ChemComm

Supporting Information (SI)

for

In vitro Chemoenzymatic and *In vivo* Biocatalytic Syntheses of New Beauvericin Analogues

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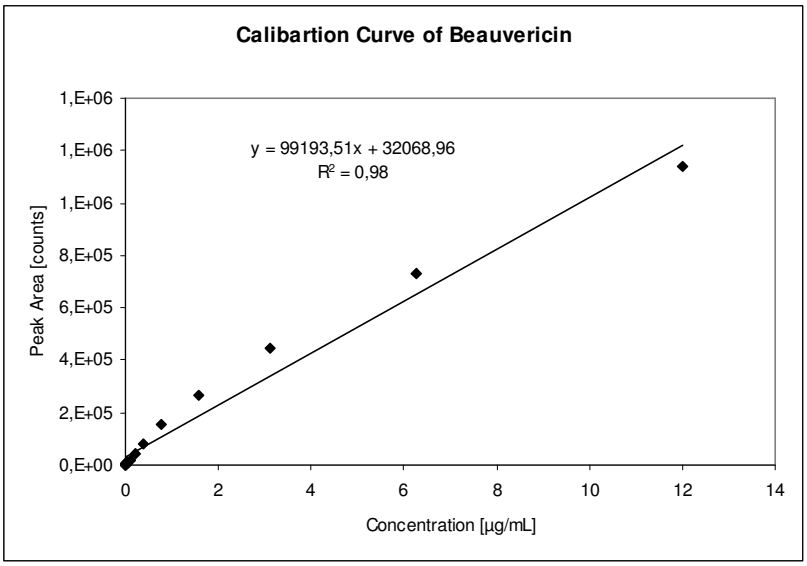
1. General techniques

Starter cultures of *E. coli bbBeas*+ expression strains were grown overnight at 37°C in LB medium (3 ml) with shaking at 200 rpm. Main stage cultures (200 ml, LB medium) were inoculated with 3 ml of the starter culture, grown at 37°C with shaking at 200 rpm to an OD_{600} of 1.0, and transferred to 16°C for induction with IPTG (200 µM, final concentration). Incubation with shaking at 200 rpm was continued for 16 hr at 16°C. Chloramphenicol (40 mg/ml, final concentration) was supplemented to all cultures. The cells were collected by centrifugation, and resuspended in fresh LB medium (20 ml) supplemented with D-Hiv and L-Phe (each at 15-30 mM, final concentrations). The resultant biotransformation reactions were incubated with shaking at 200 rpm for 48 hr at 16°C. The expression level of BbBEAS was ~0.4 mg/mL.

Quantification of *in vivo* analogue-production was performed by LC-ESI-MS/MS using an Agilent 6410 Triple Quadrupole LC/MS system in the Multiple Reaction Monitoring (MRM) mode. A 12 µg/mL stock solution of beauvericin from Sigma-Aldrich (purity ≥97 % by HPLC) was prepared in 100 % MeOH and 2x stepwise dilutions were made with MeOH to obtain the desired concentrations for the calibration standards. The concentrations of the beauvericin calibration standards ranged from 0.0015 to 12 µg/mL. Three replicates of each standard were analyzed and calibration curves, constructed by linear regression analysis, were plotted as the average peak area versus beauvericin concentrations. Results for the calibration curve showed good linearity with R^2 = 0.98. Calibration curve equations were used to calculate the concentrations of beauvericin in the samples based on their peak areas.



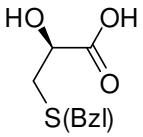
For the quantification of beauvericin analogues, cells were collected from the biocatalytic reactions by centrifugation at 4500 rpm at 4 °C for 15 minutes as described earlier. The resulting pellet was extracted with MeOH and the organic phase was collected and evaporated to dryness in a SpeedVac Vacuum Evaporation System at 45 °C. Reconstitution was done with 1 mL of MeOH, and the resulting sample was injected into the LC-ESI-MS/MS system.

Thin layer chromatography (TLC) was performed using TLC plates purchased from *Merck* (Silica gel 60, F_{254}, coating thickness 0.2 mm). The compounds were identified by radiochemical detection. The deuterated solvent for NMR-spectroscopy was dimethylsulfoxide-d_6 (99.8%), purchased from *Deutero GmbH* (Kastellaun, Germany). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a *Bruker Avance* 400 NMR-spectrometer. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; band, several overlapping signals; b, broad. Optical rotation was recorded on a p-2000 polarimeter from *Jasco Labor- und Datentechnik GmbH* (Groß-Umstadt, Germany). High-resolution mass-spectroscopy (HRMS) using ESI- and EI-techniques was performed on a LTQ Orbitrap XL apparatus by *Thermo Scientific* (Waltham, MA, USA).

2. Synthesis of α-D-hydroxy acids

The following compounds were synthesized according to published methods:
1-5, 7-9, 11, 13-33[1]; 10[2]; 6[3].

For the following substrates, synthesis followed the general procedures of Feifel *et al.* [3] and Müller *et al*.[1] Briefly, a solution of sodium nitrite (6.0 equiv) in H_2O (1.35 mL/mM) was added slowly to a solution of α-D-amino acid (1.0 equiv) in 0.5 M H_2SO_4 (2 mL/mM, 2.0 equiv) at 0°C. The reaction was stirred at 0°C for 3h and was then allowed to warm up to room temperature while stirring for 24h. The reaction mixture was extracted three times with diethyl ether (5mL/mM). The combined organic layers were washed with brine (5 mL/mM), dried over Na_2SO_4, filtered and concentrated to afford the product as the α-D-hydroxy carboxylic acid.



3-(Benzylthio)-*(R)*-2-hydroxy-propionic acid (36). D-Cysteine (Bzl)-OH (1000 mg, 4,7 mmol), 0.5 M H_2SO_4 (9.5 mmol, 19 ml), $NaNO_2$ (1958 mg, 28.4 mmol) in 6.4 ml H_2O.

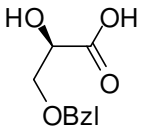
Yield: 57%; white solid.

Opt. rotation $[\alpha]_D^{20}$ = -1.2 (c = 0.66, MeOH)

^{1}H-NMR δ_H (400 MHz; DMSO-d_6) 2.58 (dd, *J* = 13.57, 6.85 Hz, 1H), 2.69 (dd, *J* = 13.60, 6.92 Hz, 1H), 3.64 (s, 2H), 4.12 (dd, *J* = 6.85, 4.84 Hz, 1H) 7.23 – 7.34 (m, 5H)

^{13}C-NMR δ_C (100 MHz; DMSO-d_6) 34.8, 35.2, 70.3, 126.8, 128.4, 128.4, 128.9, 138.3, 174.1.

