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Production, Structural Elucidation, and *In Vitro* Antitumor Activity of Trehalose Lipid Biosurfactant from *Nocardia farcinica* Strain

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The objective of this study was to isolate and identify the chemical structure of a biosurfactant produced by *Nocardia farcinica* strain BN26 isolated from soil, and evaluate its *in vitro* antitumor activity on a panel of human cancer cell lines. Strain BN26 was found to produce glycolipid biosurfactant on *n*-hexadecane as the sole carbon source. The biosurfactant was purified using medium-pressure liquid chromatography and characterized as trehalose lipid tetraester (THL) by nuclear magnetic resonance spectroscopy and mass spectrometry. Subsequently, the cytotoxic effects of THL on cancer cell lines BV-173, KE-37 (SKW-3), HL-60, HL-60/DOX, and JMSU-1 were evaluated by MTT assay. It was shown that THL exerted concentration-dependent antiproliferative activity against the human tumor cell lines and mediated cell death by the induction of partial oligonucleosomal DNA fragmentation. These findings suggest that THL could be of potential to apply in biomedicine as a therapeutic agent.

Keywords: Biosurfactants, glycolipids, trehalose tetraester, *Nocardia farcinica*, NMR, cytotoxicity

Introduction

Biosurfactants are naturally occurring surface-active biomolecules produced by microorganisms [3]. Owing to their amphiphilic character (*i.e.*, they contain both hydrophobic and polar groups), biosurfactants often attach to surfaces and concentrate at interfaces. As a result, they reduce surface and interfacial tensions, thus forming microemulsions where hydrocarbons can solubilize in water or water can solubilize in hydrocarbons. These unique properties determine their potential application in environmental remediation technologies and also in the pharmaceutical, cosmetics, and food industries as emulsifiers, surfactants, and dispersants [3].

Biosurfactants comprise a diverse group of chemical structures. Generally, the hydrophobic parts of their molecules consist of long-chain fatty acids, hydroxyl fatty acids, or α-alkyl-β-hydroxyl fatty acids, whereas the hydrophilic moieties can be carbohydrates, amino acids, cyclic peptides, phosphates, carboxylic acids, or alcohols. Biosurfactants can be categorized into five groups regarding their chemical composition: glycolipids, lipopeptides, phospholipids, fatty acids, and polymeric biosurfactants. They can also be grouped into two categories: low-molecular-mass compounds that lower surface and interfacial tensions, and high-molecular-mass compounds that bind tightly to surfaces [21].

Most known biosurfactants are glycolipids, composed of a carbohydrate head and a lipid tail. Trehalose lipids are glycolipid biosurfactants produced by most species belonging to the mycolates group such as *Mycobacterium*, *Rhodococcus*, *Arthrobacter*, *Nocardia*, and *Gordonia*. Several structural types of trehalose lipids have been elucidated, where an α,α′-trehalose moiety (hydrophilic part) is linked by an

ester bond to short simple or long complex fatty acids (hydrophobic moiety). Most of them were found to be powerful biosurfactants, lowering in their pure form the surface tension of water from 72 mN/m to values between 19 and 43 mN/m, and the interfacial tension between water and *n*-hexadecane (decane or kerosene) to less than 1 mN/m at CMC values between 0.7 and 37 mg/l [12, 15, 16, 24–26].

Recently, some biosurfactants were proved to be suitable alternatives to synthetic medicines and may be used as effective theurapeutic agents [18]. For example, certain trehalose lipids have been shown to possess properties of biomedical importance, since they can act as anticancer and immunomodulating agents [8, 22].

This paper deals with the production and structural elucidation of a trehalose lipid biosurfactant from a newly isolated strain of *Nocardia farcinica.* In addition, its *in vitro* cytotoxic activity was evaluated in a panel of human cancer cell lines.

Materials and Methods

Microorganism, Media, and Cultivation

The *Nocardia farcinica* strain BN26 employed in this study was isolated from soil polluted with hydrocarbons, by a standard enrichment technique as described previously [25]. Strain BN26 was maintained in Nutrient agar slants (Difco Laboratories) at 4°C. Inocula were prepared by transferring bacterial cells from the storage culture to 250 ml flasks containing 50 ml of Nutrient broth and incubating at 30°C and 150 rpm on a rotary shaker. Four milliliters of this mid-exponential phase culture was inoculated into 1 L flasks containing 200 ml of mineral medium (MSM) with the following composition (g/l): $K_2HPO_4{\cdot}3H_2O$, 4.8; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $Mg\ SO_4{\cdot}7H_2O$, 0.5; supplemented with trace element solution (mg/l) of $CaCl_2{\cdot}2H_2O$, 2.0; $MnCl_2.4H_2O$, 0.4; $NiCl_2{\cdot}6H_2O$, 0.4; $ZnSO_4{\cdot}7H_2O$, 0.4; $FeCl_3{\cdot}6H_2O$, 0.2; $Na_2MoO_4{\cdot}2H_2O$, 0.2; and 2% *n*-hexadecane as the carbon source. The pH of the medium was adjusted to 7.0. Cultures were incubated while shaking (150 rpm) at 30°C for 7 days. Bacterial growth was assessed by determination of the optical density (OD_{610}) of the culture.

