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ACS chemical biology

High-Throughput, Microarray-Based Synthesis of Natural Product Analogues *via in Vitro* Metabolic Pathway Construction

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ABSTRACT The generation of biological diversity by engineering the biosynthetic gene assembly of metabolic pathway enzymes has led to a wide range of "unnatural" variants of natural products. However, current biosynthetic techniques do not allow the rapid manipulation of pathway components and are often fundamentally limited by the compatibility of new pathways, their gene expression, and the resulting biosynthetic products and pathway intermediates with cell growth and function. To overcome these limitations, we have developed an entirely *in vitro* approach to synthesize analogues of natural products in high throughput. Using several type III polyketide synthases (PKS) together with oxidative post-PKS tailoring enzymes, we performed 192 individual and multienzymatic reactions on a single glass microarray. Subsequent array-based screening with a human tyrosine kinase led to the identification of three compounds that acted as modest inhibitors in the low-micromolar range. This approach, therefore, enables the rapid construction of analogues of natural products as potential pharmaceutical lead compounds.

Nature owes its unparalleled structural and functional chemical diversity to the power of multienzyme pathways that comprise the synthetic machinery of biological systems. Mankind has only been able to tap into a small part of this biocatalytic repertoire, yet this has resulted in a vast array of natural products for use as pharmaceuticals (*1*) and raw materials for chemical intermediates and polymeric materials (*2*). New and high-throughput technologies will be necessary to gain access to nature's "warehouse" of structures and functions and to be able to manipulate the synthesis of these molecules to yield novel and biologically active compounds.

Metabolic pathway engineering efforts to date have largely focused on the reconstruction and manipulation of native and artificial pathways, respectively, by extracting, transferring, and functionally expressing essential genes into suitable hosts (*3*–*5*). Extensive work has been performed to rationally and randomly engineer pathways to yield sufficient amounts of natural products and their analogues. For example, combinatorial biosynthesis has been used to generate novel analogues or intermediates of natural products (*e.g.*, erythromycin (*6*) and an intermediate of paclitaxel, among others (*7*)) and new pathways to known compounds (*e.g.*, the antimalarial compound artemisinin (*5*)) as drug lead candidates. Nonetheless, while combinatorial biosynthesis has resulted in a wide range of novel natural product derivatives, its use in drug discovery is fundamentally limited. Specifically, combinatorial biosynthesis requires precise regulatory control (*8*) over gene expression of relevant biosynthetic metabolic pathways, which may be influenced by unknown pro-

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Received for review February 14, 2007 and accepted April 26, 2007.

