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New Aromatic Nitro Compounds from *Salegentibacter* sp. T436, an Arctic Sea Ice Bacterium: Taxonomy, Fermentation, Isolation and Biological Activities

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Abstract Nineteen aromatic nitro compounds were isolated from the culture broth of an Artic sea ice bacterium. Four of these compounds are new and six compounds are reported from a natural source for the first time. The new natural products showed weak antimicrobial and cytotoxic activities. 2-Nitro-4-(2′-nitroethenyl)-phenol was the most potent antimicrobial and cytotoxic substance. Some of the compounds exhibit plant growth modulating activities. Based on its biochemical properties and the 16S rRNA gene sequence, the producing strain can be described as a distinct species within the genus *Salegentibacter*.

Keywords marine bacteria, aromatic nitro compounds, antibacterial activity, cytotoxic activity, *Salegentibacter*

Introduction

The marine environment represents a rich source of living organisms and concomitant with this biodiversity a high chemical diversity of marine natural products is found. Lately marine bacteria have roused interest due to their unique secondary metabolites which differ significantly from the ones produced by terrestrial counterparts [1, 2]. During our screening for metabolites with antimicrobial activities, *Salegentibacter* sp. T436 was found to produce a large number of aromatic nitro compounds, some of which have antimicrobial and cytotoxic activities.

In this paper we report the taxonomy of strain T436, its fermentation, the isolation and purification of the compounds based on bioactivity-guided fractionation, as well as their biological activities. The physico-chemical and chemical structures will be reported in a separate paper [3].

Materials and Methods

Producing Organism

The *Salegentibacter* strain T436 was derived from a bottom section of a sea ice floe collected from the Arctic Ocean [4] during the cruise of R.V. Polarstern, ARKXIII/2. It has been deposited in the collections of the Alfred-Wegener-Institute and the Institute of Biotechnology and Drug Research (IBWF), Germany.

Taxonomic Studies

Morphological, Biochemical, and Physiological Tests

Morphological studies were carried out using a light microscope and a phase contrast microscope on cultures grown for 3∼4 days at 22°C on modified LB agar medium (50% marine LB agar medium).

Biochemical and physiological characteristics were determined using standard procedures [5, 6]. Production of acid from different carbohydrates, utilization of organic

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compounds as sole carbon or nitrogen sources were determined by the method of Helmke & Weyland [7].

Sequencing and Analysis of 16S rDNA

Extraction of genomic DNA was carried out on 2.0 ml of a well-grown culture in modified LB medium by following the method described by Süßmuth *et al.* [6] with some modifications. For polymerase chain reaction (PCR), 100∼300 ng of extracted DNA was added to 50 μl of a reaction mixture containing 1 U of *Taq* polymerase (MBI Fermentas, St. Leon, Germany), dNTP mixture (400 μM of each type), 1 pmol of primers 16SA (5′-AGAGTTTGA-TCCTGGCTC) and 16SB (5′-AAGGAGGTGATCCAGC-CGCA), 4 mM magnesium chloride, and PCR buffer with ammonium sulfate. The primers were synthesized by MWG-biotech (Ebersberg, Germany). Amplification was done by an initial denaturation period of 3 minutes at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension period of 10 minutes. The PCR product (∼1500 bp) was detected by electrophoresis [8] and purified with a NucleoSpin® extraction kit (Macherey & Nagel, Düren, Germany) following the manufacturer's instructions. The 16S rRNA gene sequence was compared with those available in the GenBank and Ribosomal database project II (RDP).

