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X-ray crystallographic, FT-IR and NMR studies as well as anticancer and antibacterial activity of the salt formed between ionophore antibiotic Lasalocid acid and amines

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HIGHLIGHTS

- Ionophore antibiotic complexes with aromatic and aliphatic amines were obtained.
- Antimicrobial tests demonstrated activity of all compounds studied against Gram-positive bacteria.
- Lasalocid acid and its complexes can be recognized as potential anticancer drug candidates.
- A pseudo-cyclic structure was found to be strictly requested for cytotoxic activities.

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ABSTRACT

Two new complexes of the ionophore antibiotic Lasalocid acid (LAS) with phenylamine (PhA) and butylamine (BuA) were synthesized and their molecular structures were studied using single crystal X-ray diffraction and spectroscopic methods. In the solid state both amines are protonated and all (NH_3^+) protons are hydrogen bonded to etheric, hydroxyl and carboxylic oxygen atoms of the LAS anion. In chloroform solutions the structure observed in the crystal of LAS–BuA complex is preserved and an equilibrium between the LAS–PhA complex and dissociated Lasalocid acid and phenylamine is observed. *In vitro* antimicrobial tests of the complexes showed a significant activity towards some strains of Gram-positive bacteria. For the first time Lasalocid acid and its complexes with amines were tested *in vitro* for cytotoxic activity against human cancer cell lines: A-549 (lung), MCF-7 (breast), HT-29 (colon) and mouse cancer cell line P-388 (leukemia). We found that LAS and its complexes are strong cytotoxic agents towards all tested cell lines. The cytostatic activity of the compounds studied is greater than that of cisplatin, indicating that Lasalocid and its complexes are promising candidates for new anticancer drugs.

1. Introduction

Natural products have proven to be the most reliable single source of new and effective anticancer agents. Nearly 60% of anticancer and anti-infective agents that are commercially available or in the late stages of clinical trials originate from natural products [1]. Available literature data suggest that the utility of natural products as sources of novel structures, but not necessarily the final drug entity, is still topical [2,3]. Thus, in the field of anticancer drugs, in the time range from the 1940s to date, over 70% of the agents are other than synthetic, with almost 50% actually being either natural products or directly derived from them [4].

Lasalocid acid is a representative of naturally occurring polyether ionophore antibiotics [5,6]. Ionophores are lipophilic chelating agents that transport cations across lipid bilayer membranes, such as the plasma and subcellular membranes of cells. This unregulated membrane transports of various ions leads to mitochondrial injury and cell swelling, vacuolisation, and finally death [5–7]. To date, over 120 polyether ionophore structures have been characterized [8]. Ionophore antibiotics show a broad spectrum of bioactivity ranging from antibacterial, antifungal, antiparasitic, antiviral, and recently discovered anti-tumour cell cytotoxicity [9]. The discovery of strong anticancer properties of one of them

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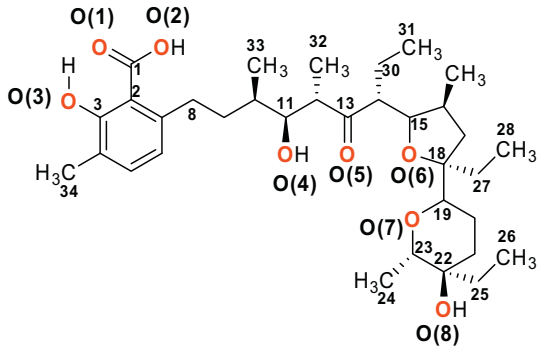

Scheme 1. Structure of Lasalocid acid (LAS).

– salinomycin has received much attention in recent years [10–15]. It has been shown that salinomycin is 100 times more effective against breast cancer stem-like cells than Taxol (paclitaxel), a commonly chemo-therapeutic drug used against breast cancer [15]. After discovery of the anticancer properties of salinomycin, which has been so far the top candidate for a chemotherapeutic agent among the ionophore antibiotics, other ionophore antibiotics have been more thoroughly explored [14]. It has been also demonstrated that several candidates for chemotherapeutic drugs can be found among other naturally occurring ionophore antibiotics such as monensin A, inostamycin and nigericin [9,16–20].

Lasalocid acid (Scheme 1), isolated from *Streptomyces lasaliensis*, is able to form pseudomacrocyclic complexes with monovalent and divalent cations and to transport these cations across cell membranes [21]. It is commercially used as a coccidiostat for poultry and as growth promoter for ruminants [22].

In previous publications, we have shown that Lasalocid acid forms complexes with allylamine [23] and tetramethylguanidine [24]. The first complex shows higher antibacterial activity than pure Lasalocid and the second complex has antibacterial activity comparable to that of Lasalocid. As an extension of these studies we synthesized and investigated two new hydrogen-bonded complexes of Lasalocid acid (LAS) with aromatic amine (phenylamine, PhA) and aliphatic amine (butylamine, BuA) by X-ray and FT-IR, and NMR spectroscopy. We determined the antimicrobial and cytotoxic activity of the Lasalocid complexes for the first time and compared them to the respective activity of uncomplexed Lasalocid.

2. Experimental

2.1. General

Lasalocid sodium salt was extracted from Avatec. Phenylamine (PhA) and *n*-butylamine (BuA) and solvents were obtained from Sigma–Aldrich or Fluka and used without any further purification.

2.2. Preparation of Lasalocid–Phenylamine (LAS–PhA) and Lasalocid–Butylamine (LAS–BuA) complexes

Lasalocid sodium salt (1.0 g) was dissolved in dichloromethane (150 ml) and stirred vigorously with a layer of diluted aqueous sulphuric acid (pH = 1.5) (100 ml). The organic layer containing Lasalocid acid (LAS) was washed three times with distilled water. Subsequently dichloromethane was evaporated under reduced pressure to dryness giving LAS (0.75 g).

The crystals of 1:1 complex of LAS–PhA and 1:1 complex of LAS–BuA were obtained by crystallization from dried acetonitrile solution using a 1:1 molar ratio of LAS (50 mg) and respective amine. Mp = 208–210 °C (LAS–PhA) and 180–182 °C (LAS–BuA).

