Insect Antifeedant Isoryanodane Diterpenes from *Persea indica*

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Three new diterpenes with the rare isoryanodane skeleton, indicol (**7**), vignaticol (**8**), and perseanol (**9**), have been isolated from *Persea indica* (Lauraceae). These compounds proved to be antifeedants against *Spodoptera litura*, perseanol being the most active.

Ryanodine (**1**) is the main representative of the ryanodane class of diterpenes. In previous works we have reported on the isolation of five diterpenes of this type, ryanodol (**2**), ryanodol 14-monoacetate (**3**), cinnzeylanol (**4**), cinnzeylanone (**5**), and *epi*cinnzeylanol (**6**) from the Canarian endemic species *Persea indica* (L.) Spreng (Lauraceae), their ecological relation with the wild rats, the insecticidal effects on *Macaronesia fortunata* and *Heliothis armigera*,[1−3] and the antifeedant activity against the insect pest *Spodoptera litura*.[4] Three avocadofurans and an alkene-γ-lactone have also been obtained from *P. indica*.[5] In this work we communicate the isolation and the structure determination of three novel diterpenes from this plant. These compounds, which have been named indicol (**7**), vignaticol (**8**), and perseanol (**9**), possess the rare isoryanodane skeleton and show antifeedant activity against *S. litura*.

Results and Discussion

The structure **7** has been assigned to indicol on the basis of the following considerations. Its molecular formula, determined by HRMS, is $C_{20}H_{32}O_4$. Three of the four oxygens of the molecule are joined to quaternary carbons. Only one proton geminal to an oxygen function was observed, doublet at δ 3.82 (2.3 Hz), in the ^{1}H-NMR spectrum. Two angular methyls (δ 1.01 and 1.14) and three secondary methyls, of which two were in an isopropyl group at δ 0.94 (6H, d) and one at δ 1.08 (3H, d), also appeared in this spectrum.

The ^{13}C-NMR spectrum was very informative, showing signals of five methyls, four methylenes, six methines, and five quaternary carbons. One methine at δ 75.8 was joined to an oxygenated function. Of the quaternary carbons, two bore one oxygen atom (δ 87.6 and 89.4), and one of them bore two oxygens (hemiacetalic function at δ 106.7). All these facts led us to consider that compound **7** possesses an isoryanodane skeleton similar to that of cinncassiol D_1 (**10**), which had been isolated from *Cinnamomum cassia*.[6]

The chemical shifts of the carbon given in Table 1 are in accordance with the structure **7**. The assignments have been made using 2D NMR methods, such as COSY, HMQC, and HMBC. The connectivities observed in the HMBC experiment are as follows: H-19 (or H-20) [C-18, C-13]; H-17 [C-13, C-12, C-11, C-7]; H-16 [C-10, C-5, C-8]; H-15 [C-3, C-2, C-1]; H-6 [C-5, C-7]. With respect to ryanodol (**2**) or cinnzeylanol (**4**), in the ^{13}C-NMR spectrum of **7** the C-18 appears at a lower field, δ 28.6, and the two methyls of the isopropyl group at a higher field, δ 21.9 and 23.7, indicating the absence in this compound of a hydroxyl group at C-13. On the other hand, the carbon resonances point to different B and C rings, which previously had been deduced from the observed coupling constant between H-1 and H-6 in the ^{1}H-NMR spectrum.

Table 1. ^{13}C-NMR Data[a] of **7**−**9**

C	7	8	9
1	53.2	52.0	81.6
2	35.7	35.9	46.9
3	34.4	34.0	30.0
4	37.5	38.2	37.4
5	87.6	88.2	83.0
6	75.8	74.4	77.6
7	89.4	87.4	89.0
8	47.9	82.0	85.1
9	41.7	42.4	44.7
10	41.8	44.2	45.5
11	106.7	103.1	104.3
12	56.5	58.7	62.8
13	45.8	81.0	82.1
14	26.0	51.0	52.6
15	15.1	14.8	18.5
16	18.5	17.8	12.6
17	8.2	9.6	10.7
18	28.6	34.0	35.2
19	21.9	18.5	18.9
20	23.7	18.6	18.9

[a] Chemical shifts are in ppm and were measured in $CDCl_3$ at 100.6 MHz.

