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Coupled Methyl-Group Epimerization and Reduction by Polyketide Synthase Ketoreductase Domains. Ketoreductase-Catalyzed Equilibrium Isotope Exchange

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Abstract

Incubation of [2-^{2}H]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SACP ([2-^{2}H]-**1a**) with the epimerizing ketoreductase domain EryKR1 in the presence of a catalytic amount $NADP^+$ (0.05 equiv) resulted in time-and cofactor-dependent washout of deuterium from **1a**, as a result of equilibrium isotope exchange of transiently generated [2-^{2}H]-2-methyl-3-ketopentanoyl-ACP (**2**). Incubations of [2-^{2}H]-(2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-SACP ([2-^{2}H]-**1b**) with RifKR7 and with NysKR1 also resulted in time-dependent loss of deuterium. By contrast, incubations of [2-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SACP ([2-^{2}H]-**1c**) and [2-^{2}H]-(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SACP ([2-^{2}H]-**1d**) with the non-epimerizing ketoreductase domains EryKR6 and TylKR1, respectively, did not result in any significant washout of deuterium. The isotope exchange assay directly establishes that specific polyketide synthase ketoreductase domains also have an intrinsic epimerase activity, thus enabling mechanistic analysis of a key determinant of polyketide stereocomplexity.

Modular polyketide synthases are responsible for the biosynthesis of a wide range of pharmacologically active compounds remarkable for their structural and stereochemical complexity. Since the characterization of the genes that encode the polyketide synthase (PKS) that is responsible for biosynthesis of 6-deoxyerythronolide B (6-dEB), the parent macrolactone of the erythromycin family of antibiotics, hundreds of microbial PKS genes have been identified.[1] During the PKS-catalyzed assembly of complex polyketides, each module is responsible for a single round of polyketide chain elongation and functional group modification that sets the oxidation level and stereochemistry of the growing polyketide chain before it is passed to the immediately downstream module prior to the succeeding round of chain elongation.[2] Since the first codification of macrolide and polyether polyether stereochemistry by Celmer,[3] numerous biosynthetic investigations have sought to elucidate the mechanistic basis for this stereochemical complexity.[4]

Specific combinations of recombinant [KS][AT] didomains and ACP domains derived from PKS modules can stereospecifically convert acyl-*N*-acetylcysteamine (SNAC) primers and methylmalonyl-CoA chain extenders to (2*R*)-2-methyl-3-ketoacyl-ACP intermediates.[5] *In*

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ASSOCIATED CONTENT

Supporting Information. Experimental procedures, including synthesis of deuterated substrates, LC-MS and GCMS data, isotope exchange data and analysis, and kinetic data, as well as structures of representative polyketides. This material is available free of charge via the Internet at http://pubs.acs.org.

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situ reduction of these ACP-linked 3-ketoacyl thioesters by recombinant KR domains in the presence of NADPH yields single diastereomers of the corresponding 2-methyl-3-hydroxyacyl-ACP products.[6a] Thus EryKR1, derived from module 1 of the 6-deoxyerythryonolide B (6-dEB) synthase, generates exclusively (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**1a**) from transiently generated (2*R*)-2-methyl-3-ketopentanoyl-ACP (**2**),[5a] while RifKR7 from module 7 of the rifamycin synthase and NysKR1 from module 1 of the nystatin synthase each generate the diastereomeric (2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP (**1b**) from **2** (Scheme 1).[6b] In like manner, EryKR6, from module 6 of the 6-dEB synthase, yields (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP (**1c**),[5a] while TylKR1, from module 1 of the tylactone synthase, converts **2** to (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**1d**).[6c] The absolute configuration of each reduced 2-methyl-3-hydroxyacyl thioester (**1a–1d**) precisely matches the stereochemistry of the known or presumed polyketide chain elongation intermediate that is generated by the responsible KR domain within its native, full-length polyketide synthase.

