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Citrus Limonoids and Their Semisynthetic Derivatives as Antifeedant Agents Against *Spodoptera frugiperda* Larvae. A Structure−Activity Relationship Study†

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The antifeedant activity of *Citrus*-derived limonoids limonin (**1**), nomilin (**2**), and obacunone (**3**) and their semisynthetic derivatives **4**−**26** was evaluated against a commercially important pest, *Spodoptera frugiperda*. Simple chemical conversions were carried out on the natural limonoids obtained from seeds of *Citrus limon*. These conversions focused on functional groups considered to be important for the biological activity, namely the C-7 carbonyl and the furan ring. In particular, reduction at C-7 afforded the related alcohols, and from these their acetates, oximes, and methoximes were prepared. Hydrogenation of the furan ring was also performed on limonin and obacunone. The known antifeedant properties of the *Citrus* limonoids are confirmed. Comparison with previously reported data shows that insect species vary in their behavioral responses to these structural modifications. Highly significant antifeedant activity ($P < 0.01$) for two natural (**1** and **3**) and three semisynthetic limonoids (**4**, **8**, and **10**) was observed against *S. frugiperda*.

KEYWORDS: ***Citrus limon*; semisynthetic limonoids; antifeedant activity; *Spodoptera frugiperda***

INTRODUCTION

Limonoids are the most representative class of secondary metabolites in the order Rutales, which includes the families Rutaceae, Meliaceae, and Simaroubaceae. They are tetranortriterpenoids with a 4,4,8-trimethyl-17-furanylsteroidal skeleton, bearing several oxygenated functions**.** These secondary metabolites exhibit a wide range of biological activities, including insect antifeedant activity (*1*) as well as antibacterial, antifungal, antiviral, and cytotoxic activity (*2*). Some have potent anti-insect properties and thus show potential for development as natural pesticides. The best known example is azadirachtin, a potent antifeedant and insect growth-regulator isolated from the seed of the Indian neem tree *Azadirachta indica* A. Juss (Meliaceae) (*3*−*5*).

In the *Citrus* genus, limonoids are present as aglycons and glucosides. To date, 36 aglycons and 17 glucosides have been isolated from *Citrus* and its hybrids (*6*−*9*). Limonin (**1**), nomilin (**2**), and obacunone (**3**) (**Figure 1**) are the main limonoid aglycons, whereas limonin 17β-D-glucopyranoside is the predominant glucoside. Limonoid aglycons are responsible for the delayed bitterness of *Citrus* juices, due to hydrolysis of the corresponding glucosides (*10*).

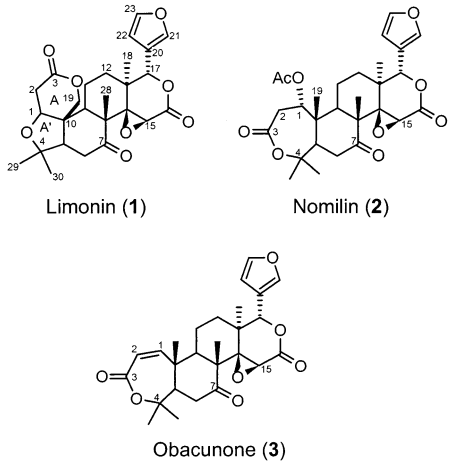

Figure 1. Natural *Citrus* limonoids.

To date, research has focused on the medicinal and insect-antifeedant properties of *Citrus*-derived limonoids. Their antitumoral properties were studied using both *in vitro* (*11*−*16*) and *in vivo* assays (*17*−*19*). The limonoids limonin (**1**), nomilin (**2**), and obacunone (**3**) are reported to deter insect feeding (*20*−

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23). However, previous data on the antifeedant activity of *Citrus* limonoids are difficult to compare, due to the heterogeneity of protocols and species of insects used (*1*, *24*−*27*), as well as the fact that structure−activity studies are rare. Data from larvae of *Leptinotarsa decemlineata* suggest that the functional groups important for the antifeedant activity are the epoxy and furan rings, while reduction of the carbonyl function at C-7 should enhance activity (*20*−*22*, *28*). In contrast, the complete hydrogenation of the furan ring caused a loss of activity toward *L. decemlineata*, even at higher doses. This latter evidence seems in contrast with data reported for other limonoids (*1*).

These natural products are readily available from waste products (seeds, peel, molasses, etc.) of the *Citrus*-processing industry, and there is increasing commercial interest in their exploitation. Brazil and the United States of America, the two main *Citrus*-processing countries, produce ca. 2×10^6 tons of waste products annually (*28*). In Southern Italy, one of the principal *Citrus*-processing countries of the Mediterranean basin, *ca*. 500 000 tons/year of waste are produced (*30*).

The present study investigates whether three limonoids (**1**−**3**) that occur in *Citrus*-processing waste influence the feeding behavior of larvae of *Spodoptera frugiperda* and whether their activity can be enhanced by simple chemical transformations.

MATERIALS AND METHODS

General Experimental Procedures. High-resolution electron impact mass spectra (HREIMS) were obtained on a Kratos M50S mass spectrometer. UV and IR spectra were recorded on Beckman model DU-65 and Perkin-Elmer model 684 spectrophotometers, respectively. Optical rotations were measured at 25 °C on a JASCO 135 instrument. ^{1}H NMR spectra were measured on a Varian INOVA operating at 500 MHz and a Bruker ARX-250 at 250 MHz, whereas ^{13}C NMR spectra were run at 63 MHz on a Bruker ARX-250 instrument. Multiplicities of ^{13}C signals were determined by distortionless enhancement of polarization transfer (DEPT) experiments. Two-dimensional (2D) NMR experiments, including correlation spectroscopy (COSY), nuclear Overhauser enhancement spectroscopy (NOESY), rotational Overhauser enhancement spectroscopy (ROESY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple-bond correlation (HMBC), were performed using standard Varian and Bruker software. All NMR experiments were carried out at constant temperature (298 K). Chemical shifts (δ) are indirectly referred to TMS using residual solvent signal. High-performance liquid chromatography (HPLC) was conducted on a Varian LC Star chromatographic system equipped with a UV−vis detector (model 9050). Preparative liquid chromatography (PLC) was conducted on a Jobin-Yvon instrument. Thin-layer chromatography (TLC) was carried out using precoated silica gel plates (Merck).

Plant Material. Seeds of *Citrus limon* were kindly supplied by Ditta Lucchesi, Aci Catena (CT), Italy.