HRMS (ESI) m/z calculated for $C_{10}H_{11}O_3S$ (M-H) 211.04344; detected 211.04242.



3-(Benzyloxy)-*(R)*-2-hydroxy-propionic acid (35). D-Serine (OBzl)-OH (2093 mg, 10 mmol), 0.5 M H_2SO_4 (20 mmol, 40 ml), $NaNO_2$ (4139 mg, 60 mmol) in 13.5 ml H_2O.

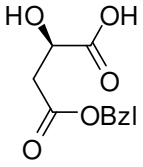
Yield: 92%; white solid.

Opt. rotation $[\alpha]_D^{20}$ = -8.3 (c = 0.72, MeOH)

^{1}H-NMR δ_H (400 MHz; DMSO-d_6) 3.60 (d, *J* = 4.30 Hz, 2H), 4.15 (t, *J* = 4.43 Hz, 1H), 4.49 (d, J = 5,40, 2H) 5.33 (br s, 1H), 7.25 – 7.35 (m, 5H)

^{13}C-NMR δ_C (100 MHz; DMSO-d_6) 70.0, 72.0, 72.3, 127.4, 127.5, 128.2, 138.3, 173.8.

HRMS (ESI) m/z calculated for $C_{10}H_{11}O_4$ (M-H) 195.06628; detected 195.06538.



3-(Benzyloxy)-*(R)*-2-hydroxy-4-oxo-butyric acid (37). D-Aspartate (OBzl)-OH (2233 mg, 10 mmol), 0.5 M H_2SO_4 (20 mmol, 40 ml), $NaNO_2$ (4139 mg, 60 mmol) in 13.5 ml H_2O.

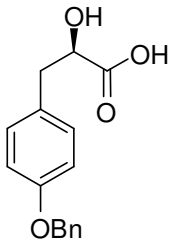
Yield: 89%; white solid.

Opt. rotation $[\alpha]_D^{20}$ = +6.9 (c = 1.2, MeOH)

^{1}H-NMR δ_H (400 MHz; DMSO-d_6) 2.60 (dd, *J* = 15.58, 7.93 Hz, 1H), 2.76 (dd, *J* = 15.58, 7.93 Hz, 1H), 4.31 (dd, *J* = 7.79, 4.84 Hz, 1H),5.10 (s, 2H), 5.57 (br s, 1H), 7.30 – 7.38 (m, 5H)

^{13}C-NMR δ_C (100 MHz; DMSO-d_6) 38.9, 65.6, 66.9, 127.8, 127.9, 128.4, 136.1, 170.2, 174.3.

HRMS (ESI) m/z calculated for $C_{11}H_{11}O_5$ (M-H) 223.06120; detected 223.06024.



1-Hydroxy-1-cyclohexane carboxylic acid (34). 1-Amino-1-cyclohexane carboxylic acid (1000 mg, 3.7 mmol), 0.5 M H_2SO_4 (7.44 mmol, 14.8 ml), $NaNO_2$ (1523 mg, 22.1 mmol) in 5 ml H_2O.

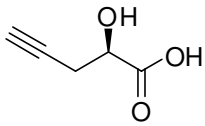
Yield: 79%; white solid.

Opt. rotation $[\alpha]_D^{20}$ = +13.1 (c = 1.05, MeOH)

^{1}H-NMR δ_H (400 MHz; DMSO-d_6) 2.70 (dd, *J* = 13.84, 8.06 Hz, 1H), 2.87 (dd, *J* = 13.84, 4.57 Hz, 1H), 4.07 (dd, *J* = 8.06, 4.57 Hz, 1H), 5.05 (s, 2H), 5.06 (s,1H), 6.88-6.92 (m,2H), 7.12-7.17 (m,2H), 7.32 – 7.43 (m, 5H)

^{13}C-NMR δ_C (100 MHz; DMSO-d_6) 40.2, 69.1, 71.2, 114.3, 114.7, 127.6, 127.7, 128.4, 130.2, 130.4, 137.3, 156.8, 175.2.

HRMS (ESI) m/z calculated for $C_{16}H_{15}O_4$ (M-H) 271.09758; detected 271.09631.



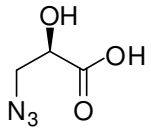
***(R/S)*-2-Hydroxy-4-pentynoic acid (12)**. (*DL*)-2-Amino-4-pentynoic acid (500 mg, 4.4 mmol), 0.5 M H_2SO_4 (8.8 mmol, 22.5 ml), $NaNO_2$ (1830 mg, 26.5 mmol) in 7.6 ml H_2O.

Yield: 97%; brown oil.

^{1}H-NMR δ_H (400 MHz; DMSO-d_6) 2.32-2.45 (m, 2H), 2.75 (t, *J* = 2.69 Hz, 1H), 4.05 (dd, *J* = 6.45, 5.37 Hz, 1H)

^{13}C-NMR δ_C (100 MHz; DMSO-d_6) 24.2, 68.8, 72.7, 81.0, 174.0.

HRMS (ESI) m/z calculated for $C_5H_5O_3$ (M-H) 113.02442; detected 113.02429.



***(R)*-2-Hydroxy-3-azidopropanoic acid (38)**. D-Azido-alanine hydrochloride salt (500 mg, 3.0 mmol), 0.5 M H_2SO_4 (6.0 mmol, 23 ml), $NaNO_2$ (1250 mg, 18.0 mmol) in 8 ml H_2O.

Yield: 46%; brown oil.