2.3. X-ray measurements

The crystals of both complexes selected for single-crystal X-ray diffraction measurements formed colourless parallelepipeds with well-developed faces. They were stable under normal conditions and their X-ray diffraction measurements were carried out on an Oxford Diffraction Super Nova diffractometer (Oxford Diffraction, Abingdon, UK) using CuKα radiation at room temperature. Their structures were solved by direct methods with SHELXS-97 and refined by full-matrix least-squares with SHELXL-97 [25]. Nine H-atoms at N(1) in LAS–PhA and LAS–BuA complexes and at O(3), O(4) and O(8) in LAS–BuA complex were located from the difference Fourier maps and refined with isotropic temperature factors. All other H-atoms were located from the molecular geometry (C—H 0.93–0.98 and O—H 0.82 Å) and their U_{iso}'s were related to the thermal vibrations of their carriers. Selected details about the crystal structure, experiment, structure solution and refinement are given in Table 1.

2.4. Spectroscopic measurements

The ^{1}H and ^{13}C NMR spectra of LASA, LAS–PhA and LAS–BuA (0.1 mol dm^{-3}) were recorded in $CDCl_3$ solutions using Bruker Avance 600 MHz spectrometer. All spectra were locked to deuterium resonance of $CDCl_3$. The ^{1}H NMR measurements were carried out at the operating frequency 600.0018 MHz and the ^{13}C NMR spectra at the operating frequency 150.885 MHz. The temperature 298.0 K and TMS as the internal standard were used in both cases. No window function or zero filling was used. The errors of the ^{1}H and ^{13}C NMR chemical shift values were 0.01 ppm and 0.1 ppm, respectively. The ^{1}H and ^{13}C NMR signals were assigned using 2-D (COSY, HETCOR, NOESY and HMBC) whose examples are shown in the Supplementary Materials. 2-D spectra were recorded using standard pulse sequences from Bruker pulse-sequence libraries.

The mid infrared region the FT-IR spectra of LAS, LAS–PhA complex and LAS–BuA complex were recorded in chloroform solution (0.1 mol dm^{-3}) and the FT-IR spectra of LAS–PhA and LAS–BuA crystals were recorded in nujol and fluorolube mulls. For solutions, a cell with Si windows and wedge-shaped layers was used to avoid interferences (mean layer thickness 170 μm). The spectra were taken with an IFS 113v FT-IR spectrophotometer (Bruker, Karlsruhe) equipped with a DTGS detector; resolution 2 cm^{-1}, NSS = 64. The Happ–Genzel apodization function was used.

The ESI-MS spectra were obtained on a Waters/Micromass (Manchester, UK) ZQ2000 mass spectrometer (single quadrupole type instrument, Z-spray, software MassLynx V3.5, Manchester, UK). The sample solutions were prepared in chloroform (concentration 5×10^{-5} mol/dm^3). The solutions of the sample were infused into the ESI source using a Harvard pump, the flow rate of 80 dm^3/min. The ESI source potentials were capillary 3 kV, lens 0.5 kV, extractor 4 V and cone voltage 10 V. The source temperature was 120 °C and the dessolvation temperature was 150 °C. Nitrogen was used as the nebulizing and dessolvating gas at the flow-rates of 100 and 300 dm^3 h^{-1}, respectively.

2.5. Antimicrobial activity

Micro-organisms used in this study were as follows: Gram-positive cocci: *Staphylococcus aureus* NCTC 4163, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Enterococcus*

Table 1
Crystal data and structure refinement for LAS–PhA and LAS–BuA.

	LAS–PhA	LAS–BuA
Empirical formula	$C_{40}H_{61}NO_8$	$C_{38}H_{65}NO_8$
Formula weight	683.90	663.91
Temperature (K)	293(2)	293(2)
Wavelength	1.54178 Å	1.54178 Å
Crystal system, space group	Orthorhombic, $P2_12_12_1$,	Orthorhombic, $P2_12_12_1$
Unit cell dimensions	a = 10.6585(1) Å	a = 10.1671(1) Å
	b = 18.9220(2) Å	b = 18.7745(1) Å
	c = 19.3361(2) Å	c = 20.4549(1) Å
Volume	3899.71(7) Å^3	3904.48(3) Å^3
Z,	4	4
Calculated density	1.165 g cm^{-3}	1.129 g cm^{-3}
Absorption coefficient	0.640 mm^{-1}	0.621 mm^{-1}
F(000)	1488	1456
Crystal size	0.25 × 0.20 × 0.10 mm	0.25 × 0.20 × 0.15 mm
θ Range for data collection	3.27–76.49°	3.19–73.82°
Limiting indices	$-13 \leqslant h \leqslant 13$, $-23 \leqslant k \leqslant 22$, $-24 \leqslant l \leqslant 24$	$-12 \leqslant h \leqslant 9$, $-23 \leqslant k \leqslant 23$, $-25 \leqslant l \leqslant 25$
Reflections collected/unique	61008/8122 R_{int} = 0.0178	30662/7816 R_{int} = 0.0121
Completeness to θ = 26.63	99.5%	99.3%
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	8122/0/454	7816/0/447
Goodness-of-fit on F^2	1.092	1.005
Final R indices [$I > 2\sigma(I)$]	R_1 = 0.0344, wR_2 = 0.1045	R_1 = 0.0421, wR_2 = 0.1375
R indices (all data)	R_1 = 0.0365, wR_2 = 0.1062	R_1 = 0.0433, wR_2 = 0.1406
Absolute structure parameter	−2.2(6)	0.09(15)
Largest diff. peak and hole	0.173 and −0.120 eÅ^{-3}	0.431 and −0.213 eÅ^{-3}

Table 2
Dimensions of the hydrogen bond (Å and °).

