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LIMONOIDS OF CALAMONDIN SEEDS

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Abstract—Five new limonoids were isolated from calamondin seeds: methyl deacetylnomilinate (**7**), calamin (**9**), retrocalamin (**10**), cyclocalamin (**11**), and methyl isoobacunoate diosphenol (**13**). Structures were assigned on the basis of ^{1}H and ^{13}C NMR spectra, and chemical conversions of **9** to **10**, **11**, and **13**. These reactions are also biogenetically plausible. Both **11** and the structurally similar limonoid rutaevin have been assigned the 5β-H configuration, which is opposite to that of all other known Rutaceae limonoids. ^{13}C NMR data are presented for 15 limonoids.

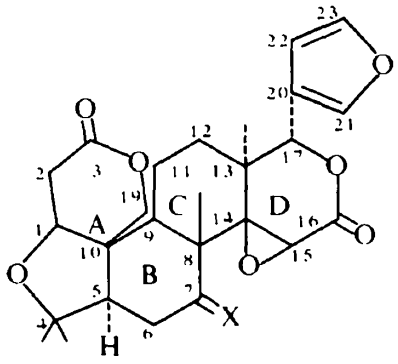
Calamondin (*Citrus reticulata* var. austera X *Fortunella* sp.)[1] seeds were reported to contain limonin by Dreyer[2] during a survey of the distribution of limonoids in the *Rutaceae*. We have now made a more detailed investigation of the calamondin limonoids and have isolated five new compounds which appear to be biogenetically related.

Tlc of a calamondin seed extract showed eight spots giving the characteristic limonoid color[3] with Ehrlich's reagent. When this extract was fractionated by silica gel column chromatography, three of the compounds were identified as limonoids previously isolated from various *Citrus* species: limonin (**1**), nomilin (**5**), and deacetylnomilin (**6**).[2] Another compound, methyl deacetylnomilinate (**7**), was the methyl ester of an acidic limonoid previously found in grapefruit seeds.[4] However, the limonoid present in highest concentration in the original extract was not recovered in the column fractions. One of the minor, less polar compounds in the extract was the major limonoid obtained from the column. This suggested that a conversion had occurred on the column. By running the column very rapidly at 5 °, this reaction was slowed enough to allow the original major limonoid to be isolated.

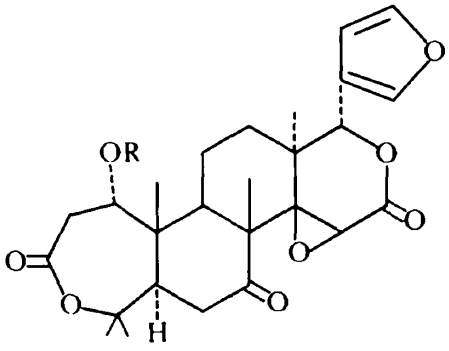
The ^{1}H NMR spectrum of this compound, which we have named calamin (**9**), was quite similar to that of **7**. It showed the usual limonoid furan and H-17 signals, a 3-proton methyl ester singlet, five C-methyl resonances, a one-proton multiplet similar in appearance and chemical shift to that of H-1 in **7**, and an H-15 epoxide signal, identifiable by its sharp ringing.[5] In addition, a one-proton doublet at 4.26 ppm collapsed to a singlet in the presence of D_2O, indicating the presence of a secondary alcohol group. The ^{13}C NMR spectra of **9** and **7** were also similar (Table 2). Both spectra showed three carbonyl resonances, assignable to ketone, ester, and lactone groups. The SFORD (single frequency off-resonance decoupled) spectra showed that both had the same number of methyl and quarternary carbons, but that **9** had one more methine carbon and one less methylene carbon than **7**. This extra methine carbon resonance was located at 80 ppm and was shown to be coupled to the secondary alcohol proton signal referred to above. Thus, **9** may be most simply formulated as derived from **7** by hydroxylation of a methylene group. However, other NMR evidence suggested that the keto group was now at a different position. The chemical shift of the H-15 epoxy proton in limonoids is known to be strongly affected by the nature of the C-7 functionality, because of the close spatial proximity of the two positions.[5] The downfield position of the H-15 resonance in **9** compared to **7** suggested a β-oriented hydroxyl instead of a ketone at the 7-position (see Table 1, limonin (**1**) vs 7α-limonol (**2**) and 7β-limonol (**3**). Likewise, the downfield shift (6.2 ppm) of the C-14 resonance in the ^{13}C spectrum of **9** compared to that of **7** favored a 7β-hydroxyl group in the former. The C-14 resonance of **3** is shifted downfield by 6.2 ppm from that of **1**. This is also in accord with the substituent effects observed in steroids; i.e., reduction of a 7-ketone causes C-14 downfield shifts of 1.7 ppm for the 7α-alcohol and 6.8 ppm for the 7β-alcohol.[6]

Accepting that the C-7 substituent was β-hydroxyl rather than carbonyl, the position of the keto group remained to be established. The only major difference between the SFORD spectra of **7** and **9**, other than the signal at 80 ppm previously referred to, was a 12 ppm downfield shift of the C-5 methine resonance. This observation indicated C-6 as the site of the keto group, as did the downfield shift of H-7 in **9** compared to methyl 7β-deacetylnomilolate (**8**) (Table 1). Thus, calamin (**9**) appeared to be the 6-keto-7β-hydroxyl analog of **7**. This assignment is further substantiated by the presence of the same structural feature in limonoids of other *Rutaceae* species: rutaevin (**14**),[7] atalantin (**16**),[8,9] and spathelin (**17**),[10] as well as by the reactions of **9** discussed below.

