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The First Hydroxylated Archazolid from the Myxobacterium *Cystobacter violaceus*: Isolation, Structural Elucidation and V-ATPase Inhibition

Dirk Menche, Jorma Hassfeld, Heinrich Steinmetz, Markus Huss, Helmut Wieczorek, Florenz Sasse

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Abstract The novel macrocyclic polyketide, 10-hydroxymethyl-archazolid-7-*O*- β -D-glucopyranoside (archazolid D), was obtained from the myxobacterium *Cystobacter violaceus*. The structure of this first hydroxylated archazolid was determined by spectroscopic analysis, in particular by HMBC, HMQC, and ROESY NMR investigations, and by degradation. This novel metabolite was evaluated for growth inhibition of murine connective tissue cells and V-ATPase inhibition in comparison to other known archazolids.

Keywords polyketide, V-ATPase inhibition, archazolid, myxobacterium, *Cystobacter violaceus*

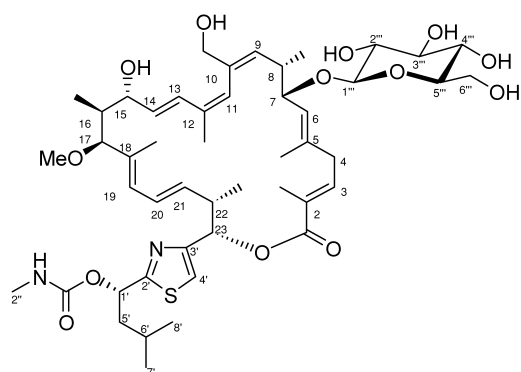
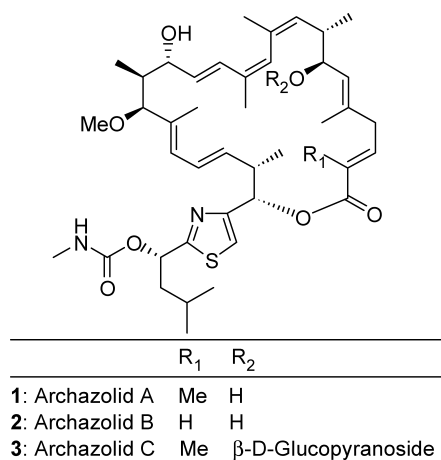
The development and molecular understanding of inhibitors for vacuolar-type ATPases (V-ATPases) presents an important research goal, as the functionality of these heteromultimeric, proton transporting proteins is associated with various diseases such as osteoporosis, renal acidosis and cancer [1]. The macrolide antibiotics archazolid A (**1**, Fig. 1) and B (**2**) [2] are a structurally novel type of

particularly efficient V-ATPase inhibitors with IC₅₀ values in the low nanomolar range, both *in vitro* [3] and *in vivo* [2]. It has been demonstrated that **1** binds to the membrane bound V_o subunit c in a reversible non-covalent manner [3]. This subunit forms an oligomer, building up a ring structure of six or more copies which transports protons across the membrane. Recently, the structure of the ring from a bacterial V-ATPase has been described at 2.1 Å resolution and showed that 10 copies form the c ring [4]. This increasing molecular understanding in combination with the very promising biological profile of the archazolids renders these macrolide antibiotics attractive structures for further development. Very recently, the full stereochemistry of the archazolids has been determined by extensive NMR studies and chemical derivatization [5], and was confirmed by total synthesis [6]. So far, however, only very limited SAR-data are available, relying on modifications of the hydroxyl groups at C-7, C-15 and C-1' [7, 8]. Herein, we describe the isolation and structure elucidation of the first archazolid with a modified carbon backbone, the hydroxylated derivative 10-hydroxymethyl-archazolid-7-*O*- β -D-glucopyranoside (archazolid D, **4**), from fermentation broths of the myxobacterium *Cystobacter violaceus* and report on the V-ATPase inhibitory effect of this novel macrolide antibiotic in comparison to other known V-ATPase inhibitors.

In a screening for novel and more efficient archazolid producing myxobacteria, we have recently identified *C. violaceus* strain Cb vi105 as the natural source of the first archazolid glycoside, archazolid-7-*O*- β -D-glucopyranoside (archazolid C, **3**, Fig. 1), which was obtained as the major metabolite from fermentation broths together with the parent compound archazolid A. A detailed analysis of this

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4: 10-Hydroxymethyl-archazolid-7-O-β-D-glucopyranoside (Archazolid D)

Fig. 1 The archazolids: potent V-ATPase inhibitors from myxobacteria.

extract for trace metabolites by LC-MS in combination with UV-VIS spectra suggested one further archazolid derivative to be present in traces, i.e. by a characteristic UV maximum around 230 nm. As this metabolite was only produced in minute amounts (<0.03 mg/liter), a 300-liter bioreactor of *C. violaceus* strain Cb vi105 was run. Fermentation was performed in the presence of 3 liters Amberlite XAD for absorption of excreted secondary metabolites. After 10 days, the adsorber resin and cell mass (4.9 kg) were obtained by centrifugation and subsequently extracted with acetone to give 13.7 g of crude extract. Consecutive gel chromatography (Sephadex LH-20), MPLC and reversed-phase HPLC yielded 4.2 mg of the novel derivative.

