OLIGOSTATINS, NEW ANTIBIOTICS WITH AMYLASE INHIBITORY ACTIVITY

II. STRUCTURES OF OLIGOSTATINS C, D AND E

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The structures of oligostatins C, D and E were studied spectrometrically and chemically. Oligostatins C, D and E are pseudo-penta, -hexa and -heptasaccharides with a nitrogen containing glycosidic bond. These antibiotics represent a new series of amylase inhibitors of the saturated basic oligosaccharide.

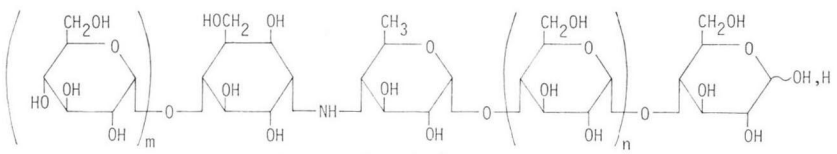
Oligostatins C, D and E produced by *Streptomyces myxogenes* nov. sp. SF-1130 are new antibiotics with activity against Gram-negative bacteria and amylase inhibitory activity. Production, isolation and characterization were reported in the previous paper[1]. In the present paper, structural elucidation of oligostatins C, D and E is described.

Structure of Oligostatin C

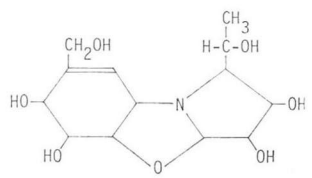
Oligostatin C (**Ic**), a basic, water-soluble, amorphous substance; m.p. 183°C (dec.), $[\alpha]_D^{23}+154°$ (*c* 1, H_2O), shows strong IR absorption maxima at 3100~3600 and 980~1180 cm^{-1} indicative of its oligosaccharide nature. PMR spectrum of **Ic** in D_2O shows a methyl signal at 1.38 ppm (d, J=6 Hz), a methine proton at 2.76 ppm (t, J=9 Hz) and complex signals at 5.2~5.5 ppm assignable to anomeric protons (J=4 Hz). Neither olefinic proton in PMR, nor sp^2 carbon in CMR is observed.

Acid hydrolysis of **Ic** gave 3 moles of D-glucose and a basic product found identical with tricyclic

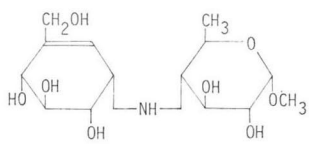
Fig. 1. Structures of oligostatins.



Oligostatin C (Ic) m = 0 n = 2
Oligostatin D (Id) m = 0 n = 3
Oligostatin E (Ie) m = 1 n = 3



II



III

Fig. 2. PMR Spectrum of **III** (100 MHz, D_2O).

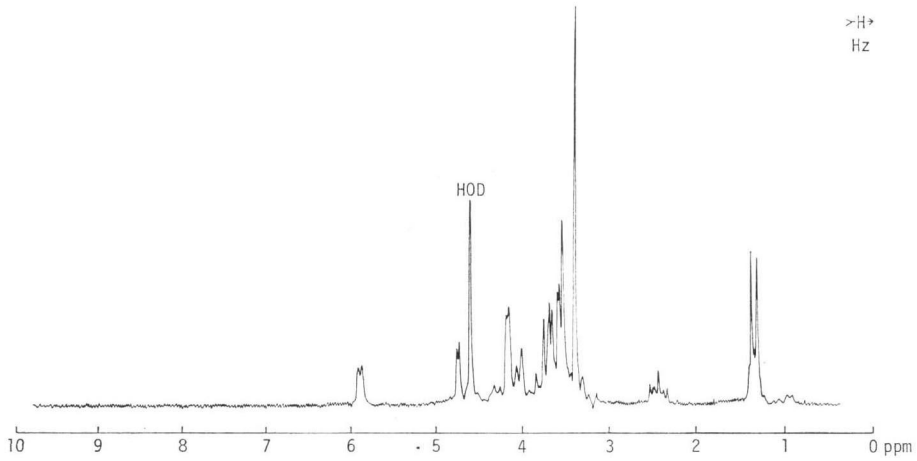


Fig. 3. CMR Spectrum of **III** (25.16 MHz, D_2O).

