stirred for 10 – 14 h at 80 °C. After the starting material had been consumed (TLC), the mixture was added to an ice/HCl mixture (3/1, three times the reaction volume). The mixture was then extraced several times with $CH_2Cl_2$. The combined $CH_2Cl_2$ fractions were dried over $MgSO_4$, and the solvent was removed by distillation. The residue was purified by chromatography on silica gel with $CH_2Cl_2$ to yield red **7b** (555 mg, 91 %).

**8b**: Bis(dicarboximide) **7b** (500 mg) was dissolved in 250 mL of toluene. The mixture was heated to 100 °C and 0.5 mL of DBU added with a syringe through a septum. The reaction mixture was stirred for 8 – 12 h at 100 °C. For the workup, the mixture was added to ice-cooled, dilute HCl and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$, and the product precipitated from $CH_3OH$ to afford orange **8b** in quantitative yield (500 mg).

Spectroscopic investigations: For the film experiments defined amounts of **8b** along with PS (Aldrich, $M_w = 280\,000$, $T_g = 100$ °C) were dissolved in $CHCl_3$ and films prepared from these by spin-coating.

For the fluorescence experiments the samples were excited at 413 nm (ca. 10 mW on an irradiation area of 2 mm in diameter) with a krypton-ion laser. The fluorescence spectra were recorded with a computer-integrated spectrometer card (Ocean Optics, model PC1000) under an angle of 30°. A cut-off filter was used to suppress any scattered light of the excitation laser.

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# The Thioesterase of the Erythromycin-Producing Polyketide Synthase: Influence of Acyl Chain Structure on the Mode of Release of Substrate Analogues from the Acyl Enzyme Intermediates**

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The polyketide core of the antibiotic erythromycin A is assembled by a polyketide synthase (PKS) from seven $C_3$ units.[1] The units are condensed to form a putative enzyme-bound heptaketide product **1**, which is then released through cyclization to the macrolactone, 6-deoxyerythronolide B (**2**) (Scheme 1). The PKS contains a separate catalytic domain for each step in the formation of **2**, housed in three gigantic multifunctional proteins, DEBS 1 – 3. The ordering of the domains can be analyzed in terms of six chain-extension modules, fronted by a loading domain for the starter acyl group, and terminated by an off-loading thioesterase (TE) responsible for release of the completed product.[2]

A pivotal step in this biosynthetic scheme is the thioesterase-catalyzed cyclization of the heptaketide **1** to form the macrolide ring **2**. Early in vitro studies with simple substrate

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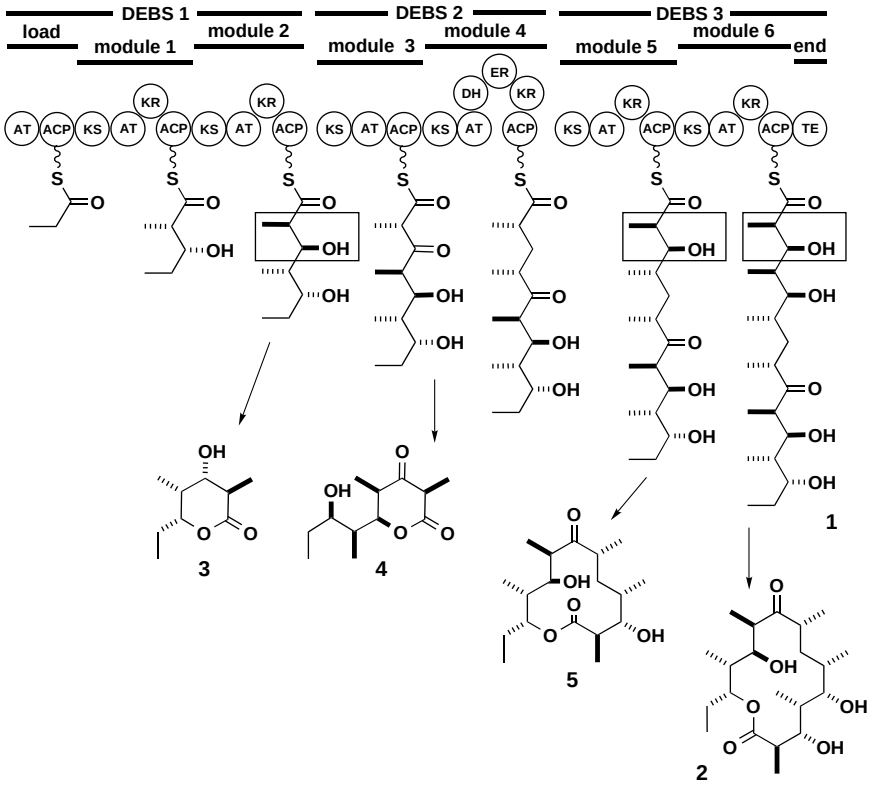


