Supplementary information

Exploiting the inherent promiscuity of the acyl transferase of the stambomycin polyketide synthase for the mutasynthesis of analogues

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Materials and Methods

Materials

All reagents and chemicals including the fed mono- and diacids were obtained from Sigma-Aldrich. Oligonucleotide primers were synthesized by Eurogentec (France). The propargyl-malonic acid and 6-bromohexyl-malonic acid were chemically synthesized as described in reference[1].

Strains and media

Unless otherwise specified, all *Escherichia coli* strains were cultured in LB medium (yeast extract 10 g, tryptone 5 g, NaCl 10 g, distilled water up to 1 L, pH 7.0) or on LB agar plates (LB medium supplemented with 20 g/L agar) at 37 °C. *Streptomyces ambofaciens* ATCC23877[2] and its derived mutants were grown in TSB (TSB powder 30 g (tryptone 17 g, soy 3 g, NaCl 5 g, K_2HPO_4 2.5 g, glucose 2.5 g), distilled water up to 1 L, pH 7.3) or on TSA plates (TSB medium supplemented with 20 g/L agar), and sporulated on SFM agar plates (NutriSoy flour 20 g, D-mannitol 20 g, agar 20 g, tap water up to 1 L) at 30 °C. All strains were maintained in 20% (v/v) glycerol in 2 mL Eppendorf tubes and stored at −80 °C. For fermentation of *S. ambofaciens* ATCC23877 and its mutants, spores were streaked on TSA with appropriate antibiotics (apramycin 50 μg mL^{-1}, kanamycin 50 μg mL^{-1}, spectinomycin 50 μg mL^{-1}) and after incubation for 48 h at 30 °C, a loop of mycelium was used to inoculate 7 mL of MP5 medium (yeast extract 7 g, NaCl 5 g, $NaNO_3$ 1 g, glycerol 36 mL, MOPS 20.9 g, distilled water up to 1 L, pH 7.4) supplemented with selective antibiotics and sterile glass beads, followed by incubation at 200 rpm and 30 °C for 24–48 h. Finally, the seed culture was centrifuged (3500 *g*) and resuspended in 2 mL fresh MP5 before being inoculated into 50 mL MP5 medium in a 250 mL Erlenmeyer flask, and cultivated at 200 rpm and 30 °C for 4 days. Monoacids and diacids were fed to the media at a final concentration of 10 mM after inoculation of seed culture at 24 h incubation.

PCR-targeting based genetic engineering to generate the mutant ATCC/OE484/Δ483

To render the BAC BAB19ZF4 proficient for selection following conjugation, its chloramphenicol resistance gene was replaced , by a spectinomycin resistance gene cassette sourced from pIJ778[3] using a PCR-targeting approach, resulting in BAC3 (**Table S3**). In order to obtain an in-frame deletion of the *samR0483* gene*,* the primer pair D483_For/D483_Rev (**Table S4**) was designed to PCR amplify the "*FRT+aac(3)IV+oriT+FRT*" disruption cassette from pIJ773[3], affording PCR fragment K7Δ483. The PCR fragment was then electro-transformed into *E. coli* BW25113/pKD20/BAC3, giving rise to the mutant BAC BAC3_K7Δ483. Subsequently, BAC3_K7Δ483 was introduced into *E. coli* ET12567/pUZ8002[4] and then *S. ambofaciens* ATCC23877 *via* intergeneric conjugation. The resulting exconjugants (ATCC/Δ483_*aac(3)IV+oriT*) were selected for apramycin resistance and spectinomycin sensitivity, attesting to successful double crossover. The correct mutations were confirmed by PCR using primer pair F483_For/F483_Rev and sequencing. Thereafter, the cassette "*aac(3)IV+oriT*" was excised by the FLP recombinase encoded by plasmid pUWL-*oriT-flp* as described previously[5], leaving an 81 bp scar sequence (mutant ATCC/Δ483). Successful removal of the cassette was verified by PCR using primer pair F483_For/F483_Rev and DNA sequencing. Finally, the LAL regulator overexpression plasmid pOE484[6] and the parental vector pIB139 which was used as a control, were transferred into mutant ATCC/Δ483, giving rise to the mutants ATCC/OE484/Δ483 and ATCC/pIB139/Δ483, respectively (**Table S2**). (Note, mutant ATCC/OE484/Δ482 was generated in previous work[6]).

Complementation of mutant ATCC/OE484/Δ483

The gene *samR0483* and the constitutive promoter *ermEp** (including an RBS) were PCR amplified using primers Comp483_For/Comp483_Rev and ermEp_For/ermEp_Rev (**Table S4**) using ATCC genomic DNA and pIB139 as template, respectively. Two PCR fragments were fused into one fragment

(*ermEp*+RBS+samR483*) by overlap extension PCR, prior to cloning into the vector pCR™-Blunt. The fragment was then excised from pCR™-Blunt by FD *Nhe*I and FD *Spe*I digestion, and then cloned into pre-digested (FD *Nhe*I and FD *Spe*I) and dephosphorylated (FastAP) vector pOSV809, giving rise to the construct pOSV809-*ermEp*-samR483.* The vector was then transformed into *E. coli* S17-1 and introduced into mutants ATCC/Δ483/OE484 and ATCC/Δ483/pIB139 via intergeneric conjugation. Kanamycin and apramycin resistant conjugants were selected and verified by PCR with primers pOSV-For/φBT1-attB-Rev and pOSV-Rev/φBT1-attB-For (**Table S4**).

Construction of mutant ATCC/OE484/Δ483/MatB_cinna

The gene *matB_cinna* was cloned into an integrative and spectinomycin resistant vector by the group of Prof. Frank Schulz (Ruhr-Universität Bochum, Germany), resulting in the plasmid pRT801_lacZ_PermE_MatB_cinna. The plasmid was then transformed into *E. coli* S17-1[7] and introduced into mutant ATCC/Δ483/OE484 via intergeneric conjugation. Spectinomycin and apramycin resistant conjugants were selected and verified by PCR with primers pOSV-For/φBT1-attB-Rev and pOSV-Rev/φBT1-attB-For (**Table S4**).

LC-ESI-HRMS analysis of fermentation metabolites

The fermentation broth was centrifuged at 4,000 *g* for 10 min. The parental stambomycins and their derivatives were then extracted from the mycelia by first resuspending the cells in 40 mL distilled water, followed by centrifugation (4,000 *g*, 10 min, repeated 3×) to remove the water-soluble components. After decanting the water, the cell pellets were weighed and extracted with MeOH by shaking at 150 rpm for 2 h at room temperature. Thereafter, the MeOH extracts were filtered to remove the cell debris, followed by rotary evaporation to dryness. The obtained extracts were dissolved in MeOH, whose volume was determined according to the initial weight of the mycelia (70 µL MeOH to 1 g of cell pellet). The resulting mycelial extracts were then centrifuged at 16,000 *g* at 4 °C for 20 min and analysed in heated positive electrospray (HESI+) mode by HPLC-HRMS on either a Thermo Scientific Orbitrap LTQXL or an Orbitrap ID-X Tribrid Mass Spectrometer using an Alltima™ C18 column (2.1 × 150 mm, 5 µm particle size). Separation was carried out with Milli-Q water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) using the following elution profile: 0–48 min, linear gradient 5–95% solvent B; 48–54 min, constant 95% solvent B; 54–60 min, constant 5% solvent B. Mass spectrometry operating parameters were: spray voltage, 5 kV; source gases were set respectively for sheath gas, auxiliary gas and sweep gas at 30, 10, and 10 arbitrary units min^{-1}; capillary temperature, 275 °C; capillary voltage, 4 V; tube lens, split lens and front lens voltages 155, −28, and −6 V, respectively. Due to the much lower sensitivity of the Orbitrap LTQXL relative to the Orbitrap ID-X Tribrid as described previously[8], we introduced a 10× correction factor to the yields determined using the Orbitrap LTQXL. Quantification was achieved by adding erythromycin (1 mM) as internal standard during the extraction process. Alternatively, the yield of stambomycins from parental and mutasynthesis strains were determined using the stambomycin standard curve generated previoulsy[8], based on the integrated areas of EIC (extracted ion chromatogram) peaks.

NMR spectra were acquired on Bruker AV 500 and AV600 (only used for deoxy-allyl-stambomycin measurements) spectrometers, using the residual solvent peaks as reference. The spectra were processed using MestReNova 15.0.1 software. Coupling constants (*J*) are given in Hz.

Isolation and purification of mutasynthetic stambomycin analogues

In order to isolate allyl- and butyl-incorporated stambomycin analogues, 85 × 50 mL flasks of fermentation culture for ATCC/Δ483/OE484/MatB_cinna and 50 × 50 mL flasks culture for ATCC/Δ483/OE484 were cultivated in the presence of 10 mM fed allyl- and butylmalonic acids, respectively. The crude allyl-fed extract (18 mL in MeOH) and butyl-fed extract (12 mL in MeOH) were subjected to Agilent preparative HPLC system and a Waters Xbridge BEH C18 column (19 × 250 mm). The two extracts were separated by

using acetonitrile/H_2O containing 0.1% formic acid with the elution profile of 0–2 min 10% acetonitrile, then 2–27 min linear gradient 5–95% acetonitrile at the flow rate of 20 mL min^{-1} to yield four purified compounds deoxy-butyl-stambomycin (36.3 mg), butyl-stambomycin (11.6 mg), C-24-demethyl-stambomycin (3.1 mg), and deoxy-allyl-stambomycin (1.8 mg). Details for the HPLC separation are also summarized in **Table S8**.

Antibacterial and antiproliferative tests

The antibacterial activities of the four purified stambomycin analogues were analysed by loading 1 µL of the compounds at 10 mM and at various dilutions (from 1:2 to 1:20) onto a lawn of the Gram-positive bacteria *Bacillus subtilis* ATCC6633 and *Micrococcus luteus* ATCC10240, or the Gram-negative bacterium *Escherichia coli* DH5α[9]. One microliter of DMSO, the solvent used to resuspend the purified analogues, was used as a control. The plates were incubated overnight at 30 °C (*B. subtilis*) or 37° C (*M. luteus* and *E. coli*). For the antiproliferative activities on tumour cells, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays were carried out to measure the effects of the stambomycin analogues on the metabolic activity of two cancer cell lines, U87-MG glioblastoma cells (brain cancer) (ATCC HTB-14) and MDA-MB-231 cells (breast cancer) (ATCC HTB-26). Briefly, U87-MG and MDA-MTB-231 cells (200 µL at 1.5×10^4 cells mL^{-1}) were seeded in 96-well plate. Drug treatment was carried out 48 h later. After 48 h, the cells were incubated with 0.625 mg ml^{-1} MTT for 3 h, and then the absorbance was measured at 560 nm using a microplate reader (Multiskan Ascent, ThermoFisher). For completeness, cell viability was assessed by cell counting (number of cells mL^{-1}) using a TC20 automated cell counter (Bio-Rad). All the assays were performed in triplicate via three independent experiments, and the concentrations that induced 50% of cytotoxicity (IC50) were calculated using the Prism 5.00 software.

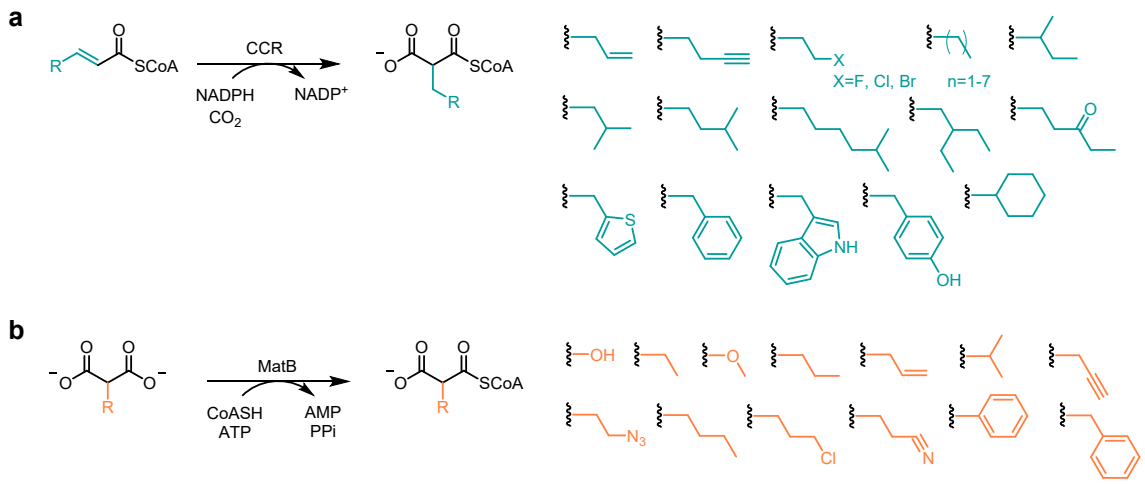


Figure S1. Biosynthesis of CoA-linked polyketide synthetase extender units. **a**) CCR enzymes (e.g. AntE[10], SalG[11] and SpnE[12], etc.) and their engineered variants catalyse the reductive carboxylation of CoA-linked α,β-unsaturated acyl-CoA via utilization of one reducing equivalent of NADPH, to afford malonyl-CoA analogous. **b**) MatB (e.g. MatB_Sc[13] from *S. coelicolor*, MatB_cinna[1] from *S. cinnamonesis* and MatB_Rt[14] from *Rhizobium trifolii*, etc.) and derived mutants are capable of generating the CoA derivatives of a broad range of malonate analogues, via an ATP-dependent reaction.

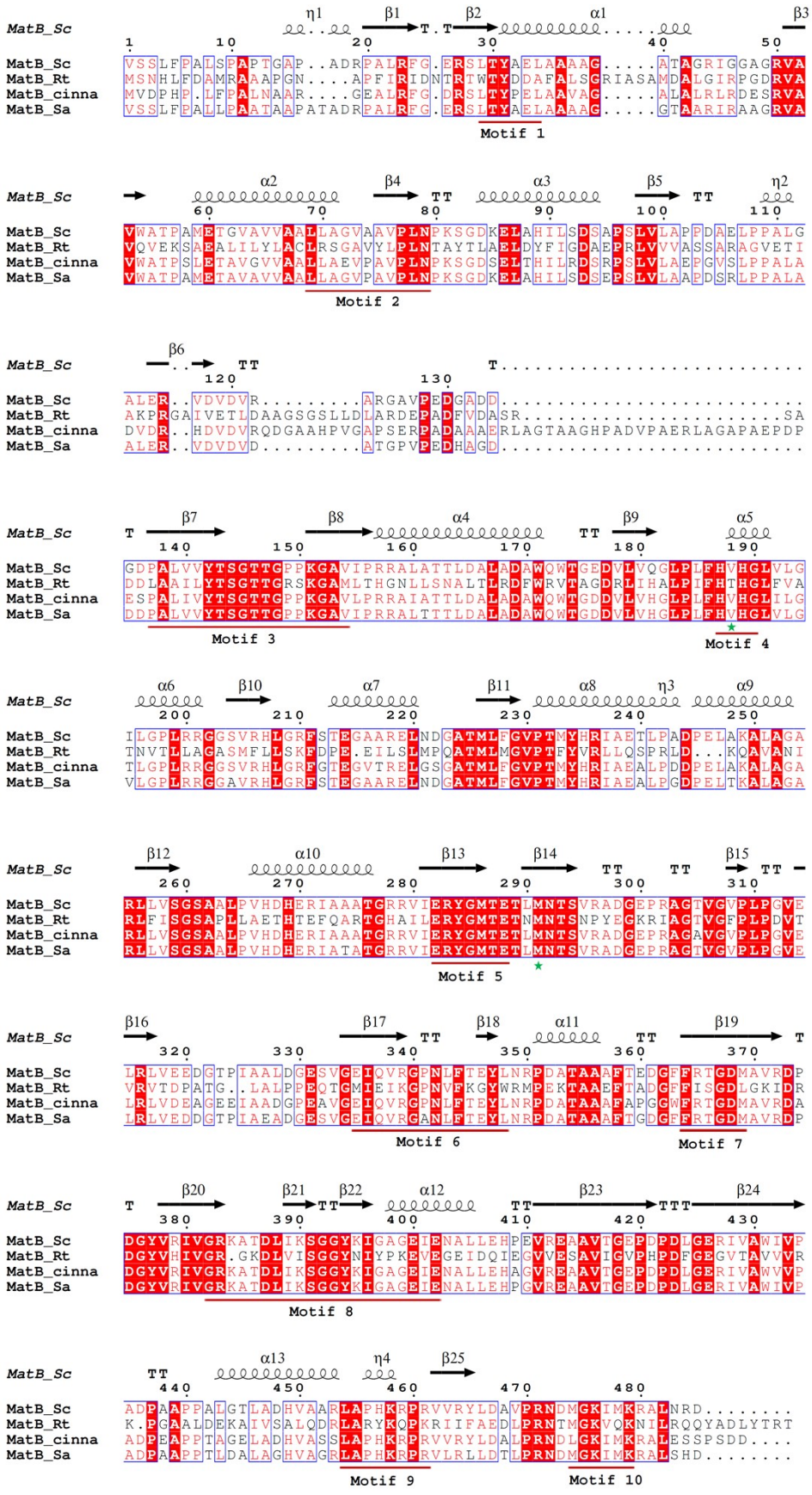
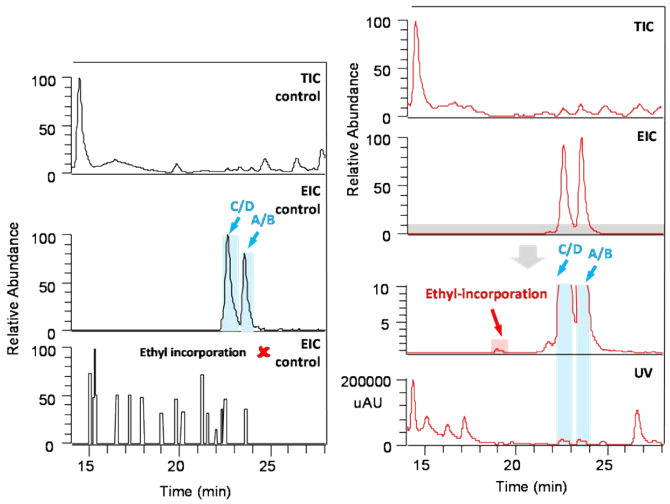
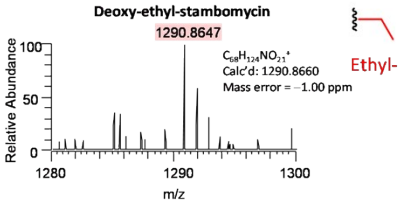

MatB_Sc
MatB_Rt
MatB_cinna
MatB_Sa
Motif 1
Motif 2
Motif 3
Motif 4
Motif 5
Motif 6
Motif 7
Motif 8
Motif 9
Motif 10

Figure S2. Sequence alignment of MatB-type enzymes. The secondary structure of MatB_Sc (PDB 3NYQ:A) is shown at the top. Residues which are strictly conserved are indicated in white on a red background, while relatively conserved residues are indicated in red. The red underlines represent conserved core motifs for adenylate-forming enzymes, of which motifs 4 and 5 are known to bind the carboxylated substrates and stabilize the formation of the adenylate intermediates[13]. The green stars refer to two hydrophobic amino acids mutated in MatB_Rt[14], which on the basis of the elucidated structure of MatB_Sc are likely to influence the promiscuity of MatB-type enzymes for α-substituted malonate derivatives. MatB_Sa, WP_053130319, MatB from *S. ambofaciens*; MatB_Sc, 3NYQ:A, MatB from *S. coelicolor*; MatB_cinna, MatB from *S. cinnamonensis* (no available accession number but the amino acid sequence was published previously[15]); MatB_Rt, AAC83455, MatB from *R. trifolii*. MatB_Sa exhibits 90%, 75% and 39% amino acid sequence identity respectively to MatB_Sc, MatB_cinna and MatB_Rt.

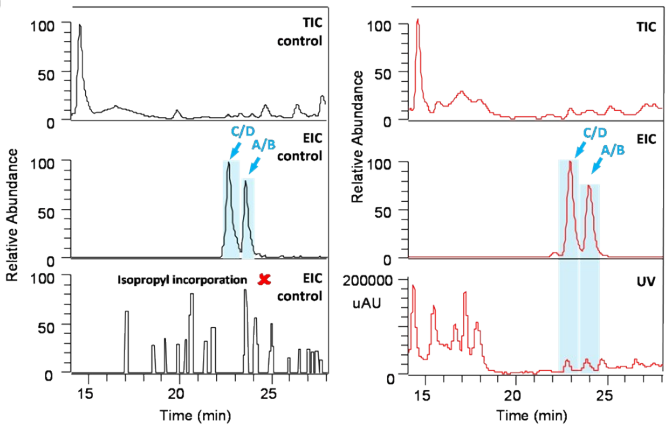
a





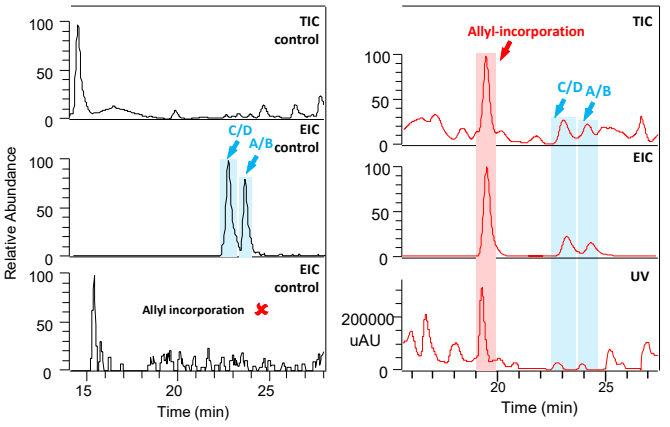
Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with ethylmalonic acid	Stambomycin C/D + A/B	149221532 (853%)
	Ethyl-stambomycin	n.d.
	Deoxy-ethyl-stambomycin (**1**)	151190 (0.86%)

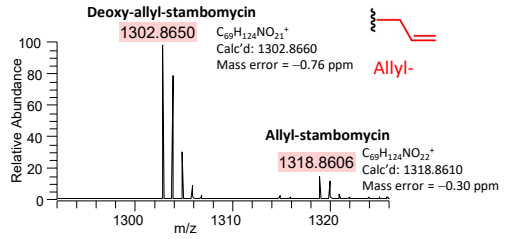
b



Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with isopropyl-malonic acid	Stambomycin C/D + A/B	63227152 (361%)
	Isopropyl-stambomycin	n.d.
	Deoxy-isopropyl-stambomycin	n.d.

