



Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Gastroprotective effects of new diterpenoid derivatives from *Azorella cuatrecasii* Mathias & Constance obtained using a β -cyclodextrin complex with microbial and chemical transformations

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ARTICLE INFO

Article history:

Received 16 March 2016

Revised 25 May 2016

Accepted 27 May 2016

Available online 28 May 2016

Keywords:

Azorella cuatrecasii

Biotransformation

Microbial transformation

Mulinane

Gastric ulcer

ABSTRACT

Mulinane diterpenoids isolated from *Azorella* species have displayed gastroprotective effects in animal models. In this study we have transformed the main constituent, mulin-11,13-dien-20 oic acid from this plant using the filamentous fungus *Mucor plumbeus* and a β -cyclodextrin inclusion complex and we have obtained two main products with good yields (33% and 15% for compound **4** and **5**, respectively) for further preparation of semisynthetic derivatives to evaluate their gastroprotective effects. In addition, one of the compounds isolated from *Azorella cuatrecasii* was new (9-*epi*-13 α -hydroxymulinene **1**). Six new derivatives **4a–4c** and **5a–5c** were then prepared by simple chemical transformations. The structures of all compounds were elucidated by spectroscopic means based on 1D and 2D-NMR techniques. Some 8 diterpenes were evaluated for their gastroprotective effects in the ethanol/HCl-induced ulcer model in mice at 20 mg/kg. The highest gastroprotective activity was shown by 7 α ,16-dihydroxymulin-11,13-dien-20-oic acid **5**, which was higher than the reference drug lansoprazole, while 16-hydroxymulin-11,13-dien-20-oic acid **4** was as active as lansoprazole.

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Plants belonging to the genus *Azorella* (Apiaceae) are popularly known as 'llareta' and play an important role in folk medicine in the Andes of South America mainly in Peru, Bolivia, the north of Chile and the west of Argentina. No data related to their folk use of *Azorella cuatrecasii* have been reported in Colombia. Previous chemical investigations of this genus have reported the presence of terpenoids,^{1–9} isoflavonoids and chromones in different *Azorella* species.^{10,11} Secondary metabolites from this plant have displayed interesting biological activities such as gastroprotective,^{10,11} trypanosomicidal,¹² trichomonocidal,² toxoplasmodicidal,¹³ antiplasmodial,³ antibacterial,¹¹ spermicidal,¹⁴ antihyperglycemic,⁷ antitubercular,^{4,5,8} antiinflammatory and analgesic activities.¹⁵ Previous microbial transformation of a diterpene known as mulin-11,13-dien-20 oic acid by *Mucor plumbeus* led to its 16-hydroxy and 7 α ,16-dihydroxy derivatives but reached very low yield.¹⁶ Therefore, this diterpenoid was included to β -cyclodextrin for the improvement of the bioconversion yield.

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From a low-polar extract of *A. cuatrecasii* a new diterpenoid (**1**) plus two known compounds¹⁰ (**2** and **3**) were isolated and identified on the basis of their spectroscopic analysis. Then, the complex mulin-11,13-dien-20-oic acid **3**/ β -cyclodextrin was subjected to biotransformation by *M. plumbeus* and a significant increase in the biotransformation yield was obtained (33% for compound **4** and 15% for compound **5**). In addition, six new derivatives (**4a–4c** and **5a–5c**) were prepared from compounds **4** and **5** by simple chemical reactions (scheme for this synthesis in Supporting information). The chemical structures of the compounds are shown in Figure 1. Compounds **1**, **4a–4c** and **5a–5c** are reported for the first time. The structural elucidation of the new compound, the biotransformation and the gastroprotective activity is explained below.

9-*epi*-13 α -Hydroxymulinene (**1**) was isolated as a gummy liquid, and had a molecular formula of C₂₀H₃₄O according to NMR and HRESIMS data. The ¹H NMR data (Table 1) showed two doublets assigned to methyl groups at δ_{H} 0.83 (d, *J* = 6.3 Hz; CH₃-18) and δ_{H} 0.93 (d, *J* = 6.3 Hz; CH₃-19) belonging to an isopropyl group, three methyl groups at δ_{H} 0.84 (s, CH₃-20), 1.02 (s, CH₃-17) and 1.33 (s, CH₃-16), a broad triplet at δ_{H} 3.48 (*J* = 7.3; 7.3 Hz, H-9)