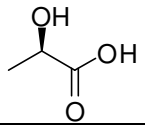
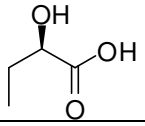
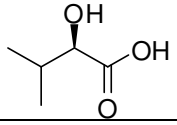
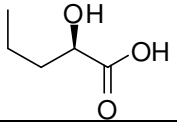
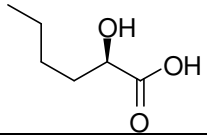
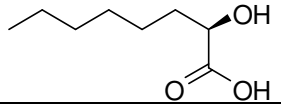
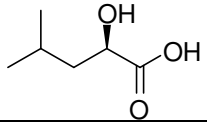
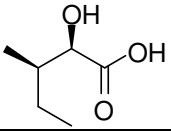
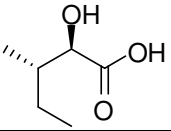
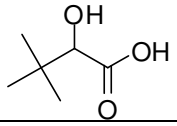
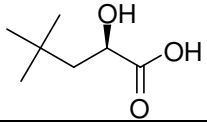
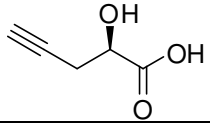
Opt. rotation $[\alpha]_D^{20}$ = +72.5 (c = 0.08, MeOH)

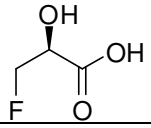
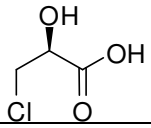
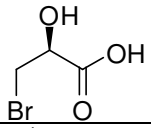
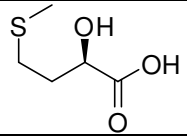
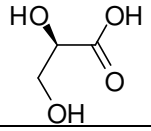
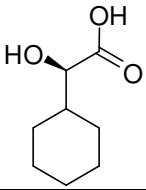
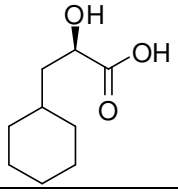
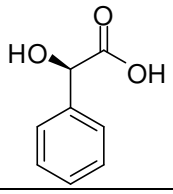
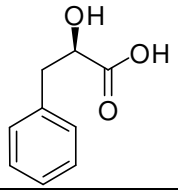
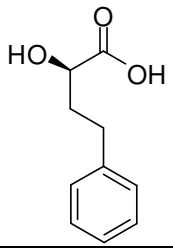
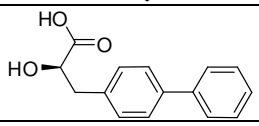
^{1}H-NMR δ_H (400 MHz; DMSO-d_6) 3.38 (dd, *J* = 12.93, 5.88 Hz, 1H), 3.47 (dd, *J* = 12.93, 3.64 Hz, 1H), 4.20 (dd, *J* = 5.88, 3.60 Hz, 1H)

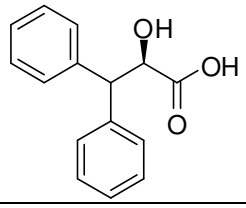
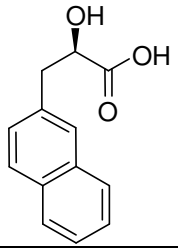
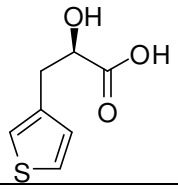
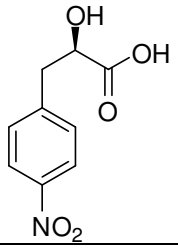
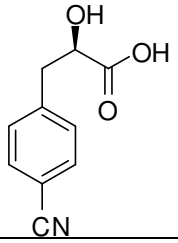
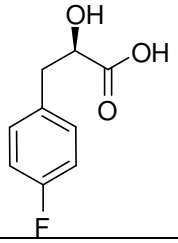
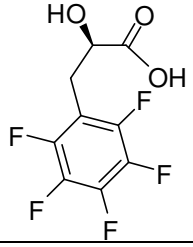
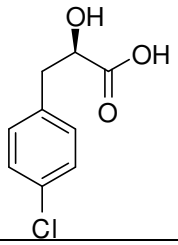
^{13}C-NMR δ_C (100 MHz; DMSO-d_6) 53.6, 69.9, 173.2.

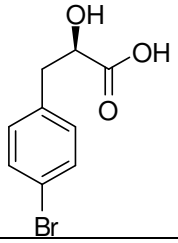
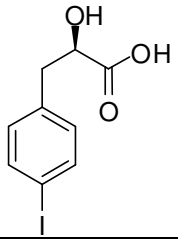
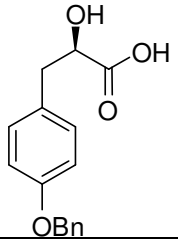
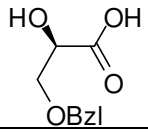
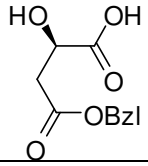
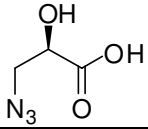
HRMS (ESI) m/z calculated for $C_3H_4N_3O_3$ (M-H) 130.02581; detected 130.02559

3. Supplementary Table 1. α-D-hydroxycarboxylic acids tested and beauvericin analogues detected during *in vivo* and *in vitro* syntheses

2-Hydroxycarboxylic acid precursor			Beauvericin analogue			Synthetic method		
No.	**Structure**	**Name**	**Mass $[M+H]^+$**	**HPLC t_R [min]**	**TLC R_F**	***In vivo, B. bassiana kivr⁻***	***In vivo, E.coli bbBeas⁺***	***In vitro,* chemo-enzymatic**
1		Lactic acid				no	no	-
2		2-Hydroxy-butyric acid	742.50	5.4	0.7	yes	yes	yes
3		2-Hydroxy-3-methyl-butyric acid	784.60	5.6	0.4	yes	yes	yes
4		2-Hydroxy-pentanoic acid	784.60	5.7		yes	yes	no
5		2-Hydroxy-hexanoic acid	826.6	6.1		yes	yes	no
6		2-Hydroxy-octanoic acid				no	no	-
7		2-Hydroxy-4-methyl-pentanoic acid				no	no	-
8		(R)-2-Hydroxy-3-methyl-pentanoic acid	826.8	6.2		yes	yes	no
9		(S)-2-Hydroxy-3-methyl-pentanoic acid	826.7	6.0		yes	yes	no
10		DL-2-Hydroxy-3,3-dimethyl-butyric acid	826.6	6.1		yes	yes	no
11		2-Hydroxy-4,4-dimethyl-pentanoic acid				no	no	-
12		DL-2-Hydroxy-pent-4-ynoic acid	772.6	5.3	0.6	yes	no	yes

13		Fluor lactate	754.3		0.5	-	no	yes
14		3-Chloro-2-hydroxy-propionic acid	802.2		0.6	no	no	yes
15		3-Bromo-2-hydroxy-propionic acid				no	no	-
16		2-Hydroxy-4-methylsulfanyl-butyric acid	880.4	5.6		yes	-	-
17		2,3-Dihydroxy-propionic acid				no	-	-
18		Cyclohexyl-hydroxy-acetic acid				no	no	-
19		3-Cyclohexyl-2-hydroxy-propionic acid				no	no	-
20		Hydroxy-phenyl-acetic acid				no	no	-
21		2-Hydroxy-3-phenyl-propionic acid				no	no	-
22		2-Hydroxy-4-phenyl-butyric acid				no	no	-
23		3-Biphenyl-4-yl-2-hydroxy-propionic acid				no	no	-

