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NEW INSECT ECDYSIS INHIBITORY LIMONOID DEACETYLAZADIRACHTINOL ISOLATED FROM AZADIRACHTA INDICA (MELIACEAE) OIL

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Abstract - Insect bioassay-guided fractionation of neem oil expressed from the fresh fruits of Azadirachta indica (Meliaceae) resulted in the isolation and structural elucidation of a new limonoid, deacetylazadirachtinol (1). In addition, four previously elucidated limonoids, including azadirachtin (2), salannin (3), 6-O-acetylnimbandiol (4), and 3-desacetylsalannin (5), were isolated from the same source, spectrally identified, and bioassayed. The structure of 1 was elucidated by spectral comparison with the known azadirachtin using their ^{1}H-^{1}H and ^{1}H-^{13}C 2D NMR spectra to assign the total ^{1}H and ^{13}C chemical shifts. Deacetylazadirachtinol was found to be as potent as azadirachtin in the inhibition of insect ecdysis when fed in artificial diet to larvae of the tobacco budworm, Heliothis virescens (Noctuidae). The other isolated limonoids were much less active against H. virescens.

Indian neem Azadirachta indica (Meliaceae) is a fast growing sturdy tree found widely in India, Pakistan and parts of Africa, and scattered in other parts of the world (e.g., Haiti)[1]. Almost every part of the neem tree, including its roots, leaves, and fruits, offers great potential for agricultural, industrial and commercial exploitation.[2] For instance, the seed oil has commercial possibilities for lighting and heating and in the manufacture of wax, lubricants and soap. In addition, neem oil holds great promise for use in insecticidal formulations because of its potent antifeedant and ecdysis (molting) inhibitory activities against pest insects.[3]

The major active constituent in neem is azadirachtin (2, see Figure 1), one of the strongest insect antifeedant and ecdysis inhibitory compounds known from a botanical source.[4] Its structure was established, mainly based on spectroscopic data, to be a complex limonoid.[5] Although its structural complexity has precluded its economical synthesis, its potent and specific effects against insects may establish it as a model compound for synthetic insecticide research.

In an ongoing program to correlate the structure of azadirachtin with its activity, we have isolated, identified, and bioassayed related limonoids from neem and chinaberry (Melia azedarach). In the present paper, we report on the isolation, structure elucidation, and biological activity of a new limonoid, deacetylazadirachtinol (1).

RESULTS AND DISCUSSION

Neem oil from the fresh fruits of A. indica collected in Haiti was partitioned into n-hexane and methanol soluble portions. Monitoring with an artificial diet feeding bioassay[6] using

agricultural pest insects, Heliothis virescens (Noctuidae) and Pectinophora gossypiella (Gelechiidae), the activity was found to reside in the methanolic portion. Subsequent fractionation of the methanolic portion on silica gel (petroleum ether/ether) was monitored by the insect bioassays. The most active fraction was further chromatographed on C_{18} reversed phase columns (methanol/water) to give five active compounds, 1 (5 mg), 2 (8 mg), 3 (10 mg), 4 (28 mg) and 5 (55 mg).

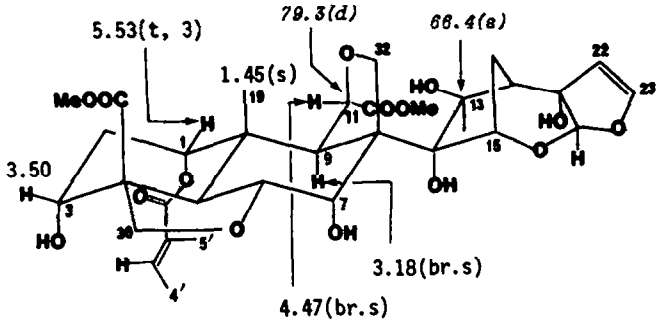
Compounds 2 (mp 156°, $[\alpha]_D$ -46°, MW 720), 3 (mp 168°, $[\alpha]_D$ +154°, MW 596), 4 (mp 178°, $[\alpha]_D$ +232°, MW 498) and 5 (mp 207°, $[\alpha]_D$ + 54°, MW 554) were identified to be the known limonoids, azadirachtin (2),[5,7] salannin (3),[8] 6-O-acetylnimbandiol (4)[9] and 3-desacetylsalannin (5)[8], respectively, by comparison of their physical and spectral data (mp, $[\alpha]_D$, MS, ^1H NMR) with published data (Figure 2).

