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ISOLATION AND CHARACTERIZATION OF TRITERPENES FROM *ZIZYPHUS GLABRATA*

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ABSTRACT

From the stem bark of *Zizyphus glabrata* a new dammarane-type triterpene, pseudojujubogenin-3-O-β-D-glucopyranoside, along with the known ceanothane triterpenes, ceanothic acid, granulosic acid were isolated. The structures of the compounds were fully characterized by detailed NMR investigations including ^{1}H and ^{13}C NMR, HSQC, COSY, HMBC and NOESY experiments. In addition, the dammarane glycoside was tested for its potential to inhibit various bacteria and was found to possess significant bactericidal activity. The ^{1}H, ^{13}C and full 2D-NMR data on granulosic acid has also been presented. This is the first report on the chemical constituents of the stem bark of *Zizyphus glabrata*.

Key words: Triterpene, *Zizyphus glabrata*.

INTRODUCTION

Zizyphus glabrata Heyne (Syn: *Z. Trinervia Roxb*), is a small tree that grows up to 30 ft in height, having olive-brown wood and commonly found in the forests of Peninsular India and Bhutan[1,2]. The leaves and aerial parts of the plant are traditionally used to treat inflammation, to relieve pain, convulsions and viral infections[3]. Plants belonging to the genus *Zizyphus* (Rhamnaceae) have been noted to produce a variety of charaterstic secondary metabolites ranging from cyclopeptide alkaloids that possess antibacterial and antifungal activities[4], and the dammarane class of triterpenes that are reported as sweetness inhibitors[5-7]. The present work describes the isolation and characterization of a new dammarane-type triterpene, pseudojujubogenin-3-O-β-D-glucopyranoside along with the ceanothane triterpenes, ceanthic acid, granulosic acid.

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EXPERIMENTAL

General experimental procedures

Melting points were measured on a Cipla I-28 digital melting point apparatus and are reported uncorrected. The IR spectra were recorded on a Buck Scientific 500 Infrared spectrophotometer. Silica gel (Acme, 60-120 mesh) for column chromatography and silica gel (Acme 0) was used for preparative thin layer chromatography. Spots on chromatogram were detected under UV light and by spraying with 5% H_2SO_4 in methanol. The NMR experiments were performed on a Bruker AVANCE DRX-500 spectrometer operating at 500.13 MHz and 125.77 MHz, respectively. Mass spectra were obtained using an Agilent 1100 series LC/MSD in APCI or API-ES mode.

Plant material

The stem bark of *Zizyphus glabrata* (1.2 kg) were collected at the Khailasa hills, India, in April 2009. The sample was authenticated by Dr. M. Venkaiah, Taxonomist, Botany Department, Andhra University, Visakhapatnam. A voucher specimen (SG/ZGL/03/ 345) has been deposited at the Herbarium, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam India (Herbarium Code = SKU).

Extraction and isolation

Powered plant material (900 g) was extracted in a Soxhlet apparatus, successively with hexane, $CHCl_3$ and MeOH and the extracts were concentrated using a rotary evaporator at a maximum temperature of 45^{o}C. The dark viscous green residue 11 g from the methanol extract was separated over silica gel eluting with different mixtures of petroleum ether-chloroform and chlororom-methanol to give 25 fractions. Fraction 12-18 were combined, purified by repeated preparative TLC that recrystallized from methanol to give **2** (43.0 mg) and **3** (38.0 mg). Fraction 20 was further purified by repeated small column chromatography and recrystallized with chloroform-methanol to give **1** (64.0 mg).

Pseudojujubogenin-3-O-β-D-glucopyranoside (1): Pale green amorphous powder, m.p. 241-243^{o}C. IR (KBr): v = 3200, 3640 (OH), 1465, 1285, 1078, 1012, cm^{-1}, ^1H NMR (500.13 MHz, d_5-pyridine, ^{13}C NMR (128.77 MHz, d_5-pyridine) COSY, HMBC and NOESY see Tables 1 and 2 and structure 1. HR-EIMS:*m/z* (%) = 650 (18) (M^+). $C_{36}H_{58}O_{10}$ (650.85) : calcd. C 66.43, H 8.98, O 24.58; found C 66.32, H 8.94, O 24.54.

