Antimicrobial Diterpene from the Brazilian Termite Nasutitermes macrocephalus (Isoptera: Termitidae: Natutitermitinae)

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Termites are insects with a complex social organization on castes among which soldiers are the only responsible for the colony defense. The soldiers of the Nasutitermes genus use chemical defense, which comprises a mixture of mono, sesqui and mainly diterpenes. The new diterpene 2β,3α-dihydroxy-trinerva-1(15),11-diene, found in Nasutitermes macrocephalus, was isolated using preparative high performance liquid chromatography (HPLC) and identified by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) techniques. The absolute stereochemistry determined was supported by ab initio calculations and by comparison of the experimental circular dichroism data with the theoretical results. The isolated diterpene was evaluated against the bacteria Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative), and the fungi Candida albicans and Aspergillus brasiliensis. The isolated diterpene presented minimal inhibitory concentrations of 62.5 and 62.5 µg mL⁻¹ against S. aureus and C. albicans, respectively.

Keywords: Nasutitermes macrocephalus, biological activities, HPLC

Introduction

Termites are insects present all over the world, with about 2750 species living in tropical and subtropical regions.1 They are social insects with castes responsible for distinct functions: workers are tasked to build the mound and to feed the others castes; king and queen respond for reproduction, resulting in eggs that become larvae that may become workers, soldiers or winged (secondary breeding).2,3 Soldiers are responsible for the defense of the mound and, depending on the species they will present mechanical and/or chemical defense.1,2,4 The Nasutitermes genus (Isoptera: Termitidae: Nasutitermitinae) is the most evolved among the termites since their soldiers use only chemical defense, avoiding contact with the enemies.5 They produce and storage a mixture of mono, sesqui and diterpenes in the frontal gland, resulting in a sticky secretion that is ejected against the enemies.5,6-10 The great success of the Nasutitermes evolution has been attributed to this defense method.2 The monoterpenes are the same compounds found in plants, like α-pinene, β-pinene, limonene and terpinolene, and they are associated with the protection of the colony from fungal infections.11 Sesquiterpenes are in minor quantity, as found in N. gracilirostris, N. torresi, N. triodiae, N. octopilis and N. macrocephalus.9,12,13 The skeletons of the diterpenes found in termites (trinervitanes, kempanes and rippertanes) are broadly described in all species,14 and are related to both species communication, as they are blind, and to defense.15 Although there are several studies on the identification of these diterpenes, just a few of them focused on the biological activities of those substances. For example, one study presented five compounds with antibacterial activity against Bacillus subtilis and Escherichia coli.10 Therefore, several molecules with interesting biological activities produced by species in the Nasutitermes genus, remains to be studied.

As a consequence, our research group recently studied the biological activities and chemical composition of the crude extract from N. macrocephalus soldiers, which inhibited the growth of methicillin resistant Staphylococcus aureus and Candida albicans.13 To continue this study, the crude extract was further fractionated to obtain a new substance that was fully characterized by...
spectrometric methods and underwent antibacterial and antifungal assays to evaluate its biological activities.

**Experimental**

**General experimental procedures**

Optical rotation was measured in a PerkinElmer 341 LC polarimeter. Electronic circular dichroism (ECD) spectrum was obtained in a JASCO, J-715, Circular Dichroism (CD) Spectropolarimeter, using acetonitrile as solvent. 1D and 2D nuclear magnetic resonance (NMR) spectra were recorded in a Bruker Ultrafield Avance 600 MHz or 400 MHz spectrometer, with CDCl₃ as solvent. EIMS analysis were performed in an HP GC-EIMS (gas chromatography with electron ionization mass spectrometry) (mass selective detector model 5937 and GC model G1530A) (70 eV) spectrometer with a HP-5 column (30 m × 250 µm × 0.25 µm). The HRESIMS (high-resolution electrospray ionization mass spectrometry) spectra in a positive mode were recorded in a Waters QTOF Micro mass spectrometer with direct infusion on the ionization source. Fractionation and isolation were performed in a preparative high performance liquid chromatograph (HPLC) Shimadzu Prominence 20A system with a Phenomenex Gemini C-18 preparative column (21.2 × 250 mm, 5 µm).

**Termite material**

The mound was collected at Fiocruz campus (22°52′53.24″S, 43°14′40.73″W, 8 m above sea level (ASL), Rio de Janeiro, RJ, Brazil) from a *Leucaena* sp. Benth. tree and identified as *Nasutitermes macrocephalus* (Isoptera: Termitidae: Nasutitermitinae) by Dsc Maurício Rocha from University of São Paulo (USP) Museum, where a specimen has been deposited (MZUSP 15803).

