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A NEW THIOL PROTECTING TRIMETHYLACETAMIDOMETHYL GROUP. SYNTHESIS OF A NEW PORCINE BRAIN NATRIURETIC PEPTIDE USING THE *S*-TRIMETHYLACETAMIDOMETHYL-CYSTEINE[1,2]

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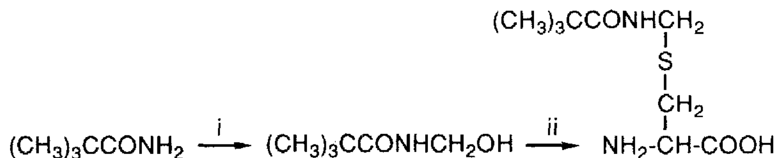
Summary: The *S*-trimethylacetamidomethyl-cysteine [Cys(Tacm)], which can be easily prepared without any serious side reaction, was stable to acidic and alkaline conditions, and readily converted to cystine by iodine oxidation. This new Cys(Tacm) derivative is successfully applied to the conventional solution synthesis of a new porcine brain natriuretic peptide.

Protection of the thiol function of cysteine is required during peptide synthesis.[3] We now report that a new derivative, *S*-trimethylacetamidomethyl-L-cysteine [Cys(Tacm)] can be easily prepared and quantitatively converted to cystine under mild conditions, and a new porcine brain natriuretic peptide (pBNP)[4] has been successfully synthesized using this Cys(Tacm) derivative by the conventional solution method.

The *S*-acetamidomethyl (Acm) group[5] stable to acidic conditions but removable with mercury ion[5] or iodine[6] is one of the widely used thiol protecting groups. However, the preparation of Cys(Acm) is rather troublesome because thiazolidine-2-carboxylic acid is formed as a by-product,[5] and the activation of Boc-Cys(Acm)-OH using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) method is accompanied by a side reaction.[7] When the *S*-benzamidomethyl (Bam) group[8] was used, these complications were not observed, but it was unstable during HF cleavage and alkaline conditions.[9] Therefore we developed a new superior thiol protecting group, *S*-Tacm group, which was stable to acidic and alkaline conditions but removable with iodine or mercury ion and did not show complications, .

The Cys(Tacm) was easily prepared without any serious side reaction in good yield as shown in Scheme 1. *N*-Hydroxymethyl trimethylacetamide was obtained almost quantitatively from trimethylacetamide (1 equiv.) in a solution of formaldehyde-water (7:13) (0.7 equiv.) in the presence of KOH (0.1 equiv.).[10,11] Subsequently, the Tacm group was incorporated into L-cysteine by treatment with *N*-hydroxymethyl trimethylacetamide (1.1 equiv.) in trifluoroacetic acid (TFA) at room temperature for 1h. The *N*-Boc derivative, Boc-Cys(Tacm)-OH, was prepared with di-*tert*-butyl dicarbonate (1.4 equiv.) in the presence of sodium carbonate and then isolated as the cyclohexylamine salt {mp.142-144°C, $[\alpha]_D^{20}$ -14.7°(c 0.7, MeOH), satisfactory elemental analyses were obtained



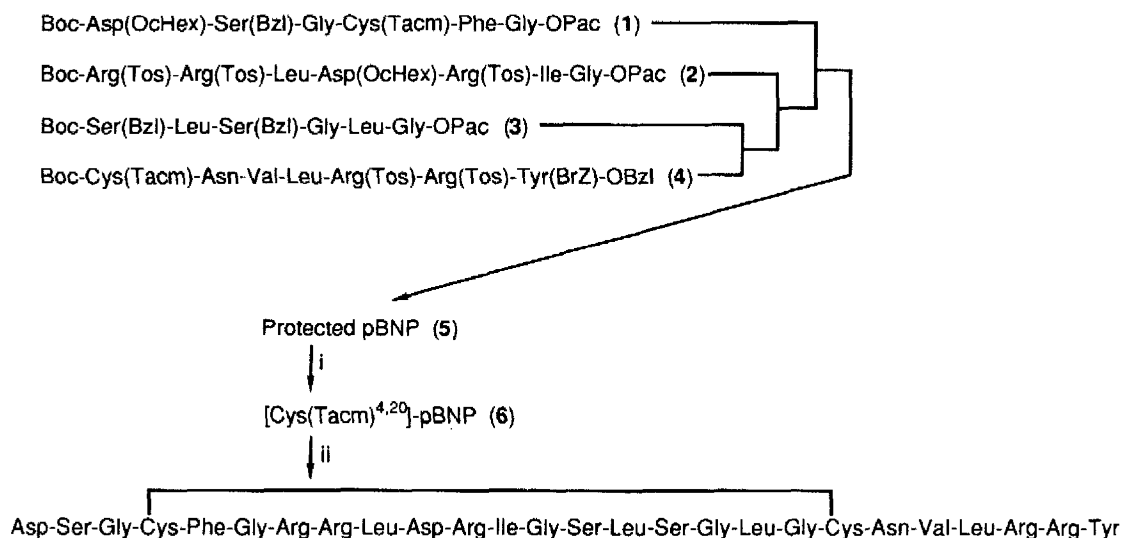
Scheme 1. *Reactants*: i, HCHO-KOH; ii, L-cysteine•HCl in TFA.

