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Mapping the Mechanism of the Resorcinol Ring Formation Catalyzed by ArsB, a Type III Polyketide Synthase from *Azotobacter vinelandii*

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Polyketides, produced by polyketide synthases (PKSs), are a large class of secondary metabolites widely found in bacteria, fungi, plants, and marine animals. Polyketide-based drugs include antibiotics, immunosuppressants, antiparasitics, and cholesterol-lowering, anticancer and antioxidant agents. PKSs are classified into three types according to their architecture. Type I PKSs are large, modular, multidomain enzymes, and type II PKSs are dissociable multienzyme complexes. In type I and II PKSs, each domain or enzyme typically performs a discrete function.[1] In contrast, type III PKSs—homodimers comprising subunits of about 45 kDa—are multifunctional.[2] Each subunit iteratively condenses a starter acyl-CoA substrate with a number of acetate units derived from malonyl-CoA (**2**, Scheme 1), and cyclizes the linear polyketide intermediate to produce polyketides with distinct ring structures.

Although structurally simpler than type I and II enzymes, type III PKSs also produce a diverse array of polyketide products. The diversity comes from the choice of the starter CoA substrate, the number of condensation steps, and the cyclization mechanism. ArsB and ArsC from *Azotobacter vinelandii* utilize long-chain fatty acyl-CoA esters (**1a**),[3] whereas chalcone synthase (CHS) and stilbene synthase (STS), the two most studied plant type III PKSs, use *p*-coumaroyl-CoA or cinnamoyl-CoA (**1b**) as the starter substrate.[4,5] Although the number of decarboxylative condensations catalyzed by type III PKSs varies from one[6] to seven,[7] the majority of type III PKSs catalyze three condensation reactions to give triketo CoA thioester intermediates (commonly called tetraketide intermediates, **3**) that can be cyclized into different six-membered ring structures (Scheme 1).[8] CHS catalyzes the C-6→C-1 Claisen acylation to give a phloroglucinol derivative (**4**), whereas ArsC catalyzes O-acylation to produce 2′-oxoalkyl-α-pyrones (**5**). In these two cyclization reactions, CoA serves as a leaving group. On the other hand, alkylresorcylic acid synthase (ARAS) connects the C-2 methylene carbon with the C-7 carbonyl carbon through an aldol condensation and also hydrolyzes the CoA thioester to afford 6-alkyl-β-resorcylic acid (**6**).[9] STS and ArsB also catalyze an aldol cyclization, but instead produce 5-substituted resorcinols (**7**; Scheme 1).

The resorcinol ring formation catalyzed by STS and ArsB involves aldol cyclization, hydrolysis of the thioester, decarboxylation, dehydration and aromatization. However, the sequence of these reactions remains unresolved. As shown in Scheme 2, the triketo CoA thioester (**3**), produced after three condensation reactions could be converted to the final 5-substituted resorcinol (**7**) through several different pathways. Resorcinol formation can either begin with hydrolysis of the thioester bond in **3** to give the triketocarboxylate **8** (pathway A), or begin with aldol cyclization of **3** to give the cyclized thioester **9** (pathway B). In the "hydrolysis first" pathway A, aldol cyclization of **8** can occur concomitantly with decarboxylation (pathway A1) to give **10**. Alternatively, the aldol cyclization of **8** can occur first (pathway A2) to give an equilibrium mixture of **11**, **12**, and possibly the cyclized dianion **13**.[10] The cyclization might then be followed by sequential β-keto decarboxylation and dehydration (pathway A2.1) or by coupled decarboxylation/dehydration (pathway A2.2) to give the final product **7**. Alternatively, the nonaromatic cyclized compound (**11**, **12**, or **13**) might undergo dehydration and aromatization to give the substituted β-resorcylic acid **16**, which is then decarboxylated to **7**. For the "aldol first" pathway B, the cyclized thioester **9** can either be hydrolyzed to give the same equilibrium mixture of **11**, **12**, and **13** (pathway B1), or **9** can undergo dehydration and aromatization to the β-resorcylic thioester **14**, which then enters into a hydrolysis/decarboxylation sequence to form **7** (pathway B2).

