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Synthesis of an Endothelin Cyclic Analogue using an Original Multidimensional Protection Scheme

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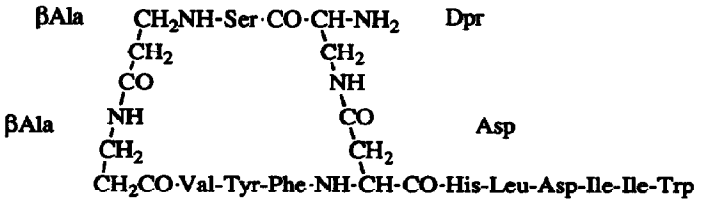
Abstract: A short cyclic analogue of endothelin 1 has been synthesized. The native disulphide bridges between Cys^3-Cys^{11} and Cys^1-Cys^{15} have been replaced respectively by two βAla and a diaminopropionic acid linked to an aspartic acid.

Endothelin ET-1, **1**, the most potent vasoconstrictor hormone is a bicyclic peptide containing four cysteines linked by two disulphide bridges.

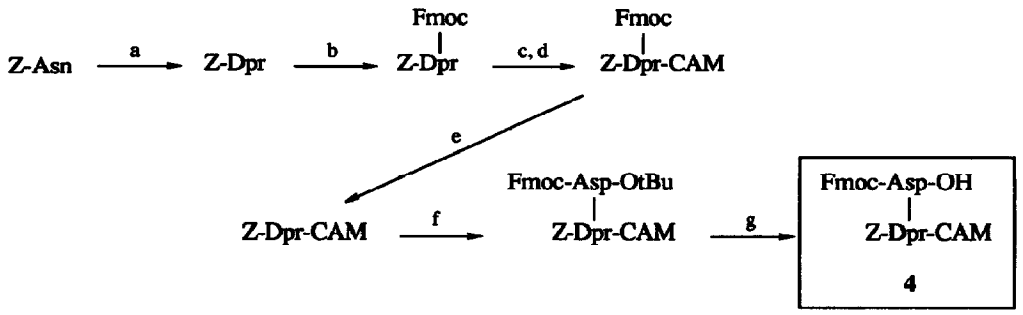
1 **2**

In the course of our investigations on the endothelin structure-activity relationships and research on new antagonists, we synthesized a cyclic analogue in which only the loop 2 of endothelin and the *C*-terminal hexapeptide of ET-1 were retained. The loop 2 was built by replacing the disulphide bridges with peptide bonds providing a structure with the same number of atoms. This choice resulted from two observations. Firstly, ET-1 and several toxins contain the same pattern Cys-Xaa-Xaa-Xaa-Cys connected to a Cys-Xaa-Cys sequence within an extended fragment[1] suggesting the importance of the loop 2 of ET-1 for its activity; a finding which would be in agreement with biological assays demonstrating the importance of disulphide bridges for the vasoconstrictor activity of ET-1[2]. Secondly, an antagonist of ET-1 has been obtained by replacing the disulphide bridge Cys^1-Cys^{15} by a peptide bond[3]. Considering these two elements, the disulphide bridges of the loop 2 of ET-1 were mimicked by two amide bonds between two βAla residues and between the side-chains of Dpr and Asp residues[4], leading to the targetted cyclic peptide **3**. In addition, this replacement results in a stable analogue and avoids possible alternative cyclization or polymerisation by formation of incorrect disulphide bridges.



