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Lutoside : an Acyl-1-(Acyl-6'-Mannobiosyl)-3-Glycerol Isolated from the Sponge-associated Bacterium *Micrococcus luteus*

Valérie Bultel-Poncé[a], Cécile Debitus[b], Alain Blond[a], Claude Cerceau[a], Michèle Guyot[a*]

[a] Laboratoire de Chimie du Museum National d'Histoire Naturelle, associé au CNRS, 63 rue Buffon, 75005 Paris, France. [b] ORSTOM Nouméa, Nouvelle-Calédonie.

Abstract. Lutoside, an unusual acyl-1-(acyl-6'-mannobiosyl)-3-glycerol **1** was isolated from the sponge-associated bacterial strain *Micrococcus luteus*. Sructure elucidation was performed by sprectroscopic analysis and chemical transformations.
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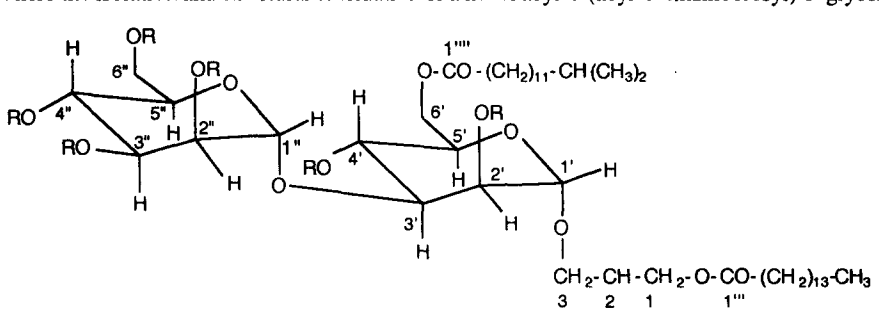
In the last few years marine bacteria emerged as a new field for the discovery of novel biologically active compounds from marine origin[1] . Isolation of these bacteria can originate mainly from sediments, but also from open oceans or marine surfaces including marine living organisms [2]. To date only a restricted number of papers are devoted to the chemical study of bacteria associated with invertebrates. Bacteria collected from sponges [3-9], tunicate [10] , soft coral [11] , jelly fish [12] have allowed isolation of antimicrobial compounds, which suggests that these bacteria may play a role in the defence mechanism of these invertebrates. Furthermore, metabolites previously ascribed to invertebrates were lately demonstrated to be biosynthesized by bacteria[3, 8, 9] .

In a program devoted to the search for bioactive compounds biosynthesized by marine invertebrate associated bacteria we studied a bacterial strain, *Micrococcus luteus* collected on a sponge *Xestospongia* sp[13]. We report here the isolation and structural elucidation of a novel acyl-1-(acyl-6'-mannobiosyl)-3-glycerol **1**.



Structure of compounds **1** : R=H and **2** : R=Ac.

The sponge *Xestospongia* sp was collected by scuba diving off Nouméa (New Caledonia) and stored in a sea water current aquarium (2 days). Sponges were rinsed with sterile sea water and brushed. The resulting suspension was then diluted in ten fold series, the dilutions were spred on Marine agar medium (Difco 2216). One of the isolated strains, *Micrococcus luteus*, was cultivated at 27°C, in 4 x 3-l batch cultures (sea water DIFCO-peptone 18g/l enriched medium) for 4 days with rotatory shaking. Cells were harvested by centrifugation at 11 000 rpm for 15 mn. The pellet was sonicated and extracted successively with methanol and dichloromethane-methanol (8/2). The organic phase was separated by centrifugation and the solvents removed under reduced pressure.

FAX : 33-1-40-79-31-47, e-mail : guyot@mnhn.fr

The extract (1.1g) was fractionated on a silicagel column using a dichloromethane-methanol gradient. The fractions eluted with dichloromethane-methanol (8/2) to (7/3) were further purified on another silica gel column eluted with chloroform-methanol (8/2) to obtain compound **1**, white amorphous solid (50 mg, 0.05% dry weight), mp 88°C, $[\alpha]_D$ +8.6 (c 0.5 methanol); IR (NaCl) 3401 cm^{-1} 1756 cm^{-1}; ^1H and ^{13}C NMR see table 1 and text ; HRFAB-MS $[M+Na]^+$ found *m/z* 887.5594, $C_{45}H_{84}O_{15}Na$ requires 887.5608.