Fermentation

Salegentibacter sp. T436 was cultured in 500-ml Erlenmeyer flasks containing 250 ml of modified LB medium composed of yeast extract 0.5%, tryptone 0.5%, NaCl 1.0% in half strength artificial sea water, pH 7.2. Half strength artificial sea water was prepared by dissolving 16.7 g of marine salt mixture, purchased from Tropic marine® (Dr. Biener, Wartenberg-Germany), in 1 liter distilled water. The incubation was carried out on a rotary shaker (121 rpm) at 21°C for 48 hours. This culture was used to inoculate a Biolafitte C6 fermentor containing 20 litres of B1-medium (A-Z amine 0.25%, beef extract 3.8%, soy meal 0.1%, yeast extract 0.25%, seaweed extract 0.25% (v/v), marine salts mixture 3.33%, adjusted to pH 8.0). The fermentations were carried out at 21°C with aeration of 3.0 litres/minute and agitation of 121 rpm.

During the fermentation process, daily samples (150∼200 ml) were taken and the culture fluid was separated from the bacterial cells by centrifugation (16000 *g*, 10 minutes). The supernatant was adjusted to pH 4 and extracted with an equal volume of EtOAc. The organic phase was dried over Na_2SO_4, concentrated *in vacuo* at 40°C and the resulting residue was dissolved in MeOH to a final concentration of 10 mg/ml. Aliquots corresponding to 300 μg of the concentrated residue were used for the evaluation of the antifungal and antibacterial activities in the agar diffusion assay using *Nematospora coryli* and *Bacillus brevis* as test organisms.

Analysis of the Fermentation Samples

Growth was monitored by OD measurement at 580 nm (1 : 10 diluted culture fluid) and the increase in colony forming units (cfu). The changes in the metabolite spectrum were monitored by analytical HPLC using 75 μl samples (RP-18, LiChroCart®, 5.0 μm, 125×4 mm; flow: 1.0 ml/minute; gradient: 0.1% H_3PO_4 - MeCN 1∼100% in 20 minutes).

Isolation and Purification of the Compounds

After 115∼137 hours of fermentation in B1 medium, when the OD started to decrease and/or the antimicrobial activity had reached its maximum, the cultures were harvested. The fluid was centrifuged; the supernatant was adjusted to pH 4 and extracted with EtOAc. After drying with Na_2SO_4, the organic phase was concentrated. The combined crude extracts from three 20 litres fermentations (8.97 g) were applied onto a silica gel column (Merck 60, 0.063∼0.2 μm; column 7×18 cm). Elution with cyclohexane - EtOAc (1 : 1) yielded 1.21 g of active fraction. Further purification of this fraction by repeated chromatography on silica gel (column size 2.5×20 cm) gave rise to two active fractions A and B: fraction A (31.2 mg) was eluted with cyclohexane - EtOAc (3 : 1), and fraction B (477 mg) with cyclohexane - EtOAc (1 : 1). Fraction A was purified by preparative HPLC (Hibar RT LiChrosorb RP-18, 7.0 μm: column 25×250 mm, flow 15 ml/minute) with a gradient of a decreasing polarity of 0.1% H_3PO_4 - MeCN as eluent to yield **2a** (4.2 mg, eluted at 48 : 52 v/v). Fraction B upon further purification by Sephadex LH-20 chromatography in MeOH (column size: 3×30 cm) and preparative HPLC (conditions as above) gave rise to **1a** (5.8 mg, eluted at 60 : 40 v/v), **5a** (1.0 mg, eluted at 59 : 41 v/v), **2c** (6.3 mg, eluted at 58 : 42 v/v), **2d** (3.4 mg, eluted at 57 : 43 v/v), **3b** (2.0 mg, eluted at 55 : 45 v/v), **3c** (5.6 mg, eluted at 54 : 46 v/v), a mixture of **1b** and **1c** (8.5 mg, eluted at 49 : 51 v/v), **8** (2.0 mg, eluted at 48 : 52 v/v), **2b** (3.3 mg, eluted at 47 : 53 v/v), **3a** (4.2 mg, eluted at 44 : 56 v/v), **7a** (7.0 mg, eluted at 42 : 58 v/v), and **7b** (1.4 mg, eluted at 40 : 60 v/v). The purity of all isolated compounds as checked by HPLC was >98%.

