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The Constituents of Conifer Needles

II *. Pinifolic Acid, a new Diterpene Acid Isolated from *Pinus silvestris* L.

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Dedicated to Professor *Holger Erdtman* on his 60th birthday

A diterpene acid, pinifolic acid, has been isolated from the needles of *Pinus silvestris* L. and shown to have structure (1 a).

In a previous investigation [1] a study was made of the low-molecular carbohydrates present in the needles of *Pinus silvestris* L. The present paper deals with the isolation of a new diterpene acid from the same source and the elucidation of its structure.

Fresh needles, collected in the autumn, were extracted with acetone and the concentrated extract was partitioned between water and chloroform. The acid fraction, obtained from the chloroform phase on extraction with sodium hydrogen carbonate, contained about 65 % of one component and gave, on recrystallisation, a pure diterpene acid, $C_{20}H_{32}O_4$, in 0.27 % yield based on the dry weight of the needles. The name pinifolic acid is proposed for this compound.

The structure (1) is assigned to pinifolic acid on the following evidence. The infrared (Fig. 1) and ultraviolet spectra of the acid indicated the presence of an isolated, unsymmetrically disubstituted double bond and catalytic hydrogenation over Adam's catalyst gave dihydropinifolic acid (2). Pinifolic acid, on dehydrogenation with palladium on charcoal at 290°, gave 1,2,5-trimethyl naphthalene identified by its characteristic ultraviolet absorption [2], together with some volatile acids, which were collected and examined. The first volatile fraction, collected after 10 min, was, according to paper chromatographic results [3], a mixture of acetic and propionic acids; the second fraction, collected after 2 h, appeared to contain very small amounts of (S)-(+)-3-methylpenta-

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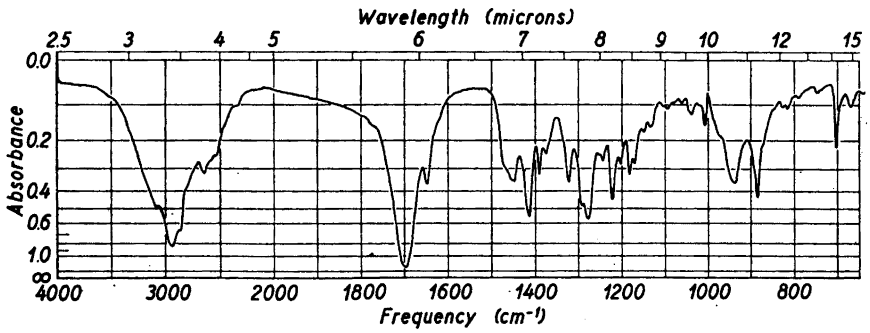
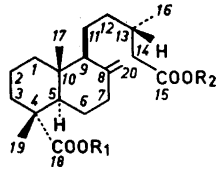


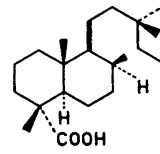
Fig. 1. Infrared spectrum of pinifolic acid.

noic acid [4-6]. In view of the stereochemical importance of this acid further investigation is in progress.

These results suggested that pinifolic acid was closely related to dihydroagathic acid (4), which was prepared from agathic acid [7] (5) by reduction with sodium in butanol [8]. Pinifolic and dihydroagathic acids were found to have similar rotations but somewhat different R_F-values and their dimethyl esters gave infrared spectra which were nearly identical above 1 400 cm^{-1}. The mass spectra of the two dimethylesters (Figs. 2 and 3), discussed below, suggested that the two acids were epimeric at C-(4). Pinifolic acid was therefore related to labdanolic acid [9] (6) in the following manner.



1 a $R_1=R_2=H$
1 b $R_1=R_2=CH_3$
1 c $R_1=CH_3$; $R_2=H$



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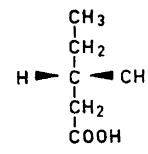

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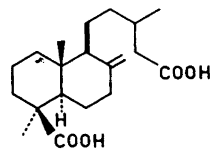

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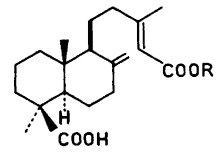
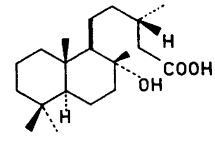


