Spirocyclic Defensive Alkaloid from a Coccinellid Beetle

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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-9b-azaphenalene and 3,4-dimethyloctahydro-8b-azaacenaphthylene.

INTRODUCTION

A highly diverse set of alkaloids, all presumably defensive in function, has been isolated from various species of coccinellid beetle (Coleoptera, Coccinellidae). A notable subset is made up of the azaphenalenes, which possess the tricyclic skeleton exemplified by hippodamine (1). Recently, Timmermans et al. 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-8b-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. 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...
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) 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_{31}N_{2}O by high resolution EI mass spectrometry [m/z 390.2646 (68%); calculated for C_{26}H_{31}N_{2}O: 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.](image)

<table>
<thead>
<tr>
<th>peak no.</th>
<th>retention time (min)</th>
<th>relative percent</th>
<th>mass spectrum</th>
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<td>201 (M^+48), 186 (100), 173 (9), 158 (13), 144 (12), 130 (8)</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>IV</td>
<td>49.31</td>
<td>35</td>
<td>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)</td>
</tr>
<tr>
<td>V</td>
<td>56.86</td>
<td>10</td>
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<tr>
<td>VI</td>
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<td>45</td>
<td>390 (M^+14), 347 (2), 191 (100), 190 (31), 176 (17), 149 (5), 148 (6)</td>
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<tr>
<td>VII</td>
<td>72.42</td>
<td>9</td>
<td>390 (M^+1), 346 (3), 207 (27), 206 (100), 188 (9), 136 (5), 44 (5)</td>
</tr>
</tbody>
</table>
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resolution mass spectrum, the intense peaks at m/z 348 (43%) and at m/z 347 (62%) (M⁺ - C₃H₅, m/z 347.2116, calculated for C₂₃H₂₂N₅O: 347.2123) indicate a clear structural difference from 4. The base peak at m/z 191 (calculated for C₁₃H₁₈N₄: 191.1674, found 191.1656) is identical to that of chilocorine A (4), but different from that of exochorine (3) (m/z 192.173, calculated for C₁₃H₁₂N₄: 192.176). This information implies the presence of a methylperhydro-9b-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 C₁₃H₂₀N: 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-9b-azaphenalene and 3,4-dimethyloctahydro-8b-azaacenaphthylene moieties, joined in a new way.

The ¹³C 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 CH₃ 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 CH₂ groups might have identical chemical shifts. This was confirmed by an HMOC 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 CH₂ (triplet), two olefinic CH (doublet), three CH (doublet), twelve CH₂ (triplet), and one CH₃ (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$_2$CH(C(H$_2$)CH$_2$CH$_2$CH$_2$CH$_2$)), the methine proton corresponding to the signal at $\delta$ 1.55 was placed next to the readily recognized methyl group ($\delta$ 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 ($\delta$ 0.81, $J$ = 12.5 Hz) and C-3 ($\delta$ 0.55, $J$ = 12.5 Hz). Similarly, the assignment of the other methine proton ($\delta$ 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 ($\delta$ 0.55, $J$ = 12.5 Hz) and C-4 ($\delta$ 1.01, $J$ = 10.0 Hz). Subsequently, all other protons in this spin system were assigned by successive correlations, in conjunction with DEPT and HMOC experiments. In the spin system C-7 – C-9 (CH$_2$CH$_2$CH$_2$), the methylene protons observed at $\delta$ 2.10 were placed in the middle position (C-8) because of its vicinal correlations with both methylene proton pairs at C-7 ($\delta$ 1.07/2.10) and C-9 ($\delta$ 0.77/2.03), respectively. The HMOC experiment showed the long range $^1$H-$^1$C correlations of H-7 ($\delta$ 2.10) to C-6 ($\delta$ 36.2), and H-8 ($\delta$ 1.83) to C-6a ($\delta$ 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 ($\delta$ 0.77) and C-1 ($\delta$ 41.2) as well as of H-8 ($\delta$ 1.83/1.93) and C-9a ($\delta$ 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 azaphenalenone 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 between coupling constants and dihedral angles that were obtained from a computer-simulated conformation of this subunit.