Extraction of *Citrus* Limonoids. Seeds were washed and then freeze dried. Dry material (150 g) was ground and then Soxhlet extracted, first with C_6H_{14} (1 L) and then with CH_2Cl_2 (1 L). This latter extract was evaporated to dryness, and the residue was dissolved in C_6H_{14} and kept at 5 °C for 24 h. The precipitate, consisting mainly of limonoids, was collected by filtration and subjected to several column chromatography separations on silica gel using increasing amounts of CH_2Cl_2 in Et_2O as the eluant. Limonin (**1**), nomilin (**2**), and obacunone (**3**) were obtained in that order and with the following yields: 0.24%, 0.20%, and 0.13%, respectively.

Preparation of Limonoid Derivatives. Limonoid derivatives were prepared by conventional methods and purified by chromatography. Known compounds were identified on the basis of comparison of physical data with those reported in the literature (*31*−*37*). The structures of compounds not previously described were deduced essentially from MS and NMR data.

$NaBH_4$ Reduction of Limonoids 1, 2, and 3. Limonin (**1**), nomilin (**2**), and obacunone (**3**) were reduced with $NaBH_4$ to give, in each case, a mixture of α- and β-isomers, which were separated by column chromatography. The procedure is exemplified by the reduction of limonin.

Limonin (**1**, 240 mg) was dissolved in CH_2Cl_2 (10 mL), and 1.6 mL of a $NaBH_4$ solution (110 mg in 5 mL of anhydrous CH_3OH) was added. The mixture was continuously stirred at −5 °C for 24 h. The reaction was stopped by adding dilute HCl, and then the mixture was extracted with CH_2Cl_2 and the residue purified by HPLC (Hypersil ODS column, 5 μm, 8.0 mm i.d.; H_2O:CH_3CN 6:4, 3 mL/min) to give limonol (15 mg) and *epi*-limonol (108 mg).

Limonol (**4**): $[\alpha]^{25}_D = -57.28$ ($c = 0.30$ in $CHCl_3$); HREIMS [M^+] 472.5347 (calcd for $C_{26}H_{32}O_8$ 472.5353); UV λ_{max} (in CH_3CN) 210 nm ($\epsilon = 3800$); IR ν_{max} (in CH_2Cl_2) 3453, 1745 cm^{-1}.

Epi-limonol (**5**): $[\alpha]^{25}_D = +7.16$ ($c = 0.80$ in $CHCl_3$); HREIMS [M^+] 472.5344 (calcd for $C_{26}H_{32}O_8$ 472.5353); UV λ_{max} (in CH_3CN) 206 nm ($\epsilon = 5900$); IR ν_{max} (in CH_2Cl_2) 3450, 1750 cm^{-1}.

7α-Nomilol (**13**): amorphous solid prepared by reduction of nomilin; $[\alpha]^{25}_D = -8.79$ ($c = 0.33$ in $CHCl_3$); HREIMS [M^+] 516.5879 (calcd for $C_{28}H_{36}O_9$ 516.5884); UV λ_{max} (in CH_3CN) 205 nm ($\epsilon = 6000$); IR ν_{max} (in CH_2Cl_2) 3448, 1753 cm^{-1}.

7β-Nomilol (**14**): amorphous solid prepared by reduction of nomilin; $[\alpha]^{25}_D = +23.82$ ($c = 0.78$ in $CHCl_3$); HREIMS [M^+] 516.5877 (calcd for $C_{28}H_{36}O_9$ 516.5884); UV λ_{max} (in CH_3CN) 206 nm ($\epsilon = 4590$); IR ν_{max} (in CH_2Cl_2) 3450, 1750 cm^{-1}.

7α-Obacunol (**19**): $[\alpha]^{25}_D = +43.83$ ($c = 0.40$ in CH_2Cl_2); HREIMS [M^+] 456.5351 (calcd for $C_{26}H_{32}O_7$ 456.5359); UV λ_{max} (in CH_2Cl_2) 249 nm ($\epsilon = 340$); IR ν_{max} (in $CHCl_3$) 3470, 1736, 1692 cm^{-1}.

7β-Obacunol (**20**): amorphous; $[\alpha]^{25}_D = +48.41$ ($c = 0.76$ in CH_2Cl_2); HREIMS [M^+] 456.5350 (calcd for $C_{26}H_{32}O_7$ 456.5359); UV λ_{max} (in CH_2Cl_2) 229.5 nm ($\epsilon = 8812$); IR ν_{max} (in $CHCl_3$) 3468, 1736, 1696 cm^{-1}.

Reaction of Limonin (1), Nomilin (2), and Obacunone (3) with Hydroxylamine. Limonoids were reacted with hydroxylamine chloridrate as described below for **1**.

Limonin (186 mg) and hydroxylamine hydrochloride (200 mg) were dissolved in C_5H_5N (4 mL) and absolute C_2H_5OH (4 mL), and the solution was refluxed for 4 h. The reaction was cooled and a saturated solution of NaCl added. The mixture was then extracted with AcOEt to obtain pure limonin-7-oxime (184 mg, 94% yield).

Limonin-7-oxime (**8**): amorphous solid; $[\alpha]^{25}_D = -159.46$ ($c = 1.72$ in CH_2Cl_2); HREIMS [M^+] 485.5334 (calcd for $C_{26}H_{31}NO_8$ 485.5341); UV λ_{max} (in CH_2Cl_2) 230 nm ($\epsilon = 525$); IR ν_{max} (in $CHCl_3$) 3460, 1741, 1597 cm^{-1}. NMR data are given in **Table 1**.

Nomilin-7-oxime (**16**): obtained from nomilin as amorphous solid in 97% yield; $[\alpha]^{25}_D = -141.62$ ($c = 0.74$ in CH_2Cl_2); HREIMS [M^+] 529.5865 (calcd for $C_{28}H_{35}NO_9$ 529.5872); UV λ_{max} (in CH_2Cl_2) 229.5 nm ($\epsilon = 1064$); IR ν_{max} (in $CHCl_3$) 3464, 1748, 1597 cm^{-1}. NMR data are given in **Table 1**.

Obacunone-7-oxime (**23**): amorphous solid obtained from obacunone in 93% yield; $[\alpha]^{25}_D = -26.69$ ($c = 0.34$ in CH_2Cl_2); HREIMS [M^+] 469.5339 (calcd for $C_{26}H_{31}NO_7$ 469.5347); UV λ_{max} (in CH_2Cl_2) 248 nm ($\epsilon = 401$); IR ν_{max} (in $CHCl_3$) 3468, 1736, 1698, 1602 cm^{-1}. NMR data are given in **Table 1**.

Reaction of Limonin (1), Nomilin (2), and Obacunone (3) with Methylhydroxylamine. Limonoid methoximes were prepared by reaction of the parent compounds with methylhydroxylamine, following the procedure illustrated below for limonin.