Ceanothic acid (2): Colourless needles from Me_2CO-methanol, m.p. 356-357^{o}C, m.p. 333-335^{o}C, dec. -$[\alpha]^{24}$ = -51.5^o (c, 1.01 in $CHCl_3$) IR and MS in agreement with the

published data [12], ^{1}H NMR (500.13 MHz, d_5-pyridine) δ = 1.09, 1.17, 1.29, 1.41, 1.44, 1.68 (6 x S, 18H, CH*Me*) 1.71, (m, 1H, 18-H), 3.22 (s, IH, 1-H), 4.84 (s, 1H, 3-H), 2.23 (d, 1H, *J* = 2.8 Hz, 5/9-H), 1.45, 1.54 (m, 2H, 6/7-H), 1.60, (m, 1H, 11-H), 2.11 (d, 1H, *J* = 11.4 Hz, 11-H), 1.34, (m, 1H, 12-H), 1.98 (d, 1H, *J* = 10.5 Hz, 12-H), 2.79 (dd, 1H, *J* = 2.8, 8.5 Hz, 13-H), 1.25, (m, 1H, 15-H), 1.92 (dd, 1H, *J* = 2.8, 10.5Hz, 15-H), 1.50, 2.61 (d, 2H, *J* = 11.4 Hz, 16 = H), 3.51 (d, 1H, *J* = 2.3, Hz, 19-H), 1.50, 2.23 (m, 1H, 21-H), 1.50, 2.23 (m, 1H, 22-H), 4.68, (s, 1H, 30-H (CH_2 = C), 4.87 (d, 1H, *J* = 10.0Hz, 30-H (CH_2 = C). ^{13}C NMR (d_5-pyridine) δ = 20.7 (24-CH_2), 15.5, 17.4, 19.2, 20.0, 31.9 (all CH*Me*), 67.4 (C-1), 85.1 (C-3), 44.2 (C-4), 57.4 (C-5), 19.5 (C-6), 35.1 (C7), 42.5 (C-8), 45.5 (C-9), 50.0 (C-10), 24.6 (C-11), 26.6 (C-12), 39.5 (C-13), 43.9 (C-14), 30.9 (C-15), 33.3 (C-16), 57.0 (C-17), 50.1 (C-18), 48.0 (C-19), 31.7 (C-21), 38.0 (C-22), 110.1 (30CH_2) 178.4 (2-COOH), 179.3 (28-COOH), NOESY correlations: H-1 ↔H-3, H-19 ↔ H_2-30, H-5 ↔ H-3, H_3-29 ↔ H_2-30A/B, H_3-23 ↔ H-3, H_3-24 ↔ H-3, H-13 ↔ H_3-26, H-13 ↔ H_2-12 and H_3-29 ↔ H-19.

Granulosic acid (3): Colourless flakes from methanol, m.p. 237-239°C, -$[\alpha]^{24}_{D}$ = -51.5°C, (1.01 in $CHCl_3$). ^{1}H NMR (500.13 MHz, d_5-pyridine), ^{13}C NMR (125.77 MHz, d_5-pyridine), COSY, HMBC and NOESY, see Table 3 and structure. 3 HR-EIMS : m.z (%) = 502 (20) (M^+). -$C_{30}H_{46}O_6$ (502.32) : calcd. C 71.68, H 9.22, O 19.10; found C 71.64, H 9.16, O 19.06.

RESULTS AND DISCUSSION

The stem bark of *Zizyphus glabrata* were extracted successively with hexane, chloroform and methanol that on concentration afforded three dark viscous semisolids. The methanolic residue was separated by silica gel column chromatography to furnish a new dammarane type triterpene glycoside together with the known ceanothane triterpenes, ceanthic acid, granulosic acid.