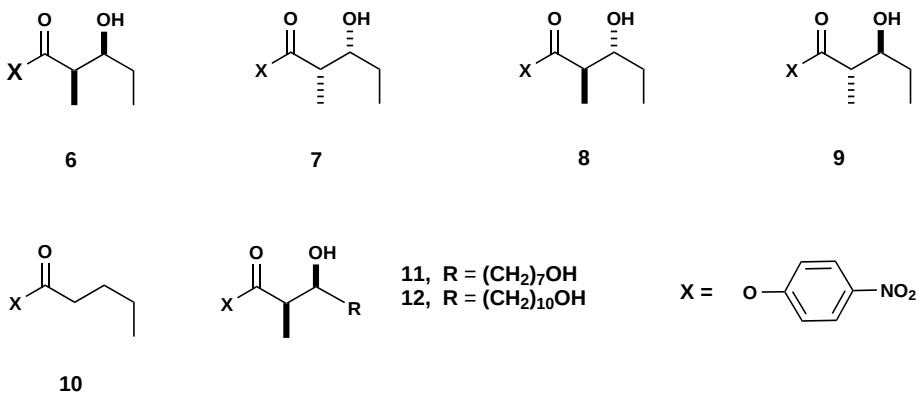

Scheme 1. Genetic engineering of the erythromycin PKS.

analogues and a purified extract of the bidomain ACP-TE derived from the end of DEBS 3 demonstrated that the first step in acyl ester cleavage involves transfer of the acyl chain to the active-site serine hydroxyl of the TE domain to form an acyl enzyme intermediate.[3] In the natural system this intermediate is then cleaved by nucleophilic attack of the C-13 hydroxyl group to release the macrocyclic lactone. For this reaction to occur, not only must the thioesterase recognize and position the C-13 hydroxyl for attack on the acyl enzyme intermediate, but the alternative hydrolytic mechanisms of release characteristic of many esterases must also be suppressed.

The TE domain has been repositioned by genetic engineering to cause premature release of the ketide chain at the triketide **3**, tetraketide **4**, and hexaketide stages **5** (Scheme 1).[4] Structurally modified versions of these products have also been produced by further genetic engineering of the PKS.[5] The success of this approach for the production of polyketides critically depends on the availability of a thioesterase capable of off-loading and/or cyclizing each novel substrate with nucleophiles that are present in vivo. Knowledge of which structural features facilitate or prevent product release by the erythromycin TE would increase the success rate of future genetic engineering by allowing designers to avoid negative results caused by the inability of the thioesterase to release a target product from the PKS.

With the exception of tetraketide **4**, all of the published novel products whose release has been catalyzed in vivo by the erythromycin TE have the natural (2*R*,3*S*)-2-methyl-3-hydroxyl substitution pattern adjacent to the acyl terminus. No conclusions can therefore be drawn concerning the importance of structure and stereochemistry at these two potentially crucial positions.[4] The most direct way to gain information on these key questions of substrate specificity is to carry out experiments with synthetic analogues in vitro. In this study we selected the four diastereoisomers **6**–**9** of the putative diketide intermediate to investigate whether stereochemical variation at the C-2 and C-3 positions might be a barrier to the release of novel substrates from the DEBS in vivo. To determine the effect of functionality at these key positions, turnover results with compounds **6**–**9** were compared to those of **10**, an unfunctionalized diketide analogue of the same chain length. Other structural analogues (**11** and **12**) with longer chains and distal hydroxyl groups capable of participating in the formation of lactone rings were also tested.