A few studies have addressed the mechanism of STS-catalyzed resorcinol ring formation. In an elegant study using deuterated malonyl-CoA and mass spectrometry, Shibuya et al.[11] demonstrated that stilbenecarboxylate (**16b**) is not an intermediate in the STS-catalyzed resorcinol ring formation and proposed that thioester hydrolysis precedes aldol cyclization and decarboxylation (pathway A). Funa et al.[3] suggested that **16a** is not an intermediate in the ArsB-catalyzed resorcinol ring formation, given that **16a** was not detected in the reaction mixture of ArsB. A parallel observation was made with STS and **16b** by Li et al.[12] Meanwhile, Austin et al.[5] proposed pathway A2.2 (coupled decarboxylation/dehydration of **12**) as the most likely mechanism for STS-catalyzed resorcinol ring formation, based on solution chemistry of biomimetic polyketide cyclization[13] and a computer-assisted docking study. However, direct evidence for the "hydrolysis first" hypothesis has been lacking. One way to elucidate the mechanism of a multistep enzymatic reaction is to examine the putative reaction intermediates. In this study, we prepared a linear triketocarboxylate 3,5,7-trioxoeicosanoic acid (C_{20}-TKA, **8a**) and incubated it with ArsB to determine the first step of the ArsB-catalyzed resorcinol ring formation.

Diketo acids and their dipotassium salts have commonly been synthesized by treating the methyl ester with ethanolic KOH.[14,15] Attempts to use the same strategy to synthesize **8a** were unsuccessful, as the methyl ester of **8a** was unstable and rapidly aldol-cyclized under the basic conditions.[10] Instead, **8a**

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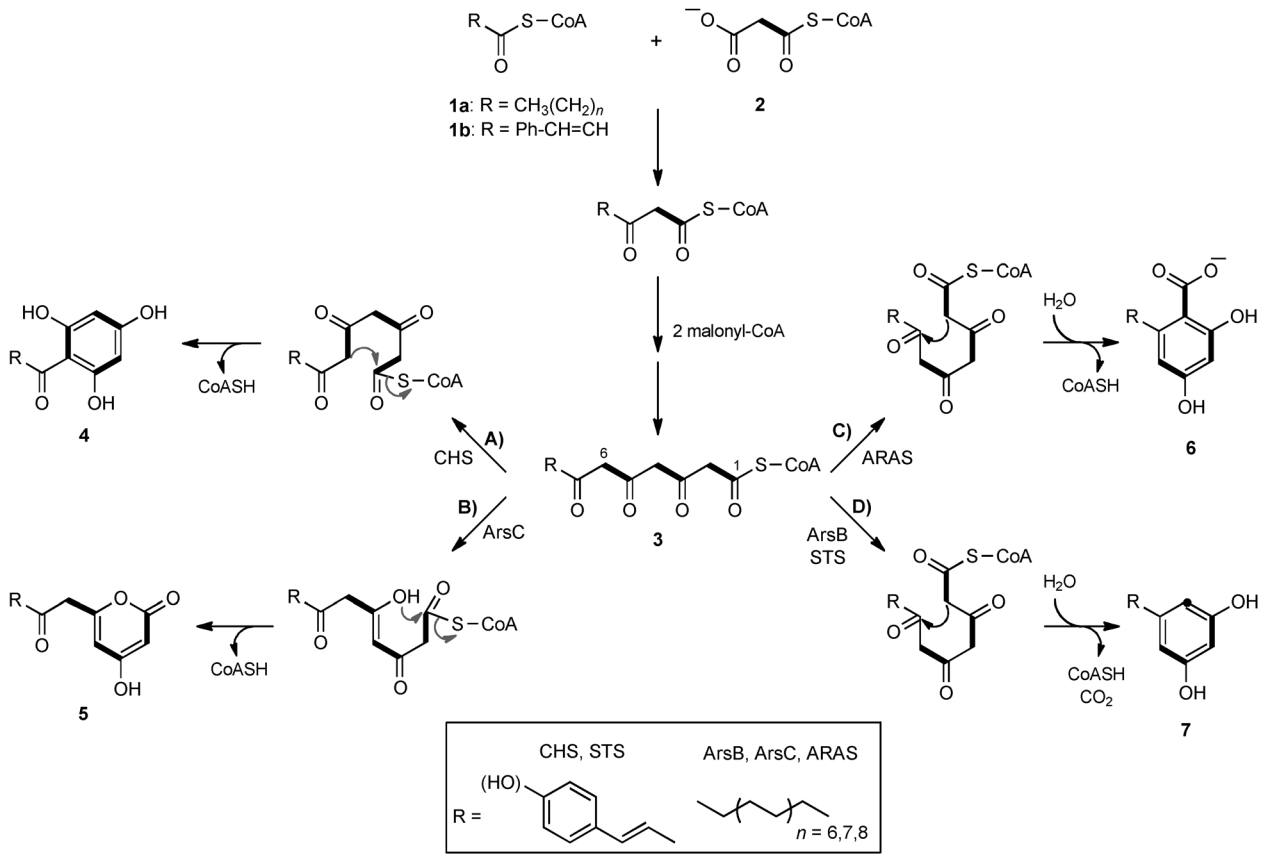


