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Spirocyclic Defensive Alkaloid from a Coccinellid Beetle[1]

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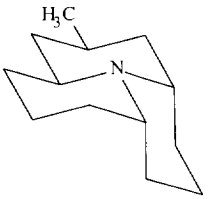
Abstract: A new alkaloid, chilocorine B, was characterized from a coccinellid beetle, *Chilocorus cacti*. This compound represents the first spirocyclic example of the new "dimeric" alkaloid family whose structures are derived from two thirteen-carbon tricyclic subunits, 2-methylperhydro-9*b*-azaphenalene and 3,4-dimethyloctahydro-8*b*-azaacenaphthylene.

INTRODUCTION

A highly diverse set of alkaloids, all presumably defensive in function, has been isolated from various species of coccinellid beetle (Coleoptera, Coccinellidae).[2-19] A notable subset is made up of the azaphenalenes, which possess the tricyclic skeleton exemplified by hippodamine (**1**).[14-19] Recently, Timmermans *et al.*[20] isolated and characterized a novel hexacyclic alkaloid, exochomine (**3**), from a European coccinellid, *Exochomus quadripustulatus*. In this "dimeric" alkaloid, the familiar hippodamine moiety is linked by a single bond to a previously unreported partner, 3,4-dimethyloctahydro-8*b*-azaacenaphthylene (**2**). We have recently described the isolation and characterization of a second "dimeric" alkaloid, chilocorine A (**4**) (originally named chilocorine), from the coccinellid *Chilocorus cacti*.[21] This heptacyclic dimer is comprised of these same two partners, linked by two bridges instead of one. A continuing search for new defensive alkaloids from *C. cacti*



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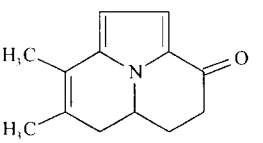

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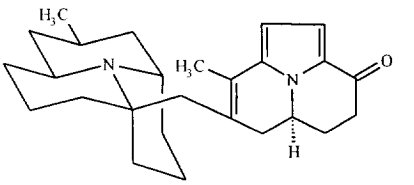

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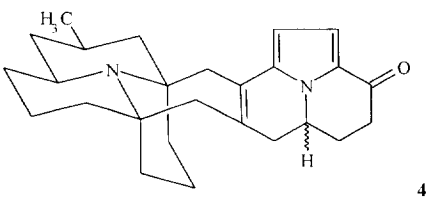

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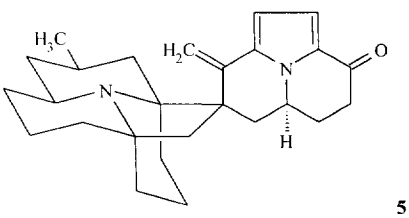

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has led us to isolate several additional examples, including chilocorine B (**5**), the characterization of which we here report.

RESULTS AND DISCUSSION

GC-MS examination of the crude alkaloid extract obtained from freshly collected specimens of *C. cacti* showed the presence of several components (**Fig. 1**). In the earlier eluting range, in addition to two previously reported alkaloid isomers (showing molecular ions at *m/z* 191)[21] possessing the skeleton of **1** (not visible in **Fig. 1** due to their very small amount), trace quantities of three new isomeric alkaloids (peaks I, II, and III, molecular ions at *m/z* 201) were detected. They may be of significance because they have a molecular weight identical to that of the hypothetical tricyclic partner **2**. Three of the four later eluting alkaloids, excluding chilocorine A (**4**) (peak VI), corresponding to peak IV (*m/z* 390), peak V (*m/z* 390) and the newly detected peak VII (*m/z* 390), were unknown. The abundant alkaloid chilocorine B (peak IV) could be isolated by flash chromatography over a silica gel column, and purified by HPLC and recrystallization. The molecular formula of this alkaloid was established as $C_{26}H_{34}N_2O$ by high resolution EI mass spectrometry [*m/z* 390.2646 (68%); calculated for $C_{26}H_{34}N_2O$: 390.2671], indicating that it is isomeric with chilocorine A (**4**). However, in its high