As before, the experiments were conducted in vitro with a truncated version of the DEBS 3 protein, consisting of the C-terminal bidomain, an acyl-carrier protein plus the thioesterase (ACP-TE), or the thioesterase alone, which were overexpressed in *Escherichia coli*.[3, 6] In this host, the acyl-carrier protein was not modified by post-translational addition of a phosphopantetheine residue, but the ability of the neighboring TE domain to function independently as a thioesterase in vitro was not affected by this deficiency. The substrates were derivatized as their *p*-nitrophenyl esters to allow convenient UV monitoring of reaction rates. Although *N*-acetylcysteamine thioesters are formally a better mimic of the natural derivative on the acyl carrier protein,[7] *p*-nitrophenyl derivatives were found in initial experiments to react more quickly,

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without significantly altering the observed trends of relative reactivity for different acyl chains.[3]

Initially the substrates **6**–**10** were incubated under standard conditions with ACP-TE at pH 7.4 in an aqueous phosphate buffer containing 1% (*v/v*) of acetonitrile to aid substrate solubility (Table 1). The rates of ester cleavage were monitored at 400 nm ($\lambda_{max}$ for the phenolate anion) and calculated from the initial linear portion of the curves. In all cases, the rates were corrected for the background rate of chemical hydrolysis in the absence of enzyme.

Table 1. Rate of release of *p*-nitrophenolate anion catalyzed by the erythromycin thioesterase.[a]

| Substrate | $v$ [nmol min$^{-1}$] with MeCN | with EtOH | Increase with EtOH [%] | Normalized increase [%][b] |
|---|---|---|---|---|
| **6** | 1.05 | 3.50 | 233 | $75.4 \times 10^3$ |
| **7** | 1.75 | 4.55 | 160 | $51.8 \times 10^3$ |
| **8** | 1.40 | 2.10 | 50 | $16.2 \times 10^3$ |
| **9** | 1.75 | 9.45 | 440 | $14.2 \times 10^4$ |
| **10** | 6.88 | 15.1 | 119 | $38.5 \times 10^3$ |

[a] Measured at 37°C for the appearance of *p*-nitrophenolate anion ($\lambda_{max}$ 400 nm) after the reaction had reached the initial linear phase; substrate concentration, 800 μM; enzyme concentration 1.31 μM; potassium phosphate (200 mM) and Tris (2.5 mM) buffer containing EDTA (50 μM) at pH 7.4. [b] With allowance for relative molarities of water and ethanol.

Although the TE released all four C-2, C-3 functionalized diketides by hydrolysis, the rates of reaction were markedly reduced relative to the unfunctionalized substrate **10** (Table 1); analogue **6**, which has the same relative configuration at the acyl terminus as the natural substrate **1**, had the lowest rate of hydrolysis. These results indicate that the erythromycin TE is optimized, through the recognition of functionality and stereochemistry at the acyl terminus of its substrates, to avoid wasteful hydrolytic release of acyl enzyme intermediates. This optimization is greatest when the C-2 methyl, C-3 hydroxyl stereochemistry of a substrate is in the natural 2*R*,3*S* configuration. However, hydrolytic release was also significantly suppressed for compounds incorporating the three unnatural configurations at these positions. It is therefore likely that macrolactones with altered stereochemistry at C-2 and C-3 can be obtained by genetic engineering.