The ^1H NMR spectrum registered in d5-pyridine was in agreement with a diacylated diosyl glycerol with the presence of a number of signals in the 4-6 ppm region, two anomeric protons at δ 5.37 and 5.96 ppm, aliphatic methines at δ 1.25 ppm and three terminal methyl signals. The ^{13}C NMR spectrum indicated the presence of signals for two carbonyl groups (δ 173.6 and 173.2 ppm), two anomeric carbons (δ 104.0 and 102.4 ppm), 9 methines and 4 methylenes in the 63-81 ppm region, long chain aliphatic carbons (δ 30.1 ppm), three methyl signals (δ 11.6, 22.8, 19.4 ppm)(Table 1).

Table 1 : ^{13}C (75 MHz) and ^1H NMR data of **1** (300 MHz) and **2** (500 MHz) (δ ppm, pyridine d-5).

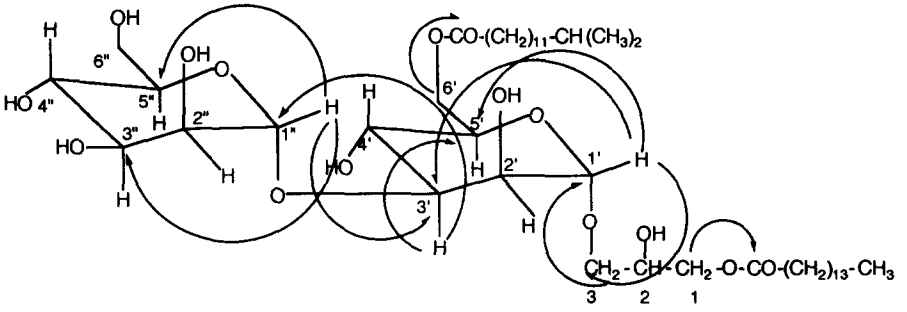
Compound 1				Compound 2			
Assign-ments	^1H	^{13}C	HMBC	Assign-ments	^1H Multiplicity - *J* (Hz)	^{13}C	HMBC
1	4.55 (m)	66.4	2, 1'''	1	4.65* 4.47 (dd, *J* = 10; 6.2)	62.6**	3, 1'''
2	4.45 (m)	68.5	-	2	5.62 (m)	70.2	3
3	4.18 (dd, *J* = 10.2; 4.4) 3.87 (dd, *J* = 10.2; 6.2)	69.7	1, 1', 2	3	4.15 (dd, *J* = 11; 4.4) 3.94 (dd, *J* = 11; 6.2)	66.8	1, 2, 1'
1'	5.37 (d, *J* = 1.3)	102.4	3, 3', 5'	1'	5.31 (d, *J* = 1.3)	98.4	3', 5', 3
2'	4.93 (dd, *J* = 3.1; 1.3)	70.7	4'	2'	5.73 (dd, *J* = 3.1; 1.3)	71.4	3', 4'
3'	4.63 (m)	81.0	1"	3'	4.65 *	75.4	1"
4'	4.70 (m)	66.9	3'	4'	5.84 (dd, *J* = 10.1; 10)	68.2	3', 5'
5'	4.45 (m)	72.6	-	5'	4.35 (m)	69.7	1'
6'	4.98 (dd, *J* = 11.2; 3.3) 4.82 (dd, *J* = 11.2; 6.7)	64.6	1''''	6'	4.67* 4.55 (dd, *J* = 12.3; 3.3)	62.9**	1''''
1"	5.96 (d, *J* = 1.8)	104.0	3', 3", 5"	1"	5.39 (d, *J* = 1.7)	99.5	3', 3", 5"
2"	4.68 (dd, *J* = 1.8, 3.2)	72.3	4"	2"	5.55 (dd, *J* = 3.1; 1.7)	70.4	4"
3"	4.58 (m)	73.0	1"	3"	5.71 (dd, *J* = 10.1; 3.1)	69.1	-
4"	4.55 (m)	69.4	3", 5"	4"	5.76 (dd, *J* = 10.1; 9.8)	66.6	-
5"	4.80 (m)	75.6	4"	5"	4.62 (m)	70.2	3"
6"	4.57 (m) 4.30 (dd, *J* = 11.3; 6.7)	62.9	5"	6"	4.63* (m) 4.45 (dd, *J* = 10; 5.8)	62.7**	-
1'''	-	173.2*		1'''		173.3	
2'''	2.37 (t, *J* = 6.7)	34.4	1'''	2'''	2.51 (t, *J* = 7.4)	39.3	1'''
3'''	1.67 (m)	25.3	1'''	3'''	1.71 (m)	25.2	1'''
(CH_2)	1.25 (22 H)	30.1		(CH_2)	1.25 (22H)	30.1	
CH_3	0.85 (t)	11.6		CH_3	0.84 (t)	11.6	
1''''	-	173.6*		1''''		173.5	
2''''	2.32 (t, *J* =6.7)	34.4	1''''	2''''	2.40 (t, *J* = 7.5)	36.7	1''''
3''''	1.65 (m)	25.0	1''''	3''''	1.66 (m)	27.4	1''''
CH iPr	1.47 (m)	34.2	CH_3 iPr	CH iPr	1.48 (m)	34.6	CH_3 iPr
(CH_2)	1.25 (18 H)	30.1		(CH_2)	1.25 (18H)	30.1	
CH_3	0.85 (d, 6H))	22.8 19.4	CH iPr	CH_3	0.85 (d, 6H)	22.8; 19.4	CH iPr
				CH_3CO	2.1-2.6 (seven s)	20.8-20.4 170-169.5	

