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Expanding the fluorine chemistry of living systems using engineered polyketide synthase pathways

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Abstract

Organofluorines represent a rapidly expanding proportion of molecules used in pharmaceuticals, diagnostics, agrochemicals, and materials. Despite the prevalence of fluorine in synthetic compounds, the known biological scope is limited to a single pathway that produces fluoroacetate. Here, we demonstrate that this pathway can be exploited as a source of fluorinated building blocks for introduction of fluorine into natural product scaffolds. Specifically, we have constructed pathways involving two polyketide synthase systems and show that fluoroacetate can be used to incorporate fluorine into the polyketide backbone *in vitro*. We further show that fluorine can be introduced site-selectively and introduced into polyketide products *in vivo*. These results highlight the prospects for the production of complex fluorinated natural products using synthetic biology.

The catalytic diversity of biological systems provides enormous potential for application of living cells to the scalable production of pharmaceuticals, fuels, and materials (1–4). However, the scope of innovation of living organisms is typically limited to functions that confer a direct advantage for cell growth, thereby maximizing biomass as the end product rather than a distinct molecule or reaction of interest. In contrast, synthetic biology approaches allow us to disconnect some of these remarkable biochemical transformations from cell survival and reconnect them differently for the targeted synthesis of alternative classes of compounds. One particularly interesting area of opportunity is the development of methods to introduce fluorine into complex small molecule scaffolds, which has become a powerful strategy for the design of synthetic pharmaceuticals. Indeed, it is estimated that 20–30% of drugs, including many of the top sellers, contain at least one fluorine atom (5–7).

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Recent innovations have expanded the scope of synthetic C–F bond forming methodologies, but the unusual elemental properties of fluorine that serve as the basis for its success also continue to restrict the range of molecular structures that can be accessed (8–11). As such, the invention of alternative routes for the site-selective introduction of fluorine into structurally diverse molecules, particularly under mild conditions, remains an outstanding challenge.

In comparison to synthetic small molecules, fluorine has limited distribution in naturally occurring organic compounds; the only organofluorine natural products characterized to date consist of a small set of simple molecules associated with the fluoroacetate pathway of *Streptomyces cattleya*, a soil bacterium that houses the remarkable ability to catalyze the formation of C–F bonds from aqueous fluoride (Figure 1A) (12, 13). Although these compounds lack the intricacy typically expected of secondary metabolites, they represent a potentially rich source of modular organofluorine building blocks for the production of complex fluorinated natural products. In this regard, the backbones of several large classes of medicinally-relevant natural products – including polyketides, isoprenoids, steroids, alkaloids, eicosanoids, leukotrienes, and others – are biosynthesized directly from the assembly and tailoring of simple acetate units (Figure 1A). Introduction of the fluoroacetate monomer in place of acetate would allow us to incorporate fluorine into the backbone of these targets and create new molecular function by combining the medicinal chemistry advantages of fluorine with the structural complexity and bioactivity of natural products. For example, the introduction of fluorine via synthetic or semisynthetic routes has enabled the improvement of the clinical properties of several natural products but remains challenging to achieve (14–17). While previous studies have shown that distal fluorine substituents can be accommodated in natural product biosynthetic pathways (18, 19), access to fluoromalonyl-CoA, a fluorinated analog of one of nature's most powerful carbon nucleophiles, as an extender unit would enable a general method for direct incorporation of fluorine into any polyketide structure.

Many acetate-based natural products, polyketides in particular, are generated through the iterative condensation of activated thioesters, resulting in reactive β-keto units that condense further to produce a wide range of structures (20, 21) (Figure 1B). The structural diversity of polyketides is especially striking given that the majority of polyketides draw on only two monomers, acetate and propionate, as the extender units that form their carbon skeletons (3, 20, 22). Although polyketide synthases (PKSs) have been observed to be promiscuous with regard to their starter units (23), the encoding of extender units has been found to be quite selective and many cellular acyl-CoAs are excluded from the backbone (22). However, progress in engineering extender unit incorporation has been made by domain engineering (23–25) or incorporation via a domain that encodes a rare extender unit (17, 26). Although fluoroacetate serves as a starter unit in nature to produce highly toxic ω-fluorofatty acids (Figure 1A) (13), fluorine has never been observed to date within the backbone, implying that chain extension reactions with the fluorinated acyl-CoA do not occur in these systems. The apparent inability of living systems to utilize fluoroacetate for the biosynthesis of complex small molecules likely results in part from the extreme properties of fluorine that affect biological as well as chemical synthesis. For example, the pK_a of the α-proton,

