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The Structure of Graucin A, a New Bitter Limonoid from *Evodia grauca* Miq. (Rutaceae)

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Graucin A, a new bitter limonoid, has been isolated from the root bark of *Evodia grauca* Miq. as well as the known limonoids limonin, rutaevin and limonin diosphenol. Its structural and stereochemical assignments were made using NMR (COSY and NOESY), and CD spectroscopy, which indicated the uncommon 5β-H configuration of this new compound. Rutaevin was also recognized to be a 5β-H limonoid.

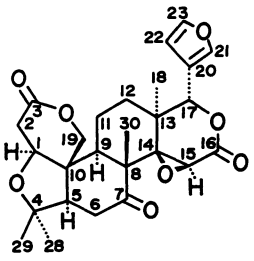
In the course of our studies on the biologically active limonoids,[1] we isolated a new limonoid graucin A (**1**) along with the known limonoid limonin (**2**),[2] rutaevin (**3**)[3] and limonin diosphenol (**4**)[3,4] as bitter components of the root bark of *Evodia grauca* Miq.(Rutaceae). The structure **1** was assigned on the basis of COSY and NOESY NMR spectra of **1** and the acetate **5**, and by the CD study on the benzoate **6**. Both graucin A and the structurally similar limonoid rutaevin (**3**) have been assigned the 5β-H configuration, which is opposite to that of many other known Rutaceae limonoids including limonin.

Some biological activities of the limonoids (**1**—**4**) were also examined.

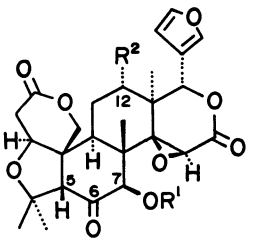
Results and Discussion

The ether extract of the root bark contained a variety of limonoids which were detected by the characteristic color with Ehrlich's reagent on TLC. Fractionation by extensive silica-gel column chromatography followed by the final HPLC purification on a normal phase column using 0.4—1.0% methanol in dichloromethane as an eluant gave the new compound **1** named graucin A along with three known limonoids previously isolated from various Rutaceae plants: limonin (**2**), rutaevin (**3**) and limonin diosphenol (**4**).

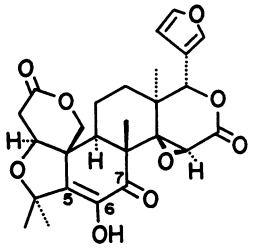
Graucin A (**1**), $C_{26}H_{30}O_{10}$, mp > 310 °C (prisms from acetonitrile), exhibited the following spectral data. SI-MS: m/z 503 $(M+1)^+$; $[\alpha]_D^{15}$ −150° (c 0.0005, CH_3CN); UV (MeOH): 209 nm (ε 6300, π-π* of furan). The IR spectrum showed the presence of hydroxyl (3450 cm^{-1}), γ-lactone (1765 cm^{-1}), δ-lactone (1740 cm^{-1}) and ketone groups (1700 cm^{-1}) and the CD spectrum also showed the presence of furan (228 nm, $\Delta\varepsilon$ −5.3; π-π*) and ketone groups (297 nm, $\Delta\varepsilon$ −1.7; n-π*). The ^{1}H NMR spectrum showed signals of four tertiary methyl groups at δ 0.61 (8-Me), 1.23 (4α-Me), 1.35 (13-Me), and 1.44 (4β-Me), a singlet at δ 4.15 (15-H), a AB quartet (J=12 Hz) at δ 4.10 (19-H_a) and 4.28 (19-H_b), and a signal at δ 5.51 (s, 17-H) weakly coupling with furan



limonin (**2**)



