Indian Journal of Natural Products and Resources
Vol. 9(3), September 2018, pp. 222-228

Bioactive metabolites from the ruminal bacterium *Enterobacter amnigenus* ZIH

Imene Zendah El Euch[1], Khaled A. Shaaban[2,3], Naheed Riaz[4], Muhammad Bahi[5] and Mohamed Shaaban[6*]

[1]Institute of Organic and Biomolecular Chemistry, University of Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany

[2]Center for Pharmaceutical Research and Innovation, [3]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, United States

[4]Department of Chemistry, Baghdad-ul-Jadeed Campus, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

[5]Chemistry Department, Faculty of Mathematics and Natural Sciences, University of Syiah Kuala, Kampus Darussalam, Banda Aceh, 23 111, Indonesia

[6]Chemistry of Natural Compounds Department, Division of Pharmaceutical Industries, National Research Centre, El-Behoosst. 33, Dokki-Cairo 12622, Egypt

Received 29 July 2017; Revised 30 July 2018

Bioassay-guided fractionation and chromatographic purification of the crude extract of ruminal bacterium *Enterobacter amnigenus* ZIH (isolated from cattle) produce one new microbial product:butyl-α-D-glucopyranoside together with eleven known compounds including (*S*)-brevinic acid, 3-(hydroxyacetyl)-indole, $N_{ß}$-acetyltryptamine, tyrosol, phenol, tryptophol, indole-3-lactic acid, uracil, adenine and two diketopiperazines *cyclo*-(Phe, Pro) and *cyclo*-(Leu, Pro). The complete NMR assignments of butyl-α-D-glucopyranoside was done - using different spectroscopic (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC, and HMBC) and spectrometric methods (ESI-MS, HRESI-MS). Compounds 1a and 2 reported for the first time from a natural source. The bacterial extract exhibited high cytotoxicity against brine shrimp and moderate antimicrobial activity against a diverse set of pathogenic bacteria strains.

Keywords: Biological activities, Brevinic acid, Butyl-glucoside, *Enterobacter amnigenus* ZIH, Ruminal bacteria.

IPC code; Int. cl. (2015.01)− A6IK, A6IK 35/00, A61P 31/00

Introduction

Animal digestive tracts are complex ecosystems with a large degree of microbial diversity[1]. Ruminal bacteria colonizing the gut are consequently numerous, having many interactions such the antagonism[2,3,4]. Research on antimicrobials produced by ruminal bacteria led to the identification of several substances with bactericidal or bacteriostatic characteristics, including organic acids[5], hydrogen peroxide and bacteriocins[6]. These findings encouraged the isolation of ruminal bacteria with potential antagonism against pathogens. In accordance, ruminal bacteria may provide alternative new sources of antibiotics and bioactive drugs[3,7-10].

Material and Methods

Optical rotation was recorded on Perkin-Elmer polarimeter (model 343; Waltham, MA, USA). The NMR spectra were measured on Varian (Palo Alto, CA, USA) Unity 300 (300.145 MHz) and Varian Inova 500 (125.7 MHz) spectrometers. ESI-MS spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorkerosine as a reference substance for HR-EIMS. HR-ESIMS were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). Flash chromatography was carried out on silica gel (230-400 mesh). RP-chromatography was carried out on RP-18 (Macherey-Nagel). R_f values were measured on Polygram SIL G/UV$_{254}$ TLC cards (Macherey-Nagel). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany).

Isolation and Taxonomy of the Producing Strain

The ruminal bacterium isolate ZIH was isolated from cattle. Its isolation and identification were performed in the first instance with standard methods of microbiology (morphological form, Gram staining,

*Correspondent author
Email: mshaaba@gmail.com

biochemical tests) and were completed accordingly with Manual Galerie API and with the Automat mini API bioMerieux[10]. The isolate ZIH was identified as *Enterobacter amnigenus*. It is a Gram-negative bacterium, oxidase negative, catalase positive, citrate positive, indole negative, and rod-shaped bacterium[11]. A reference sample of the bacterial isolate ZIH was deposited in the collection center in Laboratoire des Microorganismes et des Biomolecules Actives (LMBA), Faculté des Sciences de Tunis, Université Tunis-El Manar, Tunisia

