Supplemental Data

Analysis of Two Additional Signaling Molecules in *Streptomyces coelicolor* and the Development of a Novel Butyrolactone-Specific Reporter System

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Primers used in this work

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* The *EcoRI* site is underline.  ** The *SacI* site is underline.

Supplemental Data LC-MS and tandem MS Data

The extracted ion chromatograms (EIC) of mass range 245.17-245.18 in which SCB1/SCB2 hydrogen adducts [(M+H)+] should be present show that they eluted late during the run. The synthesized SCB1 eluted at RT 40.33 min (Table 1). In the M145 ethyl acetate extract, the m/z 245.17 eluted at RT 39.30 min and RT 40.20 min (Table 1). In the mass spectra of wild type *S. coelicolor* M145 ethyl acetate extract and the synthesized SCB1 and SCB2 in correspondence to the highest peaks detected in the EICs, the following masses were detected: 245.17 (M+H)+, 227.16 (M+H-H2O)+ and 209.15 (M+H-H2O)+. The best matching chemical formula given by the Xcalibur 2.0.5 elemental composition tool software (Thermo Fisher Scientific) for these masses are: C_{13}H_{25}O_{4} (m/z 245.17), C_{13}H_{23}O_{3} (m/z 227.16) and C_{13}H_{21}O_{2} (m/z 209.15), which confirm the mono- and di-dehydration.

In the tandem MS spectrum of m/z 245.17, the most intense peaks correspond to the dehydrated structures (m/z 227.16 and m/z 209.15). Fragmentation of mass 227.16 (M+H-H2O)+ was also performed: major peaks at m/z 169.12, 155.11 and 141.09 were found and they represent the mono-dehydrated structures of SCB1 or SCB2 after beta-elimination on the acyl chain at different position. Minor peaks at m/z 191.14, 135.12, 133.10, 107.09, 95.09 and 81.07 were also detected at much lower intensity. Predicted formulas were also given for these masses: C_{10}H_{17}O_{2} (m/z 245.17), C_{10}H_{15}O_{3} (m/z 227.16) and C_{10}H_{13}O_{2} (m/z 209.15), which confirm the mono- and di-dehydration.

MS analysis was also performed to confirm the presence of SCB3 [(M+H)+...
In the MS spectrum of M145 at the highest peak in the mass range 259.18-259.20 at RT 43.97 min, the following masses are detected: m/z 259.19 (M+H)+, which is SCB3 hydrogen adduct, 241.18 (M+H-H2O)+ and 223.17 (M+H-H2O)+ which are the mono- and di-hydrated structure of SCB3. The same masses are also found in the reference standards spectra. The best matching chemical formula given by the Xcalibur elemental composition tool for these masses are: C14H27O4 (m/z 259.19), C14H25O3 (m/z 241.18) and C14H23O2 (m/z 223.17), which are the mono- and di-dehydrated structures.

In the tandem MS spectrum of mass 259.19 the most intense peaks correspond to the dehydrated structures (m/z 241.18 and m/z 223.17). Fragmentation of mass 241.18 (M+H-H2O)+ shows as major peaks with predicted formula m/z 223.17 (C14H22O2), m/z 169.12 (C10H17O2) and m/z 155.11 (C9H15O2), which represent the mono-dehydrated structures of SCB3 after beta-elimination on the acyl chain at different positions and m/z 205.16 (C14H21O1) which is a dehydrated form of the ring-opened lactone. Minor peaks of m/z 187.15, m/z 95.09 and m/z 67.05 are visualised at lower intensity. Predicted formulas for these masses are: C14H19 (187.15), C7H14 (m/z 95.09) and C5H7 (m/z 67.05). As negative control, SMMS ethyl acetate extract and ΔscbA, a mutant which does not produce any γ-butyrolactones, ethyl acetate extract were injected. No precursor ions and no fragment ions corresponding to SCB1, SCB2 and SCB3 were detected in these MS and MS/MS analysis.