5 a R = H
5 b $R = CH_3$



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Pinifolic acid dimethyl ester on treatment with one equivalent of sodium hydroxide in methanol gave the monoester (1 c), which was reduced with sodium in ethanol or better with lithium in liquid ammonia to the hydroxy acid (7). The methyl ester of this acid was oxidised with chromium trioxide

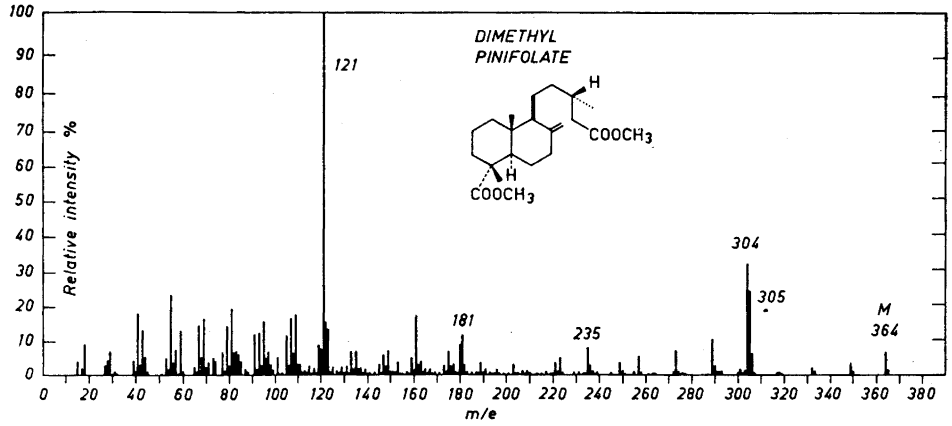


Fig. 2. Mass spectrum of dimethyl pinifolate.

in pyridine to the corresponding oxoester (8), which on Huang-Minlon reduction yielded the acid (9). The cyclohexylamine salt of this acid gave an infrared spectrum identical with that of the corresponding derivative of labd-8(20)-en-15-oate [9] and did not depress the melting point of the latter derivative although the melting points of the two samples were slightly different. The ketoacid (10), obtained on ozonolysis of the acid (9), was identical in all respects (mixed m.p., optical rotation, I.R.) with 20-nor-8-oxo-labdan-15-oic acid [9]. The corresponding oximes were also identical.

The above results settle the structure of pinifolic acid with the exception of the location of the second carboxyl group. The following data are however consistent only with structure (1a) for pinifolic acid. In view of the "finger print" character of mass spectra [10] only the isomer (1b) would be expected to give a mass spectrum (Fig. 2) with all peaks at the same mass numbers as

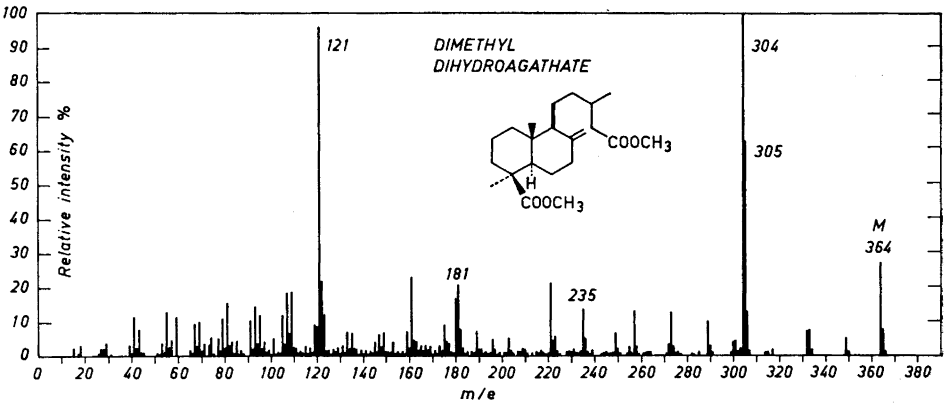


Fig. 3. Mass spectrum of dimethyl dihydroagathate.

those in the spectrum of dimethyl dihydroagathate (Fig. 3). The strong peaks at *m/e* 305 and 304 correspond, respectively, to ions obtained by elimination of the ester group at C(4) and by elimination of this ester group and hydrogen [11,12*]. The peaks at *m/e* 181 and 235 correspond to ions obtained, respectively, from ring A by rupture of the C(6)—C(7) and C(9)—C(10) bonds [13] and from rings A and B by rupture of the C(9)—C(11) bond [11].

