A BIOSYNTHETIC ROUTE TO [^{14}C]-LABELLED RHAMNOLIPIDS

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SUMMARY

After feeding 200 mg of [U-^{14}C]-D-glucose (8.25 MBq/mmol; 9.16 MBq) to a culture of *Pseudomonas aeruginosa* DSM 2659, the rhamnolipids RL-1 and RL-2 produced by the microorganism were obtained in uniformely ^{14}C-labelled form as crude fraction by extraction of the medium. The fraction was analyzed by TLC and HPLC. Cleavage with HCl gave rhamnose (identified by TLC) and β-hydroxydecanoic acid (identified by TLC and GC-MS). The radiochemical yields were 1.19 % for RL-1 and 1.15 % for RL-2; specific activities amounted to 23.191 and 27.062 MBq/mmol, respectively.

Key Words: ^{14}C-Labelled Rhamnolipids, Glycolipid, Biosurfactant, *Pseudomonas aeruginosa* DSM 2659, Biosynthesis.

INTRODUCTION

Bacteria of the species *Pseudomonas aeruginosa* are known to produce four chemically closely related rhamnolipids, when grown *e.g.* on glucose or glycerol. These glycolipids are composed of rhamnose and β-hydroxydecanoic acid subunits, and are excreted into the culture medium. The two rhamnolipids primarily produced by the strain *P. aeruginosa* DSM 2659 are displayed in Fig. 1; in the present investigation, the compounds are termed RL-1 and RL-2.[1-8] Due to their physicochemical properties, rhamnolipids belong to a class of natural products usually designated as biosurfactants. In recent years, interest in biosurfactants in general and rhamnolipids in particular is increasing, since the compounds are supposed to enhance the biodegradation in the environment of lipiphilic, especially anthropogenic chemicals, such as alkanes or polycyclic aromatic hydrocarbons.[9-12] So, rhamnolipids were shown to stimulate the uptake and degradation of n-alkanes by the producer strain itself and other *Pseudomonas* species under lab conditions.[13, 14] *Pseudomonas* strains were also reported to be particularly abundant in soils contaminated with water-insoluble compounds, such as petroleum products.[15, 16] However, detailed knowledge of the role of rhamnolipids in the environmental degradation and mineralization of lipophilic substrates is not at hand.[12]

Though concentrations are generally lower, the rhamnolipids RL-1 and RL-2 are also biosynthesized and excreted during lab cultivation of *Pseudomonas aeruginosa* DSM 2659 on n-alkanes.[17] The glycolipids consequently may be regarded as metabolites of these lipophilic substrates. As part of an ecochemical program[17] aiming at the degradation of a number of (^{14}C-labelled) n-alkanes by *P. aeruginosa* DSM 2659, we thus became concerned with the further

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fate and behaviour of both glycolipids in cultures of the producer strain. Ecochemical studies of such a kind are preferentially performed using labelled compounds; so, the preparation of ^{14}C-labelled rhamnolipids RL-1 and RL-2 was projected. Although a (chemical) synthesis of the glycolipids was published[18] and suitable ^{14}C-labelled starting materials were commercially available, this approach was regarded as insufficient for the following reasons. First, purely synthetic rhamnolipids would be labelled at only one or two defined positions. For the intended purposes however, a - preferably uniform - labelling of all subunits was requisite. Secondly, the β-hydroxydecanoic acid component of synthetic rhamnolipids would be present as racemic mixture, whereas the bacterial products are strictly in the R-configuration. Thirdly, absolute yields and specific activities of the chemically prepared glycolipids would possibly be too low for subsequent ecochemical experiments due a considerable number of steps of the synthesis. A biosynthetic approach was thus regarded as more favourable. The objective of the present investigation was to biosynthesize the rhamnolipids RL-1 and RL-2 in cultures of *Pseudomonas aeruginosa* DSM 2659 using as substrate commerially available uniformly ^{14}C-labelled glucose.

