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Salinipyrone and Pacificanone Are Biosynthetic By-products of the Rosamicin Polyketide Synthase

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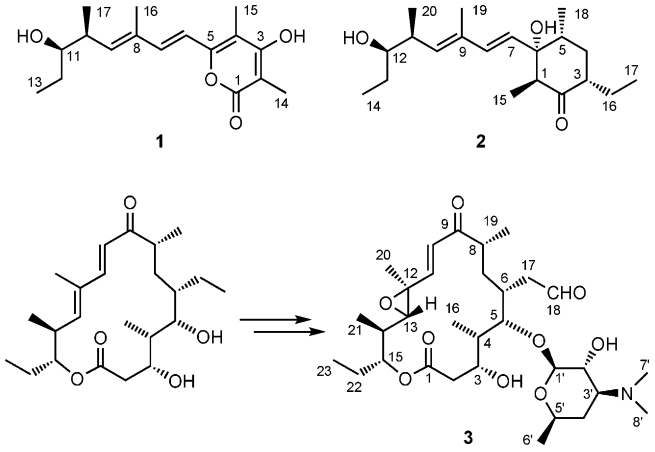
Salinipyrones and pacificanones are structurally related polyketides from *Salinispora pacifica* CNS-237 that are proposed to arise from the same modular polyketide synthase (PKS) assembly line. Genome sequencing revealed a large macrolide PKS gene cluster that codes for the biosynthesis of rosamicin A and a series of new macrolide antibiotics. Mutagenesis experiments unexpectedly correlated salinipyrone and pacificanone biosynthesis to the rosamicin octamodule Spr PKS. Remarkably, this bifurcated polyketide pathway illuminates a series of enzymatic domain- and module-skipping reactions that give rise to natural polyketide product diversity. Our findings enlarge the growing knowledge of polyketide biochemistry and illuminate potential challenges in PKS bioengineering.



Salinipyrone A (**1**) and pacificanone A (**2**) are structurally related polyketides produced by the marine bacterium *Salinispora pacifica* CNS-237.[1] These natural products share identical lipid tails yet differ in the composition of their lipid head groups: γ-hydroxypyrone versus cyclohexanone, respectively. It has been suggested that the associated polyketide synthase (PKS) might skip the penultimate extension module to yield the slightly smaller salinipyrone.[1] The concept of module skipping by assembly line, modular PKSs is best illustrated by the methymycin/picromycin system in which 12- and 14-membered ring macrolactones are generated by the same PKS complex adopting alternative conformations to enable an early chain termination in the case of the shorter methymycin aglycone.[2] Module skipping and module stuttering can lead to biosynthetic diversification in nature, as exemplified by the thalassospiramide family of calpain inhibitors.[3] We thus explored the biosynthetic basis for salinipyrone/pacificanone differentiation and were surprised to learn that, although module skipping is indeed a contributing factor, these metabolites are in fact by-products of an even larger PKS system that is associated with the rosamicin macrolide antibiotics.

To define the biosynthetic logic of salinipyrone/pacificanone construction, we examined the recently sequenced draft genome of *S. pacifica* CNS-237.[4] As **2** contains an ethyl side chain in the cyclohexanone unit, we anticipated that its biosynthetic origin would be the PKS substrate ethylmalonyl-CoA[5] and thus specifically searched for ethylmalonate-specifying acyl transferase (AT) domains within modular PKSs. We identified a single genomic example in a biosynthetic gene cluster fragmented across several contigs. Extensive primer walking completed the DNA sequence of the cluster to give the 23-ORF *spr* locus spanning 64.2 kb (GenBank: KP997155). This PKS gene cluster shares high sequence homology with that for the macrolide rosamicin A (**3**) in *Micromonospora carbonacea* subsp. *aurentiaca* NRRL 2997[6] and contains five PKS genes (*spr7–11*) consisting of eight PKS modules, three cytochrome P450 genes (*spr5–6*, *-12*),[7] one glycosyltransferase (*spr13*), and genes encoding desosamine biosynthesis (*spr4*, *14*, *19–20*, *22–23*)[8] (Table S1 in the Supporting Information).

