APORPHINE ALKALOIDS FROM Ocotea macrophylla (LAURACEAE)

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Four aporphine alkaloids from the wood of Ocotea macrophylla (Lauraceae) were isolated and characterized as (S)-3-methoxy-nordomesticine (1), (S)-N-ethoxycarbonyl-3-methoxy-nordomesticine (2), (S)-N-formyl-3-methoxy-nordomesticine (3) and (S)-N-methoxycarbonyl-3-methoxy-nordomesticine (4); alkaloids 2-4 are being report for the first time. The structure the isolated compounds were determined based on their spectral data and by comparison of their spectral data with values described in literature. The alkaloid fraction and compound 1 showed antifungal activity against Fusarium oxysporum f. sp. lycopersici and also compound 1 showed antimicrobial activity towards Staphylococcus aureus, Enterococcus faecalis as well.

Keywords: Ocotea macrophylla; aporphine alkaloids; Lauraceae.

INTRODUCTION

The Ocotea genus (Lauraceae) includes more than 350 species found in the American continent and southern Africa.¹,² In Colombia, there are 35 Ocotea species distributed throughout the country mainly in the Andean forests.¹ In traditional medicine, some Ocotea species shown different applications. O. quixos is used as disinfectant, local anesthetic and anti-diarrheic.⁴ O. lancifolia is used as antiparasitic, and O. caparrapi is used to treat insect bites, snake bites, bronchitis, and cancerous tumors.⁵,⁶

Chemically, the Ocotea genus is known mainly as a source of metabolites type furan and tetrahydrofuran lignans,⁵ bicyclo[3.2.1] octane⁶ and benzofuran neolignans,⁶ benzylisoquinoline¹¹ and aporphine alkaloids.⁷ In previous studies, four aporphine alkaloids from wood of Ocotea macrophylla were isolated and identified as nantenine, glaucine, isocorydine and dehydronantenine.⁸ In this paper, we describe isolation and structural determination of three new aporphine alkaloids 2-4 besides (S)-3-methoxy-nordomesticine 1 and the reports antibacterial and antifungal activities of compounds.

RESULT AND DISCUSSION

The ethanolic extract from the stem of O. macrophylla was subjected to an acid-base extraction to obtain an alkaloidal fraction, that was further subjected to fractionation and purification by chromatographic methods leading to the isolation of four alkaloids: (S)-3-methoxy-nordomesticine (1), (S)-N-ethoxycarbonyl-3-methoxy-nordomesticine (2), (S)-N-formyl-3-methoxy-nordomesticine (3) and (S)-N-methoxycarbonyl-3-methoxy-nordomesticine (4). The structures of the alkaloids 1-4 are shown in Figure 1 and spectroscopic data in the Table 1.

Alkaloid 1 was obtained as a yellowish oil that gives a positive reaction to Dragendorff reagent, which suggests the presence of an alkaloid. The molecular formula of 1 was established as C_{19}H_{15}NO_{4} from HRESIMS at m/z 340.1178 [M-H], indicating eleven degrees of insaturation. Its UV spectrum showed maximum absorptions at 224 and 308 nm, characteristic of aporphine alkaloids,¹⁵ and the IR spectrum displayed hydroxyl (3404 cm⁻¹), aromatic (1585 and 1467 cm⁻¹), and methylenedioxy (1039 and 935 cm⁻¹) absorptions.¹⁵ The "H NMR spectrum shows typical signals for aporphine alkaloids at: 4.74 (1H, dd, J=3.9 and 13.8 Hz, H-6a), 3.73 (1H, ddd, J=12.1, 4.3 and 2.1 Hz, H-5), 3.24 (1H, ddd, J=12.1, 8.4 and 3.6 Hz, H-5), 2.93 (2H, m, H-3, H-7) and 2.64 (2H, m, H-3, H-7). Also, two singlets were present in the aromatic region at 6.79 (H-11), 6.70 (H-8) besides the hydroxyl group at 6.30, as well as signals for a methylenedioxy group at 5.95 (1H, d, J=1.3 Hz) and 5.94 (1H, d, J=1.3 Hz) and two methoxyl groups at 3.97 and 3.87 (3H, each s). The "C NMR and DEPT showed spectra of 1 showed 19 signals, corresponding to two methoxyl groups at 60.9 and 60.3; four methylene groups at 100.9, 43.9, 35.1 and 23.8, where the first signal corresponds to a methylenedioxy group; three methines at 108.5, 108.6, and 52.8, and ten quaternary carbons, whose chemical shift are found in Table 1. The signal at δ_H 6.30 (1H, s) showed no connectivity in the HMBC experiment, indicative of the presence of a phenolic OH group, supported by IR. Analysis of the HMQC spectrum allowed the location of substituents, according to the correlations observed between the signals at δ_H 5.94 and 5.95 (for the methylenedioxy group) with the signals at δ_C 145.9 (C-9) and 146.2 (C-10), and these last two signals with the protons at δ_H 6.70 (H-8) and 7.90 (H-11), respectively, suggesting the presence of a methylenedioxy group at positions 9 and 10 and two hydrogens on the aromatic ring in a para orientation. The presence of the hydroxyl group at position 1, was determined using correlations between the signals at δ_H 6.30 and δ_C 116.5, assigned to C-11b. The location of the methoxyl groups in positions C-2 and C-3, were assigned according to the correlation of