Fermentation, isolation and purification

A 5 L culture of *Enterobacter amnigenus* ZIH was grown in 1 L erlenmeyer flasks each filled with 300 mL of LB-medium (peptone 8 g, yeast extract 5 g, NaCl 5 g/ L distilled water, adjusted to pH 7.8) on a linear shaker with 95 rpm at 37 °C for three days. After fermentation, the culture broth was used to inoculate a Braun Biostat U fermenter, filled with 20 L of LB medium, and maintained with stirring rate of 200 rpm, 37 °C, pH 6.5±1.5, and aeration of 1.5 m^3/h. The culture broth was harvested after 5 days. Then, the cells phase was filtered off by means of a pressure filter and extracted with ethyl acetate (3 × 5L) and acetone (2 × 5L). The filtrate, on another hand, was passed through an Amberlite XAD-16 column (6 × 120 cm). The adsorbed organic extract was then washed with 25 L of demineralized water, followed by elution with 15 L methanol. The methanolic extract was then concentrated *in vacuo*, and the remaining water residue was re-extracted by ethyl acetate (3× 1 L), followed by concentration *in vacuo* till dryness. On basis of their similar chromatograms according to TLC monitoring, both of the mycelium and filtrate extracts were combined yielding 3.3 g as the dark violet crude extract.

The bacterial crude extract (3.3 g) was applied to chromatographic fractionation using silica gel column chromatography eluted with DCM-MeOH of gradual increasing of polarity, and monitoring by TLC to afford four fractions I-IV. Fraction I (0.4 g) was rich in fat and undesired compounds and discarded, therefore. Purification of Fraction II (1.2 g) yielded 3-(hydroxy acetyl)-indole (14 mg), N_β-acetyltryptamine (17 mg), *cyclo*-(Phe, Pro) (8 mg), and *cyclo*-(Pro, Leu) (13 mg) as colourless solids. Fraction III (0.8 g) was applied to silica gel column eluted with DCM-MeOH with a gradual increase in the polarity, followed by purification on Sephadex (DCM/MeOH, 6:4) afforded colourless solids uracil (18 mg), phenol (16 mg), tyrosol (12 mg)and the violet pigment of brevinic acid (2, 12 mg). Finally, fraction IV (0.6 g) was sub-fractionated on Sephadex LH-20 to afford two sub-fractions, IVa (0.2 g) and IVb (0.15 g). Sub-fraction IVa was purified on Sephadex LH-20 (MeOH) to yield tryptophol (9 mg) and indole-3-lactic acid (13 mg) as colourless solids. On the other hand, the sub-fraction IVb was purified on an RP-18 column (12 × 1 cm) using 40 % aqueous methanol afforded butyl-α-D-glucopyranoside (18 mg) and adenine (13 mg).

Butyl-α-D-glucopyranoside (1a): Colourless solid, turns to light green after spray with anisaldehyde/sulphuric acid; $[\alpha]_D^{20}$ = + 42.2 (*c* 1.35, MeOH); ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; (+)-ESIMS: *m/z* 259 $[M+Na]^+$, 495 $[2M+Na]^+$; (+)-ESI-HRMS: *m/z* 259.11536 $[M+Na]^+$ (calc. 259.11521 for $C_{10}H_{20}O_6Na$).

Brevinic acid (**2**):Purple pigment; UV absorbing (254 nm); $[\alpha]_D^{20}$ = + 120.1 (*c* 1.1, MeOH); ^{1}H NMR (300 MHz, CD_3OD) and^{13}C-NMR (125 MHz, CD_3OD), see Table 2; (–)-ESI-MS: *m/z* 288 ($[M–H]^–$; (+)-ESI-HRMS: *m/z* 312.03020 (calc. 312.0301 for $C_{14}H_{11}NO_4SNa$ $[M+Na]^+$).

Table 1 — Biochemical characterization of *Enterobacter amnigenus ZIH*

Type characterized test	*Enterobacter amnigenus* ZIH reponse
Gram stain	-
Oxidase	-
Catalase production	+
Lipase	-
Motility	+
ONPG	+
Arginine dihydrolase	d
Lysine decarboxylase	-
Ornithine decarboxylase	+
Citrate utilization	+
Urea hydrolysis	-
Indole production	-
D-Glucose	+
D-Mannitol	+
Inositol	-
Sorbitol	+
Rhamnose	+
Lactose fermentation	d
Maltose	+
Sucrose fermentation	-
Melibiose fermentation	+
Raffinose fermentation	-
Xylose	+

Table 2 — ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HMBC and COSY correlations of butyl-α-D-glucopyranoside (1a) in CD_3OD in comparison with the literature data.

