Barakacin: A Thiazolyl-indole Alkaloid Isolated from a Ruminal *Pseudomonas* sp.

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A new thiazolyl-indole alkaloid, barakacin (**1**), has been isolated from the ruminal bacterium *Pseudomonas aeruginosa* strain ZIO. On the basis of detailed spectroscopic analyses and comparison with the data of related compounds, its structure has been determined as 2-{4-[bis-(1*H*-indol-3-yl)-methyl]-thiazol-2-yl}-phenol. In addition, the known compounds phenazine-1-carboxylic acid, 3-(hydroxyacetyl)-indole, indole-3-carbaldehyde, and glycolipid A were isolated. The discovery of compounds with a new skeleton emphasizes the importance for exploring new ecological niches like the rumen of bovines for the detection of new natural products. This paper describes the fermentation, isolation, structure elucidation and biological activities of compound **1**.

Key words: Barakacin, Indole Derivatives, Thiazolyl Antibiotics, Ruminal Bacterium

Introduction

The paunch or rumen – the first stomach of ruminant animals – accommodates a complex microbial community, which includes archeae, protists, bacteria, and fungi [1, 2]. The ruminal bacterial population is predominantly composed of obligate anaerobes. In this work we report on the isolation and characterization of a new metabolite of a facultatively aerobic bacterium, isolated from the rumen of a Tunesian cow and identified as *Pseudomonas aeruginosa* on the basis of the 16S rRNA gene sequence.

More than 1100 indole derivatives (peptides excluded) have been isolated from microorganisms, and many of them were reported to have potent biological activities that cover antimicrobial, antiviral, cytotoxic, insecticidal, antithrombotic, or enzyme inhibitory properties [3 – 6].

In our search for new bioactive compounds from ruminal bacteria, the extract of *P. aeruginosa* strain ZIO exhibited a strong antibacterial activity against a wide range of human-pathogenic bacteria. Extraction of the culture broth followed by a series of chromatographic steps afforded a new indole alkaloid as a yellow solid, which was identified as 2-{4-[bis-(1*H*-indol-3-yl)-methyl]-thiazol-2-yl}-phenol (**1**) (Fig. 1) and was named barakacin. Additionally, four known compounds were isolated, namely phenazine-1-carboxylic acid, 3-(hydroxyacetyl)-indole, glycolipid A, and indole-3-carbaldehyde.

Results and Discussion

Fermentation and isolation

Details of the fermentation and isolation are summarized pictorially in Fig. S1 (Supporting Information: online only). The ruminal *P. aeruginosa* ZIO grew well on peptone-yeast extract medium (PYM) at 37 °C. The fermentation was carried out on a 30-liter scale in peptone-yeast extract broth (PYM) for 3 days at 37 °C. The bacterial biomass was filtered off and extracted with organic solvents, while the culture filtrate was extracted with Amberlite XAD-16. The combined organic phases were evaporated to dryness yielding a greenish-brown crude extract.

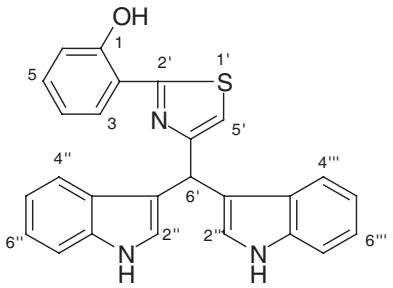


Fig. 1. Structure of barakacin (**1**).

Chromatography of the latter on silica gel resulted in four fractions I–IV. Purification of fraction II using PTLC followed by Sephadex LH-20 afforded 25 mg of **1** as yellow, green fluorescent solid (Fig. S2A) along with pentadecanoic acid methyl ester. Fractions III and IV afforded phenazine-1-carboxylic acid [7], 3-(hydroxyacetyl)-indole [8, 9], indole-3-carbaldehyde [10] and glycolipid A [11], which were identified on the basis of their MS and NMR data using AntiBase [12]. The assignment was confirmed by comparison with authentic spectra and literature data.

