Structure Elucidation and Characterization of Microbial 2-Tridecyl Sophorosides (Biosurfactants)

Verena K. Recke[a,*], Jun B. Park[a], Melanie Gerlitzki[b], Rudolf Hausmann[c], Christoph Syldatk[b], Victor Wray[d], Harukuni Tokuda[e], Nobutaka Suzuki[e], and Siegmund Lang[a]

[a] Braunschweig University of Technology, Institute of Biochemistry, Biotechnology and Bioinformatics, Department of Biotechnology, Spielmannstr. 7, D-38106 Braunschweig, Germany. Fax: (+49) 531 391 5763. E-mail: verena.recke@gmail.com or s.lang@tu-braunschweig.de

[b] Karlsruhe Institute of Technology (KIT), Institute of Process Engineering in Life Sciences, Section II: Technical Biology, Engler-Bunte-Ring 1, D-76131 Karlsruhe, Germany

[c] Hohenheim University, Institute of Food Science and Biotechnology, Department of Bioprocess Engineering, Fruhwirthstr. 12, D-70599 Stuttgart, Germany

[d] Helmholtz Centre for Infection Research, Department of Molecular Structural Biology, Inhoffenstr. 7, D-38124 Braunschweig, Germany

[e] Kanazawa University, Department of Complementary and Alternative Medicine, Clinical R & D, Graduate School of Medical Science, 13–1 Takara-machi, Kanazawa, Japan

* Author for correspondence and reprint requests

Z. Naturforsch. **68 c**, 489–498 (2013); received June 12/November 4, 2013

To produce novel types of sophorose lipids containing an odd number of carbon atoms in the lipophilic moiety, *Candida bombicola* ATCC 22214 was grown in 500-ml flask cultures with glucose as main carbon source, and additionally, 2-tridecanone as co-substrate. After solvent extraction, the crude product mixture was separated into pure fractions, and each fraction was analysed via NMR and mass spectroscopy. This effective strategy generated five new glycolipids, 2-tridecyl sophorosides, which differed in the number of glucose units, and acetyl and hydroxy groups, respectively. Based on these compounds, a proposal for the possible biosynthetic pathway was deduced. Two compounds of the mixture, mono- and diacetylated 2-tridecyl sophorosides, respectively, were able to lower the surface tension of water from 72 mN m^{-1} to 32 mN m^{-1} and the interfacial tension between water and *n*-hexadecane from 43 mN m^{-1} down to 4 and 3 mN m^{-1}. Thus, both compounds possess a very good surfactant behaviour. Moreover, it was observed that the new products inhibit the growth of particular Gram-positive bacteria, and they indicate potential for antitumour-promoting activity.

Key words: Candida bombicola, Alkyl Sophoroside, 2-Tridecanone

Introduction

Microbial glycolipids belong to the group of biosurfactants. They are low-molecular-weight compounds (< 1500 g mol^{-1}) consisting of a hydrophilic mono-, di- or oligosaccharide unit connected to a lipophilic moiety, such as long-chain hydroxy fatty acid chains (Rau *et al.,* 2005). The molecular structures can be sub-divided into non-ionic and ionic types. They may occur extracellularly (*e.g.*, rhamnolipids from *Pseudomonas aeruginosa*) or cell-wall-attached such as trehalose corynomycolates from *Rhodococcus erythropolis* (Lang, 2002). The overproduction of the biosurfactants by these microorganisms is achieved especially under nitrogen limitation in the stationary growth phase (Syldatk *et al.*, 1985).

Classical sophorolipids, known since 1961 (Gorin *et al.*, 1961), have been produced extracellularly by *Candida bombicola* using glucose as main carbon source and triglycerides (or long-chain *n*-alkanes) as co-substrates (Asmer *et al.*, 1988; Daniel *et al.*, 1998, 1999; Daverey and Pakshirajan, 2010; McGaffrey and Copper, 1995; Van Bogaert *et al.*, 2007). The microbial crude product is a mixture of various similar compounds. The hydrophilic part consists of the disaccharide sophorose which is built by two β-1,2-linked glucose molecules. The lipophilic part is made up by a terminally or subterminally hydroxylated fatty

acid, β-glycosidically linked to the sophorose unit. This biosurfactant is synthesized as a mixture of slightly different molecules. The two major points of variation are acetylation at the 6'- and/or 6"-positions of the disaccharide and lactonization (hydroxylated fatty acid) to the 4"-position of sophorose. Further minor differences are associated with the hydroxy fatty acid chain (16 or 18 carbon atoms, saturated or unsaturated chain). Van Bogaert *et al.* (2011) suggested a biosynthetic pathway for this kind of glycolipids. Enzyme-mediated, regioselective modifications of the native sophorolipids have been reported (Bisht *et al.*, 1999; Singh *et al.*, 2003).

In an effort to reduce the chain length of the lipophilic part and to exclude the occurrence of lactonic/acidic sophorolipids, Brakemeier *et al.* (1998a) reported on new sophorolipid structures when changing the co-substrate. Using 2-dodecanol, they found exclusively nonacidic 2-dodecyl sophorosides containing only twelve carbon atoms in the lipophilic moiety. These alkyl sophorosides showed a better surface activity than the classical sophorolipids and lowered the surface tension of water from 72 mN m^{-1} to 31 mN m^{-1}. Furthermore, Brakemeier *et al.* (1995, 1998b) showed that other secondary alcohols, such as 2-tetradecanol or 2-hexadecanol and ketones with an even number of carbon atoms like 2-, 3-, and 4-dodecanone, can be converted to the corresponding alkyl sophorosides by *C. bombicola*.