Limonin (95 mg) was dissolved in CH_2Cl_2 (10 mL), and then methylhydroxylamine (17 mg) dissolved in 2 mL of absolute C_2H_5OH and 2 mL of C_5H_5N was added. The mixture was kept under reflux and continuous stirring for 4 h. After the mixture cooled, a saturated solution of NaCl was added and the organic matter extracted with AcOEt. The residue was purified by preparative liquid chromatography (PLC) (silica gel, CH_2Cl_2:Et_2O 6:4) to obtain limonin-7-methoxime (73.8 mg, 73% yield).

Limonin-7-methoxime (**9**): amorphous solid; $[\alpha]^{25}_D = -134.65$ ($c = 0.945$ in CH_2Cl_2); HREIMS [M^+] 499.5602 (calcd for $C_{27}H_{33}NO_8$ 499.5610); UV λ_{max} (in CH_2Cl_2) 240 nm ($\epsilon = 25$); IR ν_{max} (in $CHCl_3$) 1730, 1633, 1600 cm^{-1}. NMR data are given in **Table 2**.

Nomilin-7-methoxime (**18**): amorphous solid prepared from nomilin in 60% yield; $[\alpha]^{25}_D = -87.21$ ($c = 0.26$ in CH_2Cl_2); HREIMS [M^+]

Table 1. NMR Data of Oximes **8**, **16**, and **23**[a]

	limonin-7-oxime (**8**)		nomilin-7-oxime (**16**)		obacunone-7-oxime (**23**)	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	4.04 bs	79.6 d	5.02 d (7.0)	71.2 d	6.53 d (12)	158.8 d
2	2.95 dd (17, 3)	36.3 t	3.22 d (15)	35.7 t	5.91 d (12)	122.9 d
	2.74 d (17)		3.08 dd (15, 7.2)			
3		170.8 s		170.1 s		168.0 s
4		81.1 s		85.8 s		85.5 s
5	1.98 m	60.4 d	2.34 dd (14, 3.2)	50.4 d	2.36 dd (14, 4)	56.1 d
6	3.57 m	19.1 t	3.74 dd (14, 3.2)	21.4 t	3.44 dd (14.2, 4)	23.0 t
	2.00 m		1.94 m		2.18 m	
7		159.1 s		159.6 s		158.6 s
8		46.1 s		46.6 s		46.4 s
9	2.45 m	50.1 d	2.44 m	45.9 d	2.10 m	50.3 d
10		46.5 s		44.7 s		43.7 s
11	1.82 m	19.8 t	1.60 m	18.0 t	1.80 m	20.2 t
			1.20 m		1.45 m	
12	1.80 m	33.2 t	1.80 m	34.0 t	1.85 m	34.2 t
	1.50 m		1.20 m			
13		38.3 s		38.2 s		38.6 s
14		65.7 s		65.8 s		65.8 s
15	3.82 s	54.6 d	3.73 s	54.3 d	3.73 s	54.4 d
16		168.2 s		168.3 s		168.1 s
17	5.47 s	78.8 d	5.45 s	78.9 d	5.51 s	78.8 d
18	1.23 s	21.6 q	1.20 s	21.9 q	1.21 s	21.9 q
19	4.40 d (13)	66.3 t	1.24 s	16.9 q	1.40 s	16.6 q
	4.20 d (13)					
20		120.5 s		120.6 s		120.7 s
21	7.40 bs	141.4 d	7.42 bs	141.3 d	7.41 bs	141.3 d
22	6.34 bs	110.1 d	6.36 bs	110.1 d	6.36 bs	110.2 d
23	7.40 bs	143.6 d	7.39 bs	143.6 d	7.41 bs	143.4 d
28	1.19 s	21.7 q	1.57 s	24.6 q	1.54 s	27.3 q
29	1.33 s	30.7 q	1.59 s	34.1 q	1.53 s	32.7 q
30	0.96 s	18.7 q	1.09 s	17.8 q	1.13 s	17.5 q
CH_3*CO*				169.8 s		
CH$_3$CO			2.03 s	21.2 q		

[a] ^{1}H NMR (250 MHz) and ^{13}C NMR (63 MHz) spectra were run in $CDCl_3$ (ppm from TMS). ^{13}C multiplicities were obtained by DEPT experiments.

543.6135 (calcd for $C_{29}H_{37}NO_9$ 543.6141); UV λ_{max} (in $CHCl_3$) 240 nm ($\epsilon = 633$); IR ν_{max} (in $CHCl_3$) 1741, 1650, 1595 cm^{-1}. NMR data are given in **Table 2**.

Obacunone-7-methoxime (**25**): obtained from obacunone as amorphous solid in 68% yield; $[\alpha]^{25}_D = -27.87$ ($c = 1.94$ in CH_2Cl_2); HREIMS [M$^+$] 483.5610 (calcd for $C_{27}H_{35}NO_7$ 483.5616); UV λ_{max} (in CH_2Cl_2) 244 nm ($\epsilon = 6047$); IR ν_{max} (in $CHCl_3$) 1733, 1645, 1607 cm^{-1}. NMR data are given in **Table 2**.

Acetylation. The starting compound (usually 10 mg or more) was treated with C_5H_5N (1 mL) and $(CH_3CO)_2O$ (1 mL). The mixtures were continuously stirred for 24 h at room temperature, and then the solvents were removed and the residue was purified by chromatography.

Limonyl acetate (**6**): prepared by acetylation of limonol in 90% yield; $[\alpha]^{25}_D = -54.31$ ($c = 0.23$ in CH_2Cl_2); HREIMS [M$^+$] 514.5719 (calcd for $C_{28}H_{34}O_9$ 514.5726); UV λ_{max} (in CH_2Cl_2) 232 nm ($\epsilon = 544$); IR ν_{max} (in CH_2Cl_2) 1744 cm^{-1}.

Epi-limonyl acetate (**7**): obtained as amorphous solid by acetylation of *epi*-limonol; $[\alpha]^{25}_D = +10.00$ ($c = 0.07$ in CH_2Cl_2); HREIMS [M$^+$] 514.5718 (calcd for $C_{28}H_{34}O_9$ 514.5726); UV λ_{max} (in CH_2Cl_2) 230 nm ($\epsilon = 1370$); IR ν_{max} (in CH_2Cl_2) 1744 cm^{-1}.

Limonin-7-oxime acetate (**10**): obtained as amorphous solid from limonin-7-oxime in 95% yield; $[\alpha]^{25}_D = -116.89$ ($c = 1.59$ in CH_2Cl_2); HREIMS [M$^+$] 527.5708 (calcd for $C_{28}H_{33}NO_9$ 527.5714); UV λ_{max} (in CH_2Cl_2) 248 nm ($\epsilon = 99$); IR ν_{max} (in $CHCl_3$) 1747, 1601 cm^{-1}.