Position	Butyl-α-D-glucopyranoside (CD_3OD)		Butyl-α-D-glucopyranoside (C_5D_5N)[24]		Butyl-β-D-glucopyranoside (1b) (C_5D_5N)[24]	
	δ_C, type	δ_H (mult.; *J* in Hz)	δ_C	δ_H (mult.; *J* in Hz)	δ_C	δ_H (mult.; *J* in Hz)
1	101.5, CH	4.75 (d, 1.5)	101.3	5.19 (d, 3.4) *	104.8	4.76 (d, 7.8)
2	74.6, CH	3.50 (m)	75.1	4.83 (brs) *	75.3	4.15 (t, 7.8, 8.9)
3	72.7, CH	3.76 (m)	76.8*	5.13 (t, 4.8, 9.6) *	78.5	4.21 (t, 8.9)
4	72.3, CH	3.76 (m)	70.2	3.68-3.65 (m, 9.1)	71.9	3.97 (t, 8.9)
5	68.6, CH	3.60 (m)	76.8*	3.64-3.61 (m, 6.1, 9.3)	78.7	3.91-3.89 (m, 2.5, 5.8)
6	62.9, CH_2	3.68 (m), 3.80 (m)	62.8	4.47 (brd, 18.10)*, 4.31 (brd, 18.0) *	63.0	4.50 (dd, 2.0), 4.32 (dd, 5.8, 11.8)
1'	68.3, CH_2	3.40 (m), 3.72 (m)	68.2	4.07-4.03 (m, 6.5, 15.5)*, 3.38 (dd, 6.5, 15.5)	69.8	4.05 (ddd, 2.6, 6.8, 15.2), 3.61 (ddd, 2.5, 7.8, 15.2)
2'	32.7, CH_2	1.60 (m)	32.7	1.77-1.75 (m, 6.5, 7.2)	32.5	1.59-1.53 (m, 6.8, 7.5, 7.8)
3'	20.5, CH_2	1.40 (m)	19.8	1.55-1.52 (m, 6.5, 7.2)	19.8	1.33-1.28 (m, 7.4, 7.5)
4'	14.2, CH_3	0.91 (t, 7.3)	4.3	1.02 (t, 7.2)	14.3	0.76 (t, 7.4)

*Highly downfield shifted (^{1}H and ^{13}C) data of the reported butyl-α-D-glucopyranoside (1a in C_5D_5N) compared with ours (CD_3OD), which might be attributed mostly to a solvent influence or unknown reason.

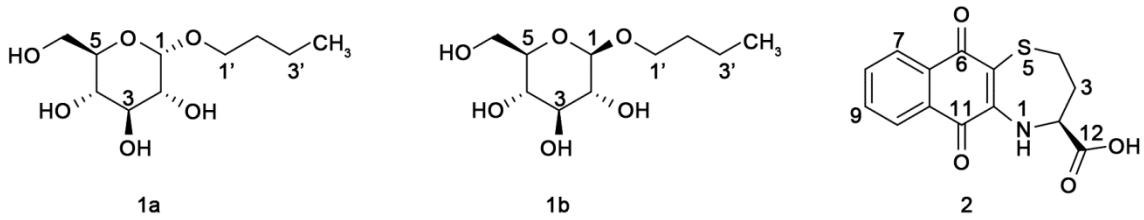

Fig. 1 — Chemical structures of compounds 1a and 2 produced by the ruminal bacterium *Enterobacter amnigenus* ZIH

Biological activity studies

Antimicrobial activity

Antimicrobial assays were conducted utilizing the agar diffusion method[12,13] against diverse sets of microorganisms including *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* Govan 978, *Fusarium culmorum*, *F. graminearum* and *Phoma tracheiphilia*. The bacterial strains were grown on nutrient agar medium, while, the fungal strains were grown on (PDA) medium. The ruminal bacterium extract was dissolved in CH_2Cl_2/10 % MeOH at a concentration of 1 mg/mL. Aliquots of 100 µL of the crude extract were served per each well. The agar plates of pathogenic microorganisms were incubated for 24 h at 37 °C for bacteria and 72 h (30 °C) for the fungi.

