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Engineered Biosynthesis of Novel Polyketides: *actVII* and *actIV* Genes Encode Aromatase and Cyclase Enzymes, Respectively

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Abstract: The early steps in the biosynthesis of the aromatic polyketide actinorhodin (**1**) are catalyzed by six gene products: the minimal polyketide synthase (PKS) (consisting of a ketosynthase, chain length determining protein, and acyl carrier protein), a ketoreductase (KR), and two additional proteins encoded by the *actVII* and *actIV* genes whose functions have not been perfectly understood. By comparing the structures of polyketides produced by a series of recombinants carrying various combinations of the six genes, the functions of the proteins encoded by *actVII* and *actIV* have been elucidated. Two novel polyketides, SEK34 (**4**) and SEK34b (**5**), were produced by a recombinant strain expressing the *act* minimal PKS, *act* KR, and *actVII* genes (but lacking *actIV*). The structures of these molecules, together with those of previously isolated polyketides from other *act* PKS enzyme combinations, suggest that the protein encoded by *actVII* functions as an aromatase responsible for catalyzing two dehydrations of the first carbocyclic ring, whereas the protein encoded by *actIV* is a cyclase that catalyzes an intramolecular aldol condensation to form the second ring. These results may be extrapolated to related aromatic PKSs to provide a conceptual framework for the rational design and engineered biosynthesis of novel polyketides by manipulating the early aromatization and cyclization steps, in addition to the chain assembly and ketoreduction steps already described.

Polyketides are a large family of structurally diverse natural products with a broad range of biological activities, including antibiotic and pharmacological properties. They are synthesized by multifunctional polyketide synthases (PKSs) which catalyze repeated decarboxylative condensations between acyl thioesters (usually acetyl, propionyl, malonyl, or methylmalonyl). As well as varying the chain length, PKSs introduce structural variability into the product by reducing certain keto groups of the nascent polyketide chain. Some PKSs are also responsible for regio-

specific cyclizations of the nascent chain.^{1,2} Over the past few years, molecular genetics has revealed a high degree of sequence similarity among PKSs.³⁻¹² There is considerable interest in the potential use of PKSs for rational design and genetically

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engineered biosynthesis of novel polyketides, capitalizing on existing knowledge of their structural similarities and differences, and the ways they control product structure.

The actinorhodin (*act*) PKS has served as an excellent model system for genetic and biosynthetic studies aimed at understanding the function and specificity of bacterial aromatic PKS enzymes. Members of this class of PKSs consist of discrete subunits for the various functions involved in chain assembly and modification. Over the past two decades, the isolation and structural characterization of intermediates or shunt products from *act* mutants of *Streptomyces coelicolor* derived by classical mutagenesis have led to the elucidation of a tentative metabolic pathway for actinorhodin biosynthesis.^{13–16} In addition, the cloning, sequencing, and use in complementation experiments of *act* genes have facilitated functional assignments to several PKS genes.^{3,4} However, until recently, the inability to characterize the biosynthetic intermediates or shunt products of some mutant classes, particularly those blocked in the early stages of biosynthesis, had prevented the emergence of a complete, detailed biosynthetic model. In particular, the enzymatic basis for the cyclizations undergone by the nascent polyketide chain has not been well understood. This problem is interesting in light of recent genetic studies of actinorhodin^{3,17} and tetracenomycin (*tcm*)^{6,7} biosynthesis, which implicate the involvement of several structurally unrelated families of proteins in this process.

Recently, we described a specially constructed host–vector system for expressing recombinant *Streptomyces* PKS gene clusters.¹⁸ Characterization of the resulting products has provided new insights into the catalytic functions and specificities of aromatic PKS enzymes. In particular, proteins involved in determining carbon chain length,^{18,19} degree and regioselectivity of ketoreduction,^{19,20} and regioselectivity of cyclization^{20–22} have been analyzed. Our studies have also confirmed and elaborated upon the earlier model of the actinorhodin biosynthetic pathway. For example, it was shown that, in the absence of a ketoreductase (KR), the *act* minimal PKS synthesizes SEK4 (2; Table 1), whereas in the presence of the *act* KR it makes mutactin (3; Table 1). The structural differences between these