The rationale of this study was to use glucose as main substrate and, for the first time, a ketone (co-substrate) with an odd number of carbon atoms – here: 2-tridecanone – for the cultivation of *Candida bombicola*, to produce 2-tridecyl sophorosides. Besides the aim to obtain novel natural products, our expection was that no, or less, by-products (classical lactonic C-18 sophorolipid) would be produced in comparison to even carbon atoms chain ketones, as in the case of an odd-numbered ketone as co-substrate more biochemical reactions are necessary to convert it into C-18 fatty acids. Additionally, we expected interesting biological activities not possessed by even carbon atoms chain alkyl sophorosides. The obtained new metabolites were purified by medium pressure chromatography and their structures elucidated by NMR and MS analysis. The novel biosurfactants were characterized by determining their effect on the surface tension of water (vs. air) as well as the interfacial tension between water and oil. Finally, the bioactivity potential of the novel glycoconjugates was investigated in antimicrobial tests and, additionally, in a bioassay for cancer chemoprevention.

Material and Methods

Microorganism and culture conditions

Candida bombicola ATCC 22214 (American Type Culture Collection, Rockville, MD, USA) was initially cultivated on 100 ml yeast medium as a preculture using a 500-ml shake flask. The main culture was performed in a 2-l shake flask containing 500 ml of the basic medium of Brakemeier *et al.* (1998a) which consisted of: 100 g l^{-1} glucose as main carbon source, 1 g l^{-1} yeast extract, 4.7 g l^{-1} sodium citrate dihydrate, 1.54 g l^{-1} NH_4Cl, 1 g l^{-1} KH_2PO_4, 0.16 g l^{-1} K_2HPO_4, 0.7 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1} NaCl, and 0.16 g l^{-1} $CaCl_2 \cdot 2H_2O$. The co-substrate 2-tridecanone (purity 99%) – purchased from Sigma-Aldrich (Steinheim, Germany) – was added to a final concentration of 5 g l^{-1} after 48 h, 72 h, and 96 h of culture as reported by Brakemeier *et al.* (1998a). After 9 d of incubation at 30 °C and 100 rpm the resulting mixture of alkyl sophorosides was extracted twice with ethyl acetate.

Separation of the different glycolipid components via medium-pressure liquid chromatography (MPLC)

After evaporation of the solvent, the crude residue was subjected to MPLC: column, Easy Vario Flash D31 Si 60 (40–63 µm) (Götec Labortechnik, Bickenbach, Germany); solvent system, $CHCl_3$/CH_3OH/H_2O (65:15:2, v/v/v); chromatography pump, B-688 (Büchi AG, Flawil, Switzerland); pressure, 4–6 bar; flow rate, 4 ml min^{-1}; fraction collector, Frac-1000 (Amersham Pharmacia, Freiburg, Germany).

Thin-layer chromatography (TLC) for qualitative analysis

For the qualitative analysis of the 2-tridecyl sophorosides, silica gel plates (normal phase) and the solvent system $CHCl_3$/CH_3OH/H_2O (65:15:2) as mobile phase were used. The detection was performed with α-naphthol/sulfuric acid reagent, and the plates were heated to 120 °C. Thereby sugar and sugar derivatives were observed as pink-violet spots (Asmer *et al.*, 1988).

Determination of molecular structures

The molecular structures of the purified glycolipid components were elucidated using a combination of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD on Bruker DPX 300, ARX 400, and DMX 600 NMR spectrometers (Bruker Daltonik GmbH, Bremen, Germany). Chemical shifts are given in ppm relative to tetramethylsilane and coupling constants in Hz. High-resolution electrospray ionization mass spectroscopy (HRESIMS) was done on an LTQ Orbitrap Velos instrument (Thermo Fisher Scientific, Schwerte, Germany) with glycerol as matrix.

Determination of physicochemical properties

The influence of the purified glycolipids on the surface tension (σ) of aqueous solutions and on the interfacial tension (γ) of water/*n*-hexadecane systems was determined at 25 °C with a tensiomat (TE1C MGW; Lauda, Lauda-Königshofen, Germany) using the ring method (Recke *et al.*, 2013).

Determination of biological activities

Agar diffusion test

The influence of the new glycolipids on the growth of different bacteria *(Bacillus subtilis* DSM 347, *Bacillus megaterium* DSM 90, *Staphylococcus capitis* subsp. *capitis* DSM 20326, *Escherichia coli* DSM 1103, *Pseudomonas aeruginosa* DSM 1117), fungi (*Ustilago maydis* DSM 4500, *Eurotium repens**, *Mycotypha microspora**), and the yeast *Candida magnoliae* (DSM 70638) was analysed using the disc test (58940) or the so-called agar diffusion test (*from the Institute of Microbiology, Department of Mycology, TU Braunschweig, Braunschweig, Germany) (Vollbrecht *et al.*, 1998, 1999).

Short-term *in vitro* bioassay for determining the antitumour-promoting activity

The antitumour-promoting activities of the glycolipids were determined using a short-term *in vitro* assay for Epstein-Barr virus (EBV) activation in Raji cells (EBV genome-carrying human lymphoblastoid cells) induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These cells were cultivated in 8% fetal bovine serum (FBS) RPMI 1640 medium and incubated ($1 \cdot 10^6$ ml^{-1}) at 37 °C for 48 h in 1 ml of medium containing 4 mM *n*-butyric acid, 32 pmol TPA in dimethyl sulfoxide (DMSO), and a known amount of the test compound in DMSO. Smears were made from the cell suspension, and the activated cells were stained by high-titer EBV-positive sera from nasopharyngeal carcinoma patients and fluorescein isocyanate-labelled anti-human IgG. Detection was achieved by the indirect immunofluorescence technique; 500 cells were counted in each assay, and experiments were performed in duplicate (Colombo *et al.*, 2002; Shirahashi *et al.*, 1993).

