Supplemental Material for

Biosynthesis of the Mitochondrial Adenine Nucleotide Translocase (ATPase) Inhibitor Bongkrekic Acid in *Burkholderia gladioli*

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Experimental

General

HPLC-MS measurements were recorded employing a Thermo Electron Surveyor HPLC with PDA and a reversed phase C18 column (Nucleosil 100, 5 µm, 125x4,6 mm) with gradient elution (MeCN/0.1 % (v/v) HCOOH 2/98 in 30 min to MeCN/0.1 % (v/v) HCOOH 98/2; flow rate 0.6 mL min^{-1}) coupled with a Finnigan LCQ benchtop mass spectrometer with an electrospray ion source and ion trap mass analyzer. HRESI-MS were recorded on a TSQ Quantum AM Ultra, Thermo Electron, Bremen. Analytical HPLC was performed on a Shimadzu HPLC system consisting of an autosampler, high pressure pumps, column oven and DAD. HPLC conditions were: C18 column (Nucleosil 100, 5 µm, 125x4.6 mm) and gradient elution (MeCN/0.1 % TFA 0.5/99.5 in 30 min to MeCN/0.1 % TFA 100/0, MeCN 100 % for 5 min), flow rate 1 mL min^{-1}. Preparative HPLC was performed on a Shimadzu HPLC system with a diode array detector using a Macherey-Nagel C18-RP column (Nucleosil 100, 5 µm, 250x20 mm; flow rate 10 mL min^{-1}, detection at 270 nm). NMR spectra were recorded on Bruker Avance DRX 500 and DRX 600 instruments. Spectra were referenced to the residual solvent signals. ^{13}C-contents were calculated based on the natural ^{13}C-content (1.1%) of unlabelled carbons. The signals of carbons in the ^{13}C spectrum of unlabelled **1** were integrated via Lorenz distribution (MestReC 4.9.9.6 software, Mestrelab Research). The obtained area was relatively referred to the area of an unlabelled carbon in the ^{13}C spectrum (17a for ^{13}C labelled acetic acid-NAC-thioester-feeding and 21a for 1-^{13}C-methyl-methionine-feeding) of unlabelled **1**.

$$\frac{AulR}{Aulx} = RF$$

The obtained response factor RF was used for calculation of the ^{13}C incorporation (% ^{13}C) of each signal in the spectrum of labelled **1**.

$$\frac{AlR}{1.1\%} = \frac{Alx * RF}{X}$$

Abbreviations:
AulR: area of the reference carbon in the ^{13}C spectrum of unlabelled **1**
AlR: area of the reference carbon in the ^{13}C spectrum of labelled **1**
Aulx: area of desired carbon in the ^{13}C spectrum of unlabelled **1**
Alx: area of desired carbon in the ^{13}C spectrum of labelled **1**
1.1 %: relative natural occurence of ^{13}C
X: % ^{13}C of desired carbon

Cultivation and extraction

B. gladioli HKI 10521 (DSM 11318) was grown on coconut medium for 7 days. Composition of medium was: commercial coconut shreds (15 g), and glycerol (Roth, 2 mL) were mixed with potato-dextrose-broth (Difco) and placed in 500 mL Erlenmeyer flasks. Amberlites XAD16 (5 g, Sigma-Aldrich) were added. After sterilization 15 flasks were inoculated with bacteria supsension (each 2.5 mL, 48 h grown on TSB medium at 28 °C and 100 rpm orbital shaking) and incubated at 28° C for 7 days at 60 rpm orbital shaking. The cultures were pooled and exhaustively extracted with ethyl acetate (4 h with 1:1.5). The combined extracts were concentrated under reduced pressure.