Resting cells were prepared by harvesting growing cells from 1 day culture in MSM with 2% *n*-hexadecane and washing them twice with fresh medium with no added nitrogen source.

Taxonomic Identification

Preliminary identification was carried out by both microscopic observations and biochemical experiments based on *Bergey's Manual of Determinative Bacteriology* [6]. Confirmation was obtained through determining the 16S rRNA gene sequence. DNA was extracted from a pure culture by a standard salting out procedure [13]. The extracted DNA was amplified by polymerase chain reaction (PCR) using designed primers for 16S rRNA of *Nocardia farcinica* (GenBank Accession No. IFM10152). The forward primers used were $496F_1$ (5′-ccggaattactgggcgtaa-3′) and $745F_2$ (5′-ccacgccgtaaacggtggta-3′) and the reverse primer was $1165R_1$ (5′-cttcacaca.tgctacaatg-3′). The PCR was performed in the following mixture: About 100 ng bacterial DNA, 10 pmol of each primer, 0.2 mmol/l of each dNTP, 1× supplied PCR buffer (including 1.25 mmol/l $MgCl_2$), 0.5 U ExPrime Taq (Genet Bio, Chungnam, Korea), and ddH_2O up to a final volume of 25 µl. The cycling conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 45 sec, annealing at 58°C for 45 sec, and synthesis at 72°C for 90 sec; final synthesis at 72°C for 5 min.

The obtained amplification products were seperated by 2% agarose gel electrophoresis and then sequenced by the same primers used in the PCR. The sequencing reaction was performed with a BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed by an automated sequencer ABI 310 Genetic Analyzer (Applied Biosystems).

Biosurfactant Production and Biodegradation Kinetics

Surface tension measurement and emulsifying activity were used for the detection of biosurfactant production. Equilibrium surface tension (ST) measurement of the supernatant fluid was carried out after centrifugation at 8,000 ×*g* for 20 min at 25°C with a Kruss T 10 tensiometer using the DU Nouy ring method. The emulsifying activity of whole cultures was estimated by adding 0.5 ml of sample fluid and 0.5 ml of hexadecane to 4.0 ml of distilled water. The tube was vortexed for 10 sec, held stationary for 1 min, and then visually examined for turbidity of a stable emulsion.

Biodegradation was measured as substrate depletion. Whole cultures were extracted with equal volumes of *n*-hexane, and residual *n*-hexadecane was quantified by gas chromatography using a Hewlett-Packard model 5859 instrument equipped with a flame ionization detector.

Isolation and Purification of the Biosurfactant

To isolate the surface active compound, the whole broth was sonificated for 2 min and extracted with 200 ml of methyl *tert*-butyl ether. The organic layer was separated from the aqueous phase and removed by rotary evaporation at 40°C under reduced pressure. The resulting crude extract was lyophilized. Then 4 g of the crude product was dissolved in a mixture of 80 ml of methanol:water (3/1 (v/v)) and washed three times with *n*-hexane to remove residual *n*-hexadecane and compounds less polar than the biosurfactants. After separation of the *n*-hexane layer, the methanol phase was removed from the aqueous phase by evaporation under reduced pressure. The aqueous solution was freeze-dried and analyzed for biosurfactant compounds by thin layer chromatography (TLC) using as the mobile phase chloroform/methanol/water (65/15/2 (v/v/v)) and spraying with anisaldehyde/sulfuric acid reagent for detection of carbohydrate-positive compounds.

One gram of the glycolipid mixture was dissolved in 2 ml of chloroform/methanol/water (65/15/2 (v/v/v)) and separated using medium-pressure liquid chromatography (MPLC). The MPLC equipment was composed of a Labor column, size B, LiChrosper Si 60,40−63 μm (Merck, Darmstadt, Germany), and a chromatography pump B 688 (Buchi, Konstanz, Germany). Chromatography was performed with chloroform/methanol/water (65/15/2 (v/v/v)) as a mobile phase, at a pressure of 2 bar and a flow rate of 1 ml/min. The eluate was fractionalized using the Pharmacia LKB Frac-100 Collector (Pharmacia LKB, Freiburg, Germany). The purification of the glycolipids was monitored off-line by thin layer chromatography. TLC was carried out on Si gel 60 plates as the stationary phase, with the same solvent system as used for MPLC, and with anisaldehyde/sulfuric acid reagent for detection of carbohydrate-positive compounds. Based on R_f values, the MPLC fractions containing the desired products were separately pooled and the solvent was evaporated under reduced pressure.

Characterization of the Purified Biosurfactant

^{1}H (1D, 2D COSY) and ^{13}C (1D, 2D HMQC, and HMBC) NMR spectra of the biosurfactant were recorded at 300 K on Bruker ARX 400 or DMX 600 NMR spectrometers (Bruker Biospin GmbH, Karlsruhe, Germany) locked to the major deuterium signal of the solvent CD_3OD / $CDCl_3$ (30:70). Chemical shifts are given in ppm relative to the residual proton signal of the methanol at 3.35 and coupling constants in Hz.

