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Chain Elongation, Macrolactonization, and Hydrolysis of Natural and Reduced Hexaketide Substrates by the Picromycin/Methymycin Polyketide Synthase**

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Macrolide polyketides are a structurally diverse group of natural products, widely used for their pharmaceutically important antimicrobial, antitumor, and immunosuppressant properties.[1] The picromycin/methymycin synthase (PICS) of *Streptomyces venezuelae*, which is responsible for the formation of the 12- and 14-membered-ring antibiotics methymycin and picromycin,[2,3] is a typical modular polyketide synthase (PKS), in which each module is responsible for a single round of polyketide-chain elongation and functional-group modification.[4–6] Each module is in turn made up of a specific combination of fatty acid synthase-like domains that catalyze the individual biochemical steps of polyketide biosynthesis.

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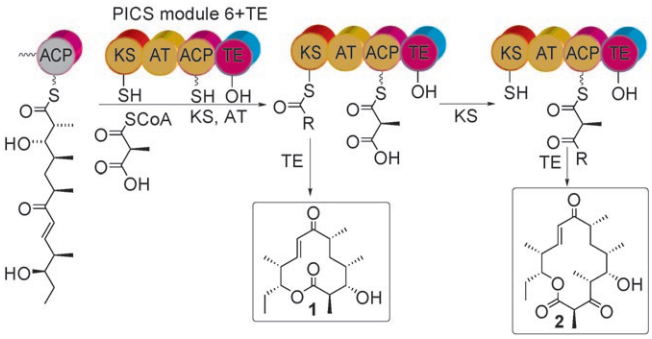
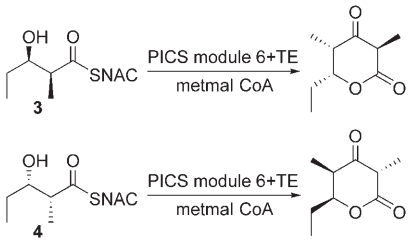
PICS module 6, a homodimer of subunit $M_w = 142$ kDa encoded by *pikAIV*, is organizationally one of the simplest known PKS modules, as it only consists of a ketosynthase (KS), a methylmalonyl transferase (AT), and an acyl carrier protein (ACP) domain as well as a C-terminal thioesterase (TE; Scheme 1). In spite of this apparent structural simplicity, PICS module 6(+TE) is one of the catalytically most intriguing PKS modules, on the basis of its demonstrated role in the formation of both 10-deoxymethynolide (**1**)[7] and narbonolide (**2**), the parent macrolide agylcones of methymycin and picromycin, respectively.[4,6,8–10]



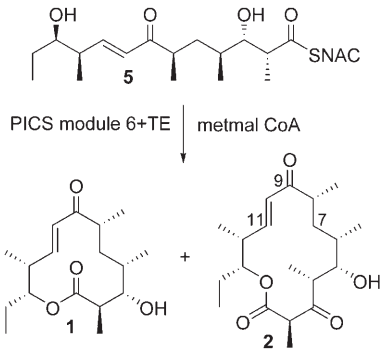
Scheme 1. Conversion of hexaketide into 10-deoxymethynolide (**1**) and narbonolide (**2**) by PICS module 6+TE.

We have reported the expression and purification of recombinant PICS module 6+TE from *Escherichia coli* and have investigated its relative substrate specificity by using *N*-acetylcysteamine (SNAC) thioesters of 2-methyl-3-hydroxypentanoic acid as diketide analogues of the natural hexaketide chain-elongation substrate.[11] It was found that PICS module 6+TE processed both *syn*-diketide diastereomers (2*S*,3*R*)-**3** and (2*R*,3*S*)-**4** with a modest 2.5:1 preference in k_{cat}/K_m for **4**, but did not process either of the two *anti* diketides (Scheme 2). Similar findings were reported independently by Sherman and co-workers,[9] who also described extensive in vivo and in vitro investigations of the biochemical basis for the competition between elongation of the natural hexaketide substrate by PICS module 6 to give **2** and direct cyclization to **1**, with the final macrolactonization being controlled in both cases by the C-terminal TE domain.[8,12] Although active KS6, AT6, and ACP6 domains were found to be essential for the formation of **1**, the precise role of these



Scheme 2. Conversion of diketide-SNAC analogues into triketide ketolactones by PICS module 6+TE. metmal CoA = methylmalonyl coenzyme A.