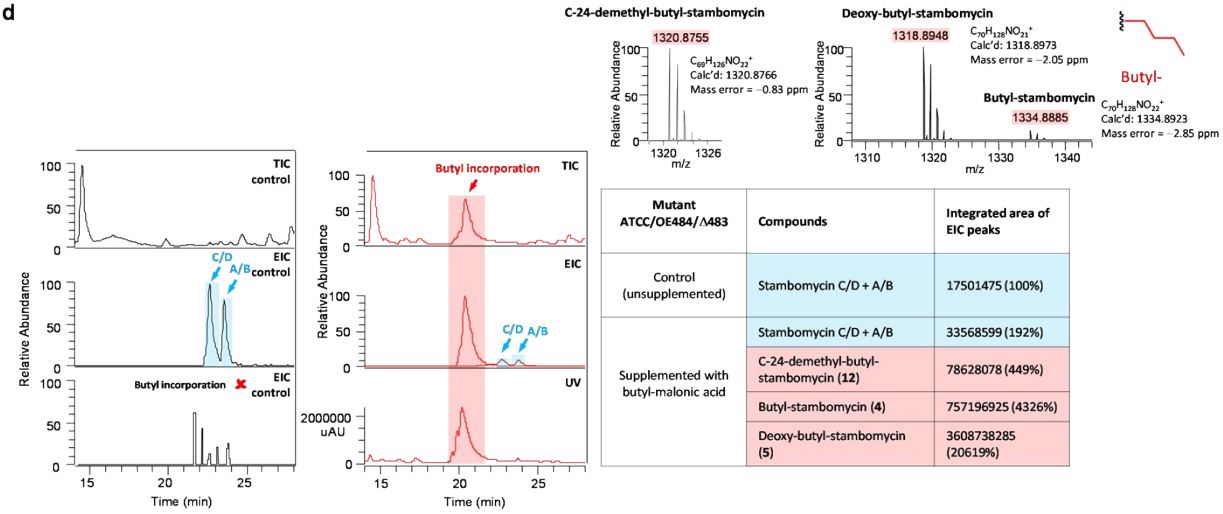
c





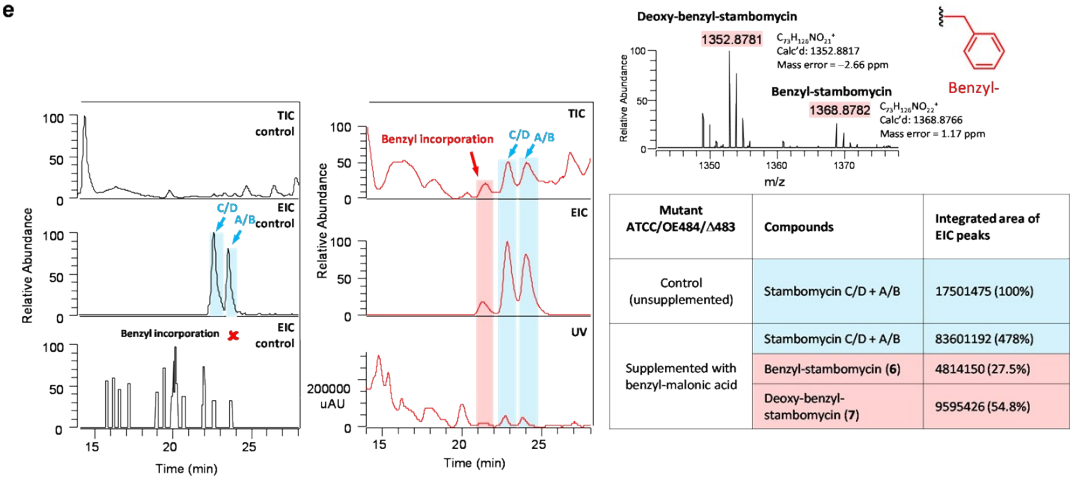
Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with allyl-malonic acid	Stambomycin C/D + A/B	33027066 (189%)
	Allyl-stambomycin (**2**)	359513 (2.05%)
	Deoxy-allyl-stambomycin (**3**)	65873199 (378%)

d



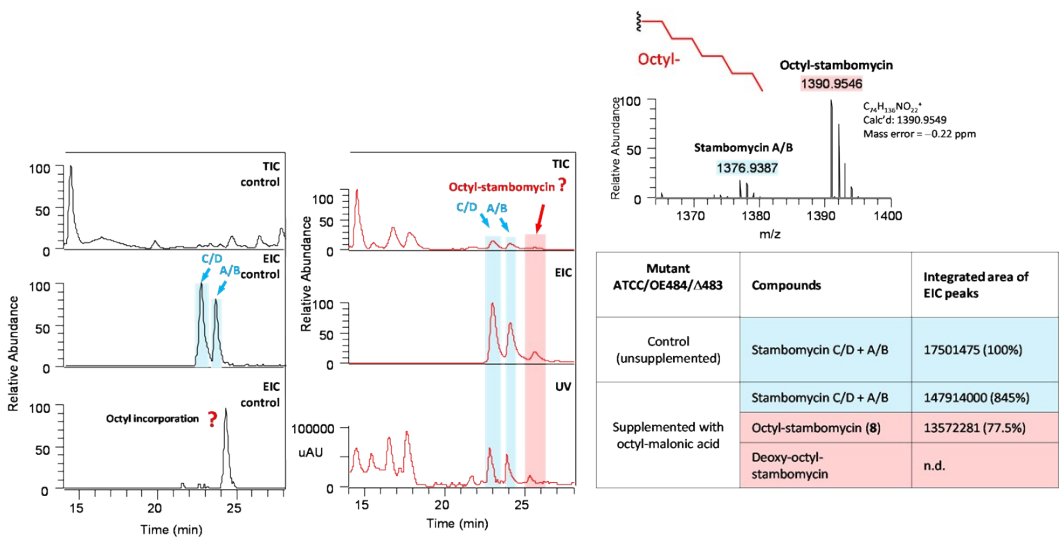
Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with butyl-malonic acid	Stambomycin C/D + A/B	33568599 (192%)
	C-24-demethyl-butyl-stambomycin (**12**)	78628078 (449%)
	Butyl-stambomycin (**4**)	757196925 (4326%)
	Deoxy-butyl-stambomycin (**5**)	3608738285 (20619%)

e



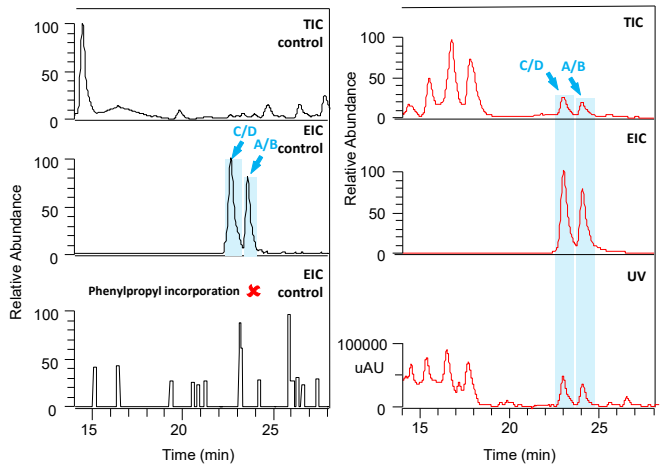
Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with benzyl-malonic acid	Stambomycin C/D + A/B	83601192 (478%)
	Benzyl-stambomycin (**6**)	4814150 (27.5%)
	Deoxy-benzyl-stambomycin (**7**)	9595426 (54.8%)

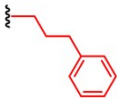
f



Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with octyl-malonic acid	Stambomycin C/D + A/B	147914000 (845%)
	Octyl-stambomycin (**8**)	13572281 (77.5%)
	Deoxy-octyl-stambomycin	n.d.

g

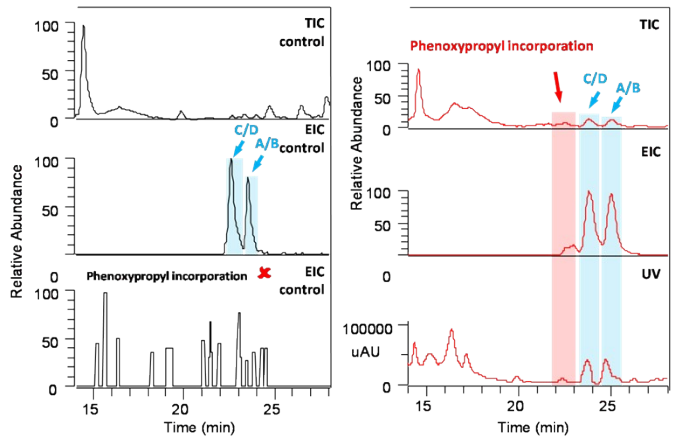


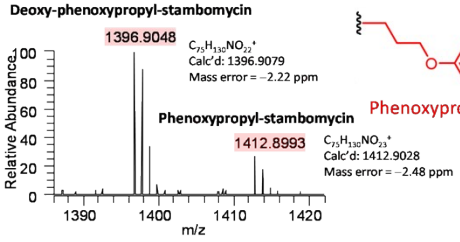


Phenylpropyl-

Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with phenylpropyl-malonic acid	Stambomycin C/D + A/B	103429282 (591%)
	Phenylpropyl-stambomycin	n.d.
	Deoxy-phenylpropyl-stambomycin	n.d.

h

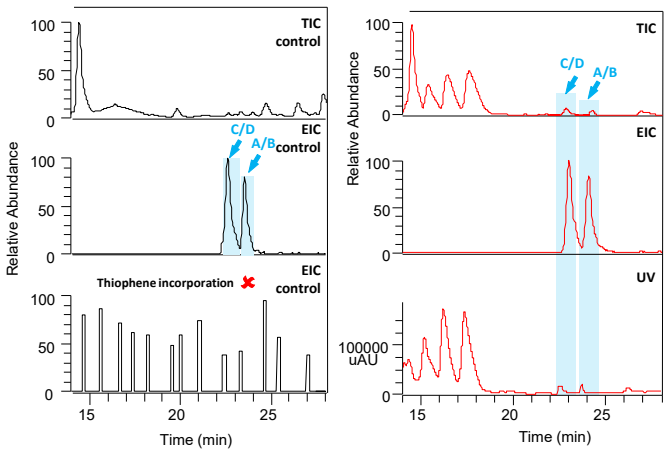




Phenoxypropyl-

Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with phenoxypropyl-malonic acid	Stambomycin C/D + A/B	40699658 (233%)
	Phenoxypropyl-stambomycin (**9**)	1916650 (10.9%)
	Deoxy-phenoxypropyl-stambomycin (**10**)	2366663 (13.5%)

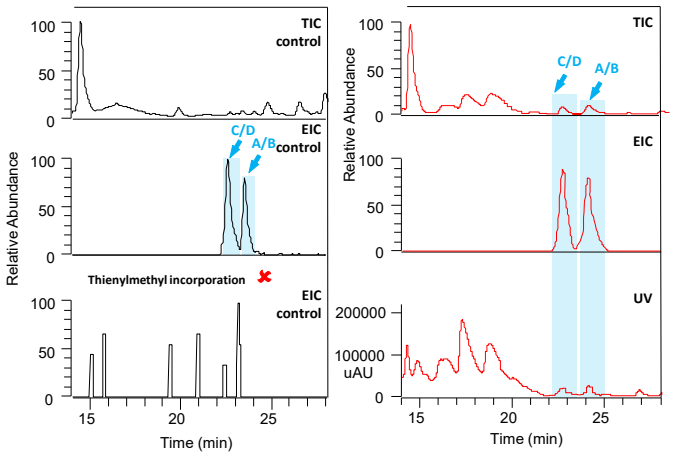
i



Thiophenyl-

Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with thiophene-malonic acid	Stambomycin C/D + A/B	14692173 (83.9%)
	Thiophene-stambomycin	n.d.
	Deoxy-thiophene-stambomycin	n.d.

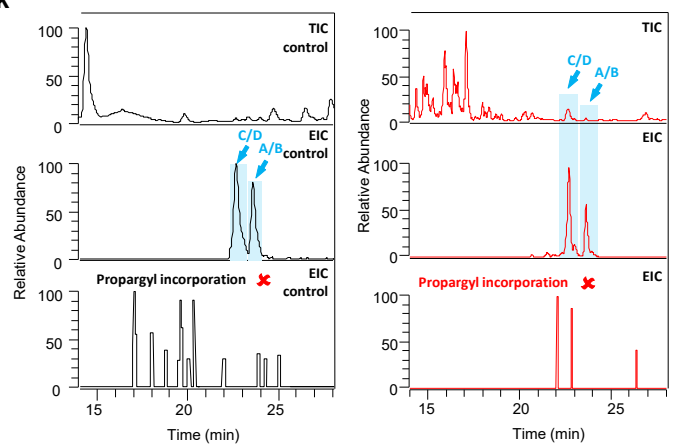
j



Thienylmethyl-

Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with thienylmethyl-malonic acid	Stambomycin C/D + A/B	17404928 (99.4%)
	Thienylmethyl-stambomycin	n.d.
	Dexoy-thienylmethyl-stambomycin	n.d.

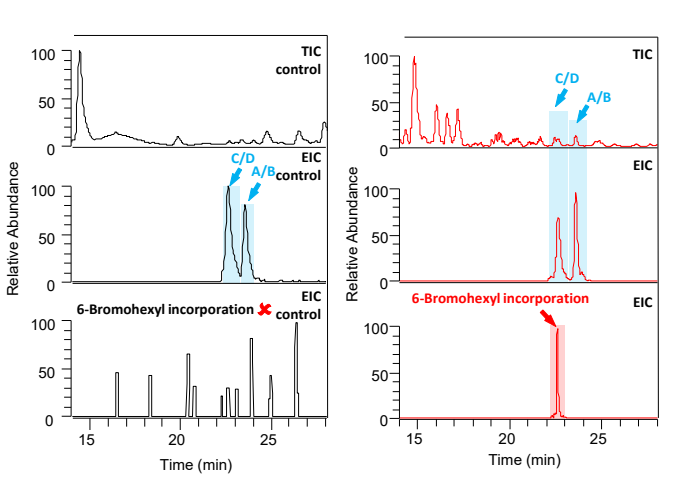
k



Propargyl-

Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with propargyl-malonic acid	Stambomycin C/D + A/B	80079185 (458%)
	Propargyl-stambomycin	n.d.
	Deoxy-propargyl-stambomycin	n.d.

l



6-Bromohexyl-

Mutant ATCC/OE484/Δ483	Compounds	Integrated area of EIC peaks
Control (unsupplemented)	Stambomycin C/D + A/B	17501475 (100%)
Supplemented with 6-bromohexyl-malonic acid	Stambomycin C/D + A/B	106923327 (611%)
	6-Bromohexyl-stambomycin (lacking 2H) (**11**)	4817471 (27.5%)
	Deoxy-6-bromohexyl-stambomycin (lacking 2H)	n.d.

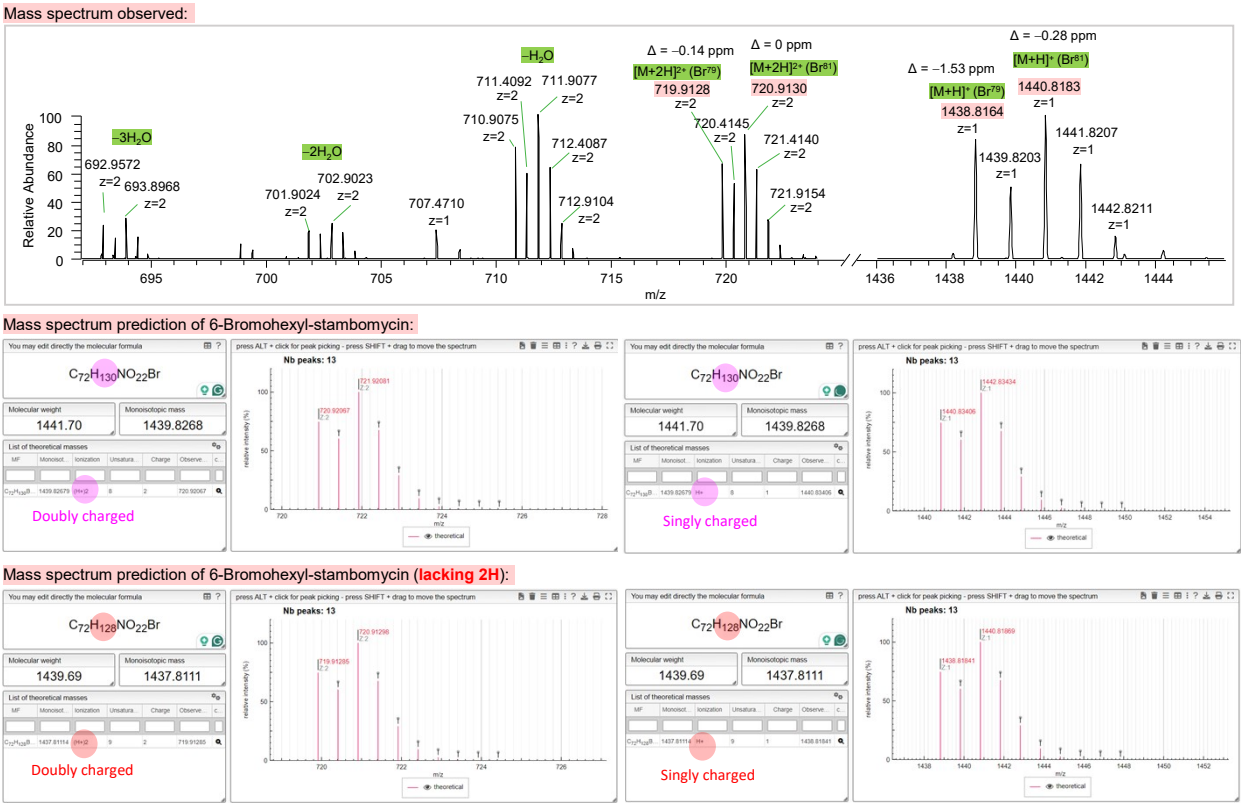


Figure S3. Analysis by mass spectrometry and UV detection of crude extract from the mutasynthesis strain ATCC/OE484/Δ483 supplemented with various malonic acid alternatives in comparison to the control ATCC/OE484/Δ483 without supplementation. In each case are shown: the TIC, EIC and UV_{238} of crude fed-extracts, the mass spectrum of the corresponding stambomycin analogues, and the production levels of both the analogues and the native stambomycins A–D based on the integrated area of EIC peaks (average of at least two independent fermentations). The relative yield of stambomycins/analogues in the fed strain is also shown, which was calculated based on the production of stambomycins (C/D + A/B) by the mutasynthesis strain ATCC/OE484/Δ483 in the absence of supplementation (set to 100%). " n.d." indicates compounds which were not detected. (Note: the measured masses for the product resulting from incorporation of 6-bromohexyl-malonic acid exhibit a strong M+2 peak, and the characteristic bromine isotopic pattern. However, the observed mass is 2 Da lower than expected, which we propose arises from over-oxidation at the C-28 position.) All experiments were conducted in triplicates (see details in **Table S7**).

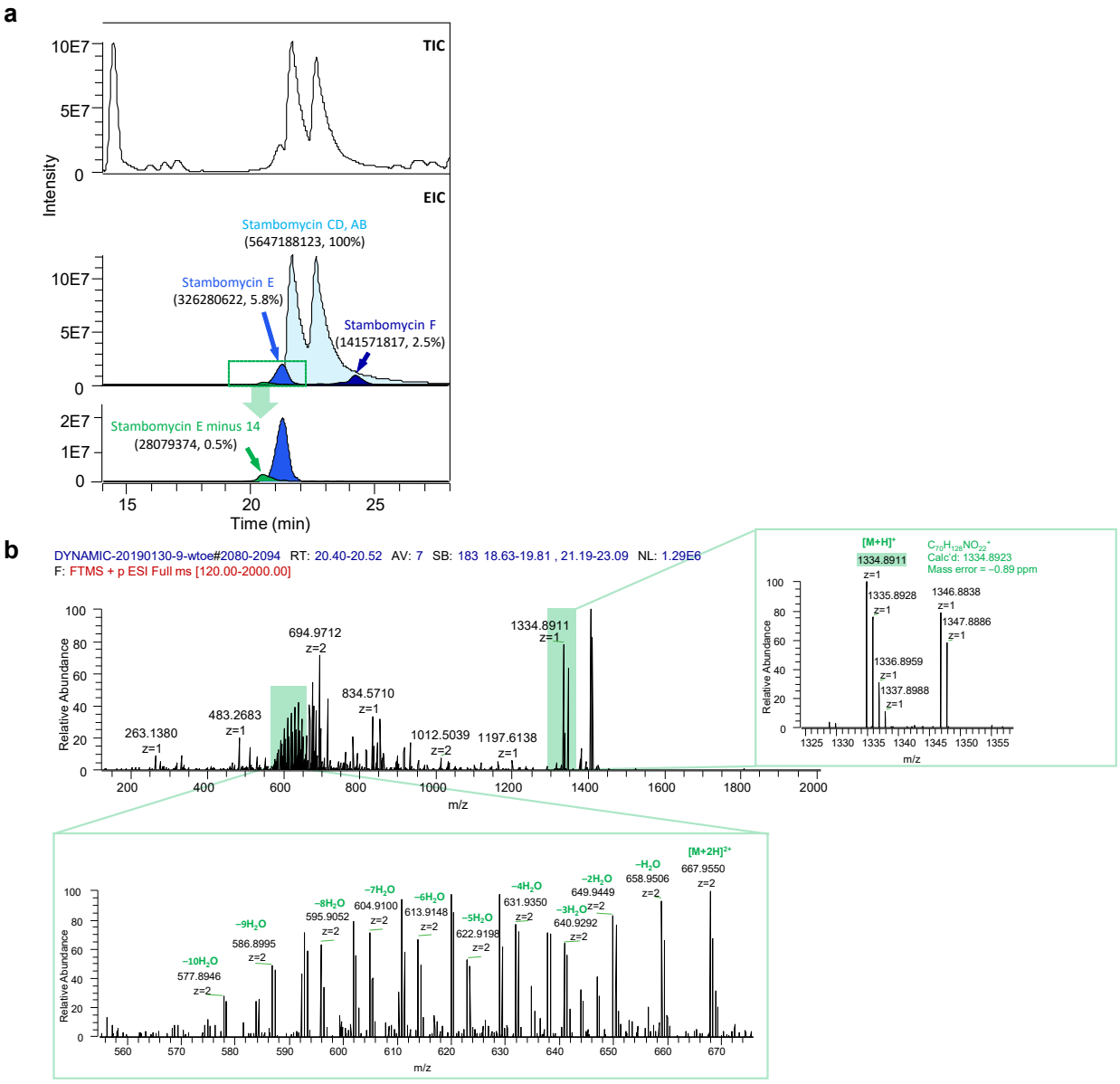


Figure S4. Analysis by mass spectrometry of the crude extract from the parental ATCC/OE484. **a**) TIC of the crude extract of ATCC/OE484 and the EIC of stambomycins A–F (representative of two replicates). The production of stambomycins E and F are only 5.8% and 2.5% of stambomycins A–D, as judged on the basis of relative peak areas. We also detected a mass consistent with stambomycin E minus 14, which we attribute to selection by module 13 of malonyl-CoA extender unit instead of the typical methylmalonyl-CoA. Nevertheless, malonyl-CoA remains favoured by AT_{13} as the yield of stambomycin E (5.8%) is 12-times higher than the putative demethyl-stambomycin E (0.5%) in the parental strain. **b**) Mass spectra of dimethyl-stambomycin E, showing the characteristic multiple water losses[8].

