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# Studies on Anti-inflammatory Agents. V.[1] A New Anti-inflammatory Constituent of *Pyracantha crenulata* Roem.[2]

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A new compound, pyracrenic acid (**1**), was isolated from the bark of *Pyracantha crenulata* Roem. of Rosaceae by tracing its granulation-suppressive activity by the fertile egg method in the course of an extensive search among crude drugs and plants for anti-inflammatory constituents.

Methylation of **1** with diazomethane-ether solution gave methyl O,O-dimethylpyracrenate (**6**), which was cleaved by hydrolysis into methyl betulinate (**7**) and O,O-dimethylcaffeic acid (**8**). Thus, **1** has the structure of 3$\beta$-(3,4-dihydroxycinnamoyl)oxylup-20(29)-en-28-oic acid. Results obtained by the cotton pellet method showed that **1** potently inhibits the formation of granulation tissue.

**Keywords**——anti-inflammatory constituent; Rosaceae; *Pyracantha crenulata* Roem.; pyracrenic acid; betulinic acid; *trans*-3,4-dihydroxycinnamic acid (=caffeic acid); fertile egg method; cotton pellet method

Extensive searches for biologically active constituents of natural resources are done not only to identify active constituents of crude drugs which have long been used for medical purposes, but also to find other plant sources of these constituents. The present anti-inflammatory screening method (fertile egg method) has been designed to ensure taxonomical impartiality by using nearly 1700 species of disparate families.[3,4]

We have already found[3,4] that some crude drugs and plants inhibit granulation tissue formation when studied by the fertile egg method. The plant *Pyracantha crenulata* Roem. (Rosaceae) is one of them. The present study deals with the isolation and structure determination of an active anti-inflammatory compound obtained from the bark of this plant, which is called "Himalaya-pirakansa" in Japanese and is native to China. It is an evergreen shrub which bears small red berries in summer, and is recently often used as a hedge. *P. angustifolia* Schneid. ("Tachibanamodoki" in Japanese), and *P. coccinea* Roem. ("Tokiwasanzashi" in Japanese) are well known plants of the same genus.

Preliminary examination of some parts of *Pyracantha crenulata* Roem. revealed that its bark exhibits the most potent inhibitory activity against granulation tissue formation.

The bark was extracted with acetone and this extract was chromatographed on a silica gel column; the p-2 fraction was re-chromatographed on a silica gel column. A p-c fraction was eventually identified and separated from the p-2 fraction, as shown in Chart 1. This fraction was recrystallized from *n*-hexane–ethanol to give colorless scaly crystals **1**, mp 310—312°C (dec.), which showed one spot on a thin-layer chromatogram (Chart 1). Compound **1** gave positive coloration with ferric chloride solution and in the Liebermann-Burchard reaction. The molecular formula $C_{39}H_{54}O_6$ was determined by mass spectroscopy (MS), elemental analysis, and molecular weight determination by the vapor pressure osmometer method. The ultraviolet absorption (UV) spectrum of **1** in ethanol had four maxima at 217, 244, 300 (shoulder) and 328 nm. The infrared absorption (IR) spectrum (potassium bromide disc) of

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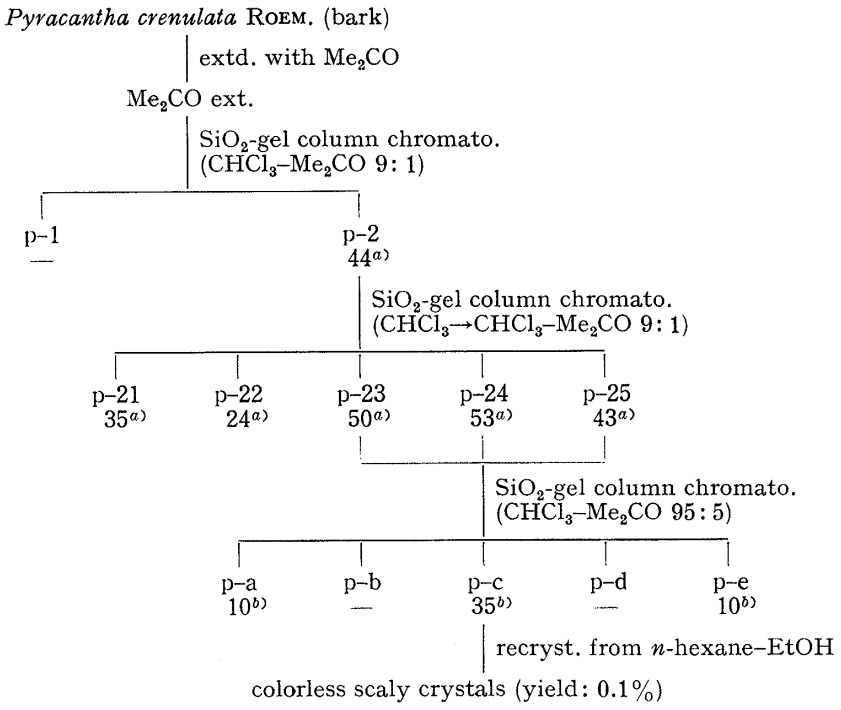


