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URODIOLENONE FROM GRAPEFRUIT JUICE, A URINARY METABOLITE FOUND IN HYPERTENSIVE SUBJECTS

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Key Word Index—*Citrus paradisi*; Rutaceae; grapefruit; urodiolenone; dihydroxy nootkatone; glucuronide; hypertension; urinary metabolite.

Abstract—Urodiolenone, a compound isolated from the urine of hypertensive subjects, is a 1:1 epimeric mixture of two dihydroxy derivatives of nookatone, a constituent of grapefruit. The structure of urodiolenone was suggested by NMR spectroscopy and confirmed by partial synthesis from nootkatone, a sesquiterpenoid ketone with a valencene skeleton. In addition, we show that urodiolenone itself is also present in the grapefruit in free form and possibly also as a glucuronide. The question whether urodiolenone is produced exogenously or endogenously is discussed.

INTRODUCTION

It has recently been reported that hypertensive subjects excrete a low M_r compound in the urine [1]. This compound, named urodiolenone, isolated after urine hydrolysis with β-glucuronidase, was found to be an α,β-unsaturated ketone with a molecular formula of $C_{15}H_{24}O_3$. Recently, urodiolenone was isolated, in comparatively large amounts, from urine of a large number of subjects with neurocirculatory asthenia and labile hypertension, as well as in cases of adrenal hyperplasia (R. Chayen, unpublished results).

In this report, we show that urodiolenone (**1**) is a dihydroxy derivative of a sesquiterpene, nootkatone (**2**) [2], a constituent of grapefruit. In addition, we show that urodiolenone itself is also present in grapefruit in the free form and possibly also as a conjugate of glucuronic acid. This finding raises the question whether the presence of urodiolenone (**1**) in urine is a direct result of diet, or from the metabolism of nootkatone (**2**), which may be specific to hypertensive people. On the other hand, a possibility exists wherein urodiolenone (**1**) is produced endogenously by hypertensive subjects.

RESULTS AND DISCUSSION

The structure determination of urodiolenone (**1**) isolated from the urine of hypertensive patients was established mainly on the basis of its ^{1}H NMR spectrum. Signal doubling for two methyl groups indicated that urodiolenone (**1**) exists as a mixture of two compounds. Also, the side chain proton resonances are doubled in the spectrum (two doublets and two quartets) pointing to a mixture of two stereoisomers. On this basis, as well as use of data from UV, IR and mass spectral studies [1], we have established that urodiolenone exists as a mixture ($\sim$1:1) of two C-11 epimers, **1a** and **1b**.

We have confirmed the structure of urodiolenone (**1**) by synthesis, starting from the readily available nootkatone (**2**) [2], a consistent of grapefruit peel. Nootkatone (**2**) was epoxidized with *m*-chloroperbenzoic acid and the resulting 1:1 mixture of two 11,12-epoxy derivatives **4** [4], was treated with perchloric acid to yield urodiolenone, identical in all respects to the 1:1 mixture of the C-11 epimers, **1a** and **1b**, isolated from urine. Since all attempts to separate this epimeric mixture failed, we separated their respective C-12 acetates, **3a** and **3b**, using analytical TLC. Following the hydrolysis of the separated acetates, their ^{1}H NMR spectrum was identical to the spectrum of the natural urodiolenone (**1**).

Since urodiolenone (**1**) is a derivative of nootkatone (**2**), a constituent of grapefruit, it became plausible that the former may also be present in grapefruit as a natural constituent. Analysis of the grapefruit juice was performed by extraction with organic solvents, followed by chromatographic separations. Fractions having the same R_f values as urodiolenone (**1**) were acetylated and separated by analytical TLC, resulting in two acetates having identical ^{1}H NMR and mass spectra as acetates **3a** and **3b**.