Published online May 25, 2007

10.1021/cb700033s CCC: $37.00

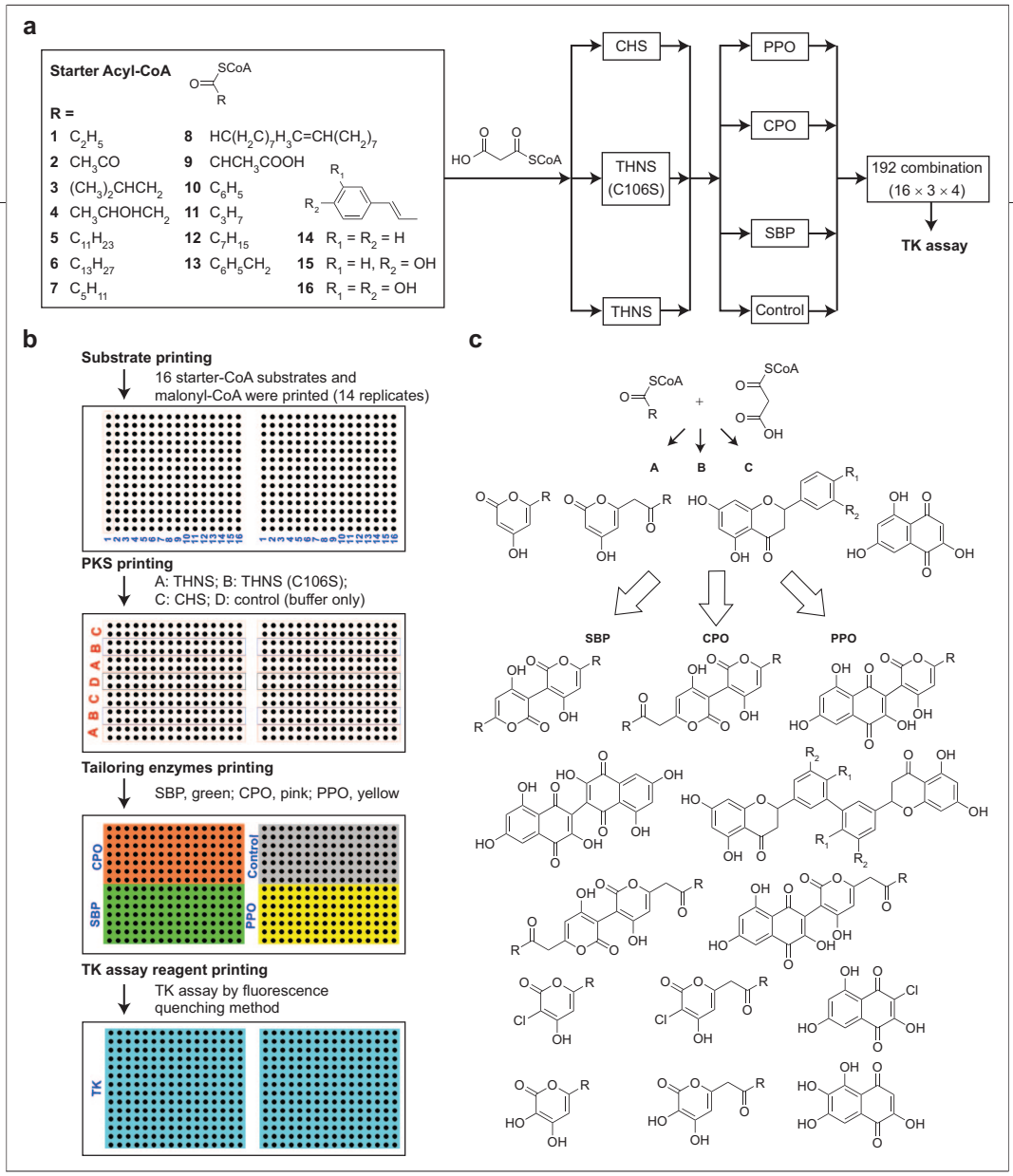

Figure 1. Strategy for generating polyketide analogues. a) Schematic of the *in vitro* metabolic pathway microarray. b) Operational design of the *in vitro* metabolic pathway microarray consisting of PKS, post-PKS tailoring enzymes, and TK assay reagents (left side) and the predicted polyketide analogues (right side).

tein–protein interactions (*9*–*11*), the presence of toxic biosynthetic intermediates and products (*12*), and the difficulty of adapting to high-throughput synthesis, which is required for discovery efforts (*13*). As a result, there is an unmet need to develop alternative techniques to synthesize natural-product-based libraries in high throughput and subject these compounds to various cell-based or cell-free screens.

To address this need, we report herein a microarray-based *in vitro* metabolic pathway engineering concept for generating a natural product analogue library. We have chosen the functionally diverse type III polyketide synthases (PKSs) to demonstrate the feasibility of this approach. These relatively small homodimeric enzymes ($M_r \approx 40$ kDa per subunit) are involved in the synthesis of aromatic and pyrone products, including the flavones, flavonones, flavonoids, stilbenes, and related compounds. The diversity of the type III PKSs results from a small number of coenzyme A (CoA) ester precursors and is therefore heavily dependent on the variation of polyketide chain length and chemistry due to the selectivity of the specific PKS, as well as modifications of the polyketide scaffold by post-PKS tailoring enzymes. Thus, further expansion of the structural and functional diversity of type III PKS-generated polyketides is desirable and may result from the introduction of unnatural precursor CoA substrates, along with tailoring enzymes that are not limited to those found in PKS-containing organisms.

