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Activating Hybrid Modular Interfaces in Synthetic Polyketide Synthases by Cassette Replacement of Ketosynthase Domains

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Summary

Unnatural combinations of polyketide synthase modules often fail to make a polyketide product. The causes of these failures are likely complex and are not yet amenable to rational correction. One possible explanation is the inability of the ketosynthase (KS) domain to extend the ketide donated to it by the upstream module. We therefore addressed the problem by exchanging KS domains of the acceptor module in a combinatorial fashion and coexpressing these chimeric modules with ketide-donor modules that naturally interact with the transplanted KS. This approach was remarkably successful in activating previously unproductive bimodular combinations, and the results augur well for the ongoing development of molecular tools to design and produce novel polyketides.

Introduction

Bacterial modular polyketide synthase (PKS) genes determine the biosynthesis of valuable natural products like erythromycin, epothilone, FK-506, and many others [1]. They encode giant proteins consisting of sets (modules) of active sites (domains) that form an enzymatic "assembly line" to build the carbon chain of the final product in a stepwise manner. The paradigm PKS gene cluster for 6-deoxyerythronolide B synthase (DEBS), which is responsible for 6-deoxyerythronolide B biosynthesis, encodes a loading module followed by six extender modules. Polyketide synthesis is initiated when the loading PKS module (LM) selects or generates the starter acyl-CoA (propionyl CoA for DEBS) and transfers its acyl group to the first of several extender modules. All canonical extender modules contain at least a ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domains. The KS receives the acyl unit from the preceding module, while the AT transfers an appropriate extender unit from its CoA ester to the prosthetic group of the ACP. The KS then catalyzes a decarboxylative Claisen condensation between the acyl-KS and the extender unit to give a β-keto-acyl-ACP. Modules may also contain enzymes that further modify the growing polyketide chain: notably, a set of reductases/dehydratase for the β-keto group. The polyketide chain is successively elongated by downstream modules and, upon completion, is cleaved from the PKS—usually by a thioesterase (TE) domain at its end [2]. Thus, the components of each PKS module "encode" the structure of the two-carbon unit in the main polyketide chain, the order of modules determines the sequence of two-carbon units in the polyketide product, and the number of modules determines the carbon chain length. Because modules may be distributed over more than one protein, both intra- and interpolypeptide acyl chain transfers can occur, promoted by appropriate docking domains to facilitate proximity [3, 4].

The modular nature of polyketide biosynthesis has facilitated genetic engineering of PKS genes to modify polyketide structure. Because PKS modules comprise natural, integrated catalytic units, rearranging intact modules is an attractive approach for combinatorial biosynthesis [3]. In theory, this approach could generate virtually any polyketide by combining modules with the desired activities, but major challenges need to be addressed. Success depends on each downstream KS accepting the ketide from a donor module through an unnatural intermodular junction, and this depends on structural recognition of the ketide by the KS, physical proximity of the ACP of the donor module and the acceptor KS, and possible protein-protein interactions between them. Carbon chain elongation then requires substrate tolerance by and catalytic activity of the acceptor KS [5].

Because current knowledge is insufficient to rationally design functional module-module interfaces a priori, we approached the problem empirically by using combinatorial biosynthesis [6]. The strategy was to make and test large numbers of module-module combinations in order to identify enough productive interfaces for assembly of diverse, complex polyketides.

A combination of several technologies was necessary to implement this approach. First, to avoid the comparative inefficiency and inconvenience of working with actinomycetes and myxobacteria, the natural polyketide producers, *Escherichia coli* was engineered to serve as a host for polyketide production [7, 8]. Second, to effect rapid genetic manipulation, we developed a generic design of PKS genes that involves optimizing codon usage for *E. coli* expression and introducing a set of standard unique restriction sites flanking catalytic domains, docking domains, and modules to allow easy interchange [6]. Third, technology was developed for the facile synthesis of modules ~5 kb-long containing unique restriction sites, so that the redesigned PKS genes could readily be obtained [9].