Positive ion electrospray mass spectra (ESI-MS) of the same were recorded on a Finnigan QTOF^2 mass spectrometer (Thermo Quest LC and LC/MS Division, CA, USA).

The adhesion was calculated as a percentage: 100 × [1 – (OD_{600} after mixing/OD_{600} before mixing)].

Cell Lines and Culture Conditions

The following cell lines were used in the experiments: HL-60 (DSMZ No: ACC 3, established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia) and its multidrug-resistant subline HL-60/Dox; BV-173 (DSMZ No: ACC 20 from the peripheral blood of a 45-year-old man with chronic myeloid leukemia in blast crisis); SKW-3 (DSMZ No: ACC 53, established from the peripheral blood of a 61-year-old man with T-cell lymphocytic leukemia), and JMSU-1 (DSMZ No: ACC 505, derived from malignant ascitic fluid of a 75-year old man with urinary bladder carcinoma).

The cells were passaged and kept in log phase as previously described [9]. Briefly, cells were grown as suspension cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma) at 37ºC in an incubator with humidified atmosphere and 5% CO_2. Cells were passaged two or three times a week.

Cytotoxicity Assessment

The cytotoxicity of the biosurfactant was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay as described by Mosmann [14]. Exponentially growing cells were seeded in 96-well microplates (100 μl/well at a density of 3.5×10^5 cells/ml for the adherent and 1×10^5 cells/ml for the suspension cell lines) and allowed to grow for 24 h prior to the exposure to the studied compounds. Stock solutions of the biosurfactants were freshly prepared in DMSO and then diluted with the corresponding growth medium. At the final dilutions, the solvent concentration never exceeded 0.5%. Cells were exposed to the tested agents for 24, 48, and 72 h, whereby for each concentration a set of eight separate wells were used. Every test was run in triplicate. After incubation with the tested compounds, MTT solution (10 mg/ml in PBS) aliquots were added to each well. The plates were further incubated for 4 h at 37ºC and the formazan crystals formed were dissolved by adding 110 μl of 5% HCOOH in 2-propanol. Absorption of the samples was measured by an ELISA reader (Uniscan Titertec) at 580 nm. The survival fraction was calculated as a percentage of the untreated control. The experimental data were processed using GraphPad Prizm software and were fitted to sigmoidal concentration/response curves.

DNA Extraction and Gel Electrophoresis

DNA was extracted from the cytosolic fraction as described previously [9]. Briefly, BV-173 cells were seeded in Petri dishes, containing about 5×10^6 cells each, and allowed to grow prior to trehalose lipid exposure. On the next day, the cells were treated with 35 or 70 μM of trehalose lipid and a non-treated control was prepared. After 24 h incubation at 37ºC and 5% CO_2, treated and untreated cells were washed in PBS and lysed in buffer (0.3% Triton X-100, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4). The lysates were centrifuged at 13,000 ×*g* for 20 min. The supernatants were kept and processed by adding 0.187 ml of 6 M NaCl solution and 0.937 ml of 2-propanol. Probes were incubated at –20ºC overnight, and thereafter centrifuged at 13,000 ×*g* for 20 min, and the DNA pellets were washed with 70% ethanol, air dried, re-dissolved in distilled water, and analyzed by electrophoresis on a 0.8% agarose gel.

Lymphocytes from Healthy Donors

Fresh human peripheral blood from healthy donors, obtained from the National centre of Blood Transfusion in Sofia, Bulgaria, was used for isolation of lymphocytes using Ficoll Paque gradient centrifugation. The lymphocytes were resuspended in RPMI-1640 Cell culture medium (without phenol red), supplemented with 10% FCS and Pen Strep (1%) at a concentration of 10^7 cells/ml, and activated by adding 3 μg/ml phytochemagglutinin, dissolved in the culture medium.

The lymphocyte cultures were treated under the same conditions as the cell lines.

Cell Surface Hydrophobicity

Cell surface hydrophobicity was determined by the bacterial adhesion to hydrocarbons (BATH) and measured spectrophotometrically as

described by Rosenberg *et al*. [20]. The difference between the OD of the aqueous phase before and after the mixing time was used to calculate the adhesion as a percentage: 100 × [1 – (OD_{600} after mixing/OD_{600} before mixing)].

Results

Identification of Strain BN26

The isolate was an aerobic, gram (+), rod-shaped, non-motile, catalase-positive, weakly acid-fast bacterium, forming aerial branched filamentous cells on solid agar plates. According to Bergey's Manual phenotypic tests, the strain was consistent with the pattern of *Nocardia* species. As proposed by Wallace and steele [27], four additional tests were used for the identification of the strain as *Nocardia farcinica*: the isolate showed comparable growth on TSA at 35ºC and 45ºC after incubation for 3 days, utilized acetamide as the sole carbon and nitrogen source, produced acid from L-rhamnose, and was negative for arylsulfatase production.