Results and Discussion

Cultivation with co-substrates with an odd number of carbon atoms and separation of the different glycolipids

Based on the experience of Brakemeier *et al.* (1998a) with the production of alkyl sophorosides by using a co-substrate with an even number of carbon atoms, *C. bombicola* was cultured in the presence of a co-substrate with an odd number of carbon atoms, 2-tridecanone. After 9 days of cultivation and extraction with ethyl acetate (two times), 1.5 g crude product were obtained from 1 l culture volume. The TLC analysis revealed that the crude product is a mixture of various compounds (Fig. 1, lane RE). For comparison, Brakemeier *et al.* (1998b) reported that they obtained 15 g l^{-1} alkyl sophorosides after 12 days of cultivation with

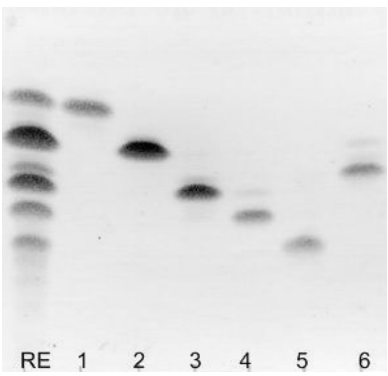

Fig. 1. Thin-layer chromatogram of the components in the culture medium of *Candida bombicola* after growth with 2-tridecanone as co-substrate. RE, crude product mixture; lanes 1–6, single components after purification by MPLC. Sample volume, 10 μl; solvent system, $CHCl_3$/CH_3OH/H_2O (65:15:2); stained with α-naphthol/sulfuric acid reagent.

Table I. Amounts and structural characteristics of certain sophorolipids of *C. bombicola* produced during cultivation on glucose (main C source) and 2-tridecanone as co-substrate. Starting material for downstream processing was 1 g crude product.

TLC lane	Amount [mg]	C-6' Position (sophorose)	C-6" Position (sophorose)	C-12 Position (fatty alcohol)	Denomination
1	50	CH_3CO	CH_3CO	-[a]	$SL_{18,\ classical}$
2	125	CH_3CO	CH_3CO	H	$SL\text{-}F_{2-13}$
3	200	H	CH_3CO	H	$SL\text{-}E_{2-13}$
4	40	CH_3CO	CH_3CO	acetyl glucose	$SL\text{-}A_{2-13}$
5	150	H	H	H	$SL\text{-}D_{2-13}$
6	20	CH_3CO	CH_3CO	OH	$SL\text{-}C_{2-13}$

[a] 17-OH octadeca(e)noic acid in C-1' position of the sophorose.

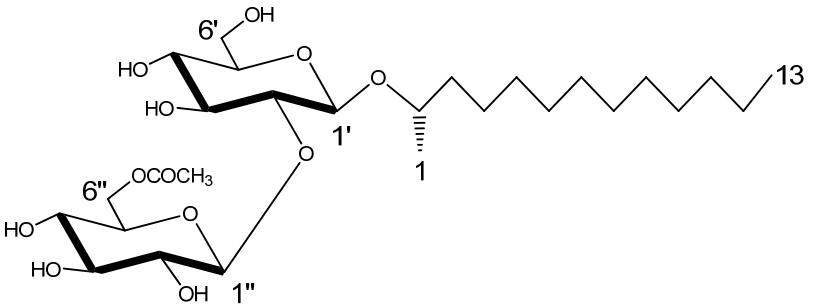

Fig. 2. Molecular structure of $SL\text{-}E_{2-13}$.

the co-substrate 2-dodecanone. By the use of 2-tetradecanol or 2-hexadecanol as co-substrate they obtained around 24 g l^{-1} alkyl sophorosides after 9 days of cultivation (Brakemeier *et al.*, 1995). Since the 2-tridecanone cultivation was a preliminary experiment, there is potential for improvement, *e.g.* the incubation time should be extended.

For separation of the constituents, 1 g crude product mixture was subjected to MPLC yielding six compounds (Fig. 1, lanes 1–6) with yields of 20 to 200 mg (the main product being $SL\text{-}E_{2-13}$, Table I), which were analysed by NMR and mass spectroscopy.

The structure of $SL\text{-}E_{2-13}$ (lane 3 in Fig. 1), is presented in Fig. 2. The positions 6' and 6" of the sophorose and the C-12 position of the fatty alcohol are highlighted, because the six compounds differed by their substituents at these positions (Table I). The denomination of the compounds (last column of Table I) followed the appropriate nomenclature of 2-dodecyl sophorosides (Brakemeier *et al.*, 1998a).

Elucidation of molecular structures

As for lane 1 of the thin-layer chromatogram (Fig. 1), initial ^1H NMR spectra confirmed our assumption that this compound was the well-known classical diacetylated sophorolipid containing 17-hydroxy-octadeca(e)noic acid at the C-1' position, and additionally, lactonically linked to the C-4' position of sophorose (data not shown). Thus our aim to exclude production of this sophorolipid type was not fulfilled.

