Incorporation of Two Oxygens from $^{18}O_2$ in the Epoxyquinone from the Dihydroxyacetanilide Epoxidase Reaction: Evidence for a Dioxygenase Mechanism

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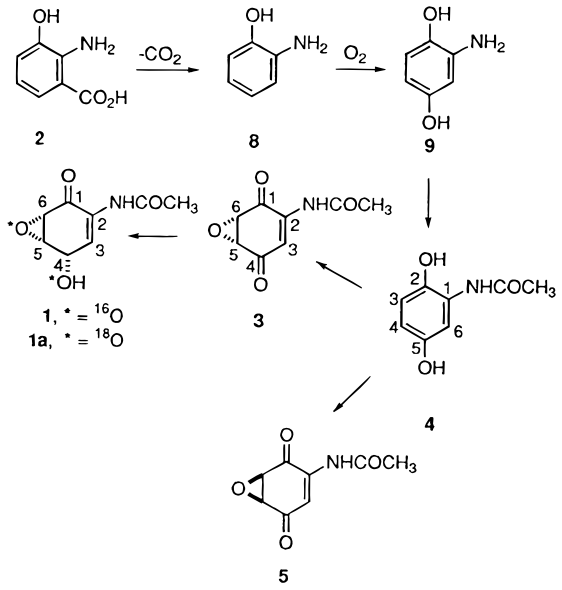
Abstract: Dihydroxyacetanilide, **4**, is oxidized to the 5*R*,6*S*-epoxyquinone, **3**, by dihydroxyacetanilide epoxidase-I (DHAE I) from *Streptomyces* LL-C10037, without the assistance of an organic cofactor. ^{13}C NMR analysis revealed that in the presence of $^{18}O_2$ a full equivalent of ^{18}O is incorporated at the epoxide. However, control reactions revealed the rapid exchange of the C-4 carbonyl with $H_2{}^{18}O$. By coupling the DHAE I reaction with 2-acetamido-5,6-epoxy-1,4-benzoquinone oxidoreductase (AEBQOR I) from the same organism, NADP, and an NADPH regeneration system based on glucose 6-phosphate dehydrogenase, the epoxyquinol LL-C10037α, **1**, was produced with ∼20% incorporation of a second ^{18}O atom at the C-4 alcohol. Therefore, DHAE I is a dioxygenase with an epoxidation mechanism essentially the same as has been observed for the dihydrovitamin K epoxidation occurring during the mammalian vitamin K-dependent glutamate carboxylase reaction.

Antibiotic LL-C10037α, **1**, produced by *Streptomyces* LL-C10037,[1] is derived from the shikimic acid pathway via 3-hydroxyanthranilic acid, **2**,[2,3] and the 5*R*,6*S*-epoxyquinone **3** (Scheme 1).[4] The latter is derived directly from 2,5-dihydroxyacetanilide, **4**, which is also the immediate precursor to the 5*S*,-6R-epoxyquinone **5** in *Streptomyces* MPP3051.[4,5] The enzymes that convert **4** to **3** and to **5**, (3-*si*,4-*re*)-2,5-dihydroxyacetanilide epoxidase (DHAE I) and (3-*re*, 4-*si*)-dihydroxyacetanilide epoxidase (DHAE II), respectively, have been purified and have been shown to carry out these 4-electron oxidations without the need for either flavin or nicotinamide coenzyme.[6] DHAE I and DHAE II required only substrate (**4**) and molecular oxygen. Similar reactions had been reported for oxidation of gentisyl alcohol by a particulate preparation from the fungus *Penicillium patulum*[7] and for dihydrovitamin K with a microsomal mammalian enzyme (dihydrovitamin K epoxidase or vitamin K-dependent glutamate carboxylase),[8−10] which suggests that numerous naturally-occurring epoxyquinones and epoxyquinols might be derived by the same novel mechanism.[6] In each case studied with $^{18}O_2$, label was found in the oxirane oxygen. Priest and Light proposed a monooxygenase reaction with epoxide formation directly from a peroxide.[7] However, Dowd and co-workers proposed a dioxygenase reaction with intervention of a dioxetane,[10] and both this latter group[11−14] and Walsh's[15] have confirmed its validity for this enzyme. Thus, **1** could be generated *via* either **6** or **7** (Scheme 2), were one of these mechanisms to apply to the DHAE reaction.