Fermentation of *Salegentibacter* sp. T436 in B2 medium (B1 medium supplemented with corn steep solids 0.5%) under the same conditions as above yielded 15.4 g oily residue from 50 litres of culture fluid. The residue was applied onto a silica gel column (7×28 cm) and yielded two active fractions: fraction A (2.35 g) eluted with

cyclohexane-EtOAc (3 : 1) and fraction B (1.1 g) eluted with cyclohexane-EtOAc (1 : 1). Chromatography of fraction A on silica gel (column size 5×8.5 cm) afforded three active fractions: fraction A1 (1.29 g) eluted with cyclohexane-EtOAc (3 : 1), fraction A2 (415 mg) eluted with cyclohexane-EtOAc (1 : 1), and fraction A3 (467 mg) eluted with cyclohexane-EtOAc (1 : 3). From fraction A1, **2a** (3.0 mg) and 2-nitro-4-(2′-nitroethenyl)-phenol (**6**, 2.1 mg) were obtained by Sephadex LH-20 chromatography in MeOH (column size 3×80 cm) and preparative HPLC (Hibar RT LiChrosorb RP-18, 7.0 μm: column 25×250 mm, flow 15 ml/minute) with a gradient of a decreasing polarity of 0.1% H_3PO_4-MeCN. Fraction A2 yielded after the same purification steps **7b** (1.4 mg, eluted at 40 : 60 v/v), and **5b** (1.3 mg, eluted at 33 : 67 v/v). Fraction A3 by preparative HPLC yielded **1a** (3.7 mg, eluted at 60 : 40 v/v), **2b** (2.2 mg, eluted at 47 : 53 v/v), and **3b** (1.2 mg, eluted at 55 : 45 v/v).

Fraction B was further purified by Sephadex LH-20 and preparative HPLC to yield **5c** (5.7 mg, eluted at 59 : 41 v/v), **4b** (2.0 mg, eluted at 44 : 56 v/v), and **4a** (1.4 mg, eluted at 32 : 68 v/v).

The purity of all compounds as checked by HPLC was >98%.

Biological Activities

Antimicrobial activities, determined in the serial dilution assay, and inhibition of germination and growth of *Setaria italica* and *Lepidium sativum* were evaluated as described by Anke *et al.* [9]. Inhibition of conidial germination of *Magnaporthe grisea* was tested in 96-well microtiter plates following the method of Kettering *et al.* [10]. Nematicidal activities against *Caenorhabditis elegans* and *Meloidogyne incognita* were determined according to Anke *et al.* [11]. Cytotoxic activity was assayed as described previously [12]. L1210 (mouse lymphocytic leukaemia), Jurkat (human acute T cell leukaemia), and Colo-320 (human colorectal adenocarcinoma) cells were grown in RPMI 1640 medium. MDA-MB-231 (human breast adenocarcinoma), HL 60 (human promyelocytic leukemia) and MCF-7 (human breast adenocarcinoma) cells were grown in DMEM medium. All media contained 10% fetal calf serum (FCS), 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate. The cells were incubated at 37°C in a humidified atmosphere containing 5.0% CO_2.

Inhibition of DNA, RNA, and protein synthesis was tested with *N. coryli* and HL 60 cells as described previously [12, 13]. Inhibition of oxygen uptake (respiration) in *Bacillus subtilis* and *Nematospora coryli* was measured with an oxygen electrode (pO_2 analysator R55, Bachofer, Reutlingen). The test substances dissolved in 10 μl MeOH were added to 10^8~10^9 cells (control 10 μl MeOH).

Results and Discussion

Strain T436 forms yellow-beige colonies on LB-agar. The cells are Gram-negative ovoid rods, 0.9~1.1 (1.4) μm long and 0.7~0.85 μm wide, nonsporogenic, non-motile and aerobic. The strain does not accumulate poly-β-hydroxybutyrate and has no arginine dihydrolase system. It is oxidase and catalase positive but lacks a β-galactosidase activity and can reduce nitrate. It is a psychrotolerant strain. The results of the biochemical and physiological characterization are summarized in Table 1.