The color reaction of **1** with anisaldehyde/H_2SO_4 (orange; Fig. S2B) and Ehrlich's reagent (red-violet; Fig. S2C) indicated an indole derivative. The UV spectra (MeOH) of **1** displayed four strong bands at λ_{max} = 221, 282, 290, and 326 nm in neutral solution. Under basic conditions in methanol, the latter band showed a bathochromic shift to λ_{max} = 361 nm. The molecular weight was determined by ESI MS: the quasi-molecular ion peaks in positive and in negative modes confirmed the molecular weight of compound **1** as 421 Dalton. High-resolution MS delivered the molecular formula $C_{26}H_{19}N_3OS$.

NMR spectra

Pictures of key NMR spectra are shown as Figs. S3–S6 in the Supporting Information.

The ^{1}H NMR spectrum of compound **1** (Fig. S3) showed the pattern of two 1,2-disubstituted aromatic systems. The first pattern consisted of two *ortho*-coupled doublets at δ = 7.50 and 7.34, and of two triplets of *meta*-coupled doublets at δ = 7.20 and 7.07. Another signal at δ = 6.82 (d, J = 1.7 Hz) and an H/D exchangeable broad signal at δ = 7.94 together with the positive reaction with Ehrlich's reagent indicated a 3-substituted indole system. Due to the signal intensity, this partial structure was present twice in the molecule in a symmetrical manner.

Two *ortho*-coupled 1 H doublets of doublets at δ = 7.62 (J = 7.8, 1.5 Hz) and 7.02 (J = 8.3, 1.1 Hz), and two triplets of doublets at δ = 7.29 (J = 7.3, 1.6 Hz) and 6.90 (J = 7.9, 1.1 Hz) indicated a further 1,2-disubstituted benzene ring. Finally, the ^{1}H NMR spectrum displayed two narrow doublets at δ = 6.89 (J = 0.7 Hz) and 6.11 (J = 0.8 Hz) along with a broadened OH singlet at δ = 12.43. The HMBC spectrum of compound **1** (Fig. S6) indicated 18 carbon signals, of which seven were due to quaternary carbon atoms, and eleven to methine carbons. All carbon signals were localized in the sp^2 region, except for the methine signal at δ = 36.6 (δ_H = 6.11). The quaternary carbon atom at δ = 168.7 could be due to a carbonyl or indicated an sp^2 carbon localized between two heteroatoms as in a thiazole moiety ($-$N=C$_q$ $-$ S$-$). This agreed with the empirical formula and the strong green fluorescence, which resembled that of aeruginoic acid [13]. The ^{13}C NMR spectrum (Fig. S4) confirmed the presence of two indole moieties in a symmetrical orientation.

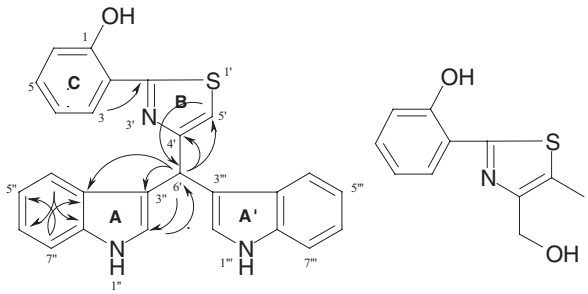
The ^{1}H-^{1}H COSY spectrum (Fig. S5) showed the expected correlations; the indole rings were further confirmed by HMBC cross signals (Fig. S6) from H-2″ (δ = 6.82) to C-3″, C-3a″, C-7a″, and C-6′, and from H-6′ (δ = 6.11) to C-2″, C-3″ and C-3a″ of the indole units (Fig. S7, fragment A). The HMBC correlations of methine H-6 (δ = 7.02) to C-2 and C-4, and from H-3 (δ = 7.62) to C-5 and the phenolic carbon C-1 (δ_C = 156.7) indicated the presence of a 2-substituted phenol (δ_{OH} = 12.43); H-3 showed an additional correlation with C-2′ (fragment C). The doublet of H-6′ at δ = 6.11 (J = 0.8 Hz) showed further couplings with C-4′ and C-5′; the H-5′ signal at δ = 6.89 (d, J = 0.7 Hz) correlated with C-2′, C-4′ and C-6′, giving substructures B1 or B2, which overlapped with atoms 6′ and 2′ in fragments A and C, respectively (Fig. S7). These correlations revealed that fragments A and C were connected indeed *via* a thiazole ring B1 or B2.