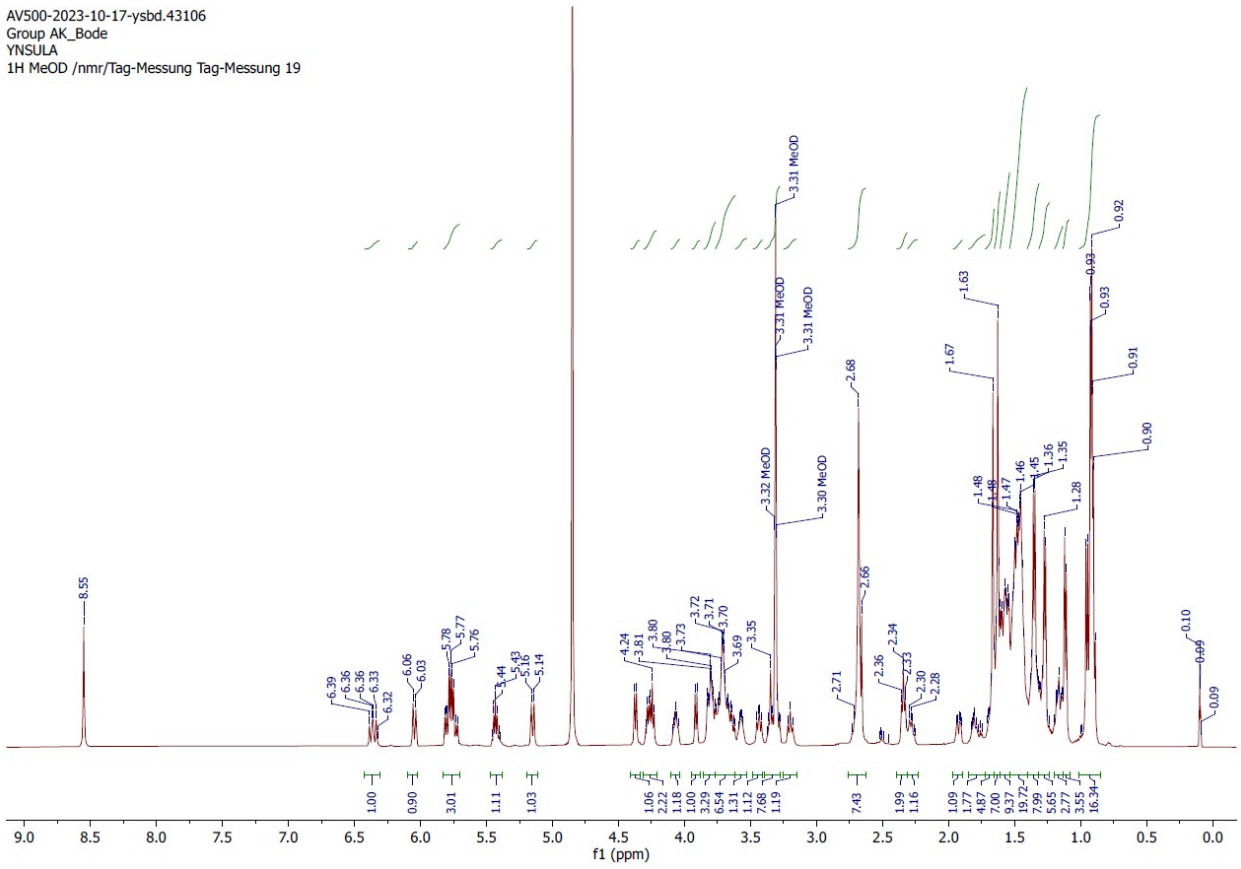


Figure S5. ^{1}H NMR spectrum of deoxy-butyl-stambomycin (**5**).

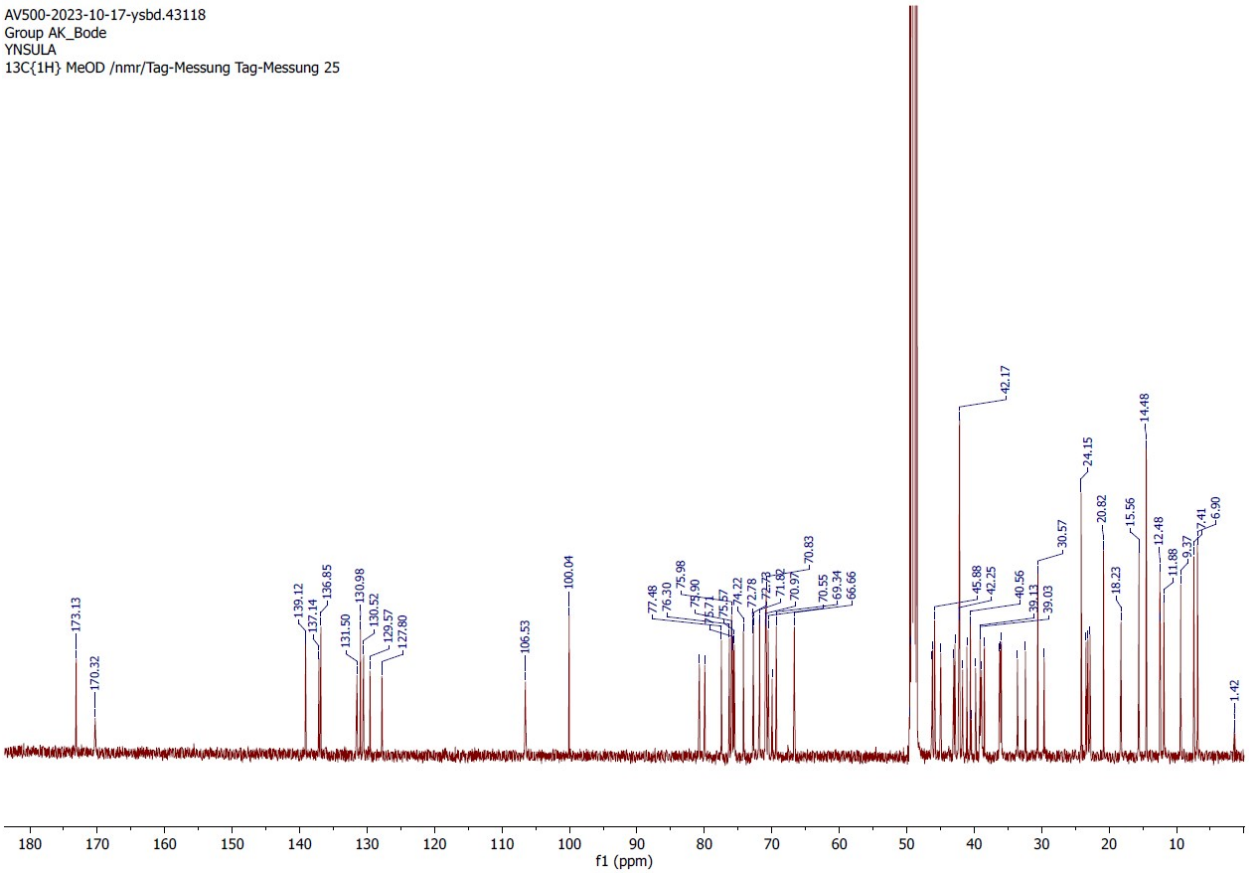


Figure S6. ^{13}C NMR spectrum of deoxy-butyl-stambomycin (**5**).

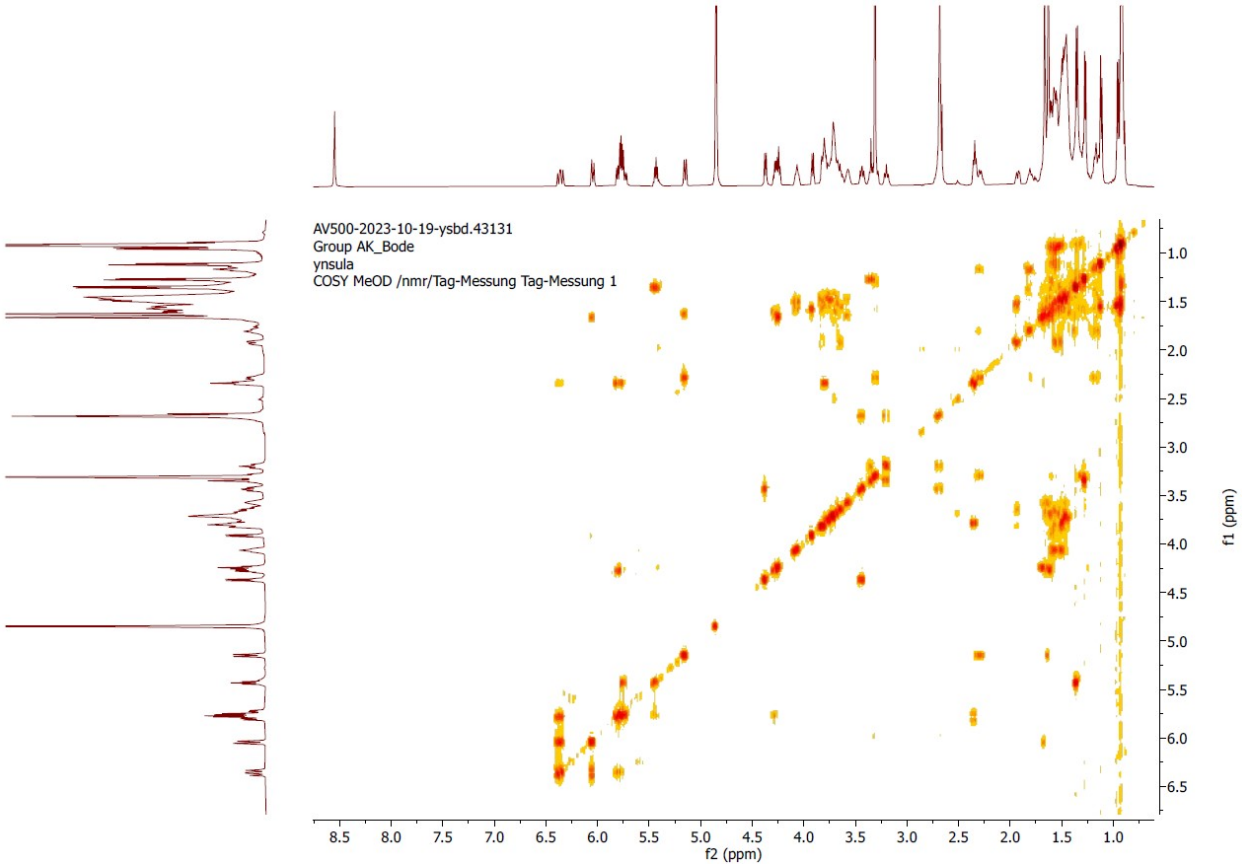


Figure S7. ^{1}H-^{1}H COSY spectrum of deoxy-butyl-stambomycin (**5**).

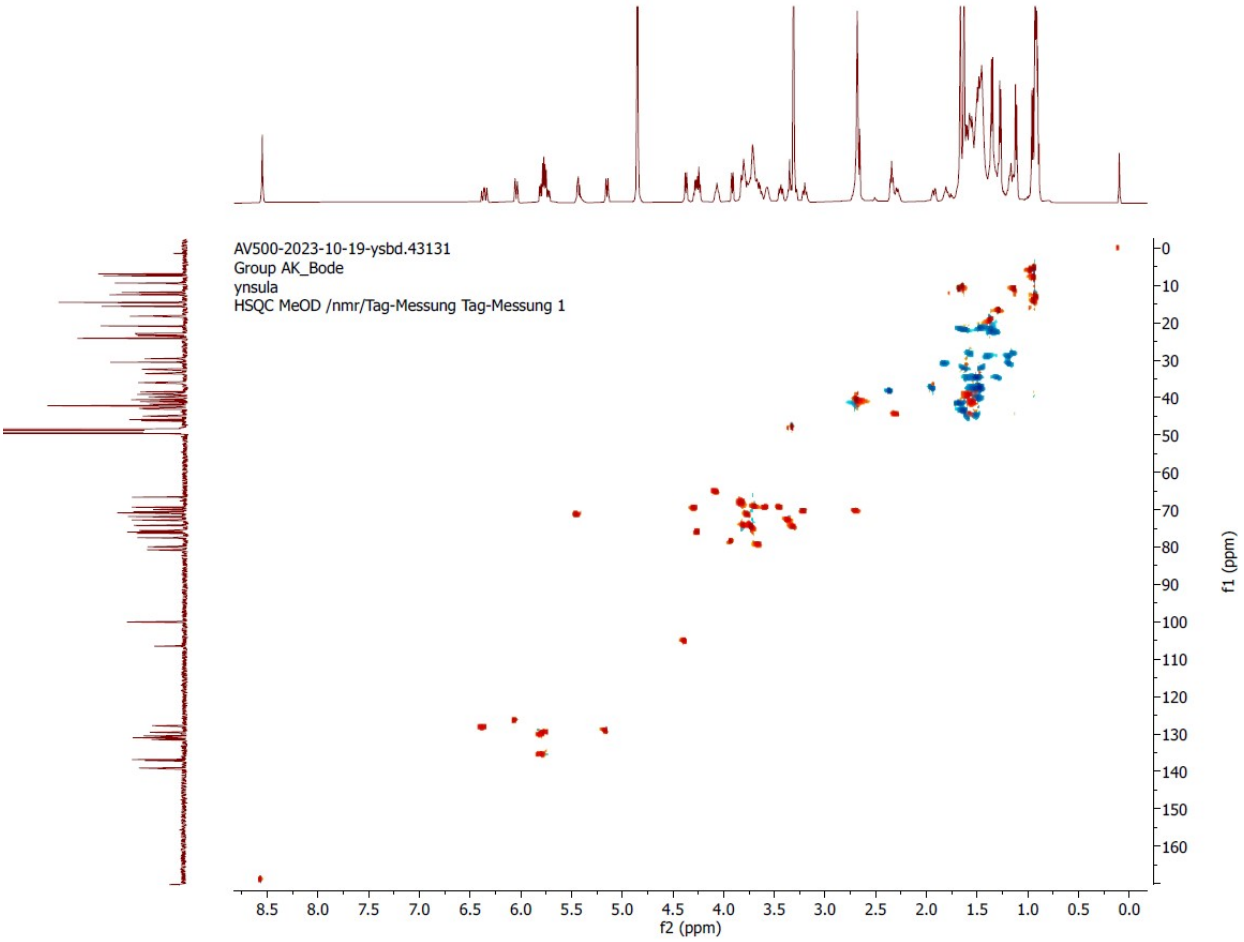


Figure S8. [^{1}H, ^{13}C]-HSQC spectrum of deoxy-butyl-stambomycin (**5**).

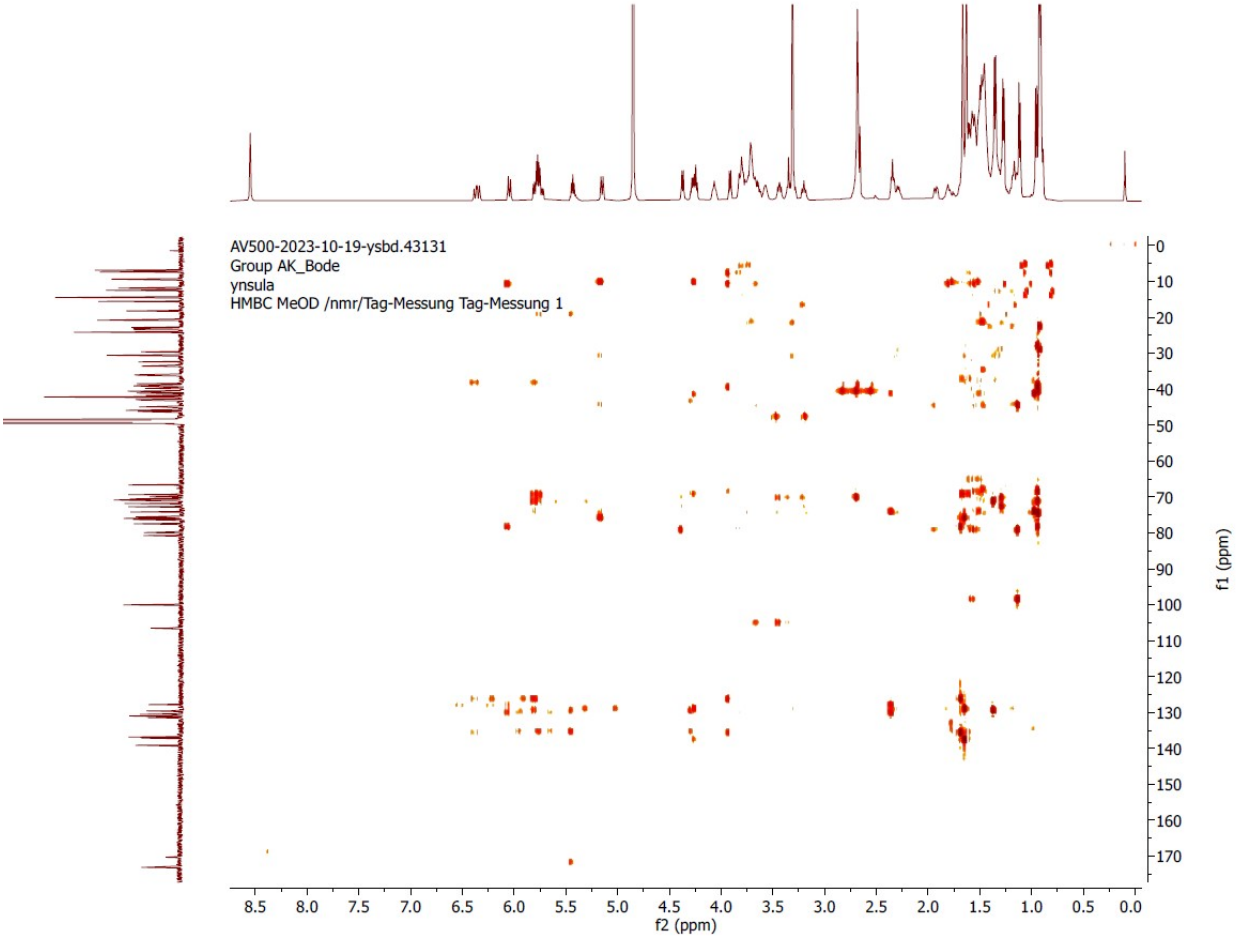
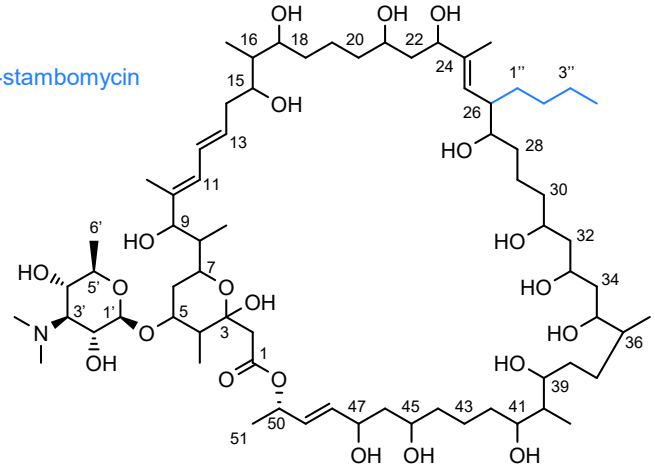


Figure S9. [^{1}H, ^{13}C]-HMBC spectrum of deoxy-butyl-stambomycin (**5**).

Deoxy-butyl-stambomycin



Position	δC (ppm)	δH (ppm)	HMBC	Position	δC (ppm)	δH (ppm)	HMBC
1	173.1			24-Me	11.8	1.63	
2	43.0	2.70	10.0	25	129.5	6.36	15.0, 10.9
3	100			26	42.2	2.64	
4	45.8	2.29		1″	32.4	1.15, 1.81	
4-Me	12.4	1.11		2″	24.1	1.30, 1.35	
5	80.7	3.65		3″	36.2	1.58	
6	39.1	1.46, 1.90		4″	15.5	0.91	
1′	106	4.37	7.4	27	76.3	3.70	
2′	70.5	3.68	10.6, 9.6	28	23.1	1.43	
3′	71.8	3.19		29	29.6	1.13, 1.56	
3′-NMe_2	42.1	2.66		30	35.9	1.46	
4′	70.8	3.43	10.3, 7.4	31	69.3	3.81	
5′	74.2	3.35	9.4	32	46.2	1.47	
6′	18.2	1.28	5.9	33	66.6	4.07	
7	69.9	3.81		34	39.7	2.35	
8	40.5	1.57		35	72.7	3.75	
8-Me	9.3	0.93		36	41.0	1.57	
9	79.9	3.91	7.3	36-Me	14.4	0.91	
10	137.1			37	30.5	1.18, 1.38	
10-Me	12.4	1.66		38	38.9	1.47, 1.94	
11	127.8	6.04	10.8	39	75.9	3.30	7.3
12	130.5	5.15	15.0, 10.2	40	41.7	1.48	
13	131.5	5.78	15.4, 8.3	40-Me	6.9	0.91	
14	40.4	2.66		41	75.9	3.70	
15	75.7	3.72		42	33.5	143, 1.60	
16	42.8	1.62		43	22.8	1.43	
16-Me	7.4	0.96		44	39.0	1.54, 1.91	
17	75.5	3.79		45	71.8	2.68	
18	36.1	1.56, 1.46		46	46.1	1.57	
19	23.4	1.59, 1.34		47	70.9	4.27	12.7, 6.7
20	38.4	1.45		48	136.8	5.77	15.4, 8.3
21	70.8	3.57		49	130.9	5.73	
22	44.9	1.6		50	72.7	5.44	6.4
23	77.4	4.23	6.7	51	20.8	1.35	6.4
24	139.1						

Figure S10. Structure and summary of the NMR data for deoxy-butyl-stambomycin (**5**). The ^{13}C NMR spectrum exhibits signals accounting for 70 carbons. Comparison of the ^{1}H and ^{13}C NMR data acquired on deoxy-butyl-stambomycin with that reported for stambomycin C[6], revealed that the two compounds share the same macrocyclic structure, except for the alkyl chain present at position C-26. The HMBC correlations of H-50 (δ_C 5.44) with the C-1 (δ_C 173.1), C-51 (δ_C 72.7), C-49 (δ_C 130.9) and C-48 (δ_C 136.8), show that a closed macrocycle is present. Connections between carbons and their respective protons were assigned based on the HSQC spectrum. The coupling constant are indicated in the table above when possible. Together, these data confirmed the identity of the metabolite as deoxy-butyl-stambomycin.

