

Characterization of the Epoxide Hydrolase NcsF2 from the Neocarzinostatin Biosynthetic Gene Cluster

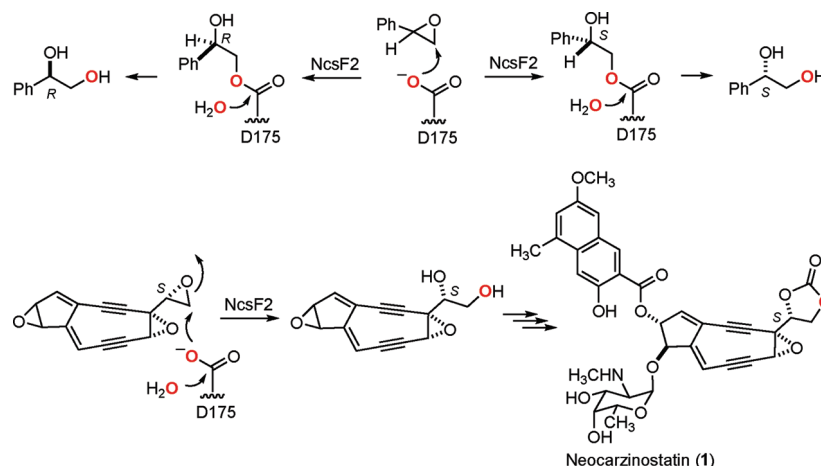
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ABSTRACT



Neocarzinostatin (1) biosynthesis is proposed to involve a vicinal diol intermediate. It is reported that NcsF2, one of two epoxide hydrolases encoded by the NCS gene cluster, catalyzes regiospecific addition of H₂O to C-2 of both (*R*)- and (*S*)-styrene oxides to afford (*R*)- and (*S*)-1-phenyl-1,2-ethanediols, respectively, supporting its proposed role in 1 biosynthesis. (*R*)-1-Phenyl-1,2-ethanediol (87% yield and 99% ee) was obtained from (±)-styrene oxide hydrolysis by cocatalysis using NcsF2 and SgcF, the complementary epoxide hydrolase from the C-1027 biosynthetic pathway.

Neocarzinostatin (NCS) is an enediyne antitumor antibiotic produced by *Streptomyces carzinostaticus* ATCC 15944 as a chromoprotein complex consisting of an apoprotein and the NCS chromophore (1).¹ Characterization of the NCS biosynthetic gene cluster from *S. carzinostaticus* led to a proposed convergent biosynthetic pathway in which the peripheral moieties are appended to an enediyne core vicinal diol intermediate to afford 1 (Figure 1A).² The presumed (*S*)-vicinal

diol is thought to arise from hydrolysis of an epoxide intermediate by one of two putative epoxide hydrolase (EH) enzymes encoded by the NCS gene cluster, NcsF1 or NcsF2.² A similar convergent logic has been proposed for the C-1027 (2) biosynthetic pathway in *Streptomyces globisporus* (Figure 1B),³ from which an EH, SgcF, has been characterized using styrene oxide as a substrate mimic.⁴ SgcF was shown to catalyze an

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(1) (a) Ishida, N.; Miyazaki, K.; Kumagai, M.; Rikimaru, M. *J. Antibiot.* **1965**, *18*, 68–76. (b) Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Otake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331–340.

(2) Liu, W.; Nonaka, K.; Nie, L.; Zhang, J.; Christenson, S. D.; Bae, J.; Van Lanen, S. G.; Zazopoulos, E.; Farnet, C. M.; Yang, C. F.; Shen, B. *Chem. Biol.* **2005**, *12*, 293–302.

(3) Liu, W.; Christenson, S. D.; Standage, S.; Shen, B. *Science* **2002**, *297*, 1170–1173.

(4) Lin, S.; Horsman, G. P.; Chen, Y.; Li, W.; Shen, B. *J. Am. Chem. Soc.* **2009**, *131*, 16410–16417.