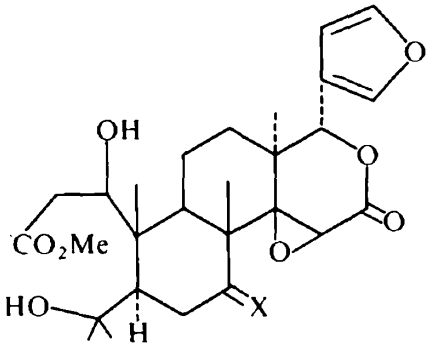
The ^{1}H NMR spectrum of the compound to which **9** was converted on the silica gel column, retrocalamin (**10**), was similar to that of **9**, except that it contained only three quarternary methyl resonances instead of five. Its ^{13}C SFORD spectrum showed the loss of three carbons, two of which were methyl and the third a quarternary oxycarbon at about 70 ppm. These three carbons must be C-4 and its two attached methyl groups in **9**, split off as acetone in a retroaldol type reaction. Both the ^{1}H and ^{13}C NMR spectra of retrocalamin are in complete accord with the proposed structure **10**. Retroaldol reactions are usually base-catalyzed, with the first step being abstraction of the hydroxyl proton, and indeed under basic conditions, e.g., heating with pyridine, **9** was converted to **10**. The



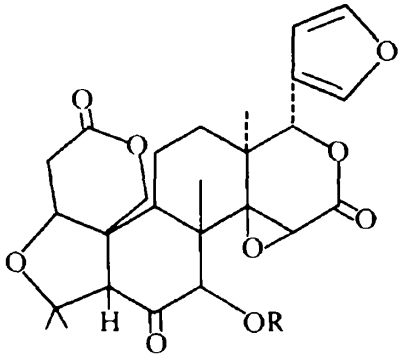
Limonin (1) X = O
7α-Limonol (2) X = α-OH, β-H
7β-Limonol (3) X = α-H, β-OH
7β-Limonyl Acetate (4) X = α-H, β-OAc



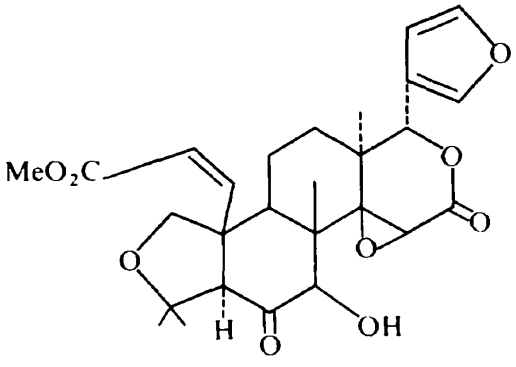
Nomilin (5) R = Ac
Deacetylnomilin (6) R = H



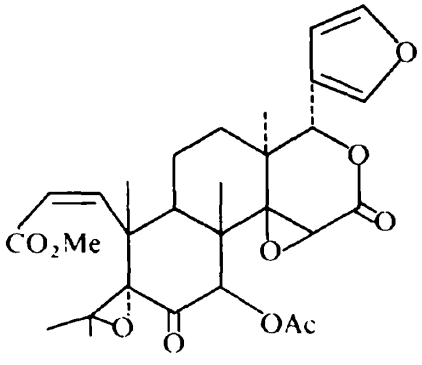
Methyl Deacetylnomilinate (7) X = O
Methyl 7β-Deacetylnomilolate (8) X = α-H, β-OH



Rutaevin (14) R = H
Rutaevin Acetate (15) R = Ac



Atalantin (16)



Spathelin (17)

Table 1. Selected resonances in ^{1}H NMR spectra of calamondin limonoids and related compounds (in ppm)[a]

roton No.	Compound 1	2[b]	3[b]	4	5	6[b]	7	8	9	10	11	12	13	14	15	16	17[c]	18	19	20	21	23[d]
H-1	4.01	4.01	3.98	4.00	5.00	3.81	4.69	4.72	4.62	3.88	4.30	4.31	3.93	4.37	4.38	6.58	6.22	3.90	3.80	3.80	4.27	6.37
H-7	--	3.51	3.81	4.93	--	--	--	3.73	4.26	4.29	4.27	5.53	--	4.37	5.60	4.75	6.25	--	3.80	4.33	--	4.33
H-15	4.01	3.92	4.61	3.67	3.79	3.78	3.71	4.47	4.34	4.35	4.14	3.81	4.13	4.16	3.81	4.42	3.83	4.37	4.50	4.39	3.57	4.24

n $CDCl_3$ at 100 MHz unless otherwise indicated.

DCl_3-$(CD_3)_2SO$.

ef. 10.

ef. 9.

ease with which this reaction occurred on the silica gel column, under essentially neutral conditions, is surprising. The conversion of **9** to **10** confirms the presence of a 6-keto group, since this reaction requires that the hydroxyl group be β to a carbonyl.

The least polar compound from the silica gel column was methyl isoobacunoate diosphenol (**13**). This compound was not previously known as a natural product, but it had been synthesized by oxygenation of isoobacunoic acid, followed by methylation.[10] The next compound from the column, cyclocalamin (**11**), was a hydroxyketone which could be converted to **13** by oxidation with CrO_3 followed by acid-catalyzed enolization. Thus, **11** was a methyl isoobacunoate (**18**) derivative with either a 6-keto-7-hydroxyl or 7-keto-6-hydroxyl structure. The carbinol and H-15 resonances in the ^{1}H NMR spectrum were very similar to those of rutaevin (**14**) (Table 1), which is the 6-keto-7β-hydroxyl analog of limonin.[7]

Likewise, a comparison of the ^{13}C spectra of cyclocalamin acetate (**12**) and rutaevin acetate (**15**) (the acetates were used because of the low solubility of rutaevin in all common NMR solvents) showed no significant differences in the chemical shifts of the C-6, C-7, C-8, C-9, C-14, and C-15 resonances (Table 2). Therefore, cyclocalamin, like rutaevin, must contain a 6-keto-7β-hydroxyl moiety.