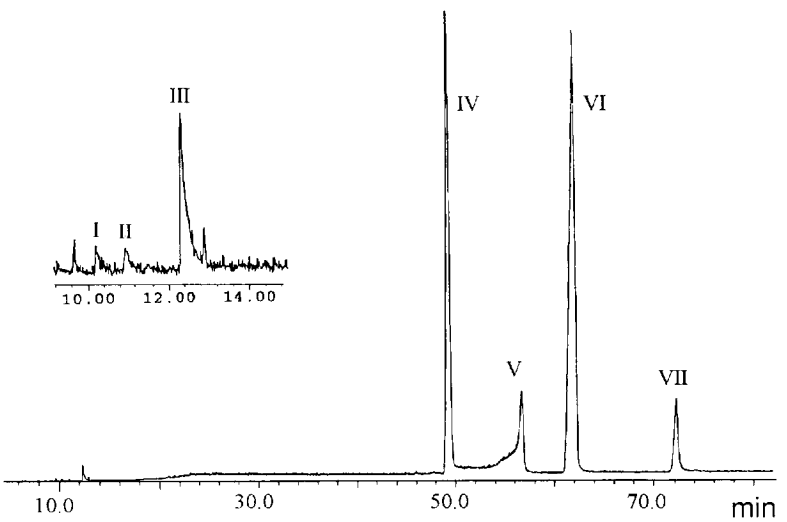

Fig. 1 A gas chromatogram obtained from a crude alkaloid extract of *C. cacti* beetles on a DB-5 capillary column.

Table 1 GC-MS Data of Alkaloids from the Crude Extract of *C. cacti.*

peak no.	retention time (min)	relative percent	mass spectrum [*m/z* (%)]
I	10.24	0.1	201 (M^+, 48), 186 (100),173 (9), 158 (13), 144 (12), 130 (8)
II	10.75	0.1	201 (M^+, 42), 186 (100), 173 (9), 158 (17), 144 (130, 130 (11)
III	12.35	0.8	201 (M^+, 100), 186 (51) 172 (17), 158 (18), 144 (39), 130 (9)
IV	49.31	35	390 (M^+, 62), 375 (5), 348 (56), 347 (67), 333 (9), 319 (6), 237 (23), 191 (100), 190 (75), 176 (21), 174 (11), 148 (8)
V	56.86	10	390 (M^+, 7), 191 (12), 190 (100), 188 (6), 174 (2), 148 (5)
VI	61.93	45	390 (M^+, 14), 347 (2), 191 (100), 190 (31), 176 (17), 149 (5), 148 (6)
VII	72.42	9	390 (M^+, 1), 346 (3), 207 (27), 206 (100), 188 (9), 136 (5), 44 (5)

resolution mass spectrum, the intense peaks at *m/z* 348 (43%) and at *m/z* 347 (62%) (M^+- C_3H_7, *m/z* 347.2116, calculated for $C_{23}H_{27}N_2O$: 347.2123) indicate a clear structural difference from **4**. The base peak at *m/z* 191 (calculated for $C_{13}H_{21}N$: 191.1674, found 191.1656) is identical to that of chilocorine A (**4**), but different from that of exochomine (**3**) (*m/z* 192.173, calculated for $C_{13}H_{22}N$: 192.176).[20] This information implies the presence of a methylperhydro-9*b*-azaphenalene ring system which, as in chilocorine A (**4**), is joined to its partner via two bonds. The peak at *m/z* 190 (64%) (*m/z* 190.1626, calculated for $C_{13}H_{20}N$: 190.1596) further supports this conclusion. Since the ultraviolet spectrum of chilocorine B resembles that of **3** and **4**,[20,21] we presumed the presence of a closely related tricyclic chromophore. From these data, we concluded that this new alkaloid is derived from 2-methylperhydro-9*b*-azaphenalene and 3,4-dimethyloctahydro-8*b*-azaacenaphthylene moieties, joined in a new way.