	R^1	R^2
1:	H	OH
3:	H	H
5:	Ac	OAc
6:	Bz	OBz
7:	Ac	H
8:	Bz	H



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protons,[1,5] along with the usual limonoid furan signals at δ 7.29 (21-H), 6.46 (22-H), and 7.29 (23-H). The 19-H_b signal showed a W-type long-range coupling with a signal at δ 4.45 (br t, 1-H), which revealed the 1-H to be in α. Furthermore, the chemical shift of the deshielded 15-epoxide proton suggested the presence of a 7β-OH group.[6] The ^{1}H NMR spectrum of **1** also showed a signal for a carbinol methine proton at δ 4.34 (d, 7α-H), which collapsed to a singlet by addition of deuterium oxide (D_2O). Another carbinol methine signal was observed at δ 4.04 (12-H) as a multiplet, which also collapsed to a doublet by the addition of D_2O. The above ^{1}H NMR spectral feautures closely resembled to those of rutaevin (**3**) except for the presence of the signals due to an additional hydroxyl group (see experimental).

Because of the low solubility of the natural compound **1** in all common NMR solvents, the acetate **5**, obtained by treating with acetic anhydride/pyridine, was used for detailed NMR studies. The ^{1}H NMR spectrum of **5** also showed close similarity to that of rutaevin acetate (**7**) except for the additional acetyl signal at 1.83 (12α-OAc), which was heavily shielded by a paramagnetic anisotropy of the furan ring (Table 1). 2D-Homonuclear J-correlation (COSY) indicated the coupling protons on the linked carbons (C-1 and 2, and C-9, 11, and 12). No couplings were observed between 11α- and 9α-H and 11α- and 12β-H, showing their dihedral angles to be close to 90°. A skew boat conformation of the C ring was suggested by that the 11α-proton showed only geminal coupling (J=15 Hz).

The configuration of the 7- and 12-hydroxyl groups in **1** was also clarified from exciton-split CD curves arising from interaction between benzoate and furan chromophores.[7] The 7,12-dibenzoate **6** exhibited a positive split CD spectrum at 242 ($\Delta\varepsilon$+4.0) and 227 nm ($\Delta\varepsilon$−4.9) like rutaevin 7-benzoate (**8**), arised from the positively coupled oscillator of furan and 7-benzoate (Fig. 2). Since both interactions of 7-OBz/12-OBz (dihedral angle is *ca.* 180°) and 12-OBz/furan (dihedral angle is *ca.* 0°) are not appreciated, the configuration of the 12-hydroxyl group could be determined to be α.

In the ^{1}H NOE experiments of **5**, irradiation of the 8-Me protons at δ 0.78 induced 19.9, 10.0 and 2.9% peak enhancements on the 15α-H, 19-H_b and 5-H signals, respectively. The NOE on 5-H revealed its configuration to be in β opposite to that of limonin (**2**), in the spectrum of which irradiation of the 8-Me protons at δ 1.07 showed following NOEs: 5.5 (6β-H), 13.5 (15-H), 2.5 (19-H_a), and 6.7% (19-H_b). Therefore, graucin A should be the 7β,12α-dihydroxy-6-oxo-5β-*H* analog of limonin. The ^{1}H NMR spectrum of **5**

Table 1. ^{1}H NMR Data of Graucin A Acetate (**5**) and Rutaevin Acetate (**7**)