Scheme 1



Culturing *S.* LL-C10037 in the presence of $^{18}O_2$ yielded **1a**, with label incorporated at both the oxirane and hydroxyl oxygens (Scheme 1).[2] However, the C-5 hydroxyl of **4** is introduced by hydroxylation of 2-aminophenol, **8**, to yield the hydroquinone **9**,[3] so the labeling in **1a** did not unambiguously require invoking[16] the Dowd dioxygenation mechanism for the epoxi-

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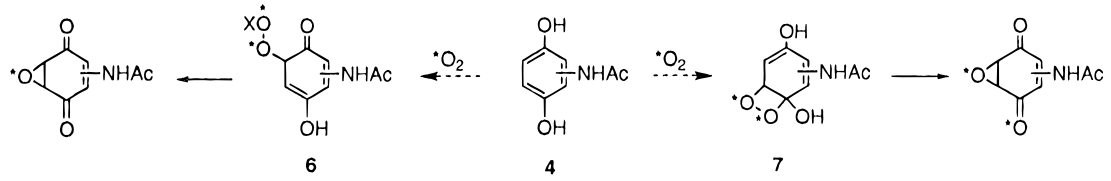
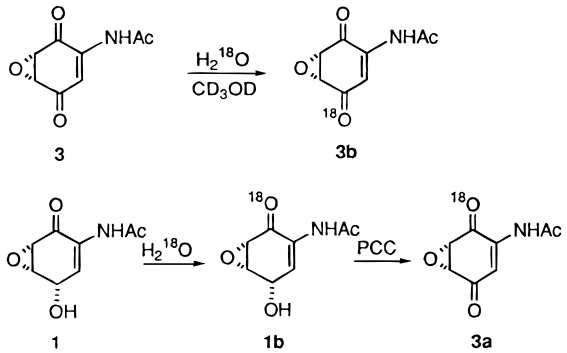
Scheme 2



Scheme 3



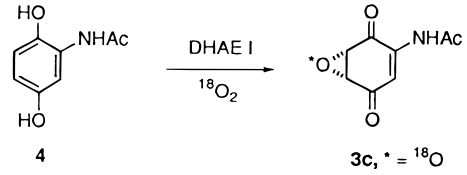
dase reaction in this pathway. We have, therefore, examined the DHAE I reaction in the presence of $^{18}O_2$.

Results and Discussion

We had previously observed that the ketone carbonyl of **1** undergoes rapid exchange with water,[2] and have observed partial[17] and complete[18,19] washout of ^{18}O-labels from quinone carbonyls that had initially been biosynthetically labeled by $^{18}O_2$ during *in vivo* feeding studies. It was thus necessary to determine the integrity of the quinone carbonyls of **3**. A sample of **3** was dissolved in methanol-d_4, $H_2{}^{18}O$ was added, and the ^{13}C NMR spectrum was then sampled every 15 min. The resonance at δ 194 was unchanged over a total of 600 min, but the δ188 resonance already appeared as two lines ($\Delta\delta$ 0.045 ppm) after only 15 min. The ^{18}O isotope-induced shifted resonance revealed that one carbonyl was subject to rapid exchange. In order to assign the carbonyl resonances, a sample of **1** was partially exchanged (20%) in the same manner, and the derived **1b** was then oxidized with PCC to yield **3a** (Scheme 3). In this latter compound, an additional, isotope-induced shifted resonance was observed at δ 194 ($\Delta\delta$ 0.049 ppm). Thus, it is the C-4 carbonyl of **3** that exchanged with the $H_2{}^{18}O$, yielding **3b**.

If the dioxygenase dioxetane mechanism is applied to the DHAE I reaction, two regiospecific oxygenations would be possible, each leading to a different labeled dioxetane (i.e. **10a** or **10b**, Scheme 4). Furthermore, the opening of each dioxetane would generate a hydrate with diastereotopic oxygens. Thus, enzyme-catalyzed dehydration could eliminate either the original or the newly introduced oxygen, leading to retention or loss of the ^{18}O label at this site, respectively. In fact, only a 17% incorporation of ^{18}O at the analogous oxygen was observed in the dihydrovitamin K epoxidase reaction, and this was explained by fortuitous non-specific chemical dehydration competing with an enzyme-catalyzed loss of the (newly-introduced) hydroxyl *syn* to the epoxide.[14,15]

