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Rhizoleucinoside, Rhamnolipid-Amino Alcohol Hybrid from the Rhizobial Symbiont *Bradyrhizobium* sp. BTAi1

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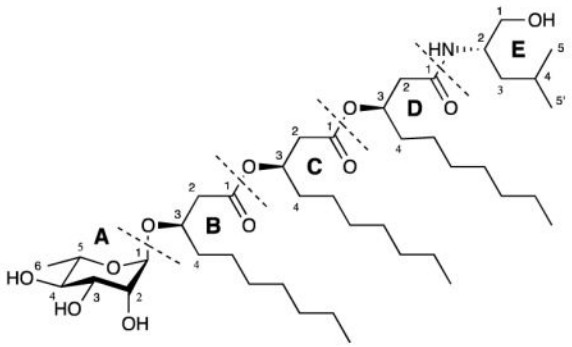
Abstract

Rhizoleucinoside (**1**), a unique rhamnolipid-amino alcohol hybrid was isolated from the rhizobial symbiont bacteria *Bradyrhizobium* sp. BTAi1. **1** features a rare rhamnolipid core attached to an unprecedented leucinol moiety. Its structure and absolute configuration were determined by spectroscopic analysis, tandem mass spectrometry, chemical degradation and application of the Marfey's method. **1** possesses moderate cytotoxicity to BV-2 murine microglia and highly aggressive proliferating immortalized (HAPI) rat microglia cells.

Graphical Abstract



Rhamnolipids are a group of biosurfactants produced by bacteria and their amphiphilic property originates from their glycolipidic structures.[1] Rhamnolipids were first isolated from the opportunistic human pathogen *Pseudomonas aeruginosa*.[2] More recent studies have

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Notes

The authors declare no competing financial interest.

Supporting Information

Experimental details, the HRESIMS, MS/MS, NMR spectra and biological activity of **1**. The Supporting Information is available free of charge on the ACS Publications website.

expanded the bacteria taxa known to produce rhamnolipids to include new families such as *Acinetobacter calcoaceticus* from the family Moraxellaceae,[3] different orders such as *Pseudoxanthomonas* sp. from the order Xanthomonadales,[4] and even different classes such as *Burkholderia* spp. from the class Betaproteobacteria.[5,6] Despite the production of rhamnolipids over disparate taxonomic groups, their biological function is still unclear. Studies suggest rhamnolipids might play multifunctional roles such as promoting the uptake of water insoluble substrates,[7] immune modulators,[8] antimicrobials,[9] and insecticides.[10] The common core structures are composed of one or two L-rhamnoses linked to one or two β-hydroxy fatty acids (C_{10}-C_{16}) through a glycosidic bond. β-Hydroxy fatty acids are linked through ester bonds while di-saccharides are linked through α-1,2 glycosidic linkages.[1]

In the study presented here, an unprecedented rhamnolipid-amino alcohol hybrid rhizoleucinoside (**1**) (Figure 1), featuring a rare Rha-C_{10}-C_{10}-C_{10} glycolipid core attached to a terminal leucinol moiety, was isolated from *Bradyrhizobium* sp. BTAi1 (ATCC BAA-1182). *Bradyrhizobium* species are symbiotic bacteria that fix nitrogen for their leguminous host plants.[11] Although the whole genome of this strain has been sequenced,[11] secondary metabolites have not been previously reported for this organism. To the best of our knowledge, this is the first example of an amino alcohol-containing rhamnolipid as well as the first rhamnolipid isolated from a bacterium belonging to the class Alphaproteobacteria.

1 was isolated as an amorphous, colorless solid, $[\alpha]^{23.2}_{D}$ −13.7 (*c* 0.1, MeOH) from culture extracts of *Bradyrhizobium* sp. BTAi1. The molecular formula of **1** was established as $C_{42}H_{79}NO_{11}$ on the basis of the HRESIMS ion at *m/z* 774.5730 $[M + H]^+$ (calcd for $C_{42}H_{80}NO_{11}$, 774.5726) and its sodium adduct ion *m/z* 796.5556 $[M + Na]^+$. The IR spectrum showed a broad absorbance for OH and NH (~3320 cm^{-1}) and sharp absorbances for C=O (1736 cm^{-1} for ester and 1646 cm^{-1} for amide) and C-O (1042 cm^{-1}).

