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Convenient Synthesis of Human Calcitonin and Its Methionine Sulfoxide Derivative

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Abstract—The human calcitonin peptide chain was assembled using Fmoc solid-phase peptide synthesis chemistry. The combinations of cleavage Reagent H with *trans*-$[Pt(en)_2Cl_2]^{2+}$ and Reagents B, K, and R with *trans*-$[Pt(CN)_4Cl_2]^{2-}$ provide convenient methods for the synthesis of human calcitonin and its methionine sulfoxide derivative; the formation of intramolecular disulfide bonds by the above Pt(IV) oxidants is essentially quantitative.

Calcitonin, a single-chain, 32-amino acid peptide with a proline amide group at its carboxyl terminus and a seven-amino acid (23-atom) cyclic loop formed by a disulfide bond between cysteines at positions 1 and 7, is a calcium-regulating peptide hormone found in many vertebrate species.[1–7] Human calcitonin (peptide **5** in Scheme 1) originates from the calcitonin-I gene on chromosome 11. Salmon, human, and porcine calcitonins are widely used clinically for treatment of Paget's disease, hypercalcemia, osteoperosis, and relief of bone pain.[1–7] Moreover, calcitonin is one of the most successful peptide drugs, with a history of more than two decades of safe and effective therapeutic uses in the treatment of bone and mineral disorders.[1–6]

Because of their therapeutic importance, much research has been carried out on the synthesis of calcitonins derived from different sources and their analogues, as well as their structure–activity relationships.[7–15] Human calcitonin was first synthesized by Sieber and co-workers in 1968.[8] In the early syntheses, several fragments, including one containing the 23-atom disulfide loop, were usually prepared separately by solution-phase methods, and the fragments were then joined to form the whole peptides.[8,9] Later, solid-phase peptide synthesis methods based on Boc and Fmoc chemistry were used to assemble the whole peptide chain in a single experiment, resulting in a much higher efficiency in terms of the whole synthesis process.[7,10–14]

Although synthesis of the linear peptide chain by solid-phase peptide synthesis methodology is now straightforward, some challenging problems of sulfur chemistry remain for the synthesis of human calcitonin (and calcitonins derived from other sources such as rabbit, rat, and pig),[2] and other peptides that contain both a disulfide bond and methionine residues. These include the slow and nonselective formation of the intramolecular disulfide loop (vide infra)[7–15] and partial oxidation of the methionine side chain during peptide synthesis by solid-phase Fmoc chemistry.[16,17] Recently, we discovered a new class of reagents [*trans*-dichloro-platinum(IV) complexes] for highly selective and efficient formation of intramolecular peptide disulfide bonds.[18–20] Also, we recently developed a cleavage reagent, Reagent H, with which methionine sulfoxides are reduced during cleavage and deprotection of the resin-bound peptide.[16]

In this letter, we describe convenient and high-yield syntheses of human calcitonin and its methionine sulfoxide derivative by combining the different oxidizing strengths of the two Pt(IV) complexes with different cleavage reagents developed for Fmoc chemistry. The purpose of the present work was to develop a high-yield synthesis of human calcitonin and its methionine sulfoxide derivative and, in the process, to develop methodology that can be generalized for the synthesis of both the native and methionine-sulfoxide forms of other peptides that contain both a disulfide loop and methionine residues such as melanin-concentrating hormone, somatostatin-28 and peptides of Atrial Natriuretic Factors. There is increased interest in the biological activity

