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Journal Name

COMMUNICATION

Overproduction and identification of butyrolactones SCB1-8 in the antibiotic production superhost *Streptomyces* M1152

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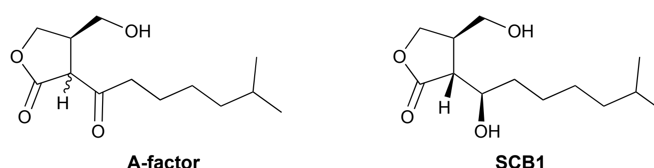
Gamma-butyrolactones (GBLs) are signalling molecules that control antibiotic production in *Streptomyces* bacteria. The genetically engineered strain *S. coelicolor* M1152 was found to overproduce GBLs SCB1-3 as well as five novel GBLs named SCB4-8. Incorporation experiments using isotopically-labelled precursors confirmed the chemical structures of SCB1-3 and established those of SCB4-8.

Introduction

Genome mining has revealed that *Streptomyces* bacteria typically harbour 20-30 machineries or gene clusters dedicated to the production of bioactive natural products. Many of these compounds find use in human or veterinary medicine as well as in agriculture.[1] Hundreds of gene clusters proposed to direct the biosynthesis of unknown (cryptic) antibiotic-like molecules are currently available in publicly accessible databases. The characterisation of these cryptic metabolites is often challenged by the presence of transcriptional repressor proteins that prevent the expression of biosynthetic genes when bacteria are grown in laboratory culture conditions.[1]

Since the discovery of the γ-butyrolactone (GBL) A-factor in 1967 and subsequent characterisation of its intracellular target, the transcriptional repressor ArpA, distinct classes of antibiotic production inducers have been discovered.[2,3] In particular butenolides and 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs) have been shown to trigger the expression of antibiotic-like biosynthetic gene clusters by interacting with specific ArpA-like transcriptional repressors.[4-6]

Early investigations of signalling molecules in *S. coelicolor* A3(2) reported at least seven GBL-like signalling molecules produced by this strain.[7] The chemical structures of *S. coelicolor* GBLs SCB1-3 were elucidated in the 2000s and the



scbA gene, an *afsA*-like butenolide synthase, was shown to direct their biosynthesis.[8-11] SCBs act as antibiotic production inducers and directly control the expression of a cluster of genes, named *cpk*, responsible for the biosynthesis of the coelimycin polyketide antibiotic in *S. coelicolor*.[12,13]

The molecular mode of action of SCBs involves direct binding to the transcriptional repressor ScbR. In the absence of SCBs, ScbR binds to specific operator sequences in particular upstream of the transcriptional activator *cpkO* and prevents coelimycin production.[14] An additional transcriptional repressor, ScbR2, is also responsible for repressing the expression of *cpk* genes.[14] Both *scbR* and *scbR2* genes are adjacent to the *cpk* gene cluster but on opposite sides (Fig. 1). Genetic inactivation of *scbR2* resulted in overproduction of coelimycin and a related yellow pigment.[15,16] In addition ScbR2 is known to bind to the *scbA-scbR* intergenic region and repress expression of *scbA*.[16] Consequently SCB1 was found to be overproduced in the *S. coelicolor* M145 *scbR2* mutant.[16]

The *S. coelicolor* superhost strain M1152, derived from *S. coelicolor* M145, has been genetically optimised for the production of antibiotic-like natural products whose biosynthesis is directed by heterologous metabolic pathways.[17] Construction of this strain involved deletion of multiple biosynthetic gene clusters, including ≈50 kb of the coelimycin gene cluster. This deletion included the gene *scbR2* but not the GBL-biosynthetic genes *scbA* and *scbB* (Fig. 1).

In this study we reveal that the engineered M1152 overproduces GBLs fortuitously. We report the overproduction of known *S. coelicolor* GBLs SCB1-3 as well as production of five new SCBs, SCB4-8 in M1152.[17] The chemical structures of SCB1-8 were determined by a combination of incorporation experiments using stable isotope-labelled precursors and mass spectrometry analyses.

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Electronic Supplementary Information (ESI) available: Experimental procedures and spectroscopic data. See DOI: 10.1039/x0xx00000x

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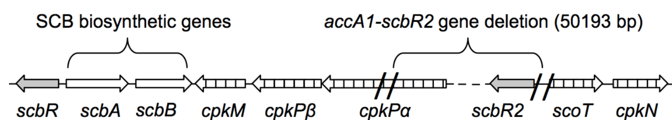

Fig. 1 Genetic organisation of the *scb/cpk* gene cluster in *Streptomyces coelicolor* highlighting the deleted DNA fragment (~50 kb) during the construction of the superhost *S. coelicolor* M1152.

Results and Discussion

Identification of SCBs in *Streptomyces* M1152

SCBs are produced at nanomolar concentration in the parent strain *S. coelicolor* M145: ≈320 µg of SCB1 were purified from a 300 L culture in the original study.[8] In *S. coelicolor* M1152 we expected the *scbR2* deletion to result in *scbAB* overexpression and in turn overproduction of SCBs. *S. coelicolor* M1152 was therefore grown on the minimal culture medium AlaMM and ethyl acetate extracts of the culture supernatant were submitted to liquid chromatography-mass spectrometry, LC-MS, analyses (Fig. 2).

Based on the calculated *m/z* values for the sodium adducts of SCB1 (**1**) and SCB2 (**2**), *m/z* = 267.1567 $[C_{13}H_{24}O_4+Na]^+$, and that of SCB3 (**3**) *m/z* = 281.1723 $[C_{14}H_{26}O_4+Na]^+$, positive ion mode chromatograms were extracted for these ions. In addition, *m/z* values consistent with derivatives carrying shorter or longer alkyl chains (*m/z* = 239.1, 253.1 and 295.2) were also extracted and revealed the presence of metabolites **4**-**8**. Each of the compounds **1-8** exhibited fragmentation patterns consistent with those previously reported for SCBs,[18] i.e. daughter ions corresponding to $[M\text{-}H_2O+H]^+$ and $[M\text{-}2H_2O+H]^+$ in addition to $[M+H]^+$ and $[M+Na]^+$ molecular ions (Fig. 2 and Fig. S1).

The high accuracy of the mass spectrometer used in these experiments combined with the isotopic pattern identified for each of these ions allowed masses to be generated within 5 ppm of those corresponding to the predicted molecular formulae for **1**-**8** (Fig. 2 and Table S1). The molecular formulae of compounds **1** and **2** matched that of SCB1-2 ($C_{13}H_{24}O_4$) while for compound **3** it was consistent with that expected for SCB3 ($C_{14}H_{26}O_4$). For the previously uncharacterised metabolites, a common $C_{12}H_{22}O_4$ molecular formula was determined for **4-6**, while **7** and **8** exhibited formulae of $C_{14}H_{26}O_4$ and $C_{11}H_{20}O_4$ respectively (Table S1). In addition to fragmentation patterns and molecular formulae, the respective retention times on reverse phase HPLC were also consistent with **1-3** corresponding to SCB1-3. The identity of SCB1 was also confirmed by HPLC purification and NMR analysis (Fig. S11 and S12).

The structural diversity between **1-8** was proposed to directly derive from the nature of the precursor incorporated into SCBs. The enzyme ScbA is the only butenolide synthase encoded in *S. coelicolor* M1152 genome and has been shown to be essential for SCB1-3 biosynthesis.[10] ScbA is an orthologue of AfsA that has been shown to catalyse the condensation of dihydroxyacetone phosphate (DHAP) with a β-ketothioester intermediate hijacked from fatty acid metabolism to yield a butenolide phosphate intermediate.[11] This intermediate is then reduced by the butenolide phosphate reductase BprA in *Streptomyces griseus* to generate A-factor.[11] Located downstream of *scbA* is *scbB*, which encodes for a homologue of BprA (76% identity, 84% similarity over 279 amino acids).

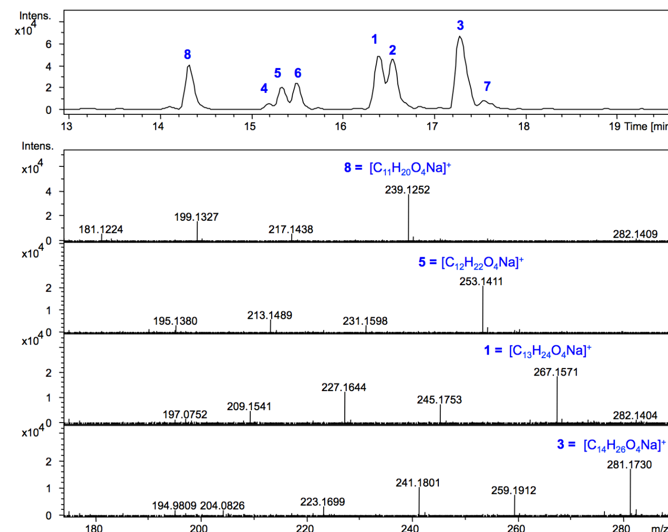

Fig. 2 LC-MS analysis representing the extracted ion chromatogram (*m/z* = 239.1, 253.1, 267.2, 281.2 in positive ion mode) corresponding to SCB sodium adducts produced by *S. coelicolor* M1152 and mass spectra (HR-MS) for compounds **8**, **5**, **1** and **3**.

