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CHEMICAL AND MICROBIOLOGICAL SYNTHESES OF INTERMEDIATES IN GIBBERELLIN BIOSYNTHESIS

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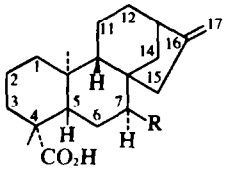
Abstract—A partial synthesis of kaurenoic acid **1** from the hydroxy acid **3** is described. The hydroxylation of the 2'-carboxyethyl ester of **1** by *Gibberella fujikuroi* has been utilized for the synthesis of 7β-hydroxykaurenoic acid **2**. An alternative synthesis of **2** is provided by the microbiological conversion of **3** to the 7β-hydroxy derivative by *Calonectria decora*.

The central role of kaurenoic acid **1**† and 7β-hydroxy-kaurenoic acid **2** in the biosynthesis of gibberellic acid is now well established.[1] The availability of both of these compounds, however, has been restricted. Kaurenoic acid has been obtained from *Gibberella fujikuroi*[2] and from a number of plant sources[3] in limited quantities and routes towards its partial[4] and total synthesis[5] have been developed. A partial synthesis of 7β-hydroxykaurenoic acid **2** from 7β-hydroxykaurenolide was reported[6] and the presence of **2** in *G. fujikuroi* cultures was subsequently established.[6] The metabolism of kaurenoic acid **1** in cell free preparations from immature seeds of *Echinocystis macrocarpa*[7] and *Cucurbita pepo*[8] leads to the formation of **2**. In view of the gibberellin-like activity of these two compounds[7,9] and their importance in the study of gibberellin biosynthesis we have devised methods of partial synthesis which allow the production of each in reasonable yields from more readily available starting materials. Thus the hydroxy acid **3** has been converted to kaurenoic acid **1**.

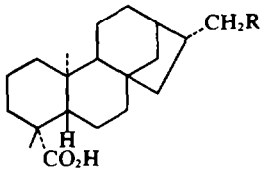
The predilection of *G. fujikuroi* for 7β-hydroxylation of the kaurene skeleton[10] has been employed for the generation of 7β-hydroxykaurenoic acid **2**. An alternative entry to the formation of **2** and the synthesis of C-20 gibberellins is provided by the direct hydroxylation at 7 of the hydroxyacid **3** by *Calonectria decora* and *Rhizopus nigricans*.

RESULTS AND DISCUSSION

The hydroxyacid **3** can be isolated[11] in large quantities from *Beyeria calycina*‡ (2% of the dry weight of the plant) and can be generated readily from the corresponding diacid, which occurs in *Ricinocarpus stylosus* (0·7% of dry weight of plant).[12] The conversion of the hydroxy acid **3** to kaurenoic acid was best achieved as follows. Formation of the tosylate **4** and treatment of this with KI/acetone proceeded smoothly to give the 17-iodo derivative **5**. The latter on treatment with KOBu^t in DMSO at 80° for 1 h afforded kaurenoic acid in 60% yields. More forcing conditions led to a mixture of endo- and exocyclic double bond isomers. Overall yields of 50% of kaurenoic acid from the hydroxy acid **3** can be obtained.



1: R = H
2: R = OH



3: R = OH
4: R = OTs
5: R = I

The observation[10] that *G. fujikuroi* can hydroxylate some kaurene derivatives at position 7 suggested a method for the production of 7β-hydroxykaurenoic acid **2**. Although this compound arises directly from hydroxylation of kaurenoic acid in *G. fujikuroi* cultures, it does not accumulate but is converted into the normal gibberellin metabolites of this mould.[1] Protection of the 19-carboxylic acid group in **1** was expected to

†Substituents which are above or below the plane of the paper are referred to as β- or α. Systematically these substituents will be *ent*-α and *ent*-β- (*The Common and Systematic Nomenclature of Cyclic Diterpenes*, 3rd revision, ed. Dr. J. W. Rowe, Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wisconsin).