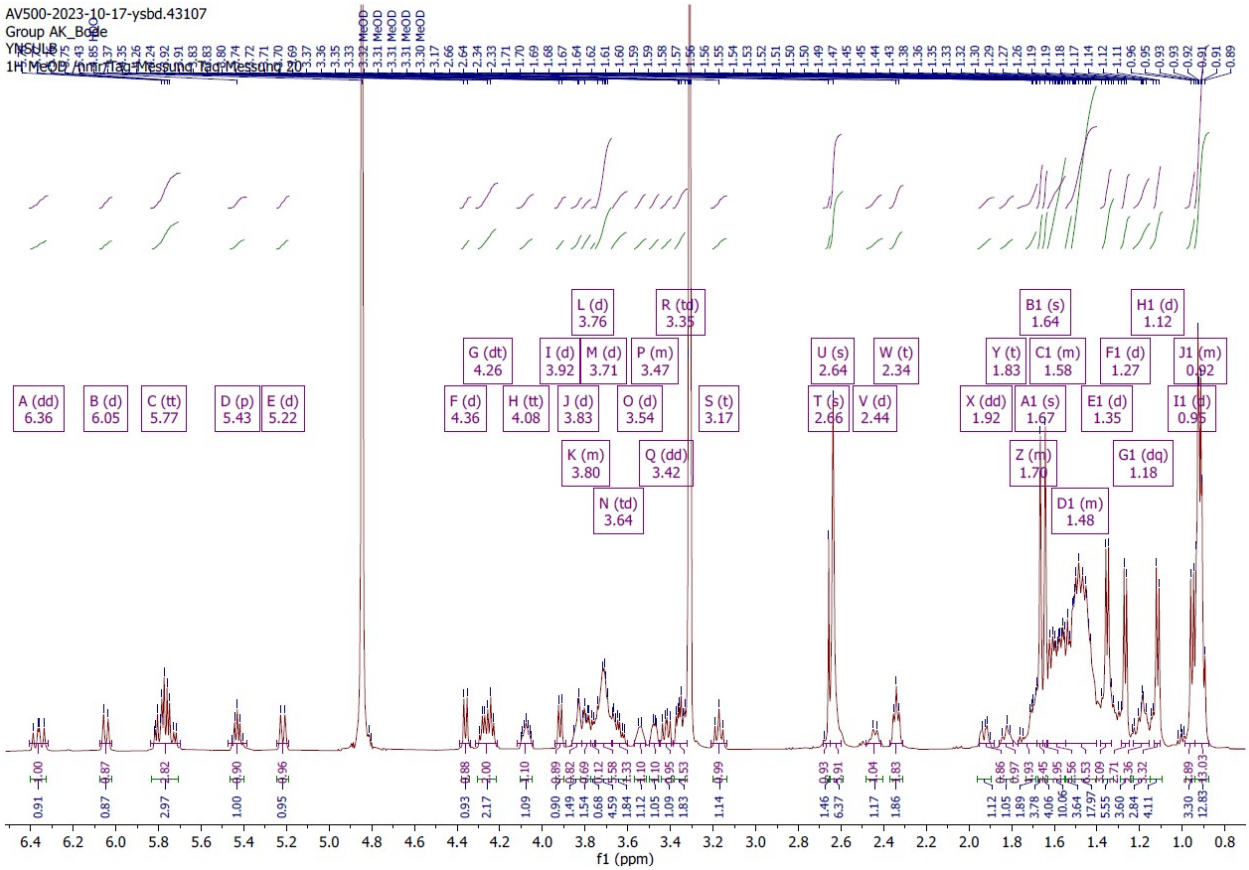


Figure S11. ^{1}H NMR spectrum of butyl-stambomycin (**4**).

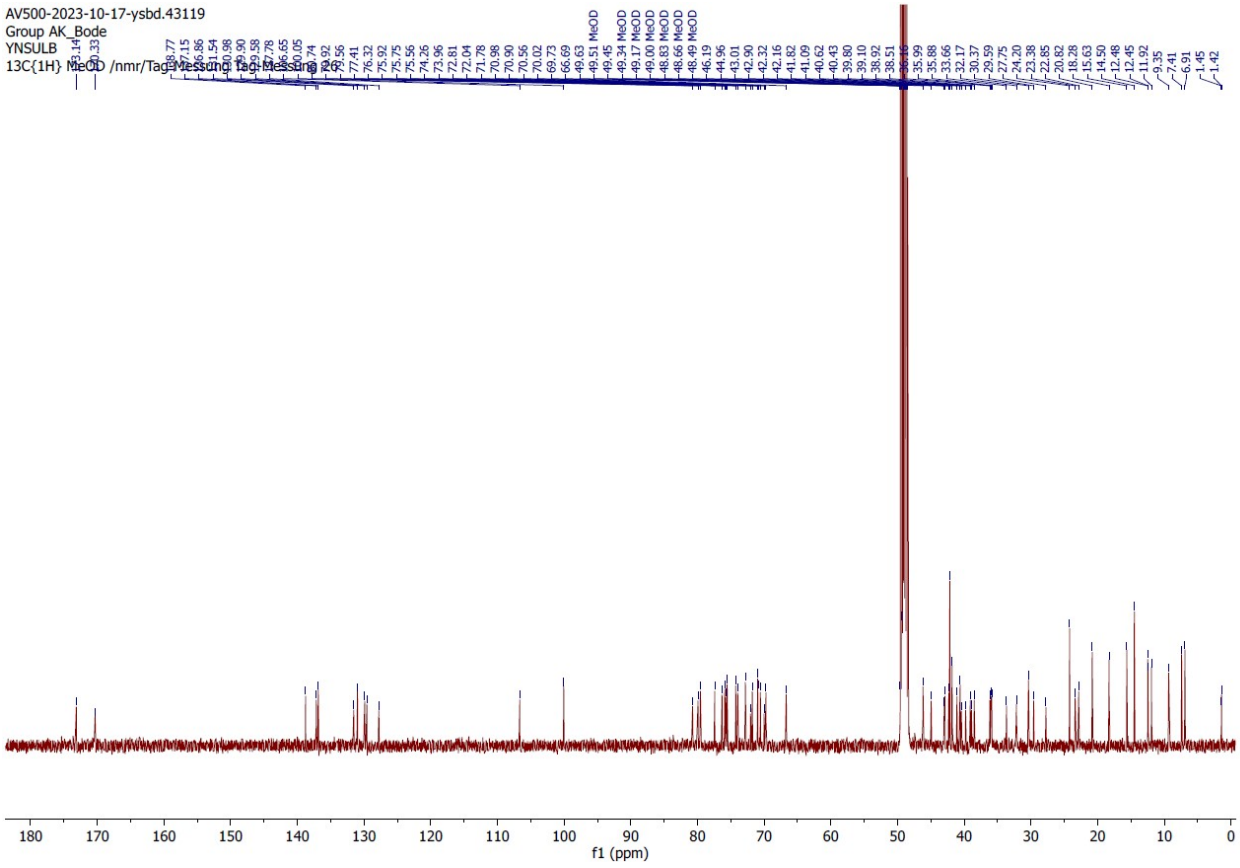


Figure S12. ^{13}C NMR spectrum of butyl-stambomycin (**4**).

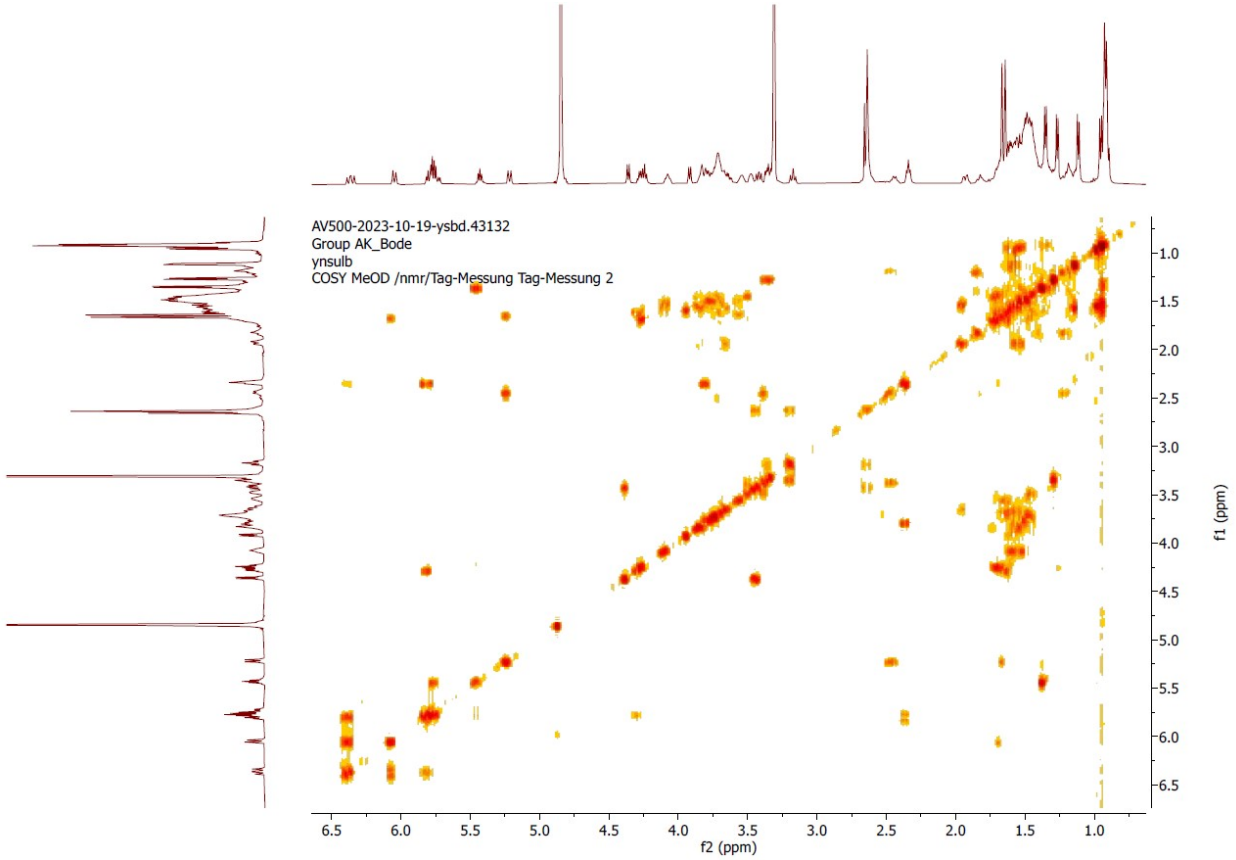


Figure S13. ^{1}H-^{1}H COSY spectrum of butyl-stambomycin (**4**).

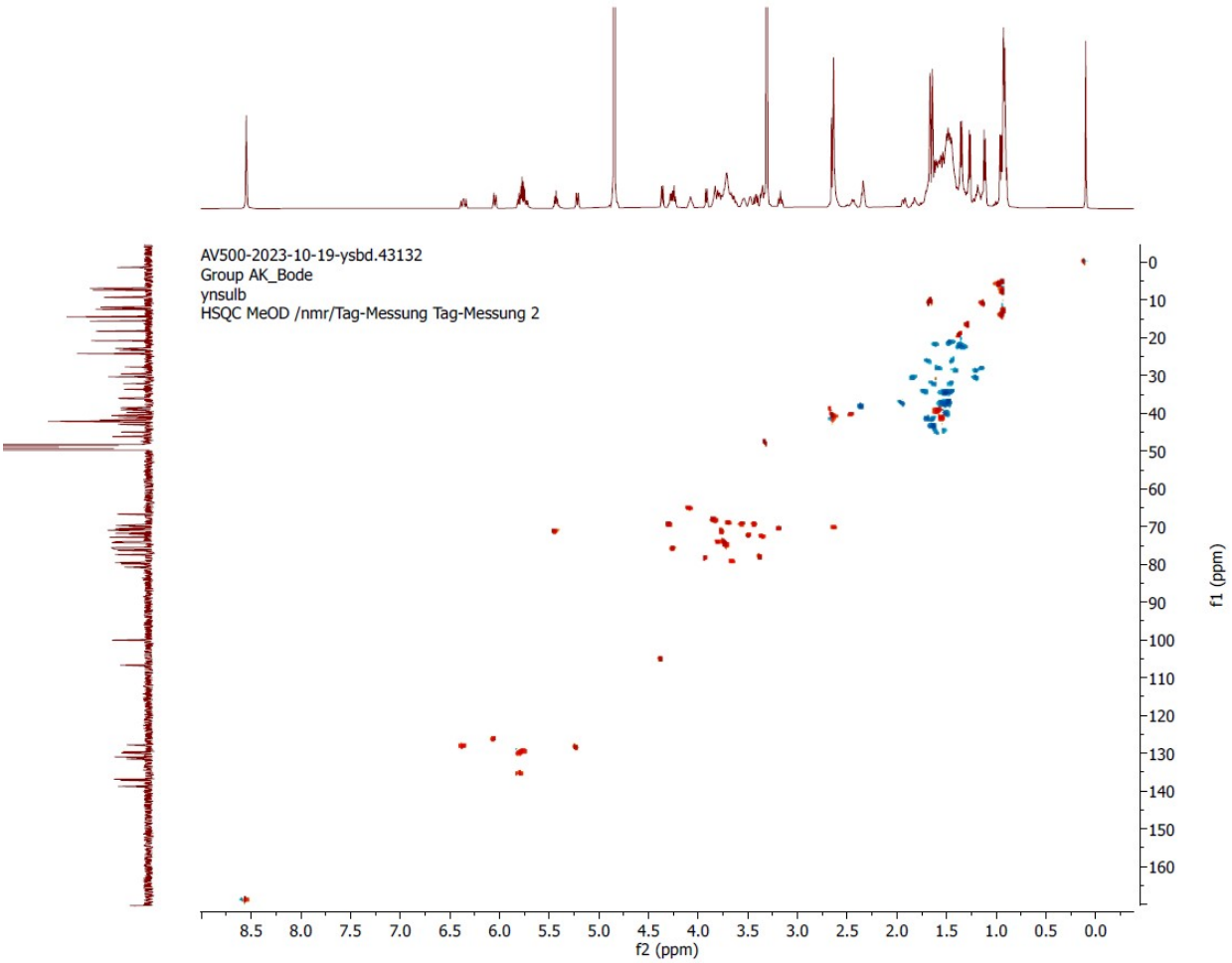


Figure S14. [^{1}H, ^{13}C]-HSQC spectrum of butyl-stambomycin (**4**).

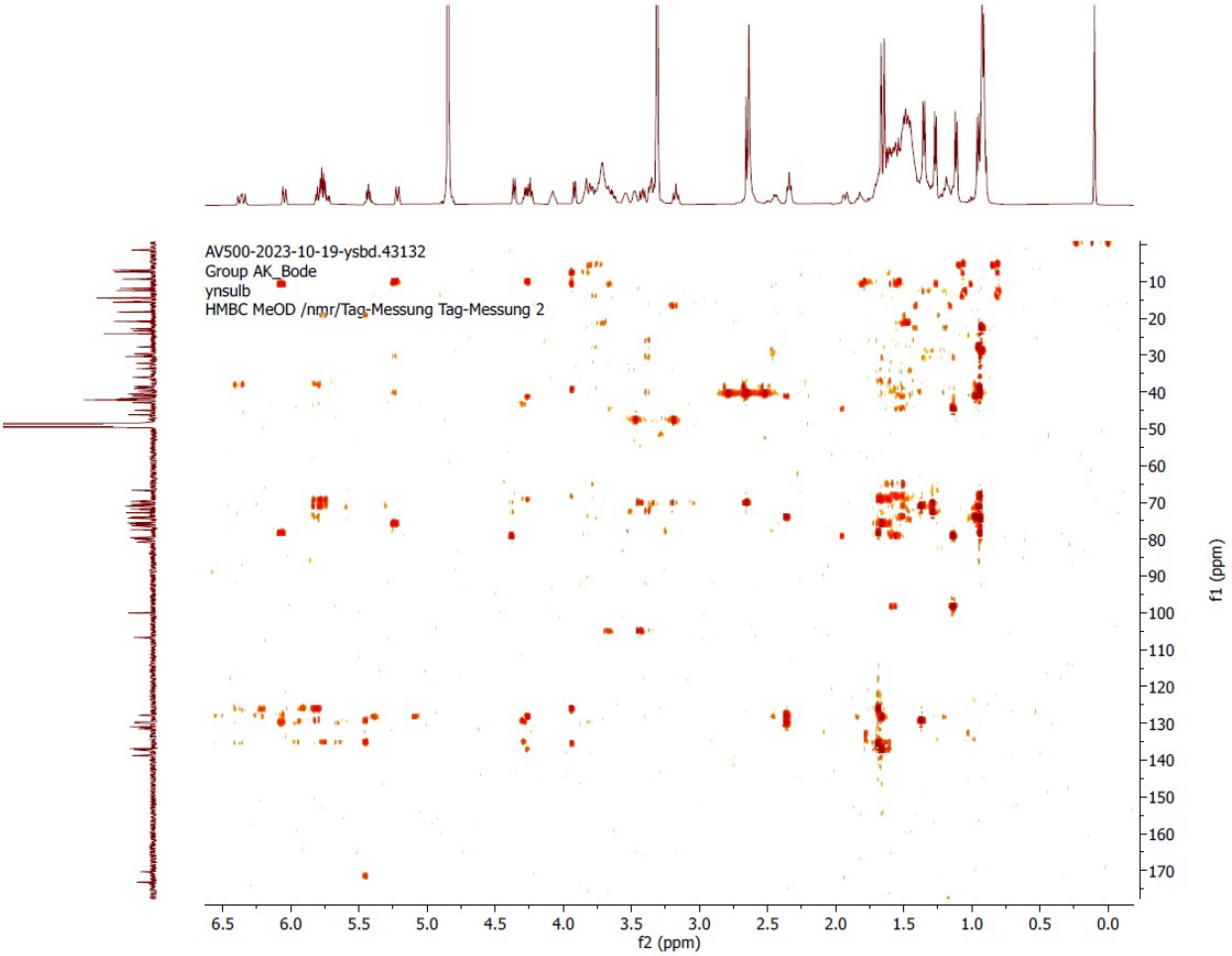
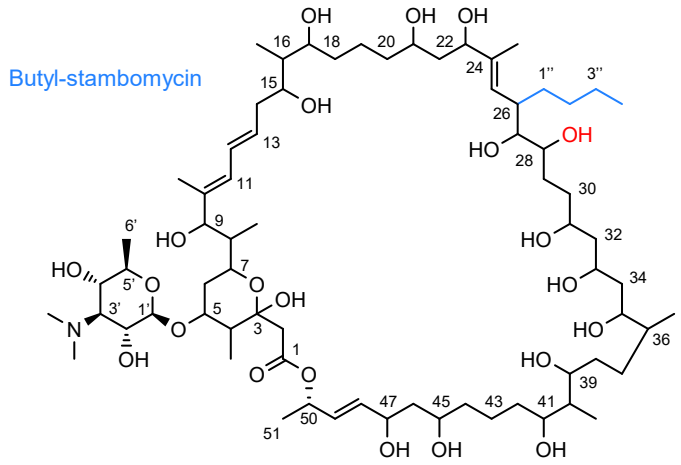


Figure S15. [^{1}H, ^{13}C]-HSQC HMBC spectrum of butyl-stambomycin (**4**).



Position	δC (ppm)	δH (ppm)	HMBC	Position	δC (ppm)	δH (ppm)	HMBC
1	173.1			24-Me	11.9	1.64	
2	43.0	1.62, 1.67		25	129.9	5.22	10.4
3	100.0			26	40.4	2.65	
4	46.1	1.51, 1.60		1″	30.3	1.18, 1.38	
4-Me	12.4	1.12		2″	24.2	1.34	
5	80.7	3.64		3″	36.1	1.47, 1.71	
6	39.1	1.47		4″	14.5	0.91	
1′	106.6	4.36	7.4	27	79.5	3.35	
2′	70.5	3.68	7.4, 10.7	28	75.9	3.71	
3′	72.0	3.16	9.4, 9.4	29	27.7	1.43, 1.68	
3′-NMe_2	42.1	2.44		30	35.9	1.47, 1.71	
4′	70.0	3.82		31	70.9	3.55	
5′	73.9	3.47	10.3, 7.4	32	46.1	1.51, 1.60	
6′	18.2	1.27		33	66.6	4.08	
7	69.7	3.81		34	41.8	1.48, 2.44	
8	41.0	1.57		35	72.8	3.74	
8-Me	9.3	0.91		36	40.6	1.57	
9	79.9	3.92	7.2	36-Me	15.6	0.94	
10	137.1			37	29.5	1.13, 1.57	
10-Me	12.4	1.66		38	33.6	1.44, 1.62	
11	128.7	6.05	10.9	39	76.3	3,75	
12	129.5	6.36	15.0, 10.9	40	42.1	1.48	
13	131.5	5.77	15.5, 7.4	40-Me	6.9	0.91	
14	39.8	2.34	6.9	41	75.7	3.71	
15	75.5	3.77		42	32.1	1.19, 1.82	
16	42.9	1.57		43	23.3	1.59	
16-Me	7.4	0.95		44	38.9	1.46, 1.94	
17	74.2	3.77		45	70.9	3.55	
18	35.8	1.47, 1.71		46	44.9	1.63	
19	22.8	1.45		47	71.7	2.61	
20	38.5	1.46		48	136.8	5.77	15.5, 7.4
21	70.9	3.43		49	130.9	5.77	15.5, 7.4
22	42.3	2.61		50	72.8	5.42	6.6, 6.4
23	77.4	4.26	6.6	51	20.8	1.35	6.6
24	138.7						

Figure S16. Structure and summary for the NMR data of butyl-stambomycin (**4**). The ^{1}H NMR data obtained on butyl-stambomycin are highly similar to those for butyl-deoxystambomycin, with the exception of the deshielded proton attached to C-28. Furthermore, the ^{13}C NMR spectrum of butyl-stambomycin also include 70 carbon signals, except that the C-28 carbon is relatively deshielded (δ_C 76.3, δ_H 3.75), consistent with the presence of the C-28 hydroxyl. The data corresponding to the alkyl chain at C-26 are similar to those of butyl-deoxystambomycin, allowing assignment of the side chain as *n*-butyl. Together, these data confirmed the identity of the compound as butyl-stambomycin.