The SCB-like molecules **1-8** observed in this culture extract were therefore proposed to each result from the incorporation of different β-ketothioester intermediates. Specific primary metabolites, namely leucine, isoleucine, valine, propionic and butyric acids, are known to act as precursor of starter units in fatty acid biosynthesis and are converted into β-ketothioester intermediates that are subsequently incorporated into butenolides and GBLs such as SCB1 (Scheme 1).

Structural characterisation of SCB1-8 using stable-isotope labelled precursors

To unambiguously determine the nature of the alkyl side chains in each of the SCB-like compounds **1-8**, *S. coelicolor* M1152 was grown in AlaMM media supplemented with 1 mM final concentration of deuterium-labelled d_8-DL-valine, d_{10}-L-leucine, d_{10}-L-isoleucine, d_5-propionic acid or d_7-butyric acid. This method was used previously to probe incorporation of specific deuterium-labelled precursor molecules into the AHFCA signalling molecules.[5] Metabolites were extracted from each of the supplemented cultures and analysed by LC-MS. As expected, feeding with each labelled precursor altered the mass spectra of specific SCB-like metabolites.

Feeding with d_8-DL-valine resulted in a proportion of compound **1** (SCB1) to specifically contain seven deuterium atoms, suggesting the intact incorporation of a β-ketothioester primed with isobutyryl-CoA. The molecular formula of **1** $[M+Na]^+$ with *m/z* = 274.2 was confirmed to be $C_{13}H_{17}D_7O_4Na$ (Fig. S5). The *m/z* values of $[M+H]^+$, $[M\text{-}H_2O+H]^+$ and $[M\text{-}2H_2O+H]^+$ presented a similar pattern where seven deuterium atoms were incorporated (Fig. S5 and Table S2). In this particular experiment, **1** was the only metabolite amongst **1-8**

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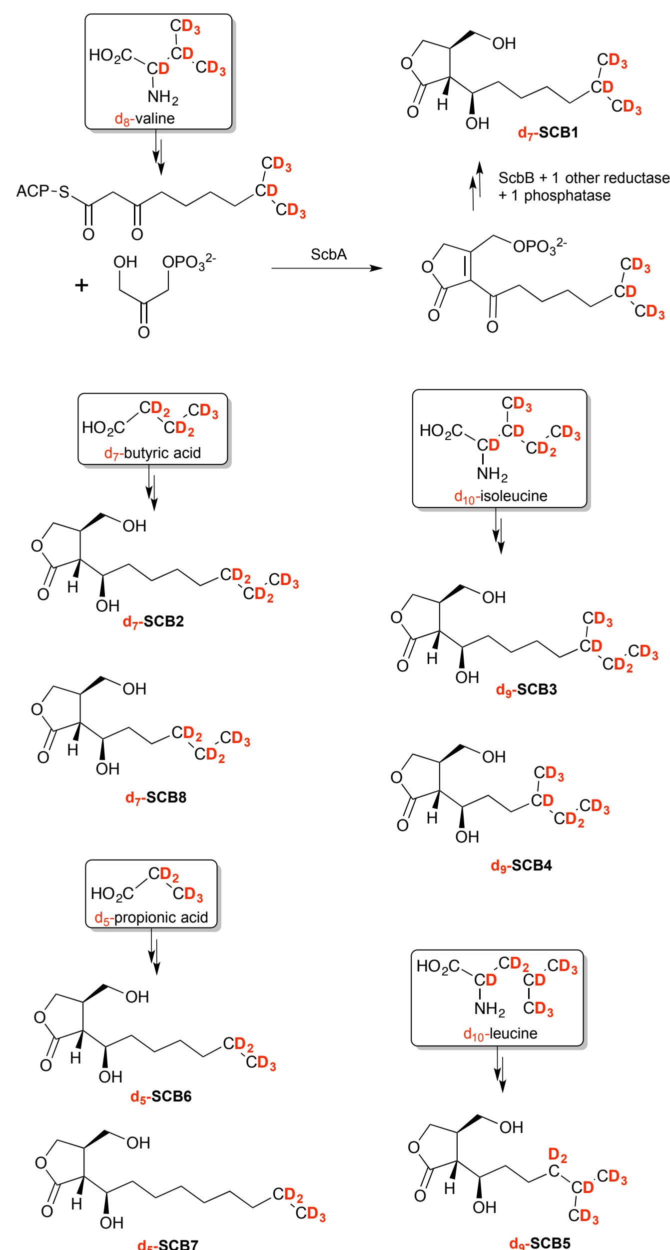
revealing an intact incorporation of the labelled precursor. This experiment confirmed that the alkyl chain of **1** terminates with an isopropyl group, which agrees with the published structure for SCB1 (Scheme 1). In a similar way the linear nature of the alkyl chain in **2**, SCB2, was confirmed by detecting the intact incorporation of seven deuterium atoms from d_7-butyric acid (Fig. S6). Compound **3**, revealing the incorporation of 9 deuterium atoms from d_{10}-isoleucine, was confirmed to correspond to the previously reported SCB3 (Fig. S7).



Scheme 1 Specific incorporation of deuterated precursors into compounds **1-8** revealing the chemical structure of SCB1-8.

Feeding with d_{10}-isoleucine also resulted in specific labelling of compound **4**. The difference between SCB3 (**3**) and **4** rises from the fact that their β-ketothioester precursors have gone through a different number of chain extensions: 3 extensions in the case of SCB3 (**3**) and only 2 for compound **4**. Metabolites **5** and **6** were found to specifically incorporate nine deuterium atoms from d_{10}-leucine and five deuterium atoms from d_5-propionic acid, respectively (Fig. S3 and S4). Compound **7** was specifically labelled upon addition of d_5-propionic acid and **8** specifically incorporated d_7-butyric acid (Fig. S8 and S2).

The structures of the new natural products **4-8**, named SCB4-8 respectively, were therefore as indicated in Scheme 1. All the molecular formulae generated from UHR-LC-MS analyses of the labelled and unlabelled compounds **1-8** are reported in Tables S1 and S2.

Compounds **4-8** have not been previously reported from natural sources, however **6-8** were previously synthesised and tested on the dissociation of ScbR from its DNA binding site using an *in vivo* kanamycin reporter assay.[9] Compounds **6** and **7** were shown to be 10 times less effective than SCB1 and **8** was 480 times less effective at stimulating dissociation of ScbR.[9]

Conclusion

In conclusion, we have established that the superhost strain *Streptomyces* M1152, increasingly used as a heterologous host for the expression of bacterial gene clusters, overproduces SCB1-3 (mg/L scale compare to μg/L scale in the wild-type strain) and at least five additional SCBs. The chemical nature of SCB1-8 was determined using a combination of incorporation experiments and mass spectrometry.

Overproduction of SCB antibiotic production inducers in *S. coelicolor* M1152 might provide this strain an advantage in triggering the expression of otherwise silent GBL-dependent metabolic pathways from heterologous origin. However, in most cases, the cost of GBL overproduction in terms of precursor supply and energy is more likely to impair the yield of metabolite of interest. In addition, we observed that **1-8** interfered significantly with the purification of novel metabolites co-eluting with SCB1-8. As a result of the present work, construction of an improved superhost strain has been undertaken. Inactivation of the biosynthetic gene *scbA* in the *S. coelicolor* M1152 background is expected to particularly improve the production of polyketide natural products as they share the same pool of precursors as those used in SCB biosynthesis.

Acknowledgements

This work was mainly supported by a University Research Fellowship (UF090255) from The Royal Society and by an EPSRC DTA studentship to JDS. The Bruker MaXis mass spectrometer used in this research was obtained with support from AWM and the ERDF. YKQ was supported by National Natural Science Foundation of China 31570031.

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Supporting information

Overproduction and identification of butyrolactones SCB1-8 in the antibiotic production superhost *Streptomyces* M1152

John D. Sidda, Vincent Poon, Lijiang Song, Weishan Wang, Keqian Yang and Christophe Corre*

CONTENTS:

1. **Experimental**
   Culture conditions
   Liquid chromatography-mass spectrometry analyses

2. **LC-MS data**
   2.1 High resolution mass spectrometry data for SCB1-8
   2.2 Low resolution mass spectrometry data for SCB1-8

3. **NMR data for SCB1**

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1. Experimental

Culture conditions

AlaMM liquid medium (50 mL) was inoculated with 20 µL spores of *Streptomyces coelicolor* M1152 and incubated (30 °C, 180 rpm). After 24 h, a solution of sterile filtered precursor (d_5-propionic acid, d_7-butyric acid, d_8-DL-valine, d_{10}-L-leucine or d_{10}-L-isoleucine) was added to give a final concentration of 1 mM. This feeding procedure was repeated at 12 h intervals until a total incubation time of 5 days, after which the metabolites were extracted from these cultures and analysed.

