Total Synthesis of Human Cholecystokinin-33

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Human cholecystokinin (hCCK-33) has been synthesized by selective esterification of the Tyr-phenolic OH group (position 27) of unsulphated hCCK-33 with pyridine–SO_3 complex after reversible masking of other functional groups by hard base (F^-)-labile protecting groups: amino functions by the fluoren-9-ylmethoxycarbonyl group and the Ser-OH group by the t-butyldiphenylsilyl group.

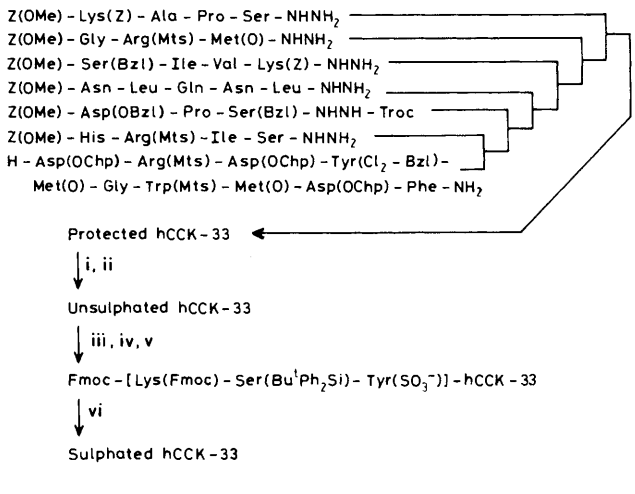
We report here the synthesis of a 33-residue peptide amide corresponding to the entire amino acid sequence of human cholecystokinin (hCCK-33), the gene structure of which has been elucidated by Takahashi *et al.*[1] From a synthetic viewpoint, CCK-33 provides considerable difficulty, since Tyr(SO_3H) is labile to acids, including trifluoroacetic acid (TFA), and sulphation with any reagent proceeds at the Ser–OH group at a much faster rate than that at the Tyr–OH group. The use of protected Ser derivatives, Ser(O–COR), results in β-elimination by base treatment.[2] Thus, it was obvious that the total synthesis of CCK-33 could not be achieved by applying the same methods employed for the synthesis of CCK-8, or even CCK-12 (positions 26—33 or 22—33).[3]

We have synthesized hCCK-33 by a new method, different from those employed for the recent syntheses of porcine CCK-33 by Kurano *et al.*,[4] and Penke *et al.*[5] In these syntheses, the protecting groups attached at 5 Ser residues (R = phenoxyacetyl or Ac) were removed, after sulphation of the Tyr residue (27-Tyr), by treatment with dilute NaOH. In our synthesis, 27-Tyr was selectively sulphated with pyridine–SO_3 complex, the other functional groups being reversibly masked by hard base (F^-)-labile protecting groups; *i.e.*, the 3-amino functions with the Fmoc group[6] and the 4-Ser-OH functions with t-butyldiphenylsilyl (Bu^tPh_2Si) group[7] (Scheme 1).

First, an unsulphated form of hCCK-33 was synthesized by the successive azide condensation[8] of seven fragments, followed by deprotection with trimethylsilyl trifluoromethanesulphonate (TMSOTf)–thioanisole/TFA[9] (1 M, 0 °C, 150 min) in the presence of *m*-cresol and ethanedithiol (EDT). Prior to deprotection, Met(O) was reduced to Met by treatment with phenylthiotrimethylsilane ($PhSSiMe_3$)[10] (20 °C, 30 min). The deprotected peptide was purified to homogeneity by ion-exchange chromatography on CM-trisacryl M, followed by high performance liquid chromatography (h.p.l.c.) on a YMC-AM302 ODS column. In this synthesis, Asp(OChp)[11] was employed to suppress base-catalysed succinimide formation of the Asp residues.[12]