However, the NMR data indicated that this structural feature was not the only difference between cyclocalamin and methyl isoobacunoate (**18**). This is best seen by comparing the ^{13}C spectra of cyclocalamin (**11**) and methyl 7β-isoobacunolate (**19**). If the only structural difference between **11** and **19** were the presence of a 6-keto group in **11**, significant changes in chemical shifts should be limited to those carbons α and β to the carbonyl, i.e. C-4, C-5, C-7, C-8 and C-10. Figure 1 shows that this is not the case, as positions remote from C-6 are strongly affected. This cannot be attributed to a simple change in conformation of the molecule due to introduction of the 6-carbonyl group, because Dreiding models show that the ring system in **19** is too rigid to allow significant deformations. The conversion of both cyclocalamin and **18** to **13** correlates their configurations at every position except C-5, whose chirality is destroyed during these reactions. All of the known *Rutaceae* limonoids have been assigned the 5α-H configuration, based upon correlations with **3**, whose stereochemistry was established by X-ray crystallography.[11] In this configuration the 5-membered ether ring is *trans*-fused to the B-ring, which is thereby locked into the chair conformation. If cyclocalamin had this structure, C-4 would be eclipsed by the 6-carbonyl group and its ^{13}C resonance should be shifted upfield, like those of carbons eclipsed by carbonyl groups in steroids.[12] In fact, C-4 is shifted downfield (Fig. 1). With a 5β-H configuration, the 5-membered ring is *cis*-fused and the molecule as a whole becomes much less rigid. With the B-ring in the chair conformation, C-4 would no longer be eclipsed by the 6-carbonyl. The B-ring could also adopt a boat conformation, but C-4 would then be eclipsed; also, H-7 would be equatorial and thus coplanar with the 6-carbonyl group, which should cause a large downfield shift of this signal in the ^{1}H NMR spectrum. In atalatin (**16**), the B-ring is in the boat form[8,9] and the H-7 resonance is observed at 4.75 ppm, compared with 4.27 ppm for cyclocalamin. Thus, the NMR data are best satisfied by a 5β-H configuration with a chair B-ring, and cyclocalamin is assigned the structure **11**. The large changes in the ^{13}C NMR of the C- and D-ring carbon resonances in comparing **11** and **19** (Fig. 1) can then be attributed to conformational changes in the molecule when the strain of the *trans*-fused B-ring junction is relieved.

The same arguments used in assigning a 5β-H configuration to **11** apply to the case of rutaevin (**14**), which was correlated with limonin via limonin diosphenol.[7] As noted above, the ^{13}C spectra of

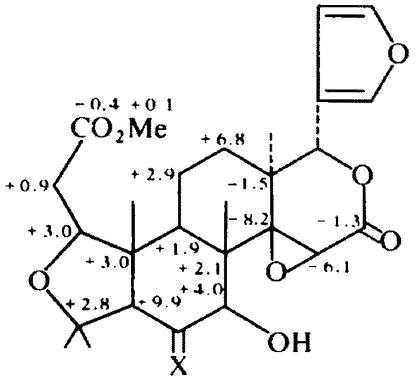


Fig. 1. ^{13}C chemical shift differences between **19** (X=H_2) and **11** (X=O) ($\delta_{11}-\delta_{19}$).

Table 2. ^{13}C NMR spectra of calamondin limonoids and related compounds (in ppm)[a]

