J|A|C|S
ARTICLES
Published on Web 12/13/2002

Assay for the Enantiomeric Analysis of [$^2H_1$]-Fluoroacetic Acid: Insight into the Stereochemical Course of Fluorination during Fluorometabolite Biosynthesis in *Streptomyces cattleya*

David O'Hagan,*,† Rebecca J. M. Goss,‡ Abdelkrim Meddour,§ and Jacques Courtieu*,§

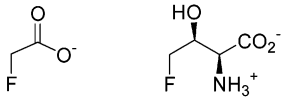
Contribution from the School of Chemistry, University of St Andrews, Centre of Biomolecular Sciences, North Haugh, St Andrews, United Kingdom, KY15 9EA, Department of Chemistry, University of Durham, Science Laboratories, South Road, Durham, United Kingdom, DH1 3LE, and Laboratoire de Chimie Structurale Organique, I.C.M.O., URA-CNRS 1384, Bat. 410 Orsay Cedex, France

Received April 24, 2002; E-mail: do1@st-andrews.ac.uk

Abstract: A sensitive method for the configurational analysis of (*R*)- and (*S*)-[$^2H_1$]-fluoroacetate has been developed using $^2H\{^1H\}$-NMR in a chiral liquid crystalline solvent. This has enabled biosynthetic experiments to be conducted which reveal stereochemical details on biological fluorination occurring during the biosynthesis of fluoroacetate and 4-fluorothreonine in the bacterium *Streptomyces cattleya*. In particular, feeding experiments to *S. cattleya* with isotopically labeled (1*R*, 2*R*)- and (1*S*, 2*R*)-[1-$^2H_1$]-glycerol **3d** and **3e** and [2,3-$^2H_4$]-succinate **4a** gave rise to samples of enantiomerically enriched [2-$^2H_1$]-fluoroacetates **1a**. The predominant enantiomer resulting from each experiment suggests that the stereochemical course of biological fluorination takes place with an overall retention of configuration between a glycolytic intermediate and fluoroacetate **1**. Consequently, this outcome suggests that the stereochemical course of the recently identified fluorinase enzyme which mediates a reaction between fluoride ion and S-adenosyl-L-methionine (SAM), occurs with an inversion of configuration.

Introduction

Fluorinated metabolites are an extremely rare group of secondary metabolites and the details of biological fluorination are unclear.[1−3]



1 **2**

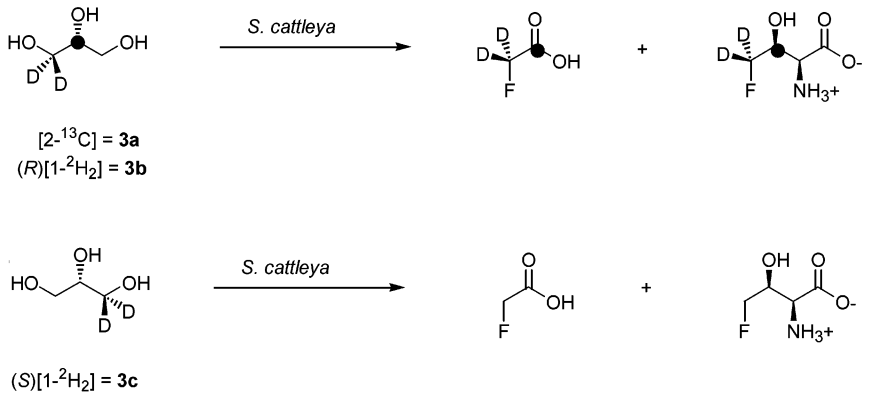
Fluoroacetate **1**, is the most widely distributed fluorometabolite found as a toxin in many tropical and sub-tropical plants.[4,5] Fluoroacetate **1** is also a metabolite of the bacterium *Streptomyces cattleya*[6] where it is produced as a co-metabolite of the amino acid 4-fluorothreonine **2**. Some recent insights into the biosynthesis of fluoroacetate **1** and 4-fluorothreonine **2**, in *S. cattleya* have been uncovered.[7−13] For example a biosynthetic feeding experiment with [2-^{13}C]-glycerol **3a**[10] resulted in isotopic incorporation into C-1 of fluoroacetate **1**. Also isotopic labeling experiments with (2*R*) and (2*S*) [$^2H_2$]-glycerols **3b** and **3c** have shown[11] that the fluoromethyl groups of fluoroacetate **1** and 4-fluorothreonine **2** are labeled exclusively from the *pro-R* hydroxymethyl group of glycerol **3** as shown in Scheme 1.

The experiment revealed that *both* deuterium atoms from (2*R*)-[$^2H_2$]-glycerol **3b** had become incorporated into the fluoromethyl groups of **1** and **2**. The *pro-R* hydroxymethyl group of glycerol **3** is activated in vivo by glycerol kinase to generate (2*R*)-glycerol-3-phosphate prior to entry into the glycolytic pathway. Accordingly, the hydroxymethyl group of glycerol **3** that is phosphorylated during glycerol activation is also that

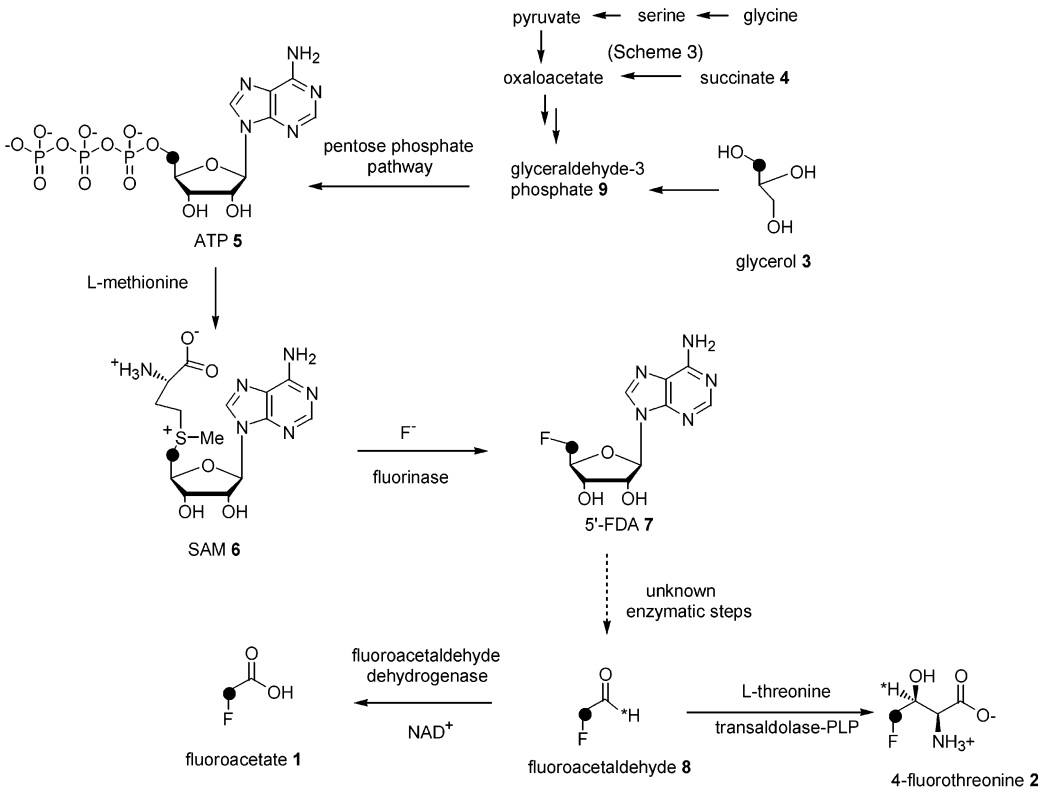
† School of Chemistry, University of St Andrews, Centre of Biomolecular Sciences.
‡ Department of Chemistry, University of Durham, Science Laboratories.
§ Laboratoire de Chimie Structurale Organique, I.C.M.O., URA-CNRS.

