proton by CYP101. This is also consistent with results from metabolic studies with mammalian CYP enzymes.[35] Furthermore, the slow rotation between the 5′ cis and 5′ trans binding orientations that are observed at lower temperatures are consistent with the small intramolecular deuterium isotope effects observed at the 5′ position.[35] The following results do not agree with experiment. The ratio of the methyl group oxidation for the (*S*)-enantiomer is predicted to be higher than that at the 5′ positions, and the methyl group of the (*S*)-enantiomer is predicted to be more reactive than the methyl group of the (*R*)-enantiomer. However, the mean values determined at 303 and 1000 K do predict the correct rank order of methyl group to 5′ oxidation. The overprediction of methyl group oxidation for the 1000 K simulation is most likely a result of the increased population of the high-energy binding mode with the methyl group approaching the oxygen (vide supra). These results might be improved if a longer simulation at a lower temperature was performed.

Conclusions

Both binding affinity and regioselectivity are predicted by the dynamics methods reported here although some discrepancies are observed. It is interesting to note that the theoretical calculations were performed prior to obtaining the experimental results and the majority of the theoretical discussion on regioselectivity was written without any knowledge of the experimental results on regioselectivity. Thus, we were able to predict the relative binding affinities, specific steric interactions, and the regioselectivity of the two enantiomers of nicotine. When these methods are combined with the quantum chemical techniques of Korzekwa et al.,[6] a complete picture of the factors that govern small molecule reactivity with the iron–oxygen species of CYP begins to emerge.

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Supplementary Material Available: The input files required to reproduce the heme and nicotine parameters (Prep files for AMBER) (4 pages). Ordering information is given on any current masthead page.

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Mechanism of Proton Abstraction in Biotin-Dependent Carboxylation Reactions†

Donald J. Kuo and Irwin A. Rose*

Contribution from the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received August 3, 1992*

Abstract: Transcarboxylase (E-biotin) in T water is diluted into methylmalonyl CoA (MMCoA) in normal water to determine by isotope capture whether enzyme or solvent provides the proton required to form the product, propionyl CoA. This half-reaction

$$\text{E-biotin·methylmalonyl CoA} \overset{H^+}{\rightleftharpoons} \text{E-biotin-}CO_2^-\text{·propionyl CoA}$$

is followed, in the presence of pyruvate, by formation of oxalacetic acid and free propionyl CoA with regeneration of E-biotin, the three events required for another cycle of reaction with MMCoA. In the absence of pyruvate, the half-reaction is completed by dissociation of propionyl CoA and the reaction is limited to one cycle by the stability of the E-biotin-CO_2. The pulse–chase experiment with T enzyme shows that close to 1 enzyme equiv of T is derived from the pulse and used in the formation of propionyl CoA in the absence of pyruvate. Therefore, an enzyme donor site is protonated in the absence of substrate for transfer after carboxyl transfer to a presumed stabilized carbanionic precursor of the product. The group appears to be *monoprotonic* for two reasons: (1) No additional T is captured with added pyruvate, which greatly increases the rate of release of the propionyl CoA and should allow the donor site to be sampled for T by subsequent cycles of reaction with MMCoA. This is especially to be expected given the slow dissociation (or exchange) rate of the first T, i.e. $k_{ex} \cong 0.2k_{cat}$ at 0 °C. (2) No additional T is captured in the absence of pyruvate when malonyl CoA instead of MMCoA is used in the chase solution. The three methyl protons of acetyl CoA should have provided an expanded pool with which T of a polyprotonic donor would have equilibrated in the one turnover. Other enzyme protons within hydrogen bond distance to the donor site must exchange rapidly before the second turnover. The earlier observation of a small amount of T transfer from 3T pyruvate to propionyl CoA in a complete reaction mixture (Rose, I. A.; O'Connell, E. L.; Solomon, F. *J. Biol. Chem.* **1976**, *251*, 902–904) probably results from enolization of the pyruvate occurring on the oxalacetate subunit in the E·MMCoA·pyruvate complex. The transfer of T would occur if, before the propionyl CoA departs, the donor site is regenerated within the ternary complex from a common pool of protons with which the abstracted T can exchange. These protons would be lost during product liberation, since only one T can be captured from ET in the presence of pyruvate.