The ^{13}C NMR (100 MHz) spectrum of chilocorine B (**Table 2**) showed only 24 signals rather than the anticipated 26, including one C=O (δ 186.3) and six olefinic carbons (δ 100-145). A DEPT experiment revealed that one of the missing carbon signals was overlapped by the CH_3 resonance at δ 22.3. The intensity of the signal at δ 36.2 in the DEPT spectrum was relatively high, suggesting that the two CH_2 groups might have identical chemical shifts. This was confirmed by an HMQC experiment in which the carbon signal at δ 36.2 could be correlated with four protons. The DEPT experiment also revealed that the structure of chilocorine B contains one terminal olefinic CH_2 (triplet), two olefinic CH (doublet), three CH (doublet), twelve CH_2 (triplet), and one CH_3 (quartet). The observation of one C=O and six olefinic carbons in a structure with eleven unsaturation equivalents requires that chilocorine B be heptacyclic.

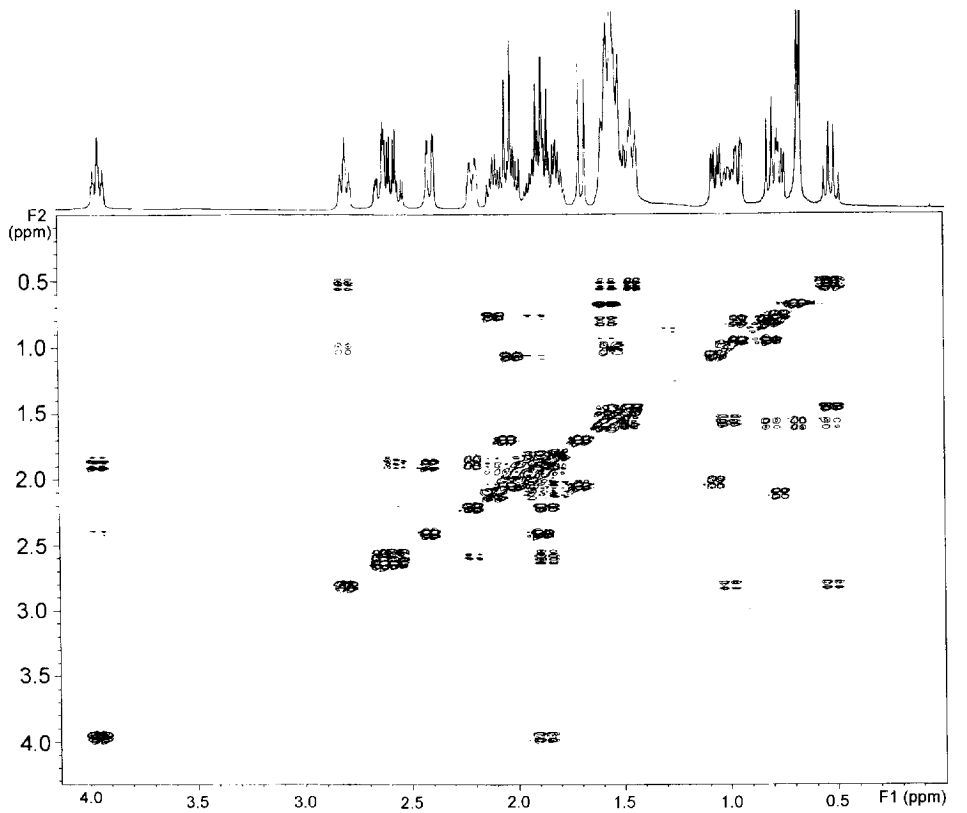

Fig. 2. A 500 MHz ^1H NMR spectrum and the corresponding DQ COSY spectrum of chilocorine B.