Carbon no.	Compound 3[b]	4[b]	5[c]	6[b]	7[b]	8[b]	9[b]	10[b]	11[c]	12[c]	13[c]	15[b]	19[c]	20[b]	21[c]
1	78.8	78.7	70.8	68.5	73.6	73.2	73.0	71.4	85.8	85.6	82.8	81.9	82.8	67.0	84.9
2	35.6	35.6	35.3	39.0*	37.1	37.0	36.9	37.0	37.6	37.6	35.9	35.8	36.7	39.0	37.4
3	170.1	170.1*	169.2	170.6	172.8	172.8	172.3	172.6	171.7	171.6	171.4	169.5	172.1	170.9	171.4
4	79.7	79.6	84.3	83.7	72.9	73.2	71.8	---	81.5	81.7	80.3	81.5	78.7	81.2	82.5
5	56.6	55.8	51.1	49.3	51.3	50.2	63.8	43.9	68.8	69.7	144.6*	64.5	58.9	59.5	71.0
6	27.9	24.1	38.9	39.2*	38.9	32.3	210.6	209.5	209.3	201.7	138.9*	200.4	28.4	209.7	199.0
7	75.4	78.0	206.9	208.4	210.8	74.6	80.5	80.5	82.6	82.6	196.1	81.5	78.6	80.9	195.3
8	45.1*	42.4*	52.9	52.0	51.8	46.1*	48.2*	46.7*	48.4*	44.8*	47.8*	43.8*	46.3*	49.7*	52.5
9	44.7	45.1	44.3	43.6	43.6	42.7	41.6	37.5	46.2	47.7	45.2	46.0	44.3	39.2	44.9
10	43.0*	45.1*	44.3	44.3	45.2	44.2*	51.0*	43.7*	46.7*	47.7*	46.9*	48.4*	43.7*	47.9*	47.4
11	16.9	17.3	16.5	16.8	18.4	17.9	17.7	17.0	20.4	20.5	19.4	19.2	17.6	15.1	20.1
12	25.5	26.9	32.2	31.2	31.4	28.7	27.5	28.9	32.1	32.4	31.1	30.8	25.3	25.8	31.5
13	38.5	38.5	37.6	36.9	36.6	37.5	38.2	38.3	38.1	38.1	37.9	37.3	39.6	38.7	37.1
14	72.9	71.3	65.6	65.8	65.4	71.0	71.6	69.8	66.4	65.8	66.0	65.4	74.6	73.1	63.9
15	55.0	54.7	53.5	52.9	52.2	53.1	54.7	53.1	51.4	51.1	52.7	50.2	57.5	55.7	51.9
16	168.0	167.0	166.9	167.0	167.1	168.1	167.6	167.6	167.1	166.8	166.9	166.5	168.4	167.7	166.1
17	77.4	77.4	78.1	77.6	77.6	77.6	77.7	77.8	78.1	78.2	77.8	77.5	78.1	77.7	77.7
19	65.3	65.1	--	--	--	--	--	--	--	--	--	69.3	--	--	--
20	120.5	120.2	120.2	120.3	120.2	120.4	120.3	120.6	120.2	119.8	120.2	119.7	120.5	120.5	119.8
21	141.7	141.7	141.1	141.3	141.4	141.5	141.5	141.5	141.1	141.1	141.1	141.6	141.3	141.7	141.0
22	110.2	110.1	109.7	110.1	110.2	110.2	110.1	110.3	109.9	109.7	109.8	110.1	109.9	110.3	109.7
23	143.2	143.3	143.3	143.1	143.3	143.2	143.2	143.3	143.2	143.3	143.2	143.3	143.0	143.4	143.2
C-Methyls	30.2	30.1	33.5	32.9	32.4	33.5	32.7	19.4	30.3	30.0	26.2	28.5	30.3	31.9	30.5
	21.4	21.4*	23.4	23.3	29.0	27.8	27.5	18.7	23.4	23.0	24.6	23.3	23.8	24.4	23.5
	18.2	18.6	20.8	20.1	20.5	19.6	19.0	13.8	21.1	20.9*	20.4	20.3*	18.8	17.9	20.6
	13.4	14.0	17.2	16.2	17.0	17.1	17.7		20.7	20.5*	18.8	14.7	14.4	15.0	20.0
			17.2	16.1	15.7	12.3	13.1		14.5	15.1	15.3		12.5	13.5	14.6
Methoxy					51.1	50.8	50.8	51.4	51.9	51.9	52.0		51.8		51.9
Acetate Methyl		21.1*	20.8							20.5*		20.1*			
Acetate Carbonyl		169.9*	169.2							169.8		170.6			

[a]At 15 MHz. Assignments of asterisked values in the same vertical column may be reversed. C-21 and C-23 assignments were based upon data of Sabata et al.[9]

[b]In $(CD_3)_2SO$.

[c]In $CDCl_3$.

rutaevin acetate (**15**) and cyclocalamin acetate (**12**) are very similar, so it seems likely that both have the same overall molecular conformation. Also, a comparison of the ^{13}C spectra of **15** and 7β-limonyl acetate (**4**) (Table 2) showed the same large differences in the C- and D-ring resonances observed above for **11** and **19**, as well as the downfield shift of C-4. Thus, all of the NMR data indicate that rutaevin is also a 5β-H limonoid.

The configuration at C-5 of calamin (**9**) was next considered. A comparison of the ^{13}C NMR spectra of **9** and its 6-methylene analog, methyl 7β-deacetylnomilolate (**8**) (Table 2), did not show the large changes in the C- and D-ring carbon signals observed in the two cases above. Significant changes were limited to the positions α and β to the 6-carbonyl group, but since **9** lacks the 5-membered ether ring present in **11** and **14**, a change of configuration at C-5 would not be expected to cause conformational changes in the molecule as a whole. However, the C-4 resonance is shifted upfield in **9**, which suggests that it is eclipsed by the 6-carbonyl. This would require an α-orientation of H-5. Further support for a 5α-configuration is provided by a comparison of the ^{13}C spectra of **9** and retrocalamin (**10**). If **9** had a 5β-H configuration, the bulky 5α-dimethyl-carbinol substituent would be axial and in a γ *gauche* relationship to H-7, which should cause the C-7 resonance to be shielded by several ppm.[13] Removal of this substituent on converting **9** to **10** should then result in a downfield shift of the C-7 resonance. With a 5α-H configuration, on the other hand, the dimethylcarbinol substituent would be equatorial and should have no significant effect on C-7. In fact, the C-7 chemical shift is the same in **9** and **10** (Table 2). This argument depends upon the B-ring having the chair conformation. The close similarity of the H-7 and H-15 resonances in **9** and **10** (Table 1) shows that the B-ring has the same conformation in both. While it could be

argued that a boat B-ring might be favored in **9** because a bulky substituent would be equatorial rather than axial, this does not apply to **10**, and there seems no reason to expect the B-ring of the latter not to be in the chair conformation. The H-7 NMR signals of **9** and **10** are also consistent with a chair B-ring rather than a boat as in atalantin (**16**) (Table 1). Therefore, **9** is assigned the 5α-H conformation.

When the ^{13}C spectrum of **9** was run in $CDCl_3$ with data accumulation over a period of 20 hr, the result was a spectrum of cyclocalamin (**11**).