16S rRNA gene sequence analysis revealed that strain T436 is very closely related to a number of unidentified Arctic sea-ice isolates assigned to the genus *Salegentibacter* (99% similarity level), a taxon newly generated by McCammon & Bowman [14]. As shown in Fig. 1, in which the phylogenetic tree is depicted, the nearest relative was *S. holothuriorum* (98% similarity level, access no. AB116148). Strain T436 differed from *S. holothuriorum* in the ability to reduce nitrate, its tolerance of high salt concentrations (13%) and the lack of β-galactosidase activity. Strain T436 was unable to utilize carbohydrates and did not grow at 37°C. Therefore, this strain represents a distinct species within the genus *Salegentibacter*.

Data from a typical fermentation scheme of strain T436 are shown in Fig. 2. The OD reached its maximum after 115 hours, which was in agreement with the number of cfu. The antifungal activity against *Nematospora coryli* had reached a plateau after 90 hours. Exhaust gas analysis during the fermentation showed a higher rate of carbon dioxide production in comparison to the oxygen consumed indicating an anaerobic like respiration and the utilization of NO_3^- as an electron acceptor under oxygen limitation (data not shown).

The cultures were harvested after 115 hours. Bioactivity guided fractionation resulted in the isolation of 19 aromatic nitro compounds (Fig. 3). Four of these are new and six were known as synthetic products and are reported here for the first time as natural metabolites (for details see Table 2). All substances were tested for their antimicrobial, nematicidal, phytotoxic, and cytotoxic activities. For comparison the previously reported compounds were also included. The results are given in Tables 3 and 4. **6** showed the highest antimicrobial and cytotoxic activities. Jurakt and L1210 cells were the most sensitive cell lines with an IC_{50} of 10~20 μg/ml. Though already known as a plant

Table 1 Biochemical and physiological characteristics of *Salegentibacter* sp. T436

Characteristic		Characteristic	
NaCl requirement	+	Compound as C- & N-source	
Salinity tolerance	13%	L-Alanine	+
Oxidation/Fermentation		L-Leucine	−
D-Glucose	−/−	L-Proline	+
D-Lactose	−/−	D-Aspartic acid	−
Sucrose	−/−	L-Lysine	−
L-Arabinose	−/−	L-Hystidine	+
D-Xylose	−/−	L-Cysteine	−
Fructose	−/−	L-Asparagine	−
Maltose	−/−	L-Phenylalanine	+
Rhamnose	−/−	L-Glutamine	+
Mannitol	−/−	L-Tyrosine	−
Glycerol	−/−	Hydrolysis of:	
Raffinose	−/−	Tween 40, 80	±
Sorbitol	−/−	Starch	−
D-Galactose	−/−	Gelatine	+
		Esculin	+
Organic compound as C-source		Esterase activity	+
Sodium acetate	+	Arginine decarboxylase	−
D-Glucuronic acid	−	Lysine decarboxylase	−
Succinic acid	−	β-Galactosidase	−
Glycine	−	H_2S production	−
DL-Lactic acid	+	Indole production	−
Oxalic acid	−	Nitrate reduction	+
Sodium glutamate	+	Motility	−
D-Aspartic acid	−	Spore formation	−
Sodium pyruvate	+	Poly-β-hydroxybutyrate	−