Although only small amounts of ^{18}O-labeled vitamin K epoxide could be generated, forcing recourse to mass spectrometric analysis, adequate quantities of **3** could be generated by DHAE I to permit direct analysis by ^{13}C NMR. DHAE I was partially purified by DE-52 anion exchange chromatography, and an aliquot was gently shaken and incubated with 20 mg of **4** under an $^{18}O_2$ atmosphere. As substrate was consumed (HPLC monitoring), additional portions of **4** were added. Within 2.5 h 140 mg of **4** had been consumed, and workup yielded 120 mg of **3c**. ^{13}C NMR analysis revealed one set of resonances (Figure 1, lower trace). When the sample was diluted with a portion of authentic unlabeled **3**, only the oxirane carbon resonances, at δ 53.6 and δ 55.1, were doubled (Figure 1, upper trace). The latter result proved that ^{18}O had been incorporated ~100% in the epoxide, but there was no evidence for a second ^{18}O-label. This reduced the likelihood of a dioxygenase mechanism involving C-2 of **4** (*vide infra*).



In order to examine the possibility of a dioxygenase mechanism involving C-5 of **4**, it would be necessary to trap any ^{18}O introduced at C-4 of the derived **3**. Otherwise, the label was sure to be lost by exchange. The approach chosen was to rapidly reduce the C-4 carbonyl to an exchange-inert hydroxyl. The last step in the biosynthesis of **1**, an NADPH-dependent reduction of **3**,[3] was added to the protocol. The epoxidase could be eluted from the DE-52 column at 200 mM KCl, and partially purified 2-acetamido-5,6-epoxy-1,4-benzoquinone oxidoreductase (AEBQOR I) subsequently eluted at 300 mM KCl. The specific activity of the epoxidase at this stage was five times that of the dehydrogenase. Success of the labeling experiment would require effectively immediate *in situ* reduction by the coupled reaction, so a large volume of dehydrogenase was needed. A considerable quantity of NADPH would also be needed, and the system was developed further to include *in situ* NADPH regeneration by glucose 6-phosphate dehydrogenase (G6PDH).[20]

In the event, a sample of **4**, 20 mL of dehydrogenase, G6PDH, and glucose 6-phosphate, **11**, were combined and carefully titrated with small aliquots of epoxidase; HPLC monitoring showed the consumption of **4** and formation of **1** without observable levels of intervening **3**. When these conditions were successfully repeated in the presence of $^{18}O_2$, additional portions of **4,** dehydrogenase, and G6P/G6PDH were periodically added. Ultimately 50 mg of **1c** were produced in 4 h (Scheme 5). ^{13}C NMR analysis revealed single resonances for all carbons except C-5, C-6, and C-4. The former two showed small signals indicating ~5% ^{16}O-epoxide, while the C-4 resonance at δ 65.58 had two lines ($\Delta\delta$ 0.017 ppm, same as that previously observed for **1a**[2]) in a ratio of 3.5:1 (Figure 2, lower trace). When this sample was diluted 1:2 with authentic unlabeled **1**, the ^{13}C NMR spectrum showed two lines for each of the two oxirane carbons

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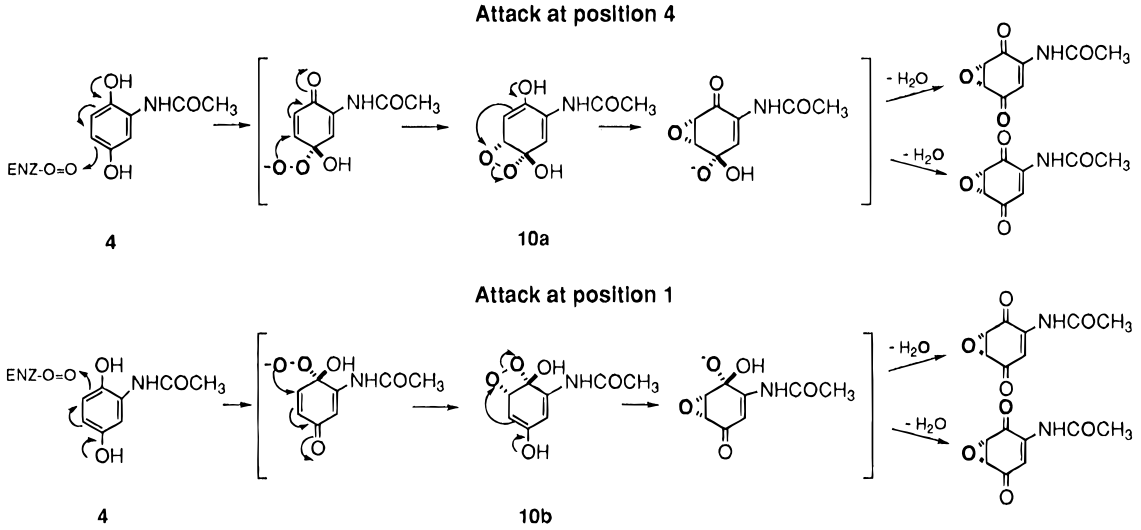
Scheme 4