	D—H···A	d(D—H)	d(H···A)	d(D···A)	<(DHA)
LAS–PhA	O(3)—H(3)···O(1)	0.82	1.72	2.448(2)	148
	O(8)—H(8)···O(1)	0.82	1.88	2.674(2)	163
	O(4)—H(4)···O(2)	0.82	1.96	2.740(2)	159
	N(1)—H(2 N)···O(8)	0.91(2)	1.88(2)	2.745(2)	160(2)
	N(1)—H(1 N)···O(2)	0.90(2)	1.94(2)	2.820(2)	168(2)
	N(1)—H(3 N)···O(6)	0.85(2)	2.13(2)	2.935(2)	159(2)
	N(1)—H(3 N)···O(5)	0.85(2)	2.56(2)	3.043(2)	118(2)
	N(1)—H(2 N)···O(7)	0.91(2)	2.50(2)	3.089(1)	123(1)
LAS–BuA	O(3)—H(3)···O(1)	1.08(4)	1.39(4)	2.460(2)	165
	O(8)—H(8)···O(1)	0.90(3)	2.03(3)	2.717(2)	132
	O(4)—H(4)···O(2)	1.00(3)	1.78(3)	2.707(2)	153
	N(1)—H(1 N)···O(8)	0.95(3)	1.96(2)	2.784(2)	144
	N(1)—H(3 N)···O(2)	0.96(4)	1.86(4)	2.813(2)	168
	N(1)—H(2 N)···O(6)	0.97(3)	2.08(3)	2.954(2)	150

hirae ATCC 10541, *Micrococcus luteus* ATCC 9341, *Micrococcus luteus* ATCC 10240; Gram-negative rods: *Escherichia coli* ATCC 10538, *Escherichia coli* ATCC 25922, *Escherichia coli* NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* NCTC 6749, *Pseudomonas aeruginosa* ATCC 27863, *Bordetella bronchiseptica* ATCC 4617 and yeasts: *Candida albicans* ATCC 10231, *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019.

Antimicrobial activity was examined by the disc-diffusion method under standard conditions using Mueller-Hinton II agar medium (Becton Dickinson) for bacteria and RPMI agar with 2% glucose (Sigma) according to CLSI (previously NCCLS) guidelines [26].

Sterile filter paper discs (9 mm diameter, Whatman No. 3 chromatography paper) were dripped with tested compound solutions (in MeOH or MeOH/DMSO 1:1) to load 400 μg of a given compound per disc. Dry discs were placed on the surface of appropriate agar medium. The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35 °C. Compounds with recognized activity in disc-diffusion tests were examined by the agar dilution method to determine their MIC – Minimal Inhibitory Concentration (CLSI) [27]. Concentrations of the agents tested in solid medium ranged from 3.125 to 400 μg/ml. The final inoculum of all studied organisms was 10^4 CFU ml^{-1} (colony forming units per ml). Minimal inhibitory concentrations were read after 18 h of incubation at 35 °C. The data on the antimicrobial activity of the compounds are summarized in Table 4.

2.6. Cytotoxic activity

2.6.1. Cell lines

To evaluate antiproliferative activity of Lasalocid acid and its complexes, six different cell lines were used: A-549 (human lung adenocarcinoma), MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), P-388 (murine leukemia), HLMEC (human lung microvascular endothelial cells) and BALB/3T3 (murine embryonic fibroblast cell line). The cell lines were obtained from the American Type Culture Collection (ATCC) and are/were maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Twenty four hours before adding the compounds tested, the cells were seeded in 96-well plates (Sarstedt, Germany) at a density of 10^4 cells per well (for HLMEC 10^3 cells per well) and cultured in RPMI 1640 medium (HLMEC, P388) (IIET, Wroclaw), OptiMEM + RPMI 1640 (1:1) medium (A-549, HT-29) (OptiMEM from Gibco), Dulbecco medium (BALB/3T3) or Eagle medium (MCF-7) (IIET, Wroclaw). RPMI medium was supplemented with 10% foetal bovine serum (HyClone, UK), 2 mM L-glutamine (HLMEC, P388) and 1 mM sodium puryvate (P388) (Sigma–Aldrich, Germany). OptiMEM + RPMI medium was supplemented with 5% foetal bovine serum, 2 mM L-glutamine (A549, HT-29) (PAA, Austria) and 1 mM sodium puryvate (HT-29). Eagle medium for MCF-7 was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, aminoacids and insulin (Sigma–Aldrich, Germany). Dulbecco medium was supplemented with 10% foetal bovine serum (PAA, Austria), 4 mM L-glutamine and glucose 4,5 g/l (Sigma–Aldrich, Germany). All media contained antibiotics: 100 μg/ml streptomycin and 100 U/ml penicillin (Polfa–Tarchomin). During entire experiment all cell lines were incubated at 37 °C in a humid atmosphere saturated with 5% CO_2.

Table 3
The most important ^{1}H and ^{13}C NMR chemical shifts δ (ppm) of LAS–PhA, and LAS–BuA complexes in $CDCl_3$ and differences Δ (ppm) between shifts and respective chemical shifts for LAS in $CDCl_3$.

	LAS		LAS–BuA		LAS–PhA					
No. atoms	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	Δ_1 ^{1}H	Δ_1 ^{13}C	Δ_2 ^{1}H	Δ_2 ^{13}C
1	–	173.2	–	175.4	–	174.4	–	2.2	–	1.2
2	–	111.0	–	115.5	–	113.1	–	4.5	–	2.1
3	–	161.6	–	161.6	–	161.6	–	0.0	–	0.0
4	–	124.2	–	123.6	–	123.8	–	−0.6	–	−0.4
5	7.17	134.5	7.05	132.0	7.15	132.1	−0.12	−2.5	−0.02	−2.4
6	6.63	121.4	6.51	120.7	6.61	119.7	−0.12	−0.7	−0.02	−1.7
7	–	144.1	–	144.2	–	144.2	–	0.1	–	0.1
11	4.08	73.0	4.30	71.7	4.26	71.9	0.22	−1.3	0.18	−1.1
12	2.84	48.8	2.76	48.7	2.79	48.8	−0.08	0.1	−0.05	0.1
13	–	214.4	–	217.8	–	216.3	–	3.4	–	1.9
14	2.62	55.0	2.48	55.9	2.53	55.4	−0.14	0.9	−0.09	0.4
15	3.88	83.8	4.05	83.1	3.88	83.4	0.17	−0.7	0.00	−0.4
18	–	86.2	–	87.2	–	86.9	–	1	–	0.7
19	3.50	70.7	3.43	70.2	3.45	70.3	−0.07	−0.5	−0.05	−0.4
22	–	72.6	–	71.1	–	71.4	–	−1.5	–	−1.2
23	3.93	76.0	3.82	76.8	3.95	76.4	−0.11	0.8	0.02	0.4
35	–	–	3.02	39.3	–	133.6	–	–	–	–
O(1)H	6.14_{br}	–	–	–	–	–	–	–	–	–
O(3)H	11.84	–	14.90	–	11.95	–	3.06		0.11	
O(4)H	6.14_{br}	–	4.87_{br}	–	3.80_{vbr}	–	−1.27	–	−2.34	–
O(8)H	6.14_{br}	–	4.07	–	3.80_{vbr}	–	−2.07	–	−2.34	–
(NH_3^+)	–	–	7.35	–	9.34	–	–	–	−0.02	–
NH_2	–	–	–	–	3.80_{vbr}	–	–	–	–	–

Δ_1 = δLAS–BuA − δLAS; Δ_2 = δLAS–PhA − δLAS.
br – broad signal; vbr – very broad signal.

