Chinese Chemical Letters Vol. 10, No. 7, pp. 549–552, 1999

Synthesis of Salmon Calcitonin Analogs Using Fmoc-based Chemistry on MBHA Resins

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Abstract: Peptide analogs of salmon calcitonin (sCT) were synthesized by using Fmoc-based chemistry on MBHA resins. Salmon calcitonin was modified by 1) cysteines at positions 1 and 7 were replaced by valine and alanine respectively to result in open chain analogs , 2) the glycine at position 30 was replaced by alanine, D-alanine and sarcosine respectively, and 3) some residues were deleted besides the above two modifications. A modified two-step deprotection / cleavage procedure, in which a solvent of TFA / TMSBr / thioanisole / EDT / m-cresol combines with HF cleavage, was adopted in SPPS.

Keywords: Salmon calcitonin analogs, Fmoc-based SPPS, MBHA resin, the two-Step deprotection / cleavage procedure.

Calcitonin is a cyclic 32 amino acid peptide hormone that has hypocalcemic activity. Although there are many differences in individual amino acids in calcitonins of different species, they all have a disulfide bridge between cysteine residues at positions 1 and 7, and the C-terminus is always proline amide. The N-terminal disulfide bridge is essential for the biological activity of human calcitonin, but it is not required for that of eel and salmon calcitonin. Although Studies have demonstrated the requirement for almost the entire peptide structure to keep the high hypocalcemic potency of calcitonins, some residue deletions, such as Ser^{2}, Asn^{3}, or -Leu^{19}-Gln^{20}-Thr^{21}-Tyr^{22}- (Leu^{19}~Tyr^{22}) sequence deletions, have little effect on the potency of salmon calcitonin[1].

Table 1 Salmon calcitonin analogs and their FAB-MS measurements

Analogs	Molecular Weight (Calculated. / Measured FAB-MS)
[Val^{1},Ala^{7}]sCT (**1**)	3397.8 / 3398.5
[Val^{1},Ala^{7},Ala^{30}]sCT (**2**)	3411.8 / 3412.3
[Val^{1},Ala^{7},Sar^{30}]sCT (**3**)	3411.8 / 3410.6
[Val^{1},Ala^{7},des-Ser^{2}]sCT (**4**)	3310.7 / 3311.5
[Val^{1},Ala^{7},des-Ser^{2},des-Asn^{3}]sCT (**5**)	3196.6 / 3196.1
[Val^{1},Ala^{7},des-leu^{19}~Tyr^{22}]sCT (**6**)	2892.2 / 2892.8
[Ala^{30}]sCT (**7**)	3445.9 / 3446.0
[D-Ala^{30}]sCT (**8**)	3445.9 / 3446.1
[Sar^{30}]sCT (**9**)	3445.9 / 3446.0
[Ala^{30},des-leu^{19}~Tyr^{22}]sCT (**10**)	2940.3 / 2941.0

To understand the structure-activity relationship of calcitonins, many analogs of calcitonins have been reported in a number of patent papers. In this paper, we report new peptide analogs (**Table 1)** of salmon calcitonin with following structure modifications: 1) cysteines at positions 1 and 7 were replaced by valine and alanine respectively to yield open-chain analogs, 2) the glycine at position 30 was replaced by alanine, D-alanine and sarcosine respectively, 3) residue Ser^2, Asn^3, or -Leu^{19}~Tyr^{22}- sequence were deleted besides the above two modifications. The experimental hypocalcemic activity results of these analogs indicated that these modifications have not greatly affected the potency of salmon calcitonin. The open-chain analogs except analog **6** have almost the same hypocalcemic activity as that of salmon calcitonin[2].

