42. Absolute Configuration of a Diterpene Lactone from *Parinari capensis*

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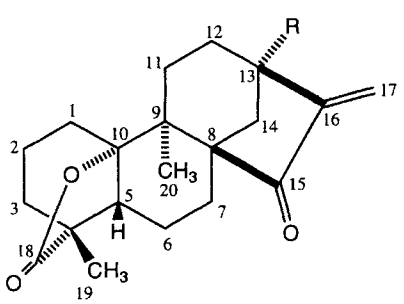
Phytochemical investigation of *Parinari capensis* (Chrysobalanaceae) led to the isolation of three diterpene lactones **1**–**3**. The structures of these compounds have been established by NMR spectroscopy and X-ray crystal-structure analysis. Addition of bromine onto the exocyclic methylidene group of **1** yielded the brominated derivative **1-Br**. X-Ray analysis of **1-Br** established unambiguously the absolute configuration, and **1** was then identified as the (4*R*,9*R*)-10-hydroxy-13-methoxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic acid γ-lactone. Diterpenes **2** and **3** were identified as the C(13)-demethoxylated and the C(13)-demethylated derivatives of diterpene **1**, respectively. Antifungal activity against *Cladosporium cucumerinum* was determined for **1**–**3**.

1. Introduction. – In the course of a systematic biochemical investigation on Zimbabwean plants used in traditional medicine, the Chrysobalanaceae species *Parinari capensis* HARV. has been investigated. The roots of this tropical bush are used by traditional healers in Zimbabwe to treat delirium [1]. In a series of preliminary screenings for different kinds of activity, it has been shown that the CH_2Cl_2 extract of the whole plant of *P. capensis* displayed interesting activity against the development of spores of the plant pathogenic fungus *Cladosporium cucumerinum* in a biautographic TLC assay [2]. Activity-guided fractionation afforded three diterpene lactones **1**–**3**.

The structure determination of compounds **1**–**3** was mainly achieved by the use of 1D- and 2D-NMR analysis, including COSY, HMBC, and HMQC experiments. While this study was in progress, a report on another Zimbabwean species of the genus,



1 R = MeO
2 R = H
3 R = OH

Parinari curatellifolia, was published [3]. This describes isolation of the same three compounds, two of which, **1** and **3**, were new naturally occurring products. However, configuration was not precised, and their ^{13}C-NMR attribution was not accurate. Therefore, we report here some details on structure elucidation, including full ^{13}C-NMR attribution, determination of the absolute configuration, together with the antifungal activity of these compounds.

2. Results. – Dried and powdered whole plant was extracted at room temperature with CH_2Cl_2. Fractionation on the CH_2Cl_2 extract by MPLC on silica gel and further purification of the fractions of silica-gel columns or by gel filtration *Sephadex LH-20* afforded compounds **1**–**3**. The EI-MS of **1** exhibited a $[M]^+$ peak at *m/z* 344 in agreement with the formula $C_{21}H_{28}O_4$. The ^{13}C-NMR ($CDCl_3$) spectrum indicated the presence of a ketone at 208.0, a carboxyl function at 180.3, as well as an exocyclic methylidene group at 147.3 (quaternary C-atom) and at 116.4 (=CH_2) ppm. In the ^{1}H-NMR, no aromatic protons were present; however, two olefinic protons were clearly visible at 5.38 and 6.14 ppm. From these data, **1** was assigned as a kaurenoid diterpene.

In the UV spectrum, one band (λ_{max} at 227 nm) suggested the ketone group to be conjugated with the exocyclic methylidene group on the five-membered ring. On the other hand, a γ-lactone moiety present near a quaternary C-atom (IR: 1764 cm^{-1}; ^{13}C-NMR: 87.2 ppm) indicated that **1** was a modified kaurenoid with the Me group at C(10) shifted to C(9) [4]. It was, therefore, identified as the 10-hydroxy-13-methoxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic acid γ-lactone. Further 2D-NMR experiments (COSY, HMBC and HMQC) allowed us the full assignment of the C-atoms (*Table*). Crystals obtained from hexane/AcOEt were subjected to X-ray analysis (*Fig. 1*). The structure of **1** was thus confirmed and relative configurations at C(4), C(5), C(8), C(9), C(10), and C(13) were determined.

