Uroterpenol β-D-Glucuronide

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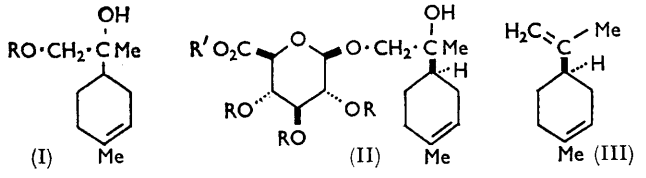
Uroterpenol (*p*-menth-1-ene-8,9-diol) is shown to occur in human urine as a β-D-glucuronide which has been isolated and characterised. The parent diol has been synthesised by oxidation of (+)-limonene with plumbic acetate and converted into a β-D-glucuronide identical with the natural product except that both materials are shown to be mixtures, in different proportions, of diastereoisomers configurationally identical at position 4 but differing at position 8.

Periodates cleave the olefinic bond in uroterpenol with unexpected ease.

After preliminary observations by Edwards and Wade,[1a] we found that human urine contains uroterpenol, shown to be *p*-menth-1-ene-8,9-diol (I; R = H), as a conjugate not then defined either by us [1b] or by Tschesche, Duphorn, and Gelissen,[2] who had made similar observations. We now offer evidence that the conjugated material is (4*R*-8-hydroxy-*p*-menth-1-ene-9-yl β-D-glucopyranosid)uronic acid (II; R = R′ = H), but is a mixture of diastereoisomers differing at position 8. It is also shown that although glycol cleavage of uroterpenol by periodates is normal, the reagent is able to effect oxidative scission of the olefinic bond with unusual ease.



Solvent extractions of human urine followed by a series of chromatographic separations on alumina and on Celite columns furnished a syrup which appeared to consist of a single substance but which failed to crystallise. Nevertheless, the substance is essentially uroterpenol β-glucuronide since enzymatic hydrolyses with limpet β-glucuronidase liberated, according to chromatographic evidence, uroterpenol only. Moreover, estimations, by means of the anisaldehyde reaction,[1b] of the uroterpenol formed in such hydrolyses indicated that, in the conjugated material, one terpenoid residue is coupled to one glucuronic acid residue. In order to obtain a substrate suitable for other analytical work, the syrup was methylated and acetylated, giving the crystalline methyl triacetylglucuronidate (II; R = Ac; R′ = Me), which possesses a tertiary hydroxyl group resistant to acetylation but absorbing near 3 mμ in the infrared spectrum and, as a broad band removed by exchange with deuterium oxide, near τ 8 in the nuclear magnetic resonance (n.m.r.) spectrum. The n.m.r. spectrum confirms through proton counts in appropriate regions that one terpenoid residue is attached to one glucuronic acid residue. Details of the mass spectrum given in the Table show that the chief fission sequences are consistent with the suggested structure and to a large extent are, as expected, a combination of those characteristic of uroterpenol and methyl (tri-*O*-acetyl-β-D-glucopyran)-uronate separately.

These conclusions have been confirmed, and the stereochemistry of the terpenoid residue clarified, by a synthesis of uroterpenol and thence of the methyl tri-*O*-acetylglucuronidate beginning with (+)-limonene, the absolute configuration of which has been discussed by Brewster [3] and is shown in (III).

Aratani [4] has reported that oxidation of limonene by pumbic acetate leads to a diol having structure (I; R = H), but we have had to rely on abstracts in English rather than the Japanese original and have been unable to duplicate this work. However, in our hands this oxidation afforded a complex mixture including the monoacetate (I; R = Ac) and the diacetate of the required diol, which was best isolated after hydrolysis of the mixture and partition between water and benzene.[1b] The diol so obtained was identical with the natural product according to analytical, chromatographic, and infrared spectroscopical criteria, and the derived *p*-nitrobenzoates (I; R = $NO_2 \cdot C_6H_4 \cdot CO$) were similarly identical. Despite that, the natural and synthetic diols differ slightly in certain respects because they are mixtures of diastereoisomers in somewhat different proportions. The n.m.r. spectra of the *p*-nitrobenzoates show this most clearly. The band centred at τ 8·71, assigned to a quaternary methyl groups, appears as a doublet in which the separation is not due to long-range coupling effects, because it is proportional to the applied field, being 1·9 and 3·2 c./sec. at 60 and 100 Mc./sec., respectively. The methylenic band near τ 5·6 is similarly split though here the effect is overlaid by spin–spin splitting of the kind usual in methylene groups attached to asymmetric carbon atoms.[5] Thus at 60 Mc./sec. this band appears as an ill-defined doublet, but at 100 Mc./sec. as a triplet.