Table 4
Antimicrobial activity of Lasalocid complexes: LAS–PhA, LAS–BuA and Lasalocid acid (LAS), diameter of the growth inhibition zone (Giz, mm) and Minimal Inhibitory Concentration (MIC, μg/ml).

	LAS		LAS–PhA		LAS–BuA	
	Giz	MIC	Giz	MIC	Giz	MIC
S. aureus NCTC 4163	30	4	28	4	25	4
S. aureus ATCC 25923	29	4	29	2	27	4
S. aureus ATCC 6538	24	4	23	2	21	4
S. aureus ATCC 29213	24	4	23	2	22	4
S. epidermidis ATCC 12228	28	4	26	4	26	8
B. subtilis ATCC 6633	31	2	28	2	26	2
B. cereus ATCC 11778	29	2	30	2	30	4
E. hirae ATCC 10541	20	8	21	4	20	8
E. faecalis ATCC 29212	25	4	25	2	25	4
M. luteus ATCC 9341	28	4	27	2	26	4
M. luteus ATCC 10240	31	4	31	2	29	4

2.6.2. The antiproliferative assays in vitro

The antiproliferative activity of all compounds tested was evaluated after 72 h exposure of cultured cells to four different concentrations (in the range 100 μg/ml–0.1 μg/ml) of each agent and to reference drug cisplatin at similar concentrations in a positive control of the test. Additionally, the cells were exposed to DMSO (Sigma–Aldrich, Germany), solvent used to dissolve the compounds tested, at the same concentrations as it was present in examined agents' concentrations.

Each compound at every concentration was tested in triplicates in a single experiment, and each experiment was repeated at least three times.

2.6.3. MTT assay

After 72 h of incubation of the agents tested with leukaemia cells, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (Sigma–Aldrich, Germany) were added to each well and cells were incubated for (another) 4 h at 37 °C as described above. Viable cells metabolize yellow MTT to navy blue formazan, so that the more viable cells in the well the more formazan is produced. Then, 80 μl of the lysing mixture, consisting of 225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate (both from Sigma–Aldrich, Germany) and 275 ml of distilled water, were added to each well. After 24 h, when the formazan crystals had been dissolved, optical densities of all solutions were read on the Multiskan RC photometer (Labsystems) at 570 nm wavelength.

2.6.4. SRB assay

Adherent cells cultured in 96-well plates were fixed by gently layering of 50% cold trichloroacetic acid TCA (POCH, Poland) on the top of culture medium and incubated at 4 °C for 1 h. All wells were washed then four times with water. Then, fixed cells were stained with 0.4% sulforodamine B solution (Sigma–Aldrich, Germany) in 1% acetic acid (POCH, Poland) for 0.5 h and then each well was washed with 1% acetic acid and protein bound dye was

Table 5
Cytotoxicity of Lasalocid acid (LAS) and its complexes with phenylamine (LAS–PhA) and *n*-butylamine (LAS–BuA) against various cell lines.

Compound	IC_{50} (μg/ml)					
	A-549	MCF-7	HT-29	HLMEC	BALB/3T3	P388
LAS	1.35 ± 0.25	4.51 ± 1.83	2.99 ± 1.07	0.45 ± 0.20	5,01 ± 1,88	2.13 ± 0.90
LAS–BuA	1.32 ± 0.10	4.17 ± 0.61	3.06 ± 0.55	0.37 ± 0.02	4,57 ± 1,17	2.14 ± 0.86
LAS–PhA	1.36 ± 0.12	4.08 ± 0.64	3.12 ± 0.54	0.39 ± 0.06	5,12 ± 1,02	2.72 ± 0.46
Cisplatin	3.35 ± 0.30	3.71 ± 0.62	11.18 ± 1.98	1.95 ± 0.13	1,81 ± 0,67	2.98 ± 0.08

Values are mean values ± SD measured using SRB or MTT assay from at least three independent experiments.

extracted with 10 mM TRIS base (Sigma–Aldrich, Germany) for optical density reading of each well in Multiskan RC photometer (Labsystems, Helsinki, Finland) at 540 nm wavelength.

The results are presented as the IC_{50} values: the concentration of the agent tested which inhibits proliferation of 50% of cells population. IC_{50} values were calculated in Cheburator 0.4, Dmitry Nevozhay software for each experiment and the results are presented as a mean IC_{50} ± standard deviation calculated from each experiments performed. The results are summarized in Table 5.

3. Results and discussion

3.1. Crystal structure of the Lasalocid–Phenylamine (LAS–PhA) and Lasalocid–Butylamine (LAS–BuA) complexes

The crystals of Lasalocid–Phenylamine (LAS–PhA) and Lasalocid–Butylamine (LAS–BuA) complexes are isostructural. Both complexes crystallise in the same space group and the crystals have very similar unit-cell dimensions (Table 1). The unit-cell dimension *a* is longer by about 0.5 Å in LAS–PhA, but parameter *c* is more than 1 Å longer in LAS–BuA, and the unit-cell volumes of both compounds differ in only 5 $Å^3$. It is characteristic that LAS anions assume very similar pseudo-cyclic conformations, with the same system of hydrogen bonds O—H···O, as illustrated in Fig. 1 and in Table 2. The shortest of O—H···O bonds links hydroxyl group O(3)—H and carboxylate oxygen O(1) (Table 2). The other two O(8)—H···O(1) and O(4)—H···O(2) intramolecular hydrogen bonds, are similar in length in LAS–PhA and in LAS–BuA complexes, too. Likewise, the complexes host the PhA and BuA cations in a similar manner (see Table S1, Supplementary material). They are included in the void inside the pseudo-cyclic structure, with practically identical position of the nitrogen atom. The location of the N-atoms is fixed by intermolecular N—H···O hydrogen bonds to LAS oxygen atoms O(2), O(6) and O(8) (Fig. 1 and Table 2). These host-guest N—H···O hydrogen bonds additionally stabilize the LAS conformation. The crystal packing of LAS–PhA and LAS–BuA is similar (Fig. 2), and dominated by van der Waals interactions.