electrophilicity of the carbonyl group, and the stability of the acyl-CoA and its corresponding carbanion are all highly impacted by fluorine substitution. Furthermore, the fluoroacetyl group bears a clear similarity to the fluoromethylketone motif used for the design of covalent inhibitors, suggesting that the irreversible alkylation of active-site nucleophiles could also create problems (27). Thus, the development of a system to incorporate fluorinated extender units could dramatically increase the range of complex structures that can be accessed but must also address the challenges involved in activating the fluoroacetate monomer for the downstream C–C bond forming chemistry involved in chain extension reactions.

Chain elongation in polyketides and related fatty acid-based natural products relies on a separate pool of extender units formed by carboxylation of acyl-CoAs at the α-position. These malonyl-CoA derivatives are then used as masked enolates for C–C bond formation following decarboxylation. The fluorinated extender, fluoromalonyl-CoA, can be made through two routes: either a two-step activation of the biogenic fluoroacetate or a direct ligation of CoA to fluoromalonate (Figure 2). We reasoned that the acetate kinase (AckA)–phosphotransacetylase (Pta) pair would be effective at fluoroacetate activation, as mutations in this gene locus have been shown to lead to fluoroacetate resistance in *Escherichia coli* (28). The enzymes from *E. coli* were thus overexpressed and characterized biochemically, confirming that AckA and Pta serve as an effective activation system to rapidly produce both acetyl- and fluoroacetyl-CoA in nearly quantitative yield (Figures S1–S2). Analysis of the kinetic parameters for these enzymes with respect to fluorinated substrates indicated that neither appears to be affected by the fluorine substituent beyond inductive effects that alter the nucleophilicity of the carboxylic acid (AckA) or electrophilicity of the carbonyl (Pta) (29). Next, we purified the individual AccABCD subunits that make up the acetyl-CoA carboxylase (ACCase) from *E. coli* and added these enzymes to the AckA–Pta system in order to carry out the carboxylation of fluoroacetate in a one-pot reaction to generate the fluoromalonyl-CoA extender unit (Figure 2A, Figure S1). Under these conditions, the ligation of CoA with AckA–Pta to produce the acyl-CoA is rapid and production of the carboxylated product is limited by the ACCase. Although the rate of conversion is 4.5-fold slower for fluoroacetate compared to acetate, the overall extent of reaction is similar for both congeners and suggests that covalent inactivation of the ACCase by fluoroacetyl-CoA is not significant. In addition to the route from fluoroacetate, we also tested a malonyl-CoA synthetase (MatB) (30) for coupling CoA directly to fluoromalonate. Although MatB exhibits a 10^3-fold selectivity for malonate over fluoromalonate, fluoromalonyl-CoA is still produced at reasonable efficiency (Figure 2B, Figures S3–S4). Both of these systems also provide *in situ* regeneration capacity that can amplify product yields from polyketide synthases and we found that either system increased polyketide production by tetrahydroxynaphthalene synthase (31) compared to simple addition of malonyl-CoA (Figure S5).

We next turned our attention to utilizing the fluoromalonyl-CoA monomer for downstream chain elongation reactions. To start, we examined the behavior of a simple polyketide synthase system with regard to one cycle of chain extension and ketoreduction, which is a key functionality of larger multimodular systems for controlling downstream cyclization and

