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### Cholecystokinin (Pancreozymin). 3.<sup>1</sup> Synthesis and Properties of an Analogue of the C-Terminal Heptapeptide with Serine Sulfate Replacing Tyrosine Sulfate

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The influence of tyrosine *O*-sulfate, the 27th residue in the sequence of cholecystokinin (pancreozymin) (CCK-PZ) on calcium outflux in isolated pancreatic cells of guinea pigs, was studied by replacing this residue in the biologically active C-terminal heptapeptide, CCK-PZ-(27–33) (I), with L-serine *O*-sulfate. The synthetic analogue Ser-(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (IV), produced the half-maximal outflux observed with I, if applied at about 250 times higher concentration. The unsulfated form of IV was about ten times less potent than unsulfated I. Thus, in the effect on the calcium outflux, serine cannot replace tyrosine without a major loss in potency; a sulfate ester group in position 27 is important but in itself not sufficient for full potency. Interestingly, if the terminal amino group of the heptapeptide is left protected by a *tert*-butyloxycarbonyl group, the potencies of the derivatives of both I and IV were slightly, but significantly, higher than those of the free peptides.

A gastrointestinal hormone stimulating the contraction of the gall bladder was discovered by Ivy and Oldberg<sup>2</sup> and was named cholecystokinin (CCK), while a factor causing the release of digestive enzymes from the pancreas was found by Harper and Raper<sup>3</sup> in the intestinal mucosa of the hog and was designated as pancreozymin (PZ). Subsequently, Jorpes and Mutt<sup>4</sup> demonstrated that a single compound is responsible for both kinds of activities. The sequence of the 33 amino acid residues in the peptide chain of CCK-PZ was elucidated by Mutt and Jorpes.<sup>5</sup> The C-terminal dodecapeptide of CCK-PZ, synthesized by Ondetti and his co-workers,<sup>6</sup> and even the C-terminal heptapeptide, Tyr(*O*-SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (I), showed the hormonal properties of the entire molecule. Similar biological activities are exhibited by caerulein, a peptide isolated from the skin of *Hyla caerulea* by Anastasi and her associates.<sup>7</sup> The C-terminal sequences of CCK-PZ and caerulein are quite similar, except that methionine in position 28 of CCK-PZ is replaced by threonine in caerulein. Among the synthetic peptides related to caerulein, the heptapeptide with the C-terminal sequence of CCK-PZ was also investigated.<sup>8</sup> A closely related sequence is found also in the antral hormone gastrin.<sup>9</sup>

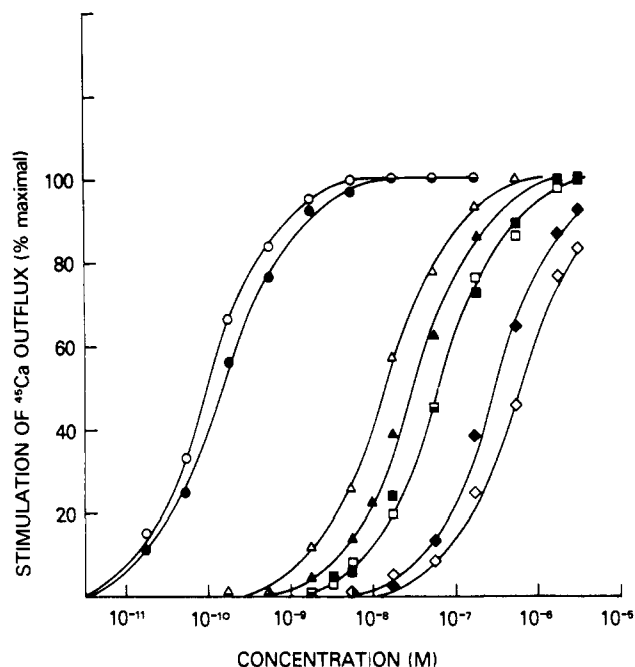
Unlike in gastrin, where the presence or absence of a sulfate ester group on the phenolic hydroxyl of tyrosine has no major influence on biological activity, in CCK-PZ the fact that a tyrosine *O*-sulfate residue and not a simple tyrosine is present is quite significant. The hormonally active C-terminal peptides tested before sulfation had only