Next, in order to sulphate 27-Tyr selectively, several model experiments were conducted by using CCK-12 (positions 22—33) and functional amino acids and their derivatives. Preferential masking of Ser-OH in the presence of Tyr was found to be possible by silylation, owing to a tolerable difference in the reaction rates at these two functional groups. For this purpose, Bu^tPh_2Si–Cl was selected as a silylating reagent. Other trialkylsilyl groups tested, trimethylsilyl and t-butyldimethylsilyl, were too labile under sulphation conditions. Z(OMe)–Ser–OMe was silylated quantitatively with Bu^tPh_2Si–Cl (20 equiv.) in the presence of imidazole (20 equiv.) within 30 min (4 °C), while Z(OMe)–Tyr–OMe was silylated in 46% yield after 4 h. Addition of phenol (20 equiv.) was sufficient to suppress *O*-silylation of Tyr (31% after 4 h and 40% after 14 h). The Bu^tPh_2Si group attached at Ser–OH was removed by treatment with tetrabutylammonium fluoride (Bu_4NF)[13] (1 M, 0 °C, 1 h) in dimethylformamide (DMF), together with the Fmoc group[14] used for temporary protection of the α- and ε-amino functions during sulphation. Considering the presence of the unmasked Trp residue in unsulphated hCCK-33, we decided to perform sulphation with pyridine–SO_3 complex in DMF, rather than with pyridinium acetylsulphate[15] in TFA. Partial oxidation of Met during the sulphation reaction was suppressed by addition of EDT.

The free form of hCCK obtained above was converted into the corresponding sulphated form by the following sequence of reactions. Treatment with Fmoc–OSu (30 equiv.) in DMF in the presence Et_3N for 2 h masked the amino functions, phenol (30 equiv.) was added to protect Tyr, and the product was precipitated with diethyl ether. Treatment with Bu^tPh_2Si–Cl (120 equiv.) in the presence of imidazole (120 equiv.) masked 4 Ser–OH functions (4 °C, 14 h). Phenol (120 equiv.) was added to minimise silylation of 27-Tyr. The product was gel-filtered on Sephadex LH-20 using DMF as an eluant. Sulphation of 27-Tyr was achieved by treatment with pyridine–SO_3 complex (100 equiv.) in pyridine–DMF (1 : 4) (25 °C, 24 h). EDT (30 equiv.) was added to protect Met. The product was gel-filtered on Sephadex LH-20. Treatment with Bu_4NF (1 M in



Scheme 1. Synthetic route to hCCK-33. *Reactants:* i, $PhSSiMe_3$; ii, TMSOTf–thioanisole/TFA (1 M); iii, Fmoc–OSu; iv, Bu^tPhSi–Cl; v, pyridine–SO_3 complex; vi, Bu_4NF. Z(OMe) = *p*-methoxybenzyloxycarbonyl, Z = benzyloxycarbonyl, Bzl = benzyl, Cl_2–Bzl = 2,6-dichlorobenzyl, Chp = cycloheptyl, Mts = mesitylenesulphonyl, Fmoc–OSu = fluoren-9-ylmethoxycarbonyl *N*-hydroxysuccinimide ester, Troc = 2,2,2-trichloroethoxycarbonyl (removed by Zn–AcOH treatment, before condensation).

DMF) removed the Fmoc and Bu^tPh_2Si protecting groups attached (25 °C, 60 min). EDT (30 equiv.) was added to quench the dibenzofulvene derived from the Fmoc group. The product was gel-filtered on Sephadex G-10 using NH_4HCO_3 (0.1 M) as an eluant.

The crude sulphated hCCK-33 thus obtained was purified by ion-exchange chromatography on CM-Trisacryl M using gradient elution with NH_4HCO_3 (0.2 M), followed by h.p.l.c. on an Asahipak ODP-50 column with isocratic elution using MeCN (28%) in aqueous $AcONH_4$ (0.1 M, pH 6.5); overall yield 15%, from unsulphated hCCK-33. When silylation was conducted at 25 °C for 4 h, the yield was 13%. The former purification was effective to remove oversulphated and unsulphated CCKs and the latter to remove the Met(O)-derivative. The purity of synthetic hCCK-33 thus obtained was ascertained by amino acid analysis, after hydrolysis with 6 M HCl and analytical h.p.l.c. on a Cosmosil 5C18 column [retention time, 14 min, on gradient elution with MeCN (20—40%, 30 min) in aqueous TFA (0.1%)]. Amino acid ratios in leucine aminopeptidase digest were; Asp 3.49 (required 4), Ser 4.22 (4), Pro 1.50 (2), Gly 2.12 (2), Ala 1.13 (1), Val 1.14 (1), Met 2.92 (3), Ile 1.96 (2), Leu 2.07 (2), Tyr(SO_3H) 0.91 (1), Lys 2.00 (2), His 0.92 (1), Trp 0.96 (1), Arg 2.87 (3), Phe 1.00 (1) (recovery 81%); Asn and Gln were not determined.