Compounds in lanes 2 and 3 of the thin-layer chromatogram were identified as the new 2-tridecyl sophorosides $SL\text{-}F_{2-13}$ and $SL\text{-}E_{2-13}$. Exemplarily, for $SL\text{-}E_{2-13}$ all NMR data are documented in Table II. The singlet at δ_H 2.10 ppm provides evidence for the presence of a single acetyl group in position C-6". The corresponding C-6"A and C-6"B protons show double doublets at δ_H 4.41 and 4.24 ppm, respectively. As for the C-6'A and C-6'B protons (OH-group attached at C-6'), the δ_H values are shifted to higher field at 3.88 and 3.70 ppm. Evidence for the exclusive positioning of the acetyl group at C-6" arose from 2D COSY* and TOCSY** through-bond correlations (*correlation spectroscopy, **total correlated spectroscopy): on the one hand, significant cross-signals were detected for the C-1" and C-6" protons, on the other hand for the C-1' and C-6'

Table II. ^{1}H (600 Mz) and ^{13}C NMR (100 Mz) data of SL-E_{2-13} with marked characteristic signals, recorded in CD_3OD. s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

Position	δ_H (ppm) / J (Hz)	δ_C (ppm)
Sophorose		
C-1'	4.49 d / 7.7	102.4
C-2'	3.42 dd / 7.8, 9.3	83.6
C-3'	3.58 t / 9.1	78.0
C-4'	3.37 m	71.4
C-5'	3.29 m	77.6
C-6'A	**3.88 dd** / 2.4, 11.9	62.7
C-6'B	**3.70 dd** / 5.7, 11.9	-
C-1"	4.59 d / 7.8	105.6
C-2"	3.29 m	76.1
C-3"	3.42 m	77.5
C-4"	3.35 m	71.4
C-5"	3.49 m	75.6
C-6"A	**4.41 dd** / 2.2, 11.9	64.9
C-6"B	**4.24 dd** / 5.4, 11.9	-
CH_3COO- (C-6')	-	-
CH_3COO- (C-6")	2.10 s	20.9 / **172.7**
Sec. alcohol		
C-1	1.25 d / 6.2	21.8
C-2	3.84 m	78.1
C-3 A	1.45 m	37.7
C-3 B	1.67 m	-
C-4–C-12	1.33–1.41 m	23.7–33.1
C-13	**0.94 t** / 6.9	**14.4**

protons. The different functionalization of C-1" and C-1' facilitated the correlation. A triplet at δ_H 0.94 ppm indicated a terminal methyl group (C-13 position) which was confirmed by ^{13}C NMR data (δ_C 14.4 ppm). Only single signals were detected for the two carbon atoms of an acetyl group, at δ_C 20.9 ppm for CH_3 and at δ_C 172.7 ppm for COOH.

In the case of SL-F_{2-13} the characteristic NMR data were as follows (Table III): One more sharp signal at δ_H 2.09 ppm suggested the presence of an additional acetyl group (at C-6' position); compared to SL-E_{2-13}, the integrals at δ_H 4.41 and 4.23 ppm were doubled; there were no signals at δ_H 3.88 or 3.70 ppm (proof for missing free OH group at C-6'). As for ^{13}C NMR, the integrals at δ_C 20.7 and 172.7 ppm were doubled (proof for two acetyl groups).

Table III also presents the most important data for the compounds in lanes 4–6.

As for SL-D_{2-13} (lane 5) the H-6'A and H-6'B protons as well as H-6"A and H-6"B protons showed signals at δ_H 3.88 and 3.70 ppm which indicated no additional acetyl groups. Absence of the characteristic singlet at δ_H 2.10 ppm confirmed our assumption.

Table III. Characteristic ^{1}H NMR [600 MHz, δ (ppm) / J (Hz)] and ^{13}C NMR [100 MHz, δ (ppm)] signals of SL-F_{2-13}, SL-D_{2-13}, SL-C_{2-13}, and SL-A_{2-13}, recorded in CD_3OD. s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

Position	SL-F_{2-13}	SL-D_{2-13}	SL-C_{2-13}	SL-A_{2-13}
^{1}H NMR				
C-6'A	4.41 dd / 2.2, 11.9	3.88 m	4.41 dd / 2.2, 11.9	4.41 dd / 2.2, 11.9
C-6'B	4.23 dd / 6.7, 11.8	3.70 m	4.22–4.25	4.22–4.26
C-6"A	4.41 dd / 2.2, 11.9	3.88 m	4.41 dd / 2.2, 11.9	4.41 dd / 2.2, 11.9
C-6"B	4.23 dd / 5.1, 11.9	3.70 m	4.22–4.25	4.22–4.26
CH_3COO- (C-6')	2.09 s	-	2.09 s	2.09 s
CH_3COO- (C-6")	2.10 s	-	2.09 s	2.09 s
CH_3COO- (C-6‴)	-	-	-	2.10 s
C-12	1.33–1.41 m*	1.31–1.45 m[a]	3.75 m	3.79 m
C-13	0.94 t / 6.8	0.94 t / 7.0	1.18 d / 6.2	1.24 d / 5.8
^{13}C NMR				
CH_3COO- (C-6')	20.7 / 172.7	-	20.7 / 172.7	20.7 / 172.7
CH_3COO- (C-6")	20.9 / 172.7	-	20.9 / 172.7	20.9 / 172.7
CH_3COO- (C-6‴)	-	-	-	20.7 / 172.7
C-12	23.7–33.1[a]	23.7–33.1[a]	68.6	78.2
C-13	14.4	14.4	21.8	22.0

[a] One signal among others for C-4–C-12.

Table IV. ESIMS data of the new 2-tridecyl sophorosides.

Compound	$[M + Na]^+_{found}$	$[M + Na]^+_{calcd.}$	Molecular formula
SL-E_{2-13}	589.3188	589.3199	$C_{27}H_{50}O_{12}$
SL-F_{2-13}	631.3293	631.3305	$C_{29}H_{52}O_{13}$
SL-D_{2-13}	547.3085	547.3089	$C_{25}H_{48}O_{11}$
SL-C_{2-13}	647.3237	647.3249	$C_{29}H_{52}O_{14}$
SL-A_{2-13}	851.3875	851.3883	$C_{37}H_{64}O_{20}$

The data of the compound SL-C_{2-13} (lane 4) were very similar to those of SL-F_{2-13}, with the exception of one important difference: At the C-13 position of the alcohol moiety of the glycolipid, the signal for the methyl group was changed to a doublet at δ_H 1.18 ppm. This indicates that one hydrogen atom of the neighbouring C-12 methylene group was replaced by another (hydroxy) group. The ^{13}C NMR signal at 14.4 ppm (C-13) was shifted to 21.8 ppm. In addition, a new signal at δ_C 68.6 ppm for a -CHOH- group appeared.