3.2. Spectroscopic studies

In Fig. 3 the FT-IR spectra of crystalline Lasalocid acid (dashed line) and its crystalline complexes with phenylamine (LAS–PhA) (solid line) and butylamine (LAS–BuA) (dashed-dotted line) are compared. We observe clear differences between these structures, especially regarding the formation of hydrogen bonds (Fig. 3b).

According to the X-ray data, in the spectrum of the LAS–PhA and LAS–BuA complexes, the bands labelled in Fig. 3b should be assigned to the inter- and intramolecular hydrogen bonds of different strength that exist within the structure of these complexes. The hydrogen bonds and their parameters are given in Table 2. The strongest hydrogen bond observed in both complexes is formed between the O(3)—H group and the O(1) atom of the carboxylic group, in LAS–BuA it is more symmetrical than in the LAS–PhA structure. These intramolecular hydrogen bonds belong to the so called quasi-aromatic hydrogen bonds [28] characterized by very low intensity and limited shape of the band assigned to the stretching protonic vibrations occurring usually in the region ca. 2200–3000 cm^{-1} [29,30]. Thus, the broad band with maxima at 2595 cm^{-1} and 2310 cm^{-1} should be assigned to the $\nu(NH_3^+)$ vibrations of the protonated and hydrogen bonded amine group.

According to the crystal data (Table 2) in the spectrum of the LAS–PhA complex, the bands with maxima at 3394 cm^{-1} and 3062 cm^{-1} are assigned to O(4)—H···$O(2)^-$ and O(8)—H···$O(1)^-$ intramolecular hydrogen bonds, respectively.

In the spectrum of LAS–BuA complex, the bands with maxima at 3379 cm^{-1} and 3166 cm^{-1} can be assigned to O(8)—H···$O(1)^-$ and O(4)—H···$O(2)^-$ intramolecular hydrogen bonds on the basis of the hydrogen bonds parameters collected in Table 2.

The most important information provided by the FT-IR spectra concerning the structures of LAS–PhA and LAS–BuA complexes is included in the region 1750–1500 cm^{-1} (Fig. 3c). In the spectrum of Lasalocid acid (dashed line) the band assigned to the ν(C=O) vibrations of the carboxylic group is observed at 1652 cm^{-1}. In the spectra of both complexes (solid and dashed-dotted lines, respectively) this band is no longer observed and instead a new complex band arises in the region below 1600 cm^{-1} with maxima at 1587 cm^{-1} and 1571 cm^{-1}. This new composite band is a superposition of the $\nu_{as}(COO^-)$, $\delta(NH_3^+)$ and ν(C=C) vibrations and its presence indicates a proton transfer from the carboxylic group of LAS to the NH_2 group of the respective amines within the complex.

The band assigned to the ν(C=O) vibrations of the ketone group in Lasalocid and its both complexes is observed at ca. 1712 cm^{-1} indicating that this group is not involved in any hydrogen bond.

Formation of protonated 1:1 LAS–BuA and LAS–PhA complexes in chloroform solution is indicated by their ESI-MS spectra (Fig. S1, supplementary data) in which at *cv* = 10 V only one signal at *m/z* = 664 and 684 respectively, characteristic of such complexes, is observed.

Comparison of the FT-IR spectra of LAS–BuA complex in chloroform and in the solid state (Fig. 4) shows small changes associated only with changes in hydrogen bond strength, indicating that in the solution the crystal structure is practically conserved. Protonic vibrations within the O(8)—H···$O(1)^-$ and O(4)–H···$O(2)^-$ hydrogen bonds are indicated by a broad band in the same region in which the respective vibrations in the spectrum of the crystalline LAS–BuA complex are found.

In Fig. 5 the spectra of LAS–PhA complex in chloroform and in the solid state and the spectrum of LAS in solution are compared showing very strong changes. The spectral features of the complex in solution compared with those in the spectrum of LAS indicate clearly a partial dissociation of the complex with formation of Lasalocid acid. This statement is proved by the appearance of a band at 1653 cm^{-1}, 3597 cm^{-1} and a shoulder at 3430 cm^{-1} as well as by the ^1H and ^{13}C NMR data of LAS and its complexes given in Table 3. Most informative are the signals of the C(1) atom of the carboxylic group and its C(2) neighbouring atom. In the ^{13}C NMR spectrum of LAS the signal of C(1) atom is found at 173.2 ppm and in the spectrum of LAS–BuA at 175.4 ppm due to the proton transfer from the carboxylic group to the amine group. In the spectrum of LAS–PhA the respective signal is observed at 174.4 ppm indicating that the proton transfer process is incomplete. The same conclusion follows from a comparison of the chemical shifts of C(2) carbon atom.

The analysis of the shift of the ^{13}C NMR signal assigned to the carbon atom of ketone group from 214.4 ppm (LAS) to 217.8 ppm (LAS–BuA complex) or 216.3 ppm (LAS–BuA complex) can indicate that oxygen atom of carbonyl group interacts with the hydrogen atoms of protonated amines by weak hydrogen bond. On the other hand, the band assigned to the C=O stretching vibrations in the FT-IR spectra of both complexes shifts very slightly toward lower wave numbers in comparison with the position of this band in the FT-IR spectrum of LAS. Thus, it seems that carbonyl group is not engaged in the hydrogen bond formation with (NH_3^+) group and the shifts of the ^{13}C NMR signal carbonyl carbon atom is connected with conformational changes of LAS molecule after complexation of the respective amines.