The low chemical shift of C-14 (δ 26.0) and the form of resonance of the two H-14, multiplets, observed in the ^{1}H-NMR spectrum, indicated the existence of tertiary hydrogens at C-8 and C-13. The β-stereochemistry assigned to these protons was based on the 2D NMR ROESY spectrum, which showed NOE effects between H-16 and H-8, H-8 and H-14, and H-14 and H-13. The spatial disposition of these centers is the same as that of cinncassiol D_1 (**10**), the structure of which had been resolved by X-ray analysis.[6] The stereochemistry assigned to H-1 in **7**, also β, was assigned in accordance with the coupling shown with H-6 (J = 2.3 Hz) and the NOE observed between these two protons. This last technique also permitted us to distinguish between the resonance of H-4(α) (δ 2.27, m) and H-4(β) (δ 1.50 m), because the former showed a NOE effect with H-10. The relatively high chemical shift of H-4(α) confirmed the position and the β-stereochemistry of the 5-hydroxyl group, which firstly we assigned by ^{13}C-NMR data.

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Another new diterpene was vignaticol (**8**). Its HRMS does not show the molecular ion, but one at 332.2004, formed by loss of two molecules of water, corresponding to the formula $C_{20}H_{28}O_4$. Another observed fragment appears at *m/z* 325.1654 ($C_{17}H_{25}O_6$), formed from the molecular ion by loss of the isopropyl group. Thus, **8** possesses the molecular formula $C_{20}H_{32}O_6$.

The ^{1}H-NMR spectrum of **8** was very similar to that of **7**, signals appearing of the two angular methyls, the isopropyl group, the methyl at C-2, and the hydrogen geminal to the oxygen at C-6. This last proton shows the same chemical shift and the same coupling constant as in **7**, indicating that the molecular environment around C-6 must be the same.

Taking into consideration the ^{13}C-NMR spectrum (Table 1) and the molecular formula, it was deduced that vignaticol (**8**) differs from indicol (**7**) in that, in the latter, two tertiary hydrogens are replaced by two hydroxyl groups. One of these is the H-13, because in **8** the C-18 resonates at a higher field (δ 34.0) than in **7**. This value is similar to that of the ryanodane diterpenes hydroxylated at C-13.[4] This last carbon appears at δ 81.0. The presence of the 13-OH also explains the facile allylic C-13,C-18 cleavage of the isopropyl group observed in the MS.

A study of the HMQC and HMBC 2D NMR spectra was in accordance with the structure given and has permitted the assignment of the carbon resonances of **8** (Table 1), with the exception of the values of the methyls of the isopropyl group, which can be interchanged. The connectivities observed in the HMBC spectrum were: H-19 (or H-20) [C-18, C-13]; H-17 [C-12, C-7, C-11, C-13]; H-16 [C-9, C-8, C-7, C-5]; H-15 [C-2, C-1]; H-14 [C-18, C-9, C-13]; H-10 [C-16, C-9, C-5].

Comparison of the ^{13}C-NMR spectra of **7** and **8** showed that the substituents of the five-membered ring C were identical in both compounds. Thus, the second hydrogen that has been substituted in **7** by another hydroxyl must be H-8. Now, in the ^{1}H-NMR spectrum of **8**, the two H-14 appear as a clean pair of doublets at δ 1.94 and 2.59 (J = 16 Hz). The first was assigned to the 14α and the second to the 14β, considering that a NOE effect was observed between H-14α and the hydrogens of the C-16 methyl group. The β-stereochemistry was assigned to the hydroxyl groups at C-8 and C-13 in **8**, as in cinnzeylanol (**4**),[4] by comparison of the ^{1}H- and ^{13}C-NMR spectra of both substances.

