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THE SYNTHESIS OF AN ACTIVE DERIVATIVE OF CYCLOMALTOHEPTAOSE FOR THE HYDROLYSIS OF ESTERS AND THE FORMATION OF AMIDE BONDS

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ABSTRACT

The synthesis is described of a derivative of cyclomaltoheptaose (β-cyclodextrin) to which the tripeptide Ser–His–Asp, the catalytic triad found in chymotrypsin, has been coupled. The derivative enhanced the rates of hydrolysis of activated esters, as measured by the release of *p*-nitrophenol, and the formation of amine bonds.

INTRODUCTION

Much attention has been given to the construction of organic models that can mimic the hydrolytic action of chymotrypsin[1–3]. The active site of chymotrypsin involves mainly three amino acid residues[4], namely, serine-195, histidine-57, and aspartic acid-102, which co-operate to increase the nucleophilicity of the hydroxyl group of serine-195 and faciltate its nucleophilic attack on the carbonyl carbon in the peptide or ester-bond to be cleaved[5]. The tripeptide Ser–His–Asp hydrolyses active esters more efficiently than does histidine alone[6,7].

Using recombinant DNA-tehniques, the preparation of a protein consisting of seven "esterase" octapeptides (–Glu–Ala–His–Ala–Ser–Phe–Phe–Phe–) fused to the N-terminal end of galactokinase has been described, and the hydrolysis of different activated esters studied[7].

The ability[8] to bind different compounds in the hydrophobic cavity makes cyclomalto-oligosaccharides (cyclodextrins, CDs) suitable for providing the substrate-binding domain in model compounds that can mimic the action of enzymes. In order to increase the nucleophilicity of the hydroxyl groups in cyclomaltohexaose (αCD) or cyclomaltoheptaose (βCD), histamine units have been coupled to the primary[9] and secondary[10–12] hydroxyl groups. *o*-(4-[5]-Mercaptomethyl-4[5]-methylimidazol-2-yl)benzoate[13] has been linked to a secondary hydroxyl group in βCD.

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Model compounds where the catalytic action is effected solely by a moiety linked to CDs have been prepared by coupling *N*-(*N*,*N*-dimethylaminoethyl)acetohydroxamic acid and *N*-(4-imidazolylmethyl)acetohydroxamic acid to αCD[14] and hydroxamate to βCD[15].

We now report the synthesis of a model for chymotrypsin, consisting of Ser–His–Asp- linked to βCD (cyclomaltoheptaose), and its use in the hydrolysis of esters and formation of amide bonds.

RESULTS AND DISCUSSION

Treatment[8,16] of βCD with tosyl chloride in pyridine at room temperature gave 6-*O*-tosyl-βCD (**1**). The presence of ditosyl[17] and polytosyl derivatives in the mixture of products was revealed by t.l.c. and h.p.l.c., and **1** was isolated by preparative t.l.c. Reaction of **1** with 2-aminoethanethiol gave 6-(2-aminoethylthio)-βCD (**2**). The tripeptide Ser–His–Asp was synthesized from the N-terminus by the classical procedure with protection of the α-amino group by the *tert*-butyloxycarbonyl (Boc) group, using OBu^t for protection of the side chains. The α-carboxyl groups were temporarily protected as methyl esters. The following steps were involved Boc-Ser(OBu^t)–OH ⇒ Boc-Ser(OBu^t)–His–OMe (**3**) ⇒ Boc-Ser(OBu^t)–