(1) O'Hagan, D.; Harper, D. B. *J. Fluorine Chem.* **1999**, *100*, 127−133.
(2) O'Hagan, D.; Harper, D. B. *Ramachardran, Asymmetric Fluoroorganic Chemistry*, Ed P. V.; ACS Symposium Series 746; 2000; pp 210−224.
(3) Harper, D. B.; O'Hagan, D. *Nat. Prod. Rep.* **1994**, *11*, 123−133.
(4) Twigg, L. E.; Wright, G. R.; Potts, M. D. *Aust. J. Botany* **1999**, *47*, 877−880.
(5) O'Hagan, D.; Perry, R.; Lock, J. M.; Meyer, J. J. M.; Dasaradhi, L.; Hamilton, J. T. G.; Harper, D. B. *Phytochemistry* **1993**, *33*, 1043−1046.
(6) Sanada, M.; Miyano, T.; Iwadare, S.; Williamson, J. M.; Arison, B. H.; Smith, J. L.; Douglas, A. W.; Liesch, J. M.; Inamine, E. *J. Antibiotics* **1986**, *39*, 259−265.
(7) Hamilton, J. T. G.; Murphy, C. D.; Amin, M. R.; O'Hagan, D.; Harper, D. B. *J. Chem. Soc., Perkin Trans. 1*. **1998**, 759−767.
(8) Hamilton, J. T. G.; Amin, M. R.; Harper, D. B.; O'Hagan, D. *Chem. Commun.* **1997**, 797−798.
(9) Schaffrath, C.; Murphy, C. D.; Hamilton, J. T. G.; O'Hagan, D. *J. Chem. Soc., Perkin Trans. 1*. **2001**, 3100−3105.
(10) Tamura, T.; Wada, M.; Esaki, N.; Soda, K. *J. Bacteriol.* **1995**, *177*, 2265−2269.
(11) Nieschalk, J.; Hamilton, J. T. G.; Murphy, C. D.; Harper, D. B.; O'Hagan *Chem. Commun.* **1997**, 799−800.
(12) Schaffrath, C.; Cobb, S. L.; O'Hagan, D. *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 3913−3915.
(13) O'Hagan, D.; Schaffrath, C.; Cobb, S. L.; Hamilton, J. T. G.; Murphy, C. D. *Nature* **2002**, *416*, 279.

Scheme 1



Scheme 2



which is fluorinated toward the end of the biosynthetic pathway. Additionally, the observed retention of both deuteriums from (2*R*)-[$^2H_2$]-glycerol **3b** at the fluoromethyl groups of **1** and **2** indicates that there is no requirement for oxidation at that carbon during metabolic conversion of the C−O to C−F bond during fluorometabolite biosynthesis. Recently, the nature of the fluorination enzyme in *S. cattleya* has been revealed[13] and it has been shown that inorganic fluoride reacts with S-adenosyl-L-methionine (SAM) **6** to generate 5′-fluoro-5′-deoxyadenosine (5′-FDA) **7** (Scheme 2).

SAM is of course derived from a reaction between ATP and L-methionine mediated by methionine adenosyltransferase; thus, glycerol becomes incorporated into the ribose ring of adenosine, via glyceraldehyde-3-phosphate incorporation into sedoheptulose-7-phosphate by transaldolase[14] and then to ribose-5-phosphate by the action of transketolase.[15] Subsequent involvement in ATP biosynthesis delivers the original *pro-R* hydroxymethyl group of glycerol to the triphosphate activated 5′-carbon of ATP **5**. Methionine adenosyltransferase mediates a nucleophilic attack of the sulfur of L-methionine to 5′-carbon center of ATP to generate SAM **6** with inversion of configuration.[16] The stereochemical course of the fluorinase reaction between SAM and fluoride ion to generate 5′-FDA **7**, remains to be determined.

Fluoroacetaldehyde **8** has been identified[12,17] as the last common biosynthetic intermediate to both fluoroacetate **1** and 4-fluorothreonine **2**. Resting cells of *S. cattleya* can efficiently oxidize fluoroacetaldehyde **8** to fluoroacetate **1** and a feeding experiment also in resting cells of *S. cattleya* with [1-$^2H_1$]-fluoroacetaldehyde **8a** resulted in a high incorporation (60%)

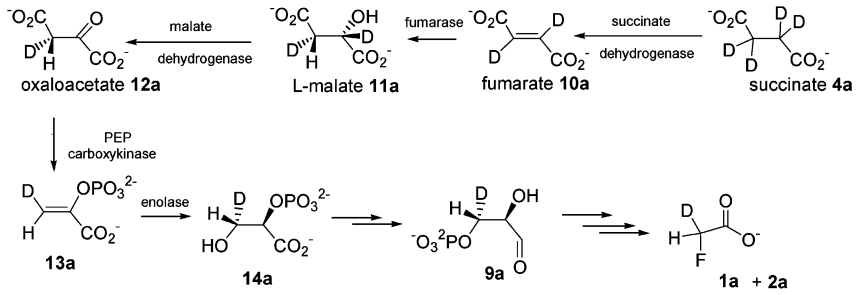
(14) Kamada, N.; Yasuhara, A.; Takano, Y.; Ikeda, M. *Appl. Microbiol. Biotech.* **2001**, *56*, 710−717.

(15) Gunsalus, I. C.; Horecker, B. L.; Wood, W. A. *Bacteriol. Rev.* **1955**, *19*, 79−128.

(16) Parry, R. J.; Minta, M. *J. Am. Chem. Soc.* **1982**, *104*, 871−872.

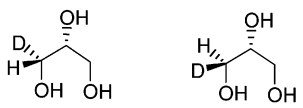
(17) Moss, S. J.; Murphy, C. D.; Hamilton, J. T. G.; McRoberts, W. C.; O'Hagan, D.; Schaffrath, C.; Harper, D. B. *Chem. Commun.* **2000**, 2281−2282.

Scheme 3



of the deuterium atom into C-3 of 4-fluorothreonine **3**, indicating the direct incorporation of fluoroacetaldehyde **8** into C-3 and C-4 of 4-fluorothreonine **2** as illustrated in Scheme 2. This pivotal role for fluoroacetaldehyde **8** has been reinforced more recently by the isolation of the two enzymes that convert fluoroacetaldehyde **8** to fluoroacetate **1**[18] and fluoroacetaldehyde **8** to 4-fluorothreonine **2**.[19] Also fluoroacetaldehyde has been identified in vitro in incubations of 5′-FDA with a cell free extract of *S. cattleya*.[12] Thus the transformation of fluoroacetaldehyde **8** to fluoroacetate **1** and 4-fluorothreonine **2** is on a secure biochemical basis. To explore the process of biological fluorination in greater detail, it became pertinent to assess the stereochemical course of the fluorination event. Clearly, an S_N2 substitution reaction by fluoride ion on SAM **6** will generate 5′-FDA **7** with inversion of configuration to generate the fluoromethyl group found in the fluorometabolites **1** and **2**. Alternatively, a sequence of events involving two S_N2 substitution reactions perhaps involving a nucleophilic substituent on the enzyme, would result in an overall retention of configuration. Finally, there is the possibility of an elimination reaction ocurring on SAM **6** to generate an enzyme bound substrate with sp^2 hybridization at C-5′. Fluoride addition could then occur to one or other face of the double bond and this would result in either an inversion or retention of configuration depending on the stereochemical course of the reaction.

To trace the stereochemical fate the *pro-R* hydroxymethyl carbon of glycerol during fluorometabolite biosynthesis, two criteria had to be met. First appropriate deuterium labeled precursors were required for feeding experiments to generate samples of chiral [$^2H_1$]-fluoroacetate **1a** in vivo. As (*R*)-[$^2H_2$] glycerol **3b** had already proven[11] to be good substrates for incorporation into the fluorometabolites **1** and **2**, appropriately labeled glycerols carrying single and stereochemically defined deuterium atoms on the *pro-R* hydroxymethyl group, **3d** and **3e**, emerged as ideal candidate precursors for this stereochemical investigation.