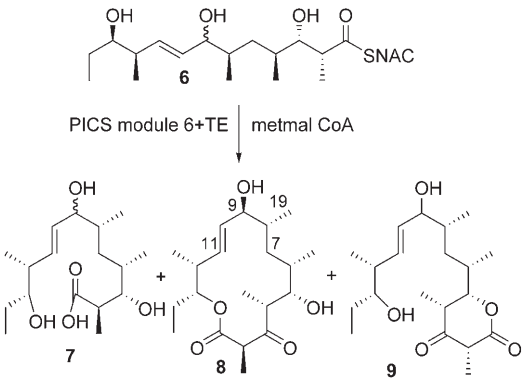
domains in the delivery of the hexaketide substrate to the active site of the TE domain remains to be established. Investigations, both by us and Sherman and co-workers, have recently established that recombinant PICS TE alone can efficiently catalyze the macrolactonization of synthetic *seco*-hexaketide acyl SNAC **5** to **1** while catalyzing the exclusive hydrolysis of the corresponding 7-dihydro-*seco*-SNAC thioester **6**.[13,14] Most recently, Sherman also reported that PICS module 6+TE can convert the *seco*-SNAC thioester **5** and methylmalonyl coenzyme A (CoA) into **2**, although they did not monitor the competing direct lactonization to **1** and reported only a
k_{cat}/K_m value for the chain elongation and the lactonization reaction.[15] We now report that in the presence of the cosubstrate methylmalonyl CoA PICS module 6+TE catalyzes competing macrolactonization and chain elongation of **5**, which is converted into a 4:1 mixture of **1** and **2** (Scheme 3).



Scheme 3. Conversion of hexaketide *seco*-SNAC thioester **5** into **1** and **2** by PICS module 6+TE.

PICS module 6+TE is also shown to convert **6** and methylmalonyl CoA into a mixture of the direct hydrolysis product **7** and the isomeric chain-extended heptaketide lactones **8** and **9** (Scheme 4).

The requisite substrates **5** and **6** were each prepared by degradation and semisynthesis from **1**, as previously described.[13] The *seco*-SNAC thioester **5** is in equilibrium with approximately 50–60% of the corresponding 3,7-hemiacetal



Scheme 4. Hydrolysis and lactonization of 7-dihydro-*seco*-SNAC-thioester **6** by PICS module 6+TE.

in $CDCl_3$.[13,15] Incubation of **5** with recombinant PICS module 6+TE and [2-^{14}C]methylmalonyl CoA produced a 4:1 mixture of **1** and **2**, as monitored by RP-HPLC with concatenated inline UV/Vis and flow-scintillation detection (Scheme 3). The identities of the enzymatic reaction products were confirmed by carrying out a preparative-scale reaction over 0.5 hours with **5** (2 mM) and methylmalonyl CoA (2 mM) in the presence of PICS module 6+TE (13.2 μM).[16] The resulting macrolide aglycones, purified by preparative RP-HPLC, displayed ^{1}H NMR and HR-ESI mass spectra that were identical to authentic samples of **1** as well as literature values for **1** and **2**.[17] The steady-state kinetic parameters for the formation of both **1** and **2** were determined by carrying out a series of incubations at concentrations of **5** that ranged from 0.5 to 4.0 mM, with the yields of **1** and **2** determined by HPLC/diode-array UV (230 nm) and phosphorimaging with TLC, respectively. The corresponding k_{cat} and K_m values were calculated by direct fitting of the plots of v versus [S] to the Michaelis–Menten equation (Table 1).[17] The relative values

Table 1: Steady-state kinetic parameters for PICS module 6+TE.

Substrate	Product	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M [M^{-1} s^{-1}]
5	**1**	17.9±1.0	3.3±0.3	91.5±10.6
		43.0±5.2[a]	3.2±0.7[a]	223.3±56.7[a]
	2	4.4±0.7	5.2±1.5	14.1±4.7
6	**7**	6.9±0.7	2.1±0.5	64.7±14.3
	8	1.2±0.1	2.1±0.4	9.5±2.0
	9	6.8±1.2	2.6±0.9	43.6±17.3

[a] Formation of **1** in the absence of methylmalonyl CoA.

of k_{cat} showed the expected 4:1 preference for the formation of the macrolactonization product **1** over chain extension and cyclization to **2**. The observed K_m values were not corrected for the presence of the corresponding hemiacetal form of **5**, as the concentration of this species in the reaction buffer was not determined and all of the substrate was consumed in the preparative-scale reaction. Interestingly, incubation of **5** with PICS module 6+TE under identical conditions but in the absence of methylmalonyl CoA gave exclusively **1**, but with approximate 2.2-fold increases in both k_{cat} and k_{cat}/K_m values relative to the cyclization to **1** in the presence of the methylmalonyl CoA cosubstrate.