**Extraction, GC-EIMS analysis, and isolation**

Soldiers (21.5 g) of *N. macrocephalus* were separated from the other castes and crushed in CH₂Cl₂ at room temperature for 5 min. The resulting mixture was filtered and dried under a nitrogen flow to yield 824.2 mg of a crude extract. An aliquot of this material (1 mg mL⁻¹ in CH₂Cl₂) was analyzed by GC-EIMS, with the injector set at 280 °C, detector at 290 °C, injection volume of 1 µL, and nitrogen as gas carrier at 1.2 mL min⁻¹. The temperature was initially maintained at 45 °C for 5 min, and then raised to 130 °C at a rate of 12 °C min⁻¹. From 130 to 230 °C and from 230 to 290 °C, the temperature was raised at rates of 33 and 1.5 °C min⁻¹, respectively. The total time of analysis amounted to 60.0 min. Part of the CH₂Cl₂ extract (602.7 mg) was dissolved in methanol (12 mL, 50.2 mg mL⁻¹) and fractionated (volume of each injection: 800 µL) on an HPLC system using CH₃CN:H₂O (75:25) at flow ratio of 20 mL min⁻¹ as mobile phase. The process was monitored at λ 210 nm for 55.0 min to select the fractions to be collected, which were dried under reduced pressure. The fraction with the isolated diterpene (14.5 min) yielded 10.0 mg.

**Antimicrobial assays**

Both the CH₂Cl₂ extract and the isolated substance underwent broth microdilution assays to determine their MIC (minimal inhibitory concentration) values.¹⁷ Briefly, after two-fold serial dilution of test samples, the wells were inoculated with 10 µL of a bacterial suspension of *E. coli* ATCC 8739 or methicillin-resistant *S. aureus* BMB 9393 (MRSA) in Müller-Hinton, or inoculated with 100 µL of RPMI-MOPS pH 7.2 containing *C. albicans* ATCC 10231 or *A. brasilensis* ATCC 16404. The microplates were incubated overnight at 37 °C for MRSA and for 48 h at room temperature (28-30 °C) for fungi. The negative control was pure medium, and positive controls comprised inoculated growth medium. The results were based on visual growth of microorganisms. The lowest concentrations of the samples that inhibited the growth of microorganisms were considered their MIC values. The visual observations were confirmed after aseptically addition of resazurin (30 µL) to the microplate wells, followed by incubation at 37 °C for 1 h.

**Computational methods**

Initially, the isolated substance underwent a conformational search using the software Open3Dalign 2.103.¹⁸ One thousand molecular dynamics simulations were carried out for the substance at 1,000 K, with a 1 fs time step for 1 ps using the MMFF94 force field, and considering the solvent (water) implicitly using the GB/SA model. The most stable conformation in each simulation was optimized with the MMFF94S force field and ranked according to the final energy. The most stable conformation and those up to 10 kcal mol⁻¹ from the most stable underwent optimization with the Hamiltonian PM7¹⁹ using the software MOPAC 2012.²⁰,²¹ In this step the solvent (acetonitrile, dielectric constant = 37.5) was implicitly considered using the conductor-like screening model (COSMO). The most stable conformation and those up to 10 kcal mol⁻¹ distant from the most stable were optimized in a density functional theory (DFT) calculation at the B3LYP/Def2-TZVP-RJICOSX²²-²⁷ level of theory using the software ORCA 2.9.²⁸ COSMO was also used...
in this calculation to consider the solvent (acetonitrile) implicitly. As no conformation could be found up to 20 kJ mol\(^{-1}\) from the most stable, only one was used in the next step that comprised time-dependent density functional theory (TD-DFT) calculation at the same level of theory. A total of 20 excited states were calculated, and only singlet excited states were considered. The DFT optimization of the conformations and TD-DFT calculation were also performed at the B3LYP/6-311G (2d,2p)\(^{20}\) level of theory. Rotational and oscillator strengths were respectively converted into CD and UV (ultraviolet) spectra using the software SPECDIS 1.51.\(^{30}\) These conversions were carried out employing Gaussian curves with half the bandwidth of the CD band at 1/e peak height (\(\sigma\)) equal to 0.29 eV.

2\(\beta,3\alpha\)-Dihydroxytrinervita-1(15),11-diene

White resin (10.0 mg); \([\alpha]\)\(_D\) \(20^\circ\) \(-5.0^\circ\) (c 0.046 CHCl\(_3\)); CD (0.33 mM, CH\(_2\)CN) \(\lambda\)\(_{max}\) / nm (\(\Delta\): 200 (14.3), 220 (\(-15.8\)), 240 (\(-0.3\)); EIMS (70 eV) \(m/\epsilon\): 304 [M\(^+\)] (1), 286 (13), 271 (19), 255 (6), 215 (4), 207 (13), 201 (5), 190 (8), 175 (19), 159 (11), 147 (12), 135 (40), 119 (27), 107 (29), 91 (30), 79 (21), 67 (22), 55 (26), 44 (100). HRESIMS, ESI (+): \(m/\epsilon\) [M + H]\(^+\) 305.2472 (calcd. for C\(_{23}\)H\(_{30}\)O\(_2\)^+ 305.2475).