Introduction

The mechanism of proton abstraction from substrates to make them acceptors for carboxylation in carboxybiotin-dependent reactions is not well understood. A carbanion intermediate mechanism is indicated for transcarboxylase (methylmalonyl CoA:pyruvate carboxytransferase, E.C. 2.1.3.1), which catalyzes conversion of added enolpyruvate to pyruvate if MMCoA is also present[1] and which produces HF and acrylyl CoA when one attempts to carboxylate β-fluoropropionyl CoA.[2] Proton activation, as shown by α-proton exchange, does not occur unless biotin-dependent enzymes are used in the carboxybiotin form.[1,3]

* Address correspondence to Dr. Irwin A. Rose, Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. Telephone: 215-728-2190. FAX: 215-728-3655.
† Dedication: We dedicate this to Harland G. Wood (1907–1991) in appreciation of his many elegant contributions to our understanding of the action of CO_2 in metabolism and especially for his contributions to structural and mechanistic studies of transcarboxylase.

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Table I. Capture of E-T of Transcarboxylase at 0 °C, pH 7.0 with or without Pyruvate

chase components (μM)		T captured, n^a
none		(0.08)
MMCoA (50)		0.76
malonyl CoA (50)		0.62
(100)		0.59
pyruvate (10 mM) + MMCoA	(5)	0.78
	(10)	0.82
	(50)	0.97

[a] Each value of n (nmol of T captured/nmol of biotin as determined with ^{14}C-carboxy MMCoA) is corrected for the control (0.08) without enzyme. In addition, when pyruvate was present, a correction was made for turnover at 9 s^{-1} for the 3 s used before quenching. No further corrections were made in the one-turnover experiments. Dilutions were 125-fold into a well-mixed chase solution at 0 °C with the noted additions as detailed in the methods section.

This, and the fact that transcarboxylase[4] and all known substrate carboxylation reactions due to biotin enzymes go with a stereochemistry of retention, has given rise to suggestions that components of the carboxybiotin itself, the ureido-oxygen[5] or the N-1 carboxylate,[6] may function as the base responsible for the proton abstraction. The data to be presented do not support these proposals.

Evidence that the abstracted proton has some stability against exchange with the medium comes from an observation with transcarboxylase with 3T pyruvate as substrate in which ~5% of the abstracted T was found in the propionyl CoA formed from methylmalonyl CoA.[7] This result is particularly surprising, since the MMCoA and pyruvate recognition sites are known to reside on different 12S and 6S subunits, which, together with the small biotin-carrying subunit, make up the components required for transcarboxylation.[8] It tends to support the possibility that a single residue, such as biotin or carboxybiotin, carries the abstracted proton in the reverse direction of the CO_2 transfer and is therefore involved in the abstraction step.[7,8]

To obtain information on the origin of the proton used to complete the biotin carboxylation half-reaction, we have used a pulse–chase procedure[9] in which enzyme in T water is diluted into a much larger volume of normal water containing the substrate to be protonated. In the present case, MMCoA was used either alone or with pyruvate. T, found in propionyl CoA, would indicate the presence of a protonated donor site on the enzyme prior to reaction with substrate, the extent of its protonation in the pulse, and perhaps the stability of T on the enzyme–substrate complexes formed before capture into product. Without pyruvate the enzyme can undergo only one turnover, which terminates with dissociation of the propionyl CoA:

$$\text{E-biotin·MMCoA} \leftrightarrow \text{E-biotin-}CO_2^-\text{·propionyl CoA} \rightarrow \text{E-biotin-}CO_2^- + \text{propionyl CoA} \quad (1)$$

With both MMCoA and pyruvate in the chase, additional propionyl CoA will be formed at the turnover rate of the enzyme. This allows the active site to be sampled for additional sources of T for the donor residue if they are sufficiently stable to survive recycling of the enzyme. These values were analyzed in relation to the quantity of enzyme sites used in each experiment. The latter value was determined independently using ^{14}C-carboxy MMCoA and the ^{14}C that could become fixed to protein.