As for SL-A_{2-13} (lane 6), the spectroscopic data also suggest a substitution of the C-12 position of the alcohol part (doublet at δ_H 1.24 ppm, shift from δ_C 14.4 to 22.0 ppm). Since there is an increase of signals for glucose-specific protons (δ_H 3.34–4.41 ppm) and carbon atoms (δ_C 65–104 ppm) and furthermore, the appearance of a third acetyl group attached to the third glucose unit, the OH-group in SL-C_{2-13} is replaced by an acetylglucose unit.

To demonstrate the accurate chain length of the fatty alcohol moiety within the new glycolipids ESIMS data are presented in Table IV. For instance, the $[M + Na]^+$ ion at *m/z* 589.3188 in the HRESI mass spectra was in accordance with the molecular formula of the proposed structure shown in Fig. 2 (SL-E_{2-13}). Also all other $[M + Na]^+$ ion values were consistent with the proposed molecular structures briefly mentioned in Table I and Table III.

A proposal for the biosynthetic pathway for the formation of 2-tridecyl sophorosides

Based on the preceding elucidation of molecular structures, a proposal for the biosynthetic pathway for the formation of 2-tridecyl sophorosides, starting with glucose and the co-substrate 2-tridecanone, is shown in Fig. 3. This pathway is based on biosynthesis schemes of Fleurackers (2006) and Van Bogaert *et al.* (2011) for classical sophorolipids (fatty acids as co-substrate), and now adapted to the uncommon ketone co-substrate.

Initially Fig. 3 suggests a ketone reduction by the yeast alcohol dehydrogenase giving a secondary alcohol. Following the left branch of the biosynthesis, two distinct glucosyltransferases attach two glucose units to the alcohol giving rise to SL-D_{2-13}, the first intermediate which we isolated from the microbial culture. An acetyltransferase catalyzes the formation of SL-E_{2-13} (monoacetylation) and SL-F_{2-13} (diacetylation). As for the right branch, a monooxygenase is supposed to be responsible for the introduction of a hydroxy group in the second subterminal position (C-12). Similar to the left side, then the glucosyltransferases 1 and 2 add two glucose molecules, and an acetyltransferase adds two acetyl groups giving rise to SL-C_{2-13}. Finally, the free C-12 OH-group is combined with a third glucose moiety which is in turn acetylated generating SL-A_{2-13}, the last compound we could isolate in sufficient amounts.

Physicochemical characterization of the new 2-tridecyl sophorosides

The measurements of the influence of the new glycolipids on the surface and interfacial activities of aqueous solutions provided interesting results according to which the crude mixture of 2-tridecyl sophorosides (including all compounds mentioned before) lowers the surface tension of water from 72 mN m^{-1} to 34 mN m^{-1}. The corresponding values caused by the pure compounds of this mixture were in the range of 32 to 41 mN m^{-1}. As for $SL_{18,\ classical}$ (5–10% content in the crude product, see Table I) a minimum surface tension value of 35 mN m^{-1} at a critical micelle concentration of 40 mg l^{-1} has been reported (Lang, 2002). As for the reduction of the interfacial tension between water and *n*-hexadecane SL-E_{2-13} and SL-F_{2-13} showed a stronger effect than the crude product (lowering from 43 mN m^{-1} to 4 and 3 mN m^{-1}, respectively, compared to 11 mN m^{-1}).

