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CHEMICAL STUDIES OF MARINE INVERTEBRATES—II*

TERPENOIDS—LVIII†

GRISEOGENIN, A NEW TRITERPENOID SAPOGENIN OF THE SEA CUCUMBER *HALODEIMA GRISEA* L.

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Abstract—Chemical degradation coupled with extensive physical measurements has led to structure V for griseogenin. The correctness of this structure has been confirmed by correlation with the known sapogenin, 22,25-oxidoholothurinogenin (I).

NUMEROUS species of sea cucumbers are endowed with special organs, the Cuvier glands, containing a powerful poison that the animal can discharge into the surrounding water, presumably as a natural defense against predators. Some of these toxins from the class *Holothurioidea* have been the subject both of physiological and chemical studies.[1,2]

Halodeima grisea L. (Phylum *Echinodermata*, class *Holothurioidea*, order *Aspidochirota*, family *Holothuriidae*) is a common species on the Brazilian coastline. It has been collected[3] by us both in Rio Grande do Norte, where it is locally abundant in the intertidal zone, and off the city of Rio de Janeiro in deeper water.

Cuvier glands of *H. grisea* were removed immediately after collection, stored in ethanol, and the glycosidic fraction hydrolysed with hydrochloric acid and then extracted with chloroform to give a crude mixture of sapogenins. Chromatography of this mixture on silicagel gave a crystalline sapogenin whose m.p., IR, UV and NMR spectra pointed to the known 22,25-oxidoholothurinogenin (I).[1,2] Identity was established by comparison of the mass spectra (Table 1) of the sapogenin and the derived 3-ketone, (II) with those of authentic samples.[1] Reduction of the sapogenin gave the known[1,2] tetraol, (III), m.p. 223–230°, whose mass spectrum (Table 2) was in accord with the structure formulated.

* Paper I, B. Tursch, H. Barreto and N. Sharapin, *Bull. Soc. Chim. Belg.* **72**, 807 (1963).

† For "Terpenoids–LVII" see J. Karliner and C. Djerassi, *J. Org. Chem.* **31**, 1945 (1966).

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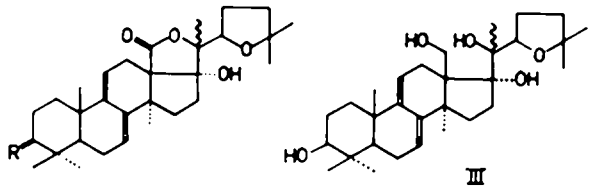
[1] For leading references see J. D. Chanley, T. Mezzetti and H. Sobotka, *Tetrahedron* **22**, 1857 (1966). We are indebted to Drs. Chanley and Sobotka for furnishing us with a manuscript of their paper prior to publication and for several comparison samples.

[2] T. Matsuno and T. Yamanouchi, *Nature, Lond.* **191**, 75 (1961).

[3] One of us (B. T.) thanks Dr. Jean Pierret and Mr. N. Sharapin for participating in the collecting trips and especially for the courage of Dr. Pierett who spent long moments stranded on a "jangada" raft on top of a surfaced *Globicephalus*, while attempting to locate deep water collecting ground in the S. Atlantic ocean.



I R=α-H, β-OAc
II R=O
IV R=α-H, β-OH

III

TABLE 1. DIAGNOSTIC PEAKS IN THE MASS SPECTRA OF TRITERPENOID LACTONES

Compound	I	II	V	VI	X	VII	VIII	IX
M^{+a}	484	482	486[b]	570	528	484	482	526
$M-H_2O$[c]	466	464	468	*d*	*d*	466	464	508
M-CO		454				*e*	*e*	498
$M-CO_2$ (?)							438	
$M-(CO_2 \cdot OH)$			425[b,f]			423[f]	421[f]	
Ring A cleavage	397[g]	397	399[b,g]	441	399	399	397	441[h]
C_{10} C_{11} cleavage	99	99						

[a] Accompanied by M-15 ($M-CH_3$).
[b] Empirical composition of ion confirmed by high resolution as follows: *m/e* 486 $C_{30}H_{46}O_4$; 425 $C_{27}H_{37}O_4$; 399 $C_{26}H_{39}O_4$.
[c] Accompanied by M-33 ($M-H_2O-CH_3$).
[d] Show M-60 (M-HOAc) and M-75 (M-HOAc-CH_3).
[e] Diketone VIII shows no loss of 28 units, while the monoketone VII shows N-30 at *m/e* 454.
[f] Appears at $M-(C_3H_7 + HOAc)$ in the acetates VI and X, while in the ketones VII and VIII the peak remains at $M-(C_3H_7 + H_2O)$ but is of low intensity and is accompanied in VIII by $M-C_3H_7$.
[g] The M-87 fragments of 22,25-oxidoholothurinogenin (I) and griseogenin (V) are formed directly from the molecular ions as evidenced by metastable peaks at 326 and 327·8 respectively. For origin *vide infra*.
[h] Accompanied by weaker peaks 42 and 60 units lower.

