

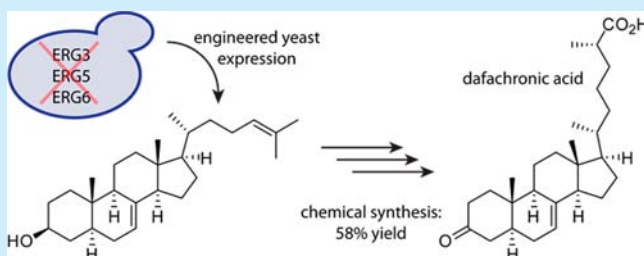
Mixed Bioengineering–Chemical Synthesis Approach for the Efficient Preparation of Δ^7 -Dafachronic Acid

Matias I. Kinzurik,[†] Lachezar V. Hristov,[†] Seiichi P. T. Matsuda,^{*,†,‡} and Zachary T. Ball^{*,†}

[†]Department of Chemistry and [‡]Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005, United States

S Supporting Information

ABSTRACT: Combining bioengineering with chemical synthesis has enabled an efficient method for producing Δ^7 -dafachronic acid, a steroidal hormone associated with nematode germline longevity. *Saccharomyces cerevisiae* was engineered to produce 7,24-cholestadienol, a convenient starting material for a four-step synthesis of Δ^7 -dafachronic acid.

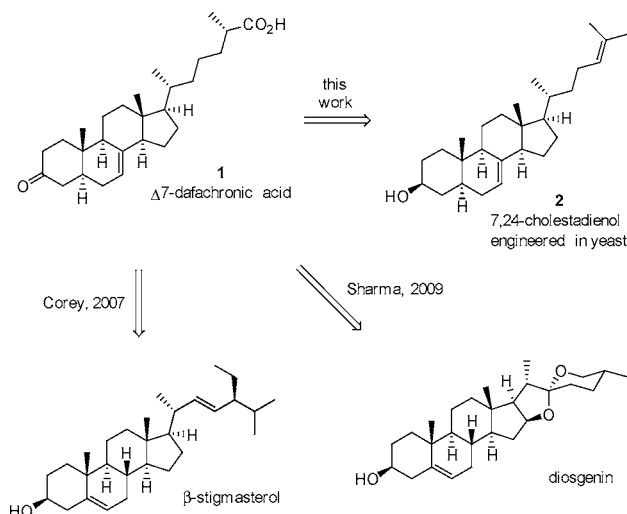


Parasitic nematodes cause serious problems for human health and agriculture. For example, the human parasites *Ancylostoma* and *Necator spp* affect one in every 10 people on the planet and are a leading cause of iron-deficient anemia worldwide,¹ while plant parasitic nematodes are an important and unsolved threat to agriculture.² The natural product Δ^7 -dafachronic acid³ (**1**) disrupts nematode development by halting the lethargic dauer developmental stage through interactions with the nuclear receptor DAF-12.⁴ DAF-12 signaling pathways are conserved across numerous parasitic nematodes,^{5–7} and so the novel mechanism of action of Δ^7 -dafachronic acid has inspired synthetic and biological study of the compound for its anti-nematode potential.^{8–14} In this paper, we report a semisynthetic route, developing engineered mutant yeast *S. cerevisiae* to produce the precursor sterol **2** that allows efficient and high-yielding access to Δ^7 -dafachronic acid (**1**).

The complexity of steroid structure has long created synthetic challenges, requiring control over stereochemistry, degree of unsaturation, and oxygenation site. To date, routes to Δ^7 -dafachronic acid have started from commercially available steroidal building blocks including β -stigmasterol,⁸ diosgenin,¹² 3β -hydroxycholesterol-5-en-24-oic acid,^{9,15} and hydoxycholesterol acid¹¹ (a selection of starting materials is shown in Scheme 1). Despite the similarities to Δ^7 -dafachronic acid, these compounds still require routes of more than 10 steps to forge the target compound (**1**), often using reagents that are challenging for efficient synthesis on scale. A central challenge in these syntheses is that readily available sterols and steroids contain functionality that is not trivial to manipulate toward Δ^7 -dafachronic acid.