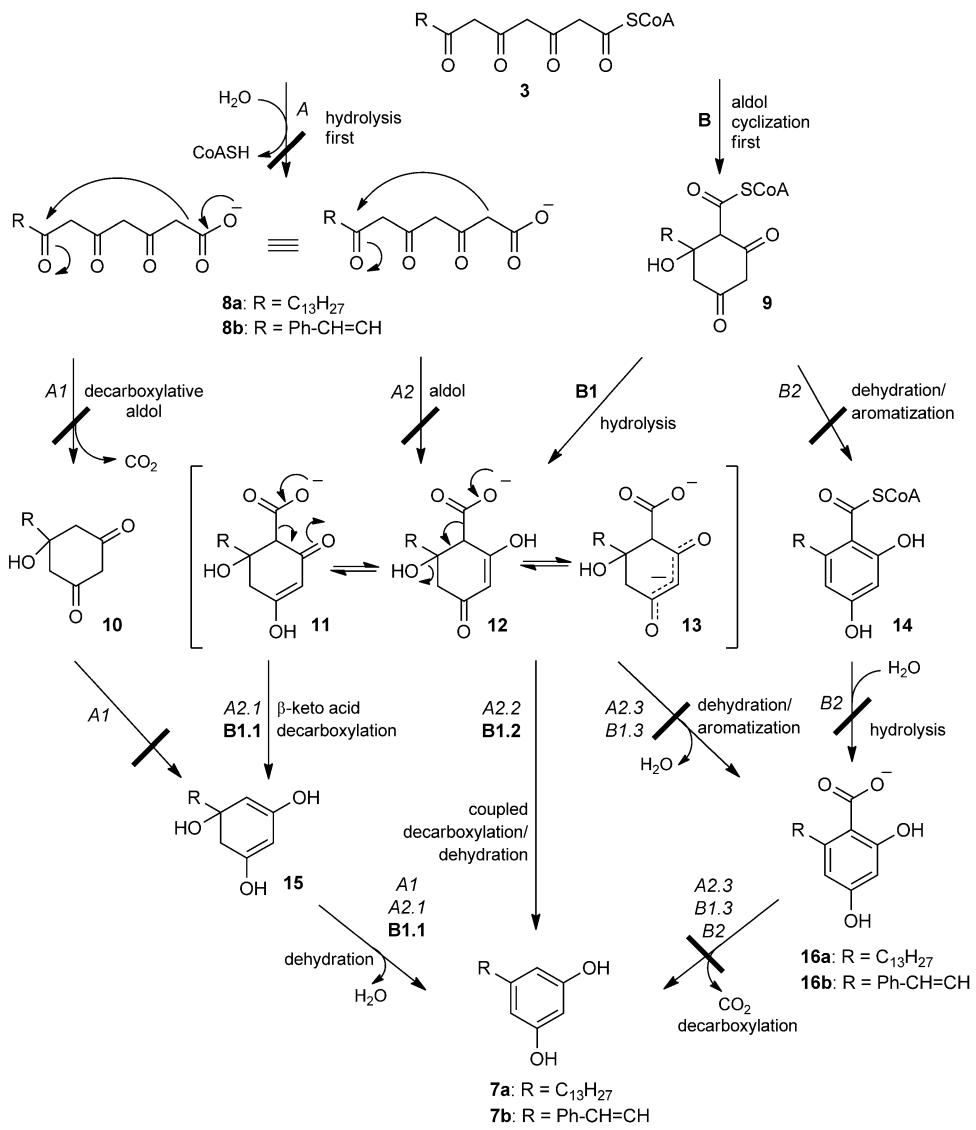
Scheme 1. Different cyclization reactions catalyzed by type III polyketide synthases.