Fig. 2 depicts the upfield section of the DQ COSY spectrum of chilocorine B (the rest is not shown since the four olefinic protons are not correlated to other protons). Analysis of the COSY spectral data revealed the presence of several isolated spin systems as shown by bold lines in **Fig. 3**. In the spin system C-1 – C-6 [-$CH_2CH(CH_3)CH_2CH\ CH_2\ CH_2$-], the methine proton corresponding to the signal at δ 1.55 was placed next to the readily recognized methyl group (δ 0.68, J = 6.5 Hz) because of its correlation observed with the methyl group, in addition to the axial-axial couplings resulting from the axial protons at C-1 (δ 0.81, J = 12.5 Hz) and C-3 (δ 0.55, J = 12.5 Hz). Similarly, the assignment of the other methine proton (δ 2.80) to the position C-3a was based on the chemical shift consideration and the observation of the axial-axial couplings to the neighboring axial protons at C-3 (δ 0.55, J = 12.5 Hz) and C-4 (δ 1.01, J = 10.0 Hz). Subsequently, all other protons in this spin system were assigned by successive correlations, in conjunction with DEPT and HMQC experiements. In the spin system C-7 – C-9 (-$CH_2CH_2CH_2$-), the methylene protons observed at δ 1.83/1.93 were placed in the middle position (C-8) because of its vicinal correlations with both methylene proton pairs at C-7 (δ 1.07/2.10) and C-9 (δ 0.77/2.03), respectively. The HMBC experiment showed the long range ^{1}H-^{13}C correlations of H-7 (δ 2.10) to C-6 (δ 36.2), and H-8 (δ 1.83) to C-6a (δ 56.9), which allowed us to connect the two spin systems via the quaternary C-6a. Furthermore, the observation of cross peaks for H-9 (δ 0.77) and C-1 (δ 41.2) as well as of H-8 (δ 1.83/1.93) and C-9a (δ 65.0) enabled us to link the other two ends of the two spin systems through the quaternary C-9a. Connecting a nitrogen atom to the three carbons C-3a, C-6a, and C-9a, which shows relatively high ^{13}C NMR chemical shift values, established the azaphenalene subunit of chilocorine B, as shown in **Fig. 3a**. The orientation assignments of the protons as indicated in **Fig. 3a** were further confirmed by using the vicinal Karplus correlation[22] between coupling constants and dihedral angles that were obtained from a computer-simulated conformation[23] of this subunit.

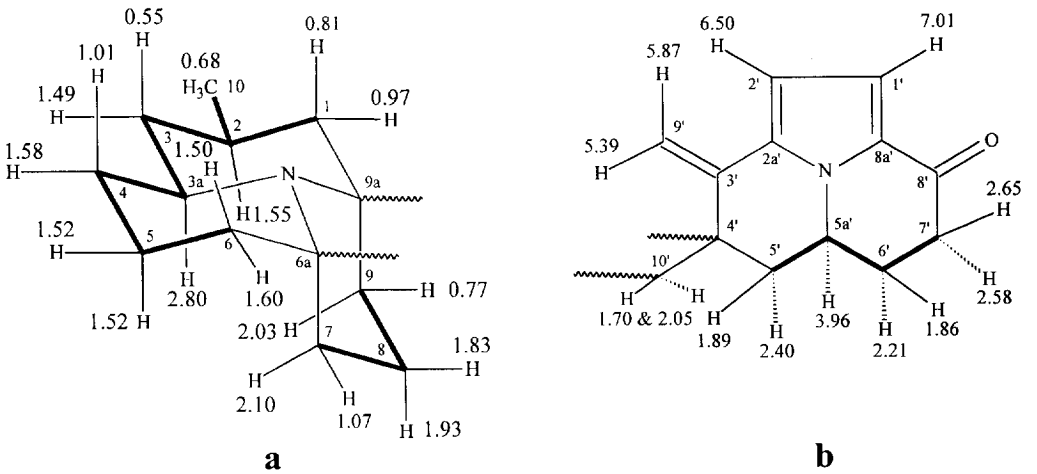

Fig. 3. The two structural subunits of chilocorine B and the corresponding ^{1}H NMR data.

