Enzyme Mechanisms

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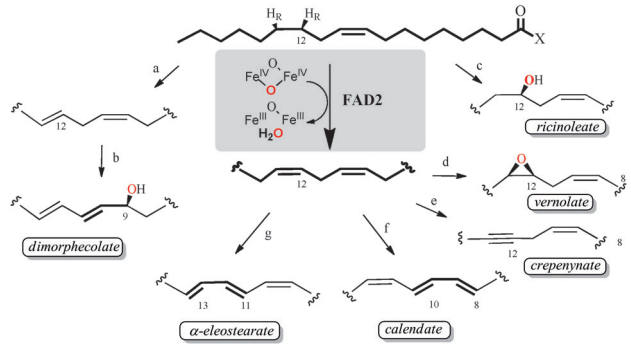
Topological Study of Mechanistic Diversity in Conjugated Fatty Acid Biosynthesis**

Palash Bhar, Darwin W. Reed, Patrick S. Covello, and Peter H. Buist**

Plant-derived seed oils are of current interest in the context of renewable hydrocarbon-type industrial feedstocks.[1,2] Functionalization of oleochemicals adds value to this commodity and is achieved by a set of O_2-dependent, non-heme diiron enzymes known as desaturases.[3–5] The FAD2 subfamily is particularly interesting because of the mechanistic diversity exhibited by this set of sequence-related membrane proteins (Scheme 1).

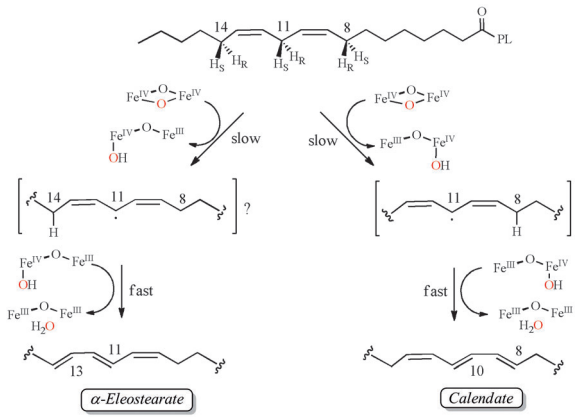


Scheme 1. Mechanistic diversity of the FAD2 family of desaturases.

The parent FAD2-catalyzed reaction involves initial pro-*R* hydrogen atom abstraction at C12 of an oleyl substrate followed by facile C13–H_R-bond cleavage to form linoleate (9*Z*,12*Z*-18:2).[6] The putative diiron oxidant is thought to be structurally related to compound Q, the species believed to be responsible for C–H activation in methane monooxygenase.[7] This is a *syn* process and is consistent with both hydrogen atoms being abstracted by the same iron-bound oxygen atom as implied in Scheme 1. Species-specific variations of this prototypical reaction include (12*E*)-olefin formation and subsequent allylic oxidation[8] (pathway a,b), 12-hydroxylation (pathway c),[9] 12,13-epoxidation (pathway d),[10] 12,13-acetylenation (pathway e),[10,11] and regioselective 1,4-dehydrogenation (pathway f,g).[12,13] The products of this phytochemical virtuosity typically exhibit antifeedant bioactivity and accumulate to concentrations as high as 90% in mature plant seeds. The oxidative power and selectivity displayed by FAD2-type enzymes is unmatched by any other known catalytic system and deserves closer scrutiny.

The high sequence similarity of FAD2 variants suggests that relatively modest changes in active site architecture are sufficient to govern reaction outcome. The membrane-bound nature of these enzymes has prevented their detailed structural characterization but one can imagine that subtle alterations to the contours of a hydrophobic binding pocket could influence substrate positioning relative to a potent oxidizing diiron(IV) species. To gain more information on this aspect of FAD2 biochemistry, we decided to focus our mechanistic study on three reactions that involve a common 11-H abstraction step. These are calendate (pathway f), α-eleostearate (pathway g), and dimorphecolate formation (pathway b). As FAD2 enzymes are highly unstable outside their cellular membrane-bound state, we took advantage of the fact that *S. cerevisiae* expression systems are available for each enzyme[8,13,14] to carry out our mechanistic studies.