rearrangements within the polyketide backbone (Figure 3A) (3, 20). We constructed a synthetic gene encoding NphT7 from *Streptomyces* sp. CL190 (32), which appears to be a free-standing ketosynthase that is related at the structural level to the ketosynthase domain of more complex polyketide synthases (Figure S6), and isolated the heterologously-expressed enzyme for biochemical characterization (Figure S1). Using a coupled assay with an *R*-hydroxyl forming acetoacetyl-CoA reductase (PhaB), we found that NphT7 is competent to catalyze the formation of acetofluoroacetyl-CoA using an acetyl-CoA starter and fluoromalonyl-CoA extender with only a five-fold defect in catalytic efficiency (k_{cat}/K_M) derived from a drop in k_{cat} with the fluorinated substrate (Figure 3). This lower turnover rate observed with the fluorinated substrate is possibly related to the reduced reactivity of the enolate species, which would be stabilized by the fluorine substituent. However, the overall yield was comparable for both fluorinated and nonfluorinated substrates, which shows that a decarboxylative Claisen condensation with fluoromalonyl-CoA can take place at a similar extent of conversion compared to malonyl-CoA. Furthermore, these experiments also show that the 2-fluoro-3-keto motif produced with the fluoromalonyl-CoA extender can be accepted by ketoreductases, as PhaB is capable of efficiently reducing the acetofluoroacetyl-CoA substrate (Figure S7). The ^{1}H and ^{19}F NMR spectra of the reduced product indicate that both diastereomers are produced in this reaction (Figure S7), which may result either from lack of stereochemical preference of NphT7 with respect to the fluorine substituent or from racemization of the product prior to reduction by PhaB. Although PhaB does not appear to show diastereoselectivity with respect to the fluorine group, the polyketide synthase ketoreductases are known to be selective with regard to their native α-substituent and could potentially carry out the stereochemical resolution of the fluorine modification upon reduction (33).

With this information in hand, we sought to extend our biosynthetic method for fluorine introduction to more complex polyketide synthase systems, which use the chain elongation reaction for the biosynthesis of many bioactive and clinically important natural products, such as erythromycin and rapamycin (3, 20). Of the multimodular polyketide systems, 6-deoxyerythronolide B synthase (DEBS) is likely the best understood and also responsible for production of the erythromycin precursor (34). We therefore focused our studies on the sixth module of DEBS, including the terminal thioesterase ($DEBS_{Mod6}$+TE) (35). Using a diketide substrate (NDK-SNAC), $DEBS_{Mod6}$+TE can catalyze a single round of chain elongation with its native methylmalonyl-CoA extender unit and then cyclize the tethered product to form a methyltriketide lactone (TKL) (Figure 4A, R = CH_3; Figure 4B, **1**; Figure S8) (36). We found that $DEBS_{Mod6}$+TE is also able to accept the fluorinated monomer in chain extension catalysis to form the 2-fluoro-2-desmethyltriketide lactone (F-TKL) and incorporate fluorine into the polyketide backbone (Figure 4B, **2**–**4**; Figure S9). The identity of the F-TKL was established by comparison to an authentic synthetic standard by reverse-phase HPLC monitored by ESI-MS and further confirmed by characterization of the isolated compound by high resolution MS, GC-MS, and ^{19}F NMR spectroscopy (Figures S10–S13). Although the 2*S* keto tautomer is generated in ≥94% diastereomeric excess (d.e.) (Figure S12), this ratio appears to be set by the compound's stereoelectronic factors rather than the stereochemical preference of $DEBS_{Mod6}$+TE, as the F-TKL is fully enolized in aqueous solution. The F-TKL can also be produced directly from fluoroacetate using the AckA–Pta/

ACCase activation system in either a multi-stage (Figure 4B, **5–6**) or single-pot reaction (Figure 4B, **7–8**) with $DEBS_{Mod6}$+TE in a similar yield to the MatB reaction, which allows us to connect fluorinated polyketide production directly to the biosynthetically available fluorinated building block (Figure 1A, Scheme S1).

In contrast to the chain extension reaction catalyzed by NphT7, $DEBS_{Mod6}$+TE does not incorporate fluorinated extender units into the triketide lactone product as efficiently as its native methylmalonyl-CoA extender. Preliminary studies indicate that the reduced efficiency of $DEBS_{Mod6}$+TE with the fluorinated extender is not due to covalent inactivation of the enzyme (Figure S14), but rather to the more complex biochemistry of polyketide synthases with regard to monomer selection (37). Extender unit hydrolysis, which occurs even for the native substrate (Table S2), appears to limit fluoromalonyl-CoA incorporation based on the observations that MatB and ATP are needed for fluoromalonyl-CoA regeneration and that fluoromalonate remains the major organofluorine species even in their presence (Figure S15). The fluoromalonyl-CoA extender is however incorporated at higher efficiency by $DEBS_{Mod6}$+TE than malonyl-CoA (R = H), which is reported to be naturally excluded by DEBS (38). In fact, $DEBS_{Mod6}$+TE produces at least 10-fold more F-TKL than H-TKL in a direct competition experiment with equimolar amounts (1 mM) of fluoromalonyl-CoA and malonyl-CoA (Table S3).

