



Research Article

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Chemical structures and biological activities of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* M14808

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ABSTRACT

P. aeruginosa M14808 was screened to produce rhamnolipid with high yield by mutation in a high magneto-gravitational environment. After silica gel columniation chromatogram, the components of the crude biosurfactant extract were analyzed by TLC, FTIR, and ESI-MS. The major components were characterized as rhamnolipids. The rhamnolipids consisted of two main types, mono- and di-rhamnolipids. Di-rhamnolipids showed significant anti-proliferative activity against human breast cancer cell line (MCF-7) at a minimum inhibitory concentration (MIC) of 1 $\mu\text{g/mL}$ as opposed to a minimum inhibitory concentration of 5 $\mu\text{g/mL}$ against human non-small lung cancer cell line (H460).

Key words: *Pseudomonas aeruginosa*; rhamnolipid; chemical structure; antitumor activity

INTRODUCTION

Microbially-derived surfactants called biosurfactants, are amphipathic molecules produced by a wide variety of bacteria, yeasts and filamentous fungi. Biosurfactants have properties of the general surfactants, which reduce the interfacial tensions between liquids, solids and gases and confer excellent detergency, emulsifying, foaming and other versatile chemical process. Compared with chemical surfactant, biosurfactants offer several advantages, such as low toxicity, inherently good biodegradability, and ecological acceptability [1]. Furthermore, antibiotic activity of some biosurfactants [2-4] and their inhibitory effect on algicidal activity [5] have been reported. These properties make a variety of potential applications, including cosmetics- pharmaceutical formulations, agricultural, food industry, oil recovery and environment protection technology [4, 6-9].

Bacteria of the genus *Pseudomonas* are known to produce a glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids [10]. Rhamnolipids, which are a group of secondary metabolites by *Pseudomonas aeruginosa*, have been widely studied and are reported to be a mixture of the homologous species RL1 (RhaC₁₀C₁₀), RL2 (RhaC₁₀), RL3 (Rha₂ C₁₀C₁₀) and RL4 (Rha₂C₁₀) [11]. Rhamnolipids are easily isolated from culture broth and can be produced using hydrophobic and hydrophilic substrates such as carbohydrates, hydrocarbons, vegetable oils or wastes from food industry [12-14]. In recent studies, Thanomsub *et al.* [15] found that Rhamnolipids had significant inhibition effect on the growth of human breast cancer cell lines (MCF-7) and insect cell line (C6/36); Stipcevic *et al.* [16, 17] reported that di-rhamnolipids had differential effects on the growth of human keratinocytes and fibroblasts and also could improve the healing of deep burns.

In this paper, we describe the biosurfactant producing strain. Also, we determine the structure and the biological activities of these biosurfactants against two cancer cell lines in vitro in order to verify.

EXPERIMENTAL SECTION

Microorganisms, cell lines and media: *Pseudomonas aeruginosa* M14808 mutated in High Magneto-Gravitational Environment was selected as the highest biosurfactant producer and stored at -80 °C in 50% glycerol. Two cancer cell lines, human non-small lung cancer cell (H460) and human breast cancer cell line (MCF-7), were applied in the study and provided by Wenzhou Medical University.

Medium (g·L⁻¹): NaNO₃ 3.0, KH₂PO₄ 2.0, K₂HPO₄ 1.0, MgSO₄·7H₂O 0.50, KCl 0.1, CaCl₂·2H₂O 0.01, FeSO₄·7H₂O 0.01, yeast extract 0.01, vegetable oil 40, 0.05 mL trace-element solution containing (g·L⁻¹) H₃BO₃ 0.26, CuSO₄·5H₂O 0.5, MnSO₄·H₂O 0.5, MoNa₂O₄·2H₂O 0.06, ZnSO₄·7H₂O 0.7. pH was adjusted to 7.0 ± 0.2.

Production and extraction of biosurfactants: *P. aeruginosa* M14808 was cultivated in 500-mL Erlenmeyer flasks containing 160 mL of medium. Cultures were incubated at 30 °C on a rotary shaker at 220 rpm for 7 days. The culture broth was centrifuged at 10000 rpm for 20 min at 4 °C. The supernatant was adjusted to pH 2.0 with 6M HCl and then incubated at 4 °C overnight. The total broth was extracted twice with chloroform–methanol (2:1, v/v). The mixture was stirred with Magnetic Stirrer for 20 min. The organic phase was concentrated by the vacuum distillation at 40 °C. After evaporation, the resulting yellowish, oily crude product containing biosurfactants was obtained.

