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Article

Biotransformation of the Diterpene *Ent*-18,19-dihydroxytrachylobane by *Rhizopus stolonifer*

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The diterpene *ent*-18,19-dihydroxytrachylobane was biotransformed for the first time by *Rhizopus stolonifer*, and yielded the new *ent*-11β,18,19-trihydroxytrachylobane derivative besides the new *ent*-kaur-11-ene diterpenes *ent*-16α,18,19-trihydroxykaur-11-ene and *ent*-18,19-dihydroxy-16α-methoxykaur-11-ene. Their structures were determined by spectrometric methods.

Keywords: *ent*-trachylobane diterpene, *ent*-kaur-11-ene diterpenes, biotransformation, *Rhizopus stolonifer*, fungus

Introduction

Secondary metabolites transformation by fungi is considered a useful tool for the production of new compounds under environmentally friendly conditions. Most biotransformation of natural products involves chemo-, regio-, and/or stereoselective reactions and yield products which are difficult to be obtained by chemical methods.[1]

Thus, as part of our research program on the production of novel compounds by microbial transformation of secondary metabolites,[2] the biotransformation of the *ent*-trachyloban diterpene **1** (Figure 1) by the fungus *R. stolonifer* was investigated. To the best of our knowledge this constitutes the first report of microbial transformation of **1**.

Studies on fungal biotransformation of *ent*-trachylobane diterpenes are reported in the literature.[3-7] Most of the products are hydroxylated *ent*-trachylobane derivatives formed by Csp^3 oxidation of carbons 1, 2, 3, 7, 17 or 19, or *ent*-kaur-11-ene diterpenes (with or without C-9 hydroxylation) formed by backbone rearrangement of the *ent*-trachylobane. In fact, the isolation of *ent*-kaur-11-ene diterpenes only from plants that also produce *ent*-trachylobane diterpenes strongly suggests that these latter compounds are probable precursors of *ent*-kaur-11-ene diterpenes.[3,5]

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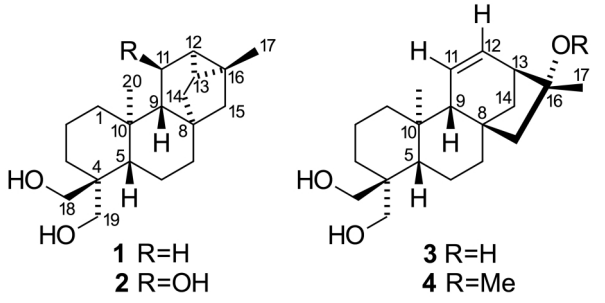

Figure 1. Chemical structures of *ent*-18,19-dihydroxytrachylobane (**1**) and its biotransformation products **2**-**4**.

Herein, we report the production of three new compounds identified as *ent*-11β,18,19-trihydroxytrachylobane (**2**), *ent*-16α,18,19-trihydroxykaur-11-ene (**3**) and *ent*-18,19-dihydroxy-16α-methoxykaur-11-ene (**4**) by fungal biotransformation of *ent*-18,19-dihydroxytrachylobane (**1**). The structures of these compounds were established mainly on the basis of their 1D and 2D nuclear magnetic resonance (NMR) spectroscopic data.

Experimental

General procedure

Melting points were determined on a Micro-Química MQAPF-302 and Mettler Toledo FP62 apparatus, and

are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer. Optical rotations were determined on a Perkin-Elmer P-2000 and Perkin-Elmer 341 polarimeters. NMR spectra (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC) were recorded in CD_3OD (Tedia®, with tetramethylsilane (TMS) as internal standard) on Bruker Avance DPX 300 (300 MHz) and Avance DPX 500 (500 MHz) spectrometers. High-resolution mass spectra (MS) were obtained on a Shimadzu LC-MS IT-TOF spectrometer equipped with an electrospray ionisation (ESI) source in positive and negative modes.