**Fig. 1**

![Chemical structures](image)

**Figure S1.**
Chemical structures of the γ-butyrolactones isolated previously from *S. coelicolor* from Anisova *et al.*, (1984).
Figure S2. 400MHz $^1$H-NMR spectrum of SCB2

The predicted structure of SCB2 is shown in the inset. The number above each signal indicates the corresponding proton in SCB2. The signals at 4.43 and 3.99 ppm corresponded to methylene protons at C-4, and those at 3.76 and 3.70 ppm to hydroxymethylene protons at C-5. The signals at 4.02, 2.78 and 2.66 ppm corresponded to three methine protons at C-1’, C-3 and C-2, respectively. The remaining signals at 1.26 - 1.30 (10 protons) seemed to come from 5 methylene groups of alkyl side chain at C-2. However, the triplet signal at 0.89 ppm corresponded to three protons of one methyl group and indicated the presence of unbranched side chain at C-2, which contrasted to the terminal isopropyl containing side chain in SCB1. The coupling constant between H-2 and H-3 ($J_{2,3}=9.2$ Hz) was different from that of VB-A ($J_{2,3}=7.4$ Hz)(Yamada et al., 1987), but agreed well with that of IM-2 ($J_{2,3}=9.3$ Hz) (Sato et al., 1989), suggesting that SCB2 possesses an IM-2-type stereochemistry for C-2, 3 and 1’.

Figure S3. The schematic map of pTE134 used for the kanamycin bioassay

Fig. 3

Supplementary Material
pTE134 is constructed with scbR (open arrow) with its own promoter region, scbRp (black arrow) and a cpkO promoter, cpkOp (black arrow) coupled with a promoterless kanamycin resistant gene (neo, solid arrow). The light gray arrow indicates hyg (omega hygromycin resistance gene), the hatched arrow represents oriT (RP4 origin of single-stranded DNA transfer), the deep gray arrow represents int (phiC31 integrase), the shaded arrow indicates a partial coding region (204 bp) of scbB and the attP site (phiC31 attP site) is indicated by a vertical black line. The 200 bp length is indicated below and the restriction enzymes used for cloning are indicated by vertical black lines (not all SacI and EcoRI sites are represented in the map).

**Fig. 4**

![A-factor bioassay](image)

**Figure S4. Kanamycin bioassay using A-factor**

Different concentrations of A-factor were spotted onto a lawn of LW16::pTE134 on DNAagar plates containing 5 µg/ml kanamycin, respectively. The plates were incubated at 30 °C for 72 hours. The concentration where a growth halo is observed indicates the ability for A-factor to bind to ScbR.
Figure S5.
Kanamycin bioassay of the SCB1 isomers, SCB2 and SCB3
SCB1 isomers, SCB2 and SCB3: the natural SCB1((-)-SCB1 [2R,3R,1’R]), its
isomers: (+)-SCB1 [2S,3S,1’S], VB type (-)-SCB1 [2R,3R,1’S] and VB type (+)-SCB1
[2S,3S,1’R], the racemic SCB2 (IM-2 C₈) and the racemic SCB3 (IM-2 C₉-sec) were
spotted onto a plate with different amounts. The bold represent the natural SCBs
identified from S. coelicolor. The name of the compound is indicated above. The
amounts of the analogues are indicated at the left. The plates were incubated at 30 °C
for 72 hours.
Figure S6. Kanamycin bioassay of the IM-2 analogues
IM-2 type series: racemic analogous possessing a C-1’-β-hydroxyl group with a different length of linear C-2 side chain were spotted with different concentrations.

Figure S7. Kanamycin bioassay of the VB analogues
VB type series: racemic analogous possessing a C-1’-α-hydroxyl group with a different length of linear C-2 side chain were spotted with different concentrations. The bold represents the natural γ-butyrolactone VB-D identified from *S. virginiae*. 
Figure S8. Kanamycin bioassay of the branched IM-2 and VB analogues
The racemic IM-2 and the racemic VB analogues with 1'-hydroxy-7'-methylloctyl or 1'-hydroxy-6'-methylloctyl side chain or 1'-hydroxy-5'-methylheptyl side chain (VB C9-i, IM-2 C9-i, VB C9-sec, IM-2 C9-sec, VB C8-sec or IM-2 C8-sec) were spotted with different concentrations.

Figure S9. Kanamycin bioassay of three antibiotic producers from the Tübingen collection
*S. antibioticus*, *S. olivaceus* ssp. *Atratu* and *S. mediteranei* were grown on SMMS for 3 days at 30 °C (upper pictures) and extracted by ethyl acetate (see Experimental procedures) and spotted onto lawns of LW16::pTE134 on DNAgar plates containing 5 µg/ml kanamycin which were incubated at 30 °C for 6 days. Only the example for *S. antibioticus* is shown for those strains that did not grow well. Details listed in Table 3.