Chemical and Physical Characterization of Four Interfacial-Active Rhamnolipids from *Pseudomonas spec.* DSM 2874 Grown on *n*-Alkanes

Christoph Syldatk, Siegmund Lang, and Fritz Wagner

Institut für Biochemie und Biotechnologie, Technische Universität Braunschweig, Bültenweg 17, D-3300 Braunschweig, Bundesrepublik Deutschland

Victor Wray and Ludger Witte

Gesellschaft für Biotechnologische Forschung, D-3300 Braunschweig-Stöckheim, Bundesrepublik Deutschland

Z. Naturforsch. **40 c,** 51–60 (1985); received August 20, 1984

Pseudomonas, Interfacial-Active, Rhamnolipids, *n*-Alkanes, Structure-Eludication

Four extracellular glycolipids produced under growth-limiting conditions were isolated from the culture broth of *Pseudomonas spec.* DSM 2874. After purification by column and thick-layer chromatography they were identified as anionic rhamnolipids. ^{1}H and ^{13}C-NMR studies showed that two of these, β(β(2-O-α-L-rhamnopyranosyloxy)decanoyl)decanoic acid and β(β(2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyloxy)decanoyloxy)decanoic acid, were identical with compounds described previously, while the other more hydrophilic compounds, β(2-O-α-L-rhamnopyranosyloxy)decanoic acid and β(2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyloxy)decanoic acid, were new compounds.

Surface and interfacial activity of the organic crude extract and of the purified components were determined in different aqueous solutions. The pH-dependence of surface and interfacial properties of the two previously described rhamnolipids (4, 20, 23) were examined in Teorell-Stenhagen-buffer (supplemented with 10% NaCl) at pH 3.0 and pH 9.0. All rhamnolipids reduced the surface-tension from 72 to about 30 mN/m and the interfacial-tension from 42 to about 1 mN/m. The critical micelle concentrations were of the order of 5 to 200 mg/l depending on the structure of the molecule.

Introduction

Today there exists a wide spectrum of surface-active compounds produced by microorganisms [1]. In recent years they have become very important, as there is a wide range of applications, such as enhanced oil recovery, for these so called biosurfactants in industry [2]. The advantages of these products over synthetic products are their biodegradability, nontoxicity and relatively simple production by microbial fermentation [3].

Pseudomonas aeruginosa and *Pseudomonas fluorescens* are known to produce different extracellular rhamnolipids when grown on *n*-alkanes, glucose or glycerol [4–8]. These glycolipids reduce remarkably the interfacial tension between aqueous solutions and *n*-hexadecane [9] and show good emulsifying power in comparison with chemical surfactants [10]. They play an important role in the growth of *Pseudomonas aeruginosa* on hydrocarbons [10, 11] and are produced in substantial amounts in the stationary phase of growth [12]. When grown on a mixture of C14-C15 *n*-alkanes as the sole carbon-source *Pseudomonas spec.* DSM 2874 synthesized two glycolipids under nitrogen-limitation [13]. In this paper we describe the production, isolation and characterization of glycolipids found in the culture medium of this microorganism. Their ability to reduce the surface and interfacial tensions of high salinity solutions has been investigated in detail as little has been reported previously in the literature.

Reprint requests to Christoph Syldatk.

0341-0382/85/0100-0051 $ 01.30/0

Materials and Methods

Microorganism

Pseudomonas spec. DSM 2874 was isolated by enrichment culture techniques from a water sample. Morphological and physiological characteristics indicated that the organism belonged to the genus of *Pseudomonas.*

Cultivation conditions

Pseudomonas spec. DSM 2874 was cultivated in a mineral salts medium, pH 6.8, containing (per

liter) 4.42 g $Na_2HPO_4 \cdot 2H_2O$, 3.4 g KH_2PO_4, 8 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g $CaCl_2 \cdot 2H_2O$, 0.2 g citric acid · $1H_2O$, 0.04 g $FeSO_4 \cdot 7H_2O$, 0.005 g $MnSO_4 \cdot H_2O$, 0.0012 g NH_4-heptamolybdate and 80 g of C14-C15 *n*-alkanes as the sole carbon source at a temperature of 30 °C. Batch cultivations were carried out in a b-20-bioreactor (Giovanola Frères, Monthey, Switzerland) equipped with an intensor system. Physiological activity was monitored by the use of a pH-electrode, a pO_2-electrode and oxygen- and carbon-dioxide-gas analyzers (Unor and Oxygor, Fa. H. Maihak AG, Hamburg, F.R.G.). pH was titrated to pH 6.8 by addition of 10% NH_4OH-solution in the initial phase of growth and later by addition of 10% NaOH-solution. The time course of the cultivation was checked by measuring dry mass-, rhamnolipid-, alkane- and ammonium-ion-concentration.

Biomass concentration was determined by the method of Rapp *et al.* [14]. The ammonium-ion-concentration in the supernatant was determined by the method of Facwett *et al.* [15]. The concentration of rhamnolipids was measured by the anthrone-method [16] after extraction. Alkane-concentration in the culture-broth was monitored after extraction by gas chromatography.

