Monitoring Biocatalytic Transformations Mediated by Polyketide Synthase Enzymes in Cell Lysate via Fluorine NMR

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Abstract: The biocatalytic employment of modular polyketide synthase enzymes in cell lysate has become a viable route to preparative quantities of synthetically valuable polyketide fragments. We report the quantitative, uninvasive, and continuous monitoring of such biocatalytic reactions by observing trifluoromethyl-bearing substrates via ^{19}F NMR spectroscopic analysis. To demonstrate the utility of this technique, we followed reactions catalyzed by a thioesterase and several ketoreductases.

Key words: biosynthesis, biocatalysis, polyketide synthase enzymes, polyketide, fluorine NMR

Modular polyketide synthases (PKSs) are enzymatic assembly lines that produce complex polyketides such as the antibacterial erythromycin and the antifungal amphotericin.[1] Employing the catalytic power of PKS enzymes to generate polyketides possessing desired substituents and stereochemistries has long been a goal in biosynthetic engineering. Recently, advances in employing isolated PKS enzymes as biocatalysts have enabled access to preparative quantities of polyketide fragments.[2] These biocatalytic reactions are performed in the cell lysate of the overexpression host (e.g., *Escherichia coli*), thus maximizing the quantity of enzyme and avoiding resource-intensive protein purification. Because cell lysate is a complex mixture of all the soluble biomolecules produced by the overexpression host, other fates are possible for a substrate entered into such a system in addition to the desired transformation. To evaluate such biocatalytic reactions we sought a quantitative and uninvasive technique that could continuously monitor both substrates and products.

To these ends, NMR spectroscopy would be useful; however, observing the conversion of substrate into product via ^{1}H NMR spectroscopic analysis is complicated by the high background generated by the hydrogen-containing components of biocatalytic reactions (e.g., biomolecules, co-substrates, buffering agent, glycerol, DMSO) and by significant noise from water even when suppression methods are employed. However, if the substrates contained a trifluoromethyl group and were observed by ^{19}F NMR spectroscopic analysis, no background would interfere with measurements because *E. coli* does not incorporate fluorine into any of its biomolecules (few organisms do).[3] Furthermore, the ^{19}F nucleus is (i) 83% as intrinsically sensitive as ^{1}H, (ii) 100% naturally abundant, (iii) isosteric with hydrogen, and (iv) very responsive to its electronic environment.[4] These properties have been exploited in drug discovery efforts; one technique, called FABS (fluorine atoms for biochemical screening), identifies inhibitors by detecting a decrease in the rate of conversion from a fluorinated substrate into its product by ^{19}F NMR spectroscopic analysis.[5]

First, we observed a hydrolysis reaction mediated by the erythromycin thioesterase (EryTE), which is known to catalyze the hydrolysis of acyl thioesters.[2b,6] We sought to determine how accurately the kinetics of EryTE-mediated hydrolysis could be measured within the cell lysate by ^{19}F NMR spectroscopic analysis compared to the more traditional technique of employing HPLC and a UV detector. Thus, 3,3,3-trifluoropropionyl-*S*-*N*-acetylcysteamine (NAC; **1**) was incubated in EryTE-containing cell lysate both in an NMR tube and in a separate vessel so that for every spectrum acquired, a sample was also quenched for later HPLC analysis. ^{19}F NMR spectroscopic analysis (without proton decoupling) yielded a triplet for each species due to splitting of the fluorine resonance by the adjacent methylene hydrogens. The triplet of substrate **1** appeared at δ = –63.00 ppm, while the 3,3,3-trifluoropropionate product (**2**) showed triplets at δ = –63.55 and –63.70 ppm, possibly due to two different interactions with counterions (Figure 1). Kinetic characterization was performed by measuring the change in concentration of **1** by both ^{19}F NMR spectroscopic analysis and reversed-phase HPLC. The determined k_{cat} and K_m parameters agreed within the error limits. Thus, ^{19}F NMR spectroscopic analysis accurately measured the kinetics of EryTE on **1** in cell lysate (k_{cat} = 0.077 ± 0.010 s^{-1}, K_m = 39.3 ± 4.5 mM, k_{cat}/K_m = 1.97 ± 0.14 $M^{-1}s^{-1}$). Previous kinetic analysis of EryTE towards similar, unfluorinated thioester substrate analogs gave comparable values.[6] Kinetic analysis is not only more facile by ^{19}F NMR spectroscopic analysis than by HPLC, but also more informative — generation of the nonchromophoric product **2** was observed in the EryTE reaction.

