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Biosynthesis of acaterin: Mechanism of the reaction catalyzed by dehydroacaterin reductase

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Abstract—Dehydroacaterin reductase is an enzyme which catalyzes the final step of acaterin biosynthesis, that is, the reduction of the C-4/C-5 double bond of dehydroacaterin. The mechanism of the reduction was investigated with a cell-free preparation obtained from the acaterin-producing microorganism, *Pseudomonas* sp. A 92. Incubation of dehydroacaterin in the presence of [4,4-$^2H_2$]NADPH or D_2O followed by ^2H NMR analysis of the resulting acaterin revealed that the deuterium atom from NADPH was incorporated into the C-5 position of acaterin, while the deuterium atom from D_2O was introduced into the C-4 position. It was further demonstrated that the pro-*R* hydrogen at C-4 of NADPH was stereospecifically utilized in this reduction.

1. Introduction

Acaterin (**1**) was isolated from *Pseudomonas* sp. A 92 as an inhibitor of acyl-CoA:cholesterol acyltransferase.[1] Dehydroacaterin (**2**) was subsequently isolated by our group and elucidated to be the immediate biosynthetic precursor of acaterin.[2] We have been studying the biosynthesis of acaterin, since little has been known on the biosynthesis of secondary metabolites with a 2-penten-4-olide skeleton.[3] In the previous papers, we demonstrated that glycerol was incorporated into the branched C_3 moiety (C-3, -4, and -5 positions) in such a manner that *sn*-C-1 of glycerol becomes C-3 of **1**,[4] and pro-*R* and pro-*S* hydrogens at *sn*-C-3 of glycerol become 5*E* and 5*Z* hydrogens, respectively, of 4-dehydroacaterin (**2**).[5] Furthermore, hydrogen atoms at *sn*-C-1 of glycerol were lost completely during the transformation.[4] On the basis of these findings, we proposed that the immediate precursor of the branched C_3 unit of **1** could be a glyceric acid equivalent, and a tetronic acid-type intermediate would be involved in the formation of 3-H type compounds.[4] Moreover, we reported evidence supporting a coupling of octanoate and a C_5 unit corresponding to the lactone part in acaterin biosynthesis.[6] In addition, we indicated that the biosynthesis of agglomerins, which belong to the same class of secondary metabolites, utilizes a glyceric acid equivalent as the immediate biosynthetic precursor of the branched C_3 unit of **1**.[7] These accumulated evidence led us to postulate biosynthetic route for acaterin (Scheme 1). More recently we have developed a cell-free system catalyzing transformation of **2** to **1** (the enzyme was named dehydroacaterin reductase) from the acaterin-producing microorganism. The availability of the in vitro system prompted us to investigate the mechanism of the reduction of the unique substrate, an α, β:γ, δ-diene system conjugated with a lactone carbonyl group. This paper describes the regiospecificity of hydrogen transfer from NADPH and water, and the stereospecificity in the utilization of the prochiral C-4 hydrogens of NADPH in the reaction catalyzed by dehydroacaterin reductase.

2. Results and discussion

The active cell-free preparation (20,000*g* supernatant) was obtained by sonicating the cells harvested in a phosphate buffer (pH 7.4) followed by centrifugation of the sonicate. Addition of NADPH showed increased activity for the conversion of **2** to **1**. The enzyme activity was conveniently determined by reversed-phase HPLC analysis of the product formed.

Dehydroacaterin was incubated with the cell-free preparation in the medium of D_2O/H_2O (2:1) in the presence of NADPH. The resulting acaterin was purified and analyzed by ^2H NMR spectroscopy. The ^2H NMR

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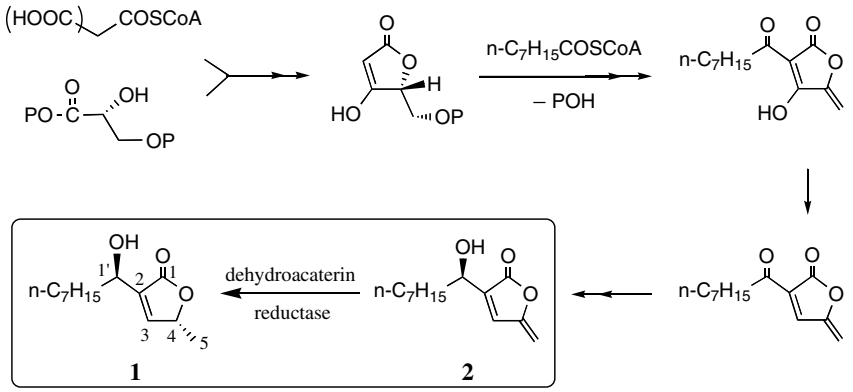