The reduction products **1a** and **1b** that are generated by EryKR1 and by RifKR7 or NysKR1, respectively, result from both epimerization of the original (2*R*)-2-methyl substituent of **2** and subsequent stereospecific ketoreduction. By contrast, EryKR6 and TylKR1 simply catalyze only stereospecific reduction on opposite faces of the 3-keto group of the ACP-bound substrate **2**, without epimerizing the 2-methyl group. The comparable rates of reduction of transiently generated **2** by EryKR1 and by EryKR6, which are 2–3 orders of magnitude faster than the estimated rate of buffer-catalyzed epimerization of the 3-ketoacyl thioester intermediate, suggests, but does not prove, that the EryKR1 domain alone is responsible for catalysis of both the epimerization and reduction steps.[5a,7]

The use of a KS domain for the *in situ* chemoenzymatic generation of the requisite (2*R*)-**2**, coupled with the modest configurational lability of this 3-ketoacyl thioester, even at neutral pH, has precluded studying KR-catalyzed reductions of **2** in isolation.[8] Although diastereospecific KR-catalyzed reduction of (2*RS*)-2-methyl-3-ketoacyl-*S*-ACP thioesters[6b] as well as of racemic (2*RS*)-2-methyl-3-ketoacyl-*S*-pantetheine and (2*RS*)-2-methyl-3-ketoacyl-SNAC substrates can also yield single diastereomers of the corresponding 2-methyl-3-hydroxyacyl thioesters,[4d,9] such experiments cannot distinguish between mere diastereospecific reduction of the appropriate (2*R*)- or (2*S*)- component of the (2*RS*)-2-methyl-3-ketoacyl thioester substrate and an active catalytic role for the KR domain in the actual epimerization of the configurationally labile 2-methyl-3-ketoacyl thioester.

In order to define rigorously the role of specific KR domains in both reduction *and* epimerization of ACP-bound substrates, we required an unambiguous analytical method that would meet the following key experimental requirements: 1) No PKS enzyme other than the relevant KR domain should be present during the assay; 2) It must be possible to prepare, if only transiently, diastereomerically pure samples of both the (2*R*)- and the (2*S*)-2-methyl-3-ketoacyl-ACP substrates; and 3) The lifetime of transiently generated 2-methyl-3-ketoacyl-ACP intermediates should be kept to a minimum, in order to avoid competing, buffer-catalyzed methyl group epimerization. Fortunately, these prerequisites can all readily be satisfied by taking advantage of the intrinsic reversibility of the reductase–dehydrogenase reaction and using LC-ESI(+)-MS-MS to monitor the KR-catalyzed, time- and cofactor-dependent equilibrium isotope exchange of reduced, configurationally stable, [2-^{2}H]-2-methyl-3-hydroxypentanoyl-ACP thioesters ([2-^{2}H]-**1a–1d**). Any loss of deuterium will be the consequence of reductase-catalyzed epimerization of the transiently formed, oxidized intermediate, [2-^{2}H]-2-methyl-3-ketopentanoyl-ACP (**2**) (Scheme 2).

To assay the expected epimerase activity of EryKR1, chemoenzymatically prepared [2-^{2}H]-(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SEryACP6 ([2-^{2}H]-**1a**) (300 μM, 45 nmol, 1

equiv),[10] was incubated with EryKR1 (75 μM, 11.25 nmol)[11] in the presence of a catalytic quantity of cofactor $NADP^+$ (15 μM, 2.25 nmol, 0.05 equiv) at room temperature in pH 7.2 phosphate buffer (Scheme 2a). Samples were withdrawn at periodic intervals from 0 to 60 min and analyzed directly by LC-ESI(+)-MSMS. The loss of deuterium from recovered **1a** was quantitated by analysis of the isotopic content of the derived 2-methyl-3-hydroxypantetheinate ejection fragment (**3**) (d_0 *m/z* 375.3; d_1 *m/z* 376.3), produced by collision-induced dissociation (Figure 1).[12] Assays of three independent incubations revealed approximate first-order loss of 55±1% of the original deuterium after 60 min incubation (Figure 2, Table S1).[13] Supplementing with additional mixtures of 11.25 nmol EryKR1 and 2.25 nmol $NADP^+$ at 1, 2, and 3 h, resulted in >95% washout of deuterium after 4 h total incubation time.