Purification of biosurfactants: The crude extract was purified by silica gel column chromatography which was eluted with chloroform and then with a sequence of chloroform–methanol 10:1 (v/v) 100 mL, 4:1 (v/v) 100 mL, 2:1 (v/v) 100 mL, 1:1 (v/v) 100 mL. The different glycolipidic types were separated by analytical thin-layer chromatography (TLC), carried out on silica gel plates GF-254 using chloroform/methanol/acetic acid (65:15:2, v/v/v) as developing solvent. Then the plates were visualized with sprayed phenol–H₂SO₄ and developed at 100 °C for 5 min.

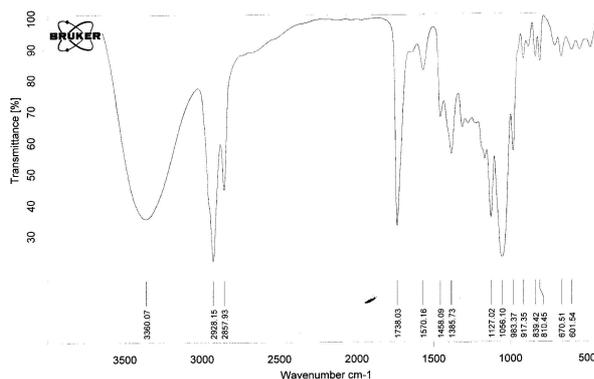
Structure characterization of the purified compounds: Fourier transform infrared spectroscopy (FTIR), FTIR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures. In this present study, a Bruker TENSOR 27 FTIR spectrometer was used to provide a chemical analysis of the components in the crude biosurfactant; Electrospray ionisation–mass spectrometry (ESI–MS), ESI tandem mass spectra were acquired by mass-selecting the target ion using a quadrupole mass analyzer. The conditions of the analyses were: TSQ Quantum ultra (AM) quadrupole instrument (Finnigan Ltd., Foster City, CA, USA). Negative ion mass spectra were used. The scanning mass range was 30~800 Da.

Antitumor activities: Antiproliferative test against the cancer cell line was performed using MTT assay. These two cell lines (MCF-7 and H460) were grown in DMEM, at 37 °C, 5% CO₂. The cancer cell line culture was supplemented with 10% fetal bovine serum and penicillin and streptomycin at 100 unit/mL and 100 µg/mL, respectively. The cell lines were pooled, diluted to the cell density 10⁵ cells/mL and dispensed into a 96-well plate. Biosurfactants were dissolved in DMSO, sonicated and then diluted 10 fold with the culture medium and added to the cells to final volume 100 µL per well. After 48 h of incubation at 37 °C, 5% CO₂, 2 mg/mL MTT were added to each well and incubated for another 4 h. Then 100 µL DMSO were added and mixed thoroughly. The absorbance was measured at 570 nm using a microplate reader (TECAN infinite M200 PRO, Austria). The positive and negative controls were included in the assay.

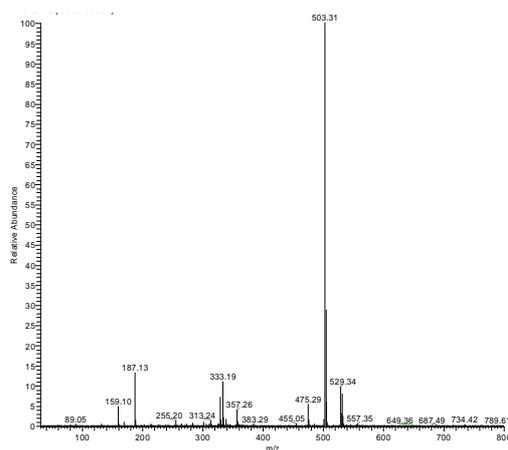
RESULTS AND DISCUSSION

Purification and detection of biosurfactants: The crude extract showed two major spots with R_f value 0.40 and 0.78 analyzed by silica gel TLC. According to the color reaction, the spots with R_f value 0.40 (named R1) and 0.78 (named R2) are glycolipids (The data was not showed). The separation of biosurfactant was accomplished by silica gel column chromatography. The fractions with similar R_f value were combined and dried under reduced pressure with a rotor evaporator at 40 °C. Sample R1 and R2 were prepared.