The concentration of urodiolenone (**1**) in the grapefruit juice was estimated, by reverse-phase HPLC, to be 250 μg. (see Experimental). A similar concentration of urodiolenone (**1**) was found using a quantitative fluorescent measurement of an eluted spot from TLC of an extract of grapefruit juice [1]. The concentration of urodiolenone (**1**), as determined by the latter method, increased fourfold, following incubation with *Helix pomatia* juice, indicating that urodiolenone (**1**) might be present as

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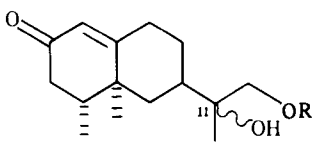
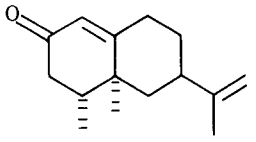


1a R = H; 11*R*(or 11*S*)
1b R = H; 11*S* (or 11*R*)
3a R = Ac; 11*R*(or 11*S*)
3b R = Ac; 11*S* (or 11*R*)



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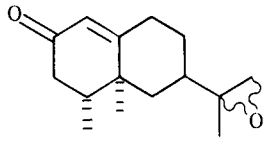

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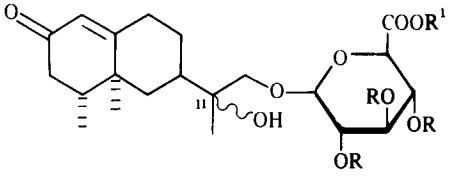
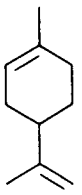


5 R = H, R^1 = H
6a R = Ac, R^1 = Me; 11*R*(or 11*S*)
6b R = Ac, R^1 = Me; 11*S* (or 11*R*)



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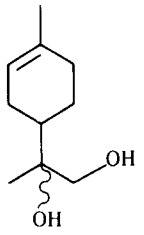

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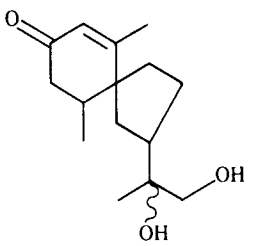

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glucuronide **5** in the grapefruit juice. We attempted to establish the presence of the glucuronide of urodiolenone in the grapefruit juice. It involved preparation of a synthetic standard compound, by condensation of urodiolenone (**1**) with the bromo-α-D-glucuronic acid methylester, using an improved Koenings–Knorr method [5]. This reaction led to formation of two epimeric glucuronic acid derivatives **6a** and **6b**, which were separated on TLC. We were unable to remove the protecting groups from the glucuronic acid without corresponding hydrolysis of the *O*-glycosidic bond. Instead, we used the acetylated epimers (**6**) as the reference compounds. Therefore, after the filtered grape-fruit juice was passed through an XAD-2 column, which was then eluted with MeOH, the methanolic fraction was acetylated and partially purified by flash column chromatography, analytical TLC and HPLC. The final separation yielded two peaks in a 1:1 ratio with retention times identical to those of the two synthetic glucuronides **6a** and **6b**. This comparison indicated the presence of urodiolenone glucuronides (**5**) in grapefruit juice.

It appears that, during the past 30 years, four other groups have described compounds, which, according to their physiochemical properties, are most likely identical with urodiolenone (**1**) [6–14]. These were isolated from urine in pregnancy [6], during heat stress [7], in essential and malignant hypertension [6–12, 14] and in Cushing's syndrome [6, 12]. The same compounds have also been found in citrus fruit [12], and it was claimed that they are excreted in the urine after ingestion of orange juice. Therefore, their presence in urine was attributed only to exogenous sources [8, 9, 11]. Their endogenous origin should not be overlooked, as potassium administration or angiotensin infusion in patients with hypertension caused a dose response increase in the excretion of these compounds by 30–100-fold under strict dietary conditions [12]. The observation of excretion of elevated amounts of urodiolenone (**1**) by hypertensive and not by normotensive subjects [1] also raises the question of the extent to which urodiolenone (**1**) is produced endogenously. It is of interest to note that an analogous 1:1 mixture of epimeric vicinal diols, uroterpenol (**8**) is excreted in large amounts in subjects with adrenal hyperplasia, as well as in pregnancy, after administration of limonene (**7**) [15–20]. It should also be noted that another 1:1 mixture of epimeric vicinal diols, **9**, possessing a biogenetically similar skel-

eton with the same substitution pattern as urodiolenone, was found in flue-cured tobacco [21] and infected potatoes [22], and is considered to be a phytoalexin (plant stress compound). Whether urodiolenone (**1**) also has a role in the reaction to stress is a matter that has yet to be determined.