3

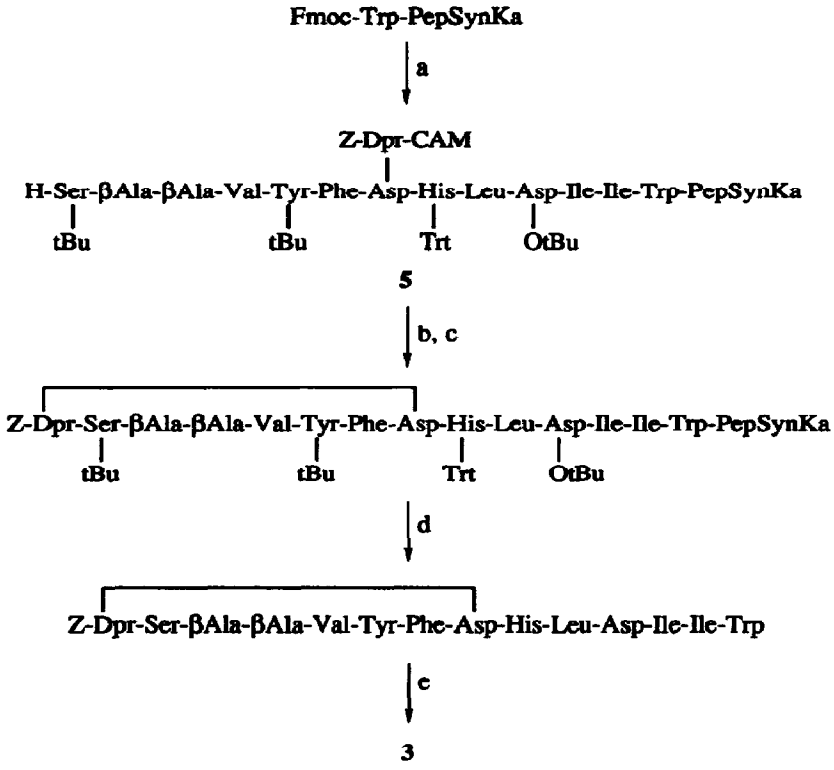
Numerous types of cyclization on peptides have been reported in the literature : side-chain to side-chain[5], *N*-terminal to side-chain[6] or head to tail types[7] or cyclization by disulphide bridges[8]. In this paper, we describe a new example of *N*-terminal to side-chain cyclization in the solid phase using Fmoc-methodology and tBu, Z, and CAM-ester[9] protecting groups for the side-chains. Our original approach is based on the use of the synthon 4 in which the amide bond between Dpr and Asp side-chains is already built[10]. This approach avoids any possibility of aspartimide formation resulting either from the activation required for the Asp side-chain to Dpr side-chain cyclization or from the base catalysed reaction of the ester protecting group of aspartic acid side-chain during the repeated treatments with piperidine required for Fmoc-protecting group removal. The synthon 4[11] was prepared from Z-Asn and Fmoc-Asp-OtBu (Scheme 1).



Scheme 1. The synthesis of the synthon 4.

a) $PhI(OAc)_2$, DMF/H_2O 60:40; b) Fmoc-OSu, $NaHCO_3$, acetone/water 50:50; c) $CsHCO_3$ in EtOH/ H_2O, evaporate under reduced pressure with $PhCH_3$; d) ICH_2CONH_2, DMF; e) Diethylamine 10% in DMF; f) Fmoc-Asp-OtBu, TBTU, DIEA; g) TFA.

The solid phase assembly of the linear peptide 5 was carried out by a conventional continuous flow procedure (Scheme 2) except when the synthon 4 was introduced. After the CAM-ester hydrolysis, the cyclization was carried out in the solid phase using TBTU/HOBt since no particular possibility of epimerization of the N^{α}-Z-protected residue was expected (Scheme 2). The peptide substitution level of 0.097 meq/g was expected to maintain a pseudo-dilution effect minimizing interchain side-reactions during the cyclization step. The crude product was dissolved in water and then lyophilized. The purification of the crude product was carried out by semi-preparative HPLC[12] and the purified peptide showed a single peak, purity > 99 %. ES-MS *m/z* 809.5 ($M+2H^+$). Amino acid analysis : Asp 1.98 (2); Ser 0.77 (1); Val 0.93 (1); Ile 1.48 (2); Leu 1.06 (1); Tyr 0.92 (1); Phe 0.94 (1); βAla 2.1 (2); His 0.94 (1); Dpr 0.8 (1); W not dosed.



Scheme 2. Synthesis, cyclization and deprotection of the cyclic peptide 3.