7β-Nomilyl acetate (**15**): amorphous solid prepared from 7β-nomilol in 80% yield; $[\alpha]^{25}_D = +25.88$ ($c = 0.68$ in CH_2Cl_2); HREIMS [M$^+$] 558.6249 (calcd for $C_{30}H_{38}O_{10}$ 558.6257); UV λ_{max} (in CH_2Cl_2) 227 nm ($\epsilon = 298$); IR ν_{max} (in $CHCl_3$) 1752 cm^{-1}.

Nomilin-7-oxime acetate (**17**): prepared in 98% yield as amorphous solid from nomilin-7-oxime; $[\alpha]^{25}_D = -102.78$ ($c = 0.40$ in CH_2Cl_2); HREIMS [M$^+$] 571.4238 (calcd for $C_{30}H_{37}NO_{10}$ 571.6245); UV λ_{max} (in CH_2Cl_2) 240.5 nm ($\epsilon = 422$); IR ν_{max} (in $CHCl_3$) 1732, 1601 cm^{-1}.

7α-Obacunyl acetate (**21**): obtained from 7α-obacunol in 84% yield; $[\alpha]^{25}_D = +22.97$ ($c = 0.18$ in CH_2Cl_2); HREIMS [M$^+$] 498.5727 (calcd for $C_{28}H_{34}O_8$ 498.5732); UV λ_{max} (in CH_2Cl_2) 240 nm ($\epsilon = 808$); IR ν_{max} (in $CHCl_3$) 1742 cm^{-1}.

7β-Obacunyl acetate (**22**): amorphous powder by acetylation of 7-β-obacunol in 61% yield; $[\alpha]^{25}_D = +30.59$ ($c = 0.09$ in CH_2Cl_2); HREIMS [M$^+$] 498.5726 (calcd for $C_{28}H_{34}O_8$ 498.5732); UV λ_{max} (in CH_2Cl_2) 225.5 nm ($\epsilon = 1547$); IR ν_{max} (in $CHCl_3$) 1736, 1701 cm^{-1}.

Obacunone-7-oxime acetate (**24**): amorphous solid from obacunone-7-oxime in 98% yield; $[\alpha]^{25}_D = -21.85$ ($c = 0.41$ in CH_2Cl_2); HREIMS [M$^+$] 511.5712 (calcd for $C_{28}H_{35}NO_8$ 511.5720); UV λ_{max} (in CH_2Cl_2) 248 nm ($\epsilon = 376$); IR ν_{max} (in $CHCl_3$) 1747, 1702, 1601 cm^{-1}.

Preparation of Perhydrolimonin (11) and *Epi*-perhydrolimonin (12). Limonin (100 mg) and palladium (10 wt %) on activated charcoal were suspended in CH_2Cl_2 (10 mL) and subjected to H_2 pressure (2 atm) for 48 h. After filtration, the resulting mixture was purified by PLC (silica gel, gradient of tetrahydrofuran in cyclohexane from 40% to 85%) to obtain 32 mg (32% yield) of perhydrolimonin and 15.5 mg (16% yield) of *epi*-perhydrolimonin.

Perhydrolimonin: (**11**): amorphous powder; $[\alpha]^{25}_D = -116.35$ ($c = 0.85$ in CH_2Cl_2); HREIMS [M$^+$] 474.5505 (calcd for $C_{26}H_{34}O_8$ 474.5512); UV λ_{max} (in $CHCl_3$) 240 nm ($\epsilon = 1887$). NMR data are given in **Table 3**.

Epi-perhydrolimonin (**12**): amorphous powder; $[\alpha]^{25}_D = -120.26$ ($c = 1.36$ in CH_2Cl_2); HREIMS [M$^+$] 474.5507 (calcd for $C_{26}H_{34}O_8$ 474.5512); UV λ_{max} (in $CHCl_3$) 282 nm ($\epsilon = 126$). NMR data are given in **Table 3**.

Preparation of 1,2-Didehydroobacunone (26). Obacunone (18 mg) and palladium (10 wt %) on activated charcoal were suspended in CH_2Cl_2 (3 mL) and subjected to H_2 pressure (1 atm) for 20 min. After filtration, the mixture was subjected to PLC (silica gel, CH_2Cl_2 as the eluant) to yield 1,2-didehydroobacunone (7 mg, 40% yield).

Table 2. NMR Data of Methoximes **9**, **18**, and **25**[a]

	limonin-7-methoxime (**9**)		nomilin-7-methoxime (**18**)		obacunone-7-methoxime (**25**)	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	3.96 bs	79.2 d	5.08 d (7)	71.3 d	6.50 d (12)	158.3 d
2	2.95 dd (16, 4)	35.8 t	3.18 dd (16, 1.5)	35.7 t	5.91 d (12)	123.0 d
	2.66 dd (16, 1.5)		3.04 dd (16, 7)			
3		169.3 s		169.7s		167.6 s
4		80.4 s		85.4 s		85.2 s
5	1.93 m	60.4 d	2.29 dd (12, 3.5)	50.5 d	2.30 dd (12.5, 4)	56.3 d
6	3.43 d (12)	19.4 t	3.60 dd (17, 3.5)	21.2 t	3.21 dd (15, 4)	23.8 t
	1.95 d (12)		1.92 m		2.15 m	
7		157.7 s		158.3 s		158.5s
8		46.1 s		46.6 s		46.4 s
9	2.39 d (10)	49.8 d	2.39 d (9)	45.8 d	1.95 m	50.2 d
10		45.8 s		44.7 s		43.6 s
11	1.74 m	19.5 t	1.52 m	19.9 t	1.80 m	20.2 t
			1.20 m		1.45 m	
12	1.50 m	33.0 t	1.80 m	34.0 t	1.87 m	34.0 t
	1.30 m		1.17 m			
13		37.9 s		38.8 s		38.4 s
14		64.8 s		65.6 s		65.6 s
15	3.82 s	54.2 d	3.72 s	54.4 d	3.70 s	54.4 d
16		166.9 s		169.6 s		167.6 s
17	5.46 s	78.2 d	5.46 s	78.8 d	5.50 s	78.8 d
18	1.24 s	21.3 q	1.20 s	22.0 q	1.24 s	22.1 q
19	4.66 d (13)	65.7 t	1.21 s	17.0 q	1.39 s	16.6 q
	4.32 d (13)					
20		120.2 s		120.7 s		120.8 s
21	7.41 bs	140.9 d	7.39 bs	141.3 d	7.42 bs	141.3 d
22	6.35 bs	109.7 d	6.34 bs	110.1 d	6.38 bs	110.2 d
23	7.39 bs	143.1 d	7.39 bs	143.6 d	7.42 bs	143.5 d
28	1.10 s	21.4 q	1.52 s	24.3 q	1.51 s	17.3 q
29	1.30 s	30.2 q	1.54 s	18.1 q	1.50 s	32.7 q
30	0.94 s	18.0 q	1.12 s	17.7 q	1.11 s	17.4 q
OCH_3	3.85 s	62.1 q	3.84 s	62.4 q	3.82 s	62.3 q
CH_3CO				169.6 s		
CH_3CO			2.00 s	21.2 q		

[a] ^{1}H NMR (250 MHz) and ^{13}C NMR (63 MHz) spectra were run in $CDCl_3$ (ppm from TMS). ^{13}C multiplicities were obtained by DEPT experiments.