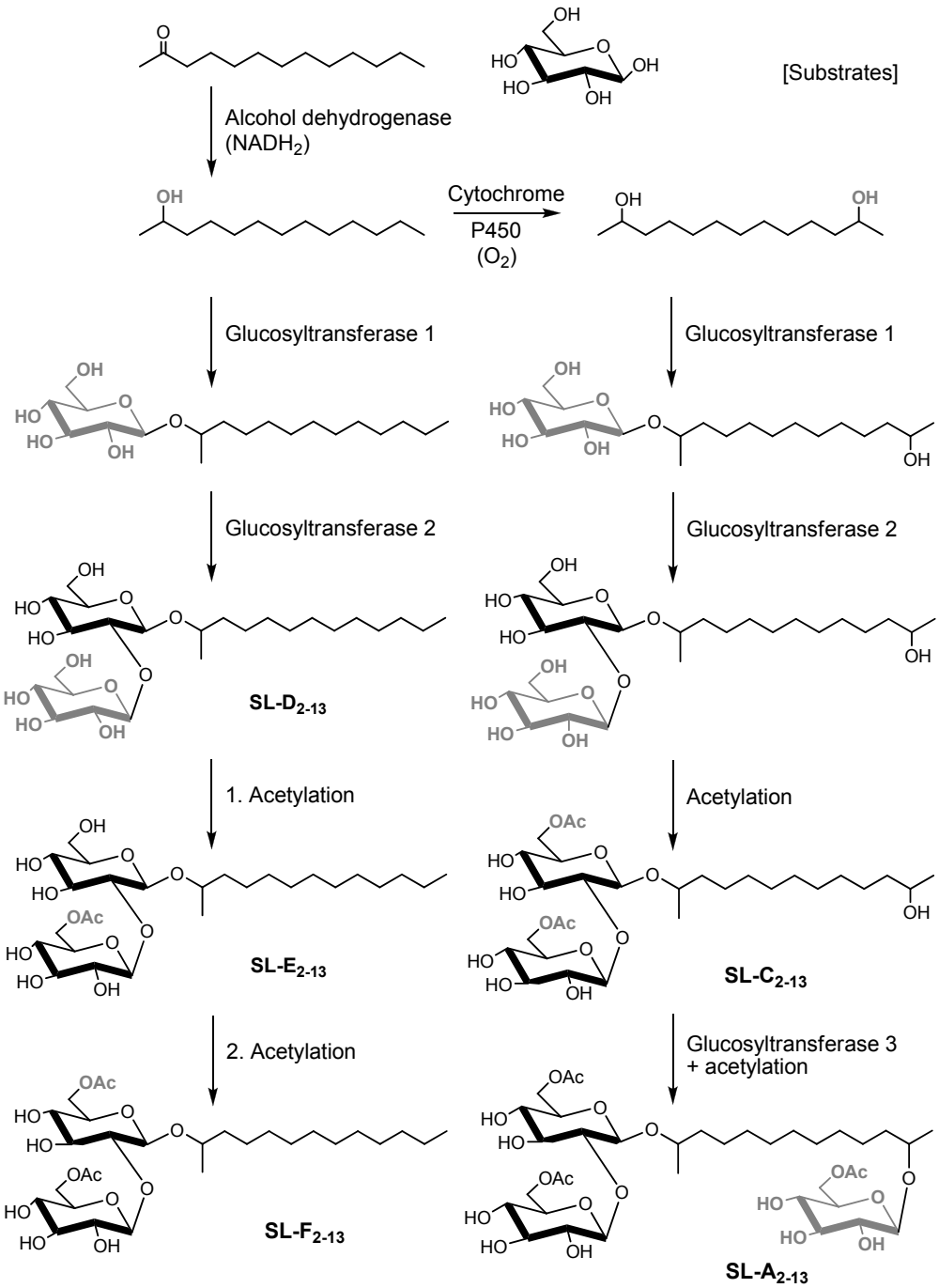

Fig. 3. Proposal for the biosynthetic pathway for the formation of 2-tridecyl sophorosides by *Candida bombicola* ATCC 22214, deduced from intermediates found in this study. Carbon sources were glucose and 2-tridecanone. [Modified scheme based on Fleurackers (2006) and Van Bogaert *et al.* (2011), suggested for the biosynthesis of classical sophorolipids.]

To compare the values of our 2-tridecyl sophorosides with those of the corresponding 2-dodecyl sophorosides (one carbon atom less) and 2-tetradecyl sophorosides (one carbon atom more), those compounds (Brakemeier, 1997; Brakemeier *et al*., 1998a) were tested as well in the surface tension measurements. According to their lower lipophilic character (SL-E_{2-12}, 12 carbon atoms) or, on the other hand, stronger lipophilic character (SL-E_{2-14}, 14 carbon atoms) these very similar glycolipids effected somewhat lower values (31 mN m^{-1}) or higher values (35 mN m^{-1}), respectively, compared to SL-E_{2-13} (13 carbon atoms) with 32 mN m^{-1}.

Nevertheless, all new glycolipids showed a good potential for surfactant applications.

Biological activities of the new 2-tridecyl sophorosides

Agar diffusion test

The new 2-tridecyl sophorosides were tested for their influence on the growth of bacteria, yeasts, or filamentous fungi using the agar diffusion test (Table V). All compounds strongly affected the growth in case of the Gram-positive bacteria *Bacillus subtilis* and *B. megaterium. Staphylococcus capitis* subsp. *capitis*, similar to pathogenic *S. aureus*, was slightly inhibited by all the new substances, with the exception of SL-D_{2-13}. Also *Candida magnoliae*, similar to pathogenic *C. albicans*, showed moderate resistance against all glycolipids. In the case of the Gram-negative bacterica *Escherichia coli* and *Pseudomonas aeruginosa* and the fungi *Eurotium repens*, *Mycotypha microspora*, and *Ustilago maydis* no growth inhibition was observed. Similar studies, but using the classical lactonic C-18 sophorolipids in solution, had shown remarkable minimum inhibitory concentrations (reducing growth to 50%) of only 6 mg l^{-1} towards *Bacillus subtilis* and *Staphylococcus epidermidis* (Lang *et al*., 1989).

Short-term *in vitro* bioassay for antitumour promoters

Chemoprevention is dedicated to identifying agents with potential preventive roles in cancer. Recently, glycoglycerolipid analogues bearing short- to medium-length lipophilic chains, have shown antitumour-promoting activity in the TPA-promoted *in vitro* Epstein-Barr virus early antigen (EBV-EA) activation test (Colombo *et al.,* 2002, 2005, 2006). Also some other compounds from natural sources, such as coumarins, sesquiterpenes or triterpenoids, may be valuable as antitumour promoters in chemical carcinogenesis (Fukuda *et al*., 2005; Ito *et al.*, 2005; Mendoza *et al.*, 2005). The mechanisms by which such compounds interfere with tumour promotion need yet to be identified.

In the present study, the 2-tridecyl sophorosides were tested for their antitumour-promoting activity using the above mentioned short-term *in vitro* assay for Epstein-Barr virus activation by TPA in Raji cells induced. Table VI shows the *in vitro* tumour inhibitory activity of the compounds relative to the control (10.1–16.3% at 1000 mol ratio/TPA). The most active compound was SL-

Table V. Agar disc diffusion test with bacteria and fungi. Amount of glycoconjugate, approx. 600 μg per disc; (++) diameter of the inhibition area > 10 mm; (+) diameter of the inhibition area < 10 mm; (–) no inhibition area. Pen G impact as comparison; diameter of the inhibition area > 15 mm vs. Gram-positive bacteria.