The dissociation of LAS–PhA complex in solution is also confirmed by the proton signals assigned to (NH_3^+) in the spectrum of the complex at 9.34 ppm and of the free, non-hydrogen-bonded NH_2 protons of the aniline molecule at 3.80 ppm. The dissociation

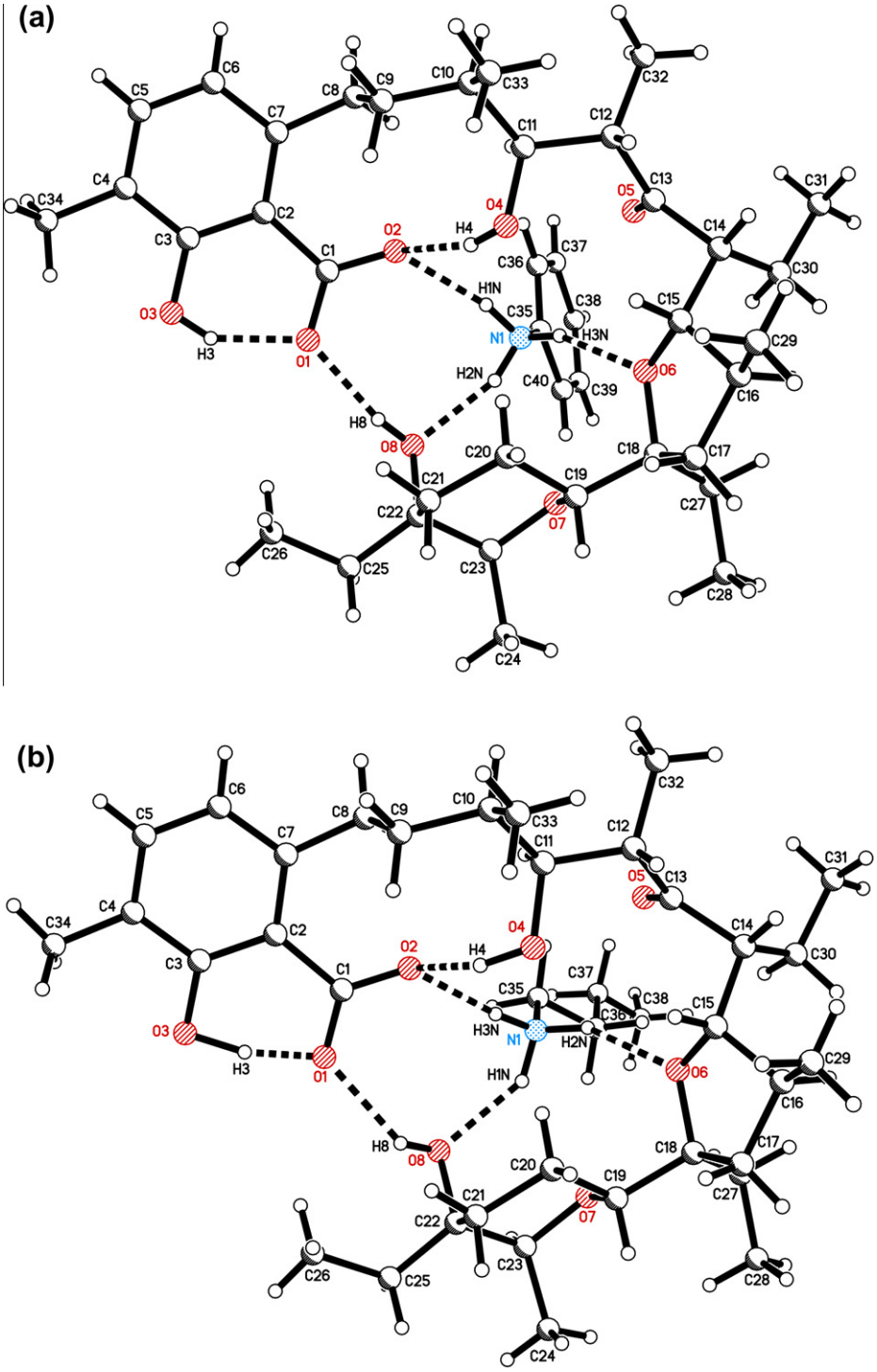

Fig. 1. A perspective view of: (a) LAS–PhA and (b) LAS–BuA complexes in the crystal structure. The hydrogen bonds have been indicated by dashed lines.

of the LAS–PhA complex is not surprising because phenylamine (aniline) belongs to very weak N-bases (pK_a = 4.6).

3.3. Antimicrobial activity evaluation of the compounds

Preliminary tests of the antimicrobial activity of Lasalocid acid have shown that it is active against Gram-positive standard bacterial strains [23]. Recently, we have proved that the complex of Lasalocid acid with allylamine exhibited higher antibacterial activity than pure Lasalocid acid [23]. This result inspired us to verify *in vitro* the antibacterial activity of other Lasalocid complexes with phenylamine and butylamine against typical Gram-positive cocci, Gram-negative rods and yeast-like organisms.

The data concerning the antimicrobial activity of the compounds studied are summarized in Table 4. Lasalocid acid (LAS), and its complexes LAS–PhA and LAS–BuA are very active against Gram-positive bacteria. LAS–PhA shows slightly higher antibacterial activity in comparison to that of Lasalocid (Table 4). The antibacterial activity of LAS and LAS–BuA is similar. Lasalocid acid, pure amines and their complexes are inactive against strains of *Candida* and Gram-negative bacteria.