Table 1. Products Isolated from Recombinant Strains Containing PKS Genes for the Early Stages of Actinorhodin Biosynthesis

gene ^a	major product	ref
<i>act</i> min PKS	SEK 4 (2)	22
<i>act</i> min PKS + <i>act</i> KR	mutactin (3)	22
<i>act</i> min PKS + <i>act</i> KR + <i>act</i> VII	SEK34 (4)	this work
<i>act</i> min PKS + <i>act</i> KR + <i>act</i> VII + <i>act</i> IV	DMAC (6)	18

^a *act* min PKS consists of three genes (the *actI* genes) encoding the ketosynthase/putative acyltransferase, chain length factor, and acyl carrier protein; the *act* KR gene is also known as *actIII* (see Figure 1).

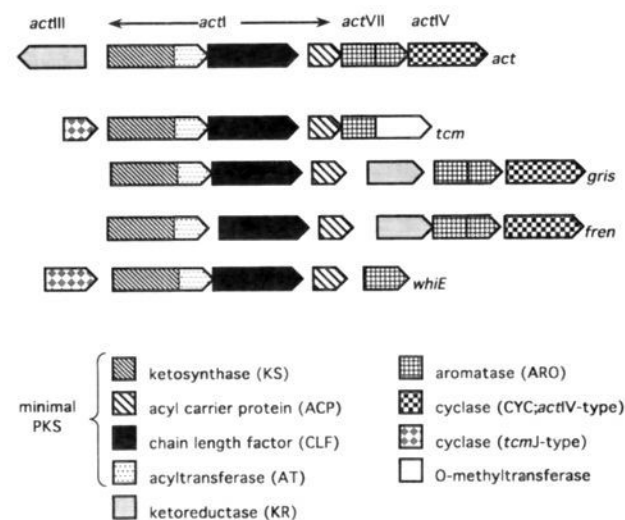


Figure 1. Related bacterial aromatic PKS gene clusters. Arrangements of genes are shown for the PKS gene clusters which produce actinorhodin (*act*) in *S. coelicolor*,^{3,4} tetracenomycin (*tcm*) in *Streptomyces glaucescens*,^{5–7} griseusin (*gris*) in *Streptomyces griseus*⁸ (Yu, T. W., personal communication), frenolicin (*fren*) in *Streptomyces roseofulvus*⁹ Bibb, M. J., personal communication), and the *whiE*-encoded spore pigment in *S. coelicolor*.²⁴ For the *act* cluster, the relevant genes or mutant classes (*actI*, -III, -IV, and -VII) according to Rudd and Hopwood²³ are also shown. Note that the region labeled “acyltransferase” is postulated to transfer the acetate starter unit from CoA to the KS.^{3,5} Also, the *actVII* gene product and its homologs, rationalized in this paper, were previously referred to as cyclases (CYC).

two molecules were rationalized by the inability of the C-11 carbonyl to enolize in the reduced backbone.^{20,22}

Here, we report the isolation of two related novel polyketides from a genetic construct containing the *act* minimal PKS, the *act* KR, and the *actVII* genes but not the *actIV* gene. The structure and deduced biosynthesis of this molecule have allowed us to revise the previously assigned roles of the *actVII* and *actIV* proteins and their homologs in other aromatic polyketide pathways and to propose a more detailed mechanistic model for the early stages of aromatic polyketide biosynthesis.

Results

The genes (*act*) involved in the initial stages of actinorhodin biosynthesis are shown at the top of Figure 1. Earlier cosynthesis studies demonstrated that the order of functions of their protein products in the pathway was I, III, VII, and IV.²³ More recently, several polyketides have been isolated and structurally characterized from transformants of *S. coelicolor* CH999¹⁹ in which genetic constructs containing all or parts of the *act* PKS gene set were expressed (see Table 1). Since the entire *act* gene cluster has been deleted from CH999, these recombinant