24		2-Hydroxy-3,3-diphenyl-propionic acid				no	no	-
25		2-Hydroxy-3-naphthalen-2-yl-propionic acid				no	no	-
26		2-Hydroxy-3-thiophen-3-yl-propionic acid				no	no	-
27		2-Hydroxy-3-(4-nitro-phenyl)-propionic acid				no	no	-
28		3-(4-Cyano-phenyl)-2-hydroxy-propionic acid				no	no	-
29		3-(4-Fluoro-phenyl)-2-hydroxy-propionic acid				no	no	-
30		2-Hydroxy-3-pentafluorophenyl-propionic acid				no	no	-
31		3-(4-Chloro-phenyl)-2-hydroxy-propionic acid				no	no	-

32		3-(4-Bromo-phenyl)-2-hydroxy-propionic acid				no	no	-
33		2-Hydroxy-3-(4-iodo-phenyl)-propionic acid				no	no	-
34		3-(4-Benzyloxy-phenyl)-2-hydroxy-propionic acid				no	no	-
35		3-Benzyloxy-2-hydroxy-propionic acid				no	no	-
36		3-Benzylsulfanyl-2-hydroxy-propionic acid				no	no	-
37		2-Hydroxy-succinic acid-4-benzyl ester				no	no	-
38		(R)-2-Hydroxy-3-azidopropanoic acid				no	no	-

(-) Hydroxycarboxylic acid not tested. Boxes filled with hatch mark: Not detected

4. Supplementary Table 2. MS/MS fragmentation analysis of beauvericin analogues obtained by *in vivo* biocatalytic synthesis with *B. bassiana kivr*$^{-}$

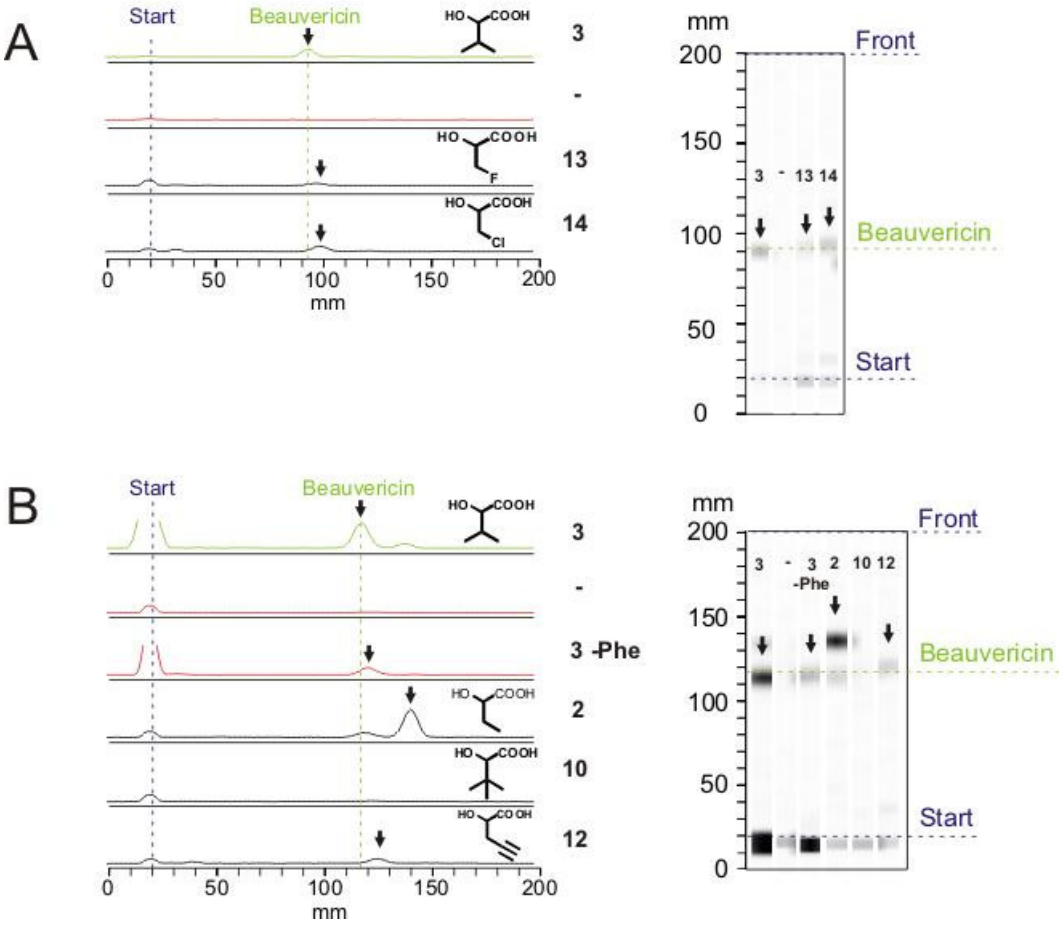
Analogue	Molecular ion [m/z]	MRM transitions [m/z]	Fragmentor Voltage [eV]	Collision Energy [eV]
Beauvericin	784.6 $[M+H]^+$	362.2	230	30
		523.2	230	
		623.3	230	
Beauvericin 2	742.5 $[M+H]^+$	334.2	230	30
		134.0	230	
		381.3	230	
Beauvericin 4	784.6 $[M+H]^+$	362.2	230	30
		523.2	230	
		623.3	230	
Beauvericin 5	826.6 $[M+H]^+$	390.3	230	30
		276.1	230	
		134.1	230	
Beauvericin 8	826.8 $[M+H]^+$	390.3	230	30
		276.1	230	
		134.1	230	
Beauvericin 9	826.7 $[M+H]^+$	390.3	230	30
		276.1	230	
		134.1	230	
Beauvericin 10	826.6 $[M+H]^+$	390.3	230	30
		276.1	230	
		134.1	230	
Beauvericin 12	772.6 $[M+H]^+$	134.2	230	30
		354.2	230	
		611.3	230	
Beauvericin 16	880.4 $[M+H]^+$	276.0	230	30
		426.1	230	
		719.3	230	