TABLE 2. DIAGNOSTIC PEAKS IN THE MASS SPECTRA OF REDUCED LACTONES (POLYOLS)

	M^+	M-18[a]	*b*	*c*		c-H_2O				
III	488	470	371	344	328	326	313	144[d]	126[d]	99[d]
XI[e]	*f*	472	371	344	328	326	313			

[a] Accompanied by $M-2H_2O$ and $M-3H_2O$.
[b] Cleavage of C_{20}–C_{22} and loss of water.
[c] Cleavage of C_{17}–C_{20} with hydrogen transfer, or cleavage of C_{20}–C_{22} and loss of C_{14} and C_{18}, with hydrogen transfer.
[d] Intense peaks probably derived from the side-chain (C_{20}–C_{27}).
[e] Empirical composition of all ions established by high resolution as follows: *m/e* 472, $C_{30}H_{48}O_4$; 371, $C_{24}H_{35}O_3$; 344, $C_{23}H_{28}O_3$; 328, $C_{23}H_{28}O_2$; 326, $C_{23}H_{30}O_2$; 313, $C_{21}H_{29}O_2$.
[f] Not observed.

Although the chemical defense mechanism of holothurians is believed to proceed through the Cuvier glands, saponins were found to be present also in the body wall of *Halodeima grisea*, as it has been shown to be the case for *Holothuria vagabunda*.[2] Whole eviscerated animals were cut into small pieces and hydrolysed directly in refluxing methanolic hydrochloric acid. Chromatography of the crude sapogenin

mixture gave a new sapogenin, griseogenin (V), $C_{30}H_{46}O_5$, m.p. 285–287°, ν_{max}^{chf} 3570–3300 (bonded and non-bonded OH), 1755 (γ-lactone), 1631 (conjugated double bonds); ν_{max}^{KBr} 825 and 803 cm^{-1} (trisubstituted double bonds). The UV spectrum (λ_{max}^{EtOH} 236, 244 and 252 mμ) was identical to that of 22,25-oxidoholothurinogenin (I) and the general similarity of the IR spectrum of compounds I and V indicated that griseogenin was a $\Delta^{7,9(11)}$-lanostadiene derivative. Acetylation of griseogenin at

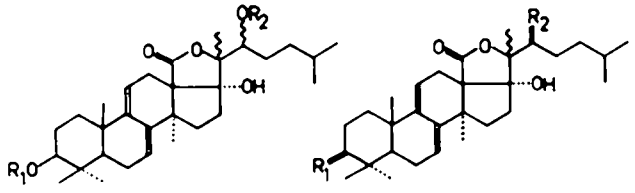
TABLE 3. NMR SPECTRA (ppm IN $CDCl_3$)

Assignment	Griseogenin (V)	Griseogenin diacetate (VI)	22,25-Oxidoholothurinogenin acetate (IV)
C_4-methyls[a]	0·91 s, 1·04 s	0·88 s, 0·97 s	0·91 s, 0·99 s
C_{26}-methyls	0·91 d (J = 8 c/s)	0·97 d (J = 8 c/s)	1·26 s, 1·28 s
C_{10}-methyl[a]	1·11 s	1·11 s	1·15 s
C_{14}-methyl[a]	1·20 s	1·14 s	1·22 s
C_{21}-methyl	1·38 s	1·46 s	1·38 s
CH-O[b]	3·24 m, 3·83 m	4·50 m, 5·1 m[c]	4·23 t, 4·56 m
Vinyl H[b]	5·27 m, 5·53 m	5·22 m, 5·50 m	5·25 m, 5·51 m

[a] Tentative assignment. [b] All one proton absorptions. [c] Overlaps vinyl absorption.

room temperature gave a diacetate (VI), m.p. 259–261°, ν_{max}^{chf} 3470 (bonded OH), 1773 (γ-lactone), 1739–1730 (acetate), 1252 cm^{-1} (acetate). The NMR spectra of griseogenin (V), its diacetate (VI) and 22,25-oxidoholothurinogenin monoacetate[1] (IV), are compared in Table 3, and while confirming the above mentioned similarity indicate absence of oxygenation at C_{25} in griseogenin.