Test organism	SL-E_{2-13}	SL-F_{2-13}	SL-D_{2-13}	SL-C_{2-13}	SL-A_{2-13}
Bacteria					
Bacillus megaterium	+	+	+	+	++
Bacillus subtilis	++	++	++	+	+
Staphylococcus capitis[a]	+	+	–	+	+
Escherichia coli	–	–	–	–	–
Pseudomonas aeruginosa	–	–	–	–	–
Yeast					
Candida magnoliae	++	+	+	+	+
Fungi					
Eurotium repens	–	–	–	–	–
Mycotypha microspora	–	–	–	–	–
Ustilago maydis	–	–	–	–	–

[a] Subsp. *capitis.*

Table VI. Antitumour-promoting activities of the new glycoconjugates: inhibition of TPA-induced activation of Epstein-Barr virus early antigen (EBV-EA).

Glycoconjugate	Percentage relative to control (percentage viability of Raji cells)[a]			
	1000	500	100	10
SL-E_{2-13}	14.6 (70) ± 0.6	55.7 ± 1.4	84.8 ± 2.3	100 ± 1.5
SL-F_{2-13}	16.3 (70) ± 0.5	58.6 ± 1.5	86.8 ± 2.2	100 ± 1.3
SL-D_{2-13}	14.0 (70) ± 0.4	55.9 ± 1.5	84.9 ± 2.0	100 ± 1.6
SL-C_{2-13}	10.1 (70) ± 0.5	50.2 ± 1.6	79.3 ± 2.1	100 ± 1.7
SL-A_{2-13}	13.2 (70) ± 0.6	54.6 ± 1.7	83.2 ± 2.2	100 ± 1.6
Heptyl-galactosyl-glyceride[b]	0.0 (70) ± 0.5	20.3 ± 1.3	38.5 ± 1.5	72.1 ± 0.4

[a] Values are EBV-EA activation (%) in the presence of different amounts of the tested glycoconjugates (mol ratio/TPA), relative to the control (100%). Activation was attained by treatment with 32 pmol TPA; the tested substances were applied in multiples of this concentration (10- to 1000-fold). The numbers in parentheses are the viability rates of the tested Raji cells.
[b] Colombo *et al.* (2002).

C_{2-13} which reduced the fraction of EBV-positive cells to 10.1% after treatment with an 1000-fold concentration (relative to TPA). For all compounds a weak cytotoxicity against Raji cells was observed (70% viability at 1000 mol ratio/TPA). Comparable results we obtained using diglucosyl glycerolipids from a marine sponge-associated *Bacillus pumilus* strain and, additionally, using di- and tetrasaccharide lipids produced by the soil bacterium *Tsukamurella* sp. (Ramm *et al.*, 2004; Langer *et al.*, 2006).

Conclusions

2-Tridecyl sophorosides were produced by the cultivation of *Candida bombicola* with glucose as substrate and 2-tridecanone as co-substrate. After solvent extraction, MPLC of the crude product mixture yielded – besides the well-known classical lactonic C-18 sophorolipid – five new compounds which were analysed by NMR and mass spectroscopy. These glycolipids differed in the number of their glucose, acetyl, and hydroxy groups, respectively. Based on these identified intermediates and additional data from the literature, a proposal for the possible biosynthetic pathway was presented. The crude mixture of 2-tridecyl sophorosides as well as the individual compounds had good surface-active and interfacial-active properties. The biological tests indicated that the new glycolipids inhibited especially Gram-positive bacteria. SL-C_{2-13} appeared to have advantageous effects in *in vitro* tests of antitumour-promoting activity.

Acknowledgements

The authors are grateful for funding of their studies by the DFG (German Research Foundation, Bonn). We also would like to thank C. Kakoschke for recording NMR spectra and A. Abrahamik for recording all HRESIMS data.

Asmer H., Lang S. Wagner F., and Wray V. (1988), Microbial production, structure elucidation and bioconversion of sophorose lipids. J. Am. Oil Chem. Soc. **65**, 1460–1466.

Bisht K. S., Grosse R. A., and Kaplan D. L. (1999), Enzyme-mediated regioselective acylations of sophorolipids. J. Org. Chem. **64**, 780–789.

Brakemeier A. (1997), Mikrobielle Alkyl-Glycoside von *Candida bombicola.* Ph. D. Thesis. Braunschweig University of Technology, Braunschweig, Germany.

Brakemeier A., Lang S., Wullbrandt D., Merschel L., Benninghoven A., Buschmann N., and Wagner F. (1995), Novel sophorose lipids from microbial conversion of 2-alkanols. Biotechnol. Lett. **17**, 1183–1188.

Brakemeier A., Wullbrandt D., and Lang S. (1998a), *Candida bombicola:* production of novel alkyl glycosides based on glucose/2-dodecanol. Appl. Biochem. Biotechnol. **50**, 161–166.

Brakemeier A., Wullbrandt D., and Lang S. (1998b), Microbial alkyl-sophorosides based on 1-dodecanol or 2-, 3- or 4-dodecanones. Biotechnol. Lett. **20**, 215–218.

Colombo D., Compostella F., Ronchetti F., Reza-Elahi S., Scala A., Toma L., Aoi W., Kuchide M., Takayasu J., Tokuda H., and Nishino H. (2002), Inhibitory effect of stabilized analogues of glycoglycerolipids on

Epstein-Barr virus activation and mouse skin tumor promotion. Cancer Lett. **186**, 37–41.

Colombo D., Franchini L., Tomma L., Ronchetti F., Nakabe N., Konoshima T., and Nishino H. (2005), Anti-tumor-promoting activity of simple models of galactoglycerolipids with branched and unsaturated acyl chains. Eur. J. Med. Chem. **40**, 69–74.

