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Constituents of Pollen. VIII.[1] Constituents of *Podocarpus macrophylla* D. Don

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The new sterols 24(ξ)-methyl-25(ξ)-cholest-5-ene-3β,26-diol and 24(ξ)-ethyl-25(ξ)-cholest-5-ene-3β,26-diol were isolated, together with *p*-coumaric acid, apigenin and amentoflavone, from pollen grains of *Podocarpus macrophylla* D. Don.

Keywords——*Podocarpus macrophylla* D. Don; Podocarpaceae; pollen grains; *p*-coumaric acid; apigenin; amentoflavone; sterol; 24(ξ)-methyl-25(ξ)-cholest-5-ene-3β, 26-diol; 24(ξ)-ethyl-25(ξ)-cholest-5-ene-3β,26-diol

Podocarpus macrophylla D. Don. (Japanese name, Inumaki) is a dioecious evergreen tree of the Podocarpaceae family, growing in forests in warm mountainous regions. The reported constituents of this tree include the insect metamorphosis hormones, ponasterone-A[3] and makisterone-A,[4a] -B, -C and -D,[4b] biflabones such as kayaflavone[5] and podocarpusflavone-A and -B[6] from the leaves and plant-growth inhibitors, inumaki-lactone-A,[7a] -B and -C[7b] from the wood. However, there has been no report on the constituents of pollen grains, the reproductive cells. Therefore, the pollen grains of *Podocarpus macrophylla* were extracted with ether and fractionated into acid, phenolic, and neutral fractions.

Gas–liquid chromatography (GLC) of the acid fraction showed the presence of formic, acetic, butyric and hexanoic acids. *p*-Coumaric acid (I) was also isolated.

The fraction soluble in sodium hydroxide was found to contain saturated fatty acids with even numbers of carbons, C_{14} to C_{34}, mainly C_{14}, C_{16} and C_{18}. The phenolic compounds were isolated by preparative thin-layer chromatography (P. TLC) and identified as apigenin (IIa) and amentoflavone (IIIa) through their acetates.

The neutral fraction was subjected to column chromatography and identified as saturated hydrocarbons of C_{15} to C_{31}, and even-numbered saturated alcohols of C_{16} to C_{30} by comparison of the GLC patterns with those of authentic samples.

The fraction eluted with benzene–chloroform (1:1) on column chromatography of the neutral portion was repeatedly purified by chromatography and high-performance liquid chromatography (HPLC) to yield colorless plates; IVa of mp 162—164° and Va of mp 148—150°.

Elemental analysis and mass spectrum (MS) of IVa showed its composition to be $C_{28}H_{48}O_2$. Its infrared (IR) spectrum showed absorption due to a hydroxyl group at 3300 cm^{-1} and that of a double bond at 1625 cm^{-1}. The proton magnetic resonance (PMR) spectrum of IVa exhibited signals due to 5 methyl protons at δ 0.70—1.29, a 2H double doublet

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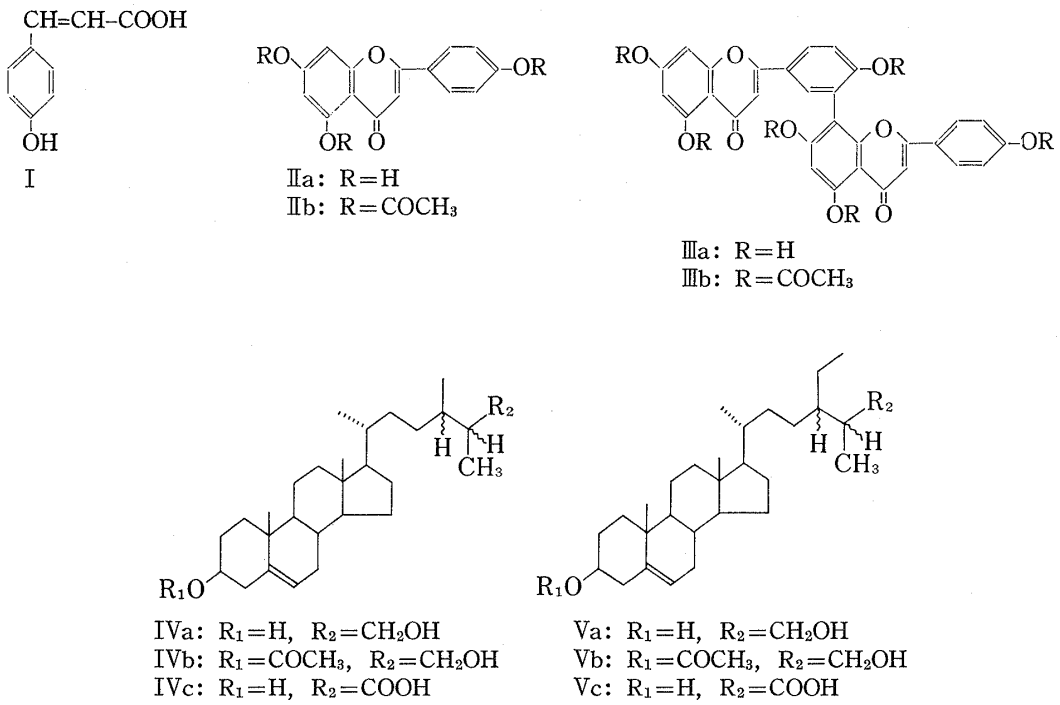