Inspection of the chemical structure of macrolide **3** revealed striking structural similarities to **1** and **2**; this suggested that all three structure types might originate from the same PKS assembly line. To evaluate this revised biosynthetic hypothesis, we first inactivated the *spr* gene cluster by inserting an apramycin resistance gene cassette (*aac(3)IV*) into the region encoding *spr8–11* by intergenic conjugation (Figure S1). As predicted, inactivation abolished the production of **1** and a

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previously unreported derivative, dehydrosalinipyrone A (**4**; Figure 1), thus confirming that the Spr PKS is responsible for their production (Table S1). Unfortunately, we could not reproduce the production of **2** in the wild-type strain under any of the fermentation conditions tested, and thus could not positively link its biosynthesis to the *spr* locus. We did, however, observe the loss of five additional compounds in the *spr* mutant never before reported from this bacterium (Figure 1 A). Upon isolation and complete spectral analysis, compounds were found to be the known rosamicin analogues **3** and 21-hydroxyrosamicin (izenamicin A3,[9] **5**) along with three new derivatives, 18-dihydro-14-hydroxyrosamicin (**6**), 18-dihydro-21-hydroxyrosamicin (**7**), and 14-hydroxyrosamicin (**8**). We predict that the conserved stereocenters in the new rosamicin analogues **6**–**8** are identical to those previously reported for **3** and that the new C14 tertiary hydroxy stereocenter in **6** and **8** is *S*, as in the macrolide mycinamicin II.[10] In the latter case, the cytochrome P450 protein MycG[11] installs the analogous 14*S* hydroxy group. Of the three cytochrome P450s encoded within the *spr* gene cluster, Spr6 is the closest in identity to MycG at 45% and is thus a likely candidate for the C14 hydroxylation. Rosamicins **5**–**8** exhibit moderate antibacterial properties (Table S10).