Table. *^{13}C-NMR Chemical Shifts* [ppm][a]) *of Diterpenes* **1**–**3**

C-Atom	1	2	3	C-Atom	1	2	3
C(1)	31.0	30.9	30.9	C(12)	34.5*	30.0*	36.0*
C(2)	19.9	20.0	19.9	C(13)	79.6	37.4	75.0
C(3)	35.1	35.3	35.1	C(14)	40.4*	29.3*	46.5*
C(4)	47.3	47.5	47.4	C(15)	208.0	210.7	208.3
C(5)	51.7	51.8	51.7	C(16)	147.3	148.9	151.2
C(6)	18.0	18.1	18.0	C(17)	116.4	115.1	115.6
C(7)	25.3	25.5	25.1	C(18)	180.3	180.6	180.7
C(8)	54.2	52.0	54.4	C(19)	16.9	17.0	16.9
C(9)	42.9	43.5	42.7	C(20)	18.4	18.5	18.5
C(10)	87.2	87.8	87.5	C(21)	50.0		
C(11)	31.5	39.0	31.9				

[a]) In $CDCl_3$. * Assignements interchangeable in each column.

The absolute configuration of **1** was determined by the technique of anomalous dispersion. The C(16)=C(17) bond was brominated, leading to the compound **1-Br** (see *Exper. Part*) whose crystal structure was determined. The anomalous dispersion effect for bromine with MoK_α radiation is significant enough as $\Delta f' = -0.347$, and $\Delta f'' =$

2.456 [5]. By measuring a complete set of *Friedel* pairs (that is, the intensity of reflections h,k,l at 2Θ, and $-h$, $-k$, $-l$ at -2Θ) for **1-Br**, it was possible to refine the *Flack* absolute structure factor x [6] [7], which have a value of $-0.004(11)$. This indicated that the coordinates correspond to the absolute structure of the molecule (x must be zero, within 3 e.s.d., for the correct enantiomorph). Thus, **1** was finally determined as (4*R*,9*R*)-10-hydroxy-13-methoxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic acid γ-lactone.

The completion of bromination was checked by EI-MS and DCI-MS. The presence of the two Br-atoms was clearly visible in the DCI/MS with $[M + NH_4]^+$ signals at m/z 520, 522, and 524. On the other hand, only $[M - Br]^+$ ions were exhibited by EI/MS at m/z 423 and 425.

The structures of diterpenes **2** and **3** were established by the same means as for **1**. Lack of the MeO unit and slight differences in the ^{13}C-NMR spectrum of **2** were in good accordance with the structure of the known 15-oxozoapatlin [4], previously isolated from *Viguiera maculata*, an Asteraceae from Mexico. An X-ray analysis undertaken on **2** confirmed the structure as the 10-hydroxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic acid γ-lactone.

EI-MS of compound **3** exhibited a major $[M]^+$ base peak at m/z 330 (14 amu less than **1**). This corresponded to a demethylated derivative of **1**. Further proofs were given by ^{13}C-NMR where signals for the C-atoms C(11)–C(14) were modified with respect to those recorded for **1**. Finally, a series of selective-INEPT experiments established clearly the structure of **3** to be the 10,13-dihydroxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic acid γ-lactone.

Bromination of **2** and **3** were not undertaken. All three compounds have a negative $[\alpha]_D$, and, on a biogenetic evidence, the two diterpenes **2** and **3** should have the same absolute configuration (4*R*,9*R*) as **1**.

The antifungal activity against the phytopathogenic fungus were determined by dilution assays using solid media [8]. In comparison with a blank, compounds **1** and **2** inhibited the growth of the fungus *Cladosporium cucumerinum* within 20 $\mu g\ ml^{-1}$, while **3** was the least active compound with a limit of activity at 100 $\mu g\ ml^{-1}$. In the determination of MIC values, the known antifungal compound amphotericine B was used as positive control and inhibited the growth of the microorganism with 1 $\mu g\ ml^{-1}$.

3. Discussion. – Activity-guided fractionation afforded three fungitoxic diterpenes from *Parinari capensis*. During this work, an American group working on the research of cytotoxic compounds isolated the same three active compounds from *Parinari curatellifolia*, also collected in Zimbabwe.

These results are interesting from a chemotaxonomic point of view, since, up to now, only works on fatty acids in some species of the genus *Parinari* were carried out [9–11]. The presence of the same three diterpene lactones, **1**–**3**, in these two species of *Parinari* is noteworthy, since this type of kaurene derivatives seems up to now to be restricted to Asteraceae species [12], particularly in the Heliantheae tribe [4] [13] [14].