Isolation and preparation of the glycolipids

After a cultivation time of 180 h cells were separated by centrifugation. The supernatant was acidified with 10% H_2SO_4 to pH 2.0 and extracted with ethyl acetate. After solvent removal by rotary evaporation the residue was separated by column chromatography on Silica Gel 60 (No. 9385, E. Merck AG, Darmstadt, F.R.G.) eluted with chloroform-ethanol-solvent mixtures varying in ratio from 20:1 to 1:1 (vol./vol.). Two main components were isolated in the case of nitrogen limited cells, while four main components were isolated from the culture broth of resting cells [17]. The components were purified by repeated preparative thick-layer-chromatography on Silica Gel 60 (No. 5745, E. Merck AG, Darmstadt, F.R.G.) in different solvent systems containing chloroform, methanol, acetic acid and distilled water.

Characterization of the main components of the organic crude extract

The main components of the organic crude extract were identified by physical and chemical methods. Analytical thin layer chromatography was carried out on Silica Gel plates (No. 5554, E. Merck AG, Darmstadt, F.R.G.) using the solvent system A: $CHCl_3$–CH_3OH-Acetic Acid (65:15:2). After horizontal development of the plates different spray reagents were used to determine functional groups of the components [18]. Sugars were determined with 4-methoxy-benzaldehyde and anthrone. Lipids were identified with 2′,7′-dichlorofluoresceine (No. 9677, E. Merck AG, Darmstadt, F.R.G.) and molybdatophosphoro acid (No. 531, E. Merck AG). Glycolipids were identified with diphenylamine [19]. Bromocresolgreen reagent (No. 1998, E. Merck AG) was used to distinguish free acid groups.

Infrared spectra were recorded with a Beckman spectralphotometer 4260 in CD_2Cl_2.

^1H and ^{13}C nuclear magnetic resonance spectra of the glycolipids and their p-bromophenacylesters were measured with a Bruker WM-400 NMR Spectrometer operating at 400 and 100 MHz respectively. Chemical shifts are reported with respect to internal tetramethylsilane.

GC-MS-measurements of the fatty acid methylesters were carried out with a Perkin-Elmer F 22 instrument which was combined with an A.E.I. MS 30 mass spectrometer.

HPLC measurements of the sugars were carried out with a Milton-Borg-instrument on a Lichrosorb-NH_2-column (No. 8011206, Fa. Knauer, F.R.G.).

The surface and interfacial tensions of the organic crude extract and the purified components were measured by the ring method with a Lauda Autotensiomat (Fa. Lauda-Wobser KG, Königshofen, F.R.G.) [14].

Chemical methods

Alkaline hydrolysis of the glycolipids, for separation of the fatty acids, was carried out by refluxing with 0.5 N sodium hydroxide solution for 5 h at 60 °C. After extracting the fatty acids with *n*-hexane their methyl esters were prepared by treatment with diazomethane.

Acid hydrolysis of the glycolipids and the products of alkaline hydrolysis was achieved by refluxing with 1 N HCl for 3 h at 90 °C.

Results

Formation of rhamnolipids

Fig. 1 shows the typical time course of glycolipid formation during a batch fermentation of *Pseudo-*

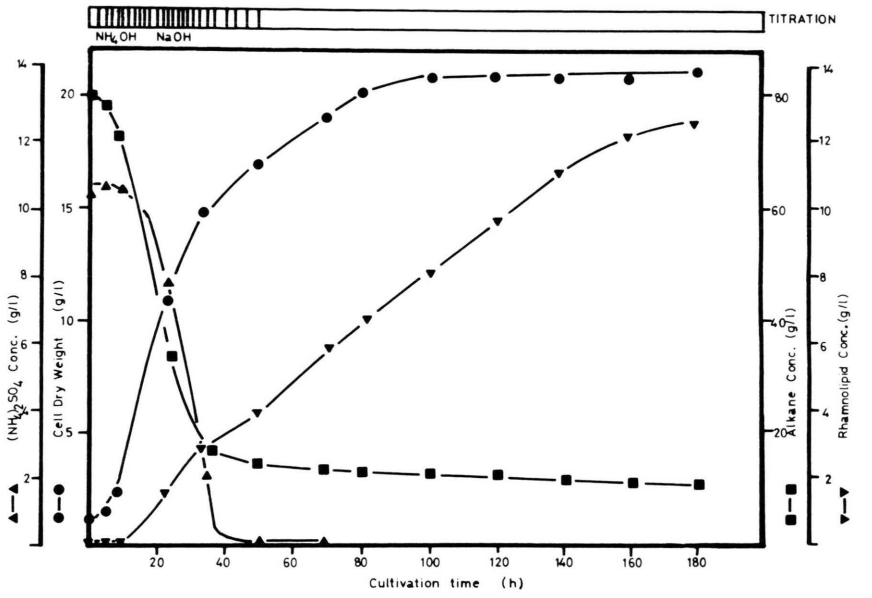


Fig. 1. Formation of rhamnolipid R1 and R3 by *Pseudomonas spec.* DSM 2874 under nitrogen-limitation. Conditions: mineral salts medium, pH 6.8; intensor system, 1000 Rpm; aeration, 0.75 VVm; $T = 30\ ^{\circ}C$.