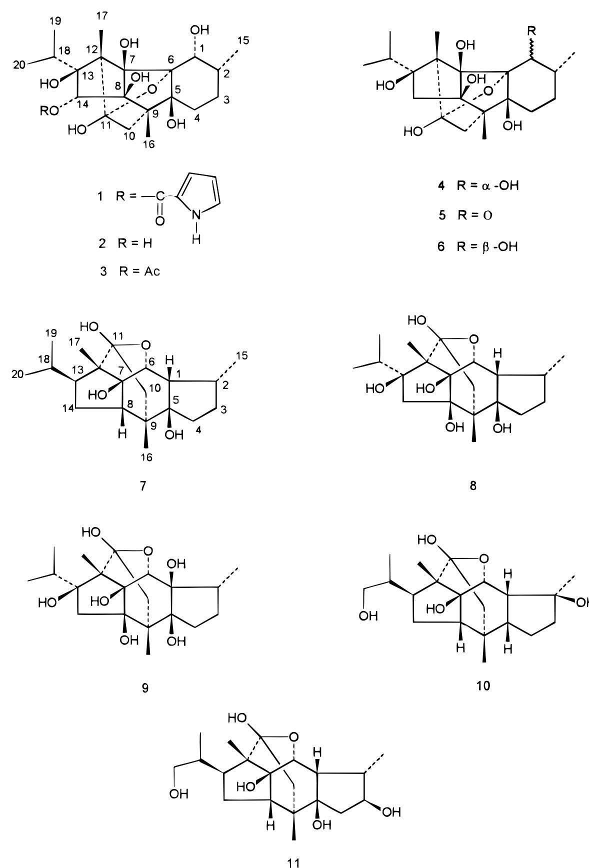
The most polar of the diterpenes with the isoryanodane skeleton isolated from *P. indica* was perseanol (**9**). Its MS showed the molecular ion at *m/z* 384, corresponding to a molecular formula of $C_{20}H_{32}O_7$. Other peaks observed in its MS spectrum were at *m/z* 366 and 341, which are originated from the molecular ion by loss of H_2O and of the isopropyl group, respectively. Then, each gave a fragment at *m/z* 323 by loss of the isopropyl group and H_2O, respectively.

Its ^{1}H-NMR spectrum was in accordance with the assigned structure. Thus, the two methyls of the isopropyl group appear at δ 0.88 and 0.94 as two doublets. Another doublet at δ 1.01 corresponds to the signal of the C-15 methyl. The two angular C-16 and C-17 methyls resonate at δ 1.04 and, 1.20 respectively. Both the H-10 and H-14 appear in this spectrum as a pair of doublets, the first at δ 1.62 and 1.87, and the second at δ 1.89 and 2.50, both with J = 16 Hz. The H-18 and H-4(α) are multiplets at δ 1.84 and 2.14, and the H-6 a singlet at δ 3.73.

The ^{13}C-NMR spectrum of **9** (Table 1) was also assigned utilizing HMQC and HMBC experiments. Differences in the chemical shift of the C-1, C-2, C-3, C-5, C-6, and C-15 resonances were observed in comparison with those of **8**, due to the introduction of a new hydroxyl group at C-1. The stereochemistry of this OH group has been given as β considering that there are no differences between the chemical shifts of H-6 in the ^{1}H-NMR spectra of **9** and **8**.

The correlations observed in the HMBC spectrum of **9** were: H-20 (or H-19) [C-18,C-13]; H-18 [C-19,C-20]; H-17 [C-12, C-7, C-13, C-11]; H-16 [C-9, C-5, C-8]; H-15 [C-3, C-2, C-1]; H-14 [C-18, C-9, C-8, C-12]; H-10 [C-9, C-8, C-12, C-11]; H-6 [C-5, C-7, C-8, C-11].

The stereochemistry of the methyl at C-2 in **7** and **8** was assigned as α. Thus, in **7** and **8** the resonances of H-1 appear at δ 1.99 and 2.00 as broad doublets, with a coupling constant of 8.2 and 8.0 Hz, respectively. The calculated coupling constant of H-1 with H-2(β), for the minimum energy conformations, was 8.9 Hz for both diterpenes. By analogy with these compounds, the stereochemistry of the C-15 methyl in **9** was also assigned as α. On the other hand, this also indicated that the stereochemistry of this methyl in cincassiol D_3 (**11**), which remained undetermined,[5] should also be α.