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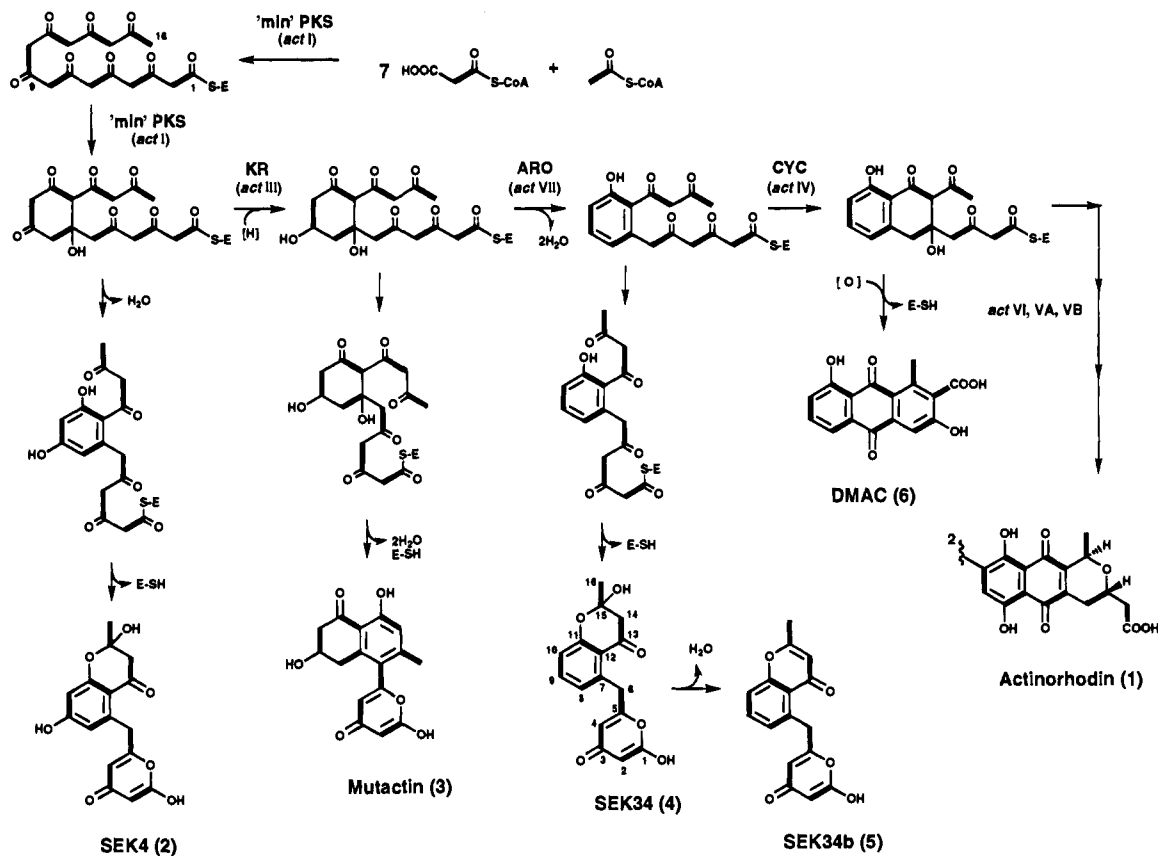


Figure 2. Structures of the novel polyketides SEK34 (4) and SEK34b (5) and the revised biosynthetic pathway for the early steps of actinorhodin (1) biosynthesis. Proposed pathways for the polyketides produced by the gene combinations in Table 1 are shown (biosynthetic intermediates shown have not been isolated and are hypothetical). After biosynthesis of the full length polyketide chain by the minimal PKS, the nascent polyketide undergoes an initial cyclization (also catalyzed by the minimal PKS), ketoreduction (catalyzed by the ketoreductase (KR)), aromatization of the first ring (catalyzed by the aromatase (ARO)), and a second intramolecular aldol condensation (catalyzed by the cyclase (CYC)) (see text for further discussion). In the absence of additional enzymes from the actinorhodin biosynthetic pathway, the intermediate generated by the cyclase is converted into 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC,6), whereas in the presence of these downstream enzymes (encoded by the *actVI*, -*VA*, and -*VB* genetic loci), the same intermediate is converted into actinorhodin (1). It should be noted that it is unknown whether ketoreduction occurs prior to or after the first ring formation.

strains contained only the *act* genes shown in Table 1. Thus, compounds produced by these strains were unaffected by the presence of any other PKS component or downstream enzyme in the actinorhodin pathway (see Figure 2).