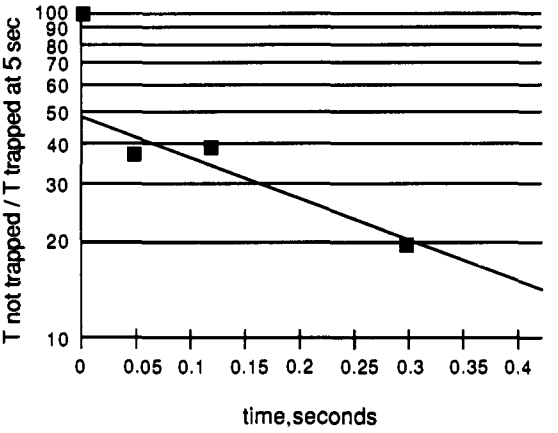

Figure 1. Time course of T capture in a single-turnover. Transcarboxylase (0.2 nmol) in T-water was diluted 40-fold in a continuous flow mixer with MMCoA (50 μM) at 25 °C. Samples were quenched at 5 s and at the times noted. At 5 s, 0.91 equiv of T were captured/equiv of E-biotin. Progress of capture is biphasic with half too slow to be in the normal reaction cycle.

Results and Discussion

T Capture in Single and Multiple Enzyme Turnovers. Table I shows that the proton that replaces the carboxyl group of MMCoA is derived from a residue that becomes labeled by brief exposure to T water. Several possibilities may be considered to explain the <1 equiv of T trapped. (1) *The donor residue may have a pK_a close to the pH of the pulse solution, leading to incomplete occupancy of the donor site in the pulse solution.* This was ruled out by finding the same amount of T capture using pulse and chase solutions at pH 6.0–7.5. (2) *The donor site may be polyprotonic*, in which case a small isotope discrimination may occur in the first cycle. Additional cycles increase the amount trapped but not to >1 equiv due to loss by exchange in each cycle. To test for a polyprotonic donor malonyl CoA, an alternative substrate with about 5% of the V_{max}/K_m value, K_m 35 μM compared to ~5 μM for MMCoA,[10] was used. In this case the three protons of bound acetyl CoA product could equilibrate with any additional protons of the donor. Equilibration would be expected in view of the absence of isotope discrimination in forming T propionyl CoA in T water with MMCoA and pyruvate as substrates. A diprotonic donor should result in 1.5 times as much T in acetyl CoA as in propionyl CoA. A triprotonic donor would give 1.8 times more T, assuming torsional freedom for both the CH_3 of the bound acetyl CoA and an NH_3^+ group, should it be the donor. As shown in Table I, malonyl CoA does not result in more T captured. Therefore, the donor is likely to be monoprotonic. (3) *T may be lost from the donor site prior to transfer.* This seems ruled out, since, with pyruvate included in the chase, a significant increase in T capture to about 1 equiv was seen (Table I).

An explanation for the effect of pyruvate can be considered in reference to eq 1. If the dissociation of propionyl CoA from complexation with E-biotin-CO_2 is slow, it could be that 5 s at 0 °C was not sufficient time to complete the turnover of all the enzyme present in the pulse. To test this, the time course for trapping with MMCoA (50 μM) was determined. In this experiment, done at 25 °C in a rapid-mixing apparatus, the fraction of the 0.91 equiv of T that was trapped at 5 s was determined at 50, 100, and 300 ms (Figure 1). T capture is clearly biphasic with the first component faster than required for the steady-state rate of 30 s^{-1} with pyruvate present followed by a very slow second rate, $t_{1/2}$ ~ 250 ms, which in the Table I experiments at 0 °C would have been much slower. The explanation for the two rates

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observed is not clear at present. One possibility might be that the T propionyl CoA present at 50 ms is largely enzyme-bound, limited in amount by an equilibrium constant close to unity for the first step of the overall reaction (eq 1). The slow phase would then represent the dissociation of propionyl CoA into the large volume of the chase. The effect of pyruvate could then be on the rate of this dissociation step. Possibly decarboxylation of the biotin triggers the release of both products, propionyl CoA and oxalacetate, by allowing the channel between the two subunits to expand. Wood and Zwolinksi[8] have discussed the need to accommodate the ready accessibility of the biotin residue to avidin on the one hand with the closed model of Fung et al.[11] based on distance measurements between a bound paramagnetic propionyl CoA analog and ^{13}C-pyruvate and Co^{2+} of the pyruvate/oxalacetate subunit. Another explanation for the two phases in the capture of T is to suppose that the enzyme of the pulse exists in two conformational states, only one of which reacts rapidly with MMCoA. A two-state model, proposed by Northrop and Wood,[12] depends on the orientation of biotin with respect to the two-substrate specific subunits. In the absence of pyruvate, orientation being random, half the enzyme might not be set for reaction with MMCoA. In this explanation, MMCoA must bind and be able to protect the donor T from exchange in both states to explain the 0.91 T eventually trapped in the absence of pyruvate (Figure 1).