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### Table 1. $^1$H and $^{13}$C NMR spectroscopic assignments of 1-4 in (CDCl$_3$, $^1$H NMR: 400 MHz and $^{13}$C NMR: 100 MHz)

<table>
<thead>
<tr>
<th>Position</th>
<th>Alkaloid 1</th>
<th>Alkaloid 2</th>
<th>Alkaloid 3a</th>
<th>Alkaloid 3b</th>
<th>Alkaloid 4</th>
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<tr>
<td></td>
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$J$ values (Hz) are in parenthesis.

The absolute configuration of C-6a was assigned as $S$, because it has a negative Cotton effect at 280 nm and a positive Cotton effect at 240 nm in CD curve. Additionally, this was confirmed by the positive value of optical rotation $\alpha_0^D = +51.7$ (c 0.38, CHCl$_3$). Therefore, alkaloid 1 was determined as (S)-3-methoxy-nordomesticine, an aporphine alkaloid reported previously from *Nectandra sinuate* also belonging to the Lauraceae family. This report corrects and completes spectroscopic data for this compound.

Alkaloid 2 was obtained as a yellow oil that gives a positive reaction to Dragendorff reagent and its optical rotation value was $\alpha_0^D = +33.3$ (c 0.60, CHCl$_3$). The UV and IR spectrum of 2 were similar to those of 1, except for the absorption bands at 282 nm and 1688 cm$^{-1}$, respectively.

The $^1$H and $^{13}$C NMR spectra showed the appearance of three new signals at $\delta_c$ 158.8 (C), 61.0 (CH$_3$), and 14.8 (CH$_3$), typical signals of an ethoxycarbonyl group attached to a nitrogen atom. The absolute configuration of C-6a was determined as $S$, because it showed the same Cotton effects as 1. Therefore the alkaloid compound 2 was identified as (S)-N-ethoxycarbonyl-3-methoxy-nordomesticine. Its ESI-MS spectrum gave a pseudomolecular ion peak at $m/z$ 414 [M+H]$^+$ corresponding to the molecular formula of C$_{20}$H$_{20}$NO$_4$. In HRESIMS negative mode was observed the pseudomolecular ion [M-H]$^-$, 368 [M+H+46]$^-$, respectively. The negative ion mode ESI spectrum showed peaks at $m/z$ 412 [M-H]$^-$ and $m/z$ 383 [M-H+29]$^-$, being the last one the loss of an ethyl group. This type of alkaloids with substituents N-ethoxycarbonyl have been isolated from *Lindera angustifolia* (Lauraceae).

Alkaloid 3 was obtained as a yellow oil, with an optical rotation value of $\alpha_0^D = +7.5$ (c 0.53 CHCl$_3$). The IR spectrum was similar to that of alkaloids 1 and 2. Additionally, there were observed the presence of absorptions at 2830, 2700, 1739 cm$^{-1}$, characteristic for formyl group. In HRESIMS negative mode was observed the pseudomolecular ion [M-H]$^-$ at $m/z$ 369.1133, corresponding to the molecular formula of C$_{20}$H$_{20}$NO$_4$. The negative ion mode of ESI-MS spectra showed the loss of a formyl group at $m/z$ 339 [M-H+29]. The
NMR profile was similar to those of the alkaloids 1 and 2. In the 1H NMR appeared different signals belonging to a mixture of two isomers in 3:1 ratio. The comparison of the major isomer with alkaloid 1 in NMR, showed the same pattern of substitution of aromatic ring, besides the appearance of two new signals at δ 8.25 (1H, s) in the 1H and at δ 161.9 in the 13C of the formyl group to 3. This functionality on the nitrogen led to formation of rotational isomers, which have been described previously for alkaloids with a N-formyl and N-acetyl group.21 The absolute configuration was determined as S, in the same manner as that for 1 and 2. Therefore, this alkaloid 3 was identified as (S)-N-formyl-3-methoxy-nordomesticine.