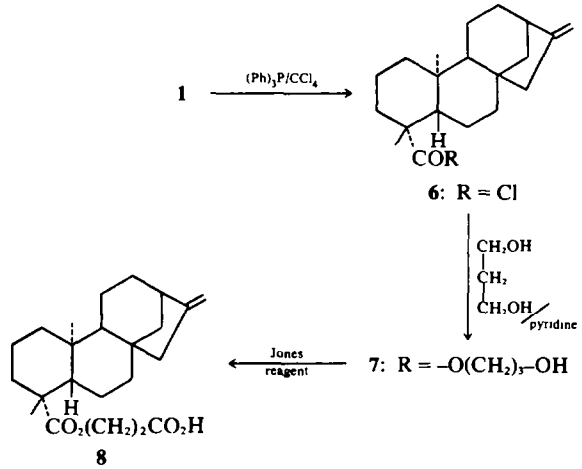
‡Previously[12] referred to as "a new Beyeria species". This species has now been classified: H. K. Airy Shaw, *Kew Bulletin* **20**, 67 (1971) No. 1, Royal Botanic Gardens, Kew, London.

inhibit conversion into the gibberellin metabolites after the initial hydroxylation. The β-hydroxypropionic acid derivative **8** was considered a suitable substrate and was synthesised as shown in Scheme I. Preparation of the acid chloride **6** was achieved by a modification of the method[13] using $(Ph)_3P/CCl_4$. Whereas the conventional method led to mixtures of products in which the double bond was either isomerised or hydrochlorinated, heating the two reagents prior to the addition of the kaurenoic acid gave good yields of the acid chloride **6**. This was treated directly with 1,3-propanediol in pyridine to give the 3′-hydroxypropanyl ester **7** which was oxidized with Jones reagent to the corresponding acid **8**.

Incubation of **8** with the mycelium of *G. fujikuroi* for 7 days yielded, after saponification of the products isolated, two major metabolites which were identified by comparison of their physical properties with those reported for 7β-hydroxy-[6] and 6β,7β-dihydroxykaurenoic acid[14] (**2** and **9** respectively). Confirmation of the structure of the metabolites was obtained by direct comparison of the products from $LiAlH_4$ reduction of their methyl esters with authentic samples of 7β,19-dihydroxy- and 6β,7β,19-trihydroxykaurene. Reasonable yields of **2** can be obtained in the hydroxylation step (38%) and the sequence provides a simple method for the preparation of 7β-hydroxykaurenoic acid **2**. This method may be compared with that[15] in which kaurenoic acid is directly hydroxylated at the 7β-position by *Rhizopus nigricans* in 25% yield.

The ability of some microorganisms to hydroxylate kauranes[15] encouraged us to screen some of these with the hydroxyacid **3**. *Calonectria decora* and *R. nigricans* to different extents utilized the substrate whereas *Aspergillus ochraceous* did not. Incubation of the hydroxy acid **3** with *Calonectria decora* yielded two major metabolites, the less polar of which was obtained by fractional crystallization from acetone. The metabolite **10** analysed for $C_{20}H_{32}O_4$ and showed an M^+ in its mass spectrum at *m/e* 336 expected for the introduction of an oxygen atom. The NMR spectrum of **10** showed signals for a carbinol methine proton at δ 3·87 (br s) which shifted to δ 4·77 in the NMR spectrum of the diacetate **11**. The $w_{1/2}$ of this signal (5·5 Hz) suggested an equatorial proton. Location of the OH group at 7 was implied from a consideration of the NMR spectral data of **10**, **11**, and the ketodiester derivative **12** obtained by Jones oxidation of **10** and subsequent methylation. Position 1 and 12 for the OH group could be excluded since the corresponding keto diesters have been reported[16,17] and have different properties to those of **12**. Bromination of the keto diester **12** gave a monobromoketone **13** the NMR spectrum of which showed a bromomethine proton as a doublet at δ 5·38 (J 6 Hz). The β-configuration of the Br atom in **13** can be assigned from a consideration of the spectroscopic data. The bromoketone **13** had IR maxima at 1730 cm^{-1} (CO absorption) similar to that (1725 cm^{-1}) of the parent ketone **12** and a UV maximum at 322 nm (ε 100) shifted from 285 nm (ε 30) in the latter. These data point to the Br atom being out of plane of the CO group. Furthermore, bromination results in an upfield shift of the C-10 Me of 27 Hz. This can be explained by assuming that the methyl group falls in the shielding zone of the 7-CO group which is possible if the B-ring attains a boat conformation.