5. Supplementary Table 3. MS/MS fragmentation analysis of beauvericin analogues obtained by *in vivo* biocatalytic synthesis with *E. coli bbBeas*$^{+}$

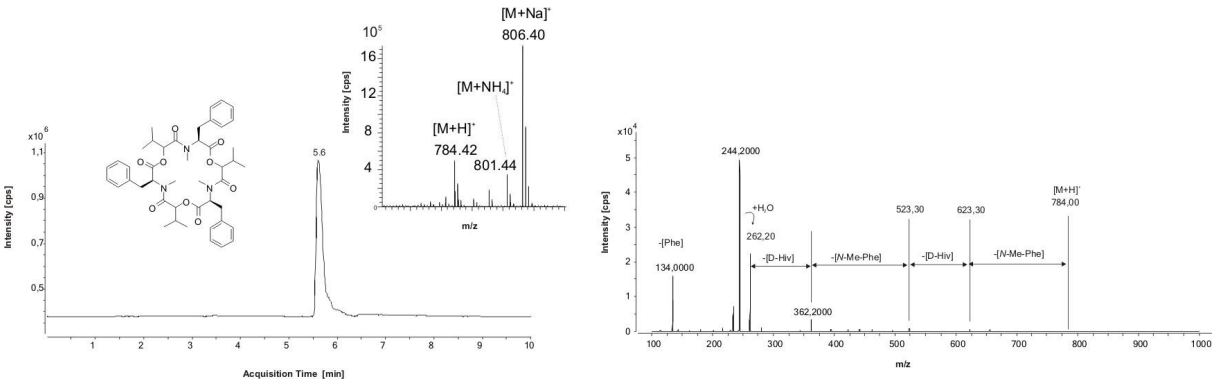
Analogue	Molecular ion [m/z]	MRM transitions [m/z]	Fragmentor Voltage [eV]	Collision Energy [eV]
Beauvericin	784.4 $[M+H]^+$	362.2	230	30
		244.2	230	
		134.0	230	
Beauvericin 2	764.4 $[M+Na]^+$	156.0	230	50
		356.2	230	
		517.2	230	
Beauvericin 4	784.4 $[M+H]^+$	362.1	230	30
		244.2	230	
		134.0	230	
Beauvericin 5	848.5 $[M+Na]^+$	687.3	230	50
		412.3	230	
		133.9	230	
Beauvericin 8	848.5 $[M+Na]^+$	133.9	230	50
		412.2	230	
		687.3	230	
Beauvericin 9	848.5 $[M+Na]^+$	412.2	230	60
		280.2	230	
		133.9	230	
Beauvericin 10	834.4 $[M+Na]^+$	280.2	230	50
		398.2	230	
		673.2	230	
Beauvericin 12	ND	ND	ND	ND
		ND	ND	
		ND	ND	
Beauvericin 16	ND	ND	ND	ND
		ND	ND	
		ND	ND	

ND = not detected

6. Supplementary Fig. 1. *In vitro* chemoenzymatic synthesis of beauvericin analogues. Line densitometric traces and X-ray photographic images of thin layer chromatographic plates for reactions with: A. Precursors 3, 13 and 14; and B. Precursors 3, 2, 10, and 12. Control reactions without hydroxycarboxylic acid (-) and without L-Phe but with precursor 3 (3-Phe) are also shown.

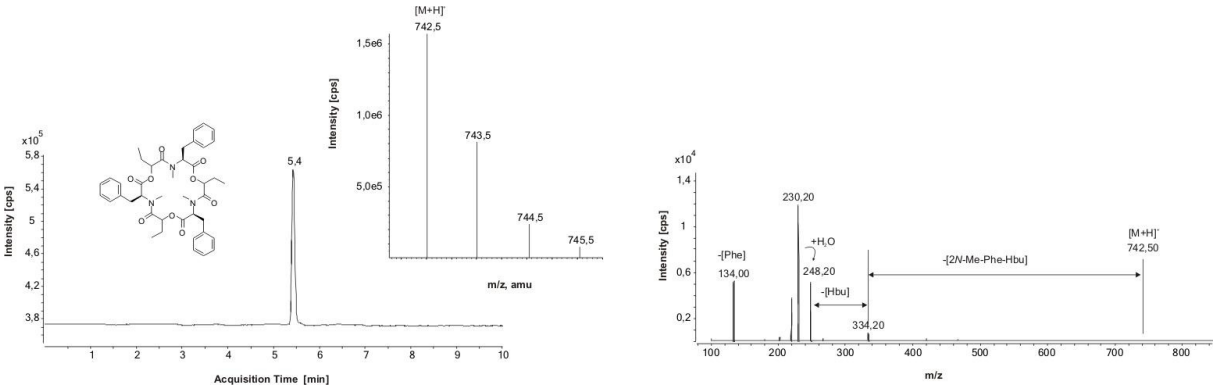


7. Supplementary Fig. 2. Structure, HPLC-ESI-MS spectrum, and HPLC-ESI-MS/MS spectrum of beauvericin from *in vitro* chemoenzymatic synthesis

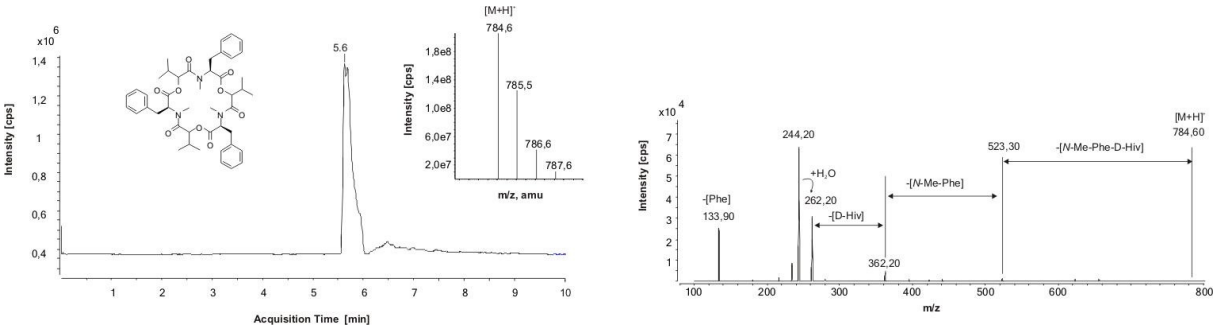


8. Supplementary Fig. 3. Structures, HPLC-ESI-MS spectra, and HPLC-ESI-MS/MS spectra of beauvericin analogues obtained by *in vivo* biocatalytic synthesis with the *B. bassiana kivr*⁻ strain