Compound 1 (mp 148°) was unknown in the literature. The IR spectrum ($CHCl_3$) of 1 showed the presence of hydroxyl (ν 3440), ester (ν 1730, 1720, 1700), double bond (ν 1690, 1650) and ether (ν 1260, 1230, 1075) groups, and the UV spectrum (EtOH) showed a peak at 222 nm due to the $\pi-\pi^*$ transition of the α,β-unsaturated carbonyl group. The molecular formula of 1 was ascertained to be $C_{33}H_{44}O_{15}$ based on the 44 protons and 33 carbons observed in the ^1H and ^{13}C NMR spectra, respectively, and also on an ion peak at m/z 645 which seemed to be caused by the fragment $[M-2H_2O + H]^+$ in the SIMS.

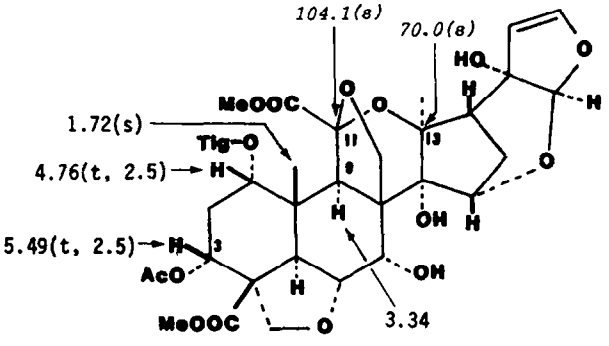
Table 1. ^1H and ^{13}C NMR data of deacetylazadirachtinol (1) and azadirachtin (2) in $CDCl_3$.

^1H	1	2	^{13}C	1	2
1-H	5.53(t,3)	4.76(t,2.5)	C-1	67.5(d)	70.51(d)
2-H	2.30(ddd,3,3,16.5)	2.30(m)	C-2	31.9(t)	29.37(t)
	2.06(m)	-	C-3	69.2(d)	66.99(d)
3-H	3.50	5.49(t,2.5)	C-4	43.9(s)	45.41(s)
5-H	3.31(d,12.5)	3.35(d,12)	C-5	35.0(d)	37.06(d)
6-H	4.57(dd,3,12.5)	4.58(dd,3,12)	C-6	76.0(d)	74.37(d)
7-H	4.70(d,3)	4.62(d,3)	C-7	73.5(d)	76.43(d)
9-H	3.18(br.s)	3.34	C-8	51.1(s)	50.19(s)
11-H	4.47(br.s)	-	C-9	43.6(d)	44.69(d)
15-H	4.61(d,3.5)	4.70(d,2.5)	C-10	53.2(s)	52.52(s)
16-H	1.68(ddd,3.5,5.5,13)	1.7	C-11	79.3(d)	104.1(s)
	1.33(d,13)	1.31(d,12)	C-12	173.3(s)*	171.1(s)*
17-H	2.34(d,5.5)	2.38(d,6.0)	C-13	66.4(s)	69.95(s)
18-H	2.04(s)	2.06(s)	C-14	69.4(s)	68.53(s)
19-H	1.45(s)	1.72(s)	C-15	74.2(d)	73.79(d)
21-H	5.66(s)	5.64(s)	C-16	24.9(t)	25.06(t)
22-H	5.05(d,3)	5.05(d,2.5)	C-17	48.8(d)	48.67(d)
23-H	6.45(d,3)	6.42(d,2.5)	C-18	18.1(q)	20.88(q)
30-H	3.82(d,9)	3.75	C-19	21.2(q)	18.40(q)
	4.05(d,9)	4.05	C-20	83.5(s)	83.55(s)
32-H	3.47(d,9.5)	3.65	C-21	109.0(d)	107.3(d)
	3.94(d,9.5)	4.16	C-22	107.4(d)	108.7(d)
3'-H	6.94(qq,7,1.5)	6.85(m,6)	C-23	146.6(d)	147.0(d)
4'-H	1.80(dq,7,1)	1.78(d,6)	C-30	73.2(t)	72.99(t)
5'-H	1.84(dq,1.5,1)	1.84	C-31	173.9(s)*	173.2(s)*
OAc	-	1.92(s)	C-32	71.3(t)	69.07(t)
OMe	3.74(s)	3.76(s)	C-1'	166.9(s)	166.1(s)
	3.74(s)	3.65(s)	C-2'	128.3(s)	128.6(s)
			C-3'	138.7(d)	137.5(d)
			C-4'	14.5(q)	14.29(q)
			C-5'	11.9(q)	11.94(q)
			OMe	52.5(q)	52.72(q)
				52.9(q)	53.52(q)
			OAc	-	21.33(q)
				-	169.5(s)