Chart 1

at δ 3.47 due to methylene protons of a primary alcohol, indicating the presence of =CH–CH_2OH group, a methine signal at δ 3.64 due to a secondary alcohol, and a 1H multiplet at δ 5.34 due to a trisubstituted double bond. MS also indicated the formation of a fragment ion of the steroidal skeleton at *m/e* 273 and a fragment ion of the side chain at *m/e* 143. These results indicate that one primary hydroxyl and 3 methyl groups are present in the side chain, and the secondary hydroxyl group and the double bond in the steroidal skeleton. The presence of fragment ions at *m/e* 137 and 119, respectively, suggest the presence of a trisubstituted double bond at C-5 and the presence of a secondary alcohol in the A-ring.

Acetylation of IVa by the conventional method gave an acetate (IVb) as colorless plates, mp 94—96°. The PMR spectrum of IVb exhibited two singlet signals at δ 2.01 and 2.03, indicating the formation of a diacetate. The position of the secondary hydroxyl group was

Table I. ^{13}C Chemical Shifts of Sterols

Carbons	IVa	Va	Carbons	IVa	Va
1	37.90	37.35	16	28.54	28.31
2	32.24	31.72	17	56.51	56.12
3	71.34	71.87	18	12.05	11.90
4	41.75	42.37	19	19.64	19.41
5	140.04	140.91	20	36.21	36.13
6	121.27	121.80	21	18.95	18.74
7	32.24	31.95	22	34.17	34.04
8	32.24	31.95	23	29.93	25.75
9	50.58	50.22	24	43.41	42.37
10	36.94	36.54	25	29.46	29.73
11	21.37	21.13	26	65.33	66.88
12	40.14	39.84	27	29.46	29.73
13	42.56	42.37	28	14.29	23.76
14	56.97	56.84	29	—	12.50
15	24.53	24.33			

considered to be C-3 in view of the formation of the fragment ion of *m/e* 119 and from biogenetic considerations. Its configuration was estimated to be β from the width of the coupling constant of the methine proton, W 1/2=14 Hz, and by comparison of its carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum with those of known compounds.[8] The primary hydroxyl group in the side chain was presumed to have been formed by oxidation of the methyl group at C-21, C-26, C-27, or C-28. Therefore, IVa was oxidized with chromium trioxide in the usual manner to yield a carboxylic acid (IVc). The MS of IVc exhibited a molecular ion peak at *m/e* 430 and a dehydration peak at *m/e* 412, and also fragment ion peaks at *m/e* 73 and 74, as observed in the determination of the position of a hydroxyl group at C-26 in 3-keto-cholest-4-en-26-ol by Schubert *et al.*,[9] so that the position of the hydroxyl group in the side chain was assigned as C-26. Consequently, the plain structure of IVa was identified as 24(ξ)-methyl-25(ξ)-cholest-5-ene-3β,26-diol.