Isotope labelling experiments

Coconut medium (90 mL) was inoculated with *B. gladioli* as described above. Labelled compounds (dissolved in water) were added through a sterile syringe filter. Both pulse- and bolus-feeding modes were employed. In the bolus-feeding mode, the whole amount of substance was added at the beginning of fermentation (T = 0 h). In pulse-feeding mode, substances were added in equal parts at T = 0, 8, 22 and 34 h. 1-^{13}C-sodium acetate (99%, Sigma-Aldrich) and 1,2-^{13}C-sodium acetate (99%, Cambridge Isotopes Laboratories) were added at a final concentration of 13.2 mM, 2-^{13}C malonic acid (99%, Cambridge Isotopes Laboratories) at 5.2 mM, 1-^{13}C-propionate (99%, Cambridge Isotopes Laboratories) at 14.7 mM, 1-^{13}C-methyl-methionine (99%, Cambridge Isotopes Laboratories) at 1.85 mM, and single and double ^{13}C labelled acetic acid-NAC-thioester at 0.9 mM.

Inhibition of β-oxidation

For inhibiton of β-oxidation, acrylic acid was dissolved in sterile destilled water (250 µL) and added to a final concentration of 3 mM in the media.

Synthesis of ^{13}C labelled acetic acid-NAC-thioester

1,2-^{13}C-acetic acid (224 µL, 3.79 mmol, 99%) was mixed with dichloromethane (10 µL) and cooled to 0 °C. EDC x HCl (799 mg, 4.17 mmol,), dimethylaminopyridine (DMAP, 93 mg, 0.76 mmol) and *N*-acetyl cysteamine (HSNAC, 676 µL, 5.69 mmol, 95%) were added and the solution was stirred for 10 min at 0 °C. After 3 d stirring at room temperature, the reaction was stopped by adding saturated NH_4Cl-solution (ca. 10 mL) and the reaction mixture was extracted with dichloromethane (5 x 20 mL). The solvent was completely removed under reduced pressure and redissolved in 2 mL dichloromethane. Further purification was accomplished by filtration over a $CuSO_4$-impregnated silica gel column. The column was washed with dichloromethane (200 mL). The crude product was purified by open column chromatography on silica gel using chloroform/methanol (98:2) as eluent. Yield was: 68%.

^{1}H NMR (300 MHz; $CDCl_3$): δ = 5.92 (br s, ^{1}H, C**H**$_2$NH), 3.4 (m, 1H,C**H**$_2$NH); 3.02-2.96 (m, 1H, C**H**CH$_2$NH); 2.32 (dd, 3H, $^{1}J(^{1}H,^{13}C)$ = 130.1 Hz, $^{2}J(^{1}H,^{13}C)$ = 6.2 Hz, ^{13}**C**H$_3$) ppm.

^{13}C NMR (300 MHz; $CDCl_3$): δ = 196.3 (d, $^{1}J(^{13}C\text{-}^{13}C)$ = 47 Hz, ^{13}**C**=O), 170.2 (s, **C**=O), 39.5 (s, CH$_2$**C**H$_2$NH), 30.6 (d, $^{1}J(^{13}C\text{-}^{13}C)$ = 47 Hz, ^{13}**C**H$_3$), 28.7 (s, S**C**H$_2$CH$_2$), 23.2 (s, C=O**C**H$_3$) ppm.

Isolation of bongkrekic acid

The oily residue of an extracted *B. gladioli* culture (extraction with ethyl acetate as described above) was reextracted with methanol (approximately 2 vol eq.) at pH 4 for 4 h. The samples were cooled to 4 °C until the coconut fat solidified. The methanolic phase was separated from the fat and concentrated under reduced pressure. The concentrate was subsequently extracted with chloroform and the solvent concentrated again under reduced pressure. The samples were further purified by preparative RP-HPLC (gradient mode MeCN/0.1% TFA 50/50 in 30 min to MeCN/0.1% TFA 83/17, MeCN 83% for 5 min). The presence of **1** was monitored by HPLC-MS. Isotope labelled **1** was identified by mass spectrometry and ^{13}C NMR spectroscopy in comparison to unlabelled BA.