a small fraction of the potency of the corresponding sulfated materials.<sup>6,8,10</sup> Also, the recently reported<sup>11</sup> synthesis of Tyr<sup>27</sup>-CCK-PZ produced a peptide which was 250 times less active than the parent hormone. Yet, while these observations provided ample evidence on the importance of the sulfate ester grouping, it was still questionable whether or not a sulfate ester in the side chain of the 27th residue is *sufficient* for full hormonal activity. The role of the structural features of *tyrosine* remained to be investigated. We decided, therefore, to synthesize the shortest part of the CCK-PZ molecule with significant biologic activity, the C-terminal heptapeptide CCK-PZ-(27–33), with serine *O*-sulfate instead of tyrosine *O*-sulfate in position 27. The potency, if any, of this analogue was expected to shed light on the role of the tyrosine moiety.

In previous studies CCK-PZ as well as synthetic C-terminal fragments have been shown to increase amylase secretion from dispersed acinar cells from the guinea pig pancreas.<sup>10</sup> The initial steps in the action of CCK-PZ on enzyme secretion are release of exchangeable cellular calcium followed by an increase in cellular 3',5'-guanosine monophosphate.<sup>12,13</sup> Previous results<sup>10,12,13</sup> indicate that stimulation of calcium outflux from pancreatic acinar cells is a sensitive, reproducible bioassay system for CCK-PZ and structurally related peptides.

**Synthesis.** The protected heptapeptide amide *tert*-butyloxycarbonyl-L-seryl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide (II) was synthesized stepwise,<sup>14</sup> starting with the C-terminal





**Figure 1.** Stimulation of  $^{45}\text{Ca}$  outflux from guinea-pig pancreatic acinar cells by analogues of the C-terminal heptapeptide of CCK-PZ. Dispersed acinar cells were preincubated in Krebs-Ringer bicarbonate (pH 7.4) containing 1% (wt/vol) albumin and 0.5 mM  $^{45}\text{Ca}$  for 60 min at 37 °C. Calcium outflux was determined from the loss of cellular  $^{45}\text{Ca}$  during a 5-min incubation after adding 5 mM EDTA. Stimulation of calcium outflux is given as the percent of maximal stimulation obtained with I at  $10^{-7}$  M. Each point was determined in duplicate and results shown are means of five separate experiments. The symbols in Figure 1 represent: O, Boc-Tyr(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (IX); ●, H-Tyr(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub>-CF<sub>3</sub>COOH (I); △, Boc-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (VIII); ▲, H-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub>-CF<sub>3</sub>COOH (VIIIa); □, Boc-Ser(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (III); ■, H-Ser(SO<sub>3</sub>H)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub>-CF<sub>3</sub>COOH (IV); ◇, Boc-Ser-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (II); ◆, H-Ser-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub>-CF<sub>3</sub>COOH (IIa).

obtained: 0.3 g; mp 210–214 °C.

To a stirred suspension of L-methionyl-L-aspartyl-L-phenylalanine amide (2.2 g) in DMF (30 mL), *tert*-butyloxycarbonyl-L-tryptophan 2,4,5-trichlorophenyl ester<sup>20</sup> (2.9 g) was added. Gradually, a clear solution formed. After 72 h, the ninhydrin reaction was still positive. A second portion of the active ester (200 mg) was added and stirring continued for an additional 48 h; the ninhydrin reaction became negative. The solvent was evaporated in vacuo and the residue triturated with ether (100 mL). It was filtered, and the solid was resuspended in EtOAc (50 mL), filtered, washed with EtOAc, and dried: 3.53 g (87%); mp 206–209 °C dec; TLC *R*<sub>f</sub> 0.49; [ $\alpha$ ]<sub>D</sub><sup>25</sup> -34.7° (c 2, DMF). According to TLC, this was a single, homogeneous compound; no succinimide derivative<sup>13</sup> could be detected.