The proximity of the Br and the C-4 βMe group is indicated by the deshielding effect of 15 Hz on the latter. Consistent with these results the coupling between the 6-H and 5-H of 6 Hz indicates a dihedral angle of 150°. This result eliminates positions 2, 3, 6, 14 and 15 as sites of hydroxylation leaving only 7 and 11 as possibilities. A decision in favour of 7 was made following decoupling experiments on the bromomethine proton signal.



SCHEME 1

These indicated that the proton was part of an AB system both protons being coupled only to each other. Attempts to dehydrobrominate not unexpectedly[18] led to the formation of the α-keto-γ-lactone **14**.

Confirmation of the proposed structure was obtained by correlation with authentic 7β-hydroxykaurenoic acid as shown in Scheme II.

The more polar metabolite was assigned the 7α-hydroxy structure **15** since oxidation with Jones reagent and methylation afforded a compound identical with the keto diester **12**. The major metabolite (20%) obtained from incubation of **3** with *R. nigricans* was identified by comparison with **10**.

The efficient utilization of the hydroxy acid **3** by *C. decora* allows an alternative entry to the production of 7β-hydroxykaurenoic acid if the sequence shown in Scheme I is undertaken with **10**. Furthermore the keto lactone, **14** could be utilized for the synthesis of C-20 gibberellin analogues.[19]

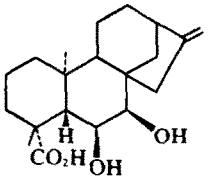
EXPERIMENTAL

General experimental details are as described previously.[20]

Synthesis of ent-kaur-16-en-19-oic acid **1**. The hydroxyacid **3** (30 g) in pyridine (400 ml) at 5° was treated with toluene-p-sulphonyl chloride (30 g) and the mixture was allowed to stand for 18 h at 5–10°. The material recovered was crystallized from $CHCl_3$-light petroleum as prisms of the tosylate **4** (34·3 g), mp 151–3°, and 190–190·5°, $[\alpha]_D$ − 51° (*c*, 0·4). (Found: C, 68·67; H, 8·37. $C_{27}H_{38}O_5S$ requires: C, 68·32; H, 8·06%). NMR (60 MHz: $CHCl_3$) δ: 7·65 (AA'BB': aromatic protons), 3·82 (d, *J* 7 Hz, 17-H_2), 2·5 (s, aromatic methyl), 1·2, 0·92 (s, 18- and 20-H_3). The tosylate **4** (33 g) in acetone and NaI (30 g) was heated under reflux for 48 h. The mixture was filtered, the acetone removed and the residue crystallized from $CHCl_3$-light petroleum to give the iodide **5** (24·8 g), mp 157–160°. NMR δ: 3·08 (d, *J* 7 Hz, 17-H_2), 1·25 and 0·92 (s, 18- and 20-H_3). The iodide **5** (10 g) in dry DMSO (400 ml) and KOBu^t (30 g) was heated at 80° for 1 h. The reaction mixture was cooled acidified and extracted with ether. The recovered product (6·6 g) was recrystallized from ether-pentane to give needles of *ent*-kaur-16-en-19-oic acid **1** (4·1 g), mp 175–177° (lit.[12] 169–171°, 179–181°) identical with authentic sample. When the reaction was attempted with KOBu^t/Bu^{t}OH a mixture (1:4) of starting material and kaurenoic acid was obtained. Temperatures above 85° in the above method afforded a mixture containing a small amount of the endocyclic double bond isomer.

2′-Carboxyethyl-ent-kaur-16-en-19-oate **8**. $P(Ph)_3$ (8 g) and CCl_4 (50 ml) were heated under reflux for 5 h under a N_2 atmosphere. Kaurenoic acid **1** (2 g) was added to the cooled solution and the reaction mixture heated under reflux for 0·5 h. After evaporation of the solvent a solution of 1,3-propanediol (25 ml) in pyridine (40 ml) was added to the residue, containing the unstable acid chloride **6** and the mixture left for 24 h at room temp. The product recovered in ether contained a mixture of $(Ph)_3PO$ and the 3′-hydroxypropanyl ester **7**. This mixture was dissolved in acetone (50 ml) and treated with Jones reagent (3·5 ml) at 0°. The reaction mixture was diluted with water, extracted with ether and the ether layer washed with 5% KOH. Recovery of the acid fraction gave a yellow oil (1·9 g) which was adsorbed on a column of silicic acid (50 g). Elution with 60–80% $CHCl_3$-light petroleum gave the acid **8** (980 mg), $[\alpha]_D$-66° (*c*, 1·0) M^+ observed 374·24532; $C_{23}H_{34}O_4$ requires 374·24569. MS: *m/e* at 374, 359, 330, 315, 302 (base peak) 287. NMR ($CHCl_3$: 60 MHz) δ: 4·73 (*m*, $w_{1/2}$ 8 Hz, 16-H_2), 4·30 (t, *J* 7 Hz, 1′-H_2), 2·68 (t, *J* 7 Hz, 2′-H_2), 1·15 (s, 19-H_3), 0·85 (s, 20-H_3).