Compound 1 was the major isolate obtained in this investigation as pale green amorphous powder, m.p. 241-243°C. It gave a positive for Liebermann Burchard test for triterpenes and Molisch test for sugars. The IR spectrum indicated the presence of a tertiary hydroxyl at 3460 cm^{-1} and the absence of a conjugated system in the molecule. The high resolution mass spectrum showed a molecular ion peak at m/z 650.85$[M]^+$, supporting the molecular formula of $C_{36}H_{58}O_{10}$ for **1**, deduced from the mass spectrum in conjunction with the ^{13}C NMR spectrum. The NMR spectrum (Table 1) exhibited signals for 36 carbons: nine methylene [two of them bearing oxygen atoms (δ = 66.2 and 68.9)], seven methnines [one oxymethine (δ = 89.0)], seven methyl carbons, an anomeric carbon δ = 107.2 bound to δ= 4.97 (1H, *d*, 7.7) according to the HSQC spectrum. Comparison of the NMR data for 1

(Table 1) with the COSY 45^o spectrum, revealed the sugar (pyranose form 0 to be glucose. The coupling constant of the anomeric proton i.e δ = 4.97 (1H, *d*, 7.7) indicated β-configuration of glucopyranosyl moiety. A ^{1}H double doublet at δ = 3.38 (J = 4.7, 11.5 Hz) characteristic for H-3 α having a sugar linked at C-3 was supported by ^2J HMBC correlations with the anomeric carbon 107.2 (G-1), and the geminal methyls [28.4 (C-28), 16.6 (C-29)] located at C-4, NOE correlation between H-3 of the genin and G-1 of the glucose confirmed the attachment of the sugar at position C-3 of the aglycone. These signals resembled a dammarane type triterpene having a single sugar unit in the A ring at 3-O-β-position and a free tertiary hydroxyl group[8-11].

The spectrum also revealed an olefinic methane, δ = 5.42 (δ=127.2) along with signals typical to that of an isobutenyl side chain. The COSY 45^0 spectrum revealed that the methyls δ=1.64 and 1.72 and resonances at δ = 25.9 and 18.7 ascribed to C-26 and C-27 were coupled to the unsaturated methane at δ = 5.42 and were assignable to H-24. The placement of the side chain at C-22 was accomplished through the HMBC experiment. The olefinic methane resonating at δ = 5.42 (H-24) showed a ^2J correlation with the carbon δ = 46.9 (C-22) and 3 *J* long-range couplings with the carbons δ = 68.9 (C-23), 25.9 (C-26), 18.9 (C-27) supporting that the side chain was located at C-22 of the three oxygen functions in 1, one was assigned to a tertiary hydroxyl group δ = 69.1 (C-20) while the two other oxygen's were directly involved in ethers of a ketal group δ = 5.03 and 4.28 (d, *J* = 8.6 Hz, H-30) with resonances δ = 68.9 and 66.2 assignable to sp^3 carbons C-23 and C-30 respectively. The relative stereochemistry at C-3/5/28 and C-18/19 were confirmed by means of the NOESY spectrum. The H-3 α proton showed strong NOE interactions with H_3-28 resonance and H-5 methine suggesting that they were α-oriented and the H-24 olefinic methane showed two interactions with the angular methyls H_3-18 and H_3-19 establishing β-orientation of the methyls.

Some key HMBC correlations (Table 2) observed were between the methyls (δ = 1.34 and 0.74) that exhibited ^3J coupling between themselves indicating their geminal nature and ^2J coupling with the oxymething C-3 and the methane C-5, while the methyl at δ = 1.44 showed ^2J correlation with the methane at C-22. The angular methyl, H_3-18 (δ = 1.02) showed ^2J correlation to the methylene at C-7 and the quaternary carbon at C-14 and ^3J couplings with the quaternary carbon C-10. On the basis of the above spectral data, compound 1 was identified as psedujujubogenin-3-O-β-D-glucopyranoside, a new natural product. ^{1}H and ^{13}C NMR resonances were assigned using COSY, HMBC and NOESY spectra and are presented in Tables 1 and 2 and on structure 1.

Table 1: ^{1}H, ^{13}C NMR and COSY spectral data for dammarane triterpene glycoside 1