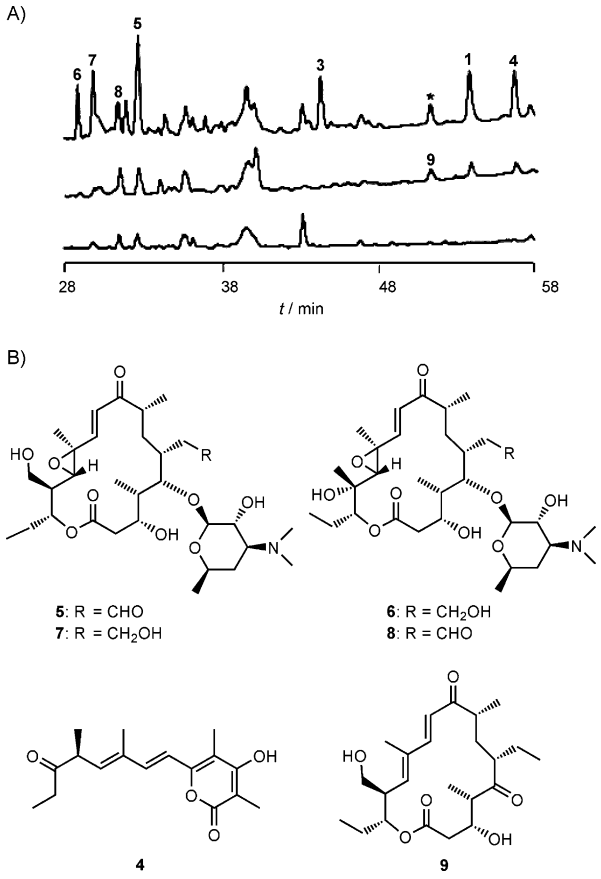
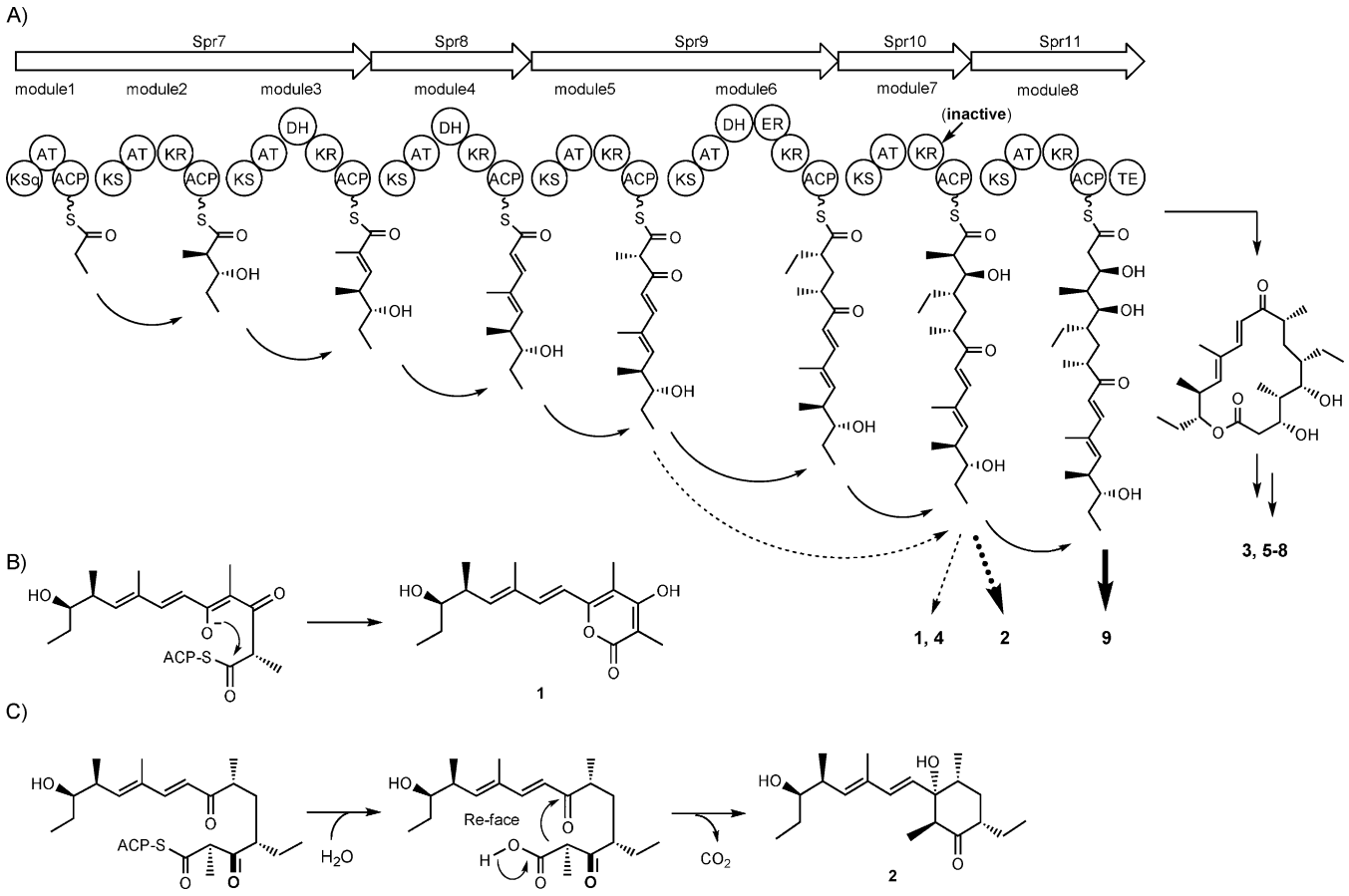


Figure 1. A) HPLC chromatograms acquired by detection at 254 nm of the metabolites from i) CNS-237 wild-type, ii) *spr10*Y1290F, and iii) Δ*spr* cultured in A1FeBC medium. * indicates the compound whose mass and UV spectra are identical to those of **1**. Although **9** is eluted at the same t_R as this compound, the peak of **9** is detected at 280 nm only in the Y1290F strain (Figure S2). B) Chemical structures of **4**–**9**.