Jones oxidation of griseogenin furnished a mixture of a monoketone (VII), whose negative ORD Cotton effect is consistent[4] with a $\Delta^{7,9(11)}$-lanostadien-3-one, and a diketone (VIII). Both showed carbonyl absorption at 1773 (γ-lactone) and ca. 1710 cm^{-1} (6-membered or open chain ketone) and hydroxyl absorption. The ketones were characterized by their mass spectra (Table 1) and by the formation of a monoacetate (IX) of the monoketone.



V R_1 = H; R_2 = H
VI R_1 = Ac; R_2 = Ac
X R_1 = Ac; R_2 = H

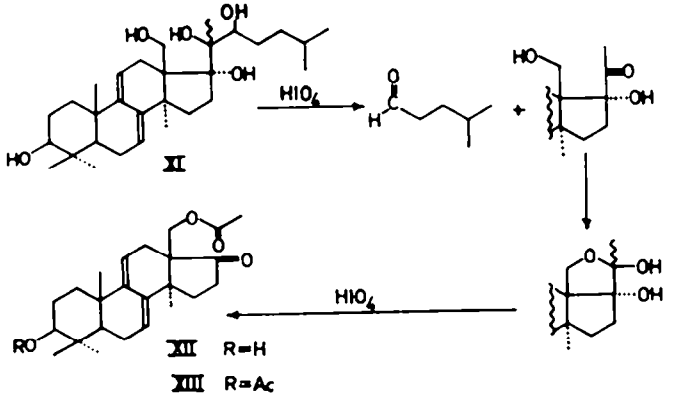
VII R_1 = O; R_2 = H,OH
VIII R_1 = O; R_2 = O
IX R_1 = O; R_2 = H,OAc

A monoacetate subsequently shown to be the 3-monoacetate (X) resulted when griseogenin was refluxed with ethyl acetate in the presence of a small amount of *p*-toluenesulphonic acid. Further acetylation of X with acetic anhydride–pyridine furnished the diacetate (VI). The five oxygen atoms of griseogenin are thus contained in a γ-lactone, two secondary (one hindered) and another alcoholic function whose resistance to oxidation and acetylation suggested its tertiary nature.

[4] C. Djerassi, O. Halpern, V. Halpern and B. Riniker, *J. Amer. Chem. Soc.* **80**, 4001 (1958).

The co-occurrence and general spectral resemblance with 22,25-oxidoholothurinogenin (I) strongly indicated the presence of a lanostane skeleton. The NMR spectrum of griseogenin (Table 3) showed the presence of seven rather than eight methyl groups and hence the carboxyl group of the lactone presumably is derived from the oxidation of one such group. The NMR spectrum of griseogenin and its acetate (Table 3) show no proton at the alcoholic terminus of this lactone which must therefore be attached to a tertiary carbon atom. In the lanostane skeleton only one position for such a lactone bridge is possible—that already exemplified in 22,25-oxidoholothurinogenin (I).

Griseogenin was resistant to LAH even under vigorous conditions but its diacetate (VI) or monoacetate (X) could be reduced smoothly in dioxan to a pentaol (XI), in which the lactone bridge has been reduced as demonstrated by the IR and mass spectra (for latter see Table 2). Cleavage of this pentaol with periodic acid in methanol-water at room temperature gave 4-methylpentanal (isocaproaldehyde) isolated as its 2,4-dinitrophenylhydrazone, m.p. 98–99° identical by IR, mixture m.p. and mass spectrum with an authentic specimen[5] and a ketoacetate. The latter had unchanged $\Delta^{7,9(11)}$-lanostadiene UV absorption at 236, 243 and 252 mμ and exhibited NMR signals for four angular methyl groups at 0·91 0·93 and 1·03 (6H) ppm, an acetate methyl at 2·0 ppm, a C*H*—OH proton at 3·26 ppm (quartet, J = 5 and 9 c/s, consistent with 3α-axial H) and vinyl absorptions at 5·43 and 5·70 ppm (multiplets). Notable was a new AB pattern at 3·66 and 4·30[6] ppm (J = 11·5 c/s), attributable to an angular CH_2OAc. The high resolution mass spectrum established the molecular formula as $C_{24}H_{34}O_4$ and in conjunction with peaks at *m/e* 313·2212 ($C_{21}H_{29}O_2$, calc. 313·2167, loss of CH_2OAc) and 298·1982 ($C_{20}H_{26}O_2$, calc. 298·1933, loss of CH_3 + CH_2OAc) led to the probable formula XII for the ketoacetate. The ORD positive Cotton effect curve of XII was identical in shape and sign to that of the known diacetate XIII, confirming the position of the carbonyl group and the stereochemistry of the C/D ring juncture. The mass spectra of XII and XIII were very similar except in the high mass range where XIII showed peaks (e.g. *m/e* 350, 355 and 368) 42 units higher than XII, due to the extra acetyl group at C_3. Finally acetylation of the griseogenin degradation product XII gave XIII identical by mixture m.p. and mass spectrum with an authentic sample.[1]