1,2-Didehydroobacunone (**26**): amorphous solid; $[\alpha]^{25}_D = -79.76$ ($c = 0.09$ in CH_2Cl_2); HREIMS [M^+] 456.5351 (calcd for $C_{26}H_{32}O_7$ 456.5359); UV λ_{max} (in CH_2Cl_2) 229.5 nm ($\epsilon = 1234$); IR ν_{max} (in $CHCl_3$) 1720, 1718 cm^{-1}. NMR data are given in **Table 3**.

Antifeedant Activity Bioassay. *Spodoptera frugiperda* larvae were reared on a wheatgerm-based diet (*38*). Final stadium larvae aged 24−36 h were used in the binary choice bioassay. They were deprived of food for 2 h before being placed individually into Petri dishes, which contained control and treatment glass-fiber disks (Whatman GF/A, 2.1 cm diameter). All disks were treated with the phagostimulant sucrose (100 μL of a 0.05 M solution) and then left to dry. An aliquot (100 μL) of one of the test compounds in $(CH_3)_2CO$ was applied to 10 treatment disks. Acetone alone (100 μL) was applied to the control disks. The disks were then dried and weighed prior to bioassay. The bioassays were observed every 30 min, and each experiment was terminated after 8 h, the time before larvae had eaten 50% of either control or treatment disks. Larvae were then removed from the Petri dishes. The disks were allowed to dry and then reweighed. The amount of control (C) and treatment (T) disk eaten in each Petri dish was used to calculate the feeding index, [(C − T)/(C + T)]%. The potency of each compound was analyzed using the Wilcoxon matched-pairs test, and the Mann−Whitney *U* test was used to determine whether the semisynthetic derivatives were more potent than the parent natural compounds (*39*).

RESULTS AND DISCUSSION

A range of three *Citrus*-derived limonoids (**1**−**3**) and 23 semisynthetic derivatives (**4**−**26**) were evaluated for antifeedant activity against larvae of the fall armyworm, *S. frugiperda*. The derivatives were obtained through simple chemical modifications of the natural limonoids and are suitable for possible large-scale applications. The conversions focused mainly on modification of the carbonyl function at C-7, namely reduction to epimeric alcohols, conversion to the corresponding oxime and methoxime, and preparation of their acetates. Catalytic hydrogenation of the furan ring was also carried out, to verify its role in the antifeedant activity of limonin.

Limonin (1) Derivatives. The reduction of the carbonyl function at C-7 in **1** was carried out through mild $NaBH_4$ treatment, as previously described for various limonoids (*31*, *36*, *37*). The two epimeric alcohols limonol (**4**) and *epi*-limonol (**5**) (**Figure 2**) were obtained with a 1:30 (α:β) ratio. These results could be explained by the strong steric hindrance on the two faces of the carbonyl function, due to the spatial location of the A and A′ rings of limonin. A number of 7α-hydroxy natural limonoids are known; among them, limonol (**4**) is present in seeds of *Citrus paradisi* (*31*). Compounds **4** and **5** were subjected to acetylation, which afforded limonyl acetate (**6**) and *epi*-limonyl acetate (**7**), respectively (**Figure 2**). Limonyl acetate (**6**) is known as a natural product, as it occurs in fruit of a *Citrus*−*Poncirus* hybrid (*31*).

To verify the effect on the antifeedant activity of a further increase of lipophilicity at C-7, attempts were made to obtain palmitate, valerate, or benzoate esters, but all these reactions showed negligible yields, probably owing to the steric hindrance of the 7-hydroxy group.

The preparation of limonin-7-oxime (**8**, **Figure 2**) from **1** was carried out as a slight modification of a previously reported protocol (*37*), which enhanced the yield and substantially reduced the reaction time. The product was identified by its

Table 3. NMR Data of Hydrogenated Derivatives **11**, **12**, and **26**[a]

	perhydrolimonin (**11**)		*epi*-perhydrolimonin (**12**)		1,2-didehydrobacunone (**26**)	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	4.08 bs	79.1 d	4.08 bs	79.5 d	1.96 m	33.2 t
					1.48 m	
2	3.00 dd (15, 4)	35.7 t	3.01 dd (15, 4)	36.1 t	2.65 m	39.8 t
	2.70 dd (15, 2)		2.71 dd (15, 1.5)			
3		168.9s		169.6 s		166.6 s
4		80.2 s		80.7 s		84.4 s
5	2.22 dd (15, 3)	60.9 d	2.23 dd (15.7, 3)	61.2 d	2.32 dd (13.5, 5)	54.6 d
6	2.83 dd (15, 14.5)	36.4 t	2.85 dd (15, 14.5)	36.8 t	2.94 dd (13.5, 13.5)	37.6 t
	2.45 dd (15, 3)		2.46 dd (15, 3.5)		2.42 dd (13.5, 5)	
7		206.1 s		206.5 s		207.4 s
8		51.6 s		51.9 s		51.3 s
9	2.53 dd (10, 3)	48.2 d	2.55 dd (15, 2)	48.5 d	2.04 m	53.0 d
10		46.0 s		46.4 s		40.2 s
11	1.79 m	19.2 t	1.80 m	19.5 t	1.80 m	18.6 t
12	1.61 m	30.1 t	1.75 m	30.6 t	1.40 m	32.3 t
13		37.7 s		38.2 s		40.1 s
14		64.9 s		65.6 s		65.5 s
15	3.80 s	53.1 d	3.83 s	53.6 d	3.66 s	53.6 d
16		166.4 s		166.8 s		166.9 s
17	4.32 d (6)	82.0 d	4.45 d (7)	84.3 d	5.45 s	78.5 d
18	1.21 s	21.5 q	1.19 s	21.7 q	1.24 s	19.4 q
19	4.77 d (13)	65.4 t	4.77 d (13)	65.8 t	1.21 s	21.6 q
	4.43 d (13)		4.44 d (13)			
20	2.35 m	39.4 d	2.39 m	39.6 d		120.7 s
21	3.86 m	70.5 t	4.02 m	70.3 t	7.41 bs	141.4 d
	3.38 m		3.62 m			
22	2.06 m	31.7 t	1.94 m	31.3 t	6.36 bs	110.2 d
	1.94 m		1.74 m			
23	3.82 m	67.9 t	3.86 m	68.1 t	7.41 bs	143.5 d
			3.75 m			
28	1.00 s	17.2 q	1.02 s	17.6 q	1.12 s	18.9 q
29	1.30 s	28.8 q	1.30 s	29.5 q	1.51 s	30.7 q
30	1.18 s	21.3 q	1.19 s	21.8 q	1.48 s	27.2 q

[a] ^{1}H NMR (250 MHz) and ^{13}C NMR (63 MHz) spectra were run in $CDCl_3$ (ppm from TMS). ^{13}C multiplicities were obtained by DEPT experiments.