The assignments of the 13 NMR spectra of these new diterpenes (Table 1) have also revealed some mistakes in the resonance values given to cincassiol D_1 (**10**) and other related diterpenes.[6] Thus, C-10 and C-14 values must be interchanged, as well as those of C-17 and C-20. The absolute configuration of these isoryanodane diterpenes has not been determined, but it is considered to

Table 2. Yield and Feeding Indexes of the EtOH Extract and the Pure Isoryanodane Diterpenes from *P. indica* against *S. litura* in a Choice Leaf-Disk Bioassay

test substance (ppm)	yield (g dry wt.)	index (FI) AVG (SE)[a]
extract	23.0	0.00
indicol (**7**)	1.16×10^{-3}	49.39 (4.93)
vignaticol (**8**)	0.83×10^{-3}	12.57 (3.44)
perseanol (**9**)	9.40×10^{-3}	11.43 (2.94)

[a] Average (AVG) and standard error (SE). Each experiment (n = 5 larvae) was repeated a minimum of five times.

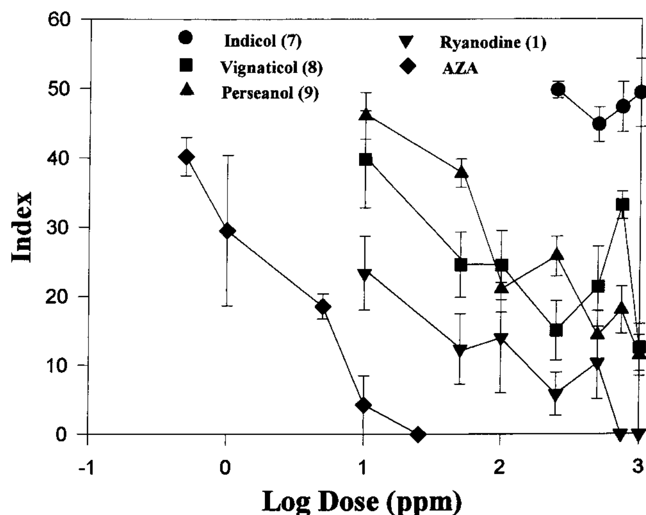


Figure 1. Dose–response curves of three isoryanodane diterpenes from *P. indica* against *S. litura* in a choice leaf-disk bioassay.

Table 3. Effective Doses and Structure–Activity Relationships of the Text Compounds

compound	log EC_{50} (ppm)[a]	95% limits (lower, upper)	substituents C-1	C-8	C-13
7	>3		H	H	H
8	1.97	(−0.79, 4.74)	H	OH	OH
9	2.23	(−0.33, 4.80)	OH	OH	OH
1[b]	1.15	(−1.61, 3.92)			
2[b]	2.18	(−0.18, 4.54)			
4[b]	1.93	(−0.41, 4.30)			
AZA[c]	0.25	(−0.08, 0.57)			

[a] Effective dose to give a 50% feeding reduction. [b] Taken from Fraga *et al.*[5] [c] Taken from Lajide *et al.*[11]

be the same as that in ryanodol,[7] which is also present in this plant.

Biosynthetically, the hydroxylation of **7** must first form **8** and subsequently **9**. The hydroxylations of C-1 and C-8 occur with retention of configuration, while that of C-13 takes place with inversion.

Continuing with the study of the strong antifeedant activity of the ryanodane diterpenes from *P. indica*,[4] we have evaluated the biological action of the isoryanodane diterpenes described here. Table 2 shows the yield and activity of the pure compounds tested at a dose of 1000 ppm. The most abundant one was **9**, followed by **7** and **8**. These compounds gave feeding index values of less than 20, with the exception of **7**.

The dose–response data (Figure 1, Table 3) indicate that **8** and **9** had a similar activity level. The effective doses of the ryanodane diterpenes ryanodol (**2**) and cinnzeylanol (**4**), the ryanodane diterpene alkaloid ryanodine (**1**), and the antifeedant limonoid azadirachtin (AZA) have been included for comparison purposes. The structure–activity relationships of the isoryanodane diterpenes studied showed that both C-8 and C-13 substituents play an important role in their antifeedant activity.

The effective dose range of the active isoryanodane diterpenes vignaticol (**8**) and (**9**) was similar to that of the major and minor ryanodane diterpenes isolated from *P. indica*, ryanodol (**2**) and cinnzeylanol (**4**), and cinnzeylanone (**5**).[4] The structure–activity relationships of the isoryanodane diterpenes studied here showed that the polarity of the pentacyclic A-ring (C-8 and C-13 substituent) determined their antifeedant activity. Another isoryanodane diterpene cincassiol D_1 (**10**) has shown anti-complement activity,[6] but this is the first report on insect antifeedant activity of isoryanodane diterpenes.