The conversion of **9** to **11** was confirmed by tlc analysis of the solution. The reaction was apparently caused by traces of acid in the $CDCl_3$, because **9** was stable in other NMR solvents. This conversion could also be accomplished by treating **9** with HCl in CH_2Cl_2 for a much shorter time. In this case a second product, which contained no methyl ester group, was also obtained. The NMR and mass spectra indicated that this compound was **20**, the 6-keto-7β-hydroxyl analog of deacetylnomilin, with a 5α-H configuration. A clue to the mechanism by which **9** was converted to **11** was provided by periodic examination of the ^{13}C spectrum of **9** in $CDCl_3$ during the first few hours of accumulation. Two olefinic resonances, at about 130 and 150 ppm, appeared gradually and then later grew weaker and finally disappeared. This phenomenon could best be interpreted as a dehydration of the tertiary 4-hydroxyl to form an α,β-unsaturated carbonyl system as in **21**, followed by Michael addition of the 1-hydroxyl group to the double bond. An alternative dehydration of the 1-hydroxyl to form a 1,2-double bond seems to be ruled out, because **7** is stable under these conditions. Further support for this mechanism is provided by the structure of atalantolide (**22**),[9] which has been isolated from *Atalantia monophylla*. This compound differs from the proposed intermediate **21** only in the presence of a 1, 2-double bond, and its C-4 and C-5 resonances are located at 152.8 and 135.8 ppm, respectively.

DISCUSSION

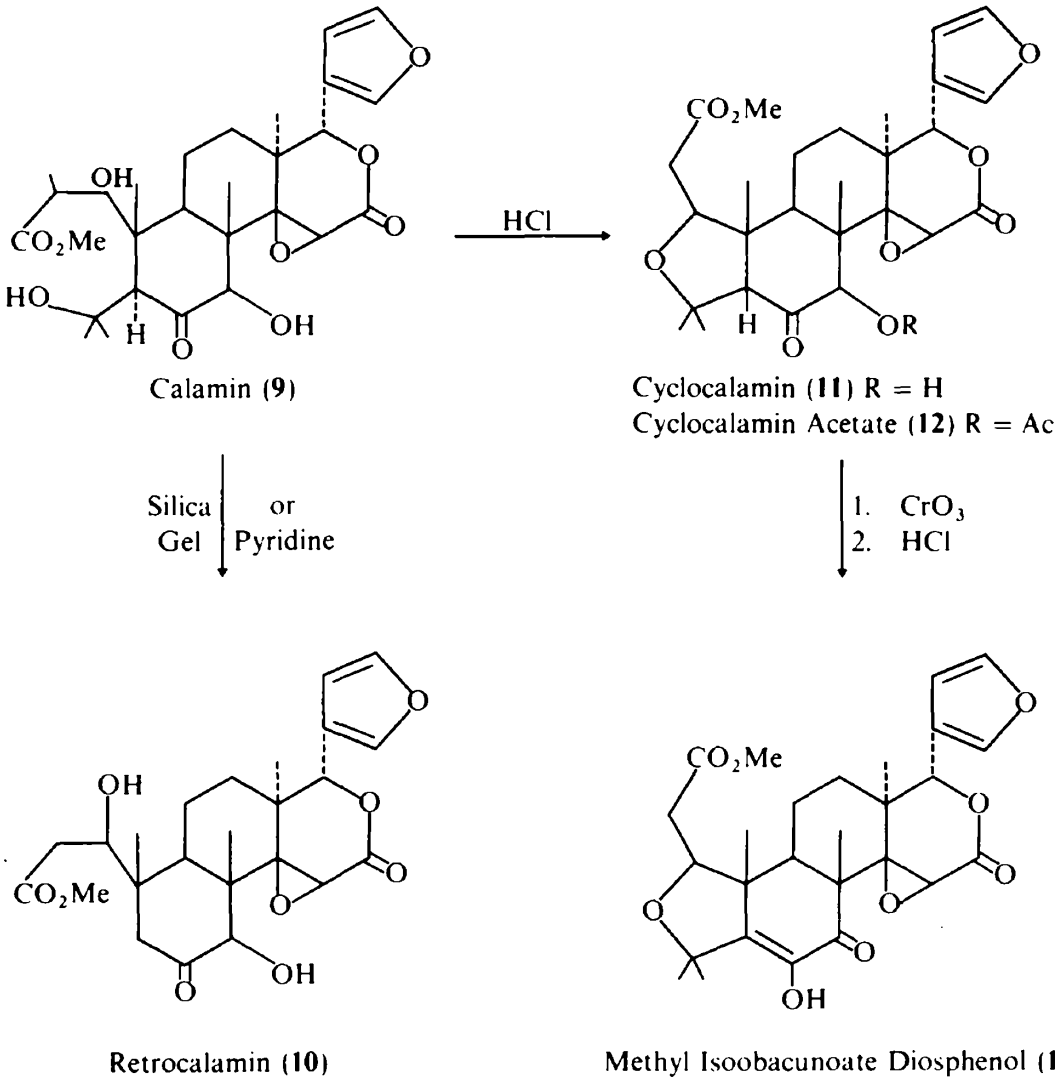
Because the ^{13}C NMR data in Table 2 played an important role in determining the structures of the calamondin limonoids, considerable care was taken to assure that the assignments were correct. With a data base of more than 30 limonoids on which SFORD spectra were run (see Ref. 8 for some not included in Table 2),§ many assignments could be made by comparison of spectra of closely related compounds. Selective proton decoupling was used whenever possible to confirm assignments. Shift reagent experiments were helpful in some instances, such as differentiation of the 6 and 12 methylene signals in 7-hydroxy limonoids. Aside from a few cases in which signals of the same SFORD multiplicity differed by less than 1 ppm, the only ambiguous assignments were those of the quarternary C-8 and C-10 resonances in the 7-hydroxy limonoids, and the olefinic resonances in **13**. However, a reversal of these assignments would not affect any of the arguments used in the structure determinations reported here. For spectral comparisons the same solvent was always used for both compounds, because differences of up to 2 ppm were observed for some limonoid resonances when the solvent was changed. Thus, some of the compounds shown in Table 2 had to be run in more than one solvent; e.g. the spectrum of **15** was also taken in $CDCl_3$ for comparison with **12**. Such additional data have not been included in the table.