***N*-tert-Butyloxycarbonylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (VI).** A sample of the protected tetrapeptide V (900 mg) was dissolved in TFA containing 5% anisole (2.5 mL). After 30 min in an ice-water bath and under nitrogen, the TFA was evaporated in vacuo and the residue triturated with ether, filtered, washed with ether, and dried to yield the tetrapeptide amide trifluoroacetate (900 mg). This was dissolved in a mixture of water (30 mL) and ethanol (20 mL). A suspension of Dowex-1 acetate (5 mL) was added, and the mixture was stirred for 20 min. A white precipitate started to separate. The suspension of the precipitate was decanted from the resin, the solid collected by centrifugation, and the supernatant used to recover more precipitate from the resin. Finally, the resin was washed with the same solvent mixture (100 mL), the combined extracts were evaporated, and the residue was combined with the material collected by centrifugation: VIa, 710 mg; mp 223–225 °C dec. The amphoter was suspended in DMF (10 mL) and

*tert*-butyloxycarbonylglycine *o*-nitrophenyl ester<sup>21</sup> (425 mg) was added. After overnight stirring, the ninhydrin reaction became negative. The solvent was removed in vacuo and the residue triturated with ether (50 mL). The product was filtered, resuspended in ethyl acetate (15 mL), stirred for 10 min, and refiltered: 750 mg (76%); mp 196–198 °C dec; TLC *R*<sub>f</sub> 0.42; [ $\alpha$ ]<sub>D</sub><sup>25</sup> -24.5° (c 2, DMF). Only a trace of the corresponding succinimide derivative<sup>13</sup> and slight impurity with *R*<sub>f</sub> 0.48 could be detected.

***N*-tert-Butyloxycarbonyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (VII).** A. The *tert*-butyloxycarbonyl group was removed from compound VI (1.55 g) with TFA containing 5% anisole (5.5 mL) as described before. The trifluoroacetate salt VIa was dissolved in DMF (15 mL); DIEA (0.64 mL) was added, followed by *tert*-butyloxycarbonyl-L-methionine *p*-nitrophenyl ester<sup>22</sup> (0.95 g). The solution was stirred at room temperature overnight; the ninhydrin and fluorescamine reactions became negative. The solvents were evaporated in vacuo, and the residue was triturated with ether, filtered, and dried: 1.57 g (86%); mp 180–182 °C dec. This contained some of the corresponding succinimide derivative.<sup>15</sup> *R*<sub>f</sub> 0.78. Crystallization from ethanol raised the melting point to 200–201 °C dec; TLC *R*<sub>f</sub> 0.42; [ $\alpha$ ]<sub>D</sub><sup>25</sup> -30.3° (c 2, DMF). Amino acid analysis: Asp 1.05, Gly 0.96, Met 2.01, Phe 1.00. Anal. (C<sub>41</sub>H<sub>56</sub>N<sub>8</sub>O<sub>10</sub>S<sub>2</sub>) C, H, N.

B. A sample of VIa (3.0 g) was dissolved in about 40% aqueous methanol (200 mL), a suspension of Dowex-1 acetate (30 mL) was added, and the amphoteric pentapeptide amide (2.56 g, mp 214–215 °C dec) was secured as described in the preparation of VI. It was suspended in DMF (30 mL), and *tert*-butyloxycarbonyl-L-methionine *p*-nitrophenyl ester<sup>22</sup> (1.7 g) was added. After stirring for 16 h, the ninhydrin reaction became negative. The solvent was evaporated in vacuo, and the residue was triturated and stirred with ether (60 mL), filtered, and suspended in EtOAc (25 mL), then stirred, filtered, and washed with EtOAc (10 mL): 2.8 g (81%); mp 187–189 °C dec; TLC *R*<sub>f</sub> 0.42; [ $\alpha$ ]<sub>D</sub><sup>25</sup> -26.3° (c 2, DMF). This product contained a very small amount of the succinimide derivative.<sup>15</sup>