Scheme 1. Postulated biosynthesis of acaterin (**1**). The present studies deal with dehydroacaterin reductase which catalyzes the conversion of dehydroacaterin (**2**) to **1**.

spectrum is shown in Figure 1. The deuterium signal resonated at δ 5.07 corresponds to the signal of H-4 of **1**, thus indicating that the deuterium atom was introduced at C-4 of **1** regiospecifically. Dehydroacaterin was then incubated in the presence of [4,4-$^2H_2$]NADPH with the cell-free preparation. The formed acaterin showed a signal at δ 1.45 in the ^{2}H NMR spectrum, which corresponds to 5-H_3 of **1**, thus indicating that the deuterium atom from NADPH was incorporated regiospecifically into the C-5 position of **1**. These results agree with the Michael addition mechanism wherein the hydride transfer from NADPH took place at the C-5 position and the resulting enolate anion was trapped by the proton from water at the C-4 position.

With the hydride transfer from NADPH having been established, we further investigated which prochiral hydrogen at C-4 of NADPH is utilized in this reduction. Incubation of dehydroacaterin in the presence of [4-pro-*R*-^{2}H]- or [4-pro-*S*-^{2}H]NADPH cofactors would solve the question. Generally, the requisite [4-pro-*R*-^{2}H]NADPH has been prepared enzymatically from $NADP^+$ with an [^{2}H]alcohol as a deuterium source in a reaction catalyzed by an alcohol dehydrogenase such as *Thermoanaerobium brockii* alcohol dehydrogenase,[8] while [4-pro-*S*-^{2}H]NADPH has been obtained with [1-^{2}H]glucose as a deuterium source in a reaction catalyzed by glucose dehydrogenase such as *Bacillus megaterium* glucose dehydrogenase.[9] Mosted et al. reported an unprecedented A-specific glucose dehydrogenase, *Thermoplasma acidophilium* glucose dehydrogenase,[10] and now this enzyme is commercially available. In the present study, [4-pro-*R*-^{2}H]- and [4-pro-*S*-^{2}H]NADPH cofactors were enzymatically prepared from $NADP^+$ with