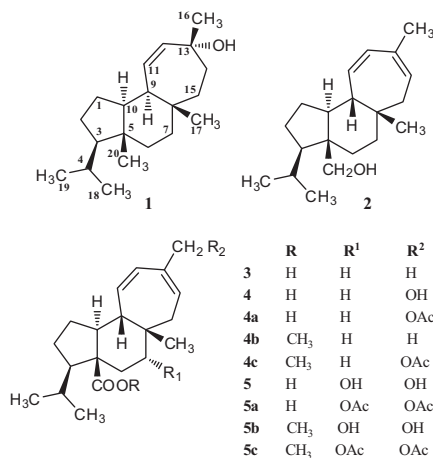


Figure 1. Chemical structures of compounds 1–5, 4a–4c and 5a–5c.

and two signals at δ_{H} 5.60 (dd, $J = 10.6$; 1.8 Hz, H-12) and 5.89 (dd, $J = 10.6$; 7.3 Hz, H-11), which indicated the presence of a 9-*epi*-mulinane skeleton published by us recently.¹¹ The ¹³C NMR spectrum showed resonances for 20 carbons. DEPT analysis indicated the presence of five methyl carbons, six methylenes, six methine carbons and three non-hydrogenated carbons. DEPT 135° spectrum showed two sp^2 methine carbons at δ_{C} 135.1 (C-12) and 136.9 (C-11), four saturated methines at δ_{C} 31.3 (C-4), 41.3 (C-9), 45.6 (C-10) and 60.8 (C-3), and six methylenes at δ_{C} 22.6 (C-1), 28.3 (C-2), 34.9 (C-6), 45.3 (C-7), 35.7 (C-14) and 35.2 (C-15). ¹³C and ¹H chemical shifts in **1** were quite similar to that reported for 7 α -acetoxy-9-*epi*-13 β -hydroxymulinane¹¹ which confirmed that **1** has a *trans-syn-trans* mulinane arrangement. Cross peak correlations in the HMQC and HMBC spectra corroborated all proposed connectivities. The unusual proton signal at δ_{H} 3.48 (H-9) showed correlations with the carbon signals at δ_{C} 39.5 (C-5), 45.3 (C-7), 135.1 (C-12) and 19.5 (C-17), and the proton signal at δ_{H} 1.94 (H-10) had cross peaks

with δ_{C} 22.6 (C-1), 39.8 (C-5), 17.9 (C-20) and 136.9 (C-11) confirming that the proton signal at δ_{H} 3.48 was at C-9. The relative configuration was assigned based on a 2D NOESY experiment. In the NOESY spectrum, correlation between δ_{H} 1.94 (H-10 α) and 3.48 (H-9) indicated that H-9 had also α -configuration. In addition, H₃-17 showed a cross peak with H₃-16 and H₃-20 indicating that methyl groups were in the β -configuration. Finally, compound **1** is the second example of a *trans-syn-trans* mulinane arrangement in nature.¹¹

16-Acetoxy-11,13-dien-20-oic acid (**4a**) was isolated as a gummy liquid. The ¹H and ¹³C NMR spectrum of **4a** was close to that of **4**. The main difference was an additional acetoxy group at δ_{H} 2.08 in the ¹H NMR spectrum. The ¹³C NMR spectrum of **4a** supported this structure with resonances at δ_{C} 171.3 and 20.9 indicating acetylation of the alcohol function at C-16.

16-Hydroxymulin-11,13-dien-20-oic acid methyl ester (**4b**) was isolated as a gummy liquid. Treatment of **4** with diazomethane afforded the corresponding methyl ester **4b**. The ¹H and ¹³C NMR spectrum of **4b** was very similar to that of **4** except for the signal attributed to the additional methyl ester. Such resonances were at δ_{H} 3.69 in ¹H NMR and δ_{C} 50.7 in ¹³C NMR.

16-Acetoxy-11,13-dien-20-oic acid methyl ester (**4c**) was isolated as a gummy liquid. The main difference between **4a** and **4c** was the presence of a methyl ester. This additional methyl ester group gave resonances at δ_{H} 3.69 in ¹H NMR and δ_{C} 50.7 in ¹³C NMR indicating methylation of the carboxylic acid group at C-20.