The ¹H NMR data of **4**, as shown in Table 1, revealed a similar pattern to that of the known archazolids: Ten one-proton signals in the unsaturated region with the expected coupling constants for 11-H, 4'-H, 6-H, 9-H, 13-H, 19-H, 3-H, 14-H, 20-H and 21-H and five further low-field

Table 1 ¹H- and ¹³C-NMR data for 10-hydroxymethyl-archazolid-7-O-β-D-glucopyranoside (**3**)^a

No.	¹³ C ^b	¹ H mult., J (Hz)
1	168.4	—
2	130.1	—
3	142.1	6.76 t, 7.4
4	40.9	2.84 m/3.06 dd, 14.0, 9.4
5	136.2	—
6	131.0	5.21 d, 9.1
7	74.0	4.09 t, 9.63
8	40.4	2.38 ddq, 9.4, 9.1, 6.8
9	133.9	5.59 d, 9.8
10	138.0	—
11	127.4	5.76 s
12	133.3	—
13	131.2	6.94 br m
14	131.0	5.66 d, 12.8
15	74.3	4.70 br s
16	42.8	1.73 m
17	89.3	3.58 m
18	135.9	—
19	131.0	5.88 d, 10.9
20	127.2	6.25 t, 12.6
21	134.7	5.63 m
22	41.0	3.16 br m
23	73.5	6.03 br s
2-CH ₃	12.9	1.93 s
5-CH ₃	16.7	1.73 s
8-CH ₃	17.5	0.82 d, 6.4
10-CH ₂ OH	68.2	3.93 d, 12.8/4.04 d, 12.8
12-CH ₃	19.9	1.97
16-CH ₃	12.5	0.74 d, 7.2
17-OCH ₃	56.4	3.20 s
18-CH ₃	11.8	1.65 s
22-CH ₃	16.9	1.23 d, 6.8
1'	77.7	6.03 dd, 8.3, 4.5
2'	174.3	—
3'	156.3	—
4'	116.4	7.15 s
5'	46.1	1.84/1.96
6'	26.5	1.81 m
7'	22.5	1.03 d, 6.0
8'	23.5	1.03 d, 6.0
1''	158.2	—
2''	27.7	2.75 s
1'''	102.2	4.23 d, 7.9
2'''	75.5	3.29 dd, 8.3, 7.9
3'''	77.9	3.34 m
4'''	71.9	3.38 m
5'''	77.8	3.21 m
6'''	62.8	3.74 dd, 11.5, 5.1/3.86 dd, 11.5, 2.5

^a Spectra were recorded in CD₃OD at 600 (¹H NMR) and 150 MHz (¹³C).

^b The ¹³C assignments were achieved by HMQC and HMBC experiments.

protons for 7-H, 15-H, 17-H, 23-H and 1'-H together with further one-proton signals in the aliphatic region (chemical shift=3 ppm) for the macrocyclic protons 4a-H, 4b-H, 8-H, 16-H, 22-H, 5'-H and 6'-H indicated a poly-unsaturated backbone closely related to **1**. Remaining ^1H NMR signals were observed in the region from δ 3.00 to 4.00, which were very similar to those of **3** and likewise suggested the presence of a hexose substituent. The *O*-glycosidic nature of this compound became also evident from a doublet at δ 4.23 (7.9 Hz), attributed to an anomeric proton (1'''-H). The signals for 2'''-H to 5'''-H were resolved in CD_3OD and assigned to glucose. The ^{13}C NMR data displayed also the expected number of carbons and chemical shifts for glucose [9], which was further confirmed by ROESY interactions and ultimately proven by acidic hydrolysis and detection as the per-TMS-silylated methyl-glycoside by GC-comparison according to the method of Chaplin [10]. The position of the glucose unit was deduced by ROESY experiments (interactions of 1'''-H with 7-H and 8-H) and by a long-range C-H correlation from (1'''-H to C-7). Therefore, the sugar unit is situated at position O-7.

The molecular ion peak for the novel metabolite was observed at m/z 916, which is 16 mass units higher than that of **3** (MW=900) and hinted at the presence of an additional oxygen atom. Furthermore, a noticeable difference in the proton NMR spectrum of **4** was the absence of one three-proton signal for an allylic methyl group. Only four-instead of five-singlets (/doublets with a small w-coupling constant of smaller than 2 Hz) at δ =1.93, 1.73, 1.97 and 1.65 were observed, which were assigned to the allylic methyl groups at C-2, C-5, C-12 and C-18 by COSY and HMBC correlations. These data suggest that **4** is an oxygenated archazolid and contains an allylic hydroxymethyl group. Based on HMBC and ROESY correlations, this hydroxymethyl group is situated at C-10, and the new hydroxylated archazolid has the constitution shown in Fig. 2. Its structure was further confirmed by TOCSY experiments and HMBC and HMQC interactions. For the new hydroxylated archazolid, we suggest the name archazolid D. The close similarity of the spectroscopic data of **4** to **3** suggests this novel analogue to reside in a solution conformation closely related to that of the other archazolids [5, 7].