Engineering mutant organisms to produce a target structure directly is a compelling alternative,^{16–18} and efforts have been made to elucidate the precise steps in the biosynthetic pathway.^{19,20} But there are limits to the reactions that can be carried out efficiently in a producing organism. For example, accumulation of carboxylate-containing steroids has been

Scheme 1. Selected Examples of Starting Material Selection for Synthesis of Δ^7 -Dafachronic Acid^a



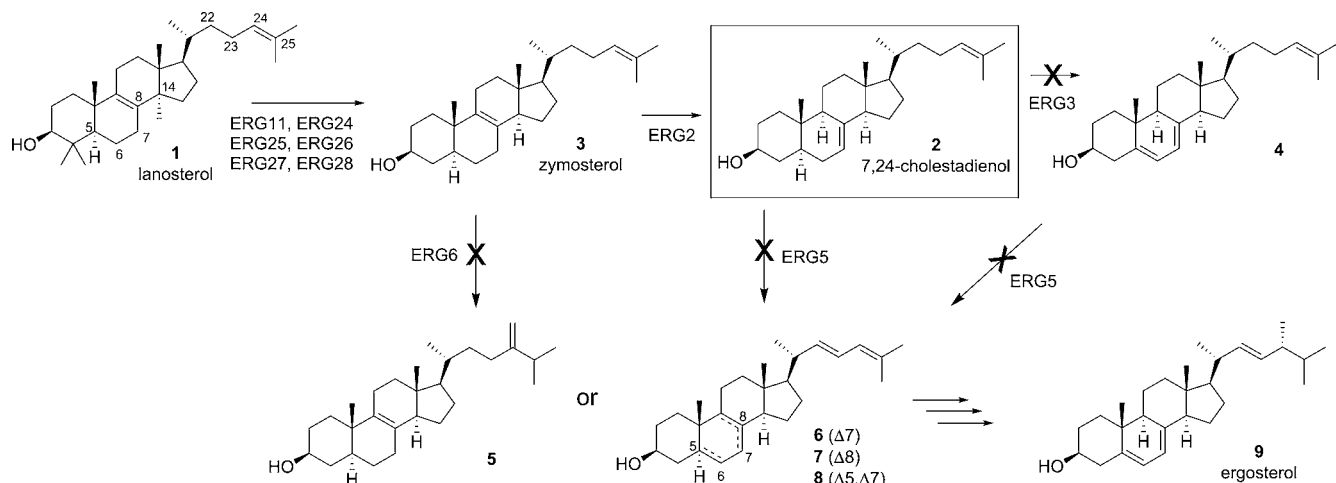
^aProducing the engineered sterol **2** minimizes the need for oxidation-state alterations.

shown to prevent yeast growth, indicating that the late stages of Δ^7 -dafachronic acid biosynthesis would likely be incompatible with efficient bioproduction.²¹ We have developed a semisynthesis of Δ^7 -dafachronic acid by manipulating yeast sterol biosynthetic pathways to bioengineer a mutant strain that could efficiently produce a compound with sufficient structural similarity and functional handles to be converted into the target structure through chemical synthesis.

The ergosterol biosynthetic pathway accesses intermediates^{22,23} that display structural features that are desirable in a

Received: March 3, 2014

Published: April 9, 2014

Scheme 2. Anticipated Control of Ergosterol Pathways in a Mutant Yeast Strain Accumulating 7,24-Cholestadienol (2)^a

^aA representative sample of plausible enzyme pathways from lanosterol. Arrows blocked with an "X" indicate plausible natural pathways that have been blocked by gene deletion in the triple mutant (ERG6/ERG5/ERG3) strain.

starting material for Δ^7 -dafachronic acid synthesis. Lanosterol is the initial carbocyclic intermediate en route to sterols (Scheme 2). Lanosterol displays the tetracyclic ring system and oxidation at C3 that are also present in Δ^7 -dafachronic acid, and the C24–C25 olefin is a valuable handle for side-chain modification. Some native yeast enzymes modify lanosterol to more closely resemble Δ^7 -dafachronic acid: demethylation at 4, 4', and 14 and isomerization of the C8–C9 olefin to C7–C8. The plausible intermediate 7,24 cholestadienol (2) would be an ideal starting material for chemical synthesis and could be obtained if enzymatic steps that diverge from Δ^7 -dafachronic acid are eliminated through mutation. However, because yeast sterol biosynthesis is not rigidly linear—the order of the transformations is variable—halting the action of a single enzyme typically is insufficient to allow accumulation of a single desired intermediate, such as 7,24 cholestadienol (2).