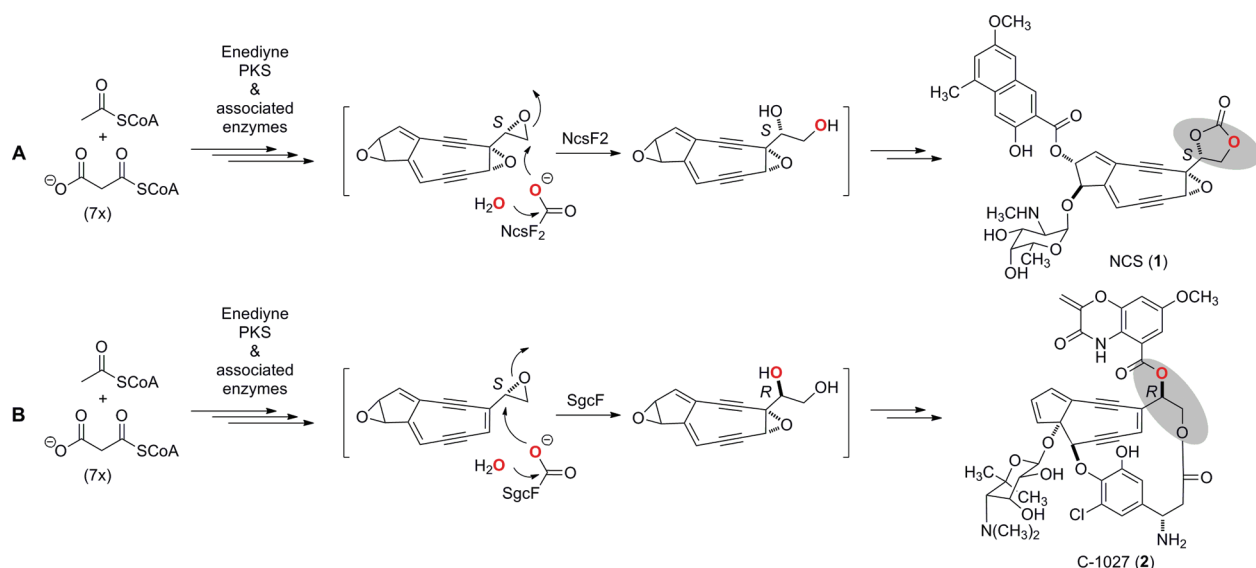


Figure 1. Proposed biosynthetic pathways featuring (A) an NcsF2-catalyzed formation of an (*S*)-vicinal diol intermediate from an (*S*)-enediynes core epoxide precursor for NCS (**1**) and (B) an SgcF-catalyzed formation of an (*R*)-vicinal diol intermediate from an (*S*)-enediynes core epoxide precursor for C-1027 (**2**). The vicinal diol moieties in **1** and **2** concerned in this study are shaded.

enantioconvergent process⁵ in which H₂O attacks primarily at C-2 for (*R*)-styrene oxide and at the more hindered C-1 for (*S*)-styrene oxide (Figure 2A).⁴ Consequently, the major product is always the (*R*)-vicinal diol, but the 37-fold higher specificity of SgcF for (*S*)-styrene oxide implicates an (*S*)-epoxide enediynes intermediate in C-1027 biosynthesis, consistent with the proposed pathway (Figure 1B).^{3,4} Here, we report that NcsF2, but not NcsF1, efficiently hydrolyzes styrene oxide. In contrast to SgcF, NcsF2 is regiospecific for C-2 such that hydrolysis of (*R*)- and (*S*)-styrene oxides yield (*R*)- and (*S*)-vicinal diol products, respectively (Figure 2B). Although hydrolysis of an (*S*)-epoxide enediynes intermediate would afford the expected (*S*)-vicinal diol (Figure 1A), the 44-fold preference of NcsF2 for (*R*)-styrene oxide is inconsistent with our biosynthetic proposal² and may be an artifact of using styrene oxide as a substrate mimic. By exploiting the complementary activities of NcsF2 and SgcF, we finally demonstrated the preparation of (*R*)-1-phenyl-1,2-ethanediol from racemic styrene oxide with excellent chemical yield and enantiomeric excess (Figure 2C).