(1R, 2R) **3d** *(1S, 2R)* **3e**

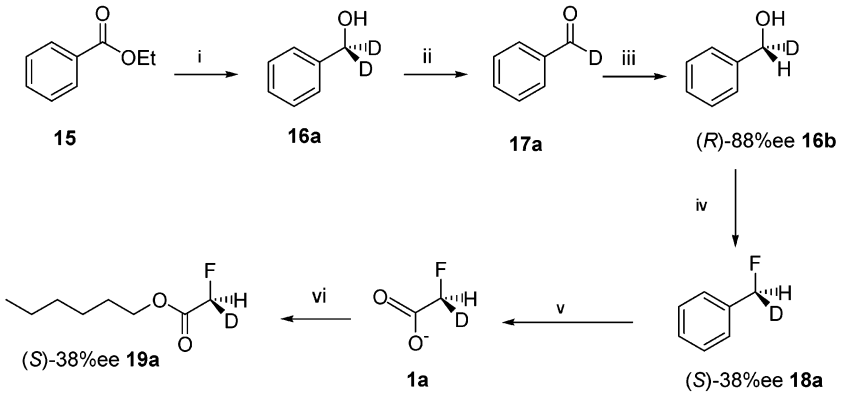
It was anticipated that the resultant fluoroacetates **1a** would retain a single, stereochemically defined deuterium atom after feeding experiments with each of these substrates. The required (1*R*, 2*R*)- and (1*S*, 2*R*)-[1-$^2H_1$]-glycerols **3d** and **3e** respectively, for such feeding experiments were accessible from a previously developed synthesis protocol.[20] [$^2H_4$]-Succinate **4a** also emerged as an alternative biosynthetic precursor in which to explore the stereochemical course of fluorination during fluorometabolite biosynthesis. It has previously been shown[7] that when [$^2H_4$]-succinate **4a** is administered to resting cells of *S. cattleya* the resultant fluoroacetate **1** and 4-fluorothreonine **2** become labeled with a *single* deuterium atom in each of the fluoromethyl groups. This is rationalized by the processing of succinate **4** along enzymes of the citric acid cycle via fumarate **10** and L-malate **11** to oxaloacetate **12**. The enzyme phosphoenylpuruvate(PEP) carboxykinase,[21] converts oxaloacetate **12** to PEP **13** and then enolase[22] generates 2-phosphoglycerate **14**, before further processing to glyceraldehyde-3-phosphate **9** as shown in Scheme 3.

The enzymatic transformations mediated by fumarase, PEP-carboxykinase and enolase all impact on the absolute configuration at the ultimate fluoromethyl groups in **1** and **2**. The stereochemical course of all four of these enzymatic reactions[21−23] is already established, and therefore, it can be deduced that the (2*R*, 3*S*)-[3-$^2H_1$]-glyceraldehyde-3-phosphate **9a** generated in vivo from [$^2H_4$]-succinate **4a** will have predominantly the (*3R*) configuration at the isotopically labeled carbon as indicated in Scheme 3. Accordingly, if the configuration of the resultant fluoroacetate **1a** can be determined then the stereochemical relationship between (2*R*, 3*R*)-[3-$^2H_1$]-glyceraldehyde-3-phosphate **9a** and the fluoromethyl carbon of [$^2H_1$]-fluoroacetate **1**, can be evaluated.

Second a method was required to assess the enantiomeric enrichment of the resultant samples of chiral [$^2H_1$]-fluoroacetates **1a**. Circular dichroism (CD) has been used to determine[24] the absolute configuration of [$^2H_1$]-fluoroacetate **1a**. The method is effective; however, such analysis requires a chemically pure and highly isotopically enriched sample of either (*R*) or (*S*)-[$^2H_1$]-fluoroacetate **1a**. For this biosynthetic study, a method of analysis was required that could reliably assay sub-milligram quantities of biosynthetically derived fluoroacetate which contained only a moderate level of isotopic enrichment (∼15%). Chiral [$^2H_1$]-fluoroacetate **1a** has been used to explore the stereospecificity of the citrate synthase mediated condensation of oxaloacetate and fluoroacetyl-CoA to generate 2-(2*R*, 3*R*)-

(18) Murphy, C. D.; Moss, S. J.; O'Hagan, D. *Appl. Environ. Microbiol*. **2001**, *67*, 4919−4921.
(19) Murphy, C. D.; O'Hagan, D.; Schaffrath, C. *Angew Chem, Int. Ed. Engl.* **2001**, *40*, 4479−4481.
(20) Nieschalk, J.; O'Hagan, D. *Tetrahedron Asymmetry* **1997**, *8*, 2325−2330.
(21) Rose, I. A.; O'Connell, E. L.; Noce, P.; Utter, M. F.; Wood, H. G.; Willard, J. M.; Cooper, T. G.; Benziman, M. *J. Biol. Chem.* **1969**, *244*, 6130−6133.
(22) Cohn, M.; Pearson, J. E.; O'Connell, E. L.; Rose, I. A. *J. Am. Chem. Soc.* **1970**, *92*, 4095−4098.
(23) Gawron, O.; Glaid, A. J.; Fondy, T. P. *J. Am. Chem. Soc*. **1960**, *83*, 3634−3640.
(24) Gartz, D.; Reed, J.; Rétey, J. *Helv. Chim. Acta*. **1996**, *79*, 1021−1025.

Scheme 4 [a]



[a] i. $LiAl^2H_4$, THF reflux, 6 h, 98%; ii. PCC, 3 Å mol sieves, CH_2Cl_2, 4 h, 25 °C, 81%; iii (−)Alpine Borane, THF reflux, 2 h, 66%; iv Et_2NSF_3, CH_2Cl_2, −78 °C to r.t, 10 h, 50%; v. $KMnO_4/KIO_4$, H_2O, 24 h, then H_2SO_4, lyophilize, dil. NaOH then freeze-dry, add unlabeled NaFAc; vi. $SOCl_2$, $C_6H_{13}OH$, 0 °C to reflux, 2 h, 50%.

fluorocitrate.[25−27] The enzyme abstracts the *pro-S* hydrogen of fluoroacetyl-CoA with a high level of selectivity and delivers a single stereoisomer of fluorocitrate. Citrate synthase offered a potential enzymatic assay for this biosynthetic study; however, in view of the small amounts of fluoroacetate that are generated from the biosynthetic experiments and the requirement to prepare samples of fluoroacetyl-CoA from that material, this approach proved impractical and was unsuccessful in our hands.

In this paper, a novel method for the assay of [$^2H_1$]-fluoroacetate **1a** chirality is described using ^2H NMR in a chiral lyotropic liquid crystalline phase prepared using poly-γ-benzyl-L-glutamate (PBLG).[28−30] When oriented in such a mesophase it has been shown that enantiotopic nuclei become non equivalent as the order parameters along enantiotopic directions are different. $^2H\{^1H\}$-NMR emerges as a powerful analytical tool for enantiomeric analysis in liquid crystalline medium.[31] The sensitivity of the technique is maximal for deuterium labeled compounds as the large quadrupolar interaction (150−200 kHz for this nucleus) induces large quadrupolar splittings. The $^2H\{^1H\}$-NMR spectrum of a racemic mixture of a monodeuterated compound dissolved in PBLG/cosolvent exhibits four peaks which are to be viewed as two sets of doublets. As the chemical shift anisotropy of deuterium is small, the two doublets are centered on the same frequency, the chemical shift of the deuterium. The inner doublet is assigned to one enantiomer and the outer one, to the other enantiomer. The quadrupolar splitting is the difference (in Hz) between the two peaks of each doublet. This splitting is extremely sensitive to temperature and to the PBLG/solvent and the PBLG/solute ratios; however, although the magnitude of the quadrupolar splittings can vary, there is generally no peak inversion/crossover for the enantiomer doublets. We have used this method to assay the chirality of fluoroacetate from three biosynthetic feeding experiments with *S. cattleya* cells, using (1*R*, 2*R*)- and (1*S*, 2*R*)-[1-$^2H_1$]-glycerols **3d** and **3e** and [$^2H_4$]-succinate **4a**. The experiments gave rise to samples of [$^2H_1$]-fluoroacetate **1a**. Derivatization of **1a** as its hexyl ester **19a** and then $^2H\{^1H\}$-NMR analysis in a chiral liquid crystalline medium (PBLG/chloroform) resulted in the successful stereochemical analyses of the fluoromethyl group.