was prepared by treatment of *tert*-butyl 3,5,7-trioxoeicosanoate with TFA (Supporting Information). The formation of **8a** was confirmed by the absence of the *t*Bu signal at 1.46 ppm in ^{1}H NMR spectrum. The *t*Bu ester derivative of **8a** proved to be more stable and did not cyclize as rapidly as the methyl ester counterpart. This is likely due to steric hindrance of the attack of C-2 on C-7 provided by the bulky *t*Bu group in the intramolecular aldol reaction step. Compound **8a** was found to be unstable in solution at 4 °C, but was stable for at least four weeks when stored as a solid at −20 °C. It was reasonably assumed that **8a** is cyclized to 6-tridecyl-β-resorcylic acid (**16a**; see below) or decarboxylated to the triketone, as these reactions are known to occur spontaneously.[16] Fast Blue salts are commonly used for the visualization of phenolic lipids including alkylresorcinols.[17] We found that **8a**, but not the diketo compounds, was stained with Fast Blue B salt (0.1% (*w*/*v*) in water) to give a reddish brown color that faded to beige/orange over time. This offered a convenient way to detect **8a** by TLC (Figure S1 in the Supporting Information).

First, the stability of **8a** in the HPLC mobile phase (acetonitrile/H_2O/acetic acid 8:2:0.01, *v*/*v*/*v*) and in the enzyme reaction buffer (0.1 M potassium phosphate (KPi), pH 7.8) was investigated. Freshly prepared **8a** remained relatively stable after incubation in the mobile phase for 6 h, thus validating HPLC as the analytical method of choice (Figure S2). After prolonged incubation (22 h), the conversion of **8a** to two other compounds was almost complete. The compound that eluted at t_R = 9.2 min exhibited chromatographic and spectroscopic parameters characteristic of an alkylresorcinol: UV (methanol): λ_{max} = 276, 282 nm;[18] stained violet with Fast Blue B salt (Figure S1); ^{1}H NMR ($CDCl_3$): δ = 2.49 (t, 2H), 6.17 (t, 1H), 6.24 ppm (d, 2H). Thus, the compound with t_R = 9.2 min was identified as 5-tridecylresorcinol (C_{13}-RL, **7a**). The compound that eluted at t_R = 10.3 min exhibited diagnostic parameters of an alkyl-β-resorcylic acid: UV (methanol): λ_{max} = 216, 258, 298 nm;[19] stained violet with Fast Blue B salt (Figure S1); ^{1}H NMR: δ = 2.90 (t, 2H), 6.25 (d, 1H), 6.28 ppm (d, 1H). Therefore, this compound was determined to be 6-tridecyl-β-resorcylic acid (C_{13}-RA, **16a**). An uncharacterized component was also detected at t_R = 11.3 min (marked with an asterisk in Figures 1 and S2); this component had a UV λ_{max} of 266 nm, and was not stained with Fast Blue B salt, unlike other polyketide-derived compounds.

When incubated in pH 7.8 buffer, **8a** could no longer be detected after 4 h (Figure 1A). Instead, **16a** was formed, and was detected typically within 10 min of incubation. Decarboxylation of **16a** to **7a** was extremely slow under these conditions, and only after 48 h incubation a small amount of **7a** was detected. The sequential conversion of **8a** to **16a** to **7a** was evident when the peak areas of the three compounds were plotted against incubation time (Figure S3), in agreement with the known solution chemistry at neutral pH.[10,16]

Next, the possible intermediacy of **8a** in ArsB-catalyzed resorcinol ring formation was examined by incubating **8a** in the presence of ArsB (200 μg). ArsB failed to convert **8a** to **7a** (Fig-



Scheme 2. Possible mechanisms of resorcinol ring formation. The pathways that are eliminated by the results obtained in this study are cross-checked and indicated in italics, the remaining candidate pathways are indicated in bold.