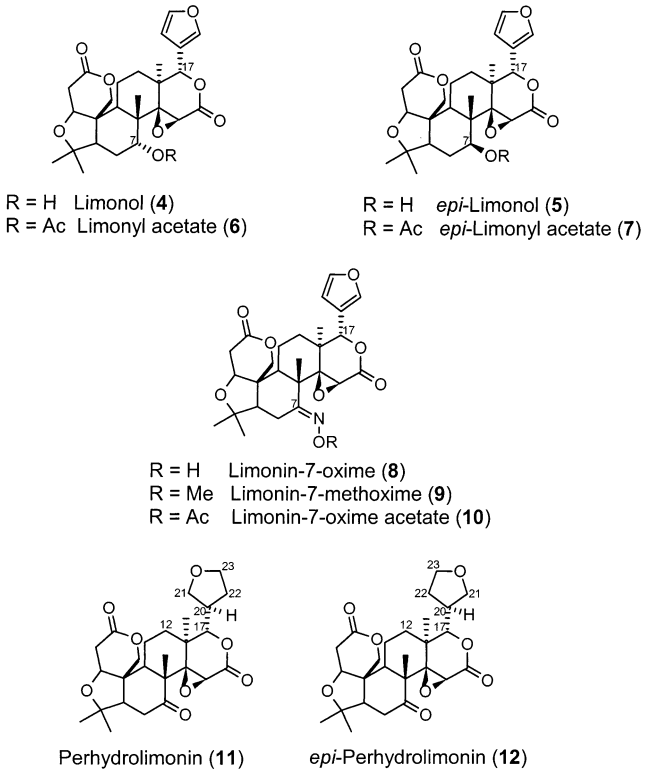

Figure 2. Limonin derivatives.

spectroscopic features. In particular, the NMR data (**Table 1**) showed the expected shifts of the proton and carbon signals influenced by the ═N—OH group. In principle, the formation of the oxime should afford two geometric isomers at the C═N double bond. We could not observe the formation of a *cis/trans* mixture; indeed, the analysis of NMR spectra showed signals associated with a single product.

To distinguish between *E* and *Z* configuration, the limonin-7-oxime (**8**) was subjected to methylation with CH_3I to obtain the corresponding limonin-7-methoxime (**9**, **Figure 2**). The analysis of ^{1}H and ^{13}C NMR spectra (**Table 2**) confirmed that the expected derivative has been obtained, showing the new methoxy signals at δ 3.85 (^{1}H) and δ 62.4 (^{13}C). A 2D NMR study allowed complete assignment of both ^{1}H and ^{13}C NMR spectra (**Table 2**). A ROESY experiment on this compound showed a strong correlation between the methoxy group at δ 3.85 and the signal at δ 3.43, assigned to one of the methylene protons in C-6. This is consistent only with an *E* configuration for the C═N double bond.

Furthermore, applying the same methodology to obtain the oxime (**8**), the reaction of limonin (**1**) with methylhydroxylamine allowed us to obtain directly the limonin-7-methoxime (**9**), whose spectral features were perfectly coincident with those of the methoxime obtained from the corresponding oxime. In both cases, a strong driving force ascribable to a considerable steric hindrance of one side of the C═O double bond of limonin (as well as of the other limonoids) led to the exclusive formation of only one geometric isomer.

As previously described for limonols, various esterifications of the oxime function have been attempted, but only the acetate was obtained in good yield. Limonin-7-oxime acetate (**10**, **Figure 2**) was purified and fully characterized.

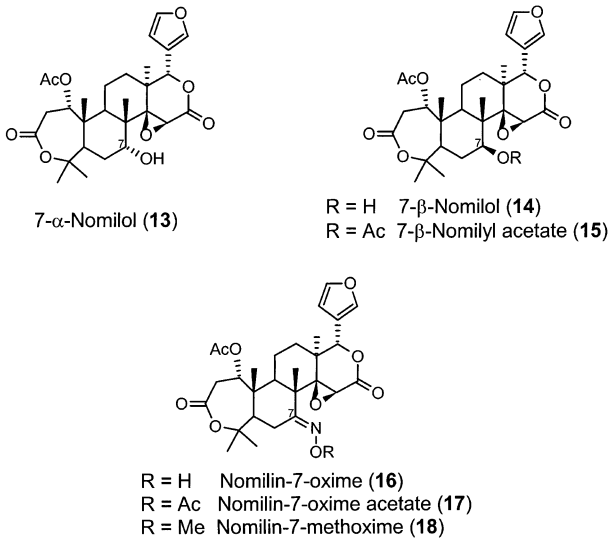

Figure 3. Nomilin derivatives.

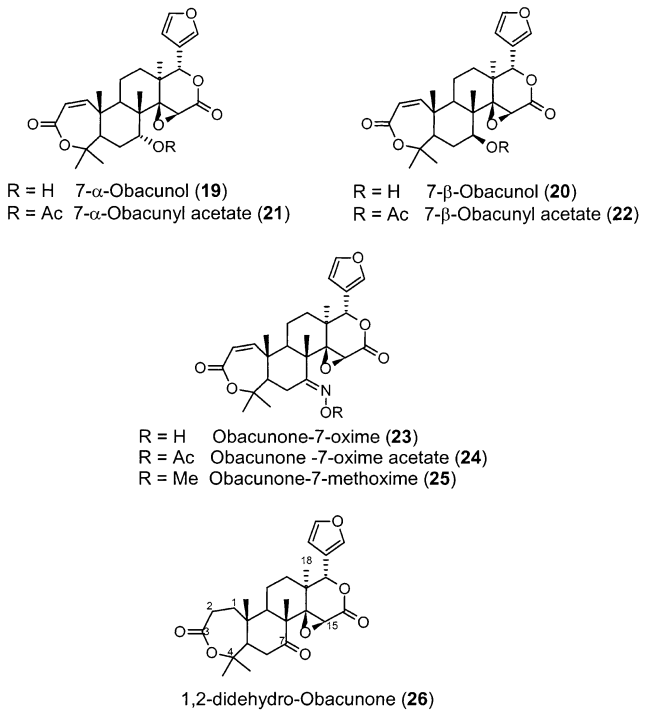

Figure 4. Obacunone derivatives.

