GLUCOSIDES OF ACIDIC LIMONOIDS IN CITRUS

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Abstract—17-*O*-β-D-Glucopyranosides of nomilinic acid, deacetylnomilinic acid, isoobacunoic acid, epiisobacunoic acid, obacunoic acid, and *trans*-obacunoic acid were isolated from grapefruit seeds. The limonoid aglycones were identified by ^{1}H and ^{13}C NMR spectroscopy

INTRODUCTION

We recently reported the discovery of 17-*O*-β-D-gluco-pyranosides of the major neutral citrus limonoids limonin (**1**), nomilin, deacetylnomilin and obacunone in grape-fruit (*Citrus paradisii*) seeds [1]. These glucosides are present in high concentrations relative to the aglycones and may be of significance in the areas of limonoid biosynthesis and metabolism, taste of citrus products, and human nutrition. We have now isolated and charac-terized six more limonoid 17-*O*-β-D-glucopyranosides from grapefruit seeds. All of them are glucosides of limonoids in which the A-ring lactone is open and C-3 is therefore a free carboxyl group

RESULTS AND DISCUSSION

As we reported previously [1], aqueous extracts of grapefruit seeds contain *ca* 15 compounds giving an Ehrlich-positive reaction on TLC which is characteristic of limonoids [2] and four of which were isolated and identified. In these compounds the D-ring lactone was open and D-glucose was attached to the 17-hydroxyl by a β-glycosidic linkage. After repeated reversed phase and ion exchange column chromatography, six of the other compounds were isolated. In each case acid hydrolysis produced D-glucose, identified by a specific enzyme reac-tion as described previously [1]. Furthermore, the sugar resonances in the ^{1}H and ^{13}C NMR spectra of these compounds were almost identical to those of the gluco-sides previously identified. Likewise, the resonances at-tributable to the limonoid positions C-12 to C-17 and the furan ring of the new compounds showed the same anomalous shifts previously observed. Thus the new compounds must also be 17-*O*-β-D-glucopyranosides.

As the limonoid aglycones of the glucosides are de-stroyed under the conditions used for acid hydrolysis, they were identified by NMR spectroscopy of the gluco-sides. Both the ^{1}H and ^{13}C NMR spectra of the major glucoside contained six C-methyl signals, one of which showed chemical shifts characteristic of an acetate methyl. A downfield one-proton signal at δ 5.81 in the ^{1}H NMR spectrum was consistent with a proton attached to a carbon bearing an acetoxy group. The only known

acetylated citrus limonoids are nomilin and nomilinic acid (**2**). The glucoside of nomilin was one of the four previously isolated, so the ^{13}C NMR spectrum of the new glucoside was compared with that of methyl nomilinate (**3**) [3]. The signals for the carbon atoms remote from the D-ring area (C-1 to C-11) were very similar for the two compounds (Table 1). The largest differences, *ca* 3 ppm, were observed for carbons C-5 and C-9, probably due to changes in the shape of the B and C rings when the D-ring is open. The chemical shift of the C-4 signal (73 ppm) clearly shows that the A-ring of the glucoside is open, making C-3 a free acid and C-4 a hydroxyl group. A lactone linkage at C-4 causes a chemical shift of *ca* 83–84 ppm for this carbon resonance, while a 1,4-cyclic ether system (A′-ring) as in **1** results in a chemical shift of *ca* 78–79 ppm. All of the signals in both the ^{1}H and ^{13}C NMR spectra are completely consistent with **2** as the aglycone, and accordingly we have assigned the structure nomilinic acid 17-*O*-β-D-glucopyranoside (**4**) to this com-pound.