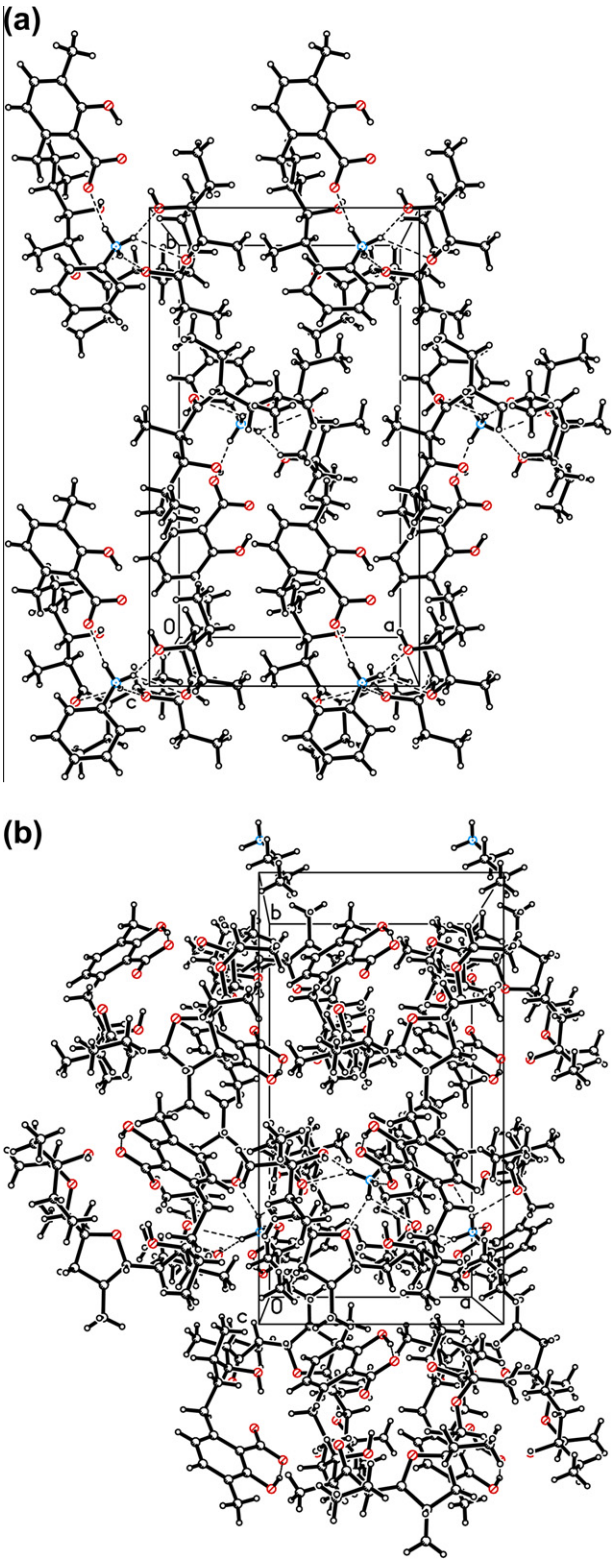

Fig. 2. Autostereographic projections [32] of complexes: (a) LAS–PhA and (b) LAS–BuA structures, viewed along the 001 crystal direction.

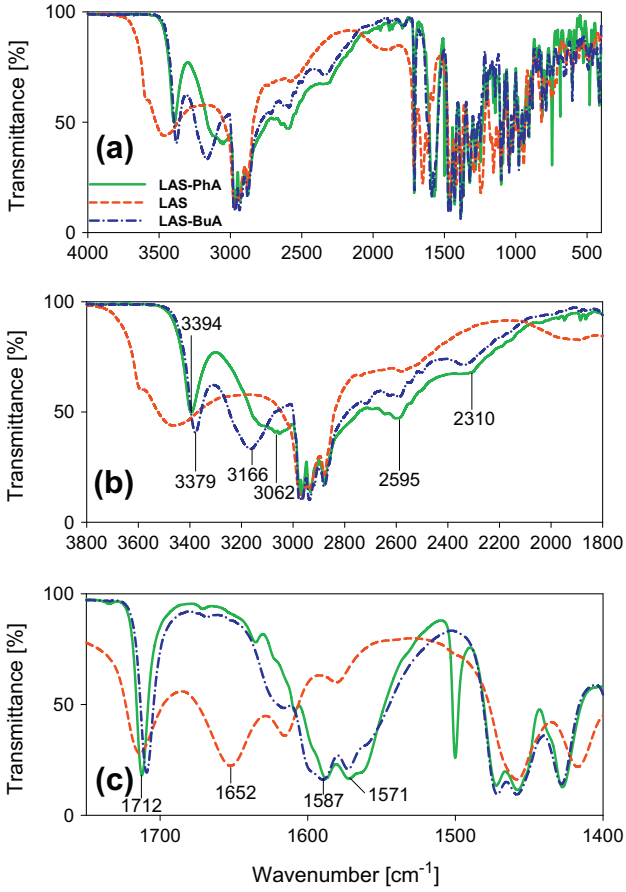

Fig. 3. The FT-IR spectra of crystals of: (---) LAS, (––) 1:1 LAS–PhA and (-·-) 1:1 LAS–BuA complexes in nujol/fluorolube mulls in the ranges: (a) 4000 – 400 cm^{-1}; (b) 3800 – 1800 cm^{-1} and (c) 1750 – 1400 cm^{-1}.

3.4. Cytotoxic activity evaluation of the compounds

The cytotoxic activity of Lasalocid acid (LAS) and its complexes (LAS–PhA and LAS–BuA) was tested against 6 different cancer cell lines: A-549 (human lung adenocarcinoma), MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), HLMEC (human lung microvascular endothelial cells), P-388 (murine leukemia), Balb3T3 (mouse embryonic fibroblasts). In view of the potential clinical application of Lasalocid in combination with different chemotherapeutic drugs, this study was undertaken to examine the antitumor activity of Lasalocid in combination with various amines such as aromatic amine – phenylamine (PhA) and aliphatic amine – butylamine (BuA), which form relatively stable and structurally well defined complexes discussed above.

As shown in Table 5, all compounds tested showed moderate to potent anticancer activities against all the cells selected. All the compounds are very promising because they are outranged of the positive control cisplatin. Lasalocid acid and its complexes showed almost equipotent activity with cisplatin only against P388 and MCF-8 cell lines. All compounds tested revealed stronger cytotoxicity against A-549, HT-29, HLMEC cell lines, about three-fold stronger than that of the positive control cisplatin.

4. Conclusions

Two new complexes of ionophore antibiotic Lasalocid acid (LAS) and amines such as phenylamine (PhA) and butylamine (BuA) have been synthesised and their molecular structures have been fully characterized using crystallographic and spectroscopic methods.

In the solid state both complexes of Lasalocid acid are completely deprotonated and the amine groups are protonated. The structures of the complexes are stabilized by the intra- and intermolecular hydrogen bonds formed between the Lasalocid anion and the respective protonated amine. The (NH_3^+) protons of the