Analytical thin-layer chromatography (TLC) was performed on pre-coated 0.25 mm thick plates of silica gel 60 F_{254}, and the spots were visualized under a UV lamp (254 nm) and by spraying with a solution of perchloric acid-vanilin in EtOH, followed by heating. High performance liquid chromatography (HPLC) analyses were done on a Shimadzu instrument equipped with a LC-20AT high-pressure pump, a SPD-M20A photodiode array detector, and a normal phase Phenomenex® (10 × 150 mm, 5 μm) column. Hexane and isopropanol from Tedia®, previously filtered on nylon membrane (0.22 μm, Phenomenex®), were used as solvents. A wavelength of 254 nm was used in the isolation of the compounds by high performance liquid chromatography with photodiode array detection (HPLC-DAD).

Potato-dextrose-broth was purchased from HIMEDIA®, and all other chemical compounds were from Vetec® and Synth®.

Fungal material

R. stolonifer (strain BRF-130) was isolated from wheat seed and identified by observation of key morphological characters at the Mycology Laboratory of the Universidade Federal do Ceará (Fortaleza-CE, Brazil). The key morphological characters observed were irregular in shape often polygonal, globose, ovoid or elliptical unicellular striate sporangiospores produced on globose sporangiophores forming a cluster with branched rhizoids at the base and chlamydospores absent in the stolons.

Biotransformation of compound **1**

Mycelial plugs (5 mm diameter) of the fungal colony previously grown for 7 days in potato-dextrose-agar were transferred to seven 250 mL Erlenmeyer flasks each containing 100 mL of potato-dextrose (24 g L^{-1}) broth. After 7 days under static condition at room temperature (ca. 28 °C), the mycelium in each flask was separated from the liquid medium by vacuum filtration (aseptic conditions), and was transferred to a 250 mL Erlenmeyer flask containing 100 mL of phosphate buffer solution (pH 7) providing a total of seven flasks. Compound **1** (30 mg *per* flask) was added to six of these flasks, and one flask (no compound added) was used as the control. After 21 days in a shaker (150 rpm and 28 °C), the mycelium was separated by vacuum filtration. The liquid portion from each flask was extracted with EtOAc (3 × 50 mL) after saturation with NaCl, and the organic layer was dried with anhydrous Na_2SO_4, filtered and concentrated. After TLC analysis (EtOAc:hexane, 8:2) all extracts (except from the control experiment) were combined to afford 88.7 mg of the total extract. Purification of this extract by HPLC (wavelength: 254 nm; flow rate: 3 mL min^{-1}; injection volume: 200 μL; sample concentration: 20 mg mL^{-1}; isocratic mixture: hexane:^{i}PrOH 28%) afforded compounds **2** (R_T: 6.19 min, 10.0 mg, 5.3%), **3** (R_T: 4.82 min, 9.8 mg, 5.3%) and **4** (R_T: 5.43 min, 6.0 mg, 3.1%).

Ent-11β,18,19-trihydroxytrachylobane (**2**)

White solid; m.p. 158.3-160.7 °C; $[\alpha]^{20}_D$ −26.73 (*c* 0.1, MeOH); IR (film on ZnSe) ν_{max} / cm^{-1} 3348, 2923, 2852, 1031; HRMS *m/z* calcd.: $C_{20}H_{32}O_3Na^+$: 343.2244; found: 343.2215 $[M + Na]^+$; ^{1}H NMR (300 and 500 MHz, CD_3OD) and ^{13}C NMR (75 and 125 MHz, CD_3OD) see Tables 1 and 2.

Ent-16α,18,19-trihydroxykaur-11-ene (**3**)

Amorphous solid; $[\alpha]^{20}_D$ −71.66 (*c* 0.1, MeOH); IR (film on ZnSe) ν_{max} / cm^{-1} 3284, 3020, 2922, 2842, 1663, 1026; HRMS *m/z* calcd.: $C_{20}H_{32}O_3Na^+$: 343.2244; found: 343.2251 $[M + Na]^+$; ^{1}H NMR (300 and 500 MHz, CD_3OD) and ^{13}C NMR (75 and 125 MHz, CD_3OD) see Tables 1 and 2.

