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NOTE

Competition and co-regulation of spirotoamide and tautomycetin biosynthesis in *Streptomyces griseochromogenes*, and isolation and structural elucidation of spirotoamide C and D

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Spirotoamide A (**1**) and B (**2**) (Figure 1a), two spiroketal-containing polyketides, were recently isolated from a microbial metabolite fraction library of *Streptomyces griseochromogenes* JC82-1223 by searching for structurally unique compounds based on a spectral database.[1] The carbon skeleton of spirotoamides was predicted to be assembled by a type I polyketide synthase (PKS) that utilizes acetyl-CoA as a starter unit and catalyzes decarboxylative condensation with four malonyl-CoAs, four methylmalonyl-CoAs and one ethylmalonyl-CoA as extender units. The spiroketal moiety of spirotoamides was speculated to be formed non-enzymatically, as no homologue was detected in the genome of *S. griseochromogenes* upon Southern analysis using *revJ*, which encodes a spiroketal synthase characterized from the reveromycin A biosynthetic machinery,[1,2] as a probe. Transfer of an amino group to the C-1 carboxyl group and the hydroxylation at C-6 or C-8 of spirotoamides were predicted to be catalyzed by a carboxamide synthase and P450 hydroxylases, respectively (Figure 2, path b).[1]

Intriguingly, under the same fermentation condition, *S. griseochromogenes* produced tautomycetin (TTN) as the major metabolite, a polyketide, featuring a unique 2,3-dialkylmaleic anhydride (DA) moiety (Figure 1a), that was originally isolated in 1989.[3,4] TTN is best known for its potent serine/threonine protein phosphatase inhibitory activity.[5–7] The *ttn* biosynthetic gene cluster has been cloned and sequenced from two producers.[8,9] On the basis of ^{13}C-labeled precursor feeding experiments and functional characterization of the *ttn* gene cluster from *S. griseochromogenes*, it has been established previously that (i) biosynthesis of the DA moiety from one molecule each of α-ketoglutarate and propionate, and its subsequent coupling with the polyketide scaffold of TTN are catalyzed by eight proteins of TtnKLMNOPRS, (ii) assembly of the polyketide scaffold of TTN from five molecules of malonyl-CoA, four molecules of methylmalonyl-CoA and one molecule of ethylmalonyl-CoA is catalyzed by the type I PKS proteins of TtnAB, affording TTN F-1 as the nascent intermediate, and (iii) TtnF, TtnD and TtnI catalyze the C-1"/C-2" dehydration, C-3" decarboxylation and C-5 oxidation, respectively, tailoring TTN F-1 en route to TTN (Figure 2, path a).[9–12]

Co-production of spirotoamides and TTN, which share essentially identical nascent polyketide intermediates for their biosynthesis (Figure 2), raises an interesting question if they are biosynthesized by the same type I PKS. That *S. griseochromogenes* produces TTN as major product (~10 mg l^{-1}) and **1** at a very low titer (~0.3 mg l^{-1}) (Figure 3, panel I) begs the question if TTN and spirotoamide biosynthesis compete for the same pool of precursors or the nascent polyketide intermediate (Figure 2). Since we have generated several TTN-nonproducing mutants in *S. griseochromogenes* in our previous efforts to study TTN biosynthesis,[9,11,12] we reasoned that these mutants could be ideal models to study the biosynthetic relationship between TTN and spirotoamides.

We first fermented selected TTN-nonproducing mutant strains SB13003 (Δ*ttnA*), SB13005 (Δ*ttnP*), SB13006 (Δ*ttnR*) and SB13007 (Δ*ttnS*), under the standard TTN production condition,[9,11,12] with the *S. griseochromogenes* wild-type strain as a control. While TTN production was completely abolished in all these mutant strains, HPLC analysis of the metabolite profiles showed that all the mutants still produced **1** (Figure 3, panels I vs II-V), establishing that spirotoamides and TTN must be synthesized by two distinct PKSs, presumably with similar modular architectures, competing for the same pool of polyketide substrates (Figure 2). This would be consistent with the fact that the titers (~5 mg l^{-1}) of **1** were significantly increased in all TTN-nonproducing mutants, which were about 16-fold higher than that (~0.3 mg l^{-1}) of the wild-type strain. The increased titers of **1** in these mutants also allowed the detection of two additional metabolites (**3** and **4**) with similar retention times and UV spectra to that of **1**. To further corroborate these findings, three