Based on the similarity of the ryanodine binding site between mammals and insects, and considering the selective toxicity of ryanodol against insects, Lehmberg and Casida[8] have proposed a different binding site for ryanodol. However, the similarity in the antifeedant effective dose range between active ryanodane and isoryanodane diterpenes and ryanodine, observed in our studies, suggests that both types of molecules could have a similar target-site in insects taste. Further research is needed in order to understand the mode of action of the isoryanodane diterpenes.

Experimental Section

General Experimental Procedures. Rotatory circular chromatographies were made in a Chromatotron from Harrison Research. ^1H- and ^{13}C-NMR spectra were recorded on Bruker WP200SY and AMX400 instruments. Mass spectra were obtained at 70 eV (probe) in a Hewlett-Packard 5995 and a Shimazu Q2000, and HRMS in a VG Micromass ZAB-2F. Conformations of minimum energy and calculated coupling constants were determined by computational methods employing the *Chem X* program of Chemical Design. Dry column chromatographies were made on Si gel Merck 0.02–0.063 mm. Ryanodine (**1**) was purchased from Sigma.

Plant Material. *P. indica* branches were collected at Monte de Las Mercedes, Tenerife, in March. Air-dried, chopped aerial parts (1.3 kg) were extracted with EtOH in a Soxhlet apparatus. The cold extract was filtered and then concentrated *in vacuo* to afford a syrupy gum (307 g). This syrup was treated with EtOAc and the solution separated by filtration. The solvent was evaporated, and the residue (138 g) was chromatographed on Si gel eluting with petroleum–EtOAc mixtures. In this way, after several rechromatographies in "dry column" mode and also using rotatory circular chromatography, the following diterpenes were obtained in polarity order: indicol (**7**) (7 mg), vignaticol (**8**) (5 mg), and perseanol (**9**) (57 mg).

Isolation and extraction of indicol (7): $[M]^+$ at m/z 336.2305; $C_{20}H_{32}O_4$ requires 336.2300; ^1H NMR (400 MHz, $CDCl_3$) δ 0.94 (6H, d, J = 6, H-19 and H-20), 1.01 and 1.14 (each 3H, s, H-16 and H-17), 1.08 (each 3H, d, J = 7 Hz, H-15), 1.50 (1H, m, H-4β), 1.61 and 1.71 (each 1H, d, J = 16 Hz, H-10), 1.73 (1H, m, H-14β), 1.79 (1H, m, H-13), 1.93 (1H, m, H-14α), 1.99 (1H, br d, J = 8, H-1), 2.09 (1H, d, J = 7 Hz, H-8), 2.27 (1H, ddd, J = 12, 8 and 12 Hz, H-4α), 2.48 (1H, m, H-2), 3.82 (1H, d, J = 2.3 Hz, H-6); EIMS m/z (rel int) 336 $[M]^+$ (3), 318 (6), 303 (9), 285 (29), 275 (36), 259 (71), 258 (53),

241 (58), 215 (29), 211 (12), 197 (15), 151 (38), 149 (34); HRMS *m/z* 336.2305 ($C_{20}H_{32}O_4$), 318.2212 ($C_{20}H_{30}O_3$), 303.1960 ($C_{19}H_{27}O_3$), 285.1839 ($C_{19}H_{25}O_2$), 275.1639 ($C_{17}H_{23}O_3$), 259.2070 ($C_{18}H_{27}O$), 241.1960 ($C_{18}H_{25}$), 215.1407 ($C_{15}H_{19}O$).