(+)-Limonene (III) has the *R*-configuration and so must the derived diol since the active centre has not been disturbed in the oxidation. Although the diol contains a second optically active feature, this will have little effect in comparison with the large contribution by the conformational asymmetry in the cyclohexene ring,[3]

[1] (*a*) R. W. H. Edwards and A. P. Wade, *J. Endocrinol.*, 1965, **33**, 153; (*b*) A. P. Wade, G. S. Wilkinson, F. M. Dean, and A. W. Price, *Biochem. J.*, 1966, **101**, 727.

[2] R. Tschesche, I. Duphorn, and K. Gelissen, *Z. physiol. Chem.*, 1966, **345**, 100.

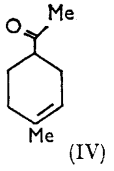
[3] J. H. Brewster, *J. Amer. Chem. Soc.*, 1959, **81**, 5493.

[4] T. Aratani, *J. Chem. Soc. Japan*, 1959, **80**, 528.

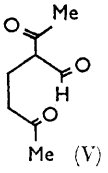
[5] K. Mislow, M. A. W. Glass, H. B. Hopps, E. Simon, and G. H. Wall, *J. Amer. Chem. Soc.*, 1964, **86**, 1710.

as is shown by the similarity of the molecular rotation (+151°) to that (+169°) of the parent diene. The natural diol has a rather lower molecular rotation (+129°) but clearly it must be assigned the *R*-configuration at position 4. The natural material may also contain small amounts of the (−)-diols, since uroterpenol may be derived from dietary limonene [1b] which, though commonly the dextrorotatory form in essential oils from citrus families and many others, is occasionally racemic or laevorotatory, as in oils from the mint families. However, repeated fractionation of the *p*-nitrobenzoate failed to effect any change in the proportions of diastereoisomers present.

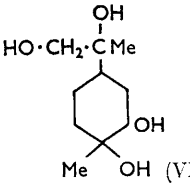
Condensed with methyl (2,3,4-tri-*O*-acetyl-1-α-bromo-1-deoxy-D-glucopyran)uronate, synthetic uroterpenol afforded a monoglucuronide derivative (II; R = Ac; R′ = Me) identical in all respects with the derivative from natural sources except for slight discrepancies in the molecular rotations and in the n.m.r. spectra that must again be attributed to diastereoisomerism. The quaternary methyl band near τ 9 shows splitting proportional to the field, and it seems from the peak heights that the minor component of the natural material is the major one of the synthetic. Repeated recrystallisation of the synthetic derivative gradually changed the peak heights in favour of the major component, and multiple chromatographic runs of the natural derivative have indicated the presence of two separable components; possibly pure diastereoisomers could be obtained in this way. However, we are not yet able to offer further details, but conclude that uroterpenol β-glucuronide is a mixture of diastereoisomers derived from (4*R*-8-hydroxy-*p*-menth-1-en-9-yl β-D-glucopyranosid)uronic acid (II; R = R′ = H), the stereochemistry at position 8 being unspecified.



(IV)



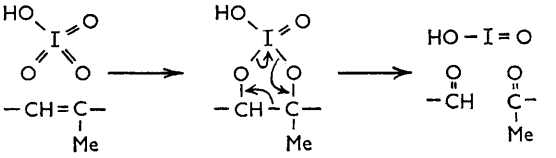
(V)



(VI)

The difficulties [1b] encountered during periodate oxidations of uroterpenol have been studied using the relatively plentiful synthetic material and found to be due to an unexpectedly easy scission of the olefinic link. This effect cannot be attributed to adventitious traces of catalysts, *e.g.*, osmium, ruthenium, or manganese oxides, because it was reproducible under conditions in which other olefins were unaffected. There is first a rapid glycol fission giving a ketone (IV), then a slower oxidation leading to the diketonic aldehyde (V) which was too sensitive to acids or bases to be characterised fully. No doubt the multiple possibilities for inter- and intra-molecular aldol condensations account for the complex results attending efforts to prepare carbonyl derivatives, etc. Fortunately, the tetrol (VI) is well known [6] and upon conventional periodate oxidation gave the same material, as indicated by chromatographic evidence; the n.m.r. spectrum furnished clear support for the presence in the oxidation product (V) of two distinct methyl ketone groupings together with one aldehyde function, and confirmed the absence of an olefinic bond.