The remainder of the chilocorine B structure consists of another subunit (**Fig. 3b**), which bears the second nitrogen atom and the four downfield protons whose signals are not depicted in **Fig. 2**. The two coupled aromatic protons (δ 7.01/6.50, J = 4.0 Hz) were placed at C-1' and C-2' based on the HMBC correlations of H-1' (δ 7.01, J = 4.0 Hz) to C-8a' (δ 128.6) and H-2' (δ 6.50, J = 4.0 Hz) to C-1' (δ 115.5); the two isolated singlet signals (δ 5.39/5.87, J = 0 Hz) were assigned to the terminal olefinic protons at C-9' (δ 114.3). In the spin system C-5'–C-7' (-$CH_2CHCH_2CH_2$-), the signal at δ 3.96 (d,d,d,d J = 12.5, 12.5, 3.0, and 2.0 Hz) was assigned to the methine proton at C-5a', again, because of chemical shift considerations and the observation of its axial-axial couplings to the two vicinal protons at C-5' (δ 1.89, J = 12.5 Hz) and C-6' (δ 1.86, J = 12.5 Hz). The remaining methylene protons (δ 2.58/2.65) correlating with the protons at C-6' were thus assigned to C-7'. The

Table 2. The ^{13}C NMR (100 MHz) and ^{1}H NMR (500 MHz) assignments for chilocorine B (**5**). Chemical shifts are given in ppm relative to the ^{13}C and ^{1}H peaks of $CDCl_3$ at 77.0 and 7.26 ppm respectively.

position	group	^{13}C data	^{1}H data			
		δ (ppm)	δ (ppm)	mult.	int.	coupling constants *J* (Hz)
1	CH_2	41.2	0.81	d,d	1H	12.5, 12.0
			0.97	d,d,d	1H	12.5, 2.0, 1.5
2	CH	27.0	1.55	m	1H	
3	CH_2	42.1	0.55	d,d,d	1H	12.5, 12.5, 12.5
			1.49	d,d,d	1H	12.5, 3.5. 3.0
3a	CH	47.5	2.80	d,d,d,d	1H	12.5, 11.5, 3.0, 2.5
4	CH_2	33.7	1.01	d,d,d,d	1H	11.5, 10.0, 6.0, 2.5
			1.58	m	1H	
5	CH_2	20.7	1.52	m	2H	
6	CH_2	36.2	1.50	m	1H	
			1.60	m	1H	
6a	C	56.9				
7	CH_2	22.6	1.07	d,d,d	1H	14.0, 7.0, 0.5
			2.10	d,d,d	1H	14.0, 13.5, 7.0
8	CH_2	19.7	1.83	d,d,d,d,d	1H	14.0, 13.5, 12.5, 7.0, 6.5
			1.93	d,d,d	1H	14.0, 7.0, 6.5
9	CH_2	22.3	0.77	d,d,d	1H	14.0, 6.5, 0.5
			2.03	d,d,d	1H	14.0, 12.5, 6.5
9a	C	65.0				
10	CH_3	22.6	0.68	d	3H	6.5
1'	CH	115.5	7.01	d	1H	4.0
2'	CH	106.0	6.50	d	1H	4.0
2a'	C	137.5				
3'	C	142.0				
4'	C	49.6				
5'	CH_2	37.7	1.89	d,d	1H	14.5, 12.5
			2.40	d,d	1H	14.5, 3.0
5a'	CH	50.3	3.96	d,d,d,d	1H	12.5, 12.5, 3.0, 2.0
6'	CH_2	31.4	1.86	d,d,d,d	1H	13.5, 12.5
			2.21	d,d,d,d	1H	13.5, 3.0, 2.5, 2.0
7'	CH_2	36.2	2.58	d,d,d	1H	17.5, 13.0, 5.0
			2.65	d,d,d	1H	17.5, 2.5, 2.0
8'	C	186.3				
8a'	C	128.6				
9'	CH_2	114.3	5.87	s	1H	0
			5.39	s	1H	0
10'	CH_2	51.7	1.70	d	1H	14.0
			2.05	d	1H	14.0

long range ^1H-^{13}C connectivities observed for the isolated CH_2 (δ 1.70/2.05, J = 14.0 Hz) to both C-3' (δ 142.0) and C-5' (δ 37.7) led to the assignment of this proton pair to C-10', which serves as a bridge connecting the two subunits. The structure of the aromatic tricyclic subunit **b** was therefore determined. Furthermore, the assignment of orientations of other protons relative to the proton at C-5a' was supported by a computer-simulated conformation.[23]