for $C_{14}H_{26}N_2O_5S{\bullet}C_6H_{13}N$} in 85% yield (based on starting cysteine).

The *S*-Tacm group was stable to acidic conditions [HF(0°C, 1h),[9,12] 1M trifluoromethanesulphonic acid (TFMSA)-thioanisole in TFA (0°C, 2h)[13]], basic conditions [0.05M NaOH in aq. MeOH (0°C, 1h), NH_2NH_2 in MeOH (r.t., 24h)][9] and Zn-90%AcOH(r.t., 3h), while Cys(Tacm) was quantitatively converted to cystine by 20% I_2/EtOH (10 equiv.) in 90% AcOH (25°C, 1h)[6] and also quantitatively deblocked by $Hg(OAc)_2$ (1 equiv.) in TFA (0°C, 30min).[5] This protecting group had stability similar to the *S*-Acm group. However, on stirring vigorously in DMF (r.t., 48h), Boc-Cys(Acm) was faintly oxidized to the corresponding sulphoxide, while the sulphoxide form of Boc-Cys(Tacm) was not detected. Also, the former was almost completely oxidized to the corresponding sulphoxide by $NaBO_3$ after 24h, whereas the latter was oxidized to less than 60 % under the same condition. The results indicate that Cys(Tacm) is less susceptible to air oxidation than Cys(Acm), presumably because of the steric hindrance of the bulky side chain.

Using this Cys(Tacm), we synthesized a 26-residue peptide, pBNP, which was newly isolated from porcine brain, sequenced and synthesized in solid phase by Matsuo et al.[4] in 1988. It exhibited interesting activities that regulated the homeostatic balance of body fluid and blood pressure. The structure of pBNP with an intramolecular disulphide linkage is remarkably similar to but definitely distinct from that of α-atrial natriuretic peptide (α-ANP).

The synthesis of pBNP was performed by the conventional solution method as illustrated in Scheme 2. The whole sequence was divided into four fragments **1**-**4** at glycine residues, since no racemization was involved in coupling reactions using 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide•HCl (water-soluble carbodiimide, WSC)-HOBt.[14] In combination with TFA-labile Boc group for N^{α}-protection, amino acid derivatives bearing protecting groups removable with HF were employed, *i.e.*, Boc-Asp(OcHex),[15] Boc-Ser(Bzl), Boc-Arg(Tos) and



Scheme 2. *Conditions*: i, HF-m-cresol-Me_2S (0°C, 1h); ii, Oxidation using I_2 in 90% AcOH (25°C 1h). Boc=*t*-butoxycarbonyl, Pac=phenacyl, cHex = cyclohexyl, Bzl = benzyl, Tos = toluenesulphonyl, BrZ = 2-bromobenzyloxycarbonyl.

Boc-Tyr(BrZ)[16] except for Boc-Cys(Tacm). Every fragment was synthesized by the known amide-forming reactions. In the synthesis of the fragments **1** and **4** containing Cys(Tacm), no side reaction was observed during the activation using DCC-HOBt procedure. After removal of C-terminal phenacyl ester (OPac) by Zn in AcOH, each fragment was successively assembled by WSC-HOBt procedure. Each protected peptide was purified by precipitation from DMF with EtOH. The homogeneity of each product was ascertained by t.l.c. and amino acid analysis after acid hydrolysis.