Alkaloid 4 was obtained as a white solid with a melting point of 222-223 °C (MeOH), and with an optical rotation of [α] D25N = +47.0 (c 0.55 CHCl3). The analysis of NMR data of 4 revealed that it has the same basic skeleton as 2, but it has a methyl ester according to the signals at δ 3.76 (3H, s) and δ 5.26 for 4 in place of the signals for ethyl group in 2. HRESIMS showed a pseudomolecular ion [M-H]- at m/z 398.1233 corresponding to the molecular formula C22H33NO. Therefore, the alkaloid 4 was identified as (S)-N-methoxy carbonyl-3-methoxy-nordomesticine.

The antifungal activity was evaluated through the disc diffusion method against Fusarium oxysporum f. sp. lycopersici,23 a phytopathogenic fungus that affects tomato crops, causing huge losses for farmers. The alkaloid fraction was active against F. oxysporum at 250 µg/mL. The inhibitory activity against the growth of the fungi was moderate at 5 µg/mL for (S)-3-methoxy-nordomesticine 1, while the other alkaloids were ineffective, suggesting that the presence of electron withdrawing substituents on nitrogen atom decrease the antifungal activity.

Although few evaluation reports of aporphines antifungal activity, there has been found that these are active against species such as Candida albicans,24,25 but inactive against fungal pathogens as Cladosporium herbarium.26 These results are a new evaluation report of antifungal activity of these alkaloids, where it is emphasized that the type of substituents on the nitrogen of aporphines has an effect on the antifungal activity they present.

The antibacterial activity was evaluated by radial diffusion method reported by Lerhrer27 against two Gram (+) standard strains: Staphylococcus aureus 6538 and Enterococcus faecalis 29212 and three Gram (-): Escherichia coli 25922 and Salmonella typhimurium, strains MS7953 and 14028s. Alkaloid 1 (2.5 µg) showed antimicrobial activity against the both Gram positive bacteria evaluated with values of 30 AU as shown Table 2.

It has been reported for the racemic mixture of compound 3-methoxy-nordomesticine, its activity against E. coli (MIC= 256 µg/mL) and S. aureus (MIC= 512 µg/mL),28 however in this case the compound 1 is not active against E. coli.

Similarly as in the antifungal activity, the results show that the nature of the substituent on nitrogen influences the antibacterial activity (Table 2).

**EXPERIMENTAL**

**General procedures**

Melting point was determined by using Mel-temp Fisher Johns, Laboratory Device. UV spectra were recorded on a Perkin Elmer Lambda 2S and CD spectra on a JASCO J-720 spectrometer. IR spectra were obtained on Perkin Elmer FT-IR Panagon 500 series 1000 as a thin film. Optical rotations were recorded on Schimdt-Haensch polarimeter. 'H (400 MHz) and 13C (100 MHz) NMR spectra, as well as 2D spectra (COSY, HMOC, and HMBCC) were performed on a Bruker Avance 400 MHz spectrometer using CDCl3, as an internal reference. HRMS were determined on a Shimadzu LCMS-IT-TOF mass spectrometer system with ESI in positive ion mode and negative ion mode. Column chromatography (CC) was carried out with silica gel (70-230 and 230-400 mesh, Merck), and analytical chromatography was performed using silica gel 60 PF254 (0.25 mm).

**Plant material**

The stems of *O. macrophylla* were collected in July 2006 from Nocaima, Colombia by W. Delgado. The botanical specimen was identified by A. Jara, and a voucher specimen (COL-517191), and was deposited in the Colombian National Herbarium of the National University of Colombia, Bogotá, Colombia.