To address the issue of site- or regioselective fluorine incorporation, we turned our attention to exploiting the greater reactivity of the fluorinated extender unit towards acylation reactions. In this regard, we hypothesized that it would be possible for a fluorinated substrate to selectively acylate either the AT or ACP domains of individual DEBS modules in the presence of a catalytically compromised or inactive AT domain, an approach that has been shown to facilitate malonyl incorporation by DEBS (39). Experiments with $DEBS_{Mod6}$+TE showed that not only does F-TKL yield increase as expected but fluorine selectivity also improves upon introduction of a key S2107A mutation, reversing the selectivity of the wild-type module (Figure 4C). Indeed, when the NDK-SNAC substrate is used with its native module, $DEBS_{Mod2}$, in conjunction with the analogous S2652A mutation, extension with fluoromalonyl-CoA to form F-TKL reaches 30% efficiency compared to methylmalonyl-CoA (Figure S16). Furthermore, we found that the standalone trans-AT from the disorazole polyketide synthase (40, 41) accepts fluoromalonyl-CoA and can further enhance F-TKL formation by the AT-null mutant (Figure 4C). Using this approach, we began to explore the possibility of site-selective fluorine incorporation with a mini-PKS model system, consisting of $DEBS_{Mod2}$ and $DEBS_{Mod3}$+TE, that was designed to carry out two chain extension reactions from the NDK-SNAC substrate (42). Using the appropriate AT-null constructs, we were able to observe exclusive production of either regioisomer of the fluoro-methyl tetraketide lactone (tetraKL). The identity of the 2-fluoro-4-methyl tetraKL and 2-methyl-4-fluoro tetraKL were established by both HR ESI-MS and LC-MS based on their different retention times, as well as their mass fragmentation patterns which are consistent with the incorporation of fluorine at the expected sites (Figure 4D, Figure S17). These studies also indicate that further chain extension after fluorine insertion can be achieved and that downstream reactions of fluorinated intermediates could potentially be tolerated. This observation is consistent with previous work that has shown

that intermediates with non-native substituents, including fluorine, can be extended and tailored to the final structure (3, 17–20, 23) and gives promise that larger fluorinated polyketide targets may be accessible through this approach.

The observed selectivity for fluoromalonyl- over malonyl-CoA extender units suggested that polyketide chain extension reactions with fluoromalonyl-CoA could possibly be catalyzed *in vivo* in *E. coli*, which contains a significant malonyl-CoA pool (~35 μM) (43) but almost no methylmalonyl-CoA (44, 45). We carried out preliminary ^{19}F-NMR studies of cells expressing MatB, NphT7 and PhaB and fed with non-toxic levels of fluoromalonate. Analysis of the media and cell extracts indicated that flux through fluoromalonyl-CoA could reach 100 μM to 1 mM, which is sufficient for use by PKSs in live cells (Table S4). Next, we tested the ability of $DEBS_{Mod6}$+TE to catalyze chain elongation in cell lysates prepared from *E. coli* BAP1 co-expressing $DEBS_{Mod6}$+TE and MatB. Under these conditions, F-TKL is produced with no observable H-TKL upon addition of only NDK-SNAC, fluoromalonate, CoA, ATP, and the ATP regeneration system (Figure S18A). Negative controls with either no $DEBS_{Mod6}$+TE/MatB expressed or no NDK-SNAC substrate show no production of F-TKL (Figure S18A). These results demonstrate that the intracellular level of expression of the $DEBS_{Mod6}$+TE and MatB enzymes is sufficient for the incorporation of the fluorinated extender unit. They also further imply that fluorine could be introduced into the polyketide backbone inside living cells, which are capable of generating ATP through normal metabolic processes. We therefore cultured *E. coli* BAP1 co-expressing $DEBS_{Mod6}$+TE and MatB and harvested the cells after induction. These cells were then fed with the fluoromalonate precursor, which resulted in the production of F-TKL upon addition of NDK-SNAC (Figure 4B, **9;** Figure S18B). The identity of the F-TKL under these conditions were established by LC-MS, co-injection with an authentic standard, as well as high resolution MS. Moreover, F-TKL can also be produced directly in cell culture with the simple addition of a mixture of both substrates to the media after induction of $DEBS_{Mod6}$+TE and MatB (Figure S18C). Taken together, these studies show that the natural selectivity of the polyketide synthase allows for the site-selective introduction of fluorine over hydrogen into the polyketide backbone inside living cells.