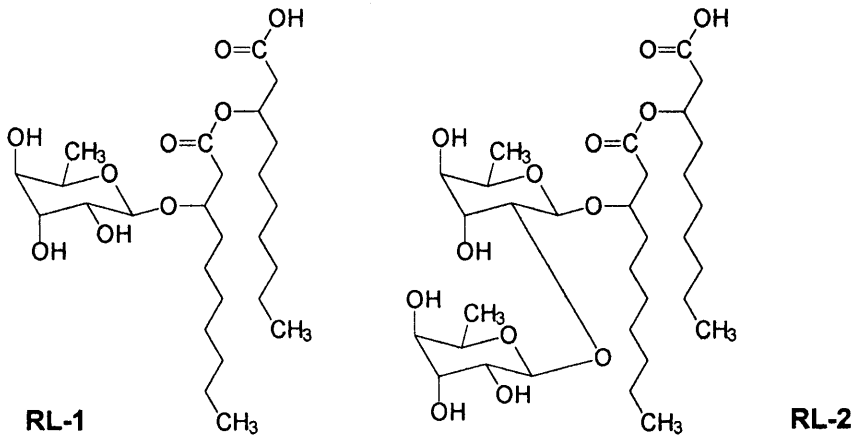

Fig. 1: Chemical structures of the main rhamnolipids produced by *Pseudomonas aeruginosa* DSM 2659.

RESULTS AND DISCUSSION

Before the labelling experiment discussed below was executed, the influence of different culture conditions of *P. aeruginosa* DSM 2659 on the production yields of the rhamnolipids RL-1 and RL-2 was investigated using non-labelled glucose. These conditions included the composition of the production medium, the time of harvesting of the bacteria employed for the subsequent production experiments, and the incubation interval used for production. The experiments further concentrated on the use of suspended and (alginate) immobilized cells. Essentially, these studies were performed in compliance with published investigations and results.[19, 20] Two important modifications however were introduced with regard to the subsequent labelling experiment. First, to obtain rhamnolipids labelled with maximum attainable specific activity, the glucose concentration in the assays was generally reduced from 2 % (routine cultivation) to 0.4 % or 0.2 % (w/v). Secondly, the experiments were performed in a closed system which rendered possible the trapping of the (gaseous) dissimilation product $^{14}CO_2$ and thus, a complete balancing of the radioactivity applied. Throughout, concentrations

of the rhamnolipids RL-1 and RL-2 were determined by HPLC according to a previously published procedure.[8] The main result of the experiments was that RL-1 and RL-2 were biosynthesized by *P. aeruginosa* grown with low glucose concentrations. The yields obtained however, were noticeably reduced to ≤ 250 mg/l (6.25 % based on mass of glucose provided in 0.4 % to the cells) as compared to about 2.5 g/l (12.5 %) usually resulting from routine cultivation.[8, 21] Prevailingly, yields obtained with suspended bacteria were higher than those with immobilized cells.

The labelling experiment was then performed with a suspension of *P. aeruginosa* DSM 2659 and 9.16 MBq (8.25 MBq/mmol; 200 mg) of [U-^{14}C]-D-glucose. After 5 days of incubation, the main portions of radioactivity were found in the nutrient solution (67.25 % of applied ^{14}C; 6.16 MBq) and in the trap for $^{14}CO_2$ (30.48 %; 2.79 MBq). Only a minor percentage remained associated with the cells (2,68 %, 0.25 MBq). Thus, the applied ^{14}C was quantitatively recovered from the assay (100.41 %). The nutrient solution was then extracted with ethyl acetate. While 63.39 % (5.81 MBq) of the applied radioactivity remained in the aqueous phase, 3.86 % (0.35 MBq) were detected in the organic layer as crude rhamnolipid fraction. Ethyl acetate was previously shown to be most efficacious concerning the extraction of rhamnolipids from aqueous solution.[8] Thin-layer chromatographic analysis of the organic extract revealed six ^{14}C zones. Main peaks 5 (R_f 0.72, 31.1 % of ^{14}C applied onto the plate) and 3 (R_f 0.31, 29.9 %) were identified as the rhamnolipids RL-1 and RL-2, respectively by cochromatography with authentic samples.[8] The identity of the other peaks remained unknown (1: R_f 0.00, 2.0 %; 2: R_f 0.14, 14.9 %; 4: R_f 0.53, 2.6 %; 6, yellow pigment: R_f 0.91, 19.5 %). According to R_f values observed, we speculated that peaks 2 and 4 were further rhamnolipids.