**Extraction and isolation**

Dried and powdered stems (2.0 kg) of *Ocotea macrophylla* were extracted with EtOH by maceration at room temperature and concentrated in vacuum to obtain an extract (102.6 g). A sample (48.9 g) of this extract was solubilized in water using ultrasound and acidified with 5% HCl to pH 2.0. The acidic suspension was then filtered and basified with 20% NaOH to pH 8.0, and successively extracted with chloroform. The chloroform fraction (3.01 g) was subjected to column chromatography with silica gel and eluted with a gradient of petroleum ether:AcO/iPr (4:6 to 0:10) and AcO/iPr:MeOH (10:0 to 1:10), obtaining four fractions (I-IV). Fraction I (347 mg) was purified by repetitive column chromatography with silica gel and eluted with a gradient of petroleum ether:AcO/iPr (4:6 to 10:0) and AcO/iPr:MeOH (10:0 to 1:10), obtaining four fractions (I-IV). Fraction I (347 mg) was chromatographed repetitively by column (CC) on silica gel and eluted with n-hexane:AcO/iPr (9:1-8:2), CHCl3, and Tol:AcO/iPr 7:3. This yielded alkaloids 1 (7 mg) and 2 (4 mg). Fraction II (250 mg) was submitted to column chromatography on silica gel and eluted with CHCl3/MeOEt (85:15), then submitted to CC on Sephadex LH-20 (CH2OH) to yield alkaloid 3 (8 mg). Fraction IV (1433 mg) was purified by repetitive column chromatography on silica gel using CH2Cl2/MeOH 97.3 and CH2Cl2, as elution systems. Finally, successive washings with MeOH yielded alkaloid 4 (98 mg).

**Table 2.** Results antibacterial activity of compounds isolated

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<th>25922</th>
<th>14028s</th>
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</table>

(S)-3-Methoxy-nordomesticine (1): Yellow oil; [α]D25N = +51.7 (c 0.38, CHCl3); UV (MeOH) 224, 308 nm; IR (film): vmax 3404,
2923, 2855, 1585, 1464, 1274, 1129, 1041, 935, 757 cm⁻¹; CD [θ]₂₅₀⁻+3345, [θ]₂₈₀⁻250; for H and ¹³C NMR spectroscopy data, see Table 1; positive ESIMS: m/z = 340 [M-H]; HR-ESIMS: m/z = 340.1178 [M-H] (calcd. 340.1185 for C₁₂H₁₂NO₅).

(S)-N-Ethoxycarbonyl-3-methoxy-nordomesticine (2): Yellow oil; [α]₀°[b] = +33.3 (c 0.60, CHCl₃); UV (MeOH) 220, 282, 308 nm; IR (film): ν₃₃₄₀, 2928, 2830, 2700, 1739, 1464, 1236, 1145, 1039, 936, 757 cm⁻¹; CD [θ]₃₃₄₀⁺3634, [θ]₂₈₀⁻100; for H and ¹³C NMR spectroscopy data, see Table 1; positive ESIMS: m/z = 414 [M+H⁺]; 382 [M+CH₃CH₂OH]; 386 [M+CH₃CH₂OH]; negative ESIMS: m/z = 412 [M-H]; 383 [M-H-CH₂]; HR-ESIMS: m/z = 412.1385 [M-H] (calcd. 412.1396 for C₁₂H₁₄NO₅).

(S)-N-Formyl-3-methoxy-nordomesticine (3): Yellow oil; [α]₀°[b] = +47.0 (c 0.53, DMSO); UV (MeOH) 220, 282, 310 nm; IR (film): ν₃₃₄₀, 2925, 1688, 1464, 1270, 1145, 1039, 936, 768 cm⁻¹; CD [θ]₃₃₄₀⁺2321, [θ]₂₈₀⁻250; for H and ¹³C NMR spectroscopy data, see Table 1; positive ESIMS: m/z = 369 [M+H⁺]; negative ESIMS: m/z = 368 [M-H]; 339 [M-H-CHO]; HR-ESIMS: m/z = 369.1133 [M-H] (calcd. 369.1134 for C₁₂H₁₂NO₅).

(S)-N-Methoxycarbonyl-3-methoxy-nordomesticine (4): Solid cream, melting point 222-223°C (MeOH); [α]₀°[b] = +47.0 (c 0.55, CHCl₃); UV (MeOH) 220, 282, 308 nm; IR (film): ν₃₃₄₀, 3372, 2927, 1685, 1466, 1272, 1146, 1039, 935, 876 cm⁻¹; CD [θ]₃₃₄₀⁺303, [θ]₂₈₀⁻10; for H and ¹³C NMR spectroscopy data, see Table 1; positive ESIMS: m/z = 400 [M+H⁺]; 368 [M+H₂CH₂OH]; negative ESIMS: m/z = 398 [M-H]; 339 [M-H-CHO]; HR-ESIMS: m/z = 398.1233 [M-H] (calcd. 398.1240 for C₁₂H₁₂NO₅). Antifungal activity