For biological evaluation of the novel analogue, first the inhibitory effect on the growth of the murine connective tissue cell line L-929 was tested. As expected for a 7-*O*-substituted analogue, **4** was less potent (IC_{50} =330 nM) as compared to the parent natural products **1** and **2** (IC_{50} =0.81/1.1 nM), however it was 5 times more active than the corresponding non-hydroxylated congener **3** (IC_{50} =1600 nM). This similarity in biological activity already suggested

that **4**, like other members of the archazolid family, should target V-ATPases. Indeed, by using established procedures it was shown that the purified V-ATPase holoenzyme from the midgut of the tobacco hornworm was inhibited with an IC_{50} value of 1.2 $\mu\text{mol}/\text{mg}$ enzyme [7, 11, 12]. Thus, **4** is much less inhibitory than **1** and **2** with an IC_{50} value of 0.6 nmol/mg enzyme. Nevertheless it inhibits the V-ATPase, albeit in the micromolar instead of the nanomolar range.

In summary, we have reported the first hydroxylated archazolid, 10-hydroxymethyl-archazolid-7-*O*- β -D-glucopyranoside (named archazolid D, **4**) from the myxobacterium *C. violaceus*. As compared to **1** and **2**, **4** exhibits only moderate activity against the V-ATPase *in vitro* and *in vivo*. However, in the cell culture assays it was shown to be more potent than the corresponding non-hydroxylated analogue, **3**. These results suggest that it might be possible to increase the potency of the non-glycosylated archazolids by structural modifications of the macrocyclic backbone and makes it very rewarding to develop synthetic analogues along these lines.

Experimental

General Experimental Procedures

Optical rotations were determined on a Perkin-Elmer 241 instrument. UV spectra were recorded on a Shimadzu UV-2102 PC scanning spectrometer. IR spectra were measured with a Nicolet 20DXB FT-IR spectrometer. NMR spectra were recorded in CD_3OD on a Bruker DMX-600 spectrometer. EI and DCI mass spectra (reactant gas ammonia) were obtained on a Finnigan MAT 95 spectrometer, high resolution data were acquired using peak matching (M/DM =10000). Pure compounds were characterized by analytical HPLC on Nucleodur C 18 (column 125 \times 2 mm, 5 μm , methanol/water=82:18, flow 0.3 ml/minute, diode array detection). The retention times for **1**, **3** and **4** were 11.8, 6.3 and 4.8 minutes.

Production of the Novel Archazolid **4**

Fermentation of *C. violaceus* strain Cb vi105, isolated at the HZI, in the presence of 3 liter Amberlite XAD-16 adsorber resin for 10 days at 30°C and preparation of the crude extract (13.7 g) from the adsorber resin proceeded according to previously reported procedures [2, 7]. **4** was isolated and purified from the crude extract by gel chromatography on Sephadex LH 20 (Fluka Steinheim, solvent: methanol, flow 7 ml/minute), reversed-phase MPLC (solvent: methanol/water=8:2, detection 230 nm, flow 65 ml/minute) and high pressure RP chromatography

(solvent: acetonitrile/water=65/35, detection 230 nm, flow 15 ml/minute). In total, 4.2 mg were obtained.

Physicochemical Properties of 4

10-Hydroxymethyl-archazolid-7-*O*- β -D-glucopyranoside (archazolid D, **4**): colourless amorphous solid; $[\alpha]_D^{22} -26.8^\circ$ (*c* 0.22, MeOH); IR ν_{\max} (film) cm^{-1} 3355, 2926, 1715, 1080; UV $\lambda_{\max}^{\text{MeOH}}$ 231 nm; NMR see Table 1; HR-MS 917.4860 $[(M+H)]^+$, $\text{C}_{48}\text{H}_{73}\text{N}_2\text{O}_{13}\text{S}$ requires 917.4833.

Cell Culture and Growth Inhibition Assay

The L-929 mouse cell line was from the German collection of Microorganisms and Cell Cultures (DSMZ) and cultivated in DME medium (GIBCO BRL) plus 10% newborn calf serum at 37°C and 10% CO_2 in a moist atmosphere. Growth inhibition was measured on microtiterplates. Aliquots of 120 μl of the suspended cells ($50,000 \text{ ml}^{-1}$) were given to 60 μl of a serial dilution of the inhibitor. After 5 days, metabolic activity per well was determined using the MTT assay [13].

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