We previously reported that incubation of **6** (7*S*/7*R* 3:1) with the recombinant PICS TE alone results exclusively in hydrolysis to the corresponding diastereomeric mixture of *seco* acids **7**.[13,14] By contrast, preparative-scale incubation of **6** (2 mM) and methylmalonyl CoA (1 mM) with PICS module 6+TE (10 μM) gave **7**, **8**, and **9** in a 6:3:4 mixture that could be readily separated by flash chromatography (Scheme 4). The NMR spectroscopic and chromatographic properties of **7** were identical to those previously determined.[13] The structure and stereochemistry of **8** and **9** were determined by ^{1}H and ^{13}C NMR spectroscopy, including HMQC and NOESY analysis.[17] Both compounds exhibited HR-ESI mass spectra consistent with the molecular composition $C_{20}H_{34}O_5$ expected for the isomeric lactones. The

^{1}H NMR spectrum of **8** closely resembled that of **2**, with several key differences. Thus, the two olefinic protons exhibited the expected upfield shift and change in multiplicity relative to the enone double bond of **2**: H10 ($\delta = 5.34$ ppm, dd, $J = 15.4$, 7.8 Hz) coupled to H11 ($\delta = 5.42$ ppm, dd, $J = 15.4$, 7.7 Hz), with similar changes in the ^{13}C NMR spectrum (C10 $\delta = 134.1$; C11 133.6 ppm). The presence of only a single resonance for allylic H9 in both the ^{1}H NMR ($\delta = 3.47$ ppm, t, $J = 8.5$ Hz—correlated to both H10 and H8 in the ^{1}H COSY spectrum) and ^{13}C NMR spectra ($\delta = 78.8$ ppm) is consistent with the formation of a single diastereomer of **8**. The configuration of **8** was readily assigned as the 9*S* isomer, as based on the NOESY spectrum, which showed crosspeaks between H9 and the H19 methyl protons ($\delta = 1.03$ ppm) as well as between H9 and both the olefinic H11 proton ($\delta = 5.42$ ppm) and one of the H7 methylene protons ($\delta = 0.74$ ppm), but not with either H8 or H10 (Figure 1). Compound **9** was assigned the isomeric δ-lactone structure based on comparison with the spectra of **8**. Thus, the ^{13}C NMR resonance for the lactonic C5 ($\delta = 82.4$ ppm) appeared downfield of the corresponding signal for C5 in **8** ($\delta = 71.7$ ppm), with the expected differences in the attached H5 protons as well (**9**: $\delta = 4.32$ ppm, dd, $J = 9.8$, 2.0 Hz; **8**: $\delta = 4.02$ ppm, dd, $J = 5.4$, 2.4 Hz). The signals for C13 (**9**: $\delta = 76.8$; **8**: 80.1 ppm) and H13 (**9**: $\delta = 3.44$ ppm, td, $J = 8.4$, 3.5 Hz; **8**: $\delta = 4.77$ ppm, td, $J = 10.3$, 3.1 Hz) also showed the expected complementary changes in chemical shift. Although **9** is most likely a single diastereomer (H9 $\delta = 3.44$ ppm, t, $J = 5.1$ Hz; C9 $\delta = 75.9$ ppm), the configuration at C9 was not assigned. The steady-state kinetic parameters for the formation of **8** and **9** (lactonization) as well as **7** (hydrolysis) were also determined (Table 1).

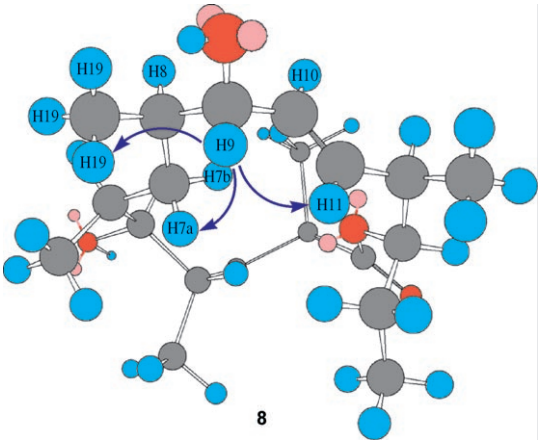


Figure 1. Key NOESY correlations for (9*S*)-9-dihydronarbonolide (**8**). (Energy-minimized conformation calculated by using the MM2 module of Chem3D.)