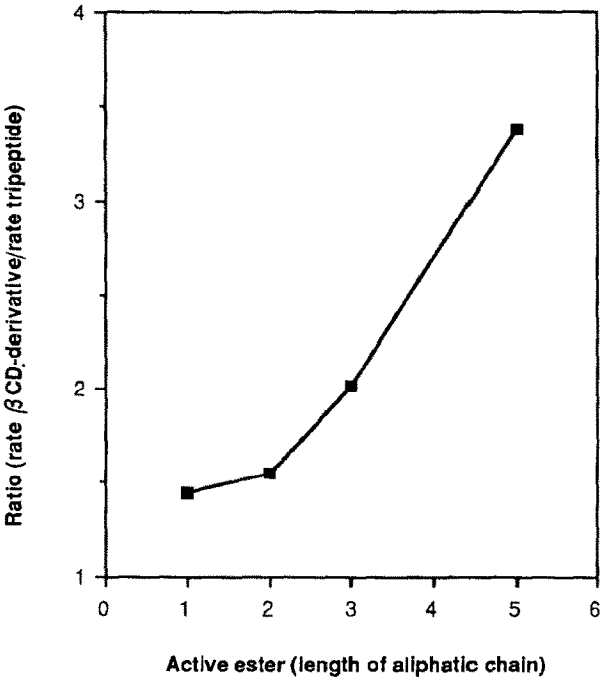

Fig. 1. Hydrolysis of active esters ($C_nH_{2n+1}COONP$) [*p*-nitrophenyl acetate (n = 1), *p*-nitrophenyl propionate (n = 2), *p*-nitrophenyl butyrate (n = 3), and *p*-nitrophenyl hexanoate (n = 5)] in aqueous 5% acetonitrile determined spectrophotometrically. The ratios are mean values (±6%) of at least three runs.

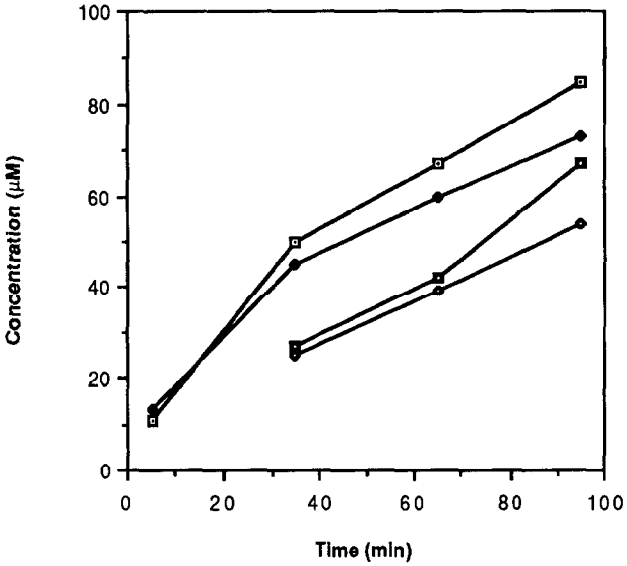

Fig. 2. The formation of *p*-nitrophenol and *N*-benzylhexanamide from *p*-nitrophenyl hexanoate and benzylamine: formation of *p*-nitrophenol in the presence of **8** (—□—) and Ser–His–Asp (—◆—), and formation of the amide in the presence of **8** (—■—) and Ser–His–Asp (—◆—).

His–OH (**4**) ⇒ Boc-Ser(OBu^t)–His–Asp(OBu^t)–OMe (**5**) ⇒ Boc-Ser(OBu^t)–His–Asp(OBu^t)–OH (**6**) (see Experimental). The tripeptide derivative **6** was then coupled to **2** to give **7**. Deprotection of **7** with trifluoroacetic acid then gave the final product 6-(Ser–His–Asp–$NHCH_2CH_2S$)-βCD (**8**).

The formation of *p*-nitrophenol, determined spectrophotometrically, during the hydrolysis of various activated esters with Ser–His–Asp or the βCD derivative (**8**) in aqueous 5% acetonitrile is shown in Fig. 1. As can be seen, **8** was the more efficient reagent, the effect being more pronounced with longer aliphatic chains since these promote binding to the βCD unit[18]. The largest difference (3.4-fold) was observed in the hydrolysis of *p*-nitrophenyl hexanoate. Hydrolyses with Ser–His–Asp plus βCD or βCD alone gave rates of the same order as those obtained using Ser–His–Asp or buffer. Thus, the increase in the rate of hydrolysis observed with **8** is not due to effects on the absorbtion spectrum of *p*-nitrophenol bound to βCD[8].

The enhancement of the rate of hydrolysis of *p*-nitrophenyl hexanoate by **8** in aqueous 50% *N,N*-dimethylformamide over a longer period of time was confirmed by h.p.l.c. (Fig. 2). In the presence of a competitive inhibitor, cyclohexanebutyric acid[10], the initial rate of hydrolysis of *p*-nitrophenyl hexanoate with Ser–His–Asp was unaffected, whereas that with **8** was only 63% of the value obtained in the absence of inhibitor.