RESULTS AND DISCUSSION

Our *in vitro* strategy (Figure 1) used three type III PKSs, including 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) from *Streptomyces coelicolor*, a site-directed mutant of THNS (C106S) that prematurely truncates polyketide extension to give pyrone products (Figure 2, Table 1), and a chalcone synthase (CHS) from alfalfa. The three PKSs are initially contacted with 16 distinct precursor acyl-CoA esters (Figure 1, panel a) along with the extender substrate, malonyl-CoA, resulting in 48 reaction combinations (16 substrates × 3 enzymes). These precursor CoA esters encompass a wide range of

structural features, including different-length alkane and alkene chains, aromatic groups, and phenolic and catecholic moieties. The resulting polyketides are then subjected to post-PKS tailoring reactions catalyzed by soybean peroxidase (SBP), chloroperoxidase (CPO), or polyphenol oxidase (PPO), giving 144 potential combinations of enzyme reactions plus the PKS-only reactions generated in the first step (Figure 1, panel a). To enable high-throughput synthesis, we have adapted this strategy to a microarray format (Figure 1, panel b) with a capacity of 448 individual reactions per slide. This approach is ideally suited for microarray-based screening of the reaction products.

To that end, we then screened the polyketide-based library as potential inhibitors of the human tyrosine kinase (TK), FynT, which is an Src family non-receptor protein kinase (*14*–*16*). TK serves as a logical screening target, given the well-known, albeit only modest, inhibition of TKs by flavonoids, which appear to possess structural features that are complementary to the kinase ATP binding site and thus may have clinical application in the treatment of chronic myeloid leukemia, gastrointestinal stromal tumors, and breast cancer (*16*). For example, the flavonoid genistein inhibits human FynT with an IC_{50} of 1.0 μM (*17*).

Microarray-Based Polyketide Synthesis and Derivatization. To establish the effectiveness of microarray PKS catalysis, different concentrations of malonyl-CoA solutions (30 nL) were first spotted onto methyltrimethoxysilane (MTMOS)-coated slides (448 total spots per slide); the hydrophobic coating prevents the aqueous-based spot from spreading on the slide surface (*18*). The substrate spots were then air-dried. PKS solutions (30 nL) containing purified THNS dissolved in aqueous buffer (containing 10% (v/v) glycerol; the solvent was added to minimize evaporation (*19*) and to prevent polyketide precipitation) were then printed over the substrate spots, and the reactions were allowed to proceed for 12 h. Following air drying of the reaction spots the autofluorescence of the expected flaviolin product was measured; higher malonyl-CoA concentrations led to higher fluorescence, which was indicative of the synthesis of higher flaviolin concentrations (Supplementary Figure 1). LC/MS analysis of the full slide extracted with ethyl acetate confirmed that the fluorescent product was the expected flaviolin (Supplementary Figure 2). Approximately 50% yield of flaviolin was obtained with 1 mM malonyl-CoA, which