Results and Discussion

According to Everaerts et al.\(^{29}\) and Braekman et al.,\(^{31}\) there is no difference in the final results of diterpene composition when crushing just the heads or the entire bodies of the termites. Therefore, the entire bodies of \(N.\) macrocephalus soldiers were employed in the present study to obtain larger mass of extracted substances. Fractionation of the CHCl\(_3\) extract yielded 10 mg of a white resin whose molecular formula (C\(_{23}\)H\(_{30}\)O\(_2\)) was determined through HRESIMS by the protonated molecule at \(m/\epsilon\) 305.2472 [M + H]\(^+\).

The structure elucidation (relative stereochemistry) of the isolated compound was carried out using 1D (\(\text{\(^1H\), \(^1\)C}\) and 2D (HSQC (heteronuclear single quantum correlation) (\(^{1}J_{	ext{HC}}\)), HMBC (heteronuclear multiple bond correlation) (\(^{2}J_{	ext{HC}}\)), COSY (correlation spectroscopy) and NOESY (nuclear Overhauser effect spectroscopy) NMR techniques (Table 1). Chemical shifts are given in parts per million (ppm). On the basis of \(^1\)C NMR data, 20 resonances were assigned to four methyl groups (\(\delta\) 22.0, 21.3, 18.1 and 16.4), six methylenes (\(\delta\) 37.8, 28.6, 40.3, 24.6, 38.4 and 28.0), six methines (\(\delta\) 76.9, 78.8, 48.8, 30.5, 129.6 and 61.8), and four quaternary carbons (\(\delta\) 130.8, 44.7, 131.3 and 130.4). The \(^1\)H NMR spectrum showed signals consistent with the presence of four methyls at \(\delta_{\text{H}}\) 1.55 (d, \(J\ 1.9\) Hz, CH\(_3\)-17), 0.96 (CH\(_3\)-18), 0.84 (d, \(J\ 6.8\) Hz, CH\(_3\)-19), and 1.66 (d, \(J\ 1.3\) Hz, CH\(_3\)-20), the first one and the last being allylic methyls, the second is bonded to a quaternary carbon, and the third bonded to a tertiary carbon.

<table>
<thead>
<tr>
<th>Position</th>
<th>(^1)C NMR (\delta_c / \text{ppm})</th>
<th>HSQC (\delta_H / \text{ppm})</th>
</tr>
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<tr>
<td>C 1</td>
<td>130.8</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>76.9</td>
<td>4.03 (1H, d, (J\ 8.7) Hz)</td>
</tr>
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<td>3</td>
<td>78.8</td>
<td>3.71 (1H, d, (J\ 8.7) Hz)</td>
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<td>4</td>
<td>44.7</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>37.8</td>
<td>1.44 (1H, m), 1.67 (1H, m)</td>
</tr>
<tr>
<td>6</td>
<td>28.6</td>
<td>1.62 (1H, m), 1.82 (1H, m)</td>
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<tr>
<td>7</td>
<td>48.8</td>
<td>1.88 (1H, m)</td>
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<td>30.5</td>
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<td>40.3</td>
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<td>24.6</td>
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<tr>
<td>11</td>
<td>129.6</td>
<td>5.28 (1H, dd, (J\ 5.0) Hz)</td>
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<tr>
<td>12</td>
<td>131.3</td>
<td>–</td>
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<tr>
<td>13</td>
<td>38.4</td>
<td>2.10 (1H, m), 2.53 (1H, m)</td>
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<td>28.0</td>
<td>2.13 (1H, m), 2.42 (1H, m)</td>
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<tr>
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<td>18</td>
<td>21.3</td>
<td>0.96 (3H, s)</td>
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<tr>
<td>19</td>
<td>18.1</td>
<td>0.84 (3H, d, (J\ 6.8) Hz)</td>
</tr>
<tr>
<td>20</td>
<td>16.4</td>
<td>1.66 (3H, d, (J\ 1.3) Hz)</td>
</tr>
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</table>