We have previously predicted two genes, *ncsF1* and *ncsF2*, within the NCS biosynthetic gene cluster, to encode EHs on the basis of bioinformatics analysis.² We first cloned, overproduced in *E. coli*, and purified both NcsF1 and NcsF2 to homogeneity (Figure S1, Supporting Information).⁶ The EH activity of both enzymes toward racemic styrene oxide was then analyzed using HPLC.^{4,6} Incubation of NcsF2 (50 μM) with 2 mM styrene oxide produced a single peak corresponding to 1-phenyl-1,2-ethanediol, and in contrast, NcsF1 produced

only a very minor peak, indicating a dramatically less efficient enzyme (Figure 3). Careful inspection of the selected EH protein sequences reveals that, in contrast to SgcF and NcsF2, NcsF1 may lack key amino acid residues conserved among EHs (Figure S2, Supporting Information). First, the acid residue of the nucleophile–His–acid catalytic triad has been replaced by Ala (Asp174–His361–Ala334) at its usual position after strand β7, but some EHs are known to harbor this residue after strand β6.⁷ Second, the two canonical EH Tyr residues that activate the epoxide oxygen, one of which is absolutely conserved,⁷ are missing in NcsF1 (Pro234 and His303), although we note that the adjacent Tyr304 may function equivalently. Thus, although NcsF1 is 53% and 59% identical to SgcF and NcsF2, respectively, its unusual sequence and relatively inefficient hydrolysis of styrene oxide suggest an alternative function for this enzyme in **1** biosynthesis, perhaps for hydrolysis of the internal epoxide of the NCS enediynes core precursor (Figure 1A).

The observed activity of NcsF2 prompted further characterization of the enzyme. We then determined the steady-state kinetic parameters of NcsF2 toward (*R*)- and (*S*)-styrene oxides using a spectrophotometric assay^{6,8} to continuously monitor the formation of 1-phenyl-1,2-ethanediol. The Michaelis–Menten equation was fitted to plots of initial velocity versus substrate concentration (Figure 4) to yield $k_{\text{cat}} = 133 \pm 4 \text{ min}^{-1}$, $K_{\text{M}} = 0.5 \pm 0.1 \text{ mM}$, and $k_{\text{cat}}/K_{\text{M}} = 266 \text{ min}^{-1} \text{ mM}^{-1}$ for (*R*)-styrene oxide and $k_{\text{cat}} = 31 \pm 2 \text{ min}^{-1}$, $K_{\text{M}} = 5.0 \pm 0.6 \text{ mM}$, and $k_{\text{cat}}/K_{\text{M}} = 6 \text{ min}^{-1} \text{ mM}^{-1}$ for (*S*)-styrene oxide, respectively. Thus, NcsF2 is 44-fold more specific for (*R*)-

(5) (a) Faber, K.; Kroutil, W. *Tetrahedron: Asymmetry* **2002**, *13*, 377–382. (b) Hwang, S.; Choi, C. Y.; Lee, E. Y. *Biotechnol. Lett.* **2008**, *30*, 1219–1225. (c) Steinreiber, A.; Faber, K. *Curr. Opin. Biotechnol.* **2001**, *12*, 552–558. (d) Bellucci, G.; Chiappe, C.; Cordoni, A. *Tetrahedron: Asymmetry* **1996**, *7*, 197–202. (e) Kroutil, W.; Mischitz, M.; Plachota, P.; Faber, K. *Tetrahedron Lett.* **1996**, *37*, 8379–8382.

(6) See the Supporting Information for full experimental details.

(7) Barth, S.; Fischer, M.; Schmid, R. D.; Pleiss, J. *Proteins: Struct. Funct. Bioinform.* **2004**, *55*, 846–855.

(8) (a) Doderer, K.; Lutz-Wahl, S.; Hauer, B.; Schmid, R. D. *Anal. Biochem.* **2003**, *321*, 131–134. (b) Mateo, C.; Archelas, A.; Furstoss, R. *Anal. Biochem.* **2003**, *314*, 135–141.