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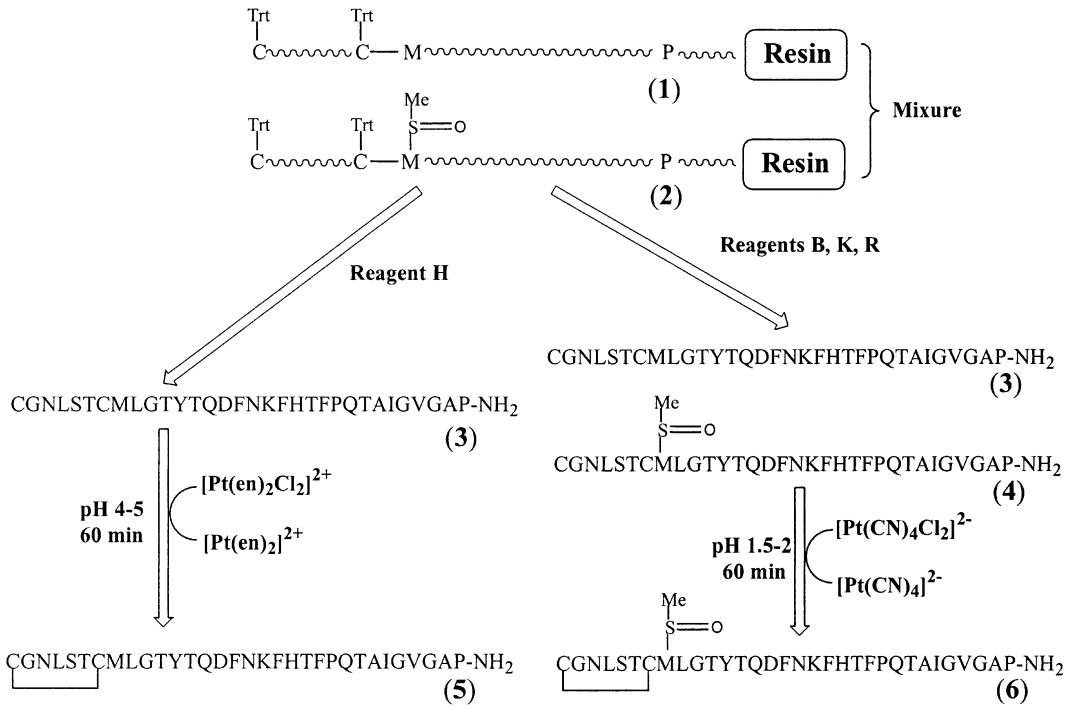

Scheme 1. Synthesis of human calcitonin (**5**) and its methionine sulfoxide derivative (**6**). Components of reagents H, B, K, and R used for peptide cleavage are described in the text.

of the methionine sulfoxide form of peptides and proteins,[21] which can be formed in vivo by oxidation of methionine residues by hydrogen peroxide, hydroxyl radicals, hypochlorite and peroxynitrite,[22] following the recent reports that the level of methionine sulfoxide reductase is decreased in Alzheimer's disease brains[23] and mice lacking the methionine sulfoxide reductase gene have decreased longevity.[24]

Assembly of the side chain protected human calcitonin peptide chain on resin, H-Cys(Trt)-Gly-Asn(Trt)-Leu-Ser(*t*Bu)-Thr(*t*Bu)-Cys(Trt)-Met-Leu-Gly-Thr(*t*Bu)-Tyr(*t*Bu)-Thr(*t*Bu)-Gln(Trt)-Asp(O*t*Bu)-Phe-Asn(Trt)-Lys(Boc)-Phe-His(Trt)-Thr(*t*Bu)-Phe-Pro-Gln(Trt)-Thr(*t*Bu)-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH-resin, was carried out on a Millipore model 9050 Plus peptide synthesizer using solid-phase, Fmoc peptide synthesis methodology. 0.40 g of Fmoc-PAL-PEG-PS resin with a loading capacity of 0.22 mmol/g and a 5-times excess of Fmoc-protected amino acids were used for the synthesis. The Fmoc group was removed from the N-terminal amino group of the resin-bound peptide with 20% piperidine in *N,N*-dimethylformamide. Entering amino acids were preactivated with 1-hydroxy-7-azabenzotriazole (HOAt) and *N,N*′-diisopropyl-carbodiimide (DIPCDI), and couplings were run for 90 min. After being dried for 24 h under vacuum, 100 mg of the peptide-bound resin was reacted with 5 mL of freshly prepared Reagent R (TFA 90%, thioanisole 5%, ethanedithiol 3%, anisole 2%)[25] for 2.5 h under a nitrogen atmosphere. The resin was removed by filtration and washed with TFA (2 × 1 mL), which was combined with the filtrate. The filtrate was then poured into 150 mL cold ether, resulting in precipitation. The precipitate was then dissolved in water and lyophilized to give 29 mg crude product. Similarly, treatment of the peptide-bound resin with Reagent B (TFA 88%, phenol 5%, triisopropylsilane 2%, and water 5%)[25] and Reagent K (TFA 82.5%, phenol 5%, thioanisole 5%, ethanedithiol 2.5%, and water 5%),[26] respectively, gave approximately the same amount of crude product.