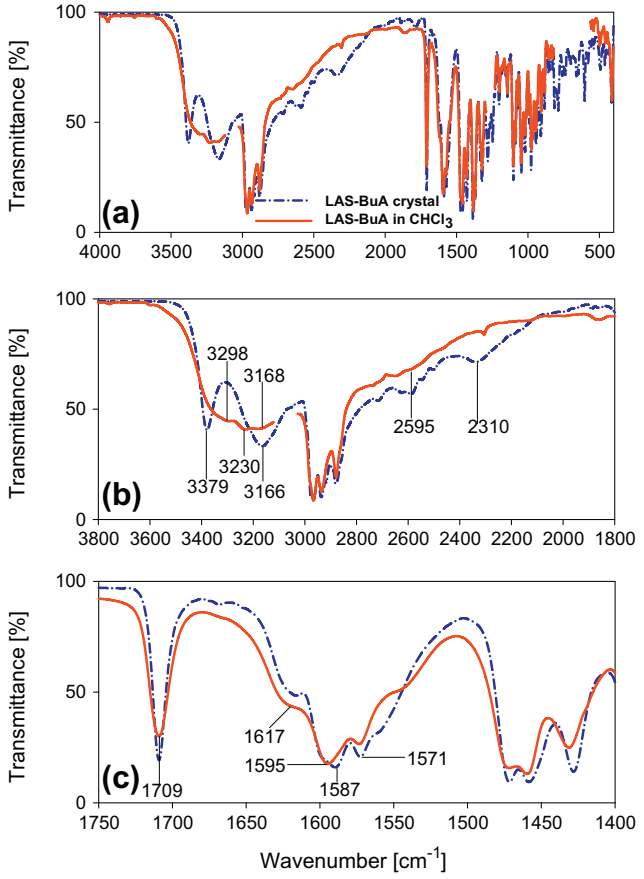

Fig. 4. FT-IR spectra of the crystals of (---) 1:1 LAS–BuA complex in nujol/fluorolube mulls and (—) LAS–BuA complex in chloroform solution: (a) 4000 – 400 cm^{-1}; (b) 3800 – 1800 cm^{-1} and (c) 1750 – 1400 cm^{-1}.

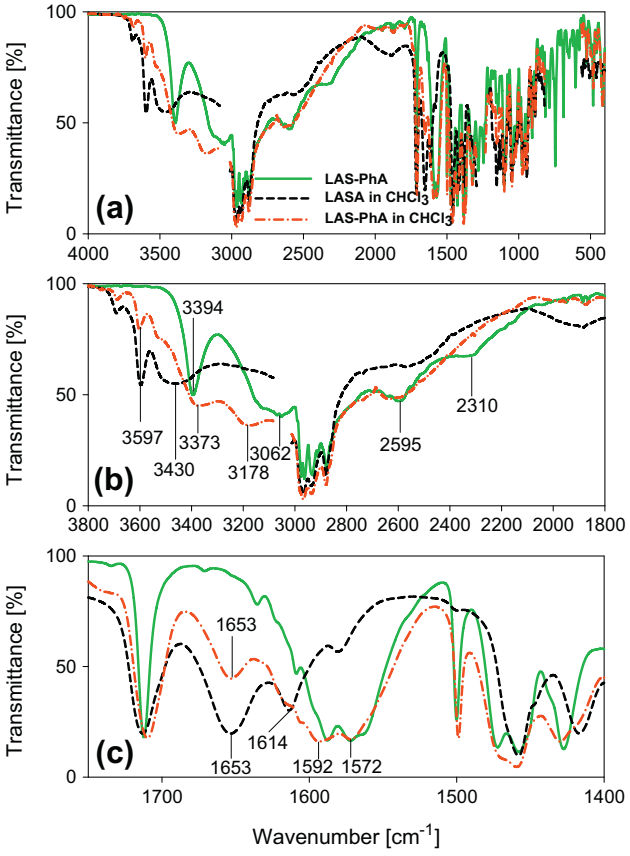

Fig. 5. The FT-IR spectra of the crystals of (—) 1:1 LAS–PhA complex in nujol/fluorolube mulls, (---) LAS–PhA complex in chloroform solution and (---) LAS in chloroform solution: (a) 4000 – 400 cm^{-1}; (b) 3800 – 1800 cm^{-1} and (c) 1750 – 1400 cm^{-1}.

protonated amine molecules are hydrogen bonded with the etheric oxygen atom O(6), the hydroxyl oxygen atom O(8), and one carboxylate oxygen atom O(2) of the LAS anion. In both complexes the O(1) oxygen atom of the carboxylate group is involved in a relatively strong intramolecular quasi-aromatic O(1)—H···O(3) hydrogen bond. The pseudo-cyclic structure of Lasalocid anion is stabilized by two O(4)—H···O(2) and O(8)—H···O(1) intramolecular hydrogen bonds binding the ends of the LAS anion.

In chloroform solutions the crystal structure of the LAS–BuA complex is preserved and the equilibrium between LAS–PhA complex and its dissociated mixture of Lasalocid acid and phenylamine is observed. Thus, in the solution the proton transfer process from Lasalocid acid to phenylamine is incomplete.

All compounds were examined for their *in vitro* antimicrobial activities against Gram-positive and Gram-negative bacteria and Candida fungi. Lasalocid complexes with amines (LAS–PhA and LAS–BuA) holding the aromatic amine moiety and the aliphatic amine moiety showed significant antibacterial activity against Gram-positive bacteria compared to that of Lasalocid. All compounds showed negative antifungal activity and antibacterial activity against Gram-positive bacteria. For the first time, cytotoxicity studies of Lasalocid and its new complexes with amines were carried out against A-549 (lung), MCF-7 (breast), HT-29 (colon) and P-388 (leukemia) cancer cell lines as well as normal BALB/3T3 (murine embryonic fibroblasts) and HLMEC (human lung microvascular endothelial) cell lines.

Lasalocid and their complexes show good cytotoxic activity and outperform the reference drug cisplatin against all cancer cell lines examined. Moreover, their high antiproliferative activity against HLMEC cell line may suggest potential antiangiogenic activity. On the other hand, their moderate activity against normal fibroblast cell line may predict its lower toxicity in further studies *in vivo*. The structure activity relationship study revealed that the compounds holding the aromatic amine species showed cytotoxicity similar to that of aliphatic amine species and slightly lower than that of pure Lasalocid acid.

The compounds reported in this paper could serve as potential leads for cytotoxic activity. Further investigation is needed to recognize the exact mechanism of action of these molecules which is clearer after their structural characterizations preformed in this paper.

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Appendix A. Supplementary material

Supplementary data (exemplary NMR, ESI-MS spectra and Tables) associated with this article can be found, in the online version.

CCDC 867826 and 867827 contains the supplementary crystallographic data for complex LAS-PhA and complex LAS-BuA. These data can be obtained free of charge via http://www.ccdc.cam.a-

c.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK or e-mail: deposit@ccdc.cam.ac.uk. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2012.07.036.

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