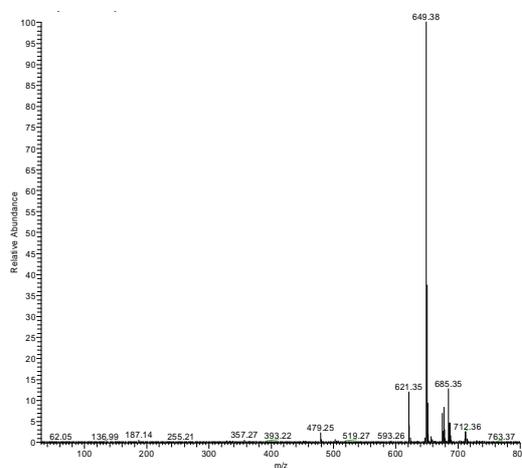
Chemical structures of biosurfactants: The chemical composition of each component fractionated from the crude biosurfactant was preliminarily investigated by using FTIR technique. The FTIR spectra of the two kinds of glycolipids (R1, R2) were partially similar, suggesting that the three fractions should have similar chemical structures (in Fig. 1).

**Fig. 1** FT-IR spectrum of glycolipid (R1, R2)

The important adsorption bands (R1, R2) located at 3360, 2928, 2857, 1738, and 1300–1100 cm^{-1} indicate that all of them have chemical structures identical to those of rhamnolipids, which are composed of rhamnose rings and long hydrocarbon chains [18]. The presence of an ester structure is clearly indicated by the absorbance bands at the wave numbers of 1738, and 1128 cm^{-1} : the first corresponds to the stretching of ester C=O and the latter to the stretching of C-O-C. The strong absorbance at the range of 3050–3650 cm^{-1} indicates the presence of hydroxyl groups in the chemical structures of the biosurfactants. The strong adsorption peaks present at 2928 and 2858 cm^{-1} are expected to be the C–H stretching vibrations of the hydrocarbon chain positions.

**Fig. 2** Average mass spectrum of sample R1

With the use of MS analyses, the chemical structures of the isolated fractions were revealed. From the mass spectra, the possible molecular weights were identified for fractions R1 and R2 respectively. Fig. 2 and Fig. 3 showed the average mass spectrum of R1 and R2.

**Fig. 3** Average mass spectrum of sample R2

The mass spectrum of sample R1 shows intense molecular ions at m/z 333, 475, 503, 529 respectively and molecular ions at m/z 503 is the predominant component. These data are consistent with the structure of mono-rhamnolipids. In the case of sample R2, the presence of signals at m/z 479, 621, 649, 677, 685 in the mass spectrum correspond to di-rhamnolipids [4, 19-21] and the predominant component is molecular ions at m/z 649.