Calamondin is a hybrid of *Citrus* and *Fortunella*, and it appears that the new limonoids come from the latter parent. Tlc of a kumquat (*F. margarita*) seed extract showed that **9** was the major limonoid present, in higher concentration than in calamondin seeds. However, the amounts of the related limonoids **10**, **11** and **13** were much lower than in calamondin, so the latter is a more favorable source of these compounds. All of the new limonoids are methyl esters, and all except **7** are oxygenated at C-6. None of the known *Citrus* limonoids contain these structural features.

The chemical conversions of calamin (**9**) to the other calamondin limonoids shown in Scheme 1 raised the possibility that **10** and/or **11** could be artifacts produced from **9** during the isolation process. However, tlc showed that both were present in the original extract prior to chromatography, and when a pure sample of **9** was subjected to the extraction conditions, it was not converted to **10** or **11**. Thus, all of the compounds isolated are natural constituents. A reasonable biosynthetic pathway for the new limonoids could proceed from **7** to **9**, followed by enzymatic transformations analogous to the chemical reactions shown in Scheme 1. The intermediate **21** proposed for the chemical conversion of **9** to **11** may also be a biosynthetic intermediate. The closely related *Atalantia* limonoid **22** has similarly been proposed as the precursor of **16** via hydroxylation at C-19 and Michael addition to the double bond.[9] Such a sequence also provides a mechanism for the inversion of configuration at C-5 in **11**. The 5β-H structure is apparently favored in **11** and **14** because a *cis*-fusion of the 5-membered ring allows a less strained conformation. In **16** a 5β-H configuration is not stereochemically feasible because C-19 is axial, and in the biosynthesis of citrus limonoids containing a 5-membered A-ring, such as **1** and **18**, the 4-hydroxyl group probably remains intact in the intermediates prior to ring closure. Thus, there would be no opportunity for inversion at C-5, and therefore these compounds have the more strained 5α-H configuration.

Most limonoids are present in very low concentrations compared to other constituents of citrus seeds, but they can readily be detected by tlc because they give a characteristic color with Ehrlich's reagent.[3] However, serious difficulties arise in isolating them in pure form because of their low concentrations. Often several chromatographic separations, both column and preparative tlc, are required. We have now developed an extraction procedure which avoids these difficulties. Citrus seeds contain an enzyme which, at pH 8, opens the D-ring lactone of limonoids to produce water-soluble salts of the 16-carboxyl group. Therefore, we homogenize the seeds with aqueous buffer and filter the homogenate to remove most other

§Taylor[14,15] has published ^{13}C NMR data for many of the Meliaceae limonoids. In general, our assignments are consistent with his.



Scheme 1. Interconversions of calamondin limonoids.

constituents. The filtrate is then acidified to close the lactone rings, and the limonoids are extracted with chloroform. Washing of the chloroform extract with bicarbonate solution removes acidic material carried along with the limonoids to this point, leaving the latter in highly concentrated form. Tlc showed that an acetone extract of calamondin seeds contained the same limonoids, in the same relative proportions, as extracts obtained by the above procedure.

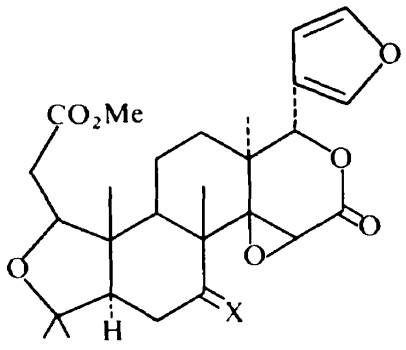
EXPERIMENTAL

Mps are uncorrected. Mass spectra were obtained on a VG Micromass 707OF instrument. Under EI conditions most limonoids do not show a molecular ion, but in the CI mode all of the compounds discussed in this paper except the tertiary alcohols **7**, **8**, and **9** gave strong M + 1 peaks. Ammonia was used as the CI reagent gas and tetraiodoethylene as the reference for accurate mass measurements. ^{1}H NMR spectra were run on a JEOL PS-100 instrument and ^{13}C NMR spectra on a JEOL FX-60 instrument. Calamondin fruits were obtained from citrus groves of the University of California at Riverside.

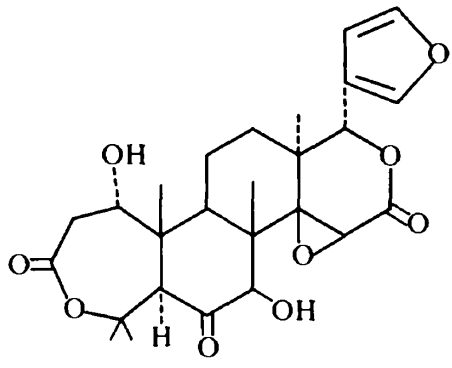
Isolation of limonoids. Calamondin seeds were homogenized in a blender with about three volumes of 0.1 M tris buffer, pH 8.0. The homogenate was allowed to stand at 25° overnight and filtered through Celite. The filtrate was acidified to pH 3 with HCl and extracted twice with $CHCl_3$. The extracts were combined, washed twice with 2% $KHCO_3$ and then with H_2O, and evaporated. After removal of most of the limonin by crystallization from CH_2Cl_2–i-PrOH, the other limonoids were isolated by column chromatography.