Incubation of **8** *with G. fujijuroi*. The method was essentially that described previously.[21] In this case the



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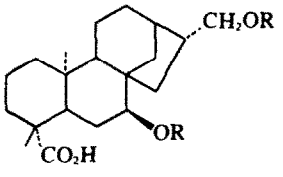
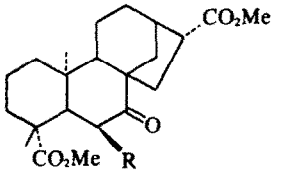
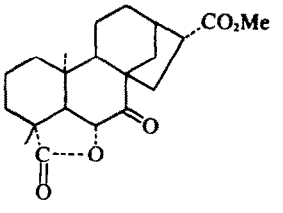


10: R = H
11: R = Ac



12: R = H
13: R = Br



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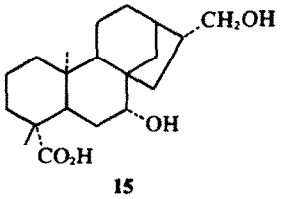

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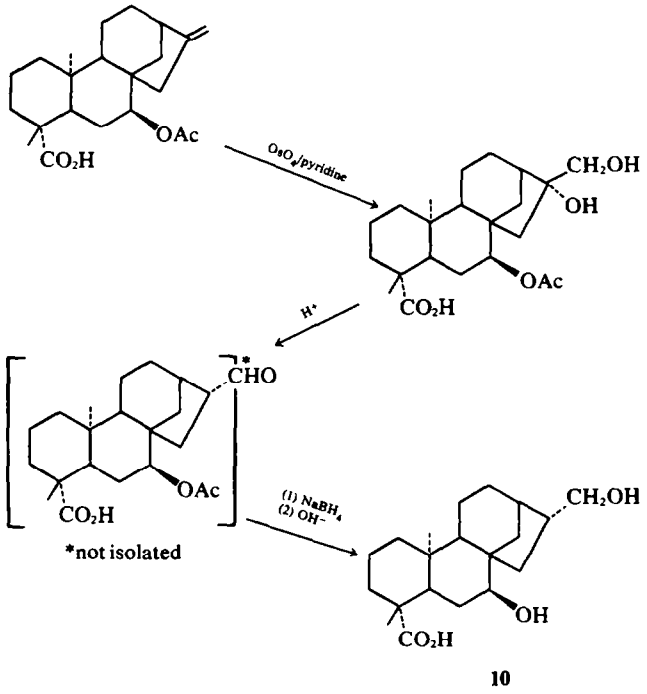

SCHEME 2

mycelium of 6 day-old *G. fujikuroi* was resuspended in 800 ml of medium (pH 7) and the substrate (120 mg) in ethanol was introduced. The metabolism was monitored after 3 and 7 days, better conversions being obtained after 7 days. The mycelium and the medium were extracted with ethyl acetate and the combined extracts washed with 5% Na_2CO_3. The acids recovered were heated with aq KOH (10%, 150 ml) for 1 h to remove the β-hydroxypropionic acid residue. The ether-soluble fraction consisted of two major products which were separated by preparative tlc (MeOH: $CHCl_3$; 1:3). (a) The less polar component (40 mg) was recrystallized from acetone-light petroleum as needles of *ent*-7α-hydroxykaur-16-en-19-oic acid **2**, mp 254–258° (lit.[6] 255–8°). NMR data were identical with those reported.[6] The hydroxyacid **2** (50 mg) was methylated with ethereal diazomethane and the product in diglyme (20 ml) was treated with $LiAlH_4$ (200 mg) and stirred for 4 h at room temperature. The recovered product (30 mg) was recrystallized from acetone-light petroleum as prisms, mp 186–189°, undepressed on admixture with an authentic sample of *ent*-7α,19-dihydroxykaur-16-ene. (b) The more polar component (15 mg) was recrystallized from acetone as prisms of *ent*-6α,7α-dihydroxykaur-16-en-19-oic acid **9** mp 232–235° (lit.[14] 234–236°, 234–237°). The NMR and IR spectra were comparable to those reported. The dihydroxyacid **9** (5 mg) was methylated and the methyl ester on reduction with $LiAlH_4$ yielded a compound (3 mg) which crystallised from acetone-light petroleum as needles, mp 189–191° undepressed on admixture with an authentic sample of *ent*-6α,7α,19-trihydroxykaur-16-ene.