*Assignments could be reversed.

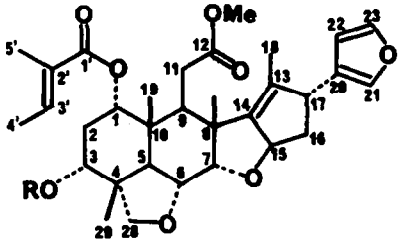


Deacetylazadirachtinol (1)

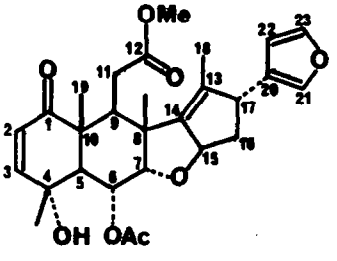


Azadirachtin (2)

Figure 1. Structures of deacetylazadirachtinol (1) and azadirachtin (2) and their partial ^{1}H and ^{13}C NMR assignment in $CDCl_3$ (^{13}C NMR assignments are in italics).



R = Ac, Salannin (3)
R = H, 3-Desacetylsalannin (5)



6-O-Acetylnimbandiol (4)

Figure 2. Structures of salannin (3), 6-O-acetylnimbandiol (4) and 3-desacetylsalannin (5).

The ^{13}C NMR spectrum of 1 suggests that 1 was closely related to azadirachtin (Table 1). In particular, the carbon signals of C(4)-C(10), C(14)-C(17) and C(20)-C(32) of azadirachtin agreed well with the corresponding signals of 1 such that the carbon skeleton of 1 was elucidated to be the same as that for azadirachtin. Furthermore, the ^{1}H-^{1}H (Figure 3) and the ^{1}H-^{13}C (Figure 4) 2D NMR spectra[10] showed that 1 also had one tigloyl group and two methoxycarbonyl groups, the same as for azadirachtin.

However, in the ^{13}C NMR of 1, the signal corresponding to the C-11 ketal carbon (104.1 ppm) was not observed, but a tertiary carbon signal appeared at 79.3 ppm. In the ^{1}H-^{13}C 2D NMR spectrum of 1, this carbon signal was coupled with the 4.47 ppm proton signal, which also showed the small vicinal coupling with the 9-H signal at 3.18 ppm in the ^{1}H-^{1}H 2D NMR spectrum. Therefore, the 79.3 ppm ^{13}C NMR signal was assigned to the 11-C. The small ^{1}H NMR coupling between the 9-H and the 11-H ($wh/2$ = 4 Hz) indicated the dihedral angle of H-C(9)-C(11)-H was approximately 90°. This indicated that the configuration of the methoxycarbonyl group attached to the 11-C was opposite to that of azadirachtin.

Also, the ^{13}C NMR chemical shift of the 13-C (66.4 ppm) was different from that of azadirachtin (69.95 ppm). This significant difference indicated that 1 had a structure in which the C(11)-O-C(13) ether linkage was reductively cleaved at the 11 position of azadirachtin. Since the 18-methyl protons showed a lower shifted ^{1}H NMR signal (2.04 ppm) in 1 by a through space effect of the 20α hydroxyl group, it was clear that the 13-C configuration of 1 was the same as that for azadirachtin. The conformation of the dihydrofuran ring of 1 was assigned β based on azadirachtin.