The biosynthetic logic of the *spr* gene cluster is consistent with rosamicin octaketides **3**, **5**–**8**, in which all eight PKS modules of Spr7–11 are progressively involved in the linear construction of the macrolide aglycone (Scheme 1 A). The smaller salinipyrone hexaketides **1** and **4**, however, are consistent with an incomplete processing by the PKS in which modules 6 and 8 are both skipped. Alternatively, the slightly larger heptaketide **2** could derive from skipping just the final PKS module 8. In both cases, the truncated products appear to arise from intermediates independent of the ketoreductase (KR) domain of Spr10 (Scheme 1 A), thus suggesting its role, both directly and indirectly, in product dispersal. To explore this concept, we inactivated the *spr10*-KR domain by introducing a chromosomal point mutation through a single reciprocal recombination event to effectively replace the catalytic Tyr1290 with phenylalanine. Detailed HPLC-MS analyses revealed that the mutant no longer produced the rosamicin octaketides **3**, **5**–**8**, but retained **1** and **4**, albeit at lower production titers (Figure 1 A). The *spr10*-Y1290F mutant also produced a new product, 18-deoxo-5-deoxy-5-oxotylonolide (**9**), in which the desosamine sugar, the C12–C13 epoxide, and the C18 hydroxy groups were missing, thus suggesting that **9** is not a substrate for post-PKS modifying enzymes. We further identified two new compounds with the same molecular composition as **2**, and the epimeric pacificanone B (Figure S3), yet due to poor production yields, we were unable to conclusively elucidate their structures.

Inspection of the wild-type *S. pacifica* CNS-237 strain in certain growth media also revealed **9** (Figure S4), albeit at lower levels than for the *spr10*-Y1290F mutant strain; this suggested that the Spr10 KR7 domain is naturally partially effective in processing Spr10-linked substrates. We suspect that unreduced β-keto intermediates linked to the module 7-acyl carrier protein (ACP7) also give rise to products in the truncated salinipyrone/pacificanone series, and that their protein offloading might be driven by the innate chemical reactivity of their associated polyketone substrates (Scheme 1 B and C). In the case of **1**, this proposal combines a rare intermodule-skipping mechanism (module 6) and a domain-skipping reaction (KR7) to yield the ACP7-bound salinipyrone intermediate, which is likely released through a spontaneous enol lactonization reaction. This biosynthetic bifurcation mediated by module and domain skipping explains the different product outcomes of the Spr PKS in yielding the salinipyrone, pacificanone, and rosamicin natural products, and represents an entirely different mechanism from the one we previously reported in the related bacterium *Salinispora arenicola* that co-produces the arenicolide and rifamycin polyketide natural products by off-PKS enzymatic processing.[12] This study further adds to our growing appreciation of the enzymatic complexity of biosynthetic assembly lines[13] and illuminates how much we still need to learn about the structural complexity of these systems to use their full potential in ongoing PKS bioengineering research.



Scheme 1. Proposed biosynthesis of **1**–**9**. A) Spr PKS organization and deduced assembly of **1**–**9**. The arrows depict the sequential synthesis of intermediates leading to the mature products **3** and **5**–**8**. Dotted arrows represent module-skipping movements leading to **1** and **4**. The bold arrow shows the assembly of **9**; the bold dotted arrow leading to **2** shows skipping of the final module. In both cases, the KR domain in module **7** is inactive. The formation of B) **1** and C) **2** involves reactions on premature ACP-bound intermediates.

Experimental Section

General: All chemicals were acquired from Fisher Scientific and Sigma–Aldrich and used without further purification. PCR was performed with PrimeStarMax DNA polymerase (TaKaRa Bio, Shiga, Japan). Gibson assembly cloning kits were from New England Biolabs. All solvents were of HPLC grade or higher. Analytical HPLC analyses were conducted with an Agilent 1200 HPLC system with diode array detection connected to a Phenomenex Luna C18(2), 10.0×250 mm, 5 μm column. LC-MS analyses were conducted with an 6530 Accurate-Mass Q-TOF MS (MassHunter software, Agilent) equipped with a dual electrospray-ionization source and a 1260 LC system (ChemStation software, Agilent) with diode array detector. Preparative HPLC was carried out by using an Agilent 218 purification system (ChemStation software, Agilent) equipped with a ProStar 410 automatic injector, an Agilent ProStar UV-Vis dual wavelength detector, and a 440-LC fraction collector connected to Phenomenex Luna C18(2), 10.0×250 mm, 5 μm column. NMR data were acquired at the UCSD Skaggs School of Pharmacy and Pharmaceutical Sciences NMR Facility on a 600 MHz Varian NMR spectrometer (Topspin 2.1.6 software, Bruker) with a 1.7 mm cryoprobe.