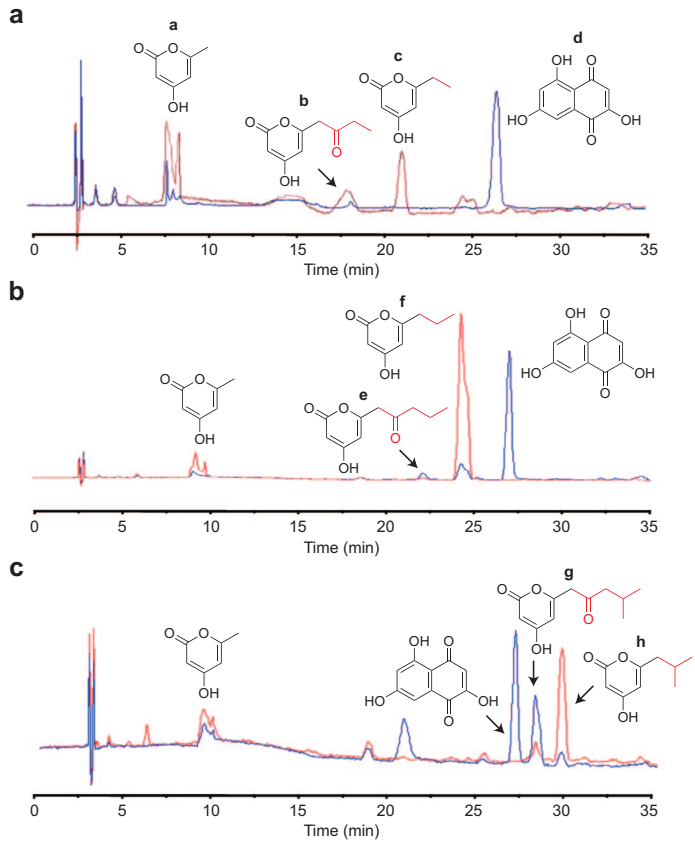

Figure 2. LC/MS analysis of products (a–h) synthesized using wild-type (blue line) and C106S mutant THNS (red line) with various substrates. a) Propionyl-CoA and malonyl-CoA. b) Butyryl-CoA and malonyl-CoA. c) Isovaleryl-CoA and malonyl-CoA. Each peak was identified *via* LC/MS and/or NMR (see Supporting Information).

was similar to that observed in solution-phase off-slide reactions.

Extension of THNS catalysis to other starter substrates, however, did not result in substantial yield of the expected pyrones. In fact, while wild-type THNS has broad starter unit substrate specificity (*20*), the major product was almost always the full pentaketide flaviolin (Figure 2 and Table 1), suggesting that the enzyme strongly prefers malonyl-CoA as both the starter unit and the extender unit. This confirmed work of Noel and colleagues, who suggested that the active cysteine residue (Cys106) could interact with the terminal carboxylate moiety (starter malonyl-derived) of di- or triketides, facilitating malonyl-CoA-primed polyketide extension beyond the triketide stage (*21*).

TABLE 1. Starter preference of THNS variants with different substrates

Starter Unit	Enzyme	TAL (μM)	Flaviolin (μM)	Tetrapyrone (μM)	Tripyrone (μM)
Propionyl-CoA	Wild-type	0.5 ± 0.1	10 ± 1.1	0.5 ± 0.1	[a]
	C106S	5.3 ± 0.5	[a]	5.3 ± 1.1	8.5 ± 1.2
Butyryl-CoA	Wild-type	0.6 ± 0.1	13.5 ± 1.6	1.2 ± 0.1	2.7 ± 0.3
	C106S	0.9 ± 0.3	[a]	0.9 ± 0.3	38 ± 1.7
Isovaleryl-CoA	Wild-type	0.9 ± 0.2	11 ± 1.3	6.8 ± 0.5	1.5 ± 0.1
	C106S	3.9 ± 0.7	[a]	2.2 ± 0.5	12 ± 1.2

[a] Not detected.

If such a hypothesis were to explain the apparent ability of malonyl-CoA to out-compete other starter units, then we would expect to observe a striking pH dependence on starter unit specificity. Therefore, we performed THNS catalysis at pH 6.5 (native conditions) and 9.0, the latter to simulate a condition where pH > pK_a (~8.4) of the Cys106 moiety (Supplementary Table 1). At the lower pH, the yield of flaviolin was ~3-fold higher than that of the combined yields of the tri- and tetrapyrones (Supplementary Table 2). However, at pH 9, the reverse was observed, with the yields of the pyrones ~17-fold higher than that of flaviolin. We therefore hypothesized that proton donation from the thiol group of Cys106 possibly enhances malonyl-CoA starter unit loading to the catalytic Cys138 residue due to the ease of thiol deprotonation of the Cys106 residue at pH 9, resulting in decreasing the binding affinity toward malonyl-CoA (Supplementary Figure 3).

Another route to altering the specificity of THNS would be to eliminate the thiol group at the 106 position. Indeed, by replacement of the thiol with a hydroxyl moiety (C106S), which would have a much higher pK_a and not be in a position to easily donate a proton to malonyl-CoA, the specificity of THNS was dramatically altered. The C106S strongly disfavored malonyl-CoA from acting as the starter unit and essentially eliminated the formation of flaviolin (Figure 2 and Table 1). As a result, the mutant enzyme generated tri- and tetrapyrones along with triacetic acid lactone (TAL).