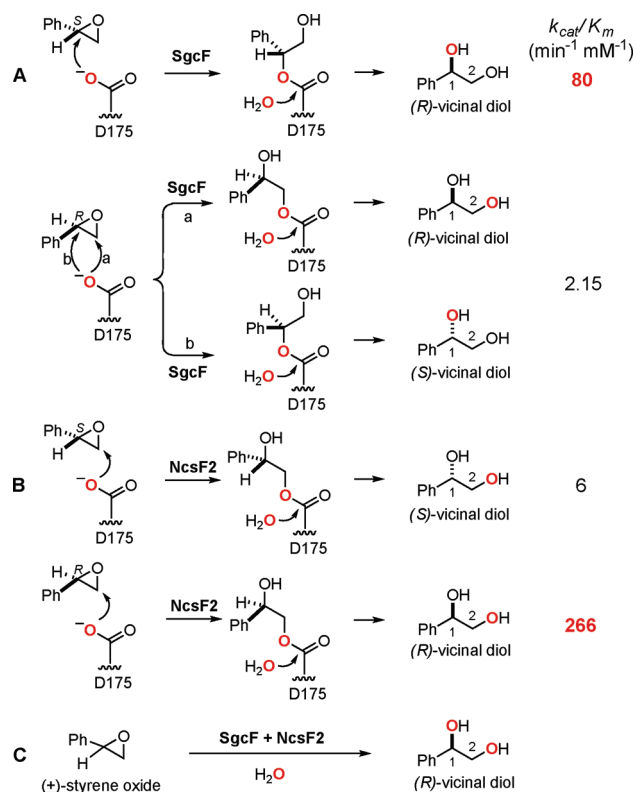


Figure 2. SgcF- and NcsF2-catalyzed hydrolysis of styrene oxide to 1-phenyl-1,2-ethanediol (vicinal diol): (A) SgcF-catalyzed regioselective hydrolysis of (*S*)-styrene oxide to (*R*)-vicinal diol and regioselective hydrolysis of (*R*)-styrene oxide to both (*R*)- and (*S*)-vicinal diol with enantioselectivity ($E = 37$) for the (*R*)-styrene oxide; (B) NcsF2-catalyzed regioselective hydrolysis of (*S*)- and (*R*)-styrene oxides to (*S*)- and (*R*)-vicinal diols, respectively, with enantioselectivity ($E = 44$) for the (*R*)-styrene oxide; (C) SgcF/NcsF2-catalyzed preparation of (*R*)-vicinal diol from racemic styrene oxide.

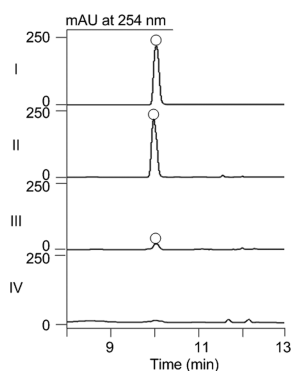


Figure 3. HPLC profiles for NcsF1- or NcsF2-catalyzed hydrolysis of racemic styrene oxide: (I) 1-phenyl-1,2-ethanediol standard (○); (II) with 50 μM NcsF2; (III) with 50 μM NcsF1; and (IV) with 50 μM NcsF2 (boiled) as a negative control.

styrene oxide (Figure 2B), in contrast to the 37-fold preference of SgcF for (*S*)-styrene oxide (Figure 2A).⁴ The opposite enantioselectivities of NcsF2 and SgcF were consistent with the respective vicinal diol stereochemistries

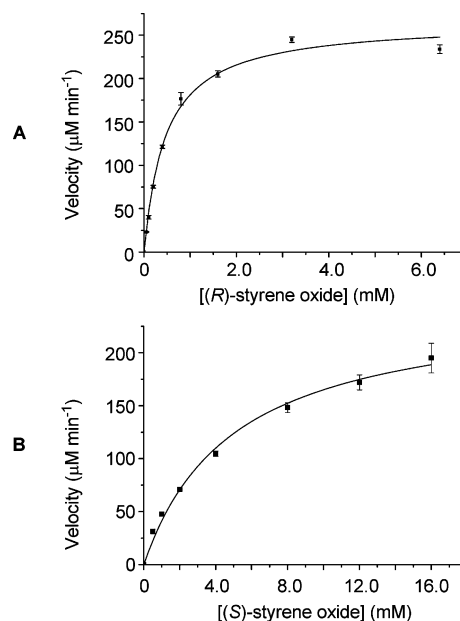


Figure 4. Kinetic analysis of NcsF2-catalyzed hydrolysis of (*S*)- and (*R*)-styrene oxide showing single substrate kinetic plots for (A) (*R*)-styrene oxide with 2 μM NcsF2 and (B) (*S*)-styrene oxide with 8 μM NcsF2.

of NCS and C-1027 (Figure 1), but only if NcsF2 was regioselective for C-2 and catalyzed stereochemical inversion of the preferred (*R*)-styrene oxide substrate to yield the (*S*)-vicinal diol.