We also developed a two-plasmid system to rapidly scan the ability of two heterologous PKS modules to interact and produce polyketide [6]. The "donor" plasmid contained the loading module of the erythromycin PKS (LM^{ery}), followed by the diketide-donating module, and terminated in the C-terminal docking domain from eryM2 (LC—the second module of the erythromycin PKS). The "acceptor" plasmid contained the eryM3 N-terminal docking domain (LN), followed by the diketide-accepting module, and terminating in TE^{ery} (Figure 1A). The plasmids were coexpressed in engineered *E. coli*

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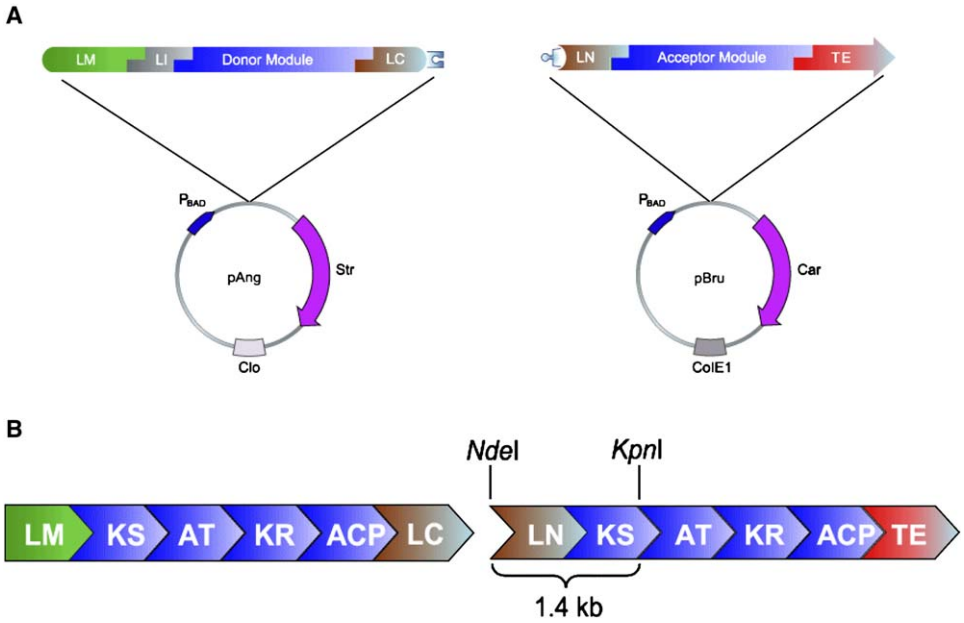

Figure 1. Expression Vectors and Cloning Scheme Used for Studying Effects of KS Domain Replacement

(A) The two compatible expression vectors used to test bimodular interactions. The pAng plasmids contain LM^{ery} followed by the donor module and the C-terminal docking domain from eryM2 (LC). The pBru acceptor plasmids contain the N-terminal docking domain of eryM3 followed by the acceptor module and TE^{ery}.

(B) Unique NdeI site at the start codon and KpnI site at the end of the KS domain of the synthetic modules used for KS exchange.

strain K207-3 [8], and cultures were analyzed for the expected triketide lactone product (TKL) by LC/MS/MS. By creating libraries of modules in each of these vectors, and by using combinatorial transformation [6], we scanned over 150 module-module combinations for activity, and, remarkably, 50% of the combinations produced the expected TKL. Since each module was active in one or more contexts, we surmised that the problem with the inactive unnatural module pairs could be the absence of an appropriate ACP-KS interaction, or inability of the KS to catalyze the extension of a particular substrate.

The present work was undertaken to develop reliable tools for converting inactive module-module interfaces to active ones. In a few cases, an inactive module-module interface has been activated by substituting the KS domain of the ketide-accepting module by the KS that is normally associated with the ketide-donating module [10–12]. However, it is currently not possible to predict whether a transplanted KS will accept and extend the ketide, much less whether it will provide the product in acceptable yield. We therefore embarked on a study in which combinatorial biosynthesis was used to activate inactive module pairs and optimize the yield of a desired polyketide product by changing KS domains. We describe herein the results of these experiments, and provide an algorithm for "resuscitating" inactive hybrid modular interfaces of PKSs and screening for those that yield the most product.