The bioactivity of these compounds should also be considered. These diterpene lactones seems to have a broad spectrum of activities, both on microorganisms such as *Cladosporium cucumerinum*, and on a panel of cultured human cell cancer cell lines [3]. Since larvicidal activity for kaurenoic-acid derivatives have been reported in the literature [15] [16], compounds **1**–**3** were then submitted to preliminary larvicidal tests against

the larvae of *Aedes aegypti*, the vector of yellow fever. In these tests [17], these products did not show any significant larvicidal activity (up to 500 ppm for **1** after 24 h; **2** and **3** were inactive in all dilution tested).

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Experimental Part

General. M.p.: *Mettler-FP-80/82* hot stage apparatus, uncorrected. α_D: *Perkin-Elmer-241* polarimeter; $[\alpha]_D^{23}$ (solvent used; *c* in g sample in 100 ml solvent). UV: *Varian DMS 100* spectrophotometer, recorded in MeOH; λ_{max} (log ε). TLC: Silica gel $60F_{254}$ Al sheets (*Merck*), detection at 254 nm and with *Godin* reagent [18]; solvent systems for TLC. $CHCl_3$/MeOH 9:1 (*A*), $CHCl_3$/AcOEt 2:1 (*B*). Medium-pressure liquid chromatography (MPLC): home-packed silica gel *60* (15–40 μm, 500 × 36 mm, *Merck*). Column chromatography (CC): silica gel *60* (40–63 μm, 750 × 25 mm i.d., *Merck*) and *Sephadex LH-20 (Pharmacia)*. Anal. HPLC has been carried out on *HP-1090* instrument equipped with a photodiode array detector. Fractions were analyzed on *Nova-pak* C_{18} columns (4 μm, 150 mm × 3.9 mm, i.d., *Waters*) with a gradient of MeCN 5–70% in 30 min, at a flow rate of 1 ml min^{-1}. IR Spectra: *Perkin-Elmer 1600 FTIR* spectrophotometer; *v* in cm^{-1}. ^{1}H- and ^{13}C-NMR: *Varian VXR 200* (200.06 MHz and 50.3 MHz, resp.) and *Varian VXR 500* (499.870 MHz and 125.704 MHz, resp.); in $CDCl_3$; chemical shifts in ppm as δ rel. to Me_4Si as internal standard, *J* in Hz. Complete attribution has been performed on the basis of 2D experiments (COSY, HMBC, HMQC, and selective INEPT). MS: *Finnigan-MAT-TSQ-700* triple stage quadrupole instrument; *m/z* (rel. intensity in %); EI-MS (ionization energy 70 eV) and DCI-MS (NH_3, positive ion mode). Bioassays: dilution assays were carried out in *LB* media (*Luria-Bertani*) for *Cladosporium cucumerinum*. The pure compounds were assayed at 1, 10, 50, and 100 μg ml^{-1}. Larvicidal activity: The pure compounds were solubilized in DMSO with a maximum of concentration of 50 μg/μl (for a final test soln. of 500 ppm). DMSO Solns. (100 μl) were added to 9.9 ml of tap water. 20 s instar larvae of *Aedes aegypti* were introduced into the test solns., and mortality was evaluated visually after 30 min and 24 h [17].

Plant Material. Whole plants of *Parinari capensis* HARV. were collected in Skyline, near Harare. A voucher specimen has been deposited at the National Herbarium of Zimbabwe, Causeway, Harare.

Extraction and Isolation. At r.t., 288 g of dry powdered whole plant were extracted with CH_2Cl_2 (3 × 2500 ml) to afford 6.5 g of extract. A portion of that extract (5.5 g) was fractionated by MPLC on SiO_2 into 18 fractions (I–XVIII) with a step-gradient elution (petroleum ether/AcOEt, 9:1 → 1:2) and finally MeOH. Compound **1** (380 mg) was obtained from *Fr. VII* by gel filtration on *Sephadex LH-20* ($CHCl_3$/MeOH 1:1). Fractionation of *Fr. V* on silica gel using $CHCl_3$/AcOEt 9:1 afforded pure **2** (220 mg). *Fr. XV* was subjected to gel filtration on *Sephadex LH-20* ($CHCl_3$/MeOH 1:1), and afforded 5 fractions (XV.1–XV.5). Compound **3** (35 mg) was obtained from *Fr. XV.2* by gel filtration on *Sephadex LH-20* ($CHCl_3$/MeOH 1:1).