[1] We are indebted to Dr. J. S. Mills of the National Gallery, London, for this sample; J. S. Mills, *J. Chem. Soc.* 2196 (1956).

[6] The downfield portion was split at 100 Mcs by 1 c/s possibly due to the presence of two conformers.

The formation of the ketoacetate XII may be rationalized on the basis of structure XI for the pentaol and hence structure V for griseogenin itself, in which the lactone ring requires the 17β-configuration for the side chain.

Definite structures can now be proposed for the monoketone (VIII), its acetate (IX) and griseogenin monoacetate (X) on the basis of their mass spectra (Table 1). The peak at *m/e* 399 in griseogenin (M-87) moves to *m/e* 397 in the diketone VIII (M-85) and to *m/e* 441 in the diacetate VI (M-129), but remains at *m/e* 399 in the monoketone (VII). Since high resolution experiments (Table 1) show that a C_5O moiety is expelled, this cannot arise from the side chain which would require a C_6O fragment expulsion. By analogy to the documented[7] cleavage of the 1–2 and 4–5 bonds in 4,4-dimethyl-3-ketosteroids, it is almost certain that a similar fragmentation obtains in this instance. The observed mass shifts in the entry of Table 1 labelled "Ring A cleavage" are only consistent with the variations in the C-3 functionality in the monoacetate and monoketone. Thus both the monoketone (VII) and its acetate (IX) show M-85 fragments locating the keto group at C-3, while in the monoacetate (X), the loss of 129 mass units locates the acetate function also at C-3.

The present structural argument rests on comparison with a degradation product (XIII) of 22,25-oxidoholothurinogenin (I). A direct correlation with lanosterol is the object of present work in the authors' laboratories.

EXPERIMENTAL[8]

Isolation of 22,25-*oxidoholothurinogenin*. Cuvier glands of *H. grisea* (333 g) were removed immediately after collection, stored in EtOH, the total residue including glands defatted with benzene (benzene soluble residue 1·5 g) and extracted with water. The aqueous extract was submitted to hot acid hydrolysis (30% HCl, 24 hr) and then extracted with chf to give a crude mixture of sapogenins (9·5 g). Chromatography of this mixture on silicagel (78 g) gave crude cholesterol eluted with benzene, followed by I[1] (105 mg), m.p. 300–301° after recrystallization from chf–hexane. Jones oxidation of I gave II[1], m.p. 298·5°, while LAH reduction gave the known tetraol III,[1] m.p. 223–230°. For mass spectra which identify these compounds see Table 1.

Isolation of griseogenin (22ξ-*hydroxyholothurinogenin*, V). Whole specimens of *H. grisea* (250) from which the Cuvier glands had been removed were cut into small pieces and hydrolysed directly in a refluxing mixture of 70% MeOH and 30% conc HCl during 4 hr. The mixture was filtered and the filtrate extracted with chf, and the chf evaporated giving a crude sapogenin mixture (15·5 g). Chromatography on silicagel (277 g) gave by gradient elution with benzene–ether a crystalline solid which, after recrystallization from chf–hexane, gave V (677 mg) m.p. 285–287°, with transition point at 260–270°, $[\alpha]_D^{26}$ −22° (*c* 0·399 in chf). For IR see text, UV absorption was observed at λ_{max}^{EtOH} 236, 244 and 252 mμ (ε 10,320; 11,260; 8,440); for NMR see Table 3 and for low resolution mass spectrum, Table 1; the high resolution mass spectrum showed: *m/e* 486·334155 (M^+, $C_{30}H_{46}O_4$ requires 486·334505), 425·268747 ($C_{27}H_{37}O_4$ requires 425·269169), 399·253443 ($C_{25}H_{37}O_4$ requires 399·253519).