Elemental analysis and the MS of Va indicated its composition to be $C_{29}H_{50}O_2$. Its IR spectrum showed absorptions due to a hydroxyl group at 3250 cm^{-1} and a double bond at 1620 cm^{-1}. Its PMR spectrum showed signals of 5 methyl protons at δ 0.66—1.28. One of them at δ 0.87 was a triplet with a coupling constant of 6.5 Hz, indicating the presence of an ethyl group. There was also a 2H double doublet of methylene protons at δ 3.50 due to a primary alcohol, indicating the presence of =CH–CH$_2$OH, a methine proton due to secondary alcohol at δ 3.65, and a 1H multiplet at δ 5.32 due to a trisubstituted double bond. The MS exhibited a molecular ion peak at *m/e* 430, with fragment ions at *m/e* 273, 255 and 175, indicating that the primary alcohol is present in the side chain, and the secondary alcohol and the double bond in the steroidal skeleton. In addition, formation of fragment ions at *m/e* 137 and 119 indicates that the hydroxyl group is in the A-ring and the double bond is at C-5, as in Va. The position of the secondary hydroxyl group was considered to be C-3 based on the MS and biogenetic considerations. Its configuration was estimated to be β from the width of the coupling constant, W 1/2=14 Hz, of the methine proton at δ 3.65 in the PMR spectrum and by comparison with the ^{13}C-NMR spectra of known compounds.[8]

Acetylation of Va by the conventional method gave the acetate (Vb) as colorless plates, mp 60°. The PMR spectrum of Vb showed singlet signals at δ 2.03 and 2.05, indicating the formation of a diacetate. The position of the primary alcohol in the side chain was determined to be at C-26 by the method of Schubert *et al.*[9] as in the case of IVa. Consequently, the plain structure of Va is 24-(ξ)-ethyl-25(ξ)-cholest-5-ene-3β,26-diol.

The ^{13}C-NMR chemical shift values of C-20, C-21, C-22 and C-23 in the steroidal skeleton and side chain of IVa and Va agree well with the values reported by Koizumi *et al.*[8] and by Wright *et al.*[10] for campesterol and β-sitosterol, respectively. Accordingly, the methyl group of Va was estimated to be at C-24. The configuration is assumed to be *S* at the C-20 methyl group on the basis of its chemical shift value, the same as in campesterol and β-sitosterol. If IVa and Va are considered to be the C-26 oxidation products of campesterol and β-sitosterol, respectively, then the configuration at C-24 should be *S* for IVa and *R* for Va. This requires confirmation. A 26-hydroxysterol has been isolated by Schubert *et al.*[9] in the form of 3-keto-cholest-4-en-26-ol from microorganisms and also synthesized by Varma *et al.*[11] from kryptogenin diacetate, but this is the first example of its isolation from pollen grains, and IVa and Va are new compounds.

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Experimental

Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. IR, PMR, ^{13}C-NMR, MS spectra and optical rotations were taken on Hitachi EPI-G3, JEOL JNM-4H-100, Hitachi R-900, JEOL JMS-01-SG-2 and Erma D-913A spectrometers, respectively. Column chromatography was done on Al_2O_3 (Wako, 300 mesh) and silica gel (Wako, C-200). TLC was carried out on Merck Kieselgel 60, and spots were detected with 5% $FeCl_3$ (phenolic derivatives) and 10% H_2SO_4 (alcohols and steroids), using S-1 [phenolic derivatives, $CHCl_3$–MeOH (5: 1)], S-2 [flavonoid acetates, $CHCl_3$–MeOH (50: 1)], S-3 [alcohols, sterols, $CHCl_3$–AcOEt (8: 1)] as developing solvents. GLC was carried out on a Hitachi 063 gas–liquid chromatograph using a stainless steel column (3 mm×1 m) packed with 2% SE-30 and 10% SE-30 on Chromosorb-W (60—80 mesh) with N_2 carrier gas at a flow rate of 30 ml/min. HPLC was carried out on a Hitachi 635A machine using a stainless steel column (8 mm×50 cm) packed with Hitachi gel 3011 and eluted with MeOH at a flow rate 2 ml/min.

Extraction and Fractionation of Components——Pollen grains (1677 g) collected in June, 1976, at Tohgane City, Chiba Prefecture, were extracted with ether in a Soxhlet apparatus. The extract (262.0 g) was dissolved with ether and the ether solution was shaken with 5% $NaHCO_3$ and 5% NaOH.

Formic, Acetic and Other Organic Acids——The 5% $NaHCO_3$ extract was treated with dil. HCl and extracted with ether. The ether extract was dried over Na_2SO_4 and the ether was evaporated off. The residue (2.08 g) was subjected to GLC (10% SE-30, column temp 20—150°, 10°/min) and components of the residue were identical with authentic samples of the following lower saturated fatty acids: HCOOH, CH_3COOH, C_2H_5COOH, C_3H_7COOH.

