homogenized and extracted. Measurement by RIA was carried out after isolation of the 5α-androst-16-en-3α-ol fraction on silica-gel (see table). The remaining extract was also purified by TLC after addition of ^{3}H-5α-androst-16-en-3α-ol as an internal standard. TMS derivatives were prepared and analyzed by GC-mass spectrometry as described elsewhere[13] (LKB 2091 instrument 1.5 m GC glass column with 1% OV 3 on chromosorb WHP) modified by a temperature program of 150–200 °C (3 °C/min). The identity with authentic 5α-androst-16-en-3α-ol was confirmed by gas-chromatographic retention time and mass spectrometric behaviour[3]. These data establish the presence of 5α-androst-16-en-3α-ol in truffles. The organoleptic examination of thin layer chromatograms of truffleextracts indicates that the fungus contains at least 1 additional musk compound of similar polarity, the flavour of which has a more herbal quality. Attempts for identification are in progress. It is remarkable that the concentration of 5α-androst-16-en-3α-ol in truffles surpasses its level in boar plasma 2-fold[14]. The biological role of this boar sex pheromone might explain the efficient interest of pigs in search of this delicacy.

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Enhancing effect of heparin on aprotinin activity

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Summary. Preincubation of heparin with aprotinin enhanced the inhibitory effect of aprotinin on the esterolytic activity of trypsin, but did not change its effect on the proteolytic activity of trypsin or on the esterolytic and proteolytic activities of chymotrypsin.

Aprotinin is used in the therapy of various pathological states such as acute pancreatitis, owing to its inhibitory effects on trypsin, chymotrypsin, plasmin and kallikrein[1]. It is widely distributed in ruminant tissues, especially bovine tissues[1], and in previous work[2,3] we confirmed that it is present in mast cells of bovine tissues. Heparin is a main component of mast cell granules[4] and the existence of a proteinase such as a trypsin-like or chymotrypsin-like enzyme in the cells has also been reported[5–8]. This paper describes the effect of heparin on the inhibition of trypsin and chymotrypsin by aprotinin.

Materials and methods. A commercial sample of Trasylol [10,000 kallikrein inhibitor units (KIU) per ml; Bayer AG, FRG], was used as aprotinin, and the following reagents were used: tosyl-L-arginine methyl ester hydrochloride (TAME), and benzoyl-L-tyrosine ethyl ester (BTEE) (Protein Research Foundation, Japan); casein according to Hammarsten (Wako Pure Chemicals, Japan); trypsin (EC 3.4.21.4) and α-chymotrypsin (EC 3.4.21.1) – both 3 times crystallized – (Miles Laboratories, USA); diphenylcarbamyl chloride (DPCC)-treated trypsin – type XI – and tosyl-L-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin – type VII – (Sigma USA); Novoheparin 130 IU/mg (Novo Industry, Denmark).

Aprotinin was preincubated with various amounts of heparin at 37 °C for 6, 24 and 48 h, and then the mixture was incubated with enzyme and its substrate. The inhibitory effects of aprotinin on the esterolytic activities of trypsin and α-chymotrypsin were determined by the method of Simlot and Feeney[9]. In this case, the preincubation mixture was incubated with trypsin (5 μg/ml) or α-chymotrypsin (15 μg/ml) at 37 °C for 5 min and then the substrate (TAME and BTEE, respectively) was added. For determination of the inhibitory effects of aprotinin on the caseinolytic activities of trypsin and α-chymotrypsin, the preincubation mixture of heparin and aprotinin was incubated with DPCC-treated trypsin (5 μg/ml) or TLCK-treated α-chymotrypsin (5 μg/ml) and casein (0.3%, w/w), prepared by the method of Kunitz[10], at 37 °C for 10 min. The protein was precipitated with trichloroacetic acid (final concentration 3%, w/w), and the mixture was centrifuged. Aliquots of the clear supernatant (2 ml) were mixed with 2.5 ml of 1 N sodium hydroxide, 1 ml of 20% (w/w) sodium carbonate and 0.5 ml of phenol reagent. The mixture was allowed to stand at room temperature for 10 min, and then the amount of amino groups liberated from casein was determined by measuring the absorption at 650 nm.

