Synthesis and Incorporation of an *N*-Acetylcysteamine Analogue of Methylmalonyl-CoA by a Modular Polyketide Synthase

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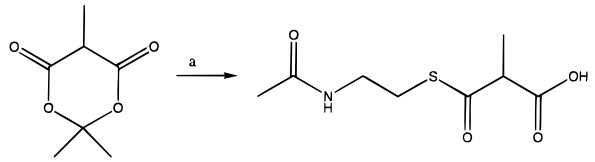
The structurally broad class of natural products called polyketides has found wide use in human therapy, animal health, and agriculture. Their biosynthetic diversity is generated by repetitive condensations of simple monomers by polyketide synthases (PKSs) that mimic fatty acid synthases, but with additional processing reactions.[1] For instance, the deoxyerythronolide-B synthase (DEBS) catalyzes the chain extension of a propionyl primer with six methylmalonyl coenzyme A (MeMalCoA) derived extender units to produce the erythromycin core.[1] Genetically engineered PKSs now provide the promise of new polyketide-based drugs.[2] Although "unnatural" natural products can be synthesized in vivo and in vitro,[3] the cost of the required coenzyme A thioesters needed for chain extension prohibits larger scale in vitro synthesis of polyketides or in vivo synthesis in organisms such as *E. coli* that do not synthesize an endogenous supply of MeMalCoA. A more economical and easily modifiable source of methylmalonyl extender units is key to the realization of preparative scale syntheses of engineered polyketides. Here we report the one-step synthesis of a new truncated MeMalCoA derivative (**2**) and its efficient acceptance as a substrate analogue by a module of the DEBS protein complex to make a polyketide product. Our result demonstrates the remarkable tolerance of extender unit acyl transferase domains for CoA mimetics.

An obvious simplification of the MeMalCoA structure is truncation of the CoA side chain to *N*-acetylcysteamine (NAC). Previous work had demonstrated that NAC thioesters can be incorporated both in vivo[4] and in vitro[5] as primers for polyketide synthesis. Unlike CoA esters, these analogues can be taken up by cells from exogenous media and can be processed by the desired enzymes. However, whereas SNAC-derived primers go directly to the ketosynthase (KS) portion of the PKS,[6] CoA-derived extender units must be accepted via extender unit acyltransferase (AT) domains. Therefore, a key question in the use of SNAC extender units was whether the AT domains would accept these derivatives and acylate the required acyl carrier protein (ACP).

Scheme 1. Synthesis of MeMalSNAC[a]



1 **2**

[a] Conditions: (a) TMS−SNAC, catalytic TMSOTf, CH_2Cl_2, 70%.

The preparation of MeMalSNAC had not been reported previously.[7] While the multistep route used to make MeMalCoA via the thiophenyl ester[8] would be viable to produce the NAC analogue, we desired a shorter sequence in which the difficult monothioesterification of a diacid could be avoided. Meldrum's acid is a commonly used substrate for acylation chemistry, because nucleophilic opening and decarboxylation subsequently yield β-keto esters.[9,10] Additionally, the acid can serve as a way of differentiating the two ends of malonic acid. Thus, direct nucleophilic attack of Meldrum's acid is a common route to various monoesters.[11] Unfortunately, treatment with NAC failed to afford the desired thioester. Clearly, a more reactive nucleophile was in order, but the low pK_a (4.97)[12] of Meldrum's acid prohibited use of preformed thiolates. Previous work with amines and alcohols[13] pointed to the possibility of opening up Meldrum's acid under milder conditions using silylated nucleophiles. Indeed, we found the same method to work effectively with a trimethylsilylated thiol nucleophile made with catalytic trimethylsilyl triflate[14] without addition of heat. Conveniently, methyl Meldrum's acid is readily available and less expensive per mole than methylmalonic acid. The same reaction sequence succeeded with methyl Meldrum's acid as with the unmethylated case (Scheme 1). We thereby had a one-step route to the desired MeMalSNAC (**2**).[15]