A distinction between the 2,4- and 2,5-disubstituted thiazoles B1 and B2 (Fig. S7) was not possible on the basis of 2D correlations. However, comparison with pulicatin C (**2**) [14] indicated a close similarity for the phenylthiazole part, and the indole signals matched those of vibrindole A (3,3′-bisindolylmethane) [15] perfectly (Table 1 and Fig. 2). As the shift of thiazol carbons are scarcely influenced by sp^3 C residues, the above spectral infor-

Table 1. ^{13}C NMR (125 MHz) and ^{1}H HMR (300 MHz) data of barakacin (**1**) and pulicatin (**2**) in $CDCl_3$[a]. The indole part of **1** was compared with vibrindole A (3,3′-bis-indolylmethane); δ values in ppm, J in Hz.

Position	Barakacin (**1**)		**2**
	δ_H (mult., J in Hz)	δ_C	δ_C
1	–	156.7	156.6
1-OH	12.43 (s)	–	–
2	–	117.2	115.4
3	7.62 (dd, 7.8, 1.5)	126.9	126.8
4	6.90 (td, 7.9, 1.1)	119.3	119.2
5	7.29 (td, 7.3, 1.6)	131.5	131.2
6	7.02 (dd, 8.3, 1.1)	117.6	117.5
2′	–	168.7	166.3
4′	–	157.9	149.5
5′	6.89 (d, 0.7)	113.1	128.6
6′	6.11 (d, 0.8)	36.6	58.5/28.0[b]
(NH)	7.94 (2H, s)	–	–
2″, 2‴	6.82 (2H, d, 1.7)	123.2	121.1
3″, 3‴	–	117.4	121.8
3a″, 3a‴	–	126.7	127.0
4″, 4‴	7.50 (2H, d, 7.9)	119.5	119.7
5″, 5‴	7.07 (2H, td, 7.1, 1.0)	119.4	119.0
6″, 6‴	7.20 (2H, td, 7.1, 1.1)	122.0	121.7
7″, 7‴	7.34 (2H, d, 8.1)	111.2	111.0
7a″, 7a‴	–	136.5	136.6

[a] Referenced to $CDCl_3$ with $\delta_H = 7.27$ and $\delta_C = 77.00$; [b] 58.5: C-6′ of **2**; 28.0: CH_2 and values below are of vibrindole A.



1 **2**

Fig. 2. HMBC correlations (→) connecting fragments A–C of barakacin (**1**) and the structure of pulicatin C (**2**).

mation and the molecular formula finally established the structure of barakacin as 2-{4-[bis-(1*H*-indol-3-yl)-methyl]-thiazol-2-yl}-phenol (**1**, Fig. 1). It is the first microbial thiazolyl-indolylmethane alkaloid.

Biological activity

The inhibitory effect of *P. aeruginosa* strain ZIO was investigated against a collection of human-pathogenic bacteria using the agar diffusion method (Fig. S8 and Table S1). The supernatant of the culture showed inhibition of a broad spectrum of Gram-positive and Gram-negative bacteria (Table S1). Although a pronounced antibacterial activity of many indole-alkaloids against Gram-positive bacteria has been reported, barakacin (**1**) was inactive at 40 μg per paper disc against the tested organisms. The strong activity of the crude extract of *P. aeruginosa* against the tested strains was due to phenazine-1-carboxylic acid.

Barakacin (**1**) showed, however, a weak and unselective cytotoxic activity against human cancer cell lines LXFA 629L, LXFL 529L (lung), MAXF 401NL (breast), MEXF 462NL (melanoma), RXF 944L (kidney), and UXF 1138 (uterus) with a mean IC_{50} value of 2.8 μg mL^{-1} (mean $IC_{70} = 5.4\,\mu$g mL^{-1}).