Results and discussion. To exclude the possible effect of mutual contamination of the commercial trypsin and α-chymotrypsin, the synthetic substrates TAME and BTEE were used for determination of the esterolytic activities of trypsin and α-chymotrypsin, respectively, and in measurement of the caseinolytic activities of these enzymes, DPCC-treated trypsin and TLCK-treated α-chymotrypsin, respectively, were used. In the present study, linear inhibition of the esterolytic activity of trypsin was observed with up to 5 KIU/ml of aprotinin and of that of α-chymotrypsin with up to 10 KIU/ml of aprotinin; linear inhibition of the caseinolytic activity of both enzymes was observed with up

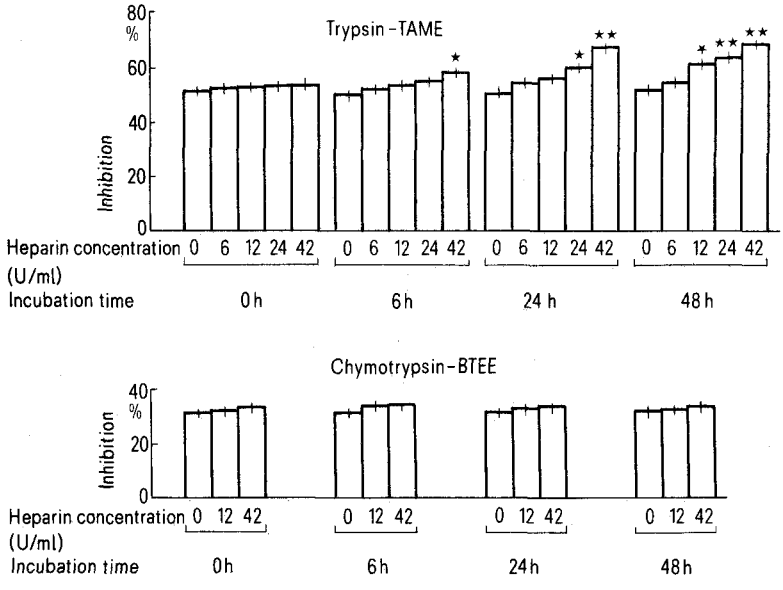

Figure 1. Various concentrations of heparin were incubated with or without aprotinin at 37 °C for the times shown. Then the enzyme and substrate (2 mM TAME or 0.5 mM BTEE) were added and incubation was continued. Enzymatic activity was measured by the method of Simlot and Feeney. The percentage inhibition was determined from the difference in enzymatic activities with and without aprotinin. Columns show mean values ± SE for 4 experiments. Significant differences from the control value (heparin concentration, 0) were determined by Student's t-test: $*p < 0.05$; $**p < 0.01$.

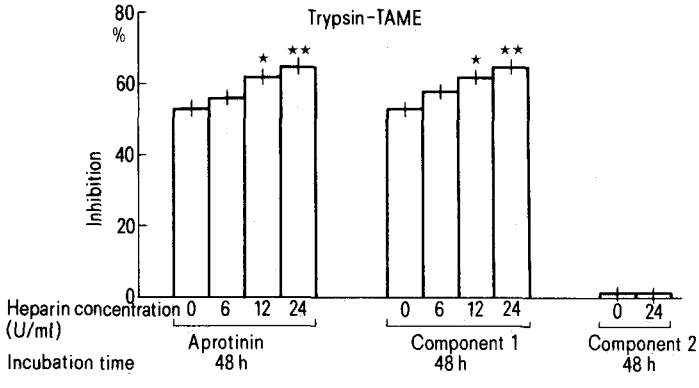

Figure 2. A commercial product of aprotinin used in this study was separated into components 1 and 2 on a Sephadex G-50 column. Various concentrations of heparin were preincubated with or without these components at 37 °C for 48 h and then the solution were incubated with trypsin and TAME (2 mM). Other explanations, as for figure 1.