Cytotoxic activity

The cytotoxicity assay was performed using brine shrimp according to Takahashi *et al.* and Sajid *et al.* screening[14,15].

Results and Discussion

In our plan to investigate secondary metabolites from microorganisms, the ruminal bacterium *Enterobacter amnigenus* ZIH was proved to exhibit moderate bioactivity against a set of microorganisms, including *Staphylococcus aureus* ATCC 25 923 and *Pseudomonas aeruginosa* Govan 978 and high cytotoxicity against brine shrimp. On the other hand, the bacteria extract was inactive against the phytopathogenic fungi: *Fusarium culmorum*, *Fusarium graminearum* and *Phoma tracheiphilia*. In accordance, purification of the bacterial extract utilizing a series of chromatographic techniques afforded the new microbial; butyl-α-D-glucopyranoside (Fig. 1a) along with the patented unusual nitrogen/sulphur containing (*S*)-brevinic acid (Fig. 1b). In addition, ten known compounds namely;3-(hydroxyacetyl)-indole, *N*ß-acetyl tryptamine, tyrosol, phenol, tryptophol, indole-3-lactic acid, uracil, adenine, and *cyclo*-(Phe, Pro) and *cyclo*-(Leu, Pro) have been isolated and identified from the strain crude extract. The chemical structure of butyl-α-D-glucopyranoside (1a) has been confirmed on the basis of comprehensive 1D and 2D NMR and mass spectrometry data analysis. Complete 1D and 2D NMR assignments for the known

metabolite brevinic acid (2) was also provided for the first time.

Identification of the bacterial strain ZIH was basically dependent on the determination of the cell form, Gram staining and biochemical testing (Table 1) following Bergey's Manual of Systematic Bacteriology[16,17], proving it as *Enterobacter amnigenus*.

Chemistry of the isolated compounds

Structures of the known compounds:3-(hydroxyacetyl)-indole[18], $N_{ß}$-acetyltryptamine[19], tyrosol[20], phenol, tryptophol, indole-3-lactic acid, uracil and adenine, together with the diketopiperazines*cyclo*-(Phe, Pro) and *cyclo*-(Pro, Leu)[21], have been assigned on the bases of their NMR and mass spectral data and comparison with the corresponding literature data.

Butyl-α-D-glucopyranoside

Compound 1a was obtained from fraction IV using Sephadex LH-20 and RP-18 chromatographic columns as polar colourless solid. By monitoring TLC, it was not UV absorbing. However, it showed a green spot after spraying with anisaldehyde/sulphuric acid. The molecular weight of compound 1a was determined as 236 Da according to ESI-MS, and its molecular formula was established as $C_{10}H_{20}O_6$ by HR-ESIMS, bearing only one DBE. This referred to (a) as non-aromatic or olefinic nature.

The ^{1}H NMR/HMQC spectra of (1a**)** (Table 2) displayed a doublet at δ 4.75 (J= 1.5 Hz), which might be attributed to an anomeric proton of a sugar moiety (H-1). In addition, multiplets of four oxygenated methines were concluded at δ 3.76-3.50 (H-2, H-3, H-4, H-5). Furthermore, two oxygenated methylenes were appeared at (*δ3.40, 3.72[H_2-1'], and 3.68, 3.80[H_2-6])* as two AB systems. In addition, two multiplets of further two methylene groups (*δ1.60[H_2-2'] and 1.40[H_2-3']) and* a triplet methyl signal (*δ*0.93*[H_3-4']*) were displayed.

According to ^{13}C NMR/HMQC experiments, ten carbon signals were displayed including one anomeric carbon*[C-1]* (*δ*101.5), four oxygenated methines*[C-2~C-5]* (*δ*74.6~68.6), and two oxymethylenes (*δ*68.3*[C-1']* and 62.9*[C-6]*), respectively. The remaining three carbon signals were specific for two aliphatic methylene carbons (*δ*32.7*[C-2']*, 20.5*[C-3']*) and a methyl carbon at *δ*14.2*[C-4']*. Based on the above spectroscopic discussion, and search in different Data-Bases (AntiBase, Dictionary of Natural Products [DNP][22] and SciFinder (https://www.cas.org/products/scifinder) proved the novelty of 1a. Consequently, the structure of butyl-glucopyranoside was intensively applied to full assignment using ^{1}H-^{1}H COSY and HMBC experiments (Fig. 2, Table 2).