Isolation of *p*-Coumaric Acid (Ia)——After treatment of the 5% $NaHCO_3$ extract with dil. HCl the precipitated crystals were recrystallized from H_2O. A crystalline compound (0.19 g) which showed one spot on TLC (S-1) was obtained. Colorless needles, mp 214°. IR ν_{max}^{KBr} cm^{-1}: 3350, 1675, 1630, 1600, 1515, 1450, 1310, 1245, and 1210. The mixed melting point on admixture with an authentic sample of *p*-coumaric acid showed no depression, and the IR spectra and TLC properties of the two samples were identical.

Higher Saturated Fatty Acids——Part of the 5% NaOH (8.01 g) extract was treated with dil. HCl and the precipitated crystals (100 mg, fatty acids) were esterified with CH_3I and KOH. The methyl esters were subjected to GLC (2% SE-30, column temp. 50—260°, 20°/min) and components of the mixture were identical with authentic samples of the following saturated fatty acids: $C_{13}H_{27}COOH$, $C_{15}H_{31}COOH$, $C_{17}H_{35}COOH$, $C_{19}H_{39}COOH$, $C_{21}H_{43}COOH$, $C_{23}H_{47}COOH$, $C_{25}H_{51}COOH$, $C_{27}H_{55}COOH$, $C_{29}H_{59}COOH$, $C_{31}H_{63}COOH$, $C_{33}H_{67}COOH$.

Isolation of Apigenin (IIa) and Amentoflavone (IIIa)——Part of the 5% NaOH extract was treated with dil. HCl and the precipitated crystals (1.65 g) were separated by preparative TLC [Solvent, $CHCl_3$–MeOH (4: 1)].

Apigenin (IIa)——The crystalline material (71 mg) obtained on preparative TLC was recrystallized from 80% EtOH, mp>300°, yellow granular crystals; $FeCl_3$ test, dark green; Mg+HCl test, rose. UV λ_{max}^{EtOH} nm (log ε): 270 (4.12) and 338 (4.14). IR ν_{max}^{KBr} cm^{-1}: 3300, 1648, 1605, 1582, 1545 and 1491.

Acetylation of Apigenin (IIa)——A solution of 60 mg of IIa, 1 ml of pyridine and 0.3 ml of Ac_2O was allowed to stand for 16 hr at room temperature, then poured into ice-water. The white powder (61 mg) that appeared was collected and recrystallized from AcOEt to give 5-0, 7-0, 4′-0-triacetylapigenin (IIb) as colorless columnar crystals, mp 182°. IR ν_{max}^{KBr} cm^{-1}: 1766, 1650, 1630, 1612, 1376, 1210, 1180, 1137, 1084, 1030, 1014, 895 and 840. MS *m/e*: 396 (M^+), 354 (M^+-COCH_2), 312 ($M^+-COCH_2\times2$) and 270 ($M^+-COCH_2\times3$). PMR ($CDCl_3$) δ: 2.31 (3H×2, s), 2.40 (3H, s), 6.59 (1H, s), 6.89 (1H, d, J=2 Hz), 7.20 (2H, d, J=8 Hz), 7.30 (1H, d, J=2 Hz) and 7.84 (2H, d, J=8 Hz). The mixed melting point on admixture with an authentic sample of 5-0, 7-0, 4′-0-triacetylapigenin showed no depression, and the IR and PMR spectra and TLC properties of the two samples were identical.

Isolation of Amentoflavone——Crystalline material (IIIa, 84 mg) obtained on preparative TLC was recrystallized from pyridine–MeOH, mp>300°, yellow granular crystals; $FeCl_3$ test, dark green; UV λ_{max}^{EtOH} nm (log ε): 341 (4.35). IR ν_{max}^{KBr} cm^{-1}: 3200, 1660, 1605, 1350, 1160 and 830. The IR spectrum was identical with that of an authentic sample of amentoflavone.