Griseogenin-3,22-*diacetate* (VI). A soln of griseogenin (132 mg) in pyridine (2 ml) and Ac_2O (2 ml) was kept at room temp for 18 hr and the excess anhydride destroyed by addition of water. Extraction with chf and recrystallization of the residue (172 mg) from benzene gave VI m.p. 259–261°, ν_{max}^{chf} 1773 s, 1730 br, 1625 w, 1255 br cm^{-1}. The mass spectrum (Table 1) showed the molecular ion peak at *m/e* 570. (Found: C, 71·01; H, 8·76. $C_{34}H_{50}O_7$ requires: C, 71·51; H, 8·84%; M, 570.)

Jones oxidation of griseogenin. Griseogenin (121 mg) was oxidized by dropwise addition of the Jones reagent (CrO_3, 100 g, and H_2SO_4 (80 g) diluted with water to 375 ml) in acetone (20 ml) at 0° during 1 min giving after dilution with water and extraction with chf a crystalline residue which was chromatographed on silicagel, giving by elution with benzene–AcOEt, 9:1, griseogen-3,22-dione (VIII, 6 mg) m.p. 229–234°, ORD in dioxan, *c* 0·52, $[\phi]_{589}^{26}$ 0°, $[\phi]_{314}$ +1,020°, $[\phi]_{300}$ 0°, $[\phi]_{288}$ −23,374°

[7] R. H. Shapiro and C. Djerassi, *Tetrahedron* **20**, 1987 (1964).

[8] M.ps (Kofler apparatus) are uncorrected.

(trough), $[\phi]_{344}$ +44,522° (peak); ν_{max}^{chf} 3570 (sharp), 1773 s, 1710 s cm^{-1}; UV spectrum identical to that of V, the mass spectrum (Table 1) showed the molecular ion at *m/e* 482. $C_{30}H_{42}O_6$ requires: M 482.

Contined elution (see above) with benzene–AcOEt, 4:1, gave VII (29 mg), m.p. 214°, ORD in dioxan, *c* 0·92, $[\phi]_{590}^{ss}$ −2,004°, $[\phi]_{344}$ −21,352° (trough), $[\phi]_{333}$ +56,940° (peak); ν_{max}^{chf} 3520 m, 1773 s, 1701 cm^{-1}; the mass spectrum (Table 1) showed the molecular ion at *m/e* 484. $C_{30}H_{44}O_6$ requires: M 484.

Griseogen-3-one (VII, 29 mg) acetylated at room temp in Ac_2O–pyridine for 18 hr gave, after recrystallization of the product from benzene–AcOEt, griseogen-3-one 22-acetate IX (13 mg), m.p. 223–227°, ν_{max}^{chf} 3530 m, 1767 s, 1740 sh, 1700 s cm^{-1}, whose mass spectrum (Table 1) showed the molecular ion at *m/e* 526. $C_{32}H_{46}O_7$ requires: M 526.

Griseogenin 3-acetate (X). Griseogenin (102 mg) was heated under reflux during 6 hr with AcOEt (10 ml) containing a trace of *p*-toluenesulphonic acid. After addition of water the organic phase was separated to give by evaporation and recrystallization of the residue from benzene–AcOEt, X (72 mg), m.p. 267–269·5°, ν_{max}^{chf} 3360 br, 1760 s, 1712 s, 1250 s cm^{-1}, UV spectrum identical to that of V. The mass spectrum (Table 1) showed the molecular ion at *m/e* 528. $C_{32}H_{48}O_7$ requires: M 528.

Lithium aluminium hydride reduction of griseogenin 3,22-diacetate. The diacetate VI (172 mg) was heated under reflux in dioxan with excess LAH during 4·5 hr. The excess hydride was destroyed with sat $MgSO_4$aq and the product isolated by repeated extraction with chf. Recrystallization from chf–MeOH gave XI (3β,17α,18,20ξ,22ξ-pentahydroxylanosta-7,9(11)-diene; 99 mg), m.p. 228–234°, ν_{max}^{KBr} 3360 br, 1626 br, w cm^{-1}. For low resolution mass spectrum see Table 1. The high resolution mass spectrum showed no molecular ion peak. Fragmentation peaks were observed at *m/e* 472·355377 ($C_{30}H_{48}O_4$, M-H_2O, requires 472·355240), 371·25802 ($C_{24}H_{35}O_3$ requires 371·25861), 344·23499 ($C_{23}H_{32}O_3$ requires: 344·23513), 328·23971 ($C_{23}H_{32}O_2$ requires 328·24022), 326·22425 ($C_{23}H_{30}O_2$ requires 326·22457), and 313·21620 ($C_{21}H_{29}O_2$ requires 313·21674).