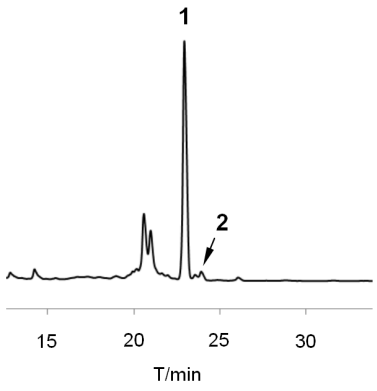

Figure S1. HPLC profile of crude extract of a *B. gladioli* culture in coconut medium (7d); **2**: isobongkrekic acid.

Table S1. ^{13}C-contents after feeding 1-^{13}C-methyl-methionine.

Position	**% ^{13}C**
6a	21.5
17-OCH_3	24.6
18a	22.2

Table S2. Analysis of NMR data of **1**.

Position	$\delta^{13}C$ [ppm]	$\delta^{1}H$ [ppm]	Multiplicity	*J* [Hz]
1	177.0			
2	40.7	α: 3.44	d	16.0
		β: 3.30		
3	148.9			
4	125.2	7.41	d	16.0
5	145.5	6	dd	8.2; 16.0
6	38.8	2.33	m	
6a	20.4	1.22	d	6.7
7	40.0	α: 2.14	m	
		β: 1.89	m	
8	128.1	5.3	partial overlap with position H-15	
9	131.5	5.39	partial overlap with position H-15	
10	32.5	2.02	m	
11	33.1	2.09	m	
12	135.9	5.72	partial overlap with position H-23	
13	124.6	6.25	dd	11.8; 14.7
14	131.3	6.04	partial overlap with position H-5	
15	124.3	5.34	partial overlap with positions H-9 and H-8	
16	32.1	α: 2.42	m	
		β: 2.25	m	
17	79.9	4.31	dd	5.0; 9.1
17-OCH_3	56.7	3.19	s	
18	148.5			
18a	18.6	1.88	s	
19	124.1	6.23	d	12.1
20	134.5	7.61	d	12.2
21	125.1			
21a	11.7	1.89	s	
22	174.7			
23	118.3	5.69	s	
24	170.9			

Table S3. Calculated ^{13}C contents and couplings constants of the satellite signals after feeding 1,2-^{13}C-acetyl-SNAC and acrylic acid. *Reference used for calculation of ^{13}C-contents. ** Low signal intensity.

position	%^{13}C	J_{cc} in HZ
1	1.34	53.1**
2	1.33	53.2
3	1.67	52.6
4	1.35	51.0
5	1.72	43.1
6	1.32	43.4
6a	1.1*	-
7	1.24	44.0
8	1.26	44.5
9	1.49	41.4
10	1.22	42.8
11	1.29	43.2
12	1.61	42.6
13	1.38	54.7
14	1.79	56.3
15	1.43	39.9
16	1.36	41.6
17	1.65	44.4
17-OCH_3	1.08	-
18	1.93	44.1
18a	1.18	-
19	1.29	57.2
20	1.58	57
21	1.5	70.2
21a	1.89	-
22	1.58	70.2
23	1.26	72.6
24	(1.19)	-