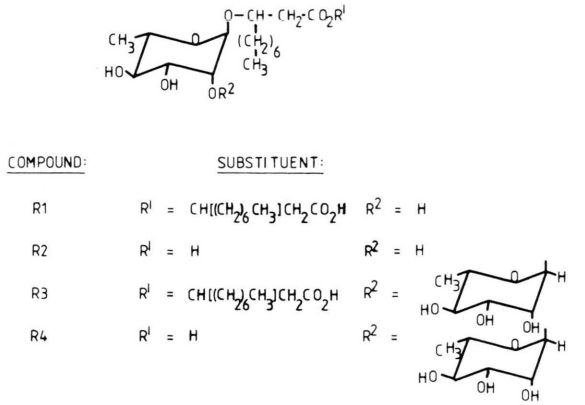


Fig. 2. Structures of the isolated rhamnolipids R1–R4 formed by *Pseudomonas spec.* DSM 2874.

monas spec. DSM 2874 in a 20 l-bioreactor with an intensor system. An exponential phase of growth was not observed because of an oxygen-limitation which occurred after 6 h. Glycolipid production began after 10 h and continued during the stationary phase of growth which was caused by a nitrogen-limitation after 40 h. After the occurrence of nitrogen-limitation the content of *n*-alkanes in the supernatant diminished quite slowly, while the glycolipid formation was linear up to 140 h. After 180 h 12.8 g/l glycolipid were formed. The cell dry weight amounted to 20.8 g/l. The specific formation was 0.63 g rhamnolipid/g cell dry weight. Similar experiments were performed with resting cells. Extensive work has been carried out on the optimization of these processes and they will be discussed elsewhere [17].

After separating the biomass by centrifugation the supernatants were acidified and extracted. The organic crude extract contained two main components in the case of nitrogen-limited cells (R1 and R3) and four main components in the case of resting cells (R1, R2, R3 and R4) [17]. After analytical thin-layer-chromatography and detection with different spray-reagents all compounds turned out to be anionic glycolipids. They had the following R_F-values in solvent system A: R1:0.82, R2:0.44, R3:0.29, R4:0.11. After column- and repeated thick-layer-chromatography the structures of the purified glycolipids were elucidated by the spectroscopic and analytical methods described below.

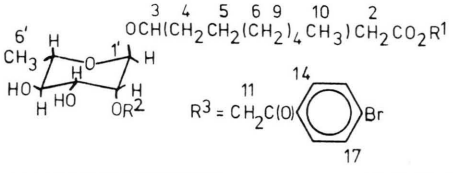
Structure elucidation

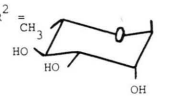
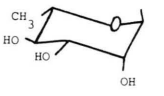
The two main components, R1 and R3, isolated under nitrogen limiting conditions [13], were identical with compounds described previously [4], while two further compounds, R2 and R4, isolated from the culture broth of resting cells are novel and analogues of R1 and R3. The structure of R1 and R3 have been deduced previously from tedious chemical analyses [4, 20] without the aid of instrumental

techniques. High field ^1H and ^{13}C NMR spectroscopy of the pure compounds or derivatives, coupled with combined gas chromatography-mass spectrometric analysis of the lipid moiety, affords a quick and reliable analysis of the number, nature and position of each substituent in the molecule.

Broadening of the signals in the nmr spectra of the pure compounds was observed, presumably due to the presence of small amounts of paramagnetic impurities or micelle formation, and prevented spectral interpretation of R3. This phenomenon has been observed previously in related systems when free carboxyl groups are present in the molecule [21]. Formation of the *p*-bromophenacyl derivatives of R1 and R3 overcame this difficulty and allowed unambiguous analyses to be made. ^1H and ^{13}C NMR data for R1, R2 and R4, together with the *p*-bromophenacyl derivatives of R1 and R3, are given in Tables I and II respectively. Comparison of the integral in the ^1H spectra of the isolated methyl signal of the lipid moiety at 0.85 with respect to an isolated sugar proton gave the molar ratio of lipid to sugar, while the presence of a low field multiplet at 5.2 to 5.4 for H3, caused by esterification of the

Table I. ^1H data for Rhamnolipids R1, R2, R3 and R4.