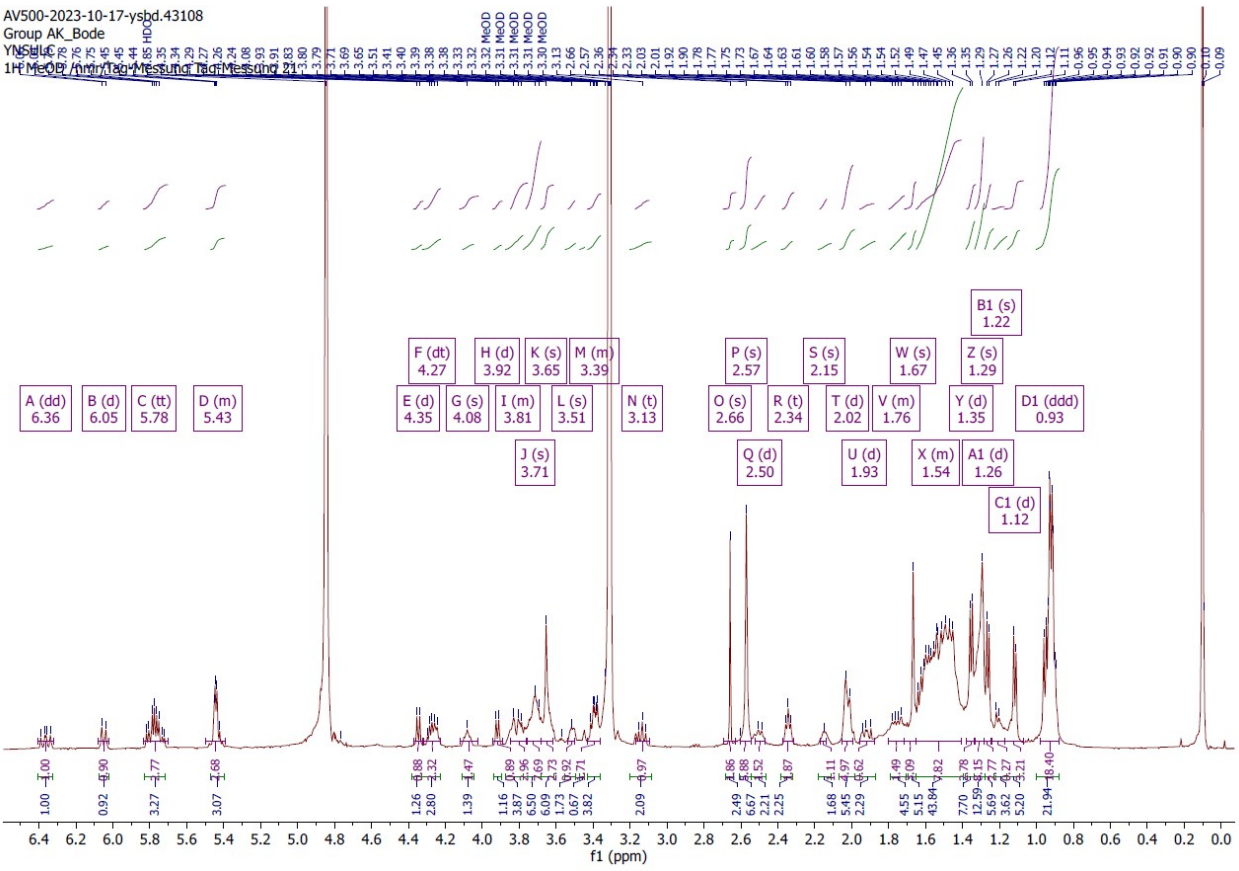


Figure S17. ^{1}H NMR spectrum of C-24-demethyl-butyl-stambomycin (**12**).

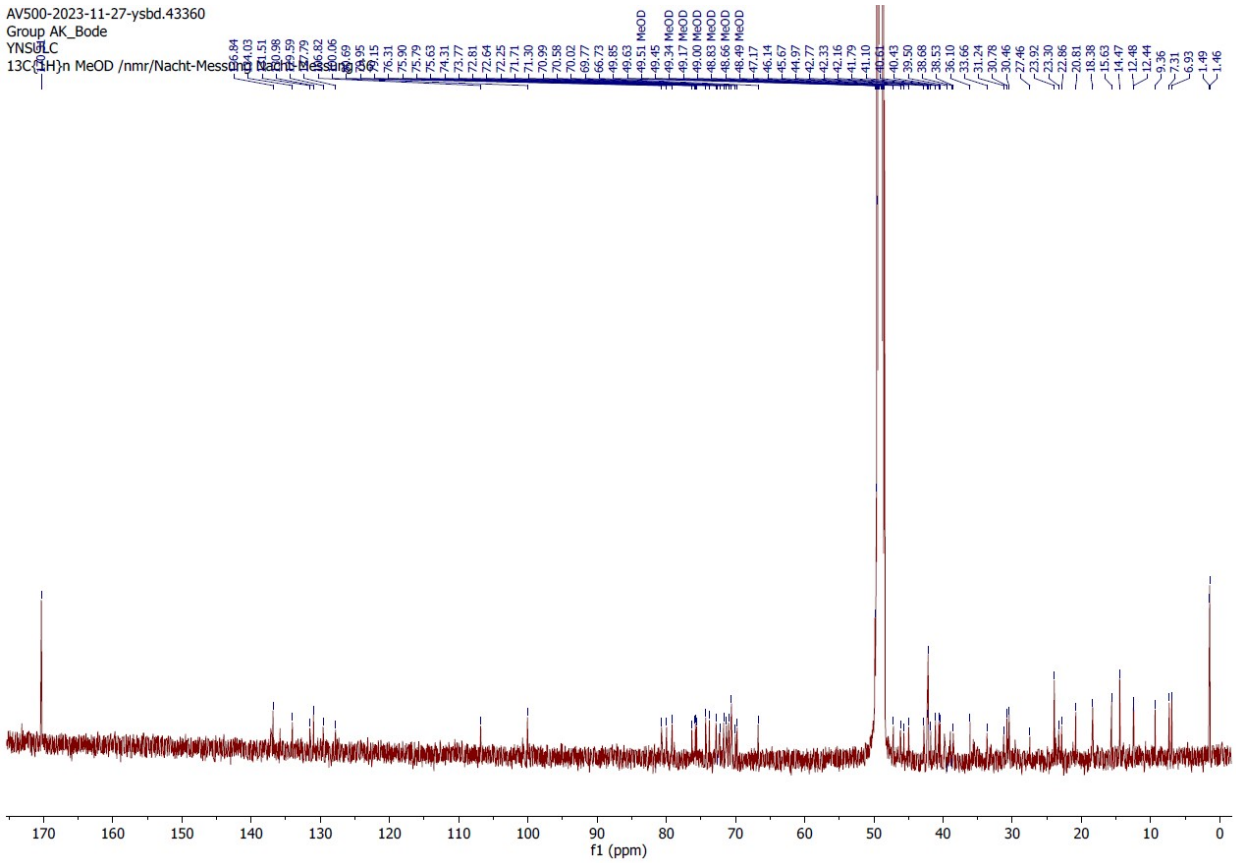


Figure S18. ^{13}C NMR spectrum of C-24-demethyl-butyl-stambomycin (**12**).

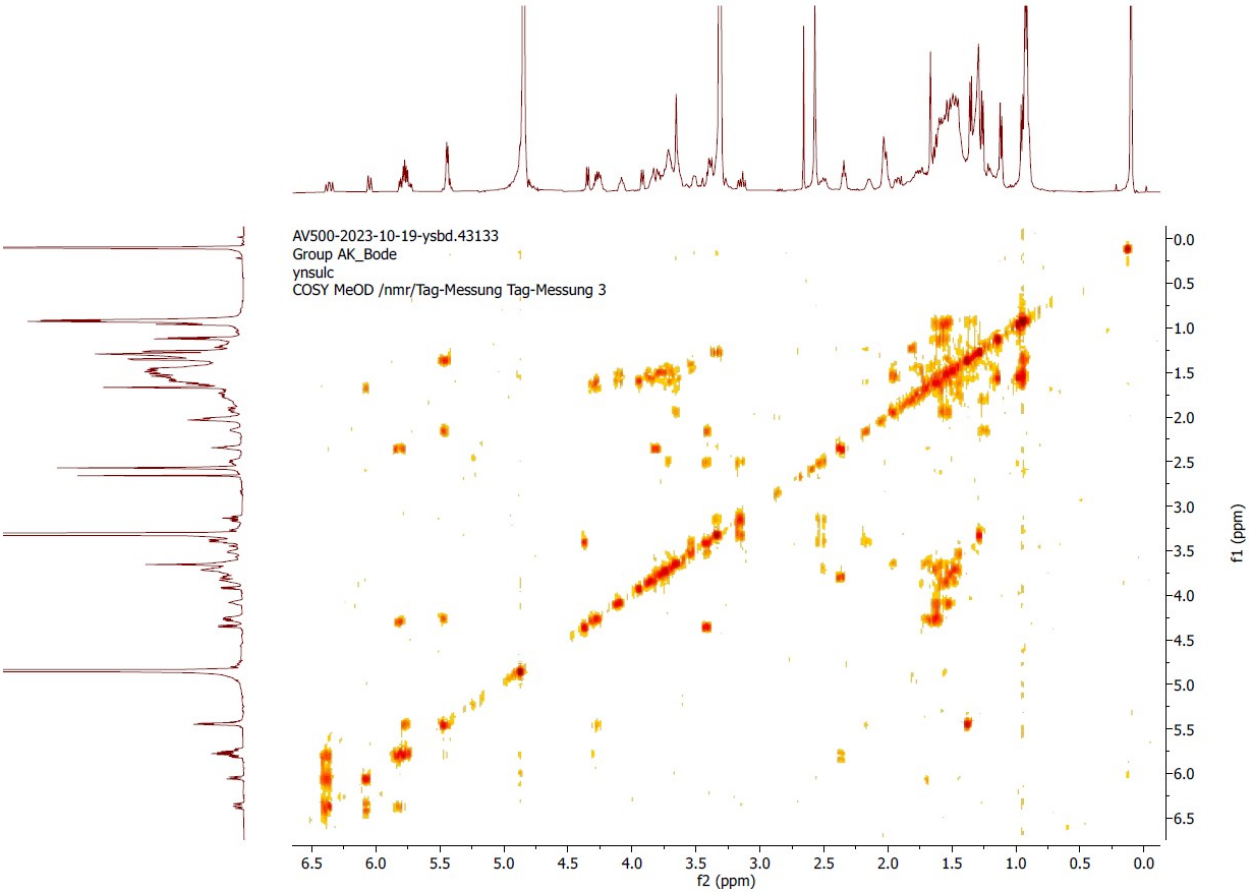


Figure S19. ^{1}H-^{1}H COSY spectrum of C-24-demethyl-butyl-stambomycin (**12**).

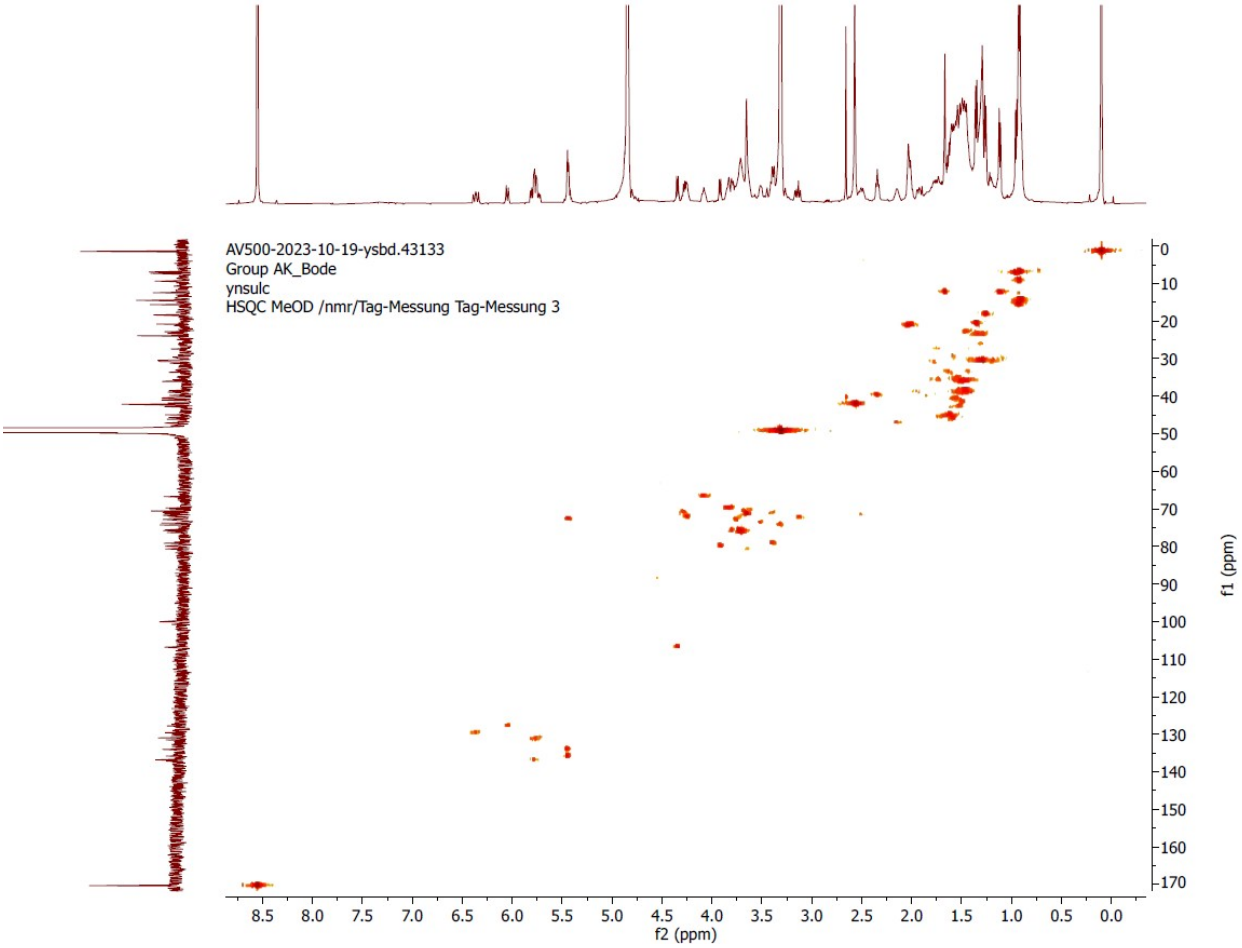


Figure S20. [^{1}H,^{13}C]-HSQC spectrum of C-24-demethyl-butyl-stambomycin (**12**).

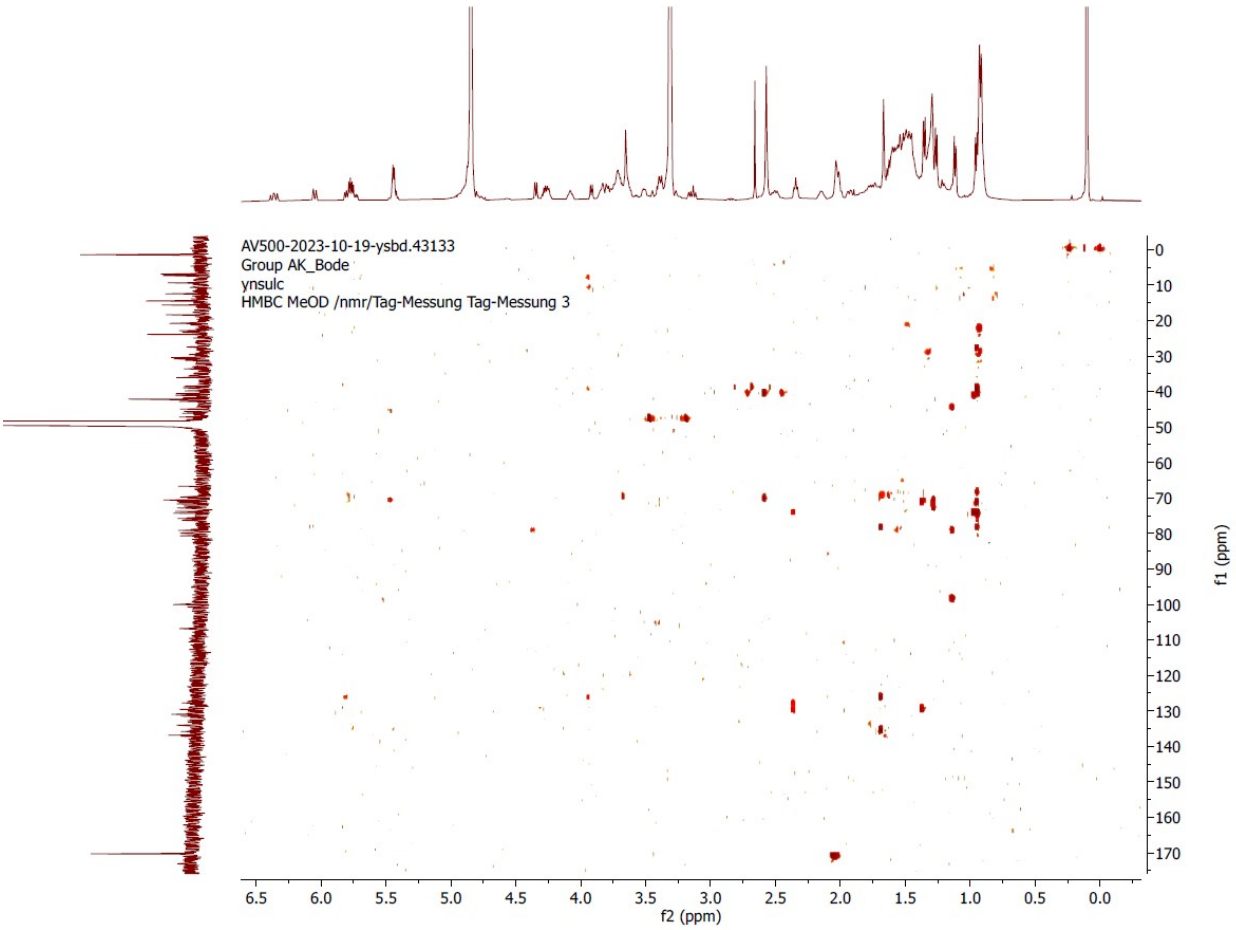
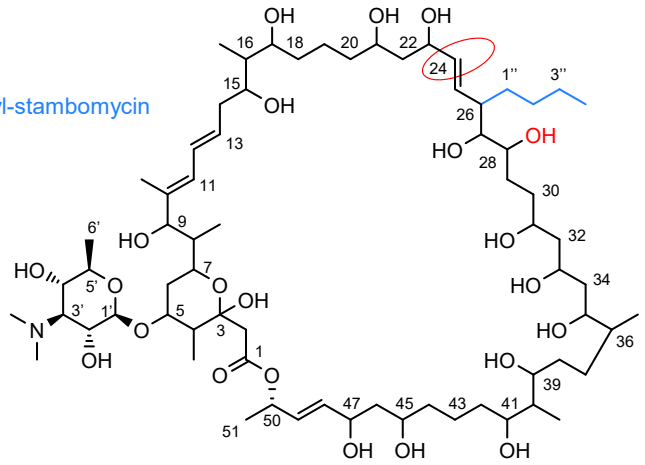


Figure S21. [^{1}H,^{13}C]-HMBC spectrum of C-24-demethyl-butyl-stambomycin (**12**).

C-24-demethyl-butyl-stambomycin



Position	δC (ppm)	δH (ppm)	HMBC	Position	δC (ppm)	δH (ppm)	HMBC
1	171.6			25	130.9	5.78	15.4, 8.0
2	44.9	1.60-1.68		26	40.4	1.54	
3	100.0			1″	30.4	1.29	
4	46.1	1.54		2″	23.9	1.34	
4-Me	12.4	1.12		3″	36.1	1.54	
5	80.6	3.65		4″	14.4	0.93	
6	38.9	1.46		27	79.1	3.39	
1′	106.8	4.35	7.5	28	75.9	3.39	
2′	70.5	3.66		29	27.4	1.42, 1.76	
3′	71.7	3.64		30	35.4	1.47, 1.71	
3′-NMe_2	42.1	2.50		31	70.9	4.27	
4′	70.0	3.81		32	45.6	1.54	
5′	72.8	3.71		33	66.7	4.08	
6′	18.3	1.26		34	41.7	1.54	
7	69.7	3.81		35	72.8	3.81	
8	41.1	1.57		36	40.6	1.54	
8-Me	9.3	0.93		36-Me	15.6	0.93	
9	79.9	3.92	7.3	37	30.7	1.29	
10	136.8			38	33.6	1.54, 1.67	
10-Me	12.4	1.67		39	75.7	3.68	
11	127.7	6.05	10.8	40	42.1	1.54	
12	129.5	6.36	15.0, 11.0	40-Me	6.9	0.93	
13	134.0	5.43		41	73.7	3.51	
14	39.8	2.34	6.9	42	31.2	1.76	
15	75.6	3.70		43	23.3	1.34	
16	42.7	1.53		44	38.6	1.54	
16-Me	7.3	0.93		45	71.3	3.71	
17	74.3	3.39		46	47.1	2.15	
18	35.6	1.54		47	72.2	3.13	
19	22.8	1.54		48	135.8	5.43	
20	38.5	1.54		49	131.5	5.78	15.4, 8.0
21	70.9	3.65		50	72.2	3.13	9.4, 9.4
22	42.3	1.54		51	20.8	1.35	6.4
23	76.3	3.72	7.6				
24	137.1	5.78	15.4, 8.0				

Figure S22. Structure and summary for the NMR data of C-24-demethyl-butyl-stambomycin (**12**). The NMR data obtained on this compound are overall similar to those for butyl-stambomycin and deoxy-butyl-stambomycin, except that C-24-demethyl-butyl-stambomycin is missing a methyl signal at (δ_H 1.64) corresponding to the methyl at C-24, and the chemical shift of an olefinic proton (δ_H 5.22 in the case of butyl-stambomycin and deoxy-butyl-stambomycin) has shifted to (δ_H 5.43). Additionally, coupling constants of 15.4 and 8.0 were observed for H-25, consistent with the presence of protons on both H-24 and H-26. Together, these data confirmed the identity of the metabolite as C-24-demethyl-butyl-stambomycin.