The next glucoside showed ^{1}H and ^{13}C NMR spectra quite similar to that of the first one, except that the no acetate methyl signal was present and the carbinol proton signal was further upfield, at δ 4.10 The C-4 resonance at δ 73 showed that again C-3 was a carboxyl and C-4 a hydroxyl group. This suggested that the aglycone was deacetylnomilinic acid (**5**) [3]. When the ^{13}C NMR spectrum of the glucoside was compared with that of methyl deacetylnomilinate (**6**), as in the previous case a close correspondence was observed for C-1 to C-11 (Table 1). Therefore we have assigned the structure deacetylnomilinic acid 17-*O*-β-D-glucopyranoside (**7**) to this compound.
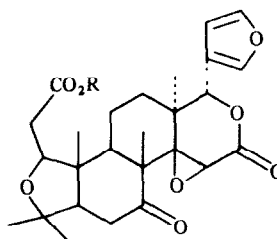
The ^{1}H NMR spectrum of the next glucoside to be considered was very similar to that of **7**, in this case the carbinol proton resonance being located at δ 3 91. How-ever, the C-4 signal was at δ 78, indicating the presence of an A′-ring. The most likely aglycone with these character-istics is isoobacunoic acid (**8**) [3], and indeed a com-parison of the ^{13}C NMR spectrum of the glucoside with that of methyl isoobacunoate (**9**) showed close similar-ities for C-1 to C-11 (Table 1). All of the resonances in the NMR spectra are consistent with the structural assign-ment of isoobacunoic acid 17-*O*-β-D-glucopyranoside (**10**) to this glucoside.
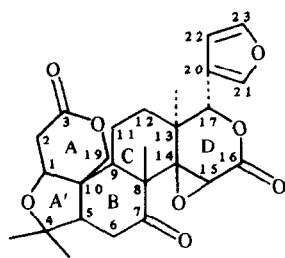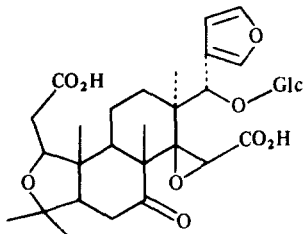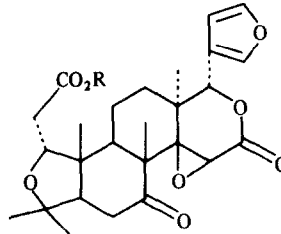
1   Limonin
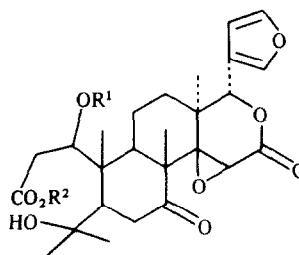
2   Nomilinic acid   R¹ = Ac, R² = H
3   Methyl nomilinate   R¹ = Ac, R² = Me
5   Deacetylnomilinic acid   R¹ = H, R² = H
6   Methyl deacetylnomilinate   R¹ = H, R² = Me
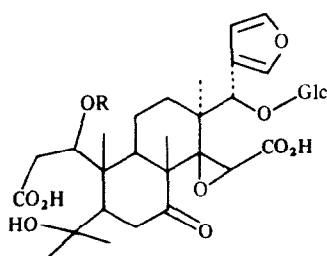
4   Nomilinic acid 17-O-β-D-glucoside   R = Ac
7   Deacetylnomilinic acid 17-O-β-D-glucoside   R = H
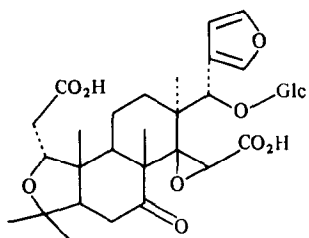
8   Isoobacunoic acid   R = H
9   Methyl isoobacunoate   R = Me

10   Isoobacunoic acid 17-O-β-D-glucoside

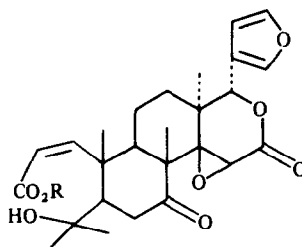11   Epiisoobacunoic acid   R = H
12   Methyl epiisoobacunoic acid   R = Me