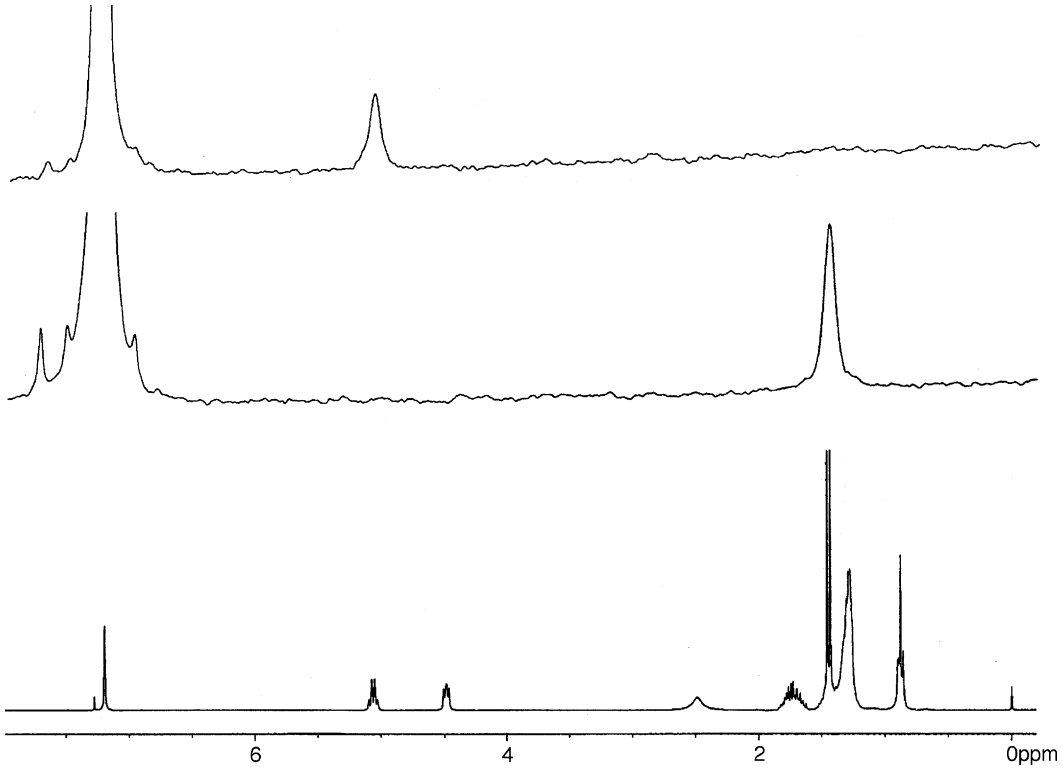


Figure 1. ^{2}H NMR spectra (61 MHz, $CHCl_3$) of acaterin obtained from dehydroacaterin with a cell-free preparation. Upper: incubated in D_2O/H_2O in the presence of NADPH. Middle: incubated in H_2O in the presence of [4,4-$^2H_2$]NADPH. Lower: ^{1}H NMR spectrum of non-labeled acaterin.

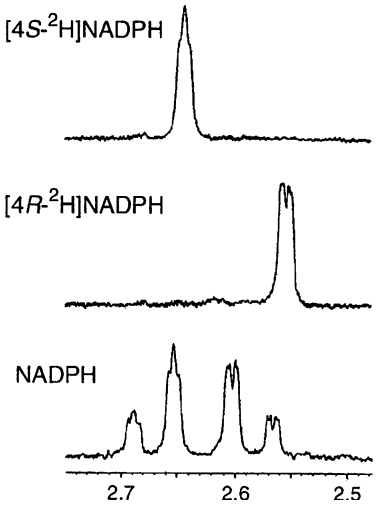


Figure 2. ^{1}H NMR spectra (500 MHz, D_2O) of the C-4 hydrogen of [4-pro-*S*-^{2}H]- and [4-pro-*R*-^{2}H]NADPHs.

[1-^{2}H]glucose as a common deuterium source by the action of *T. acidophilium*[10] (A-specific) and *Cryptococus uniguttulas*[10] (B-specific) glucose dehydrogenases, respectively. The ^{1}H NMR spectra of the labeled NADPH molecules are shown in Figure 2, which indicated excellent stereochemical purity at the C-4 position.[10]

Meanwhile, purification of dehydroacaterin reductase has progressed to some extent. Thus, the cell-free preparation was fractionated by $(NH_4)_2SO_4$ precipitation and the precipitates were further fractionated through a DEAE column to give a partially (16-fold) purified dehydroacaterin reductase fraction. Dehydroacaterin was then incubated with this enzyme preparation in the presence of [4-pro-*R*-^{2}H]- or [4-pro-*S*-^{2}H]NADPH cofactors. The resulting acaterin was purified and analyzed by ^{2}H NMR spectroscopy. As shown in Figure 3, acaterin obtained in the presence of [4-pro-*R*-^{2}H]NADPH showed a deuterium peak at δ 1.45, while instead no signal was detected near δ 1.45 in the ^{2}H NMR spectrum of acaterin obtained in the presence of [4-pro-*S*-^{2}H]NADPH. It is therefore concluded that dehydroacaterin reductase catalyzes stereospecific transfer of the 4-pro-*R* hydrogen (A-specific) of NADPH to the C-5 position of dehydroacaterin.