The enzyme ERG6 promotes the methylation of the side chain. Deletion of that enzyme is desirable to retain the C24–C25 olefin, which is an excellent handle for side-chain modification. An ERG6 knockout²⁴ strain generated sterol products with the C24–C25 olefin rather than the terminal methylene, but the dominant products retained C5–C6 and C22–C23 olefins (3, 4, and 8, see Table 1), which are absent in

to the desired 7,24-cholestadienol (2) as the primary sterol product (77%), along with minor amounts of zymosterol (20%). These two sterols comprise $\geq 97\%$ of the non-saponifiable lipids isolated from the strain (Figure 1), as judged by GC–MS and NMR analyses.

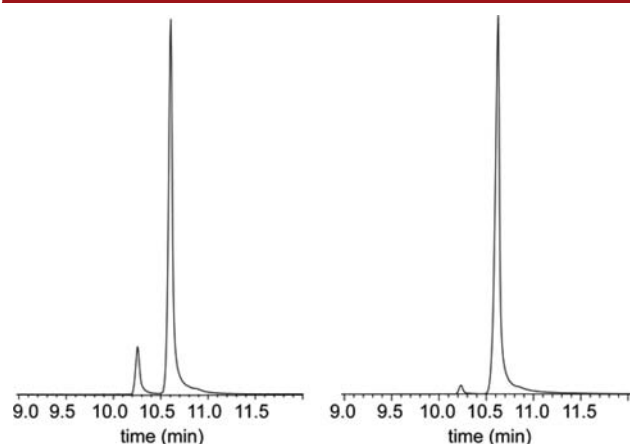


Figure 1. GC analysis of crude nonsaponifiable-lipid products isolated from the triple mutant (ERG6/ERG3/ERG5) strain, before (left, 77% purity) and after (right, 91% purity) a single recrystallization from methanol. The desired 7,24-cholestadienol (2, t_R 10.6 min) was the primary lipid produced, along with small amounts of zymosterol (3, t_R 10.3 min).

Table 1. Sterol Composition of Mutant Yeast Strains

sterol	gene deletions		
	ERG6	ERG6/ ERG5	ERG6/ERG3/ ERG5
7,24-cholestadienol (2)	3	3	77
zymosterol (3)	27	29	20
5,7,24-cholestatrienol (4)	30	60	0
5,7, 22, 24-cholestatetraenol (8)	29	0	0
all other sterols	11	8	≤ 3

Δ^7 -dafachronic acid. ERG5 was then deleted by PCR-mediated homologous recombination. The resultant ERG6/ERG5 double-knockout strain²⁵ lost the ability to form the side-chain diene and primarily accumulated sterol 4.

Removing an additional downstream desaturase (ERG3) resulted in the triple-knockout mutant²⁶ (ERG6/ERG5/ERG3) that successfully funneled the majority of lanosterol metabolites

The clean conversion of lanosterol to these two sterols indicates that among sterol biosynthetic enzymes not deliberately disrupted, only the sterol isomerase ERG2 leaves meaningful amounts of its starting material unreacted. Delta-8 sterols are frequently recovered in modest amounts from yeast.²⁷ Efforts to engineer yeast to redirect the ergosterol pathway in favor of other desired structures may result in sickly strains that require supplementation of the media with sterols and even then exhibit decreased productivity.²⁷ In contrast, the triple mutant strain reported here is remarkably robust without supplementing the growth media with sterols, and sterol 2 was produced in concentrations of 28 mg/L of culture, performed on 24-L scale. Pure material could be obtained by HPLC

purification; alternatively, a single recrystallization from methanol afforded sterol **2** in 91% purity (69% mass recovery). The subsequent chemical synthesis has been successfully applied to both HPLC-purified sterol **2** and to sterol **2** contaminated with small amounts of isomer **3**, in which case the mixture of Δ^7 - (**2**) and Δ^8 -isomers (**3**) is carried throughout the synthesis.