Metabolites were extracted with 1:1 volume of ethyl acetate, dried with $MgSO_4$ and ethyl acetate removed by under reduced pressure. The remaining residue was resuspended in 50:50 HPLC water/methanol.

2. LC-MS data

20 µL of prepared extracts were injected through a reverse phase column (Zorbax C_{18}, size 46 x 150 mm, particle size 5 µm) connected to an Agilent 1100 HPLC. The outflow was routed to a Bruker High Capacity Trap (HCT) + ion trap mass spectrometer with an electrospray source, operating in positive ion mode. A 5 min isocratic elution (95:5 solventA/solvent B) was followed by gradient elution to 0:100 solvent A/solvent B over 25 min. Solvents A and B were water (0.1 % HCOOH) and methanol (0.1 % HCOOH), respectively.

The high-resolution data were obtained by performing UPLC-MS through a reverse phase column (Zorbax Eclipse Plus C_{18}, size 2.1 x 100 mm, particle size 1.8 µm) connected to a Dionex 3000RS UHPLC coupled to Bruker Ultra High Resolution (UHR) Q-TOF MS MaXis mass spectrometer with an electrospray source. Sodium formate (10 mM) was used for internal calibration and a m/z = 50 to 3000 scan range used. A 5 min isocratic elution (95:5 solventA/solvent B) was followed by gradient elution to 0:100 solvent A/solvent B over 15.3 min. Solvents A and B were water (0.1 % HCOOH) and acetonitrile (0.1 % HCOOH), respectively.

2. LC-MS data

2.1 High resolution mass spectrometry data for SCBs 1-8

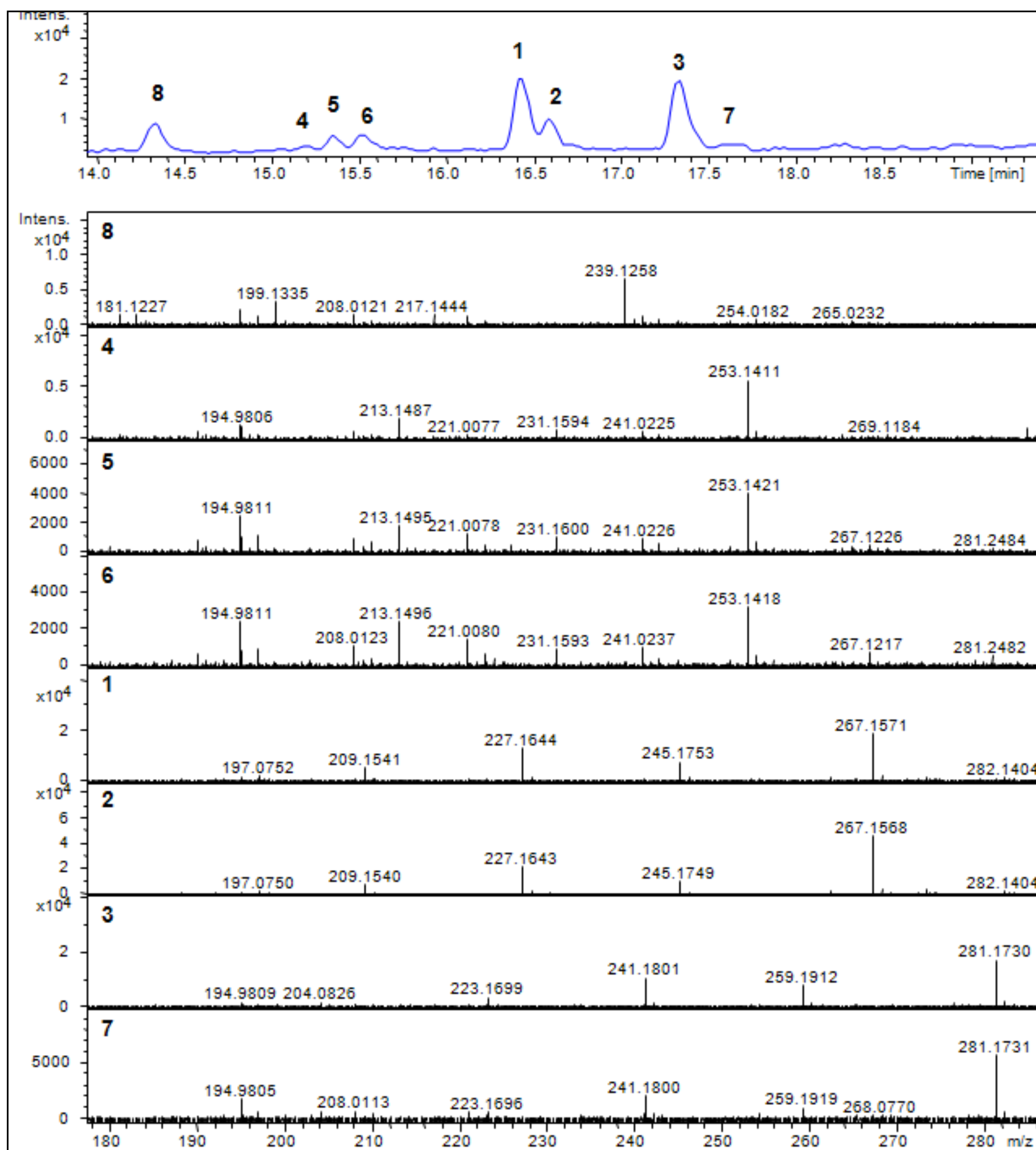

Figure S1. High resolution mass spectra of SCBs 1-8 present in culture extract of *S. coelicolor* M1152 grown for five days

Table S1. UHR-MS assignments for molecular ions and fragments observed for SCBs **1-8** extracted from *S. coelicolor* M1152 after being grown in AlaMM for five days

retention time / min	*compound*	*Molecular formula*	*Observed* m/z	*Calculated* m/z	*error / ppm*	*assignment*
14.3	SCB 8 (**8**)	$\mathbf{C_{11}H_{20}NaO_4}$	239.1258	239.1254	1.9	$[M+Na]^+$
		$\mathbf{C_{11}H_{21}O_4}$	217.1444	217.1434	4.6	$[M+H]^+$
		$\mathbf{C_{11}H_{19}O_3}$	199.1335	199.1329	3.1	$[M-H_2O+H]^+$
		$\mathbf{C_{11}H_{17}O_2}$	181.1227	181.1223	3.4	$[M-2H_2O+H]^+$
15.2	SCB 4 (**4**)	$\mathbf{C_{12}H_{22}NaO_4}$	253.1411	253.1410	0.4	$[M+Na]^+$
		$\mathbf{C_{12}H_{23}O_4}$	231.1594	231.1591	1.5	$[M+H]^+$
		$\mathbf{C_{12}H_{21}O_3}$	213.1487	213.1485	1.1	$[M-H_2O+H]^+$
		$\mathbf{C_{12}H_{19}O_2}$	195.1377	195.1380	1.2	$[M-2H_2O+H]^+$
15.3	SCB 5 (**5**)	$\mathbf{C_{12}H_{22}NaO_4}$	253.1421	253.1410	4.2	$[M+Na]^+$
		$\mathbf{C_{12}H_{23}O_4}$	231.1600	231.1591	3.8	$[M+H]^+$
		$\mathbf{C_{12}H_{21}O_3}$	213.1495	213.1485	4.7	$[M-H_2O+H]^+$
		$\mathbf{C_{12}H_{19}O_2}$	195.1385	195.1380	2.6	$[M-2H_2O+H]^+$
15.5	SCB 6 (**6**)	$\mathbf{C_{12}H_{22}NaO_4}$	253.1418	253.1410	3.1	$[M+Na]^+$
		$\mathbf{C_{12}H_{23}O_4}$	231.1593	231.1591	1.0	$[M+H]^+$
		$\mathbf{C_{12}H_{21}O_3}$	213.1496	213.1485	4.9	$[M-H_2O+H]^+$
		$\mathbf{C_{12}H_{19}O_2}$	195.1388	195.1380	4.4	$[M-2H_2O+H]^+$
16.4	**SCB 1** (**1**)	$\mathbf{C_{13}H_{24}NaO_4}$	267.1571	267.1567	1.7	$[M+Na]^+$
		$\mathbf{C_{13}H_{25}O_4}$	245.1753	245.1747	2.3	$[M+H]^+$
		$\mathbf{C_{13}H_{23}O_3}$	227.1644	227.1642	1.1	$[M-H_2O+H]^+$
		$\mathbf{C_{13}H_{21}O_2}$	209.1541	209.1536	2.2	$[M-2H_2O+H]^+$
16.5	**SCB 2** (**2**)	$\mathbf{C_{13}H_{24}NaO_4}$	267.1568	267.1567	0.5	$[M+Na]^+$
		$\mathbf{C_{13}H_{25}O_4}$	245.1749	245.1747	0.6	$[M+H]^+$
		$\mathbf{C_{13}H_{23}O_3}$	227.1643	227.1642	0.6	$[M-H_2O+H]^+$
		$\mathbf{C_{13}H_{21}O_2}$	209.1540	209.1536	1.7	$[M-2H_2O+H]^+$
17.3	**SCB 3** (**3**)	$\mathbf{C_{14}H_{26}NaO_4}$	281.1730	281.1723	2.5	$[M+Na]^+$
		$\mathbf{C_{14}H_{27}O_4}$	259.1912	259.1904	3.0	$[M+H]^+$
		$\mathbf{C_{14}H_{25}O_3}$	241.1801	241.1798	1.1	$[M-H_2O+H]^+$
		$\mathbf{C_{14}H_{23}O_2}$	223.1699	223.1693	2.8	$[M-2H_2O+H]^+$
17.5	SCB 7 (**7**)	$\mathbf{C_{14}H_{26}NaO_4}$	281.1731	281.1723	2.7	$[M+Na]^+$
		$\mathbf{C_{14}H_{27}O_4}$	259.1919	259.1904	5.9	$[M+H]^+$
		$\mathbf{C_{14}H_{25}O_3}$	241.1800	241.1798	0.9	$[M-H_2O+H]^+$
		$\mathbf{C_{14}H_{23}O_2}$	223.1696	223.1693	1.7	$[M-2H_2O+H]^+$