We next sought to monitor a more advanced biocatalytic transformation driven by an NADPH-regeneration system comprised of glucose dehydrogenase, $NADP^+$, and glucose.[2a,7] Thus, the conversion of 3-oxo-5,5,5-trifluoropentanoyl-*S*-NAC (**3**) into (3*R*)-hydroxy-5,5,5-trifluoro-

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pentanoyl-*S*-NAC (**4**) by the KR from the first module of the tylosin PKS (TylKR1) was observed by ^{19}F NMR spectroscopic analysis (Figure 2). The triplet from substrate **3** appeared at δ = –63.18 ppm, while the triplet from product **4** (confirmed by mass spectrometry and NMR analysis) appeared at δ = –63.60 ppm. The rate of the reaction measured by ^{19}F NMR spectroscopic analysis concurred with the rate measured by HPLC (at 30 mM of **3**, V_o was calculated to be 0.20 ± 0.02 mM·min^{-1} by ^{19}F NMR spectroscopic analysis and 0.23 ± 0.05 mM·min^{-1} by HPLC), approximately matching the rate anticipated from previous kinetic studies of TylKR1 on (2*RS*)-methyl-3-oxopentanoyl-*S*-NAC (0.81 mM·min^{-1}).[7a]

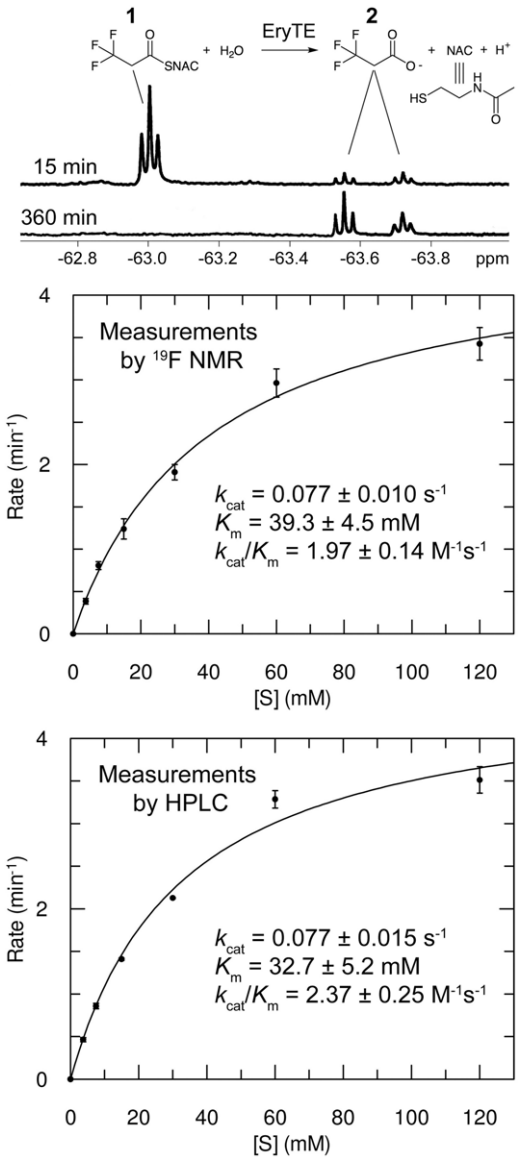

Figure 1 Accurate kinetic characterization of EryTE-mediated hydrolysis of fluorinated **1** in cell lysate via ^{19}F NMR spectroscopic analysis