We therefore examined the stereochemistry of the NcsF2 reaction products using chiral HPLC (Figure 5)^{4,6} and surprisingly found no inversion of stereochemistry. Specifically, NcsF2 transformed (*R*)-styrene oxide to (*R*)-1-phenyl-1,2-ethanediol (Figure 5A, panel II, 98% ee) and (*S*)-styrene oxide to (*S*)-1-phenyl-1,2-ethanediol (Figure 5A, panel V, 90% ee). Similarly, NcsF2 hydrolyzed the racemic substrate to afford racemic product (Figure 5A, panel III). This finding strongly suggested that, unlike SgcF (Figure 2A), NcsF2 catalyzes addition of water exclusively at C-2 for both enantiomers (Figure 2B).

We next verified the C-2 regioselectivity of NcsF2 using an ^{18}O -labeling experiment.^{4,6} Hydrolysis of (*R*)- or (*S*)-styrene oxide by NcsF2 was performed in the presence of [^{18}O]- H_2O , and the products were analyzed by ^{13}C NMR. As expected, a 2.4 Hz chemical shift was observed exclusively at the C-2 position for the 1-phenyl-1,2-ethanediol product resulting from either the (*R*)- or (*S*)-styrene oxide substrate (Figure 6). In contrast to SgcF, which adds H_2O to either C-1 or C-2 depending on the stereochemistry of the substrate (Figure 2A),⁴ NcsF2 is regioselective for C-2 of styrene oxide (Figure 2B).

Finally, the complementary activities of NcsF2 and SgcF were exploited to prepare enantiopure (*R*)-1-phenyl-1,2-ethanediol from racemic styrene oxide (Figure 2C). For example, SgcF has 37-fold higher specificity for (*S*)-styrene oxide, which is transformed almost exclusively to (*R*)-1-phenyl-1,2-ethanediol, while the slower reacting (*R*)-styrene

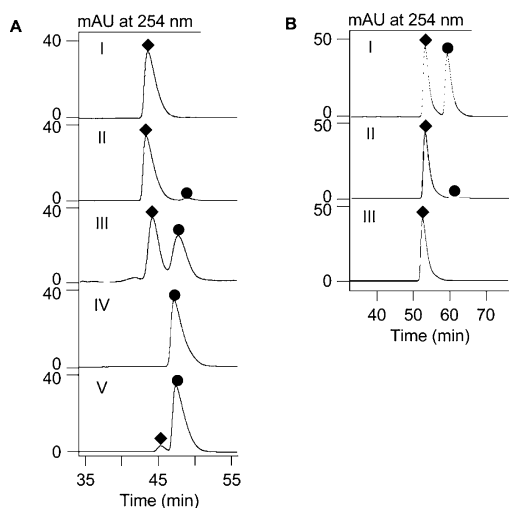


Figure 5. Chiral HPLC analysis for determination of stereochemistry of 1-phenyl-1,2-ethanediol resulting from NcsF2- or NcsF2/SgcF-catalyzed hydrolysis of styrene oxide.^{6,10} A: (I) (*R*)-1-phenyl-1,2-ethanediol standard; (II) NcsF2 with (*R*)-styrene oxide; (III) NcsF2 with ($\pm$)-styrene oxide; (IV) (*S*)-1-phenyl-1,2-ethanediol standard; (V) NcsF2 with (*S*)-styrene oxide. B: (I) ($\pm$)-1-phenyl-1,2-ethanediol standard; (II) NcsF2/SgcF with (*R*)-styrene oxide; (III) (*R*)-1-phenyl-1,2-ethanediol standard. (◆), (*R*)-1-phenyl-1,2-ethanediol; (●), (*S*)-1-phenyl-1,2-ethanediol.