Results and Discussion

Construction and Expression of Chimeric PKS Modules

The previously reported [6] pBru acceptor expression vectors used here contain synthetic ORFs encoding nine PKS modules—eryM1, eryM2, eryM3, eryM5, eryM6, epoM7, sorM6, gdmM3, and rifM5—flanked by the N-terminal docking domain of eryM3 at the 5′ end, and the DEBS TE at the 3′ end, all under control of the P_{BAD} promoter. The ORFs were designed with a unique NdeI site at the start codon and a unique KpnI site at codons for the completely conserved GT dipeptide found immediately downstream of the KS domain (Figure 1B) [6]. The docking domain and KS domains of the 9 ORFs were then removed by NdeI/KpnI digestion and replaced with the docking domain-KS fragments obtained by NdeI/KpnI digestion of eryM2, eryM3, eryM5, and eryM6, to produce 32 chimeric PKS modules with heterologous KS domains. When the 9 ORFs with native modules and the 32 hybrid ORFs were expressed from the P_{BAD} promoter at 22ºC in *E. coli* K207-3, all gave similar levels of soluble protein in the range of 60–96 mg/l (see Table S1 in the Supplemental Data). The high levels of protein expression are undoubtedly due to the codon-optimized PKS genes, which produce 5- to 10-fold more PKS than wild-type sequences [13].

Activity of Modules Containing Natural and Heterologous KS Domains

Extension of a diketide thioester by an acceptor module requires: (1) substrate recognition and acylation of the acceptor KS domain; (2) Claisen condensation between the acyl-KS and the acyl-ACP of the same module; and (3) modification of the β-keto group of the ketide chain by the reduction/dehydration domains of the module. In order to assess the intrinsic activity of individual acceptor modules, module-TE fusions expressed in *E. coli* K207-3 were treated with the (2S,3R)-2-methyl-3-hydroxyhexanoic acid *N*-acetylcysteamine thioester (SNAC) **1**, and media were analyzed for production of

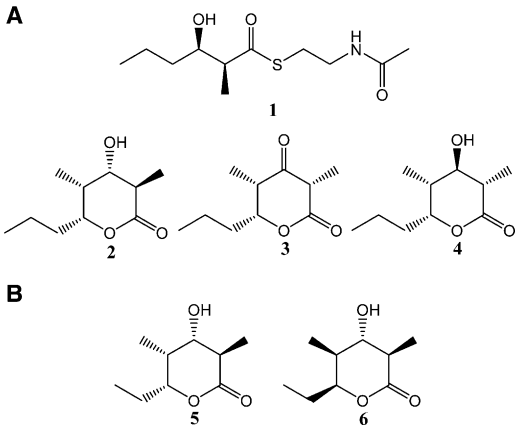

Figure 2. TKL Products Expected to Be Produced in this Study

(A) TKL products expected from the single extension of the SNAC 1 substrate by the modules used in this study.

(B) Structures of TKL products expected from the bimodular combinations.

the expected TKL **2**, **3**, or **4** by LC/MS/MS (Figure 2A). Additional products may be present in some cases, but were not looked for, since the issue of potential side products was beyond the scope of this work.

We used the diketide SNAC **1** derived from a butyryl rather than the more common propionyl starter unit to avoid ambiguities as to the origin of TKL products if modules were capable of using MeMal-CoA for both loading and iterative extension—a phenomenon referred to as "stuttering" [6, 14, 15]. That is, the TKL product of stuttering cannot be distinguished from that derived from the propionyl-diketide, whereas it is easily differentiated by LC/MS/MS from that emanating from the butyryl diketide SNAC **1**. It has previously been shown that propionyl-, butyryl-, and pentanoyl-diketide SNACs are all effective in producing TKL products with various PKS modules used here as sources for KS domains [16–18].

As shown in Table 1, eryM2-TE^{ery}, eryM5-TE^{ery}, and eryM6-TE^{ery} efficiently catalyze the conversion of **1** to provide the corresponding TKL **2**, while eryM3-TE converts the same SNAC to TKL **3**. In contrast, although eryM1 correctly extends propionic acid *N*-acetylcysteamine thioester [19], corresponding to its natural substrate propionyl-CoA, it does not extend the diketide moiety of **1** to the expected TKL product **4**. Instead, a small amount (0.8 mg/l) of the corresponding keto lactone **3** is produced. Thus, the KS of eryM1 can accept diketide **1** and catalyze the Claisen condensation, but the KR of the module does not effectively reduce the β-keto group of the extended diketide.

The KS domains of eryM2, eryM3, eryM5, and eryM6 were exchanged to explore the effects of interchanging KSs among already functional modules (Table 1, columns 2–5). SNAC **1** was fed to cultures expressing each of the wild-type and chimeric modules, which are predicted to produce TKL **2** from eryM2-, eryM5-, and eryM6-TEs, and TKL **3** from eryM3-TE. In all cases, the chimeric modules produced less TKL than the native modules, although this reduction was less marked for substitutions into the eryM3-TE module and the hybrid of eryM5 containing eryM3 KS. Since the introduced KSs accept the SNAC efficiently in their natural modules, the lower yields obtained with the chimeric modules are likely due to problems with the subsequent KS-catalyzed Claisen condensation or unproductive interactions resulting from KS-AT fusion junctions.