In addition to the consideration of the strong diamagnetic shielding effect of the aromatic subunit **b** on some protons of subunit **a** (H-1, H-3, H-9, and H-10), ^1H-^{13}C long range correlations observed for the proton at H-7 (δ 2.10) with C-10' (δ 51.7) as well as the proton at H-9 (δ 2.03) with C-4' (δ 49.6) suggested that the only logical way of linking subunits **a** and **b** would be via two bridges, which allows for two final possible diastereomeric spirocyclic structures, **5** and **5a**. Since the relative stereochemistry at position 5a' could not be solved by ^1H NMR analysis only, a single crystal X-ray crystallographic analysis seemed appropriate. Re-isolation of the defensive alkaloids from 200 beetles provided a sample which, after careful chromatography and recrystallization from $MeOH/CH_2Cl_2$, afforded a crystal of chilocorine B. The structure and stereochemistry of this alkaloid was then fully determined by a single crystal X-ray diffraction analysis. The computer-generated molecular structure from the X-ray crystallographic study is depicted in **Fig. 4**, and the complete assignments of ^1H and ^{13}C NMR data are list in **Table 2**.

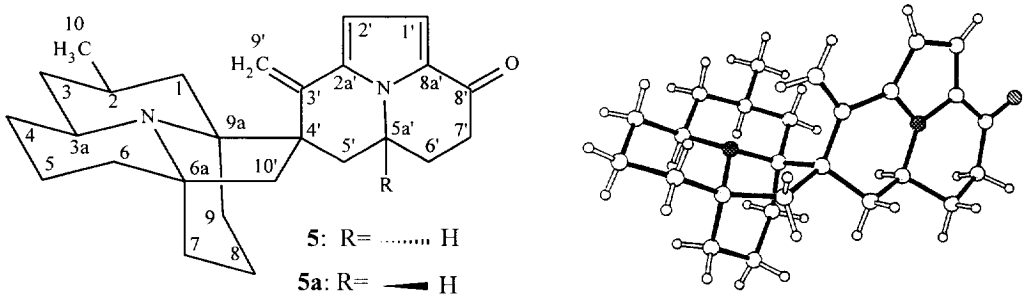

Fig. 4 The molecular structure of chilocorine B (**5**) showing its correct relative configuration.

In summary, a new spirocyclic alkaloid, chilocorine B (**5**), has been isolated from *Chilocorus cacti*, and is shown to have a structure closely related to that of chilocorine A (**4**). This spirocyclic compound is among the most complex of the alkaloids identified so far from insects. Bioassays have shown *Chilocorus cacti* to be rejected by lycosid spiders. While we have not as yet tested for the deterrency of chilocorine A and B, we suspect these alkaloids to be responsible, in part at least, for the unacceptability of the beetles.

EXPERIMENTAL

The Beetle. Chilocorus cacti were of the same source (Texas) as in our previous study.[21]

Extraction, Isolation and Purification of Alkaloids. Chilocorus cacti beetles (200 specimens) were immersed in 2% sulfuric acid in CH_3OH (10 ml), crushed, and left for 2 h at room temperature. The supernatant was removed and the residue was re-extracted with the same acidic solvent (2 x 10 ml). The combined extract was concentrated to *ca.* 2 ml and diluted with water (10 ml). The aqueous solution was extracted with ether (6 x 10 ml). The alkaloids were then released from the aqueous solution by adding concentrated KOH solution. The alkaloids were extracted with CH_2Cl_2 (3 x 10 ml). The CH_2Cl_2 extract was washed with water and concentrated to *ca.* 1 ml for further chromatographic separation and spectroscopic analysis.

The alkaloids were subjected to flash chromatography over silica gel (60 μm, EM Science, Gibbstown, NJ). The column (25 cm x 3 mm, silica gel: 0.6 g) was eluted with $CH_2Cl_2/MeOH/NH_4OH$ (9.5:0.45:0.05 to

7.0:2.95:0.05) and fractions were monitored by GC analysis which indicated that the least polar and UV active fraction contained *ca.* 75% of chilocorine B and *ca.* 25% of the unknown alkaloid corresponding to peak V in **Fig. 1**. Purification by HPLC [column: 25 cm x 4.6 mm ID Supelcosil LC-Si; mobile phase: CH_2Cl_2:MeOH (from 100:0 to 50:50) containing 0.15% NH_4OH], followed by crystallization from CH_2Cl_2/MeOH (1:1), gave a white crystalline solid (**5**) (*ca.* 300 μg).