Results and Discussion

Enantiomeric Assay of [$^2H_1$]-Fluoroacetate for Biosynthetic Experiments. To develop an assay for [$^2H_1$]-fluoroacetate **1a** a suitable reference sample of known chirality was required. The hexyl ester of fluoroacetate was prepared as shown in Scheme 4.

A sample of [1-^2H]-benzaldehyde **17a** was generated by the reduction of ethyl benzoate **15** to **16a** with lithium aluminum-[$^2H_4$]-hydride and then oxidation of **16a** to **17a** with pyridinium chlorochromate. Asymmetric reduction of [1-^2H]-benzaldehyde **17a** using β-3-pinanyl-9-BBN ((*S*)-Alpine Borane)[32] has previously been shown[33] to give the (*R*)-[7-$^2H_1$]-benzyl alcohol **16b** as the predominant product. The enantiomeric excess of this product was conveniently determined by chiral liquid crystal $^2H\{^1H\}$-NMR[28] (Figure 1a) and shown to be 88%ee.

Treatment of this alcohol with diethylaminosulfur trifluoride (DAST)[34] generated a sample of [7-$^2H_1$]-benzylfluoride **18a**. This compound, which now carries a chiral fluoromethyl group, was assayed for its enantiomeric excess (38%ee) using chiral liquid crystal $^2H\{^1H\}$-NMR analysis. The enantiomers of [7-$^2H_1$]-benzylfluoride **18a** were readily resolved as shown in Figure 1b. The spectrum contains an "extra" set of doublets due to total D-F coupling (9.6 Hz). This established for the first time that chiral liquid crystal $^2H\{^1H\}$-NMR was sufficiently discriminating to resolve a chiral fluoromethyl group. DAST reactions on secondary alcohols are well-known to suffer from a lack of stereochemical integrity,[35] although the predominant stereoisomer generally results from a configurational inversion. In this case, stereochemical inversion will generate the (*S*) isomer of **18a** as the major product enantiomer. The loss in stereochemical integrity can be attributed to a significant level

(25) Dummel, R. J.; Kun, E. *J. Biol. Chem.* **1969**, *244*, 2966−2969.
(26) Keck, R.; Haas, H.; Rétey, J. *FEBS Letts.* **1980**, *114*, 287−290.
(27) Marletta, M. A.; Srere, P. A.; Walsh, C. *Biochemistry* **1981**, *20*, 3719−3723.
(28) Meddour, A.; Canlet, C.; Blanco, L.; Courtieu, J. *Angew. Chem., Int. Ed.* **1999**, *38*, 2391−2393.
(29) Lesot, P.; Merlet, D.; Loewenstein, A.; Courtieu, J. *Tetrahedron Asymmetry* **1998**, *9*, 1871−1881.
(30) Canet, I.; Courtieu, J.; Loewenstein, A.; Meddour, A.; Pechine, J. M. *J. Am. Chem. Soc.* **1995**, *117*, 6520−6526.
(31) Tavasli, M.; Courtieu, J.; Goss, R. J. M.; Meddour, A.; O'Hagan, D. *Chem. Commun*. **2002**, 844−845.
(32) Midland, M. M.; Tramontano, A.; Kazubski, A.; Graham, R. S.; Tsai, D. J. S.; Cardin, D. *Tetrahedron* **1984**, *40*, 1371−1380.
(33) Midland, M. M.; Greer, S.; Tramontano, A.; Zedric, S. A. *J. Am. Chem. Soc*. **1979**, *101*, 2352−2355.
(34) Middleton, W. J. *J. Org. Chem*. **1975**, *40*, 574−578.
(35) Madiot, V.; Lesot, P.; Gree, D.; Courtieu, J.; Gree, R. *Chem. Comm*. **2000**, 169−170.

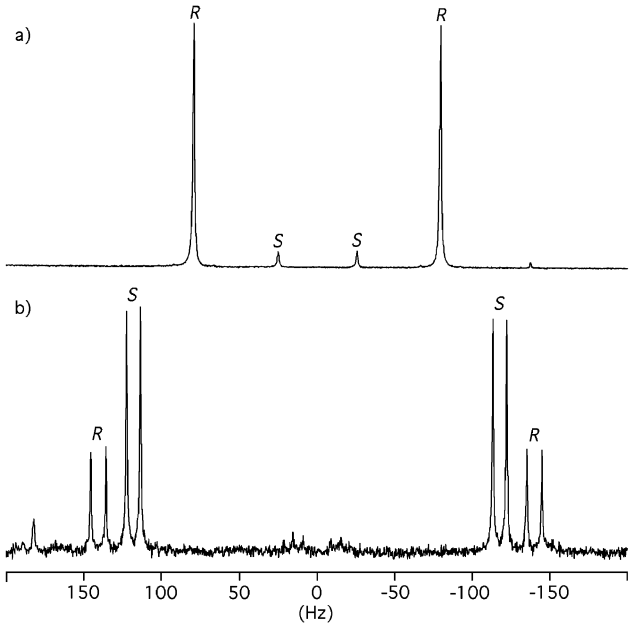


Figure 1. $^2H\{^1H\}$-NMR spectra of (a); (*R*)-[1-$^2H_1$]-benzyl alcohol (88%ee) **16b** and (b) (*S*)-[1-$^2H_1$]-benzylfluoride (38%ee) **18a**, in a chiral liquid crystalline medium of chloroform and poly-γ-benzyl-l-glutamate (PBLG). In (b) the additional doublets arises from ^{19}F-^2H coupling (9.6 Hz).

of S_N1 character during the DAST reaction, a feature that is almost certainly accommodated by the benzylic nature of the substrate. There was no improvement in the enantiomeric excess when this reaction was carried out at −50 °C, although the reaction was much slower. Fluorination using Ishikawa's reagent (*N*,*N*-diethyl-1,1,2,3,3,3-hexafluorylpropylamine)[36] was also explored, however the product [7-$^2H_1$]-benzylfluoride **18a**, which was subjected to the same stereochemical analysis, had an identical enantiomeric enrichment of 38%ee also in favor of (*S*) **18a**. Thus, there was no advantage in using this alternative fluorination reagent. Oxidation of the aromatic ring of [7-$^2H_1$]-benzylfluoride **18a** to generate a sample of [$^2H_1$]-fluoroacetate **1a**, was accomplished using aqueous $KMnO_4/KIO_4$. The product was secured after acidification and lyophilisation. The lyophilisate was then neutralized with dil. NaOH and the neutral solution freeze-dried to leave sodium [$^2H_1$]-fluoroacetate **1a** as a clean white amorphous residue. This material was diluted with unlabeled sodium fluoroacetate **1** such that the ^2H-isotope enrichment was ∼1%. The increased mass provided by the unlabeled "carrier" material allowed for easier chemical derivatization. Dilution of the deuterium label in the sample was not a significant limitation and demonstrates that this assay method can be used very effectively for low levels of deuterium enrichment because the enantiomeric assay is based on $^2H\{^1H\}$-NMR analysis of the isotopically labeled molecules only. Hexyl [2-$^2H_1$]-fluoroacetate **19a** was prepared by direct treatment of sodium [$^2H_1$]-fluoroacetate **1a** with thionyl chloride followed by the addition of hexanol. The product **19a** was purified by chromatography prior to $^2H\{^1H\}$-NMR analysis in a chloroform/PBLG mesophase. Figure 2 shows the resultant $^2H\{^1H\}$-NMR spectrum of a separately prepared *racemic* sample to **19a**. The spectrum contains three sets of signals (doublets) which arise from the quadrupolar splitting from three different species

(36) Takaoka, A.; Iwakiri, H.; Ishikawa, N. *Bull. Chem. Soc., Jpn.* **1979**, *52*, 3377−3380.