Compound	Substituent	
R1	$R^1 = CH(CH_2CO_2H)(CH_2)_6CH_3$	$R^2 = H$
R1 ester	$R^1 = CH(CH_2CO_2R^3)(CH_2)_6CH_3$	$R^2 = H$
R2	$R^1 = H$	$R^2 = H$
R3 ester	$R^1 = CH(CH_2CO_2R^3)(CH_2)_6CH_3$	
R4	$R^1 = H$	

1 A. Chemical shifts of lipid moiety (δ) [a]

	Solvent	CH_3(10)	CH_2(6–9)	CH_2(5)	CH_2(4)	CH(3)	CH_2(2)
R1	$CDCl_3$	0.850(t) 0.841(t)	1.34 – 1.12(m)	1.65 –	1.34(m)	4.237(m) 5.415(m)	2.57 – 2.27(m) [c]
R1 ester	$CDCl_3$	0.865(t)	1.40 – 1.21(m)	1.65 –	1.40(m)	4.096(m) 5.24(m)	2.730(dd) A [d, c, e] 2.683(dd) B 2.489(dd) A′ 2.398(dd) B′
R2	$CDCl_3$	0.828(t)	1.32 – 1.18(m)	1.421(m)	1.586(m)	4.072(m)	2.450(dd) A [b] 2.377(dd) B
R3 ester	$CDCl_3$	0.858(t) 0.847(t)	1.38 – 1.09(m)	1.69 –	1.38(m)	4.048(m) 5.234(m)	2.767(dd) A [d, c] 2.689(dd) B 2.546(dd) A′ 2.436(dd) B′
R4	CD_3OD	0.939(t)	1.53 –	1.23(m)	1.597(m)	4.127(m)	2.71 – 2.35(m)

Table I. (continued)

1 B. Chemical shifts of sugar moiety (δ) [a]

	1′	2′	3′	4′	5′	6′	Coupling constants [Hz]
R1	4.831	3.781	3.73 – 3.62	3.393(t)	3.73 – 3.62	1.249(d)	(9 – 10) 7.1, 7.1; (3′-4′) 9.5; (4′-5′) 9.5; (5′-6′) 6.5
R1 ester	4.822(d)	3.761(dd)	3.618(dd)	3.280(t)	3.648(dq)	1.274(d)	(2A-2B) – 16.2; (2A-3) 8.2; (2B-3) 4.4; (2A′-2B′) – 15.4; (2A′-′3) 8.4; (2B′-′3) 4.3; (9 – 10) 7.3, 7.3; (1′-2′) 1.7; (2′-3′) 3.9; (3′-4′) 8.7; (4′-5′) 9.5; (5′-6′) 6.1
R2	4.839	3.784	3.66 – 3.58	3.351(t)	3.66 – 3.58	1.221(d)	(2A-2B) – 14.3; (2A-3) 3.5; (2B-3) 9.9; (9-10) 7.1; (1′-2′) 0.6; (3′-4′) 9.5; (4′-5′) 9.5; (5′-6′) 5.9
R3 ester	{4.903 {4.886	4.123 3.746	{3.78 – 3.54	3.467 3.335	3.78 – 3.54	1.38 – 1.09}	(2A-2B) – 15.7; (2A-3) 7.2; (2B-3) 5.5; (2A′-2B′) – 15.3; (2A′-′3) 7.3; (2B′-′3) 3.3; (9-10) 6.9, 7.0; (3′-4′) (4′-5′) (3″-4″) (4″-5″) all ca. 9.3
R4	{4.974 {f	4.021 3.797	{g g	g 3.429	g g	1.292(d) (×2) }	(3″-4″) ~ 9.4; (4″-5″) ~ 9.4; (5′-6′) 6.5; (5″-6″) 6.5

[a] Abbreviations are: q = quartet, t = triplet, d = doublet and m = multiplet.
[b] Analysed as an ABX system.
[c] Two fatty acid chains present but absolute assignments not possible.
[d] Two ABX systems present the X parts of which are denoted by 3 and ′3, being in either the first or second fatty acid chain.
[e] Other signals at 7.715 (H-14, H-18); 7.567 (H-15, H-17); 5.263, 5.230 (CH_2-11, $J = -16.5$) of *p*-bromophenacyl moiety.
[f] One of H-1′ signals hidden by residual water signal.
[g] All other sugar signals at 3.77 – 3.67.
[h] Other signals at 7.755 (H-14, H-18); 7.606 (H-15, H-17); 5.302, 5.274 (CH_2-11, $J = -16.7$).

β-hydroxyl function, characterized the presence of a second fatty acid chain.

Complete ^{1}H spectral assignment of the *p*-bromophenacyl derivative of R1 by homonuclear decoupling unambiguously identified rhamnose as the sugar moiety. Although the anomeric linkage was ambiguous as both α and β-linkages show a small $J(1'-2')$. The presence of rhamnose in the other compounds was detected in each case by the characteristic methyl doublet at ca. $\delta = 1.2$.