In order to determine the specific activity of the rhamnolipids, the organic extract of the media was subjected to HPLC analysis.[8] Identification of RL-1 and RL-2 was executed using both radioisotope and UV detection (including the respective UV spectra). Quantitative evaluation of the UV chromatograms demonstrated that totals of 4.70 µmol (RL-1; $C_{26}H_{48}O_9$, 504 g/mol) and 3.88 µmol (RL-2; $C_{32}H_{58}O_{13}$, 650 g/mol) had been isolated from the assay. In relation to the corresponding amounts of radioactivity detected by TLC (0.109 MBq and 0.105 MBq, respectively), the specific activity of RL-1 thus amounted to 23.191 MBq/mmol, that of RL-2 to 27.062 MBq/mmol. Since in the bacteria, both the carbohydrate and β-hydroxydecanoic acid moiety of the rhamnolipids are ultimately derived from the carbon source glucose (C_6), the (theoretical) maximum labelling of RL-1 (C_{26}) was 35.75 MBq/mmol and that of RL-2 (C_{32}) 44.00 MBq/mmol. The present results indicate that noticeably portions of the rhamnolipids or crucial precursors of the glycolipids were present in the microorganisms before the ^{14}C-labelled substrate was added. This supposition is corroborated by different yields obtained in the labelling experiment. Concerning radioactivity, 2.34 % of the applied ^{14}C was recovered in the rhamnolipids, while as to mass of carbon (added as glucose), a total of 3.70 % was found in RL-1 and RL-2.

The crude rhamnolipid fraction was also treated with HCl in order to hydrolyze glycosidic and ester linkages between carbohydrate and fatty acid subunits. After extraction of the reaction mixture, TLC analysis demonstrated that only one ^{14}C compound was present in the aqueous layer which cochromatographed with non-labelled rhamnose. An analogous examination of the ethyl acetate phase (23.700 dpm) revealed a ^{14}C peak cochromatographing with an authentic sample of β-hydroxydecanoic acid (R_f 0.48; synthesized as described[17]). After derivatization with BSTFA, the fatty acid was qualitatively and quantitatively identified by GC-MS; m/z (relative intensity): 331 (1, $M^{+\bullet}$), 317 (28), 275 (21), 233 (79), 201 (50), 189 (19), 147 (100), 133 (13), 117 (8), 101 (9), 73 (85), 69 (11), 43 (5). Quantitative evaluation showed that 10.05 µg (0.0588 µmol) had been liberated from the aliqot (100.000 dpm) of the crude rhamnolipid fraction, which resulted in a specific activity of 6.718 MBq/mmol (theoretical, maximum labelling: 13.750 MBq/mmol).

We arrived at the following conclusions. Both subunits of the rhamnolipids RL-1 and RL-2 were ^{14}C-labelled; in all probability, the radioactive label was uniformly distributed across the carbon skeleton of the glycolipids. The yield obtained in the labelling experiment was lower than that resulting from the corresponding preliminary (non-labelled) investigation; this requires further studies in order to optimize the general procedure. Since the crude rhamnolipid fraction was sufficient for the projected experiments, RL-1 and RL-2 were not purified and separated. This may be achieved by preparative TLC or column chromatography.[8] On the whole, the investigation demonstrated that ^{14}C-labelled rhamnolipids with specific activities and yields adequate for most ecochemical experiments can be obtained using a biosynthetic approach. The procedure may also be applied to other biosurfactants, *e.g.* sophorose lipids.

EXPERIMENTAL

General. Thin-layer chromatography (TLC) was performed on silica gel plates (SIL G-25; Macherey-Nagel; Düren, Germany developed in solvent systems chloroform:methanol:acetic acid, 65:15:2 (v/v/v; rhamnolipids and rhamnose), n-butanol:acetic acid: water, 4:4:2 (v/v/v; rhamnose), or diethylether:n-hexane:acetic acid, 10:5:1 (v/v/v; β-hydroxydecanoic acid). Radioactive zones were detected by means of a Tracemaster 40 radiochromatogram scanner (Berthold; Wildbad, Germany); non-labelled carbohydrates and rhamnolipids were visualized using p-anisaldehyde (1 ml dissolved in 250 ml of acetic acid and 5 ml of H_2SO_4; development at 100°C) or thymol (0.5 g dissolved in 95 ml of ethanol and 5 ml of H_2SO_4; development at 100°C). High-performance liquid chromatography (HPLC) was executed as described.[8] ^{14}C-labelled compounds were additionally analyzed by means of a Radioisotope Detector 171 (Beckman; München, Germany). Gaschromatography/mass spectroscopy (GC-MS) was carried out on a HP 5890 Series II gas chromatograph coupled to a HP 5971A mass selective detector (Hewlett-Packard; Waldbronn, Germany); column: HP 5 (Hewlett-Packard); carrier gas: He (1 ml/min); temperature program: 85°C for 3 min, 85 - 200°C at 10°C/min, 200 - 280°C at 20°C/min, hold 280°C for 3 min; injector: 250°C; interface: 300°C; splitless injection. EI-MS spectra were recorded at 70 eV. Prior to GC-MS analysis, β-hydroxydecanoic acid was derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA (Fluka; Buchs, Switzerland) at 60°C for 30 min. Portions of ^{14}C associated with the bacteria were determined by combustion analysis using a biological oxidizer OX 500 (Zinsser; Frankfurt, Germany). Liquid scintillation counting (LSC) was performed by means of a Canberra Packard Tricarb 1500 analyzer (Dreieich, Germany).