We began by studying the mechanistic relationship between calendate and α-eleostearate biosynthesis (Scheme 2). Using the KIE (kinetic isotope effect) method, we have previously determined that the site of initial H abstraction is at C11 in the 1,4-dehydrogenation process leading to calendate.[15] We hypothesized that α-eleostearate formation in the seeds of the tung tree (*Aleurites fordii* Hemsl.) is also initiated at C11, but in this case, the putative radical intermediate would undergo rapid C14–H cleavage rather than at C8 as shown in Scheme 2.



Scheme 2. Biosynthesis of calendate and α-eleostearate.

[*] P. Bhar, Prof. P. H. Buist
Department of Chemistry, Carleton University
1125 Colonel By Drive, Ottawa, ON K1S 5B6 (Canada)
E-mail: peter_buist@carleton.ca
D. W. Reed, Dr. P. S. Covello
NRC Plant Biotechnology Institute
110 Gymnasium Place, Saskatoon, SK S7N 0W9 (Canada)
E-mail: patrick.covello@nrc-cnrc.gc.ca

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The site of initial oxidation (cryptoregiochemistry) for α-eleostearate formation was elucidated by measuring the primary deuterium kinetic isotope effects on each of the two C–H cleavage steps. This is a general approach that has been validated and applied successfully to some sixteen desaturases.[5] The method is based on the premise that the cleavage of the first C–H bond in desaturase-mediated reactions is energetically more difficult than the subsequent collapse of the putative radical intermediate by β-C–H scisson. In principle, only the first step should be rate-limiting and subject to a primary deuterium KIE. Typically KIE values are determined by intermolecular competitive experiments using 1:1 mixtures of non-deuterated and regiospecifically dideuterated substrates.[16]

A mixture consisting of approximately equivalent amounts of saponified methyl esters of non-labeled and [11,11-D_2]linoleates or [14,14-D_2]linoleates[17] was introduced, in triplicate, as a solution in 10% Tergitol (ca. 5 mg each) to growing cultures (50 mL) of the pDR100/INVSC1 strain of *S. cerevisiae* (see the Supporting Information), a transformant that functionally expresses the $\Delta^{11,13}$-desaturase enzyme from *Aleurites fordii* Hemsl.[13] The incubation conditions (20 °C for 3 days and another 3 days at 15 °C) were identical to those used previously in the study of calendate biosynthesis.[15] The cellular fatty acid fractions were isolated from the harvested cells by hydrolysis/methylation and the deuterium content of methyl α-eleostearate products was assessed by GC-MS. This analysis showed that in both incubations, the product consisted of a mixture of D_0 and D_1 species derived from D_0 substrate and D_2 substrate respectively (See Table 1).

Table 1: Intermolecular isotopic discrimination in the *Aleurites fordii* Hemsl. $\Delta^{11,13}$-desaturase-mediated 1,4-desaturation of [11,11-D_2]linoleate and [14,14-D_2]linoleate.

	Linoleic acid substrate		α-Eleostearic acid product		KIE
	% D_0	% D_2	% D_0	% D_1	
C11	49.7 ± 1.1	50.3 ± 1.1	82.8 ± 1.0	17.2 ± 1.0	4.9 ± 0.3[a]
C14	53.1 ± 0.7	46.9 ± 0.7	55.9 ± 1.0	44.1 ± 1.0	1.12 ± 0.04[a]

[a] The standard deviation in KIE is based on the results of at least three incubations.

The product kinetic isotope effects (k_H/k_D)[18] were calculated as:

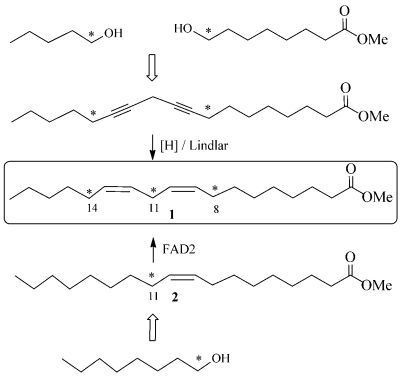
$$\frac{k_H}{k_D} = \frac{\%D_0(P)/\%D_1(P)}{\%D_0(S)/\%D_2(S)} \quad (1)$$

where P is product and S is substrate.

Using the formulation described above, the presence of a large primary deuterium isotope effect (4.9 ± 0.3) for the carbon–hydrogen bond cleavage at C11 was calculated, whereas the carbon–hydrogen bond cleavage at C14 was found to be relatively insensitive to isotopic substitution (KIE = 1.12 ± 0.04).