When given non-malonyl-CoA starter units, the C106S mutant yielded only pyrone products in the microarray. For example, reactions with butyryl-CoA resulted in the formation of the expected butyl tripyrone (Supplementary Figure 2) in an on-slide yield of ~70%, nearly identical to that obtained in solution under similar reaction conditions (data not shown). Similar pyrone synthesis was achieved with propionyl-CoA or isovaleryl-CoA using the C106S mutant; in no case was flaviolin generated (Table 1). In addition to the mutant THNS, CHS cannot accept malonyl-CoA as a starter substrate. Hence, the reaction with coumaryl-CoA and malonyl-CoA as starter and extender substrates, respectively, yielded naringenin (Supplementary Figure 2) in an on-slide yield of ~30%. Thus, all three enzymes (wild type (wt)-THNS, C106S THNS, and CHS) were active on 30 nL reaction spots on a glass microarray.

Protein Kinase Assay on the Microarray Platform. The second microarray component is the subsequent TK screen. We adapted the FynT assay to an array-based platform by modifying a microtiter-plate-based fluorescence quenching method (using the custom IQ reagent from Pierce) that employs a specific quencher of fluorogenic phosphorylated peptide substrates. When phosphorylated peptidic standard and quenching reagent were sequentially spotted onto an MTMOS-coated glass slide (Figure 3, panel a), the resulting fluorescence quenching correlated to the degree of phosphorylation. Thus, the TK assay technique is feasible on the microarray platform. To assess TK activity on the microarray, we printed 30 nL of the peptidic substrate onto an MTMOS-coated glass slide followed by addition of 30 nL of TK enzyme solution. As a result of incubation at RT for 3 h and subsequent quenching, spots containing active TK showed weaker fluorescence than spots that did not contain TK, indicating that TK was active on the microarray (Figure 3, panel b). Inhibition of TK was then assessed using PP2, a well-known Fyn inhibitor (*17*), which was spotted initially onto the MTMOS-coated glass slide and dried (Figure 3, panel c). The on-array calculated IC_{50} value was 14 nM, which was similar to well-based values (*17*).

High-Throughput Generation and Screening of Polyketide Library. Having established PKS activity and the functionality of the TK screen on a microarray, we proceeded to combine polyketide synthesis, tailoring enzyme catalysis, and TK inhibition assays all on the same microarray platform. First, PKS catalysis was performed in 30 nL spots, as described above, using THNS, THNS (C106S), and CHS with 16 acyl-CoA starter substrates and malonyl-CoA as the extender substrate, as laid out (Figure 1). Following 12 h reactions, an additional 30 nL of SBP, CPO, or PPO solutions were added to specific spots on the slide, as outlined (Figure 1), and the reactions were performed for an additional 3 h followed by air drying. This sequential spotting technique, therefore, essentially represents a synthetic *in vitro* metabolic pathway microarray that couples enzymatic reactions not normally associated with PKS pathways to generate analogues of polyketides. In this case, a large

number of possible polyketide-based products could be generated (Figure 1).

To confirm on-slide compound generation from two-step enzyme reactions, we carried out nine different combinatorial enzyme reactions out of the 192 possible combinations. Various polyketide analogues were identified from sequential PKS and post-PKS tailoring enzyme reactions (Supplementary Table 3). From this limited confirmation, we demonstrated on-slide formation of SBP-coupled polyketide dimers, halogenated polyketides from CPO catalysis, and hydroxylated polyketides from PPO reactions.