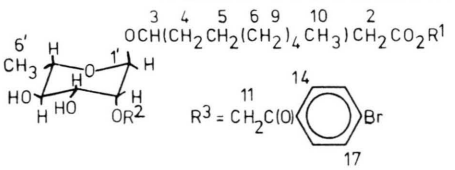
The number and intensity of the signals in the ^{13}C NMR spectra confirmed the number and nature of the lipid and sugar moieties present. Comparison with literature data allowed assignment of the ^{13}C signals and the shifts of the sugar carbons were characteristic of an α-glycosidic linkage [22]. The low field shift of H2′ and the corresponding carbon, C2′, of the lipid-bound sugar were indicative of the linkage position of the second sugar moiety in R3 and R4.

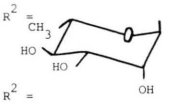
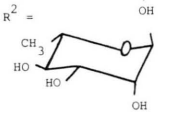
The nature of the lipid and sugar moieties were confirmed by hydrolysis of R1 and R3. The water soluble sugar component after acidic hydrolysis was identical with rhamnose on tlc with various solvent systems and on hplc. Two hydrocarbon-soluble compounds were produced upon partial alkaline hydrolysis of either R1 or R3. Combined gas chromatography-mass spectrometric analysis of their methyl esters indicated that these were the methyl esters of β-hydroxydecanoic acid and β-(β-hydroxydecanoyloxy)-decanoic acid; Table III.

Surfactant properties of the rhamnolipids

After product isolation the surface and interfacial properties of the anionic rhamnolipids R1 – R4 were determined after emulsification in different aqueous solutions. The aim was to determine the critical micelle concentration and to demonstrate possible dependence on salt concentrations or pH conditions. The results are summarized in Table IV. All of the anionic rhamnolipids exhibited significant surface and interfacial active properties. The minimum surface tensions of the rhamnolipids R1 – R4 were in the range of 25 to 31 mN/m and the minimum interfacial tensions in the range of 8 to smaller than 1 mN/m. Fig. 3 and Fig. 4 show the influence of different pH-conditions on the surface and interfacial behaviour of rhamnolipid R1 and R3. The CMC value of the surface tension of

Table 2. ^{13}C chemical shift data (δ) for Rhamnolipids R1, R2, R3 and R4.



Compound	Substituent	
1	$R^1 = CH(CH_2CO_2H)(CH_2)_6CH_3$	$R^2 = H$
2 ester	$R^1 = CH(CH_2CO_2R^3)(CH_2)_6CH_3$	$R^2 = H$
2	$R^1 = H$	$R^2 = H$
3 ester	$R^1 = CH(CH_2CO_2R^3)(CH_2)_6CH_3$	
4	$R^1 = H$	

Carbon	R1 $CDCl_3$ [a]	R2 ester $CDCl_3$	R2 $CDCl_3$	R3 ester $CDCl_3$	R4 CD_3OD
Aglycon					
1	171.02 [e] 174.70	171.08 170.46	174.70	171.06 170.21	9
2	39.59 39.23	39.99 38.77	40.00	40.13 38.85	43.55 [h]
3	70.57 71.20 [b]	70.62 71.36	71.18 [b]	71.49 [b] 71.41	76.29 [h]
4	31.66 34.56	34.05 32.75	31.72 [c]	33.95 33.13	34.25
5	25.08 24.59	25.13 24.74	24.58	25.17 24.83	25.89
6	29.72 [d] 29.21 [d]	29.69 [d] 29.25 [d]	29.13 [d]	29.73 [d] 29.24 [d]	30.79 [d]
7	29.41 [d] 29.09 [d]	29.36 [d] 29.15 [d]	29.57 [d]	29.38 [d] 29.17 [d]	30.25 [d]
8	31.75 31.75	31.81 31.81	32.29 [c]	31.86 31.80	32.89
9	22.60 22.60	22.65 22.65	22.55	22.65 22.65	23.57
10	14.03 14.03	14.08 14.08	13.96	14.11 14.11	14.29
Sugar					
1′	95.38	97.22	96.57	97.10 102.59	98.93 104.09
2′	71.36 [b]	71.90 [b]	71.41 [b]	79.71 70.82 [b]	80.46 72.22 [b]
3′	71.68 [b]	73.19 [b]	72.52 [b]	70.82 [b] 70.73 [b]	72.40 [b] 72.03 [b]
4′	73.52	73.81	72.84 [b]	73.59 72.79	74.47 74.00
5′	67.84	68.24	68.28	68.97 68.71	70.22 70.08
6′	17.26	17.38	17.21	17.75 17.57	18.01 18.01

Table III. Mass spectral data for the methyl esters of the fatty acid moieties of R1 and R3.