Microorganism, cultivation and media. The microorganism *Pseudomonas aeruginosa* DSM 2659 was provided by Deutsche Stammsammlung für Mikroorganismen (Braunschweig, Germany), and was routinely cultivated in 3M Medium at 37°C on a rotary shaker at 200 rpm using glucose (2 %; w/v) as carbon source.[3,8] Medium 2M employed for the production of the ^{14}C-labelled rhamnolipids consisted of 2.5 g/l NaCl and 0.37 g/l $CaCl_2$ $2H_2O$.[19,20] For the labelling experiment, 50 ml of a routine culture of *P. aeruginosa* (45 h of cultivation; optical density, OD_{595} = 5.56) was harvested by centrifugation (10 min at 7.500 x g). The supernatant was discarded, while the pellet was washed with 0.9 % (w/v) NaCl solution. After a further centrifugation step, the bacteria were resuspended in 20 ml of 2M medium.

Radiochemical. [U-^{14}C]-D-Glucose (9.16 MBq, 6.25 mg, 266,4 MBq/mmol, 4.81 MBq/ml of water) obtained from Sigma Chemical Company (Deisenhofen, Germany) was diluted with 193.75 mg of α-D-(+)-glucose to a specific activity of 8.25 MBq/mmol (total of 1.11 mmol). For the labelling experiment, the entire mixture was dissolved in 30 mL of sterile 2M medium.

Biosynthesis of the ^{14}C-labelled rhamnolipids. The experiment was performed in a closed, air-tight system which consisted of a 100 ml Erlenmeyer flask with a thread connected to a trap

for $^{14}CO_2$ (Fig. 2). After sterilization of the entire system, the trap was filled - from bottom to top - with 0.3 g of glass wool, 10 g of soda lime ($^{14}CO_2$), 0.2 g of glass wool, 4.0 g of soda lime (atmospheric CO_2), and 0.2 g of glass wool. Subsequently, the solution of ^{14}C-glucose and the bacterial suspension were introduced into the flask (resulting glucose concentration: 0.4 %, w/v). The assay was incubated for 5 days at 36°C and 150 rpm in a water bath, while the whole system was aerated with moistened air (100 ml/min) *via* a sterile filter (0.2 µm).

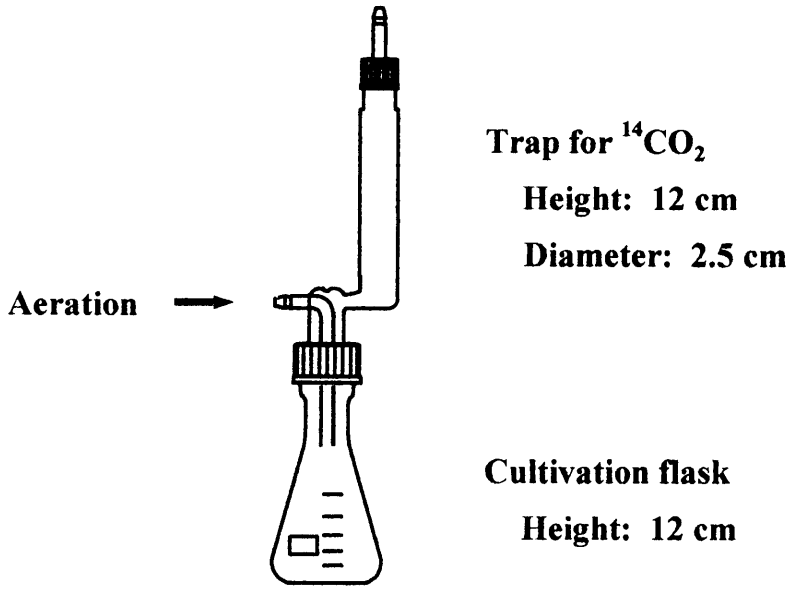

Fig. 2: Closed cultivation system used for the biosynthesis of the ^{14}C-labelled rhamnolipids by *Pseudomonas aeruginosa* DSM 2659.