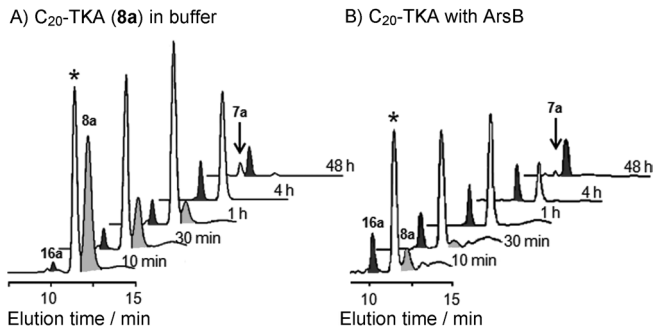


Figure 1. Time-course HPLC analysis of the reaction mixture of C_{20}-TKA (**8a**) in the A) absence and B) presence of ArsB. In all chromatograms, 6-tridecyl-β-resorcylic acid (C_{13}-RA, **16a**) is dark-shaded, and C_{20}-TKA (**8a**) is light-shaded. 5-Tridecylresorcinol (C_{13}-RL, **7a**) is indicated with an arrow, the unidentified contaminant is indicated with an asterisk.

ure 1B). The amounts of **7a** formed after 48 h of incubation in the absence or presence of ArsB were similar, thus indicating that **7a** was formed by nonenzymatic decarboxylation of **16a**, even in the presence of ArsB. Interestingly, the initial rate of the aldol cyclization and aromatization of **8a** to **16a** was enhanced by ArsB (Figure 1). This rate enhancement was also observed with ArsC, a type III PKS that does not produce alkylresorcinols but produces alkylpyrones (Scheme 1).[3] During the initial 10 min of incubation, both ArsB and ArsC facilitated the formation of **16a** from **8a** by 13-fold (Figure 2A, left). Furthermore, both enzymes appeared to stabilize **16a** and hindered the decarboxylation of **16a** to **7a**; less **7a** was formed in the presence of either enzyme after prolonged incubation (12 h; Figure 2A, right). The identical effects of ArsB and ArsC on the conversion of **8a** to **16a** to **7a** strongly suggested that these effects are not due to the catalytic activity of ArsB. It is likely that ArsB and ArsC provided a favorable environment, presumably at the acyl binding site, that was conducive to an entropically favored aldol cyclization of **8a** (cage effect). The aldol cyclization occurs spontaneously in the presence of ArsB or ArsC, but, as the results indicate, relatively faster than in solution. Observations that both denatured ArsB (Figure 2B) and BSA (data not shown) failed to facilitate the formation of **16a** from **8a** provided support for the notion of a cage effect. Clearly, ArsB did not catalyze either the aldol cyclization and aromatization of **8a** to **16a** or the decarboxylation of **16a** to **7a**.

It could be thought that ArsB and ArsC, but not BSA, contain other site(s) that bind **8a** and exert a cage effect. Such non-active-site binding could have limited the availability of **8a** to the active site, keeping it from being catalytically converted to **7a**. On the other hand, if **8a** is not an intermediate but the observed conversion to **16a** occurs at the active site, **8a** would exhibit an inhibitory effect on the ArsB reaction in a similar manner to other structurally related compounds. Compound **8a** inhibited the ArsB reaction by 44% at 0.35 mM. It was a stronger inhibitor than stearic acid (32% inhibition at 1 mM)

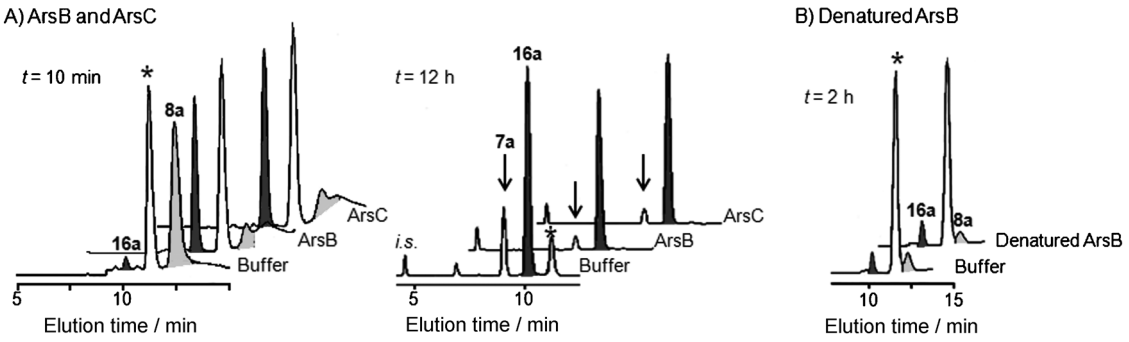


Figure 2. HPLC analysis of the reaction mixture of C_{20}-TKA (**8a**) and A) ArsB and ArsC, or B) denatured ArsB. In all chromatograms, 6-tridecyl-β-resorcylic acid (C_{13}-RA, **16a**) is dark-shaded, and C_{20}-TKA (**8a**) is light-shaded. 5-Tridecylresorcinol (C_{13}-RL, **7a**) is indicated with arrow, the unidentified contaminant is indicated with asterisk. i.s. = internal standard (olivetol), which was added to the reaction mixture prior to ethyl acetate extraction to control the extraction efficiency.