Control experiments carried out in the absence of added $NADP^+$ resulted in <10% loss of deuterium from **1a** after 1 h, attributable to the presence of trace amounts of residually bound $NADP^+$ cofactor in the purified EryKR1 preparation. Omission of EryKR1 abolished isotope exchange altogether. With increased concentrations of $NADP^+$, LC-ESI(+)-MS-MS allowed detection of small quantities of the oxidized intermediate, d_0- and d_1-2-methyl-3-ketopentanoyl-ACP (**2**), as evidenced by the characteristic pantetheinate ejection fragments at *m/z* 373.3 and 374.3. To verify the structure and stereochemistry of the recovered isotope–exchanged sample of (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-EryACP6 (**1a**), a 15-min incubation mixture was treated with aq. NaOH for 20 min at 65 °C thereby quenching the enzyme reaction and hydrolyzing the acyl-ACP thioester. The resulting acid was methylated with trimethylsilyldiazo-methane ($TMSCHN_2$) (Scheme 3). Chiral GC-MS analysis, as previously described,[5a,6b] confirmed that the recovered product consisted of diastereomerically pure methyl (2*S*,3*R*)-2-methyl-3-hydroxypentanoate (**4a**) (d_0 ~25%, d_1 ~75%) (Figure S10).

Both NysKR1 and RifKR7 have each been implicated in both reduction and epimerization of (2*R*)-2-methyl-3-ketoacyl-ACP substrates to give the diastereomeric (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP product.[6b] Accordingly, NysKR1 and RifKR7 were individually incubated with [2-^{2}H]-(2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP (**1b**, 1 equiv) in the presence of catalytic $NADP^+$ (0.05 equiv) under the same conditions used for the incubations with EryKR1 (Scheme 2b). Monitoring of each incubation by LC-ESI(+)-MS-MS confirmed the predicted enzyme-dependent equilibrium isotope exchange of the reduced substrate, as exemplified by the loss after 1 h of 53±1% and 48±2%, respectively, of the original deuterium label in the recovered (2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP (**1b**) (Figures 1 and 2, Table S1). The loss of the label was calculated from the isotopic distribution of the derived pantetheinate ejection fragment **3**. Chiral GC-MS analysis of the derived methyl ester (2*S*,3*S*)-**4b** confirmed the retention of the original configuration of (2*S*, 3*S*)-**1b** during the equilibrium isotope exchange experiment (Scheme 3, Figure S11).

In contrast to the behavior of these 3 epimerizing KR domains, analogous 60-min incubations of EryKR6 and TylKR1 with their cognate reduced diketides, [2-^{2}H]-(2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP (**1c**) and [2-^{2}H]-(2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP (**1d**), respectively, resulted in insignificant washout of deuterium from recovered substrate, as established by time-dependent LC-ESI(+)-MS-MS analysis of the derived pantetheinate ejection fragment **3** (Scheme 2c and 2d, Figure 2, Table S1). These results not only confirm the expected absence of epimerase activity associated with either EryKR6 or TylKR1, but also serve as important controls that definitively rule out adventitious buffer-catalyzed or ACP-promoted exchange of the labile, transiently generated [2-^{2}H]-(2*S*)-2-methyl-3-ketopentanoyl-ACP intermediate **2** as the cause of deuterium washout during the incubations with the epimerizing ketoreductase domains EryKR1, RifKR7, and NysKR1.[14]