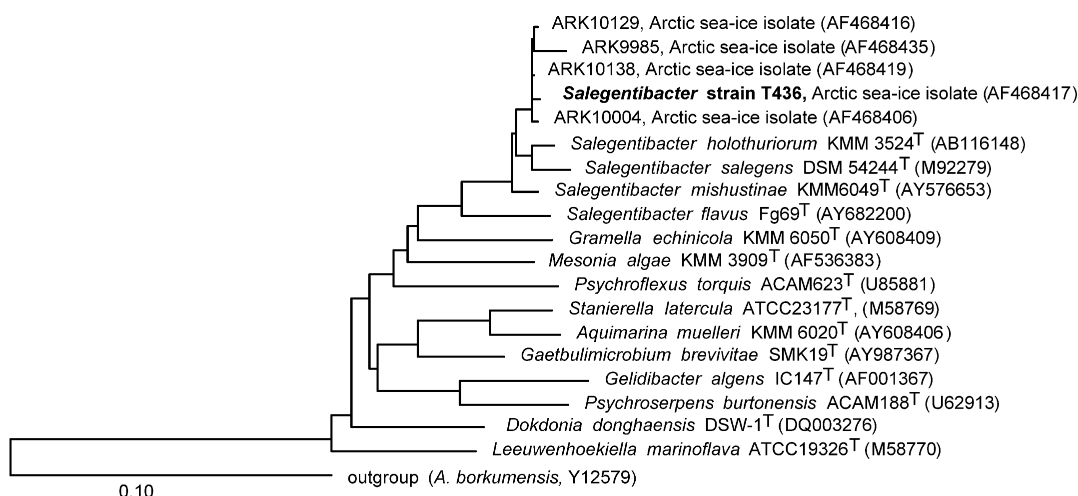

Fig. 1 Phylogenetic tree of strain T436.

Phylogenetic tree reconstructed by using the maximum likelihood algorithm based on the 16S rRNA gene sequence of strain T436 and related members of the *Bacteroidetes* phylum. The scale bar indicates 10% estimated sequence divergence.

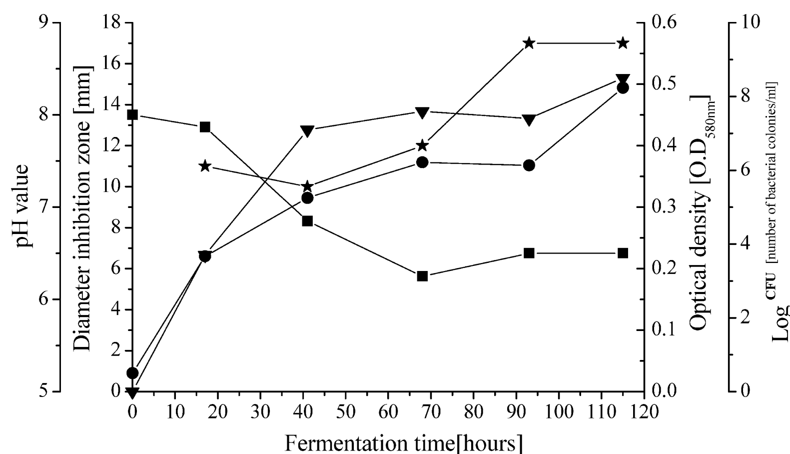

Fig. 2 Fermentation of *Salegentibacter* sp. T436 in 20 liters of B1-medium.
★ Activity against *Nematospora coryli*, ● optical density, ▼ CFU [number of bacterial colonies/ml], ■ pH value.