A striking feature of the pulse–chase experiments is the low amount of MMCoA (<5 μM) required to do the trapping—possibly 5 times less than its K_m in the presence of pyruvate. This may mean that exchange and dissociation of the donor proton from the enzyme is ~5 times slower than the 9 s^{-1} value for k_{cat} at 0 °C. This is suggestive of monoprotonic donors such as serine or tyrosine-OH. Exchange of T from E·MMCoA must be much slower than this to explain the high amount trapped at saturation. Although pyruvate increases the turnover, this is not the basis for the increase in T trapped, since, from Figure 1, it is seen that the full amount of T would have been trapped in time without pyruvate. The pyruvate effect on T capture could simply be an allosteric consequence of its binding to the neighboring subunit, and indeed Northrop and Wood[12] observed changes in the isotope exchange rate between propionyl CoA and MMCoA in the presence of pyruvate analogs.

The observation of 5% intermolecular transfer from T pyruvate to propionyl CoA[7] implies that the pyruvate has enolized while in the complex of E·MMCoA or carboxybiotin enzyme·α-carbanion propionyl CoA, possibly indicating the source of protons used to regenerate the donor for the next turnover. Ninety-five percent of the T that reaches this point has exchanged with the medium at some step in the overall reaction from T pyruvate to T propionyl CoA. However, no more than a few percent of the T from ET of the pulse is lost in the chase containing H-pyruvate and MMCoA. Clearly, the opportunity for exchange must occur prior to labeling a residue that is specific for the half-reaction of the CoA substrates. This would rule out highly symmetrical models for proton transfer such as those in which biotin or an amino acid residue of the biotin-carrying subunit acts as the sole proton-abstracting group and proton carrier between the subunits. More likely, the two half-reactions are served by residues particular to each subunit. In this case, exchange occurs from the pyruvate/oxalacetate specific site or in the process of transfer across the intersubunit space. The finding that no more than 0.1 enzyme equiv of T is lost in the pulse–chase experiment at 25 °C with k_{cat} = 30 s^{-1} cannot be ascribed to slow exchange of T from this space, since the MMCoA in H water of the chase must enter the active site to affect the T capture. Therefore, intermolecular transfer may require a carrier. The contrast in the extent of exchange in the intermolecular transfer implies that the pulsed T must be particularly isolated from exchange in the MMCoA/propionyl CoA half-reaction, possibly by a kinetic barrier to conformational change.

Materials and Methods

Transcarboxylase from *Propionibacterium shermanii* was the generous gift of Dr. Harland G. Wood. Its activity was determined in a standard assay at 25 °C KPO_4 (0.35 M, pH 7.0), pyruvate (10 mM), NADH (0.2 mM), (*R,S*)-MMCoA (0.1 mM), and ~1 unit of malate dehydrogenase.