Almost every known PKS harbors at least one epimerizing KR domain. (For representative polyketides and the relevant KR domains, see Figure S14.) The specific catalytic strategy by which a KR domain mediates the epimerization of a 2-methyl-3-ketoacyl-ACP through the presumed intermediacy of a conjugated enol or enolate[15] remains to be elucidated. Detailed comparisons of the structures of several KR proteins as well as extensive sequence alignments of KR domains of known or inferred stereospecificity have allowed correlation of a small number of conserved sequence motifs with the diastereospecificity of individual ketoreductase reactions.[16,17] On the other hand, these motifs do not reveal any obvious acidic or basic residues uniquely associated with epimerizing KR domains. The use of the equilibrium isotope exchange assay to monitor epimerization, combined with targeted mutation of epimerizing KR domains, should allow systematic analysis of the molecular basis of KR-catalyzed methyl-group epimerization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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2. Each homodimeric Type I PKS module harbors 1) an acyl carrier protein (ACP) domain with a 18 Å phosphopantetheinyl group to which the growing polyketide chain is tethered as an acyl thioester, 2) an acyltransferase (AT) domain that loads specific malonyl or (2*S*)-methylmalonlyl chain extenders onto the terminal thiol of the ACP side chain, and 3) a ketosynthase (KS) domain that catalyzes the chain extension reaction, a decarboxylative condensation between the ACP-bound malonyl or methylmalonyl residue and an acyl thioester donated by the immediately upstream PKS module so as to generate a 3-ketoacyl-ACP or (2*R*)-2-methyl-3-ketoacyl-ACP. Additional β-carbon-modifying domains that may be found within a PKS module are a ketoreductase (KR), a dehydratase (DH), and an enoylreductase (ER. The resultant extended and modified polyketide chain is then passed to the immediately downstream module for a further round of chain elongation and processing.

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7. Although methyl 2-methylacetoacetate shows $t_{0.5}$ 4.7 min for deuterium exchange at pD 7.2, corresponding to k_{exch} 0.15 min^{-1}, the corresponding (2*R*)-2-methyl-3-ketoacyl-ACP triketides undergo no more than 5–15% buffer-catalyzed epimerization after 60 min at pH 7.2, corresponding to a $k_{epim\text{-}buff}$ <0.003 min^{-1} (ref 5a,6a). The remarkable role of the ACP in slowing epimerization of bound substrate by a factor of >500 has not as yet been satisfactorily explained.
8. The demonstrated (2*R*)-methyl configuration of the transiently generated 2-methyl-3-ketoacyl-ACP intermediate (**2**) is independent of the which module the KS domain has come from (cf ref 5a,6a), implying that KS domains do not play an active role in any eventual epimerization of the C-2 methyl group.
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10. Purified samples of [2-^{2}H]-**1a–1d** were prepared from the corresponding diastereomerically pure, synthetic [2-^{2}H]-2-methyl-3-hydroxypentanoic acids via the corresponding –SCoA esters, using *apo*-EryACP6 and the surfactin phoshopantetheinyl transferase Sfp. See Supporting Information for experimental details.
11. The reductase activity of each of the recombinant KR domains was assayed using the standard reference substrate (9*RS*)-*trans*-1-decalone with continuous UV monitoring of the rate of consumption of NADPH (cf ref 16). All KR domains had V_{max} within 20% that of EryKR1, V_{rel} 100: NysKR1, V_{rel} 88; RifKR7, V_{rel} 93; EryKR6, V_{rel} 107; TylKR1, V_{rel} 121 (See Figure S13 for rates of KR-catalyzed oxidation).
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13. The observed rate of EryKR1-catalyzed deuterium washout does not represent the intrinsic first-order rate for the EryKR1-catalyzed epimerization reaction. The concentration of $NADP^+$ (15 μM) is ~100 times lower than the K_m for $NADP^+$ of 1.3±0.3 mM for the EryKR1-catalyzed oxidation of the model substrate (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC. The observed rate of EryKR1-catalyzed equilibrium isotope exchange (~0.01 min^{-1}) is thus <1% of the true V_{max} for EryKR1-catalyzed 2-methyl epimerization.
14. Both EryKR6 and TylKR1 catalyze the reversible oxidation of their cognate substrates **1c** and **1d**, respectively, as established by measurement of the kinetics of NADPH formation as well as direct LC-MS-MS detection of the corresponding [2-^{2}H]-**2** (Figures S12 and S13). Each KR is also completely stereospecific for oxidation of only the natural diastereomer of its reduced substrate (Figures S12). The measured low level (2–8%) washout of deuterium from incubation of the non-epimerizing EryKR6 and TylKR1 domains with the exchange-inert **1c** and **1d**, which probably represents buffer-catalyzed exchange of the transiently generated 3-ketoacyl-ACP **2**, also provides evidence for the formation of **2** by both EryKR6 and TylKR1.
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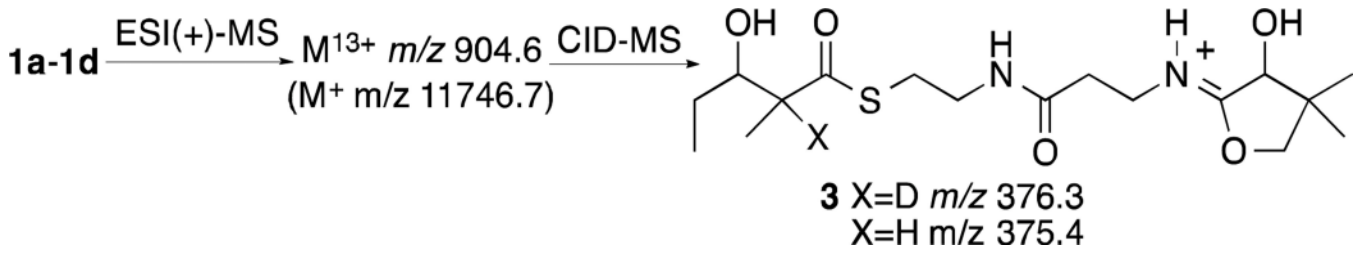