Periodate oxidation of olefins under vigorous conditions has already been reported by Chatterjee and Majumdar,[7] whose method has been used for converting the exocyclic methylene group of hypoglycin A into formaldehyde.[8] Since manganese and iodine are related elements, the reaction might be patterned upon similar oxidations by permanganate ion [9] and formulated thus:



We favour the view that an intermediate complex is formed and collapses by an internal mechanism, because this would give "iodous acid" which would undergo disproportionation to iodine and iodic acid, both of which products have been observed. We have not yet found another simple olefin that is as easily attacked as uroterpenol, so the complex formation may be more involved than that shown, but the ease with which the reagent attacks phenols,[10] enols,[11] and indoles [12] is in general agreement with these views, since such compounds contain unsaturated bonds activated for oxidation by electron relase from oxygen or nitrogen atoms.

EXPERIMENTAL

Chloroform was distilled from phosphorus pentoxide immediately before use. Triply rectified ethanol (commercial) was used; other solvents were distilled before use. Magnesium sulphate was dried at 120° for 12 hr. and used for drying solutions before concentration.

N.m.r. spectra at 100 Mc./sec. were determined by means of a Varian HA-100 spectrophotometer. Mass spectra were measured on an A.E.I. MS9 mass spectrometer at 70 ev and with a source temperature near 200°. Other spectra were obtained as described previously.[1b]

Alumina adsorption columns (diam. 2 cm.) were made from alumina (50 g.) containing water (5% v/w) and packed as a slurry in a mixture of ether and ethanol (3 : 1 v/v).

Celite partition columns were prepared from Celite 535 (Johns–Manville Co. Ltd.). This was purified by boiling with water and then with methanol, followed by treatment

[6] K. Milas and S. Sussman, *J. Amer. Chem. Soc.*, 1937, **59**, 2345.
[7] A. Chatterjee and S. G. Majumdar, *Analyt. Chem.*, 1956, **28**, 878.
[8] H. V. Anderson, J. L. Johnson, J. W. Nelson, E. C. Olson, M. E. Speeter, and J. J. Vavra, *Chem. and Ind.*, 1958, 330.
[9] K. B. Wiberg and K. A. Saegebarth, *J. Amer. Chem. Soc.*, 1957, **79**, 2822.
[10] E. Adler and R. Magnusson, *Acta Chem. Scand.*, 1959, **13**, 505.
[11] M. L. Wolfrom and J. M. Bobbitt, *J. Amer. Chem. Soc.*, 1956, **78**, 2489.
[12] L. J. Dolby and D. L. Booth, *J. Amer. Chem. Soc.*, 1966, **88**, 1049.

with concentrated hydrochloric acid for 12 hr. Finally, it was washed with water until free from chloride ions and dried at 120°. Celite saturated with the selected stationary phase (0·6 ml./g.) and suspended in the mobile phase was packed into a column (diam. 2 cm.) using about 40 g. of support for crude glucuronide fractions and 20 g. for purer fractions.

Solvent systems for partition chromatography were equilibrated overnight and had the following compositions (by vol.): system E, ethylene dichloride–t-butyl alcohol–acetic acid–water, 9 : 1 : 3 : 7; system T, toluene–t-butyl alcohol–acetic acid–water, 8 : 2 : 3 : 7; system L, light petroleum (b. p. 80—100°)–benzene–methanol–water, 8 : 2 : 7 : 3.