The ^1H (Table 1) and HSQC NMR spectra of **1** in DMSO-d_6 showed a methine proton at δ_H 4.62 bound to a di-oxygenated carbon at δ_c 100.2, four methine protons between 3.15 and 3.53 ppm correlating with oxygenated sp^3 carbons between 69.1 and 78.8 ppm, three exchangeable protons between 4.48 and 4.69 ppm, and one methyl group (δ_H 1.08/δ_c 18.2). gCOSY and zTOCSY NMR spectra revealed a spin system (Figure 2) comprising anomeric proton H-A1 (δ_H 4.62), H-A2 (δ_H 3.53), H-A3 (δ_H 3.35), H-A4 (δ_H 3.15), H-A5 (δ_H 3.44) and H-A6 (δ_H 1.08), suggesting the presence of a 6-deoxyhexosyl subunit. This subunit was designated here as subunit A. Subunit A was determined to be rhamnopyranosyl by High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) analysis of the hydrolyzed **1** in comparison with monosaccharide standards. The absolute stereochemistry of subunit A was determined after complete hydrolysis of **1**. By measurement of optical rotation, rhamnose purified from **1** hydrolysates was determined to be L-rhamnose $[\alpha]^{20}_{D}$ +7.9 (*c* 0.1, H_2O).[12] The diequatorial coupling between anomeric proton H-A1 and H-A2 ($J_{1,2}$ = 1.3 Hz)[13] indicated the glycosidic bond of L-rhamnopyranosyl moiety was in the α conformation.

The ^1H, gCOSY and HSQC NMR spectra of **1** also showed one oxygenated methylene (δ_H Ha 3.16/Hb 3.27/δ_c 64.2), one methine (δ_H 3.76/δ_c 49.0) bound to a secondary amine at δ_H

7.54, one aliphatic methylene (δ_H 1.23/δ_c 40.3), one aliphatic methine (δ_H 1.53/δ_c 24.7) and two aliphatic methyl groups (δ_H 0.79/δ_c 22.2 and δ_H 0.84/δ_c 23.8). This subunit was designated as subunit E. The gCOSY and zTOCSY NMR spectra revealed a spin system along the hydroxyl methylene H-E1 (δ_H H_a 3.16/H_b 3.27), amine bound methine H-E2 (δ_H 3.76), H-E3 (δ_H 1.23), H-E4 (δ_H 1.53) and the two methyls H-E5 (δ_H 0.79) and H-E5′ (δ_H 0.84), suggesting the presence of a leucinol subunit. Correlation between the amine proton (δ_H 7.54) and carbonyl carbon (δ_c 168.7) in the HMBC spectrum indicated that the leucinol subunit connected to the rest of **1** through an amide bond. The chirality of leucinol purified from **1** hydrolysates was determined by reaction with Marfey's reagent and comparison with the retention time of its FDAA derivative with L-leucinol and D-leucinol standards FDAA derivatives. Marfey's method has been previously used to determine the stereochemistry of amino alcohols such as alaninol and valinol.[14] To further validate this method for leucinol, L-leucinol and D-leucinol standards were derivatized with Marfey's reagent and analyzed by reversed phase HPLC. By comparison of the HPLC retention time, the amino alcohol subunit in **1** was determined to be L-leucinol.