***N*-tert-Butyloxycarbonyl-L-serine 2,4,5-Trichlorophenyl Ester.** An ethyl acetate solution (40 mL) containing *tert*-butyloxycarbonyl(*O*-benzyl)-L-serine (Beckman, 2.95 g, 10 mmol) and 2,4,5-trichlorophenol (2.4 g, 12 mmol) was cooled in ice, and DCC (2.06 g, 10 mmol) was added in portions. After 20 min, the ice bath was exchanged for the water bath of room temperature and stirring continued for 3 h more. Glacial AcOH (1 mL) was added and after 5 min the solution was filtered to removed the *N,N'*-dicyclohexylurea. The solvent was removed and the residue applied to column of silica gel (220 g, Baker) in chloroform. The elution was carried out with the same solvent. After the void volume (140 mL), 8-mL fractions were collected. The active ester was recovered from fractions 1–35 as an oil, contaminated with some trichlorophenol. One-third of this material was dissolved in EtOAc containing 1% HOAc (30 mL), 10% Pd-on-charcoal catalyst (400 mg) was added, and the mixture was hydrogenated for 18 h. On removal of the catalyst and the solvents, the product crystallized in a desiccator. On trituration with ether-hexane (1:1, 24 mL), the active ester was obtained in pure form: 195 mg; mp 104–105 °C; TLC *R*<sub>f</sub> 0.49 (CHCl<sub>3</sub>-MeOH 19:1); [ $\alpha$ ]<sub>D</sub><sup>25</sup> -41° (c 2, DMF). Anal. (C<sub>14</sub>H<sub>16</sub>O<sub>5</sub>Cl<sub>3</sub>N) H, N; C: calcd, 43.7; found, 44.2.

Two further crops (250 and 245 mg) with the same melting point were obtained from the ether-hexane mother liquor: total yield, 54%.

***N*-tert-Butyloxycarbonyl-L-seryl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (II).** The *tert*-butyloxycarbonyl group was removed from compound VII (600 mg) with TFA containing 5% anisole (3 mL), as described before. The trifluoroacetate salt VIIa was converted to the amphoter as described in the previous paragraphs: 480 mg; mp 202–203 °C. The amphoteric hexapeptide (480 mg, 0.61 mmol) was suspended in DMF (5 mL) and *tert*-butyloxycarbonyl-L-serine 2,4,5-trichlorophenyl ester (260 mg, 0.67 mmol) was added, and the mixture was stirred overnight. The ninhydrin reaction was negative. The solvent was removed, the residue triturated with ether, and the separated solid filtered and dried: 575 mg (97%). A sample (50 mg) was triturated with EtOAc-pyridine-AcOH-H<sub>2</sub>O (60:20:6:11, 1 mL) to yield 28 mg; mp 195–196 °C dec; TLC *R*<sub>f</sub> 0.34; [ $\alpha$ ]<sub>D</sub><sup>25</sup> -27.5° (c 2, DMF). Amino

acid analysis: Asp, 1.05; Ser 0.91, Gly 0.96, Met 2.09, Phe 1.00. Anal. ( $C_{44}H_{61}N_9O_{12}S_2$ ) H, N; C: calcd, 54.4; found, 53.9.

**N-tert-Butyloxycarbonyl-(O-sulfate)-L-seryl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (III).** A sample of II (200 mg) was dissolved in dry pyridine (2 mL) and DMF (5 mL) and pyridine-sulfur trioxide complex (1.2 g) was added. This mixture was stirred for 48 h maintaining the anhydrous conditions. The product (170 mg) was isolated as described by Ondetti et al.<sup>6</sup> An aliquot of the crude material (50 mg) was applied to a column of silica gel (Baker, 18 × 0.6 cm) poured from a slurry in EtOAc-pyridine-AcOH-H<sub>2</sub>O (60:20:6:11) and the same solvent mixture was used for elution. Fractions of 2 mL were collected. Fractions 4-9 contained the desired sulfated material: 23 mg; mp 172-176 °C dec; TLC  $R_f$  0.29 (double development);  $[\alpha]_D^{25}$  -24.3° (c 2, 0.1 M NH<sub>4</sub>OH). The UV spectrum showed the presence of 1 mol of pyridine. Anal. ( $C_{44}H_{61}N_9O_{15}S_3 \cdot C_5H_5N \cdot 3H_2O$ ) C, H, N.

A sample of III (10 mg) was deblocked with TFA containing 5% anisole (0.1 mL) to yield the trifluoroacetate IV: 9 mg. Hydrolysis of this sample with aminopeptidase M (Rohm)<sup>23</sup> gave Asp 0.92, Gly 0.86, Met 1.99, Phe 1.00. Ser-O-sulfate was eluted before Asp; their peaks had about the same area. TLC gave  $R_f$  0.13 (double development).