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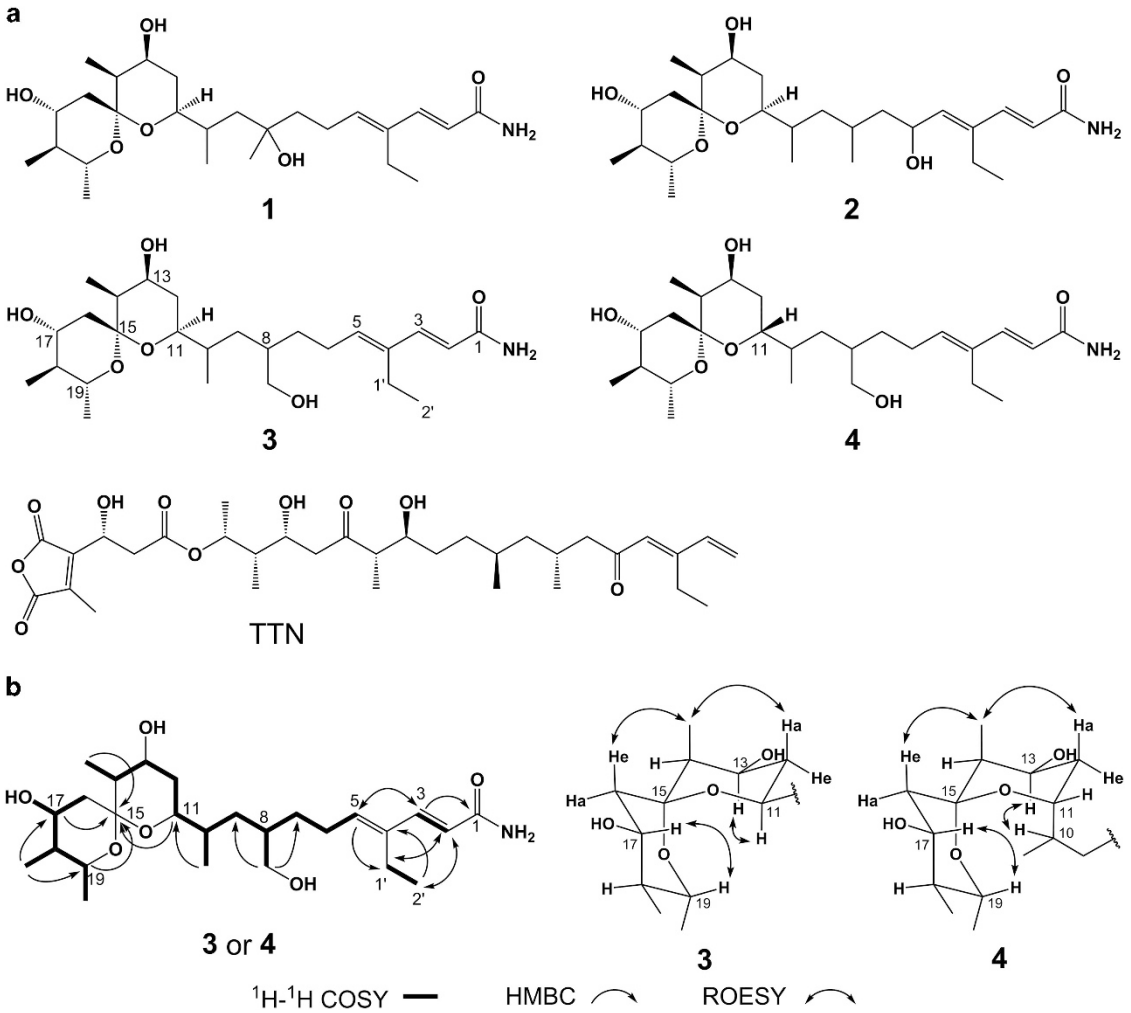

Figure 1 (**a**) Structures of spirotoamide A (**1**), B (**2**), C (**3**) and D (**4**), and tautomycetin (TTN) produced by *S. griseochromogenes* strains. While the absolute stereochemistry of TTN is known,[9,11,12] only the relative stereochemistry of the spiroketal moiety of spirotoamides is determined as depicted.[1] (**b**) Key ^{1}H-^{1}H COSY, HMBC and ROESY correlations supporting the structures of **3** and **4**.