The data presented above (that is, one large KIE at C11 and one small KIE at C14) clearly demonstrate that α-eleostearate formation is initiated by hydrogen abstraction at C11 and completed by a second facile hydrogen abstraction at the C14 position (Scheme 2). It is significant that in both calendate and α-eleostearate formation, the oxidant attacks 11-H of linoleate first. This implies that the different regiochemical outcome (8,10-diene vs. 11,13-diene) formation is simply dictated by oxidant access to C8 versus C14 of a common bisallylic radical intermediate in the second H-abstraction event. This might be achieved by regioselective cleavage of either iron–oxygen bond in the first hydrogen abstraction step with concomitant movement of the iron(IV) hydroxy group towards either the C1 or C18 terminus of substrate (Scheme 2).

To further investigate the topology of 1,4-desaturation, we wished to determine the stereochemistry of calendate and α-eleostearate formation. To this end, we prepared the six possible stereospecifically labeled 8-[D_1], 11-[D_1], and 14-[D_1]linoleates and examined the fate of the deuterium label upon 1,4-desaturation of the appropriate set of compounds by *Calendula officinalis* $\Delta^{8,10}$-desaturase and *Aleurites fordii* Hemsl. $\Delta^{11,13}$-desaturase. The Crombie-based[19] synthetic route to the required linoleate isotopomers featured coupling of acetylide synthons with the tosylates of enantioenriched (90–95% *ee*) [D_1]alcohol intermediates[20] (Scheme 3); details are given in the Supporting Information.



Scheme 3. Synthesis of enantioenriched [D_1]linoleates **1** from corresponding [D_1]alcohols. Asterisks denote the position of the D_1-containing stereocenter.

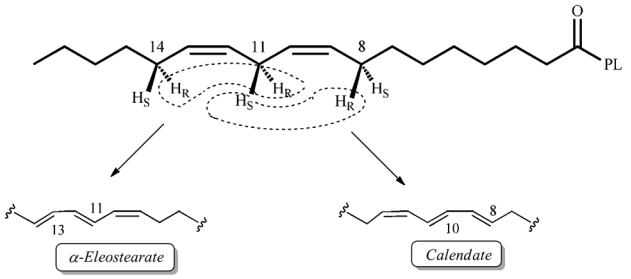
The saponified methyl esters of (8*R*)- and (8*S*)-[8-D_1]-**1**, (11*R*)- and (11*S*)-[8-D_1]-**1** were administered separately, in triplicate, to growing cultures of the pYJ/INVSC1 strain of *S. cerevisiae* (50 mL) expressing linoleate $\Delta^{8,10}$-desaturase (the enzyme responsible for calendate formation).[15] The corresponding experiment involved incubation of (11*R*)- and (11*S*)-[8-D_1]-**1**, (14*R*)- and (14*S*)-[11-D_1]-**1** with the pDR100/INVSCI strain of *S. cerevisiae* that expresses the $\Delta^{11,13}$-desaturase enzyme forming α-eleostearate.[13] The incubation conditions were identical to those used in the KIE experiments (see above). The cellular fatty acid fractions were isolated by hydrolysis/methylation and analyzed by GC-MS to determine the fate of the deuterium label in each case (Table 2).

Table 2: Isotopic content of D_1 substrates and $\Delta^{8,10}$- or $\Delta^{11,13}$-desaturated products.

D_1 species	Substrates[a]		Products[a]			
			calendate		α-eleo stearate	
	% D_0	% D_1	% D_0	% D_1	% D_0	% D_1
(8*R*)-[8-D_1]-**1**	1.2	98.8	**73.2**	26.8		
(8*S*)-[8-D_1]-**1**	1.1	98.9	9.6	**90.4**		
(11*S*)-[11-D_1]-**1**	0.8	99.2	**61.4**	38.6	8.2	**91.8**
(11*R*)-[11-D_1]-**1**	2.5	97.5	5.1	**94.9**	**74.9**	25.1
(14*S*)-[14-D_1]-**1**	1.3	98.7			10.7	**89.3**
(14*R*)-[14-D_1]-**1**	1.2	98.8			**94.3**	5.7

[a] The average standard deviation in the percent isotopic content of each species ±0.4.

