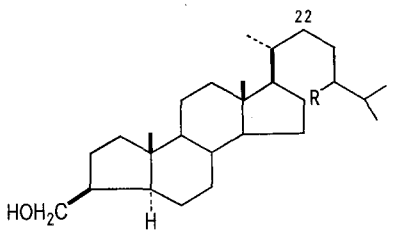
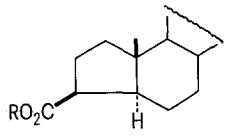
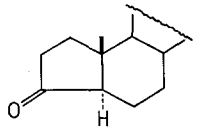


1, R=H
2, R=H, Δ^{22}
3, R=Me
4, R=Me, Δ^{22}
5, R=Et
6, R=Et, Δ^{22}



7, R=H
8, R=Me



9

A-nor-5α-cholestane nucleus, and suggest that in the sponge these unique A-nor-stanols (**1–6**) arise mainly by modification (ring-A contraction) of dietary sterols.

To obtain some information on the nature of this ring contraction, portions of the labelled stanols deriving from 290 h incubation with [4-^{14}C]-cholesterol (ca. 10 mg) were added to a mixture of carrier A-nor-stanols (ca. 300 mg), hydrogenated on palladium-charcoal and converted to the nor-ketones (part structure **9**). The conversion was accomplished (see reference [7]) by oxidation with dicyclohexylcarbodiimide/dimethylsulphoxide of the free stanols to the corresponding aldehydes, which were then treated with isopropenyl acetate and sulphuric acid, and the resulting enolacetates oxidized with ozone to yield the nor-ketones. The stanol mixture had a specific radioactivity of 4.22×10^4 dpm/mg and all of this was recovered in the nor-ketone mixture (specific activity 4.31×10^4 dpm/mg), showing that in the ring-contraction carbon-4 of the cholesterol nucleus is not lost, nor does it furnish the 3β-hydroxymethyl carbon of the A-nor-cholestane skeleton.

Riassunto. La spugna *Axinella verrucosa* trasforma il [4-^{14}C]-colesterolo nel 3β-idrossimetil-A-nor-5α-colestano (**1**), mentre utilizza 1′[1-^{14}C]-acetato per la sintesi dei 3β-idrossimetil-A-nor-sterani (**1–6**) in misura trascurabile. Si suggerisce che questi unici stanoli si originino principalmente per modificazione di steroli dietarici. Il carbonio-4 del nucleo del colesterolo non è nè perso nè dà origine al carbonio 3β-idrossimetilico dello scheletro A-nor-colestanico.

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Synthesis and Activity of Nonapeptide Fragments of Soybean Bowman-Birk Inhibitor

Bowman-Birk inhibitor (BBI)[1] has been known as a double-headed proteinase inhibitor which inhibits trypsin and chymotrypsin at two non-overlapping reactive sites[2]. The primary structure of BBI has been determined by ODANI and IKENAKA[3], Lys-Ser (16–17) and Leu-Ser (43–44) bonds having been estimated as antitryptic and antichymotryptic sites respectively[3,4]. The same authors have further divided the inhibitor molecule, consisting of 71 amino acid residues, into 2 fragments: one consists of 38 residues and the other 29 residues, each of which retains the inhibitory activity on trypsin or chymotrypsin, respectively[5].

In order to find out a smaller active fragment, if any, and to investigate structure-activity relationship of natural proteinase inhibitor, we attempted to synthesize heterodetic cyclic peptides with a disulfide bond. The present communication reports the syntheses and inhibitory properties of cyclic nonapeptides, X-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-Y (Ia: X=Ac, Y=NH_2. Ib: X=H, Y=OH), which correspond to the nonapeptide sequence of -Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys- (14–22) of BBI containing the antitryptic site.

