Pergamon

0031-9422(95)00612-5

Phytochemistry, Vol. 41, No. 2, pp. 473–475, 1996

0031-9422/96 $15.00 + 0.00

HYDROXYLATION OF SODIC GRINDELATE BY *CUNNINGHAMELLA ECHINULATA*

C. R. CARRERAS, J. RODRIGUEZ, H. J. SILVA, P. ROSSOMANDO, O. S. GIORDANO and E. GUERREIRO

Química Orgánica-Intequi-Conicet-Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera 5700, San Luis, Argentina

(*Received in revised form* 12 *July* 1995)

Key Word Index—*Cunninghamella echinulata*; fungus; biotransformation; sodic grindelate; 2β-hydroxygrindelic acid; 3β-hydroxygrindelic acid; 6,8(17)-dehydrogrindelic acid.

Abstract—A new grindelane derivative: 2β-hydroxygrindelic acid and the known grindelanes 3β-hydroxygrindelic acid and 6,8(17)-dehydrogrindelic acid were obtained by microbial transformation of sodic grindelate using cultures of *Cunninghamella echinulata*.

INTRODUCTION

It has been shown that fungi can be used for the transformation of grindelic acid into different derivatives, namely 3β-hydroxy, 3-keto and 18-hydroxy. For 6,8(17)-dehydrogrindelic acid and methyl 7,8-epoxigrindelate, only the 3β-hydroxy was obtained [1–3]. Some of these naturally occurring monohydroxylated derivatives has been reported to display antifeedant activities [4]. Previous studies from this laboratory have described the isolation of grindelic acid as the major constituent of *Grindelia pulchella* Dun. and *Grindelia chiloensis* Cabr. [5, 6]. Considering the abundance of both species in the region of Cuyo (Argentina) and the biological properties of the hydroxylated derivatives, it was important to produce them by microbial transformation. The present study describes the production of a new hydroxylate grindelic acid derivative by cultures of *Cunninghamella echinulata* (NRRL 3655) and its structural elucidation.

RESULTS AND DISCUSSION

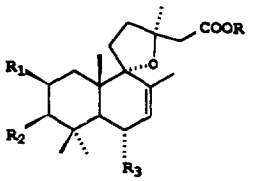
Preliminary experiments were focussed on the identification of microbial cultures capable of hydroxylating grindelic acid (**1**). Several strains of moulds belonging to the genus *Cunninghamella* and *Aspergillus*, commonly used in biotransformation studies for their hydroxylating capacity, were cultivated in the presence of the substrate. According to the results obtained using TLC for analysis, *C. echinulata* (NRRL 3655) was selected.

The substrate was always added as the sodic salt due to the insolubility of grindelic acid in aqueous solutions. In order to provide sufficient material for structure elucidation the biotransformation of sodic grindelate was carried out at preparative scale. After incubation for 5 days at 28°, no initial substrate was detected in the filtrate and cell mass.

The crude metabolites obtained by ether extraction from the acidified culture medium was methylated with CH_2N_2 and then purified by a combination of silica gel column chromatography and HPLC, which resulted in the isolation of three grindelate derivatives (**2a**–**4a**).

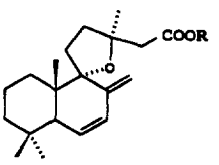
One metabolite was identified as methyl 3β-hydroxy grindelate (**3a**) by comparison of NMR data with published values [7], the second as methyl 6,8(17)-dehydrogrindelate (**4a**) by comparison with an authentic sample [5]. It is probable that **4** resulted from the dehydration of 6-hydroxygrindelic acid, during extraction in the acidified medium. The third metabolite (**2a**), an oil of molecular formula $C_{21}H_{34}O_4$, could not be identified from existing literature data.