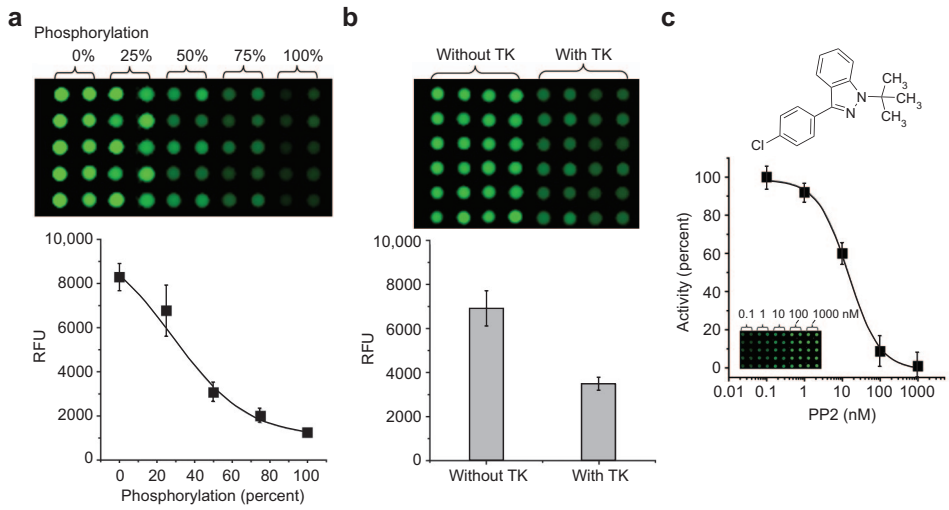

Figure 3. Demonstration of array-based TK inhibition assay. a) Scan of 60 nL of fluorophore-labeled peptidic standard (30 μM) with different degrees of phosphorylation following fluorescence quenching. The two columns represent 10 replicates of spots containing the peptide standard. b) Scan of 30 nL of TK spots printed atop 30 nL of fluorophore-labeled peptidic substrate (KVEKIGEGTYGVVYK) following fluorescence quenching. The 4 × 6 spot array represents 24 replicates of peptide phosphorylation reactions. Peptidic substrate with lower degrees of phosphorylation showed stronger fluorescence. c) Determination of IC_{50} value using PP2; fluorophore-labeled peptidic substrate (30 nL) and TK (30 nL) were sequentially printed atop PP2 spots (10 replicates).

Subsequent screening of the full polyketide-based library on the microarray was performed as described above for the model TK assay inhibition with PP2. Several spots on the microarray (Figure 4, panel a) showed significant fluorescence over that of the slide background, indicating the presence of potential TK inhibitors. The three most fluorescent spot regions corresponded to reactions of native THNS followed by SBP (region 1), native THNS and THNS (C106S) followed by SBP (region 2), and THNS (C106S) followed by CPO using KCl as halide donor (region 3).

The identities of the three "hits" were determined by resynthesizing the *in vitro* synthetic pathway in 2 mL reactions and isolating the products *via* semipreparative HPLC (see Supporting Information). As a result of this scale-up, the three products were isolated as biflaviolin (1, **i**), Bi-TAL (2, **j**), and 3-chloro TAL (TAL-Cl; 3, **k**) (Supplementary Figure 4 and Supporting Information). Known concentrations in 30 nL of each of the three isolated compounds were then spotted into individual elements of a 50-spot microarray and dried. The TK assay was then performed, as described above, to enable calculation of IC_{50} values (Figure 4, panel b). The three compounds had IC_{50} values ranging from 2.0 ± 0.04 μM (for biflaviolin) to ~5 μM for Bi-TAL, which indicated moderate TK inhibition.

In conclusion, we have developed a multienzyme-containing microarray for the high-throughput synthesis of polyketide-derived products and their subsequent screening for TK inhibition. Using a similar approach, we may be able to reconstruct and manipulate natural and unnatural biosynthetic pathways to generate new functional diversity. Many pathways of secondary metabolism serve as excellent candidates for our strategy, including other polyketides, isoprenoids, lignans, and others, and rapid microarray-based screens can be developed on the basis of key targets, for example, caspase family enzymes, kinases, phosphatases, histone deacetylases, as well as cell-based assays. The design of *in vitro* biosynthesis coupled with high-throughput technology thus serves as a new platform for the synthesis and identification of novel biologically active compounds.