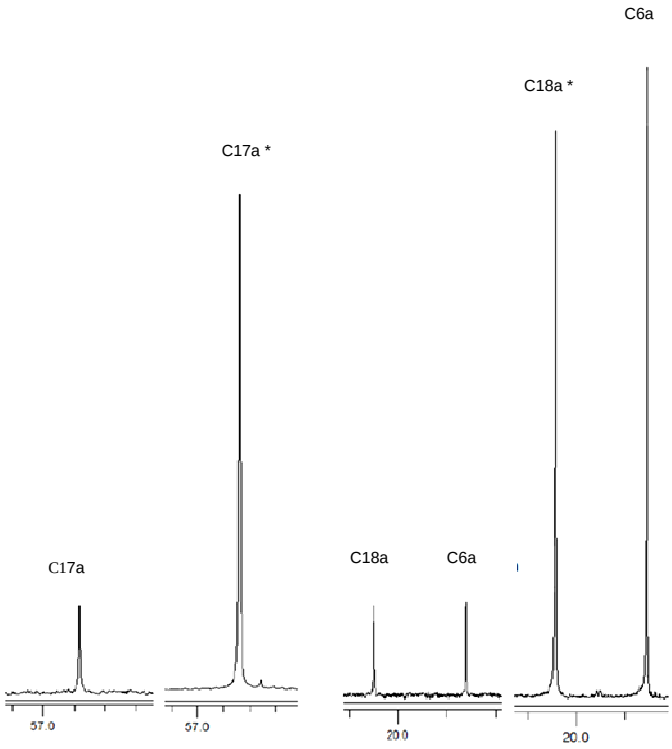

Figure S2. ^{13}C NMR signals of ^{13}C-methionine-enriched carbons (*) of **1** in comparison with reference signals.

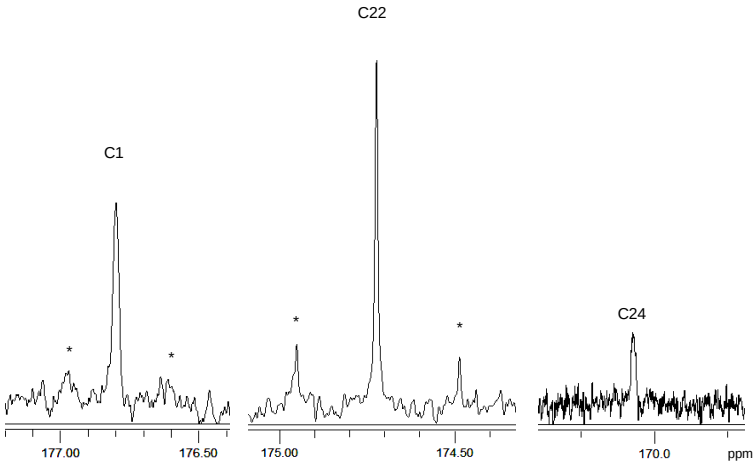

Figure S3. ^{13}C NMR signals and satellites of 1,2-^{13}C-acetate-enriched carbons of **1**.

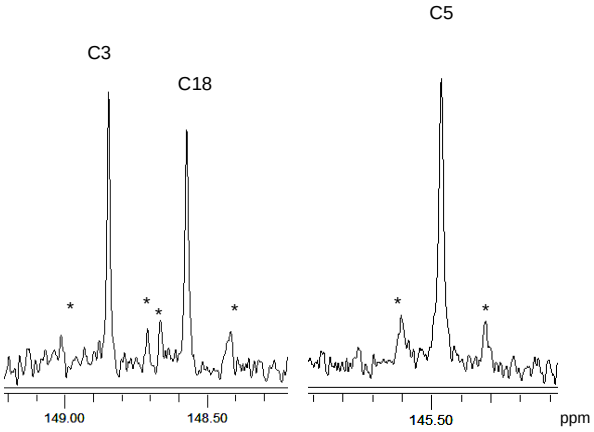

Figure S4. ^{13}C NMR signals and satellites of 1,2-^{13}C-acetate-enriched carbons of **1**.