Table 2. Growth and ecdysis inhibitory effects of neem oil limonoids fed in artificial diet to first instar larvae of Heliothis virescens.

Test Compound	EC_{50} [a] (ppm)	LC_{50} [b] (ppm)	95% [c] Confidence Limits
Deacetylazadirachtinol (1)	0.17		0.12-0.23
		0.80	0.66-0.97
Azadirachtin (2)	0.07		0.05-0.10
		0.80	0.46-1.39
6-O-Acetylnimbandiol (4)	4.4		2.8-7.0
		21.0	15.2-29.0
3-Desacetylsalannin (5)	170	d	113-257
Salannin (3)	170	e	138-210

[a] EC_{50} is the effective concentration in ppm of additive necessary to reduce larval growth to 50% of the control values.

[b] LC_{50} is the lethal concentration in ppm of additive necessary to kill (usually by inhibiting ecdysis, the final stage of molting) 50% of the treated insects.

[c] The 95% confidence limits were determined using the method of Litchfield and Wilcoxon[13].

[d] No mortality was detected up to 200 ppm, the highest concentration tested.

[e] No mortality was detected up to 400 ppm, the highest concentration tested.

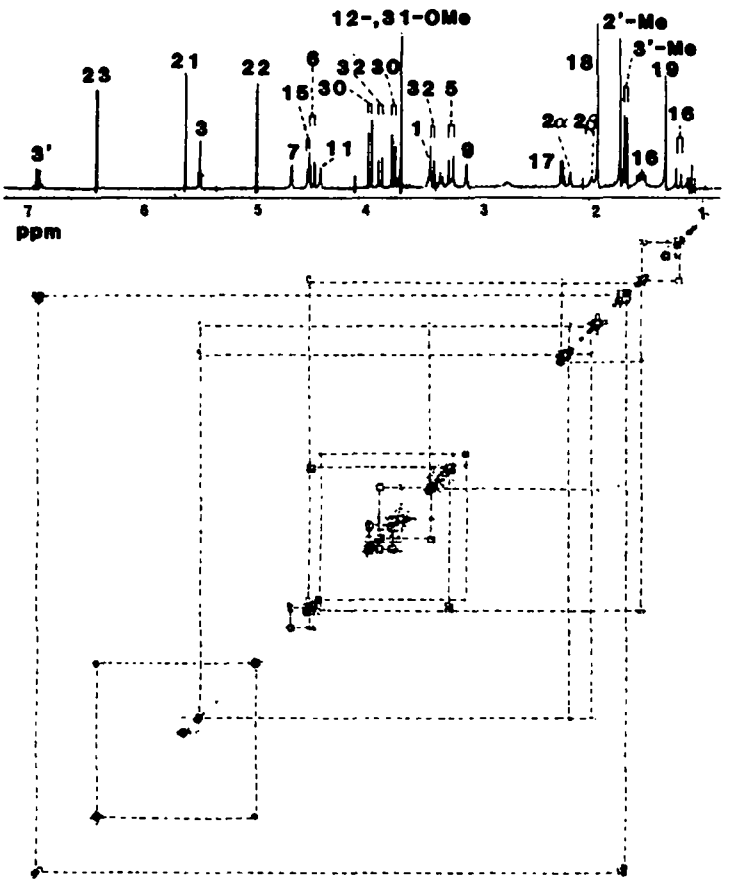


Figure 3. ^{1}H-^{1}H 2D NMR spectrum of deacetylazadirachtinol (1) in $CDCl_3$.

The lack of ^{1}H and ^{13}C NMR signals in 1 corresponding to the acetoxyl group in azadirachtin, the one appearance of the ^{1}H NMR signal assigned to the proton vicinal to an ester group (5.53 ppm), and the ^{1}H NMR signal at 3.50 ppm together indicated that 1 contained a 3α hydroxyl group and a 1α tigloyl group by comparison with azadirachtin. The significant low field shift of the 1β proton (5.53 ppm) compared to that of azadirachtin (4.76 ppm) was consistent with the removal of the anisotropic effect caused by the 11 methoxycarbonyl group. The 19 methyl proton signal shifting upfield (1.45 ppm) compared to that of azadirachtin (1.72 ppm) can also be explained by the difference of the configuration at the 11 position.