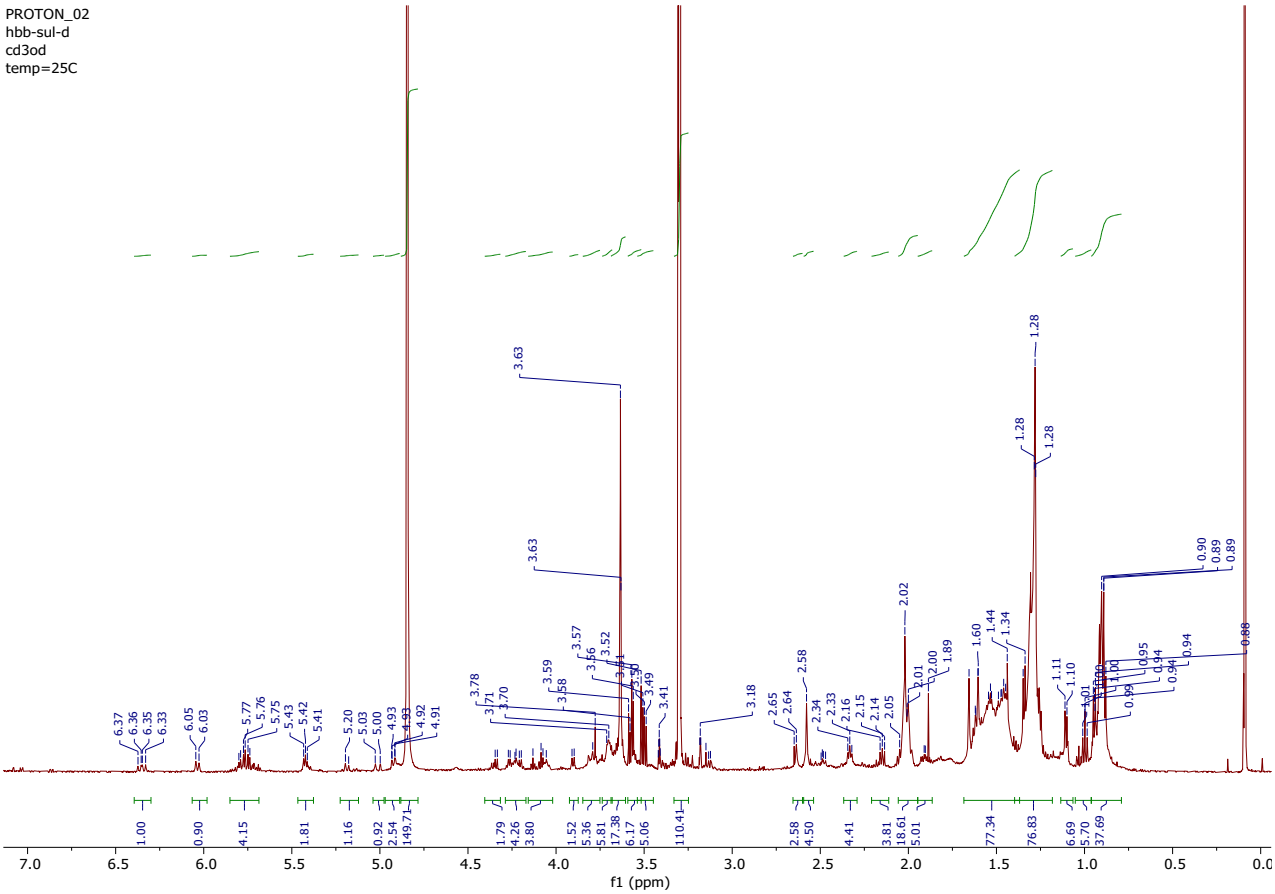


Figure S23. ^{1}H NMR spectrum of deoxy-allyl-stambomycin (**3**).

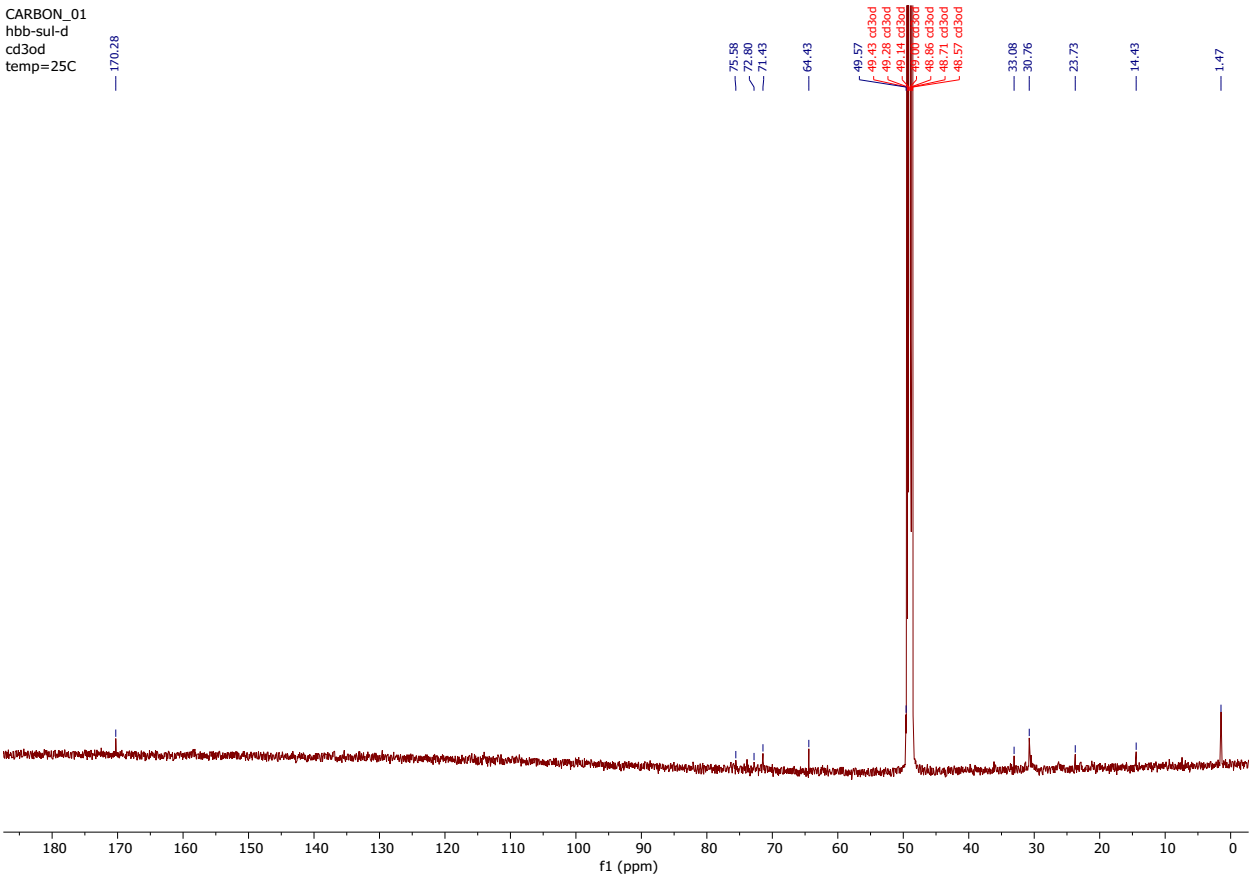


Figure S24. ^{13}C NMR spectrum of deoxy-allyl-stambomycin (**3**).

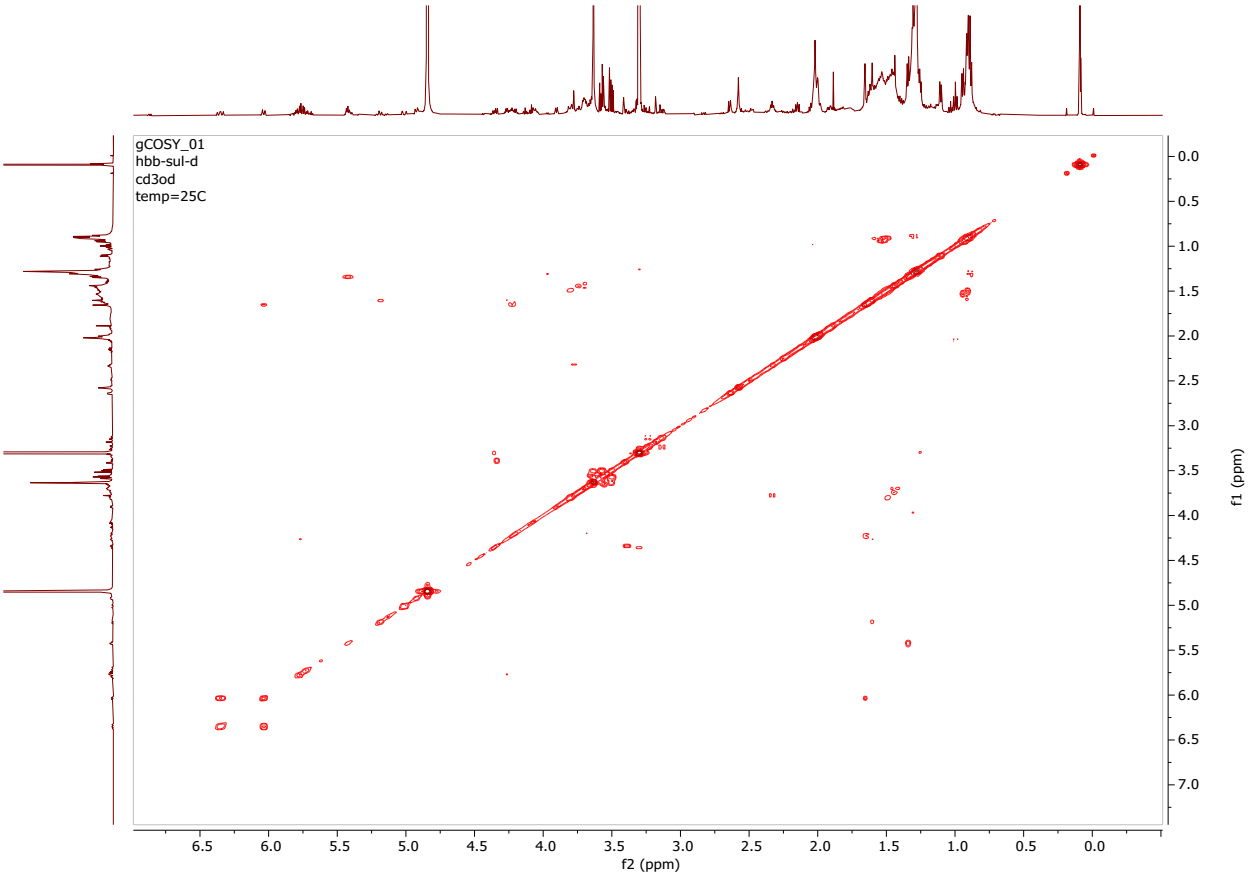


Figure S25. ^{1}H-^{1}H COSY spectrum of deoxy-allyl-stambomycin (**3**).

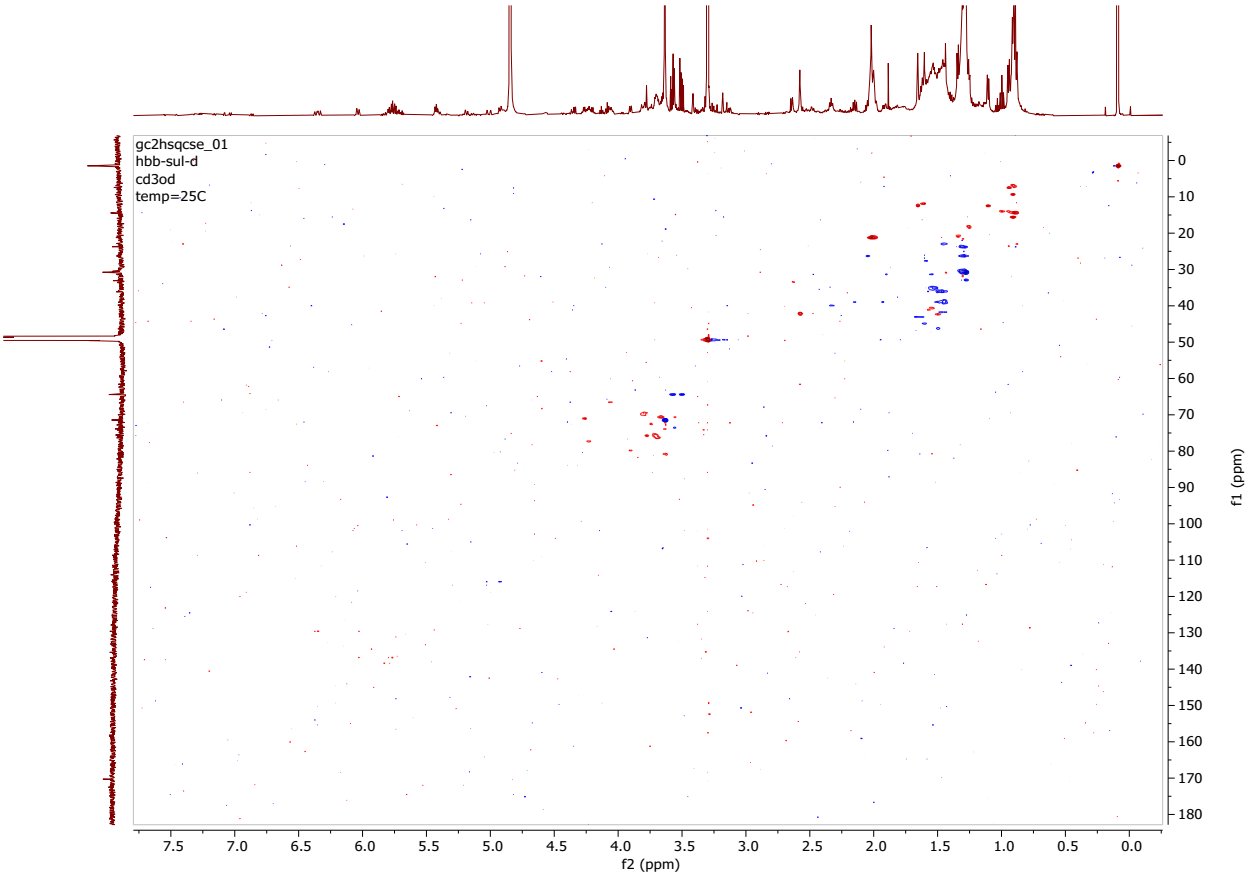


Figure S26. [^{1}H,^{13}C]-HSQC spectrum of deoxy-allyl-stambomycin (**3**).

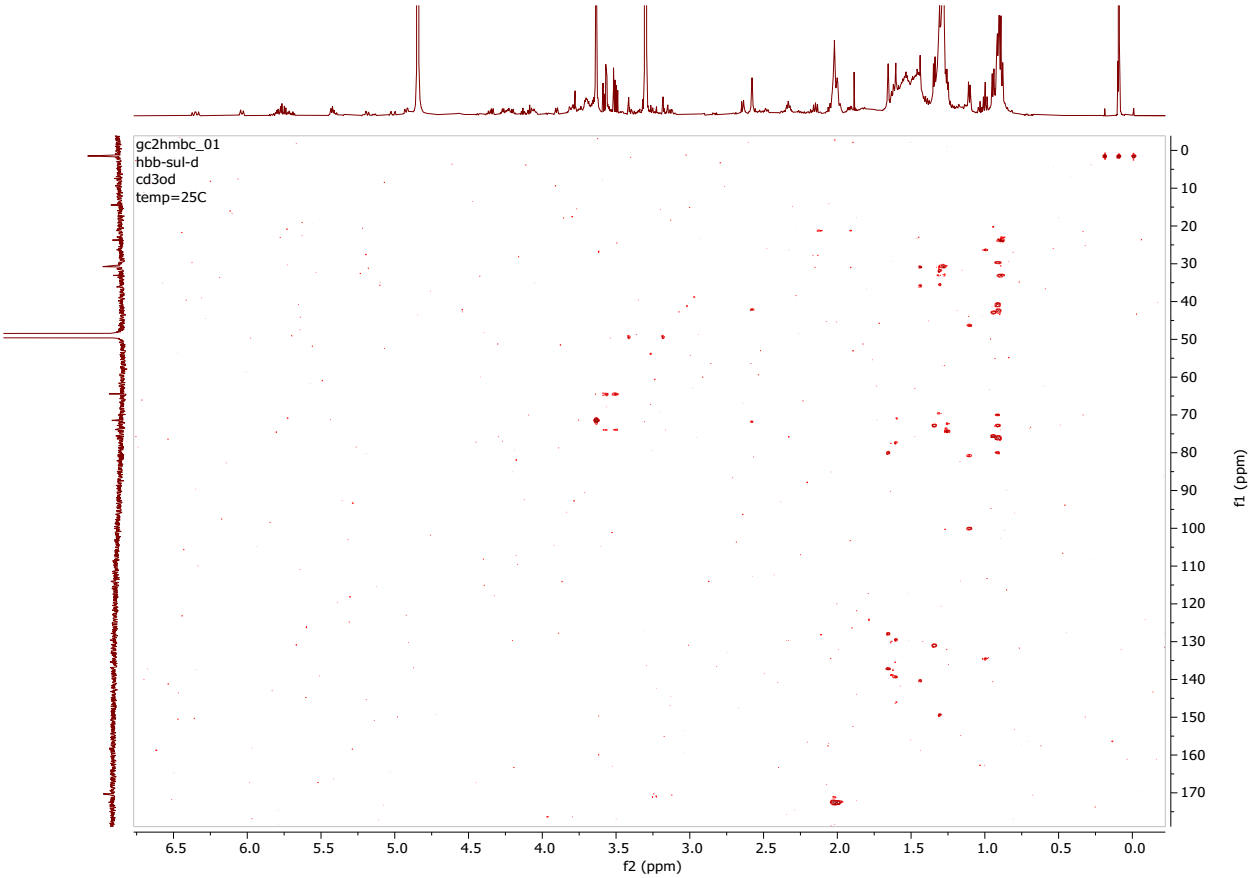
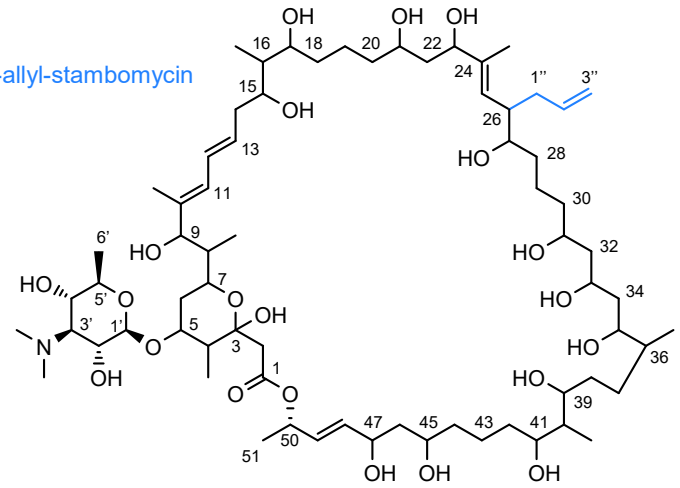


Figure S27. [^{1}H,^{13}C]-HMBC spectrum of deoxy-allyl-stambomycin (**3**).

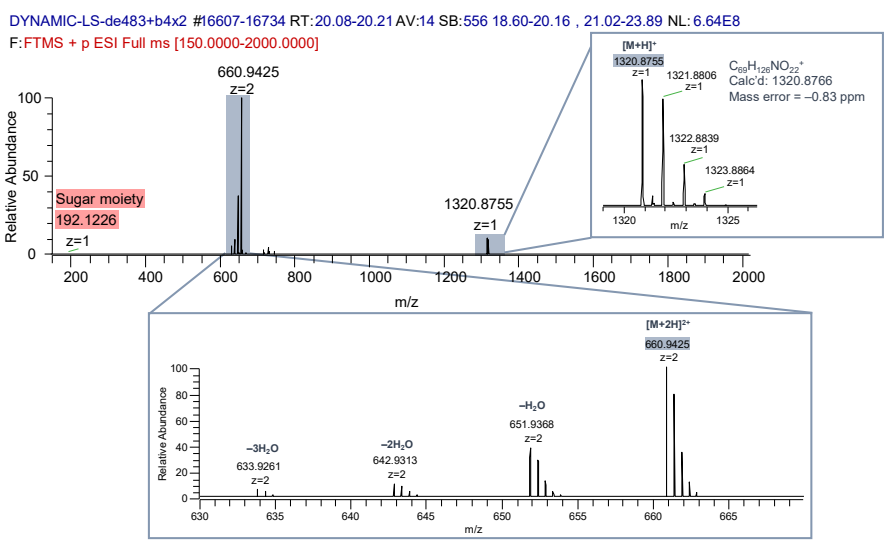
Deoxy-allyl-stambomycin



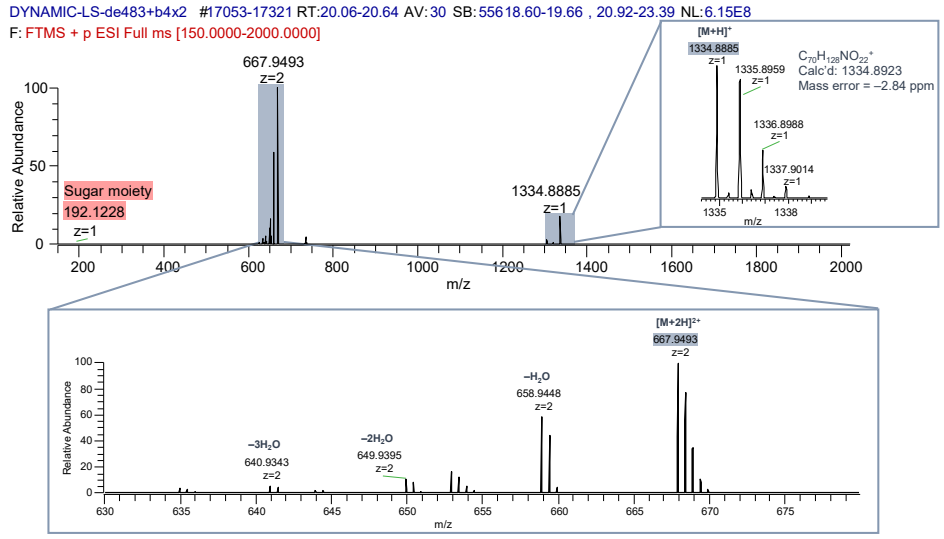
Position	δC (ppm)	δH (ppm)	HMBC	Position	δC (ppm)	δH (ppm)	HMBC
1	172.2			24-Me	11.8	1.60	
2	43.0	1.62		25	129.6	6.34	15.5, 10.9
3	100.5			26	42.1	2.57	
4	45.0	1.60		1″	33.1	1.28	
4-Me	12.4	1.10		2″	134.4	5.2	
5	80.8	3.63		3″	115.9	4.93	10.0
6	39.7	1.80		27	76.2	3.69	
1′	106.7	4.33		28	22.9	1.46	
2′	70.8	3.55	11.1, 6.0	29	30.7	1.28	
3′	71.6	3.64		30	35.0	1.53	
3′-NMe_2	42.2	2.57		31	69.3	3.79	
4′	71.4	3.64		32	46.2	1.50	
5′	73.4	3.63		33	67.4	3.61	
6′	18.2	1.26		34	38.9	1.93	
7	69.8	3.81		35	72.7	3.73	
8	41.6	1.47		36	41.0	1.57	
8-Me	9.2	0.91		36-Me	14.7	0.89	
9	79.9	3.90	7.4	37	30.7	1.28	
10	136.8			38	38.9	1.46, 1.94	
10-Me	12.4	1.10		39	75.9	3.70	
11	127.8	6.02	10.9, 14.9	40	41.7	1.57	
12	130.6	5.74		40-Me	6.7	0.89	
13	131.5	5.78		41	72.3	3.31	
14	41.6	1.44		42	33.7	159	
15	75.8	3.70		43	21.2	2.01	
16	42.8	1.53		44	39.0	1.46, 1.93	
16-Me	7.3	0.94		45	71.8	3.64	
17	76.7	3.69		46	46.2	1.50	
18	36.0	1.57, 1.47		47	70.4	3.67	
19	23.7	1.30		48	136.8	5.77	
20	38.5	1.44		49	130.9	5.74	
21	70.8	3.55		50	72.7	5.42	6.3
22	45.0	1.60		51	20.8	1.34	6.3
23	77.1	4.24					
24	139.1						