The SPPS of calcitonins and its peptide analogs described in many patent papers were almost on the Boc-based chemistry. The SPPS protocol described in the present paper was carried out by using the Fmoc-based chemical strategy on MBHA resins. MBHA resin is a formal amide resin used in Boc-based chemistry, but it was found that synthesis of peptide amides using Fmoc-based chemistry on MBHA resins resulted in In peptides chain elongation, coupling was completed by using the DCC / HOBt coupling reagent (3 equiv.) once for most residues, but for some difficult coupling residues, a further coupling with BOP reagent was needed after the DCC / HOBt coupling. Fmoc group was removed after each coupling cycle by using 25% piperidine in DMF for 15min. Quantitative ninhydrin analysis was performed to monitor the completion of the coupling after each coupling cycle[4].

For the side-chain deprotection and cleavage of peptides from MHBA resins, a modified two-step deprotection / cleavage procedure[5] in which a solvent of TFA / TMSBr / thioanisole / EDT / m-cresol combines with common HF cleavage was used. In this modified procedure, after the removal of the N-terminal Fmoc group, 0.3 g~1.0 g peptide - MBHA resin was fist treated with a mixed solvent of TFA (7.5 ml), TMSBr (1.36 ml), thioanisole (1.2 ml), EDT (0.6 ml), m-cresol (0.2 ml) at -5~0°C for 1.5~2 h . The solvent containing the reagents was removed by filtration and the resin was washed by TFA twice and completely washed by DCM, DCM / CH_3OH (1:1) and DMF. After dried, the resin was subjected to HF (10 ml), anisole (0.5 ml), thioanisole (0.5 ml), EDT (0.2 ml) at -5 ~ 0°C for 1 h. The two-step deprotection / cleavage procedure has been reported to be similar to the low-high HF method in many SPPS, but we found that the low-high HF method could not accomplish the side-chain deprotection completely in the present Fmoc-based chemistry on MBHA resins. The most obvious problem was one Trt group still remained on the peptide chain when it was cleaved by the low-high HF method. For example, the profile **A** in **Figure 1** shows the RP-HPLC chromatogram of crude peptide **3** cleaved by low-high HF method, the second main peak (retention time 33.3 min) is the side product with one Trt group still remains on peptide chain. **Figure 2** shows the FAB-MS measurement of the Trt-containing side product of analog **3.** The measured $(M+H)^+$ result 3652.3 is consistent with the theoretical molecular weight of $[M+243(Trt)]^+$. For the cleavage of other peptides, the two-step deprotection / cleavage procedure resulted in complete deprotection and cleavage and fewer side products (The RP-HPLC profiles of analog **2**, **5**, **7** cleaved by this method are showed in **Figure 1**). The disulfide bridges in analog **7**, **8**, **9** and **10** were formed by the potassium ferricyanide oxidation method in a very diluted aqueous solution[4].

Initial purification of crude peptides was performed by gel filtration on a Sephadex G-25 column, eluted with 0.5 N acetic acid, monitoring the elution by UV absorption at 214 nm. Fractions were collected, lyophilized and subjected to a semi-preparative RP-HPLC. Peptides were analyzed and characterized by analytical RP-HPLC, FAB-MS (results are showed in **Table 1**) and amino acid analysis (results are not given here).

The analytical RP-HPLC chromatograms of peptides given in the present paper were performed on a Nucleosil C_{18} column (5 u, 4.6x250 nm) with a Waters 600E gradient liquid chromatogram system, combined with a SP UV-1000 detector at 214 nm and SP 4600 Integrator. Peptides were eluted with 0.1% TFA / 70% acetonitrile in water using a linear gradient from 0% to 80% of 0.1% TFA / 70% acetonitrile within 30 min and then back to 100% of 0.1%TFA water in 5 min at a flow rate of 1.5 ml/min.

Figure 1 RP-HPLC chromatograms of some crude peptides. A) Analog **3** by the low-high HF method, B) Analog **2** by the two-step deprotection / cleavage procedure, C) Analog **5** by the two-step deprotection / cleavage procedure, D) Analog **8** by the two-step deprotection / cleavage procedure, oxidized and purified by Sephadex G-25.