Macrolactonization depends not only on the suppression of hydrolytic release, but on the formation of an ester bond. As the TE has been shown to favor ethanol over water as the nucleophile for the deacylation step,[3] it was of interest to see whether this preference could be used to increase the turnover of the C-2, C-3 functionalized substrates. Evidence for the formation of esters over free carboxylic acids would further support the case that the TE is optimized for macrolactonization. The substrates **6**–**10** were therefore incubated as above, but with 1% (*v/v*) ethanol, and again the reaction rates were monitored by UV and corrected for background chemical turnover. Addition of only 1% (ca. 1:300 molar) ethanol greatly enhanced the rates of enzyme-catalyzed turnover of three of the four functionalized diketides relative to the unfunctionalized analogue (Table 1). This preference is particularly evident when the rates of reaction are calculated in terms of moles of nucleophile (water or ethanol) present in the medium. Interestingly, the best substrate was not **6**, the stereochemical mimic of the natural substrate **1**, but **9** (7.70 nmoles min$^{-1}$), which has the same configuration at C-3 but the opposite at C-2. Compound **6** reacted at a medium rate (2.45 nmoles min$^{-1}$), as did its enantiomer **7** (2.80 nmoles min$^{-1}$). The substrate with the lowest turnover (**8**, 0.70 nmoles min$^{-1}$) is the enantiomer of **9**, the best substrate. The important conclusion is that all four stereoisomers are effective substrates and stereochemical variation of the methyl group at C-2, or the hydroxyl group at C-3, is no barrier to product release in vivo as a macrolactone given a suitable distal carbinol center.

In order to confirm that ethanol was being used by the TE in preference to water, the assays with ethanol were performed on a larger scale, and the products analyzed by $^1$H NMR (for turnover), and by methylation followed by gas chromatography (for partitioning between diketide acid and ethyl ester). By GC analysis, the ethyl ester analogues were formed in all four enzyme reactions with the diketide stereoisomers, but no such products were generated in the control reactions in the absence of the TE. When allowance was made for the amount of hydrolysis in control reactions, it was again clear that ethanolysis was the preferred mechanism of release in all cases. As in the UV assays, the highest turnover was observed for substrate **9** ($51.6 \pm 0.6$% in 1 h under standard conditions), medium rates for compounds **6** and **7** ($22 \pm 1$% and $19 \pm 1$% respectively), and a low turnover for analogue **8** ($5 \pm 2$%).

To address the esterase's preference for ethanol on a mechanistic level, we monitored the thioesterase by electrospray mass spectrometry (ESMS) during its reaction with analogue **9** in the presence of added ethanol or acetonitrile (9% *v/v*). Although it was not possible to analyze the protein under the original assay conditions, the relative substrate and enzyme concentrations were maintained in order to permit comparison. Spectra were transformed to produce single peaks with *m/z* values corresponding to singly charged ions. After five minutes of incubation, only a single peak consistent with the ACP-TE acylated by the acyl group of the substrate was observed, with or without ethanol present. As formation of the acyl enzyme intermediate is obviously not a barrier to turnover in the case of C-2, C-3 functionalized substrates, deacylation must be the rate-limiting step. It seems likely that recognition of functionality and/or stereochemistry at the C-2 and C-3 positions causes the TE enzyme to contract around the acylated substrates, excluding water from the active site and preventing the hydrolytic release seen in earlier work with simpler substrates.[3] Presumably ethanol occupies the site normally filled by the carbinol alcohol, and is not similarly excluded.

It was also of interest to see if macrolactonization might be the favored method of release over ethanolysis given a suitable distal hydroxyl group in the substrate. We therefore investigated two compounds (**11** and **12**) with the natural (2*R*,3*S*)-2-methyl-3-hydroxyl substitution pattern and an additional ω-hydroxyl functionality to enable lactonization. By $^1$H NMR spectroscopy, the longer chain compound **12** was not converted into product at a measurable rate. The shorter

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analogue **11** did react satisfactorily under standard conditions. The principal mechanism of release was again ethanolysis (>95%), while hydrolysis accounted for the remainder of product turnover. A further attempt to encourage lactonization by substituting acetonitrile for ethanol gave less than 0.1% lactone or free acid; as the acyl enzyme intermediate was detected by ESMS for both substrates **11** and **12** under these conditions, the barrier to release of the products must lie in the deacylation step. The absence of lactone in these experiments shows that in vivo suppression of hydrolysis is not sufficient to ensure macrolactonization, and that additional structural features of the chain in the natural substrate **1** are required to ensure that the TE domain folds correctly.