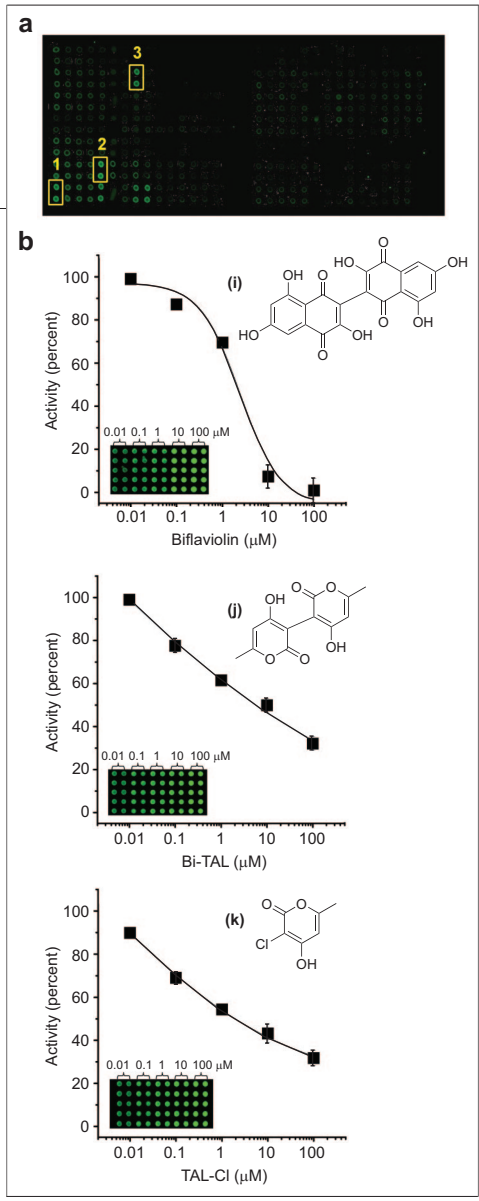

Figure 4. High-throughput TK assay of polyketide analogue library generated by the *in vitro* metabolic pathway microarray (Figure 1). a) Scan of TK assay performed on different polyketide analogues generated by PKS and post-PKS tailoring reactions. The three bright spots in yellow boxes represent "hits". b) Determination of IC_{50} values against TK for biflaviolin (i), bi-TAL (j), and TAL-Cl (k).

METHODS

Reagents. Propionyl-CoA (**1**), acetoacetyl-CoA (**2**), isovaleryl-CoA (**3**), hydroxybutyl-CoA (**4**), lauroyl-CoA (**5**), myristoyl-CoA (**6**), hexanoyl-CoA (**7**), oleoyl-CoA (**8**), benzoyl-CoA (**9**), butyryl-CoA (**10**), octanoyl-CoA (**11**), methylmalonyl-CoA, (**12**), and phenylacetyl-CoA (**13**) were purchased from Sigma Chemical Co. (St. Louis, MO). Cinnamoyl-CoA (**14**), coumaryl-CoA (**15**), and cafferoyl-CoA (**16**) were chemically synthesized (*22*). Malonyl-CoA was also purchased from Sigma. SBP and PPO (tyrosinase) were purchased from Sigma. CPO was purchased from Fluka. TK (Fyn) was purchased from Invitrogen. All other solvents and reagents were commercially available and used without further purification. Src TK inhibitor, PP2, was obtained from Calbiochem. The inhibitor stock solution was prepared in DMSO.

Expression, Purification, and Mutagenesis. Generation and isolation of THNS were described in our earlier study (*20*). The plasmid containing CHS was kindly provided by Joseph Noel (*23*). The QuickChange site-directed mutagenesis kit (Stratagene) was used to generate point mutations in THNS. Mutations were introduced using the following oligonucleotides: 5′-primer, ATCTACGTCTCCTCCACGGGCTTCATG; 3′-primer, CATGAAGCCCGTGGAGGAGACGTAGAT (codons encoding the mutations are underlined). The entire coding sequence of THNS (C106S) was verified by nucleotide sequencing. The PKS enzymes were expressed and purified as previously described (*20*).

Preparation of Enzyme and Substrate Stock Solutions. Three PKSs (THNS, THNS (C106S), and CHS) were purified with the aid of the ProBond His-tag purification system (Invitrogen, Carlsbad, CA). Due to incompatibility of phosphate buffer in the TK assay, we dialyzed the purified enzymes (1 mg mL^{-1}) with 20 mM HEPES buffer (pH 7.0) and then added glycerol (20%, v/v, in the final enzyme stock solution). All substrate stock solutions were also prepared in 20 mM HEPES buffer (pH 7.0) and adjusted to 10 mM substrate concentration.