Figure S28. Structure and summary for the NMR data for deoxy-allyl-stambomycin (**3**). Globally, the NMR data resemble those for deoxy-butyl-stambomycin, with the exception of those corresponding to the side chain attached to C-26. Specifically, we observe differences for the chemical shifts of C-2″ (δ_C 134.4) and C-3″ (δ_C 115.9) corresponding to the double bond of the allyl group. Although many of the expected ^{13}C signals are missing from ^{13}C NMR spectrum, close inspection of the cross peaks of the [^1H,^{13}C]-HSQC and [^1H,^{13}C]-HMBC spectra allowed their assignment. Together, these data confirmed the identity of the compound as deoxy-allyl-stambomycin.

a


DYNAMIC-LS-de483+b4x2 #16607-16734 RT:20.08-20.21 AV:14 SB:556 18.60-20.16 , 21.02-23.89 NL:6.64E8
F:FTMS + p ESI Full ms [150.0000-2000.0000]
660.9425
z=2
Sugar moiety
192.1226
z=1
1320.8755
z=1
Relative Abundance
m/z
[M+H]+
1320.8755
z=1
1321.8806
z=1
1322.8839
z=1
1323.8864
z=1
C68H128NO22+
Calc'd: 1320.8766
Mass error = –0.83 ppm
[M+2H]2+
660.9425
z=2
–H2O
651.9368
z=2
–2H2O
642.9313
z=2
–3H2O
633.9261
z=2

b


DYNAMIC-LS-de483+b4x2 #17053-17321 RT:20.06-20.64 AV:30 SB:556 18.60-19.66 , 20.92-23.39 NL:6.15E8
F:FTMS + p ESI Full ms [150.0000-2000.0000]
667.9493
z=2
Sugar moiety
192.1228
z=1
1334.8885
z=1
Relative Abundance
m/z
[M+H]+
1334.8885
z=1
1335.8959
z=1
1336.8988
z=1
1337.9014
z=1
C70H128NO22+
Calc'd: 1334.8923
Mass error = –2.84 ppm
[M+2H]2+
667.9493
z=2
–H2O
658.9448
z=2
–2H2O
649.9395
z=2
–3H2O
640.9343
z=2

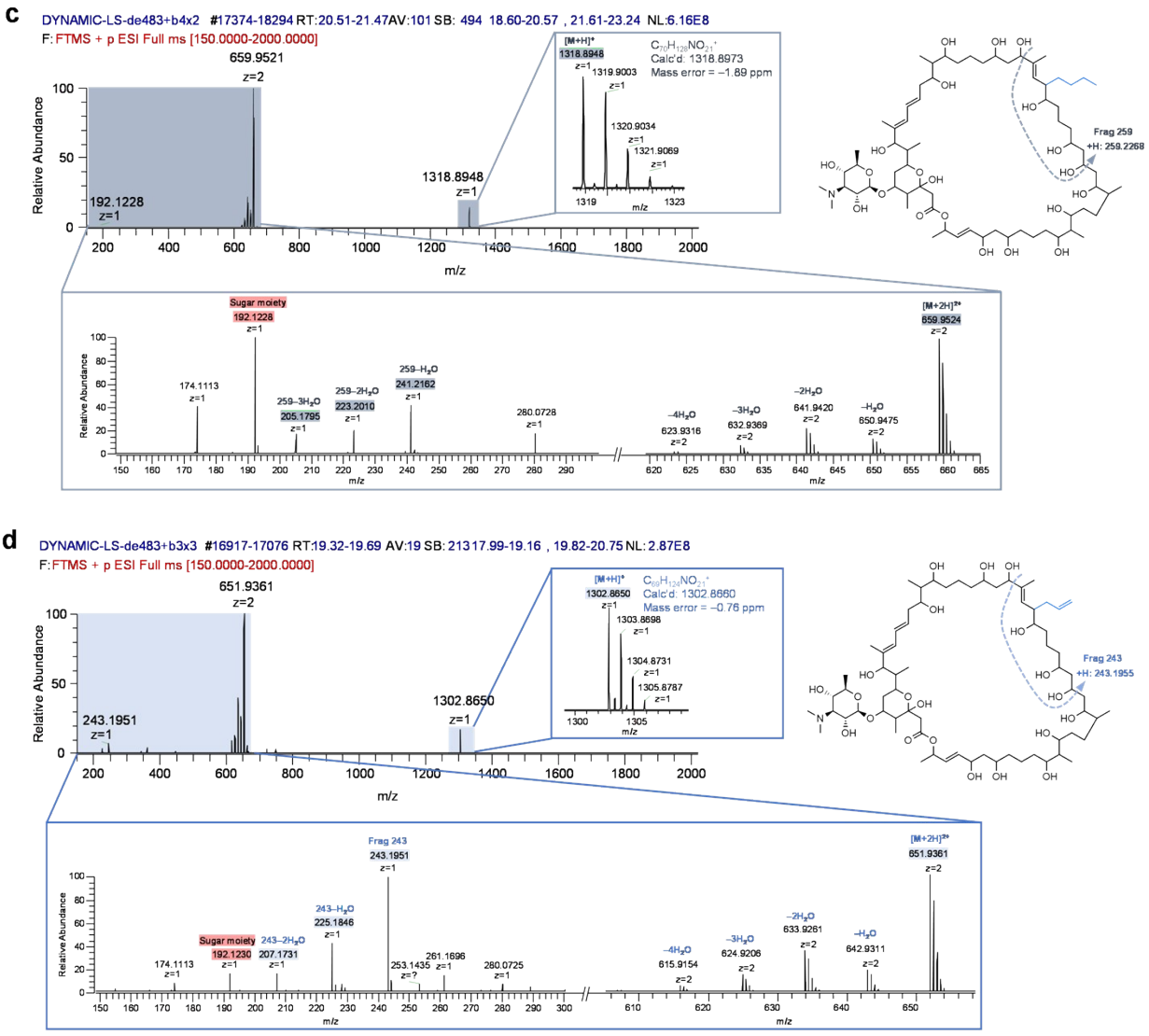


Figure S29. MS analysis of stambomycin analogues (**a**) C-24-demethyl-butylstambomycin (**12**), (**b**) butyl-stambomycin (**4**), (**c**) deoxy-butyl-stambomycin (**5**) and (**d**) deoxy-allyl-stambomycin (**3**). Similar to stambomycins A–F as shown previously[8], fragments were also identified which are diagnostic for the C-26 side chains (deoxy-allyl-stambomycin: fragment 243 and derived water loss fragments; deoxy-butyl-stambomycin: fragment 259 and derived water loss fragments). Together, these data provide further support for the structural assignments.

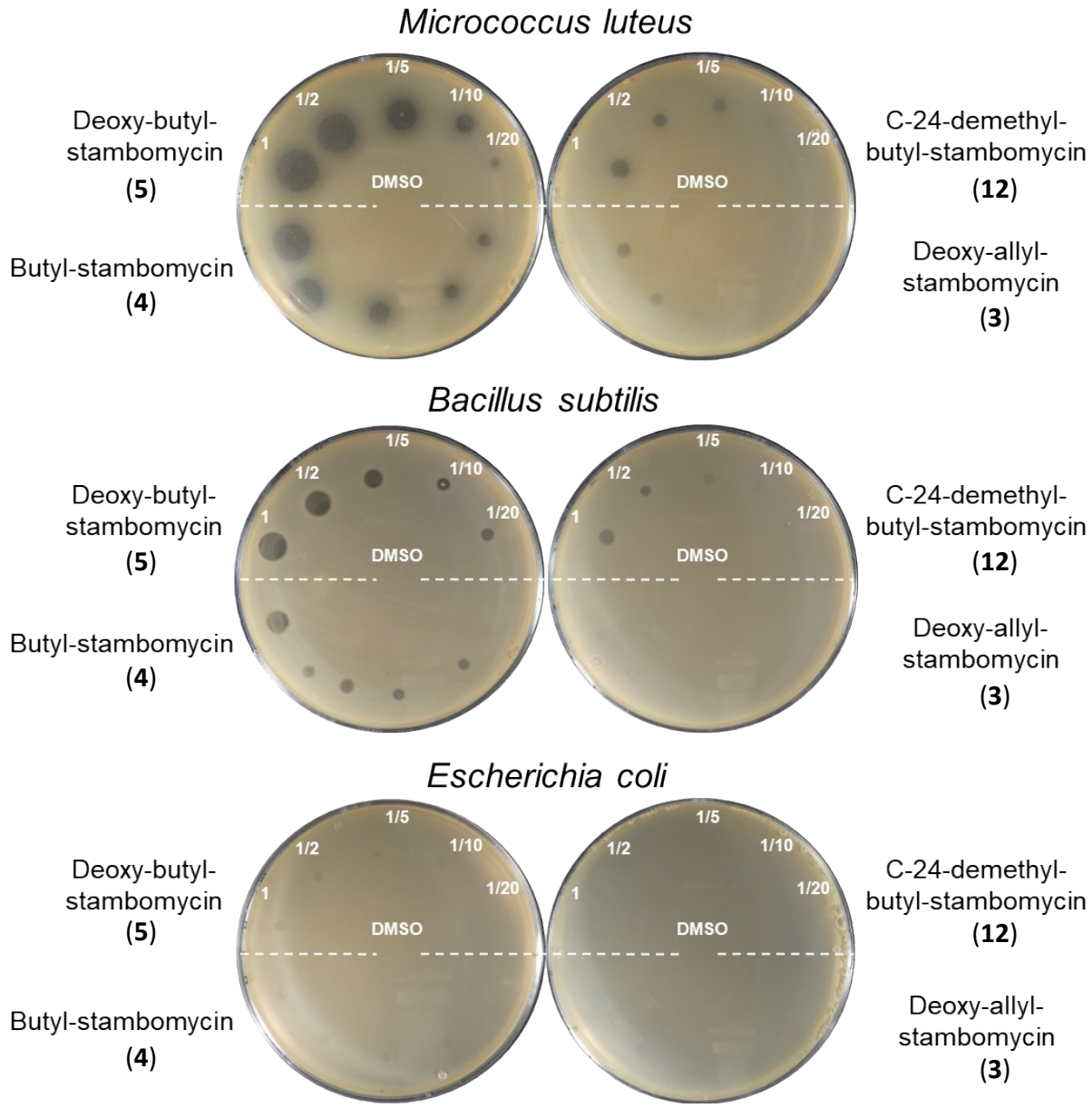


Figure S30. Antibacterial activities of the four purified stambomycin analogues (**3**, **4** ,**5** and **12**). For each analogue, 1 µL of sample (from a 10 mM stock solution in DMSO) either undiluted or at various dilutions as shown, was plated onto a confluent lawn of indicator strain (the Gram-positive bacteria *M. luteus* and *B. subtillis* or the Gram-negative bacterium *E. coli*). Pure DMSO (1 µL) was used as a control (plated in the centre).

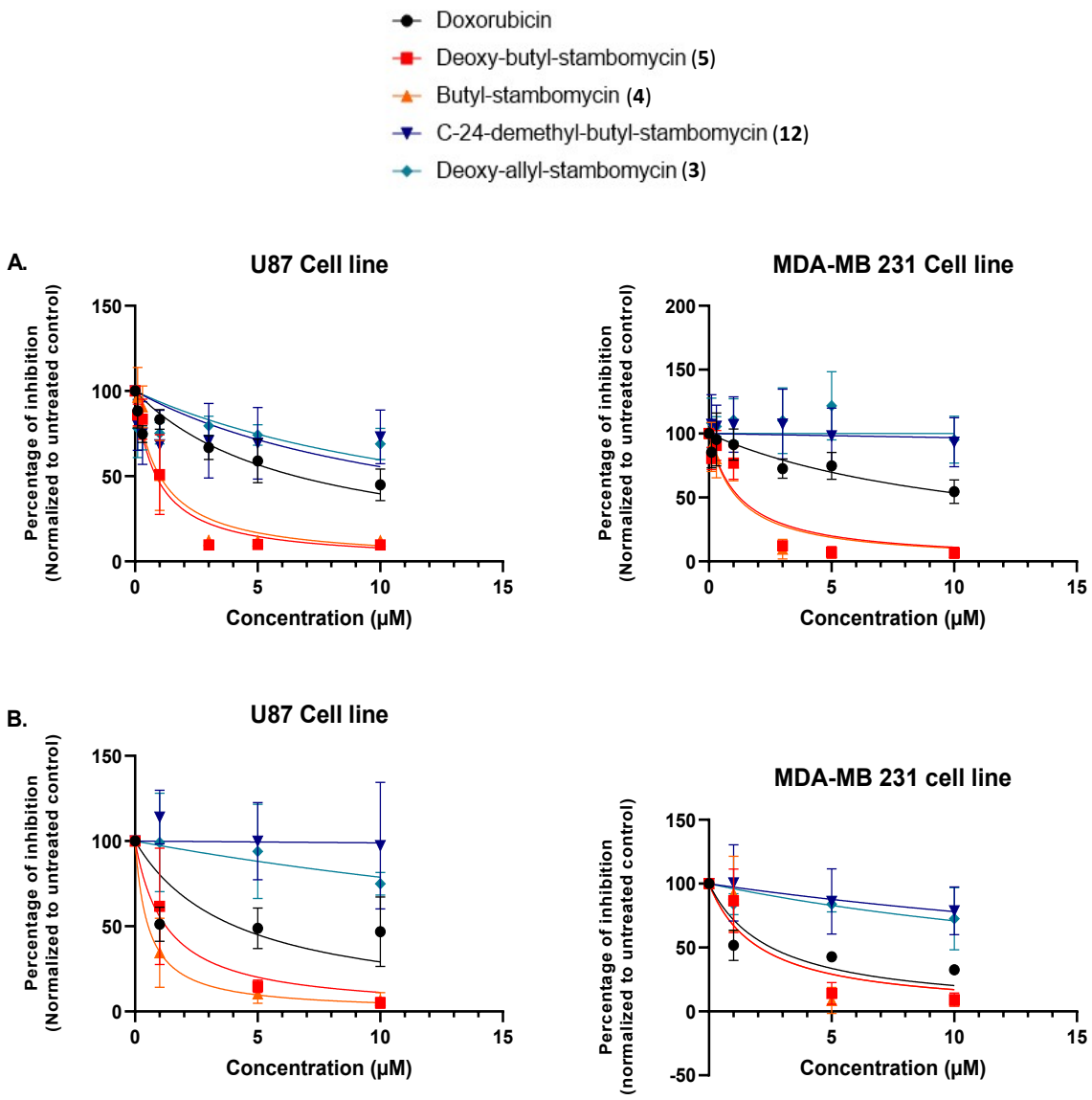


Figure S31. Antiproliferative activities of the four purified stambomycin analogues (**3**, **4** ,**5** and **12**) against two human cancer cell lines, U87-MG glioblastoma cells and MDA-MB-231 cells. **A.** MTT assays and **B.** Cell counting. In both cases, the percentage of inhibition is normalised relative to the untreated sample. The clinical anticancer agent doxorubicin was used as a control. All the assays were performed in triplicate in three independent experiments.

Table S1. Homologs of SamR0482 and SamR0483 in *S. ambofaciens* ATCC23877 revealed by BlastP analysis.

AA identities	Genome coordinates	Features	Accession number
Homologs of SamR0482 in *S. ambofaciens* ATCC23877			
29.8%	SAM23877_0423	AntF (acyl-CoA ligase, involved in antimycin biosynthesis pathway)	AKZ53472.1
34.5%	SAM23877_6654	Cgc22 (acyl-CoA synthetase, involved in congocidine biosynthesis pathway)	AKZ59699.1
27.8%	SAM23877_2750	FadD (Long-chain-fatty-acid-CoA ligase)	AKZ55799.1
28.1%	SAM23877_3675	AlkK (Medium-chain-fatty-acid-CoA ligase)	AKZ56720.1
Homologs of SamR0483 in *S. ambofaciens* ATCC23877			
52%%	SAM23877_0214 and SAM23877_7459	AlpX (putative carboxyl transferase, involved in kinamycin biosynthesis)	AKZ53263.1
59%	SAM23877_4747	PccB (propionyl-CoA carboxylase β chain)	AKZ57792.1
52%	SAM23877_5290	PccB (propionyl-CoA carboxylase β chain)	AKZ58335.1
31%	SAM23877_2811	Propionyl-CoA carboxylase	AKZ55860.1
29%	SAM23877_3346	Methyl crotonyl-CoA carboxylase carboxyl transferase	AKZ56393.1
26%	SAM23877_2503	Acetyl-coenzyme A carboxylase carboxyl transferase	AKZ55552.1

Table S2. List of strains and mutants.

Strains/mutants	Description and use (resistance)	Reference
Streptomyces		
ATCC/OE484	*S. ambofaciens* ATCC23877 carrying the integrative plasmid pIB0484 (strain overexpressing the LAL regulator SamR0484) ($Apra^R$)	[6]
ATCC/OE484/Δ482	PCR targeting mutant of ATCC/OE484 with the gene *samR0482* (SAM23877_7109, AKZ60152.1) replaced by a "*FRT+aadA+oriT+FRT*" cassette ($Spec^R$, $Apra^R$)	[16]
ATCC/OE484/Δ483	PCR-targeting mutant of ATCC/OE484 with the gene *samR0483* (SAM23877_7108, AKZ60151.1) replaced by a scar ($Apra^R$)	This study
ATCC/pIB139/Δ483	PCR-targeting mutant of ATCC/pIB139 with the gene *samR0483* replaced by a scar ($Apra^R$)	This study
ATCC/OE484/Δ483::*samR0483*	ATCC/OE484/Δ483 complemented with the conjugative and integrative plasmid pOSV809-PermE_*samR0483* (Kan^R, $Apra^R$)	This study
ATCC/pIB139/Δ483::*samR0483*	ATCC/pIB139/Δ483 complemented with the conjugative and integrative plasmid pOSV809_PermE_ *samR0483* (Kan^R, $Apra^R$)	This study
ATCC/OE483/Δ483/MatB_cinna	ATCC/OE484/Δ483 complemented with the conjugative and integrative plasmid pRT801_lacZ_PermE_MatB_cinna ($Spec^R$, $Apra^R$)	This study
E. coli		
DH5α	General cloning strain	[17]
BW25113/pKD20	PCR-targeting mutagenesis strain containing a λRED recombination plasmid pKD20 (Amp^R)	[18]
ET12567/pUZ8002 (ETU)	Non-methylating strain containing a mobilization plasmid for conjugation with *Streptomyces* (Kan^R, Cm^R)	[19]
S17-1	The conjugative donor strain containing the *tra* gene on the chromosome	[20]

Table S3. List of BACs and plasmids.

BACs/plasmids	Properties and use (resistance)	Reference
BAC3	BAB19ZF4 from the genomic library of *S. ambofaciens,* in which the chloramphenicol resistance gene is replaced by a spectinomycin resistance gene ($Spec^R$)	This study
BAC3_K7Δ483	Mutant of BAC3 with gene *samR0483* replaced by a *"FRT+aadA+oriT+ FRT"* cassette ($Spec^R$, $Apra^R$)	This study
pIB139	Conjugative and integrative vector (φC31 *attP-int, ermEp**) ($Apra^R$)	[21]
pOE484	pIB139+*samR0484* ($Apra^R$)	[6]
pIJ778	Origin of "*FRT+aadA+oriT+FRT*" cassette, ($Spec^R$)	[3]
pIJ773	Origin of "*FRT+aac(3)IV+oriT+FRT*" cassette, ($Apra^R$)	[3]
pUWL-*oriT-flp*	Conjugative plasmid for "*FRT+aac(3)IV +oriT +FRT"* cassette excision (recombinase FLP) (Hyg^R)	[5]
pOSV809	Conjugative and integrative vector (φBT1 *attP-int*) (Kan^R)	[22]
pOSV809_PermE_SamR483	For complementation of the Δ*samR0483* mutant strain (Kan^R)	This study
pRT801_lacZ_PermE_MatB_cinna	Overexpression of MatB_cinna from *Streptomyces cinnamonensis* ($Spec^R$)	Gift from F Schulz, Ruhr-Universität Bochum, Germany

Table S4. List of primers.

Oligo name	Sequence	Usage
Spec_For	GAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAA TGAAGTCTACACGAACCCTTTG	Replacement of the chloramphenicol gene by spectinomycin
Spec_Rev	AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG CCCTTATTTGCCGACTACCTTGG	Replacement of the chloramphenicol gene by spectinomycin
D483_For	GGAGGCAGGGTCGTCGTGTTGGAAGGTAGGGCTGGT ATGATTCCGGGGATCCGTCGACC	Deletion of *samR0483*
D483_Rev	GCCCCCGTACAGGAGCGGCCGGCACGACGAACGAC GTCATGTAGGCTGGAGCTGCTTC	Deletion of *samR0483*
F483_For	CGGTTCCGTGCCGTCCGATA	Verification of deleted *samR0483*
F483_Rev	AGACCTCCAGCGGCAAGGTG	Verification of deleted *samR0483*
ermEp_For	TGCGGCCGCTGCTAGCCGAGTGTCCGTTCGAGTGGC GGCTTG	For amplification of *ermEp**+RBS
ermEp_Rev	GGTCCTCCTGTGGAGTGGTGTGGATCCTACCAACCG GCACGATTG	For amplification of *ermEp**+RBS
Comp483_For	CACCACTCCACAGGAGGACCATGTCGCTCCAGGAGC CTGTCTCGC	For amplification of *samR0483*
Comp483_Rev	GCGGCCGCTACTAGTTCACAGGGGAATGTTCCCGTG TTTGCG	For amplification of *samR0483*
Ver483_For	CAAATGTAGCACCTGAAGTC	Verification of plasmid pOSV809-PermE_SamR483
Ver483_Rev	GTTCGGCCCCTTTTTTGGCC	Verification of plasmid pOSV809-PermE_SamR483
pOSV_For	GACTTCGCCCATCATGCGCTC	Verification of mutant ATCC/OE483/Δ483/*samR0483* and ATCC/pIB139/ Δ483:: *samR0483*
pOSV_Rev	GTGCTCAACGGGAATCCTGCTC	Verification of mutant ATCC/OE483/Δ483/*samR0483* and ATCC/pIB139/ Δ483:: *samR0483*
φBT1-attB_For	GACCTTGTGCTTGGTCGTCTTC	Verification of mutant ATCC/ OE483/Δ483/*samR0483* and ATCC/pIB139/Δ483::*samR0483*
φBT1-attB_Rev	GTAGATCGACAGGGCCATCCAC	Verification of mutant ATCC/ OE483/Δ483/*samR0483* and ATCC/pIB139/Δ483::*samR0483*

Key: "NNNNNNN" and "NNNNNNN" represent sequences identical to those at the right or left ends of the disruption cassette in the PCR-targeting system. "GCTAGC" is the restriction recognition site of *Nhe*I, and "ACTAGT" is that for *Spe*I.