The structure of the new limonoid was thus defined as shown in Figure 1 and named deacetylazadirachtinol. The ^{1}H and ^{13}C NMR assignments of deacetylazadirachtinol were greatly facilitated by the ^{1}H-^{1}H and ^{1}H-^{13}C 2D NMR spectra. The carbon sequence, C(1)-C(3), C(5)-C(7), C(9)-C(11), C(15)-C(17) and C(22)-C(23), corresponding to the continuous proton systems, were readily established by the ^{1}H-^{13}C 2D NMR spectrum. Especially, the unequivocal distinction between the carbon signals, C(1)/C(3), C(18)/C(19), C(21)/C(22), C(30)/C(32) and C(4')/C(5'), had been achieved without the aid of any other combined NMR techniques. For example, the 4' methyl signal at 1.80 ppm (dq, J=7 Hz, 1 Hz) and the 5' methyl signal at 1.84 ppm (dq, J=1 Hz, 1.5 Hz) were observed to correlate with the carbon resonances at 14.5 and 11.9 ppm respectively, thus providing an unequivocal assignment of the two vinyl methyl signals. In a similar manner, the

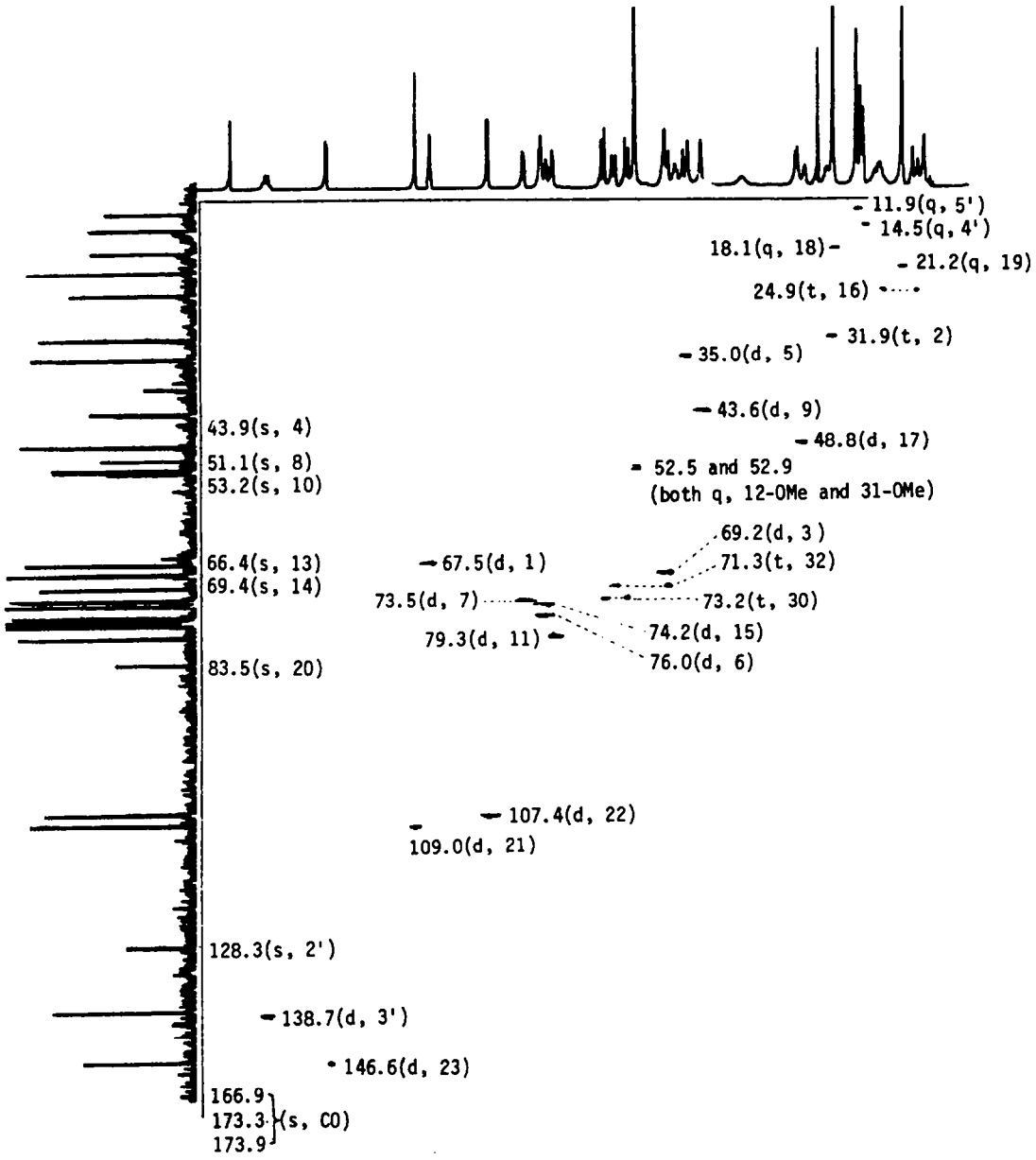


Figure 4. ^{1}H-^{13}C 2D NMR spectrum of deacetylazadirachtinol (1) in $CDCl_3$.

assignment of the two methylene carbon signals at C(30) and C(32) was also achieved, confirming the uncertain assignment made previously. The total assignments of ^{1}H and ^{13}C NMR signals in deacetylazadirachtinol is listed in Table 1.

The biological activity of deacetylazadirachtinol (1) fed in artificial diet to H. virescens is shown in Table 2 together with the activity of the other limonoids isolated from neem. Although deacetylazadirachtinol (1) was about 2.5 fold less active than was azadirachtin (2) as an insect growth inhibitor (EC_{50} = 0.17 and 0.07 ppm, respectively), the two compounds had the same insecticidal activity (LC_{50} = 0.80 ppm). Thus, both compounds inhibited the final stage of the insect molting process (ecdysis), resulting in death, at the same dietary concentration.