H	Compound **5**					Compound **7**				
	δ	Mult	J/Hz	Coupled to[a]	NOE[b]	δ	Mult	J/Hz	Coupled to[a]	NOE[b]
1	4.21	brt	3	2a, 2b, (19b)	9, 28	4.37	brt	2	2a, 2b, (19b)	28
2a	2.55	dd	16, 3	2b, 1	2b	2.64	dd	16.5, 3	2b, 1	2b
2b	2.88	dd	16, 3	2a, 1	2a	2.91	dd	16.5, 3	2a, 1	2a
5	3.10	s			29, 30	3.06	s			29, 30
7	5.63	brs		30	18	5.61	brs		30	18
9	3.08	d	12	11β	1, 7, 18	2.90	d	12	11β	18
11α	1.67	brd	15	11β, (12β)		1.75	brdd	13, 7.5	11β, 12α, (12β)	
11β	2.18	ddd	15, 12, 5	11α, 9, 12		1.83	m		9, 11α, 12α, 12β	
12α						1.6	m		11α, 11β, 12β	
12β	5.00	brd	5	11β, (11α)	17	1.95	brdd	13.5, 7	12α, 11β, (11α)	
15	3.81	s			30, 7-OAc	3.82	s			30
17	5.52	brs		(21, 22)	12β, 21, 22	5.48	brs		(21, 22)	21, 22
19a	4.04	d	12.5	19b	19b	4.15	d	12.5	19b	19b
19b	4.30	brd	12.5	19a, (1)	5, 19a, 30	4.37	brd	12.5	19a, (1)	19a, 30
21	7.41	m		22, 23, (17)	17, 18	7.42	m		22, 23, (17)	18
22	6.46	m		21, 23, (17)	17, 18, 23	6.35	m		21, 23, (17)	18
23	7.42	m		21, 22	22	7.43	m		21, 22	22
18	1.41	s			7, 9, 21, 22	1.32	s			7, 9, 21, 22
28	1.32	s			1	1.30	s			1
29	1.42	s			5	1.40	s			5
30	0.78	brs		7	5, 15, 19b	0.78	brs		7	5, 15, 19b
OAc	2.30	s			15	2.29	s			
	1.83	s								

a) Distinguished couplings are listed and numerals in parenthesis denote small coupling. b) Numerals denote the proton exhibiting the NOE based on 2D-NOE.

(Table 1) well supports the structure **1** and the cross-relaxation correlated 2D-^{1}H NOE (NOESY) of **5** (4α-Me/1-H, 4β-Me/5-H, 8-Me/5-H, 19-H$_b$, 15-H, 13-Me/7-H, 9-H, 21-H, 22-H, 7-OAc/15-H, and 9-H/1-H, 7-H) revealed the gross conformation (Fig. 1).

The mutual similarities of the ^{1}H NMR spectra of graucin A (**1**) and rutaevin (**3**) and their acetates **5** and **7** suggested that rutaevin was also a 5β-H limonoid in contrast to the previously formulated structure. It had been determined by Dreyer as 6-oxoepilimonol possessing a 5α-H from the correlation with limonin (**2**) via limonin diosphenol (**4**), $\Delta^{5,6}$-6-hydroxylimonin, and analogy with other many Rutaceae limonoids.[3] The NOE different spectra of **3** and **7** showed 2.8% and 3.1% NOEs between 8-Me and 5-H, respectively, which assigned a 5β-H configuration to **3** as pointed out from the ^{13}C NMR study by Bennett and Hasegawa.[8] Further NOE data also supported the revised structure; NOEs between methyl and proton in **3**: 4α-Me/1-H (9.5%), 4β-Me/5-H (12.0%), 8-Me/15-H (18.4%), 19-H$_a$ (3.6%), 19-H$_b$ (11.3%), and 13-Me/7-H (5.8%), 9-H (6.1%), 21-H (4.2%), 22-H (6.1%); and Table 1 for **7**. The 5β-H configuration is apparently favored in **1** and **3** because a cis-fusion of the 5-membered A ring allows a less strained conformation.

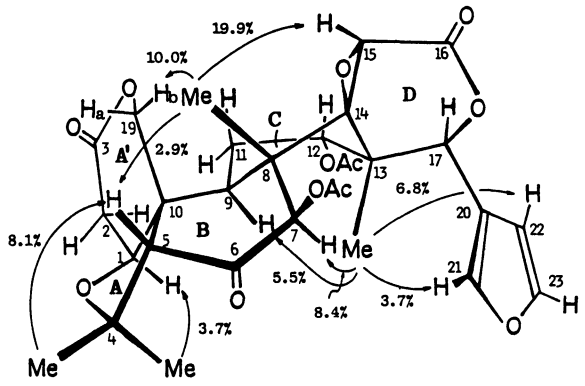

Fig. 1. Graucin A acetate (**5**), stereochemistry and NOEs between methyl and proton.