Metabolism of ent-17-hydroxykauran-19-oic acid **3**. (a) *C. decora*: Culture medium (5 × 400 ml) was inoculated with spores of *C. decora* and shaken for two days. The hydroxyacid **3** (1 g) in ethanol (50 ml) and progesterone (50 mg) was added in two portions, 6 h elapsing between additions. The flasks were shaken for three days after which the mycelium was filtered off and extracted with ethyl acetate. The aqueous medium was extracted with ethyl acetate and the combined washings were dried over $MgSO_4$. Evaporation of the solvent gave an oily residue (1·1 g) which after dissolving in acetone precipitated crystals of *ent*-7α,17-*dihydroxykauran*-19-*oic acid* **10** (200 mg) which recrystallized from acetone as prisms, mp 250–1°, $[\alpha]_D$-51° (*c*, 0·6). (Found: C, 71·02; H, 9·57. $C_{20}H_{32}O_4$ requires: C, 71·39; H, 9·59%). ν_{max}^{Nujol} 3400–3200 (OH), 3000, 1680 cm^{-1} (CO_2H). MS: *m/e* 336 (M^+: 10%), 318 (100), 300 (12), 287 (12), 182 (20), 168 (80), 164 (95), 151 (50). NMR (C_5D_5N: 90 MHz) δ 3·87 (br, s, $w_{1/2}$ 5·5 Hz, 7-H), 3·70 (d, J 7 Hz, 17-H_2), 1·41 (s, 18-H_3), 1·23 (s, 20-H_3).

Preparative TLC on the mother liquors afforded a further amount of **10** (50 mg) and *ent*-7β,17-*dihydroxykauran*-19-*oic acid* **15** (150 mg) which crystallized from acetone-light petroleum as prisms, mp 230-2°, $[\alpha]_D^{EtOH}$-83° (*c*, 0·6) (Found: C, 71·18; H, 9·90. $C_{20}H_{32}O_4$ requires: C 71·39; H, 9·59%). ν_{max}^{Nujol} 3360 (OH), 3000 and 1680 cm^{-1} (CO_2H). MS: *m/e* 336 (M^+; 5%), 318 (100), 300 (22), 272 (25), 205 (20), 164 (40), 123 (90). NMR (C_5D_5N: 60 MHz) δ 3·68 (d, J 7 Hz, 17-H_2), 3·60 (br. s, $W_{1/2}$ 15 Hz, 7-H), 1·32 (s, 18-H_3), 1·15 (s, 20-H_3). (*b*) *R. nigricans*: Similar incubation of **3** with *R. nigricans* afforded **10** in 20% yield.

Derivatives of **10**.

(a) The dihydroxy acid **10** (40 mg) was treated with pyridine/Ac_2O overnight. The product recovered was crystallised from $CHCl_3$-light petroleum as needles of the *diacetate* **11** (35 mg), mp 148–150°, $[\alpha]_D^{CHCl}$-23° (*c*, 0·2), (Found: C, 68·29; H, 8·51. $C_{24}H_{36}O_6$ requires: C, 68·54; H, 8·63%). ν_{max} 1725 cm^{-1} (acetate) MS: *m/e* 378 (M^+; 8%),