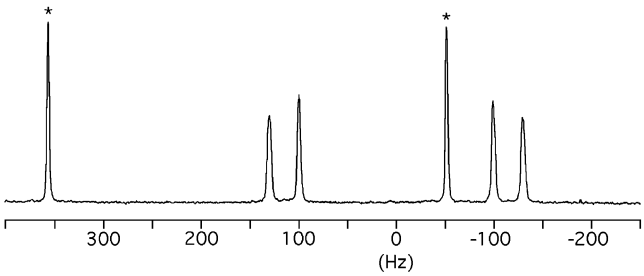


Figure 2. $^2H\{^1H\}$-NMR spectra of racemic hexyl [1-$^2H_1$]-fluoroacetate **19a** in a chiral liquid crystalline medium of chloroform and PBLG showing clear resolution of the enantiomers. The natural abundance signals from chloroform are labeled with an asterisk.

including the solvent (natural abundance $CDCl_3$ signals are marked with an asterix). Each of the doublets associated with the enantiomers is clearly resolved.

The corresponding $^2H\{^1H\}$-NMR spectrum for the synthetic sample prepared as Scheme 4 and enriched with the (*S*)- **19a** is shown in Figure 3a. Again the enantiomers of hexyl [$^2H_1$]-fluoroacetate **19a** are clearly resolved in the $^2H\{^1H\}$-NMR spectrum using this technique and indicate that the sample of hexyl [$^2H_1$]-fluoroacetate has an enantiomeric excess of (*S*)-38%ee. There was no further racemisation of the sample during the permanganate/periodate oxidation and it is assumed that the predominant enantiomer of **1a** has the (*S*) configuration following through from (*S*)-[$^2H_1$]-benzylfluoride **18a** (38%ee). The ease of the $^2H\{^1H\}$-NMR assay method and its ability to resolve the enantiomers of hexyl [2-$^2H_1$]-fluoroacetate **19a** in such a straightforward manner now offered a practical method by which to assay the enantiomeric purity of [$^2H_1$]-fluoroacetates **1a** derived from biosynthetic feeding experiments.

Synthesis and Stereochemical Analysis of (1*R*, 2*R*)- and (1*S*, 2*R*)- [1-$^2H_1$]-Glycerols 3d and 3e. The synthesis of (1*R*, 2*R*)- and (1*S*, 2*R*)- [1-$^2H_1$]-glycerols **3d** and **3e** has previously been described,[20] however access to chiral liquid crystal $^2H\{^1H\}$-NMR analysis of the product glycerols has provided a more penetrating insight into the nature of the products formed than that previously evaluated. The synthesis of the glycerols relied on either the reduction of [3-$^2H_1$]-propargyl alcohol **20a** with $LiAlH_4$ and then a H_2O quench, or the reduction of unlabeled propargyl alcohol **20** with $LiAlH_4$ followed by a D_2O quench, as shown in Scheme 5.

The preparation of (1*R*, 2*R*)-[1-$^2H_1$]-glycerol **3d** gives a satisfactory product sample (>95%ee). In the resultant $^2H\{^1H\}$-NMR of glycerol **3d** shown in Figure 4a, there is only one observable deuterium labeled species, with none of the corresponding (1*S*, 2*R*) diastereoisomer **3e** apparent. This is consistent with our earlier observation[20] where reduction of [3-$^2H_1$]-propargyl alcohol **20a** with $LiAlH_4$ generated a single product **21a**, whereas reduction of propargyl alcohol followed by a D_2O quench (Scheme 5b) gave a more complex product mixture. Accordingly in the case of (1*S*, 2*R*)-[1-$^2H_1$]-glycerol **3e**, $^2H\{^1H\}$-NMR analysis (Figure 4b) of the product mixture derived from $LiAlH_4$ reduction of propargyl alcohol **20** followed by a D_2O quench generated a predominant cis product **21b** (78%) and a minor trans product **21a** (12%). $^2H\{^1H\}$-NMR also confirmed a significant level (10%) of allyl alcohol **21c** with isotope delivered to the C-2 position as shown in Scheme 5. Compound **21c** arises from hydride delivery to the terminal acetylene carbon of propargyl alcohol **20**, with deuterium

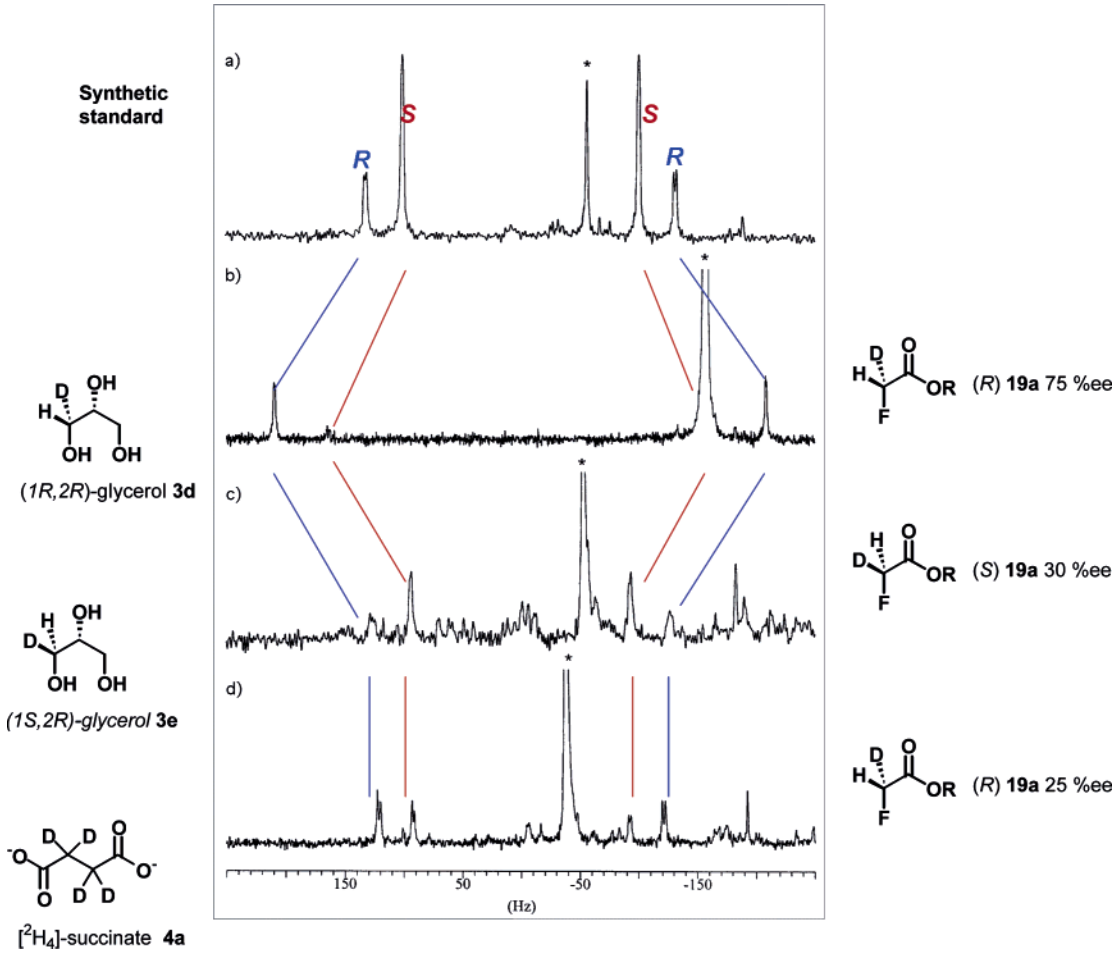


Figure 3. $^2H\{^1H\}$-NMR spectra of hexyl [1-$^2H_1$]-fluoroacetates **19a** in a chiral liquid crystalline medium of chloroform and PBLG. (a) Synthetic (*S*)-**19a** (38%ee) prepared as Scheme 4; (b) (*R*)-**19a** (75%ee) after a feeding experiment to *S. cattleya* cells with (1*R*, 2*R*)-glycerol **3d**; (c) (*S*)-**19a** (30−40%ee) after a feeding experiment to *S. cattleya* cells with (1*S*, 2*R*)-glycerol **3e**; (d) (*R*)-**19a** (25%ee) after a feeding experiment to *S. cattleya* cells with [$^2H_4$]-succinate **4a**: The natural abundance signals from chloroform are labeled with an asterisk.