It is clear that calendate derived from (8*R*)-[8-D_1]linoleic acid and (11*S*)-[11-D_1]linoleic acid proceeded with overall loss[21] of deuterium during the enzymatic reaction. Deuterium was retained in the calendate product derived from (8*S*)-[8-D_1]linoleic acid and (11*R*)-[11-D_1]linoleic acid. Precisely the opposite trends were observed for α-eleostearate formation. That is, the majority[21] of the α-eleostearate sample derived from (11*R*)-[11-D_1]linoleic acid is nondeuterated, whilst α-eleostearate product derived from (11*S*)-[11-D_1]linoleic acid is largely monodeuterated. α-Eleostearate samples derived from (14*R*)-[14-D_1]linoleic acid and (14*S*)-[14-D_1]linoleic acid were mainly nondeuterated (deuterium loss) and monodeuterated (deuterium retention), respectively. The results are summarized in Scheme 4.

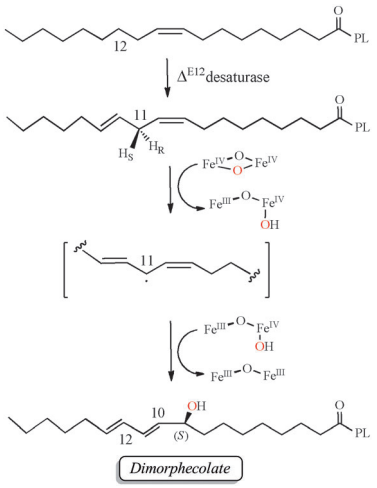


Scheme 4. Stereochemistry of calendate and α-eleostearate formation.

Therefore, the facial selectivity of C8 and C11 hydrogen removal during calendate formation is the same as that observed for the parent Δ^{12}-desaturase (FAD2) assuming an extended substrate conformation in both cases (compare Scheme 1 and 2). It is interesting to note that the enantioselectivity of calendate formation is also identical to that observed for an insect $\Delta^{10,12}$-desaturase;[22] this suggests that the overall contours of the 1,4-desaturase binding site of these enzymes are similar.

However the enantiofacial selectivity of hydrogen removal for $\Delta^{11,13}$-desaturation is opposite to that $\Delta^{10,12}$-desaturation (calendate formation) and indeed that of the parent Δ^{12}-desaturase (FAD2) and all other desaturases studied to date, as has been summarized previously.[4,5] This was an unexpected result. The only other reported case of a switch in desaturase enantioselectivity involved a change in substrate structure (Δ^{13}-desaturation of an 11-yne versus 11-ene in the moth *T. pityocampa*)[23] with concomitant alteration in substrate conformation. In our case, an alteration in active site geometry is presumably forcing a conformational change, as will be discussed in more detail below.

The third FAD2 variant we analyzed is found in *Dimorphotheca sinuata* (African daisy) and catalyzes the formation of dimorphecolic acid ((*S*)-10*E*, 12*E*-9-hydroxyoctadecadienoic acid), a unique hydroxylated conjugated fatty acid.[24] Molecular biological studies reveal that two FAD2-like enzymes (DsFAD2-1 and DsFAD2-2) work in tandem fashion to produce dimorphecolic acid from oleic acid by the 12*E* isomer of linoleate as shown in Scheme 5.[8] By analogy with



Scheme 5. Biosynthesis of dimorphecolic acid.

calendate and α-eleostearate formation, we reasoned that dimorphecolate would arise by regioselective hydroxy group capture (S_H2) by an allylic radical intermediate derived by initial 11-H abstraction (Scheme 5). The anticipated *syn* relationship between hydrogen removal and hydroxy group rebound could be tested by incubating the two enantiomers of 11-[D_1]oleate with a *S. cerevisiae* strain (DsFAD2-1,DsFAD2-2/INVSc1; see the Supporting Information) co-expressing the genes that encode for the Δ^{E12}-desaturase (DsFAD2-1) and $\Delta^{10,9OH}$-hydroxylase (DsFAD2-2).[8]

Methyl (11*S*)- and (11*R*)-[11-D_1]octadec-9*Z*-enoate were available as synthetic intermediates from the two mechanistic studies described above (Scheme 3). These were incubated, in triplicate, as the free acids with growing cultures of the DsFAD2-1,DsFAD2-2/INVSc1 strain of *S. cerevisiae* (50 mL) at 28 °C for 7 days. The cellular fatty acid fraction of the harvested yeast cells were collected by hydrolysis/methylation procedure and treated with BSTFA/pyridine to produce the trimethylsilyl derivative of methyl dimorphecolate.