Chart 1. Isolation of an Active Anti-inflammatory Constituent from Bark of *P. crenulata* ROEM.

*a*) Inhibition ratio (%) of granulation tissue formation at a dose of 400 $\mu$g per disc (fertile egg method).
*b*) Inhibition ratio (%) of granulation tissue formation at a dose of 200 $\mu$g per disc (fertile egg method).

**1** showed a hydroxyl band at 3430 $cm^{-1}$, a carbonyl band at 1698 $cm^{-1}$, $\alpha,\beta$-unsaturated carbonyl bands at 1650, 1280, 1180 $cm^{-1}$ and aromatic nucleus bands at 1619 and 1513 $cm^{-1}$. The nuclear magnetic resonance (NMR) spectrum in $d_6$-acetone exhibited signals for six methyl protons at $\delta$ 0.86, 0.90 (due to protons of two methyl groups), 0.96, 1.02 and 1.68 (due to methyl protons of the isopropenyl group), two end methylene protons, the existence of which is supported by the absorption bands at 972 and 800 $cm^{-1}$ in the IR spectrum of **1**, at $\delta$ 4.55 and 4.68, two *trans* olefinic protons at $\delta$ 6.20 (1H, d, $J$=16 Hz) and 7.80 (1H, d, $J$=16 Hz) and ABX-pattern proton signals assignable to the 1,2,4-trisubstituted benzene ring at $\delta$ 6.78 (1H, d, $J$=8 Hz), 6.98 (1H, dd, $J$=2 and 8 Hz), and 7.10 (1H, d, $J$=2 Hz).

Hydrolysis of **1** with potassium hydroxide in ethanol gave a crystalline product **2**, mp 291—297°C (dec.), which showed positive coloration in the Liebermann-Burchard reaction and no coloration with ferric chloride solution. This compound **2** was deduced to be betulinic acid on the basis of the physicochemical properties of **2** and its derivatives prepared as shown in Chart 2 and the experimental section. This assumption was confirmed by mixed melting point determination, and by the identity of IR spectra, MS, and *Rf* values on TLC with those of authentic betulinic acid.[5]

Simple methylation of **1** with diazomethane in ether yielded the trimethylated compound **6**, mp 184—186°C, $C_{42}H_{60}O_6$ ($M^+$ 660), which could be smoothly saponified to afford methyl betulinate (**7**), mp 219—221°C, and O,O-dimethylcaffeic acid (**8**), mp 190—191°C. Thus, compound **1** was shown to be 3$\beta$-(3,4-dihydroxycinnamoyl)oxylup-20(29)-en-28-oic acid. This is a new constituent from *Pyracantha crenulata* ROEM., hence it was named pyracrenic acid.

It potently inhibited the formation of granulation tissue when studied by the fertile egg method, as shown in Table I. Administered orally to intact rats at a dose of 50 mg/kg, it exhibited inhibitory activity against granulation tissue formation induced by subcutaneous implantation of cotton pellets (Table II).