F. oxysporum was obtained from the culture collection of University of Cundinamarca (Department of Agronomy, Laboratory of Phytopathology). PDA was used as medium for antifungal activity assays. The culture medium was inoculated with 100 µL of a solution of 10⁶ spores. The samples were prepared in solutions of different concentrations, corresponding to 50, 25, and 10 µg/mL of the alkaloid fraction and 5, 2.5, 1.0, 0.5, 0.2, and 0.1 µg/mL of the pure alkaloids. Ten microliters of samples were applied on the filter paper discs and placed on the inoculated medium. The plates were sealed and left in an incubator for 3 days at 25°C. Clear zones appearing against a growing fungus indicated the minimal amount of fraction or alkaloid required to inhibit the fungi growth. Three replicates were made for each treatment. Benomyl (benzimidazole – 5 µg) was used as a positive control, and acetone served as a negative control.²³

Antibacterial assays

The antibacterial activity was evaluated by radial diffusion method adapted from the methodology previously published by Lehrer.²⁷ The compounds were evaluated against two Gram (+) strains: Staphylococcus aureus ATCC 6538 and Streptococcus fecalis ATCC 29212 and three Gram (-) strains: Escherichia coli ATCC 25922 and Salmonella tiphymurium, ATCC 14028s and Salmonella tiphymurium MS7953. A colony isolated from each strain, ATCC 6538 and .c 878 with PBS and centrifuging. Finally the sediment was re-suspended in PBS and the optical density determined in 620 nm to calculate the number of CFU (colony forming units) per milliliter. It disperses a certain volume that contains 4 x 10⁷ CFU in each dish. The measured volume was mixed and homogenized in 15 mL agarose fused to more or less 45°C. This bacterial suspension was served in petri dishes and left to solidify at room temperature, after which were made of 2 mm diameter holes with a sterile punch.

The test samples were prepared dissolving 1 mg of the pure compound in 500 µL of DMSO, which are placed 8 µL of the sample in duplicate and incubated at 37°C for 30 min. After this time the nutrient medium was added, which contains molten agar agar and TSB, incubated for 18 h at 37°C and then diameter of inhibition zones was measured by the activity of the compound. Positive controls used were different antibiotics, Ampicillin (50 mg/mL), Kanamycin (10 mg/mL) and Tetracycline (4.12 mg/mL) at a dilution 1:100 in PBS and was used as negative controls DMSO and PBS, each control 8 µL be served by each well. The diameters of inhibition zones were measured in millimeters and the results were reported as units of activity according to the ratio which stipulates that 1 Unit of Action (UA) is equal to 0.1 mm of the inhibition zone.

SUPPLEMENTARY MATERIAL

1D and 2D NMR spectra for compounds 1-4. This material is available free of charge at http://quimicanova.sbq.org.br, in .PDF format.

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REFERENCES

Aporphine alkaloids from Ocotea macrophylla (Lauraceae)

APORPHINE ALKALOIDS FROM *Ocotea macrophylla* (LAURACEAE)

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Figure 1S. The $^1$H NMR data of 1 (CDCl3, 400 MHz)
Figure 2S. The $^{13}$C NMR data of 1 (CDCl$_3$, 100 MHz)

Figure 3S. The DEPT data of 1
Figure 4S. The $^1$H-$^1$H COSY data of 1

Figure 5S. The HMQC data of 1
Figure 6S. The HMBC data of 1

Figure 7S. The $^1$H NMR data of 2 (CDCl$_3$, 400 MHz)
Figure 8S. The $^{13}$C NMR data of 2 (CDCl$_3$, 100 MHz)

Figure 9S. The DEPT data of 2
Figure 10S. The $^1$H-$^1$H COSY data of 2

Figure 11S. The HMQC data of 2
Figure 12S. The HMBC data of 2

Figure 13S. The $^1$H NMR data of 3 (CDCl$_3$, 400 MHz)
Figure 14S. The $^{13}$C NMR data of 3 (CDCl$_3$, 100 MHz)

Figure 15S. The DEPT data of 3
Figure 16S. The $^1$H-$^1$H COSY data of 3

Figure 17S. The HMQC data of 3
Figure 18S. The HMBC data of 3

Figure 19S. The $^1$H NMR data of 4 (CDCl$_3$, 400 MHz)
Aporphine alkaloids from *Ocotea macrophylla* (Lauraceae)

Figure 20S. The $^{13}$C NMR data of 4 (CDCl₃, 100 MHz)

Figure 21S. The DEPT data of 4
Figure 22S. The $^1$H-$^1$H COSY data of 4

Figure 23S. The HMOC data of 4
Figure 24S. The HMBC data of 4