to 4 KIU/ml of aprotinin. Therefore, when synthetic substrates were used, 3 KIU/ml of aprotinin for trypsin and 5 KIU/ml of aprotinin for α-chymotrypsin were added, and when the caseinolytic activities of these enzymes were examined 2 KIU/ml aprotinin was added. The concentration of heparin added did not per se have any effect on the esterolytic or proteolytic activity of trypsin or α-chymotrypsin. When mixtures of aprotinin and heparin were not preincubated, the inhibitory activity of the aprotinin was not affected, but after preincubation for 6, 24 or 48 h, an enhanced inhibitory effect of aprotinin on the esterolytic activity of trypsin was observed (fig. 1). In contrast, no change in the inhibitory activity of aprotinin on the esterolytic activity of α-chymotrypsin (fig. 1) or the caseinolytic activity of trypsin or α-chymotrypsin was observed after preincubation with heparin for 48 h. The aprotinin used in this study contained at least 2 components[2] with mol.wts of about 6500 (comp. 1) and about 900 (comp. 2). The former component showed the inhibitory activity of aprotinin on trypsin[2]. Based on activity and mol.wt component 1 obviously is aprotinin whereas component 2 is an impurity in the commercial product. The 2 components were separated on Sephadex G-50 as described previously[2]. An amount of component 1 with the same inhibitory activity as the original aprotinin on the esterolysis of TAME with trypsin was used. Component 2 was adjusted to the same volume in saline as component 1. Figure 2 shows that the change in the inhibitory activity of aprotinin on the esterolytic activity of trypsin after preincubation with heparin for 48 h was due to component 1 per se, not to component 2 or a combination of component 1 with component 2. Since the heparin used in the present experiments did not have any inhibitory activity on the esterolytic or caseinolytic activity of trypsin or chymotrypsin, and since aprotinin binds to heparin because of its basicity in vitro[11], it seems likely that some conformational change in the aprotinin molecule occurs during preincubation with heparin, and that this change leads to enhancement of the inhibitory activity of aprotinin on the esterolytic activity of trypsin. The enzymes such as thrombin, plasmin and kallikrein which hydrolyze arginine esters are inhibited by antithrombin, and the enhancement by heparin of the inhibitory effect of antithrombin on these enzymes has been reported[12]. In the present experiments, with longer times of preincubation with aprotinin heparin further enhanced the inhibitory effect of aprotinin on the esterolytic activity of trypsin. As aprotinin is an intracellular inhibitor which is distinct from secretory protease inhibitors of the Kazal type, its function must be endocellular[1]. Thus it seems probable that in bovine mast cells, aprotinin, which is highly basic, forms an ionic complex with heparin and that this complex regulates the enzymatic activity of the protease present in the granules.

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Constituents of *Cannabis sativa* L. XXI: Estrogenic activity of a non-cannabinoid constituent[1]

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Summary. A non-cannabinoid phenol (4,4,dihydroxy-5-methoxybibenzyl) increased uterine weight in prepubescent female rats, suggesting non-cannabinoids contribute to the estrogenic effects of *Cannabis*.

Cannabis preparations have been reported to produce female characteristics in male rodents[4], depress plasma testosterone levels[5] and to produce excessive enlargement of male mammary glands[6]. Whether or not Δ^9-tetrahydrocannabinol produces these effects remains controversial[4,5]. Most research is involved only with Δ^9-tetrahydrocannabinol[7]. However, over 400 other compounds are known to occur in *Cannabis* and some of these resemble known estrogenic agents more closely than does Δ^9-tetrahydrocannabinol. Examples of such compounds are the non-cannabinoid phenolic spiro-indans and dihydrostilbenes recently isolated in our laboratories.