Isolation of Uroterpenol β-Glucuronide.—Urine (*ca.* 2 l.), known to contain not less than *ca.* 20 mg. uroterpenol, with the pH adjusted to 3—4, was mixed with ammonium sulphate (50 g./100 ml.), and the solution extracted with ether–methanol (3 : 1 v/v; 3 × 0·5 vol.). The extract was filtered and passed down an alumina column, and the eluate was discarded. Conjugated sulphates were eluted with 50% aqueous ethanol [13] (250 ml.); they usually gave a negative anisaldehyde reaction.[1a] The column was then washed with n-butanol–water (9 : 1 v/v; 200 ml.) and the washings were discarded. Finally, the glucuronides were eluted with n-butanol–0·1N-ammonia [14] (85 : 15 v/v; 1750 ml.) and after collection of an initial volume (750 ml.) of eluate the rest was collected automatically in 10 ml. fractions which were estimated (see below) for uroterpenol; those giving strong positive reactions were combined and concentrated under reduced pressure. The residue was dissolved in water (100 ml.), filtered, and shaken with ethyl acetate (3 × 100 ml.) which removed most of the pigment and some other material. Although the loss in weight was about 20%, the loss of uroterpenol was less than 5%. Evaporation of the aqueous solution left a gum suitable for partition chromatography.

The gum was dissolved in the stationary phase (system E) and transferred to the column by adding the solution (0·6 ml./g.) to Celite (*ca.* 3·0 g.) and packing this on the top; the column was then developed with the mobile phase. After collection of 400 ml. of eluant, fractions (10 ml.) were collected automatically and estimated for uroterpenol. This appeared after about 700 ml. had been accumulated and was completely removed from the column by about another 1 l. The eluate was concentrated to a greenish gum which was re-chromatographed on a smaller column using system T; the volume of mobile phase required to elute all the glucuronide was about 1·5 l. Uroterpenol β-glucuronide was obtained after evaporation of solvent as a syrup (10—15 mg.) that could not be purified further but did not crystallise.

Location of Uroterpenol Glucuronide in Column Fractions.—From each fraction a portion (0·1 or 0·05 ml.) was removed and portions from five successive tubes (1—5, 6—10, etc.) were combined; the solvent was removed, and the residue subjected to the anisaldehyde reaction. Fractions giving a positive response were generally combined for preparative purposes, but to confirm the identification in representative fractions uroterpenol was liberated by enzymic hydrolysis (suitably scaled down), extracted, partitioned between benzene and water, and examined chromatographically on silica thin layer plates using at every stage techniques already described.[1b]

Methyl (Uroterpenyl 2′,3′,4′-Tri-O-acetyl-β-D-glucopyranosid)uronate (II; R = Ac; R′ = Me).—The syrupy uroterpenol β-glucuronide (*ca.* 15 mg.) was treated in ethanol (2 ml.) with freshly made ethereal diazomethane [15] and kept in ice for 2 hr. Removal of the solvents under reduced pressure left a gummy methyl ester which was treated in pyridine (0·2 ml.) with acetic anhydride (0·5 ml.) for 14 hr. at 20°. Water (10 ml.) was added, and after 20 min. the mixture was extracted with ethyl acetate (3 × 5 ml.). The extracts were combined and washed with N-hydrochloric acid (2 ml.), saturated aqueous sodium hydrogen carbonate (2 ml.), and water (2 × 3 ml.). The solvent was evaporated off and the residue purified by chromatography on Celite (40 g.) using system L; about 1 l. of mobile phase passed through the column before the derivative was detected by the anisaldehyde test, and a further 700 ml. was needed to complete the elution. The gum left after evaporation of the eluate was taken up in ethyl acetate, the solution filtered, and the solvent removed. Acetone (1 ml.) was added, followed by light petroleum (b. p. 40—60°; 5 ml.), and the mixture was kept at −5° until it deposited a solid that, recrystallised twice from the same solvent, afforded *methyl (uroterpenyl 2′,3′,4′-tri-O-acetyl-β-D-glucopyranosid)uronate* as needles, m. p. 155—157°, $[\alpha]_D^{23}$ −11·5° (*c* 0·45 in $CHCl_3$) (Found: C, 56·6; H, 6·9. $C_{23}H_{34}O_{11}$ requires C, 56·8; H, 7·0%). The infrared spectrum (paraffin mull) was very similar to that of methyl (methyl tri-*O*-acetyl-β-D-glucopyranosid)uronate, apart from hydroxylic absorption near 3500 cm.$^{-1}$. The mass spectrum is given in the Table. The n.m.r. spectrum ($CDCl_3$) included three discrete, equally intense bands at τ 8·95 (quaternary methyl), 8·39 (olefinic methyl), and 6·30 (methoxy methyl). A very intense band at τ 8·02 corresponded to acetyl methyl groups, and a broad, concentration-dependent, one-proton band near τ 8 is attributed to a hydroxylic proton since equilibration with deuterium oxide removed it.