In order to determine the specific catalytic properties of the *actVII* and *actIV* products, the plasmid pSEK34, containing the *actI* (minimal PKS), -*III* (KR), and -*VII* genes, was constructed. Introduction of this plasmid by transformation into *S. coelicolor* CH999 resulted in the production of two novel polyketides, SEK34 (4) and its dehydrated analog SEK34b (5). Overall, the ^1H - and ^{13}C -NMR spectra of both molecules were consistent with those of SEK4 (2), an analogous polyketide which has been previously characterized.²⁰ Each contained the characteristic chemical shifts associated with pyrone rings²⁴ also observed in SEK4 (2)²⁰ and mutactin (3).^{15,22} ^1H -coupling patterns also suggested the presence of a reduced aromatic ring (Table 2). The results of sodium [1,2- ^{13}C]acetate feeding experiments were consistent with the acetate incorporation pattern depicted in Figure 2. Both molecules are colorless (white precipitate). SEK34, although unstable in water, was the major polyketide isolated in approximately a 10:1 ratio to SEK34b (4). SEK34b (5) is presumably formed via a dehydration of SEK34 (4) that occurs spontaneously in culture or as a result of workup conditions.

(24) The 2-hydroxy-4-pyrone configuration was chosen over a 4-hydroxy-2-pyrone on the basis of the ^{13}C -NMR chemical shifts of related molecules.^{15,25}

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Given the structural similarity between SEK4 (2)²⁰ and SEK34 (4), it is reasonable to argue that the cyclization pathways of these two molecules are similar (Figure 2). Although no unreduced polyketide analogous to SEK34b has yet been characterized from strains that produce SEK4, mass spectroscopy has suggested the existence of such a molecule (Fu, H., personal communication). In both SEK4 (2) and SEK34 (4), the first cyclization is an intramolecular aldol condensation between the C-7 carbonyl and the C-12 methylene, catalyzed by the minimal PKS.^{20,22} This is followed by a hemiketal formation between C-11 and C-15 and by pyrone ring formation at the carboxyl end of the polyketide chain. A prerequisite for hemiketal formation is the enolization of the C-11 carbonyl. Herein lies the difference between a reduced and unreduced polyketide chain. Whereas the functional expression of the *actVII* gene is required for enolization of the *reduced* intermediate (Figure 2), the corresponding reaction appears to be uncatalyzed in the case of its *unreduced* counterpart. Thus, the *actVII* gene product is deduced to be an aromatase responsible for converting the C-11 carbonyl into its enolic form via dehydration across the C-8/C-9 bond or the C-7/C-12 bond or both. This is consistent with the observation that the *act* minimal PKS and *act* KR alone produce mutactin (3)^{15,22} (Table 1), whose structure can be rationalized as arising due to the inability of the C-11 carbonyl to enolize, which in turn leads to the alternative C-6/C-15 aldol condensation. Furthermore, since the *actIV* gene product is required for catalysis of the "natural" C-5/C-14 aldol condensation, it must be the second ring cyclase

Table 2. ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data from SEK34 (4) and SEK34b (5)^a

carbon ^b	SEK34 (4)			SEK34b (5)		
	^{13}C δ (ppm)	J_{CC} (Hz)	^1H δ (ppm) (m, J_{HH} (Hz), area)	^{13}C δ (ppm)	^1H δ (ppm) (m, J_{HH} (Hz), area)	
1	163.8	80.0	11.58 (s, 1OH)	163.8	11.52 (s, 1OH)	
2	88.2	79.6	5.18 (s, 1H)	88.0	5.14 (s, 1H)	
3	170.4	57.5		170.6		
4	99.5	58.1	5.54 (s, 1H)	99.6	5.46 (s, 1H)	
5	165.6	51.3		165.7		
6	37.5	50.6	4.12 (d, 16.2, 1H) 4.21 (d, 15.8, 1H)	37.3	4.43 (s, 2H)	
7	136.1	58.3		135.8		
8	124.5	58.5	6.97 (d, 8.4, 1H)	128.6	7.56 (d, 7.6, 1H)	
9	135.0	56.5	7.50 (dd, 7.8, 7.9, 1H)	133.4	7.72 (dd, 7.5, 8.4, 1H)	
10	118.0	56.9	6.90 (d, 7.4, 1H)	118.0	7.30 (d, 7.3, 1H)	
11	159.8	60.9		157.4		
12	118.5	61.6		120.8		
13	193.1	39.7		178.3		
14	49.5	39.9	2.63 (d, 15.9, 1H) 3.06 (d, 15.8, 1H)	111.1	6.16 (s, 1H)	
15	100.8	46.2	6.99 (s, 1OH)	165.4		
16	27.5	47.2	1.59 (s, 3H)	19.5	2.35 (s, 3H)	