Construction of the *spr* gene inactivation plasmid: A 3.0 kb DNA fragment containing the *spr8* coding region was amplified by PCR with primers D1f, 5′-**accat gatta cgcca agctt** GATTC CCGTC GTTAG CAATG TGACC-3′, and D1r, 5′-**cagct ccagc ctaca** GACTG GTAGC CGACT GTCGA TCTAG-3′ (overlap sequences in bold), and the chromosome of CNT-237; a 1.1 kb DNA fragment containing *aac(3)IV* and OriT was amplified by PCR with primers AprOriTf, 5′-**AGTCG GCTAC CAGTC** TGTAG GCTGG AGCTG CTTC-3′, and AprOriTr, 5′-**GAAAC CCATG GCTGA CATTA** TTCCG GGGAT CCGTC GACC-3′ (overlap sequences in bold), and pIJ773; and a 3.0 kb DNA fragment containing the Spr11-coding region was amplified by PCR with primers D2f, 5′-**ggtcg acgga tcccc ggaat** AATGT CAGCC ATGGG TTTCA TTCGG-3′, and D2r, 5′-**aaaac gacgg ccagt gaatt c**CGCG TATTC GGTCA TCATC GACAA G-3′ (overlap sequences in bold), and the chromosome of CNT-237. The three amplified fragments were assembled with a linear pUC19, which was amplified by PCR with primers pUC19f, 5′-gaatt cactg gccgt cgttt tacaa c-3′, and pUC19r, 5′-aagct tggcg taatc atggt catag c-3′, resulting in pUC19-Δ*spr*.

Construction of *spr10*-KR point-mutation plasmid: A 3.0 kb DNA fragment containing the *Spr10* coding region was amplified by PCR with primers Spr10 KR1f, 5′-**ccccc gggct gcagg aattc** CCGGT ACGGC GATCA ACCAG GAC-3′, and Spr10 KR1r, 5′-**GCCCC G*AATG* CTGAT** TGCCC GCCGC TACCC CACAC GCCCG CGTTT GAG-3′ (overlap sequences in bold, the mutated sequence for Y1290F is italicized), and the chromosome of CNT-237; and a 3.0 kb DNA fragment containing the *spr10* coding region was amplified by PCR with primers Spr10 KR2f, 5′-**GGGCA ATCAG CA*TTC*** GGGGC CGCCA ACGCA GCCCT GGACG CAC-3′ (overlap sequence in bold, the mutated sequence for Y1290F is italicized), and Spr10 KR2r, 5′-**ccagc ctaca catcg aattc** AGCGG TGCGA TCTCT TCCTC GCTG-3′ (overlap

sequence in bold), and the chromosome of CNT-237. The two amplified fragments were assembled with a linear pIJ773, which was amplified by PCR with primers pIJ773f, 5′-gaatt cctgc agccc ggggg atc-3′, and pIJ773r, 5′-gaatt cgatg tgtag gctgg ag-3′, resulting in pIJ773-*spr10*KRm.

Introducing plasmids into *S. pacifica* CNT-237 for the inactivation of *spr* and the point mutation of the Spr10 KR: pUC19-Δ*spr* and pIJ773-*spr10*KRm were separately introduced into *Escherichia coli* S17–1 cells by electroporation and then transferred into *S. pacifica* CNS-237 by intergeneric conjugation. Mutants in which the apramycin resistance cassette was integrated into the *S. pacifica* chromosome were selected on A1 agar by overlaying plates with apramycin (200 μg mL^{-1}) and nalidixic acid (100 μg mL^{-1}). Following incubation at 28 °C for 7–9 days, exconjugants were randomly selected, and those exhibiting an Apr^r (for pUC19-Δ*spr*) or Apr^s (for pIJ773-*spr10*KRm) phenotype were evaluated by PCR and DNA sequencing for double-crossover mutation, respectively. Primer set-1 (f: 5′-AGACC GCTGT GGAAG AACAG ATCG-3′ and r: 5′-gaact tcgaa gcagc tccag cctac-3′) and primer set-2 (f: 5′-cttat tcgca cctgg cggtg ctcaa cg-3′ and r: 5′-CGCTA ACGGG GGATG TCATA ATCTG C-3′) were used for the PCR for the evaluation of Δ*spr* strain. A 595 bp fragment amplified by KRpcrf, 5′-gaact tcgaa gcagc tccag cctac-3′, and KRpcrr, 5′-CGCGG TGAAC AGATC CCAAT CCACG TC-3′, was sequenced for the evaluation of the *spr10-Y1290F* strain.