I $CH_3OCOCH_2CH(C_7H_{15})OH$ [a]			II $CH_3OCOCH_2CH(C_7H_{15})OCOCH_2CH(C_7H_{15})OH$ [a]		
m/e	I_{rel} [%]	Assignment	*m/e*	I_{rel} [%]	Assignment
201	> 1	$[C_{11}H_{22}O_3-H]^+$	354	1	$[C_{21}H_{40}O_5-H_2O]^+$
184	1	$[C_{11}H_{22}O_3-H_2O]^+$	323	3	$[C_{21}H_{40}O_5-H_2O-CH_3O^\bullet]^+$
152	3	$[C_{11}H_{22}O_3-H_2O-CH_3OH]^+$	201	27	$[CH_3OCOCH_2CH(C_7H_{15})O^\bullet]^+$
103	100	$[CH_3OCOCH_2CHOH^\bullet]^+$	184	58	$[CH_3OCOCH=CHC_6H_{13}]^+$ [b]
			153	100	$[CH_3OCOCH=CHC_6H_{13}-CH_3O^\bullet]^+$
			152	56	$[CH_3OCOCH=CHC_6H_{13}-CH_3OH]^+$

[a] The retention indices of I and II on methyl silicon phase SE 30 were 1435 and 2340, respectively.
[b] McLafferty-rearrangement product.

Table IV. Surface and interfacial [a] active properties of surfactants from *Pseudomonas species* DSM 2874 at 40 °C.

Surfactant	Solution	Minimum surface tension [mN/m]	CMC [mg/l]	Minimum interfacial tension [mN/m]	CMC [mg/l]
crude product [b]	distilled water	26	25	< 1	80
containing	5% NaCl solution	26	10	< 1	15
rhamnolipid 1 and 3	deposit water [c]	26	20	< 1	20
	buffer pH 3.0 [d]	27	10	< 1	20
	buffer pH 9.0 [d]	27	20	< 1	20
crude product [e]	distilled water	27	25	< 1	20
containing	deposit water	28	20	< 1	6
rhamnolipid 1–4					
rhamnolipid 1	distilled water	31	20	8	100
	5% NaCl solution	26	15	< 1	30
	deposit water [c]	26	20	4	40
	buffer pH 3.0 [d]	26	2	< 1	40
	buffer pH 9.0 [d]	26	20	< 1	20
rhamnolipid 2	deposit water [c]	25	200	< 1	200
rhamnolipid 3	distilled water	31	20	3	20
	5% NaCl solution	28	10	< 1	10
	deposit water [c]	27	10	< 1	5
	buffer pH 3.0 [d]	28	10	< 1	5
	buffer pH 9.0 [d]	30	40	3	30
rhamnolipid 4	deposit water [c]	30	200	< 1	200

[a] Against *n*-hexadecane.
[b] After extraction and separation of non-utilized *n*-alkanes.
[c] Composition: NaCl, 100 g/l; CaCl, 18 g/l; $MgCl_2$, 10 g/l.
[d] Teorell-Stenhagen buffer, supplemented with NaCl, 10% (w/v).
[e] C-source for production: glycerol.

[a] Solvent contains ca. 20% CD_3OD.
[b, c, d] Assignments interchangeable.
[e] Upper line is for middle moiety where specific assignments are possible.
[f] Assignment of the *p*-bromophenacyl signals are as follows: 66.25(11); 191.14(12); 132.83(13); 129.36(14, 18); 132.35(15, 17); 129.36(16).
[g] Not observed.
[h] Signals broadened.
[i] Assignment of the *p*-bromophenacyl signals are as follows: 66.25(11); 191.38(12); 132.92(13); 129.44(14, 18); 132.32(15, 17); 129.25(16).

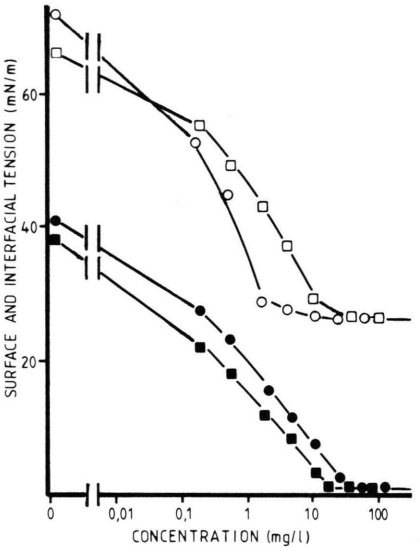


Fig. 3. Surface and interfacial tension of rhamnolipid R1 in Teorell-Stenhagen buffer* pH 3 (○) and pH 9 (□) at 40 °C. Open symbols show surface tensions and solid symbols the corresponding interfacial tension.

* Supplemented with NaCl, 10% (w/v).

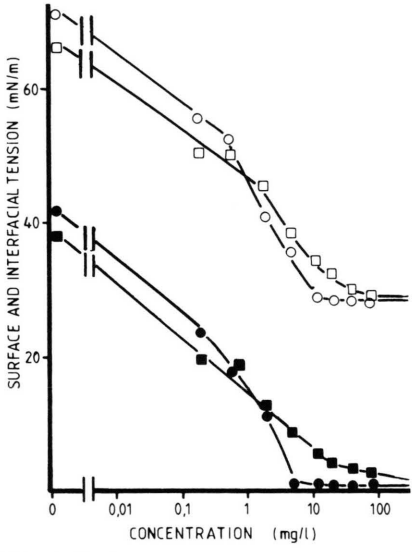


Fig. 4. Surface and interfacial tension of rhamnolipid R3 in Teorell-Stenhagen buffer* pH 3 (○) and pH 9 (□) at 40 °C. Open symbols show surface tensions and solid symbols the corresponding interfacial tension.