The isotopic content of the latter was evaluated by analyzing the base-peak ion cluster (M-157: ([Me-$(CH_2)_4$CH=CH–CH=CH–CH–O–Si$(CH_3)_3]^+$)) at *m*/*z* 225. The molecular ion cluster of cellular oleate (*m*/*z* 296) and the Δ^{E12}-desaturated intermediate ((*m*/*z* 294) was used to deter-

Table 3: Isotopic content of cellular stereospecifically labeled monodeuterated oleates, 12*E* isomer of linoleate intermediate and dimorphecolate product isolated from *S. cerevisiae* strain, DsFAD2-1,DsFAD2-2/INVSC1, expressing Δ^{E12}-desaturase and $\Delta^{10,9OH}$-hydroxylase.

Substrate	Cellular oleate[a]		12*E* isomer of linoleate[a]		Dimorphecolate[a]	
	% D_0	% D_1	% D_0	% D_1	% D_0	% D_1
(11*S*)-[11-D_1]-**2**	39.1	60.9	22.4	77.6	84.4	15.6
(11*R*)-[11-D_1]-**2**	39.4	60.6	38.3	61.7	48.3	51.7

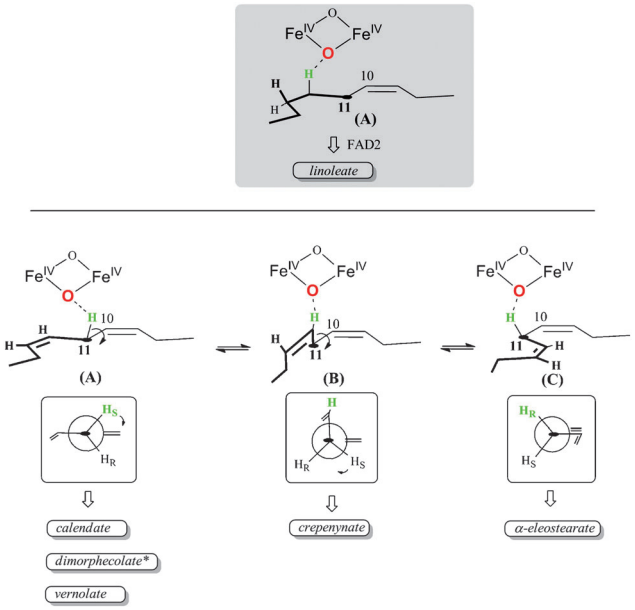
[a] The average standard deviation for each species is ±1.6 (expressed as a percentage).

mine the deuterium content of these compounds. The analytical data is displayed in Table 3.

Dimorphecolate derived from (11*S*)-[11-D_1]oleic acid has a markedly lower percent D_1 content than that derived from the (*R*)-enantiomer. The high percent D_0 content in both samples could be traced to the contribution from endogenous D_0 oleate to the 9*Z*,12*E*-octadecadienoate substrate pool (Table 3). The apparent stereoselectivity for loss of deuterium from the pro*S* C11 position is 80% [(100-100 (15.6/77.6)]; a value of 84% [(100(51.7/61.7)] was calculated for the retention of deuterium from the pro *R* C11 position. The operation of a large deuterium KIE in the removal of hydrogen/deuterium from the pro *S* C11 position can be inferred from the elevated D_1/D_0 ratio (77.6:22.4) of the 12-*E*-isomer of linoleate (9*Z*,12*E*-octadecadienoate) intermediate in the incubation of (11*S*)-[11-D_1]-**2** incubation compared to that observed in the (11*R*)-[11-D_1]-**2** experiment (61.7:38.4; Table 3). This implies that (11*S*)-[11-D_1]-9*Z*,12*E*-octadecadienoate reacts slower than (11*R*)-[11-D_1]-9*Z*,12*E*-octadecadienoate relative to endogenous [D_0]oleate. This hypothesis was tested by incubating a mixture of 11-[D_2]oleate versus oleate bearing a remote 18-[D_3] label and evaluating the D_1/D_3 ratio of the dimorphecolate product. An estimated value of 7.8 ± 0.8 for the KIE for C11H abstraction was obtained (data not shown).