The molecular formula and the strong hydroxyl absorption at 3470 cm^{-1} in the IR (KBr) spectrum, suggested that **2a** was a mono-hydroxy derivative of grindelic acid. The ^1H and ^{13}C (DEPT) NMR signals indicated the presence of a secondary hydroxyl group (δ_H4.35, δ_C68.8). In accordance with previously reported data [1] and the base peak at *m/z* 210 corresponding to the right-hand half of the RDA fragment, required the hydroxyl group to be in the ring A. The signal at δ4.35 can only be assigned to an equatorial H-2 based on the coupling pattern which appeared as *dddd* $J_{2,1\alpha} = J_{2,1\beta} = J_{2,3\alpha} = J_{2,3\beta} = 3.7$ Hz. This was confirmed by the COLOC technique optimized to 8 Hz, that emphasizes the transoid CH relationship over three bonds [8], observing the correlation between H-2α (4.35), C-4 (32.4) and C-10 (40.1). Furthermore, the relative positions of C-18, C-19 and C-20 were unambiguously established by the combined NOESY phase sensitive [9] and COLOC techniques. The H-19 signal (1.15) showed dipolar couplings



	R	R_1	R_2	R_3
1	H	H	H	H
2	H	OH	H	H
3	H	H	OH	H
5	H	H	H	OH

1a-4a R=Me



4
R=H

(NOE) with the signals at 0.9 (H-18) and 1.08 (H-20). H-19, in the COLOC of **2a**, can be assigned by its correlation with C-4, C-3 (46.8), C-18 (33.7) and C-5 (42.4). H-18 also showed the same correlation with C-4, C-3, C-5 and C-19 (24.9), while H-20 showed correlation with C-5, C-9 (90.8), C-10 and C-1. Further evidence from the COSY and HETCOR experiments support the structural assignment of **2a**. Taking into consideration the above data, the structure of **2a** was elucidated as methyl 2β-hydroxy grindelate.

EXPERIMENTAL

General. ^1H and ^{13}C NMR spectra were determined at 200.1 and 50.3 MHz respectively, in $CDCl_3$. The 2D pulses sequences were provided by BRUKER. IR spectra were obtained as KBr disk. MS were obtained by direct inlet system at 70 eV. CC and TLC were performed with silica gel 60 (70–230 mesh) and on a precoated Kieselgel 60 F_{254} plates. For TLC the following solvent systems were used: 1) benzene–dioxane–HOAc (30:5:1), 2) *n*-hexane–EtOAc (3:2), detection was achieved by spraying with H_2SO_4–HOAc–H_2O (4:20:1) followed by heating. Prep HPLC was performed on a PREPEX 25-40 Si (250 × 10 mm) column, using *n*-hexane–EtOAc (3:1); flow rate: 1 ml min^{-1} and refractive index detector.

Isolation of substrate. Grindelic acid was isolated from aerial parts of *Grindelia pulchella* (2.5 kg) by the method of ref. [5].

Microorganisms. The organisms utilized were *Cunninghamella echinulata* (NRRL 3655), *C. echinulata* (AMRRL 740), *Aspergillus niger* (ATCC 16888) and *A. niger* (ATCC 11394).

Fermentation. The screening experiments were carried out in indented conical flasks (125 ml) with 30 ml of culture medium placed on a rotary shaker at 180 rpm and incubated at 28°. Media were adjusted to pH 6.2–6.5 and sterilized at 121° for 15 min. A two-stage fermentation procedure was utilized. Sterile modified Czapek medium [10] supplemented with yeast extract (5 g l^{-1}) was inoculated with surface growth from agar slants and incubated for 72 h (first-stage cultures). The first stage culture broth (2 ml) was then inoculated into the same medium without the yeast extract (second-stage cultures). Sodic grindelate was added to 24 hr old second-stage cultures at 0.5 mg ml^{-1}. Controls of inoculated media without substrate and sterile media with substrate were run in each assay. Samples from the second-stage cultures were taken at 24 hr intervals for analysis. The samples (3 ml) were acidified to pH 3 with 6M HCl and extracted with 0.5 ml Et_2O, followed by centrifugation and subsequent analysis of the ethereal layers by TLC (solvent 1). The prep. scale experiment was conducted in a 2 l fermentor containing 1 l culture sol and incubated at 28°. Sodic grindelate was added at a final concn of 1 mg ml^{-1} and the culture was sampled daily for TLC, beginning 24 hr after addition of the substrate until a complete biotransformation was achieved.