Comparative metabolite profiling between wild-type and mutant *S. pacifica* strains: Wild-type *S. pacifica* CNS-237 and the mutants prepared above were grown in liquid seawater-based A1 + BFe medium (1.0 L; 10 g starch, 4 g yeast extract, 2 g, peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3{\cdot}4H_2O$, 100 mg KBr) for 11 days at 28 °C with shaking at 230 rpm. An aliquot (20 mL) of the whole culture was extracted with EtOAc, and the resulting extract was concentrated to dryness in vacuo, then dissolved in CH_3OH (500 μL). HPLC analyses were conducted with a flow rate of 1.5 mL min^{-1} and a gradient of 10–100% CH_3CN (0.1% TFA)/water (0.1% TFA) for 90 min, and LC-MS analyses were conducted with a flow rate of 0.7 mL min^{-1} and mobile phase gradient from 10–100% CH_3CN (0.1% formic acid)/water (0.1% formic acid) for 23 min with positive mode ESI.

Preparation of 5–8: *S. pacifica* CNS-237 was cultured in five 2.8 L Fernbach flasks, each containing seawater-based A1-BFe medium (1.0 L) and shaken at 230 rpm at 27 °C. After seven days of cultivation, HP-20 resin was added to adsorb the organic products, and the culture and resin were stirred for 1.5 h. The resin was filtered on a glass column, washed with deionized water, and eluted with CH_3OH. The methanol was removed under reduced pressure, and the resulting aqueous layer was extracted with CH_2Cl_2/CH_3OH (100:5). The extract was subjected to silica-gel column chromatography by using a CH_2Cl_2/CH_3OH stepwise gradient system (100:5, 100:7.5, 100:10 and 100:20). The fraction containing **7** and **8** (100:10) was purified by reversed-phase preparative HPLC by using a gradient of 10–55% CH_3CN (0.1% TFA)/water (0.1% TFA) for 2× 30 min to afford 0.3 mg of **8** and 0.2 mg of **7**. The fraction containing **5** and **6** (100:20) was purified by reversed-phase preparative HPLC with a gradient of 10–50% CH_3CN (0.1% TFA)/water (0.1% TFA) for 30 min to afford 0.5 mg of **6** and 0.1 mg of **5**.

Preparation of 3: *S. pacifica* CNS-237 was cultured in three 2.8 L Fernbach flasks, containing seawater-based A1-BFe medium (1.0 L) and shaken at 230 rpm at 27 °C. After seven days of cultivation, the whole culture was extracted by EtOAc, and the resulting extract was concentrated to dryness in vacuo. The extract was subjected to silica-gel column chromatography by using a stepwise gradient system of CH_2Cl_2/CH_3OH (100:0, 100:1, 100:2, 100:3, 100:4 and 100:5). The fraction containing **3** (100:2) was purified by reversed-phase preparative HPLC with a gradient of 10–60% CH_3CN (0.1% TFA)/water (0.1% TFA) for 40 min to afford 0.5 mg of **3**.

Preparation of 1 and 4: *S. pacifica* CNS-237 was cultured in a 2.8 L Fernbach flask, containing seawater-based A1-BFe medium (1.0 L) and shaken at 230 rpm at 27 °C for three days. An aliquot (25 mL) of the culture was transferred into another 2.8 L Fernbach flask, containing seawater-based A1-BFe medium (1.0 L) and shaken at 200 rpm at 27 °C. After seven days of cultivation, the whole culture was extracted with EtOAc, and the resulting extract was concentrated to dryness in vacuo. The extract was subjected to silica-gel column chromatography by using a stepwise gradient system of CH_2Cl_2/CH_3OH (100:0, 100:1, 100:3, 100:4 and 100:5). The fractions containing **1** (100:4 and 100:5) were purified by reversed-phase preparative HPLC with 45% CH_3CN (0.1% TFA)/water (0.1% TFA) for 40 min to afford 0.4 mg of **1**. The fractions containing **4** (100:3) were purified by reversed-phase preparative HPLC with 50% CH_3CN (0.1% TFA)/water (0.1% TFA) for 40 min to afford 0.3 mg of **4**.