additional mutant strains SB13014 (Δ*ttnF*), SB13013 (Δ*ttnD*) and SB13017 (Δ*ttnI*) were fermented, in which TTN production was abolished but biosynthesis of the TTN polyketide backbone remained intact.[9,11,12] HPLC analysis of the metabolite profiles of these mutants confirmed the accumulation of TTN biosynthetic intermediates TTN F-1, TTN D-1, -2, -3, -4 and TTN I-1, respectively, in titers (5–10 mg l^{-1}) that were comparable to TTN in the wild-type strain, with no apparent change on spirotoamide production (Figure 3, panels VI-VIII).

Prompted by the finding that TTN and spirotoamide are biosynthesized by two distinct type I PKSs, yet are always co-produced under the fermentation conditions examined, we next asked if the two biosynthetic machineries are co-regulated. We have previously identified *ttnQ* from the *ttn* biosynthetic gene cluster, encoding a member of the LuxR family of transcription factors, and established it as a positive regulator for TTN biosynthesis by gene inactivation (that is, SB13002 (Δ*ttnQ*)) and complementation (that is, SB13008 (Δ*ttnQ*+*ttnQ*)) experiments.[9,13] SB13002 and SB13008 were re-fermented under the standard TTN production condition,[9,11,12] with the *S. griseochromogenes* wild-type strain as a control. HPLC analysis of metabolite profiles confirmed that production of both TTN and spirotoamides was completely abolished in SB13002 and restored in SB13008 (Figure 3, panels IX and X). Taken together, these findings support the proposal that TtnQ regulates the biosynthesis of both TTN and spirotoamides in *S. griseochromogenes*. Cross-talk among pathway-specific regulators for secondary metabolite biosynthesis in actinomycetes is known but rare[14,15] and could potentially be exploited to activate cryptic gene clusters for the discovery of new natural products.[16–18]

We finally scaled up the fermentation of *S. griseochromogenes* SB13007 (5-L) and isolated the two new spirotoamides (**3,** 5.4 mg and **4,** 2.3 mg), together with the known spirotoamide (**1,** 13.7 mg). The ^{1}H and ^{13}C NMR spectra of **1**, **3** and **4** were obtained in CD_3OD (Table 1 and Supplementary Figures S1–S17), and their structures were elucidated on the basis of 1D and 2D NMR. Compound **1** was confirmed to be spirotoamide A by analysis of HRESIMS data and ^{1}H and ^{13}C NMR spectra (Supplementary Figures S1–S3), as well as comparison to the spectroscopic data reported previously.[1]

The molecular formula of **3** was deduced as $C_{26}H_{45}NO_6$ based on the HRESIMS spectrum that afforded an $[M+Na]^+$ ion at *m/z* 490.3138 (calculated $[M+Na]^+$ ion for $C_{26}H_{45}NO_6$ at *m/z* 490.3139) (Supplementary Figure S4). The overall structure of **3** was very similar to **1**, as it yielded a ^{1}H NMR spectrum that was almost identical to that of **1**, with the exception of the absence of the singlet 8-CH_3 signal.