The fully protected peptide (**5**) was treated with HF in the presence of *m*-cresol and dimethylsulphide (0°C, 1h) to remove all protecting groups except for two Tacm groups. The di-Tacm-peptide (**6**) was purified by gel-filtration on Sephadex G-25 (1N AcOH) and ion-exchange chromatography on CM-cellulose using gradient elution with 0.25M $AcONH_4$, followed by fast protein liquid chromatography on a YMC-ODS-AQ-120 (S-50)-column. The purity of di-Tacm-peptide (**6**) was ascertained by amino acid analyses after hydrolyses with 6N HCl [amino acid ratios: Asp 3.01, Ser 2.72, Gly 5.05, Val 0.96, cystine 0.21, Ile 0.97, Leu 4.00, Tyr 0.94, Phe 1.01, Arg 5.02 (recovery 86%)] and with aminopeptidase M [amino acid ratios: Asp 1.95,[17] Gly 5.06, Val 0.78, Ile 0.87, Leu 4.00, Tyr 1.23, Phe 1.18, Arg 5.26 (recovery 68%); Ser, Asn and Cys(Tacm) were not determined], analytical high performance liquid chromatography (h.p.l.c.) on a YMC-AM302-ODS column (4.6 x 150mm) [retention time, 19.6min, on gradient elution with MeCN (10-60%, 30min) in 0.1% aq. TFA, 0.7ml/min] and fast atom bombardment mass spectrometry (FAB-MS) {observed mass values [3096.7(MH^+) and 3097.7(base peak in molecular ion region)] agreed well with the theoretical values [3096.631(MH^+) and 3097.634(base peak)] corresponding to [Cys(Tacm)^{4,20}]-pBNP ($C_{132}H_{222}N_{44}O_{38}S_2$)}.

The product (**6**) thus obtained was diluted (0.05mM) with 90% AcOH and oxidized by adding 20% I_2/EtOH (10 equiv.). After stirring for 1h at 25°C, this solution was evaporated under reduced pressure and purified by gel-filtration on Sephadex G-25 (1N AcOH). The crude oxidized peptide was further purified by h.p.l.c. on a YMC-D-ODS-5 column to give a homogeneous peptide (overall yield 12% from the deprotection, oxidation, and purification steps). The purity of synthetic pBNP {$[\alpha]_D^{20}$ -50° (c 0.1, 1N AcOH)} was confirmed by t.l.c. [silica, Bu^nOH : AcOH : pyridine : H_2O 4:1:1:2, Rf 0.35; Bu^nOH : AcOH : pyridine : H_2O 30:20:6:24, Rf 0.69], amino acid analysis after acid hydrolysis with 6N HCl [amino acid ratios: Asp 3.06(3), Ser 2.86(3), Gly 4.95(5), Val 0.96(1), cystine 0.73(1), Ile 0.98(1), Leu 4.00(4), Tyr 0.95(1), Phe 0.98(1), Arg 5.08(5) (recovery 90%)], analytical h.p.l.c. on a Cosmosil $5C_{18}ST$ column (4.6 x 150mm)[retention time, 18.5min, on gradient elution with MeCN (10-60%, 30min) in 0.1% aq. TFA, 0.7ml/min] and a TSK-gel G-2000SW (7.5 x 600mm)[retention time, 46.2min,[18] 0.1M $AcONH_4$ (pH 4.0), 0.5ml/min], and FAB-MS {observed mass values [2868.4(MH^+) and 2869.4(base peak in molecular ion region)] agreed well with the theoretical values [2868.447(MH^+) and 2869.449 (base peak)] corresponding to pBNP ($C_{120}H_{198}N_{42}O_{36}S_2$)}. The chick rectum relaxant activity of the synthetic pBNP was 2.9 times more potent than that of α-rat ANP, in reasonable agreement with the literature value.[4] The synthetic pBNP also possessed physicochemical properties identical with the pBNP alternatively synthesized by essentially the same procedure using *S*-2,4,6-trimethylbenzyl-L-cysteine.[19]

The biologically active pBNP was obtained in highly purified form with sufficient characterization and these excellent results clearly show that the new *S*-Tacm group is useful for peptide synthesis.

References and Notes

1. This paper is dedicated to Professor Haruaki Yajima on this occasion of his retirement from Kyoto University in March 1989.

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