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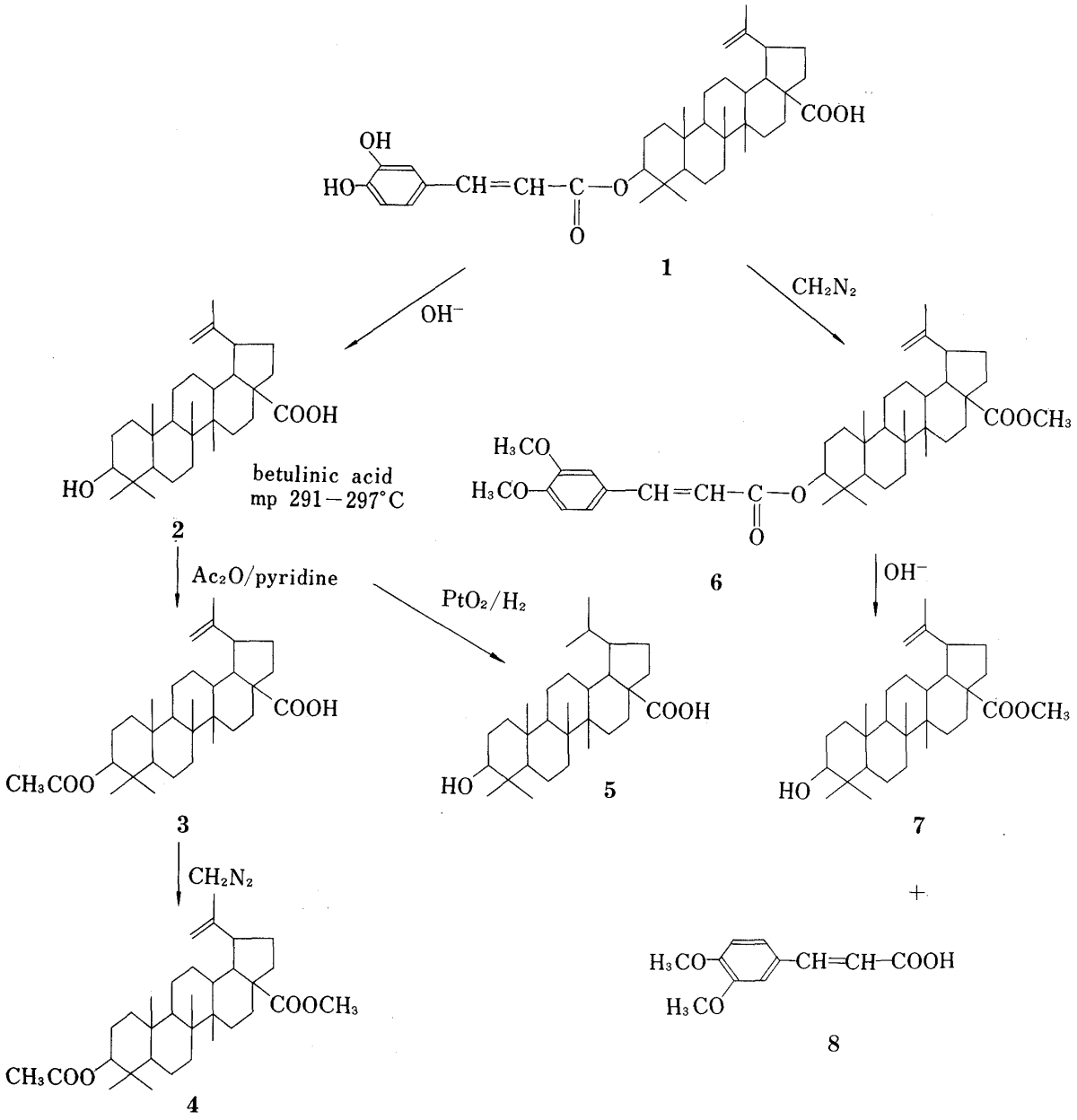


Chart 2

TABLE I. Anti-inflammatory Activity of Pyracrenic Acid (1) in the Fertile Egg Method

| Test compd. | Dose (μg/disc) | Granulation tissue[a] | | Survival ratio of embryo |
|---|---|---|---|---|
| | | Dry wt. ± S.E. (mg) | Inhibition (%) | |
| Control | | 5.8±0.9 | | 17/20 |
| Pyracrenic acid (1) | 100 | 5.0±1.0 | 13.8 | 19/20 |
| | 200 | 3.6±0.7[b] | 38.0 | 16/20 |
| | 400 | 3.3±0.6[b] | 43.1 | 18/20 |
| Berberine chloride | 25 | 3.2±0.7[b] | 44.8 | 20/20 |

*a*) Average of 20 chick embryos.
*b*) Significant at the 5% level in comparison with the control.