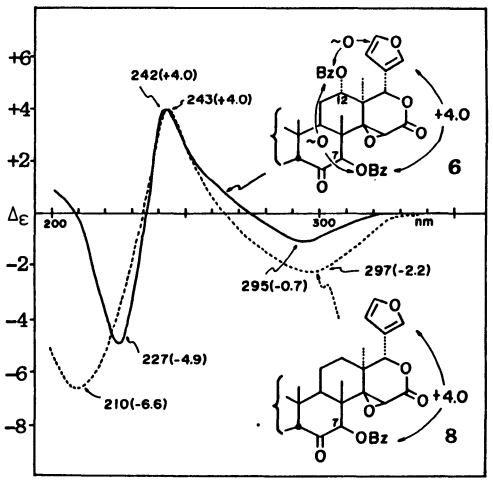

Fig. 2. CD spectra of 7,12-dibenzoylgraucin A (**6**) and 7-benzoylrutaevin (**8**).

Although there have been reported many Rutaceae limonoids, the 5β-H compounds are rare.[8] Among the compounds isolated in this work, limonin (**2**) and limonin diosphenol (**4**) were active as insect antifeedant against the larvae of *Spodoptera litura* Fab. with the leaf disk choice test. On the other hand, in antimicrobial tests against bacteria, yeasts and fungi rutaevin (**3**) showed weak antibacterial activity against *Bacillus subtilis*.

Experimental

Mps are uncorrected. UV spectra were measured in methanol (MeOH) and ^{1}H NMR spectra in chloroform-*d* with TMS as internal standard (δ=0 ppm) were measured at 360 MHz.

Plant Material. Roots of the plant were collected at Kagoshima University and identified by Dr. Sako, Kagoshima University.

Extraction and Isolation. Fresh root bark (1.0 kg) was extracted with MeOH (3×31). After concn to 200 ml, water (300 ml) was added to afford 5.2 g of a ppt which was applied onto a silica-gel column. The column was eluted with a dichloromethane (CH_2Cl_2)-MeOH gradient to yield a complex limonoid mixture and compound **1** (42 mg). The mixture was separated by repeated passage through a HPLC, semiprep silica-gel column, using 0.4—1% MeOH-CH_2Cl_2 as the solvent to give **2** (320 mg), **3** (26 mg) and **4** (21 mg).

Graucin A (1). Prisms from acetonitrile; mp >310 °C; $[\alpha]_D^{15}$ −150° (*c* 0.0005, CH_3CN); UV 209 nm (ε 6200); IR (Nujol) 3450, 1765, 1740, 1700, 1503, 1110, 1050, 1010, 875 cm^{-1}; CD(MeOH) 228 (Δ_ε−5.3), 297 nm (Δ_ε−1.7); SIMS *m/z*503 (M+H)$^+$, 645, 595, 553, 461, 277, 185, 93, 75, 57; ^{1}H NMR δ=0.61 (3H, s; 8-Me), 1.23 (3H, s; 4α-Me), 1.35 (3H, s; 13-Me), 1.44 (3H, s; 4β-Me), 2.63 (1H, dd, *J*=15 and 3 Hz; 2-H$_a$), 2.91 (1H, dd, *J*=15 and 3 Hz; 2-H$_b$), 2.98 (1H, br d, *J*=12 Hz; 9-H), 3.13 (1H, s; 5-H), 3.87 (1H, d, *J*=3 Hz; -OH), 4.04 (1H, m; 12β-H), 4.10 (1H, d, *J*=12 Hz; 19-H$_a$), 4.15 (1H, s; 15-H), 4.28 (1H, d, *J*=12 Hz; 19-H$_b$), 4.34 (1H, m; 7-H), 4.37 (1H, d, *J*=7 Hz; -OH), 4.45 (1H, m; 1-H), 5.51 (1H, s; 17-H), 6.46 (1H, m; 22-H), 7.29 (2H, m; 21- and 23-H). Found: C, 62.12; H, 6.04%. Calcd for $C_{26}H_{30}O_{10}$: C, 62.14; H, 6.02%.