The next glucoside showed a ¹H NMR spectrum closely resembling that of 10, with the carbinol proton signal slightly downfield at δ4 02. The C-4 resonance, at δ79, again indicated the presence of an A′-ring. A possible candidate for the aglycone in this case would be the C-1 isomer of 8, epiisoobacunoic acid (11) [3]. The main differences between the ¹H NMR spectra of the methyl esters of 8 and 11 are downfield shifts of protons 1-H and 15-H in the latter case Downfield shifts of these two signals are also observed in comparing the proton spectra of 10 and the present glucoside (Table 2) The largest difference between the ¹³C NMR spectra of the methyl esters of 8 and 11 is a 4 ppm upfield shift of the C-5 resonance for the latter, which is consistent with a 5 ppm upfield shift observed for this carbon signal in comparing the ¹³C NMR spectra of 10 and the present glucoside (Table 3). Furthermore, a comparison of the ¹³C NMR spectra of methyl epiisoobacunoate (12) with that of the present glucoside showed good agreement for C-1 to C-

11. These data enable us to assign the structure epiisoobacunoic acid 17-O-β-D-glucopyranoside (13) to this glucoside

The major diagnostic feature in the ¹H NMR spectrum of the next glucoside was a two proton AB quartet near δ6, ascribable to a double bond. The C-4 resonance was located at δ72, showing the absence of an A-ring lactone and an A′-ring As no H-1 carbinol signal was observed, the double bond can be confidently assigned to the 1,2-position This leads to obacunoic acid (14) as the most likely aglycone of this glucoside The chemical shifts of H-1 and H-2 in the glucoside (δ5 86 and 5 74) are close to those for methyl obacunoate (15) (δ6.01 and 5 71), and the coupling constant (J = 13 Hz) is the same in both cases Likewise, the resonances for C-1 to C-11 of the glucoside correspond closely to those of 15, except for C-1 and the bridgehead C-9 (Table 1). A similar difference in the C-1 resonance was observed previously in comparing obacunone with obacunone glucoside [1] Accordingly
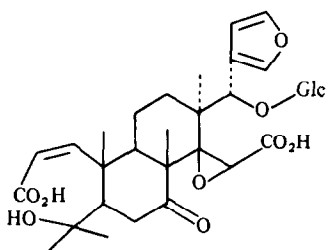
13 Epiisoobacunoic acid 17-O-β-D-glucoside

14 Obacunoic acid R = H
15 Methyl obacunoate R = Me

16 Obacunoic acid 17-O-β-D-glucoside
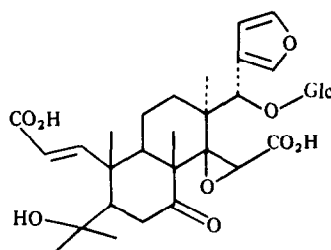
17 *trans*-Obacunoic acid 17-O-β-D-glucoside

Table 1 ^{13}C NMR chemical shift differences between aglycone methyl esters and glucosides*

| Aglycone ester | 3 | 6 | 9 | 12 | 15 |
Glucoside	4	7	10	13	16
C					
1	−0 4	−0 4	−0.6	−0.7	−4.7
2	−0 2	0 7	0.9	0.1	1.7
3	1 1	1.4	0 9	1 0	1.0
4	0.2	0.4	0.0	−0.5	−1.3
5	−3.3	−4 1	−3.2	−4 3	−1 8
6	0.3	0.6	0.7	0.2	0.4
7	2.4	2 5	0.7	0.2	3.6
8	−0.5	−0.5	0.3	−0.8	−1.5
9	−3.1	−3 9	−0.5	−1.8	−5.4
10	−1.2	−0.5	0.1	−0.2	−1.3
11	−1 1	−1.5	−0.3	−1.7	−2.7
12	−4.3	−4.4	−1.5	−3 3	−6.1
13	6 8	7.1	6 4	6.9	6.5
14	4 8	5 1	4 7	4.7	5.5
15	5 5	6.0	2.6	3.4	5.1
16	2 2	2 0	2 8	3.0	2 0
17	0.0	0.1	0.0	−0.2	−0.3
20	5 4	5.6	5.7	5 6	5.3
21	−1.7	−1.8	−1.0	−1.3	−1.3
22	2 5	2.5	3 1	3.0	2 7
23	−0.8	−0.8	−0.1	−0.2	−0.4