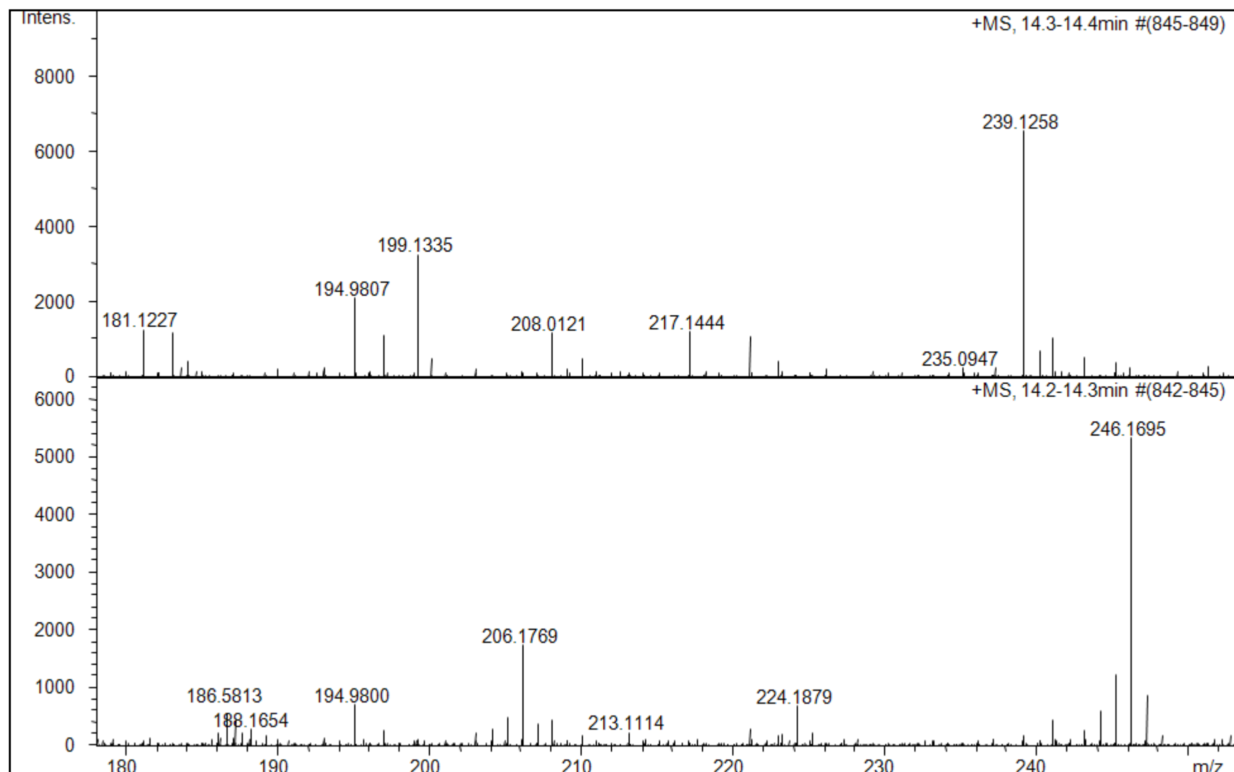

Figure S2. High resolution mass spectra of SCB8 (**8**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_7-butyric acid was added to the media (bottom panel)

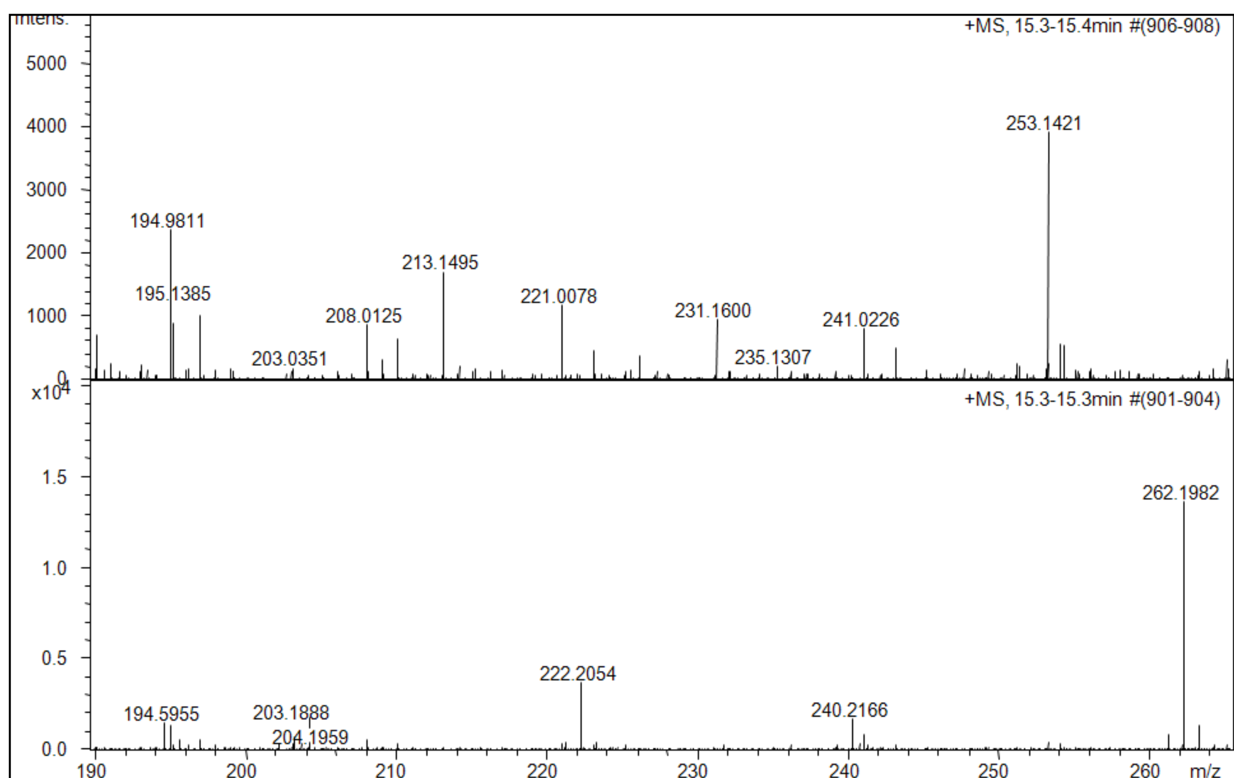

Figure S3. High resolution mass spectra of SCB5 (**5**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_{10}-leucine was added to the media (bottom panel)