The observed k_{cat} and k_{cat}/K_m values for chain-elongation/cyclization of **5** to **2** (Table 1) are nearly two orders of magnitude greater than the corresponding steady-state parameters previously determined for the diketide analogue (2*R*,3*S*)-**4** ($k_{cat} = 0.057 \pm 0.003$ min^{-1} and $k_{cat}/K_m = 0.027$ M^{-1} s^{-1}); some of the difference in the k_{cat}/K_m values rises from an approximate tenfold decrease in the K_m value relative to (2*R*,3*S*)-**4** (35 ± 4 mM).[9,11] It would be expected that the natural hexaketide intermediate, which is delivered to PICS module 6 as the corresponding acyl thioester bound to the ACP of PICS module 5, would have an even lower native K_m value.[18] Although both the KS6 or ACP6 domains have been reported to be essential for in vivo macrolactonization of the hexaketide produced by PICS module 5,[8] it is not clear whether the observed dominant macrolactonization of **5** by PICS module 6 + TE involves mandatory transfer to the TE domain by way of transient covalent attachment to either the KS6 or ACP6 domains. Irreversible formation of the acyl-KS species has been shown to be the sole determinant of the k_{cat}/K_m value for the processing of substrates by PKS modules.[19] The fact that the k_{cat}/K_m values measured for lactonization and for chain-elongation of **5** are different may suggest that these two reactions do not involve partitioning of a common hexaketide KS intermediate. Indeed, recombinant PICS TE alone catalyzes the efficient conversion of **5** into **1**.[13,14] Interestingly, the observed steady-state kinetic parameters for the conversion of **5** into **1** by PICS module 6 + TE in the absence of methylmalonyl CoA are effectively the same as those that we previously determined for the macrolactonization of **5** by recombinant PICS TE ($k_{cat} = 54.4 \pm 5.8$ min^{-1}, $K_m = 4.1 \pm 0.8$ mM, $k_{cat}/K_m = 221 \pm 50$ M^{-1} s^{-1}).[13] The k_{cat} value for the formation of **1** by PICS module 6 + TE in the absence of methylmalonyl CoA is larger than the combined k_{cat} value for both the lactonization and chain elongation by the PICS module 6 + TE in the presence of methylmalonyl CoA, thus suggesting that the presence of a methylmalonyl thioester attached to the ACP of module 6 may inhibit access of the hexaketide substrate to the active site of the downstream TE domain. Alternatively, it may reflect slower cyclization of the heptaketide substrate than of the hexaketide substrate by the TE domain.

The chain elongation/lactonization of **6** into **8** and **9** by PICS module 6 + TE also takes place at rates comparable to those observed for the processing of **5**, the analogue of the natural hexaketide substrate. Interestingly, although a mixture of isomeric lactones is observed, chain elongation appears to be diastereoselective. The formation of the two lactones from **6** is in contrast to the exclusive hydrolysis of the same substrate by recombinant PICS TE alone,[13,14] thus indicating that both the PICS KS6 domain and the TE domain can process reduced hexaketide and heptaketide subtrates, respectively.

In summary, we have shown that recombinant PICS module 6 + TE is capable of mediating both chain elongation and lactonization as well as competing lactonization or hydrolysis of both the natural hexaketide substrate **5** and the reduced 7-dihydro analogue **6** and have established the full set of steady-state kinetic parameters for both sets of reactions. The observed 4:1 ratio of lactonization to chain elongation for the processing of **5** may represent the intrinsic ratio of these two processes that leads to the characteristic formation of 12- and 14-membered-ring macrolides, thereby definitively excluding the necessity for N-terminal truncation

of PICS module 6 for formation of the hexaketide macrolactonization product 10-deoxymethynolide (**1**), as has been suggested at one time.[12]

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[17] See the Supporting Information for details of preparative and kinetic experiments, as well as complete spectroscopic data for new compounds.
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