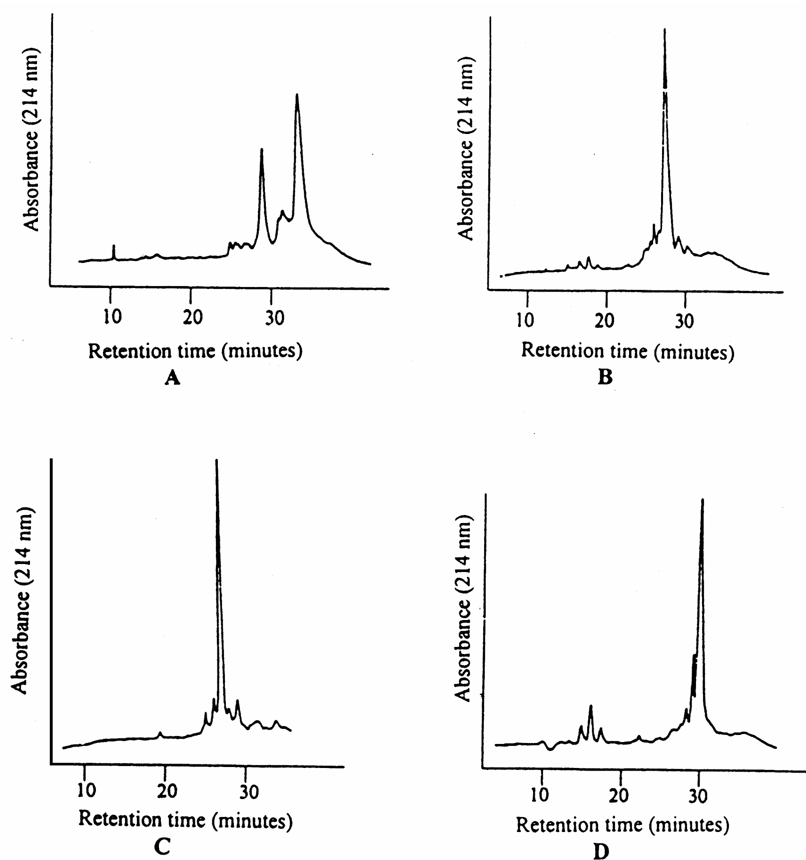
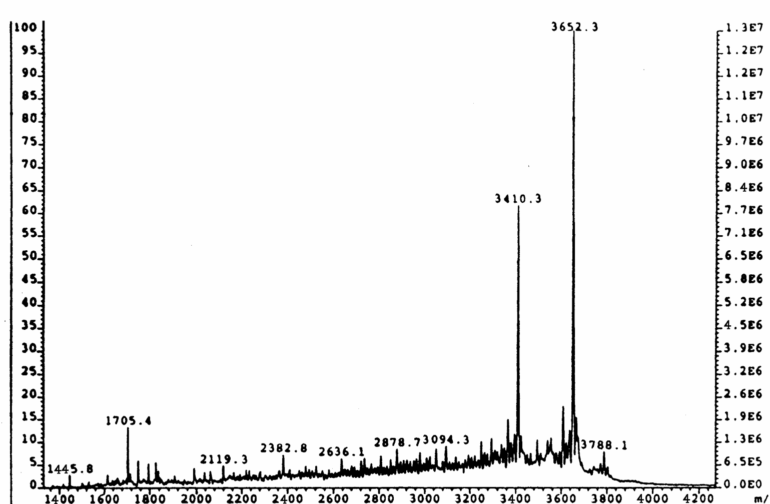


Figure 2 FAB-MS of the Trt-containing side product of analog **3**



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Abbreviations: Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature. Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; BOP, benzotriazolyl N-oxytrisdimethylaminophosphonium hexafluorophos-phate; DCC, dicyclohexylcarbodiimide; DMF, N,N-dimethylformamide; EDT, 1,2-ethanedithiol; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazol; MBHA, 4-methyl-benzhydrylamine; RP-HPLC, reversed-phase high performance liquid chromatography; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TMSBr, trimethylsilyl bromide; Trt, triphenylmethyl.

Received 26 January 1999