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Table II. Effect of Pyracrenic Acid (1) on Granulation Tissue Formation as determined by Means of the Cotton Pellet Method in Rat

| Test compd. | Daily dose[a] (mg/kg) | Mean body wt.[b] gain (g) | Granulation tissue[c] Dry wt. ± S.E. (mg) | Granulation tissue[c] Inhibition (%) |
|---|---|---|---|---|
| Control | | 39 | 36.1±1.8 | |
| Pyracrenic acid (1) | 25 | 37 | 29.8±3.7 | 17.5 |
| | 50 | 28 | 26.8±2.7[d] | 25.8 |
| Berberine chloride | 25 | 40 | 31.8±2.7 | 11.9 |
| Prednisolone | 2.5 | 23 | 19.9±2.2[d] | 44.9 |

*a*) Administration route: *per os*.
*b*) Initial body weights (male rats): 220—250 g.
*c*) Average of 12 pellets.
*d*) Significant at the 5% level in comparison with the control.

TLC of crude extracts indicated that pyracrenic acid (**1**) is also present in substantial quantity in the leaf, besides the bark, of *P. crenulata* and *P. angustifolia*.

Several studies have been done on isolating and identifying the constituents of the genus *Pyracantha*: morolic acid has been obtained from *P. coccinea* by Ryabinin and co-workers,[6] ursolic acid from *P. angustifolia* by Shimada and Kano,[7] sitosterol, stigmasterol, campesterol, $C_{23}H_{48}$, $C_{25}H_{52}$ (alkanes), docosan-1-ol, and eicosan-1-ol from *P. angustifolia* by Suzuki and co-workers,[8] *d*-sorbitol from *P. crenulata* and *P. angustifolia* by Strain,[9] pro-$\gamma$-carotene and prolycopene from *P. angustifolia* by Zechmeister and co-workers,[10] and $\alpha$-,$\beta$-,$\gamma$-carotene from *P. coccinea* by Karrer and Rutschmann.[11] However, no one has correlated the constituents to the anti-inflammatory activities. This is thus the first report on the biological action of the constituents of the genus *Pyracantha*.

## Experimental

Melting points were taken on a micro hot-stage and are uncorrected. IR spectra were taken with a Hitachi EPI-G2 spectrometer, UV spectra with a Hitachi EPS-3T spectrometer, and specific rotations with a Perkin-Elmer 141 spectrometer. Mass spectra were determined with a Hitachi RMU-6D mass spectrometer. NMR spectra were taken with a Varian HA-100 spectrometer using tetramethylsilane as an internal standard. Abbreviations used are: s=singlet, d=doublet, t=triplet, q=quartet, and sh=shoulder. Unless otherwise stated, silica gel 60 (70—230 mesh ASTM, Merck) treated with hydrochloric acid and activated by heating was used for column chromatography, during which the column was shielded by light aluminum foil. TLC was carried out using silica gel 60 $F_{254}$ (Art. 5717, Merck) plates.

**Extraction and Isolation**——The bark (10 kg) of *Pyracantha crenulata* Roem., collected at Ichijoji, Sakyo-ku in Kyoto in 1975, was extracted with chloroform (40 liters) at room temperature for 72 h. The chloroform extract was concentrated to leave a greenish residue (80 g, yield: 0.8%). The starting debris was further refluxed with acetone (60 l) to give the acetone extract (120 g, yield: 1.2%). The combined extracts were chromatographed on a silica gel (3.0 kg) column with chloroform–acetone (9: 1) and gave the p-1 fraction (60 g, yield: 0.6%) and the p-2 fraction (35 g, yield: 0.35%). The p-2 fraction, which was shown to inhibit the formation of granulation tissue by the fertile egg method (Chart 1), was re-chromatographed on a silica gel column to afford 4.2 g of p-21 fraction, 5.9 g of p-22 fraction, 5.6 g of p-23 fraction, 3.3 g of p-24 fraction and 0.6 g of p-25 fraction. The combined fractions from p-23 to p-25 were chromatographed on silica gel with chloroform–acetone (95: 5) to give fractions p-a, p-b, p-c, p-d, and p-e. The p-c fraction, which showed potent anti-inflammatory activity, was concentrated to give a faintly yellowish solid (15.8 g, yield: 0.16%). Recrystallization from *n*-hexane–ethanol gave pyracrenic acid (**1**) (10.7 g, yield: 0.1%).