The other limonoids tested were less active. For example, 6-O-acetylnimbandiol (4) was more than 60-fold and 25-fold less active as an insect growth inhibitor and insecticide, respectively, than was azadirachtin. Nevertheless, 4, with an α,β-unsaturated ketone in the A-ring, was about 39-fold more active as an insect growth inhibitor than was 3-desacetylsalannin (5), which has a tiglate group and a hydroxyl group in the A-ring or was salannin (3), which is acetylated at the 3-position. Neither 5 nor 3 were lethal to the H. virescens larvae at the highest concentrations tested (200 ppm for 5, 400 ppm for 3). 4 did prove lethal to 50% of the tested insects (LC_{50}) at 21 ppm.

Comparison of the activity of 1 and 2 seems to indicate that the ketal in the C-ring of azadirachtin is not necessary for potent ecdysis inhibitory activity, at least against H. virescens. This may be in agreement with the observation of Dreyer[11] that many of the most potent of the insect feeding deterrent limonoids are either of the C-ring secotype or with features which allow C-ring opening.

When fed to H. virescens at lower dietary concentrations than those which caused mortality, 1 was found to be less active (2.5 fold) as a growth inhibitor than was 2. The reason for this is unknown, although the functionality (acetoxyl vs hydroxyl) at position 3 in the A-ring is probably not involved. For example, no difference in insect growth and ecdysis inhibitory activities was found with 3-deacetylazadirachtin when compared to those activities of azadirachtin. Similarly, no difference was found in the insect growth inhibitory activity of 3-deacetylsalannin (5) when compared to that activity of salannin (3) (Table 2).