* Supplemented with NaCl, 10% (w/v).

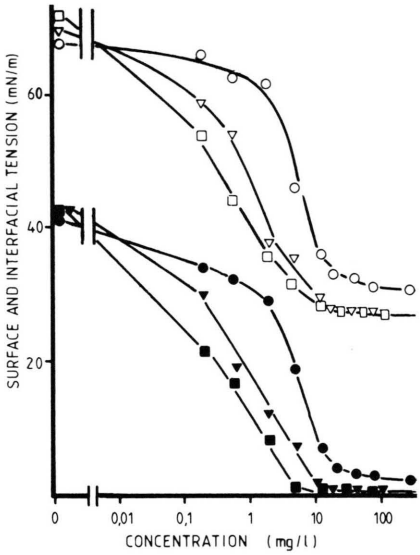


Fig. 5. Surface and interfacial tension of rhamnolipid R3 in distilled water (○), 5% NaCl solution (▽) and synthetic deposit water* (□) at 40 °C. Open symbols show surface tensions and solid symbols the corresponding interfacial tension.

* Composition: NaCl, 100 g/l; $CaCl_2$, 28 g/l; $MgCl_2$, 10 g/l.

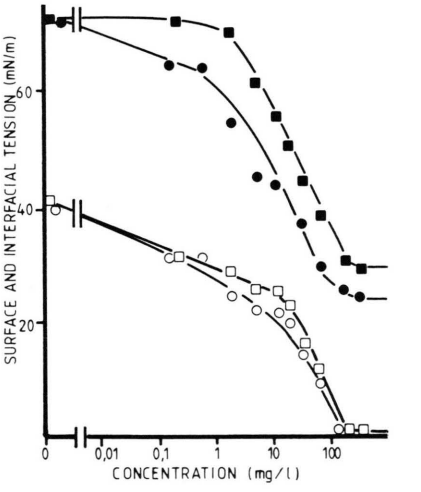


Fig. 6. Surface and interfacial tension of rhamnolipid R2 (○) and R4 (□) in synthetic deposit water* at 40 °C. Solid symbols show the surface tensions and open symbols the corresponding interfacial tension.

* Composition: NaCl, 100 g/l; $CaCl_2$, 28 g/l; $MgCl_2$, 10 g/l.

rhamnolipid R1 is lowered from 20 mg/l at pH 9.0 to 2 mg/l at pH 3.0, while the pH only slightly alters the interfacial behaviour of rhamnolipid R1. The CMC values of the surface and interfacial tension of rhamnolipid R3 are lowered from 30–40 mg/l at pH 9.0 to 5–10 mg/l at pH 3.0.

Fig. 5 demonstrates the influence of different salt concentrations on the surface and interfacial tension of rhamnolipid R3. Higher salt concentrations lower the minimum surface tension from 31 to 27 mN/m and the CMC values of the surface tension from 20 mg/l to 10 mg/l compared to distilled water solution. The minimum interfacial tension is lowered from 3 to < 1 mN/m. The CMC values of the interfacial tension are lowered from 20 mg/l to 5–10 mg/l in 5% NaCl solution and deposit water compared to distilled water solution. Fig. 6 gives the results of the surface and interfacial measurements of rhamnolipids R2 and R4 in synthetic deposit water. The values for critical micelle concentrations of 200 mg/l indicate that the combination of one or two sugars with only one β-hydroxydecanoic acid leads to higher CMC values.

Discussion

Rhamnolipids, produced by *Pseudomonas aeruginosa*, play an important role in hydrocarbon fermentation [10]. Although they are one of the important factors required for *n*-paraffin uptake by *Pseudomonas aeruginosa* [11], they are not produced in larger amounts before the stationary phase of growth [12]. For example, an overproduction of rhamnolipids can be caused by a nitrogen-limitation [13]. Evidently small amounts of rhamnolipids are sufficient to allow the growth of *Pseudomonas aeruginosa* on hydrocarbons. This supposition is in harmony with the results of the measurements of surface and interfacial active properties of the rhamnolipids. Small amounts of only 15 to 80 mg/l of crude product containing rhamnolipid R1 and R3 were sufficient to reduce the interfacial tension between *n*-hexadecane and different aqueous solutions from 42 to about 1 mN/m.

While the two main components, R1 and R3, isolated under nitrogen limiting conditions were identical with compounds described previously in the literature [4, 20, 23], two new rhamnolipids, R2 and R4, could be isolated from the culture broth of resting cells. The conditions for optimum production will be discussed elsewhere [17]. The rhamnolipids R2 and R4 possess only one β-hydroxydecanoic acid in comparison with rhamnolipids R1 and R3.