Ent-18,19-dihydroxy-16α-methoxykaur-11-ene (**4**)

White solid; m.p. 123.6-124.5 °C; $[\alpha]^{20}_D$ −7.96 (*c* 1.82, MeOH); IR (film on ZnSe) ν_{max} / cm^{-1} 3373, 2920, 2851, 1440, 1019; HRMS *m/z* calcd.: $C_{21}H_{34}O_3Na^+$: 357.2400; found: 357.2403 $[M + Na]^+$; ^{1}H NMR (300 and 500 MHz, CD_3OD) and ^{13}C NMR (75 and 125 MHz, CD_3OD) see Tables 1 and 2.

Results and Discussion

The *ent*-18,19-dihydroxytrachylobane (**1**) was recently described as the major compound in the hexane extract of roots of *Croton floribundus*.[8] This secondary metabolite was submitted to biotransformation by *R. stolonifer*, and yielded the new compounds **2** (5.3%), **3** (5.3%) and **4** (3.1%) as products.

^{1}H NMR spectrum of compound **2** showed very similar signals to those described for the *ent*-18,19-dihydroxy-trachylobane (**1**). This spectrum showed the shielded signals at δ 0.83 (m, H-13) and 1.02 (t, *J* 4.0 Hz, H-12) of the tetrasubstituted cyclopropane ring, the two angular methyl groups at δ 1.15 (s, 3H-17) and 0.90 (s, 3H-20), and the deshielding signals at δ 3.76 (d, 1H, *J* 11.2 Hz, H-19α), 3.50 (d, 1H, *J* 11.2 Hz, H-19β), 3.52 (d, 1H, *J* 11.0 Hz, H-18α) and 3.46 (d, 1H, *J* 11.0 Hz, H-18β), relative to two oxymethylene groups attached to the quaternary carbon at C-4. The only light difference was the presence of the additional signal at δ 4.11 (s, 1H, H-11) that was attributed to one oxymethine group.

The ^{13}C NMR spectrum of **2** also resembled that of compound **1**, except that the chemical shift of the C-11 (δ 73.17) was deshielded compared to the same carbon in **1** (δ 21.1), Table 2. These data led to the deduction that the methylene carbon C-11 in compound **1** was hydroxylated in **2**.

On the basis of the long-range correlations observed in the HMBC spectrum (Figure 2), the assignment of the relative position of the hydroxyl group at C-11 was determined by correlations of the hydrogen at δ 4.11 (H-11) from oxymethine group with the carbons at δ 25.65 (C-13), 48.12 (C-8) and 25.10 (C-16), and between the hydrogen at δ 0.83 (H-13) with the carbon at δ 73.17 (C-11). The relative stereochemistry of the hydroxyl group was established by the NOESY experiment. In particular, the β-orientation of hydroxyl group was determined by the diagnostic NOE cross-peaks observed between the carbinol methine at δ 4.11 (H-11) and the methyl group at δ 0.90 (CH_3-20), Figure 2. From the foregoing evidence, compound **2** was identified as the new *ent*-11β,18,19-trihydroxytrachylobane, and its molecular formula $C_{20}H_{32}O_3$ was confirmed by HRMS.

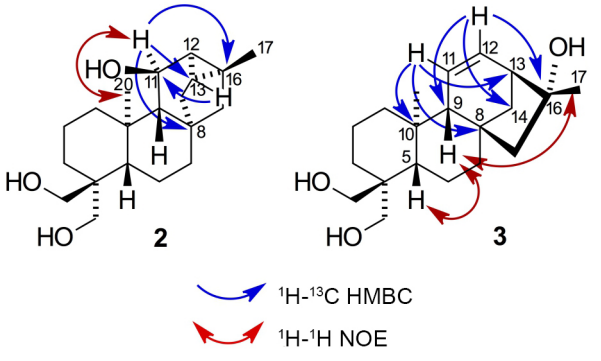

Figure 2. Long-range ^{1}H-^{13}C (HMBC) and ^{1}H-^{1}H (NOE) correlations observed for compounds **2** and **3**.