A RP-HPLC chromatogram of the crude product obtained from the Reagent R cleavage is shown on the top part of Figure 1. The major peak was assigned to peptide **4** in Scheme 1 based on its mass as determined by MALDI-TOF mass spectrometry.[27] The much smaller peak identified in the chromatogram was assigned to peptide **3** in Scheme 1.[27] Peak area measurements, indicate an HPLC purity of ca. 60% for the sum of peptides **4** and **3**.[28] The crude peptide obtained by cleavage with Reagents B and K gave HPLC chromatograms similar to that in Figure 1, indicating oxidation of the methionine residue of the resin-bound peptide during peptide chain assembly (i.e., the resin-bound peptide is a mixture of **1** and **2** in Scheme 1).

The methionine sulfoxide was quantitatively reduced during cleavage and deprotection with Reagent H (TFA 81%, phenol 5%, thioanisole 5%, ethanedithiol 2.5%, dimethylsulfide 2%, water 3%, ammonium iodide 1.5% w/w).[16] Thirty-nine milligrams of crude product was obtained from the cleavage of 100 mg peptide/resin, as compared to the ~29 mg obtained with Reagents R, B and K. The additional 10 mg of crude product is ammonium iodide that precipitated with the peptides. A chromatogram of the crude product obtained by cleavage with Reagent H is shown in the top part of Figure 2.

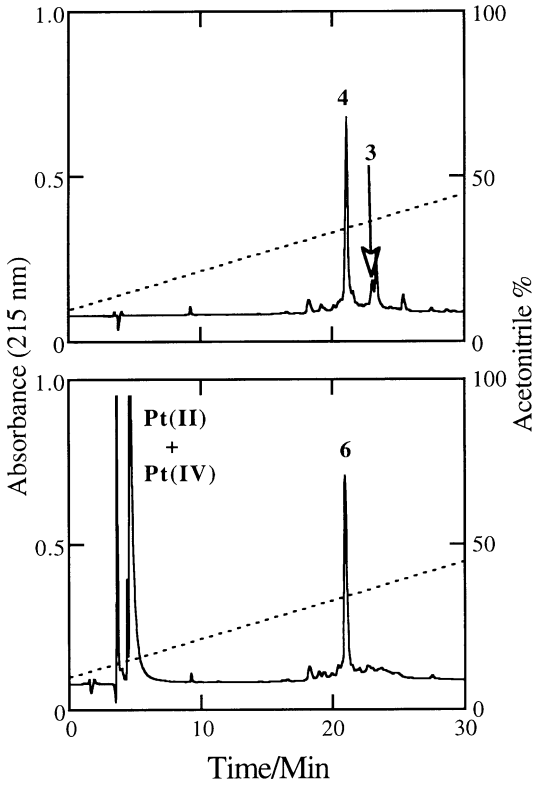

Figure 1. Chromatograms of crude product obtained from Reagent R cleavage in water (1.71 mg/mL) (top) and a reaction mixture containing crude product (1.71 mg/mL) and 2.0 mM *trans*-$[Pt(CN)_4Cl_2]^{2-}$ in dilute HCl (pH 2) after reaction for 60 min (bottom). A 10 × 250 mm Vydac C18 column was used with an acetonitrile–water gradient mobile phase containing 0.1% TFA (dashed lines indicate the acetonitrile gradient). Peak assignments are given in Scheme 1. Peptide injections are the same volume for the two chromatograms.