**Pyracrenic Acid (1)**——Colorless scaly crystals, mp 310—312 °C (dec.) (from *n*-hexane–ethanol). Ferric chloride reaction, dark green; Liebermann-Burchard reaction, reddish purple; slightly soluble in dimethyl sulfoxide, dioxane, hot ethanol, insoluble in *n*-hexane, ethyl acetate, ethyl ether, and water, but freely soluble in pyridine. *Anal.* Calcd for $C_{39}H_{54}O_6$: C, 75.04; H, 9.18. Found: C, 75.24; H, 8.99. UV $\lambda_{max}^{ethanol}$ nm ($\varepsilon$): 217 (30700), 244 (21300), 300 (sh), 328 (37400). Mass spectrum: 618 ($M^+$). Molecular weight as determined by the vapor pressure osmometer method: 614.

**Betulinic Acid (2) from Hydrolysis of Pyracrenic Acid (1)**——Compound **1** (500 mg) was added to potassium hydroxide–ethanol (1: 20) solution, and was left standing at room temperature for 16 h. Usual work-up gave 220 mg of colorless needles. Recrystallization from methanol gave betulinic acid (**2**), mp 291—297°C

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(lit.[12] mp 299—301°C); identified by comparison with an authentic sample [mixed melting point, IR (KBr disc), MS, and TLC]. *Anal.* Calcd for $C_{30}H_{48}O_3 \cdot 1/2H_2O$: C, 77.37; H, 10.59. Found: C, 77.33; H, 10.53. UV $\lambda_{max}^{methanol}$ nm ($\varepsilon$): 195 (8900). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3450 (OH), 1695 (C=O), 1640 (C=C), 885 (end methylene). NMR ($d_5$-pyridine)$\delta$: 0.82, 0.95, 1.03, 1.05, 1.17 (each 3H, s, $CH_3 \times 5$), 1.76 (3H, s, $=\!\!-CH_3$), 3.38 (1H, t, $J=7$ Hz, C-3-H), 4.70, 4.86 (each 1H, m, two protons of end methylene), 11.2 (1H, broad s, COOH). MS *m/e*: 456 ($M^+$: 90%), 441 ($M^+ - CH_3$: 10%), 438 ($M^+ - H_2O$: 10%), 423 ($M^+ - CH_3 - H_2O$: 24%), 412 ($M^+ - CO_2$: 10%), 411 ($M^+ - COOH$: 10%), 395 ($M^+ - 61$: 10%), 248 ($M^+ - 208$: 90%), 207 ($M^+ - 249$: 90%), 189 (207 $- H_2O$: 100%). $[\alpha]_D^{22}$ +6.8° ($c=0.1$, in pyridine) [lit.[13] $[\alpha]_D^{23}$ +7.9° ($c=0.057$, in pyridine)].

**3-O-Acetylbetulinic Acid (3)**——A solution of **2** (25 mg) in a mixture of acetic anhydride (0.5 ml) and pyridine (0.5 ml) was left standing at room temperature for 6 h. Usual work-up gave a crystalline residue. Recrystallization from ethyl acetate afforded colorless needles of 3-O-acetylbetulinic acid (3) (19 mg), mp 283—285°C (lit.[12] mp 285—287°C). *Anal.* Calcd for $C_{32}H_{50}O_4$: C, 77.06; H, 10.11. Found: C, 77.01; H, 10.31. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1735 (C=O), 1690 (C=O), 1640 (C=C), 885 (end methylene). NMR ($CDCl_3$) $\delta$: 0.80 (each 3H, s, $CH_3 \times 3$), 0.89 (3H, s, $CH_3$), 0.93 (3H, s, $CH_3$), 1.64 (3H, s, $=\!\!-CH_3$), 1.98 (3H, s, $OCOCH_3$), 4.44 (1H, t, $J=7$ Hz, C-3-H), 4.56, 4.70 (each 1H, m, two protons of end methylene). MS *m/e*: 498 ($M^+$: 18%), 483 ($M^+ - CH_3$: 5%), 454 ($M^+ - CO_2$: 7%), 438 ($M^+ - CH_3COOH$: 35%), 423 ($M^+ - CH_3COOH - CH_3$: 15%), 249 ($M^+ - 249$: 20%), 189 ($M^+ - 309$: 55%).