Comparison of the ^{1}H NMR data of compound **3** with those of **1** revealed some major differences between them, particularly in relation to the absence of the shielded cyclopropane protons, and the presence of two additional olefinic protons at δ 5.92 (t, 1H, *J* 8.2 Hz, H-12) and 5.55 (dd, 1H, *J* 8.2 and 3.6 Hz, H-11). These foregoing evidences suggested the opening of the cyclopropane ring on **3** and the possible formation of an *ent*-kaur-11-ene-derivative, as already reported during the biotransformation reactions of other trachyloban diterpenes.[3-5]

Further evidences for this suggestion were possible by analysis of ^{13}C NMR (broad band and DEPT) spectra that showed signals relative to a disubstituted double bond at δ 133.95 (C-12) and 128.28 (C-11), and the additional signal of one oxymethine group at δ 84.42 (C-16). The deshielded values of the carbon resonances at δ 59.73 (C-15), 51.18 (C-13) and 25.92 (C-17), compared to those data of compound **1**, indicated these carbons in a geminal position to the oxygenated function.

The HMBC correlations (Figure 2) observed between the olefinic hydrogens at δ 5.92 (H-12) with the carbons at δ 84.42 (C-16), 63.97 (C-9) and 35.18 (C-14), as well as the correlation of the other olefinic at δ 5.55 (H-11) and with the carbons at δ 51.18 (C-13), 44.51 (C-8) and 39.31 (C-10) undoubtedly determined the location of the double bond position at C_{11}-C_{12} and the hydroxyl group at C-16, respectively. The relative stereochemistry of the hydroxyl group at C-16 was established by the NOESY experiment, which showed cross-peaks between the hydrogens CH_3-17, H-9 and H-5 and indicated that they were β-oriented (Figure 2). The above data established the structure of compound **3** as a rearranged diterpene skeleton named *ent*-16α,18,19-trihydroxykaur-11-ene. This structure is in agreement with the molecular formula $C_{20}H_{32}O_3$, which was determined by HREIMS analysis. Additionally, all ^{13}C NMR data of compound **3**, especially from the rearranged moiety (carbons 11-17), are in accordance with those reported for *ent*-kaur-11-ene-derivatives.[3,4]

The ^{1}H NMR spectrum of compound **4** indicated a close relationship with those observed for **3**, through the signals relative to double bond at δ 5.59 (dd, *J* 8.5 and 3.7 Hz, H-11) and 5.89 (t, *J* 8.5 Hz, H-12), and the two oxymethylenes at δ 3.56 (d, *J* 11.3 Hz, H-18α) and 3.47 (d, *J* 11.3 Hz, H-18β), and 3.54 (d, *J* 12.1 Hz, H-19α) and 3.49 (d, *J* 12.1 Hz, 19β). A slight difference was found by the presence of the extra singlet at δ 3.13 (s, OCH_3) relative to one methoxyl group.

The proposition that hydroxyl group of compound **3** was methoxylated on **4** was suggested by comparison of their ^{13}C NMR data, that displayed one additional signal at δ 49.82 relative to the methoxyl group. The long-range connectivities in the HMBC spectrum between the methoxyl hydrogens at δ 3.13 (OCH_3) with the carbon at

Table 1. ^{1}H NMR data of *ent*-18,19-dihydroxytrachylobane (**1**) and its biotransformation products **2**-**4**