The results of our stereochemical investigation confirm our hypothesis that dimorphecolate biosynthesis involves H-abstraction and hydroxy group rebound from the same side of the olefinic plane. The enantioselectivity for 11-H abstraction was the same as for calendate formation. This implies that both enzymes utilize a similar substrate conformation; the unusually high sequence similarity between the two enzymes has been noted.[8] The switch that controls delivery of a hydroxy group to C9 of the putative allylic radical intermediate versus H-abstraction at C8 is probably purely geometric given that both processes are expected to be highly exergonic. The latent ability of desaturases to act as allylic hydroxylators was revealed in a recent serendipitous discovery by Shanklin and co-workers: a site-directed mutagenized, triple mutant T117R/G188L/D280K of castor stearoyl-ACP Δ^9-desaturase oxidizes (*Z*)-9-octadecenoyl-ACP to produce 9-hydroxy-(10*E*)-octadecenoic acid ($-CH_2-CH=CH-CH_2- \rightarrow -CH=CH-CH(OH)-CH_2-$).[25] We find it significant that for both the FAD2 variant (dimorphecolate formation) and the Shanklin Δ^9-desaturase mutant, allylic hydroxylation is initiated at C11, the carbon atom proximal to the site of initial attack for the parent enzyme (C12 for FAD2 and C10 for stearoyl-ACP Δ^9-desaturase). This implies that the conformation of the substrates in the parent and variant/mutant in these cases is probably very similar.

In summary, we have shown how three homologous FAD2-type enzymes complete an initial 11-H abstraction event to give trienoic (calendate and α-eleostearate) and hydroxylated dienoic (dimorphecolate) products with overall *syn*-stereochemistry. To correlate these reactions with others in the FAD2 family, one can postulate the existence of three possible[26] substrate conformations (Scheme 6). As a 9*Z*



Scheme 6. Top: Prototypical substrate conformation for FAD2 parent operating on oleate. Bottom: Three possible substrate conformations for FAD2 variants operating on linoleate. *Dimorphecolate is derived from the 12*E* isomer of linoleate.

double bond is a necessary feature of all FAD2 substrates, the simplifying assumption can be made that the position of the C1–C10 portion is relatively fixed and that the C11–C18 half of fatty acid is more mobile. The extended (quasi-*anti*) conformation (**A**) is the most common and leads to calendate, dimorphecolate, and vernolate formation by oxidative attack from above the olefinic plane. It is presumably also the conformation used in the parent FAD2-catalyzed reaction. The energetically difficult, C12-initiated[11] dehydrogenation of *Z*-alkenes to form an alkyne (crepenynate formation) would proceed by abstraction of vinyl hydrogen atoms from conformation **B** where the C12–C13 double bond is pointing towards the oxidant. This conformation effectively blocks competing oxidative pathways that are in principle energetically more favorable, such as H abstraction at C11. Finally we have uncovered evidence for the presence of a third conformation (**C**) that appears to be involved in α-eleostearate formation. It would be of interest to ascertain whether this novel conformation is also involved in the formation of the 12*E*-isomer of linoleate from oleate[13] and the 13*Z* isomer of α-eleostearate (punicic acid) from linoleate.[27] The topolog-

ical considerations summarized above should play an essential part in completing our understanding of the factors controlling reaction outcome. More precise information on how the active site of each FAD2 variant is sculpted to influence the course of oxidation must await structural characterization of these membrane-bound enzymes.

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[17] Methyl [11,11-D_2]linoleate and [14,14-D_2]linoleate were prepared in straightforward fashion by alkynylation of the tosylate of [1,1-D_2]-2-octyn-1-ol or [4,4-D_2]-2-octyn-1-ol with the acetylide salt of methyl 9-decynoate followed by Lindlar reduction of the skipped diyne product (see the Supporting Information).
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[21] The presence of 5–10% of D_0 product in the cases where deuterium is retained overall can be attributed to the 90–95% *ee* of the D_1 substrates. In the case of calendate formation from (11*S*)-[D_1] substrate and eleostearate formation from (11*R*)-[D_1] substrate, where deuterium is lost overall, the apparent reduced stereoselectivity can be accounted for in terms of the known large primary deuterium kinetic isotope effect associated with C11–H bond cleavage.[15] That is, the minor enantiomer in these samples, where deuterium is a spectator atom, would react substantially faster than the major species and lead to enhanced percentage D_1 content in the product. The appearance of comparatively less yet substantial amounts of [D_1]calendate in the (8*R*)-[8-D_1] incubation is puzzling but may simply be due to the unusually low stereoselectivity in the acetylide coupling step leading to this particular substrate.
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