Comparative sequence analysis of the 16S rRNA (690 bp) in the GenBank database was performed by a BLAST search and manually reading through the GenBank accession number IFM10152. Since the gene sequence comparison demonstrated 99% similarity to *Nocardia farcinica* AB023371, the identity of strain BN26 was confirmed.

Biosurfactant Production

Strain BN26 was cultivated with 2% *n*-hexadecane as a model compound for microbial degradation of middle molecular weight hydrocarbons. Biosurfactant production was detected by ST measurements and emulsification activity of the whole broth. *Nocardia farcinica* grew exponentially during the first 48 h after inoculation with a rather long lag phase (Fig. 1). The ST of the culture dropped rapidly after the first 24 h of incubation, to reach its lowest point of 24.3 mN/m after 36 h. The reduction of ST of the culture and formation of emulsions with hexadecane indicated biosurfactant production. The hydrophobicity measurements showed that strain BN26 was highly hydrophobic at this time (logarithmic growth), since 72% of the cells adhered to the substrate. In the stationary phase, cell surface hydrophobicity began to decline and at the end of cultivation was 29%. The most significant consumption of the substrate was in the log phase, since at the beginning of linear growth 70% of the initial amounts of hexadecane was already metabolized.

To investigate whether nitrogen limitation favors biosurfactant production, parallel hexadecane-degrading cultures were started using resting cells, incubated in MSM

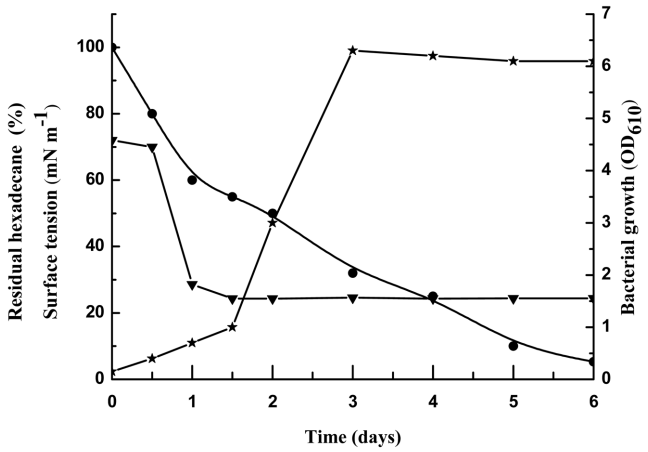

Fig. 1. Residual hexadecane (●) and surface tension (▼) measurements during bacterial growth (★) of *Nocardia farcinica* BN26 on mineral salt medium with 2% *n*-hexadecane as the carbon source.
Values are the average from triplicate treatments.

lacking a nitrogen source. The surface tension of the cultures dropped to 29.3 mN/m after a 32 h period, indicating biosurfactant formation. Data obtained from analytical TLC showed identical spots (R_f 0.41) from cultures with and without nitrogen limitation.

Isolation and Structure Elucidation of the Purified Trehalose Lipid

Glycolipids were extracted from whole cultures grown on hexadecane and purified using medium-pressure liquid chromatography. In order to determine the structure of the purified biosurfactant produced by *Nocardia farcinica* BN26, detailed mass spectrometric and NMR studies, which included ^{1}H, ^{13}C, COSY, DEPT, and HMBC analyses, were carried out and indicated the compound was a trehalose lipid. The ^{1}H NMR spectrum of the purified biosurfactant showed a typical glycolipid-type structure. The analysis of the COSY spectrum, together with the magnitudes of the vicinal coupling constants, allowed unambiguous assignment of the two glucose units of trehalose. The resonances of H-2, H-3, H-4, and H-2′ of this unit were found at around 4.8–5.4 ppm, and were to low field due to acylation of the corresponding four hydroxyl groups. The ^{1}H spectrum showed triplet signals at 0.80 ppm for three terminal CH_3 groups and characteristic patterns in the COSY spectrum of medium-length straight-chain fatty acids. A cluster of signals at 2.7 to 2.5 ppm integrating for four protons and with no cross peaks in the COSY spectrum was assigned to the CH_2 groups of the succinic acid residue (Fig. 2). The positions of