In accordance, the ^{1}H-^{1}H COSY confirmed the existence of an *n*-butanol-partial structure. On the other hand, the ^{1}H-^{1}H COSY, as well as HMBC correlations, established the existence of a glycoside moiety substituted at the anomeric carbon C-1 (*δ*101.5). Consequently, proton signals of the butanol oxy-methylene (H_2-1', *δ*3.40,3.72) displayed a ^3J HMBC correlation with the anomeric carbon C-1 (*δ*101.5), confirming their direct attachment (Fig. 2, Table 2). Therefore, compound a was finally assigned as butyl-glucopyranoside (1a). The absolute configuration of the sugar moiety in (1a) was confirmed as *α*-D-glucoside on the basis of the small coupling constant of the sugar anomeric proton H-1 (J = 1.5 Hz), the optical rotation [*α* = + 42.2 (*c* 1.35, MeOH); + 135.4 (*c* 4.0, H_2O)], and by comparison with related sugars from literatures, and hence butyl-α-D-glucopyranoside (1a) ((Fig. 1)) was unequivocally deduced. Synthetically, butyl-α-D-glucopyranoside was reported previously by Pigman and Laffre, 1951[23]. Naturally, compound (1a) ((Fig. 1) was recently reported from the medicinal plants *Winchia calophylla* (Apocynaceae)[24], *Tilia amurensis*[25] and *Allium tuberosum*[26]. In contrast, (1a) is reported herein to first time from microorganisms. Comparison of the literature reported NMR data of the two stereomeric isomers; butyl-α-D-glucopyranoside (1a) and butyl-*β*-D-glucopyranoside (1b) (Fig. 1) with ours (Table 1), confirmed definitely the latter as α-configuration. However, the NMR data for the reported were measured in a rather different solvent (C_5D_5N), exhibiting a high deviation in their NMR (^{1}H, ^{13}C) chemical shifts than ours, and were not correctly assigned as they failed to 2D NMR spectroscopy[24].

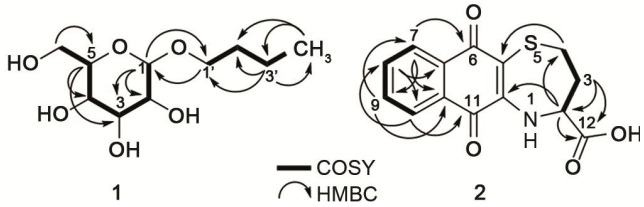

Fig. 2 — ^{1}H-^{1}H COSY (━) and selected HMBC (→) correlations of (a) Butyl-α-D-glucopyranoside and (b) Brevinic acid .

(S)-Brevinic acid

As a middle polar purple pigment, compound 2 was obtained from fraction III using a series of chromatographic techniques. It showed no colour change after exposition to aqueous sodium hydroxide or sulphuric acid, excluding its nature as *peri*-hydroxyquinone[27] or *peri*-hydroxypyrone systems[28]. The molecular mass of 2 was deduced as 289 Da on the bases of positive and negative modes of ESI-MS. The odd number of the molecular mass is an indicator for the existence of an odd number of nitrogen atoms in 2. HRESI-MS confirmed such suggestion, exhibiting the molecular formula as $C_{14}H_{11}NO_4S$, containing 10 DBE.

According to ^{1}H NMR, four aromatic proton signals were revealed being for 1,2-disubstituted aromatic residue, which have been appeared as two doublets of doublet (dd, δ_H 7.94[H-10], 7.91[H-7], *J* ~7.4, 1.7 Hz) and two triplets of doublet (td, 7.64[H-8], 7.61[H-9], *J* ~7.4, 1.6 Hz), and their neighbourhood was established by ^{1}H-^{1}H COSY (Table 2, Fig. 2). In the aliphatic region, three spin systems were visible being for 1H as multiplet (δ_H5.22, H-2), and four 1H signals being for two methylene groups, one of them displayed an AB system (δ_H 3.68,3.09, *J*~16.0, 6.0 Hz, H_2-4), while signals of the other methylene were visible as multiplets (δ_H2.50, 2.10, H_2-3). Based on ^{1}H-^{1}H COSY, a direct attachment between both methylene groups was established, and between the multiplet methyleneH_2-3 (δ_H2.50, 2.10) and those of the multiplet methine signal H-2 (δ_H5.22). The high chemical shift of the last methine proton (H-2) is attributed mostly to its adjacent to an oxygen or nitrogen and/ or flanked by an electron withdrawing group (e.g. COOH).