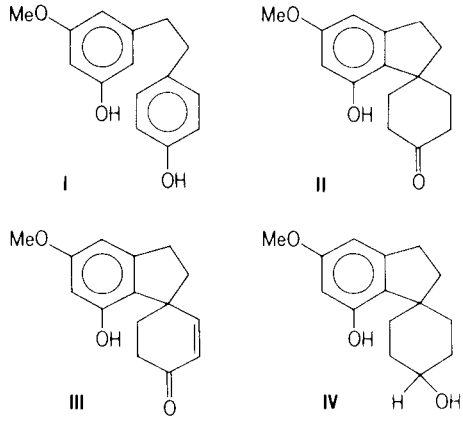
The first of the non-cannabinoid phenols isolated was cannabispiran[8], which previously may have been misidentified as (−)-Δ^8-tetrahydrocannabinol since both compounds have the same relative retention time in GC analysis. Several spiro-indans other than cannabispiran have since been isolated[8]. The spiro-indans are structurally similar to synthetic compounds which potentiate estrogenic effects[9]. Four dihydrostilbenes have subsequently been reported to occur in *Cannabis*. The dihydrostilbenes are structurally similar to 4-{2-(4-hydroxyphenyl)ethyl}-phenol, which has weak estrogenic activity[10].

As part of our continuing investigation of compounds from *Cannabis* we wished to study the estrogenic effects of cannabispiran (**II**), dehydrocannabispiran (**III**) and β-cannabispiranol (**IV**). Preliminary studies of the estrogenic effects of cannabispiran, dehydrocannabispiran and β-cannabispiran were begun, but were hampered because ample amounts of these compounds were not available from plant extracts. Therefore, a program to synthesize cannabispiran and related compounds was begun.

The dihydrostilbene derivative of *Cannabis* (**I**) has been proposed as a biogenic precursor of cannabispiran[8]. It is also an intermediate in the total synthesis of cannabispiran[10] and, as such, has been successfully synthesized in g amounts. The subject of this paper is our evaluation of some aspects of the estrogenic effects of **I** in female rats.

Compound **I** was tested alone and in combination with diethylstilbesterol (DES) by measuring its effects on uterine weight in prepubescent female Sprague-Dawley derived rats. 4 groups of rats were tested in a 2×2 factorial design which was repeated for each of 3 test doses. The 4 groups were dosed as follows: 1. Test dose of **I**; 2. Vehicle control; 3. Test dose of **I** and 0.01 mg/kg of DES; and 4. 0.01 mg/kg of DES. The rats were given i.p. injections in corn oil on 3 consecutive days beginning when they were 23 days old. On the 4th day they were sacrificed, and their uteri were removed, freed from fat, blotted and weighed. Uterine ratios were calculated for each animal as uterine weight in mg per 100 b.wt. Uterine ratios were analyzed using 2×2 analysis of variance to determine the effects of compound **I** alone and its ability to potentiate the effects of DES. Compound **I** was tested at 10, 50, and 250 mg/kg.

Compound **I**, a non-cannabinoid, produced effects similar



Uterine ratios for groups of 8 prepubertal rats. Data expressed as mean ± SEM

Dose of **I**	**I**	Vehicle (control)	I + DES	DES (control)
10 mg/kg[a]	79 ± 16	78 ± 6	289 ± 14	242 ± 39
50 mg/kg[b]	79 ± 16	72 ± 12	331 ± 21	250 ± 18
250 mg/kg[c]	99 ± 16	72 ± 3	295 ± 18	258 ± 19

[a] Significant effect for DES ($p < 0.001$), nonsignificant effect for **I** and interaction. [b] Significant effects for DES ($p < 0.001$) and for **I** ($p < 0.01$), significant interaction ($p < 0.05$). [c] Significant effect for DES ($p < 0.001$) and for **I** ($p < 0.05$), nonsignificant interaction.