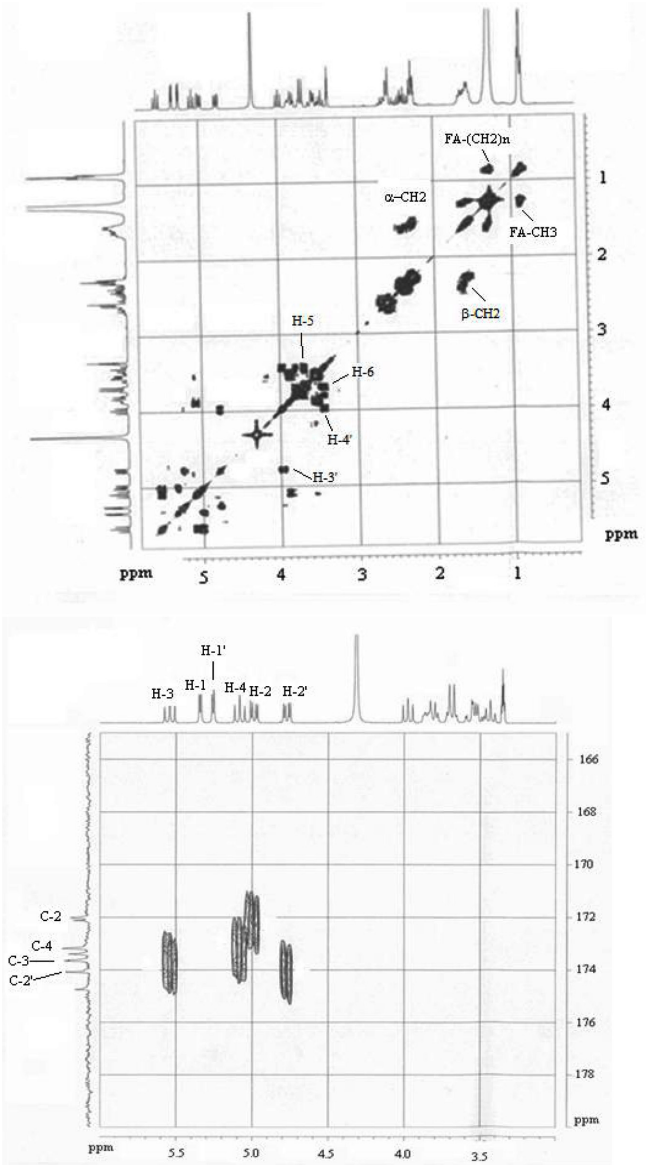

Fig. 2. 2D COSY (above) and partial low-field carbonyl correlations in the heteronuclear multiple bond correlation spectrum (below) of trehalose tetraester produced by *Nocardia farcinica* BN26.

Table 1. ^{1}H NMR data of trehalose 2,3,4,2′-tetraester in $CDCl_3$/CD_3OD.

Functional groups	^{1}H NMR δ (ppm)		J (Hz)	^{13}C NMR δ (ppm)
Saccharide				
	H-1	5.34 d	(1-2) 3.6	91.9
	H-2	4.98 dd	(2-3) 10.3	[b]
	H-3	5.54 t	(3-4) 9.4	70.0
	H-4	5.08 t	(4-5) 9.8	68.8
	H-5	3.85 m	(5-6A) 2.4	[b]
	H-6A	3.57 dd	(5-6B) 4.6	60.7
	H-6B	3.50 dd	(6A-6B) 12.5	
	H-1′	5.26 d	(1′-2′) 3.7	92.6
	H-2′	4.77 dd	(2′-3′) 9.8	72.7
	H-3′	3.98 t	(3′-4′) 9.1	[b]
	H-4′	3.43 t	(4′-5′) 9.1	[b]
	H-5′	3.69m	(5′-6A′)[a]	73.2
	H-6′A	3.80 m	(5′-6B′)[a]	61.8
	H-6′B	3.69 m	(6A′-6B′)[a]	
Succinoyl group				
	-C=O (C-2)			172.0
	-COOH			174.8
	-CH_2-	2.70-2.50		[c]
Acyl groups				
	-C=O (C-3)			173.7
	-C=O (C-4)			173.2
	-C=O (C-2′)			174.1
	-$\underline{CH_2}$CO-	2.48-2.23		34.4
	-$\underline{CH_2}CH_2$CO-	1.70-1.50		[c]
	-$(CH_2)_n$-	1.37-1.22		[c]
	-CH_3	0.80 t	(J = 6.5)	14.2, 14.1

[a]Not evaluated as second-order spectrum.

[b]71.3, 71.0, 70.8, 70.8.

[c]The remaining CH_2 signals are found at 32.1-28.7, 25.1, 25.0, 22.9, and 22.8.

the various acyl units were determined from an analysis of the long-range ^{1}H-^{13}C correlations in the HMBC spectrum (Fig. 2). The ^{13}C spectrum showed five signals for CO carbonyl carbons. The signal at 172.0 ppm correlated with H-2 at 4.98 ppm, with the protons of the succinoyl group at 2.70 to 2.50 ppm. Other signals at 174.1, 173.7, and 173.2 were assigned to the carbonyl carbons in fatty acyl groups positioned at C-2′, C-3, and C-4 as correlations were observed with H-2′ at 4.77 ppm, H-3 at 5.54 ppm, and H-4 at 5.08 ppm, respectively, and with protons at 2.48–2.23 ppm (α-CH_2CO) and 1.70–1.50 ppm (β-CH_2CH_2CO) derived from the fatty acyl groups, respectively (Table 1).