* May be reversed ** Signals overlapped

A COSY experiment allowed to assign, on one hand starting from the two anomeric protons H-1' and H-1", respectively, the protons H-2', H-3', H-2", H-3", and on the other hand, protons H-1, H-2 and H-3 corresponding to the glycerol moiety. The downfield shift of the H-2 proton suggests that the hydroxyl at C-2 was not acylated. Direct ^1H-^{13}C (HMQC) and long range ^1H-^{13}C correlations (HMBC), allowed assignments of all protons and carbons of the molecule (Table 1). HMBC experiments furnished correlations between H-1"

and C-3' and H-3' and C-1" which established unambiguously the 1"-3' linkage of the two sugar moieties. Correlations between H-1' with C-3 showed that the glycerol moiety was attached to the 1' anomeric carbon. Furthermore, long range correlations of the C-6' protons and the C-1 proton with respectively a carbonyl at δ 173.2 and one at δ 173.6 ppm, clearly demonstrated that the two acyl groups were located on the C-1 and C-6' oxygens (Scheme 1). Correlations observed between H-1' with C-5' and H-1" with C-5", allowed assignment of H-5' and H-5". The COSY experiments furnished the chemical shifts of H-6', H-6", H-4' and H-4".

Acetylation of **1** (Ac_2O-pyr) led to a heptaacetyl derivative **2**. ^1H NMR and COSY experiments showed the downfield shifts of protons 2, 2', 4', 2', 3",4", 5", 6" and confirmed our previous assessment that the second acyl group was located at the 6' position, hence that OH-2 was not acylated. Complete assignments of protons and carbons resulted from the combination of heteronuclear correlations HMQC and HMBC (Table 1). Evidence of the presence of two mannose units came from the coupling constants observed for the osidic protons in **1** and **2**. The weak coupling constants observed between H-1' and H-2' (1.3 Hz) and H-1" and H-2" (1.7 Hz) respectively, were in agreement with two sets of equatorial protons. In contrast, the high values of the coupling constants observed for the H-4' and the H-4" protons indicated an axial position and suggested the presence of two mannose units. Furthermore ^{13}C NMR data are fully consistent with those given for manα-1-3-manα-4glcNAc.[14]



Scheme 1. Selected ^1H-^{13}C long-range correlations for **1**.