H	**1**[a]	**2**[b]	**3**[c]	**4**[b]
1	1.52 (d, *J* 11.1 Hz, Hα) 0.78 (td, *J* 7.5 and 2.4 Hz, Hβ)	1.51 (br s, Hα) 0.78 (d, *J* 3.5 Hz, Hβ)	1.79 (d, *J* 13.4 Hz, Hα) 1.03 (td, *J* 12.9 and 3.3 Hz, Hβ)	1.81 (br s, 2H)
2	1.55 (m, Hα) 1.35 (m, Hβ)	1.42 (br s, *J* 2.4 Hz, 2H)	1.62 (m, Hα) 1.47 (m, Hβ)	1.48 (br s, 2H)
3	2.00 (d, *J* 11.6 Hz, Hα) 0.96 (m, Hβ)	1.47 (br s, 2H)	1.74 (d, *J* 1.6 Hz, Hα) 1.20 (br s, Hβ)	1.35 (s, 2H)
5	0.94 (m, 1H)	1.23 (s, 1H)	1.34 (m, 1H)	1.35 (s, 1H)
6	1.60 (m, 2H)	1.68 (s, Hα) 1.07 (s, Hβ)	1.60 (m, Hα) 1.34 (m, Hβ)	1.35 (s, Hα) 1.61 (s, Hβ)
7	1.35 (m, 2H)	2.04 (br s, Hα) 1.99 (br s, Hβ)	1.69 (d, *J* 10.3 Hz, Hα) 1.59 (d, *J* 4.1 Hz, Hβ)	1.61 (br s, 2H)
9	1.14 (m, 1H)	1.29 (m, 1H)	1.46 (br s, 1H)	1.48 (br s, 1H)
11	1.88 (td, *J* 13.0 and 3.0 Hz, Hα) 1.64 (ddd, *J* 13.6, 6.1 and 1.8 Hz, Hβ)	4.11 (s, 1H)	5.55 (dd, *J* 8.2 and 3.6 Hz, 1H)	5.59 (dd, *J* 8.5 and 3.7 Hz, 1H)
12	0.56 (d, *J* 7.5 Hz, 1H)	1.02 (t, *J* 4.0 Hz, 1H)	5.92 (t, *J* 8.2 Hz, 1H)	5.89 (t, *J* 8.5 Hz, 1H)
13	0.80 (dd, *J* 7.5 and 2.4 Hz, 1H)	0.83 (m, 1H)	2.18 (dd, *J* 6.2 and 3.2 Hz, 1H)	2.45 (m, 1H)
14	2.02 (m, Hα) 1.14 (m, Hβ)	1.68 (s, Hα) 1.07 (s, Hβ)	1.83 (d, *J* 10.9 Hz, Hα) 1.62 (m, Hβ)	1.77 (s, 2H)
15	1.36 (d, *J* 11.2 Hz, Hα) 1.23 (d, *J* 11.2 Hz, Hβ)	1.68 (s, Hα) 1.07 (s, Hβ)	1.47 (br s, 2H)	1.59 (br s, Hα) 1.61 (br s, Hβ)
17	1.12 (s, 3H)	1.15 (s, 3H)	1.27 (s, 3H)	1.29 (br s, 3H)
18	3.88 (d, *J* 10.5 Hz, Hα) 3.33 (d, *J* 10.5 Hz, Hβ)	3.52 (d, *J* 11.0 Hz, Hα) 3.46 (d, *J* 11.0 Hz, Hβ)	3.54 (d, *J* 11.1 Hz, 2H)	3.56 (d, *J* 11.3 Hz, Hα) 3.47 (d, *J* 11.3 Hz, Hβ)
19	3.91 (d, *J* 10.5 Hz, Hα) 3.71 (d, *J* 10.5 Hz, Hβ)	3.76 (d, *J* 11.2 Hz, Hα) 3.50 (d, *J* 11.2 Hz, Hβ)	3.77 (d, *J* 3.0 Hz, 2H)	3.54 (d, *J* 12.1 Hz, Hα) 3.49 (d, *J* 12.1 Hz, Hβ)
20	0.90 (s, 3H)	0.90 (s, 3H)	0.96 (s, 3H)	0.95 (s, 3H)
OCH_3	–	–	–	3.13 (s)

[a]500 MHz, $CDCl_3$; [b]300 MHz, CD_3OD; [c]500 MHz, CD_3OD.