Preparation of 9: The *spr10Y1290F* strain was cultured in four 2.8 L Fernbach flasks, each containing seawater-based A1-production medium (1.0 L; 10 g starch, 4 g yeast extract, 2 g, peptone, 1 g $CaCO_3$) and shaken at 230 rpm at 27 °C. After seven days of cultivation, the whole culture was extracted with EtOAc, and the resulting extract was concentrated to dryness in vacuo. The extract was subjected to silica-gel column chromatography by using a stepwise gradient system of CH_2Cl_2/CH_3OH (100:0, 100:1, 100:3 and 100:5). The fraction containing **9** (100:3) was purified by reversed-phase preparative HPLC with 40% CH_3CN (0.1% TFA)/water (0.1% TFA) for 40 min to afford 0.8 mg of **9**.

Spectroscopic data

*Salinipyrone A (**1**):* Yellow oil; $[\alpha]_D^{25.9} = -76.2$ ($c = 0.33$, CH_3OH); ^1H and ^{13}C NMR: see Table S2; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{17}H_{25}O_4$: 293.1747 $[M+H]^+$; found: 293.1760.

*Rosamicin A (**3**):* White powder; $[\alpha]_D^{26.6} = -25.4$ ($c = 0.30$, EtOH); ^1H and ^{13}C NMR: see Table S3; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{31}H_{52}NO_9$: 582.3636 $[M+H]^+$; found: 582.3660.

*Dehydrosalinipyrone A (**4**):* Yellow oil; $[\alpha]_D^{26.3} = +30.6$ ($c = 0.33$, CH_3OH); ^1H and ^{13}C NMR: see Table S4; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{17}H_{23}O_4$: 291.1590 $[M+H]^+$; found: 291.1605.

*18-Dihydro-21-hydroxyrosamicin (**5**):* White powder; $[\alpha]_D^{27.4} = -18.8$ ($c = 0.30$, EtOH); ^1H and ^{13}C NMR: see Table S5; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{31}H_{54}NO_{10}$: 600.3742 $[M+H]^+$; found: 600.3751.

*18-Dihydro-14-hydroxyrosamicin (**6**):* White powder; $[\alpha]_D^{27.4} = -27.8$ ($c = 0.30$, EtOH); ^1H and ^{13}C NMR: see Table S6; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{31}H_{54}NO_{10}$: 600.3742 $[M+H]^+$; found: 600.3768.

*21-Hydroxyrosamicin (izenamicin A3) (**7**):* White powder; $[\alpha]_D^{27.4} = -34.9$ ($c = 0.30$, EtOH); ^1H and ^{13}C NMR: see Table S7; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{31}H_{52}NO_{10}$: 598.3586 $[M+H]^+$; found: 598.3580.

*14-Hydroxyrosamicin (**8**):* White powder; $[\alpha]_D^{27.4} = -2.4$ ($c = 0.30$, EtOH); ^1H and ^{13}C NMR: see Table S8; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{31}H_{52}NO_{10}$: 598.3586 $[M+H]^+$; found: 598.3584.

*18-Deoxo-5-deoxy-5-oxotylonolide (**9**):* White powder; $[\alpha]_D^{27.4} = +33.6$ ($c = 0.30$, EtOH); ^1H and ^{13}C NMR: see Table S9; HR-ESI-MS (positive): *m/z*: calcd (%) for $C_{23}H_{36}O_6Na$: 431.2410 $[M+Na]^+$; found: 431.2426.

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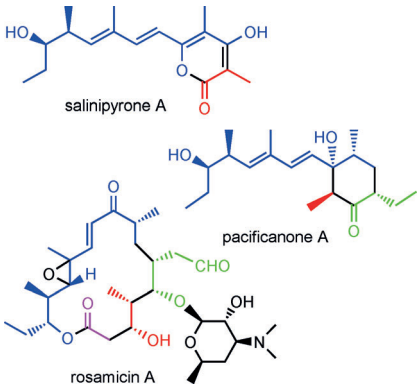
Salinipyrone and Pacificanone Are Biosynthetic By-products of the Rosamicin Polyketide Synthase



Bifurcated polyketide synthesis: Salinipyrones and pacificanones are structurally related polyketides from *Salinispora pacifica* CNS-237. Mutagenesis experiments correlated their biosyntheses to the rosamicin octamodule PKS. This bifurcated polyketide pathway illuminates a series of enzymatic domain- and module-skipping reactions that give rise to polyketide product diversity.