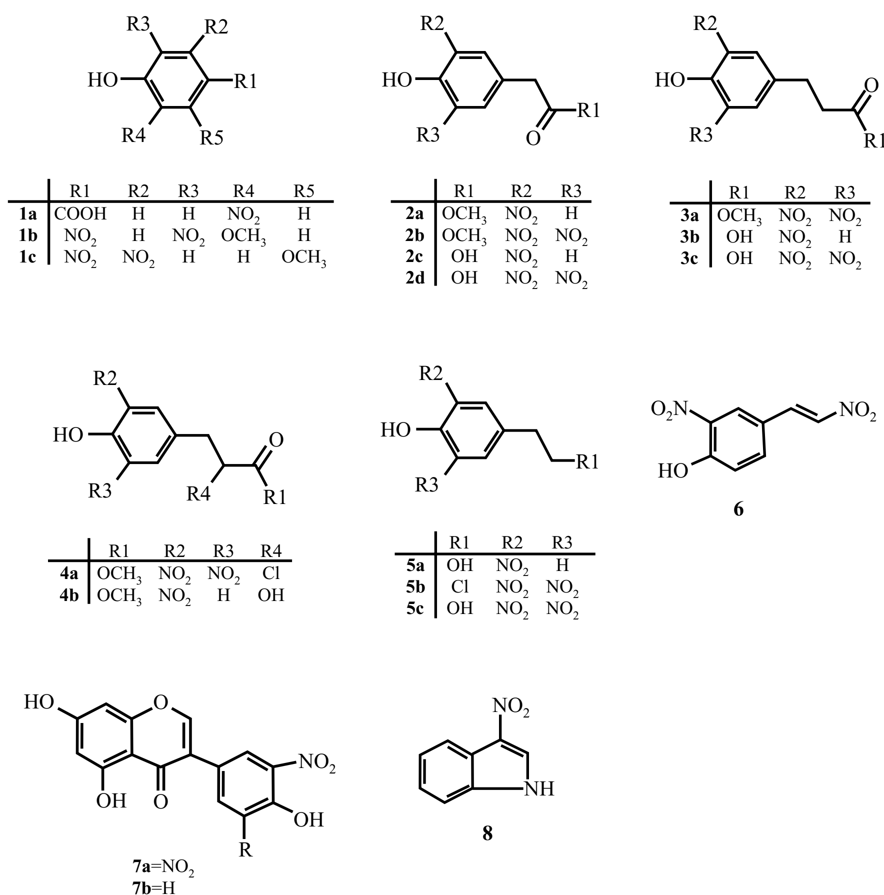

Fig. 3 Structures of the aromatic nitro compounds from *Salegentibacter* sp. T436.

metabolite, this compound has not previously been evaluated for biological activities [15]. The antimicrobial and cytotoxic activities of **6** are probably the result of an inhibition of respiration, for example in *N. coryli*, a 50% inhibition of oxygen consumption was observed at 3.6 μg/ml. This is in accordance with a nonselective inhibition of incorporation of precursors into DNA, RNA and protein. The concentrations for a 50% inhibition were

Table 2 Compounds from *Salegentibacter* sp. T436

Compound	Chemical name	Note[a]	Reference
1a	4-Hydroxy-3-nitrobenzoic acid	S	30
1b	4,6-Dinitroguiacol	K	28
1c	4,5-Dinitro-3-methoxyphenol	K	28
2a	(4-Hydroxy-3-nitrophenyl)-acetic acid methyl ester	K	27
2b	(4-Hydroxy-3,5-dinitrophenyl)-acetic acid methyl ester	S	Commercial
2c	(4-Hydroxy-3-nitrophenyl)-acetic acid	K	29
2d	(4-Hydroxy-3,5-dinitrophenyl)-acetic acid	S	Commercial
3a	(4-Hydroxy-3,5-dinitrophenyl)-propionic acid methyl ester	N	—
3b	(4-Hydroxy-3-nitrophenyl)-propionic acid	K	29
3c	(4-Hydroxy-3,5-dinitrophenyl)-propionic acid	S	Commercial
4a	2-Chloro-3-(4-hydroxy-3,5-dinitrophenyl)-propionic acid methyl ester	N	—
4b	2-Hydroxy-3-(4-hydroxy-3-nitrophenyl)-propionic acid methyl ester	N	—
5a	2-(4-Hydroxy-3-nitrophenyl)-ethanol	K	27
5b	2-(4-Hydroxy-3,5-dinitrophenyl)-ethyl chloride	N	—
5c	2-(4-Hydroxy-3,5-dinitrophenyl)-ethanol	S	Commercial
6	2-Nitro-4-(2′-nitroethenyl)-phenol	K	15
7a	3′,5′-Dinitrogenistein	K	26
7b	3′-Nitrogenistein	K	26
8	3-Nitro-1*H*-indole	S	31

[a] S: known as synthetic compound (not from natural source), K: known as natural substance, N: new compound.