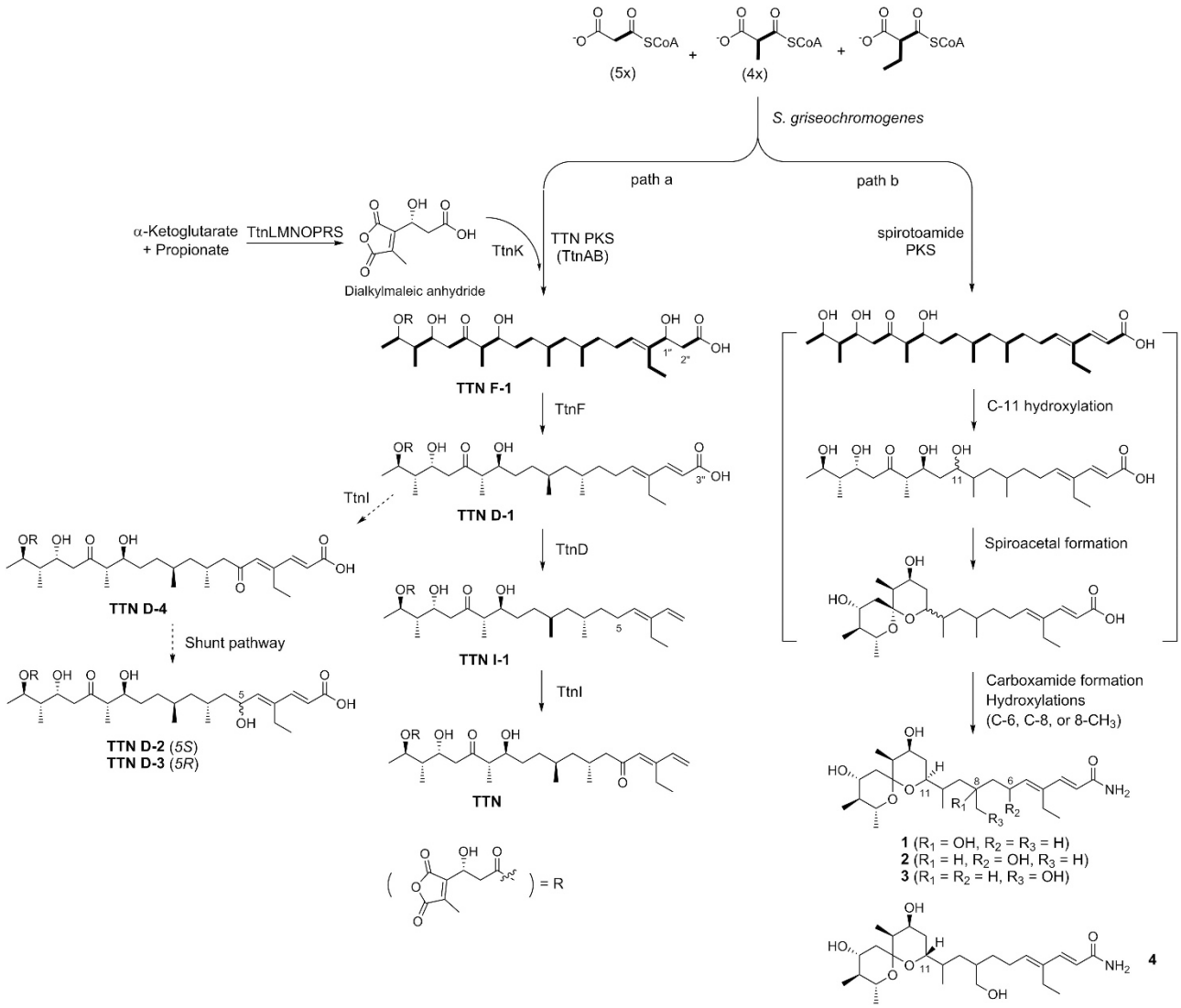

Figure 2 Biosynthetic parallels between TTN (path a) and spirotoamide (**1**-**4**) (path b) biosynthesis in *S. griseochromogenes*. The proposed pathway for TTN is based on extensive *in vivo* and *in vitro* experiments.[9,11,12] The proposed spirotoamide biosynthetic pathway is purely speculative, although a post-PKS, non-stereospecific oxidation step for the introduction of the –OH group at C-11 as depicted would account for the formation of all the spirotoamides isolated.

Instead, an extra 8-CH_2OH group was observed in **3** at δ_H 3.55 (1H, dd, $J = 11.0$, 3.4 Hz), δ_H 3.40 (1H, dd, $J = 11.0$, 5.4 Hz) and δ_C 65.2, indicating that the 8-CH_3 group in **1** might have been replaced by an 8-CH_2OH group in **3** (Supplementary Figures S5–6). This was unambiguously confirmed by HMBC correlations from the 8-CH_2OH at δ_H 3.55/3.40 to C-7 at δ_C 32.9 and C-9 at δ_C 35.3, respectively (Figure 1b and Supplementary Figure S8). The relative configurations of the ring system of **3** were determined to be the same as those of **1** by ROESY experiments and the spin-spin splitting pattern of the protons on the spiroketal moiety. Thus, H-12a at δ_H 1.37, H-13 at δ_H 4.27, and H-17 at δ_H 3.52 were all established as being in an axial position due to the observation of a large J^3-value (12.4 Hz). In the ROESY spectrum (Supplementary Figure S10), correlations were observed between H-13 and H-11 at δ_H 3.30, H-17 and H-19 at δ_H 3.28, 14-CH_3 at δ_H 0.88 and H-12a, and 14-CH_3 and H-16e at δ_H 2.09 (Figure 1b). These spectral data supported that both tetrahydropyran rings adopted a chair conformation and the spiroketal moiety of **3** shared the same relative configurations as those of **1**. The conjugated double bonds at δ^2 and δ^4 were both assigned as *E*-configurations by the ROESY correlations between H-3 at δ_H 7.10 and H-5 at δ_H 5.84, H-2 at δ_H 6.01 and H-2′ at δ_H 1.05, and H-2 and H-1′ at δ_H 2.33, which were also consistent with the large $J_{H\text{-}2,H\text{-}3}$ value (15.9 Hz) and the relatively high-field chemical shift of C-1′ at δ_C 20.8 (Table 1 and Supplementary Figure S10). These results led to the final structural assignment of **3** as shown in Figure 1a.