Isolation and extraction of vignaticol (8): $[M - 2H_2O]^+$ at *m/z* 332.2004; $C_{20}H_{28}O_4$ requires 332.1987; ^1H NMR (400 MHz, $CDCl_3$) δ 1.00 and 1.03 (each 3H, d, $J = 6$ Hz, H-19 and H-20), 1.08 (each 3H, d, $J = 7.6$ Hz, H-15), 1.18 and 1.34 (each 3H, s, H-16 and H-17), 1.51 (1H, m, H-4β), 1.72 and 1.93 (each 1H, d, $J = 16$ Hz, H-10), 1.85 (1H, m, H-18), 1.94 and 2.59 (each 1H, d, $J = 16$ Hz, H-14β and 14α), 2.00 (1H, br d, $J = 8$ Hz, H-1), 2.25 (1H, ddd, $J = 12$, 8 and 2 Hz, H-4α), 2.56 (1H, m, H-2), 3.89 (1H, d, $J = 2$ Hz, H-6); EIMS *m/z* (rel int) 350 $[M - H_2O]^+$ (2), 332 (30), 325 (13), 307 (18), 289 (19), 273 (14), 271 (16), 261 (13), 241 (73), 223 (77), 191 (22), 165 (51), 163 (30); HRMS (*m/z*) 332.2004 ($C_{20}H_{28}O_4$), 325.1651 ($C_{17}H_{25}O_6$); 241.1410 ($C_{13}H_{21}O_4$), 223.1339 ($C_{13}H_{19}O_3$), 153.0904 ($C_9H_{13}O_2$).

Isolation and extraction of Perseanol (9): $[M - H_2O]^+$ at *m/z* 366.2044; $C_{20}H_{30}O_6$ requires 366.2042; ^1H NMR (200 MHz, saturated soln $CDCl_3$) δ 0.95 (6H, t, $J = 6$ Hz, H-19 and H-20),1.10 (3H, d, $J = 7$ Hz, H-15), 1.19 and 1.33 (each 3H, s, H-16 and H-17), 1.66 and 2.09 (each 1H, d, J =16 Hz, H-14), 1.67 and 1.89 and 2.62 (each 1H, d, $J = 16$ Hz, H-10), 3.91 (1H, s, H-6), 2.55, 2.72, 3.02, 3.87, 3.92 and 4.25 (each 1H, br s, OH); ^1H NMR (400 MHz, CD_3OD) δ 0.88 and 0.94 (each 3H, d, $J = 6$ Hz, H-19 and H-20), 1.01 (3H, d, $J = 7$ Hz, H-15), 1.04 and 1.20 (each 3H, s, H-16 and H-17), 1.62 and 1.87 (each 1H, d, $J = 16$ Hz, H-10), 1.84 (1H, m, H-18), 1.89 and 2.50 (each 1H, d, $J = 16$ Hz, H-14), 2.14 (1H, m, H-4), 3.73 (1H, s, H-6); EIMS *m/z* (rel int.) 384 $[M]^+$ (4), 366 (1), 348 (11), 341 (67), 330 (40), 323 (38), 305 (33), 287 (25), 269 (11), 263 (10), 259 (19), 253 (24), 245 (21), 241 (10), 235 (21), 231 (10), 227 (10), 223 (22), 217 (21), 205 (24), 193 (32). HRMS (*m/z*) 366.2046 ($C_{20}H_{30}O_6$), 348.1940 ($C_{20}H_{28}O_5$), 341.1548 ($C_{17}H_{25}O_7$), 330.1800 ($C_{20}H_{26}O_4$), 323.1458 ($C_{17}H_{23}O_6$), 287.1316 ($C_{17}H_{19}O_4$), 1651282 ($C_{11}H_{17}O$).

Insect Bioassays. *S. litura* (Noctuidae) larvae, from a laboratory colony, were reared on artificial diet[9] at 22 °C, 70% rh, and 16:8 h light period. This bioassay was designed to quantify the feeding deterrence of the plant extract and the pure compounds. The bioassay was performed with third-instar larvae as described by Escoubas *et al.*[10] A feeding index (FI) was calculated for each treatment at 1000 ppm for comparison of the activities, and an arbitrary level (FI < 20) was used as the criterion to determine very effective deterrents. FI = 100 × %T/(%T + %C), where %T = % of treated disks consumed, and %C = % control disks consumed. The index varies from 0 (total inhibition) to 100 (total stimulation). A dose range of 10, 25, 50, 75, 100, 250, 500, 750, and 1000 ppm was used with the effective antifeedants to estimate their % [1 − (%T/%C)] × 100 and calculate their relative potencies (EC_{50}, effective dose to give a 50% feeding inhibition) with standard regression analysis (log dose over % feeding inhibition).

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