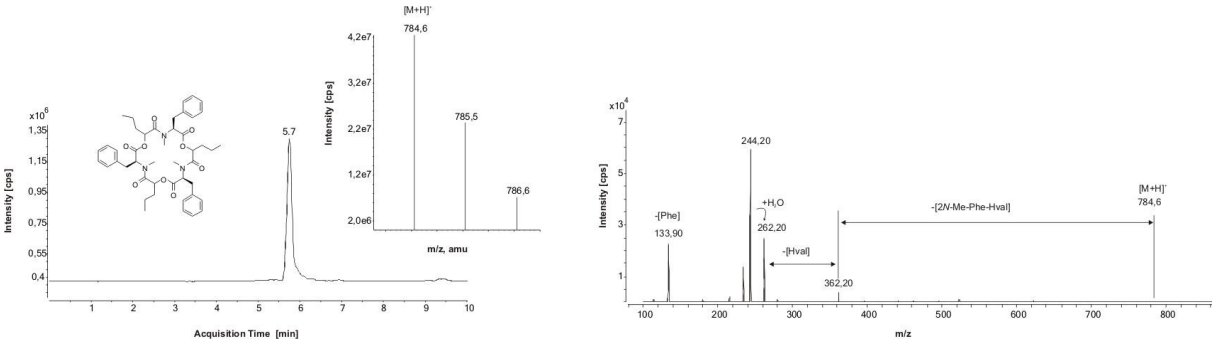
a. Beau-2



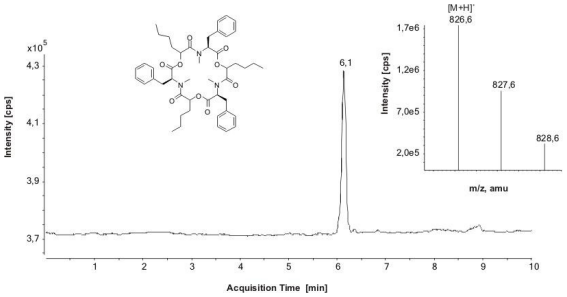
b. Beau-3

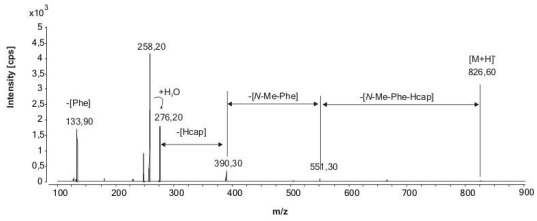


c. Beau-4

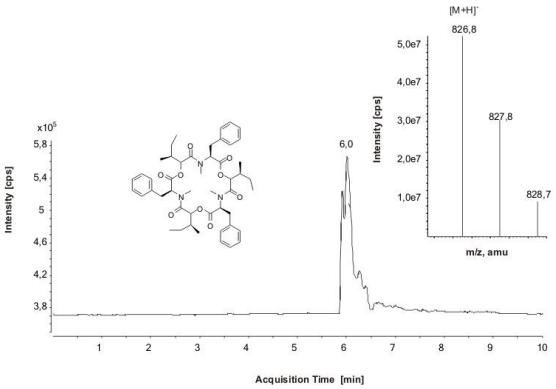


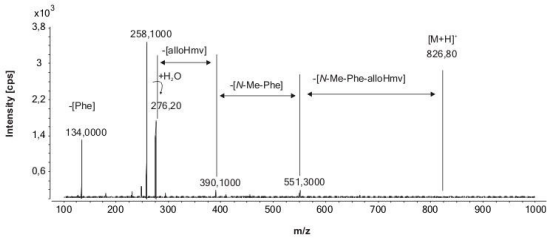
d. Beau-5



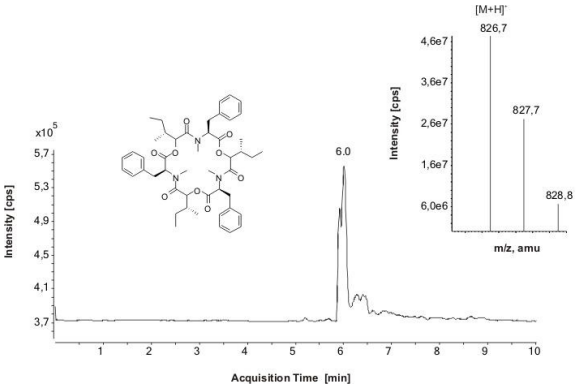


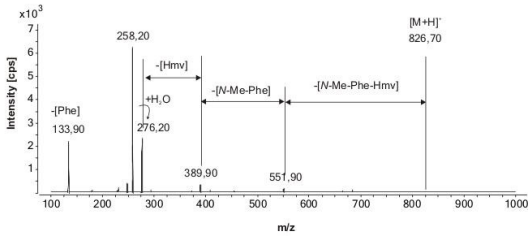
e. Beau-8



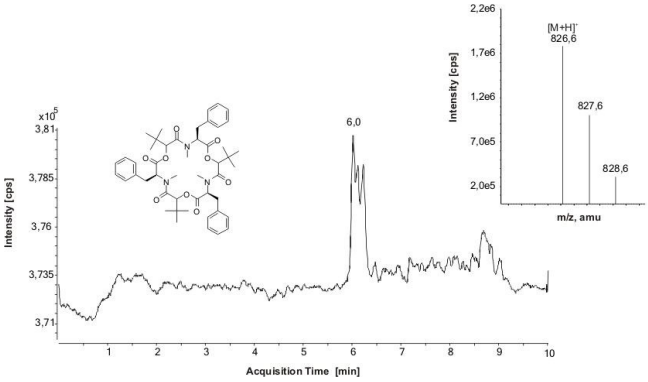


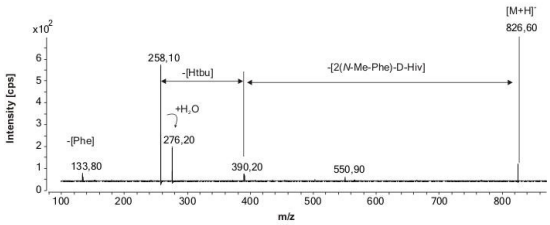
f. Beau-9



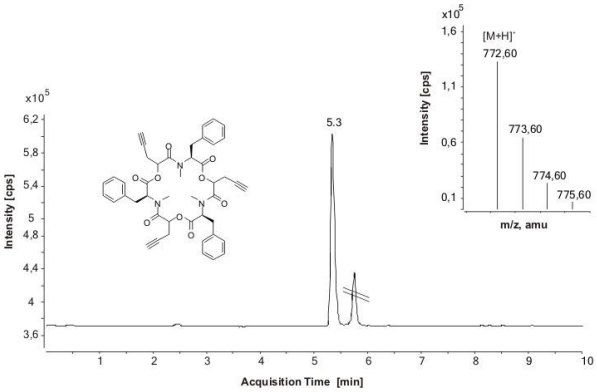