In terms of pancreatic protein output[16] and capillary blood flow[17] in dogs (n = 4), the relative potencies of synthetic hCCK-33 and unsulphated hCCK-33 with respect to those of synthetic CCK-8 (taken as 1 on a molar basis, sample purchased from Peptide Institute Inc., Osaka, Japan) were 0.92 and 0.074, respectively. It has been reported that CCK-8 is 2.5 times more potent on a molar basis than porcine CCK-33.[18] We conclude that we obtained a highly active preparation of hCCK-33 by the methods described here, without exposing the peptide to strong base.

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References

1 Y. Takahashi, K. Kato, Y. Hayashizaki, T. Wakabayashi, E. Ohtsuka, S. Matsuki, M. Ikehara, and K. Matsubara, *Proc. Natl. Acad. Sci. USA*, 1985, **82**, 1931.

2 J. I. Harris and J. S. Fruton, *J. Biol. Chem.*, 1951, **191**, 143; H. Zahn and E. Schnabel, *Liebigs Ann. Chem.*, 1957, **605**, 212; K. Inoue and H. Otsuka, *Bull. Chem. Soc. Jpn.*, 1961, **34**, 1.

3 M. A. Ondetti, J. Pluscec, E. F. Sabo, J. T. Sheehan, and N. Williams, *J. Am. Chem. Soc.*, 1970, **92**, 195; M. A. Ondetti, B. Rubin, S. L. Engel, J. Pluscec, and J. T. Sheehan, *Am. J. Dig. Dis.*, 1970, **15**, 149.

4 Y. Kurano, T. Kimura, and S. Sakakibara, *J. Chem. Soc., Chem. Commun.*, 1987, 323.

5 B. Penke, M. Zarandi, J. Zsigo, G. K. Toth, and K. Kovacs, Peptide 1986, ed. D. Theodoropoulos, Walter de Gruyter, Berlin, 1987, p. 447.

6 L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, 1970, **92**, 5748.

7 S. Haressian and P. Lavalee, *Can. J. Chem.*, 1975, **53**, 2975.

8 J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, 1961, **26**, 2333.

9 N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funakoshi, Y. Hayashi, Y. Kuroda, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, 1987, 274.

10 N. Fujii, S. Kuno, A. Otaka, S. Funakoshi, K. Takagi, and H. Yajima, *Chem. Pharm. Bull. Jpn.*, 1985, **33**, 4587.

11 N. Fujii, M. Nomizu, S. Futaki, A. Otaka, S. Funakoshi, K. Akaji, K. Watanabe, and H. Yajima, *Chem. Pharm. Bull. Jpn.*, 1986, **34**, 864.

12 M. Bodanszky, J. C. Tolle, S. S. Deshmane, and A. Bodanszky, *Int. J. Pept. Protein Res.*, 1978, **12**, 57, and references cited therein.

13 E. J. Corey and B. B. Snider, *J. Am. Chem. Soc.*, 1972, **94**, 2549.

14 Quantitative removal of the Fmoc group with Bu_4NF was independently reported by M. Ueki and M. Amemiya, *Japan Symposium on Peptide Chemistry*, 1987, Abstract, p. 264.

15 B. Penke, F. Hajnal, J. Lonovics, G. Holzinger, T. Kadar, G. Telegdy, and J. Rivier, *J. Med. Chem.*, 1984, **27**, 845.

16 D. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.

17 M. D. Stern, P. D. Bowen, P. Parma, R. W. Osgood, R. L. Bowman, and J. H. Stein, *Am. J. Physiol.*, 1979, **236**, F80.

18 G. F. Stening and M. I. Grossman, *Am. J. Physiol.*, 1969, **217**, 262.