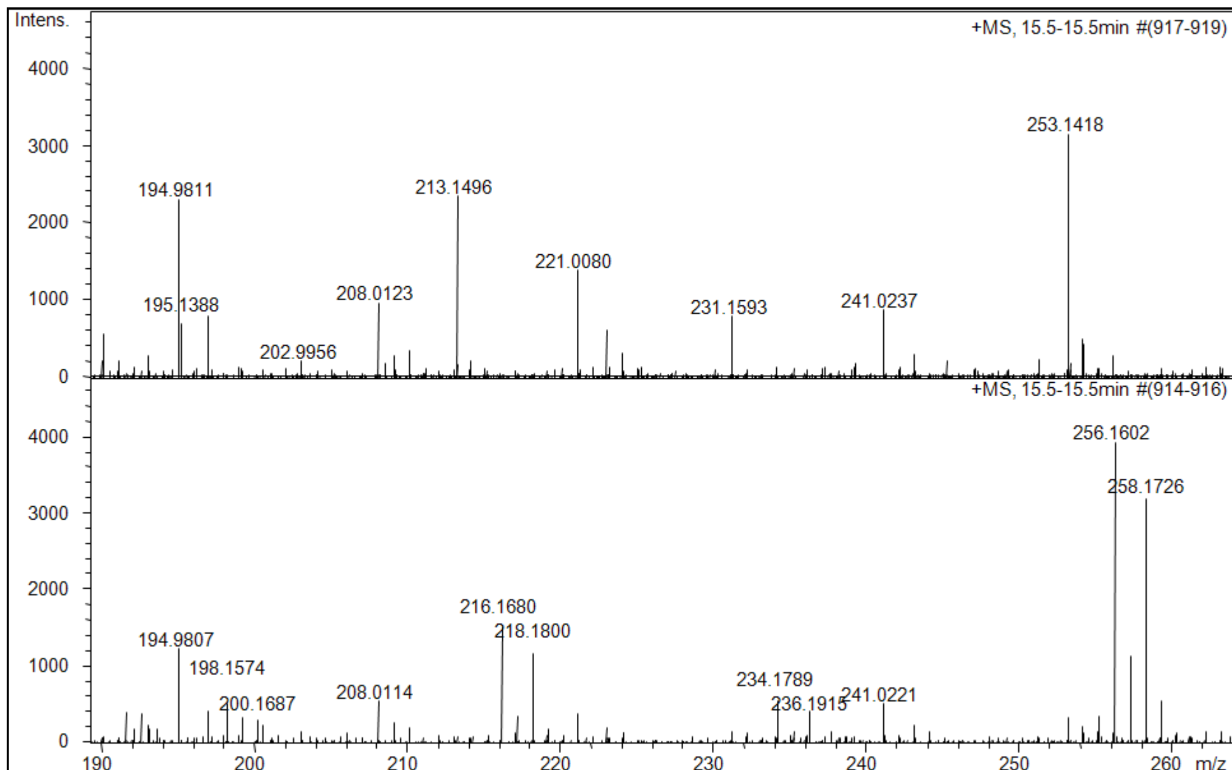

Figure S4. High resolution mass spectra of SCB6 (**6**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_5-propionic acid was added to the media (bottom panel)

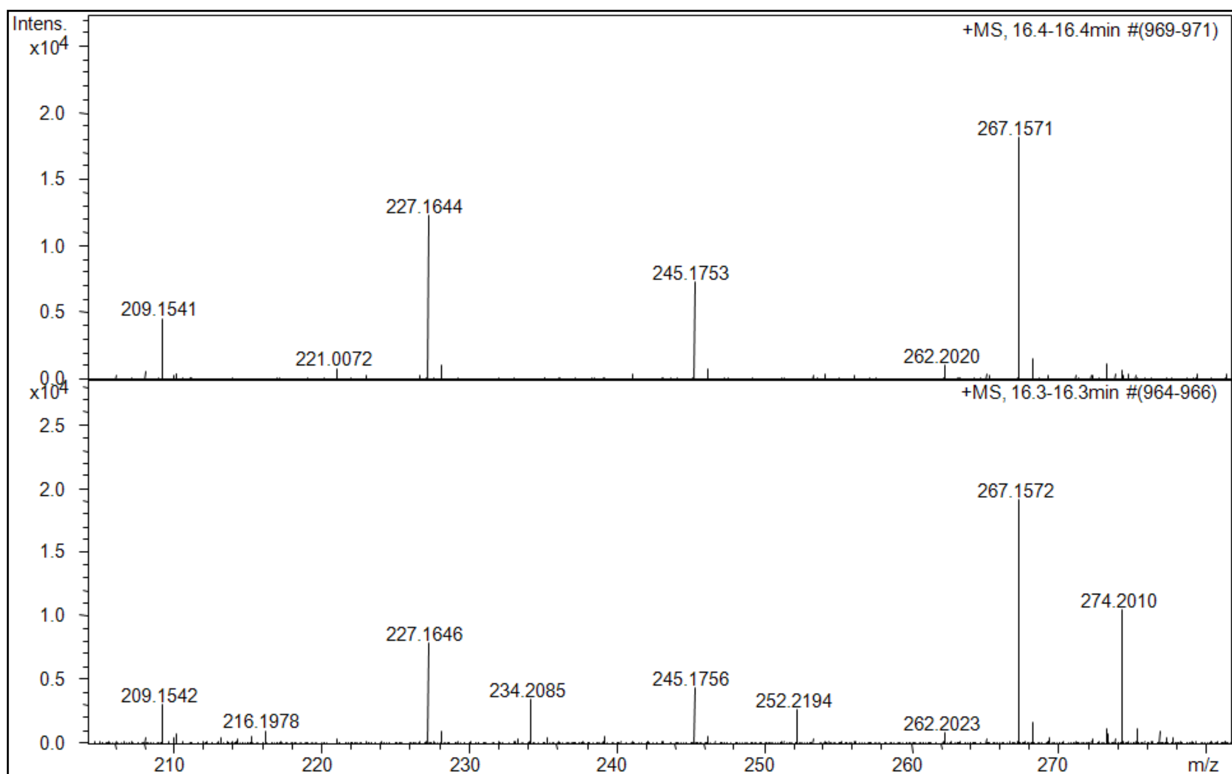

Figure S5. High resolution mass spectra of SCB1 (**1**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_8-valine was added to the media (bottom panel)

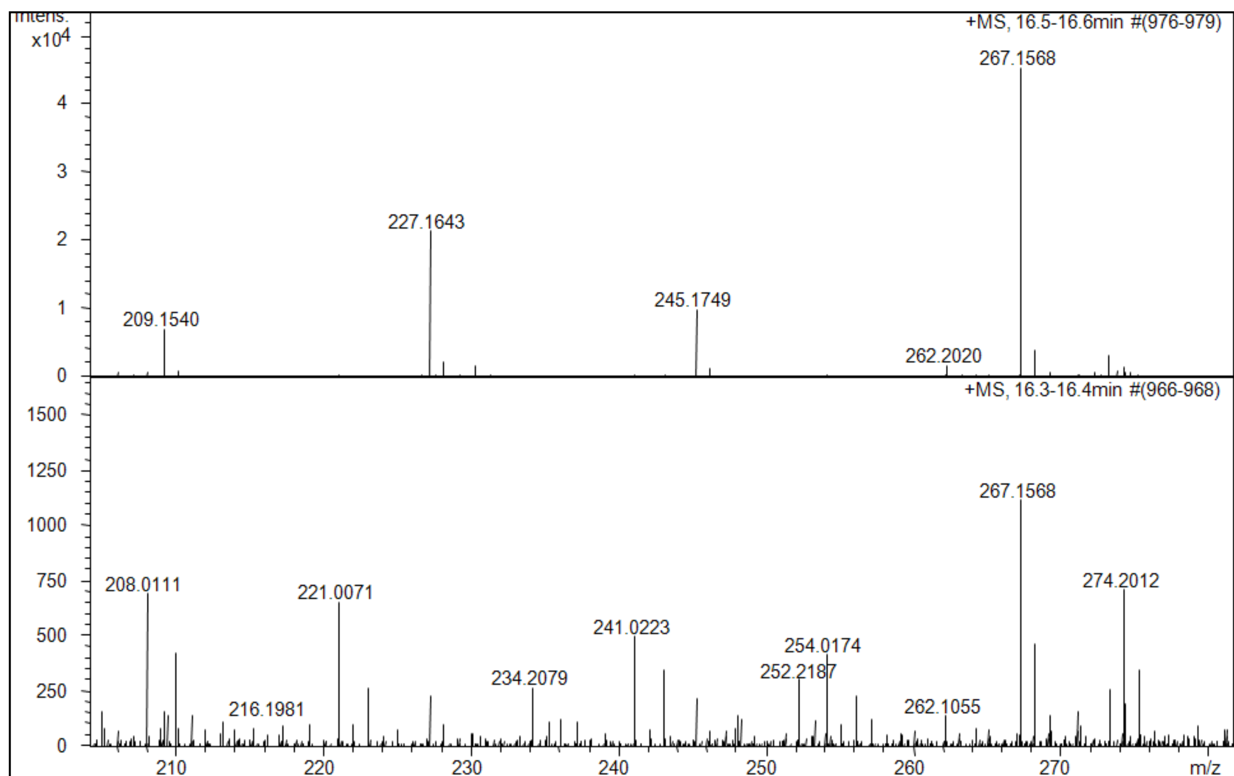

Figure S6. High resolution mass spectra of SCB2 (**2**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_7-butyric acid was added to the media (bottom panel)