We then sought to determine whether the terminal fluorine atoms in an ε-trifluoro, α-substituted β-ketothioester were sensitive enough to distinguish stereochemical differences generated at the α- and β-carbon atoms by stereocontrolled, KR-mediated reductions. Thus, 2-methyl-3-oxo-5,5,5-trifluoropentanoyl-*S*-NAC (**5**) was incubated with each of the four KRs: AmpKR2 (the KR from the second module of the amphotericin PKS), RifKR7 (the KR from the seventh module of the rifamycin PKS), TylKR1, and EryKR1 (the KR from the first module of the erythromycin PKS), which are known to set (2*R*,3*S*), (2*S*,3*S*), (2*R*,3*R*), and (2*S*,3*R*) stereochemistries, respectively, when reducing 2-methyl-3-oxopentanoyl-*S*-NAC (Figure 3).[2a,7,8] The triplet for substrate **5** appeared at δ = –63.23 ppm. Notably, the triplets for the *syn*- and *anti*-products were well-resolved (0.34 ppm); the triplet for the *syn*- products **6** and **9** appeared at δ = –63.68 ppm, and the triplet for *anti*- products **7** and **8** appeared at δ = –63.34 ppm. That the diastereomers could be readily distinguished within cell lysate highlights the sensitivity of this technique.

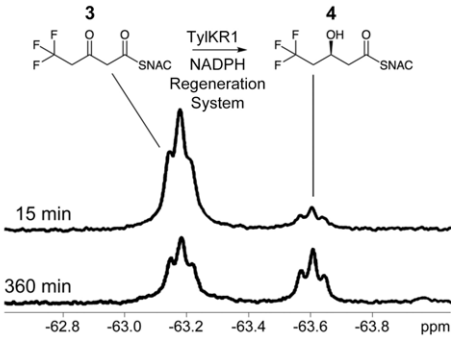

Figure 2 Monitoring biocatalysis mediated by TylKR1 in cell lysate

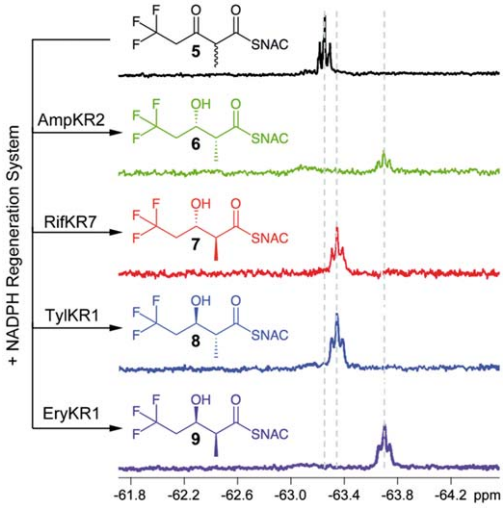

Figure 3 ^{19}F NMR resolution of diastereomers generated by stereocontrolled, KR-mediated biocatalysis in cell lysate

Whether the trifluoromethyl-bearing substrate analogs would be processed in the same manner as the nonfluorinated substrates normally entered into these biocatalytic reactions was initially unclear; however, EryTE and the KRs were shown to catalyze the desired transformations, stereoselectively in the case of the KRs.[2a] Studies report-

ing the acceptance of monofluorinated thioesters by the erythromycin PKS and the studies presented here suggest that PKS enzymes are generally tolerant towards fluorinated substrates and that biocatalytic syntheses of polyketides can be reliably evaluated by ^{19}F NMR spectroscopic analysis.[9] The ability to follow nonchromophoric substrates by ^{19}F NMR spectroscopic analysis enables monitoring of substrates and products in biocatalytic reactions generating reduced polyketides such as triketide lactone chiral building blocks. The rapid detection of thioester hydrolysis or other undesired transformations will facilitate the optimization of biocatalytic reactions performed in cell lysate.

We also seek to observe and optimize biocatalytic transformations mediated by other PKS enzymes such as dehydratases (DHs), which convert β-hydroxy intermediates into α,β-unsaturated intermediates, enoylreductases (ERs), which stereoselectively reduce α,β-unsaturated intermediates, ketosynthases (KSs), which form a carbon–carbon bond to elongate an intermediate, and PKS modules, which both elongate and process intermediates. The monitoring of whole-cell biocatalysis in which fluorinated precursors are fed to microbes expressing PKS enzymes may also be possible through in-cell NMR spectroscopic analysis.[10]

In summary, we have established a facile and powerful technique to monitor reactions in complex media that will aid in the optimization of biocatalytic transformations that generate preparative quantities of complex polyketides.

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