Figure 1.
LC-ESI(+)-MS-MS analysis of the acylpantetheinate ejection fragment **3** produced by collision-induced dissociation (CID) of the primary M^{13+} ion from [2-^{2}H]-**1a–1d**.

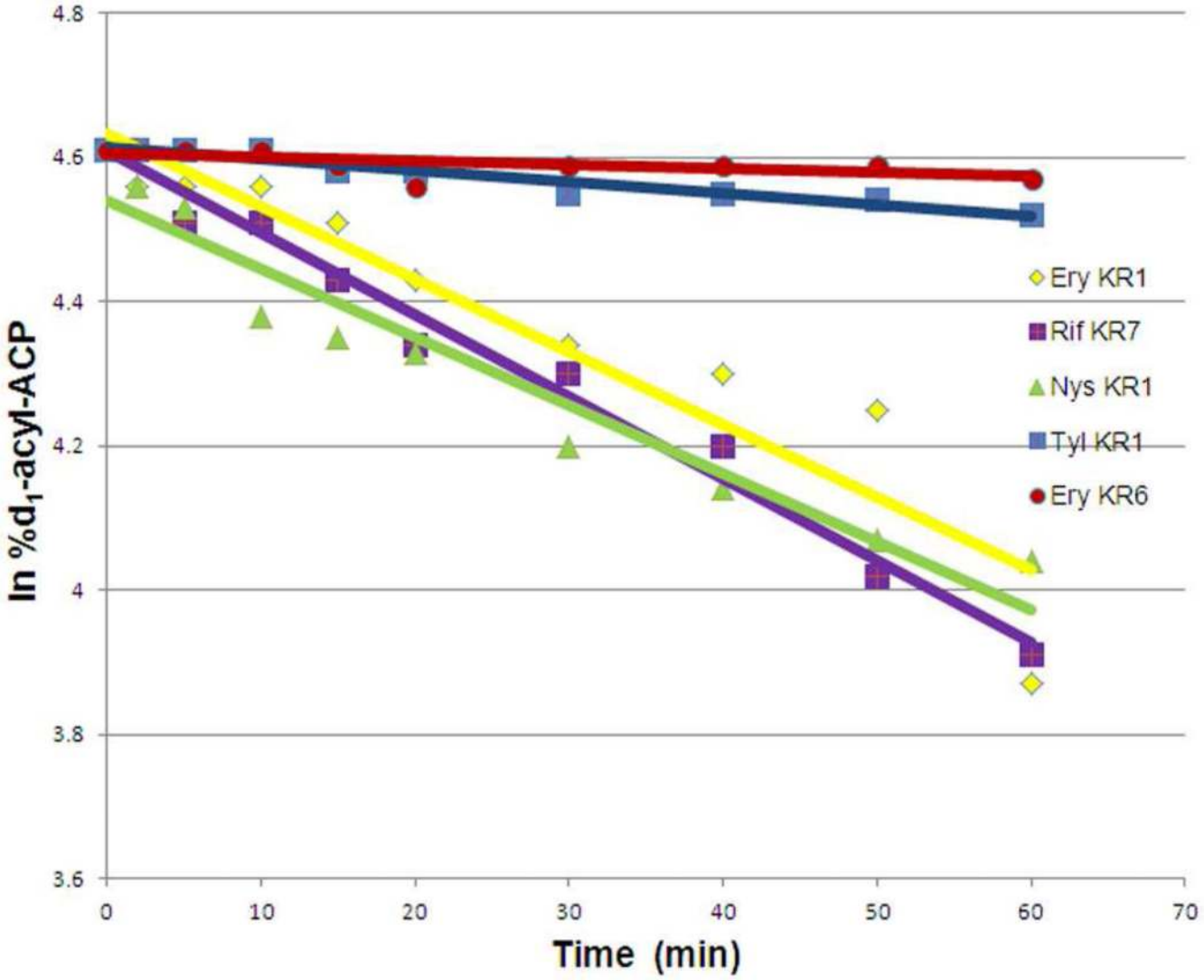