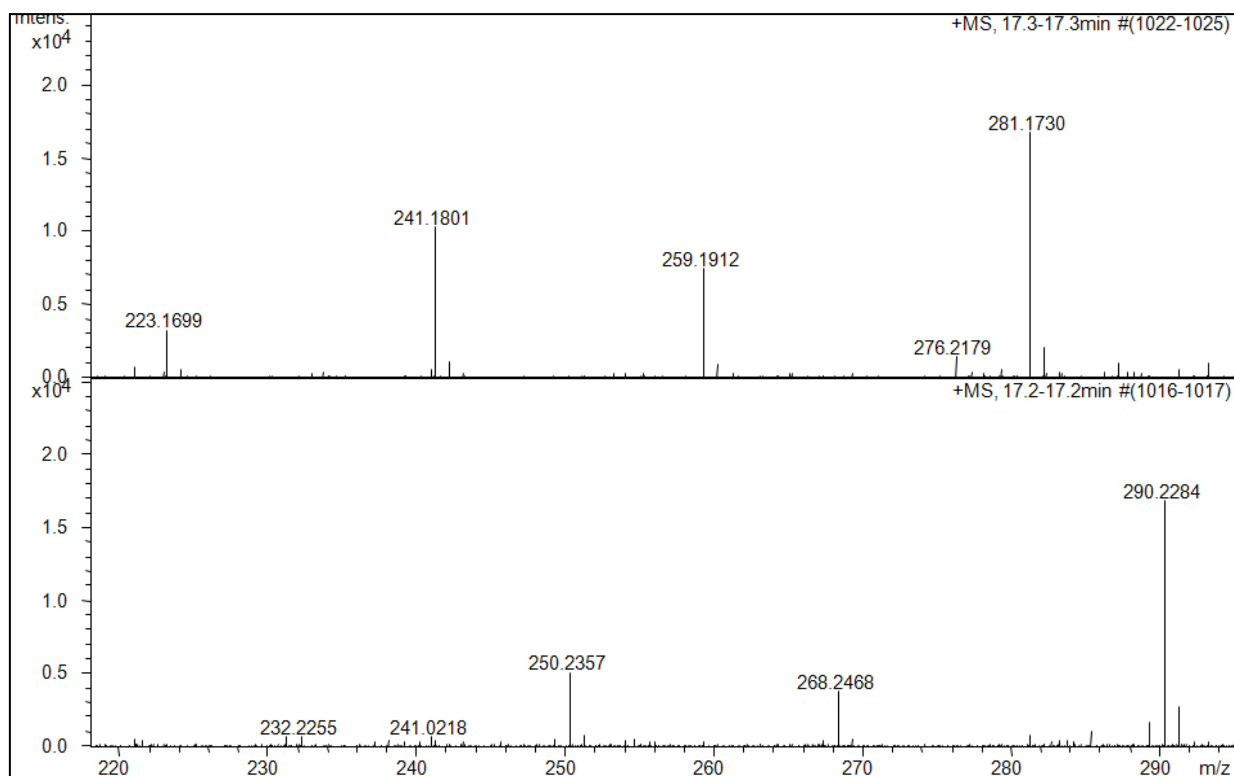

Figure S7. High resolution mass spectra of SCB3 (**3**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_{10}-isoleucine was added to the media (bottom panel)

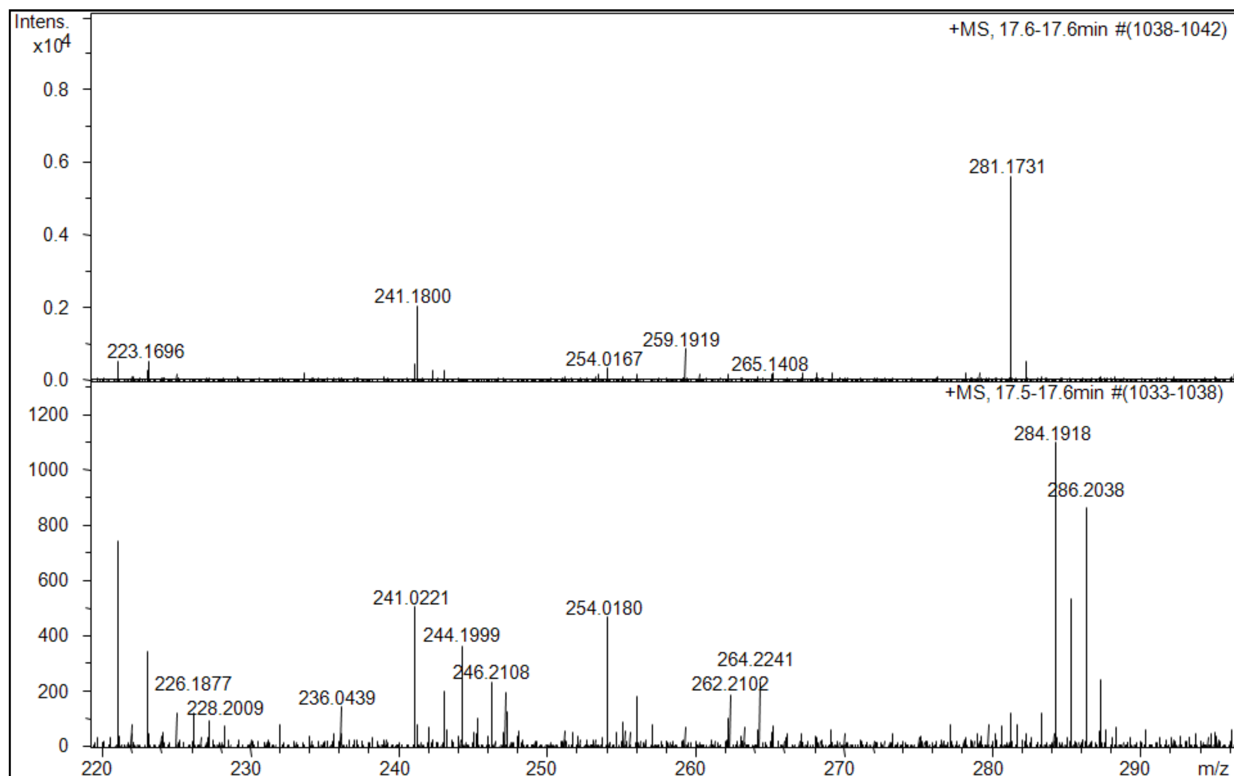

Figure S8. High resolution mass spectra of SCB7 (**7**) extracted from *S. coelicolor* M1152 grown on AlaMM (top panel) and AlaMM when d_5-propionic acid was added to the media (bottom panel)

Table S2. UHR-MS assignments for molecular ions and fragments observed for metabolites **1-8** when fed with different precursor molecules (corresponding to the spectra shown in Figures S1-S8)

retention time / min	*compound*	*Molecular formula*	*Observed* m/z	*Calculated* m/z	*error / ppm*	*assignment*
14.3	d_7-SCB 8 (**8**)	$\mathbf{C_{11}H_{13}D_7NaO_4}$	246.1695	246.1693	0.6	$[M+Na]^+$
		$\mathbf{C_{11}H_{14}D_7O_4}$	224.1879	224.1874	2.5	$[M+H]^+$
		$\mathbf{C_{11}H_{12}D_7O_3}$	206.1769	206.1768	0.7	$[M-H_2O+H]^+$
		$\mathbf{C_{11}H_{10}D_7O_2}$	188.1654	188.1662	4.7	$[M-2H_2O+H]^+$
15.2	SCB4 (**4**)	intensity of peaks too low to generate molecular formulae – see low resolution data				
15.3	d_9-SCB 5 (**5**)	$\mathbf{C_{12}H_{13}D_9NaO_4}$	262.1982	262.1975	2.5	$[M+Na]^+$
		$\mathbf{C_{12}H_{14}D_9O_4}$	240.2166	240.2156	4.3	$[M+H]^+$
		$\mathbf{C_{12}H_{12}D_9O_3}$	222.2054	222.2050	1.7	$[M-H_2O+H]^+$
		$\mathbf{C_{12}H_{10}D_9O_2}$	204.0199	204.1944	7.0	$[M-2H_2O+H]^+$
15.5	d_5-SCB 6 (**6**)	$\mathbf{C_{12}H_{17}D_5NaO_4}$	258.1726	258.1724	0.7	$[M+Na]^+$
		$\mathbf{C_{12}H_{18}D_5O_4}$	236.1915	236.1905	4.3	$[M+H]^+$
		$\mathbf{C_{12}H_{16}D_5O_3}$	218.1800	218.1799	0.3	$[M-H_2O+H]^+$
		$\mathbf{C_{12}H_{14}D_5O_2}$	200.1687	200.1693	3.2	$[M-2H_2O+H]^+$
15.5	d_3-SCB6 (**6**)	$\mathbf{C_{12}H_{19}D_3NaO_4}$	256.1602	256.1599	1.3	$[M+Na]^+$
		$\mathbf{C_{12}H_{20}D_3O_4}$	234.1789	234.1779	4.0	$[M+H]^+$
		$\mathbf{C_{12}H_{18}D_3O_3}$	216.1680	216.1674	3.0	$[M-H_2O+H]^+$
		$\mathbf{C_{12}H_{16}D_3O_2}$	198.1574	198.1568	3.0	$[M-2H_2O+H]^+$
16.4	d_7-**SCB 1** (**1**)	$\mathbf{C_{13}H_{17}D_7NaO_4}$	274.2010	274.2006	1.6	$[M+Na]^+$
		$\mathbf{C_{13}H_{18}D_7O_4}$	252.2194	252.2187	2.7	$[M+H]^+$
		$\mathbf{C_{13}H_{16}D_7O_3}$	234.2085	234.2081	1.7	$[M-H_2O+H]^+$
		$\mathbf{C_{13}H_{14}D_7O_2}$	216.1978	216.1975	1.1	$[M-2H_2O+H]^+$
16.5	d_7-**SCB 2** (**2**)	$\mathbf{C_{13}H_{17}D_7NaO_4}$	274.2012	274.2006	2.1	$[M+Na]^+$
		$\mathbf{C_{13}H_{18}D_7O_4}$	252.2187	252.2187	0.1	$[M+H]^+$
		$\mathbf{C_{13}H_{16}D_7O_3}$	234.2079	234.2081	0.7	$[M-H_2O+H]^+$
		$\mathbf{C_{13}H_{14}D_7O_2}$	216.1981	216.1975	2.4	$[M-2H_2O+H]^+$
17.3	d_9-**SCB 3** (**3**)	$\mathbf{C_{14}H_{17}D_9NaO_4}$	290.2284	290.2288	1.4	$[M+Na]^+$
		$\mathbf{C_{14}H_{18}D_9O_4}$	268.2468	268.2469	0.1	$[M+H]^+$
		$\mathbf{C_{14}H_{16}D_9O_3}$	250.2357	250.2363	2.3	$[M-H_2O+H]^+$
		$\mathbf{C_{14}H_{14}D_9O_2}$	232.2255	232.2257	1.0	$[M-2H_2O+H]^+$
17.5	d_5-SCB 7 (**7**)	$\mathbf{C_{14}H_{21}D_5NaO_4}$	286.2038	286.2037	0.1	$[M+Na]^+$
		$\mathbf{C_{14}H_{22}D_5O_4}$	intensity of peaks too low to generate molecular formulae			
		$\mathbf{C_{14}H_{20}D_5O_3}$	246.2108	246.2112	1.8	$[M-H_2O+H]^+$
		$\mathbf{C_{14}H_{18}D_5O_2}$	228.2009	228.2006	1.2	$[M-2H_2O+H]^+$
17.5	d_3-SCB 7 (**7**)	$\mathbf{C_{14}H_{23}D_3NaO_4}$	284.1918	284.1912	2.2	$[M+Na]^+$
		$\mathbf{C_{14}H_{24}D_3O_4}$	262.2102	262.2092	3.7	$[M+H]^+$
		$\mathbf{C_{14}H_{22}D_3O_3}$	244.1999	244.1987	5.0	$[M-H_2O+H]^+$
		$\mathbf{C_{14}H_{20}D_3O_2}$	226.1877	226.1881	1.9	$[M-2H_2O+H]^+$