Colombo D., Franchini L., Toma L., Ronchetti F., Tanaka R., Takayasu J., Nishino H., and Tokuda H. (2006), Cyclic and branched acyl chain galactoglycerolipids and their effect on anti-tumor-promoting activity. Eur. J. Med. Chem. **41**, 1456–1463.

Daniel H. J., Otto R. T., Reuss M., and Syldatk C. (1998), Sophorolipid production with high yields on whey concentrate and rapeseed oil without consumption of lactose. Biotechnol. Lett. **20**, 805–807.

Daniel H.-J., Otto R. T., Binder M., Reuss M., and Syldatk C. (1999), Production of sophorolipids from whey: development of a two-stage process with *Cryptococcus curvatus* ATCC 20509 and *Candida bombicola* ATCC 22214 using deproteinized whey concentrates as substrates. Appl. Microbiol. Biotechnol. **51**, 40–45.

Daverey A. and Pakshirajan K. (2010), Kinetics of growth and enhanced sophorolipids production by *Candida bombicola* using a low-cost fermentative medium. Appl. Biochem. Biotechnol. **160**, 2090–2101.

Fleurackers S. J. J. (2006), On the use of waste frying oil in the synthesis of sophorolipids. Eur. J. Lipid Sci. Technol. **108**, 5–12.

Fukuda Y., Sakai K., Matsunaga S., Tokuda H., and Tanaka R. (2005), Cancer chemopreventive activity of lupane- and oleanane-type triterpenoids from the cones of *Liquidamber styraciflua*. Chem. Biodivers. **2**, 421–428.

Gorin P., Spencer J., and Tulloch A. (1961), Hydroxy fatty acid glycosides of sophorose from *Torulopsis magnoliae*. Can. J. Chem. **39**, 846–855.

Ito C. M., Itoigawa M., Ju-ichi N., Sakamoto H., Nishino H., Tokuda H., and Furukawa H. (2005), Antitumor-promoting activity of coumarins from citrus plants. Planta Med. **71**, 84–87.

Lang S. (2002), Biological amphiphiles (microbial biosurfactants). Curr. Opin Colloid Interface Sci. **7**, 12–20.

Lang S., Katsiwela, E., and Wagner F. (1989), Antimicrobial effects of biosurfactants. Fat. Sci. Technol. **9**, 363–366.

Langer O., Palme O., Wray V., Tokuda H., and Lang S. (2006), Production and modification of bioactive biosurfactants. Proc. Biochem. **41**, 2138–2145.

McGaffrey W. C. and Cooper D. G. (1995), Sophorolipids production by *Candida bombicola* using self-cycling fermentation. J. Ferment. Bioeng. **79**, 146–151.

Mendoza C. R., Jiménez I. A., Tokuda H., Kushida H., and Bazzocchi I. L. (2005), Antitumor-promoting effects of new sesquiterpenes from *Crossopetalum tonduzii*. Chem. Biodivers. **2**, 286–294.

Ramm W., Schatton W., Wagner-Döbler I., Wray V., Nimtz M., Tokuda H., Enjyo F., Nishino H., Beil W., Heckmann R., Lurtz V., and Lang S. (2004), Diglucosyl-glyercolipids from marine sponge-associated *Bacillus pumilus* strain AAS3: their production, enzymatic modification and properties. Appl. Biochem. Biotechnol. **64**, 497–504.

Rau U., Nguyen L. A., Schulz S., Wray V., Nimtz M., Roeper H., Koch H., and Lang S. (2005), Formation and analysis of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. Appl. Microbiol. Biotechnol. **66**, 551–559.

Recke V. K., Gerlitzki M., Hausmann R., Syldatk C., Wray V., Tokuda H., Suzuki N., and Lang S. (2013), Enzymatic production of modified 2-dodecyl-sophorosides (biosurfactants) and their characterization. Eur. J. Lipid Sci. Technol. **115**, 452–463.

Shirahashi H., Murakami N., Watanabe M., Nagatsu A., Sakakibara J., Tokuda H., Nishino H., and Iwashima A. (1993), Isolation and identification of anti-tumor-promoting principles from the fresh-water cyanobacterium *Phormidium* tenue. Chem. Pharm. Bull. **41**, 1664–1666.

Singh S. K., Felse A. P., Nuñez A., Foglia T. A., and, Gross R. A. (2003), Regioselective enzyme-catalyzed synthesis of sophorolipid esters, amides, and multifunctional monomers. J. Org. Chem. **68**, 5466–5477.

Syldatk C., Lang S., Wagner F., Wray V., and Witte L. (1985), Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* spec. DSM 2874 grown on *n*-alkanes. Z. Naturforsch. **40c**, 51–60.

Van Bogaert I. N. A., Saerens K., De Muynck C., Develter D., Soetaert W., and Vandamme E. J. (2007), Microbial production and application of sophorolipids. Appl. Microbiol. Biotechnol. **76**, 23–34.

Van Bogaert I. N. A., Zhang J., and Soetaert W. (2011), Microbial synthesis of sophorolipids. Process Biochem. **46**, 821–833.

Vollbrecht E., Heckmann R., Wray V., Nimtz M., and Lang S. (1998), Production and structure elucidation of di- and oligosaccharide lipids (biosurfactants) from *Tsukamurella* sp. nov. Appl. Microbiol. Biotechnol. **50**, 530–537.

Vollbrecht E., Rau U., and Lang S. (1999), Microbial conversion of vegetable oils into surface-active di-, tri-, and tetrasaccharide lipids (biosurfactants) by bacterial strain *Tsukamurella* spec. Lipid **10**, 389–394.