*Figures were obtained by subtracting the chemical shifts of the glucosides from those of the aglycone methyl esters Compound **17** is not included in the Table because the aglycone was not available

Table 2 ¹H NMR spectra of limonoid glucosides*

H	4	7	10	13	16	17
α-Furans	7 48	7 47	7 52	7 53	7 48	7 48
	7 41	7 40	7 41	7 41	7 39	7 41
β-Furan	6 51	6 51	6 55	6 55	6 51	6 51
H-17	5 21	5 21	5 21	5 25	5 20	5 19
H-1	5 81 m	4 10 m	3 91 m	4 02 m	5 86 d (13 2)	6 83 d (16 0)
H-2	—	--	—	—	5 74 d (13 2)	5 57 d (16 0)
H-15	2 81	2 78	3 17	3 31	2 90	2 95
C-Me	1 44	1 42	1 39	1 34	1 44	1 42
	1 16	1 15	1 18	1.25	1 08	1 04
	1 15	1 07	1 01	1 07	1 08	1 04
	0 87	0 97	0 83	1 03	0 96	0 90
	0 77	0 78	0 75	0 81	0 76	0 75
Acetate Me	1 96	-	--	—	-	-
Sugar H-1	4 13 d (7 3)	4 13 d (7 3)	4 13 d (7 3)	4 14 d (7 3)	4 13 d (7 3)	4 11 d (7 3)

*Samples were run in DMSO-d_6 at 90°, at 270 MHz Coupling constants (in Hz) are in parentheses

Table 3 ¹³C NMR spectra of limonoids*

C	3	12†	15†	4	7	10	13	16	17
1	76 2	83 6	159 9	75 8	73 2	81 6	82 9	155 2	158 3
2	38 5	36 7	118 1	38 7	37 8	36 9	36 8	119 8	119 1
3	171 1	170 9	166 6	172 2	174.2	172 1	171 9	167 6	167 1
4	73 0	80 4	73 6	73 2	73 3	78 1	79 9	72 3	72 5
5	51 6	55 6	55 5	48 3	47 2	56 5	51 3	53 7	54 8
6	35 0	36 4	38 2	35 3	39 5	36 7	36 6	38 6	38 2
7	209 9	208 0	209 3	212 3	213 3	208 6	208 2	212 9	212 0
8	51 9	51 1	52 8	51 4	51 3	50 5	50 3	51 3	51 1
9	43 4	43 1	45 9	40 3	39 7	44 8	41 3	40 5	42 6
10	45 7	46 9	45 3	44 5	44 7	45 4ᵃ	46 7	44 0ᵃ	44 0ᵃ
11	18 4	17 9	19 5	17 3	16 9	17 0	16 2	16 8	17 4
12	31 2	30 1	32 8	26 9	27.0	26 5	26 8	26 7	26 6
13	36 8	38 1	37 4	43 6	43 7	45 0ᵃ	45 0	43 9ᵃ	43 6ᵃ
14	65 7	66 5	65.6	70 5	70 5	71 1	71 2	71 1	70 9
15	52 8	54 0	53 3	58 3	58 2	57 3	57 4	58 4	58 3
16	166 8	167 0	167 2	169.0	169 1	169 8	170 0	169 2	169 2
17	77 8	77 7	78 2	77 8	77 7	77 9	77 9	77 9	77 7
20	120 3	120 1	120 5	125 7	125 8	125 7	125 7	125 8	125 6
21	143 2	142 9	142 9	141 5	141 5	141 6	141 6	141 6	141 6
22	110 2	109 6	111 0	112 7	112 7	112 6	112 6	112 7	112 6
23	141 4	140 9	141 0	140 6	140 6	140 7	140 7	140 6	140 6
C-Methyls	32 4	31 6	32 5	31 7	32 5	30 1	32 1	30 2	30 1
	28 7	23 5	29 0	28 0	27 7	25 5	25 4	29 9	28 8
	20 2	20 6	20 4	24 9	24 7	23 9	24 3	24 3	25 0
	16 0	19 4	20 0	16 3	16 3	19 7	19 7	19 4	17 0
	16 0	17 7	16 6	15 0	15 3	9 7	16 2	16 9	14 5
O-Methyl	51 2	51 7	51 7	---	-	—			
Acetate carbonyl	169 5	-		169 2	-	—	--	-	
Acetate methyl	20 7	—	--	20 6	---	--	--	—	--
Glucose C-1			---	104 5	104 4	104 4	104 3	104 5	104 4
Glucose C-6	-	—	—	61 6	61 6	61 6	61 6	61 6	61 7
Glucose C2-C4	—	—	--	77 0	77 0	77 0	76 9	77 0	77 1
	—	—	—	76 1	76 1	76 2	76 1	76 0	76 2
	—	—	—	74 1	74 1	74 2	74 2	74 1	74 2
	—	—	—	70 6	70 7	70 7	70 6	70 7	70 7