EXPERIMENTAL

Methods. Flash chromatography was performed using Kieselgel 60, mesh 43–60 (Merck). TLC was performed on analytical and prep. plates (Kieselgel 60 F_{254}). Analytical HPLC: Silicon-60 5μ column at 240 nm, reverse phase HPLC: 18C-Nucleosil 10μ column at 254 nm. NMR: $CDCl_3$ assignments marked by an asterisk are interchangeable. Mps: uncorr.

Isolation of urodiolenone **1** *from urine.* Urine (5 ml) was adjusted to pH 4.5–4.7 with glacial acetic acid and 0.5 ml of 2M acetate buffer pH 4.5 was added. After the addition of 0.1 ml *Helix pomatia* juice (Sigma, *ca* 100000 units of β-glucuronidase activity per ml), the urine was incubated at 50° for 20 hr. The cooled urine was extracted with 15 ml Et_2O, and the Et_2O extract washed with 2 ml 0.5 N NaOH, with H_2O and then evapd. The dried extract was dissolved in 0.1–0.2 ml $CHCl_3$ and chromatographed on a TLC silica gel plate (Merck-60F_{254}) by the method of Kraiem *et al* [3]. Thus, the chromatogram was developed first in $CHCl_3$–EtOH (94:6), followed by $CHCl_3$–CH_2Cl_2–EtOH (45:45:10). After drying, the UV-absorbing spot running between tetrahydrocorticosterone and tetrahydro-11-dehydrocorticosterone was cut out from the plate and eluted twice with 3.5 ml $CHCl_3$–EtOH (6:1). The dried eluate was rechromatographed and developed by EtOAc–$CHCl_3$–H_2O (90:10:1) and the spot of urodiolenone was eluted as before. Following solvent evaporation, a fluorimetric assay was performed by treating the residue with 3 ml H_2SO_4–EtOH (3:1) ('Fluorescence agent FDPC', BDH). Fluorescence was measured after 15 min at activation and emission wavelengths of 445 and 495 nm, respectively. For large scale preparation of urodiolenone, urine collections from patients known to excrete large amounts of urodiolenone were passed down a column of Amberlite XAD-2 (BDH) in order to absorb the glucuronide and then processed further, as described previously [1].

Synthesis of urodiolenone (**1**). (a) Nootkatone (**2**) (16.00 g) in CH_2Cl_3 (1 l) was left with *m*-chloroperbenzoic acid (15.55 g) for 16 hr at 4°, the reaction mixture was filtered and the solvent removed by evaporation. The residue was redissolved in Et_2O, successively washed with aq. satd Na_2SO_3, with 10% Na_2CO_3 and with H_2O. Flash chromatography, using EtOAc–C_6H_{14} (1:3) as eluant, yielded epoxides **4** (12 g, 70% yield) as white needles (from C_6H_{14}). Mp. 90–91° (lit. 92°) [4], NMR: δ0.94–1.07 (9H, H-13, H-14, H-15), (*m*, 2H, H-12), 5.76 (*s*, 1H, H-1)

(b) A soln of the epoxides (**4**) (5 g) in H_2O–dioxane (1:1, 400 ml) was adjusted to pH 2 with conc perchloric acid. After 16 hr, the reaction mixture was neutralized with satd $NaHCO_3$ and lyophilized. The powdered residue was stirred overnight in EtOAc, filtered and the solvent removed under vacuum, leaving a yellow, oily residue. Flash chromatography, using EtOAc, yielded urodiolenone (**1**) (3.26 g, 60% yield) as a colourless oil. UV: λ_{max} 237 nm (EtOH, ε, 16000) and 231 nm (Et_2O, ε 16000). NMR: δ0.95–1.11 (9H, H-13, H-14, H-15), 3.54 (*m*, 2H, H-12), 5.76: (*s*, 1H, H-1).

