, **1991**, *44*, 1342; b) structural determination: S. Nakajima, K. Niiyama, M. Ihara, K. Kojiri and S. Suda, *ibid.*, **1991**, *44*, 1348; c) pharmacological evaluation: M. Ihara, T. Fukuroda, T. Saeki, M. Nishikibe, K. Kojiri, H. Suda and M. Yano, *Biochem. Biophys. Res. Commun.*, **1991**, *178*, 132.
3. Thereafter, some peptidic and non-peptidic ET_A-selective antagonists are reported: M. J. Spinella, A. B. Malik, J. Everitt and T. T. Andersen, *Proc. Natl. Acad. Sci. U.S.A.*, **1991**, *88*, 7443; M. Fujimoto, S. Mihara, S. Nakajima, M. Ueda, M. Nakamura and K. Sakurai, *FEBS Letter*, **1992**, *305*, 41; S. Miyata, N. Ohhata, H. Murai, Y. Masui, M. Ezaki, S. Takase, M. Nishikawa, S. Kiyoto, M. Okuhara and M. Kohsaka, *J. Antibiot.*, **1992**, *45*, 1029.
4. Initial structure-activity relationships of some synthetic CPP analogues: K. Ishikawa, T. Fukami, T. Nagase, K. Fujita, T. Hayama, K. Niiyama, T. Mase, M. Ihara and M. Yano, *J. Med. Chem.*, **1992**, *35*, 2139.
5. Cysteic acid and its N^{α}-Boc, -Z, or -phthaloyl derivatives are prepared and used as a water soluble protective group of cysteine in liquid phase peptide synthesis, see; A. Hubbuch, W. Danho and H. Zahn, *Liebigs Ann. Chem.*, **1979**, 776; *Idem*, *Peptides* (M. Goodman and J. Meinhofer, eds), Proc. 5th Am. Peptide Symp., 540, Wiley, New York, 1977.
6. Physical and spectral data for 3: mp: >300 °C; ^{1}H NMR (DMSO-d_6, δ ppm): 2.87 (2H, d, J=5.4Hz, CH_2-SO_3Na), 3.96 (1H, dt, J=6.7Hz, 5.4Hz, -NH-C<u>H</u>-COONa), 4.11-4.25 (3H, m, C<u>H</u>-C<u>H</u>$_2$OCO-), 6.78 (1H, d, J=6.7Hz, -NH-), 7.32 (2H, t, J=7.3Hz, aromatics), 7.40 (2H, t, J=7.3Hz, aromatics), 7.69 (2H, d, J=7.3Hz, aromatics), 7.87 (2H, d, J=7.3Hz, aromatics); IR (KBr): 3430, 1701, 1608, 1422, 1245, 1194, 1050, 756 and 738 cm^{-1}; High Resolution FAB-MS: Found: 436.0441; Calcd for $C_{18}H_{15}NO_7SNa_2$ + H: 436.0443; Elemental Analysis: Found: C, 48.45; H, 3.63; N, 3.44; S, 7.46%; Calcd for $C_{18}H_{15}NO_7SNa_2 \cdot 1/2H_2O$: C, 48.64; H, 3.63; N, 3.15; S, 7.22%.
7. Spectral data for 4: ^{1}H NMR (DMSO-d_6, δ ppm): 0.93 (12H, t, J=7.2Hz, C<u>H</u>$_3$-$(CH_2)_3$-N), 1.31 (8H, sextet, J=7.2Hz, CH_3-C<u>H</u>$_2$-$(CH_2)_2$-N), 1.46-1.65 (8H, m, CH_2-C<u>H</u>$_2$-CH_2-N), 2.70-2.91 (2H, m, CH_2-SO_3-), 3.05-3.23 (8H, m, -CH_2-N), 3.98-4.28 (4H, m, C<u>H</u>-C<u>H</u>$_2$-OCO, NH-C<u>H</u>-COOH), 7.00-7.13 (1H, m, -NH-), 7.33 (2H, t, J=7.4Hz, aromatics), 7.41 (2H, t, J=7.4Hz, aromatics), 7.70 (2H,d, J=7.4Hz, aromatics), 7.89 (2H, d, J=7.4Hz, aromatics), 11 .05 (1H, brs, -COOH).
8. Fmoc-D-Cys($ONBu_4$)-$ONBu_4$ was prepared using Bu_4NOH as a base, with moderate to low yields (30-50%). Furthermore, exchange of tetrabutylammonium carboxylate to carboxylic acid by passage of the solution through an ion exchange column of Amberlite IRC-50 (H^+-form) was a failure because a hydrophobic tetrabutylammonium cation of the sulfonic acid residue was also trapped with an ion exchange resin. The entity recovered from the eluate was mainly the free acid, not 4.
9. Detailed structure-activity relationships will be submitted elsewhere.

(Received in Japan 7 September 1992)