The KS of eryM1 was likewise exchanged for the KSs from eryM2, M3, M5, and M6 that all appropriately extend the diketide component of SNAC **1** in their native contexts. However, as with the KS in its native module, the keto-lactone **3** was the sole TKL produced by eryM1 chimeras with KSs originating from eryM2, eryM3 or eryM6 (Table 1, column 1). Thus, as with the natural KS of eryM1, KS hybrids of eryM1 accept and extend the diketide moiety of SNAC **1**, but do not appropriately reduce the β-carbon ketone.

Treatment of cells harboring unaltered epoM7-TE^{ery}, sorM6-TE^{ery}, gdmM3-TE^{ery}, and rifM5-TE^{ery} with **1** gave low but detectable yields of the TKL product **2** (Table 2, top row). It is not surprising that the KSs of these four modules process the diketide component of **1** poorly, since the (2*S*,3*R*)-ketide component of the SNAC **1** does not resemble any of their natural substrates [6]. It is also interesting to note that even though the KR of epoM7-TE is suspected to be inactive [20], epoM7-TE gave a product that, by LC/MS/MS, had identical mass, fragmentation pattern, and retention time as an authentic standard of TKL **2**, a result that has also been previously observed [6]. Thus, it is quite reasonable to conclude that the KR of epoM7-TE is active in its present context.

Chimeras of the four poorly active modules, epoM7, sorM6, gdmM3, and rifM5, were then examined. Each native KS of these modules was substituted by the

Table 1. Production, in mg/l, of Triketide Lactones **2** or **3** by Native and Chimeric Ery Module-Tes after Feeding with Diketide SNAC **1**

	Module-TE				
KS Source	(KS^{eryMx})-eryM1-TE[a]	(KS^{eryMx})-eryM2-TE[b]	(KS^{eryMx})-eryM3-TE[c]	(KS^{eryMx})-eryM5-TE[b]	(KS^{eryMx})-eryM6-TE[b]
KS^{eryM2}	(0.05)	11[d]	19	0.1	0.01
KS^{eryM3}	(0.4)	—[e]	21[d]	1	2
KS^{eryM5}	—[e]	—[e]	16	3[d]	—[e]
KS^{eryM6}	(0.6)	0.01	14	0.01	21[d]

[a] Parenthesized yields are for unexpected keto-lactone **3** instead of expected TKL **4**.

[b] Product is TKL **2**.

[c] Product is keto-TKL **3**.

[d] TKLs produced by wild-type module.

[e] No TKL detected; <0.01 mg/l.

Table 2. Production of Triketide Lactone **2**, in mg/l, by Native and Chimeric Module-Tes after Feeding with Diketide SNAC **1**

KS Source	Module-TE			
	(KS^{eryMx})-epoM7-TE	(KS^{eryMx})-sorM6-TE	(KS^{eryMx})-gdmM3-TE	(KS^{eryMx})-rifM5-TE
Native KS	0.1	0.02	0.03	0.01
KS^{eryM2}	0.2	11	12	11
KS^{eryM3}	—[a]	11	13	8
KS^{eryM5}	0.08	0.9	0.01	0.01
KS^{eryM6}	0.2	5	10	2

[a] No TKL detected; <0.01 mg/l.

four KSs known to accept the diketide **1** (i.e., KSs from eryM2, eryM3, eryM5, and eryM6) in an attempt to identify chimeric modules capable of extending the unnatural diketide substrate. Since none of the heterologous KSs increased the efficiency of epoM7 (Table 2, column1), efforts were focused on the other three modules. Substitution of the KS of sorM6 by eryM5 gave a 40-fold increase in TKL production, but the eryM5 KS was ineffective in other contexts studied (Table 2, row 4). Strikingly, activities of modules derived from sorM6, gdmM3, and rifM5 were dramatically increased by replacing their KS domains with those from eryM2, eryM3, and eryM6 (Table 2, rows 2, 3, 5), with 160- to 1300-fold increases in TKL yields over those of the natural modules. Indeed, most chimeras created by KS exchange of the poorly active sorM6-TE, gdmM3-TE, and rifM5-TE modules were much more active than the native modules, and many were about as efficient in extending the SNAC as the most active native ery modules.