3. Conclusion

The present study established the regiochemistry of the introduction of hydrogen atoms from NADPH and water in the dehydroacaterin reductase-catalyzed reduction. In addition, it was determined that the pro-*R* hydrogen at C-4 of NADPH (A-specific) was utilized stereospecifically in this reduction. To our knowledge, regiochemistry in the reduction of α,β;γ,δ-unsaturated carbonyl compounds has been studied only in one case. The reduction of (*E*,*E*)-2,4-dienyoyl CoA catalyzed by 2,4-dienoyl CoA reductases from *Escherichia coli* and bovine liver mitochondria was reported to proceed according to the Michael addition mechanism wherein the hydride attacks the C-5 position of the substrate.[11,12] Interestingly, it was reported that in the case of *E. coli* (*E*,*E*)-2,4-dienoyl CoA reductase neither pro-*R* nor pro-*S* hydrogen of NADPH was incorporated into the product.[12] The crystal structure of the *E. coli* reductase (an iron–sulfur flavoprotein) was recently published[13] and the reason the C-4 hydrogen of NADPH is not directly transferred into the substrate was postulated. In contrast, it was reported that the pro-*S* hydrogen at

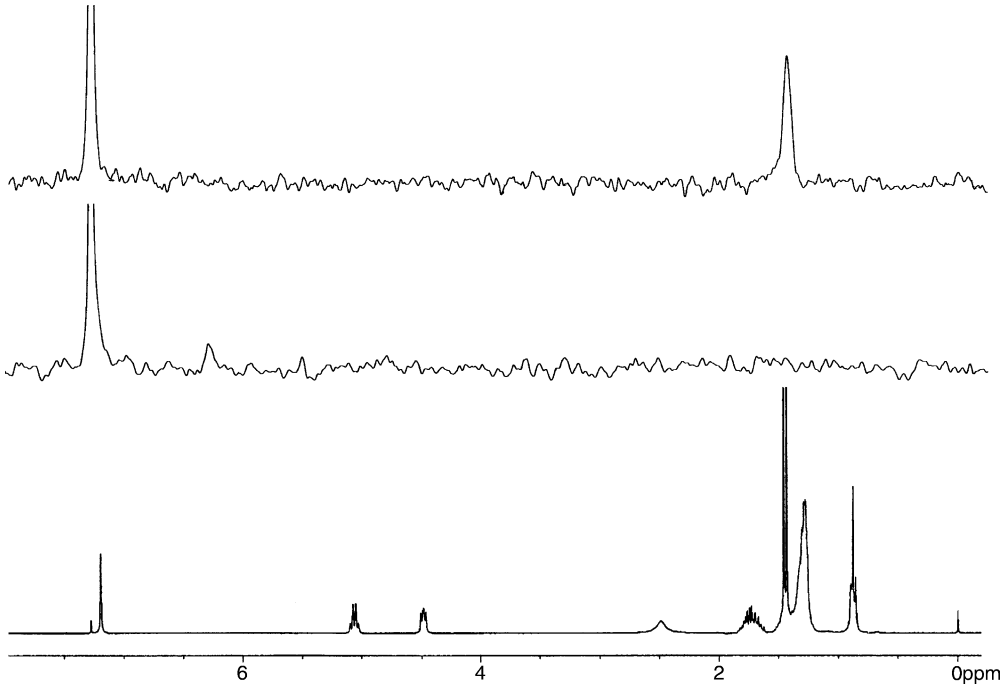
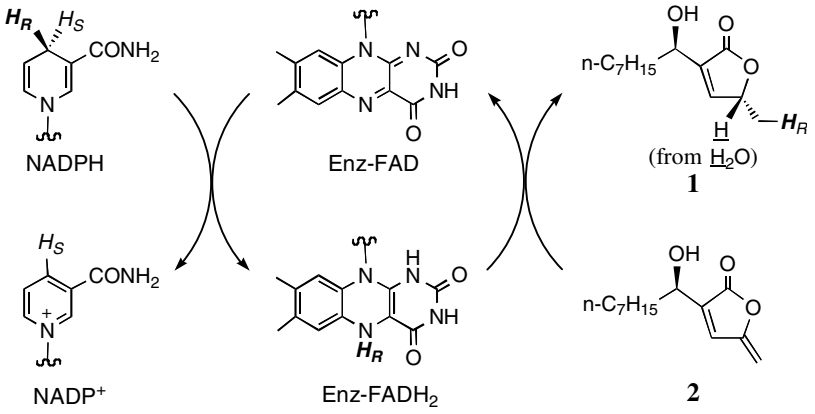


Figure 3. ^{2}H NMR spectra (76 MHz, $CHCl_3$) of acaterin obtained from dehydroacaterin upon incubation with a partially purified enzyme solution. Upper: incubated in the presence of [4-pro-*R*-^{2}H]NADPH. Middle: incubated in the presence of [4-pro-*S*-^{2}H]NADPH. Lower: ^{1}H NMR spectrum of non-labeled acaterin.