Acetylation of Amentoflavone (IIIa)——A solution of 31 mg of IIIa, 2 ml of pyridine and 1 ml of Ac_2O was allowed to stand for 18 hr at room temperature, then poured into ice-water. The white powder (27 mg) that appeared was collected and recrystallized from AcOEt to give 5-0, 7-0, 4′-0, 5″-0, 7″-0, 4‴-0-hexaacetylamentoflavone (IIIb) as colorless granules, mp 234°, *Anal.* Calcd for $C_{42}H_{30}O_{16}$: C, 63.80; H, 3.80. Found: C, 63.76; H, 3.74. IR ν_{max}^{KBr} cm^{-1}: 1770, 1650, 1365, 1183, 1092, 1080, 1045, 1028, 1010, 906 and 812. MS *m/e* 706 ($M^+-COCH_2\times2$), 664 ($M^+-COCH_2\times3$), 538 ($M^+-COCH_2\times6$), 121, 60, 45, 43, 42, (base peak) and 41. PMR ($CDCl_3$) δ: 2.05 (3H, s), 2.09 (3H, s), 2.27 (3H, s), 2.32 (3H, s), 2.44 (3H, s), 2.49 (3H, s), 6.66 (1H, s, H-3″), 6.68 (1H, s, H-3), 6.84 (1H, d, J=3 Hz, H-6), 7.02 (1H, s, H-6″), 7.05 (2H, d, J=9 Hz, H-3‴, 5‴), 7.27 (1H, d, J=3 Hz, H-8), 7.48 (2H, d, J=9 Hz, H-2‴,6‴), 7.52 (2H, t, J=9 Hz, H-3′,5′) and 8.04 (1H, d, J=3 Hz, H-2′). The mixed melting point on admixture with an axthentic sample of 5-0, 7-0, 4′-0,

5″-0, 7″-0, 4‴-0-hexaacetylamentoflavone showed no depression and the IR spectra and TLC properties of the two samples were identical.

Separation of Hydrocarbons, Alcohols and Sterols——The neutral portion (21.0 g) was chromatographed on alumina (250 g) and silica gel (100 g), and the columns were eluted successively with hexane, benzene, $CHCl_3$ and MeOH.

Hydrocarbons——The fraction (48 mg) eluted with hexane was subjected to GLC (2% SE-30, column temp. 50—260°, 20°/min), and components were identical with authentic samples of the following hydrocarbons: $C_{15}H_{32}$, $C_{16}H_{34}$, $C_{17}H_{36}$, $C_{18}H_{38}$, $C_{19}H_{40}$, $C_{20}H_{42}$, $C_{21}H_{44}$, $C_{22}H_{46}$, $C_{23}H_{48}$, $C_{24}H_{50}$, $C_{25}H_{52}$, $C_{26}H_{54}$, $C_{27}H_{56}$, $C_{28}H_{58}$, $C_{29}H_{60}$, $C_{30}H_{62}$ and $C_{31}H_{64}$.

Saturated Alcohols——The fraction (55.14 g) eluted with benzene–$CHCl_3$ (1: 1) was repeatedly chromatographed on silica gel and eluted with benzene–$CHCl_3$ (2: 1). The eluted fraction was subjected to GLC and components were identical with authentic samples of the following saturated alcohols: *n*-$C_{16}H_{33}OH$, *n*-$C_{18}H_{37}OH$, *n*-$C_{20}H_{41}OH$, *n*-$C_{22}H_{45}OH$, *n*-$C_{24}H_{49}OH$, *n*-$C_{26}H_{53}OH$, *n*-$C_{28}H_{57}OH$ and *n*-$C_{30}H_{61}OH$.

Isolation of 24(ξ)-Methyl-25(ξ)-cholest-5-ene-3β,26-diol (IVa) and 24(ξ)-Ethyl-25(ξ)-cholest-5-ene-3β,26-diol (Va)——The fraction (0.54 g) eluted with benzene–$CHCl_3$ (1: 2) was repeatedly chromatographed on silica gel, and the eluted material (0.22 g) was separated by HPLC.

24(ξ)-Methyl-25(ξ)-cholest-5-ene-3β,26-diol (IVa)——The substance (63 mg) isolated by HPLC was recrystallized from acetone, yielding a crystalline compound (IVa) which showed a single peak on GLC and HPLC. VIa: Colorless plates, mp 162—164°; $[\alpha]_D^{21}$ −36.47° (c=0.85, $CHCl_3$–MeOH). *Anal.* Calcd for $C_{28}H_{48}O_2$: C, 80.77; H, 11.54; Found: C, 80.64; H, 11.62. MS *m/e*: 416 (M^+), 398 (M^+-H_2O), 383 ($M^+-H_2O-CH_3$), 305, 273, 255, 231, 213, 173, 161, 143, 137, 119. High resolution MS, Calcd for $C_{28}H_{48}O_2$ (M^+): 416.3654. Found: 416.3655. IR ν_{max}^{KBr} cm^{-1}: 3300, 2960, 2920, 2880, 2845, 1625, 1455, 1380, 1370, 1343, 1055, 1040, 1022, 840 and 800. PMR ($CDCl_3$–CD_3OD) δ: 0.70 (3H, s), 0.92 (3H×2, d, J=5 Hz), 1.02 (3H, s), 1.29 (3H, br. s), 3.47 (2H, d.d, J=6, 6 Hz), 3.64 (1H, m, $W1/2$=14 Hz), 5.34 (1H, m, $W1/2$=10 Hz).