To close, we have demonstrated that we can expand the fluorine chemistry of living systems using engineered pathways to link simple biogenic organofluorine building blocks into more complex fluorinated small molecule targets. Because of the modular nature of the biosynthetic pathways used to produce polyketides and related acetate-derived natural products, these findings open the door to general strategies for exploring the fluorine synthetic biology of complex natural products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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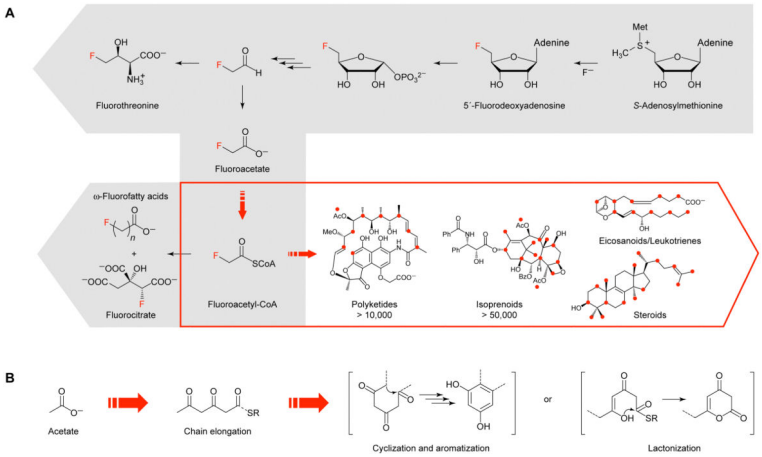
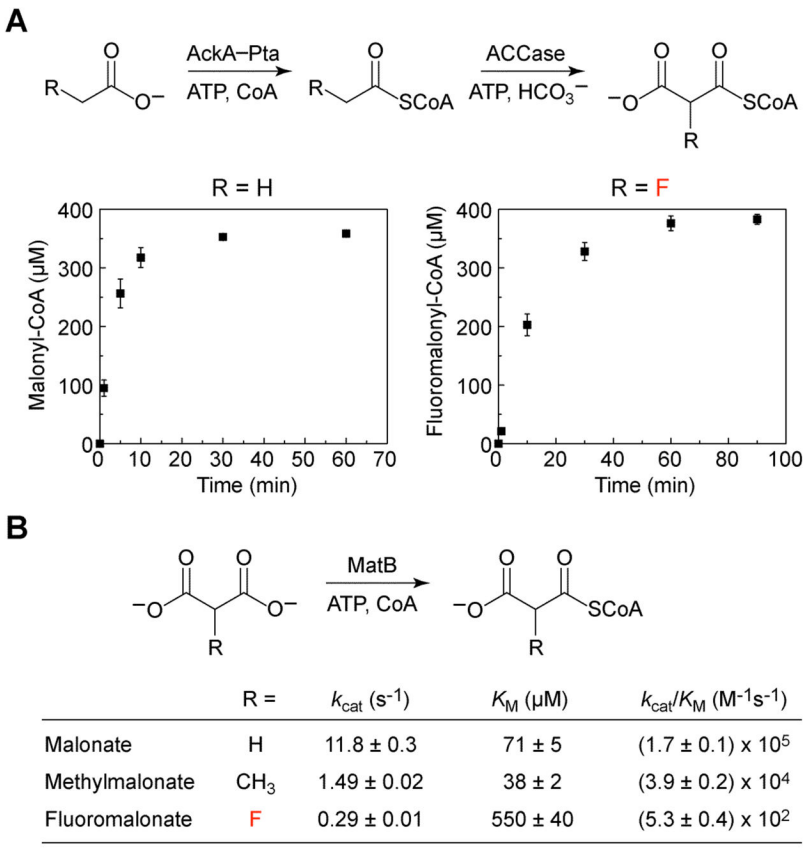


Fig. 1.