Uroterpenol from (+)-Limonene.—(+)-Limonene (III) supplied by Eastman Chemicals was distilled immediately before use and then had b. p. 58°/10 mm., $[\alpha]_D^{25}$ 123°. Plumbic acetate (400 g.; damp with acetic acid; *ca.* 2 moles) was added during 35 min. to a stirred solution of limonene (50 g.) in benzene (750 ml.), and stirring was continued for a further 4 hr. while the temperature was kept at 65° ± 3°. Insoluble materials were removed and the filtrate washed with much water and dried. The solvent was evaporated off under reduced pressure to leave a syrupy residue (100 g.) which was shown by thin layer chromatography on silica from benzene–ethyl acetate (9 : 1 v/v) to consist of four major components. In order of decreasing mobility these were (i) limonene, (ii) an ester believed to be an acetoxylimonene but not further studied, (iii) uroterpenol diacetate, and (iv) uroterpenol monoacetate (I; R = Ac). Apart from component (ii), these were identified (comparison with authentic specimens) by chromatographic and spectroscopic techniques. Chromatographic separations on a larger scale proved troublesome, so the whole of the syrup was hydrolysed with 2% ethanolic potassium hydroxide (2 l.) at 60° for 35 min. The resulting solution was filtered, diluted with water (10 l.), and extracted with methylene chloride (5 × 1 l.). Evaporation of

[13] J. J. Barlow and A. E. Kellie, *Biochem. J.*, 1959, **71**, 86.
[14] O. Crépy, M. F. Jayle, and F. Meslin, *Acta Endocrinol.*, 1957, **24**, 233.
[15] T. J. de Boer and H. J. Backer, *Org. Synth.*, 1963, vol. IV, p. 250.

Mass spectral fragmentation of methyl (uroterpenyl 2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (A) analysed in terms of the spectra of uroterpenol (B) and of methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyran)-uronate (C) separately

Main fragments *m/e*	Assignments A	B	C
(486)	*M* (not observed)		
468	$M - H_2O$		
455	$M - CH_3O$		
(334)			*M* (not observed)
317			$M - OH$
275			$317 - CH_2{\cdot}CO$
257			$*317 - CH_3{\cdot}CO_2H$
215			$*257 - CH_2{\cdot}CO$
197			$257 - CH_3{\cdot}CO_2H$
173			$*215 - CH_2{\cdot}CO$
(170)		*M* (not observed)	
155 (base peak)			$*197 - CH_2{\cdot}CO$
139		$M - CH_2OH$	
135		$M - H_2O - OH$	
134		$M - 2H_2O$	
127			$*155 - CO$
121		$*139 - H_2O$	
119		$134 - CH_3$	
105		$134 - C_2H_4$	
95		$M - MeC(OH){\cdot}CH_2OH$	
93		$*95 - 2H$	
		$*121 - CO$	
91		$*93 - 2H$	

* Indicates assignments supported by appropriate metastable peaks.

the extract left an oil (48 g.) that was dissolved in benzene (400 ml.) and extracted with water (5 × 400 ml.). The combined aqueous extracts were shaken with methylene chloride (2 × 500 ml.) and the organic layers were combined, dried ($MgSO_4$), and concentrated under reduced pressure giving *uroterpenol* (I; R = H) as an oil (22 g.), b. p. 100° (block temp.)/0·1 mm., $[\alpha]_D^{25}$ −89° (*c* 0·441 in $CHCl_3$) (Found: C, 70·3; H, 10·4. $C_{10}H_{18}O_2$ requires C, 70·55, H, 10·65%). Prepared in pyridine from *p*-nitrobenzoyl chloride, the p-*nitrobenzoate* was repeatedly crystallised from aqueous methanol, light petroleum (b. p. 60—80°), or cyclohexane and formed needles, m. p. 80·5—81·5°, mixed m. p. 78·5—80° (with a natural specimen, m. p. 78·6—79·5°), $[\alpha]_D^{25}$ +30° (*c* 0·23 in $CHCl_3$) (Found: C, 64·2; H, 6·6; N, 4·6. $C_{17}H_{21}NO_5$ requires C, 63·9; H, 6·65; N, 4·4%).