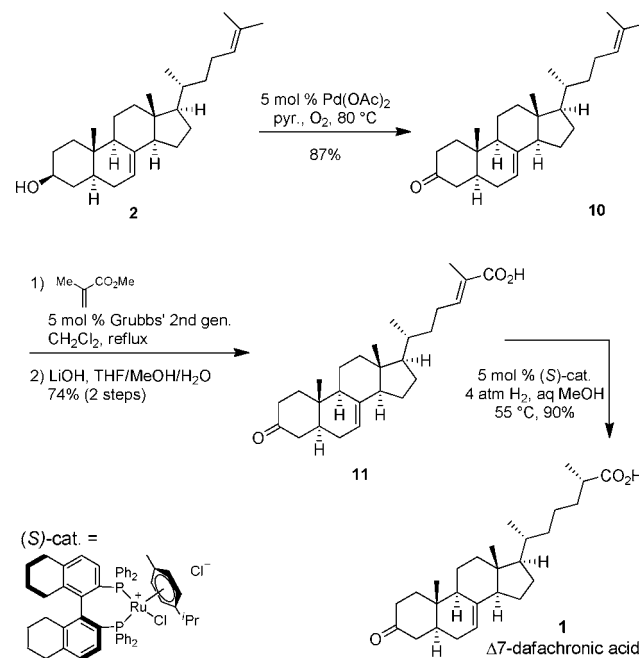
With a biosynthetic route to a suitable precursor in hand, we set about developing a synthesis of Δ^7 -dafachronic acid from sterol **2** that would be efficient and compatible with larger scale production. The ketone at C3 was first installed by palladium-catalyzed oxidation in an O_2 atmosphere, affording ketone **10**.^{28,29} This transformation was performed first because subsequent side-chain functionalization chemistry was incompatible with the C3 hydroxyl. While approaches to side chain allylic functionalization exist, such as selenium dioxide oxidation^{30,31} or circuitous multistep methods,³² we were interested in finding a more environmentally friendly route with improved yield. There is some precedent for palladium-mediated side chain allylic functionalization with linear polyisoprene substrates,^{33,34} but we were unable to oxidize the terminal methyl groups under a variety of conditions.

We turned instead to olefin metathesis. Despite the dearth of examples in the literature of trisubstituted olefin synthesis from trisubstituted starting materials,^{35,36} we were pleased to find that cross-metathesis with Grubbs' second-generation catalyst and methyl methacrylate provided the desired ester product, which was then saponified to unsaturated acid **11**. In initial studies, we found that methacrolein was also a competent substrate for this reaction, affording the corresponding enal product in 82% yield.

Finally, directed hydrogenation^{37,38} with $[RuCl(p\text{-cymene})-(S)-(-)\text{-H}_8\text{-BINAP}]\text{Cl}$ yielded the desired product with 95:5 diastereoselectivity, consistent with literature precedent.¹¹ For the case at hand, the distal nature of the C24 stereocenter meant that we were unable to assess diastereomeric purity by chromatographic means or by 1D ^1H NMR. Instead, integrating a ^1H – ^{13}C HSQC spectrum of the reaction product solved this problem, allowing reliable diastereoselectivity determination without resorting to circuitous derivatization methods. Action of the enantiomeric (*R*)-catalyst provided the analogous (2*S*) compound in 91% yield and 93:7 diastereoselectivity for spectroscopic comparison and biological testing.

The combination of engineered yeast and synthetic chemistry offers remarkably efficient access to Δ^7 -dafachronic acid (**1**) (Scheme 3). Four synthetic steps produce the target in 58% overall yield, featuring three transition-metal-catalyzed reactions and a simple ester saponification. The only stoichiometric reagents in the sequence are O_2 , H_2 , LiOH, and methyl methacrylate. At the same time, thoughtful manipulation of the *S. cerevisiae* ergosterol pathway allows production of 7,24-cholestadienol (**2**), which replicates many of the challenging features of Δ^7 -dafachronic acid, including C7–C8 unsaturation. The synthetic precursor 7,24-cholestadienol (**2**) comprises 77% of the nonsaponifiable lipids extracted from cultured yeast and is produced in culture at 28 mg/L. This productivity is sufficient for initial biological experiments and may serve as a starting point for engineering optimization, which in other contexts has shown the ability to bring about huge increases in the yield of engineered steroidal products in yeast.³⁹ The result of this work is a synthetic route that is more efficient than what is possible with synthetic or bioengineering approaches alone. Engineering yeast to produce

Scheme 3. Synthesis of Δ^7 -Dafachronic Acid (**1**)



sterol starting materials may be a valuable approach for the preparation of related Δ^7 -dafachronic analogues, of interest for medical and biological purposes,⁴⁰ with subtle variation in unsaturation and oxidation state.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and characterization of all synthetic products. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: matsuda@rice.edu.