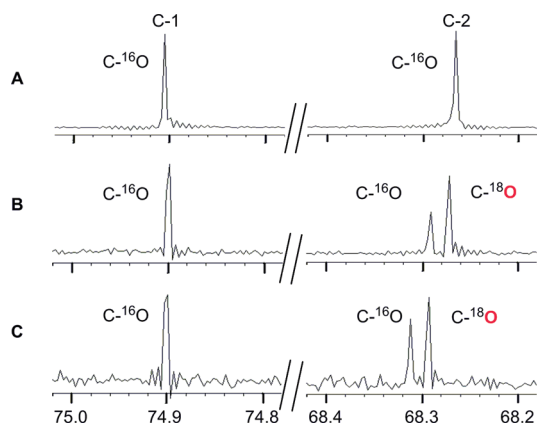


Figure 6. ¹³C NMR spectra for C-1 and C-2 of 1-phenyl-1,2-ethanediol produced by NcsF2-catalyzed hydrolysis of (A) racemic styrene oxide in H₂O, (B) (*R*)-styrene oxide in [¹⁸O]-H₂O (~80% final concentration), and (C) (*S*)-styrene oxide in [¹⁸O]-H₂O (~60% final concentration).

oxide produces a minor but significant amount of unwanted (*S*)-vicinal diol (Figure 2A). Conversely, NcsF2 preferentially hydrolyzes (*R*)-styrene oxide to afford the (*R*)-vicinal diol, but slower reacting (*S*)-styrene oxide yields unwanted (*S*)-vicinal diol product (Figure 2B). Thus, for a given enzyme, the “slow” enantiomer that yields unwanted product is the substrate for the other enzyme, which rapidly generates the correct product. These complementary specificities and product stereochemistries predict that the two enzymes

together should generate (*R*)-1-phenyl-1,2-ethanediol in high enantiomeric excess (Figure 2C). Indeed, we carried out the reaction with a 3.5:1 ratio of SgcF/NcsF2 with 14 mM styrene oxide and observed (*R*)-1-phenyl-1,2-ethanediol in 87% yield with 99% ee (Figure 5B).⁶ Such systems employing complementary enzymes have previously been used to generate enantiomerically enriched products.⁹ For example, a potato EH and an engineered EH from *Agrobacterium radiobacter* AD1 together transformed racemic styrene oxide to (*R*)-1-phenyl-1,2-ethanediol with 98% ee after complete conversion.^{9a} Interestingly, these cases resulted from deliberate efforts involving screening many enzymes to obtain a suitable biocatalytic process. In contrast, our results arose fortuitously from studies of enediyne biosynthesis and highlight the biocatalytic potential available to be harnessed from our ever-expanding knowledge of natural product biosynthetic machineries.

In conclusion, we have demonstrated that the NcsF2 enzyme is active as an epoxide hydrolase toward styrene oxide, an activity consistent with its proposed function in hydrolyzing a terminal epoxide enediyne intermediate. We further showed that NcsF2 regioselectively adds H₂O to the less hindered C-2 position of styrene oxide such that the stereochemistry is retained in the vicinal diol product. Surprisingly, the enantioselectivity of NcsF2 for (*R*)-styrene oxide to (*S*)-styrene oxide (*E* = 44) is inconsistent with preferential production of an (*S*)-vicinal diol biosynthetic intermediate, as would be expected on the basis of the structure of **1**. However, NcsF2 remains our best candidate for this function, and its unexpected selectivity may reflect the inadequacy of styrene oxide as a substrate mimic. Moreover, the natural enediyne core epoxide precursor is likely a single enantiomer, in which case regioselective hydrolysis of an (*S*)-epoxide enediyne intermediate would produce the expected (*S*)-vicinal diol stereochemistry (Figure 1A).

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Supporting Information Available: Detailed experimental procedures, SDS-PAGE (Figure S1), and sequence alignment (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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(9) (a) Cao, L.; Lee, J.; Chen, J. W.; Wood, T. K. *Biotechnol. Bioeng.* **2006**, *94*, 522–529. (b) Pedragosa-Moreau, S.; Archelas, A.; Furstoss, R. *J. Org. Chem.* **1993**, *58*, 5533–5536. (c) Manoj, K. M.; Archelas, A.; Baratti, J.; Furstoss, R. *Tetrahedron* **2001**, *57*, 695–701.

(10) The discrepancy in retention times between A and B results from the use of a new OD-H column in the latter.