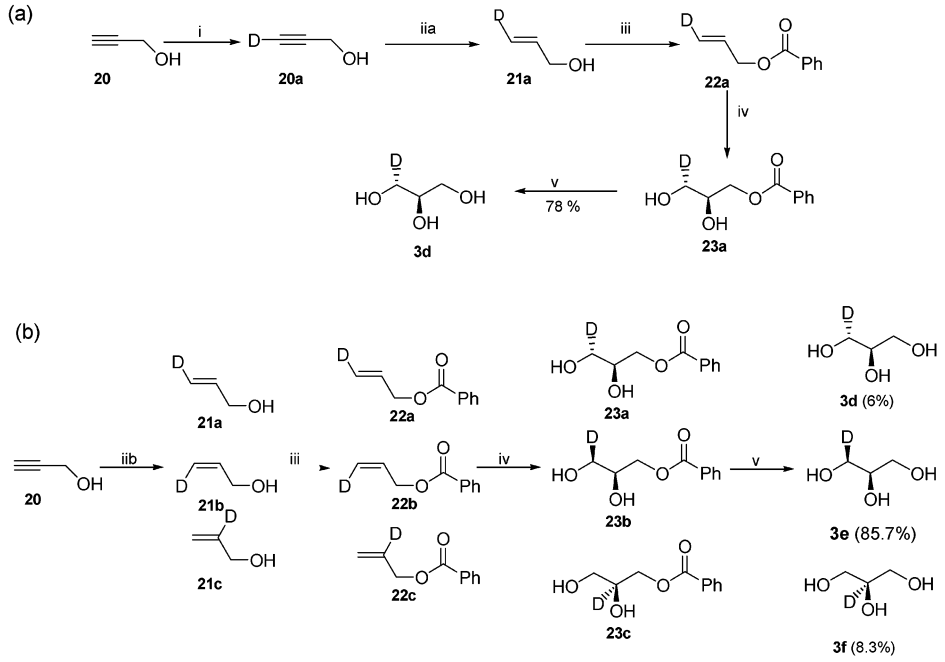
delivered to the central carbon after the D_2O quench. The resultant [$^2H_1$]-allyl alcohol mixture **21a**−**c** was then converted to the corresponding benzoyl esters **22a**−**c** followed by AD-mix-β dihydroxylation.[37] This process afforded the benzoate diols **23a**−**c** as a solid material. As previously described, recrystallization of this benzoate product significantly improved the stereoisomeric excess of the major (2*R*) alcohol over the (2*S*) products. The labeled glycerols were then generated by hydrolysis of the benzoate diols **23a**−**c**, and were purified by silica gel chromatography. The chiral liquid crystal $^2H\{^1H\}$-NMR spectrum for the resultant (1*S*, 2*R*)-[1-$^2H_1$]-glycerols **3e** (**3d** and **3f**) is shown in Figure 4b.

In Figure 4b, there are three clear doublets arising from three deuterium labeled glycerols in the product mixture. The intensities of these signals correlate closely with those found in the $^2H\{^1H\}$-NMR of the [$^2H_1$]-allyl alcohol intermediates, prepared upstream of the glycerol products. Accordingly the signals are assigned to (1*S*, 2*R*)-[1-$^2H_1$]-glycerol **3e** (85.7%, 97 Hz), the corresponding diastereoisomer (1*R*, 2*R*)-[1-$^2H_1$]-glycerol **3d** (6%, 133.5 Hz) and then the product of C-2 labeling, [2-$^2H_1$]-glycerol **3f** (8.3%) which has the largest quadrupolar splitting (262.6 Hz). The presence of this latter glycerol in the sample, although undesirable in general terms, was judged not to present a significant complication to our interpretation of the biosynthetic experiment because the C-2 carbon atom of glycerol becomes oxidized during the biosynthesis of fluoroacetate. Accordingly, ^2H-isotope at the C-2 position of glycerol **3f** is liberated in a biosynthetic experiment. However, the presence of the two diastereoisomers **3e** and **3d** in a ratio of 93.5:6.5 indicated a stereochemical excess (de) of 87% at the *pro-R* hydroxymethyl group of glycerol. It is not clear why the stereochemical integrity of the hydroxymethyl group is lower in this case, but the conclusion is consistent with the experimental observation after several preparations. With the samples of (1*R*, 2*R*)-[1-$^2H_1$]-glycerol (>95% de) **3d** and (1*S*, 2*R*)-[1-$^2H_1$]-glycerol of **3e** (87% de) in hand, biosynthetic feeding experiments to resting cells of *S. cattleya* were carried out and the results are described below.

Feeding Experiments of (1*R*, 2*R*)- and (1*S*, 2*R*)-[1-$^2H_1$]-glycerols 3d and 3e into Fluoroacetate 1 in Resting Cells of *S. cattleya*. The isotopically labeled (1*R*, 2*R*)- and (1*S*, 2*R*)-[1-$^2H_1$]-glycerols **3d** and **3e** were separately incubated with washed resting cells of *S. cattleya* prepared from a batch culture which had grown for 8 d.[9] The washed cells were then incubated with the glycerols **3d** and **3e** to a final concentration of 9mM. After 48 h, each experiment was worked up by freeze-drying the cell supernatant, followed by acidification and lyophilization. The

(37) Sharpless, K. B.; Amberg, W.; Bennani, Y. L.; Crispino, G. A.; Hartung, J.; Jeing, K.-S.; Kwong, H.-L.; Morikawa, K.; Wang, Z.−M.; Xu, D.; Zhang, X.−L. *J. Org. Chem.* **1992**, *57*, 2768−2771.

Scheme 5 [a]



[a] i. BuLi, THF, −78 °C; ^2H_2O quench, r.t. ii for (a). $LiAlH_4$, THF, 0 °C; stir, r.t., 3 h; H_2O quench, trans 100%. Ii for (b). $LiAlH_4$, THF, 0 °C; stir, r.t. 3 h; ^2H_2O quench, cis 78%, trans 12%, $CH_2CH^2HCH_2OH$ 10%. iii. PhCOCl, 30%NaOH, $Et_3BnN^+Cl^-$, DCM; for (a), steps ii-iii 32%; for (b), steps i-iii 38%. iv. AD-mix-β, *t*-BuOH/H_2O 1:1, 0 °C, 4 h; crystallization Et_2O, petroleum ether (40−60), for (a), 65%; for (b), 64%. v. NaOH (1 equiv), H_2O, acetone, for (a), 81%, 69%de; for (b), 78%, >95%de.

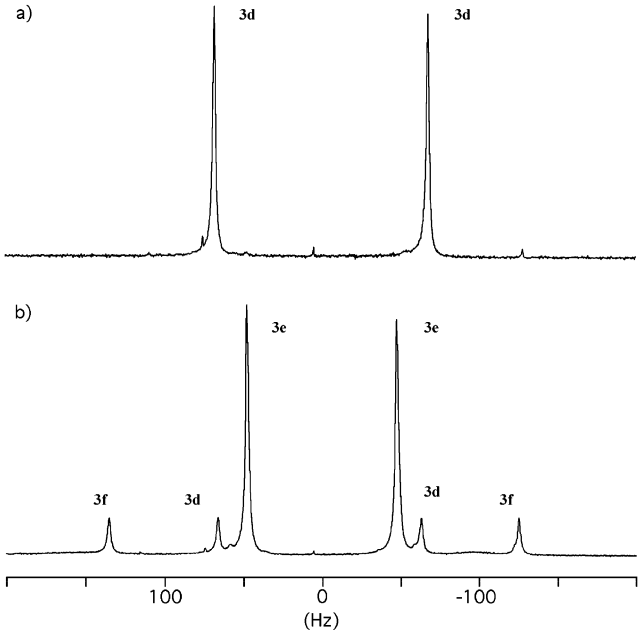


Figure 4. $^2H\{^1H\}$-NMR spectra, in a chiral liquid crystalline medium of DMF and PCBLL, of the synthetic samples of [1-$^2H_1$]-glycerols **3** prepared as Scheme 5. (a) The $^2H\{^1H\}$-NMR spectrum of (1*R*, 2*R*)-[1-$^2H_1$]-glycerol **3d**, de >95%. (b) The $^2H\{^1H\}$-NMR spectrum has signals for (1*S*, 2*R*)-[1-$^2H_1$]-glycerol **3e** (de 87%) as well as **3d** and **3f**.