2.2 Low resolution mass spectrometry data for SCB1-8 (1-8)

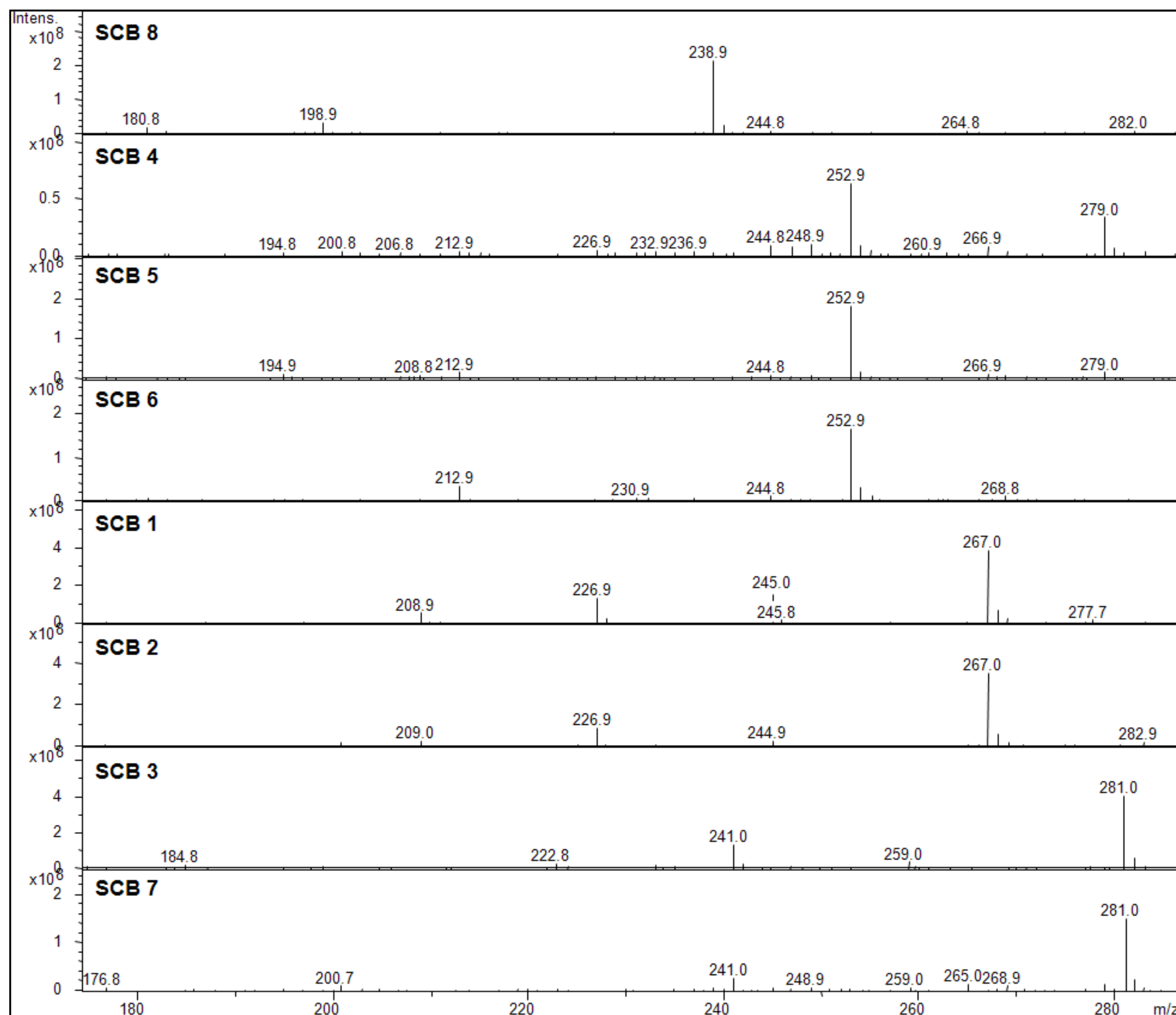

Figure S9. Low resolution mass spectra for metabolites **1**-**8** (SCB1-8) present in culture extract of *S. coelicolor* M1152 grown for five days.