7 α ,16-Diacetoxy-11,13-dien-20-oic acid (**5a**): The ¹H and ¹³C NMR spectrum of **5a** was close to that of **5**. The main differences were two additional acetoxy groups at δ_{H} 2.07 (6H) in the ¹H NMR spectrum. The ¹³C NMR spectrum of **5a** supported this new structure with resonances at δ_{C} 171.1, 170.1, 21.0 and 20.9 indicating acetylation of the alcohol groups at C-7 and C-16.

7 α ,16-Dihydroxymulin-11,13-dien-20-oic acid methyl ester (**5b**) was isolated as a viscous liquid. Treatment of **5** with diazomethane afforded the corresponding methyl ester **5b**. The ¹H and ¹³C NMR spectrum of **5b** was similar to that of **5** except for an additional methyl ester (δ_{H} 3.69 in ¹H NMR and δ_{C} 51.0 in ¹³C NMR).

7 α ,16-Diacetoxy-11,13-dien-20-oic acid methyl ester (**5c**) was isolated as a gummy liquid. The main difference between **5a** and **5c** was the presence of a methyl ester group. This additional methyl ester group gave chemical shifts at δ_{H} 3.75 in ¹H NMR and δ_{C} 51.1 in ¹³C NMR indicating methylation of the carboxylic acid group at C-20.

The ¹H and ¹³C NMR data of compound **1** are presented in Table 1, while the data for compounds **4a–4c**, and **5a–5c** is shown in Supporting information.

Regarding to biotransformation of compound **3**, we found that this compound was lipophilic and sparingly soluble in H₂O. To overcome this limitation, a β -cyclodextrin-inclusion complex was prepared and added to the culture medium to perform the microbial transformation.¹⁷ Using this methodology we have obtained 33% and 15% of yield instead of 0.8% and 0.75% for compounds **4** and **5** respectively.¹⁶

As shown in the Table 2, diterpenoids **4**, **5**, **4a**, **4b**, **5a** and **5b** at 20 mg/kg, p.o. showed gastroprotective activity on the HCl/EtOH-induced gastric lesions model in mice. The best gastroprotective effect was observed for compound **5** (69%), which was more active than lansoprazole (57%). The protection displayed by **4** (59%) was similar to that observed with lansoprazole at 20 mg/kg while compounds **4a**, **4b**, **5a**, and **5b** protected by 43%, 18%, 48%, and 36% respectively. The derivatives **4c** and **5c** were inactive on this model. Compound **1** and **2** were not tested for their gastroprotective activity, while compound **3** was evaluated previously.¹¹

It is well known that the impairment of the balance between aggressive (stress, chemical agents, drugs, gastric acid and pepsin

Table 1
NMR data of **1** and the main 2D correlations (400 MHz, CDCl₃)

C	¹ H NMR	¹³ C NMR	HMBC	NOESY
1	1.42 ⁺ m 1.38 ⁺ m	22.6 t		
2	1.84 ⁺ m 1.25 ⁺ m	28.3 t		
3	1.07 ⁺ m	60.8 d		
4	1.43 ⁺ m	31.3 d		
5	-	39.8 s	-	
6	1.76 ⁺ m 1.72 ⁺ m	34.9 t		
7	1.37 ⁺ m 1.28 ⁺ m	45.3 t		
8	-	34.3 s		
9	3.48 brt (7.3; 7.3)	41.3 d	C-5, C-7, C-12, C-17	H-10
10	1.94 m	45.6 d	C-1, C-4, C-11, C-20	H-9
11	5.89 dd (10.6; 7.3)	136.9 d	C-10, C-13	
12	5.60 dd (10.6; 1.8)	135.1 d	C-9, C-13, C-16	
13	-	72.2 d		
14	1.58 ⁺ m 1.56 ⁺ m	35.7 t	C-12	
15	1.82 ⁺ m 1.61 m	35.2 t		
16	1.33 s	34.1 q	C-12, C-13, C-14	H ₃ -17
17	1.02 s	19.5 q	C-7, C-9, C-15	H ₃ -16, H ₃ -20
18	0.83 d (6.3)	22.6 q	C-3, C-4, C-19	
19	0.91 d (6.3)	22.4 q	C-3, C-4, C-18	
20	0.84 s	17.9 q	C-5, C-6, C-10	H ₃ -17

⁺ Overlapped signals. The chemical shift of these protons was measured on HMQC.

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