Pulse–chase experiments made use of two mixing methods. For Table I, the apparatus used, designed and constructed by Dr. Steven H. Seeholzer of this laboratory, consists of two micropipet tips connected to solenoids that block or unblock the flow of N_2 (10 psi) at times that are defined by electronic control. Typically, the pulse solution contained T water (~2000 cpm/natom), KPO_4 (250 mM, pH 7.0), DTT (10 mM), BSA (1 mg/mL), and 0.2 nmol of transcarboxylase per 8 μL, kept at 0 °C for several minutes before use. The pulse solution in one tip was force-ejected into 1 mL of magnetically stirred chase solution. The chase solution (1 mL) at 0 °C contained KP_i (pH 7.0, 50 mM) and substrates MMCoA with or without pyruvate or malonyl CoA alone. TCA (250 μmol) was delivered from the second tip to quench at 5 s in the absence of pyruvate or at 3 s when pyruvate was included in the chase solution. Ten milliliters of water, 1 μmol of CoA, and the amount of triethanolamine required to neutralize were added. A sample was taken for total T counts to correct for incomplete introduction of enzyme and for use in determining the specific activity of the water in the reaction mixture. T product was recovered quantitatively, free of T water as follows: the diluted, quenched sample was added to 2 mL of DE52, previously washed with 1 N HCl and water, in a 1.5-cm-diameter column and washed with water until the effluent was free of counts, followed by 5 mL of 1 N HCl used to elute product quantitatively. Pyruvate is not retained by the column under these conditions.[7] Before counting, the samples were concentrated to dryness in vacuo. In experiments in which pyruvate is included in the chase, the steady-state rate of incorporation of label was determined in a control in which substrates were added after the 135-fold dilution of the pulse. The enzyme rate was used to correct the experiments for counts incorporated from solution during the chase interval.

For more rapid mixing and the more precise quenching times required to follow the progress of T capture in a single turnover, the Up-Date (Madison, WI) rapid-mixing apparatus was used, modified only by using a 50 μL Hamilton syringe for the pulse and the 2 mL syringe provided for the chase solution. The syringes were advanced at 2 cm/s, and reaction time was adjusted by choosing different lengths of aging tubes. Reactions were quenched in a rapidly stirred TCA solution into which the mixture was injected.

The precise content of sites capable of carboxylation by MMCoA was determined with ^{14}C-carboxy-labeled MMCoA prepared enzymatically as follows.

Preparation of ^{14}C-Methylmalonyl CoA. The reaction mixture (0.1 mL) contained KP_i (10 mM, pH 7.0), D,L-aspartic acid-*4*-^{14}C (1 mM, 4660 cpm/nmol as determined below), α-ketoglutarate (10 mM), propionyl CoA (1 mM), transaminase (5 units), and transcarboxylase (0.015 unit). The reaction was quenched after 2 h at 25 °C with 50 μmol of TCA. The solution was diluted to 25 mL, neutralized with 50 μmol of triethanolamine, and added to a 1 × 8 cm DE52 column. Fractions of 5 mL were collected using a linear gradient made with 150 mL each of 3 mM HCl and 0.1 M LiCl in 3 mM HCl.[7] The ^{14}C-containing peak, following the propionyl CoA, was concentrated in vacuo, removing acid, diluted to 10 mL, and recycled on a short DE52 column to remove salt. The 1 N HCl, used for elution, was removed in vacuo. The product, which would now be D,L-MMCoA due to spontaneous epimerization,[13] was dissolved and stored at −80 °C.

The specific activity of the aspartic acid used in the preparation was determined as follows: the D,L-aspartic acid-*4*-^{14}C was converted to L-malate (50%) by the coupling of transaminase and malate dehydrogenase. The malate formed was calculated from the decrease of NADH and its ^{14}C content determined as follows: the incubation was acidified to 0.05 N HCl and 10 μmol of malate added. Pyridine nucleotides were removed with charcoal. Fumarase was added, and fumarate formation was followed to equilibrium at 250 nm. This solution was acidified to inactivate the fumarase, 100 μmol of unlabeled fumarate was added, and the fumaric acid was recovered by crystallization in ~0.25 N TCA. After two additional crystallizations in 0.25 N TCA, the fumarate was counted and determined from its 250-nm absorbance and molar extinction of 1450 M^{-1} cm^{-1}. The total radioactivity of the initial malate was determined to be 4660 cpm/nmol from counts in total fumarate and the determined equilibrium constant of the fumarase reaction of 4.4.