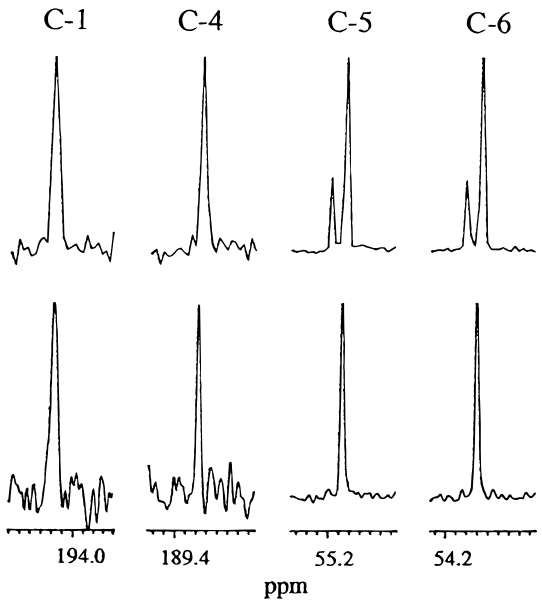


Figure 1. Portions of the 100.6-MHz ^{13}C NMR spectra of **3c** (lower trace) and of **3c** + **3** (upper trace). Tick marks are 0.5 Hz apart.

as well as for C-4 (Figure 2, upper trace). The relative intensity of the ^{18}O isotope-shifted resonance for the latter carbon was now reduced, as expected. The EI mass spectrum of **1c** showed peaks in the molecular ion region at *m/z* 183 ($^{18}O_0$), 185 ($^{18}O_1$), and 187 ($^{18}O_2$) with relative intensities of 0.98:7.85:1.16 after subtracting M + 1 contributions measured for a sample of unlabeled **1** (or a ratio of 0.95:7.60:1.45 if the theoretical M + 1 value were used). This indicates 12−15% doubly ^{18}O-labeled material, which can be considered a lower limit for the presence of ^{18}O at C-4, and is not too different than the 21% indicated by ^{13}C NMR.

Conclusions

We have now demonstrated conclusively that DHAE I is a dioxygenase and appears to utilize the same remarkable epoxidation mechanism previously observed for the dihydrovitamin K epoxidation that occurs during the mammalian vitamin K-dependent glutamate carboxylase reaction. It is reasonable to assume that this family of "hydroquinone dioxygenase (epoxidizing)" enzymes includes DHAE II, the gentisyl alcohol epoxidase (patulin pathway), and numerous others, such as for terremutin and terreic acid,[21] nanaomycin D,[22] frenolicin,[23] etc. Our mechanism results suggest an evolutionary relationship between DHAE I and the epoxidase domain of the vitamin K enzyme. The dihydrovitamin K epoxidation provides the "base strength amplification" proposed by Dowd to be essential for carboxylation of glutamate residues of prothrombin, which is essential to the blood-clotting cascade. We have recently cloned the DHAE I gene on a 7.1 kb fragment by a reverse genetics approach.[24] Subcloning is in progress and sequence comparisons will soon be possible to determine whether these epoxidation activities are related only at the mechanistic level, or whether they share structural homology. If the latter, one may ask whether a fusion was generated that involved an ancestral simple hydroquinone epoxidase. If so, what was the ancestor of the carboxylase domain?

Experimental Section

General. Water was purified with a MilliQ System, Millipore Corp. HPLC analyses were performed on a Waters 600E HPLC instrument with a Linear UVIS 200 detector and an HP 3396A integrator. Reverse phase C_{18} (Econospere, 5 μm, 250 × 4.6 mm, Alltech Assoc.) columns were used. Bacterial fermentations were carried out in a rotary incubator (Lab-Line incubator shaker). Cell disruption was performed with a sonicator (Model W-225R, Heat Systems-Ultrasonic, Inc.). Refrigerated centrifugations were done in an IEC B-20a centrifuge. $^{18}O_2$ (95−98%), $^{18}H_2O$ (50%), and $^{18}H_2O$ (98%) were purchased from Cambridge Isotope Laboratories, Andover, MA. G6PDH was purchased from Sigma, St. Louis, MO. DE-52 anion exchange resin was purchased from Whatman.

