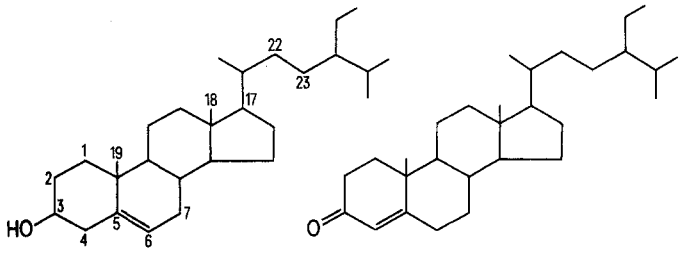
Metabolite 1 (yield 12%), m.p. 122–124 °C, $[\alpha]_D^{25}+57°$ (c, 1.01) was identified as 4,22-stigmastadien-3-one (**VI**).
Metabolite 2 (yield 2.2%), $C_{29}H_{46}O_2$ (M^+ 426), m.p. 212–214 °C, $[\alpha]_D^{25}+14°$ (c, 0.72) was characterized as 4,22-stigmastadien-3-one-6β-ol (**VII**).
Metabolite 3 (yield 1.8%), $C_{29}H_{48}O_2$ (M^+ 428), m.p. 205–208 °C, $[\alpha]_D^{25}-62.5°$ (c, 0.25), ν_{max}^{nujol} 3400, 3300, 1050, 1020 and 870 cm^{-1}, PMR ($CDCl_3$) δ 3.85 (1 H, m, 7-*H*), 3.52 (1 H, m, 3-*H*), 5.53 (1 H, m, 5-*H*) and 5.11 (2 H, m, 22-*H* and 23-*H*) formed a dibenzoate, m.p. 165–166 °C, $[\alpha]_D^{25}-90°$ (c, 0.18) and an amorphous diacetate indicating it to be a diol. In analogy with **IV**, obtained from β-sitosterol this metabolite 3 was expected to be 5,22-stigmastadien-3β, 7α-diol (**VIII**) which is a hitherto unreported compound. This was in fact found to be so. Stigmasteryl acetate on oxidation with CrO_3-pyridine in methylene chloride[10] afforded 3β-acetoxy-5,22-stigmastadien-7-one, m.p. 181–183 °C, $[\alpha]_D^{25}-100.5°$ (c, 0.48). This keto-acetate on LAH reduction yielded 2 products. The minor product, which was assigned the α-configuration in analogy with the cholesterol series[11] was found to be identical with metabolite 3. The major product, m.p. 215–217 °C, $[\alpha]_D^{25}+32.5°$ (c, 0.35), dibenzoate m.p. 177–178 °C, $[\alpha]_D^{25}+103°$ (c, 0.38) was assigned the β-configuration. The molecular rotation differences ($M_D^{\beta}-M_D^{\alpha}$) of 407 for the diols and of 1226 for the dibenzoates are comparable with the corresponding values, viz. 378 and 1215, observed in the cholesterol series[12].



I **V** Δ^{22} **II** **VI** Δ^{22}
IV 7α–OH **VIII** Δ^{22}, 7α–OH **III** 6β–OH **VII** Δ^{22}, 6β–OH

1 The soil pseudomonad strain (BS-305) is maintained at 4 °C on nutrient-agar slants.
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The C-terminal sequence of human and porcine antithrombin III and its homology with human α-1-proteinase inhibitor

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Summary. The C-terminal amino acid sequences of human and of porcine antithrombin III have been determined as Gly-Arg-Val-Ala-Asn-Pro-Cys-Val-Lys and Gly-Arg-Val-Ala-Asn-Pro-Cys, respectively. These sequences are highly homologous with the C-terminal sequence of human α-1-proteinase inhibitor.

