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

Beatriz Sepúlveda [a], Cristina Quispe [b], Mario Simirgiotis [c], Olimpo García-Beltrán [d,*], Carlos Areche [e,*]

[a] Departmento de Química, Universidad Andrés Bello, Viña del Mar, Chile
[b] Facultad de Ciencias de la Salud, Universidad Arturo Prat, Iquique, Chile
[c] Laboratorio de Productos Naturales, Departamento de Química, Facultad de Ciencias Básicas, Universidad de Antofagasta, Antofagasta, Chile
[d] Facultad de Ciencias Naturales y Matemáticas, Universidad de Ibagué, Carrera 22 Calle 67, Ibagué 730001, Colombia
[e] Departamento de Química, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

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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 cuatrecasasii* was new (9-*epi*-13α-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 7α,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.

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 cuatrecasasii* 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] trichomonicidal,[2] toxoplasmocidal,[13] antiplasmodial,[3] antibacterial,[11] spermicidal,[14] antihiperglicemic,[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 7α,16-dihydroxy derivatives but reached very low yield.[16] Therefore, this diterpenoid was included to β-cyclodextrin for the improvement of the bioconversion yield.

From a low-polar extract of *A. cuatrecasasii* a new diterpenoid (**1**) plus two known compounds[10] (**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*-13α-Hydroxymulinene (**1**) was isolated as a gummy liquid, and had a molecular formula of $C_{20}H_{34}O$ according to NMR and HRESIMS data. The ^{1}H NMR data (Table 1) showed two doublets assigned to methyl groups at δ_H 0.83 (d, J = 6.3 Hz; CH_3-18) and δ_H 0.93 (d, J = 6.3 Hz; CH_3-19) belonging to an isopropyl group, three methyl groups at δ_H 0.84 (s, CH_3-20), 1.02 (s, CH_3-17) and 1.33 (s, CH_3-16), a broad triplet at δ_H 3.48 (J = 7.3; 7.3 Hz, H-9)

* Corresponding authors. Tel.: +56 2 29787218; fax: +56 2 2713888.
E-mail addresses: jose.garcia@unibague.edu.co (O. García-Beltrán), areche@uchile.cl (C. Areche).

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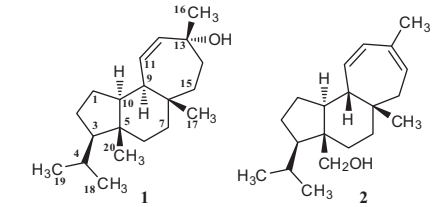

	R	R¹	R²
3	H	H	H
4	H	H	OH
4a	H	H	OAc
4b	CH_3	H	H
4c	CH_3	H	OAc
5	H	OH	OH
5a	H	OAc	OAc
5b	CH_3	OH	OH
5c	CH_3	OAc	OAc

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 an 9-*epi*-mulinane skeleton published by us recently.[11] The ^{13}C 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). ^{13}C and ^{1}H chemical shifts in **1** were quite similar to that reported for 7α-acetoxy-9-*epi*-13β-hydroxymulinane[11] 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-10α) and 3.48 (H-9) indicated that H-9 had also α-configuration. In addition, H_3-17 showed a cross peak with H_3-16 and H_3-20 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]

Table 1
NMR data of **1** and the main 2D correlations (400 MHz, $CDCl_3$)

C	^{1}H NMR	^{13}C NMR	HMBC	NOESY
1	1.42* m	22.6 t		
	1.38* m			
2	1.84* m	28.3 t		
	1.25* m			
3	1.07* m	60.8 d		
4	1.43* m	31.3 d		
5	-	39.8 s	–	
6	1.76* m	34.9 t		
	1.72* m			
7	1.37* m	45.3 t		
	1.28* m			
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	35.7 t	C-12	
	1.56* m			
15	1.82* m	35.2 t		
	1.61 m			
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.

16-Acetoxymulin-11,13-dien-20-oic acid (**4a**) was isolated as a gummy liquid. The ^{1}H and ^{13}C 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 ^{1}H NMR spectrum. The ^{13}C 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 ^{1}H and ^{13}C 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 ^{1}H NMR and δ_C 50.7 in ^{13}C NMR.

16-Acetoxymulin-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 ^{1}H NMR and δ_C 50.7 in ^{13}C NMR indicating methylation of the carboxylic acid group at C-20.

7α,16-Diacetoxymulin-11,13-dien-20-oic acid (**5a**): The ^{1}H and ^{13}C 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 ^{1}H NMR spectrum. The ^{13}C 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.

7α,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 ^{1}H and ^{13}C NMR spectrum of **5b** was similar to that of **5** except for an additional methyl ester (δ_H 3.69 in ^{1}H NMR and δ_C 51.0 in ^{13}C NMR).

7α,16-Diacetoxymulin-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 ^{1}H NMR and δ_C 51.1 in ^{13}C NMR indicating methylation of the carboxylic acid group at C-20.

The ^{1}H and ^{13}C 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 H_2O. To overcome this limitation, a **3**/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 2
Gastroprotective effect of compound **1**–**4**, **4a**–**4c**, **5a**–**5c** and lansoprazole at 20 mg/kg on HCl/EtOH-induced gastric lesions in mice

Compound	*n*	Lesion index (mm)	% Lesion reduction	*p*
4	7	15.4 ± 2.6	59*	<0.01
4a	7	21.6 ± 2.5**	43*	<0.01
4b	7	31.0 ± 2.1**	18*	<0.01
4c	7	36.8 ± 3.1**	3	<0.01
5	7	11.8 ± 2.9**	69*	<0.01
5a	7	19.6 ± 3.2**	48*	<0.01
5b	7	24.3 ± 3.8**	36*	<0.01
5c	7	35.6 ± 3.9**	6	<0.01
Lansoprazole	7	16.1 ± 2.5	57*	<0.01
Control	7	37.7 ± 4.3	—	—

The results are expressed as mean ± SD $^{*}P$ <0.01; significantly different compared with the control and $^{**}P$ <0.01 significantly different compared with lansoprazole (ANOVA followed by Dunnett's test). *n* = number of mice.

secretion) and defensive factors (endogenous mechanisms) in the gastric mucosa could lead to gastric ulcers.[18] As the results suggest, compound **4** and **5** (which include an alcohol and carboxylic acid groups respectively) can increase the gastroprotective effect suggesting a relationship between the polarity of the compounds and their gastroprotective activity. However, compounds **4a**–**4b** and **5a**–**5b** presented a lower gastroprotective effect than lansoprazole suggesting that if the hydroxy group is acylated the activity decrease. Furthermore, no activity was observed if the hydroxy group is acylated and also the carboxylic acid group is methylated.

In conclusion, a β-cyclodextrin inclusion complex allowed the improvement of the yields of two main products in the biotransformation of the diterpene mulin-11,13-dien-20 oic acid by *Mucor plumbeus* (33% and 15% for compounds **4** and **5**, respectively). In addition, the compound 9-*epi*-13α-hydroxymulinene was isolated for the first time from *A. cuatrecasasii*, while six new derivatives (**4a**–**4c** and **5a**–**5c**) were prepared by simple chemical transformations for the first time. The compounds were evaluated regarding their gastroprotective activity in the ethanol/HCl-induced ulcer model in mice at 20 mg/kg. The highest activity was shown by 7α,16-dihydroxymulin-11,13-dien-20-oic acid **5**, which was higher than the reference drug lansoprazole. The strategy employed in this work allowed the gastroprotective evaluation of six new mulinane derivatives from a parent compound obtained from *A. cuatrecasasii*.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.05.081.

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