The presence and position of the ester groups in α,α′-trehalose were evident from the cross peakes in the 2D COSY and HMBC spectra. Analysis of the data from MS measurements indicated the presence of two major components, identified as α,α′-trehalose tetraesters with a

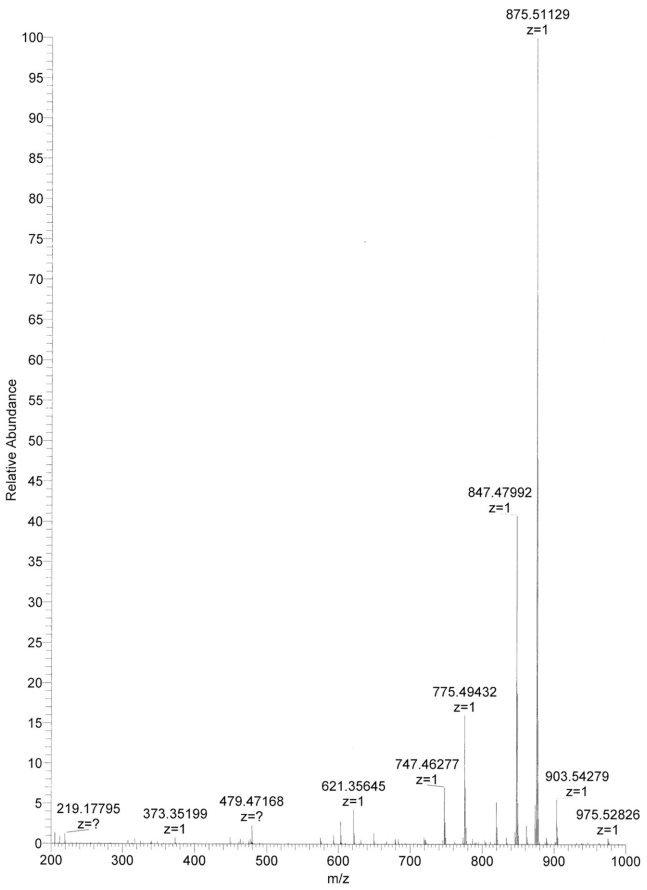

Fig. 3. Negative-ion electrospray mass spectra of the biosurfacant produced by *Nocardia farcinica* BN26.

molecular weight of 876 and 848, respectevily (Fig. 3). The elimination of the pseudo molecular ion of 100 *m/z* in each tetraester of trehalose evidenced the presence of succinic acid. The most abundant component with a molecular weight of 876 was esterified at C-2′, C-3, or C-4 with an octanoic and two decanoic acids. The other one, of lower molecular weight of 848, was esterified at the same positions with two octanoic acids and one decanoic acid (Fig. 4).

Cytotoxicity Assessment

The cytotoxic potential of THL was assessed by the MTT reduction assay in a panel of malignant cell lines originating from leukemia and solid tumors. The results of the chemosensitivity following 24, 48, and 72 h treatment were evaluated using the concentration-response curves presented in Fig. 5. As seen from Table 2, THL exhibited *in vitro* cytotoxic effects in micromolar concentrations, causing concentration-dependent cytotoxicity on all human tumor cell lines investigated. It demonstrated more pronounced

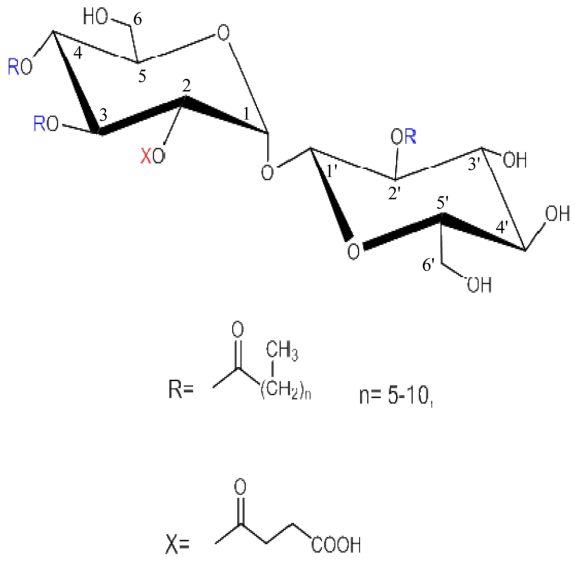

Fig. 4. Chemical structure of trehalose tetraester.

cytotoxic effects against malignant cells of lymphoid origin (BV-173 and SKW-3 cells). THL was found to be effective against HL-60 AML-derived cells as well. The congener sub-line HL-60/Dox with expression of the MRP-1 efflux pump was less sensitive, and IC_{50} values increased with the incubation time periods. Medium *in vitro* cytotoxic effect of THL was found for cells derived from the poorly differentiated transitional cell carcinoma of the urinary bladder (line JMSU-1).

Proapoptic Effect of THL

One of the tests for registration of apoptosis are the apoptotic DNA fragments obtained as discrete multiples of 180 bp subunit, which are detected as DNA ladders of oligonucleosomal fragments on agarose gels by electrophoretic separation. DNA fragments isolated from BV-173 cells treated with 25 and 70 μM RLA for 72 h produced some traces of apoptotic DNA (Fig. 6). This partial apoptotic laddering suggests also the interference of other forms of

Table 2. IC_{50} values (μM) of THL on leukemic and urinary bladder carcinoma cell lines according to the dose-response curves derived from MTT-dye reduction assays.