Separation of epimeric urodiolenones **1a** and **1b**.(i) A mixture of the two epimeric diols of urodiolenone (**1**) (130 mg) was dissolved in dry pyridine (3 ml) and Ac_2O (6 ml). After stirring for 16 hr at room temp. the soln was evapd under vacuum and flash chromatographed, using Et_2O, to yield a 1:1 mixture of acetates (**3**) (135 mg)„ 88% yield) as a colourless oil. UV λ_{max} 238 nm (EtOH, ε 16000). NMR δ0.94–1.14 (*m*, 9H H-13, H-14, H-15), 3.12 (*s*, 3H, OAc), 4.08 (*s*, 2H, H-12), 5.76 (*s*, 1H H-1), which were separated on analytical TLC by developing them five times successively in Et_2O. NMR Acetate **3a**: δ0.99 (*d*, J = 6.8 Hz, 3H, H-14) 1.11 (*s*, 3H, H-15*), 1.15 (*s*, 3H, H-13*), 2.14 (*s*, 3H OAc), 4.06 (*s*, 2H, H-12), 5.77 (*s*, 1H, H-1). Acetate **3b**: δ0.97 (*d*, J =6.8 Hz 3H, H-14), 1.08 (*s*, 3H, H-15*), 1.15 (*s*, 3H, H-13*), 2.14 (*s*, 3H, OAc), 4.13 and 4.01 (*q*, J = 11.4 Hz, 2H, C-12), 5.77 (*s*, 1H, H-1).

(ii) Acetate **3a**: (18 mg) was dissolved in MeOH (1 ml), and treated with 10% NaOH under Ar atmosphere. The progress of the reaction was monitored by TLC and upon completion, the mixture was neutralized with 10% aq. acetic acid, and the solvent removed under vacuum. The residue was dissolved in H_2O, extracted with CH_2Cl_2 and filtered through a silica gel plug, yielding urodiolenone (**1a**) as a colourless oil (11 mg, 73% yield). Acetate **3b** was hydrolysed in a similar way to yield urodiolenone (**1b**) as a colorless oil. Urodiolenone (**1a**) δ0.97 (*d*, J = 6.8 Hz, 3H H-14), 1.10 (*s*, 3H H-15*) 1.11 (*s*, 3H, H-13*), 3.60 and 3.46 (*q*, J = 10.6 Hz, 2H H-12), 5.76 (*s*, 1H, H-1). Urodiolenone **1b**: δ0.99 (*d*, J = 6.6 Hz 3H, H-14), 1.09 (*s*, 3H H-15*), 1.10 (*s*, 3H, H-13*), 3.62 and 3.46 (*q*, J = 10.8 Hz, 2H, H-12) 5.76 (*s*, 1H, H-1).

The determination of the presence of urodiolenone (**1**) *in grapefruit juice.* Grapefruit juice concn (500 ml) was diluted with H_2O (2 l) and divided into two equal portions. Each portion was extracted × 3 with EtOAc. The extract was dried over Na_2SO_4 and the solvent removed under red. pres. at room temp. The resulting finely powdered residue was fluxed with CH_2Cl_2 for 6 hr. Cooling, filtration and evapn of the solvent yielded a residue (2.5 g), which was subjected to prep. flash chromatography using EtOAc. Elution peaks having the same retention times as urodiolenone (**1a** and **1b**) in the reverse phase HPLC (CH_3CN–H_2O, 9:1) were combined. After removal of the solvents, the resulting residue was submitted once more to a similar separation by reversed phase HPLC (EtOAc–CH_2Cl_2). The fractions containing diols **1a** and **1b** were evapd to dryness, acetylated and chromatographed on analytical TLC plates. Bands with the same R_f values as the synthetic acetates **3a** and **3b** were isolated and their identity was established by comparing their ^{1}H NMR and mass spectra.