The formation of *p*-nitrophenol and *N*-benzylhexanamide from *p*-nitrophenyl hexanoate and benzylamine in aqueous 50% *N,N*-dimethylformamide during 90 min (Fig. 2) in the presence of Ser–His–Asp or **8** was faster with the latter compound.

In deacylations catalyzed by **8**, the question of the formation of the acyl-tripeptide–βCD complex remains to be investigated.

Compound **8** is an improved enzyme mimic when compared to the systems that involve aliphatic acids co-immobilized with histamine on Sephadex beads, which were used as hydrophobic affinity ligands for substrates[19], or where CD preparations carry only a single histamine unit[9–12]. Our studies were initiated to focus attention on the potential of compounds such as **8** for elucidating enzyme-like mechanisms and for applications in synthesis rather than just for the hydrolysis of activated esters[20].

EXPERIMENTAL

General methods. — Amino acid derivatives were purchased from Bachem AG and Ser–His–Asp from Serva. βCD was kindly provided by Stadex AB. Satisfactory elemental analyses could not be obtained for the hygroscopic compounds **1, 2, 7**, and **8**. Their homogeneity was established by chromatography, and the molecular weights by f.a.b.-m.s. (VG-ZABSE instrument, thioglycerol matrix).

T.l.c. was performed on silica gel plates with fluorescent indicator (Merck), and column chromatography on Kiselgel 60 (Merck), using *A* (3:3:2:5 conc. ammonia–EtOAc–water–1-propanol), *B* (45:4:1 chloroform–MeOH–HOAc), *C* (20:5:1 chloroform–MeOH–HOAc), and *D* (6:4:1 chloroform–MeOH–HOAc).

H.p.l.c. was performed on an LKB 2150-52 system with a Nucleosil-C_{18} (5 μm) column connected to a Hitachi Chromatopac C-R3A integrator and elution with *E* (1:9 acetonitrile–50mM ammonium acetate, pH 5.0) and acetonitrile at 0.75 mL/min. Elution was monitored at 260 nm, Spectrophotometric analyses were performed on a Hitachi U-3200 spectrophotometer. N.m.r. spectra were recorded with a Varian XL-300 instrument. Amino acid analyses were performed after hydrolysis (6M HCl, 110°, 24 h) at the Department for Amino Acid Analyses, Biomedical Centre, Uppsala, Sweden. Optical rotation data and elemental analyses for the βCD derivatives are corrected for water content (determined at Mikrokemi Co., Uppsala).

Boc-Ser(OBu^t)–His–OMe (**3**). — Boc-Ser(OBu^t)–OH·dicyclohexylamine (900 mg, 2.03 mmol), dicyclohexylcarbodi-imide (461 mg, 2.23 mmol), and 1-hydroxybenzotriazole (342 mg, 2.23 mmol) were dissolved in *N,N*-dimethylformamide (20 mL). After 20 min, His–OMe·2 HCl (541 mg, 2.23 mmol) and triethylamine (623 μL, 4.47 mmol) were added. After 2 days, the mixture was filtered, EtOAc (10 vol.) was added, and the product was extracted into acidified water. The extract was neutralized with sodium hydrogencarbonate and extracted with EtOAc, and the extract was concentrated. Column chromatography (solvent *B*) of the residue gave **3** (567 mg, 68%), R_F 0.15 (solvent *B*). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.18 (s, 9 H, OBu^t), 1.45 (s, 9 H, Boc), 3.17–3.19 (m, 2 H, His β-CH_2), 3.45–3.50 (q, 1 H, Ser β-H), 3.72 (s, 3 H, OMe), 3.75–3.79 (q, 1 H, Ser β-H), 4.16–4.23 (m, 1 H, Ser α-H), 4.76–4.83 (m, 1 H, His α-H), 6.82 [s, 1 H, imidazole H-4(5)], 7.61 (s, 1 H, imidazole H-2).

Boc-Ser(OBu^t)–His–OH·HOAc (**4**). — To a solution of **3** (567 mg, 1.38 mmol) in 1,4-dioxane (10 mL) was added M NaOH (1.4 mL, 1.4 mmol) during 20 min. After an additional 45 min, the solution was concentrated *in vacuo*. Column chromatography (solvent *C*) gave **4** (600 mg, 95%), R_F 0.15 (solvent *C*), $[\alpha]_D$ +28.5° (*c* 0.8, methanol).