Pinifolic acid on titration [14] had the apparent dissociation constants shown in Table 1.

Table 1. Apparent dissociation constants in methylcellosolve/water.

	$pK_1^*{}_{MCS}$	$pK_2^*{}_{MCS}$	$pK^*{}_{MCS}$	Reference
Pinifolic acid (1)	7.24	8.35		Present work
Dihydroagathic acid (4)	7.34	8.85		Present work
Methylsuccinic acid	5.98	8.37		15
Dehydroabietic acid			7.92	16, 17
Agathic acid monomethyl ester (5 b)			8.50	17, 18
Acid (11)			9.49	16

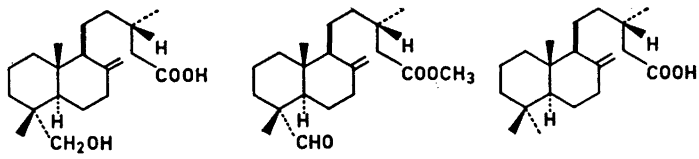
These values clearly eliminate C(10) and C(13) as possible points of attachment of the second carboxyl group. The difference (0.50 pK^*_{MCS}-units) observed between the $pK^*_{2,MCS}$-values for dihydroagathic acid and pinifolic acid is in excellent agreement with the difference (0.50 pK^*_{MCS}-units) calculated by Simon's method [17] on the basis of structures (4) and (1).

Pinifolic acid on treatment with methanol containing catalytic amounts of sulphuric acid gave the dimethyl ester (1b) in nearly quantitative yield while agathic acid (5) on the same treatment only yielded the monoester (5b) [18]. Similarly pinifolic acid gives a dicyclohexylamine salt while dihydroagathic acid yields the monocyclohexylamine salt.

The stereochemistry of pinifolic acid (1) at C(4) follows from the above discussion and at C(5), C(10), C(9), and C(13) from the correlation with labdanolic acid [9] (6). The β-orientation of the side chain at C(9) in labdanolic acid has been deduced from the stability to alkaline treatment of the 20-nor-8-oxoacid (10) obtained on ozonolysis [19] — a proof recently strengthened by the isolation of the less stable isomer of an analogous compound (12) by ozonolysis [20]. Labdanolic acid has been assigned the (S)-configuration [6] at C(13) by analysis of molecular-rotation data [21], the reliability of which is increased by the fact that neither methyl labdanolate nor methyl 13-epilabdanolate exhibit intramolecular hydrogen bonding [22].

The methyl group at C(8) in dihydropinifolic acid is assumed to be β-oriented since the addition of hydrogen should take place preferentially from the sterically less hindered α-side [9].

* Peaks corresponding to ions obtained by removal of the ester group in the side chain of compounds such as methyl cativate, methyl eperuate and methyl labdanolate are absent or of extremely low intensity (below 1 %).



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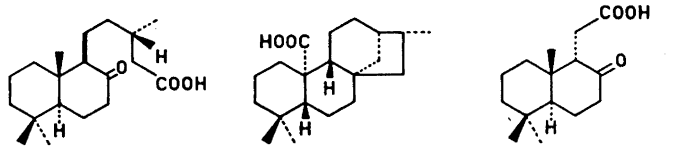

10 11 12

EXPERIMENTAL

Melting points were taken on a Kofler micro hot stage and are corrected. Melting points of the cyclohexylamine salts are dependent upon the rate of heating. Infrared spectra were recorded on a Perkin-Elmer No. 21 instrument (NaCl prism). Ultraviolet spectra were measured on a Beckman recording spectrophotometer, model DK-2. The paper chromatographic studies of the acids were made on dimethyl sulfoxide impregnated papers (Whatman No. 1) using isopropyl ether or ether as solvents [3]. The mass spectra were recorded on an instrument with a heated, all glass inlet system [23] at about 200°. The energy of the electrons was 70 eV. Microanalyses by Dr. A. Bernhardt, Mülheim.