*Samples were run in DMSO-d_6 at 90°, at 67 8 MHz, unless otherwise specified
† In CDCl₃ at 25°
ᵃAssignments in the same vertical row may be reversed

we have assigned the structure obacunoic acid 17-*O*-β-D-glucopyranoside (16) to this compound.

The ^{1}HNMR spectrum of the final glucoside was very similar to that of 16, except that the two protons of the AB quartet were more widely separated and the coupling constant was larger (16 Hz). This suggested that the double bond was *trans* rather than *cis* as in 14. The *trans*-isomer of 14 is not a known compound; to our knowledge the only known limonoids containing a *trans*-1,2-double bond are methyl anhydroisolimonate [4] and methyl 19-hydroxyobacunoate [5]. The coupling constant between H-1 and H-2 in both of these compounds is 16 Hz, as in the present glucoside. The ^{13}CNMR chemical shifts for all of the carbons of this glucoside except C-1 are very close to those of 16 (Table 3), as would be expected if they differ only in the configuration of the double bond. Thus we assign the structure *trans*-obacunoic acid 17-*O*-β-D-glucoside (17) to this compound.

Four of the acidic limonoid aglycones (2, 5, 8, and 11) were previously isolated from grapefruit seeds [3]. Although 14 and *trans*-obacunoic acid have never been isolated, indirect evidence for the presence of the former in citrus has been obtained When [^{14}C]-obacunone was administered to a young lemon tree, it was partially converted to [^{14}C]-14 [6]

These glucosides were present in much higher concentrations than the aglycones, as was the case for the glucosides previously isolated [1]. Some of the glucosides have a slightly bitter taste, although much less than for those aglycones which are bitter. A systematic study of the taste properties of the glucosides is currently in progress.

EXPERIMENTAL

General. Amberlite XAD-2 resin, 20–60 mesh, was obtained from Sigma (St. Louis, MO), and the same resin, 150–300 mesh,

from Accurate Chemical & Scientific (Westbury, NY). Column fractions were monitored by TLC and HPLC. ^{13}CNMR spectral assignments were made on the basis of SFORD and DEPT spectra, selective heteronuclear decoupling, and comparison with spectra of related limonoids for which assignments had previously been made [7, 8]. The spectrum for compound 9 is in ref. [7], and that for 6 is in ref [8].

Isolation of glucosides Grapefruit seed meal was washed thoroughly with hexane, followed by Me$_2$CO. The glucosides were then extracted from the residue with MeOH. All extractions were performed at 50–60°. The glucosides were first fractionated on a 5 × 40 cm column of coarse XAD-2 resin, eluting with a linear gradient formed from 2 l each of 5 and 65% MeOH in H$_2$O Individual fractions from this column were further purified by chromatography on a 2.5 × 90 cm column of fine XAD-2 resin, eluting with a linear gradient formed from 1 5 l of 5 and 50% MeCN in H$_2$O. Fractions containing a single glucoside were then further purified by chromatography on a 2.5 × 30 cm column of DEAE Sephacel (Pharmacia), eluting with a linear gradient formed from 1 l each of H$_2$O and 0.1 M HCl. Finally, the eluent fraction containing the glucoside was freed of HCl by passage through a column of 40 μm C-18 adsorbent (Baker), followed by elution with MeOH

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