360 (42), 314 (20), 301 (40), 300 (100), 285 (18), 259 (25). NMR ($CHCl_3$; 60 MHz) δ: 4·77 (br. s, $W_{1/2}$ 5·5 Hz, 6-H), 3·87 (d, 17-H_2), 2·08 (s, 2OCOCH_3), 1·18 (s, 18-H_3), 0·98 (s, 20-H_3). (b) The dihydroxy acid **10** (60 mg) in acetone was titrated with a slight excess of Jones reagent at room temperature. The compound recovered with ether was methylated with CH_2N_2. The *dimethyl ester* **12** obtained was crystallized from MeOH-H_2O as needles, mp 119–120°, $[\alpha]_D$-59° (*c*, 0·3). (Found: C, 70·47; H, 8·38. $C_{22}H_{32}O_5$ requires: C, 70·18; H, 8·57%). $\nu_{max}^{CS_2}$ 1725 (ketone) and 1690 cm^{-1} (ester carbonyl). λ_{max}^{EtOH} 285 nm (ϵ 30) MS: *m/e* 376 (M^+, 75%), 344 (18), 317 (83), 316 (100), 284 (15), 256 (15), 209 (15), 190 (15), 167 (18). NMR ($CHCl_3$: 60 MHz) δ; 3·70 (s, 2x-OCH_3), 1·19 (s, 18-H_3), 1·5 (s, 20-H_3). Similar oxidation of *ent*-7β,17-dihydroxykauran-19-oic acid **15** gave a compound which after methylation proved identical with the keto diester **12**, mp and mixed mp 119°.

Bromination of the keto diester **12**. The compound **12** (50 mg) in $CHCl_3$ (5 ml) was treated with an excess of Br_2 in acetic acid for 2 h. The compound recovered crystallised from ether-light petroleum as needles of dimethyl-*ent*-6α-*bromo*-7-*oxo*-*kauran*-17,19-*dioate* **13** (35 mg), mp 150–152°, $[\alpha]_D^{CHCl_3}$ + 47° (*c*, 1·0). (Found: C, 57·94; H, 7·08. $C_{22}H_{31}O_5Br$ requires: C, 58·01; H, 6·87%). ν_{max}^{EtOH} 322 nm (ϵ 100). MS: *m/e* 454,456 (M^+: 5%), 376 (48), 375 (100), 358 (10), 316 (30), 303 (15), 255 (10). NMR ($CDCl_3$: 90 MHz) δ 5·38 (d, J 6 Hz, 6-H), 3·67 (s, 2x-OCH_3), 2·34 (d, J 6 Hz, 5-H), 1·44 (s, 18-H_3), 0·57 (s, 20-H_3).

Formation of lactone **14** *from* **13**. The bromo keto diester **14** (50 mg), LiCl (100 mg) and DMF (10 ml) was refluxed for 2 h. The product recovered crystallized from acetone-light petroleum as needles of the *lactone* **14** (30 mg), mp 200–202°, $[\alpha]_D^{CHCl_3}$ + 16° (*c*, 0·2). (Found: C, 69·34; H, 7·82. $C_{21}H_{28}O_3$ requires: C, 69·97; H, 7·83%). $\nu_{max}^{CS_2}$ 1780 (5 membered ring lactone) 1730 cm^{-1} (carbonyl). MS: *m/e* 360 (M^+: 5%), 317 (50), 316 (100), 257 (20), 165 (23), 137 (90), 125 (95), 123 (95), 123 (95). NMR ($CHCl_3$: 60 MHz) δ: 4·83 (d, J 6·5 Hz, 6-H), 3·68 (s, –OCH_3), 1·30 (s, 18-H_3), 0·71 (s, 20-H_3).

Synthesis of ent-7α,17-*dihydroxykauran*-19-*oic acid* **10**. The acid **2** (50 mg) was acetylated with Ac_2O/pyridine and the acetate (55 mg) treated with OsO_4 in pyridine overnight. The compound recovered (50 mg) showed M^+ and *m/e* 394 and a base peak at *m/e* 363 (M^+–CH_2OH). Without purification the diol was dissolved in THF containing conc. H_2SO_4 (0·5 ml) and heated under reflux for 0·5 h. The compound recovered was dissolved in ethanol and treated with $NaBH_4$ overnight. Treatment of the recovered hydroxy acetate in ethanolic NaOH and work up gave a compound which was purified by preparative TLC and crystallised from acetone as prisms, mp 248–250°, identical with **10** (NMR, MS, TLC, mp and mixed mp).

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