Isolation of pinifolic acid (1a). Needles of *Pinus silvestris* were collected from all parts of the branches in the middle of November. The needles (1 530 g corresponding to 650 g dry material) were covered with acetone and boiled under reflux for 30 min. The needles were removed by filtration, milled in a Wiley-mill and extracted repeatedly (4 × 24 h) with acetone in a Soxhlet apparatus until the concentration of the extract was negligible. The combined acetone solution on cooling gave some greasy material (waxes), which did not contain any pinifolic acid and was subsequently removed by filtration. The acetone solution was concentrated to a small volume and partitioned between water and chloroform. The chloroform phase was extracted with aqueous sodium bicarbonate (10 %). The bicarbonate solution was acidified with dilute sulphuric acid (10 %) and extracted with ether. The ether solution was washed with water, dried (Na_2SO_4) and concentrated to give a lightbrown, partly crystalline residue (2.62 g). One crystallisation from acetone/isopropyl ether gave a paper chromatographically pure product, (1.76 g, 0.27 % of the dry weight of the needles, m.p. 189—191°) which on further recrystallisation gave pure pinifolic acid, m.p. 194—195°, $[\alpha]_D$ + 26° (EtOH, *c* 1.5). (Found: C 71.3; H 9.6; O 19.3; equiv. wt. 167. $C_{20}H_{32}O_4$ requires C 71.4; H 9.6; O 19.0; equiv.wt. 168). The dicyclohexylamine salt had m.p. 164—171°. (Found: N 5.2. $C_{32}H_{58}O_4N_2$ requires N 5.4).

Dihydropinifolic acid (2). Pinifolic acid (60 mg) in ethanol (95 %, 10 ml) was hydrogenated over Adam's catalyst (20 mg) and consumed 1 double bond equivalent of hydrogen in one hour, whereafter the uptake ceased. The catalyst was removed by filtration and the filtrate concentrated to give a crystalline residue which on recrystallisation gave dihydropinifolic acid (47 mg) m.p. 218—219 °, $[\alpha]_D$ + 30° (EtOH, *c* 1.5). (Found: C 70.7; H 10.1. $C_{20}H_{34}O_4$ requires C 71.0; H 10.1).

Dimethyl pinifolate (1b). (*a*) Pinifolic acid (200 mg) on treatment with an ethereal solution of diazomethane (0.5 M, 15 ml) followed by filtration through alumina (neutral activity II, 3 g) gave dimethyl pinifolate (210 mg), n_D^{25} 1.498, $[\alpha]_D$ + 27° ($CHCl_3$, *c* 1.0). (Found: C 72.1; H 10.0. $C_{22}H_{36}O_4$ requires C 72.5; H 10.0). $\nu_{max}^{CCl_4}$ 890, 1 043, 1 060, 1 102, 1 129, 1 147, 1 171, 1 193, 1 232, 1 364, 1 389, 1 437, 1 488, 1 645, 1 727 (broad), 2 880, 2 940, 3 100 cm^{-1}.

(*b*) Pinifolic acid (40 mg), methanol (3 ml) and conc. sulphuric acid (3 drops) were boiled under reflux for 2 h. Dilution with water (20 ml), extraction with ether and filtration of the concentrated ethereal solution through alumina gave dimethyl pinifolate (36 mg), identical with the above product.

Dehydrogenation of pinifolic acid. Pinifolic acid (500 mg) and palladium-charcoal (10 %, 500 mg) were heated at 290° in a small distillation flask. Gas evolution became negligible after 2 h, when 110 ml (3.2 moles/mole acid) had been produced, and the reaction was interrupted. The distillate (*ca.* 5 mg), removed after 10 min, contained according to paper chromatographic results (R_F 0.12 and 0.21, isopropyl ether), acetic and propionic acid. The distillate, (*ca.* 6 mg) removed after 2 h, contained a small amount of 1,2,5-trimethyl naphthalene and an acid, characterised by smell and paper chromatography (R_F 0.47, isopropyl ether) as a caproic acid. The latter was purified by preparative paper chromatography and afforded an acid, $[\alpha]_D$ + 8° (EtOH, *c* 1.0), which gave mass and infrared spectra differing only with respect to the intensity of some peaks from the corresponding spectra of (R)-(−)-3methylpentanoic acid. A third, main fraction (*ca.* 150 mg) was obtained by reducing the pressure to 10 mm and gave on redistillation 1,2,5-trimethyl naphthalene (76 mg) identified by its ultraviolet spectrum, $\lambda_{max}^{light\ petroleum}$ 226 mμ (log ε 4.76), 231 (4.93), 278 (3.78), 289 (3.84), 299 infl. (3.68), 309 (3.18), 324 (3.01). Heilbronner, Frölicher and Plattner [2] give $\lambda_{max}^{light\ petroleum}$ 226 mμ (log ε 4.73), 230 (4.96), 278 (3.78), 288 (3.87), 300 infl. (3.68), 324 (2.96). The additional band at 309 mμ in our spectrum was also observed by Cocker and Halsall [9] at 310 mμ (log ε 3.16).