Determination of Carboxybiotin Produced from ^{14}C-MMCoA. ^{14}C-carboxyl-labeled methylmalonyl CoA (4660 cpm/nmol) was incubated

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with 0.37 unit of transcarboxylase in 20 μL containing KP_i (250 mM, pH 7.0) and DTT (10 mM) at 25 °C. After 1 min the incubation was centrifuged through two spun-dry DE52 (1 mL wet) beds in sequence in the cold within 10 min. The ^{14}C radioactivity and enzyme activity of the second filtrate were compared. This ratio was determined as a function of the MMCoA concentration used to label the enzyme: 0.05, 0.1, and 0.25 mM. The limit, determined graphically, was 1280 cpm/unit of activity; i.e. 0.275 nmol of biotin could be carboxylated by MMCoA per unit of activity. This value is ~40% greater than the highest value reported by Wood et al.[10] on the basis of the counts of labeled biotin incorporated and the highest enzyme activity that was obtained in the same assay. The difference can be attributed to loss during storage of the assay rate, which depends on the coordinated function of the two half-reactions, without loss of capacity for the MMCoA/propionyl CoA half-reaction.[8]

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Marked Dependence of Enzyme Prochiral Selectivity on the Solvent

Fernand Terradas,[†] Michelle Teston-Henry,[‡] Paul A. Fitzpatrick, and Alexander M. Klibanov*

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received September 8, 1992*

Abstract: Prochiral selectivity of various hydrolytic enzymes (lipases and proteases) in organic solvents was investigated in transformations involving a 2-substituted 1,3-propanediol or its diester. In two instances, a significant dependence of enzyme prochiral selectivity on the solvent was found: transesterification of diol **1** with vinyl butyrate catalyzed by *Aspergillus oryzae* protease in anhydrous solvents and hydrolysis of diester **3** catalyzed by *Pseudomonas* sp. lipase in hydrated organic solvents (monoester **2** was a product in both reactions). The latter process, where the *pro-S* selectivity of the enzyme varied from around 3 in some solvents to greater than 30 in others, was examined in more detail. A mechanistic model was proposed that predicted an inverse correlation between lipase's prochiral selectivity and solvent hydrophobicity, as well as particular effects of substrate structure variation and an additive on the prochiral selectivity; all these predictions were confirmed experimentally. Subtilisin Carlsberg lacked appreciable prochiral selectivity in either transesterification or hydrolysis reactions regardless of the solvent; this was rationalized by means of interactive computer modeling based on the X-ray crystal structure of this serine protease.

Introduction

The ability of enzymes to catalyze useful synthetic transformations in neat organic solvents is now beyond doubt.[1] Perhaps the most exciting and significant development in this emerging area is a recent discovery that enzyme specificity, in particular enantioselectivity[2] and regioselectivity,[3] can be profoundly affected simply by switching from one organic solvent to another. This paves the way to altering specificity of a given enzyme at will and provides a valuable alternative to enzyme screening.

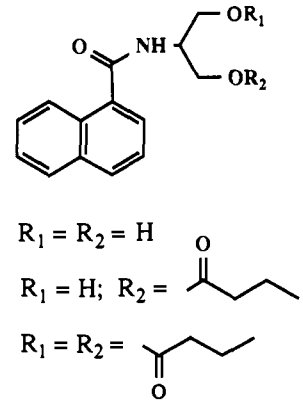
Among other biocatalytic asymmetric processes in non-aqueous media,[1] prochiral conversions catalyzed by hydrolases have been profitably utilized.[4] In the present study, we demonstrate that prochiral selectivity of enzymes also can be controlled by the solvent and that this dependence can be mechanistically rationalized.

Results and Discussion

In order to test the possibility of affecting enzyme prochiral selectivity by the solvent, we synthesized the prochiral diol **1**. The bulky and hydrophobic naphthoyl moiety was deliberately introduced into the C-2 position of the diol to allow it to serve as an orienting anchor in the active center of an enzyme. Initially, we investigated the kinetics of the transesterification reaction between **1** and vinyl butyrate[5] catalyzed by different hydrolytic enzymes in anhydrous tetrahydrofuran. The progress of the transesterification was followed by HPLC using a chiral column in order to separate enantiomers of the monobutyryl ester **2**.

Chromatogram *a* in Figure 1 corresponds to chemically prepared, racemic **2**. One can see two twin peaks; since a chiral HPLC column was employed, they must represent *R* and *S* enantiomers of **2**. It was not known at that point which peak

Chart I



corresponded to what enantiomer; therefore, for the time being, we shall refer to them simply as "first" and "second" peaks.

[†] A Swiss National Science Foundation/Foundation for the 450th Anniversary of Lausanne University Postdoctoral Fellow.

[‡] A Bourse Lavoisier/Rhône-Poulenc (France) Postdoctoral Fellow.

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