![Diagram](image)

**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 (H-1' $\delta$ 7.01/6.50, $J$ = 4.0 Hz) were placed at C-1' and C-2' based on the HMBC correlations of H-1' ($\delta$ 7.01, $J$ = 4.0 Hz) to C-8a' ($\delta$ 128.6) and H-2' ($\delta$ 6.50, $J$ = 4.0 Hz) to C-1' ($\delta$ 115.5); the two isolated singlet signals (H-5 $\delta$ 5.39/5.87, $J$ = 0 Hz) were assigned to the terminal olefinic protons at C-9' ($\delta$ 114.3). In the spin system C-5' – C-7' (CH$_2$CHCHOCH$_2$), the signal at $\delta$ 3.96 (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' ($\delta$ 1.89, $J$ = 12.5 Hz) and C-6' ($\delta$ 1.86, $J$ = 12.5 Hz). The remaining methylene protons (H-2.58/2.65) correlating with the protons at C-6' were thus assigned to C-7'.
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 (-CH₂CH(CH₃)CH₂CH₂CH₂CH₂CH₂-), 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 HMOC experiments. In the spin system C-7 – C-9 (-CH₂CH₂CH₂-), the methylene protons observed at δ 1.93/1.93 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 ¹H-¹³C 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 ¹³C 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 between coupling constants and dihedral angles that were obtained from a computer-simulated conformation of this subunit.

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' (–CH₂CH₂CH₂CH₂–), the signal at δ 3.96 (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'.
long range $^1$H-$^{13}$C connectivities observed for the isolated CH$_3$ (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.

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), $^1$H-$^{13}$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 $^1$H 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$_2$Cl$_2$, 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 $^1$H and $^{13}$C NMR data are list in Table 2.

![Diagram of chilocorine B](image)

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. Extraction, Isolation and Purification of Alkaloids. *Chilocorus cacti* beetles (200 specimens) were immersed in 2% sulfuric acid in CH$_3$OH (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$_2$Cl$_2$ (3 x 10 ml). The CH$_2$Cl$_2$ 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$_2$Cl$_2$/MeOH/NH$_2$OH (9.5:0.45:0.05 to
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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: CH2Cl2:MeOH (from 100:0 to 50:50) containing 0.15% NH4OH], followed by crystallization from CH2Cl2:MeOH (1:1), gave a white crystalline solid (5) (ca. 300 µg).

Alkaloid 5 (ca. 300 µg) and CH2Cl2 (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 CH2Cl2 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 C26H14N2O: 390.2671), 347.2116 (M' - C4H7, 62, calcd for C23H12N2O: 347.2132), 237.1193 (M' - C10H9N, 17, calcd for C14H10N: 237.1154), 191.1656 (M' - C13H11NO, 100, calcd for C13H11NO: 191.1674), 190.1626 (M' - C13H11NO, 64, calcd for C13H11NO: 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 13C 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% CH2Cl2 and 25% MeOH containing 0.15% NH4OH as the mobile phase.

X-Ray Crystallography. A plate-shaped (0.10 x 0.35 x 0.40 mm3) colorless crystal of chilocorine B was mounted in a glass capillary. Data were collected on a R3m Siemens four-circle diffractometer using graphite-monochromated CuKα radiation. The crystal belongs to orthorhombic space group P212121, with cell parameters a = 9.236(2), b = 15.299(2), c = 16.516(2) Å. Intensities of 1399 independent reflections were measured by w/2θ 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 Σw[(Fo - Fc)2] (SHELXL) with anisotropic thermal parameters for all non-hydrogen atoms. At convergence (mean shift/esd = 0.001) the residual R1 = 6.8% for 963 F0>4σ(F0). No peak greater than 0.17e/Å3 was found in the final difference Fourier map.

ACKNOWLEDGMENTS

We wish to thank Professor Jon C. Clardy and Dr. Emil Lobkovsky (Cornell University) for carrying out the X-ray crystallographic analysis, and Dr. Victor French (Texas A&M University) for kindly providing assistance to M. A. H. to locate collection sites. High resolution mass spectra were obtained in the mass spectrometry laboratory of the University of Illinois, on an instrument purchased in part with a grant from the Division of Research Resources, NIH (RR 04648). This study was supported by NIH grants AI 12020 (J. M.) and AI 02908 (T. E.), and by BARD grant # US-2359-93C (M. A. H.).
REFERENCES AND NOTES

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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