Position	δH	δC	COSY	Position	δH	δH	COSY*
1	a) 0.81 (m, 1H)	39.0	H-1b, H-11a	16		110.6	
	b) 1.49 (m, 1H)		H-1a, H-2b				
2	a) 1.77-1.90 (m,1H)	26.9	H-3α	17α	1.72 (m,1H)	53.9	H-13β
	b) 2.29 (m, 1H)		H-1b				
3α	3.38 (dd,1H,4.7,11.5)	89.0	H-2a, H-2b	18β	1.02 (s, 3H)	19.0	H-7
4		37.6[a]		19β	1.02 (s, 3H)	17.1	H-7
5α	0.70 (m, 1H)	56.3	H-28α	20		69.1	
6	1.37 (m, 2H)	18.5	H-15b	21	1.44 (s, 3H)	29.8	H-13β
7	1.54 (m, 2H)	36.2	H-18, H-19	22	2.16 (m,1H)	46.9	
8		37.4		23	5.03 (m,2H)	68.9	H-15a, H-24
9	0.81 (m, 1H)	53.2		24	5.42 (d,1H,8.0)	127.2	H-26, H-27
10		37.4[a]		25		135.4	
11	a) 1.37 (m, 1H)	21.8	H-17α	26	1.64 (s, 3H)	25.9	
	b) 1.49 (m, 1H)		H-11a, H-17α				
12	a) 1.77-1.90 (m,1H)	28.7		27	1.72 (m,3H)	18.7	H-23
	b) 1.97 (m, 1H)		H-11a				
13β	2.72 (m, 1H)	38.6		28α	1.34 (m, 3H)	28.4	H-5α
14		53.5		29β	0.74 (s, 3H)	16.6	H-1b
15	a) 1.77-1.90 (m,1H)	39.9	H-13β	30	4.28 (d, 2H, 8.6)	66.2	
	b) 2.20 (d, 1H, 8.3)						
β-D-Glucose							
G-1	4.97 (d, 1H, 7.7)	107.2	G-2	G-4	4.20 (dd,1H,8.6,9.1)	72.2	
G-2	4.07 (t, 1H, 8.7)	76.0	G-5, G-6b	G-5	4.25 (t, 1H, 8.6)	79.0	

Cont…

Position	$^{\delta}$H	$^{\delta}$C	COSY	Position	$^{\delta}$H	$^{\delta}$H	COSY*
G-3	4.02 (m, 1H)	78.6	G-4	G-6	a) 4.63 (dd,1H,2.4,11.7)	63.4	G-6b
					b) 4.42 (dd,1H,5.5,11.7)		

*Assignments were confirmed by 2D NMR experiments (HSQC, HMBC and 2D-NOESY);
[a] signals are interchangeable, coupling constants 'J" in Hertz.

Table 2: Key HMBC correlations observed for the dammarane triterpene glycoside 1

Position	^{2}J	^{3}J
H-1	26.9 (C-2)	
H-2	89.0 (C-3)	
H-3	107.2 (G-1), 28.4 (C-28), 16.6 (C-29)	
H-5		89.0 (C-3)
H-12	38.6 (C-13)	53.9
H-13	53.5 (C-14)	(C-17)
H-18	36.2 (C-7), 53.5 (C-14)	69.1 (C-20)
H-19	56.3 (C-5)	37.6 (C-10)
H-21	46.9 (C-22)	38.6 (C-4), 21.8 (C-11)
H-22	53.9(C-17), 69.1 (C-20)	
H-23	110.6 (C-16)	127.2 (C-24)
H-24	46.9 (C-22)	68.9 (C-23), 25.9 (C-26)
		18.9 (C-27)
H-26/27	127.2 (C-24)	
H-28	89.0 (C-3), 56.3 (C-5)	16.6 (C-29)
H-30	53.5 (C-14)	38.6 (C-13)
G-1	89.0 (C-3)	78.6 (G-3)
G-3	78.6 (G-4)	107.2 (G-1), 79.0 (G-5)
G-4	79.0 (G-5)	63.4 (G-6)