Acetylation of 24(ξ)-Methyl-25(ξ)-cholest-5-ene-3β,26-diol (IVa)——A solution of 35 mg of IVa, 3.5 ml of pyridine, and 0.3 ml of Ac_2O was allowed to stand for 16 hr at room temperature then poured into ice-water. The white powder (34 mg) that appeared was collected and recrystallized from acetone to give 3β,26-diacetoxy-24(ξ)-methyl-25(ξ)-cholest-5-ene (IVb) as colorless plates, mp 92—94°; $[\alpha]_D^{30}$ −29.40° (c=1.02, $CHCl_3$). *Anal.* Calcd for $C_{32}H_{52}O_4$; C, 76.80; H, 10.40. Found: C, 76.54; H, 10.51. MS *m/e* 440 (M^+-CH_3COOH), 380 ($M^+-CH_3COOH\times2$), 255, 213, 158, 147, 143, 133, 107, 105, 95, 81, 55, 43. IR ν_{max}^{KBr} cm^{-1}: 2940, 2845, 1740, 1620, 1465, 1380, 1365, 1240 and 1030. PMR ($CDCl_3$) δ: 0.65 (3H, s), 0.89 (3H×2, d, J=5 Hz), 1.00 (3H, s), 1.23 (3H, br. s), 2.01 (3H, s), 2.03 (3H, s), 3.92 (2H, d.d, J=6, 6 Hz), 4.58 (1H, m, $W1/2$=22.5 Hz), 5.35 (1H, m, $W1/2$=10 Hz).

Oxidation of 24(ξ)-Methyl-25(ξ)-cholest-5-ene-3β,26-diol (IVa)——A solution of 13 mg of IVa, 1.5 ml of pyridine, and 15 mg of CrO_3 was allowed to stand for 15 hr at room temperature, then poured into 50% MeOH. The reaction mixture were extracted with ether, and after usual work-up, the crude product (8 mg) was obtained as an amorphous compound. Three spots appeared on TLC and the material was chromatographed on silica gel (0.2 g), eluting sxccessively with benzene, $CHCl_3$ and MeOH. The fraction (2 mg) eluted with $CHCl_3$ gave a single peak on GLC. MS *m/e*: 430 (M^+), 412 (M^+-H_2O), 397 ($M^+-H_2O-CH_3$), 273, 255, 119, 74 ($C_3H_6O_2$), 73 ($C_3H_5O_2$) and 44 (CO_2). High resolution MS, Calcd for $C_{28}H_{46}O_3$ (M^+): 430.3447. Found: 430.3447.

24(ξ)-Ethyl-25(ξ)-cholest-5-ene-3β,26-diol (Va)——The substance (50 mg) separated by HPLC was recrystallized from acetone, yielding a crystalline compound (Va) which gave a single peak on GLC and HPLC. Va: Colorless plates, mp 148—150°; $[\alpha]_D^{18}$ −23.50° (c=2.00, $CHCl_3$–MeOH). *Anal.* Calcd for $C_{29}H_{50}O_2$; C, 80.93; H, 11.63; Found. C, 80.61, H, 11.86. MS *m/e*: 430 (M^+), 415 (M^+-CH_3), 412 (M^+-H_2O), 397 ($M^+-H_2O-CH_3$), 273, 255, 157, 137, 119. High resolution MS, Calcd for $C_{29}H_{50}O_2$ (M^+): 430.3811. Found: 430.3810. IR ν_{max}^{KBr} cm^{-1}: 3250, 2960, 2925, 2913, 1620, 1460, 1375, 1055, 1020 and 955. PMR ($CDCl_3$) δ: 0.66 (3H, s), 0.87 (3H, t, J=13 Hz), 0.99 (3H, s), 1.28 (3H×2, br. s), 3.50 (3H, d.d, J=5, 5 Hz), 3.65 (1H, m, $W1/2$=14 Hz), 5.32 (1H, m, $W1/2$=9 Hz).