Methyl (Uroterpenyl 2′,3′,4′-Tri-O-acetyl-β-D-glucopyranosid)uronate (II; R = Ac, R′ = Me) *from Synthetic Uroterpenol.*—Synthetic uroterpenol (1·0 g.), silver oxide (1·5 g.), and $MgSO_4$ (3·5 g.) were stirred in chloroform (15 ml.) at 22°. Iodine (0·25 g.) was added, followed after 10 min. by methyl (2,3,4-tri-*O*-acetyl-1-α-bromo-1-deoxy-D-glucopyran)uronate (2·0 g.) dissolved in chloroform (10 ml.), according to the method given by Talley.[16] Stirring was continued for a further 24 hr. with the exclusion of light, then the mixture was filtered through silica and all organic material washed out of the residue with chloroform. All filtrates were combined and evaporated leaving a crude product which was purified by crystallisation from aqueous methanol. Further purification from acetone–light petroleum (b. p. 60—80°) (1 : 5 v/v) afforded the methyl uronate as needles (0·7 g.), m. p. 154—156°, mixed m. p. 153—155° (with a natural sample, m. p. 155—157°), $[\alpha]_D^{25}$ +8·0° (*c* 0·113 in $CHCl_3$) (Found: C, 56·85; H, 7·0. $C_{23}H_{34}O_{11}$ requires C, 56·8; H, 7·0%). The mass spectrum and the infrared spectrum were identical with those of a sample of the uronate from natural sources. The n.m.r. spectrum included bands at τ 1·8 (4 aromatic H), 4·6 (one vinylic H), 5·62 (2H in $\cdot CH_2O\cdot$), 8·4 (3 olefinic methyl H), and 8·76 (protons of one quaternary methyl group), along with a concentration-dependent band near τ 8 assigned to tertiary hydroxyl.

Periodate Oxidations.—For reference purposes, *p*-menthane-1,2,8,9-tetrol (VI) was prepared from (+)-limonene by Milas' method.[6]

Synthetic uroterpenol (50 mg.) in t-butyl alcohol (1·2 ml.) and water (12 ml.) was treated with sodium metaperiodate in aqueous solution (10% w/v; 2·4 ml.) and kept at room temperature for 5 min. The organic products were isolated by extraction into methylene chloride, washed with water, dried, and recovered by evaporation of the solvents under reduced pressure. The residual oil (40 mg.) was shown by chromatography on silica plates to consist almost entirely of one product. Removed from the plate, this product gave a 2,4-dinitrophenylhydrazone, m. p. 118—120°, identical with that of 4-acetyl-1-methylcyclohexene (IV) described previously.[1b] The n.m.r. spectrum agreed with this structure; a three-proton band at τ 8·35 corresponded to the olefinic methyl group, another at τ 7·85 to the acetyl methyl group, and a one-proton multiplet centered at τ 4·65 to a vinylic proton adjacent to methylene and in a non-conjugated system.

The foregoing experiment was repeated with longer oxidation periods and at higher temperatures, the most extreme conditions used being 30 min. at 60°. Under these conditions chromatography showed the absence of ketone (IV) and the presence of material running relatively slowly, close to uroterpenol. The new substance appeared to be chromatographically pure, but all efforts to effect purification in other ways, *e.g.*, by distillation, gave complex mixtures. Complex mixtures also resulted when efforts were made to obtain carbonyl derivatives or when the substance was treated with acids or bases. The sensitivity to acids explains why these mixtures were obtained directly in previous work [1b] when periodic acid was used for the oxidation instead of sodium periodate. However, the substance recovered from the silica plates formed an oil which gave a satisfactory n.m.r. spectrum after volatile materials had been removed at 22°/5 mm. (3 hr.). Structure (V) was indicated by a one-proton band at τ 0·4 (unaffected by D_2O) appropriate to aldehyde hydrogen, by two three-proton singlets at τ 7·78 and 7·88 assigned to two non-equivalent methyl ketone groupings, and by an absence of absorption corresponding to vinylic hydrogen. Periodate oxidation of *p*-menthane-1,2,8,9-tetrol (VI) for 5 min. at room temperature gave a substance shown chromatographically and spectroscopically to be the diketonic aldehyde (V) from uroterpenol.

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[16] E. A. Talley, "Methods in Carbohydrate Chemistry," eds. R. L. Whistler and M. L. Wolfrom, Academic Press, London, 1963, vol. II, pp. 1210, 1224.