In addition to the resonances belonging to rhamnopyranosyl and leucinol subunits, ^{1}H and ^{13}C NMR spectra showed three sets of resonances sharing highly similar structural features. The ^{13}C NMR spectrum showed three carbonyl carbons at δ_c 168.7, δ_c 169.4 and δ_c 170.3, three oxygenated methine carbons at δ_c 70.3, δ_c 71.8 and δ_c 74.3, three α-methylene carbons at δ_c 39.0, δ_c 40.4 and δ_c 40.6, and three long aliphatic chains with highly overlapped resonances, consistent with three β-hydroxyl fatty acid subunits designated here as B, C and D. The gCOSY and zTOCSY spectra revealed partial features of the spin systems. For subunit B, a partial spin system was observed along α-methylene protons B-H2 (δ_H H_a 2.41/H_b 2.46), oxygenated methine B-H3 (δ_H 3.85), aliphatic methylene B-H4 (δ_H 1.43) and a group of methylene resonances between δ_H 1.12 ppm and δ_H 1.32 ppm. Similarly, two other spin systems were assigned along C-H2 (δ_H H_a 2.45/H_b 2.53), oxygenated methine C-H3 (δ_H 5.04) and aliphatic methylene C-H4 (δ_H 1.52) for subunit C; and D-H2 (δ_H H_a 2.29/H_b 2.33), oxygenated methine D-H3 (δ_H 5.08) and aliphatic methylene D-H4 (δ_H 1.46) for subunit D, respectively. HMBC correlations from B-H3 to A-C1 and from A-H1 to B-C3 indicated that the α-rhamnopyranosyl group was connected to subunit B via a glycosidic bond. The ester bond between subunit C and B was deduced from HMBC correlations B-H2 and C-H3 to the carbonyl carbon at δ_c 170.3. Similarly, the ester bond between subunit D and C was deduced from HMBC correlations C-H2 and D-H3 to carbonyl carbon at δ_c 169.4. The amide proton (δ_H 7.54) from subunit E and α-methylene protons D-H2 (δ_H H_a 2.29/H_b 2.33) showed HMBC correlations to the carbonyl carbon at δ_c 168.7, providing the connectivity between subunits D and E.

The length of each fatty acid chain for subunits B, C and D could not be resolved by NMR data alone. To address this issue, tandem mass spectrometry (MS/MS) was performed on the precursor ion *m/z* 774.5674 $[M + H]^+$. As illustrated in Figure 3, fragment ions at *m/z* 628.5108 and m/z 610.5000 were detected as loss of the rhamnose residue (−146 Da) and with additional loss of H_2O (−164 Da), respectively, indicating fragmentation across the glycosidic bond. Minor fragment ions at *m/z* 458.3804 and *m/z* 440.3707 were detected as loss of one terminal fatty acyl chain ($C_{10}H_{18}O_2$, −170 Da) from product ions *m/z* 628.5108

and m/z 610.5000, respectively. Similarly, fragment ions m/z 288.2508 and m/z 270.2414 were detected as further loss of a terminal fatty acyl chain ($C_{10}H_{18}O_2$, −170 Da). The N-(3-hydroxydecanoyl)-leucinol (*m/z* 288.2508) and its dehydrated ion were left with no further visible fragmentations. From these data, all subunits B, C and D in **1** have C_{10} backbones.

To determine the absolute configuration of the oxygenated methines, the 3-hydroxydecanoic acids were isolated following complete hydrolysis of **1**. The optical rotation of the 1:1:1 mixture was measured as $[\alpha]^{25}_{D}$ −18.2 (*c* 0.1. $CHCl_3$), which is in agreement with the reported optical rotation of (*R*)-3-hydroxydecanoic acid ($[\alpha]^{25}_{D}$ −17.5(*c* 0.9 $CHCl_3$).[15] Therefore, all three 3-hydroxyldecanoic acid subunits possess the *R*-configuration, in agreement with other bacterial rhamnolipids.[1]

1 showed cytotoxicity with IC_{50} of 6.9 μM in BV-2 (murine microglia) and IC_{50} of 22 μM in HAPI (rat microglia). **1** was also tested for antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* PAO1, but no visible growth inhibition was observed.