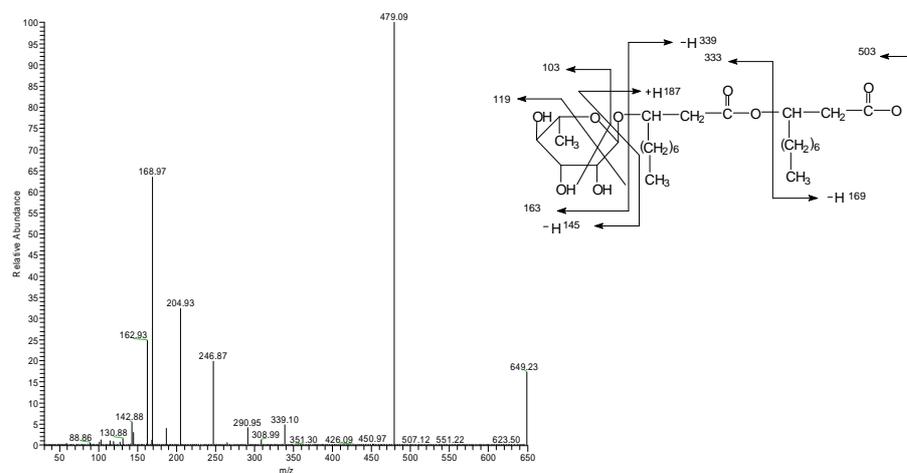


Fig. 4 MS/MS spectrum of $RhC_{10}C_{10}$

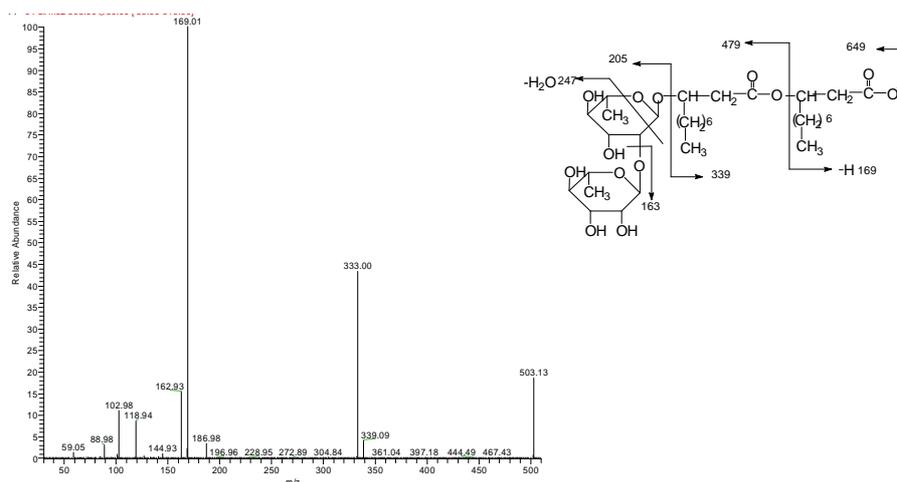


Fig. 5 MS/MS spectrum of and $Rh_2C_{10}C_{10}$

In order to confirm the structures and the positions of the fatty acids, these molecular ions were submitted to the tandem-MS mode. The main ion of R1 (m/z 503, $Rha-C_{10}-C_{10}$) showed daughter ions at m/z 333, corresponding to the rupture of the ester link, m/z 169, corresponding to the released fatty acids, and m/z 163, corresponding to the rupture of the link in the rhamnose-alkylic chain (in Fig. 4).

The major molecular ion with m/z 649 ($Rha_2-C_{10}-C_{10}$) in sample R2 was also analyzed by tandem-MS. It gave daughter ions that indicate the rupture of the ester bond between the alkyl chains, releasing Rha_2-C_{10} (m/z 479) and hydroxyl decanoate (m/z 169). A daughter ion with m/z 163, corresponding to a single rhamnose, was also identified (in Fig. 5) [22]. Tandem-MS was also performed on the other ions of R1 and R2 in Tab. 1.

Antitumor activity analysis: The R1, R2 and the crude extract of this strain culture were applied in anti-tumor experiments. The result was that R1 and the crude extract didn't have anti-tumor activity (Tab. 2). Interestingly, R2 showed significant inhibitory effect against MCF-7 and H460, at the minimum inhibitory concentration 1 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$ respectively. The MS analysis result could infer that di-rhamnolipids were the dominant component of R2. Nevertheless, no inhibition effects were observed with the biosurfactant crude extract. This may be due to the relatively low concentration of di-rhamnolipids in the total biosurfactant crude extract. The inhibitory activity of R2 was obviously observed in dose dependent manner as shown in Fig. 6 (p -value <0.05).

Tab. 1 Structure of the rhamnolipids from sample R1 and R2

NO	Rhamnolipid	m/z	Main fragmentations	NO	Rhamnolipid	m/z	Main fragmentations
1	RhC _{8:2}	301	159, 187, 131	11	Rh ₂ C ₈ C ₁₀	621	451, 169, 247
2	RhC ₈	305	163, 141	12	Rh ₂ C ₁₀ C ₈	621	479, 141, 247
3	RhC _{10:2}	329	159, 139, 171	13	Rh ₂ C ₈ C _{12:1}	647	195, 452
4	RhC ₁₀	333	169, 163	14	Rh ₂ C ₁₀ C ₁₀	649	479, 169, 247
5	RhC ₈ C ₁₀	475	169, 141, 305	15	Rh ₂ C ₁₀ C _{12:1}	675	479, 365, 193, 247, 149
6	RhC ₁₀ C ₈	475	333, 311, 141	16	Rh ₂ C _{12:1} C ₁₀	675	505, 365, 169, 247
7	Rh ₂ C ₁₀	479	309, 169, 247	17	Rh ₂ C ₁₀ C ₁₂	677	479, 367, 195, 247, 151
8	RhC ₁₀ C ₁₀	503	169, 163, 333, 103	18	Rh ₂ C ₁₂ C ₁₀	677	507, 367, 169, 247
9	RhC ₁₀ C _{12:1}	529	333, 365, 193	19	Rh ₂ C ₁₀ C _{14:1}	703	480
10	RhC _{12:1} C ₁₀	529	359, 65, 169				

Tab. 2 Biological assay of the biosurfactants produced by *P. aeruginosa* M14808 against various cell lines

Compound	MCF-7	H460
R1	NS	NS
R2	1 µg/mL	5 µg/mL
N1	NS	NS
Crude extract	NS	NS

NS means non-significant difference in the cell proliferative activity of the tested compounds compared to negative control. Numerical values represent the minimum inhibitory concentration that gave a significant proliferative activity different from controls.