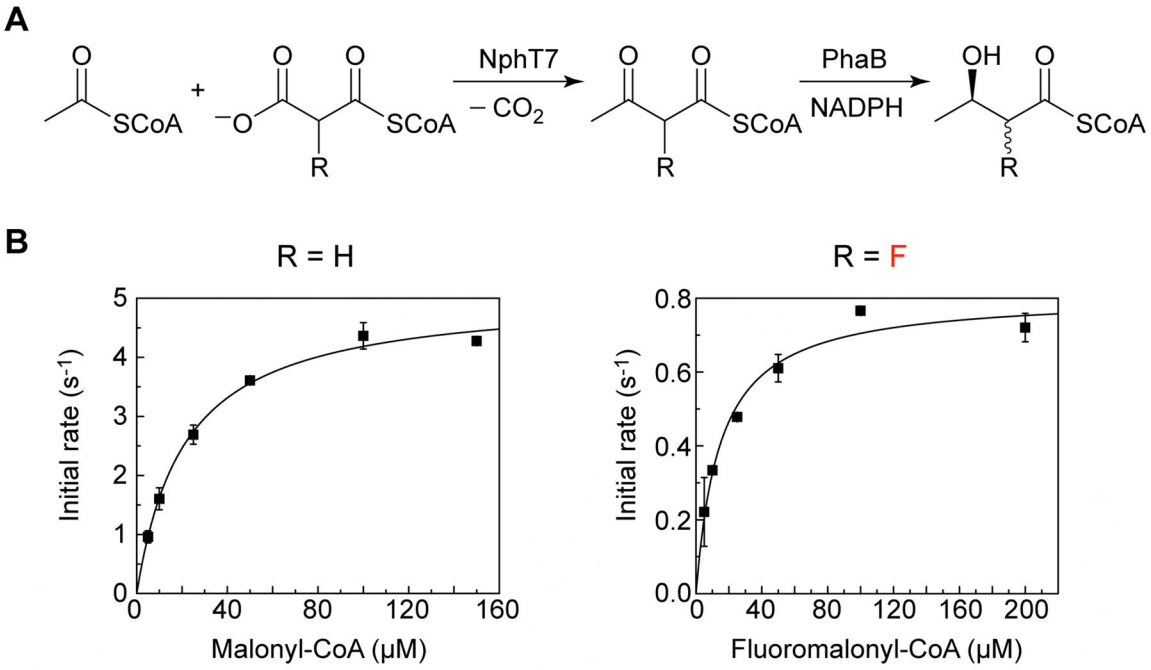
Synthetic biology of fluorine. (**A**) The fluoroacetate pathway and its metabolites represent the known scope of biological fluorine chemistry, starting with fluoride and *S*-adenosylmethionine, to produce fluoroacetate and fluorothreonine as the end products (right to left, grey box). This scope could be greatly expanded by engineering downstream pathways to use fluoroacetate as a building block for introduction of fluorine site-selectively into large families of natural products constructed from acetate backbones (left to right, red box). Red dots represent positions that could in principle be fluorinated by incorporation of a fluoroacetate monomer without altering the carbon skeleton, including locations where fluorine would replace a methyl group derived from propionate or where downstream tailoring steps have occurred on the final structure. (**B**) Assembly of acetate units in the biosynthesis of polyketide natural products.



	R =	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
Malonate	H	11.8 ± 0.3	71 ± 5	(1.7 ± 0.1) x 10^5
Methylmalonate	CH_3	1.49 ± 0.02	38 ± 2	(3.9 ± 0.2) x 10^4
Fluoromalonate	F	0.29 ± 0.01	550 ± 40	(5.3 ± 0.4) x 10^2

Fig. 2.

Enzymatic production of activated extender units for C–C bond formation reactions. (**A**) Formation of malonyl-CoA (left) and fluoromalonyl-CoA (right) from 500 μM CoA and either acetate or fluoroacetate, respectively (AckA, acetate kinase; Pta, phosphotransacetylase; ACCase, acetyl-CoA carboxylase). Values are reported as the mean ± s.d. (n = 3). (**B**) Kinetic parameters for malonate activation (MatB, malonyl-CoA synthetase). Kinetic parameters are reported as mean ± s.e. (n = 3) as determined from non-linear curve-fitting. Error in the k_{cat}/K_M parameter was obtained from propagation of error from the individual kinetic terms.