The coupling of a methyl group (C-18, \(\delta_{\text{H}}\) 0.96) bonded to a tertiary carbon bearing a proton with a signal at \(\delta_{\text{H}}\) 1.60 (H-8), with which the methyl protons were coupled with constant of 6.8 Hz, was confirmed in the COSY experiment (\(^{3}J_{\text{HH}}\)). The signal associated with the methine double bond proton at \(\delta_{\text{H}}\) 5.28 (dd, \(J\ 5.0/5.8\) Hz H-11), connected to C-11 (\(\delta_c\) 129.6) according to the HSQC experiment, was coupled to C-9 (\(\delta_c\) 40.3, \(^{3}J_{\text{CH}}\)), C-10 (\(\delta_c\) 24.6, \(^{3}J_{\text{CH}}\)), C-13 (\(\delta_c\) 38.4, \(^{3}J_{\text{CH}}\)), and C-20 (\(\delta_c\) 16.4, \(^{3}J_{\text{CD}}\)), according to the HMBC experiment. This result was important to confirm the \(^1\)H and \(^1\)C chemical shifts attributed to these groups. Although the methine proton at \(\delta_{\text{H}}\) 1.88 (H-7) showed a multiplet, the methine proton at \(\delta_{\text{H}}\) 2.13 (H-16) exhibited a doublet with a constant of 7.3 Hz. The coupling of these hydrogens was also confirmed in the COSY (\(^{3}J_{\text{HH}}\)) spectrum. The carbinolic protons presented a coupling...
constant of 8.7 Hz, that is characteristic of H-axial–H-axial coupling in a saturated six members ring. This corroborates the *trans* relationship between the hydroxyl groups. NOE correlations observed through the NOESY spectrum show interactions of H-3 with H-6a and H-14a (both up), confirming the relative configurations of both C-3 and C-8 (both hydroxyl and methyl groups in axial position). H-2 correlations with H-16 and H-18 in the NOESY spectrum also confirmed the relative configurations of C-2, C-16 and C-18 (Figure 1). NOESY spectrum also shows interaction of H-8 with H-16, also confirming the configurations of C-8 and C-16. In conclusion, interpretation of the NMR data leads to the structure 2β,3α-dihydroxy-trinervita-1(15),11-diene, which is a new diterpene (Figure 1).

There was no doubt about the relative configuration of the isolated substance. Therefore, aiming to discover its absolute configuration by comparing its experimental and theoretical ECD spectra (Figure 2), a conformational search was performed using molecular mechanics methods, which afforded eight conformations. The energy difference between the most and the less stable was 8.8046 kcal mol\(^{-1}\). When all these conformations underwent optimization through semiempirical calculations only four of them presented total energies corresponding to above 1% of the Boltzmann distribution. Therefore, only these four conformations were further optimized through DFT calculations, according to which one had energy corresponding to more than 99% of the Boltzmann distribution at both levels of theory employed.

The root-mean-square deviation between atomic positions of superimposed conformations resulting from both DFT calculations, accounting to more than 99% of the Boltzmann distribution, was only 0.074 Å. Thus, these conformations are practically the same and are in agreement with the NMR data. For example, in the conformation obtained at the B3LYP/Def2-TZVP/RIJCOSX level of theory, the distance between H-3 with H-6a is 1.942 Å, while between H-3 and H-8 is 2.761 Å. Regarding H-2, its distances from H-16 and H-18 are 3.917 and 2.274 Å, respectively (Figure 3). All these values are in accordance with the observed NOE.

Both theoretical ECD spectra, obtained through TD-DFT calculations at both levels of theory for the isolated substance, were in accordance with the experimental one, suggesting that the attributed configuration was correct. Based on the data described, the configuration of the substance was established as (2R,3R,4S,7S,8R,11E,16S)-2,3-dihydroxytrinervita-1(15),11-diene.
The isolated compound was evaluated against a series of fungal and bacterial strains. The bacteria methicillin-resistant Staphylococcus aureus BMB 9393 (clinical isolate) (MRSA) and Escherichia coli ATCC 8739 were chosen for antibacterial assay due to the association with hospital acquired infections worldwide and gastrointestinal infections, respectively.\(^2\) Candida albicans ATCC 10231 and Aspergillus brasiliensis ATCC 16404 were used in the antifungal assay, since they are responsible for candidiasis and lung infections, respectively.\(^3\) The isolated diterpene showed activity against both MRSA (MIC = 62.5 µg mL\(^{-1}\)) and C. albicans (MIC = 62.5 µg mL\(^{-1}\)), and no activity against the Gram-negative bacterium E. coli was observed. It was also inactive against the filamentous fungus A. brasiliensis. The positive controls ciprofloxacin and amphotericin B presented MIC values of 0.5 and 0.125 µg mL\(^{-1}\), for antibacterial and antifungal activity, respectively. This result contributes to know the role of diterpenes produced by the termites, and help to understand the chemical ecology of termites.

**Conclusions**

In this study a diterpene trinervitene was isolated from termite soldiers of Nasutitermes microcephalus and presented antimicrobial activities against S. aureus and C. albicans.

This work contributes to the knowledge of termite chemical composition, which is fundamental to understand the success of termites development and adaptation to many different environments.

**Supplementary Information**

Supplementary data (chromatogram, mass spectrum, \(^1\)H and \(^13\)C NMR spectra, COSY, HSQC, HMBC correlation maps, and results through molecular mechanics) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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