Extraction and work-up of assay. After termination of the labelling experiment, the pH of bacterial suspension was adjusted to 3 - 4 with 2 M HCl. Subsequently, the medium was extracted 3 times with 50 ml of ethyl acetate. The combined organic phases containing the ^{14}C-labelled rhamnolipids were dried over Na_2SO_4, taken to dryness *in vacuo*, and the resulting residue was dissolved in ethanol. Bacteria and remaining medium were separated by centrifugation. The microorganisms contained in the pellet were resuspended in *ca.* 1 ml of water and applied to filter paper. After drying, the filter paper and adhering bacteria were subjected to combustion analysis. Radioactive CO_2 absorbed by the soda lime was released over a period of 2 h by treatment with 8 M HCl in a closed, N_2-flushed apparatus, and was directly absorbed into Oxysolve C-400 scintillation cocktail (Zinsser).

Analysis. For a complete balance, the amounts of ^{14}C contained in all soluble fractions - including samples obtained by combustion and $^{14}CO_2$ liberation - were determined by LSC. The ethanolic solution of the ^{14}C-labelled rhamnolipids was analyzed by TLC and HPLC. An aliquot (100.000 dpm) of the solution was also subjected to hydrolyic conditions (2M HCl, 2 h under reflux). The reaction mixture was adjusted to pH 3 with saturated $NaHCO_3$ solution and extracted with ethyl acetate. Then, the β-hydroxydecanoic acid moiety of the rhamnolipids contained in the organic phase was examined by TLC and GC-MS, while the aqueous phase was analyzed for rhamnose by TLC.

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REFERENCES

1. Hauser G. and Karnowsky M.L. - J. Biol. Chem. 229: 91 (1957).
2. Itoh S., Honsa H., Tomita F. and Suzuki T. - J. Antibiot. 24: 855 (1971).
3. Guerra-Santos L., Käppeli O. and Fiechter A. - Appl. Environ. Microbiol. 48: 301 (1984).
4. Syldatk C., Lang S. and Wagner F. - Z. Naturforsch. 49c: 51 (1985).
5. Parra J.L., Guinea M., Mansera M., Robert M., Mercade M., Comelles F. and Bosch M.P. J. Am. Oil Chem. Soc. 66: 141 (1989).
6. Parra J.L., Pastor J., Comelles F., Manresa M.A. and Bosch M.P. - Tenside Surf. Det. 27: 302 (1990).
7. Rendell N.B., Taylor G.W., Somerville M., Todd H., Wilson R. and Cole P.J. - Biochem. Biophys. Acta 1045: 189 (1990).
8. Schenk T., Schuphan I. and Schmidt B. - J. Chromatogr. A 693: 7 (1995).
9. Jain D.K., Lee H. and Trevors J.T. - J. Ind. Microbiol. 10: 87 (1992).
10. Müller-Hurtig R., Wagner F., Blaszczyk R. and Kosaric N. - in "Biosurfactants: Production, Properties, Applications", Ed. Kosaric N., Marcel Dekker Inc. (1993), pp. 447.
11. Hommel R.K. - in "Biochemistry of Microbial Degradation", Ed. Ratledge C., Kluwer Academic Publishers (1994), pp. 63.
12. Arino S., Marchal R. and Vandecasteele J.-P. - Appl. Microbiol. Biotechnol. 45: 162 (1996).
13. Zhang Y. and Miller R. - Appl. Environ. Microbiol. 58: 3276 (1992).
14. Zhang Y. and Miller R. - Appl. Environ. Microbiol. 60: 2101 (1994).
15. MacElwee C.G., Lee H. and Trevors J.T. - J. Ind. Microbiol. 5: 25 (1990).
16. Ridgway H.F., Safarik J., Phipps D., Carl P. and Clark D. - Appl. Environ. Microbiol. 56: 3565 (1990).
17. Schenk T. - Dissertation, Rheinisch-Westfälische Technische Hochschule, Aachen, Germany (1997).
18. Westerduin P., de Haan P.E., Dees M.J. and van Boom J.H. - Carbohydr. Res. 180: 195 (1988).
19. Matulovic U. - Dissertation, Technische Universität, Braunschweig, Germany (1987).
20. Pang X. - Dissertation, Universität, Bonn, Germany (1992).
21. Reiling H.E., Thanei-Wyss U., Guerra-Santos L.H., Hirt R., Käppeli O. and Fiechter - Appl. Environ. Microbiol. 51: 985 (1986).