Antithrombin III (AT-III) is a plasma glycoprotein which plays an important role in the regulation of blood coagulation[1]. A deficiency of this protein leads to recurrent thromboses[2]. The inhibition of thrombin by AT-III is reported to involve a carboxylic ester formation between enzyme and inhibitor, preceded by a peptide bond cleavage at the reactive site of the inhibitor[3]. However, its exact location has not been elucidated yet. We present here data showing human and porcine AT-III contain an identical carboxy-terminal sequence which is very similar to that of human α-1-proteinase inhibitor (α-1-PI), which suggests a location for the reactive sites of the inhibitors.
Human and porcine AT-III were prepared by affinity chromatography on heparin-agarose as described elsewhere[4]. S-pyridylethyl (PE) human AT-III was digested with cyanogen bromide and the resultant peptides were fractionated by gel filtration on Sephadex G-50 (figure 1). 9 UV-absorbing peaks were obtained. Peak VII (shown by the arrow) was a mixture of 2 small peptides. It was gel-filtered on a Bio-Gel P-6 column (1.2 × 137 cm), equilibrated with 0.1 M formic acid. 2 completely separated peaks were obtained, the former of which (C-VII-1) consisted of 9 amino acids and contained no homoserine or its lactone, indicating that the peptide was derived from the C-terminal end of the protein (table). Gel filtration of the cyanogen bromide digest of S-pyridylethyl porcine AT-III on the same column of Sephadex G-50 gave 8 peaks with

Amino acid compositions of the carboxy-terminal peptides of human and porcine antithrombin III

	Human AT-III		Porcine AT-III	
	C-VII-1	Sequence	C-VII	Sequence
Asp	0.99	1	1.04	1
Pro	1.03	1	1.01	1
Gly	1.00	1	1.02	1
Ala	0.97	1	1.00	1
Val	1.96	2	0.94	1
Lys	0.92	1	0	
PECys	0.55	1	0.67	1
Arg	0.86	1	0.98	1
Total		9		7
Yield		56% (0.40 µmole)		69% (0.63 µmole)

a similar elution profile. Peak VII contained a pure peptide (C-VII) consisting of 7 amino acids devoid of homoserine or its lactone (table).

Amino acid sequences of purified C-terminal cyanogen bromide fragments were determined by manual Edman degradation. 7 steps of the Edman degradation of human AT-III-C-VII-1 peptide gave the sequence Gly-Arg-Val-Ala-Asn-Pro-PECys with repetitive yields of 80%. Digestion of S-pyridylethyl human AT-III (60 nmoles) with acid carboxypeptidase *[Penicillium janthinullum]* (Takara Shuzo Co., Shiga, Japan) yielded lysine (0.35, 0.53 and 0.69 moles/mole after 5, 15 and 30 min, respectively) and valine (0.09, 0.16 and 0.20 moles/mole), giving the C-terminal sequence of human AT-III as Val-Lys. From the results of the Edman degradation and acid carboxypeptidase digestion, and the amino acid composition of the peptide, the whole sequence of the peptide was determined as Gly-Arg-Val-Ala-Asn-Pro-PECys-Val-Lys.

6 steps of the Edman degradation of porcine AT-III-C-VII peptide established the sequence Gly-Arg-Val-Ala-Asn-Pro-PECys with repetitive yields of 83%. C-terminal PECys was assigned subtractively from the amino acid composition of the peptide, and confirmed by the amino acid analysis of the remaining sample after the 6th step of the Edman degradation.

Digestion of porcine AT-III with acid carboxypeptidase released no amino acid from the C-terminus of the protein, which is quite reasonable from the C-terminal sequence, -Pro-Cys, elucidated by the Edman degradation. The results of sequence analysis are shown in figure 2, with the C-terminal sequence of human α-1-PI. The N-terminal methionine was assigned to each peptide because these 3 peptides were obtained from the cyanogen bromide digest of each protein. It is evident from these data that the C-terminal sequence of human and porcine AT-III is identical, although porcine AT-III lacks 2 of the C-terminal amino acids, Val and Lys, of human AT-III. Furthermore, these C-terminal sequences are found to be homologous with that of human α-1-PI which has recently been reported by Morii et al.[5] and Travis and Johnson[6]. Both AT-III and α-1-PI have an ability to inhibit the activity of various serine proteases and have some inhibition specificity in common. Therefore, it is possible to speculate that both plasma inhibitors may have homologous amino acid sequences around the reactive site as many protease inhibitors from animal organs and plant seeds have[7]. The reactive site of α-1-PI is reported to be located very close to one end of the molecule[8,9], and the amino acid sequences at the N-terminal ends of human AT-III and α-1-PI have been reported[4,5]. However, no sequence similarity was found between the N-terminal ends of the 2 inhibitors. The sequence homology shown in figure 2, therefore, is apparently unique for the C-terminal ends of the 2 proteins and gives further support for the location of the reactive sites of both inhibitors in this region.