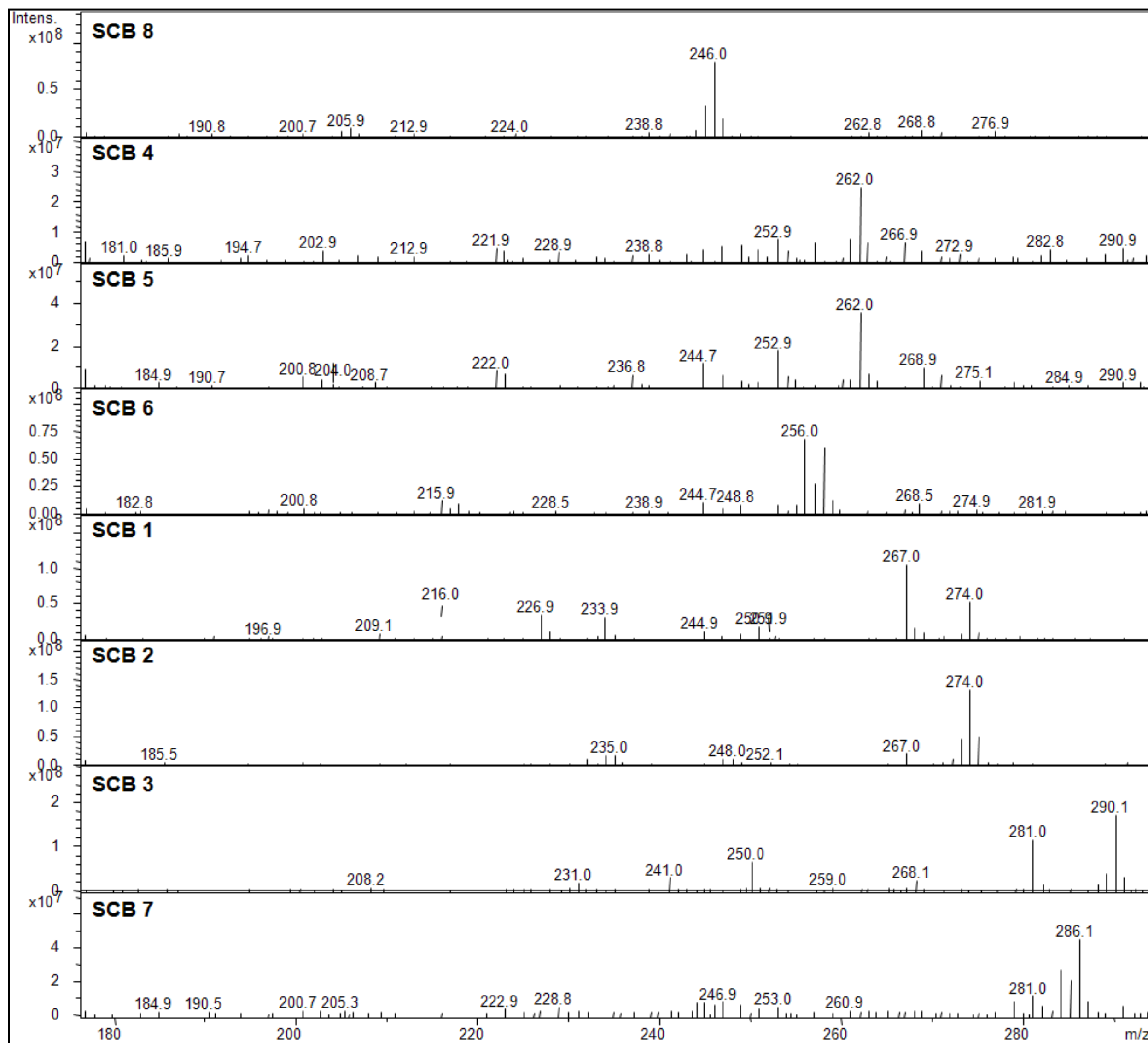

Figure S10. Low resolution mass spectra for metabolites **1-8** (SCBs 1-8) present in culture extract of *S. coelicolor* M1152 grown for five days in AlaMM enriched with d_5-proionic acid (SCBs 6 and 7); d_7-butyric acid (SCBs 2 and 8); d_8-valine (SCB 1); d_{10}-leucine (SCB 5) and d_{10}-isoleucine (SCBs 3 and 4)

3. NMR data for SCB1

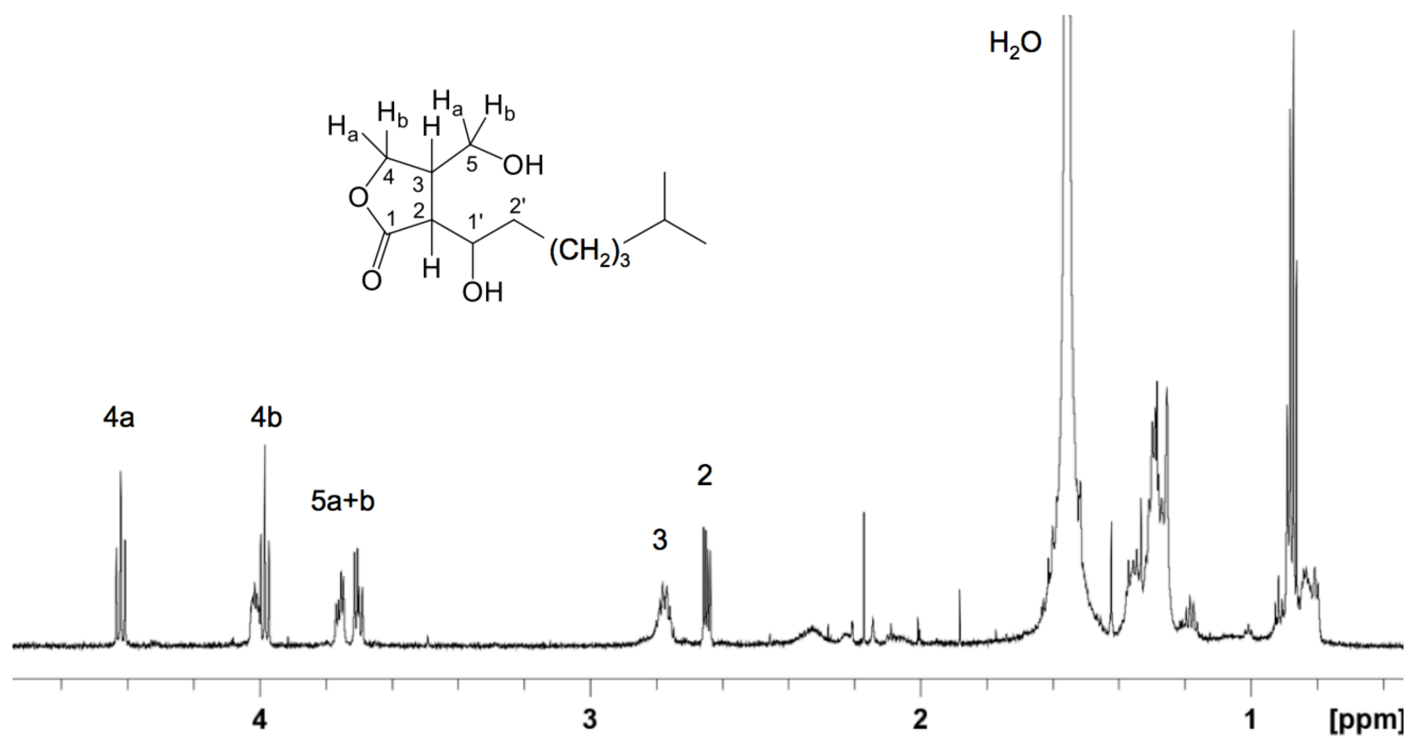

Figure S11. ^{1}H-NMR spectrum (700 MHz, $CDCl_3$) for SCB1 isolated from *S. coelicolor* M1152.

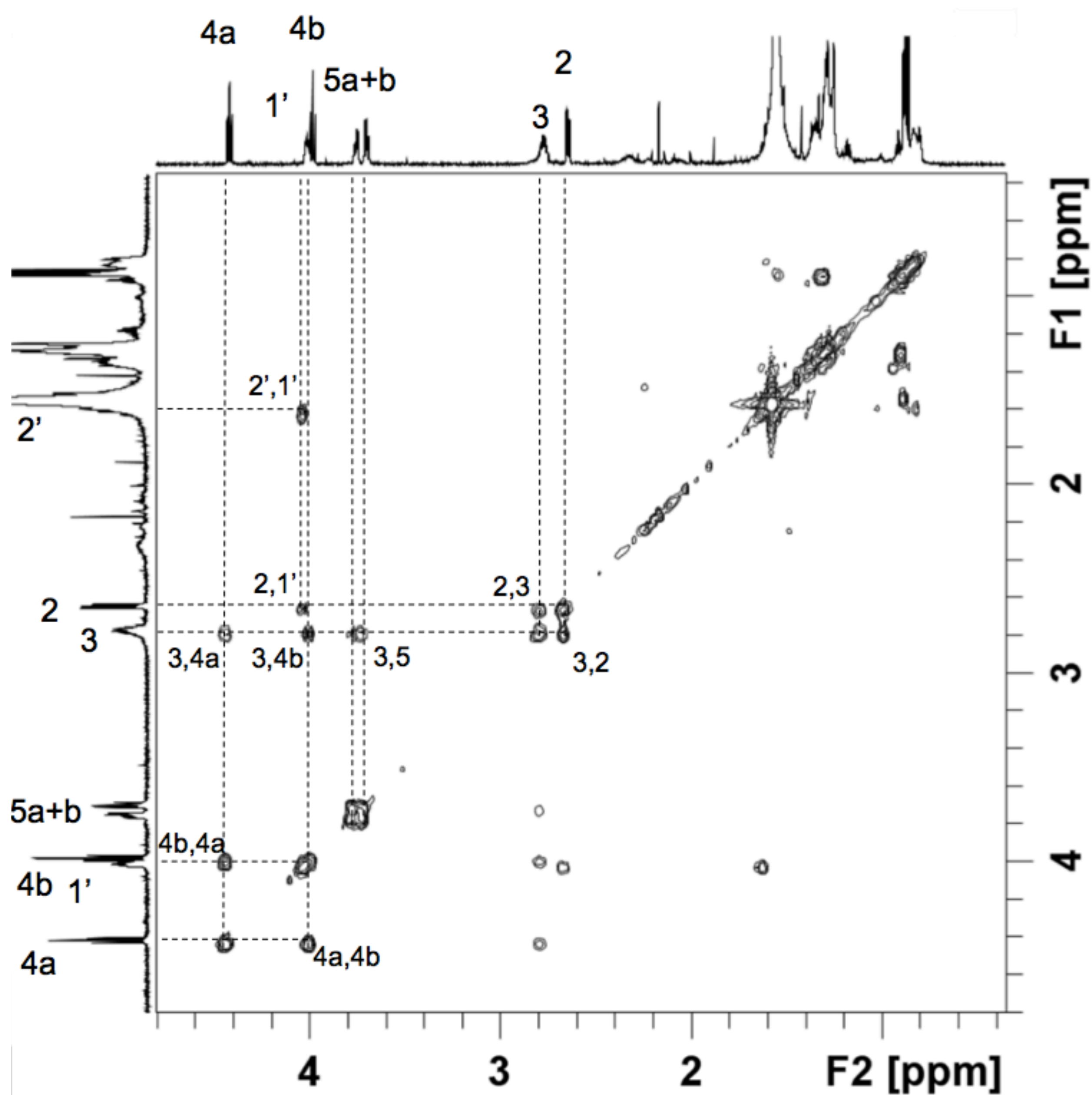

Figure S12. COSY spectrum for SCB1 isolated from *S. coelicolor* M1152; selected correlations observed in the COSY spectrum are highlighted by dashed lines.