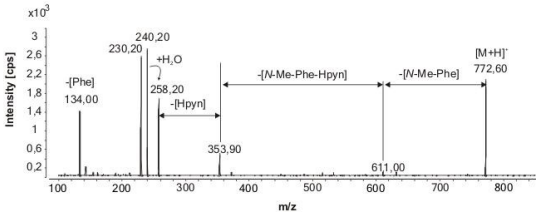
g. Beau-10



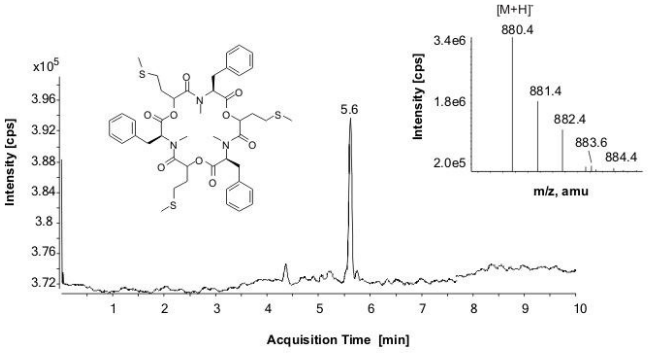


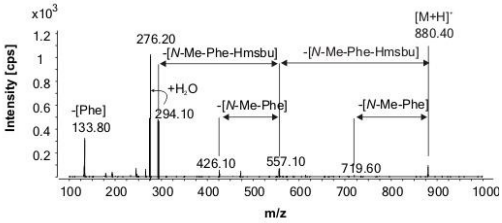
h. Beau-12





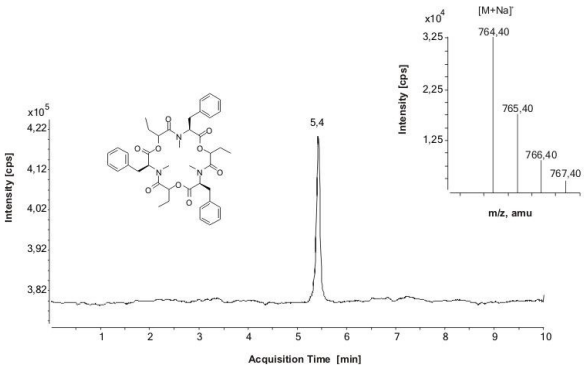
i. Beau-16

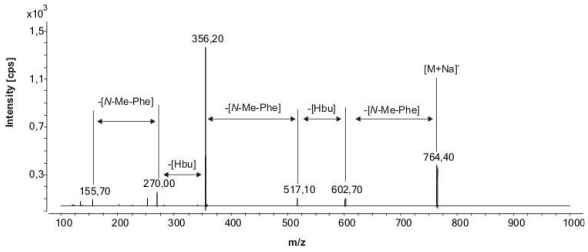




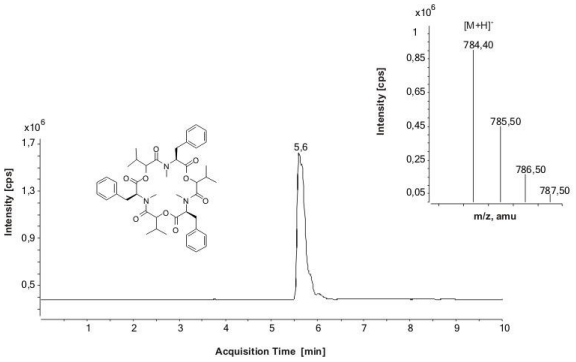
9. Supplementary Fig. 4. Structures, HPLC-ESI-MS spectra, and HPLC-ESI-MS/MS spectra of beauvericin analogues obtained by *in vivo* biocatalytic synthesis with the *E. coli bbBeas*$^{+}$ strain