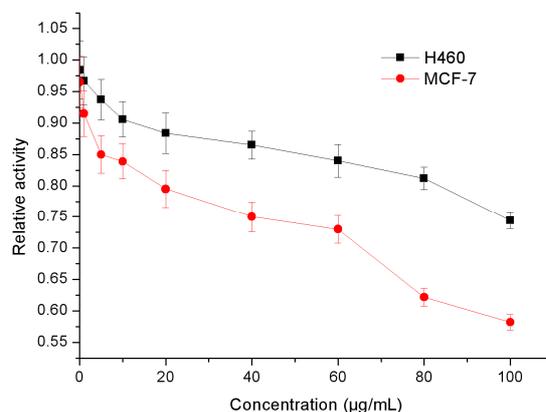


Fig 6 Dose response effect of R2 on the proliferation of MCF-7 & H460 cells after 48 h incubation

CONCLUSION

After the characterization of the purified metabolins of *P. aeruginosa* M14808 by TLC, FTIR and ESI-MS, it was found that there were two major products, mono- rhamnolipids, di-rhamnolipids. Rha-C₁₀-C₁₀ and Rha₂-C₁₀-C₁₀ were respectively verified as the major components of the mono- and di-rhamnolipids. Di-rhamnolipids showed significantly anti-proliferative activity while the other two fractions and the crude extract didn't have this potential. The MIC of di-rhamnolipids to MCF-7 and H460 was 1 µg/mL and 5 µg/mL, respectively. Thus, di-rhamnolipids produced by *P. aeruginosa* M14808 could have potential applications as anticancer drug.

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REFERENCES

- [1] J.D. Desai, I.M. Banat. *Microbiology and Molecular Biology Reviews*. **1997**, 61(1): 47-64.
- [2] M.S.C. Pedras, N. Ismail, J.W. Quail, et al. *Phytochemistry*. **2003**, 62(7): 1105-1114.
- [3] M. Abouseouda, R. Maachib, A. Amranec, et al. *Desalination*. **2008**, 223(1-3): 143-151.
- [4] M. Benincasa, A. Abalos, I. Oliveira, et al. *Antonie van Leeuwenhoek*. **2004**, 85(1): 1-8.
- [5] X. Wang, L. Gong, S. Liang, et al. *Harmful Algae*. **2005**, 4(2): 433-443.
- [6] K. Das, A.K. Mukherjee. *Bioresource Technology*. **2007**, 98(7): 1339-1345.
- [7] C.N. Mulligan. *Environmental Pollution*. **2005**, 133(2): 183-198.

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- [8] A. Abalos, A. Pinazo, M.R. Infante, et al. *Langmuir*. **2001**, 17(5): 1367-1371.
- [9] J.C. Mata-Sandoval, J. Karns, A. Torrents. *Environment Science and Technology*. **2000**, 34(23): 4923-4930.
- [10] S. Lang, D. Wullbrandt. *Applied of Microbiology & Biotechnology* **1999**, 51(1): 22-32.
- [11] S.G.V.A.O. Costa, M. Nitschke, R. Haddad, et al. *Process Biochemistry*. **2006**, 41(2): 483-488.
- [12] L. Sim, O.P. Ward, Z-Y Li. *Journal of Industrial Microbiology and Biotechnology*. **1997**, 19(4): 232-238.
- [13] M. Benincasa, J. Contiero, M.A. Manresa, et al. *Journal of Food Engineering*. **2002**, 54(4): 283-288.
- [14] R.M. Patel, D.A.J. Desai. *Journal of Basic Microbiology*. **1997**, 37(4): 281-286.
- [15] B. Thanomsub, W. Pumeechockchai, A. Limtrakul, et al. *Bioresource Technology*. **2007**, 98(5): 1149-1153.
- [16] T. Stipcevic, T. Piljac, R.R. Isseroff. *Journal of Dermatological Science*. **2005**, 40(2): 141-143.
- [17] T. Stipcevic, A.Piljac, G.Piljac. *Burns*. **2006**, 32(1): 24-34.
- [18] O. Pornsunthorntawee, P. Wongpanit, S. Chavadej, et al. *Bioresource Technology*. **2008**, 99(6): 1589-1595.
- [19] W. Shen, S. Yang, X. Li. *China Biotechnolog*. **2005**, 25(10): 83-87.
- [20] E. Haba, A. Abalos, O. Jáuregui, et al. *Journal of Surfactants and Detergents*. **2003**, 6(2): 155-161.
- [21] E. Haba, A. Pinazo, O. Jauregui, et al. *Biotechnology and Bioengineering*. **2003**, 81(3): 316-322.
- [22] S.A. Monteiro, G.L. Sasaki, L.M. Souza, et al. *Chemistry and Physics of Lipids*. **2007**, 147(1): 1-13.