Limonin (2). Prisms from MeOH; mp 289—293 °C; $[\alpha]_D^{24}$ −136° (*c* 0.004, acetone); UV 208 nm (ε 7300); IR (Nujol) 1760, 1705, 1503, 878 cm^{-1}; CD (MeOH) 230 (Δ_ε−4.9), 293 nm (Δ_ε−2.3); EIMS *m/z*470 (M$^+$), 455, 426, 413, 347, 135, 108, 94; ^{1}H NMR δ=1.07 (3H, s; 8-Me), 1.18 (3H, s; 4β-Me), 1.18 (3H, s; 13-Me), 1.30 (3H, s; 4α-Me), 1.52 (1H, m; 12α-H), 1.78 (1H, m; 12β-H), 1.83 (1H, m; 11α-H), 1.90 (1H, m; 11β-H), 2.23 (1H, dd, *J*=16 and 3 Hz; 5-H), 2.47 (1H, dd, *J*=14.5 and 3.5 Hz; 6α-H), 2.55 (1H, dd, *J*=12 and 2.5 Hz; 9-H), 2.68 (1H, dd, *J*=17 and 2 Hz; 2-Ha), 2.86 (1H, dd, *J*=15.5 and 14.5 Hz; 6β-H), 2.98 (1H, dd, *J*=17 and 4 Hz; H-2$_b$), 4.03 (1H, m; 1-H), 4.03 (1H, s; 15-H), 4.47 (1H, d, *J*=13; 19-H$_a$), 4.77 (1H, br d, *J*

=13; 19-H_b), 5.47 (1H, br s; 17-H), 6.34 (1H, ddd, J=2, 1, and 1Hz; 22-H), 7.40 (1H, dd, J=2 and 1.5 Hz; 23-H), 7.42 (1H, dd, J=1.5 and 1 Hz; 21-H). NOEs (no indication in parentheses based on 2D-NOE): 9-H/1-H, 5-H, 13-Me, 13-Me/9-H (5.8%), 21-H (4.3%), 22-H (5%), 4α-Me/5-H (6.9%), 1-H (2.5%), 4β-Me/19-H_b, 8-Me/6β-H (5.5%), 15-H (13.5%), 19-H_a (2.5%), 19-H_b (6.7%).

Rutaevin (3). Needles from MeOH; mp >300 °C; $[\alpha]_D^{24}$ −146° (c 0.0005, MeOH); UV 208 nm (ε 9400); IR (Nujol) 3420, 1770, 1740, 1710, 1504, 880 cm^{-1}; CD (MeOH) 230 ($\Delta\varepsilon$−4.1), 295 nm ($\Delta\varepsilon$−1.0); FDMS m/z 487 (M^+), 410, 369, 327, 244, 190, 127, 85; ^1H NMR δ=0.65 (3H, s; 8-Me), 1.21 (3H, s; 4α-Me), 1.36 (3H, s; 13-Me), 1.44 (3H, s; 4β-Me), 1.6 (1H, m; 12α-H), 1.73 (1H, br dd, J=13 and 8 Hz; 11α-H), 1.85 (1H, m; 11β-H), 1.95 (1H, m; 12β-H), 2.64 (1H, dd, J=15 and 3 Hz; 2-H_a), 2.79 (1H, br d, J=12 Hz; 9-H), 2.92 (1H, dd, J=15 and 3 Hz; 2-H_b), 3.12 (1H, s; 5-H), 3.89 (1H, d, J=3 Hz; -OH), 4.15 (1H, d, J=12.5 Hz; 19-H_a), 4.16 (1H, s; 15-H), 4.28 (1H, br d, J=12.5 Hz; 19-H_b), 4.35 (1H, br d, J=3 Hz; 7-H), 4.37 (1H, br t, J=3 Hz; 1-H), 5.48 (1H, br s; 17-H), 6.35 (1H, m; 22-H), 7.43 (1H, m; 23-H), 7.44 (1H, m; 21-H). NOEs: 13-Me/7-H (5.8%), 9-H (6.1%), 21-H (4.2%), 22-H (6.1%), 4α-Me/1-H (9.5%), 4β-Me/5-H (12.0%), 8-Me/15-H (18.4%), 19-H_a (3.6%), 19-H_b (11.3%).