Boc-Ser(OBu^t)–His–Asp(OBu^t)–OMe (**5**). — A solution of **4** (500 mg, 1.09 mmol), dicyclohexylcarbodi-imide (494 mg, 2.4 mmol), 1-hydroxybenzotriazole (324 mg, 2.4 mmol), and triethylamine (0.46 mL, 3.6 mmol) in *N,N*-dimethylformamide (10 mL) was kept for 20 min and Asp(OBu^t)–OMe·HCl (528 mg, 2.4 mmol) was added. After 3 days, the mixture was filtered, diluted with EtOAc, washed with water, and concentrated *in vacuo*. Column chromatography (EtOAc and EtOAc/MeOH) of the oily residue gave **5** (440 mg, 69%), R_F 0.60 (solvent *C*), $[\alpha]_D$ −7° (*c* 0.85, methanol). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.17 (s, 9 H, SerO*Bu^t*), 1.43 (s, 18 H, Boc and AspO*Bu^t*), 2.74–2.81 (m, 2 H, Asp β-CH_2), 3.15–3.17 (m, 2 H, His β-CH_2), 3.45–3.48 (m, 1 H, Ser β-H), 3.72–3.78 (m, 1 H, Ser β-H), 3.74 (s, 3 H, OMe), 4.10–4.18 (m, 1 H, Ser α-H), 4.73–4.79 (m, 2 H, His α-H, Asp α-H), 5.43–5.45 (m, 1 H, Boc-N*H*), 6.92 [s, 1 H, imidazole H-4(5)], 7.73 (s, 1 H, imidazole H-2). Relative amino acid composition: Ser, 1.00; His, 1.00; and Asp, 1.00.

Anal. Calc. for $C_{27}H_{45}N_5O_9$ (583): C, 55.6; H, 7.72; N, 12.0. Found: C, 55.3; H, 7.87; N, 11.8.

Boc-Ser(OBu^t)–His–Asp(OBu^t)–OH (**6**). — This compound, made in a manner analogous to that for **4**, had R_F 0.71 (solvent *D*).

6-O-Tosylcyclomaltoheptaose (**1**). — A solution of dry βCD (9.08 g, 8.0 mmol) and tosyl chloride[21] (10.64 g, 55.7 mmol) in dry pyridine (140 mL) was stored overnight at room temperature, then poured into ice–water (600 mL). The insoluble material was collected and the filtrate was concentrated *in vacuo*. The residual oil was diluted with aqueous 95% ethanol (300 mL), and the precipitated mixture (13 g) of βCD and tosyl derivatives was collected and subjected to preparative t.l.c. (solvent *A*). Compound **1**, R_F 0.28, was extracted with *N,N*-dimethylformamide. The extract was concentrated *in vacuo* and **1** was precipitated with ether (350 mg, 3.4%); $[\alpha]_D$ +112.8° (*c* 0.4, *N,N*-dimethylformamide). ^{1}H-N.m.r. data (CD_3OD): δ 2.54 (s, 3 H, Ts-*Me*), 3.47–3.56 (m, 14 H, H-2,4), 3.71–3.94 (m, 28 H, H-3,5,6,6), 4.96–5.00 (m, 7 H, H-1), 7.44–7.47 (d, 2 H, *o*-H of Ts), 7.78–7.81 (d, 2 H, *m*-H of Ts). F.a.b.-mass spectrum: *m/z* 1289 ($[M + 1]^+$).

6-(2-Aminoethylthio)cyclomaltoheptaose (**2**). — Compound **1** (350 mg, 0.27 mmol), 2-aminoethanethiol hydrochloride (280 mg, 2.46 mmol), and ammonium hydrogencarbonate (700 mg) were dissolved in 1:3 *N,N*-dimethylformamide–water under nitrogen. The solution was kept at 60° for 4 h, then concentrated *in vacuo*. Elution of the residual oil in 4 batches from a column (2.5 × 70 cm) of Sephadex G-10 with 3:7 MeOH–water gave **2** (139 mg, 43%), R_F 0.14 (solvent *A*), $[\alpha]_D$ +137.8° (*c* 0.8, water). ^{1}H-N.m.r. data (D_2O): δ 2.83–2.98 (m, 4 H, CH_2S, H-6,6), 3.10–3.13 (m, 2 H, CH_2N), 3.49–3.67 (m, 14 H, H-2,4), 3.80–3.97 (m, 26 H, H-

3,5,6,6), 5.03–5.05 (m, 6 H, H-1), 5.09–5.10 (m, 1 H, H-1). F.a.b.-mass spectrum: *m/z* 1194 ([M + 1]$^+$).