^a Chemical shift assignments are based on NMR data from analogous molecules.^{15,20} ^b Carbons are labeled according to Figure 2.

(Figure 2). It is still unclear, however, whether this enzyme is involved in the subsequent dehydration following this cyclization.

Discussion

Although the combined application of genetic and chemical methods has led to significant advances in our understanding of actinorhodin biosynthesis, until recently, very little information was available regarding early steps in the pathway. For example, on the basis of the structure of the earliest available shunt product, mutactin (3),¹⁵ it had been proposed that the *actVII* gene product was a cyclase and a first ring dehydratase^{16,17} (hence, its previous designation by us and others as the *act* cyclase (CYC)), whereas the *actIV* gene product was a second ring dehydratase.¹⁶ The structures of SEK4 (2)²⁰ and SEK34 (4) now provide a more complete picture of the early steps in actinorhodin biosynthesis, including evidence for the catalytic functions of the *actVII* and *actIV* proteins. These insights may also be applicable to other aromatic PKSs since homologs of the *act* aromatase and cyclase are present in several other aromatic PKSs (Figure 1). In particular, PKS gene clusters involved in the biosynthesis of granaticin (*gra*), frenolicin (*fren*), tetracenomyacin (*tcm*), griseusin (*gris*), and the *S. coelicolor* spore pigment (*whiE*) all contain *actVII* (aromatase) homologs.^{3,6,8,9,26,27} In addition, the *gra*, *fren*, and *gris* PKSs contain *actIV* (cyclase) homologs,^{8,9,27} whereas the *tcm* and *whiE* gene clusters contain members of an unrelated family of cyclases (typified by *tcmI* and *tcmJ*).⁷ We have recently demonstrated that the *act* aromatase does not recognize polyketide chains longer than 16 carbons (e.g., 18- or 20-carbon chains).²² Therefore, our results presented here demonstrate the existence of yet another biosynthetic degree of freedom for the rational design and engineered biosynthesis of novel polyketides by using alternative aromatases and cyclases in combination with variation in minimal PKS and ketoreductase components.

An interesting feature of the *act*, *gra*, *fren*, and *gris* aromatases is the intragenic homology between the N- and C-terminal halves of the proteins.⁹ In contrast, only the N-terminal half of the *tcm* aromatase (*tcmN*) is homologous to these aromatases, while the C-terminal half encodes a putative *O*-methyltransferase⁶ (Figure 1). This internal "duplication"

motif is also absent in the *whiE* spore pigment aromatase, which contains only a single region of homology, resembling the N-terminal halves of the *tcmN* product and the other aromatases⁹ (Figure 1). Perhaps these differences may be explained by the number of dehydrations required for aromatization of the first ring in reduced versus unreduced polyketides since neither the *tcm* nor the *whiE* PKS clusters include a ketoreductase. However, this cannot explain our observation that the first ring in unreduced octaketide (and decaoctetide) intermediates can aromatize even in the absence of an aromatase.²²

Sequence analysis has revealed strong similarities between the *actIV* cyclase (aldolase) and class II β -lactamases,³ specifically in regions believed to be involved in Zn^{2+} binding. It is therefore intriguing that a major class of bacterial aldolases are also zinc-dependent enzymes.²⁸

A remarkable feature of our findings is the apparent preference toward a single cyclization pattern (Figure 2) even in the absence of specific cyclizing enzymes. In each case, a predominant polyketide is isolated in preference to alternatively cyclized products. Furthermore, the observed cyclizations are consistent with those of other novel polyketides (e.g., RM18, RM20, RM20b, SEK15) that have been produced in the absence of functional cyclases.^{18–22} This is a useful property for the rational design of novel polyketides incorporating different cyclizations. Whether cyclizations in the absence of cyclases are purely controlled by the chemical properties of the nascent polyketide or are influenced by other proteins in the PKS complex is still not known. Further studies with aromatase and cyclase homologs may provide additional insights into the cyclizations of aromatic polyketides as well as tools for the design and engineered biosynthesis of novel polyketides.