Although the limonoids based on the salannin skeleton were much less active than those based on the azadirachtin skeleton, their activity seemed to be enhanced by the presence of an α,β-unsaturated ketone in the A-ring. For example, 4 was more potent than 3 or 5 as a growth inhibitor and insecticide. This is consistent with the observation that many of the most potent of the growth-inhibitory limonoids from the Rutaceae and Meliaceae have an α,β-unsaturated ketone or lactone, both potential alkylating centers, in the A-ring.[12]

The present results are useful in attempts to correlate the insect ecdysis inhibitory activity of azadirachtin with its structure. However, further research is needed before compounds can be designed which are less structurally complex than azadirachtin, yet retain its potent and specific insecticidal effect. One of our approaches to fully understand the structure-activity relationships of azadirachtin will be to continue to isolate (or, in some cases, generate) compounds closely related structurally to azadirachtin (e.g., deacetylazadirachtinol) in order to assess their relative activity against pest insects.

EXPERIMENTAL SECTION

General Methods. Melting points were determined using a Sybron Thermolyne MP-12615 and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 and 241 MC polarimeter. UV spectrum was recorded on a Hitachi 100-80 spectrophotometer and IR spectrum was on a Nicolet 7199 FT-IR. MS spectra were obtained on a Hitachi RMU 6-MG apparatus. CD spectrum was measured with a Jasco J-40 spectropolarimeter. ^{1}H–^{1}H and ^{1}H–^{13}C NMR spectra were determined on a Varian XL-400.

Materials. The neem oil (300 g), which was expressed from fresh fruits of A. indica collected in Haiti, was separated into n-hexane (291 g) and methanol (9 g) soluble fractions. The methanol fraction was chromatographed on a silica gel column (silica gel 60, E. Merck) using n-hexane/ether and petroleum ether/ether solvent systems, and then purified by C_{18} reversed-phase low pressure liquid chromatography (pump: Fluid Metering, Inc., column: SR 10/50, Pharmacia Fine Chemicals packed with Lichroprep RP-18, particle size 25-40 μm, E. Merck) using a H_2O–MeOH mixture solvent to give 1(5 mg), 2(8 mg), 3(10 mg), 4(28 mg) and 5(55 mg).

Deacetylazadirachtinol (1). Mp 148° C; CD (MeOH) Δε-4.6(237 nm) UV (EtOH) 222 nm (ε 20000); IR ($CHCl_3$) 3440, 1730, 1720, 1700, 1690, 1650, 1260, 1230 and 1075 cm^{-1}; ^{1}H NMR ($CDCl_3$) is listed in Table 1.; ^{13}C NMR ($CDCl_3$) is also listed in Table 1.; SI-MS, m/z 645 ($M-2H_2O+1$), 185.

Azadirachtin (2). Mp 156° C; $[\alpha]_D^{24}$ -45.5° (c 0.06, $CHCl_3$); ^1H NMR ($CDCl_3$) in Table 1; ^{13}C NMR ($CDCl_3$) in Table 1; EI-MS, m/z 702 ($M-H_2O$), 669, 642, 624.

Salannin (3). Mp 168° C; $[\alpha]_D^{24}$ +153.8° (c 0.08, $CHCl_3$); ^1H NMR ($CDCl_3$)δ0.98 (s, 3H, 19), 1.22 (s, 3H, 29), 1.30 (s, 3H, 30), 1.69 (s, 3H, 18), 1.81 (d, 3H, J=9 Hz, 4'), 1.95 (s, 6H, 5' and OAc), 2.10-2.35 (m, 6H, 2α,β, 11_{ab}, 16_{ab}), 2.75 (dd, 1H, J=6 Hz, 3 Hz, 9), 2.81 (d, 1H, J=12 Hz, 5), 3.24 (s, 3H, OMe), 3.58 (d, 1H, J=7 Hz, 28_a), 3.60 (br.d, 1H, J=9 Hz, 17), 3.70 (d, 1H, J=7 Hz, 28_b), 3.98 (dd, 1H, J=12 Hz, 3 Hz, 6), 4.19 (br.s, 1H, wh/2=6 Hz, 7), 4.79 (br.s, 1H, wh/2=9 Hz, 3), 4.97 (br.s, 1H, wh/2=9 Hz, 1), 5.45 (t-like, 1H, J=9 Hz, 15), 6.30 (br.s, 1H, wh/2=6 Hz, 22), 6.96 (q, 1H, J=9 Hz, 3'), 7.27 (br.s, 1H, wh/2=6 Hz, 23), 7.32 (br.s, 1H, wh/2=6 Hz, 21); SI-MS, m/z 597 (M+1), 565, 515, 495, 479, 437, 419, 259, 185, 147, 119.