*E-mail: zbl@rice.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge financial support from the Robert A. Welch Foundation Research Grant No. C-1680 (Z.T.B.) and Research Grant No. C-1323 (S.P.T.M.).

■ REFERENCES

- (1) Hotez, P. J.; Bethony, J. M.; Diemert, D. J.; Pearson, M.; Loukas, A. *Nat. Rev. Microbiol.* **2010**, 8, 814–826.
- (2) (a) Elling, A. A. *Phytopathology* **2013**, 103, 1092. (b) Zhao, L.; Zhang, S.; Wei, W.; Hao, H.; Zhang, B.; Butcher, R. A.; Sun, J. *Curr. Biol.* **2013**, 23, 2038–2043.
- (3) Dumas, K. J.; Guo, C.; Wang, X.; Burkhart, K. B.; Adams, E. J.; Alam, H.; Hu, P. J. *Dev. Biol.* **2010**, 340, 605.
- (4) Martin, R.; Entchev, E. V.; Kurzchalia, T. V.; Knölker, H. J. *Org. Biomol. Chem.* **2010**, 8, 739–750.
- (5) Ogawa, A.; Streit, A.; Antebi, A.; Sommer, R. J. *Curr. Biol.* **2009**, 19, 67.
- (6) Wang, D. D.; Cheng, X. Y.; Wang, Y. S.; Pan, H. Y.; Xie, B. Y. *Forensic Pathol.* **2013**, 43, 144.