(4R,9R)-10-Hydroxy-13-methoxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic Acid γ-Lactone (**1**). Colorless crystals from hexane/AcOEt. M.p. 145–148°. TLC(*A*): R_f 0.78(*B*), R_f 0.80. $[\alpha]_D^{23} = -51.2$($CHCl_3$; *c* = 1.010). UV: 227(3.96). IR: 2932*m*, 1764*s*, 1720*s*, 1641*m*, 1441*m*, 1376*m*, 1280*m*, 1248*m*, 1211*m*, 1134*m*, 1114*m*, 975*m*, 938*m*, 579*w*, 409*w*. ^{1}H-NMR ($CDCl_3$): 6.14(*s*, H_a–C(17)); 5.38(*s*, H_b–C(17)); 3.26(*s*, MeO(21)); 2.52(*dd*, *J* = 4.2, 13.9, H–C(5)); 1.28(*s*, CH_3(20)); 1.11(*s*, CH_3(19)). ^{13}C-NMR ($CDCl_3$; see *Table*). EI-MS: 344 (100, $[M]^+$), 301 (35), 300 (59), 241 (29), 91 (47).

Crystallographic Data for Compound **1**. $C_{21}H_{28}O_4$, orthorhombic, space group $P2_12_12_1$, *a* = 6.9563(6), *b* = 10.2274(7), *c* = 25.748(3) Å, *Z* = 4, 3768 reflections measured, 3222 independent reflections (R_{int} = 0.027), 2812 observed reflections [$I > 2\sigma(I)$], final *R*1 = 0.0431, *Rw*2 = 0.0888, Goodness of fit 1.115, residual density max/min 0.137/ – 0.128 e $Å^{-3}$. Absorption coefficient μ = 0.085 mm^{-1}; no correction for absorption was applied.

Suitable crystals of **1** were grown from hexane/AcOEt as colorless square rods. Intensity data were collected at r.t. on a *Stoe AED2* four-circle diffractometer using MoK_α graphite monochromated radiation (λ = 0.71073 Å) with $w/2\Theta$ scans in the 2Θ range 5–51°. The structure was solved by direct methods using the programme SHELXS-86 [19]. The refinement and all further calculations were carried out using SHELXL-93 [20]. All of the H-atoms were included in calculated positions and allowed to ride on the corresponding C-atom. The non H-atoms were refined anisotropically, using weighted full-matrix least-squares on F^2.

The bond lengths and angles are normal within experimental error. No attempt was made to determine the absolute configuration of the molecule. Full tables of atomic parameters and bond lengths and angles may be

obtained from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB2 1EZ (UK) on quoting the full journal citation. The molecular structure and crystallographic numbering scheme of **1** is illustrated in *Fig. 1* drawn using Xtal GX [21]. Further details may be obtained from *H. St-E.*

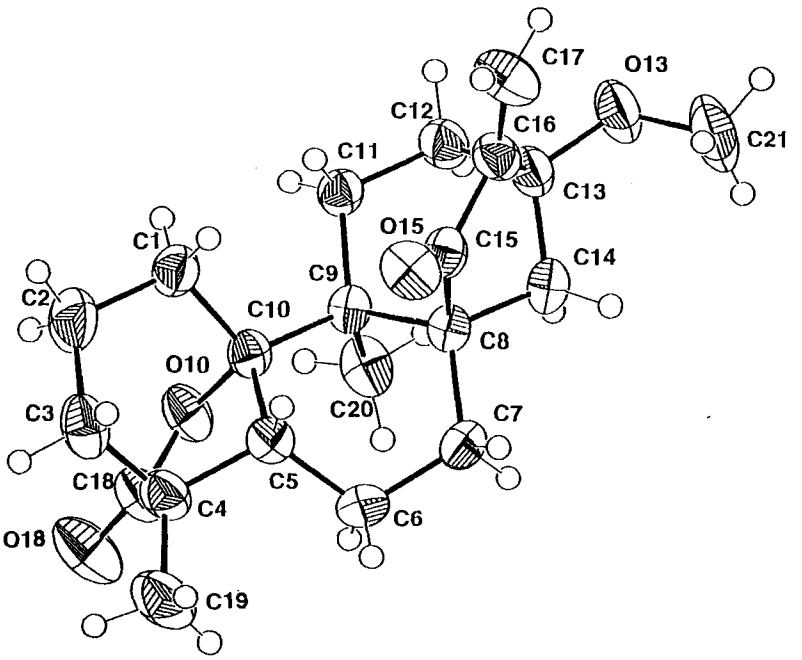

Fig. 1. *A perspective view of molecule* **1** *showing the numbering scheme used* (thermal ellipsoids at 50% probability level)