6-[Boc-Ser(OBu^t)–His–Asp(OBu^t)NHCH$_2$CH$_2$S]cyclomaltoheptaose (**7**). — A solution of **6** (96 μmol), dicyclohexylcarbodi-imide (20 mg, 96 μmol), 1-hydroxybenzotriazole (13 mg, 96 μmol), and **2** in *N,N*-dimethylformamide (2.0 mL) was stored at room temperature overnight, then filtered, and concentrated *in vacuo*. The resulting oil was purified on a Sephadex column (as above) to give **7** (67 mg, 76%), R_F 0.67 (solvent *A*), which did not react with ninhydrin; $[\alpha]_D$ +59.7° (*c* 0.8, water). Relative amino acid composition: Ser, 1.00; His, 0.98; and Asp, 0.95. F.a.b.-mass spectrum: *m/z* 1746 ([M + 2]$^+$).

6-(Ser–His–Asp–NHCH$_2$CH$_2$S)cyclomaltoheptaose (**8**). — Compound **7** (10 mg, 5.7 μmol) was treated with 9:1 trifluoroacetic acid–dichloromethane at room temperature for 2 h. The solution was concentrated *in vacuo* followed by lyophilization to give **8** (9.5 mg, 96%), R_F 0.17 (solvent *A*), $[\alpha]_D$ +72.3° (*c* 0.6, water). ^{1}H-N.m.r. data (D_2O): δ 2.66–2.97 (m, 6 H, Asp-β-CH_2, CH_2S, H-6,6), 3.02–3.43 (m, 6 H, CH_2N, Ser β-CH_2, His β-CH_2), 3.41–3.72 (m, 14 H, H-2,4), 3.66–4.08 (m, 26 H, H-3,5,6,6), 4.06–4.13 (m, 2 H, Ser α-CH_2), 4.61–4.67 (m, 1 H), 4.93–5.06 (m, 5 H, H-1), 5.05–5.10 (m, 2 H, H-1,1'), 7.29 [s, 1 H, imidazole H-4(5)], 8.60 (s, 1 H, imidazole H-2). Relative amino acid composition: Ser, 1.00; His, 0.98; and Asp, 0.96. F.a.b.-mass spectrum: *m/z* 1533 ([M + 1]$^+$).

Hydrolyses of activated esters. — The hydrolysis of $C_nH_{2n+1}COONP$ (n = 1, 2, 3, and 5) with Ser–His–Asp or **8** was followed spectrophotometrically at 405 nm. Reaction solutions consisted of 5:95 acetonitrile–0.1M sodium phosphate buffer (pH 8.0) containing 0.1mM activated ester and 0.2mM Ser–His–Asp or **8**. Ser–His–Asp and **8** were omitted from the blanks. The inhibitor, cyclohexanebutyric acid, was added in phosphate buffer as appropriate to a final concentration of 3.0mM.

Synthesis of N*-benzylhexanamide.* — The reaction mixture comprised 1:1 *N,N*-dimethylformamide–0.1M sodium phosphate buffer (pH 8.0) containing 0.1mM *p*-nitrophenyl hexanoate, 2mM benzylamine, and 0.2mM Ser–His–Asp or **8**. Aliquots were taken after 5, 35, 65, and 95 min, and the synthesis of *N*-benzylhexanamide and *p*-nitrophenol and the consumption of *p*-nitrophenyl hexanoate were determined by h.p.l.c. A gradient mobile phase of (*E*) and acetonitrile [from start to 2 min, 60% (*E*); 2–15 min, 60–20% (*E*); 15–20 min, 20% (*E*); and 20–25 min 20–60% (*E*)] was applied. The capacity factors were *p*-nitrophenol, 2.85; benzylamine, 3.47; *N*-benzylhexanamide, 4.59; and *p*-nitrophenyl hexanoate, 7.55. The spontaneous reaction in the phosphate buffer was high, but somewhat lower than that for the reaction in the presence of Ser–His–Asp.

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