This conclusion is supported by the fact that a peptide (3000–4000 dalton) was recently isolated from the α-1-PI/chymotrypsin complex, and a similar one also from the α-1-PI/elastase complex. Partial characterization indicated clearly that this peptide was derived from the C-terminal end of α-1-PI[9,10].

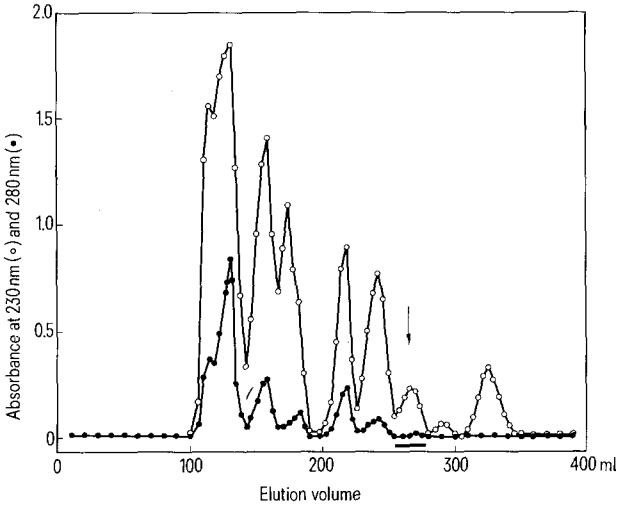

Fig. 1. Gel filtration pattern of the cyanogen bromide digest of S-pyridylethyl human antithrombin III. Human AT-III (60 mg) was pyridylethylated by the method of Friedman et al.[11] and the protein was separated from salt and excess reagent by gel filtration on a column of Sephadex G-50 (1.5 × 46 cm) equilibrated with 0.1 M formic acid. Cyanogen bromide cleavage was carried out by dissolving 53 mg of the salt-free S-pyridylethyl AT-III and 120 mg of cyanogen bromide in 3 ml 70% formic acid, and the reaction was allowed to proceed for 24 h at 4 °C. After lyophilization, the digest was fractionated by gel filtration on a column of Sephadex G-50 (1.5 × 200 cm), equilibrated with 0.1 M formic acid. The flow rate was 6 ml/h and fractions were collected in 2.1 ml/tube. The solid bar shows the fractions of peak VII that were combined and lyophilised.

Human AT-III	(Met)-Gly-	Arg-	Val-	Ala-	Asn-Pro-	Cys-Val-	Lys
Porcine AT-III	(Met)-Gly-	Arg-	Val-	Ala-	Asn-Pro-	Cys	
Human α-1-PI	(Met)-Gly-	Lys-	Val-	Val-	Asn-Pro-	Thr-Gln-	Lys

Fig. 2. The C-terminal sequence of human and porcine antithrombin III and human α-1-proteinase inhibitor. The N-terminal sequence analysis was performed by a manual Edman degradation method[12,13], using 0.2 and 0.3 µmoles samples of human and porcine AT-III C-terminal peptide, respectively. Phenylthiohydantoin amino acids were identified by TLC[14] and by amino acid analysis after back hydrolysis to free amino acids at 150 °C for 6 h in 6N HCl containing 0.3% $SnCl_2$[15]. Amino acid residues that are identical among the 3 proteins are shown in blocks and those conservatives are enclosed in broken squares.

Note added in proof: While this paper was in press, R. Carrell et al. (Biochem. biophys. Res. Commun. *91*, 1032 (1979)) reported the carboxy-terminal sequence of human α-1-antitrypsin and its homology with antithrombin III, and H. Jörnvall et al. (FEBS Lett. *106*, 358 (1979)) showed the thrombin cleavage site in bovine antithrombin was located near the C-terminal end of the molecule. These facts perfectly coincide with our results and discussion in this paper.

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