Dihydroagathic acid. Agathic acid was reduced by adding sodium (3.7 g) to a stirred, refluxing solution of the acid (1.0 g) in butanol (redist., 100 ml) as rapidly as possible. The reaction mixture was boiled under reflux for 45 min, and then water (200 ml) was added and most of the butanol removed by distillation under reduced pressure. The product was diluted with water (300 ml) and extracted with ether, then acidified with dilute sulphuric acid (10 %) and re-extracted with ether to give crude dihydroagathic acid (1.0 g). The crude acid, dissolved in ether (50 ml) was precipitated by addition of cyclohexylamine (2 ml) in ether (10 ml). After one recrystallisation from acetone-methanol the crystalline product (1.10 g) had m.p. 140—180°; on repeated recrystallisation from the same solvent mixture the monocyclohexylamine salt (0.7 g), m.p. 158—188°, $[\alpha]_D$ + 28° (MeOH, *c* 1.2). (Found: C 71.7; H 10.2. $C_{36}H_{66}O_4N$ requires C 71.7; H 10.4) was obtained. Acidification of the salt (300 mg) with dilute hydrochloric acid (10 %) followed by ether extraction gave dihydroagathic acid (4), (230 mg) as a glass, softening point 70—74°, $[\alpha]_D$ + 49° (EtOH, *c* 0.8). (Found: C 71.2; H 9.7. $C_{20}H_{32}O_4$ requires C 71.4; H 9.6). The dimethylester had n_D^{25} 1.4958, $[\alpha]_D$ + 58° ($CHCl_3$, *c* 0.8) $\nu_{max}^{CCl_4}$ 889, 1 031, 1 049, 1 151, 1 195, 1 278, 1 316, 1 331, 1 363, 1 382, 1 437, 1 463, 1 644, 1 720 (broad), 2 840, 2 425, 3 075 cm^{-1}.

18-Hydroxy-labd-8(20)-en-15-oic acid (7). Pinifolic acid dimethyl ester (208 mg), methanol (10 ml) and aqueous potassium hydroxide (1.00 M, 0.60 ml) was left at room temperature (30 min) and then boiled under reflux (15 min). The reaction mixture was cooled, diluted with water (100 ml) and, after addition of aqueous sodium hydroxide (10 %, 3 ml), extracted with ether. The aqueous phase was acidified with dilute sulphuric acid (10 %) and again extracted with ether. The two ether solutions on drying(Na_2SO_4) and evaporation of the solvent gave, respectively, starting material (17 mg) and the paper chromatographically pure monoester (1 c) (183 mg), n_D^{25} 1.4986, $[\alpha]_D$ + 23° ($CHCl_3$, *c* 1.0). This was used in the next step without further purification.

(i) A solution of pinifolic acid monomethyl ester (180 mg) in absolute ethanol (0.5 ml) was added dropwise with stirring to sodium (400 mg) at 130°. Ethanol (4 ml) was then added dropwise over a period of 2 h. After addition of water (2 ml) the reaction mixture was boiled under reflux (1 h), cooled, extracted with ether, acidified with dilute sulphuric acid (10 %) and re-extracted with ether to give a mixture of acids (164 mg). This mixture was chromatographed on silica gel (20 g) to give the hydroxy acid (7) (120 mg) and pinifolic acid (40 mg).

(ii) Pinifolic acid monomethyl ester (630 mg) in dry ether (5 ml) and liquid ammonia (*ca.* 250 ml) was treated with small pieces of lithium until a permanent blue colour was present. The mixture was left for 5 min, then absolute ethanol was added dropwise over a period of 20 min to the solution, which was kept blue by further addition of

lithium. The ammonia was evaporated, the residue diluted with aqueous sulphuric acid (10 %, 100 ml) and extracted with ether. After evaporation of the solvent the residue (556 mg) was adsorbed on silica gel (150 g). Elution with chloroform-ethanol (20:1) gave the paper chromatographically pure hydroxy acid (7), (486 mg), n_D^{25} 1.508, $[\alpha]_D$ + 33° ($CHCl_3$, *c* 0.8), $\nu_{max}^{CCl_4}$ 3 640, 1 703, 1 642, 888 cm^{-1}. (Found: C 74.5; H 10.7. $C_{20}H_{34}O_3$ requires C 74.5; H 10.6). The methylester had n_D^{25} 1.5070, $[\alpha]_D$ + 30° ($CHCl_3$, *c* 1.0).