6-O-acetylnimbandiol (4). Mp 178.0° C; $[\alpha]_D^{24}$ +232.3° (c 0.03, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.23 (s, 3H, 19), 1.35 (s, 3H, 30), 1.37 (s, 3H, 29), 1.67 (s, 3H, 18), 2.00-2.08 (m, 1H, 16_a), 2.20 (s, 3H, OAc), 2.18-2.23 (m, 2H, 16_b, 11_a), 2.77 (t, 1H, J=5 Hz, 9), 2.91 (dd, J=5 Hz, 17 Hz, 11_b), 3.07 (d, 1H, J=13 Hz, 5), 3.27 (s, 1H, OH), 3.62 (s, 3H, OMe), 3.64 (br.d, 1H, J=7 Hz, 17), 4.07 (d, 1H, J=3 Hz, 7), 5.44 (dd, 1H, J=13 Hz, 3 Hz, 6), 5.53 (br. t, J=7 Hz, 15), 5.78 (d, J=10 Hz, 2), 6.30 (br. s, wh/2=3 Hz, 22), 6.47 (d, J=10 Hz, 3), 7.21 (br. s, 1H, wh/2=3 Hz, 23), 7.30 (br. s, 1H, wh/2=4 Hz, 21); SI-MS, m/z 499 (M+1), 467, 407, 369, 277, 185.

3-Desacetylsalannin (5). Mp 207.0° C; $[\alpha]_D^{24}$ +54.2° (c 0.05, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.97 (s, 3H, 19), 1.18 (s, 3H, 29), 1.30 (s, 3H, 30), 1.64 (s, 3H, 18), 1.85 (d, 3H, J=7 Hz, 4'), 1.92 (s, 3H, 5'), 2.08 (ddd, 1H, J=3 Hz, 3 Hz, 16 Hz, 2β), 2.12-2.38 (m, 5H, 2α, 11_{ab}, 16_{ab}), 2.42 (d, 1H, J=10 Hz, OH), 2.64 (dd, 1H, J=10 Hz, 3 Hz, 9), 2.73 (d, 1H, J=13 Hz, 5), 3.20 (s, 3H, OMe), 3.62 (br.d, 1H, J=8 Hz, 17), 3.65 (d, 1H, J=7 Hz, 28_a), 3.88 (ddd, 1H, J=3 Hz, 3 Hz, 10 Hz, 3), 4.01 (dd, 1H, J=4 Hz, 13 Hz, 6), 4.13 (d, 1H, J=7 Hz, 28_b), 4.18 (d, 1H, J=4 Hz, 7), 5.02 (t, 1H, J=3 Hz, 1), 5.39 (br. t, 1H, J=7 Hz, 15), 6.27 (br. s, 1H, wh/2=3 Hz, 22), 6.91 (qd, 1H, J=7 Hz, 1 Hz, 3'), 7.25 (br. s, 1H, wh/2=3 Hz, 23), 7.31 (s, 1H, 21); EI-MS, m/z 554 (M^+), 522, 471, 421, 283, 173, 83, 55.

Bioassays. As previously described,[6] an artificial diet feeding bioassay with newly-hatched larvae of the tobacco budworm, H. virescens, and the pink bollworm, P. gossypiella, was used to detect insect growth and ecdysis inhibitory activities in the plant extracts and to monitor the fractionation of the extracts. The same bioassay (with H. virescens only) was used to determine the potency of the isolated constituents. Potency was determined either as the effective concentration (EC_{50}) of additive necessary to reduce larval growth to 50% of the control values or as the lethal concentration (LC_{50}) of additive necessary to kill (usually by inhibiting ecdysis, the final stage of molting) 50% of the treated insects. EC_{50} and LC_{50} values were determined from log probit paper and analyzed statistically by the method of Litchfield and Wilcoxon[13].

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