Material and method[6]. The protected nonapeptide resin, Boc-Cys(4-OMe-Bzl)-Thr(Bzl)-Lys(2,4-Cl_2Z)-Ser(Bzl)-Asn-Pro-Pro-Gln-Cys(4-OMe-Bzl)-resin (II) was synthesized by Merrifield's solidphase method in a stepwise fashion starting with 2.19 g of Boc-Cys(4-OMe-Bzl)-resin containing 1 mmole of S-4-OMe-Bzl-cysteine. The Boc-amino acids with protected side chains were: Lys(2,4-Cl_2Z)[7], Ser(Bzl), Thr(Bzl) and Cys(4-OMe-Bzl). The coupling reactions to form peptide bonds were mediated by DCC in CH_2Cl_2 for 4 h, except in the case of Boc-Thr (Bzl) and Boc-Cys(4-OMe-Bzl), which were allowed to react for 12 h. Introduction of Boc-Gln and Boc-Asn was carried out over 12 h with the corresponding *p*-nitrophenyl esters in addition to hydroxybenzotriazole[8]. Boc groups were removed with 1 *N* HCl-AcOH, exceptionally with 50% trifluoroacetic acid in CH_2Cl_2 for Boc-Gln and Boc-Asn residues. The weight of finally obtained II was 3.24 g. The weight gain of 1.05 g (0.77 mmol), at this stage, indicated a 77% incorporation of protected peptide based on the initial Boc-Cys (4-OMe-Bzl) content in the resin.

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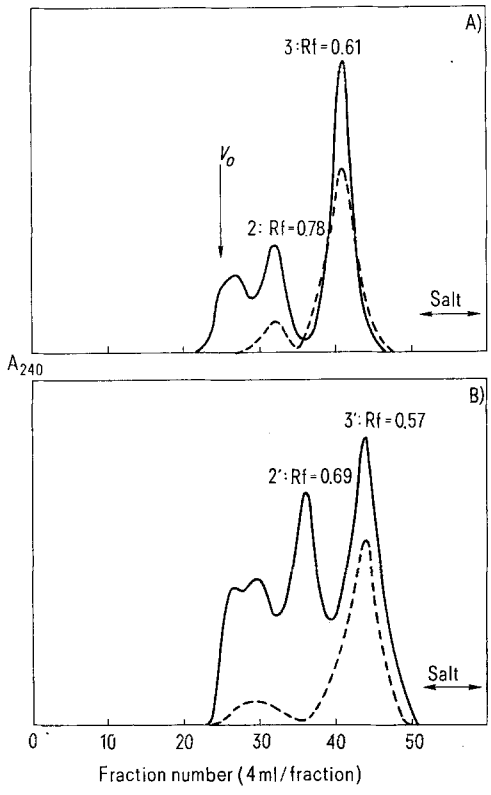

Fig. 1. Gel filtration on Sephadex G-25. A) Crude cyclized acetyl nonapeptide amide (—) and reduced one (- - -). B) Crude cyclized nonapeptide (—) and reduced one (- - -).

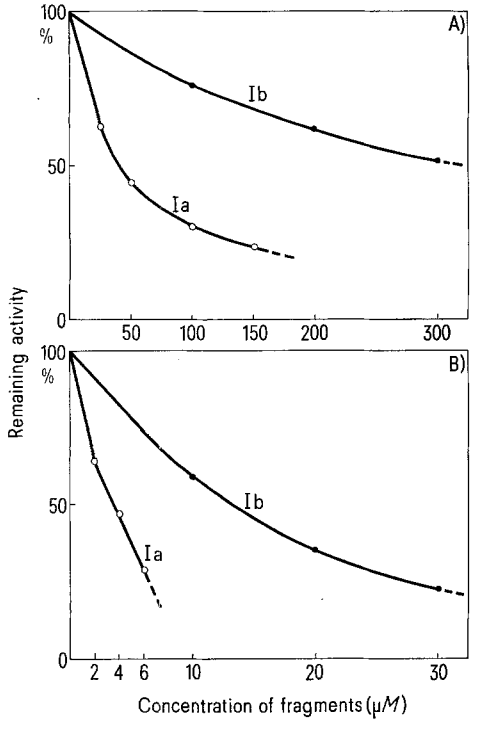

Fig. 2. Inhibition by synthetic fragments for tryptic activities. A) for esterase activity: 0.05 *M* *Tris*-HCl containing 0.01 *M* $CaCl_2$ at pH 8.0 and 25 °C; Tos-Arg-OMe, 1 m*M*; enzyme, 0.153 μg/ml. B) for peptidase activity: the same buffer at pH 8.5 and 30 °C; Gly_2-Lys-Gly_3, 3 m*M*; enzyme, 4.76 μg/ml.