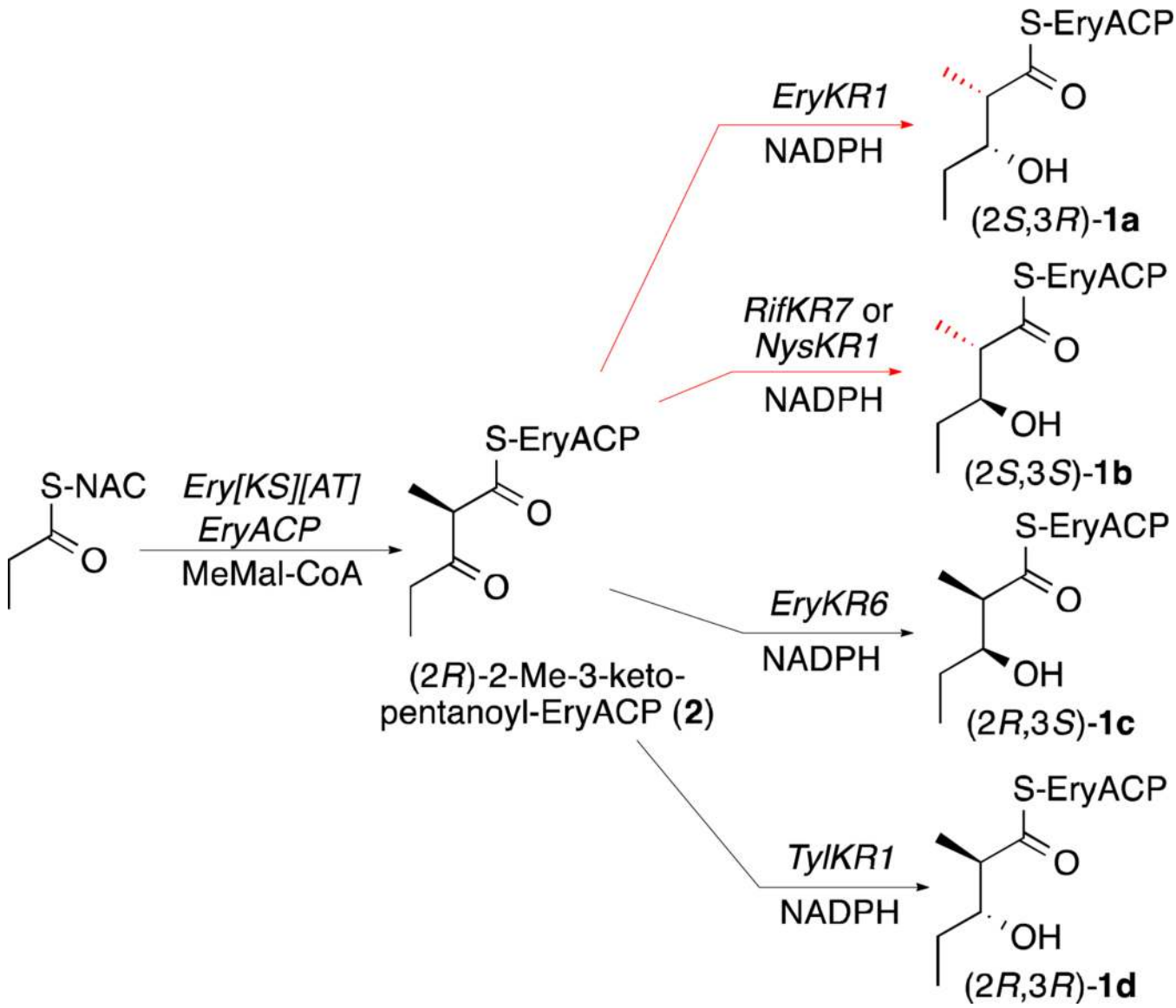
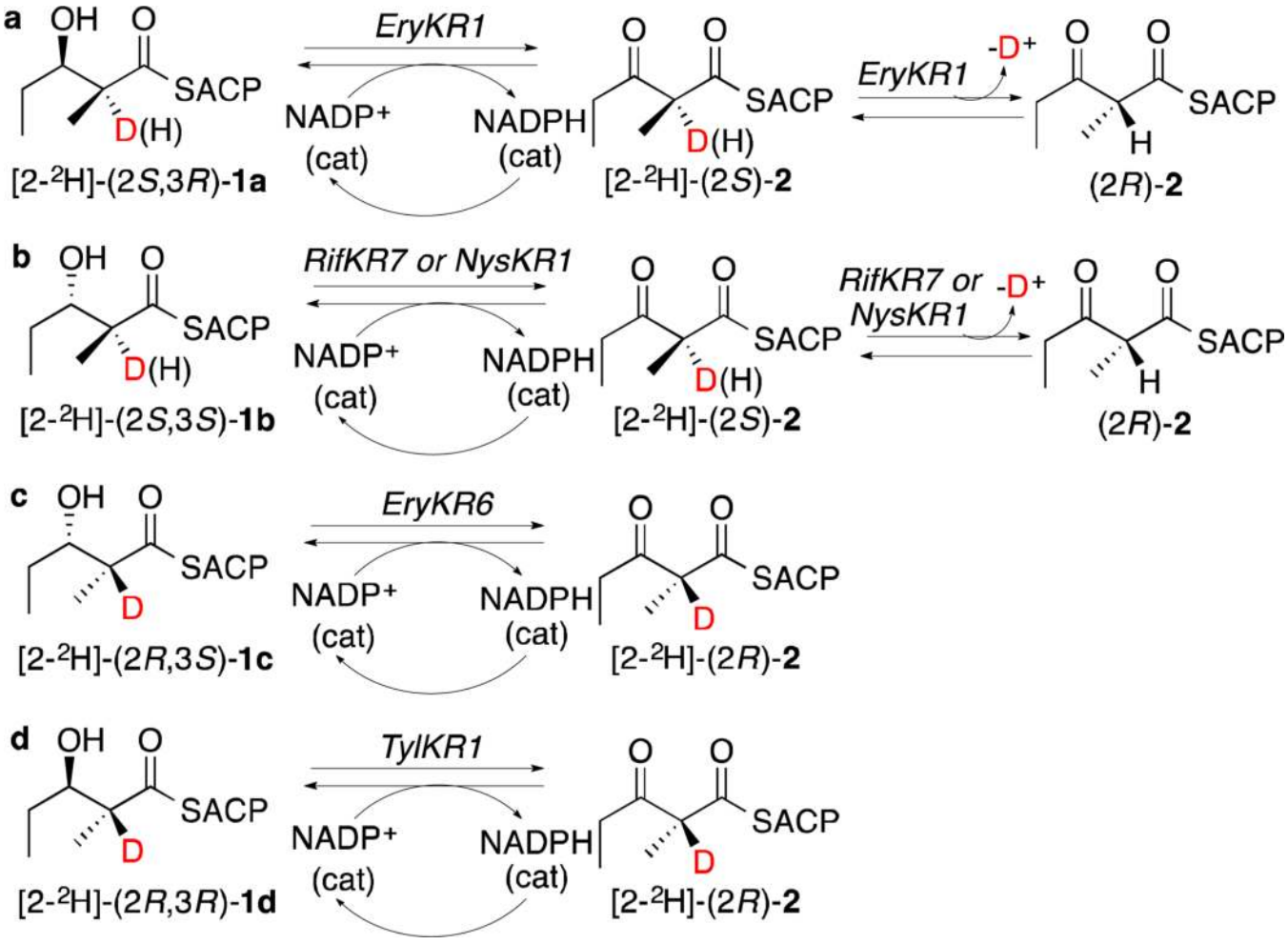