Scheme 2. Reactions catalyzed by dehydroacaterin reductase.

C-4 of NADPH was stereospecifically transferred directly to C-5 of the substrate in the reaction catalyzed by the bovine enzyme, which is free from FAD.[11] The deuteride transfer from [4-pro-*R*-^{2}H]NADPH to acaterin proceeded practically in a stoichiometric manner, as revealed by ^{1}H NMR analysis of the produced acaterin.[14] Dehydroacaterin reductase was found to contain tightly bound FAD (unpublished data). It is thus likely that the hydride transfer from NADPH to acaterin takes place via the flavin cofactor and this was confirmed by spectroscopic data monitoring the reduction of FAD (data not shown). We propose that the reactions catalyzed by dehydroacaterin reductase proceed as shown in Scheme 2. Quite recently we have completed the purification of dehydroacaterin reductase. Details of the purification and the properties of dehydroacaterin reductase will be reported elsewhere.

4. Experimental

4.1. General

^{2}H NMR spectra were recorded on a JEOL LA-400 or Bruker DRX500 spectrometer using $CHCl_3$ as a solvent. A signal of natural abundance deuterium of the solvent was used as internal standard (δ 7.26). Protein concentration was determined by the Lowry procedure with ovalbumin as standard.

4.2. Materials

Dehydroacaterin was prepared by the fermentation of *Pseudomonas* sp. A92 as described previously.[6] $NADP^+$ and NADPH were purchased from Wako Pure Chemical Industries (Osaka). [1-^{2}H]Glucose and *T. acidophilum* and *Cryptococus uniguttulas* dehydrogenases were obtained from Sigma, Toyopearl DEAE-650M from Tosoh (Tokyo). [4,4-$^2H_2$]NADPH was kindly provided by Ms. K. Takahashi, Kyoritsu College of Pharmacy.

4.3. Cell-free preparation

Maintenance of *Pseudomonas* sp. A92 and fermentation conditions are described previously.[6] In general, 48-h cultures of the strain, grown in nine 500-mL baffled Erlenmeyer flasks, each containing 100 mL of liquid medium, were harvested by centrifugation (4000*g*, 10 min at 4 °C). The cells (wet wt., 6.4 g) were washed with 0.1 M sodium phosphate buffer (pH 7.4), resuspended in the phosphate buffer (20 mL), and sonicated with a Bronson Sonifier 250 for 10 min × 3 times at 0 °C (ice water bath). The sonicate was volumed up to 60 mL and centrifuged at 8000*g* for 10 min. The supernatant was then centrifuged at 20,000*g* for 30 min and the resulting supernatant was used as the cell-free preparation (60 mL, 22 mg/mL protein). A portion of this preparation was used for the study to elucidate the regiospecificity of the reaction. Enzyme activity was determined by incubating dehydroacaterin in an enzyme solution followed by analyzing an aliquot of the AcOEt extract by HPLC (Shimadzu Shim-Pack CLC ODS column 15 cm × 0.6 cm i.d.; solvent, MeOH/H_2O = 6:1; flow rate 1.0 mL/min; UV detection at 230 nm; typical retention times for **1** and **2** were 4.5 and 5.1 min, respectively).

The cell-free preparation was fractionated by $(NH_4)_2SO_4$ precipitation (30–55%) in a standard manner. The precipitates were recovered by centrifugation (20,000*g*, 10 min) and redissolved in a minimum amount of 20 mM Tris–HCl buffer (pH 7.5), and dialyzed against the same buffer for 4 h at 4 °C. The solution were applied to a DEAE column (2.5 × 25 cm). The column was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.5). The active fractions, eluted with ca. 0.2 M NaCl, were combined and concentrated to a reduced volume (3 mL) through a membrane filter (Vivaspin 20 Polyethersulfone VS2001) by centrifugation at (6000*g*, 1 h). The concentrated enzyme solution was volumed up to 10 mL with 0.1 M sodium phosphate buffer (pH 7.4). This enzyme solution (2.3 mg/mL protein) was used for the study of the utilization of the C-4 hydrogen of NADPH.