Table 3 Antimicrobial activity of metabolites from *Salegentibacter* sp. T436 in the serial dilution assay

Organisms	Compound								
	1a	**2a**	**2b**	**3a**	**3c**	**6**	**5b**	**5c**	**8**
	MIC [μg/ml]								
Fungi:									
Candida albicans ATCC 90028	—[a]	—	—	—	—	20	—	—	—
Paecilomyces variotii ETH 114646	—	—	—	—	—	50	—	—	—
Penicillium notatum[b]	—	100	—	—	—	25	—	—	—
Mucor miehei Tü 284	—	100	—	—	—	12.5	100	100	—
Magnaporthe grisea 70-15	50	—	25	25	10	5	25	—	—
Nematospora coryli ATCC 10647	100	—	—	100	100	0.5～1	50	—	100
Ustilago nuda CBS 118.19	—	—	—	—	—	6～7	—	—	100
Bacteria:									
Bacillus brevis ATCC 9999	—	100	—	100	—	12.5	50	100	100
Bacillus subtilis ATCC 6633	—	100	100	100	—	12.5	50	100	100
Micrococcus luteus ATCC 381	—	100	—	100	100	12.5	50	100	100
Escherichia coli K12[b]	—	—	—	—	100	12.5	—	—	—
Proteus vulgaris DSM 30119	—	—	—	—	—	20	—	—	—

[a] —: not active at 100 μg/ml, [b] strains from the collection of the IBWF.

Table 4 Cytotoxic activities of the metabolites from *Salegentibacter* sp. T436

Cell line	Compound						
	2c	**3a**	**3c**	**5b**	**6**	**7b**	**8**
				IC_{50} [μg/ml]			
L1210 ATCC CCL 219	100	100	>100	80	20	50	>100
Jurkat DSMZ ACC 282	>100	>100	60	>100	10	60	>100
MDA-MB-321 ATCC HTB-26	>100	>100	>100	>100	30	>100	100
MCF-7 DSMZ ACC 115	>100	>100	>100	>100	>100	>100	>100
Colo-320 DSMZ ACC 144	>100	>100	>100	80	17	>100	>100

2.8 μg/ml for DNA and RNA and 3.4 μg/ml for protein synthesis.

The structurally closely related β-nitrostyrene SL-1, isolated from *Streptomyces lavendulae* [16] showed moderate antibacterial activity, and antifungal activity restricted towards some *Trichophyton* species (MIC 12.5~25 μg/ml) [17]. Moreover, SL-1 was cytotoxic for L1210 cells with IC_{50} value of 1.0 μg/ml. Comparison of the biological activities of **6** with those of SL-1 indicates that presence of a second nitro group on the phenyl ring enhances the antimicrobial activity but reduces its cytotoxic effect.

The novel compounds **4a** and **4b** were not active in any test system up to 100 μg/ml. Compounds **3a** and **5b** showed only very weak antimicrobial activities. Among the isolated compounds, only the mixture of **1b** and **1c** showed moderate nematicidal activity against *M. incognita* with a LD_{90} value of 25 μg/ml.

Most of the nitro-aromatic compounds were phytotoxic starting at 70 μg/ml. Although **1a** was phytotoxic at a higher concentration (300 μg/ml), it promoted the growth of roots and shoots at lower concentration but inhibited the development of chloroplasts. Similar results were also obtained during a previous study on nitro-benzoate derivatives [18]. The presence of a nitro group in 3-position and a hydroxyl group in 4-position was essential for the phytotoxic activity of these compounds regardless of the side chain in the 1-position [18]. Our tests revealed that the presence of a second nitro group on the aromatic ring decreases the phytotoxic activities (data not shown).

A number of antibiotics bearing nitro groups were reported from bacteria, such as chloramphenicol [19] and pyrrolnitrin [20]. Aromatic nitro compounds are rare structural elements in nature [21] and their synthesis is not fully understood. Several biosynthetic pathways were proposed. One possibility is the direct nitration of aromatic compounds in the presence of KNO_3 in the medium [22] or by enzymatic oxidation of the amino groups [23]. Nitration can also be the result of peroxynitrite dependent or independent pathways [24, 25].

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