The peptide resin II (1.0 g) was treated with NH_3/MeOH-DMF (2:1) for 48 h and crude protected nonappetide amide, Boc-Cys(4-OMe-Bzl)-Thr(Bzl)-Lys(2,4-Cl_2Z)-Ser(Bzl)-Asn-Pro-Pro-Gln-Cys(4-OMe-Bzl)-NH_2 (III) obtained, was purified twice by Sephadex LH-20 column (4 × 170 cm) using MeOH as a solvent; yield of pure III, 250 mg (61% based on II and 47% based on the amino acid-resin); mp 118–120 °C; $[\alpha]_D^{20}$ −42.7° (c 0.5, DMF); Rf (TLC) (solvent, $CHCl_3$-MeOH (4:1)), 0.60. Anal. Calcd. for $C_{81}H_{105}O_{19}N_{13}S_2Cl_2 \cdot 2H_2O$: C, 56.04; H, 6.10; N, 10.49%. Found: C, 56.04; H, 6.13; N, 10.64%.

After removal of the Boc group of III (174 mg) with 1 *N* HCl-AcOH (4 ml), the nonapeptide amide hydrochloride obtained was acetylated by acetic anhydride in pyridine. Recrystallization from DMF-water gave pure protected acetyl nonapeptide amide, Ac-Cys(4-OMe-Bzl)-Thr(Bzl)-Lys(2,4-Cl_2Z)-Ser(Bzl)-Asn-Pro-Pro-Gln-Cys(4-OMe-Bzl)-NH_2 (IV): yield, 152 mg (90%); mp 182–184 °C; $[\alpha]_D^{20}$ −45.7° (c 0.5, DMF); Rf (TLC) 0.49. Anal. Calcd. for $C_{78}H_{99}O_{18}N_{13}S_2Cl_2 \cdot 2H_2O$: C, 55.84; H, 6.19; N, 10.85%. Found: C, 55.58; H, 6.11; N, 10.82%.

The deblocking of IV (130 mg) was achieved by treatment with HF in the presence of anisole[9], and the peptide obtained was oxidized with potassium ferricyanide[10]. The crude product thus obtained was purified by gel filtration on Sephadex G-25 (2 × 80 cm), the eluate with 1 *M* AcOH being collected in each 4 ml fraction. A plot of the UV-absorbance values at 240 nm of the various fractions showed the presence of 3 peaks supposedly corresponding to polymer, dimer and required cyclic acetyl nonapeptide amide Ia as shown in Figure 1A. Rf (Gel) means the ratio of void volume (V_0) to the elution volume. The peak 3 was lyophilized to afford 45 mg (48%) of white fluffy powder, which showed a single spot (R 0.44 × Lys) by ninhydrin and NaCN-nitroprusside reagents[11] on a paper electrophoregram, the solvent used being HCOOH-AcOH-MeOH-water (1:3:6:10) at pH 1.8, and a single peak in CM cellulose column (0.9 × 50 cm) chromatography with 0.2 *M* pyridinium acetate buffer at pH 5.0. Amino acid analysis gave: Asp, 1.00; Glu, 1.02; Ser, 0.94; Thr, 0.95; Pro, 2.08; Cys, 1.78; Lys, 1.00; NH_3, 2.82.

The other cyclic nonapeptide Ib was directly prepared from II (500 mg) by treatment with HF and following potassium-ferricyanide oxidation. The crude Ib was purified by gel filtration (Figure 1B), and the peak 3' was lyophilized to afford 28.2 mg of fluffy white powder, the purity being ascertained by paper electrophoresis (R 0.58 × Lys) and CM cellulose chromatography as mentioned for Ia. Amino acid analysis gave: Asp, 1.00; Glu, 1.04; Ser, 0.93; Thr, 0.95; Pro, 2.10; Cys, 1.46; Lys, 1.03; NH_3, 1.87. The yield of Ib was 20% based on the weight gain on the resin.

The inhibition of tryptic hydrolysis of Tos-Arg-OMe by Ia and Ib was assayed following the literature[12]. In case of Gly_2-Lys-Gly_3[13] digestion, an increase of triglycine, one of the digested products, was quantitatively estimated by the use of an amino acid analyzer[14].