4.4. Incubation in D_2O

A portion (6.5 mL) of the 20,000*g* supernatant was mixed with 0.1 M sodium phosphate buffer (pH 7.4) (13 mL) prepared in D_2O. To this solution was added

NADPH (22 mg)/H_2O (1.1 mL) and **2** (2.0 mg)/acetone (0.6 mL). The mixture was incubated at 30 °C for 2 h aerobically. AcOEt (20 mL) was added and the mixture was vortexed, and centrifuged at 1500*g* for 10 min to get a clear organic layer. The AcOEt layer was set aside and the H_2O layer was re-extracted with AcOEt. The combined organic layer was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by p-TLC to give a pure product (2.1 mg). The ^2H NMR spectrum of the sample is shown in Figure 1.

4.5. Incubation in the presence of [4,4-$^2H_2$]NADPH

[4,4-$^2H_2$]NADPH (64 mg)/H_2O (3.2 mL) and **2** (6.7 mg)/acetone (2.0 mL) were added to a portion (35 mL) of the 20,000*g* supernatant. The mixture was incubated and worked up as described above to give acaterin (2.3 mg). The ^2H NMR spectrum of the sample is shown in Figure 1.

4.6. Preparation of [4-pro-*R*-^{2}H]- and [4-pro-*S*-^{2}H]NADPHs

To a solution of $NADP^+$ (4.6 mg) and [1-^{2}H]glucose (36 mg) in 50 mM sodium phosphate buffer (1.0 mL, pH 7.0) was added a glucose dehydrogenase solution from *T. acidophilum* Recombinant (2.5 μL, 9.9 mg protein/mL). The mixture was incubated at 37 °C for 1 h. The reaction mixture was centrifuged at 6000*g* for 1 h using a membrane filter to yield the filtrate (1 mL) containing ^2H-labeled NADPH (2.5 mg, determined by UV measurement). This was used for incubation without further purification.

In the other run, the filtrate was further purified in order to record the ^1H NMR spectrum. The filtrate was applied to a DEAE column (1 × 10 cm) and the column was eluted with an increasing concentration of NH_4HCO_3 from 0 to 0.5 M in 0.1 M potassium phosphate buffer (1.0 mL, pH 7.0). The fractions containing NADPH (eluted at ca. 0.2 M) were combined and freeze-dried. The resulting solid was dissolved in distilled water (0.5 mL) and diluted with methanol (5 mL) to cause precipitation of most of the inorganic salt. The methanol layer was taken up and concentrated to give a purified [4-pro-*S*-^{2}H]NADPH. The sample was analyzed by ^1H NMR spectroscopy (Fig. 2). By replacing the glucose dehydrogenase with *Cryptcocus uniguttulatus* glucose dehydrogenase (0.5 mg) (pH 8.0, sodium phosphate buffer was used instead of pH 7.0), [4-pro-*S*-^{2}H]NADPH (2.5 mg) was obtained from 4.6 mg $NADP^+$. The ^1H NMR spectrum of the labeled sample purified by a DEAE column as described above is shown in Figure 2.

4.7. Incubation of [4-pro*R*-^{2}H]- and [4-pro*S*-^{2}H]NADPHs

To the enzyme solution after the DEAE column (10 mL, 2.3 mg/mL protein) were added [4-pro*R*-^{2}H]NADPH (2.5 mg)/50 mM sodium phosphate buffer (1.0 mL) and **2** (1.0 mg)/acetone (0.1 mL). The mixture was incubated at 30 °C for 1 h and worked up as described above. The purified product (0.6 mg) was analyzed by ^2H NMR spectroscopy (Fig. 3). [4-pro*S*-^{2}H]NADPH (2.5 mg)/50 mM sodium phosphate buffer (1.0 mL) and **2** (1.0 mg)/acetone (0.1 mL) were similarly incubated with the enzyme solution (10 mL) to give **1** (0.6 mg). The ^2H NMR spectrum of **1** is shown in Figure 3.

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14. The proton signal at C-5 of **1** was observed as 2.15 units of hydrogen (theoretically 2.0 units), thus indicating that 85% of 4-pro-*R*-^{2}H was transferred to the C-5 position.