Total Synthesis of Porcine Cholecystokinin-33 (CCK-33)

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Porcine cholecystokinin-pancreozymin (CCK), which is a 33 amino acid peptide having a sulphated tyrosyl residue at position 27, was synthesized by the classical solution procedure applying our maximum protection strategy, for which the phenoxyacetyl group was used for the first time for protection of Ser residues.

Cholecystokinin-pancreozymin (CCK) was first isolated from hog upper intestine, and it was shown to have a peptide structure with 33 amino acid residues (CCK-33) having a sulphated Tyr at position 27.[1–3] This peptide contains five Ser, three Met, and one Trp residue among the amino acid residues. The combination of these amino acid residues makes the chemical synthesis of this peptide extremely difficult. Two possible routes may be considered: one is to start the synthesis with Tyr(SO_3^-), and the other is to incorporate the sulphate ester group into Tyr after completion of the whole sequence. In the former, the sulphate group must be retained throughout the synthesis, and it should be borne in mind that this group is relatively stable under basic conditions, but is extremely unstable under acidic conditions.[4] In the latter case, at least

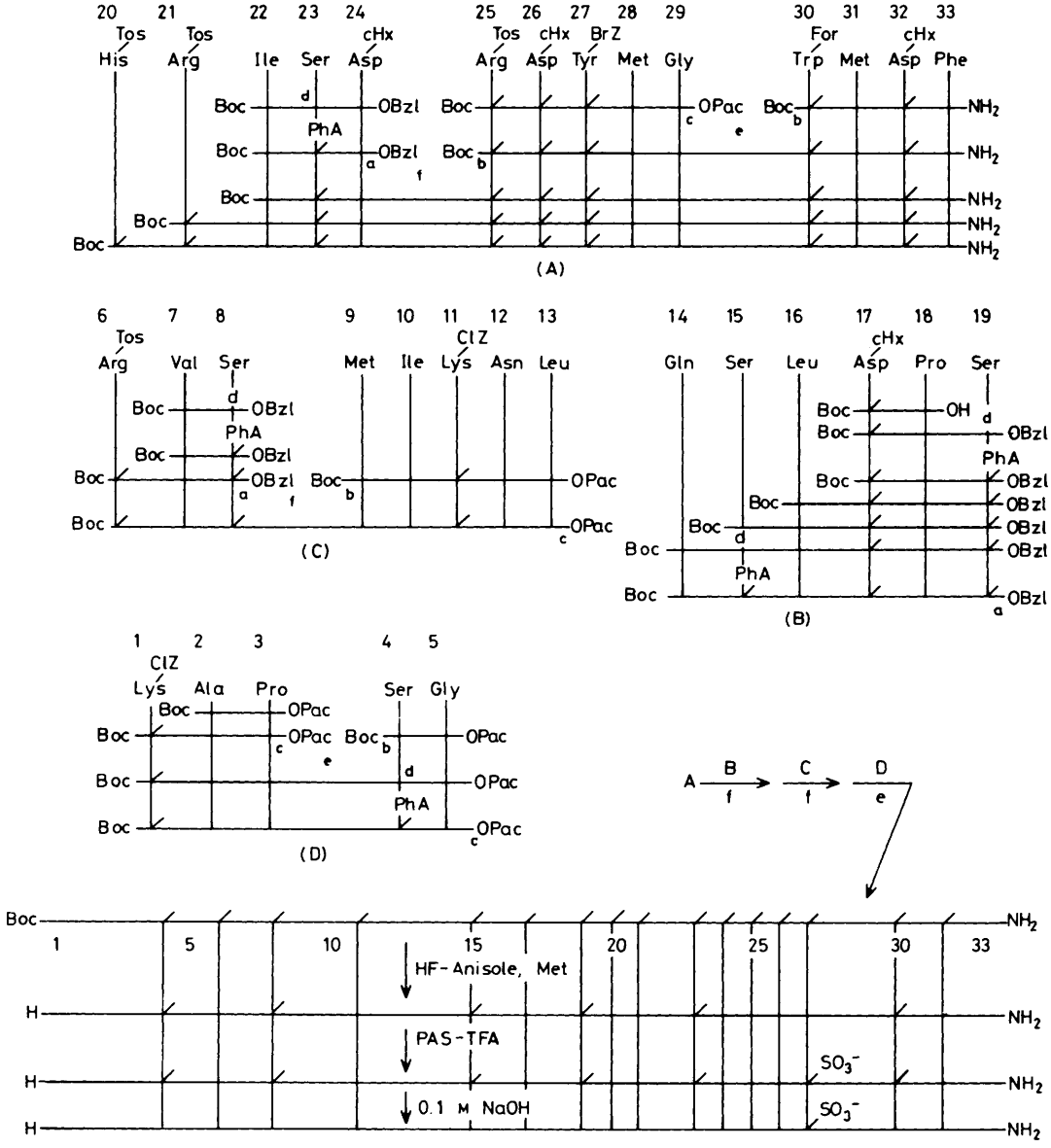

Figure 1. Reaction scheme for synthesis of porcine CCK-33. Peptide segments were elongated stepwise by coupling Boc-amino acids by the WSCI–HOBT method unless otherwise indicated. a, catalytic hydrogenolysis; b, TFA; c, Zn–acetic acid; d, phenoxyacetic anhydride; e, WSCI–HOBT; f, WSCI–HOOBT. OPAc = phenacyl.

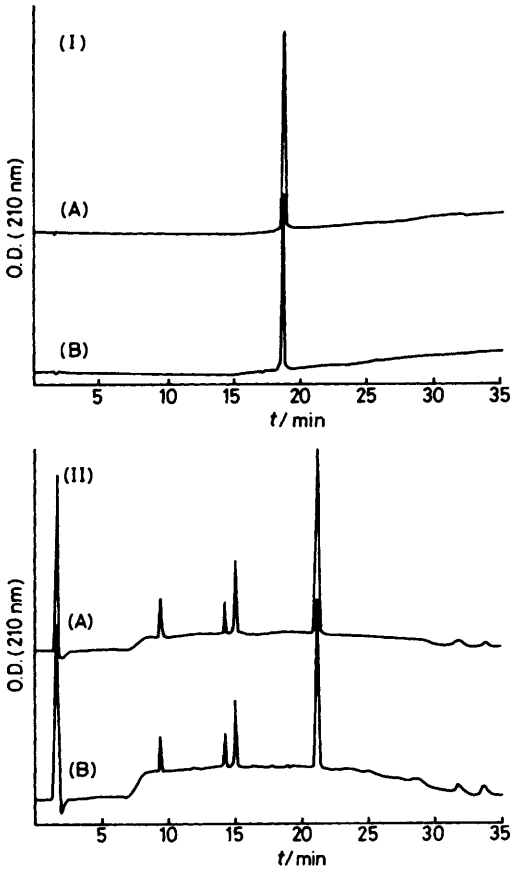

Figure 2. Comparison of synthetic and natural CCK-33 by h.p.l.c.: (A): synthetic; (B): natural. (I): reversed phase system. Column: YMC Pack A-302 ODS (Yamamura Chem. Inst., Kyoto) 4.6 × 150 mm, gradient: 10—60% MeCN in 0.1 M NaCl (pH 2.4), flow rate: 1 ml/min. (II): Tryptic peptide mapping on reversed phase system. Column: Nucleosil 5C18 (4 × 150 mm), gradient: 1—60% MeCN in 0.1% TFA, flow rate: 1 ml/min.

Ser and Trp residues have to be protected during the sulphation reaction and deprotected under basic conditions. Construction of the 33-peptide molecule under the latter conditions seemed the easier, so we chose the second method.