Catalytic hydrogenation of **1** afforded, as expected, a mixture of C-20 epimeric perhydro derivatives, namely perhydrolimonin (**11**) and *epi*-perhydrolimonin (**12**) (**Figure 2**). Although difficult, the separation of these epimers was achieved through HPLC. Compound **11** showed signals easily attributable to the tetrahydrofuran ring in the aliphatic region of the ^{1}H and ^{13}C NMR spectra (**Table 3**). The reported assignments were based on the integrated analysis of homo- and heteronuclear 2D NMR spectra, establishing the structure of **11**, apart from the C-20 configuration. Unambiguous determination of the relative stereochemistry at C-20 in **11** and **12** was not a trivial task, due to the conformational mobility of the tetrahydrofuran ring with respect to the core of the limonin skeleton. Thus, MM2 (87) calculations were used to minimize the structures of both epimers. The torsional energy profile of the $H_{17}-C_{17}-C_{20}-H_{20}$ bond was analyzed by using the dihedral driver tool of Macromodel (Version 7.2), setting a 0−360° interval and a 5° increment. From the results of this study, omitted here for the sake of brevity, we obtained for each compound a couple of very similar energy minimum structures, assumed as preferred conformations (Supporting Information). Two NMR ROESY experiments were carried out on **11** and **12**, and the resulting data (Supporting Information) were compared with the above-determined stereostructures, checking selected internuclear distances for each couple of conformers. This study allowed unambiguous assignment of C-20 relative configuration for **11** and **12**. In particular, a strong ROE correlation between the H_2-12 signal at δ 1.61 and the H_2-21 methylene at δ 3.86 and 3.31 established the relative configuration of the C-20 position, as reported in structure **11**. In contrast to **11**, and in agreement with the epimeric C-20 configuration, **12** showed a correlation of the methyl group at C-18 (δ 1.19) with one of the H_2-22 signals (δ 1.94).

Nomilin (2) Derivatives. Reactions carried out on **2** and some of its derivatives were adversely affected by the low stability of nomilin. A low total conversion was observed during the $NaBH_4$ reduction of **2**, affording two epimeric 7-hydroxyderivatives, namely 7α-nomilol (**13**) and 7β-nomilol (**14**) (**Figure 3**) in an α:β ratio of ca. 1:2. The acetylation of **14** afforded the expected 7β-nomilyl acetate (**15**, **Figure 3**), as confirmed by spectral analysis and comparison with data reported for the natural 7β-nomilyl acetate isolated from *Dictamnus albus* (*31*). When submitted to standard acetylation conditions, the epimer **13** gave 7α-obacunyl acetate (**21**, **Figure 4**), as confirmed by spectral analysis. Nomilin-7-oxime (**16**, **Figure 3**) was obtained by applying the procedure used for limonin. Spectral characterization of this new product confirmed its structure (**Table 1**). In analogy to the reaction with limonin, the formation of a *cis/trans* mixture was not observed. Acetylation of **16** gave the corresponding nomilin-7-oxime acetate (**17**, **Figure 3**), in agreement with spectral analysis.

The procedure used to obtain **9** allowed us to prepare nomilin-7-methoxime (**18**, **Figure 3**) with 60% yield, but a further product could be isolated from the reaction mixture. The main methoxime **18** was fully characterized through homo- and heteronuclear 2D NMR experiments. Analogously, MS and NMR studies established the minor product as obacunon-7-methoxime (**25**, **Figure 4**), thus confirming the easy conversion of nomilin to obacunone.

Obacunone (3) Derivatives. Reduction of C-7 carbonyl in **3**, carried out in analogy to the reductions of **1** and **2**, gave the expected alcohols, 7α-obacunol (**19**) and 7β-obacunol (**20**) (**Figure 4**), with an α:β ratio ca. 1:1. The physical data of **19** were in agreement with those reported for the natural 7α-obacunol previously isolated from *C. paradisi* seeds (*31*). Standard acetylation of both **19** and **20** afforded, respectively, 7α-obacunyl acetate (**21**) and 7β-obacunyl acetate (**22**) (**Figure 4**).

The procedures reported above for the preparation of oximes were applied to obacunone, and obacunone-7-oxime (**23**, **Figure 4**) was obtained and submitted to spectral analysis. Compound **23** showed NMR data (**Table 1**) compatible with the expected structure. Acetylation of **23** afforded obacunone-7-oxime acetate (**24**, **Figure 4**). The reaction of **3** with methylhydroxylamine yielded obacunone-7-methoxime (**25**, **Figure 4**), characterized on the basis of NMR study (**Table 2**). Both **23** and **25** were obtained only as the *trans* geometrical isomer at the C=N double bond, as expected from the above-reported preparations of oximes and methoximes from **1** and **2**.

Catalytic hydrogenation of **3** afforded, similarly to the hydrogenation of **2**, an unresolvable mixture of two C-20 epimers. After spectroscopic examination, this mixture revealed

Table 4. Antifeedant Activity of *Citrus* Limonoids and Their Semisynthetic Derivatives against Fifth Instar Larvae *Spodoptera frugiperda*[a]

compound	feeding index[b] (sem)
Limonin and Derivatives	
limonin (**1**)	87 (5.7)**a
limonol (**4**)	68 (4.0)**b
epi-limonol (**5**)	56 (4.8)*c
limonyl acetate (**6**)	45 (4.8)*c
epi-limonyl acetate (**7**)	26 (13.8)c
limonin-7-oxime (**8**)	68 (5.8)**b
limonin-7-oxime acetate (**10**)	45 (2.5)*c
limonin-7-methoxime (**9**)	76 (12.7)**a
perhydrolimonin (**11**)	29 (4.8)*c
epi-perhydrolimonin (**12**)	45 (7.8)*c
Nomilin and Derivatives	
nomilin (**2**)	56 (4.9)*a
7-α-nomilol (**13**)	46 (12.8)*a
7-β-nomilol (**14**)	35 (8.7)*b
7-β-nomilyl acetate (**15**)	46 (11.8)*a
nomilin-7-oxime (**16**)	35 (21.9)*b
nomilin-7-oxime acetate (**17**)	41 (5.4)*b
nomilin-7-methoxime (**18**)	38 (11.7)*b
Obacunone and Derivatives	
obacunone (**3**)	68 (4.8)**a
7-α-obacunol (**19**)	53 (7.9)*b
7-β-obacunol (**20**)	35 (3.8)*c
7-α-obacunyl acetate (**21**)	24 (12.8)c
7-β-obacunyl acetate (**22**)	26 (16.4)c
obacunone-7-oxime (**23**)	25 (12.5)c
obacunone-7-oxime acetate (**24**)	21 (10.2)c
obacunone-7-methoxime(**25**)	29 (8.8)*c
1,2-didehydro-obacunone (**26**)	53 (12.9)*b