Cell line	24 h	48 h	72 h
SKW-3	20.7 (19.8-21.7)	25.4 (24.2-26.6)	28.9 (26.8-31.2)
BV-173	24.7 (23.9-25.5)	21.3 (19.5-23.3)	18.7 (17.4-20.2)
HL-60	25.5 (24.1-27.1)	30.0 (25.8-35.1)	31.3 (27.7-35.4)
HL-60/Dox	31.3 (29.8-32.9)	36.7 (35.3-38.1)	61.3 (53.5-70.2)
JMSU-1	36.0 (33.0-39.6)	60.7 (55.9-66.0)	53.3 (48.9-59.2)

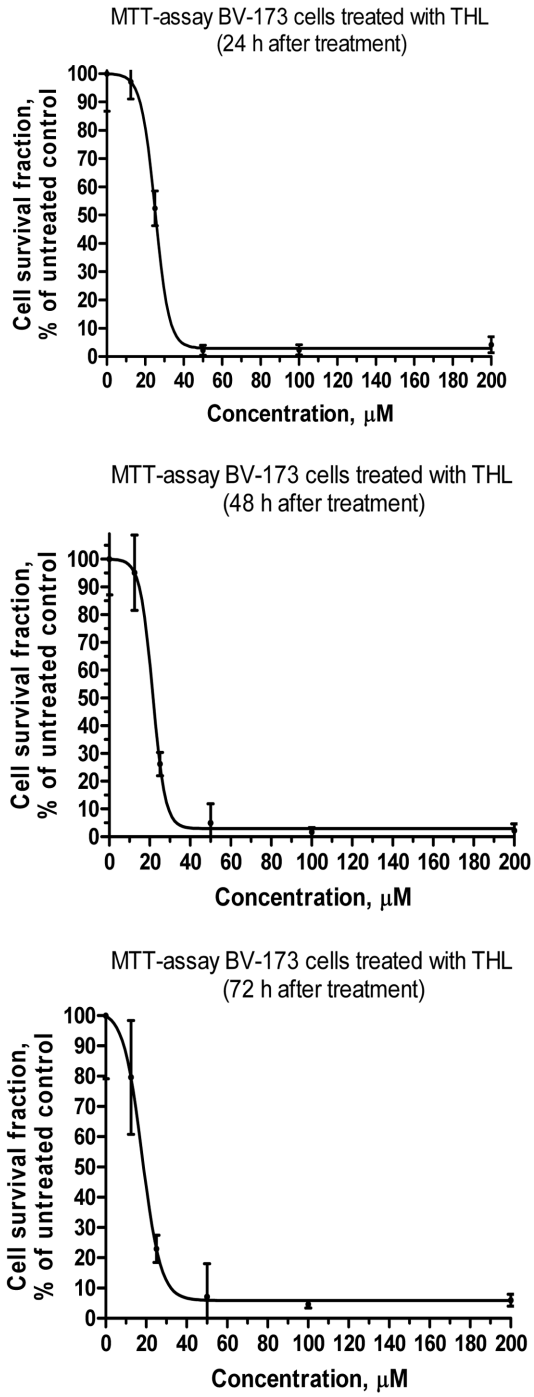

Fig. 5. *In vitro* cytotoxic effect of trehalose tetraester on BV-173 cell viability.
The cells were incubated for 72 h. Values are means ± SD from three experiments.

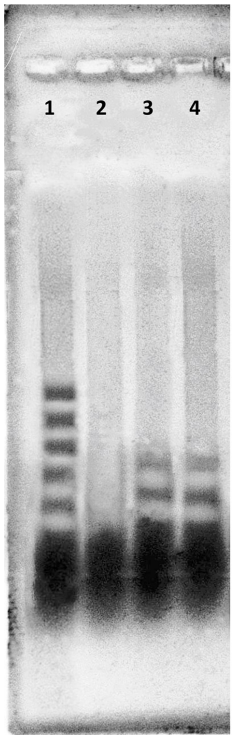

Fig. 6. Proapoptotic effect of THL on BV-173 cells.
Lane 1, marker (DNA ladder); lane 2, control cells; lane 3, THL (25 μM); and lane 4, THL (70 μM).

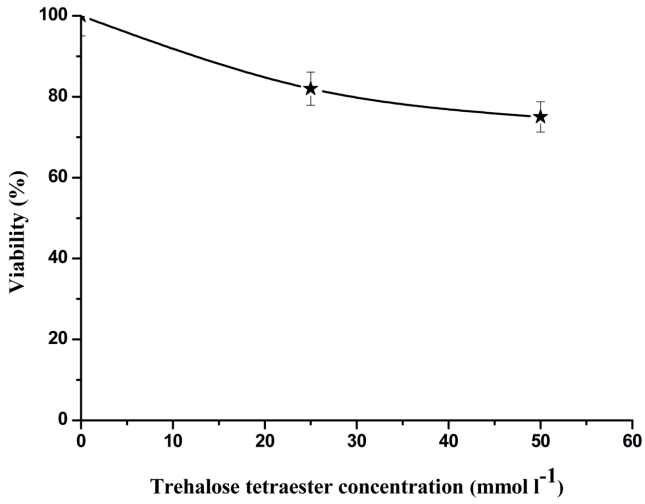

Fig. 7. Viability of healthy lymphocytes after 72 h treatment with 70 μM trehalose biosurfactant produced by *Nocardia farcinica* BN26.

cell death apart from apoptosis, such as autophagy or necrosis. The results obtained indicate that THL is an agent that, at least in part, acts through induction of apoptosis.