Table S5. Calculated and observed masses of all metabolites in this study.

Compound	Chemical formula	Retention Time (min)	Calculated Mass $[M+2H]^{2+}$	Observed Mass $[M+2H]^{2+}$ (mass error in ppm)	Calculated Mass $[M+H]^{+}$	Observed Mass $[M+H]^{+}$ (mass error in ppm)
Stambomycin F	$C_{74}H_{135}NO_{22}$	24.2	694.9815	n.d.	1390.9549	1390.9517 (–2.30 ppm)
Stambomycin A/B	$C_{73}H_{133}NO_{22}$	22.8	688.9736	688.9697 (–5.66 ppm)	1376.9392	1376.9358 (–2.47 ppm)
Stambomycin C/D	$C_{72}H_{131}NO_{22}$	21.7	681.9659	681.9620 (–5.72 ppm)	1362.9236	1362.9207 (–2.13 ppm)
Stambomycin E	$C_{71}H_{129}NO_{22}$	21.3	674.9539	674.9531 (–1.19 ppm)	1348.9058	1348.9046 (–1.11 ppm)
Demethyl-stambomycin E	$C_{70}H_{127}NO_{22}$	20.5	667.9502	667.9493 (–1.35 ppm)	1334.8923	1334.8911 (–0.89 ppm)
Ethyl-stambomycin	$C_{68}H_{123}NO_{22}$	n.d.	653.9345	n.d.	1306.8610	n.d.
Deoxy-ethyl-stambomycin (**1**)	$C_{68}H_{123}NO_{21}$	19.1	645.9345	n.d.	1290.8660	1290.8647 (–1.00 ppm)
Isopropyl-stambomycin	$C_{69}H_{125}NO_{22}$	n.d.	660.9419	n.d.	1320.8766	n.d.
Deoxy-isopropyl-stambomycin	$C_{69}H_{125}NO_{21}$	n.d.	652.9445	n.d.	1304.8817	n.d.
Allyl-stambomycin (**2**)	$C_{69}H_{123}NO_{22}$	19.3	659.9345	n.d.	1318.8610	1318.8606 (–0.30 ppm)
Deoxy-allyl-stambomycin (**3**)	$C_{69}H_{123}NO_{21}$	19.5	651.9370	651.9361 (–1.38 ppm)	1302.8660	1302.8650 (–0.76 ppm)
C-24-demethyl-butyl-stambomycin (**12**)	$C_{69}H_{125}NO_{22}$	19.9	660.9425	660.9420 (–0.75 ppm)	1320.8766	1320.8755 (–0.83 ppm)
Butyl-stambomycin (**4**)	$C_{70}H_{127}NO_{22}$	20.3	667.9502	667.9493 (–1.35 ppm)	1334.8923	1334.8885 (–2.84 ppm)
Deoxy-butyl-stambomycin (**5**)	$C_{70}H_{127}NO_{21}$	20.9	659.9527	659.9521 (–0.91 ppm)	1318.8973	1318.8948 (–1.89 ppm)
Benzyl-stambomycin (**6**)	$C_{73}H_{125}NO_{22}$	21.5	684.9580	684.9417 (–23.8 ppm)	1368.8766	1368.8782 (1.17 ppm)
Deoxy-benzyl-stambomycin (**7**)	$C_{73}H_{125}NO_{21}$	21.5	676.9423	676.9438 (2.22 ppm)	1352.8817	1352.8781 (–2.66 ppm)
Octyl-stambomycin (**8**)	$C_{74}H_{135}NO_{22}$	25.4	695.9811	695.9805 (–0.86 ppm)	1390.9549	1390.9546 (–0.22 ppm)
Deoxy-octyl-stambomycin	$C_{74}H_{135}NO_{21}$	n.d.	687.9836	n.d.	1374.9599	n.d.
Phenylpropyl-stambomycin	$C_{75}H_{129}NO_{22}$	n.d.	698.9555	n.d.	1396.9079	n.d.
Deoxy-phenylpropyl-stambomycin	$C_{75}H_{129}NO_{21}$	n.d.	690.9602	n.d.	1380.9130	n.d.
Phenoxypropyl-stambomycin (**9**)	$C_{75}H_{129}NO_{23}$	22.5	706.9554	n.d.	1412.9028	1412.8993 (–2.48 ppm)
Deoxy-phenoxypropyl-stambomycin (**10**)	$C_{75}H_{129}NO_{22}$	22.5	698.9555	n.d.	1396.9079	1396.9048 (–2.22 ppm)
Thiophenyl-stambomycin	$C_{70}H_{121}NO_{22}S$	n.d.	680.9123	n.d.	1360.8174	n.d.
Deoxy-thiophenyl-stambomycin	$C_{70}H_{121}NO_{21}S$	n.d.	672.9148	n.d.	1344.8225	n.d.
Thienylmethyl-stambomycin	$C_{71}H_{123}NO_{22}S$	n.d.	687.9201	n.d.	1374.8330	n.d.
Deoxy-thienylmethyl-stambomycin	$C_{71}H_{123}NO_{21}S$	n.d.	679.9227	n.d.	1358.8381	n.d.
Propargyl-stambomycin	$C_{69}H_{121}NO_{22}$	n.d.	658.9263	n.d.	1316.8453	n.d.
Deoxy-propargyl-stambomycin	$C_{69}H_{121}NO_{21}$	n.d.	650.9289	n.d.	1300.8504	n.d.
6-Bromohexyl-stambomycin (**11**) (lacking 2H)	$C_{72}H_{128}NO_{22}Br$	22.6	719.9129 (Br^{79}) 720.9130 (Br^{81})	719.9128 (Br^{79}) (–0.14 ppm) 720.9130 (Br^{81}) (–0 ppm)	1438.8184 (Br^{79}) 1440.8187 (Br^{81})	1438.8164 (Br^{79}) (–1.53 ppm) 1440.8183 (Br^{81}) (–0.28 ppm)
Deoxy-6-bromohexyl-stambomycin (lacking 2H)	$C_{72}H_{128}NO_{21}Br$	n.d.	711.9154 (Br^{79}) 712.9155 (Br^{81})	n.d.	1422.8235 (Br^{79}) 1424.8238 (Br^{81})	n.d.

n.d. = not detected

Table S6. Quantification of stambomycins in parental and mutasynthesis strains.

Strains (culture volume)	Integrated peak areas for stambomycins A, B, C, D (extracted ion (EI) = 1362.9236, 681.9659, 1376.9392, 688.9736)	Volume of final extract in MeOH (µL)	Calculated titer (mg L^{-1})	Average ± deviation (parental level, %)
20190130-wtOE (50 mL)	4672442552	300	28.2	30 ± 2 (100%)
20190913-wtOE (50 mL)	6621933694	240	31.7	
20190130-de482OE#1 (50 mL)	3117290008	240	15.0	15.2 ± 0.3 (50.8%)
20190130-de482OE#2(50 mL)	2550403337	300	15.4	
20190130-de483OE (50 mL)	22253469	270	0.12	0.09 ± 0.04 (0.31%)
20190206-de483OE (50 mL)	23710170	270	0.13	
20190226-de483OE#1 (50 mL)	10444147	220	0.049	
20190226-de483OE#2 (50 mL)	23826563	240	0.12	
20190430-de483OE (50 mL)	12248726	200	0.052	
20190913-de483OE (50 mL)	12525726	330	0.088	
20191108-de483OE::483#1 (50 mL)	2125375050	200	8.56	9.2 ± 0.9 (30.8%)
20191108-de483OE::483#2 (50 mL)	2337747862	210	9.88	

Table S7. Quantification of stambomycins and stambomycin analogues produced by the mutasynthesis strain ATCC/OE484/Δ483 supplemented with various malonic acid alternatives. The Extracted ions (EIs) used for integration of stambomycins (A–D) and stambomycin analogues are shown in **Table S5**.

Strains (culture volume)	Peak areas for stambomycins A, B, C, D	Integrated peak areas for Stambomycin Analogues	Integrated peak areas for deoxy-Stambomycin Analogues	C-24-demethyl-Stambomycin Analogues	Cell pellets (g)	Volume of final extract in MeOH (µL)
20191108-de483OE+ethylmalonic acid#1 (50 mL)	132876313	n.d.	132598	n.d.	2.6	180
20191108-de483OE+ethylmalonic acid#2 (50 mL)	143567324	n.d.	167324	n.d.	2.7	180
20191108-de483OE+ethylmalonic acid#3 (50 mL)	171220959	n.d.	153648	n.d.	3.0	210
Average ± deviation	**149221532 ± 82602738**	**n.d.**	**151190 ± 17493**	**n.d.**		
20190704-de483OE+isopropylmalonic acid#1 (50 mL)	63133287	n.d.	n.d.	n.d.	2.9	200
20190704-de483OE+isopropylmalonic acid#2 (50 mL)	56732414	n.d.	n.d.	n.d.	2.7	190
20190704-de483OE+isopropylmalonic acid#3 (50 mL)	69815755	n.d.	n.d.	n.d.	2.7	190
Average ± deviation	**63227152 ± 6542175**	**n.d.**	**n.d.**	**n.d.**		
20200121-de483OE+allylmalonic acid#1 (50 mL)	33235281	314576	57623911	n.d.	2.9	200
20200121-de483OE+allylmalonic acid#2 (50 mL)	36372141	373641	63992453	n.d.	2.9	200
20200121-de483OE+allylmalonic acid#3 (50 mL)	29473776	390322	76003233	n.d.	3.0	210
Average ± deviation	**33027066 ± 28801362**	**359513 ± 36365569**	**65873199 ± 9332886**	**n.d.**		
20200121-de483OE+butylmalonic acid#1 (50 mL)	31098375	735657630	3911597542	83759031	3.3	230
20200121-de483OE+butylmalonic acid#2 (50 mL)	33518173	693736419	3639924533	81335171	3.1	215
20200121-de483OE+butylmalonic acid#3(50 mL)	36089249	842196726	3274692780	70790032	2.9	200
Average ± deviation	**33568599 ± 1537021995**	**757196925 ± 1630548827**	**3608738285 ± 1944062494**	**78628078 ± 6895288**		
20191108-de483OE+benzylmalonic acid#1 (50 mL)	85980132	5598326	8973467	n.d.	2.8	195
20191108-de483OE+benzylmalonic acid#2 (50 mL)	75184735	4353298	9037041	n.d.	2.9	200
20191108-de483OE+benzylmalonic acid#3 (50 mL)	89638709	4490826	10775770	n.d.	2.9	200
Average ± deviation	**83601192 ± 38443275**	**4814150 ± 2731834**	**9595426 ± 1022702**	**n.d.**		
20191108-de483OE+octylmalonic acid#1 (50 mL)	132683570	11976573	n.d.	n.d.	3.0	210
20191108-de483OE+octylmalonic acid#2 (50 mL)	147359538	13467812	n.d.	n.d.	3.1	215
20191108-de483OE+octylmalonic acid#3 (50 mL)	163698892	15272458	n.d.	n.d.	2.8	195
Average ± deviation	**147914000 ± 74240730**	**13572281 ± 1650424**	**n.d.**	**n.d.**		
20191108-de483OE+phenylpropyl malonic acid#1 (50 mL)	106794320	n.d.	n.d.	n.d.	3.2	220
20191108-de483OE+phenylpropyl malonic acid#2 (50 mL)	108547211	n.d.	n.d.	n.d.	2.8	195
20191108-de483OE+phenylpropyl malonic acid#3 (50 mL)	94946315	n.d.	n.d.	n.d.	2.9	200
Average ± deviation	**103429282 ± 7398560**	**n.d.**	**n.d.**	**n.d.**		
20200121-de483OE+phenoxylpropyl malonic acid#1 (50 mL)	40974329	1978456	2389963	n.d.	2.9	200
20200121-de483OE+phenoxylpropyl malonic acid#2 (50 mL)	43458721	1829874	2558642	n.d.	3.1	215
20200121-de483OE+phenoxylpropyl malonic acid#3 (50 mL)	37665924	1941620	2151384	n.d.	3.2	220
Average ± deviation	**40699658 ± 19334974**	**1916650 ± 282660**	**2366663 ± 204626**	**n.d.**		
20191108-de483OE+thiophenyl malonic acid#1 (50 mL)	13290899	n.d.	n.d.	n.d.	2.8	195
20191108-de483OE+thiophenyl malonic acid#2 (50 mL)	16067843	n.d.	n.d.	n.d.	2.9	200
20191108-de483OE+thiophenyl malonic acid#3 (50 mL)	14717777	n.d.	n.d.	n.d.	2.8	195
Average ± deviation	**14692173 ± 1388649**	**n.d.**	**n.d.**	**n.d.**		
20191108-de483OE+thienylmethyl malonic acid#1 (50 mL)	18904562	n.d.	n.d.	n.d.	2.9	200
20191108-de483OE+thienylmethyl malonic acid#2 (50 mL)	17273940	n.d.	n.d.	n.d.	2.8	195
20191108-de483OE+thienylmethyl malonic acid#3 (50 mL)	16036282	n.d.	n.d.	n.d.	2.8	195
Average ± deviation	**17404928 ± 1438619**	**n.d.**	**n.d.**	**n.d.**		
20210119-de483OE+propargyl malonic acid#1 (50 mL)	85631272	n.d.	n.d.	n.d.	3.1	215
20210119-de483OE+propargyl malonic acid#2 (50 mL)	79409817	n.d.	n.d.	n.d.	2.7	190
20210119-de483OE+propargyl malonic acid#3 (50 mL)	75196466	n.d.	n.d.	n.d.	2.8	195
Average ± deviation	**80079185 ± 5249508**	**n.d.**	**n.d.**	**n.d.**		
20210119-de483OE+6-bromohexyl malonic acid#1 (50 mL)	96347831	4567832	n.d.	n.d.	2.9	200
20210119-de483OE+6-bromohexyl malonic acid#2 (50 mL)	122541322	5237893	n.d.	n.d.	3.2	220
20210119-de483OE+6-bromohexyl malonic acid#3 (50 mL)	101880828	4646688	n.d.	n.d.	3.0	210
Average ± deviation	**106923327 ± 56603651**	**4817471 ± 366224**	**n.d.**	**n.d.**		

n.d. = not detected

Table S8. Quantitative analysis by LC-HRMS using erythromycin (1 mM) as internal standard. $[M+H]^+$ = 734.4683 was used as the extracted ion (EI) for peak integration of erythromycin. EIs used for integration of stambomycins (A–D) and stambomycin analogues are shown in **Table S5**.

Strains (culture volume)	Integrated peak areas for Erythromycin ($\times 10^9$)	Integrated peak areas for stambomycins A, B, C, D ($\times 10^9$)	Integrated peak areas for Stambomycin Analogues ($\times 10^9$)	Cell pellets (g)	Volume of final extract in MeOH (µL)	Amount of stambomycins relative to internal standard	Amount of stambomycin analogues relative to internal standard
de483OE+ethylmalonic acid#1 (50 mL)	73.10	6.49	0.08	2.8	200	0.0888	0.0011
de483OE+ethylmalonic acid#2 (50 mL)	68.30	5.69	0.13	2.5	180	0.0833	0.0019
de483OE+ethylmalonic acid#3 (50 mL)	71.20	4.83	0.12	2.7	190	0.0678	0.0017
Average ± deviation						**0.08 ± 0.0109**	**0.0016 ± 0.0004**
de483OE::matB_cinna+ethylmalonic acid#1 (50 mL)	71.60	3.70	0.21	2.5	180	0.0517	0.0029
de483OE::matB_cinna+ethylmalonic acid#2 (50 mL)	68.80	2.69	0.23	2.65	190	0.0391	0.0033
de483OE::matB_cinna+ethylmalonic acid#3 (50 mL)	69.50	3.90	0.25	2.7	190	0.0561	0.0036
Average ± deviation						**0.049 ± 0.0088**	**0.0033 ± 0.0003**
de483OE+allylmalonic acid#1 (50 mL)	67.90	8.73	4.40	3.0	210	0.1286	0.0648
de483OE+allylmalonic acid#2 (50 mL)	67.40	8.69	4.80	2.8	200	0.1289	0.0712
de483OE+allylmalonic acid#3 (50 mL)	70.20	6.61	5.17	2.67	190	0.0942	0.0736
Average ± deviation						**0.1172 ± 0.02**	**0.0699 ± 0.0046**
de483OE::matB_cinna+allylmalonic acid#1 (50 mL)	70.00	4.13	9.25	2.7	190	0.0590	0.1321
de483OE::matB_cinna+allylmalonic acid#2 (50 mL)	71.30	5.45	9.91	2.6	190	0.0764	0.1390
de483OE::matB_cinna+allylmalonic acid#3 (50 mL)	72.30	6.23	8.38	2.65	190	0.0862	0.1159
Average ± deviation						**0.0739 ± 0.0138**	**0.129 ± 0.0119**
de483OE+butylmalonic acid#1 (50 mL)	61.60	0.52	54.50	3.0	210	0.0084	0.8847
de483OE+butylmalonic acid#2 (50 mL)	63.90	0.41	53.90	2.8	200	0.0064	0.8435
de483OE+butylmalonic acid#3 (50 mL)	64.50	0.54	58.70	3.1	205	0.0084	0.9101
Average ± deviation						**0.0077 ± 0.0011**	**0.8794 ± 0.0336**
de483OE::matB_cinna+butylmalonic acid#1 (50 mL)	63.20	0.06	44.20	2.7	190	0.0009	0.6994
de483OE::matB_cinna+butylmalonic acid#2 (50 mL)	67.20	0.06	49.20	2.7	190	0.0009	0.7321
de483OE::matB_cinna+butylmalonic acid#3 (50 mL)	66.30	0.08	46.40	2.8	195	0.0012	0.6998
Average ± deviation						**0.001 ± 0.0002**	**0.7105 ± 0.0188**
de483OE+benzylmalonic acid#1 (50 mL)	71.40	7.48	0.06	2.7	190	0.1048	0.0008
de483OE+benzylmalonic acid#2 (50 mL)	67.30	6.94	0.04	2.9	200	0.1031	0.0006
de483OE+benzylmalonic acid#3 (50 mL)	67.60	6.01	0.06	2.7	190	0.0889	0.0009
Average ± deviation						**0.0989 ± 0.0087**	**0.0008 ± 0.0001**
de483OE::matB_cinna+benzylmalonic acid#1 (50 mL)	60.70	0.82	0.13	2.8	200	0.0135	0.0022
de483OE::matB_cinna+benzylmalonic acid#2 (50 mL)	64.20	0.75	0.09	2.9	200	0.0117	0.0014
de483OE::matB_cinna+benzylmalonic acid#3 (50 mL)	65.30	0.89	0.10	2.8	200	0.0136	0.0015
Average ± deviation						**0.0129 ± 0.0011**	**0.0017 ± 0.0004**
WTOE+butenoic acid#1 (50 mL)	60.30	44.90	n.d.	3.5	250	0.7446	n.d.
WTOE+butenoic acid#2 (50 mL)	62.50	37.70	n.d.	3.1	215	0.6032	n.d.
WTOE+butenoic acid#3 (50 mL)	65.30	37.79	n.d.	2.7	200	0.5787	n.d.
Average ± deviation						**0.6422 ± 0.0896**	**n.d.**
WTOE+butyric acid#1 (50 mL)	68.60	59.60	8.31	3.0	210	0.8688	0.1211
WTOE+butyric acid#2 (50 mL)	67.90	55.60	6.74	2.9	200	0.8189	0.0993
WTOE+butyric acid#3 (50 mL)	66.50	56.52	8.08	3.1	215	0.8499	0.1215
Average ± deviation						**0.8459 ± 0.0252**	**0.114 ± 0.0127**
WTOE+phenyl propanoic acid#1 (50 mL)	65.60	9.10	n.d.	2.8	200	0.1387	n.d.
WTOE+phenyl propanoic acid#2 (50 mL)	65.30	8.54	n.d.	2.9	200	0.1308	n.d.
WTOE+phenyl propanoic acid#3 (50 mL)	67.20	8.52	n.d.	2.9	200	0.1268	n.d.
Average ± deviation						**0.1321 ± 0.0061**	**n.d.**

n.d. = not detected

Table S9. Conditions for the purification of mutasynthetic stambomycin analogues.

1. Prep HPLC	
Solvents	ACN/H_2O +0.1%FA
0 min	10%
2 min	10%
27 min	50%
Column	Waters XBridge BEH C18 19x250mm
Detection UV	205 mAU
Retention time of deoxy-butyl-stambomycin (**5**)	18.5 min
Retention time of butyl-stambomycin (**4**)	17.6 min
Retention time of C-24-demethyl-butyl stambomycin (**12**)	17.1 min
Retention time of deoxy-allyl-stambomycin (**3**)	16.9 min
Amounts of purified compounds	
Deoxy-butyl-stambomycin (**5**)	36.3 mg
Butyl-stambomycin (**4**)	11.6 mg
C-24-demethyl-butyl-stambomycin (**12**)	3.1 mg
Deoxy-allyl-stambomycin (**3**)	1.8 mg

Table S10. IC_{50} values of the stambomycin analogues for two human cancer cell lines. The U87-MG (ATCC, HTB-14) cell line correspond to glioblastoma cells (brain cancer) and MDA-MB-231 (ATCC, HTB-26) cells are commonly used to model late-stage breast cancer. The clinical anticancer agent doxorubicin was used as a control. IC_{50} indicates the concentration needed to inhibit the growth of 50% of cells in the population.

	IC_{50} (μM)	U87 cell line					MDA-MB-231 cell line				
		Doxorubicin	Deoxy-butyl-stambomycin	Butyl-stambomycin	C-24-demethyl-butyl-stambomycin	Deoxy-allyl-stambomycin	Doxorubicin	Deoxy-butyl-stambomycin	Butyl-stambomycin	C-24-demethyl-butyl-stambomycin	Deoxy-allyl-stambomycin
MTT assay	Experiment 1	9.4	1.1	1.3	6.7	12.7	8.9	1.0	0.7	> 1 mM	> 1 mM
	Experiment 2	5.9	0.8	0.7	5.5	25.4	9.5	1.5	1.5	26.4	47.5
	Experiment 3	5.2	0.8	1.1	74.5	10.6	18.6	1.2	1.3	71.4	> 1 mM
	Mean ± SD	**6.8 ± 2.3**	**0.9 ± 0.2**	**1.0 ± 0.3**	**28.9 ± 39.5**	**16.2 ± 8.0**	**12.3 ± 5.4**	**1.2 ± 0.3**	**1.2 ± 0.4**	**48.9**	**47.5**

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