Microarray of PKS and Post-PKS Tailoring Enzymes. Sixteen starter substrate solutions were arrayed on an MTMOS-coated glass slide (30 nL per spot, 448 spots per slide) using an MicroSys5100-4SQ microarrayer (Genomic Solutions) (*18*). The extender substrate (malonyl-CoA) solution was then printed over the starter spots. After printing the substrate solutions, PKS solutions (30 nL) were arrayed atop each substrate spot while maintaining the array chamber at 90% relative humidity to prevent evaporation of the enzyme solution. The glass slide was then quickly sealed with a 1-mm-thick silicone gasket and cover slide, which allowed the PKS reactions to continue for 12 h in the microarray without drying. Post-PKS tailoring enzyme solutions were prepared as follows. For SBP, 0.1 mg mL^{-1} enzyme was dissolved in 20 mM HEPES buffer (pH 6.0) containing 0.5 mM H_2O_2. For CPO, 0.5 mg mL^{-1} enzyme was dissolved in 20 mM HEPES buffer (pH 6.0) containing 0.5 mM H_2O_2 and 4 mM KCl. Finally, for PPO, 0.5 mg mL^{-1} enzyme was dissolved in 20 mM HEPES buffer (pH 7.0). Following the aforementioned PKS reactions, 30 nL of specific post-PKS tailoring enzyme solutions were printed atop specific PKS reaction spots, and the slides were incubated for an additional 3 h with the cover slide and gasket in place.

High-Throughput TK Assay. To assess the activity of TK in the microarray format, we modified a homogeneous assay based on fluorescence quenching of the phosphotyrosine group of a fluorophore-labeled peptidic substrate (KVEKIGEGTYGVVYK). A calibration curve was obtained using phosphorylated peptide standard (Pierce; Ex = 532 nm, Em = 590 nm). This was accomplished by printing 60 nL of the peptidic standard (30 μM in the TK buffer) and then 30 nL of fluorescence quencher, IQ working solution (1×), atop the standard spots. On-array TK assays were performed with 30 nL of peptidic substrate (30 μM in TK buffer) printed on the slide, followed by printing 30 nL of TK (Fyn) in the TK buffer (100 μM ATP, 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$) and incubating the slide with the cover slide and gasket in place at RT for 3 h. Then 30 nL of the IQ working solution (1×) was added, and the fluorescence of the spots was analyzed on the microarray scanner (GenePix 4000B, Axon Instruments, Inc.). The on-slide IC_{50} value of the known TK (Fyn) inhibitor, PP2, was determined using a PP2 stock solution that was serially diluted with the TK buffer and then added to the MTMOS slide and dried. Peptide substrate (30 nL) in 30 μM TK buffer and TK (Fyn, 48 μg mL^{-1}) solution were sequentially printed atop the PP2 spots, incubating the slide with the lid at RT for 3 h, printing IQ working solution (1×, 30 nL), and imaging the fluorescence signals with the microarray scanner (Ex = 532 nm, Em = 590 nm). For screening of the polyketide analogues, the TK assay solution was printed atop the microarray spots containing polyketide analogues synthesized from the multistep enzyme reactions and then following the aforementioned TK assay protocols.

Product Scale-Up and IC_{50} Determination. To identify the active TK inhibitors and to validate the inhibitory effect of these compounds, we scaled up the relevant reactions in 2 mL vials containing the corresponding starter acyl-CoA (5 mM), malonyl-CoA (5 mM), and the corresponding PKS enzyme (1 mg mL^{-1}). After incubating the reaction mixture for 12 h at RT, 2 mL of the corresponding post-PKS tailoring enzyme solution was added to the reaction mixture and incubated for an additional 3 h. After isolating the corresponding reaction products by preparative HPLC (see Supporting Information), we determined the IC_{50} value of each product isolated for the TK (Fyn) by adding 30 nL of various concentrations of products (0.01–100 μM) to a glass microarray followed by adding sequentially 30 nL of peptide solution and 30 nL of TK solution, incubating the array for 3 h at RT, and then printing 30 nL of the IQ working solution (1×) as described above.

Acknowledgment: This work was supported by the National Institutes of Health (Grants GM66712 and ES012619). We thank J. Noel for kindly supplying the gene for CHS. We thank L. Coulombel and S. McCallum for NMR analysis.

Supporting Information Available: This material is free of charge *via* the Internet.

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