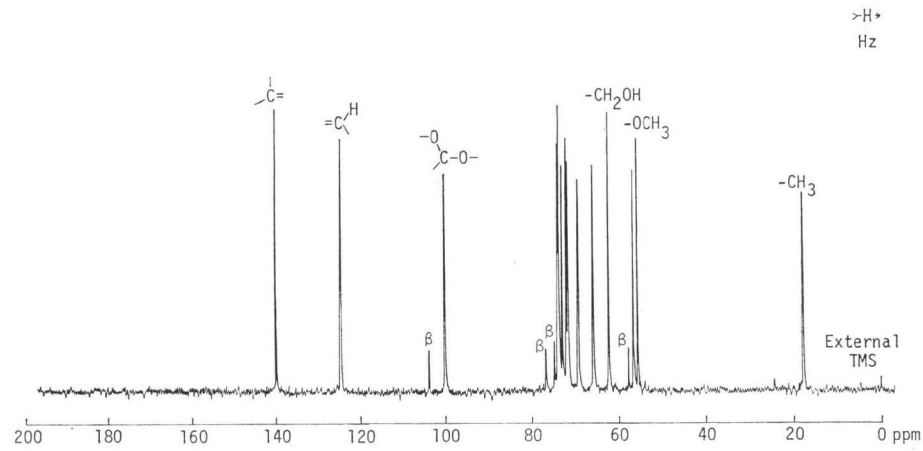
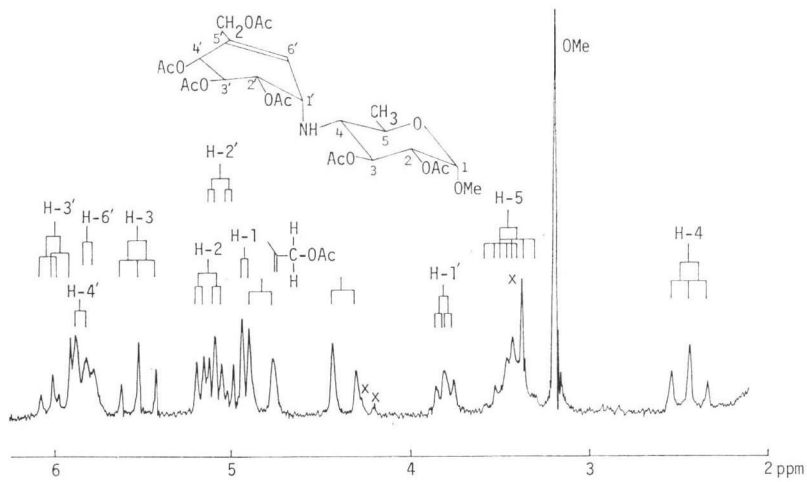


Fig. 4. PMR Spectrum of **III** hexaacetate (100 MHz, C_6D_6+1 drop of MeOH-d_4).



compound (**II**)[2] by direct comparison. Isolation of the tricyclic compound suggested the close similarity of **Ic** to BAYe 4609. On the other hand, methanolysis of **Ic** gave a new crystalline methyl glycosidic product, mainly in a form of α-glycoside. This was designated methyl dehydro-oligobiosaminide (**III**) ($C_{14}H_{25}NO_8$) which was a key degradation product for the structure determination. PMR and CMR spectra of **III** are shown in Figs. 2 and 3. The structure of the hexaacetate of **III** was determined by PMR double resonance experiment as shown in Fig. 4. A triplet at 2.34 ppm and a double doublet at 3.74 ppm could be assigned to methine protons bearing nitrogen atoms from their chemical shifts and by the fact that they were sharpened by addition of methanol-d_4. Starting from these two signal readily assignable, double resonance experiments were successfully carried out to obtain the 4-amino-4,6-dideoxy-hexose and amino-cyclohexene structures. Since **III** possessed only one nitrogen atom, these two ring systems should share that nitrogen to build a pseudonitrogen disaccharide as shown in Fig. 1.

Of particular importance was that **II** and **III** did possess a double bond while **Ic** originally did not. This suggested that **Ic** underwent β-elimination during methanolysis or acid hydrolysis to give an unsaturated product. Since **Ic** did not possess an ester group nor a halogen atom, it was most rational that an hydroxyl group, preferably axially-oriented, was originally attached to C-6′ of cyclitol moiety and that β-elimination took place under acidic condition. Therefore, in oligostatin C, the cyclitol moiety should be present as a saturated one.