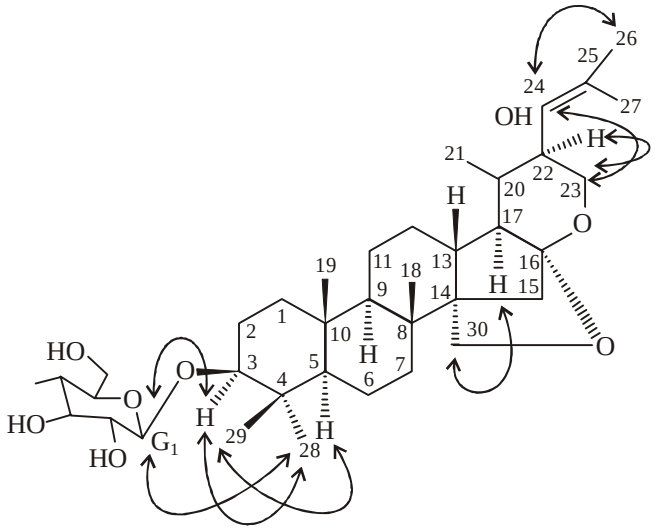

Fig. 1: Important NOESY interactions of 1

Compound 2 : Ceanothic acid was characterized by analysis of NMR spectra and comparison with the published data[12-14]. The dammaranetype triterpene glycoside is the major compound in *Zizyphus xylopyra*. Jujubogenin glycosides, jujuboside A, C and lotoside I, II have been reported from *Zizyphus lotus*[15] however, this is the first report of a pseudojujubogenin glycoside isolated from the genus, *Zizyphus*. The ceanothane triterpene, 3-O-protocatechuoylceanothic acid has been reported from *Zizyphus jujuba*[16]. The isolation of the dammarane – type glycoside from a plant of the *Zizyphus* genus is not surprising, but it is remarkable to note that *Zizyphus xylopyra* produces both dammarane and the ceanothane class of terpenoids. Compound 1 was tested for its potential to inhibit various bacteria by established methods[17]. It inhibited the growth of *Bacillus pumilus, Staphylococcus aureus, Escherichia coli* and *Proteus vulgaris* with MICs being 51.2, 102.2, 12.8, 25.6 μg/mL, respectively. The dammarane and ceanothane terpenoids have been reported to possess potent anti-inflammatory activity[18], the biological activities of the three compounds are of interest and are presently taken up for investigation.

Compound 3 was obtained as colourless flakes, m.p. 237-239^{0}C. The HR – EI mass spectrum showed a molecular ion peak at m/z 503.69 $[M]^+$ that correspondent to the molecular formula $C_{30}H_{46}O_6$. The ^{13}C NMR (Table 1) displayed 30 carbon resonances, while the HSQC experiment confirmed that 22 out of the 30 carbons were directly attached to protons. The *J* modulated ^{13}C experiment revealed the presence of five methyls, ten methylenes, seven methines and eight quaternary carbons that included resonances for two carboxylic functions δ = 178.0 and 179.2. The ^{1}H NMR (Table 1) showed five tertiary

methyl singlets, a hydroxymethyl group δ = 3.70, 4.64 (d, *J* = 9.0 Hz), an oxymethine δ = 4.96 and a saturated methane δ = 3.26 and a series of multiplets from δ = 1.49 to 1.59. These features revealed the backbone of 3 as a ceanothic acid derivative. This presumption was further confirmed when the ^{1}H and ^{13}C NMR chemical shifts of the ceanothane triterpenes 2 and 3 were found to be almost superimposable[12,13]. However, there were minor changes for signals in the ^{1}H and ^{13}C NMR of the A and B rings of the ceanothane skeleton which were also observed in the COSY and the HMBC experiments. The remaining assignments of the ^{1}H resonances in 3 were made by comparison with those published for 3a in the literature. The ^{13}C assignments were based on the HMBC experiments using ^{2}J and ^{3}J correlations while the ^{1}J C-H interactions observed in the HSQC spectrum allowed unambiguous assignments of the hydroxymethyl and methylene protons in 3. The relative stereochemistry at H-1/3/9/13 and H_3-23/25/26/27 and the carboxylic group at H-2/28 were finally determined by 2D NOESY experiments as shown on 3. Accordingly, the structure of 3 was determined as 24-Hydroxyceanothic acid, known as granulosic acid. Although dimethyl granulosate 3a has been previously reported from the hearwood's of *Colubrina granulosa* [12] and also from *Paliurus ramosissimus*[13], the present study constitutes the first isolation of 3 as a pure natural product and is reported as a rare ceanothane triterpene. In addition to its ^{1}H NMR data, ^{13}C and full 2D-NMR spectral data has been presented for the first time (see Table 3 and Structure 3)

Table 3: ^{1}H, ^{13}C NMR, HMBC, COSY, and NOESY spectra data for the ceanothane triterpene 3

H/C	$^{\delta}$H	$^{\delta}$C	HMBC	COSY	NOESY
1β	3.26 (s,1H)	66.8	C-2, C-3, C-5		H-25β
			C-10, C-25		
2-COOH		178.0			
3α	4.96 (s, 1H)	86.2	C-2, C-10, C-23		
4		48.9			
5	2.31 (m,1H)	57.6	C-4, C-6		
6a/b	1.49 (m,2H)	18.8	C-5, C-10		
7	a) 1.49 (m,1H)				
	b) 1.41 (m,1H)	35.5	C-4, C-5, C-24		
8		42.4			