Addition of Br_2 on **1**. A soln. of **1** (30 mg) in $CHCl_3$ (5 ml) was placed under constant agitation in a tricol. A soln. of Br_2 (2 drops of Br_2 in 10 ml of $CHCl_3$) was droped slowely into the sample, to avoid an increase of the temp. The reaction was followed by CCM (SiO_2; $CHCl_3$, detection with *Godin*). After 1 h, **1** was not more detectable, and the reaction was stoped. The soln. was purified by CC on silica gel (40–63 μm, 460 × 15 mm i.d., $CHCl_3$/AcOEt 2:1) and then crystallized in hexane/AcOEt to afford 25 mg of **1-Br.**

Mass Analysis of **1-Br.** EI-MS: 425(47, $[M]^+ = [(344 + {}^{81}Br)]^+$), 423(48, $[M]^+ = [(344 + {}^{79}Br)]^+$), 344(100, $[M - Br]^+$), 300(72), 241(45), 91(49). DCI-MS: 524($[M + NH_4]^+ = [(344 + 2\ {}^{81}Br)]$), 522($[M + NH_4]^+ = [(344 + {}^{79}Br + {}^{81}Br)]$), 520($[M + NH_4]^+ = [(344 + 2\ {}^{79}Br)]$), 490, 488, 362($[(M - 2Br) + NH_4]^+$), 345.

Crystallographic Data for Compound **1-Br** (+ EtOAc). $C_{21}H_{27}Br_2O_4 \cdot C_4H_8O_2$, monoclinic, space group $P2_1$, $a = 12.9704(14)$, $b = 7.503(3)$, $c = 13.3116(10)$ Å, $Z = 2$, 5076 reflections measured including *Friedel* pairs, 4693 independent reflections ($R_{int} = 0.0159$), 4329 observed reflections [$I > 2\sigma(I)$], final $R1 = 0.0391$, $Rw2 = 0.0811$, Goodness of fit 1.098, residual density max/min 0.627/ – 0.432 e Å^{-3}. Absorption coefficient $\mu = 3.158$ mm^{-1}; an empirical absorption correction was applied using DIFABS [22] as implemented in PLATON [23], transmission factors min/max = 0.524/1.000.

Suitable crystals of **1-Br** were grown from hexane/AcOEt as colorless hexagonal plates. Intensity data were collected at 223 K on a *Stoe AED2* four-circle diffractometer using MoK_α graphite monochromated radiation ($\lambda = 0.71073$ Å) with $w/2\Theta$ scans in the 2Θ range 5–51°. The structure was solved by direct methods using the programme SHELXS-86 [19]. The refinement and all further calculations were carried out using SHELXL-93 [20]. All of the H-atoms were included in calculated positions and allowed to ride on the corresponding C-atom. The non H-atoms were refined anisotropically, using weighted full-matrix least-squares on F^2.

The bond lengths and angles are normal within experimental error. The absolute structure parameter for the model was –0.004(11), hence the coordinates correspond to the absolute structure of the molecule.

Full tables of atomic parameters and bond lengths and angles may be obtained from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB2 1EZ (UK) on quoting the full journal citation. The molecular structure and crystallographic numbering scheme of **1-Br** is illustrated in *Fig. 2* drawn using Xtal GX [21]. Further details may be obtained from the author *H. St-E.*