Methyl 18-oxo-labd-8(20)-en-15-oate (8). The hydroxyester (110 mg) in pyridine (2 ml) was added to chromium trioxide (110 mg) in pyridine (2.5 ml) and the mixture was left overnight at room temperature. Methanol (1 ml) was added and after 1 h the reaction mixture was diluted with aqueous sodium hydroxide (1 %, 50 ml) and extracted with ether. The ether solution was extracted with dilute hydrochloric acid (10 %) and water to give, after drying (Na_2SO_4) and removal of the solvent, a residue (88 mg), which was adsorbed on alumina (3 g, activity II). Elution with light petroleum gave the aldehydo-ester (8), (80 mg), n_D^{25} 1.4988, $[\alpha]_D$ + 31° ($CHCl_3$, *c* 0.9), $\nu_{max}^{CCl_4}$ 2 675, 1 722 (broad).

Labd-8(20)-en-15-oic acid (9). The oxoester (8) (55 mg), hydrazine (95—97 %, 0.3 ml), sodium diethyleneglycolate (from 300 mg sodium) in diethyleneglycol (5 ml) were kept under nitrogen at 160° for 30 min and then at 200—210° for 4 h. The reaction mixture was diluted with water (2 ml), boiled under reflux for 1 h, poured into water (150 ml), acidified with dilute sulphuric acid (10 %) and extracted with ether to give a highly viscous product (40 mg). This was dissolved in ether (5 ml) and gave on addition of cyclohexylamine (0.1 ml) a crystalline precipitate which was removed by filtration, washed with ether and recrystallised from ethyl acetate to give the cyclohexylamine salt (37 mg), m.p. 136—145°, $[\alpha]_D$ + 13° ($CHCl_3$, *c* 0.8), mixed m.p. 135—145° with the cyclohexylamine salt of labd-8(20)-en-15-oate (lit. values m.p. 123—136°, $[\alpha]_D$ + 22°)[9]. The two samples gave identical infrared spectra. Acidification of the salt (37 mg) with dilute hydrochloric acid (10 %) followed by ether extraction gave the acid (8), (27 mg), $[\alpha]_D$ + 24° ($CHCl_3$, *c* 1.0) (lit. value $[\alpha]_D$ + 25°)[9]. The infrared spectra of the two samples of the free acids could not be compared since only a small amount (1.3 mg) of the cyclohexylamine salt of Cocker and Halsall's preparation was available to us.

20-Nor-8-oxo-labdan-15-oic acid. The methyl ester of the acid (9) (25 mg), prepared with diazomethane, was dissolved in methylene chloride and treated with ozone at —70° until the solution was saturated. The excess ozone was removed with dry nitrogen and the solvent evaporated *in vacuo*. The residue was dissolved in a mixture of ethyl acetate (2 ml) and acetic acid (2 ml) and a small amount of activated zinc dust was added. The reaction mixture was stirred for 1 h at room temperature, diluted with water (100 ml) and extracted with ether to give a residue (16 mg) after evaporation. This residue was dissolved in methanol (4 ml) and boiled under reflux for 15 min with aqueous sodium hydroxide (40 %, 1 ml). The solution was diluted with water (50 ml), extracted with ether, acidified and re-extracted with ether to give an acidic material (14 mg), which was absorbed on silica gel (3 g). Elution with chloroform-ethanol (100:1) gave 20-nor-8-oxo-labdan-15-oic acid (10), (11 mg), m.p. and mixed m.p. 107—108° $[\alpha]_D$ —40° ($CHCl_3$, *c* 1.0). (lit. value m.p. 110.5—111° $[\alpha]_D$ —40°)[9]. The infrared spectrum was identical with that of authentic material. Oxime, m.p. and mixed m.p. 184—186° (lit. value 188—189°).

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