Cont…

H/C	$^{\delta}$H	$^{\delta}$C	HMBC	COSY	NOESY
9	2.18 (m,1H)	45.7	C-6, C-8, C-10 C-11, C-25		H-1β, H-27α
10		50.4			
11	a) 1.59 (m,1H)	24.7	C-13		
	b) 2.10 (m,1H)			H-25β	
12	a) 1.98 (m,1H)	26.6	C-14	H-26β	
	b) 1.33 (m,1H)				
13β	2.78 (m,1H)	39.5	C-8, C-18		
14		43.9			
15	a) 1.22 (m,1H)	30.9			
	b)1.90 (m,1H)				
16	a) 1.49 (m,1H)	33.3	C-8, C-17, C-18, C-20	H-21, H-29	H-16b
	b) 2.60 (m,1H)		C-28		
17			57.0		
18	1.69 (m,1H)	50.1			H-30
19α	3.51 (m,1H0	48.0	C-30	H-21, H-22, H-29	
20		151.6			
21	a) 2.23 (m,1H0	31.7	C-18,C-28	H-21b, H-22	H-19, H-21b, H-21a
	b) 1.49 (m,1H)				
22	a) 2.23 (m,1H)	37.9	C-28		
	b) 1.55 (m,1H)				
23α	1.81 (s,3H)	26.1	C-3, C-17, C-24		H-3α,24-OH
24β	4.64 (d,2H,9.0)	67.0	C-23		24-OH
24-OH	3.70 (d,1H,9.0)		C-4, C-23	H-24	
25β	1.47 (s,3H)	19.4	C-9		H-24β
26β	1.13 (s,3H)	17.4	C-7, C-8		H-13β

Cont…

H/C	$^{\delta}$H	$^{\delta}$C	HMBC	COSY	NOESY
27α	1.06 (s,3H)	15.4	C-13,C-14, C-15		H-6, H-7
28-COOH		179.2			
29	1.67 (s,3H)	20.1	C-19,C-20, C-20, C-30		H-18,H-30a
30	a) 4.68 (d,1H, 13.6)	110.0	C-12, C-19, C-20, C-27	H-29, H-30b	H-19
	b) 4.87 (s,1H)	C-19, C-29			

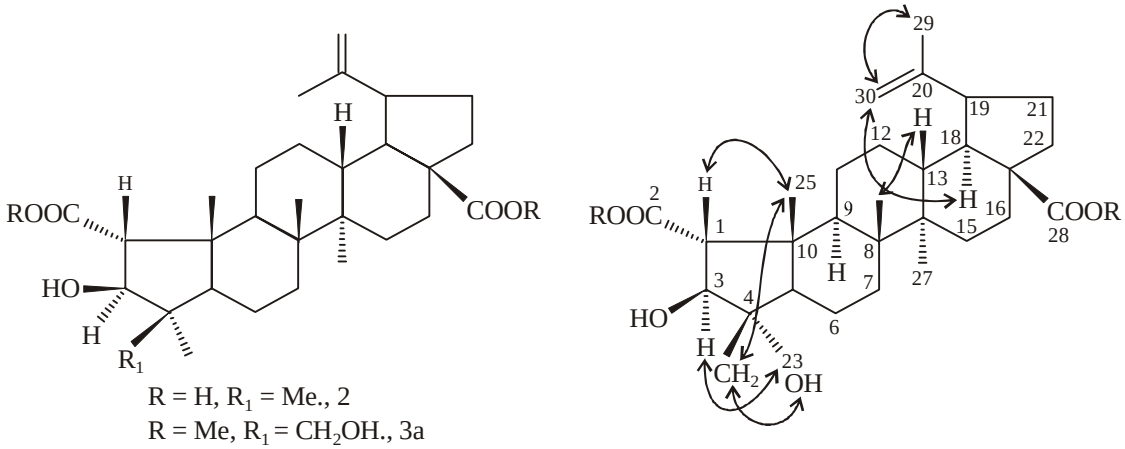

Fig. 2: Important NOESY interactions of 3

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