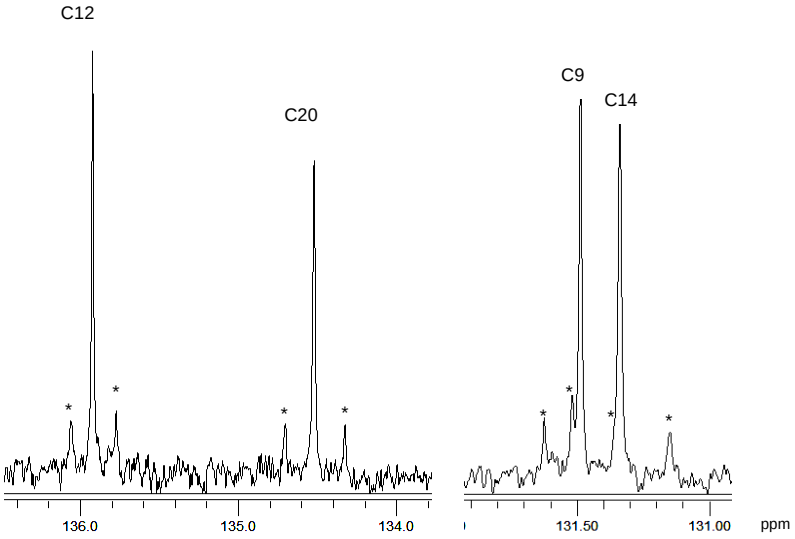

Figure S5. ^{13}C NMR signals and satellites of 1,2-^{13}C-acetate-enriched carbons of **1**.

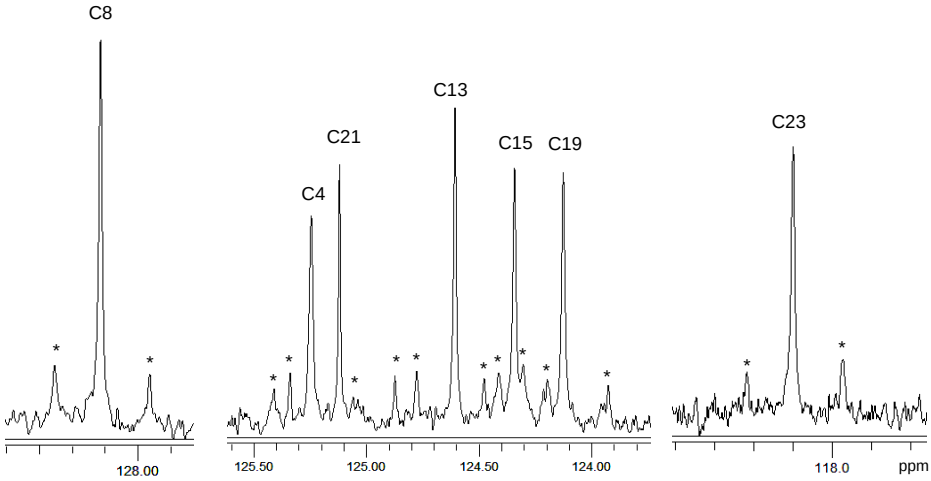

Figure S6. ^{13}C NMR signals and satellites of 1,2-^{13}C-acetate-enriched carbons of **1**.

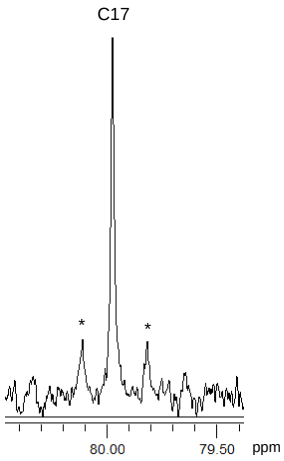

Figure S7. ^{13}C NMR signals and satellites of 1,2-^{13}C-acetate-enriched carbons of **1**.