Experimental Section

For material and methods, see Ref. [16].

PY medium

A solution of 5 g peptone extract, 8 g yeast extract and 5 g NaCl in 1 l of tap water was set to pH = 7 with 2 N NaOH and sterilized for 30 min at 121 °C. For a solid medium, 18 g agar was added prior to sterilization.

Taxonomic characteristics of strain ZIO

The bacterial strain ZIO was isolated from ruminal material (obtained from a Tunesian cow) and maintained on PY agar. The strain was taxonomically affiliated on the basis of the sequence of the 16S rRNA gene. The strain has a 99.9% 16S rRNA gene sequence identity with the *Pseudomonas aeruginosa* strain NCM2.S1 (accession no. AP012280 of the whole genome) and the strain M18 (accession no. CP002496 of the whole genome). It is > 98% identical with the type strain of *Pseudomonas aeruginosa* (strain RH815, accession no. X06684). The strain ZIO is deposited in the microbial collection at the Institute of Organic and Biomolecular Chemistry, Georg-August University of Göttingen, Germany, with the voucher number ZIO.

Fermentation and isolation

The *P. aeruginosa* strain ZIO was pre-cultivated on PY agar at 37 °C. Pieces (1 × 1 cm) of well grown agar plates were used to inoculate 120 of 1-L Erlenmeyer flasks, each containing 250 mL of PY broth. The fermentation was carried out at 180 rpm on a linear shaker for 3 days at 37 °C. The brown culture broth was harvested and filtered after addition of 1 kg Celite to separate the bacterial biomass, which was extracted with ethyl acetate, while the culture filtrate

was passed through Amberlite XAD-16 adsorption resin. The XAD column was washed with 25 L demineralized water and eluted with 15 L methanol. The eluate was concentrated under reduced pressure, and the aqueous residue was finally extracted with ethyl acetate. The Celite/biomass mixture was extracted with ethyl acetate (3 times) and acetone (2 times); the combined organic phases were then evaporated to dryness. Both the water and the biomass extracts were combined based on their chromatographic similarity, yielding 10.8 g of a greenish-brown crude extract.

Chromatography on silica gel (column 3 × 100 cm) using a CH_2Cl_2-MeOH gradient (0 % – 60 % MeOH) monitored by TLC resulted in six fractions I–IV. Purification of fraction II using PTLC followed by Sephadex LH-20 afforded 25 mg of compound **1** as a yellow solid.

*Barakacin (**1**)*

Yellow solid, orange with anisaldehyde/H_2SO_4, red-violet with Ehrlich's reagent, R_f = 0.90 (CH_2Cl_2/2 % MeOH); 0.61 (CH_2Cl_2). – UV/Vis (MeOH): λ_{max} (log ε) = 221 (4.77), 283 (4.28), 290 (4.25), 326 nm (4.10); (MeOH/HCl): 220 (4.77), 283 (4.22), 290 (4.24), 344 nm (4.04); (MeOH/NaOH): 224 (4.76), 284 (4.21), 290 (4.18), 361 nm (3.92). – NMR data: see Table 1 and Figs. S3–S6 in the Supporting Information. – MS ((+)-ESI): m/z (%) = 865 (100) $[2\,M + Na]^+$, 444 (10) $[M + Na]^+$, 422 (15) $[M + H]^+$. – MS ((−)-ESI): m/z (%) = 841 (92) $[2\,M - H]^-$, 420 (100) $[M–H]^-$. – HRMS ((+)-ESI): m/z = 422.13214 (calcd. 422.13216 for $C_{26}H_{20}N_3OS$, $[M + H]^+$).

Supporting Information

Details of the fermentation, isolation, and TLC, ^{1}H NMR, ^{13}C NMR, COSY, and HMBC spectra including ^{1}H-^{1}H COSY and HMBC correlations, and details of the antimicrobial activity are provided as Supporting Information online only (http://www.znaturforsch.com/ab/v67b/c67b.htm).

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