For isolation of calamin (**9**), an extract weighing 800 mg was chromatographed on a 100-g silica gel column, packed in hexane-EtOAc (20:80). The column was eluted as rapidly as possible in a 5° room, with increasing amounts of EtOAc in hexane. Under these conditions column resolution was not good, but a fraction consisting mainly of **9** (87 mg) was obtained. Two crystallizations from MeOH gave 45 mg of **9**, m.p. 162–165°; ^{1}H NMR ($CDCl_3$) δ 7.41 (d, 2 H, 1 Hz, α-furans), 6.34 (d, 1 H, 1 Hz, β-furan), 5.59 (s, 1 H, H-17), 4.62 (m, 1 H, H-1), 4.34 (s, 1 H, H-15), 4.26 (d, 1 H, 6 Hz, H-7), 3.69 (s, 3 H, Me ester), 3.55 (d, 1 H, 6 Hz, OH), 2.88 (s, 1 H, H-5), 1.53 (s, 3 H, Me), 1.35 (s, 3 H, Me), 1.28 (s, 3 H, Me), 1.13 (s, 3 H, Me), 0.80 (s, 3 H, Me). (Found: C, 62.3; H, 6.98. $C_{27}H_{30}O_{10}$ requires: C, 62.3; H, 6.97%).

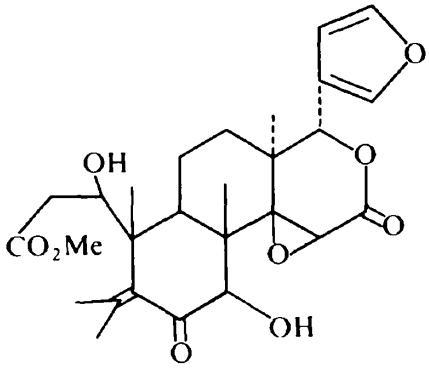
For isolation of the other limonoids, 460 mg of extract (from 400 g of dried seeds) was chromatographed on a 70 g



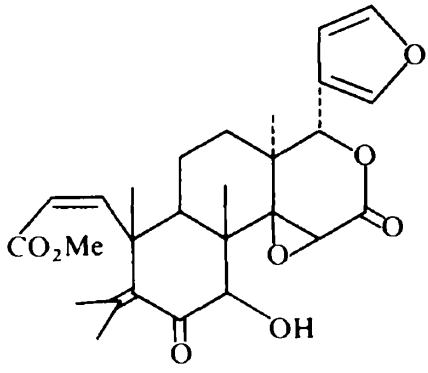
Methyl Isoobacunoate (**18**) X = O
Methyl 7β-Isoobacunolate (**19**) X = α-H, β-OH



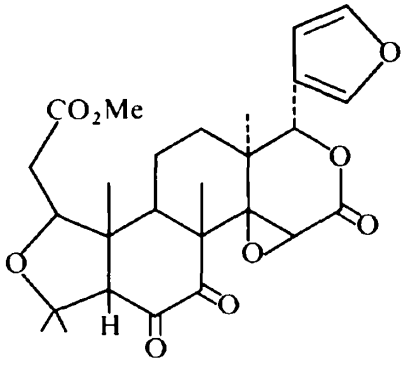
20



21



Atalantolide (**22**)



7-Dehydrocyclocalamin (**23**)

column of silica gel, packed in hexane-EtOAc (20:80). Fractions were eluted with gradually increasing amounts of EtOAc in hexane. The first compound eluted (15 mg) was identical (tlc and ^1H and ^{13}C NMR) with methyl isoobacunoate diosphenol (**13**).[10] The following fraction contained a mixture (57 mg) of **13** and cyclocalamin (**11**). This was separated by chromatography on a 40-g column of silica gel, packed in CH_2Cl_2–Et_2O (99:1). Elution with CH_2Cl_2–Et_2O (98:2) gave 4 mg of **13**, and **11** (44 mg) was eluted with CH_2Cl_2–Et_2O (97:3). Attempts to crystallize **11** were unsuccessful: ^1H NMR ($CDCl_3$) δ 7.41 (d, 2 H, 1 Hz, α-furans), 6.37 (d, 1 H, 1 Hz, β-furan), 5.47 (s, 1 H, H-17), 4.30 (t, 1 H, 6 Hz, H-1), 4.27 (s, 1 H, H-7), 4.14 (s, 1 H, H-15), 3.70 (s, 3 H, Me ester), 1.43 (s, 3 H, Me), 1.35 (s, 3 H, Me), 1.14 (s, 3 H, Me), 1.03 (s, 3 H, Me), 0.67 (s, 3 H, Me). Accurate mass measurement–Found: 503.2319. $C_{27}H_{35}O_9$ requires: 503.2281

The next material eluted from the hexane-EtOAc column was retrocalamin (**10**) (94 mg), which was purified by crystallization from MeOH and then from C_6H_6 to give 33 mg, m.p. 197–199°; ^1H NMR ($CDCl_3$) δ 7.39 (d, 2 H, 1 Hz, α-furans), 6.34 (d, 1 H, 1 Hz, β-furan), 5.53 (s, 1 H, H-17), 4.35 (s, 1 H, H-15), 4.29 (s, 1 H, H-7), 3.88 (m, 1 H, H-1), 3.70 (s, 3 H, Me ester), 1.34 (s, 3 H, Me), 0.90 (s, 3 H, Me), 0.79 (s, 3 H, Me). (Found: C, 62.4; H, 6.53. $C_{24}H_{30}O_9$ requires: C, 62.3; H, 6.54%). Accurate mass measurement-Found: 463.1923. $C_{24}H_{31}O_9$ requires: 463.1968.

The next fraction contained 68 mg of a mixture of limonin (**1**) and methyl deacetylnomilinate (**7**). Column chromatography of this material on silica gel with CH_2Cl_2–MeOH gave 18 mg of **1** and 40 mg of **7**, both identical (tlc and ^1H NMR) with authentic samples.

The last two fractions from the hexane-EtOAc column contained 80 mg of nomilin (**5**) and 22 mg of deacetylnomilin (**6**), both identical (tlc and ^1H NMR) with authentic samples.