(Figure 3A), but a weaker inhibitor than iodoacetamide (95% inhibition at 0.05 mM). More importantly, although the diketo analogue, 3,5-dioxooctadecanoic acid (C_{18}-DKA) showed a comparable inhibition (40% at 0.5 mM), its methyl ester, methyl 3,5-dioxooctadecanoate, was not inhibitory at 1 mM. Because ArsB activity was sensitive to organic solvents, it was not possible to measure the effects of the inhibitors at higher concentrations. Instead, the inhibitory effect of a water-soluble analogue, the dipotassium salt of C_{18}-DKA (C_{18}-DKAS), was investigated in more detail. C_{18}-DKAS showed a concentration-dependent inhibition, and its K_i value was determined to be 0.65 mM when fitted to a competitive inhibition model ($r^2 = 0.96$; Figure 3B). The observed inhibition by **8a** was mostly due to **8a** itself, as **16a**, which should have been formed during the inhibition assay, was a weaker inhibitor (40% inhibition at 1 mM). On the other hand, C_{18}-DKA and C_{18}-DKAS were solely responsible for the observed inhibitory effect, as they were recovered unchanged after incubation (data not shown). Comparable inhibition of the ArsB reaction by **8a** and its structural analogues, C_{18}-DKA, C_{18}-DKAS, and stearic acid, but not by methyl 3,5-dioxooctadecanoate indicates specific binding of **8a** and the analogues to ArsB. Although it cannot be completely excluded, the probability that **8a** inhibited ArsB activity by binding to a non-active site is very low. Rather, **8a** and the analogues most likely inhibited ArsB activity by competitively binding to the enzyme active site. The relatively weak affinity of **8a** and **16a** for ArsB, as evidenced by their weak inhibition and the high K_i value of C_{18}-DKAS, also agrees with the notion that neither **8a** nor **16a** is an intermediate in the ArsB reaction.

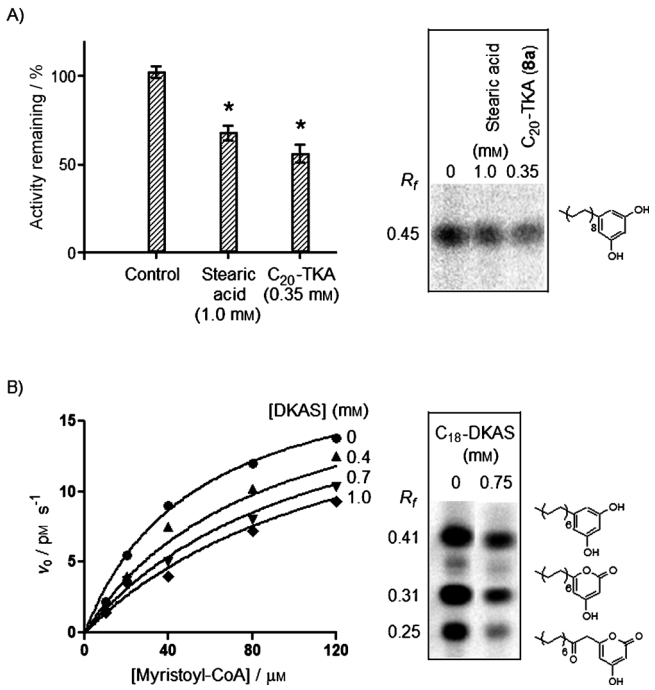


Figure 3. Inhibition of ArsB activity by C_{20}-TKA and its analogues. A) ArsB-catalyzed formation of 5-heptadecylresorcinol from stearoyl-CoA (50 μM) and [2-^{14}C]malonyl-CoA (10 μM) was measured in the presence of stearic acid (1 mM) or C_{20}-TKA (0.35 mM); mean ± SEM ($n = 4$); *$P < 0.05$, Student's t-test. A representative radio thin-layer chromatogram of the products generated by ArsB is shown right. B) Michaelis–Menten plots of the ArsB-catalyzed formation of 5-tridecylresorcinol at different concentrations of C_{18}-DKAS. Myristoyl-CoA was used as the substrate. ArsB produces tri- and tetraketide pyrones as well as alkylresorcinol with medium-chain acyl-CoA starter substrates, as shown in the representative radio-thin layer chromatogram.[3]