**Methyl Betulinate 3-Acetate (4)**——A solution of excess diazomethane in ether was added to a solution of **3** (30 mg) in carbon tetrachloride (10 ml), and the mixture was left standing at room temperature for 30 min. Usual work-up gave colorless needles of methyl betulinate 3-acetate (**4**) (40.2 mg), mp 206—209°C (lit.[14] mp 206—209°C). *Anal.* Calcd for $C_{33}H_{52}O_4$: C, 77.29; H, 10.22. Found: C, 77.45; H, 10.17. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1735 (C=O), 1640 (C=C), 890 (end methylene). NMR ($CDCl_3$) $\delta$: 0.80 (each 3H, s, $CH_3 \times 3$), 0.89 (3H, s, $CH_3$), 0.93 (3H, s, $CH_3$), 1.64 (3H, s, $=\!\!-CH_3$), 1.98 (3H, s, $OCOCH_3$), 3.60 (3H, s, $COOCH_3$), 4.44 (1H, t, $J=7$ Hz, C-3-H), 4.56, 4.70 (each 1H, m, two protons of end methylene). MS *m/e*: 512 ($M^+$: 45%), 498 ($M^+ - CH_3$: 5%), 452 ($M^+ - CH_3COOH$: 70%), 437 ($M^+ - CH_3COOH - CH_3$: 5%), 392 ($M^+ - CH_3COOH \times 2$: 15%), 249 ($M^+ - 263$: 50%), 189 (100%).

**Dihydrobetulinic Acid (5)**——A solution of **2** (50 mg) in ethyl acetate (10 ml) was shaken with platinum dioxide (11 g) at room temperature for 5 h under one atmosphere of hydrogen. Usual work-up gave colorless scaly crystals of **5** (48 mg), mp 312—314°C. *Anal.* Calcd for $C_{30}H_{50}O_3 \cdot C_2H_5OH$: C, 76.14; H, 11.18. Found: C, 75.89; H, 11.28. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1690 (C=O). NMR ($d_5$-pyridine) $\delta$: 0.82, 0.92 (each 3H, d, $J=7$ Hz, secondary $CH_3 \times 2$), 0.83, 0.96, 1.18 (each 3H, s, $CH_3 \times 3$), 1.02 (each 3H, s, $CH_3 \times 2$), 3.38 (1H, t, $J=7$ Hz, C-3-H). MS *m/e*: 458 ($M^+$: 40%), 443 ($M^+ - CH_3$: 5%), 440 ($M^+ - H_2O$: 30%), 425 ($M^+ - H_2O - CH_3$: 20%), 413 ($M^+ - COOH$: 16%), 397 ($M^+ - 61$: 20%), 207 ($M^+ - 251$: 80%), 189 ($M^+ - 269$: 70%).

**Methyl O,O-Dimethylpyracrenate (6)**——A solution of excess diazomethane in ether was added to a solution of **1** (500 mg) in carbon tetrachloride–ethanol (1: 1), and the mixture was left standing at room temperature for 30 min. Usual work-up gave colorless crystals (340 mg), which were recrystallized from ethanol–ether to give colorless scaly crystals of methyl O,O-dimethylpyracrenate (**6**) (280 mg), mp 184—186°C. *Anal.* Calcd for $C_{42}H_{60}O_6$: C, 76.32; H, 9.15. Found: C, 75.99; H, 9.11. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1710 (C=O), 1635 (C=C), 880 (end methylene). NMR ($CDCl_3$) $\delta$: 0.86, 0.88, 0.96 (each 3H, s, $CH_3 \times 3$), 0.92 (each 3H, s, $CH_3 \times 2$), 1.68 (3H, s, $=\!\!-CH_3$), 3.63 (3H, s, $COOCH_3$), 3.88 (6H, s, $OCH_3 \times 2$), 4.59 (1H, t, $J=7$ Hz, C-3-H), 4.59, 4.70 (each 1H, m, two protons of end methylene), 6.16 (1H, d, $J=16$ Hz, Ar–CH=C<u>H</u>–), 6.80 (1H, d, $J=8$ Hz, arom. proton), 7.05 (1H, dd, $J=8$ Hz, 2 Hz, arom. proton), 7.10 (1H, d, $J=2$ Hz, arom. proton), 7.81 (1H, d, $J=16$ Hz, Ar–C<u>H</u>=CH–). MS *m/e*: 660 ($M^+$).