[a] Expressed as feeding index, [(C − T)/(C + T)]%, with standard error of the mean (SEM) in parentheses. All compounds were tested at 100 ppm and $n = 10$. [b] Significance of feeding index: **, $P < 0.01$ and *, $P < 0.05$, Wilcoxon matched-pairs test. Within each group of compounds the values of the feeding indexes have been compared with that of the corresponding natural compound; values followed by a similar letter do not differ; a > b = $P < 0.05$, a > c = $P < 0.01$, Mann–Whitney *U* test.

that the 1,2 double bond had also been saturated. Using milder hydrogenation conditions, it was possible to get the 1,2-didehydroobacunone (**26**, **Figure 4**), as confirmed by spectral analysis. In fact, the ^{1}H NMR spectrum of **26** (**Table 3**) showed the absence of the 1,2 double bond signals and the presence of new signals accounting for two methylenes groups, resonating at δ 1.96−1.48 (H_2-1) and 2.65 (H_2-2). Analogously, in the ^{13}C NMR spectrum (**Table 3**) two CH_2 signals were observed at δ 33.2 and 39.8.

Antifeedant Activity of Natural and Semisynthetic Limonoids. The antifeedant activities of the natural *Citrus* limonoids **1**−**3** and their semisynthetic derivatives **4**−**26** at 100 ppm are summarized in **Table 4**. The natural limonoids showed potent antifeedant activity, with limonin (**1**) being the most active against *S. frugiperda*. This contrasts with the data for *S. frugiperda* of Champagne et al. (*1*), who reported the ED_{50} values for limonin, nomilin, and obacunone as 756, 72, and 70 ppm, respectively. However, the results are not directly comparable, as we have used an inert substrate treated with sucrose as a test compound to evaluate the activity of the compounds, whereas other authors have applied or incorporated the compounds into insect food. Our short-term assay was designed to evaluate whether plant-derived compounds play a primary role in modulating feeding behavior. Activity in these assays often correlates with neural input from gustatory sensilla. When compounds are incorporated into food, the behavior output could be due to primary activity or via some secondary feedback associated with the toxicity of the test compound.

In our experiments, limonol (**4**) was active but less so than limonin, whereas limonol decreased the consumption and growth rates of *L. decemlineata* more than limonin (*24*). In fact, Liu and co-workers (*25*) showed that the derivatives of limonin with a more polar function at either C-7 or C-6 depressed feeding of *L. decemlineata*. In addition, we found that limonol (**4**) was more active toward *S. frugiperda* than its epimer *epi*-limonol (**5**), whereas *epi*-limonol has been reported to be more active than limonol against *L. decemlineata* (*21*). Limonin-7-oxime (**8**) was as active as limonol, whereas acetates **6**, **7**, and **10** are less active than the parent compounds. Interestingly, limonin-7-methoxime (**9**) shows the highest activity (FI = 76%) among all the semisynthetic derivatives tested. Both the epimers **11** and **12**, with hydrogenation of the furan moiety, have antifeedant activity against *S. frugiperda*. This is in contrast to data reported for *L. decemlineata*, that showed that perhydro derivatives are devoid of any antifeedant activity (*21*).

Nomilin (**2**) displays the lowest activity among the three main natural *Citrus* limonoids (**Table 4**). The importance of the configuration and composition of the substituent at C-7 is unclear. The reduction of the C-7 carbonyl function did not always result in a decrease in activity, as 7-nomilol (**13**) and 7-nomilyl acetate (**14**) are as potent as nomilin, whereas the other derivatives are less active. It appears that, with nomilin, modification of the functional group at C-7 did not influence the antifeedant activity of the compound as much as occurred with the derivatives of either limonin (**1**) or obacunone (**3**).

Obacunone (**3**) shows significant antifeedant activity toward *S. frugiperda*, but all the derivatives were less active (**Table 4**). Reduction of the C-7 carbonyl function caused a decrease in activity, and the configuration of the hydroxy group at C-7 influenced activity, as the 7α epimer (**19**) was more active (FI = 53%) than the 7β (**20**) (FI = 35%). Acetylation of the polar function at C-7 in **21**, **22**, and **24** resulted in a loss of activity. Interestingly, the reduction in activity associated with hydrogenation of the furan ring is not observed in the partially hydrogenated 1,2-didehydroobacunone (**26**), that is as active as 7α-obacunol (**19**).

These data represent the results from the first homogeneous structure−activity study into the antifeedant activity of the three main *Citrus* limonoids, **1**−**3**, and their semisynthetic derivatives. Limonin (**1**), nomilin (**2**), and obacunone (**3**) displayed antifeedant activity against *S. frugiperda*, thus confirming their probable role as chemical defense agents in *Citrus*−herbivore interactions. In comparison with previously reported data, these results show that different insect species may have different responses to structural modifications (*1*, *21*, *25*), in which case structure−activity relations may be highly species-specific. Thus, the assessment that the introduction of polar groups in the limonoid skeleton at C-7 would result in an enhancement of antifeedant activity was not confirmed for *S. frugiperda*, as indicated for instance by comparison of FI values for limonin (**1**), limonin-7-oxime (**8**), and limonin-7-methoxime (**9**). The last one was the most active semisynthetic compound.

In conclusion, we obtained significant antifeedant activity ($P < 0.01$) for two natural (**1** and **3**) and three semisynthetic limonoids (**4**, **8**, and **9**) against the commercially important pest, *S. frugiperda*. Although the antifeedant activity of natural *Citrus* limonoids could not be enhanced through chemical modification, the derivative might exhibit potent antifeedant activity against other pests. The limonoids justify further exploitation as pest

control agents, especially as they are readily available as waste products of the *Citrus* industry.

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Supporting Information Available: Summary list of natural *Citrus* limonoids and their semisynthetic derivatives; NMR data of **5**, **7**, **10**, **13**, **14**, **15**, **17**, **20**, **22**, **24**; ROESY data of **11** and **12**; energy profile as a function of torsion angle ($H_{17}-C_{17}-C_{20}-H_{20}$); and stereoviews of the lowest-energy conformers for **11** and **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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