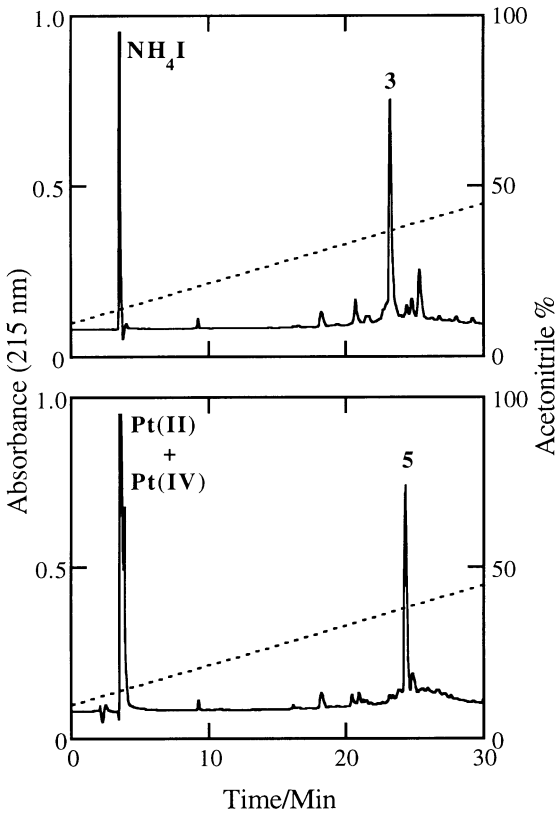

Figure 2. Chromatograms of crude product obtained from Reagent H cleavage in water (2.0 mg/mL) (top) and a reaction mixture containing crude product (2.0 mg/mL) and 3.0 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ in a pH 4.0 phosphate buffer after reaction for 60 min (bottom). A 10 × 250 mm Vydac C18 column was used with an acetonitrile–water gradient mobile phase containing 0.1% TFA (dashed lines indicate the acetonitrile gradient). Peak assignments are given in Scheme 1. Peptide injections are the same volume for the two chromatograms.

The major peak at 23.2 min was assigned to peptide **3** in Scheme 1, based on its mass,[27] with an HPLC purity of ca. 57%.[29]

The crude peptide obtained by cleavage with Reagent H was oxidized with *trans*-$[Pt(en)_2Cl_2]^{2+}$ to form the intramolecular disulfide bond.[19,20] The reaction mixture contained pH 4.0 phosphate buffer, 3.0 mM Pt(IV) complex and 2.0 mg/mL of crude peptide; the oxidation was complete in 1 h. A chromatogram of the reaction mixture is shown in the bottom part of Figure 2; the major peak at 24.3 min was assigned to peptide **5** (human calcitonin) in Scheme 1 based on mass spectrometry.[27] Based on peak area measurements, oxidation of **3** to **5** was essentially quantitative, indicating that *trans*-$[Pt(en)_2Cl_2]^{2+}$ is highly selective and efficient for formation of the intramolecular disulfide bond in human calcitonin. In the early synthesis of human calcitonin, the disulfide bond formation was achieved by iodine oxidation with a careful control of oxidation time;[8] but it was found in later experiments that I_2 can also oxidize the methionine side chain.[9,28] It also was observed that $[Fe(CN)_6]^{3-}$ can react with the methionine and tyrosine side chains of peptides,[28] although $[Fe(CN)_6]^{3-}$ has been used for disulfide bond formation in the synthesis of calcitonins without methionine.[11,15] Thus, disulfide bonds are generally formed by air oxidation in the synthesis of human calcitonin and analogues.[9,12–14] However, with air oxidation, very dilute solutions must normally be used, the reaction is slow and solution pH must be carefully controlled.[12]