To test the ability of a modular PKS to accept MeMalSNAC as a substrate, MeMalSNAC was used in a reaction with the purified third polypeptide of DEBS (DEBS 3).[5a] DEBS 3 contains the two final modules, 5 and 6, of the erythromycin PKS. The (2*S*,3*R*)-[2,3-$^{13}C_2$]diketide (**3**)[17] was employed as a primer so that product formation, if any, could easily be verified directly by ^{13}C NMR. MeMalSNAC (**2**) was utilized in the enzymatic reaction at a concentration of 1 mM, which is comparable to the concentrations of SNAC-derived primer units that are typically used in cell-free assays. The ^{13}C NMR of the ethyl acetate extract of the enzymatic reaction mixture clearly showed formation of the known triketide lactone **4**[18] (Scheme 2), thereby confirming the acceptance of MeMalSNAC as a substrate by DEBS3, most likely by module 6. A pair of doublets (J = 35 Hz) was present in the spectrum at 36 and 81 ppm, corresponding to C-4 and C-5

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(15) Physical data for **2**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.50 (br s, 1 H), 6.41 (br s, 1 H), 3.63 (q, J = 7.0 Hz, 1 H), 3.42−3.34 (m, 2 H), 3.11−2.93 (m, 2 H), 1.99 (s, 3 H), 1.45 (d, J = 7.0 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.7, 172.8, 172.7, 54.8, 40.1, 29.2, 23.4, 14.7; HRMS (FAB$^+$; NBA/NaI) calculated for $(C_8H_{13}NO_4S)H^+$ 220.0644, observed 220.0641.
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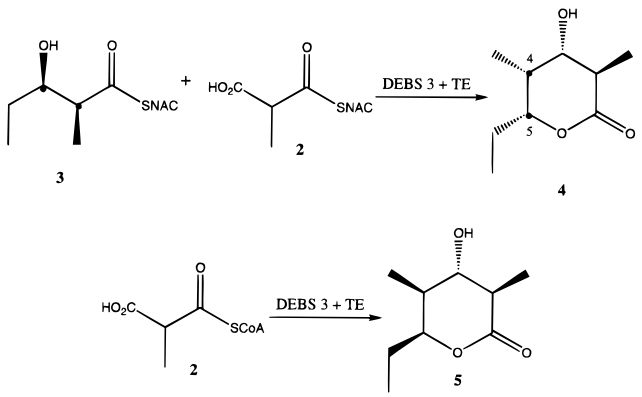
Scheme 2. Extension of Doubly ^{13}C-Labeled Diketide Primer



of **4**, respectively. The amount of polyketide product obtained per mg protein is estimated to be at least 10 nmol, which is comparable to that produced in the presence of MeMalCoA under similar conditions. Although the concentration of MeMalSNAC was approximately 5−10-fold higher in the above reaction, on a per mole basis MeMalSNAC is about 4000 times cheaper than the corresponding coenzyme A derivative.

It is noteworthy that DEBS **3** can convert the (2*S*, 3*R*)-diketide **3** into the triketide lactone **4** which is epimeric at C-4 and C-5 to the triketide lactone **5** produced by DEBS 3 in the presence of MeMalCoA alone.[16] Under the very reasonable assumption that the chain elongation, reduction, and lactonization that generates triketide lactone **4** is mediated by module 6, it appears that the ketosynthase of this module (KS6) can accept and elongate both the (2*S*, 3*R*)-2-methyl-3-hydroxypentanoyl thioester and its (2*R*,3*S*)-enantiomer as substrates. This result is yet another example of the remarkable tolerance of modular PKSs toward unnatural substrates.

The synthesis and successful incorporation of MeMalSNAC as an extender unit for a polyketide synthase not only provide a less costly means for polyketide production, but also allow the synthesis and testing of other modified extender units in combination with protein engineering. Chemistries that modify Meldrum's acid to make not only monomethylated but also other alkylated derivatives are well established[19] and should easily lend themselves to the synthesis of extender units bearing nonnatural side chains. Engineering their incorporation into polyketides would combinatorialize a presently untapped portion of their biosynthetic pathway that could lead to an even greater pool of structural diversity.

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