lyophilizate was then neutralized (dil NaOH) and freeze-dried to secure the resultant fluoroacetates as their sodium salts. After the addition of cold carrier, the fluoroacetates were converted to their corresponding hexyl [2-$^2H_1$]-fluoroacetates **19a**, and each sample was analyzed by chiral liquid crystal $^2H\{^1H\}$-NMR. The resultant spectra are shown in Figure 3b and 3c. The feeding experiment with (1*R*, 2*R*)-[1-$^2H_1$]-glycerol **3d** gave rise to a sample of hexyl [2-$^2H_1$]-fluoroacetate **19a** of 75%ee as illustrated in Figure 3b. The quadrupolar splitting in Figure 3b is larger than that observed in Figure 3a, 3c, and 3d as the PBLG wt % in $CHCl_3$ was higher (14.3%) in the sample recorded in Figure 3b than those samples (11.3%) recorded in Figure 3a, 3c, and 3d. By comparison with the synthetic reference sample (Figure 3a) it is concluded that this sample of **19** had a predominant (*R*) configuration. The complementary feeding experiment using (1*S*, 2*R*)-[1-$^2H_1$]-glycerol **3e** gave rise to a sample of hexyl [2-$^2H_1$]-fluoroacetate **19a** with a lower enantiomeric excess of 30−40%ee in favor of the (*S*) configuration as illustrated in Figure 3c. Despite a difference in the calculated %ee of the resultant hexyl [$^2H_1$]-fluoroacetates **19a** and **19b** recovered from each experiment, the predominant absolute stereochemistry was opposite in each case and thus the experiments are mutually reinforcing. It can be concluded that the replacement of the original C−O bond of the *pro-R* hydroxymethyl group of glycerol by the C−F bond in fluoroacetate **1**, has occurred with a predominant *retention of configuration* in each case.

Enantiomeric Assay of [$^2H_1$]-Fluoroacetate from a Potassium [$^2H_4$]-Succinate Feeding Experiment to *S. cattleya*. Potassium [$^2H_4$]-succinate **4a** was administered at a final concentration of 9 mM to resting cells prepared from an 8 day old culture of *S. cattleya*.[9] The cells were incubated for 48 h after which time the cells were worked up as before. Lyophilization after acidification and then neutralization (dil NaOH) followed by freeze-drying generated a sample of sodium [2-$^2H_1$]-fluoroacetate **1a**. ^{19}F NMR analysis indicated a deuterium enrichment of 15% in the resultant sodium [$^2H_1$]-fluoroacetate as evidenced by a deuterium induced shift ($\delta \approx 0.5$ ppm) to lower frequency of the natural abundance ^{19}F NMR signal (−218 ppm) corresponding to fluoroacetate (and also to that corresponding to 4-fluorothreonine at −231 ppm). Cold "carrier" sodium fluoroacetate (50 mg) was added to this material to

adjust the level of isotope incorporation to ~2% enrichment. The material was then converted to hexyl [2-$^2H_1$]-fluoroacetate **19a** as previously described and was dissolved in a chloroform solution of PBLG for $^2H\{^1H\}$-NMR analysis. The resultant $^2H\{^1H\}$-NMR spectrum is shown in Figure 3d. The enantiomeric purity of the [$^2H_1$]-fluoromethyl group of fluoroacetate **1** isolated from the [$^2H_4$]-succinate feeding experiment is low, however it can be estimated that there is a bias in favor of the (*R*) enantiomer (25%ee) of [2-$^2H_1$]-fluoroacetate by comparison with the spectrum of the synthetic (*S*)-[$^2H_1$]-fluoroacetate (38%ee) (Figure 3a). In view of the known stereochemical course of the various enzymes that process succinate (Scheme 3) such an outcome indicates again a predominant *retention of configuration* in going from a (*R*)-[1-$^2H_1$]-glycolytic phosphate intermediate such as **14** or **9** to (*R*)-[$^2H_1$]-fluoroacetate. The low enantiomeric excess most probably arises from the extent of biochemical processing that succinate has undergone as it is directed toward fluorometabolite biosynthesis (Scheme 3). For example, [3-$^2H_1$]-oxaloacetate **12a** shown in Scheme 3 is an intermediate between [$^2H_4$]-succinate **4a** and the glycolytic phosphates **14** and **9a**. It is anticipated that the stereogenic center at C-3 of [3-$^2H_1$]-oxaloacetate **12a** will be susceptible to significant in vivo racemization and could account for the low %ee in this experiment.

Conclusion

An assay to determine the configuration of (*R*) and (*S*) [2-$^2H_1$]-fluoroacetates **1a** has been developed based on $^2H\{^1H\}$-NMR in a lyotropic liquid crystalline phase. The assay has been used to determine the enantiomeric excess of samples of [2-$^2H_1$]-fluoroacetate of low isotope incorporation (~1%), as the method analyses only those molecules carrying a deuterium atom. The method has been used to explore the stereochemical course of fluorination during the biosynthesis of fluoroacetate **1** from glycerol and succinate in *S. cattleya*. Three isotopically labeled precursors, (1*R*, 2*R*)-[1-$^2H_1$]-glycerol **3d**, (1*S*, 2*R*)-[1-$^2H_1$]-glycerol **3e** and [$^2H_4$]-succinate **4a**, all gave rise to samples of [2-$^2H_1$]-fluoroacetate **1a**, generated in vivo during incubations with washed resting cells of *S. cattleya*. In all cases, there was some loss of stereochemical integrity in that the resultant [2-$^2H_1$]-fluoroacetates were not enantiomerically pure. However, the configurational data obtained after the feeding experiments with (1*R*, 2*R*) and (1*S*, 2*R*)-[1-$^2H_1$]-glycerols **3d** and **3e** were complementary and support a predominant overall retention of configuration as the *pro-R* hydroxymethyl group of glycerol is biotransformed to the fluoromethyl group of fluoroacetate **1**. In the case of **3e**, the lower calculated %ee relative to **3d** (30%ee versus 75%ee) may be due to the low signal-to-noise ratio of this sample. In light of the recent identification of the fluorinase[13] from *S. cattleya* which mediates a reaction between fluoride ion and SAM (Scheme 2), it follows that the administered glycerols became incorporated into the ribose ring of ATP. SAM was then generated from ATP by combination with L-methionine with concomitant stereochemical inversion at the original *pro-R* hydroxymethyl carbon of glycerol. The overall retention of configuration observed in these whole cell experiments from glycerol or by labeling glycolytic intermediates from succinate forces the conclusion that enzymatic C−F bond formation also occurs with an *inversion of configuration* between SAM and fluoroacetate, perhaps as a result of an S_N2 type reaction. These results do not exclude the intermediacy of a double bond in an elimination process, followed by fluoride ion addition; however, this appears to be a less likely scenario. This stereochemical study sheds some light on the mechanism of biological fluorination but a more detailed analysis of the reaction mechanism must await studies on the purified fluorination enzyme.

Experimental Section

General Procedures. All reagents were obtained from Aldrich Chem. Co. Ltd. unless otherwise stated, and were used without further purification. Solvents were dried and distilled prior to use and all reactions were carried out under an atmosphere of N_2. ^1H- and ^{13}C-NMR spectra were recorded on a Varian Gemini 300 MHz (^1H at 299.98 MHz, ^{13}C at 75.431 MHz) spectrometer. $^2H\{^1H\}$-NMR spectra were recorded on Bruker DRX-400 NMR and Unity Inova 500 MHz spectrometers.