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The kinetic parameters of the fragments in inhibition of tryptic activities[a]

	I_{50}[b] (μM)		K_i[a] (μM)	
	Tos-Arg-OMe	Gly_2-Lys-Gly_3	Tos-Arg-OMe	Gly_2-Lys-Gly_3
Ia	40	3.6	15	1.5
Ib	330	13	99	3.8

[a]The kinetic measurement was carried out under the conditions described in Figure 2: the substrate concentration was selected in the range of 0.33–1.0 mM for esterase activity, and 1.0–3.0 mM for peptidase activity. [b]The substrate concentration was 1 mM and 3 mM for Tos-Arg-OMe and Gly_2-Lys-Gly_3, respectively.

Results and discussion. The gel filtration of crude Ia on Sephadex G-25 gave 3 peaks. Materials obtained from peak 2 and 3 were reduced separately with β-mercaptoethanol at pH 8.6 and applied to the column giving a main peak at the same position to peak 3; Rf (Gel) 0.61 (Figure 1A), positive by nitroprusside. The similar results were obtained for the crude cyclic nonapeptide Ib (Figure 1B). On paper electrophoregrams the peak 2 moved to cathode, R 0.49 × Lys, and peak 3, R 0.44 × Lys. Reduced peak 2 or 3 showed the same mobility as peak 3 itself. Similar results were obtained for the materials related to Ib. These observations suggest that the peak 3 and 3′ are monomers and peak 2 and 2′ are presumed to be dimers.

Figure 2 shows that both of Ia and Ib inhibit esterase and peptidase activities of trypsin. However, these small fragments did not form stable 1:1 complex with trypsin. The value of I_{50}[15], the concentration of an inhibitor in which tryptic activity was suppressed to 50%, was given in the Table for each fragment.

The results of kinetic measurements indicate that the mode of inhibition was competitive. The K_i values of fragment Ia and Ib were determined by Dixon's plot and shown in the Table. The difference of K_i values of fragments between esterase and peptidase activity seems to be attributable to that of the K_m value of each substrate. After Ia (10 mg) was treated with trypsin at pH 3.75 as described in the literature[16], the digested fragment (Ia*) which had been hydrolized at Lys-Ser (16–17) bond was isolated by gel filtration as described above (Rf (Gel) 0.69) and ascertained by paper electrophoresis (R 0.63 × Lys). Ia* did not exhibit any inhibitory activity. The fragment Ia did not inhibit chymotryptic activity when Gly_2-Tyr-Gly_3[17] was used as a substrate. Another nonapeptide (41–49) containing antichymotryptic site and its analogs are under synthesis in this laboratory.

Zusammenfassung. Zwei Nonapeptidfragmente des Bowman-Birk Inhibitors wurden mit Hilfe der Merrifield-Synthese hergestellt und deren Trypsinhemmende Aktivität bestimmt.

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18 November 1974.

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The Association of Enzymic Activities in Subfractions of Isolated Rat Liver Golgi

The Golgi apparatus has been implicated in the biosynthesis of glycoproteins and lipoproteins and in sulphate metabolism[1–3]. It is tempting to speculate that the different enzymic activities are localized to discrete areas of this complex organelle. In our experiments it has been possible to show some separation of activities after mild homogenization and centrifugation in sucrose gradients.

Materials and methods. The Golgi fraction was isolated from male Wistar rats (200–220 g), fasted 16–18 h, by the procedure described previously[4]. Golgi fractions from 2–3 livers were collected in a volume of not more than 2 ml and were homogenized at 80 rpm with 3 up and down passes with a tygon pestle having a clearance of 0.006 inches. This homogenate was placed on a sucrose gradient of densities 1.20, 1.18, 1.16 and 1.14, containing 5 mM $MgCl_2$, 3 mM mercaptoethanol and 37.5 mM *Tris*-maleate buffer, pH 6.4. The gradients were centrifuged at 30,000 rpm for 3 h in SW-50.1 Beckman rotor. 3 bands were isolated and kept frozen at −18 °C until enzymes were assayed. About 25–30% of the original Golgi protein was recovered with a distribution of 66 in the first, 23 in the second, and 11% in the third band. The enzymes assayed have been chosen for their reported presence or suggested involvement in the activities in the Golgi-GERL[5] system[1–3], as well as non-Golgi enzymes to monitor purity of the preparations that were assayed.

Enzyme assays. The following enzymes were assayed. Acid phosphatase (EC: 3.1.3.2)[6]; alkaline phosphatase (3.1.3.1)[7]; arylsulfatase-A (3.1.6.1) and arylsulfatase-B (3.1.6.1)[8,9]; thiamine pyrophosphatase[10]; galactosyl-

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