Limonin Diosphenol (4). Needles from acetone; mp 273—278 °C; $[\alpha]_D^{24}$ −193° (c 0.002, acetone); UV 207 (ε 7900), 278 nm (ε 7900); IR (Nujol) 3450, 1740, 1690, 1660, 880 cm^{-1}; CD (MeOH) 208 ($\Delta\varepsilon$−4.2), 275 ($\Delta\varepsilon$+10.7), 320 nm ($\Delta\varepsilon$−8.5); ^1H NMR δ=1.04 (3H, s; 8-Me), 1.16 (3H, s; 4β-Me), 1.49 (3H, s; 13-Me), 1.54 (3H, s; 4α-Me), 2.64 (1H, dd, J=13 and 3 Hz; 9-H), 2.84 (1H, dd, J=17 and 5 Hz; 2-H_a), 2.91 (1H, dd, J=17 and 3 Hz; 2-H_b), 4.07 (1H, br t, J=3 Hz; 1-H), 4.12 (1H, s; 15-H), 4.62 (1H, d, J=13 Hz; 19-H_a), 4.66 (1H, d, J=13 Hz; 19-H_b), 5.43 (1H, s; 17-H), 6.23 (1H, s; -OH), 6.38 (1H, m; 22-H), 7.40 (1H, m; 23-H), 7.41 (1H, m; 21-H).

7, 12-Diacetylgraucin A (5). Graucin A (**1**) (8 mg) was acetylated with Ac_2O-pyridine at room temperature and the product was purified by chromatography over SiO_2. Recrystallization from MeOH afforded diacetate **5** (6 mg) as needles, mp >300 °C. UV 213 nm (ε 8000); IR (CH_2CI_2) 1770—1740, 1720, 1700, 1225, 1060, 875 cm^{-1}; SIMS m/z 587 $(M+H)^+$, 609, 551, 527, 418, 207, 185, 115, 93, 61, 43.

7,12-Dibenzoylgraucin A (6). Graucin A (**1**) (3 mg) was benzoylated with benzoyl chloride in pyridine at 60 °C and the product was chromatographed over SiO_2 and purified by HPLC to give **6** (2 mg). 7,12-dibenzoylgraucin A (**6**): UV 207 (ε 9400), 232 nm (ε 18000); IR (CH_2Cl_2) 1770, 1750, 1720, 1600, 1245, 1180, 1100, 880 cm^{-1}; CD (MeOH) 227 ($\Delta\varepsilon$−4.9), 242 ($\Delta\varepsilon$+4.0), 295 nm ($\Delta\varepsilon$−0.7); SIMS m/z 711 $(M+H)^+$.

7-Acetylrutaevin (7). Rutaevin (**3**) (20 mg) was acetylated with Ac_2O-pyridine and the product was purified by chromatography. Recrystallization from MeOH afforded **7** (18 mg) as needles, mp 184—188 °C; $[\alpha]_D^{24}$ −101° (c 0.0005, MeOH); UV 209 nm (ε 9400); IR (CH_2Cl_2) 1770—1740, 1715, 1700, 1220, 1055, 875 cm^{-1}; CD (MeOH) 297 nm ($\Delta\varepsilon$−0.56), 227 nm ($\Delta\varepsilon$−2.29).