Experimental Section

Bacterial Strains and Culture Conditions. *S. coelicolor* CH999¹⁸ was used as a host for transformation. DNA manipulations were performed in *Escherichia coli* MC1061. Plasmids were passaged through *E. coli* ET12567 (*dam dcm hsdS Cm*)²⁹ to generate unmethylated DNA before their use to transform *S. coelicolor*. *E. coli* strains were grown under standard conditions.³⁰ *S. coelicolor* strains were

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grown on R2YE agar plates³¹ rather than in liquid media because of the apparently more abundant production of metabolites on agar media.

Manipulation of DNA and Organisms. Standard *in vitro* techniques were used for DNA manipulations.³⁰ *E. coli* was transformed with a Bio-Rad *E. coli* Pulser electroporating apparatus using protocols provided by Bio-Rad. *S. coelicolor* was transformed by the standard procedure,³¹ and transformants were selected using 2 mL of a 500 µg/mL thiostrepton overlay.

Construction of the pSEK34 Plasmid. pSEK34 was constructed by deleting the 650 bp *NruI* (internal site in *actIV*)/*EcoRI* fragment in pRM5 (the *EcoRI* site is recovered). This results in the deletion of 39 amino acids at the C-terminus of the *actIV* cyclase. The construction of pRM5 has been described elsewhere.¹⁸

Production, Purification, and Characterization of SEK34 (4) and SEK34b (5). Nine confluent lawns of CH999/pSEK34 were grown at 30 °C on agar plates (~35 mL/plate) for 8 days. The agar was finely chopped and extracted twice with approximately 250 mL of ethyl acetate/1% acetic acid. The solvent was dried under vacuum, and the residue was resuspended in 5 mL of ethyl acetate. After a few minutes, large quantities (~200 mg) of a white precipitate formed which was filtered, washed with ethyl acetate, and characterized as SEK34 (4) (about 90% pure) by NMR spectroscopy. The filtrate was flashed through a silica gel (Baker 40 µm) chromatography column in ethyl acetate/1% acetic acid. Further purification was achieved by HPLC using a 20–60% acetonitrile/water/1% acetic acid gradient on a preparative reversed-phase (C-18) column (Beckman). Absorbance was monitored at 280 and 410 nm. A major fraction identified as SEK34b

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(5) (~20 mg) was isolated. Attempts to purify the SEK34 precipitate by HPLC resulted in most of it being converted to SEK34b.

Sodium [1,2-¹³C₂]Acetate Feeding Experiments. Four confluent lawns of *S. coelicolor* CH999/pSEK34 were grown on agar plates as described above. Each plate contained approximately 35 mL of R5 media (31) with 50 mg/mL thiostrepton and 0.5 g/L sodium [1,2-¹³C₂]-acetate (Sigma). Isolation of SEK34 (4) was carried out as described above. ¹³C NMR data indicated approximately 1–2% enrichment (estimated by comparing peak areas to the natural abundance ¹³C peak area).

Mass and NMR Spectroscopy. High resolution fast atom bombardment (FAB) mass spectroscopy was used for analysis of compounds. For SEK34 (4), strong signals were observed at *m/e* 325.0680 (M + Na⁺, 325.0688 calculated) and 285 (dehydrated product). For SEK34b, a strong signal was observed at *m/e* 285.0770 (M + H⁺, 285.0763 calculated). NMR spectra were recorded on a Varian XL-400. ¹³C spectra were acquired with continuous broad-band proton decoupling. All compounds were dissolved in DMSO-*d*₆ (Sigma, 99+ atom % D), and spectra were referenced internally to the solvent. Hydroxyl resonances were identified by adding D₂O (Aldrich, 99 atom % D) and checking for disappearance of the signal.

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