**Methyl Betulinate (7) and O,O-Dimethylcaffeic Acid (8) from Hydrolysis of 6**——A solution of **6** (470 mg) in potassium hydroxide–ethanol (1: 20) was stirred at room temperature for 48 h. Usual work-up gave a crude solid (350 mg), whose recrystallization (from ethyl acetate) gave colorless crystals of methyl betulinate (**7**) (210 mg), mp 219—221°C (lit.[15] mp 221—224°C). *Anal.* Calcd for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71. Found: C, 78.96; H, 10.75. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1735 (C=O), 1645 (C=C), 885 (end methylene). NMR ($CDCl_3$) $\delta$: 0.84, 1.00, 1.18 (each 3H, s, $CH_3 \times 3$), 0.98 (each 3H, s, $CH_3 \times 2$), 1.72 (3H, s, $=\!\!-CH_3$), 3.38 (1H, t, $J=7$ Hz, C-3-H), 3.65 (3H, s, $COOCH_3$), 4.68, 4.83 (each 1H, m, two protons of end methylene). The aqueous layer was made acidic (pH 2) with dil. hydrochloric acid, and extracted three times with ether (150 ml). The ethereal layer was treated as usual to give a crude crystalline solid (180 mg), recrystallization of which (from methanol–water) gave colorless needles of O,O-dimethylcaffeic acid (**8**) (100 mg), mp 190—191°C (lit.[16] mp 186°C), which was not depressed upon admixture with a synthetic specimen prepared through the known procedure.[17] The IR and MS were also identical with those of the authentic sample. *Anal.* Calcd for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found: C, 63.49; H, 5.77. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1680 (C=O), 1625 (C=C). NMR ($d_6$-dimethyl sulfoxide) $\delta$: 3.77, 3.88 (each 3H, s, $OCH_3 \times 2$), 6.37 (1H, d, $J=16$ Hz, Ar–CH=C<u>H</u>–), 6.90 (1H, d, $J=8$ Hz, arom. proton), 7.12 (1H, dd, $J=2$ Hz, 8 Hz, arom. proton), 7.24 (1H, d, $J=2$ Hz, arom. proton), 7.49 (1H, d, $J=16$ Hz, Ar–C<u>H</u>=CH–). MS *m/e*: 208 ($M^+$: 100%). $UV_{max}^{methanol}$: 231, 287, 315 nm.

**Biological Tests**——1) Fertile Egg Method: This method was described in the previous paper.[3] Fertile eggs, collected on the day after they had been laid by a strain of White Leghorn, were incubated at 37°C for 9 days. After confirming the survival and position of the chick embryo, a hole was drilled over the air sac, suction was applied to it with a rubber teat, and the chorio-allantoic membrane was made to fall. Next,

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part of the shell and shell membrane, circumscribed by a triangle, was carefully removed, and a sterile filter paper disc stamped ($\phi$ 12 mm) out of Toyo Roshi No. 51 paper was inserted. The open part in the shell was sealed with cellophane tape and the eggs were reincubated at 37°C for 4 days. At the end of this period, the granulation tissue together with the disc was taken out of the shell, dried overnight at 50°C, and desiccated at room temperature. Evaluation of the anti-inflammatory activity by this method was performed by calculating the inhibitory ratio of granulation tissue formation of the drug against the control. The inhibition ratio (%) of the test drug was calculated as follows:

$$\text{Inhibition ratio (\%)} = \frac{C-T}{C} \times 100$$

where, $C$ is the mean dry weight (mg) of granulation tissue formed in control eggs and $T$ is the mean dry weight (mg) of granulation tissue formed in the test eggs.

2) Cotton Pellet Method[18]: Male Wistar rats, weighing 220—250 g, were lightly anesthetized with ether and were placed in a prone position on a board, then the skin of the dorsum was shaved with an electric clipper. A horizontal incision was made on the dorsal skin and two sterile cotton pellets were implanted symmetrically under the arms of each rat. Procain penicillin G (3 million units) was then applied to the incision and the wound was carefully sutured. This day was considered day 0 of implantation. Test compounds were administered orally once a day for 7 days and the control group received solvent. Eight days later the animals were sacrificed and the pellets were removed together with granulation tissue, then dried at 80—100°C for 48 h, and weighed. In order to assess the actual amount of granulation tissue present, the weight of the dried cotton pellet, previously determined to be $30 \pm 1$ mg, was subtracted from the total weight of the dried cotton pellet plus granulation tissue.

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