Oligostatin C gave one mole of sorbitol and two moles of glucose (GC-analysis) when oligostatin C was reduced by $NaBH_4$ and then hydrolyzed. Furthermore, GC-MS analysis on the product obtained by permethylation, hydrolysis, reduction with $NaBH_4$ and acetylation of oligostatin C revealed 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol but no 4-*O*-methylated derivative[3]. This indicated that no glucose was attached to non-reducing side of the saturated cyclitol moiety. CMR spectrum of **Ic** suggested that all glucosidic bonds were α, $1\rightarrow4$ because no signal was observed assignable to glucosidic linkages other than α, $1\rightarrow4$[4]. Glycosidic bond between oligobiosamine and glucose was considered also to be α, because in CMR spectrum of **Ic**, no signal assignable to β was observed at around 103 ppm (β anomer of **III** showed a signal of C-1 at 103.0 ppm at pH 7.5). From these results, the structure of oligostatin C could be determined as **Ic** in Fig. 1.

Structures of Oligostatins D and E

Physico-chemical properties of oligostatins D (**Id**) and E (**Ie**) were very close to those of oligostatin C, but D and E contained 1 and 2 moles more of glucose respectively. By comparative study, oligostatin D turned out to be a homolog of oligostatin C possessing 4 moles of glucose all in the reducing side of oligobiosamine as shown in Fig. 1.

In case of oligostatin E, GC-MS analysis of hydrolysate of the permethylated derivative revealed that at least one mole of glucose was present in the non-reducing side of oligobiosamine. Although it was difficult to unequivocally determine how many glucoses were attached to oligobiosamine, MS analysis of peracetate (strong peak at m/z 331 and very weak peak at m/z 619) favored that one mole of glucose was present as non-reducing end unit. Resistance of oligostatin E to β-amylase also was consistent with the above suggestion.

These results lead to the structure **Ie** for oligostatin E as shown in Fig. 1.

Discussion

From the first report of BAYe 4609 by SCHMIDT *et al.*[2], a family of amylase inhibitors of basic

oligosaccharide nature has been growing steadily. As analogous inhibitors, S-AI[5], BAYe 4609[2] and trestatin[8] have been known. It was reported that S-AI had inhibitory activity on microbial, plant and mammalian α-amylases, BAYe 4609 and trestatin exhibited also specific inhibitory activity against microbial and mammalian α-amylases. On the other hand, oligostatin had unique inhibitory spectrum with strong inhibitory activity against mammalian α-amylase and glucoamylase, but with weak activity against bacterial liquefying amylase and α-amylase of *Aspergillus oryzae*. From the preliminary study on structure-activity relationship of oligostatins and related compounds, the following results were suggested: a) amylase inhibitory activity was greatly dependent on the number of glucose residue, b) the presence of glucose in non-reducing side of the cyclitol moiety weakened the inhibitory activity against glucoamylase.

Oligostatin, the first saturated member of this family, occupys a unique position in the inhibitors of basic oligosaccharide nature, and will add important information not only for structure-activity relationship but also for biosynthesis of this type of inhibitors.

Experimental

PMR spectra at 100 MHz and CMR spectra at 25.16 MHz were obtained on a Varian XL-100-12 spectrometer. ^{13}C Chemical shifts are expressed in ppm from the ^{13}C resonance of TMS. Gas chromatography was carried out on a Hewlett-Packard Model 402 gas chromatograph equipped with hydrogen flame ionization detectors was used. The column was a glass tube (ϕ3 mm$\times$120 cm) packed with 1% OV-17 on Chromosorb W. Helium was used as a carrier gas with a flow rate of 37 ml/minute. The column temperature was 150°C. GC-MS analysis was carried out on a Hitachi M-80 mass spectrometer, using a column (ϕ3 mm$\times$100 cm) packed with 2% OV-1 on Chromosorb W. Helium was used as a carrier gas with a flow rate of 40 ml/minute. The column temperature was 120~200°C (10°C/minute).

Hydrolysis of Oligostatin C and Isolation of **II**

Oligostatin C (4.0 g) was dissolved in 50 ml of 6% sulfuric acid solution and refluxed for 4 hours. The solution was neutralized with 2 N KOH and applied on a column containing 50 ml of activated carbon. The column washed with water and then eluted with 30% ethanol solution. The solution was concentrated to give a crude product of **II** (320 mg). The crude product was chromatographed on Avicel (16 cm$\times$60 cm) using a mixture of ethyl acetate - pyridine - water (10: 4: 3) as developing solvent to give a white powder of **II** (100 mg): $[\alpha]_D^{20}$ +10.7° (*c* 0.5, H_2O), PMR (100 MHz, D_2O, ppm from external TMS); 1.29 (d, *J*=6.0 Hz, 3H, CH–CH_3), 2.92 (bd, *J*=6.0 Hz, 1H, N–CH), 3.80 (broad, 1H, N–CH), 3.90, 4.50 (8H, O–CH, O–CH_2), 4.88 (d, *J*=4.0 Hz, 1H, O–CH–N), 5.72 (d, *J*=2.0 Hz, 1H, C=CH), CMR (D_2O, ppm from external TMS); 20.8, 62.9, 63.1, 68.3 (×2), 70.5, 71.3, 76.7, 77.0, 77.6, 95.2, 122.7, 139.1.