Similar reduction of X gave the same pentaol.

Periodic acid cleavage of the pentaol (XI). The pentaol XI (20 mg) dissolved in MeOH (10 ml)–water (0·5 ml) was allowed to react with periodic acid (0·5 ml 50% aqueous soln) during 90 min. The excess periodic acid was then reduced with sodium thiosulphate and the mixture steam distilled into a soln of 2,4-dinitrophenylhydrazine (4 g) in H_2SO_4 (20 ml) and water (30 ml). The orange ppt (5 mg) was collected and recrystallized from hexane and then EtOH to give 4-methylpentanal 2,4-dinitrophenylhydrazone (isocaproaldehyde-DNP) m.p. 98–99° identified with an authentic specimen[4] by mixture m.p., IR and mass spectral comparison. The mass spectrum showed (ion source inlet) *m/e* 280 (M^+, 51), 265 (M-CH_3, 6), 224 (M-C_4H_8, 39), 206 (M-C_4H_8 + H_2O, 100).

The soln containing non-steam-volatile material was extracted with dichloromethane giving a residue which after chromatography on silicagel and recrystallization from chf–hexane–AcOEt yielded XII (18-acetoxy-3-hydroxy-4,4,14α-trimethylandrostan-17-one, 4 mg), m.p. 163–165°, ORD in dioxan, *c* 0·48, $[\phi]_{589}$ +232°, $[\phi]_{331}$ +7,905° (peak), $[\phi]_{313}$ +6,510° (shoulder), $[\phi]_{300}$ −4,960° (trough); ν_{max} 3450 br, 1745 s, 1733 sh cm^{-1}; UV absorption at 235, 242 and 252 mμ was similar to that of V. For NMR absorption see text; the mass spectrum showed (ion source inlet) *m/e* 386·246 ($C_{24}H_{34}O_4$ requires 386·2457), 326·2331 (M-HOAc, $C_{22}H_{30}O_2$ requires 326·2246), 313·2212 (90%, M-CH_3OAc, $C_{21}H_{29}O_2$ requires 313·2167), 313·1845 (10%, $C_{20}H_{25}O_2$ requires 313·1804), 308·2184 (M-HOAc-H_2O, $C_{22}H_{28}O$ requires 308·2140), 299·2051 (M-CH_3—CH_3OAc + H, $C_{20}H_{27}O_2$ requires 299·2011), 298·1982 (M-CH_3—CH_3OAc, $C_{20}H_{26}O_2$ requires 298·1933), 293·1957 (M-CH_3—HOAc—H_2O, $C_{21}H_{25}O$ requires 293·1905), 275·1801 (M-CH_3—HOAc—$2H_2O$, $C_{21}H_{23}$ requires 275·1800).

Acetylation of the ketoacetate (XII). The ketoacetate XII (4 mg) was acetylated at room temp in Ac_2O (0·1 ml)–pyridine (0·1 ml) during 18 hr, giving, after chromatography on silicagel in benzene and recrystallization from hexane, the keto-diacetate XIII (2 mg), m.p. 160–163°, ORD in dioxan, *c* 0·43, $[\phi]_{319}$ +10,800° (peak), $[\phi]_{310}$ +8,900° (shoulder), $[\phi]_{291}$ −5,200° (trough). An authentic specimen[1] did not depress the m.p. and showed ORD in dioxan, *c* 1·87 ,$[\phi]_{589}$ +385°, $[\phi]_{319}$ +10,050 (peak), $[\phi]_{310}$ +8,350 (shoulder), $[\phi]_{300}$ −5,200 (trough). The UV absorption did not differ from that of XII, and the mass spectrum, identical to that of the authentic sample,[1] showed (ion source inlet) *m/e*: 428 (M^+, 4), 413 (M-CH_3, 2), 410 (M—H_2O, 2), 368 (M-HOAc, 11), 355 (M-CH_2OAc, 69%), 350 (M-HOAc—H_2O, 27), 341 (M-CH_3—CH_3OAc + H, 10), 340 (M-CH_3—CH_3OAc, 8), 312 (11), 293 (M-CH_3—2HOAc, 34), 275 (M-CH_3—2HOAc—H_2O, 23).

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