According to ^{13}CNMR and HMQC spectroscopic data (Table 3), fourteen carbon signals have been exhibited for compound b, as matched with the molecular formula, among them four sp^2-methine carbons (δ_C135.3[C-8], 133.4[C-9], 127.3[C-10], 126.6[C-7]), three carbonyl signals being mostly for two quinone carbonyls (δ_C182.6[C-6], 180.2[C-11]) and carbonyl of ester, amide or carboxylic acid (δ_C177.0[C-12]). Further four quaternary sp^2-carbon signals were observed and one of them (δ_C147.9[C-11a]) is likely attached to a hetero atom (Nitrogen or Oxygen). In the aliphatic region, three carbon signals were established being for a nitrogenous methine (δ_C57.9, [C-2]) and two methylene carbons (δ_C32.9[C-3], 32.2[C-4]).

Table 3 — ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of brevinic acid (2) in CD_3OD

Position	δ_C, type	δ_H (mult.; *J* in Hz)
2	57.9, CH	5.22 (m)
3	32.9, CH_2	2.10 (m), 2.50 (m)
4	32.2, CH_2	3.09 (dd, 16.0, 6.0) 3.68 (dq, 16.0, 6.0)
5a	114.5, C	
6	182.6, C	
6a	134.2, C	
7	126.6, CH	7.91 (dd, 7.4, 1.5)
8	135.3, CH	7.64(td, 7.4, 1.6)
9	133.4, CH	7.61 (td, 7.4, 1.4)
10	127.3, CH	7.94 (dd, 7.4, 1.8)
10a	131.6, C	
11	180.2, C	
11a	147.9, C	
12	177.0, C	

Consequently, based HMBC/COSY correlations (Fig. 2), structure of 2 was finally confirmed as 5,11-dioxo-5,7,8,9,10,11-hexahydro-6-thia-10-aza-cyclohepta[b]naphthalene-9-carboxylic acid; (*S*)-brevinic acid, unusual nitrogen and sulphur-containing compound. Brevinic acid (2) has been previously patented as a bioactive metabolite produced by *Brevibacterium Juvum* AJ 3869[29], and it was readily synthesized from 2,3-dichloronaphthoquinone and L-homocysteine[30]. As its importance from the biological activity point of view, several derivatives of it have been recently created[31]. It is worthy to refer herein that brevinic acid (2) is reported herein to first time as a natural product according to our careful searching in different databases including SciFinder.

Biological activities

The crude extract of *E.amnigenusmoderate* ZIH has been tested against a set of microorganisms and brine shrimp for cytotoxicity. The bacterial extract displayed moderate activity against Gram-positive (*Staphylococcus aureus* ATCC 25923, [15 mm]), and Gram-negative (*P. aeruginosa* Govan 978, [15 mm]) bacteria, while it found inactive against *E. coli* ATCC 2592 and *P. aeruginosa* ATCC 27853. Additionally, the strain extract showed no activity against the pathogenic fungi *F. culmorum*, *F. graminearum* and *P. tracheiphilia*. On the other hand, the bacterial extract displayed high cytotoxicity against brine shrimp with a mortality ratio of 90 % at a concentration of 40 µ/mL. Study of the antimicrobial activity of compounds 1a and 2 were carried against

Table 4 — Antimicrobial activities of the bacterial strain ZIH extract and compound 1-2 using agar diffusion method (mm diameter)

Ext/Comp	Sta[a]	EC[b]	Psag[c]	Psa[d] ATCC27853	Fusc[e]	Fusg[f]	Phot[g]
ZIH extract	15	-	15	-	-	-	-
1	-	-	-	-	-	-	
2	13	-	13	-	-	-	

[a]*Staphylococcus aureus* ATCC 25 923,[b]*Escherichia coli* ATCC 2592, [c]*Pseudomonas aeruginosa* Govan 978, [d]*Pseudomonas aeruginosa* ATCC 27853,[e]*Fusarium culmorum*, [f]*Fusarium graminearum*, [g]*Phoma tracheiphilia*

test organisms mentioned above, revealing a moderate activity for 2 against *S. aureus* ATCC 25923, (13 mm), and Gram-negative *P. aeruginosa* Govan 978 (13 mm), meanwhile compound a was inactive (Table 4) against the whole tested organisms. Based on our cytotoxic assaying, compounds 1a and 2 missed any activity.