Synthesis of a Reference Sample of Hexyl (*S*)-[2-$^2H_1$]-Fluoroacetate 19a. (Caution. All derivatives of fluoroacetate are extremely toxic!) (*R*)-[7-$^2H_1$]-Benzyl alcohol **16b** was prepared after treatment of [7-^2H]-benzaldehyde **17a** with (*S*)-Alpine Borane following the method of M. M. Midland et al.[32] This generated a sample of (*R*)-[7-$^2H_1$]-benzyl alcohol **16b** with a 88%ee as judged by chiral liquid crystal $^2H\{^1H\}$-NMR analysis (Figure 1a).[31] The resultant (*R*)-[7-$^2H_1$]-benzyl alcohol (0.49 g, 4.5 mmol) was added to a stirred and cooled (−78 °C) solution of diethylaminosulfur trifluoride (DAST) (0.7 mL, 5.8 mmol) in dichloromethane (40 mL). The reaction was allowed to stir and come to ambient temperature over 10 h. Water (40 mL) was added and the organic phase was washed with water (3 × 30 mL), dried over $MgSO_4$ and the solvent removed under reduced pressure on a cool rotary evaporator. Care was taken not to exceed a water bath temperature of 20 °C to minimize evaporation of the benzyl fluoride product. (δ_F ($CDCl_3$): −207.20 (1F, d.t, J_{HF} 47.6, J_{DF} 7.3, CDHF); δ_H($CDCl_3$): 5.4-(1H, d, J_{HF} 47.8, CDHF) 7.41 (5H, s, Ar−H). The resultant (*S*)-[7-^2H]-benzylfluoride **18a** had a 38%ee as judged by chiral liquid crystal $^2H\{^1H\}$-NMR analysis (see Figure lb). This material was not purified further and was taken directly through the oxidation reaction. Accordingly, (*S*)-[7-^2H]-benzylfluoride (398 mg, 2.2 mmol) was added to a stirred solution of potassium permanganate (105 mg, 0.67 mmol) and potassium periodate (39.2 g, 170 mmol) in water (200 mL). The mixture was stirred at ambient temperature for 48 h and then most of the water was removed under reduced pressure. The remaining solution was acidified with sulfuric acid (2 M), and then the [$^2H_1$]-fluoroacetic acid product was lyophilized. The lyophilizate was neutralized using sodium hydroxide solution (0.1 M) and the water removed by freeze-drying to furnish sodium (*S*)-[2-$^2H_1$]-fluoroacetate **1a** as a white amorphous residue. δ_F($CDCl_3$): −217.4 (1F, d.t, J_{HF} 47.8, J_{DF} 7.15, CDHF). This material was diluted with unlabeled sodium fluoroacetate (400 mg, 4 mmol), thionyl chloride (0.5 mL, 5 mmol) was added and the suspension heated under reflux for 2 h. Hexanol (2 mL, 16 mmol) was added and the reaction stirred for a further 20 min. The reaction was quenched by the addition of water and the product extracted into dichloromethane. The solvent was dried ($MgSO_4$) and hexyl fluoroacetate **19** was purified over silica gel (100%, dichloromethane) to afford hexyl fluoroacetate containing ~1% hexyl (*S*)-[2-$^2H_1$]-fluoroacetate **19a** as a colorless oil (405 mg, 50%). δ_F ($CDCl_3$): −230.4 (1F, d.t, J_{HF} 46.5, J_{DF} 7.34, CDHF, 1%), −229.9 (1F, t, J_{HF} 47.6, CH_2F, 99%), δ_H ($CDCl_3$): 0.79 (3H, t, J 6.0, CH_3), 1.20 (6H, m), 1.56 (2H, m), 4.09 (2H, t, J 6.8, CH_2O), 4.72 (2H, d, J_{HF} 47.0, CH_2F). δ_C ($CDCl_3$): 13.8, 22.4, 25.3, 28.4, 31.3, 65.3, 77.4 (d, $^1J_{CF}$ 181.6, CH_2F) 167.8 (d, $^2J_{CF}$ 22.3, C═O).

Glycerols 3d and 3e were Prepared as Previously Described.[20]

Preparation of Washed Resting Cells of *S. cattleya*. A strain of *S. cattleya* NRRL 8057 was obtained from The Queen's University of Belfast, Microbial Biochemistry Section, Food Science Department, Belfast (originally from United States Department of Agriculture,

Agricultural Research Service, Midwest Area Northern Regional Research Laboratories, Peoria, Illinois, USA). Cultures were maintained on agar slants containing soybean flour (2% w/v), mannitol (2% w/v), agar (1.5% w/v) and tap water, grown at 28 °C until sporulation could be detected as previously described. The resultant static cultures were stored at 4 °C. Seed cultures were prepared by transfer of spores and aerial mycelia from a static culture, into a conical flask (500 mL) plugged with cotton wool and containing chemically defined medium (90 mL). After being shaken for 4 d at 28 °C and 180 rpm, an aliquot (0.3 mL) of the vegetative mycelium was used to inoculate the batch cultures. The cultures were then incubated at 28 °C and 180 rpm for 6−8 days. After growth for 6−8 d, cells were harvested by centrifugation, and the pellet was washed three times with 50 mM sterile MES buffer pH 6.5. After the final wash the bacterial pellet was resuspended in 50mM MES buffer (45 mL) pH 6.5 at a concentration of 0.18 wet wt mL^{-1}. This cell suspension was used for the subsequent isotopically labeled precursor feeding experiments.

Feeding Experiments with (1*R*, 2*R*)-[1-$^2H_1$]-Glycerol 3d, (1*S*, 2*R*)-[1-$^2H_1$]-Glycerol 3e and [$^2H_4$]-Succinate 4a. A solution of NaF (3 mL, 40 mM) was added to the washed cells (45 mL) prepared as described above and then the suspension was diluted with MES buffer (42 mL). The labeled precursors (1*R*, 2*R*)-[1-$^2H_1$]- **3d** and (1*S*, 2*R*)-[1-$^2H_1$]- glycerol **3e** dissolved in 50 mM MES buffer and the pH adjusted to 6.5 with 2M KOH solution. This solution was then added to the cell suspension of *S. cattleya* such that the final glycerol concentration was 9 mM. Similarly a solution of potassium [$^2H_4$]-succinate **4a** (3 mL, 40 mM) prepared from [$^2H_4$]-succinic acid dissolved in 50 mM MES buffer and the pH adjusted to 6.5 with 2 M KOH solution, was added to the *S. cattleya* cell supension by filter sterilisation such that the final concentration of labeled succinate was 9 mM. In each case incubations were carried out at 28 °C for 48 h on an orbital shaker (175 rpm). For resting cell experiments, an aliquot (5 mL) of the cell suspension was used and supplemented with a putative precursor by filter sterilisation. The total volume was adjusted to 23 mL with 50 mM MES buffer pH 6.5 and incubated in 75 mL conical flasks plugged with cotton wool at 28 °C on an orbital shaker at 200 rpm for 48 h.

Chiral Liquid Crystal $^2H\{^1H\}$-NMR Analyses of Hexyl [2-$^2H_1$]-Fluoroacetates 19. *Poly*-γ-benzylglutamate (120 mg, MWt 150 000−350 000, Sigma Chem. Co. Ltd.,) and a sample (∼50−60 mg) of hexyl [2-$^2H_1$]-fluoroacetate **19a** were placed into a 5 mm NMR tube. Dry chloroform (0.8−0.54 mL) was added until a 10−15wt % of PBLG was achieved. To aid complete dissolution of the polymer and dispersion of the solute through the gel the sample was repeatedly centrifuged (20 times) on a low speed (600 rpm, ∼40 relative centrifugal force) benchtop centrifuge, turning the tube upside-down between each centrifugation. $^2H\{^1H\}$-NMR spectra were recorded at 61.4 Hz on Bruker DRX-400 spectrometer with a ^2H-probe. The temperature was held at 303 K with proton broad band decoupling and samples were spun at 14 Hz. In a typical experiment eg (*R*)-**19a**, Figure 3b, the sample contained 11 mgs of (*R*)-**19a** and had a 14.3wt % fraction of PBLG in chloroform. The sample was run for 6 h involving 10 000 transients. The quadrupolar splitting varies with the PBLG/$CHCl_3$ wt fraction, temperature and concentration of the solute, thus these are not constant for every experiment. The experiments shown in Figure 3a, 3c and 3d had PBLG/$CHCl_3$ wt fractions of 11−12% and thus the quadrupolar splittings are smaller (i.e., ∼230 Hz versus ∼440 Hz for the (*R*)-enantiomer) in these three spectra compared with that observed (*R*)-**19a** in Figure 3b.

Acknowledgment. We thank the Isle of Man Education Authority for a studentship (R.J.M.G.) and the EPSRC/CNRS CERC-3 program for an Anglo-French research grant.

JA026654K