Purification of DHAE I and AEBQOR I. The following buffers were used in the purification and DHAE I and AEBQOR I. buffer I: 50 mM potassium phosphate, 10% glycerol, 10 g/L soluble polyvinylpyrrolidone (10 000 MW, pH 6.5). Buffer II: 50 mM potassium phosphate, 10% glycerol, pH 6.5. Buffer III: buffer II with 50 mM KCl. Buffer IV: buffer II with 100 mM KCl. Buffer V: buffer II with 200 mM KCl. Buffer VI: buffer II with 300 mM KCl.

Step 1: Preparation of Cell-Free Extract. Cells from 6.0 L of 96-h fermentations were grown and harvested as reported earlier.[6] The washed cells (200 g, wet weight) were suspended in 400 mL of buffer I (4 °C). To this suspension polyvinyl polypyrrolidone (2.25 g) and washed Amberlite XAD-4 resin (2.25 g) were added.[25] The cellular

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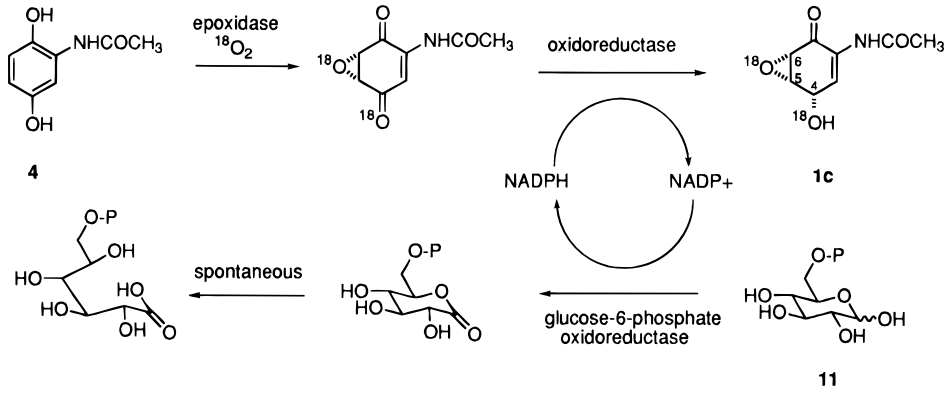
Scheme 5



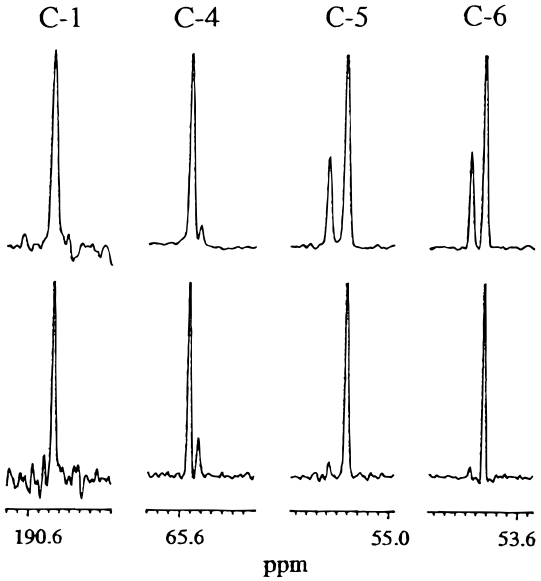


Figure 2. Portions of the 100.6 MHz ^{13}C NMR spectra of **1c** (lower trace) and of **1c** + **1** (upper trace). Tick marks are 0.5 Hz apart.

suspension was equally distributed into four beakers, and each portion was disrupted by sonication (maximum power, 90% duty, pulsed for 3 × 20 s). Cell debris was removed by centrifugation (4 °C, 13800*g*, 20 min), and the supernatants were combined to afford a crude cell-free extract (CFE, 500 mL).