Conclusion

Through a bioassay-guided fractionation and chromatographic purification of the bioactive metabolites from ruminal bacteria to first time so far, the ruminal bacterium *E. amnigenu* ZIH crude extract was found to produce a new microbial butyl-α-D-glucopyranoside (1a) in addition to the patented unusual nitrogen and sulphur containing brevinic acid (2) along with ten other known compounds. We report herein the full assignment butyl-α-D-glucopyranoside and for the first time of brevinic acid, using various spectroscopic and spectrometric techniques (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC, HMBC, and HRESI-MS). The bacterial extract exhibited moderate antimicrobial activity against the bacterial strains: *P. aeruginosa* Govan 978 and *S. aureus* ATCC 25923, but negative results against *F. culmorum*, *F.graminearum* and *P. tracheiphilia*. Additionally, the extract exhibited a potent cytotoxicity against brine shrimp. Based on this study, ruminal bacteria are considered as a new promising source of diverse bioactive compounds, and hence it should be taken in consideration in the next stage of searching for novel drugs able to share in the overcoming the newly discovered diseases.

Acknowledgement

The authors are deeply thankful to Prof. Dr. Hartmut Laatsch, Institute of Organic and Biomolecular Chemistry, Göttingen, for his unlimited support and laboratory facilities and spectroscopic measurements. We thank Dr. H. Frauendorf and Mr. R. Machinek for MS and NMR measurements. The authors would like to acknowledge Ms. F. Lissy for biological activity testing and Mr. A. Kohl for technical assistance.

References

1 Peterka M, Tepsic K, Accetto T, Kostanjsek R, Ramsak A, *et al*., Molecular microbiology of gut bacteria: genetic diversity and community structure analysis, *Acta Microbiol Immunol Hung*, 2003, **50**(4), 395-406.

2 Walter J, Britton R A and Roos S, Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm, *Proc Natl Acad* Sci U S A, 2011, **108**(1), 4645-4652.

3 Dehority B A and Tirabasso P A, Antibiosis between ruminal bacteria and ruminal fungi, *Appl Environ Microbiol*, 2000, **66**(7), 2921-2927.

4 Beier R C, Pillai S D and Phillips T D, Preharvest and postharvest food safety: Contemporary issues and future directions (Blackwell Publishing and the Institute of Food Technologists) 1st edn, 2004, 1-436.

5 Koppova I, Lukas F and Kopeny J, Effect of fatty acids on growth of conjugated-linoleic-acids-producing bacteria in rumen, *Folia Microbiol*, 2006, **51**(4), 291-293.

6 Gilliland S E, Health and nutritional benefits from lactic acid bacteria, FEMS *Microbiol Rev*, 1990, **7**(1-2), 175-188

7 Dehority B A, Pectin-fermenting bacteria isolated from the bovine rumen, *J Bacteriol*, 1969, **99**(1), 189-196

8 Russell J B and Mantovani H C, The bacteriocins of ruminal bacteria and their potential as an alternative to antibiotics, *J Mol Microbiol Biotechnol*, 2002, **4**(4), 347-355.

9 Akin D E and Benner R, Degradation of polysaccharides and lignin by ruminal bacteria and fungi, *Appl Environ Microbiol*, 1988, **54**(5), 1117-1125.

10 Zendah El Euch I, Isolation, purification and structure elucidation of new secondary metabolites from terrestrial, marine, and ruminal microorganisms. PhD Thesis, Georg-August University Göttingen, Göttingen, Germany, 2012.

11 Sanders W E and Sanders C C, *Enterobacter spp.*: Pathogenspoised to flourish at the turn of the century, *Clin Microbiol Rev*, 1997, **10**(2), 220-241.