Based on the results that ArsB and ArsC, but not denatured ArsB and BSA, facilitated the conversion of **8a** to **16a** and that ArsB and ArsC hindered the decarboxylation of **16a** to **7a**, we conclude that neither **8a** nor **16a** is an intermediate in ArsB-catalyzed resorcinol ring formation, and that aldol cyclization occurs prior to thioester hydrolysis. This eliminates any pathway that involves **8a** or **16a** as an intermediate; that is, pathways A1, A2, B1.3, and B2 (Scheme 2). The remaining plausible pathways are B1.1 and B1.2. Obtaining direct evidence for either "aldol first" pathway might not be easy. One could consider feeding a synthetic sample of the cyclized dianion **13** to ArsB to see if ArsB directly converts it to alkylresorcinol. However, one should be careful in interpreting the results. The configuration of the two chiral centers (C-2 and C-7) of synthetic **13** can differ from that of enzyme-produced **13**, and this can lead to a false-negative conclusion.[4,10,20]

An enigma in the mechanism of aldol-cyclizing type III PKSs such as ArsB and STS is how the enzyme controls the timing of

the hydrolysis of **3**. Premature hydrolysis of the growing ketide intermediates will result in mono-, di-, and triketo acids that might be dead-end products. In other words, the aldol switch needs to stay "off" until the final condensation reaction (for the "hydrolysis-first" pathway) or until aldol cyclization (for the "aldol-first" pathway). In the proposed "aldol-first" ArsB reaction pathway, the formation of the ring structure could be the switch trigger.

STS might or might not share the same ring-formation mechanism with ArsB. Similar experiments with **8b** could be devised to study STS-catalyzed resorcinol ring formation. Our attempts on this line of work did not result in conclusive evidence for the intermediacy of **8b** in the STS reaction mainly because of the chemical instability of **8b**. However, STS can produce short-chain alkylresorcinols by using, for example, hexanoyl-CoA as the starter substrate.[21] Therefore, suitable **8b** analogues can be synthesized to test the "hydrolysis-first" hypothesis for STS-catalyzed resorcinol ring formation. Austin et al.[5] found a thiolase-like, hydrogen-bonding-activated water molecule next to the catalytic Cys in the crystal structure of STS and proposed that this "aldol switch" was responsible for the hydrolysis of the tetraketide thioester intermediate (**3**). It is worth noting that, using LC-MS, Tosin et al.[22] detected a dehydrated, possibly cyclized, tetraketide CoA ester species from the reaction of STS and cinnamoyl-CoA. If this compound is indeed cyclized, it would indicate an alternative "aldol-first" pathway for STS in which the aldol cyclization of **3** is followed sequentially by dehydration, hydrolysis, β-keto acid decarboxylation and aromatization.

As a single active site is responsible for substrate selection, chain elongation, and cyclization in type III PKS reactions, it is not always possible to study cyclization reactions separately without affecting other reactions through, for example, site-directed mutagenesis. The short chain length of polyketide intermediates of type III PKS systems as compared to type I and II PKSs allows the intermediates to be prepared synthetically for mechanistic studies. ArsB, ArsC and other related fatty acyl-CoA-accepting type III PKSs such as sorghum alkylresorcinol synthase[23] and rice ARAS[9] present good model systems for studying the mechanisms of different polyketide cyclization reactions. It has been decades since the solution chemistry of polyketo acids was delineated and the possibility of these compounds being intermediates in polyketide biosynthesis was discussed (reviewed in ref. [13]). This study demonstrates (to the best of our knowledge for the first time) the feasibility of using a triketo acid derivative to investigate the cyclization mechanism of polyketide synthase.

Experimental Section

Materials and syntheses: Expression plasmids, pET16b-ArsB and pET16b-ArsC were provided by Dr. Nobutaka Funa (University of Tokyo).[3] [2-^{14}C]Malonyl-CoA (54 mCi mmol^{-1}) was from PerkinElmer, and acyl-CoA esters and other chemicals were from Sigma–Aldrich. 3,5-Dioxooctadecanoic acid (C_{18}-DKA), its dipotassium salt (C_{18}-DKAS), and methyl 3,5-dioxooctadecanoate were prepared as described.[15] The syntheses and characterization of *tert*-butyl 3,5,7-trioxoeicosanoate and 3,5,7-trioxoeicosanoic acid (**8a**, C_{20}-TKA) are described in the Supporting Information.