Activity of Bimodules with Active Chimeric Modules as Acceptors

Having identified chimeric PKS modules derived from sorM6, gdmM3, and rifM5 that showed good activity when processing a chemically synthesized precursor, we proceeded to determine whether these modules would accept a substrate produced and offered by appropriate upstream modules. For these experiments, diketide-donating modules eryM1, eryM2, and eryM5, which offer 2*S*,3*R* (eryM1) or 2*R*,3*S* (eryM2 and eryM5) substrates to downstream modules, were flanked by the ery LM on the N terminus and the LC on the C terminus [6]. These pAng donor plasmids were coexpressed with selected pBru acceptor plasmids containing sorM6, gdmM3, and rifM5 with heterologous KS domains, and TKL products were analyzed by LC/MS/MS (Table 3). As shown previously, combinations of these donor modules with sorM6, gdmM3, and rifM5 containing their native KSs do not produce TKL [6]. In the present work, the bimodular combinations tested were chosen to maintain a cognate donor ACP-acceptor KS pair, known to be functional in transfer of the donated substrate (i.e., a 2*S*,3*R* or 2*R*,3*S* diketide) at the module-module interface [6]. Thus, the LM^{ery}-eryM1 donor module was coexpressed with the three chimeric acceptor modules containing the KS from eryM2; the LM^{ery}-eryM2 was coexpressed with the three chimeric modules containing the KS from eryM3; and LM^{ery}-eryM5 with the three chimeras containing the KS from eryM6. The bimodular combinations involving LM^{ery}-eryM1 were tested for production of TKL **5**, while those involving LM^{ery}-eryM2 and LM^{ery}-eryM5 were tested for the presence of **6** (Figure 2B). Remarkably, all 9 combinations were active, and each of the 3 inactive native modules was converted to at least 1 chimera that produced relatively substantial yields (5–10 mg/l) of the expected products (Table 2).

Significance

An algorithm has been established to resuscitate inactive polyketide synthase (PKS) module-module interfaces. First, the ketosynthase (KS) domain of the substrate-accepting module-thioesterase (TE) is replaced by a library of KSs, normally downstream of modules that will be used to donate the ketide. Second, each member of the set of chimeras is tested for intrinsic activity by feeding a diketide-SNAC resembling the substrate made and presented by the donor unit. This establishes the ability of the chimeric module to accept and extend the substrate, isolated from potential problems of interaction with the upstream modules. Finally, the active modules are coexpressed with the donor modules that are cognate with the KS transplanted to the accepting module, and generation of an active bimodular interface is demonstrated by

Table 3. TKL Production by Bimodules Containing KS Substitutions in the Acceptor Module

Donor modules	Acceptor Modules								
	[KS^{eryM2}] sorM6-TE	[KS^{eryM2}] gdmM3-TE	[KS^{eryM2}] rifM5-TE	[KS^{eryM3}] sorM6-TE	[KS^{eryM3}] gdmM3-TE	[KS^{eryM3}] rifM5-TE	[KS^{eryM6}] sorM6-TE	[KS^{eryM6}] gdmM3-TE	[KS^{eryM6}] rifM5-TE
LM^{ery}-eryM1[a]	4	8	4	—	—	—	—	—	—
LM^{ery}-eryM2[b]	—	—	—	10	5	3	—	—	—
LM^{ery}-eryM5[b]	—	—	—	—	—	—	0.6	1	0.2

Unfilled cells correspond to module pairs that do not contain cognate donor ACP and acceptor KS and were not tested.
[a] Product is TKL **5**.
[b] Product is TKL **6**.

production of the expected product. The feasibility of this approach has been validated by resuscitating nine bimodular variants involving three of four ModTEs that were inactive in previously studied combinations. The constructed chimeras not only gained the ability to efficiently extend the diketide component, SNAC 1, but gave excellent yields of triketide lactone product (TKL) products when used as the acceptor module in bimodular combinations. The activation of hybrid modular interfaces by KS replacements provides a powerful tool to achieve the goal of generating, to order, novel polyketides of potential value as drugs or as synthons for making them.