Methanolysis of Oligostatin C and Isolation of **III**

Oligostatin C (4.0 g) was refluxed in MeOH (200 ml) with Amberlyst 15 (Rohm & Haas Co.) (30 g) for 16 hours. Amberlyst resin was collected by filtration, then eluted with 0.5 N NH_4OH (100 ml) to give a crude product (250 mg) of **III**. The crude product was chromatographed on Avicel (15 mm$\times$300 mm) using a mixture of ethyl acetate - pyridine - water (10: 4: 3) as developing solvent to give **III** (90 mg). This was crystallized from acetone to afford colorless needles of **III** (80 mg): m.p. 225°C (dec.), $[\alpha]_D^{20}$ +88.7° (*c* 1, H_2O), PMR (100 MHz, D_2O, ppm from external TMS); 1.32 (d, *J*=5.9 Hz, 3H, 5-CH_3), 2.43 (m, 1H, H-1), 3.40 (s, 3H, OCH_3), 4.74 (d, *J*=2.2 Hz, 1H, H-1), 5.89 (bd, *J*=3.6 Hz, 1H, H-6′), CMR (D_2O, ppm from external TMS); 17.5, 55.0, 56.0, 61.6, 65.2, 68.5, 70.8, 71.2, 72.2, 73.0, 73.2, 99.2, 123.6, 138.9.

Hexaacetate was prepared by treating **III** with acetic anhydride in pyridine at room temperature: syrup. EI-MS *m/z* 587 (M^+), $[\alpha]_D^{20}$ +97° (*c* 1, EtOH), PMR (100 MHz, C_6D_6+1 drop of CD_3OD, ppm from TMS); 1.22 (d, *J*=5.9 Hz, 3H, 5-CH_3), 1.66, 1.83 (×3), 1.95, 1.99 (s, 3H×6, CH_3CO–), 2.34 (t, *J*=1.0 Hz, 1H, H-4), 3.12 (s, 3H, CH_3O–), 3.32 (m, 1H, H-5), 3.74 (dd, *J*=5.1 Hz, 4.5 Hz, 1H, H-1′), 4.34 and 4.75 (AB 2H, *J*=13.3 Hz, –CH_2–OAc), 4.86 (d, *J*=4.1 Hz, 1H, H-1), 5.02 (dd, *J*=10.0 Hz, 4.5 Hz, 1H, H-2′), 5.08 (dd, *J*=10.5 Hz, 4.1 Hz, 1H, H-2), 5.48 (t, *J*=10.5 Hz, 1H, H-3), 5.74 (d, *J*=5.1 Hz, 1H, H-6′), 5.81 (d, *J*=8.0 Hz, 1H, H-4′), 5.90 (dd, *J*=10.0 Hz, 8.0 Hz, 1H, H-3′).

Glucose-chain Analysis of Oligostatin by GC and GC-MS

a) The solution of oligostatin (1 mg) in 1 ml of water was treated with sodium borohydride (1 mg) at 37°C for 1 hour. The excess sodium borohydride was decomposed by Dowex 50W×2 and the solution was evaporated to dryness. The residue was hydrolyzed with 0.5 N HCl in dry methanol (5 ml) under reflux for 16 hours. After concentration, to the residue in pyridine (0.2 ml) was added hexamethyldisilazane (20 μl) and trimethylchlorosilane (10 μl). The mixture was vigorously shaken for 5 minutes, and subjected to gas chromatography under previous condition.

b) Oligostatin (5 mg) was methylated with methylsulfinyl carbanion by HAKOMORI's method[7]. The permethyled oligostatin was hydrolyzed with 8% sulfuric acid at 100°C for 4 hours. The solution was neutralized with saturated barium hydroxide solution and the resulting precipitate was removed by filtration. The clear solution was concentrated and treated with 5 mg of sodium borohydride at 37°C for 1 hour. The excess reagent was decomposed by Dowex 50W×2 and the solution was evaporated to dryness. A mixture of the residue, acetic anhydride (0.5 ml) and pyridine (0.5 ml) was heated at 80°C for 1 hour. The reaction mixture was evaporated to dryness and then dissolved in chloroform in order to analyze by GC-MS. GC-MS analysis was carried under previous condition.

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