Compound **4** was assigned the same molecular formula of $C_{26}H_{45}NO_6$ as **3** on the basis of the $[M+Na]^+$ ion at *m/z* 490.3138 (calculated $[M+Na]^+$ ion for $C_{26}H_{45}NO_6$ at *m/z* 490.3139) (Supplementary Figure S11). It was apparent on the basis of the 1D and 2D NMR spectra, including ^1H-^1H COSY, HMBC and HSQC, that **4** had a nearly identical structure as **3** (Supplementary Figures S12-17). While the rest of the NMR data of **4** were almost identical to those of **3**, the resonance signals at positions C-8 to C-12, including the 10-CH_3, showed significant differences between the two compounds. In the ROESY experiment (Supplementary Figure S17), the correlations between H-10 at δ_H 1.56 and H-13 at δ_H 4.30, 14-CH_3 at

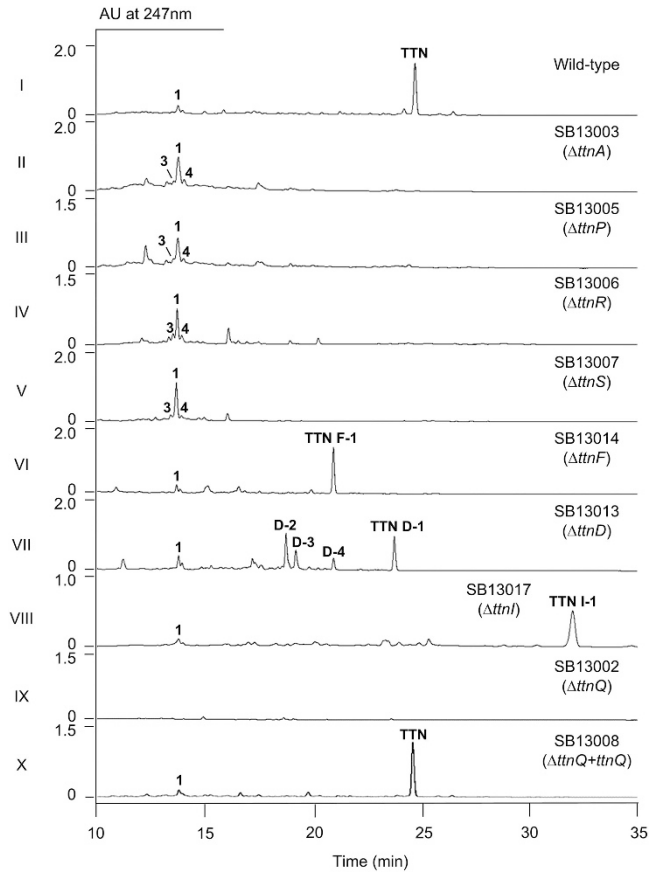

Figure 3 HPLC analysis of metabolite profiles from *S. griseochromogenes* wild-type and recombinant strains.

δ_H 0.89 and H-12a at δ_H 1.29, 14-CH_3 and H-16e at δ_H 2.08, and H-17 at δ_H 3.50 and H-19 at δ_H 3.35 indicated that **4** possessed a different relative configuration at C-11 than **1** and **3** (Figure 1b). Therefore, **4** was assigned to be the 11-epimer of **3** as shown in Figure 1a.