Table 2. ^{13}C NMR data of *ent*-18,19-dihydroxytrachylobane (**1**) and its biotransformation products **2**-**4**

C	**1**[a]	**2**[b]	**3**[c]	**4**[b]
1	39.0	40.17	40.69	40.71
2	17.3	18.56	18.79	18.79
3	30.4	30.70	30.63	30.64
4	41.7	43.33	43.56	43.56
5	53.5	51.23	49.81	49.91
6	20.7	20.81	21.33	21.32
7	39.3	32.26	42.39	42.44
8	40.6	48.12	44.51	44.14
9	53.5	56.40	63.97	64.02
10	38.2	39.25	39.31	39.32
11	21.1	73.17	128.28	129.00
12	20.7	32.06	133.95	133.64
13	24.4	25.65	51.18	45.97
14	33.5	20.81	35.18	34.83
15	50.5	46.76	59.73	57.45
16	22.6	25.10	84.42	90.10
17	20.7	20.81	25.92	19.93
18	74.0	69.76	69.79	69.79
19	65.0	64.18	64.18	64.18
20	15.1	15.94	18.64	18.65
OCH_3	–	–	–	49.82

[a]125 MHz, $CDCl_3$; [b]75 MHz, CD_3OD; [c]125 MHz, CD_3OD.

δ 90.10 (C-16) confirmed this proposition. As observed on the **3**, the relative stereochemistry of the methoxyl group at C-16 was defined as α-oriented by the same correlations observed on compound **3**, in the NOESY experiment.

In addition to the NMR data, analysis of **4** by HRMS allowed the establishment of its molecular formula as $C_{21}H_{32}O_3$. Thus, the structure of compound **4** was determined to be the new *ent*-18,19-dihydroxy-16α-methoxykaur-11-ene. Methylation of the hydroxyl group at C-16 of an *ent*-kaur-11-ene diterpene by *Rhizopus arrhizus* has been reported previously.[4]

Conclusion

In summary, the *ent*-trachyloban diterpene *ent*-18,19-dihydroxytrachylobane (**1**) was biotransformed by *R. stolonifer*, and produced the new *ent*-11β,18,19-trihydroxytrachylobane (**2**), and the new *ent*-kaurene diterpenes *ent*-16α,18,19-trihydroxykaur-11-ene (**3**) and *ent*-18,19-dihydroxy-16α-methoxykaur-11-ene (**4**). The formation of derivative **2** involved the first hydroxylation of C-11 of *ent*-trachyloban diterpene skeleton by fungus, and compounds **3** and **4** were probably produced by backbone

rearrangement of **2**. These results corroborate the potential application of microbial transformation of natural products for the formation of new compounds.

Supplementary Information

The 1D and 2D NMR spectra data associated with this article are available free of charge at http://jbcs.sbq.org.br as a PDF file.

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References

1. Liu, J-H.; Yu, B-Y.; *Curr. Org. Chem.* **2010**, *14*, 1400**.**
2. Nunes, F. M.; Santos, G. F.; Saraiva, N. N.; Trapp, M. A.; Mattos, M. C.; Oliveira, M. C. F.; Rodrigues-Filho, E.; *Appl. Catal., A* **2013**, *468*, 88.
3. Silva, E. A.; Takahashi, J. A.; Oliveira, A. B.; *J. Braz. Chem. Soc*. **2002**, *13*, 101.
4. Leverrier, A.; Martin, M-T.; Servy, C.; Ouazzani, J.; Retailleau, P.; Awang, K.; Mukhtar, M. R.; Guéritte, F.; Litaudon, M.; *J. Nat. Prod.* **2010**, *73*, 1121.
5. Fraga, B. M.; Gonzalez-Vallejo, V.; Guillermo, R.; *J. Nat. Prod.* **2011**, *74*, 1985.
6. Fraga, B. M.; Bressa, C.; Gonzalez-Vallejo, V.; González, P.; Guillermo, R.; *Phytochemistry* **2012**, *81*, 60.
7. Diaz, C. E.; Fraga, B. M.; Gonzalez, A. G.; Gonzalez, P.; Hanson, J. R.; Hernandez, M. G.; *Phytochemistry* **1984**, *23*, 2813.
8. Uchôa, P. K. S.; Silva-Jr, J. N.; Silveira, E. R.; Lima, M. A. S.; Braz-Filho, R.; Costa-Lotufo, L. V.; Araújo, A. J.; Moraes, M. O.; Pessoa, C.; *Quim. Nova* **2013**, *36*, 778.

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