The 33-peptide was constructed by the classical solution procedure applying our maximum protection strategy[5] as shown in Figure 1. For the synthesis, Asp was protected by a cyclohexyl (cHx) group, Arg and His by a tosyl (Tos) group, Lys by a 2-chlorobenzyloxycarbonyl (ClZ) group, Tyr by a 2-bromobenzyloxycarbonyl (BrZ) group, and Trp by a formyl (For) group. Several possible protective groups for Ser were tested for stability and removability, and finally a phenoxyacetyl (PhA) group was chosen since it was stable in HF and trifluoroacetic acid (TFA) during deprotection of other protecting groups but was readily removed in 0.1 M aq. ammonia within 30 min at room temperature;[6] O–Ac or Bz groups were not removed under these conditions. Thus, Ser was initially incorporated into segments without protection of this side chain and then protected with PhA at an appropriate stage by treatment with phenoxyacetic anhydride in pyridine at 0 °C for 30 min. For incorporation of a PhA group into Ser-containing peptides, it must be remembered that if a Ser residue is located at the N-terminus, an incorporated PhA group will quickly migrate to the α-amino group when the N^{α}-protecting group is removed. Protected amino acids and

Table 1. Ratio of amino acids in hydrolysates of synthetic CCK-33.

	6 M HCl 110 °C, 24 h	APM digestion[a] 37 °C, 20 h
Trp	0.84 (1)	0.96 (1)
Lys	2.04 (2)	2.00 (2)
His	0.98 (1)	0.95 (1)
NH_3	4.10 (3)	
Arg	3.18 (3)	2.91[b] (3)
Asp	5.11 (5)	3.76 (4)
Ser	4.36 (5)	
Ser + Asn		5.60 (6)
Glu	1.02 (1)	
Pro	1.99 (2)	2.08 (2)
Gly	2.07 (2)	1.96 (2)
Ala	1.04 (1)	1.07 (1)
Val	1.00 (1)	1.03 (1)
Met	2.92 (3)	2.90 (3)
Leu	2.04 (2)	2.04 (2)
Ile	1.94 (2)	1.95 (2)
Tyr	1.01 (1)	
Phe	1.00 (1)	1.00 (1)
Gln		1.18 (1)
$Tyr(SO_3^-)$		0.92 (1)

[a] The peptide was digested with aminopeptidase M (APM) in the presence of thermolysin in a 50 mM acetate buffer (pH 7.0) containing 2 mM $CaCl_2$. [b] This value contains that for Cit which was formed from Arg by a contaminated enzyme.

protected peptides with Gly or Pro at the C-terminus were coupled with water-soluble carbodiimide (WSCI), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and 1-hydroxybenzotriazole (HOBT) as reagents (WSCI–HOBT method). The coupling of protected peptides with L-amino acid residues at the C-terminus was carried out with WSCI together with 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBT),[7] which is known to minimize racemization of the activated amino acid residues (WSCI–HOOBT method). The fully protected peptide thus obtained was deprotected by the HF procedure in the presence of anisole and Met. During the deprotection reaction, the PhA and For groups were not removed. The Tyr residue was then sulphated with 40 equiv. of pyridinium acetylsulphate (PAS reagent)[8] in TFA in an ice bath for 2 h. After precipitation by adding water, the product was treated with 40 equiv. of 0.1 M NaOH in dimethyl sulphoxide in an ice bath for 10 min to remove the remaining PhA and For groups. The crude product was purified by CM-cellulose chromatography and then by reversed phase h.p.l.c. to produce a homogeneous preparation of the final product; $[\alpha]_D^{27}$ −70.0° (*c* 0.20, 1 M AcOH). The yield from the fully protected peptide was *ca.* 5%. This low yield must be due to oxidation of Met residues during the sulphation reaction; we are now trying to improve these conditions.

This material gave reasonable results on amino acid analyses after acid hydrolysis and APM digestion as shown in Table 1. At this stage, possible racemization of Ser residues was examined by gas-chromatography on a capillary column of Chirasil-Val III[9] using an acid hydrolysate of the final product. Results showed that the D-serine content in the hydrolysate was <2%. The elution profile of this material on h.p.l.c. and its tryptic peptide mapping on h.p.l.c. were identical with those of natural CCK-33 as shown in Figure 2. The homogeneity of these materials was also confirmed by ion-exchange h.p.l.c. (data not shown). Thus, we concluded that the total synthesis of CCK-33 has been achieved by the present procedure.

We thank Professor Viktor Mutt of Karolinska Institute for a generous gift of isolated porcine CCK-33.

Received, 14th August 1986; Com. 1168

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