Acetylation of 24(ξ)-Ethyl-25(ξ)-cholest-5-ene-3β,26-diol (Va)——A solution of 35 mg of Va, 3.5 ml of pyridine and 0.3 ml of Ac_2O was allowed to stand for 17 hr at room temperature then poured into ice-water. The white powder (37 mg) that appeared was collected and recrystallized from acetone to give 3β,26-diacetoxy-24(ξ)-ethyl-25(ξ)-cholest-5-ene (Vb) as colorless plates, mp 60°; $[\alpha]_D^{17}$ −50.93° (c=1.08, $CHCl_3$). *Anal.* Calcd for $C_{33}H_{54}O_4$, C, 77.04; H, 10.51. Found: C, 76.98; H, 10.67. MS *m/e*: 454 (M^+-CH_3COOH), 394 ($M^+-CH_3COOH\times2$) 255, 253, 213, 199, 185, 171, 159, 158, 147, 145, 119, 109, 107, 105, 83, 81, 59, 55. IR ν_{max}^{KBr} cm^{-1}: 2945, 2905, 2838, 1740, 1625, 1460, 1380, 1225 and 1020. PMR ($CDCl_3$) δ: 0.70 (3H, s), 0.90 (3H, t, J=13 Hz), 1.03 (3H, s), 1.30 (3H×2, br. s), 2.03 (3H, s), 2.05 (3H, s), 3.96 (2H, d.d, J=5, 5 Hz), 4.58 (1H, m, $W1/2$=22.5 Hz), 5.37 (1H, m, $W1/2$=9 Hz).

Oxidation of 24(ξ)-Ethyl-25(ξ)-cholest-5-ene-3β,26-diol (Va)——A solution of 11 mg of Va, 1.5 ml of pyridine, and 15 mg of CrO_3 was allowed to stand for 17 hr at room temperature, then poured into 50% MeOH. The reaction mixture were extracted with ether and after usual work-up, the crude product (8 mg) was obtained as an amorphous compound. The material showed three spots on TLC and was chromatographed on silica gel (0.2 g), eluting successively with benzene, $CHCl_3$ and MeOH. The fraction (2 mg) eluted with

$CHCl_3$ gave a single peak on GLC. MS *m/e* 444 (M^+), 426 (M^+-H_2O), 273 ($M^+-C_{10}H_{19}O_2$), 255, 137, 119, 74 ($C_3H_6O_2$), 73 ($C_3H_5O_2$) and 44 (CO_2). High resolution MS, Calcd for $C_{29}H_{48}O_3$ (M^+): 444.3603. Found: 444.3600.

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Inverse Substrates. IX.[1] Amidinophenyl Esters derived from Amino Acids and Peptides: Synthesis and Properties as Trypsin Substrates

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Methods for the preparation of *p*-amidinophenyl esters from amino acid derivatives are described. Blocking of the amidino function with the benzyloxycarbonyl group and its deblocking by catalytic hydrogenation after coupling were satisfactory. *p*-Amidinophenyl esters are of special interest because of their susceptibility to the hydrolytic enzyme, trypsin, and some parameters of the hydrolysis of these compounds by trypsin are presented.

Keywords——*p*-amidinophenyl ester; synthetic substrates; ester formation; trypsin; enzyme kinetics; deacylation rate constant

In our previous papers,[3] it was shown that esters of *p*-amidinophenol are specifically hydrolyzed by trypsin. These esters are characterized by their linkage, *i.e.*, the site-specific group (charged amidinium) for the enzyme is not located in the acyl moiety but in the leaving portion. A new term, "inverse substrates", was proposed for these esters, having regard to their specific binding and efficient acylation processes, comparable to those for normal-type substrates.[3a] This has provided, for the first time, a general method for the *specific* introduction of an acyl group carrying a *non-specific* residue into the trypsin active site without recourse to a cationic acyl moiety characteristic of conventional substrates.[3d] It is of interest to investigate the behavior of trypsin towards "inverse substrates" derived from a variety of amino acids and preptides, including amino acids of the D-series. Analysis of the deacylation process in the trypsin-catalyzed hydrolysis of this entirely new type of substrates could shed light on functions of the enzyme which are not manifested with typical substrates. This report is mainly concerned with the synthesis of these "inverse substrates" from various amino acids and some peptides. Their behavior towards trypsin is also described briefly.

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