The crude product obtained by Reagent R cleavage was oxidized with *trans*-$[Pt(CN)_4Cl_2]^{2-}$ to simultaneously form the intramolecular disulfide bond and convert the methionine residue in **3** to the sulfoxide form. *trans*-$[Pt(CN)_4Cl_2]^{2-}$ is a stronger oxidizing agent than *trans*-$[Pt(en)_2Cl_2]^{2+}$ and will both oxidize thiols to intramolecular disulfide bonds and methionine to methionine sulfoxide, but it is not so strong that it oxidizes tyrosine and other amino acid side chains.[18,30] The reaction mixture contained dilute HCl (pH 2), 2.0 mM $[Pt(CN)_4Cl_2]^{2-}$ and 1.71 mg/mL crude product; oxidation was complete in 60 min. A chromatogram of the reaction mixture is shown in the bottom part of Figure 1. On the basis of a monoisotopic mass of 3431.7,[27] the peak at 20.9 min was assigned to the methionine sulfoxide form of human calcitonin, peptide **6** in Scheme 1. The chromatograms in Figure 1 also illustrate that *trans*-$[Pt(CN)_4Cl_2]^{2-}$ simultaneously transforms both peptides **3** and **4** to peptide **6**, and that the transformation is essentially quantitative. Oxidation of the crude products obtained from cleavage with Reagents B and K gave similar results.

Oxidation of the crude product obtained from Reagent H cleavage with $[Pt(CN)_4Cl_2]^{2-}$ resulted in a more complicated situation due to the rapid oxidation of iodide in the crude product to iodine, which in turn reacted with the peptide.

In summary, human calcitonin and its methionine sulfoxide derivative can be conveniently synthesized in high-yield by using various combinations of the oxidation reagents *trans*-$[Pt(en)_2Cl_2]^{2+}$ and *trans*-$[Pt(CN)_4Cl_2]^{2-}$ and the different cleavage reagents, as outlined in Scheme 1. This methodology is expected to be widely useful for synthesis of other peptides that contain both intramolecular disulfide bonds and methionine residues and their methionine sulfoxide derivatives.