- (7) Wang, Z.; Zhou, X. E.; Motola, D. L.; Gao, X.; Suino-Powell, K.; Conneely, A.; Ogata, C.; Sharma, K. K.; Auchus, R. J.; Lok, J. B.; Hawdon, J. M.; Kliewer, S. A.; Xu, H. E.; Mangelsdorf, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 9138.
- (8) Giroux, S.; Corey, E. J. *J. Am. Chem. Soc.* **2007**, *129*, 9866.
- (9) Martin, R.; Schmidt, A. W.; Theumer, G.; Krause, T.; Entchev, E. V.; Kurzchalia, T. V.; Knölker, H. J. *Org. Biomol. Chem.* **2009**, *7*, 909.
- (10) Samaja, G. A.; Castro, O.; Alvarez, L. D.; Dansey, M. V.; Escudero, D. S.; Veleiro, A. S.; Pecci, A.; Burton, G. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 2893.
- (11) Gioiello, A.; Sabbatini, P.; Rosatelli, E.; Macchiarulo, A.; Pellicciari, R. *Tetrahedron* **2011**, *67*, 1924.
- (12) Sharma, K. K.; Wang, Z.; Motola, D. L.; Cummins, C. L.; Mangelsdorf, D. J.; Auchus, R. J. *Mol. Endocrinol.* **2009**, *23*, 640.
- (13) Giroux, S.; Bethke, A.; Fielenbach, N.; Antebi, A.; Corey, E. J. *Org. Lett.* **2008**, *10*, 3643.
- (14) Giroux, S.; Corey, E. J. *Org. Lett.* **2008**, *10*, 801.
- (15) Martin, R.; Schmidt, A. W.; Theumer, G.; Kurzchalia, T. V.; Knölker, H. J. *Synlett* **2008**, 1965.
- (16) Wriessneger, T.; Pichler, H. *Prog. Lipid Res.* **2013**, *52*, 277.
- (17) Fischer, M. J. C.; Meyer, S.; Claudel, P.; Bergdoll, M.; Karst, F. *Biotechnol. Bioeng.* **2011**, *108*, 1883.
- (18) Dai, Z.; Liu, Y.; Zhang, X.; Shi, M.; Wang, B.; Wang, D.; Huang, L.; Zhang, X. *Metab. Eng.* **2013**, *20*, 146.
- (19) Wollam, J.; Magner, D. B.; Magomedova, L.; Rass, E.; Shen, Y.; Rottiers, V.; Habermann, B.; Cummins, C. L.; Antebi, A. *PLoS Biol.* **2012**, *10*, e1001305.
- (20) Wollam, J.; Magomedova, L.; Magner, D. B.; Shen, Y.; Rottiers, V.; Motola, D. L.; Mangelsdorf, D. J.; Cummins, C. L.; Antebi, A. *Aging Cell* **2011**, *10*, 879.
- (21) Gachotte, D.; Gaylor, J.; Nickel, E.; Bart, M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *96*, 13794.
- (22) Lees, N. D.; Bard, M.; Kirsch, D. R. *Crit. Rev. Biochem. Mol. Biol.* **1999**, *34*, 33.
- (23) Espenshade, P. J.; Hughes, A. L. *Annu. Rev. Genet.* **2007**, *41*, 401.
- (24) Valachovic, M.; Bareither, B. M.; Bhuiyan, M. S. A.; Eckstein, J.; Barbuch, R.; Balderes, D.; Wilcox, L.; Sturley, S. L.; Dickson, R. C.; Bard, M. *Genetics* **2006**, *173*, 1893.
- (25) Bard, M.; Woods, R. A.; Barton, D. H. R.; Corrie, J. E. T.; Widdowson, D. A. *Lipids* **1977**, *12*, 645.
- (26) ERG4 acts on the C24 methylene product of the (now deleted) ERG6 enzyme and so does not affect sterol 2.
- (27) Xu, R.; Wilson, W. K.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **2002**, *124*, 918.
- (28) Nishimura, T.; Onoue, T.; Ohe, K.; Uemura, S. *J. Org. Chem.* **1999**, *64*, 6750.
- (29) Nishimura, T.; Onoue, T.; Ohe, K.; Uemura, S. *Tetrahedron Lett.* **1998**, *39*, 6011.
- (30) Nishitoba, T.; Sato, H.; Oda, K.; Sakamura, S. *Agric. Biol. Chem.* **1988**, *52*, 211.
- (31) Singh, C.; Dev, S. *Tetrahedron* **1977**, *33*, 817.
- (32) Kennedy, E. M.; P'Pool, S. J.; Jiang, J.; Sliva, D.; Minto, R. E. *J. Nat. Prod.* **2011**, *74*, 2332.
- (33) McMurtry, J. E.; Kočotovský, P. *Tetrahedron Lett.* **1984**, *25*, 4187.
- (34) Speziali, M. G.; Robles-Dutenhefner, P. A.; Gusevskaya, E. V. *Organometallics* **2007**, *26*, 4003.
- (35) Chatterjee, A. K.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **2002**, *41*, 3171.
- (36) Vyvyan, J. R.; Loitz, C.; Looper, R. E.; Mattingly, C. S.; Peterson, E. A.; Staben, S. T. *J. Org. Chem.* **2004**, *69*, 2461.
- (37) Uemura, T.; Zhang, X.; Matsumura, K.; Sayo, N.; Kumobayashi, H.; Ohta, T.; Nozaki, K.; Takaya, H. *J. Org. Chem.* **1996**, *61*, 5510.
- (38) Ohta, T.; Miyake, T.; Takaya, H. *J. Chem. Soc., Chem. Commun.* **1992**, 1725.
- (39) Paddon, C. J.; Westfall, P. J.; Pitera, D. J.; Benjamin, K.; Fisher, K.; McPhee, D.; Leavell, M. D.; Tai, A.; Main, A.; Eng, D.; Polichuk, D. R.; Teoh, K. H.; Reed, D. W.; Treynor, T.; Lenihan, J.; Jiang, H.; Fleck, M.; Bajad, S.; Dang, G.; Dengrove, D.; Diola, D.; Dorin, G.; Ellens, K. W.; Fickes, S.; Galazzo, J.; Gaucher, S. P.; Geistlinger, T.; Henry, R.; Hepp, M.; Horning, T.; Iqbal, T.; Kizer, L.; Lieu, B.; Melis, D.; Moss, N.; Regentin, R.; Secrest, S.; Tsuruta, H.; Vazquez, R.; Westblade, L. F.; Xu, L.; Yu, M.; Zhang, Y.; Zhao, L.; Lievense, J.; Covello, P. S.; Keasling, J. D.; Reiling, K. K.; Renninger, N. S.; Newman, J. D. *Nature* **2013**, *496*, 528.
- (40) Saini, R.; Boland, S.; Kataeva, O.; Schmidt, A. W.; Kurzchalia, T. V.; Knolker, H.-J. *Org. Biomol. Chem.* **2012**, *10*, 4159.