12 Tagg J R and Mc Given A R, Assay system for bacteriocins, *App Environ Microbiol*, 1971, **21**, 943.

13 Moreno I, Lerayer A L S and de Freitas Leitao M F, Detection and characterization of bacteriocin-producing Lactococcus lactis lactis strains, *Review Microbiol*, 1999, **30**, 130-136.

14 Takahashi A, Kurasawa S, Ikeda D, Okami Y and Takeuchi T, Altemicidin, a new acaricidal and antitumor substance, *J Antibiot*, 1989, **32**, 1556-1561

15 Sajid I, Fondja Y C B, Shaaban K A, Hasnain S and Laatsch H, Antifungal and antibacterial activities of indigenous Streptomyces isolates from saline farmlands: Prescreening, ribotyping and metabolic diversity, *World J Microbiol Biotechnol*, 2009, **25**(4), 601-610

16 Bergey's Manual of Systematic Bacteriology, *Int J Syst Bacteriol*, 1985, 408.

17 Bergey's, Manual of Systematic Bacteriology, vol I, edited by Noel R. Krieg, (The Williams & Wilkins Co., Baltimore), 1984, 964.

18 Shaaban M, Bioactive secondary metabolites from marine and terrestrial bacteria: Isoquinolinequinones, bacterial compounds with a novel pharmacophor. PhD Thesis, Goerg-August University, Göttingen, Germany, 2004.

19 Maeda U, Hara N, Fujimoto Y, Srivastava A, Gupta Y K, *et al*., N-fatty acyl tryptamines from *Annona reticulata*, *phytochemistry*, 1993, **34**(6), 1633-1635.

20 Lingappa B T, Prasad M, Lingappa Y, Hunt D F and Bieman K, Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus Candida albicans, *Science*, 1969, **163**(3863), 192-194.

21 Ayer W A, Browne L M, Feng M, Orszanska H and Saeedi-Ghomi H, The chemistry of the blue stain fungi. Part 1. Some metabolites ofCeratocystis species associated with mountain pine beetle infected lodgepole pine, *Can J Chem*, 1986, **64**(5), 904-909

22 Chapman & Hall Chemical Database, Dictionary of natural products. CD-ROM. Boca Raton: CRC Press, 2014.

23 Pigman W and Laffre R O, The preparation of n-butyl α-D-glucoside, *J Am Chem Soc*, 1951, **73**(10), 4994-4995.

24 Zhu W M, Li S L, Zhao Q and Hao X J, Butyl acetals from medicinal plant, Winchia calophylla (Apocynaceae), *Acta Bot Yunnanica*, 2007, **29**(6), 708-712.

25 Kim K H, Moon E, Cha J M, Lee S, Yu J S, et al., Antineuroinflammatory and antiproliferative activities of constituents from Tilia amurensis, *Chem Pharm Bull*, 2015, **63**(10), 837–842.

26 Gao Q, Li X B, Sun J, Xia E D, Tang F, et al., Isolation and identification of new chemical constituents from Chinese chive (Allium tuberosum) and toxicological evaluation of raw and cooked Chinese chive, *Food Chem Toxicol*, 2018, **112**, 400-411.

27 Shaaban K A, Shaaban M, Grün-Wollny I, Maier A, Fiebig H H, et al., Julichrome Q6 glucuronide, A monomeric subunit of the Julimycin B-I complex from a terrestrial *Streptomyces* sp, *J Nat Prod*, 2007, **70**(10), 1545-1550

28 Shaaban M, Shaaban K A and Abdel-Aziz M S, Seven naphtho-γ-pyrones from the marine-derived fungus *Alternaria alternata*: structure elucidation and biological properties, *Org Med Chem Lett*, 2012, **2**, 6.

29 Eguchi A, Suzuki Y, Onuma T, Yokokawa Y, Hirata T, et al., Naphthoquinone amino acid derivatives, JP Pat 52084220. 1976; JP 1976375, Assignee: Ajinomoto Co., Inc., Japan, https://scifinder.cas.org/scifinder/ view/ scifinder/scifinderExplore.jsf

30 Eguchi C and Yokokawa Y, New purple pigment produced by Brevibacterium-fluvum, *Heterocycles*, 1984, **21**(2), 470-470

31 Fricke J N, Chemical derivatization of the natural product collinolactone for target identification and structure activity relationships (SAR). PhD Thesis, Tübingen University, Germany, 2012. https://archives.drugabuse.gov/sites/ default/files/monograph79.pdf