Synthesis of β-D-*glucuronides* **6**. A soln of urodiolenone (**1**) (8 g) was treated in 20 ml Et_2O, with bromoyluronate [5] (329 mg) and then with freshly prepared dry Ag_2CO_3 (0.8 g) and dry $MgSO_4$ (0.5 g), in the dark. After stirring for 36 hr, the reacted silver salt was filtered off and repeatedly washed with CH_2Cl_2. Evaporation of the solvent, followed by flash chromatography (C_6H_{14}–EtOAc, 3:2) yielded a colourless oil (210 mg). Separation on analytical TLC with Et_2O afforded two β-D-glucuronides **6a** and **6b**. Glucuronide **5a**: NMR: δ0.98 (*d*, J = 6.8 Hz, 3H, H-14), 1.06 (*s*, 3H H-15*), 1.09 (*s*, 3H, H-13*), 2.03 (*s*, 6H, 2 × OAc), 2.06 (*s*, 3H, OAc), 3.69 and 3.65 (*q*, J = 9.9 Hz 2H, H-12), 3.76 (*s*, 3H, OMe), 4.06 (*m*, 1H, H-5), 4.61 (*d*, J = 7.5 Hz, 1H, H-1), 5.05 (*m*, 1H, H-2), 5.26 (*m*, 2H, H-3 and H-4), 5.74 (*s*, 1H, H-1). Glucuronide **5b**: NMR: δ0.96 (*d*, J = 6.8 Hz, 3H, H-14), 1.06 (*s*, 3H, H-15*), 1.08 (*s*, 3H, H-13*), 2.03 (*s*, 3H, OAc), 2.04 (*s*, 3H, OAc), 3.68 (*m*, 2H, H-12), 3.76 (*s*, 3H, OMe), 4.08 (*m*, 1H, H-5), 4.61 (*d*, J = 7.5 Hz, 1H, H-1), 5.05 (*m*, 1H, H-2), 5.27 (*m*, 2H, H-3 and H-4), 5.75 (*s*, 1H, H-1).

The determination of urodiolenone glucuronides (**5**) *in grapefruit juice.* Grapefruit juice concn (450 ml), diluted to 2 l was centrifuged for 30 min at 2300 *g* and then for 30 min at 4000 *g*. Suction filtration of the mother liquor through a Celite column afforded a clear, light yellow liquid. The filtrate (1.4 l) was divided into four portions which were successively passed through an XAD-2 column at a rate of approximately 150 ml per hr. After each passage, the column was regenerated by washing with

distilled MeOH (1.1 l), followed by basic MeOH (200 ml, 2% NaOH) until the eluant appeared colourless. The basic methanolic fractions were combined, neutralized with AcOH and the solvents removed by evapn and lyophilization. The residue was redissolved in MeOH (200 ml), cooled in an ice-bath and an ethereal soln of CH_2N_2 was added dropwise, followed by stirring for 12 hr. The solvent was evapd, the residue dried under vacuum, dissolved in dry pyridine (50 ml) and Ac_2O (100 ml), left overnight at room temp. followed by evapn under vacuum with repeated additions of toluene. Flash chromatography of the mixture, using EtOAc afforded, after evapn of the solvent, an oily yellow liquid (24 mg) which was separated on analytical TLC, developed × 6 in Et_2O. Two broad bands with R_f-values similar to the synthetic glucuronides **6a** and **6b** were isolated and separated on HPLC hexane–CH_2Cl_2 (1:1) with 1.5% *iso*-PrOH]. The elution profile of each band showed, among others, two peaks with the same retention times as synthetic glucuronides **6a** and **6b**, respectively.

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