In summary, we identified a novel rhamnolipid-amino alcohol hybrid rhizoleucinoside (**1)** from *Bradyrhizobium* sp. BTAi1. Its uniqueness lies in the incorporation of an amino alcohol moiety and the unprecedented rhamnolipid core structure that includes three sequential β-hydroxydecanoic acids. While the ecological role of this molecule requires further investigation, evidence suggests its possible role in establishing the symbiosis between plant host and bacterium. The first step to forming the symbiotic relationship between rhizobial bacteria and plant involves invasion of root cells.[16] Legume immune systems can be triggered during the infection but become rapidly suppressed.[17] Recent studies[18,19] have suggested that Nod factors (NFs), a group of signal molecules widely produced by rhizobium, may be responsible for plant immunity suppression. Interestingly, as a unusual phenomenon, *Bradyrhizobium* sp. BTAi1 is reported to lack the genes *nodABC*, which are essential for the production of NFs.[11] Alhede *et al*[20] postulates that rhamnolipids produced by the pathogen *P. aeruginosa* function as a shield to thwart the immune responses during infections of a human host. Further investigation will be required to determine if rhizoleucinoside plays a role for *Bradyrhizobium* sp. BTAi1 in suppressing the host plant defense system and facilitating the establishment of symbiosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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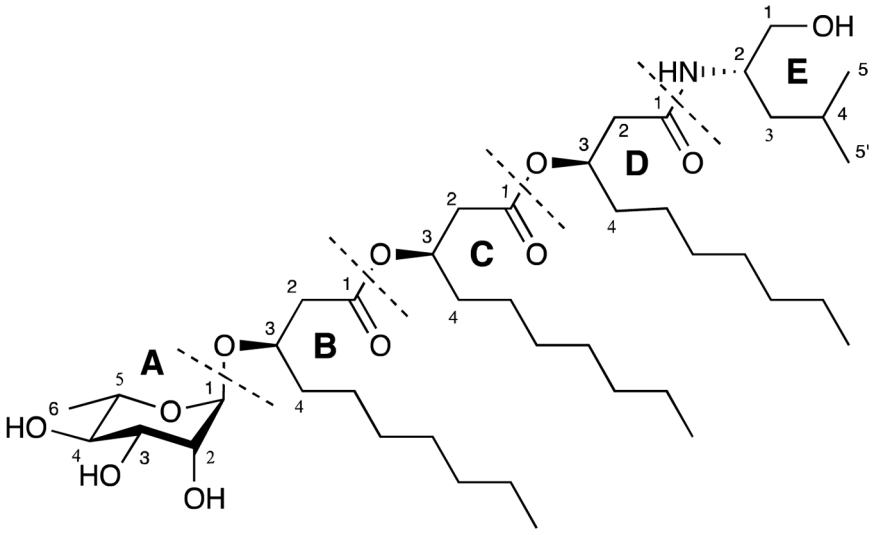

Figure 1.
Structure of rhizoleucinoside (**1**).

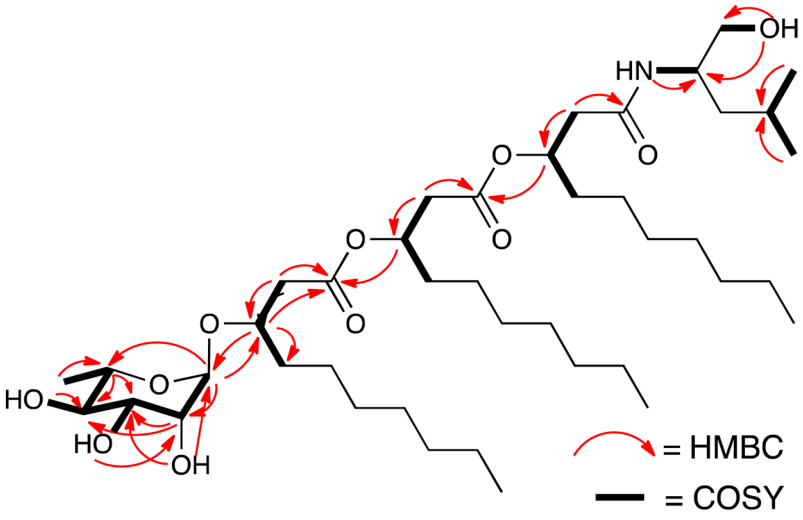

Figure 2.
Key ^{1}H-^{1}H COSY and HMBC correlations of **1**.