Experimental Procedures

Host and Vectors

The *E. coli* polyketide production strain K207-3 (BL21DprpBCD::T7prom prpE, T7prom accA1-pccB, T7prom sfp), as well as the pAng plasmids containing LM^{ery}-Mod-LC with eryM1 (pKOS422-108-1), eryM2 (pKOS422-99-2), or eryM5 (pKOS422-126-2), and the pBru plasmids containing LN-Mod-TE^{ery} with eryM1 (pKOS422-114-1), epoM7 (pKOS422-114-2), eryM5 (pKOS422-114-3), gdmM3 (pKOS422-114-4), rifM5 (pKOS422-114-6), eryM2 (pKOS422-100-1), eryM6 (pKOS422-100-2), sorM5 (pKOS422-100-3), or eryM3 (pKOS422-177-1), have been previously described [6, 8].

Chimeric Plasmids

DNA manipulation procedures were performed as previously described [21]. Fragments containing the 1.4 kb N-terminal docking domain and KS domains were obtained by digestion of pBru plasmids with NdeI and KpnI, and purified by gel electrophoresis. Alternatively, the corresponding N-terminal docking domains and KS domains to be replaced were removed from pBru plasmids containing LN-Module-TE^{ery} ORFs by digestion with NdeI and KpnI, and the remaining ~8.5 kb vector fragment was purified by gel electrophoresis. Appropriate 8.5 and 1.4 kb fragments were then ligated to give the hybrid expression plasmids containing a heterologous KS, and the sequence of each construct was verified. The expression plasmids used in this study are listed in Table S2.

Diketide Feeding to Modules

K207-3 bacteria harboring pBru expression plasmids were grown in 2.5 ml LB with carbenicillin (50 μg/ml) at 37°C to an OD_{600} = 0.5. Cultures were induced with IPTG (0.5 mM) and arabinose (2 mg/ml), and 0.5 ml of a mixture of sodium glutamate (50 mM), sodium succinate (50 mM), sodium propionate (5 mM), and (2S,3R)-2-methyl-3-hydroxyhexanoic acid N-acetylcysteamine thioester **1** (1 mM) (2S, 3R-SNAC) [22] was added. After incubation at 22°C for 72 hr with agitation, bacteria were removed by centrifugation, and supernatants were acidified with phosphoric acid to pH 2.5 and analyzed after at least 30 min for TKL production by LC/MS/MS [6].

Protein Expression Analysis

Samples (1 ml) of each culture were centrifuged at 14,000 × g for 3 min, resuspended in 1 ml 20 mM Tris, 150 mM NaCl, pH 7.5, and lysed by sonication. After 10 min of centrifugation at 14,000 × g, soluble fractions equivalent to 10 μl cell suspension were separated on NuPAGE Novex 3%–8% Tris-acetate gels (Invitrogen), stained with Sypro-Red Staining (Molecular Probes), and quantified with a Typhoon scanner with BSA standards (Table S1).

Activity of Bimodular Combinations

K207-3 bacteria harboring pAng donor plasmids and pBru acceptor plasmids were grown in 3 ml LB containing carbenicillin (50 μg/ml) and streptomycin (20 μg/ml) at 37°C to an OD_{600} = 0.5. Cultures were induced with IPTG (0.5 mM) and arabinose (2 mg/ml), and 0.5 ml of a mixture of sodium glutamate (50 mM), sodium succinate (50 mM), and sodium propionate (5 mM) was added. After 72 hr at 22°C, cultures were processed as above and analyzed for TKL production by LC/MS/MS [6].

Quantification of Products by LC/MS/MS

TKL concentrations in samples were estimated by using calibration curves developed for each TKL. Standards for TKLs **2**, **5**, and **6** were prepared as previously described [16, 17, 23]. The identity of TKL **3** was verified by comparison of its product-ion scan (see Figure S1 in the Supplemental Data) with that of the known propyl-analog [18] and analysis, as previously described [24]. The TKL **3** standard used for LC/MS quantitation was prepared by extending SNAC **1** with purified eryM3-TE [18]. To determine the concentration of the TKL **3** standard, identical reactions were performed by using unlabeled and [2-^{14}C]methylmalonyl-CoA; the radioactive TKL was separated and quantified by TLC with a Typhoon imager against a standard curve of [2-^{14}C]methylmalonyl-CoA, as previously described [25].

Supplemental Data

Supplemental Data, including supplemental figure and tables, are available online at http://www.chembiol.com/cgi/content/full/13/5/469/DC1/.

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