**N-tert-Butyloxycarbonyl-L-tyrosyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (VIII).** A. The trifluoroacetate salt VIIa (3.4 g) was dissolved in DMF (33 mL), and DIEA (1.28 mL) was added, followed by *tert*-butyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester<sup>24</sup> (1.76 g). The solution was stirred at room temperature overnight. The solvents were removed in vacuo; the residue was triturated with a mixture of EtOAc (15 mL) and ether (75 mL) for 10 min and filtered. It was resuspended in EtOAc (20 mL), stirred, filtered, and dried: 3.8 g (95%); mp 178-179 °C; TLC  $R_f$  0.42;  $[\alpha]_D^{25}$  -26° (c 2, DMF). A small amount of a faster moving material ( $R_f$  0.78), presumably the succinimide derivative,<sup>15</sup> could be detected. Amino acid analysis: Asp 1.06, Gly 0.97, Met 2.11, Tyr 0.99, Phe 1.00. Anal. ( $C_{50}H_{65}N_9O_{12}S_2$ ) C, H, N.

B. A sample of compound VIIa (4.1 g) was converted to its amphoteric form as described before. This material (3.5 g) was suspended in DMF (21 mL); *tert*-butyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (1.98 g) was added and stirred for 48 h, when the ninhydrin reaction became negative. The solvent was removed in vacuo and the residue triturated and stirred with ether (150 mL) for 30 min. It was then filtered, resuspended in EtOAc (20 mL), stirred for 15 min, filtered, and dried: 4.3 g (90%); mp 181-182.5 °C; TLC  $R_f$  0.42;  $[\alpha]_D^{25}$  -26.6° (c 2, DMF). This product contained only a trace of the succinimide derivative.<sup>15</sup>

**L-Tyrosyl-(O-sulfate)-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (I).** The protected heptapeptide amide VIII (400 mg) was sulfated, as described for III, to yield 360 mg. A sample (100 mg) of the sulfated crude product was dissolved in the solvent system EtOAc-pyridine-HOAc-H<sub>2</sub>O (60:20:6:11, 1 mL) and applied to a column of silica gel (Baker, 36 × 0.6 cm). Fractions of 3 mL were collected. They were examined by TLC and also by plotting the weight of fractions. Fractions 3-8 contained the unsulfated starting material and other minor faster moving impurities (9 mg), fractions 9-16 contained the desired Boc-heptapeptide amide sulfate IX (53 mg), and fractions 17-30 contained materials with lower  $R_f$  values (30 mg). The solid obtained from fractions 9-16 showed a strong absorption in IR at 1040 cm<sup>-1</sup>, characteristic for the phenol-sulfate esters (cf. ref 6): TLC  $R_f$  0.23.

A second sample (100 mg) of the crude sulfated Boc-heptapeptide was deblocked with TFA containing 5% anisole (1.5 mL) and the product chromatographed on a silica gel column (10 g), in the system used for the protected peptide. Fractions of 3 mL were collected, and the elution was monitored by TLC. Unsulfated heptapeptide amide (VIIIa, 30 mg) was collected from fractions 12-17, and the sulfated heptapeptide amide X (50 mg) was secured from fractions 22-28: mp (183) 189-206 °C dec; TLC  $R_f$  0.13. The UV spectrum showed the presence of 1 mol of pyridine. Anal. ( $C_{45}H_{57}N_9O_{13}S_3 \cdot C_5H_5N \cdot CF_3COOH \cdot 3H_2O$ ) C, H, N; F: calcd, 4.47; found, 3.94.

**Method for <sup>45</sup>Ca Outflux.** Outflux of <sup>45</sup>Ca from pancreatic acinar cells was determined as described previously.<sup>10</sup> Dispersed

acinar cells from guinea pig pancreas were prepared as described previously<sup>10</sup> and were suspended in Krebs-Ringer bicarbonate (pH 7.4; equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) containing 1% (wt/vol) bovine serum albumin and 0.5 mM <sup>45</sup>Ca. Cells were preincubated for 60 min at 37 °C and gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Cellular <sup>45</sup>Ca was determined at the end of the preincubation and again after a 5-min incubation with 5 mM ethylenediaminetetraacetate (EDTA). EDTA chelates extracellular <sup>45</sup>Ca and abolishes calcium influx; therefore, the loss of cellular radioactivity during the 5-min incubation is a measure of calcium outflux.

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