7-Benzoylrutaevin (8). Rutaevin (**3**) (15 mg) was benzoylated with benzoyl chloride in pyridine at 60 °C. Final purification of the product by HPLC afforded **8** (11 mg). Compound **8**, mp 285—288 °C; UV 209 (ε 9700), 234 nm (ε 14000); IR (CH_2Cl_2) 1780, 1750, 1730, 1605, 1503, 885 cm^{-1}; CD (MeOH) 210 ($\Delta\varepsilon$−6.6), 243 ($\Delta\varepsilon$+4.0), 297 nm $\Delta\varepsilon$−2.2); ^1H NMR δ=0.92 (3H, s; 8-Me), 1.36 (3H, s; 4α-Me), 1.40 (3H, s; 13-Me), 1.42 (3H, s; 4β-H), 1.6 (1H, m; 12α-H), 1.8 (1H, m; 11α-H), 1.85 (1H, m; 11β-H), 1.95 (1H, m; 12β-H), 2.66 (1H, dd, J=15 and 3 Hz; 2-H_a), 2.93 (1H, dd, J=15 and 3 Hz; 2-H_b), 3.00 (1H, br d, J=12 Hz; 9-H), 3.12 (1H, s; 5-H), 3.79 (1H, s; 15-H), 4.20 (1H, d, J=12 Hz; 19-H_a), 4.36 (1H, br d, J=12 Hz; 19-H_b), 4.41 (1H, br t, J=3 Hz; 1-H), 5.47 (1H, s; 17-H), 5.90 (1H, s; 7-H), 6.35 (1H, m; 22-H), 7.40 (1H, m; 21-H), 7.42 (1H, m; 23-H), 7.52 (2H, t, J=7 Hz; 3′-and 5′-H), 7.65 (1H, tt, J=7.5 and 1.5 Hz; 4′-H), 8.06 (2H, dd, J=7 and 1.5 Hz; 2′-and 6′-H).

Limonin Diosphenol (4) from Limonin (2). Limonin (**2**) (100 mg) suspended in t-butyl alcoholic N-potassium t-butoxide (20 ml) was shaken with oxygen in a hydrogenetion apparatus for 2 h. After addition of water, the soln was acidified with 6M-hydrochloric acid (1 M=1 mol dm^{-3}) and extracted with chloroform ($CHCl_3$). The $CHCl_3$ soln was washed with aq sodium hydrogencarbonate and water, and then shaken with aq 4M-sodium hydroxide. Acidification of this extract with 6M-hydrochloric acid gave a ppt which was extracted with $CHCl_3$ again. The $CHCl_3$ soln was washed with water, and the solvent was removed. Recrystallization from acetone gave **4** (43 mg) as needles, mp 273—278 °C.[9]

Limonin Diosphenol (4) from Rutaevin (3). Pyridinium chlorochromate (80 mg) was added to a stirred soln of rutaevin (**3**) (44 mg) in CH_2Cl_2. After stirring over night at room temperature, MeOH was added and stirred for additional 1 h, and then the soln was evapolated in vacuo to yield a brown residue which was chromatographed over SiO_2 to give **4** (30 mg).

Biological Activities. Insect Antifeedant Activity. The limonoids **1**—**4** were tested against the larvae of the pest insect *Spodoptera litura* Fab. with the leaf disk method.[10] Limonin (**2**) and limonin diosphenol (**4**) showed the activity at 1000 ppm concn. **Antimicrobial Activity.** Effects of the compound **1**—**4** on growth of microorganisms were tested by the broth dilution method by Dr. M. Taniguchi (Osaka City University). Among the compounds rutaevin (**3**) was active against a bacterium *Bacillus subtilis* at 100 μg ml^{-1} concn. Test organisms; bacterium; *S. aureus, B. subtilis, E. coli* and *Ps. aeruginosa*: fungus; *Mucor mucedo, Rh. chinensis, Asp. niger* and *P. crustosum*: yiest; *S. cerevisiae, C. utilis, Schiz. pombe* and *H. anomala*.

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