	R =	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
malonyl-CoA	H	5.1 ± 0.1	21 ± 2	(2.4 ± 0.2) x 10^5
fluoromalonate	F	0.81 ± 0.03	15 ± 2	(5.4 ± 0.7) x 10^4

Fig. 3.

A chain extension and ketoreduction cycle with a fluorinated extender using a simple polyketide synthase, NphT7. (**A**) Reactions catalyzed by NphT7 and PhaB. (**B**) Steady-state kinetic parameters for NphT7-catalyzed C–C bond formation measured using a coupled assay with PhaB. Data points are reported as the mean ± s.d. (n = 3). Kinetic parameters are reported as mean ± s.e. (n = 3) as determined from non-linear curve-fitting. Error in the k_{cat}/K_M parameter was obtained from propagation of error from the individual kinetic terms.

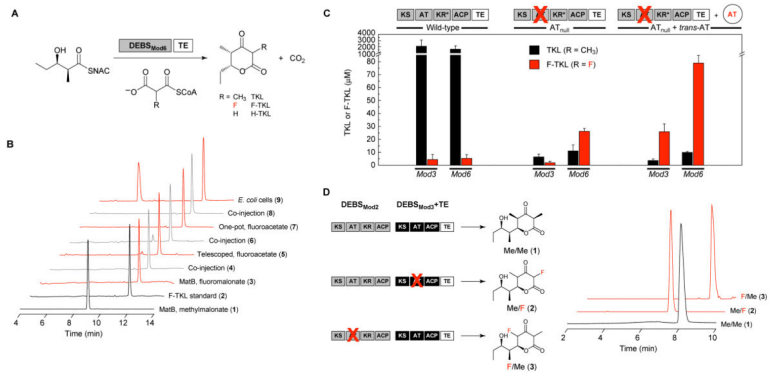


Fig. 4.

Production of fluorinated polyketides *in vitro* and *in vivo* by DEBS$_{Mod6}$+TE. (**A**) Reaction catalyzed by DEBS$_{Mod6}$+TE using the NDK-SNAC substrate with various extender units (NDK-SNAC, native diketide *N*-acetylcysteamine thioester, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester). (**B**) Chain extension by DEBS$_{Mod6}$+TE to form triketide lactones monitored by LC-MS (TKL, *m/z* = 169; F-TKL, *m/z* = 173). CoA, ATP, and ATP regeneration system are included in all *in vitro* reactions. Data are normalized with respect to the TKL peak. (**C**) Selectivity of DEBS$_{Mod6}$+TE and DEBS$_{Mod3}$+TE for methylmalonyl-CoA vs. fluoromalonyl-CoA extender unit as monitored by TKL (*m/z* = 169) and F-TKL (*m/z* = 173) formation. Conditions include wild-type modules, AT^0 modules, and AT^0 modules in conjunction with the *trans*-AT from the disorazole PKS (DszsAT). Values are reported as the mean ± s.d. (*n* = 3). (KR*, the KR domain of Mod3 is inactive). (**D**) LC-MS traces showing regioselective tetraketide lactone formation using the DEBS mini-PKS consisting of DEBS$_{Mod2}$ and DEBS$_{Mod3}$+TE (Me/Me, 2-methyl-4-methyl-tetraketide lactone, *m/z* = 227; Me/F, 2-fluoro-4-methyl-tetraketide lactone, *m/z* = 231; F/Me, 2-methyl-4-fluoro-tetraketide lactone, *m/z* = 231). Me/Me was produced using DEBS$_{Mod2}$/DEBS$_{Mod3}$+TE and methylmalonate (**1**). Me/F was produced using DEBS$_{Mod2}$/DEBS$_{Mod3}$AT^0+TE, DszsAT, methylmalonyl-CoA, and fluoromalonate (**2**). F/Me was produced using DEBS$_{Mod2}$AT^0/DEBS$_{Mod3}$+TE, methylmalonyl-CoA, and fluoromalonate (**3**). Data are normalized with respect to the Me/Me peak. All reactions contained MatB and the ATP regeneration system.