Conversion of cyclocalamin (**11**) *to methyl isoobacunoate diosphenol* (**13**). A solution of 3 mg of **11** in 0.2 ml acetone was cooled to 10° and 7 μl of Jones' reagent[16] added gradually. After 20 min at 10° the excess CrO_3 was reduced with a drop of i-PrOH. Conventional workup gave 3 mg of 7-dehydrocyclocalamin (**23**); ^1H NMR ($CDCl_3$) δ 7.41 (d, 2 H, 1 Hz, α-furans), 6.37 (s, 1 H, 1 Hz, β-furan), 5.43 (s, 1 H, H-17), 4.27 (m, 1 H, H-1), 3.70 (s, 3 H, Me ester), 3.57 (s, 1 H, H-15), 2.86 (s, 1 H, H-5), 1.46 (s, 3 H, Me), 1.19 (s, 3 H, Me), 1.13 (s, 6 H, Me), 1.03 (s, 3 H, Me). Accurate mass measurement–Found: 501.2173. $C_{27}H_{33}O_9$ requires: 501.2124.

This material was dissolved in 0.2 ml of MeOH and 0.2 ml of conc. HCl added. After 1 hr at 25° the solution was diluted with 1 ml of H_2O and extracted twice with 0.5 ml portions of C_6H_6. The extracts were combined, washed with 0.2 ml of H_2O, and evaporated, giving 2.5 mg of a compound identical with **13** (tlc and ^1H NMR).

Conversion of calamin (**9**) *to cyclocalamin* (**11**). To a solution of 6 mg of **9** in 0.5 ml CH_2Cl_2 was added 0.5 ml of a saturated solution of HCl in CH_2Cl_2. After 2 hr at 25° the solution was washed free of acid with several 0.3 ml portions of H_2O and then evaporated to dryness, leaving 6 mg. Tlc showed a mixture of two major components. This material was chromatographed on a 3-g column of silica gel, packed in cyclohexane-EtOAc, 80:20. Elution with cyclohexane-EtOAc (70:30) gave 1.5 mg of **11**, identical (tlc and ^1H NMR) with an authentic sample. Elution with cyclohexane-EtOAc (30:70) gave 2 mg of the more polar product (**20**); ^1H NMR ($CDCl_3$) δ 7.41 (d, 2 H, 1 Hz, α-furans), 6.34 (d, 1 H, 1 Hz, β-furan), 5.62 (s, 1 H, H-17), 4.39 (s, 1 H, H-15), 4.33 (s, 1 H, H-7), 3.80 (d, 1 H, 7 Hz, H-1), 1.77 (s, 3 H, Me), 1.41 (s, 3 H, Me), 1.32 (s, 3 H, Me), 1.00 (s, 3 H, Me), 0.89 (s, 3 H, Me). Accurate mass measurement-Found. 489.2162. $C_{26}H_{33}O_9$ requires: 489.2124.

Methyl 7β-deacetylnomilolate (**8**). To a solution of 25 mg of **7** in 1 ml MeCN was added a solution of 12 mg $NaBH_4$ in 0.1 ml H_2O. After 1 min at 25°, 2 ml of 10% KH_2PO_4 was added, followed by 6 ml of CH_2Cl_2. The CH_2Cl_2 layer was separated and the aqueous layer extracted with two 2 ml portions of CH_2Cl_2. The CH_2Cl_2 solutions were combined, washed with 5 ml H_2O, and evaporated to give 18 mg. After separation from a little 7α-isomer by preparative tlc on Silica Gel G with hexane–Et_2O (20:80), 10 mg of amorphous **8**, homogeneous by tlc, was obtained; ^1H NMR ($CDCl_3$) δ 7.39 (d, 2 H, 1 Hz, α-furans), 6.33 (d, 1 H, 1 Hz, β-furan), 5.58 (s, 1 H, H-17), 4.72 (m, 1 H, H-1), 4.47 (s, 1 H, H-15), 3.73 (m, 1 H, H-7), 1.29 (s, 3 H, Me), 1.23 (s, 6 H, Me), 1.18 (s, 3 H, Me), 1.07 (s, 3 H, Me). No M + 1 peak was observed by MS under CI conditions, but an M-17 peak, representing the loss of H_2O from the M + 1 ion, was present. Accurate mass measurement–Found: 489.2551. $C_{27}H_{37}O_8$ requires: 489.2488.

Methyl 7β-isoobacunolate (**19**). To a solution of 60 mg of **18** in 1 ml C_6H_6 was added a solution of 80 mg of $NaBH_4.MeN(CH_2CH_2NMe_2)_2$ (obtained from Alfa Division of Ventron Inc.) in 2 ml C_6H_6. After 20 min 1 ml of H_2O was added, and 3N HCl was then added dropwise with stirring until evolution of H_2 ceased and the aqueous layer was acidic. The C_6H_6 layer was separated and the aqueous layer was extracted with 1 ml C_6H_6. The C_6H_6 solutions were combined and evaporated. The product (56 mg) was crystallized from C_6H_6 to give 42 mg of **19**, m.p. 120–122°; ^1H NMR ($CDCl_3$) δ 7.39 (d, 2 H, 1 Hz, α-furans), 6.31 (d, 1 Hz, β-furan), 5.62 (s, 1 H, H-17), 4.50 (s, 1 H, H-15), 3.80 (m, 2 H, H-1 and H-7), 3.66 (s, 3 H, Me ester), 1.21 (s, 6 H, Me), 1.14 (s, 3 H, Me), 1.08 (s, 3 H, Me), 0.98 (s, 3 H, Me). (Found: C, 66.4, H, 7.46. $C_{27}H_{36}O_8$ requires: C, 66.4; H, 7.43%). Accurate mass measurement-Found: 489.2495. $C_{27}H_{37}O_8$ requires: 489.2488.

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