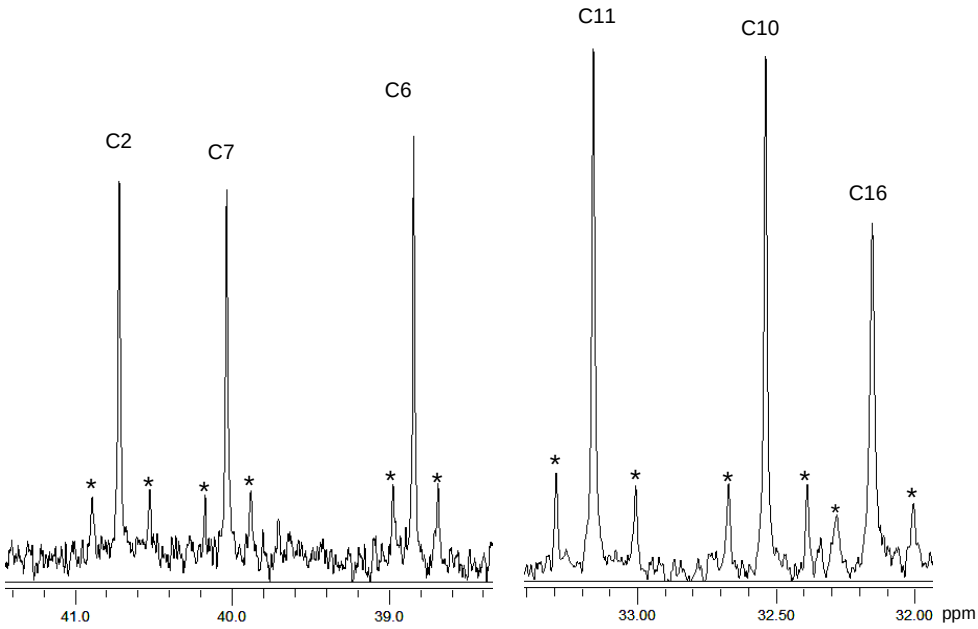

Figure S8. ^{13}C NMR signals and satellites of 1,2-^{13}C-acetate-enriched carbons of **1**.

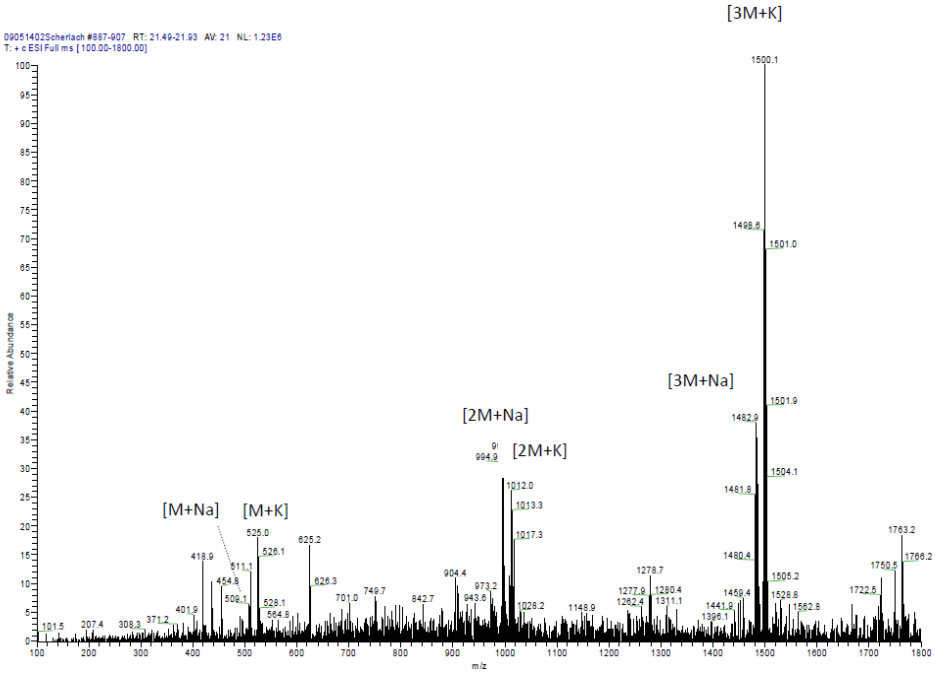

Figure S9. MS showing of ^{13}C-enriched **1** (from ^{13}C-Met labelling)