Step 2: DE-52 Anion-Exchange Chromatography. Prior to use, DE-52 resin (80 g) was washed sequentially with 0.2 L of the following: milliQ H_2O, 1 M KCl, and buffer I. The washed resin in buffer I (2:1) was gravity packed in a 10 × 100 cm BioRad Econo column. Freshly prepared cell free extract was added to the column. The resin was washed sequentially with 0.125 L of the following: buffer II, buffer III, and buffer IV. In a typical preparation, DHAE I was eluted with buffer V (125 mL) and collected in 60 mL (1.04×10^{-4} μmol·min^{-1}·μL^{-1}), followed by elution of AEBQOR I with the addition of buffer VI (125 mL), which was collected in 120 mL (2.06×10^{-5} μmol·min^{-1}·μL^{-1}). The two enzymes were stored up to 8 weeks, at −80 °C, without significant loss of activity.

Determination of Oxygen Exchangeability in 3. In an NMR tube, **3** (25 mg) was dissolved in methanol-d_4, and the ^{13}C NMR spectrum (300 MHz) was obtained. $H_2{}^{18}O$ (200 μL, 50%) was added and the spectrum was obtained every 15 min for 10 h.

^{18}O Exchange of 1 to 1b and PCC Oxidation of 1b to 3a. To dry **1** (35 mg, 0.2 mmol) were added 60 μL of $H_2{}^{18}O$ (98%) and 100 μL of MeOH. After 24 h at 25 °C, the mixture was concentrated *in vacuo* and the newly afforded **1b** was dissolved in dry CH_2Cl_2 (25 mL). NaOAc (16.0 mg, 0.2 mmol) and PCC (82 mg, 0.37 mmol) were added. The resulting solution was briefly sonicated and allowed to stir for 1.5 h (25 °C). The rusty brown reaction mixture was passed through a 1-in. column of 50:50 Florisil:Celite packed in a Pasteur pipet. The CH_2Cl_2 fractions containing the product were combined and concentrated *in vacuo* to provide a yellow solid, **3a**.

Oxidation of 4 to 3c with DHAE I. A 1-L Erlenmeyer flask was modified by removing the top half, generating a new constriction, and refitting with a ground glass 24/40 joint. This modification afforded a flask with less head space while retaining the same amount of surface area. To this flask DE-52 purified DHAE I (100 mL, 4 °C) was diluted 1:4 with O_2-free buffer II (previously sparged with helium at 4 °C for 1 h). A rubber septum was fitted over the joint, and a vacuum was generated using a high vacuum pump. The enzyme mixture was allowed to come to 25 °C while *in vacuo* (20 min). $^{18}O_2$ (1 L) was added and the flask was placed in a rotary shaker (25 rpm, 30 °C). **4** (20 mg) was added as a solution (20 mL of 1 mg/100 mL of O_2-free H_2O) and a 400-μL aliquot was taken for a zero time starting point. In 15 min **4** had been completely converted (HPLC analysis) and more **4** (20 mg) was added. The reaction was checked periodically and more **4** was added as needed. In 2.5 h 140 mg of **4** had been converted to **3c** and the reaction was stopped. KH_2PO_4 was added to bring the pH to 4.7 and the solution was brought to 50% $(NH_4)_2SO_4$ saturation. Ethyl acetate was used to extract **3c** (5 × 150 mL). The EtOAc extract was dried over anhydrous $MgSO_4$ and concentrated *in vacuo* affording a burgundy oil. Approximately 40% of the crude **3c** was further purified by flash silica (40% EtOAc/hexanes) yielding **3c** as a yellow solid (50 mg).

Conversion of 4 to 1c with DHAE I/AEBQOR I/G6PDH. The same modified flask used in the conversion of **4** to **3c** was used. DE-52 purified AEBQOR I (20 mL), DE-52 purified DHAE I (50 μL), and O_2-free buffer II (300 mL) were placed in the flask (4 °C). The flask was brought to 25 °C *in vacuo* and $^{18}O_2$ (400 mL) was added. G6PDH (23.96 units in buffer II), G-6-P (145.72 mg), and $NADP^+$ (91.7 mg) were added and the flask was placed in a shaker (25 rpm, 30 °C). After 5 min, **4** was added to start the reaction. Immediately after addition of **4** a zero time aliquot (400 μL) was removed. The reaction was monitored by HPLC and more G-6-P, $NADP^+$, and **4** were added as needed. After 4 h 30 mg of **4** had been converted to **1c** and the reaction was stopped. Workup yielded 24 mg of **1c**.

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