Extraction and Purification. Culture medium and micelia were separated by filtration. The filtrate was acidified to pH 3 with 6M HCl before extraction with Et_2O (20% of vol. × 3). The combined ethereal layers were evaporated by dryness. The dry residue was taken up into $CHCl_3$ impregnated with a small amount of silica gel and followed by further removal of solvent *in vacuo*. The resultant material was washed successively with *n*-hexane and *n*-hexane–EtOAc gradient. The *n*-hexane–EtOAc (4:1) and (3:2) eluates were evaporated and dissolved in Et_2O and treated with excess CH_2N_2–Et_2O. The solvent was evaporated and the crude material chromatographed on silica gel column eluted with *n*-hexane–EtOAc (9:1 and 7:3, respectively). The first gave 135 mg of **4a** and the second one afforded a mixture of **2a** and **3a**. The mixture was subjected to prep. HPLC to give the pure grindelic derivatives **2a** (104 mg) and **3a** (169 mg).

Methyl 2β-hydroxygrindelate **2a**. Oil, ^1H NMR: δ ($CDCl_3$) 0.90 (3H, *s*, H-18), 1.08 (3H, *s*, H-20), 1.15 (3H, *s*, H-19), 1.32 (3H, *s*, H-16), 1.77 (3H, *s*, H-17), 2.61 (1H, *d*, H-14, J = 14.3 Hz), 2.76 (1H, *d*, H-14, J = 14.3 Hz), 3.65 (3H, *s*, OMe), 4.35 (1H, *dddd*, H-2), 5.54 (1H, *br s*, H-7). ^{13}C NMR: 38.1 (C-1 or C-12), 68.8 (C-2), 46.8 (C-3), 32.4 (C-4), 42.4 (C-5), 23.9 (C-6), 126.6 (C-7), 134.4 (C-8), 90.8 (C-9), 40.1 (C-10), 28.8 (C-11), 38.2 (C-12 or C-1), 81.6 (C-13), 47.9 (C-14), 171.8 (C-15), 27.4 (C-16), 21.3 (C-17), 33.7 (C-18), 24.9 (C-19), 18.8 (C-20), 51.4 (OMe); $[\alpha]_D$ − 106.3° ($CHCl_3$, c = 0.38). MS *m/z* (rel. int.): 350 [M +] (< 1%), 332 (2), 301 (1), 277 (2), 210 (100), 178 (3), 136 (13), 122 (3), 121 (5), 107 (7), 96 (7).

Acknowledgements—This work was supported by CONICET and the Universidad Nacional de San Luis.

REFERENCES

1. Hoffmann, J. J., Punnapayak, H., Jolad, S. D., Bates, R. B. and Camou, F. A. (1988) *J. Nat Prod.* **51**, 125.
2. Hoffmann, J. J., Jolad, S. D., Bates, R. B. and Camou, F. A. (1988) *Phytochemistry* **27**, 2125.
3. Aladesanmi, A. J. and Hoffmann, J. J. (1991) *Phytochemistry* **30**, 1847.

4. Rose, A. F., Jones, K. C., Haddhon, W. F. and Dreyer, D. L. (1981) *Phytochemistry* **20**, 2249.
5. Guerreiro, E., Kavka, J., Saad, J. R., Oriental, M. A. and Giordano, O. S. (1981) *Rev. Latinoamer. de Química* **12**(2), 77.
6. Guerreiro, E., Kavka, J. and Giordano, O. S. (1982) *Rev. Latinoamer. de Química* **73**, 72.
7. Bohlmann, F., Ahmed, M., Borthakur, N., Wallmeyer, M., Jakupovic, J., King, R. M. and Robinson, H. (1982) *Phytochemistry* **21**, 167.
8. Breitmaier, E. (1993) *Structure Elucidation by NMR in Organic Chemistry*, p. 46. J. Wiley & Sons, New York.
9. Bodenhausen, G., Kogler, H. and Ernst, R. R. (1984) *J. Magn. Res.* **58**, 370.
10. Prema, B. R. and Bhattacharya, P. K. (1962) *Appl. Microbiol.* **10**, 524.