a) Synthesis scale 700 mg of resin, substitution level 0.097 meq/g. The peptide chain was assembled by consecutive addition of the N^{α}-Fmoc protected amino acids using the continuous flow Milligen Pepsynthesizer 9050, double coupling of 30 min each step except for the synthon 4 (two couplings of one hour each). The activations of carboxylic moieties were performed with 3 eq of Fmoc-amino acid, 3 eq of TBTU, 3 eq of HOBt and 6 eq of DIEA in DMF. Fmoc-protecting groups were removed by treatment with piperidine 20% in DMF; b) NaOH 3 eq in iPrOH/H_2O 7:3, 1 h, r.t. with periodic shakings, the resin was neutralized with 1.75 N AcOH 5 eq, washed (AcOH 5 eq in iPrOH/H_2O 7:3, iPrOH/H_2O 7:3, iPrOH, Et_2O) and dried under vacuum; c) TBTU 3 eq, HOBt 3eq, DIEA 6 eq, 2 h, three times (negative Kaiser test[13] after the second two hours coupling), the resin was washed with DMF, DCM, iPrOH, AcOH, iPrOH, Et_2O and then dried under vacuum; d) Peptide cleavage and side-chain deprotection : K reagent (TFA 85%, H_2O 5%, phenol 5%, thioanisole 2,5% and ethanedithiol 2,5%), 90 min, r.t., the solution was filtered, the solvent removed under reduced pressure and the peptide was precipitated with Et_2O, and then dissolved in H_2O/AcOH 90:10 and lyophilized; e) TFA/ TFMSA/thioanisole/ethanedithiol.

In conclusion, our method has allowed the synthesis of a cyclic peptide mimicking a *N*-terminal disulphide bridge by the Dpr and Asp residues using Fmoc chemistry. Though the method is presently limited to peptides involving a *N*-terminal cysteine, it could be generalized by the replacement of the Z-protecting group in the synthon **4** by a truly orthogonal protecting group (allyloxycarbonyl group using DBU[14] instead of piperidine for Fmoc group removal or Dde protecting group[5c] for example) which may allow the synthesis of the peptide to be continued after the cyclization step.

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4. Abbreviations for amino acids follow the recommendations of IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.*, **1984**, *138*, 9-37). Additional abbreviations used are : CAM : carboxyamidomethyl ester; DBU : 1,8-diazabicyclo[5,4,0]undec-7-ene; Dde : 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidine)ethyl; DIEA : diisopropylethylamine; DMF : *N,N*-dimethylformamide; Dpr : L-2,3-diaminopropionic acid; ET-1 : endothelin 1; Fmoc : fluorenylmethyloxycarbonyl; HOBt : 1-hydroxybenzotriazole; HOSu : *N*-hydroxysuccinimide; TBTU : *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TFA : trifluoroacetic acid; TFMSA : trifluoromethanesulfonic acid; Z : benzyloxycarbonyl.
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10. Though the use of Boc-Dpr-CAM Fmoc-Asp instead of the Z-protected synthon could have simplified peptide deprotection, the latter was preferred owing to its easier synthesis expected.
11. The synthon **4** was obtained as a white solid after purification by flash chromatography, R_f 0.21 (CH_2Cl_2, MeOH, AcOH, 16 : 1 : 1), in a pure form as shown by analytical HPLC R_t 19.0 min [C18 column; buffer A, 0.1% aq. TFA; B, MeCN (0.08% TFA); linear gradient 10-60% B over 20 min, 1ml/mn]. m.p. 189-191°C. FAB-MS *m/z* 633 ($M+H^+$). $[\alpha]_D^{25}$=-42° (c 0.8, DMSO). ^1H NMR [360 MHz, $(CD_3)_2SO$] δ 2.50 (dd, J_1 = 15 Hz, J_2 = 9 Hz, 1H, β-CH_2 Asp), 2.62 (dd, J_1 = 15 Hz, J_2 = 5 Hz, 1H, β-CH_2 Asp), 3.35-3.44 (m, 1H, β-CH_2 Dpr), 3.52-3.60 (m, 1H, β-CH_2 Dpr), 4.15-4.30 (m, 3H, CH-CH_2 Fmoc), 4.30-4.40 (m, 2H, α-CH Asp, α-CH Dpr), 4.47 and 4.50 (AB, *J* = 15 Hz, 2H, CH_2 CAM), 5.05 (s, 2H, CH_2 Z), 7.25-7.48 (m, 11 H), 7.50 (d, *J* = 8 Hz, 1H), 7.65-7.80 (m, 3H), 7.88 (d, *J* = 8 Hz, 2H, ArH), 8.08 (t, *J* = 6 Hz, 1H, β-NH Dpr), 12.4 (broad, COOH).
12. HPLC purification : Waters model 510 high pressure pump, Waters automated gradient controller model 680, Waters 486 tunable absorbance detector with Deltapack C18 300 Å (19x300 mm) column; Buffer A 0,1 % TFA in water; B 0,08 % TFA in CH_3CN; linear gradient 10 to 70 % in 90 min; UV detection at 280 nm.
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