Cytotoxic Effect of THL on Human Lymphocytes from Healthy Donor

The viability of lymphocytes from a healthy donor was used as a control for the cytotoxic effect of the studied

compound. Fig. 7 shows the percentage of lymphocyte viability after 72 h treatment with 25 and 70 μM THL. A weak, but statistically significant ($p < 0.05$) reduction of cell viability to 80% was observed after 72 h treatment with the higher concentration of 50 μM THL. In contrast, the IC_{50} for the cell line BV-173 was 18.7 μM.

Discussion

A biosurfactant-producing bacteruim, *Nocardia farcinica* strain BN26, was obtained in this study. The strain was identified as *Nocardia farcinica* on the basis of gram-staining, cellular morphology, acid-fast staining, phenotypic characteristics, and comparative 16S rRNA gene sequencing. It produces a mixture of two glycolipid-type homologs when grown aerobically on *n*-hexadecane. The biosurfactant was extracted and purified from whole cultures and characterized by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. The two major components consisted of α,α-trehalose tetraesters with molecular weights of 876 and 848. Similar chemical structures of trehalose tetraesters were identified in rhodococci and related genera as well [4, 12, 17, 25, 26].

Experimental data show that strain BN26 extensively metabolizes *n*-hexadecane, with a growth curve similar to other hydrocarbon-degrading bacteria [2, 5]. The genera belonging to the mycolates group such as *Nocardia, Rhodococcus,* and *Mycobacterium* possess a particular outer cell wall layer containing mycolic acids, which play an important role in the uptake of hydrophobic compounds and promote considerable cell surface hydrophobicity [1]. During exponential growth in mineral salt medium with hydrocarbons, BN26 cells become increasingly hydrophobic, and high cell adhesion to the substrate could be demonstrated both by BATH assay measurements and by visual inspection of the cultures. Under these conditions, growth was restricted to the hydrocarbon-aqueous interface, where there is direct access to the hydrocarbon. Later, in the phase of linear growth, cells developed more hydrophilic surfaces, most probably due to the cell-bound biosurfactants that expose their hydrophilic moieties towards the water phase, thus allowing cell contact with the hydrophilic outer layer of the hydrocarbon-containing micelles. Similar changes in cell surface hydrophobicity and adhesion tendency during growth on hydrocarbons were observed with a strain of *Gordonia* sp. [5]. These data confirm the suggestion that microorganisms can use their biosurfactants to regulate their cell surface properties in order to attach or detach from surfaces according to need [1].

To date, there are very few studies carried out on the potential use of trehalose lipid biosurfactants as anticancer agents. For example, Isoda *et al*. [7] investigated the biological activities of seven glycolipids, including succinoyl-trehalose lipids STL-1 and STL-3. It was found that STLs possess differentiation-inducing activity in the HL-60 human promyelocytic leukemia cell line, which was attributed to a specific interaction with the plasma membrane instead of a simple detergent-like effect. It was also found that the biological effects of STL-3 and its analogs depended on the structure of the hydrophobic moiety [23]. In other experiments, a trehalose lipid biosurfactant from *Rhodococcus ruber* stimulated interleukin-1β and tumor necrosis factor-α production of human monocytes [11].

The experimental data presented in this study indicate that THL is a biologically active compound with cytotoxic properties against malignant cells. THL causes concentration-dependent and incubation-dependent cytotoxic effects on BV-173 cells, a concentration-dependent cytotoxic effect on SKW-3 cells and, to a lesser extent, on HL-60 cells. The estimated IC_{50} values in the congener sub-line HL-60/Dox suggest that at least in part THL is a substrate for MRP-1 (a second multidrug-resistant protein with clinical significance). Furthermore, our initial experiments confirm that THL from *Nocardia farcinica* strain BN26 induces partial apoptotic laddering in cell line BV-173. A possible explanation for this partial DNA laddering is first, that apoptosis and autophagy are closely interconnected types of programmed cell death, and second, that apoptotic cells in the absence of phagocytosis proceed to secondary necrosis, which has many morphological features of primary necrotic cells. Moreover, necrosis can be recognized as a terminal stage of apoptosis and autophagy [10]. Finally, THL showed weak cytotoxicity to normal human lymphocytes, indicating a lower adverse effect on normal cells. On the basis of the observed differential cytotoxic activity, it can be concluded that THL deserves further detailed pharmacological and toxicological evaluation as an antineoplastic drug candidate.

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