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References and Notes

1. Copp, D. H. *Clin. Invest. Med.* **1994**, *17*, 268.
2. Basava, C. In *Peptides: Design, Synthesis, and Biological Activity*; Basava, C., Anantharamaiah, G. M., Eds.; Birkhäuser: Boston, 1994; p 209.
3. Becker, K L.; Nylén, E. S.; Cohen, R.; Snider, R. H., Jr. In *Principles of Bone Biology;* Bilezikian, J. P., Raisz, L. G., Rodan, G. A., Eds; Academic: San Diego, 1996; p 471.
4. Gennari, C.; Agnusdei, D.; Camporeale, A. *Calcif. Tissue Int.* **1991**, *49*, S9.
5. Potts, J. T., Jr. *Bone Mineral* **1992**, *16*, 169.
6. Wallach, S.; Farley, J. R.; Baylink, D. J.; Brenner-Gati, L. *Calcif. Tissue Int.* **1993**, *52*, 335.
7. Inoue, A.; Shikano, M.; Komatsu, Y.; Obata, J.; Ochiai, J.; Nishide, H.; Ito, N.; Nagao, H.; Kondo, K.; Tunemoto, D.; Hemmi, H.; Numao, N. *Eur. J. Biochem.* **1991**, *201*, 607.
8. (a) Sieber, P.; Brugger, M.; Kamber, B.; Riniker, B.; Rittel, W. *Helv. Chim. Acta* **1968**, *51*, 2057. (b) Kamber, B.; Brücker, H.; Riniker, B.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1970**, *53*, 556. (c) Sieber, P.; Riniker, B.; Brugger, M.; Kamber, B.; Rittel, W. *Helv. Chim. Acta* **1970**, *53*, 2135. (d) Kamber, B.; Hartman, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1980**, *63*, 899.
9. Nakagawa, Y.; Morikawa, T.; Sakakibara, S. *Pept. Chem.* **1977**, *15*, 189.
10. Shen, J.; Du, Y.; Zheng, S.; Xu, D. *Shengwu Huaxue Yu Shengwu Wuli Xuebao* **1985**, *17*, 703.
11. Moe, G. R.; Kaiser, E. T. *Biochemistry* **1985**, *24*, 1971.
12. (a) Kapurniotu, A.; Taylor, J. W. *J. Med. Chem.* **1995**, *38*, 836. (b) Kapurniotu, A.; Kayed, R.; Taylor, J. W.; Voelter, W. *Eur. J. Biochem.* **1999**, *265*, 606. (c) Taylor, J. W.; Jin, Q. K.; Sbacchi, M.; Wang, L.; Belfiore, P.; Garnier, M.; Kazantzis, A.; Kapurniotu, A.; Zaratin, P. F.; Scheideler, M. A. *J. Med. Chem.* **2002**, *45*, 1108.
13. Sasaki, J.; Matsumura, Y.; Murayama, E.; Ohba, M. *Pept. Chem.* **1995**, *33*, 313.
14. Yu, C.; Zhou, G. M.; Li, B. L.; Wu, X. F.; Cui, D. F. *Shengwu Huaxue Yu Shengwu Wuli Xuebao* **1999**, *31*, 553.
15. Pan, H.; Wang, L.; Chen, Z. *Zhongguo Yaowu Huaxue Zazhi* **1997**, *7*, 294.
16. Huang, H.; Rabenstein, D. L. *J. Pept. Res.* **1999**, *53*, 548.
17. Fülöp, L.; Penke, B.; Zarandi, M. *J. Pept. Sci.* **2001**, *7*, 397.
18. Shi, T.; Rabenstein, D. L. *J. Org. Chem.* **1999**, *64*, 4590.
19. Shi, T.; Rabenstein, D. L. *J. Am. Chem. Soc.* **2000**, *122*, 6809.
20. Shi, T.; Rabenstein, D. L. *Tetrahedron Lett.* **2001**, *42*, 7203.
21. Levine, R. L.; Moskovitz, J.; Stadtman, E. R. *IUBMB Life* **2000**, *50*, 301.
22. Vogt, W. *Free Radic. Biol. Med.* **1995**, *18*, 93.
23. Gabbita, S. P.; Aksenov, M. Y.; Lovell, M. A.; Markesbery, W. R. *J. Neurochem.* **1999**, *73*, 1660.
24. Moskovitz, J.; Bar-Noy, S.; Williams, W. M.; Requena, J.; Berlett, B. S.; Stadtman, E. R. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12920.
25. Solé, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399.
26. King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255.
27. Molecular weights were measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with a Voyager-DE™ STR spectrometer from PerCeptive Biosystems; α-cyano-4-hydroxycinnamic acid was used for the matrix. Monoisotopic masses obtained for the peptides in Scheme 1 were: $[\mathbf{3}+H]^+$, 3918.5 (theoretical: 3418.6); $[\mathbf{4}+H]^+$, 3434.6 (theoretical: 3434.6); $[\mathbf{5}+H]^+$, 3416.5 (theoretical: 3416.6); $[\mathbf{6}+H]^+$, 3432.7 (theoretical: 3432.6).
28. Eichler, J.; Houghten, R. A. *Protein Pept. Lett.* **1997**, *4*, 157.
29. It should be noted that, although the results demonstrate that Reagent H is very useful for conversion of the mixture of **1** and **2** to peptide **3** in Scheme 1, it has several drawbacks for peptide workup: (i) it is not homogeneous with ammonium iodide mainly remaining in suspension and (ii) the reagent has a malodorous smell due to the dimethylsulfide, thioanisole and ethanedithiol (there may be a synergistic effect).
30. Shi, T.; Berglund, J.; Elding, L. I. *J. Chem. Soc., Dalton Trans.* **1997**, 2073.