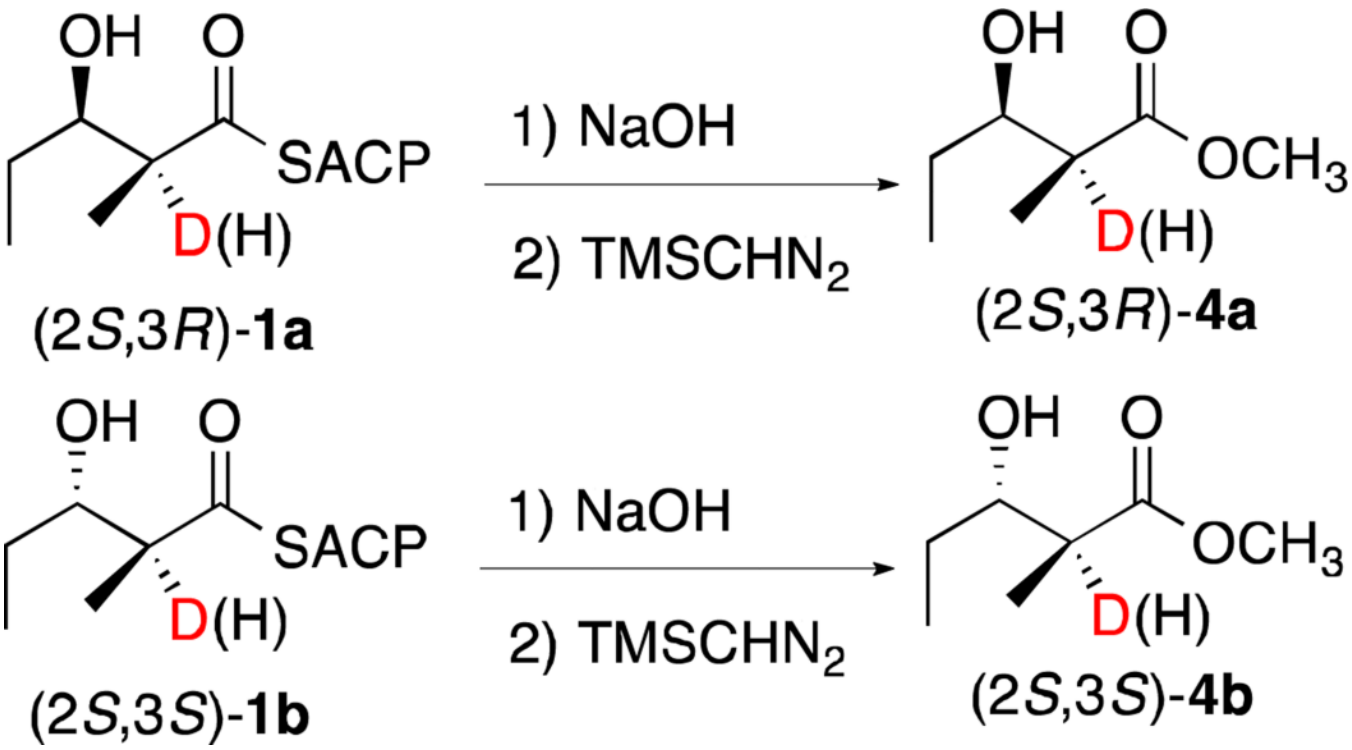
Figure 2.
Plot of ln %d_1-acyl-ACP (**1**) vs time (min) for incubation of **1a – 1d** with KR domains and cat. $NADP^+$.



Scheme 1.



Scheme 2.



Scheme 3.