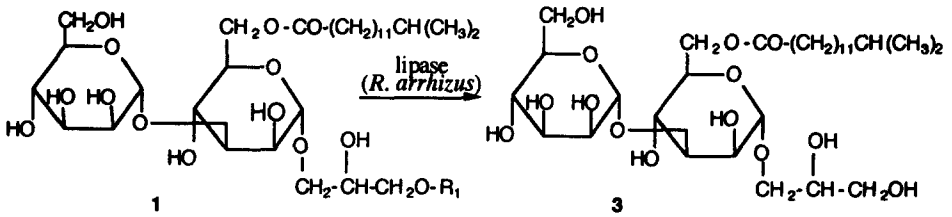
Examination of the protons ascribed to the long-chain fatty acids in **1** and **2** revealed the presence of three methyl signals, one as a triplet (3H), the other two as a doublet (6H) coupled with a multiplet at δ 1.47 ppm which suggested that one of the long-chain acyl ended with an isopropyl group.

Alcaline hydrolysis (KOH 5% in MeOH, rt., 12 hours) was performed. The resulting mixture was acidified and extracted with dichloromethane. The crude extract was subjected to a separation on a silicagel column, to afford after elution with hexane-ethyl acetate (9/1) a mixture of two aliphatic methyl esters. GCMS analysis pointed to the structure of pentadecanoic acid methyl ester and 13-methyl-tetradecanoic acid methyl ester.

In order to identify the acid substituents at C-1 and C-6', a selective enzymatic hydrolysis of **1** was undertaken using a lipase from *Rhizopus arrhizus* according to the experimental conditions known to produce *sn*-1 monodeacylation[15]. The resulting mono-acyl derivative **3** was purified first by gel filtration on Sephadex LH20, then on a silicagel column eluted with chloroform-methanol 8/2.

^1H NMR and ^1H-^1H (COSY) correlations clearly showed that only O-CH_2-1 was deacylated because the CH_2-1 signal has shifted from δ 4.55 ppm to 4.18 ppm, the O-CH_2-6' shift remaining unchanged. Signals for one long-chain CH_2 at δ 2.27 (2H), 1.65 (2H), 1.25 ppm (18H), the presence of a doublet for two CH_3 at δ

0.83 ppm and a multiplet for one CH at δ 1.45 ppm indicated that the 13-methyl-tetradecanoic acid is located at the 6' position (Scheme 2, table 2).



Scheme 2. Enzymatic hydrolysis of **1** using lipase from *R. arrhizus*.

The stereochemistry at C-2 was assigned to be *R*, from comparison of the coupling constant values between H-2/H-3a and H-2/H-3b respectivelly J = 4.4 and 6.2 Hz, with those given for (2*R*) and (2*S*) 1-acyl-galactosyl glycerol [16].

1 is the second example of a naturally occuring acyl glycerol acylated at the *sn*-1 and 6' positions, the first one was very recently reported from the coryneform bacterium *Arthrobacter atrocyaneus* [17].

Table 2 - ^{1}H NMR data of compound **3** (300 MHz) (δ ppm, pyridine-d_5)

1	4.18 (dd)	2"	4.71**
2	4.35 (m)*	3"	4.42 (m)
3	3.92 (dd)-4.30*	4"	4.62 (m)
1'	5.38 (d)	5"	4.82 (m)
2'	4.92 (dd)	6"	4.33* ; 4.57
3'	4.66 (m)	2"''	2.27 (t)
4'	4.75 (m)**	3"''	1.65 (m)
5'	4.53 (m)	CH_2	1.25 (18H)
6'	4.97 (dd), 4.79 (dd)	CH iPr	1.45 (m)
1"	5.95 (d)	CH_3	0.83 (d, 6H)

*,** Signals overlapped

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