Spirotoamides A (**1**) and B (**2**) were reported to have no cytotoxic, antibacterial or antifungal activities.[1] We subjected the two new spirotoamides **3** and **4**, together with **1**, to antibacterial assays against the selected Gram-positive strains *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 23857 and *Mycobacterium smegmatis* ATCC607 and the Gram-negative *Escherichia coli* ATCC 25922, with tetracycline as the positive control. The assays were carried out by following previously reported methods and performed in the 96-well plates in duplicate with Müller-Hinton broth.[19,20] None of the compounds showed any activity at concentrations up to 100 μM.

In summary, we report here that (i) spirotoamides and TTN are biosynthesized in *S. griseochromogenes* by two distinct type I PKSs, competing for the same pool of acyl-CoA precursors, with TTN as the dominant metabolite under the fermentation conditions examined, (ii) the biosynthesis of spirotoamides and TTN are co-regulated by TtnQ, a transcriptional activator previously characterized from the *ttn* cluster, and cross-talk among pathway-specific regulators for secondary metabolite biosynthesis could potentially be exploited to activate cryptic gene clusters for the discovery of new natural products, and (iii) spirotoamide production can be significantly increased upon inactivation of the TTN biosynthetic machinery, leading to the isolation and structural elucidation of two new analogues, named spirotoamide C (**3**) and D (**4**), together with the known spirotoamide A, none of which, however, showed any antibacterial activity against the selected Gram-positive and Gram-negative bacteria.

Table 1 ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data for spirotoamides C (**3**) and D (**4**) in CD_3OD[a]

	3		***4***	
Position	δ_C	δ_H *(mult, J in Hz)*	δ_C	δ_H *(mult, J in Hz)*
1	171.8, s	—	171.8, s	—
2	118.3, d	6.01, d (15.9)	118.3, d	6.01, d (15.9)
3	146.4, d	7.10, d (15.9)	146.4, d	7.10, d (15.9)
4	140.3, s	—	140.2, s	—
5	141.7, d	5.84, t (7.6)	141.8, d	5.83, t (7.5)
6	26.8, t	2.27, m	27.0, t	2.26, m
7	32.9, t	1.63, m	32.1, t	1.54, m
		1.34, m		1.48, m
8	39.1, d	1.59, m	44.2, d	1.55, m
9	35.3, t	1.58, m	38.5, t	1.45, m
		1.02, m		1.33, m
10	36.4, d	1.65, m	31.9, d	1.56, m
11	73.7, d	3.30, m	69.4, d	3.72, m
12[b]	31.3, t	12e, 1.55, dd (12.0, 4.8)	35.3, t	12e, 1.32, m
		12a, 1.37, dd (12.4,12.0)		12a, 1.29, m
13	67.1, d	4.27, dt (12.4, 4.8)	67.1, d	4.30, td (11.7, 4.8)
14	43.6, d	1.84, m	43.6, d	1.83, m
15	102.2, s	—	102.2, s	—
16[b]	43.3, t	16e, 2.09, dd (12.4, 4.8)	43.3, t	16e, 2.08, dd (12.4, 4.8)
		16a, 1.22, brt (12.4)		16a, 1.21, brt (12.4)
17	70.8, d	3.52, td (12.4, 4.8)	70.9, d	3.50, ddd (10.8, 10.8, 4.8)
18	46.4, d	1.12, m	46.4, d	1.10, m
19	71.2, d	3.28, m	71.1, d	3.35, m
1'	20.8, t	2.33, q (7.9)	20.7, t	2.32, q (7.7)
2'	14.0, q	1.05, t (7.9)	14.0, q	1.05, t (7.7)
8-CH_2OH	65.2, t	3.55, dd (11.0, 3.4)	62.8, t	3.57, dd (10.5, 5.9)
		3.40, dd (11.0, 5.4)		3.54, dd (10.5, 4.9)
10-CH_3	16.3, q	0.92, d (6.9)	20.0, q	0.93, d (6.7)
14-CH_3	7.4, q	0.88, d (7.2)	7.4, q	0.89, d (7.2)
18-CH_3	13.5, q	0.96, d (6.6)	13.5, q	0.96, d (6.6)
19-CH_3	19.5, q	1.16, d (6.5)	19.5, q	1.15, d (6.5)