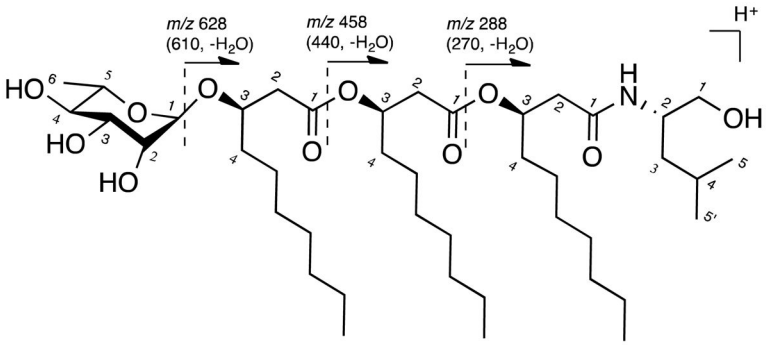

Figure 3.
Fragments of **1** by ESI-MS/MS.

Table 1

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of 1 in DMSO-d_6

no.	δ (ppm), type	δ $_H$ (ppm) (mult, *J* (Hz))	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC
A1	100.2, CH	4.62, d (1.3)	A2	A2, A5, B3
A2	71.2, CH	3.53, m	A1, A2, A2-OH	A3, A4
A2-OH		4.65, d (5.6)	A2	A3
A3	71.0, CH	3.35, m	A2, A4, A3-OH	
A3-OH		4.48, d (6.1)	A3	A2
A4	72.3, CH	3.15, m	A3, A5, A4-OH	
A4-OH		4.69, d (4.8)	A4	A4
A5	69.2, CH	3.44, m	A4, A6	A3, A4,
A6	18.2, CH_3	1.08, d (6.2)	A5	A5
B1	170.3, qC			
B2	39.0, CH_2	2.41, dd (14.0, 7.5), 2.46	B3	B1, B3
B3	74.3, CH	3.85, m	B2, B4	A1, B1, B4
B4	33.8, CH_2	1.43, m	B3	
B5-B9	22.5–31.6, CH_2	1.19–1.27, m		
B10	14.5, CH_3	0.84, m		
C1	169.4, qC			
C2	40.6, CH_2	2.45, m, 2.53, m	C3	C1, C3
C3	70.3, CH	5.04, m	C2, C4	B1
C4	33.5, CH_2	1.52, m	C3	
C5-C9	22.5–31.6, CH_2	1.18–1,25, m		
C10	14.5, CH_3	0.84, m		
D1	168.7, qC			
D2	40.4, CH_2	2.29, dd (14.0, 5.5), 2.33. dd (14.0, 7.9)	D3	D1, D3
D3	71.8, CH	5.08, m	D2, D4	C1
D4	33.5, CH_2	1.46, m	D3	
D5-D9	22.5–31.6, CH_2	1.17–1.24, m		
D10	14.5, CH_3	0.84, m		
E1	64.2, CH_2	3.16, m, 3.27, m	E1-OH	
E1-OH		4.56, t (5.5)	E1	E1, E2
E2	49.0, CH	3.76, m	E2-NH	
E2-NH		7.54, d (8.7)	E2	E2
E3	40.3, CH_2	1.23, m		
E4	24.7, CH	1.53, m		
E5	22.2, CH_3	0.79, d (6.5)	E4	E4
E5′	23.8, CH_3	0.84, d	E4	E4