Alkaloid **5** (*ca.* 300 μg) and CH_2Cl_2 (10 μl) were added to a 100 μl vial and after the solid was dissolved completely, MeOH (10 μl) was injected. This open vial was placed into a larger vial (5 ml) which was then closed tightly and kept at 0 °C for 48 h. Slow evaporation of the more volatile CH_2Cl_2 kept a crystal growing in the methanol-enriched solvent system. A single, transparent, plate-shaped crystal was obtained for X-ray crystallographic analysis after removal of the mother liquor using a syringe.

Gas Chromatography-MS. The low resolution EI mass spectra for these alkaloids were obtained on an HP 5890 gas chromatograph linked to an HP 5970 mass selective detector (MSD). Analysis was performed using a 30 m x 0.23 mm fused-silica column coated with DB-5 [temperature program: 200 °C (4 min), 10 °C/min, 270 °C]. Several peaks were observed from the crude alkaloid extract (**Table 1**). High-resolution GC-MS data were obtained on a VG 70-VSE instrument (resolution = 5000). For chilocorine B (**5**): HREIMS *m/z* (%) 390.2646 (M^+, 68, calcd for $C_{26}H_{34}N_2O$: 390.2671), 347.2116 (M^+-C_3H_7, 62, calcd for $C_{23}H_{27}N_2O$: 347.2123), 237.1193 (M^+-$C_{10}H_{19}N$, 17, calcd for $C_{16}H_{15}NO$: 237.1154), 191.1656 (M^+-$C_{13}H_{13}NO$, 100, calcd for $C_{13}H_{21}N$: 191.1674), 190.1626 (M^+-$C_{13}H_{14}NO$, 64, calcd for $C_{13}H_{20}N$: 190.1596).

NMR Spectroscopy. The ^1H NMR as well as the DQ COSY, HMQC, and HMBC spectra of chilocorine B were obtained using a Varian Unity 500 spectrometer. The ^{13}C NMR and DEPT spectra were obtained using a Varian XL 400 instrument at 100 MHz.

Ultraviolet Spectroscopy. Ultraviolet spectra were obtained using a diode-array detector (HP) linked to an HP 1090 HPLC instrument. A 25 cm x 4.6 mm ID Supelcosil LC-Si (5 micron) (Supelco, Bellefonte, PA) column was eluted with 75% CH_2Cl_2 and 25% MeOH containing 0.15% NH_4OH as the mobile phase.

X-Ray Crystallography. A plate-shaped (0.10 x 0.35 x 0.40 mm^3) colorless crystal of chilocorine B was mounted in a glass capillary. Data were collected on a R3m Siemens four-circle diffractometer using graphite-monochromated CuK_α radiation. The crystal belongs to orthorhombic space group $P2_12_12_1$ with cell parameters a = 9.236(2), b = 15.299(2), c = 16.516(2) Å. Intensities of 1399 independent reflections were measured by $\omega/2\theta$ scan, and Lorentz and polarization but not absorption corrections were applied. Three standard reflections were monitored every 97 reflections; no systematic intensity variations were found. The structure was solved by direct methods (SHELXS). A solvent methanol molecule was found at a H-bonded distance from chilocorine B's carbonyl oxygen. Hydrogen atoms were introduced through geometrical considerations. The structure was refined by full-matrix least-squares techniques minimizing $\Sigma w(||F_o|-|F_c||)^2$ (SHELXL) with anisotropic thermal parameters for all non-hydrogen atoms. At convergence (mean shift/esd = 0.001) the residual R_1 = 6.8% for 963 $F_o > 4\sigma(F_o)$. No peak greater than 0.17e/ $Å^3$ was found in the final difference Fourier map.

ACKNOWLEDGMENTS

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23. The computer program PC Model (Serena Software, Bloomington, IN) was used to obtain the computer-simulated conformations of the two subunits.

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