[a]^{1}H and ^{13}C assignments were based on COSY, HSQC and HMBC spectra, as well as in comparison with the assignments for spirotoamide A (**1**) and B (**2**) reported previously.[1]
[b]H-12a and H-12e or H-16a and H-16e referred to the axial or equatorial Hs at C-12 or C-16, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Nogawa, T. *et al.* Spirotoamides A and B, novel 6,6-spiroacetal polyketides isolated from a microbial metabolite fraction library. *J. Antibiot.* **65**, 123–128 (2012).
2 Takahashi, S. *et al.* Reveromycin A biosynthesis uses RevG and RevJ for stereospecific spiroacetal formation. *Nat. Chem. Biol.* **7**, 461–468 (2011).
3 Cheng, X. C. *et al.* A new antibiotic, tautomycetin. *J. Antibiot.* **42**, 141–144 (1989).
4 Cheng, X. C., Ubukata, M. & Isono, K. The structure of tautomycetin, a dialkylmaleic anhydride antibiotic. *J. Antibiot.* **43**, 890–896 (1990).
5 Mitsuhashi, S. *et al.* Tautomycetin is a novel and specific inhibitor of serine/threonine protein phosphatase type 1, PP1. *Biochem. Biophys. Res. Commun.* **287**, 328–331 (2001).
6 Oikawa, H. Synthesis of specific protein phosphatase inhibitors, tautomycin and tautomycetin toward structure-activity relationship study. *Curr. Med. Chem.* **9**, 2033–2054 (2002).
7 Chen, X., Zheng, Y. & Shen, Y. Natural products with maleic anhydride structure: nonadrides, tautomycin, chaetomellic anhydride, and other compounds. *Chem. Rev.* **107**, 1777–1830 (2007).
8 Choi, S., Hur, Y., Sherman, D. H. & Kim, E. Isolation of the biosynthetic gene cluster for tautomycetin, a linear polyketide T cell-specific immunomodulator from *Streptomyces* sp. CK4412. *Microbiology* **153**, 1095–1102 (2007).

9 Li, W. *et al.* Characterization of the tautomycetin biosynthetic gene cluster from *Streptomyces griseochromogenes* provides new insight into dialkylmaleic anhydride biosynthesis. *J. Nat. Prod.* **72**, 450–459 (2009).
10 Ubukata, M., Cheng, X., Uzawa, J. & Isono, K. Biosynthesis of the dialkylmaleic anhydride-containing antibiotics, tautomycin and tautomycetin. *J. Chem. Soc., Perkin Trans. 1* **19**, 2399–2404 (1995).
11 Luo, Y. *et al.* Functional characterization of *ttnD* and *ttnF*, unveiling new insights into tautomycetin biosynthesis. *J. Am. Chem. Soc.* **132**, 6663–6671 (2010).
12 Yang, D., Li, W., Huang, S.-X. & Shen, B. Functional characterization of *ttnI* completing the tailoring steps for tautomycetin biosynthesis in *Streptomyces griseochromogenes*. *Org. Lett.* **14**, 1302–1305 (2012).
13 Nah, J. *et al.* Identification and characterization of wblA-dependent *tmcT* regulation during tautomycetin biosynthesis in *Streptomyces* sp. CK4412. *Biotechnol. Adv.* **30**, 202–209 (2012).
14 Huang, J. *et al.* Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. *Mol. Microbiol.* **58**, 1276–1287 (2005).
15 Vicente, C. M. *et al.* Pathway-specific regulation revisited: cross-regulation of multiple disparate gene clusters by PAS-LuxR transcriptional regulators. *Appl. Microbiol. Biotechnol.* **99**, 5123–5135 (2015).
16 McKenzie, N. L. *et al.* Induction of antimicrobial activities in heterologous streptomycetes using alleles of the *Streptomyces coelicolor* gene *absA1*. *J. Antibiot.* **63**, 177–182 (2010).
17 Gao, C., Hindra, Mulder, D., Yin, C. & Elliot, M. A. Crp is a global regulator of antibiotic production in *Streptomyces*. *mBio* **3**, e00407–e00412 (2012).
18 Rutledge, P. J. & Challis, G. L. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat. Rev. Microbiol.* **13**, 509–523 (2015).
19 Ma, M. *et al.* Angucyclines and angucyclinones featuring C-ring cleavage and expansion from *Streptomyces* sp. CB01913. *J. Nat. Prod.* **78**, 2471–2480 (2015).
20 Wiegand, I., Hilpert, K. & Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protocols* **3**, 163–175 (2008).

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