The results imply that intermediates with 2-methyl, 3-hydroxyl functionality in any stereochemical configuration may be released by lactonization given an appropriately placed and structurally suitable distal hydroxyl group. However, if lactonization is prevented by the absence of a suitable hydroxyl group or other cause, such acyl enzyme intermediates would not be rapidly cleaved by the alternative in vivo mechanism of hydrolysis. The relative inefficiency of hydrolytic release with these substituted analogues is a potentially serious limitation to the range of novel products that are expected to be released in vivo by the erythromycin thioesterase.

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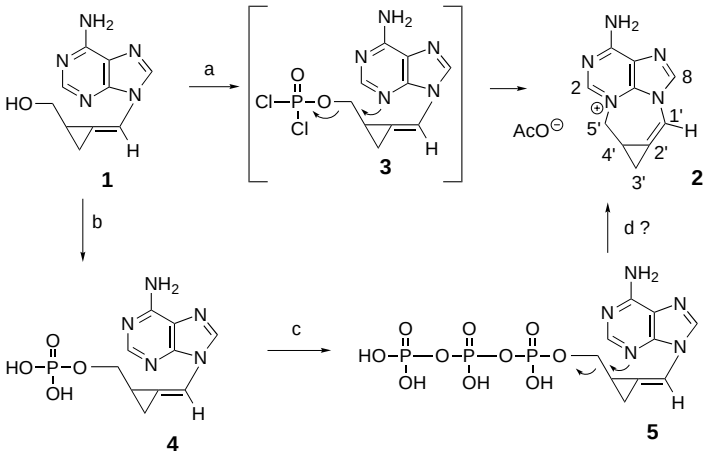
## 3,5′-Anhydrosynadenol: A Polycyclic Anhydronucleoside Analogue**

Yao-Ling Qiu and Jiri Zemlicka*

Recently we reported new nucleoside analogues that exhibit broad-spectrum antiviral activity and contain a methylenecyclopropane unit.[1, 2] Of these compounds, (*Z*)-9-[(2-hydroxymethyl)cyclopropylidenemethyl]adenine (synadenol; **1**) exhibits potent activity[1] against human and murine cytomegalovirus (HCMV and MCMV), Epstein–Barr virus (EBV), human herpes virus 6 (HHV 6), and hepatitis B virus (HBV), and a moderate effect against human immunodeficiency virus 1 (HIV-1). The $^1$H NMR spectra and biological activity indicated that **1** and related compounds can be regarded as analogues of nucleosides. Like nucleosides, the base (adenine) can exist in two conformations, *anti* and *syn*.[3] The $^1$H NMR spectra indicated that the adenine component of **1** has mainly the *anti* conformation in solution, as in nucleosides.[1]

Here we report that the *syn* form of **1** can be converted into the anhydronucleoside analogue **2**; this strengthens the analogy to nucleosides.[4] Reaction of **1** with $POCl_3$/$PO(OMe)_3$ led to a smooth cyclization and gave anhydrosynadenol (**2**), which was isolated as the acetate in 87% yield (Scheme 1). The dichlorophosphate **3** is a likely intermediate in this transformation. In nucleosides this reaction leads only to 5′-*O*-phosphorylation without formation of anhydronucleoside.[5, 6] Trisimidazolylphosphine oxide[7] in pyridine also gave **2** as the sole product. The ease of cyclization is surprising given that methylenecyclopropanes are generally assumed to be destabilized by substantial ring strain.[8] However, according to recent ab initio calculations,[9] the loss of strong




Scheme 1. a) $POCl_3$, $PO(OMe)_3$; b) Dowex 2 ($AcO^-$); c) intracellular phosphorylation; d) intracellular inactivation.

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