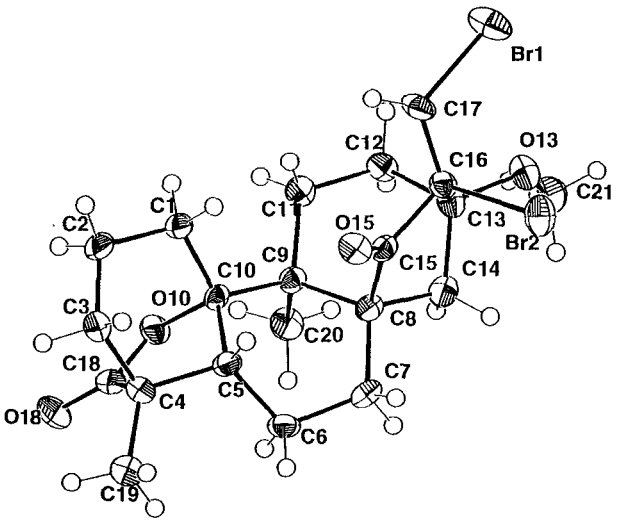

Fig. 2. *A perspective view of molecule* **1-Br** *showing the numbering scheme used* (thermal ellipsoids at 50% probability level)

(−)-10-Hydroxy-9-methyl-15-oxo-20-norkaur-16-en-18-ioc Acid, γ-Lactone (**2**). Colorless crystals from hexane/AcOEt. M.p. 142–146° ([4]: 164–165°). TLC (*A*): R_f 0.80; (*B*): R_f 0.70. $[\alpha]_D^{23} = -80.6$ ($CHCl_3$, $c = 0.88$) ([4]: −74.7°). UV: 232(3.88). IR: 2925*m*, 1759*s*, 1719*m*, 1644*m*, 1447*m*, 1381*m*, 1248*m*, 1203*m*, 1136*m*, 952*m*, 938*m*. ^{1}H-NMR ($CDCl_3$): 5.96(*d*, $J = 1$, H_a–C(17)); 5.25(*d*, $J = 1$, H_b–C(17)); 2.52(*dd*, $J = 4.2$, 13,9, H–C(4)); 1.54(*s*, Me(20)); 1.49(*s*, Me(19)). ^{13}C-NMR ($CDCl_3$; see *Table*). EI-MS: 314(100, M^+), 271(67), 270(53), 255(35), 91(43).

Crystallographic Data for Compound **2**. $C_{20}H_{26}O_3$, orthorhombic, space group $P2_12_12_1$, $a = 8.265(3)$, $b = 8.3494(18)$, $c = 24.276(10)$ Å, $V = 1674.2(12)$ Å^3, $Z = 4$, 3428 reflections measured, 2936 independent reflections ($R_{int} = 0.043$), 2365 observed reflections [$I > 2\sigma(I)$], final $R1 = 0.0722$, $Rw2 = 0.153$, Goodness of fit 1.117, residual density max./min. 0.229/ − 0.193 e Å^{-3}. Absorption coefficient $\mu = 0.082$ mm^{-1}; no correction for absorption was applied.

Suitable crystals of **2** were grown from hexane/AcOEt as colorless blocks. Intensity data were collected at r.t. on a *Stoe AED2* four-circle diffractometer using MoK_α graphite monochromated radiation ($\lambda = 0.71073$ Å) with $w/2\Theta$ scans in the 2Θ range 5–50°. The structure was solved by direct methods using the programme SHELXS-86 [19]. The refinement and all further calculations were carried out using SHELXL-93 [20]. All of the H-atoms were included in calculated positions and allowed to ride on the corresponding C-atom. The non-H-atoms were refined anisotropically, using weighted full-matrix least-squares on F^2.

The bond lengths and angles are normal within experimental error. No attempt was made to determine the absolute configuration of the molecule. Full tables of atomic parameters and bond lengths and angles may be obtained from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB2 1EZ (UK) on quoting the full journal citation. Further details may be obtained from the author *H. St-E.*

(−)-10,13-Dihydroxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic Acid γ-Lactone (**3**). White amorphous powder. M.p. 175–180°. TLC (*A*): R_f 0.69, (*B*): R_f 0.37. $[\alpha]_D^{23} = -47.3$ ($CHCl_3$; $c = 0.785$). UV: 227(3.99). IR: 3421*m*, 2943*m*, 1760*s*, 1718*s*. ^{1}H-NMR ($CDCl_3$): 6.07(*s*, H_a–C(17)); 5.49(*s*, H_b–C(17)); 1.28(*s*, Me(20)); 1.10(*s*, Me(19)). ^{13}C-NMR ($CDCl_3$; see *Table*). EI-MS: 330(100, $[M]^+$), 287(45), 286(61), 241(47), 91(73).

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