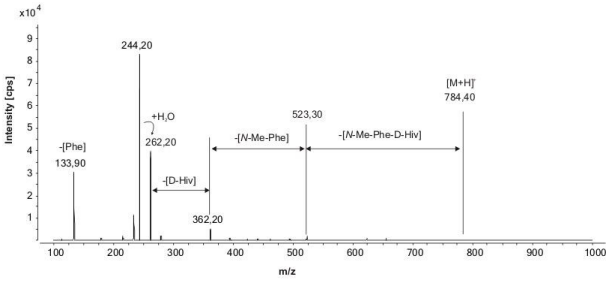
a. Beau-2



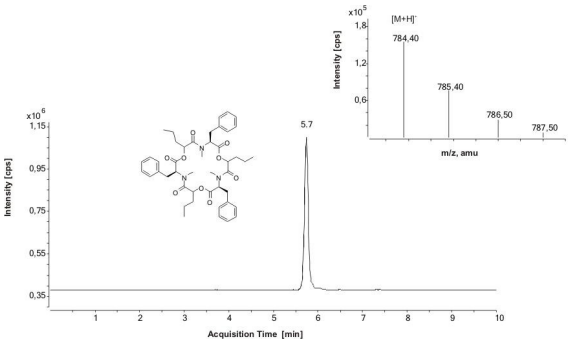


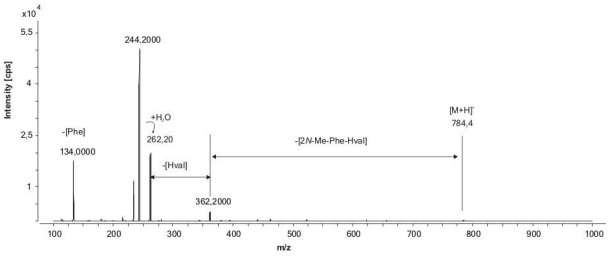
b. Beau-3



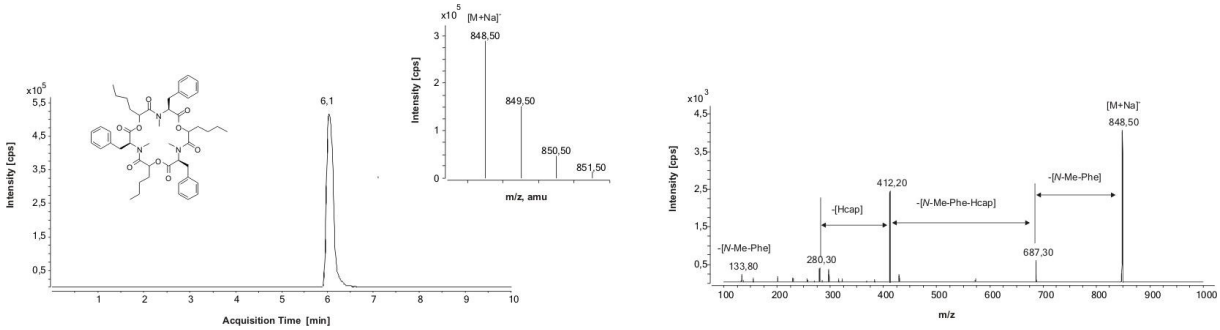


c. Beau-4

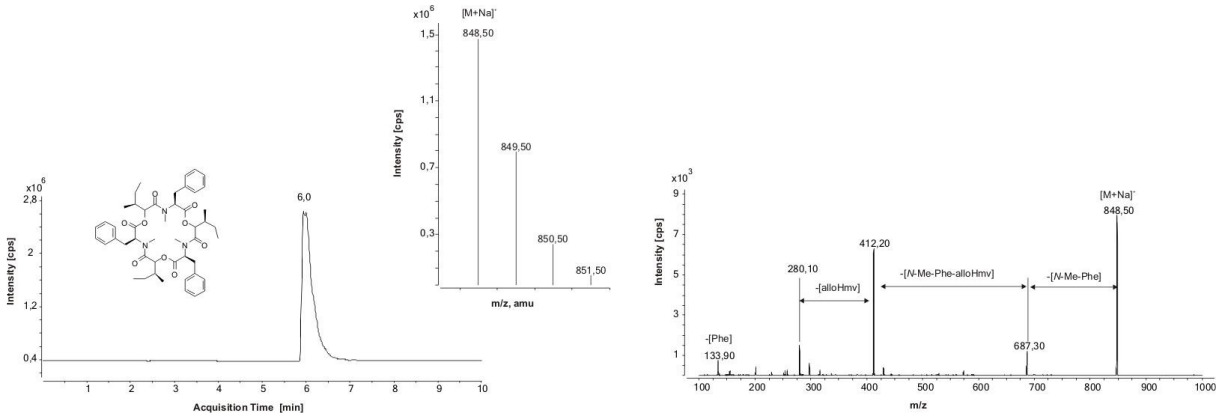




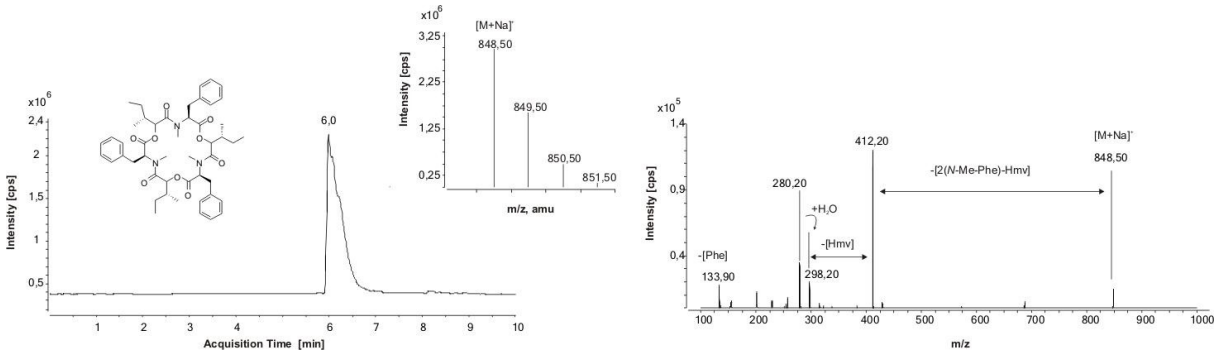
d. Beau-5



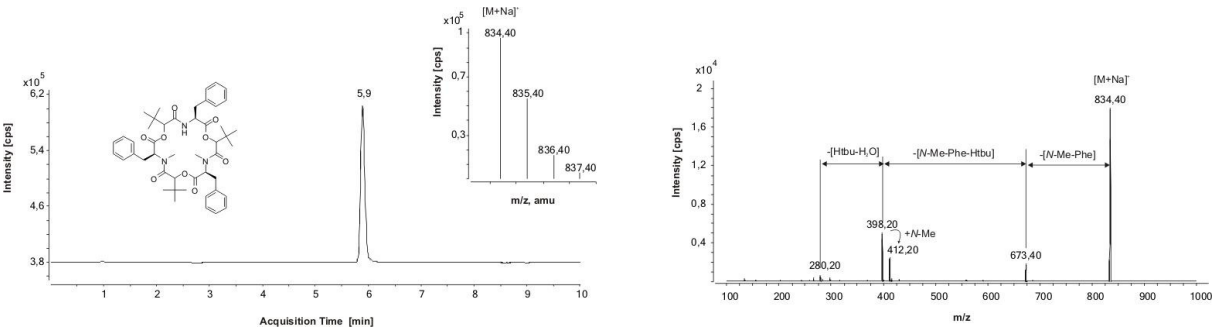
e. Beau-8



f. Beau-9



g. Beau-10



10. Supplementary References

[1] J. Müller, S. C. Feifel, T. Schmiederer, R. Zocher and R. D. Süssmuth, *ChemBioChem*, 2009, **10**, 323-328.

[2] H. Quast, H- Leybach, *Chem. Ber.* 1991, **124**, 849-859.

[3] S. C. Feifel, T. Schmiederer, T. Hornbogen, H. Berg, R. D. Süssmuth and R. Zocher, *ChemBioChem*, 2007, **8**, 1767-1770.