In the literature little data have been reported on surface and interfacial active properties of rhamnolipids. There are some data on the surface and interfacial active properties of culture broths of *Pseudomonas spec.* grown on various media containing hydrocarbons [24], while other data describe the emulsifying power of a rhamnolipid called GS [10], which is identical with R3, compared with synthetic surfactants. The CMC values of the purified rhamnolipids R1–R4 were in the range of 2 to 200 mg/l. The minimum surface tensions changed from 25 up to 31 mN/m, while the minimum surface tensions towards *n*-hexadecane were in the range 8 to smaller than 1 mN/m. The ability of low pH values and high salinities to reduce the critical micelle concentrations and the minimum surface and interfacial tension of the rhamnolipids R1 and R3 is probably caused by the anionic character of these.

In synthetic deposit water the more hydrophilic rhamnolipids R2 and R4 had CMC values of 200 mg/l while the more hydrobic rhamnolipids R1 and R3 had CMC values of 10 to 20 mg/l. This indicates that the combination of one or two sugars with only one β-hydroxydecanoic acid leads to higher CMC values.

Today there is a wide range of commercially important surfactant functions [2], ranging from emulsification, deemulsification, foaming, antifoaming, detergency, solubilization to wetting and spreading, with the production of microbial biosurfactants gaining in importance. Probably a major future application of surfactants will be their use in tertiary oil recovery. We are currently engaged in flooding experiments using the rhamnolipids and this work will be described in the near future.

Acknowledgements

We thank H. Puke and V. Borras from the Institut für Zuckertechnologie, Braunschweig, F.R.G., for carrying out the HPLC measurements.

[1] D. G. Cooper and J. E. Zajic, Adv. Appl. Microbiol. **26,** 229 (1980).
[2] N. Kosaric, N. C. C. Gray, and W. L. Cairns. In: H. Dellweg (eds.) Biotechnology **Vol. 3,** p. 576, Verlag Chemie, Weinheim 1983.
[3] D. F. Gerson and J. E. Zajic, Proc. Biochem. **14,** 20 (1979).
[4] S. Itoh, H. Honda, F. Tomita, and T. Suzuki, J. Antibiot. **24,** 855 (1971).
[5] G. Hauser and M. L. Karnowsky, J. Biol. Chem. **229,** 91 (1957).
[6] T. Suzuki and S. Itoh, Offenlegungsschrift 2150 375, Deutsches Patentamt 1972.
[7] M. Yamaguchi, A. Sato, and A. Yukuyama, Chem. Ind. **17,** 741 (1976).
[8] T. Hirayama and J. Kato, FEBS Letters **139,** 81 (1982).
[9] S. Lang, A. Gilbon, C. Syldatk, and F. Wagner, in: K. L. Mittal (ed.), Surfactants in Solution. **Vol. 2,** p. 1365, Plenum Publishing Company, New York 1983.
[10] K. Hisatsuka, T. Nakahara, N. Sano, and K. Yamada, Agr. Biol. Chem. **35,** 686 (1971).
[11] S. Itoh and T. Suzuki, Agr. Biol. Chem. **36,** 2233 (1972).
[12] G. Hauser and M. L. Karnowsky, J. Bacteriol. **68,** 645 (1954).
[13] F. Wagner, U. Behrendt, H. Bock, A. Kretschmer, S. Lang, and C. Syldatk. In: (J. E. Zajic, D. G. Cooper, T. R. Jack, and N. Kosaric, eds.), p. 55, microbial enhanced oil recovery, Pennwell Books, Tulsa, Oklahoma 1983.
[14] P. Rapp, H. Bock, V. Wray, and F. Wagner, J. Gen. Microbiol. **115,** 491 (1979).
[15] J. K. Facwett and J. E. Scott, J. Clinic. Path. **13,** 156 (1960).
[16] J. D. Hodge and B. T. Hofreiter, Methods in Carbohydrate Chemistry **1,** 380 (1962).
[17] C. Syldatk, S. Lang, U. Matulovic, and F. Wagner, Z. Naturforsch. **40c,** 61 (1985).
[18] E. Stahl, Dünnschichtchromatographie, Springer Verlag, Berlin 1972.
[19] H. Jatzkewitz, Hoppe-Seylers Z. Physiol. Chem. **320,** 251 (1960).
[20] J. R. Edwards and J. A. Hayashi, Arch. Biochem. Biophys. **111,** 415 (1965).
[21] E. Ristau, Ph. D. Thesis. University of Braunschweig 1983.
[22] W. Bremser, L. Ernst, B. Franke, R. Gerhards, and A. Hardt, Carbon-13-NMR spectral data, spectra No. 20908 and 20909, Verlag Chemie, Weinheim 1984.
[23] F. G. Jarvis and M. J. Johnson, J. Am. Chem. Soc. **71,** 4124 (1949).
[24] D. G. Cooper, S. N. Liss, R. Longay, and J. E. Zajic, J. Ferment. Technol. **59,** 97 (1981).