Enzyme purification and assay: Recombinant ArsB and ArsC were produced as His_{10}-tagged proteins in *E. coli* BL21(DE3) cells and purified by Ni^{2+}-affinity chromatography as described previously.[21] Enzyme reaction was carried out either in KPi (0.1 M, pH 7.8) or in KPi buffer (0.1 M, pH 7.2) containing 10% glycerol and 0.1% Triton X-100 (assay buffer). Reaction mixture (100 μL) containing enzyme (20–30 μg), [2-^{14}C]malonyl-CoA (50 μM, 13.5 mCi mmol^{-1}), and myristoyl-CoA or stearoyl-CoA as the starter substrate (100 μM) was incubated at 37 °C for 30 min. Reaction products were separated by silica TLC, and enzyme activity was quantified by using a phosphorimaging system as described.[15]

Enzymatic conversion of C_{20}-TKA, and HPLC analysis of reaction products: Freshly prepared C_{20}-TKA (**8a**; 0.75 mM) was incubated in KPi buffer (0.1 M, pH 7.8) at 37 °C in the presence of ArsB, ArsC, denatured ArsB (90 °C, 4 min) or BSA (each 100–200 μg). Aliquots were removed at time intervals and extracted with ethyl acetate. Extracts were dried under vacuum and redissolved in HPLC mobile phase (acetonitrile/H_2O/acetic acid 8:2:0.01, *v/v/v*). Reaction products were analyzed by HPLC by using a Waters instrument equipped with a Phenomenex Luna 5μ C5 100A column (250× 4.6 mm), a Waters 510 HPLC pump, and a Waters 717 plus autosampler. Detection was made on an Agilent 1100 Series system at 280 nm. The isocratic elution program was run at 0.9 mL min^{-1} with the mobile phase.

Inhibition of ArsB by C_{20}-TKA and related compounds: The inhibitory effects of different compounds on the activity of ArsB were tested in the enzyme assay buffer by using [2-^{14}C]malonyl-CoA (10 μM, 54 mCi mmol^{-1}) and myristoyl-CoA or stearoyl-CoA as the starter substrate (50 μM). Triton X-100 was added to increase the solubility of inhibitors. The enzyme reaction profile was not affected by the addition of the detergent. After 30 min of reaction, the remaining enzyme activity was measured as described above. Inhibitors were dissolved in ethanol, except iodoacetamide and C_{18}-DKAS, which were dissolved in the assay buffer. The final concentration of ethanol in the reaction mixture was 1%. For K_i determination, the production of 5-tridecylresorcinol was measured after 20 min of reaction of ArsB (20 μg) with myristoyl-CoA (10 ~ 120 μM) and [2-^{14}C]malonyl-CoA (10 μM) in the absence and presence of C_{18}-DKAS (0.4, 0.7, and 1.0 mM). The initial velocity data were fitted to a Michaelis–Menten inhibition model provided in GraphPad Prism v.5 (La Jolla, USA).

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Keywords: aldol reaction · biosynthesis · cyclization · enzyme catalysis · polyketide synthases

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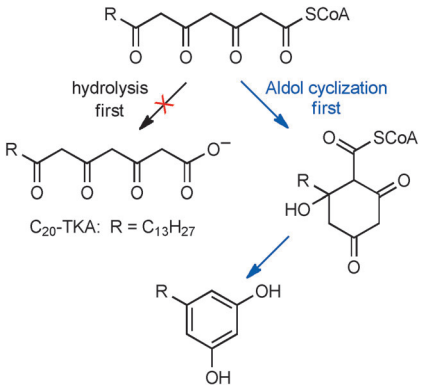
Who's first? Aldol cyclization occurs before hydrolysis in the resorcinol ring formation catalyzed by the type III polyketide synthase, ArsB. Synthetic C_{20}-TKA was not converted to alkylresorcinol by ArsB, but rather inhibited the enzyme activity, thus indicating that C_{20}-TKA is not an intermediate in ArsB-catalyzed alkylresorcinol formation.



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Mapping the Mechanism of the Resorcinol Ring Formation Catalyzed by ArsB, a Type III Polyketide Synthase from *Azotobacter vinelandii*

