Enzymatic Fluorination in *Streptomyces cattleya* Takes Place with an Inversion of Configuration Consistent with an S_N2 Reaction Mechanism

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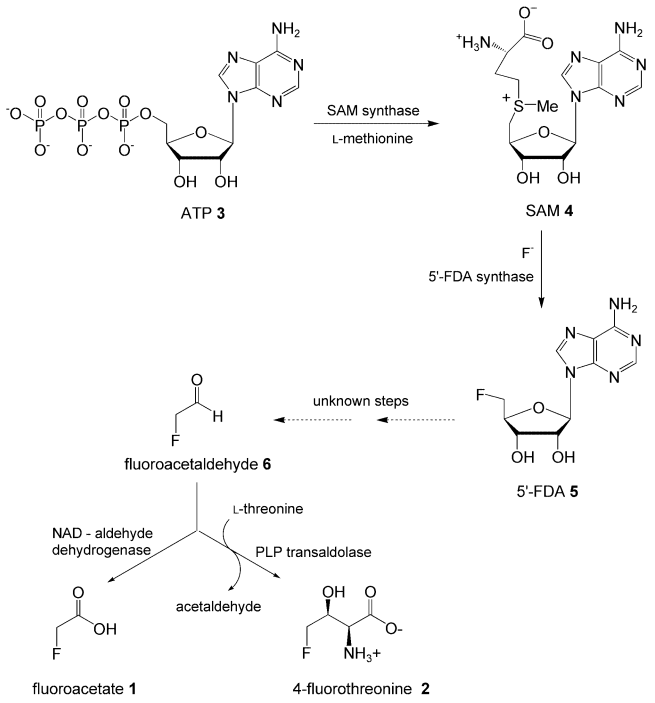
Dedicated to Professor Heinz G. Floss on the occasion of his 70th Birthday

The stereochemical course of the recently isolated fluorination enzyme from Streptomyces cattleya *has been evaluated. The enzyme mediates a reaction between the fluoride ion and S-adenosyl-L-methionine (SAM) to generate 5′-fluoro-5′-deoxyadenosine (5′-FDA). Preparation of (5′R)-[5-$^2H_1$]-ATP generated (5′R)-[5-$^2H_1$]-5′-FDA in a coupled enzyme assay involving SAM synthase and the fluorinase. The stereochemical analysis of the product relied on ^2H NMR analysis in a chiral liquid-crystalline medium. It is concluded that the enzyme catalyses the fluorination with an inversion of configuration consistent with an S_N2 reaction mechanism.*

Introduction

In 1986, a group from Merck reported that *Streptomyces cattleya* had the rare capacity to biosynthesise organofluorine metabolites.[1] The toxin fluoroacetate (**1**) and the antibiotic 4-fluorothreonine (**2**) were isolated and identified as secondary metabolites of this organism. Biosynthesis studies have since revealed some of the details of the biosynthetic pathway to the organofluorine natural products of *S. cattleya*, and these are summarised in Scheme 1.[2–5] Fluoroacetaldehyde (**6**) has been identified as the last common biosynthetic intermediate to both fluoroacetate and 4-fluorothreonine.[2,5] Cell-free extracts of *S. cattleya* are able to efficiently convert fluoroacetaldehyde to fluoroacetate, and an NAD-dependant aldehyde dehydrogenase capable of catalysing this reaction has been isolated and characterised.[6] This enzyme had a clear preference for fluoroacetaldehyde over acetaldehyde as a substrate and would appear to mediate the final transformation in fluoroacetate biosynthesis in the organism. The enzyme responsible for the transformation of fluoroacetaldehyde to 4-fluorothreonine has also been characterised.[7] This is a pyridoxal phosphate-dependant transaldolase that converts fluoroacetaldehyde and L-threonine to 4-fluorothreonine and acetaldehyde. This novel PLP-dependant enzyme does not utilise glycine as the amino acid donor, and it is interesting that for every molecule of 4-fluorothreonine that is biosynthesised, *S. cattleya* sacrifices a molecule of L-threonine, its amino acid analogue. The evolutionary significance of this interconversion of threonines is not clear, but it presumably confers some advantage to the organism's survival.



Scheme 1. *Current understanding of the metabolites and enzymes involved in the biosynthesis of fluoroacetate* ***1*** *and 4-fluorothreonine* ***2*** *in* S. cattleya.

The most intriguing enzyme on the fluorometabolite biosynthesis pathway in *S. cattleya* is 5′-fluoro-5′-deoxyadenosine synthase (5′-FDAS), the first committed enzyme on the pathway and the one responsible for converting inorganic fluoride to organic fluorine.[8] The enzyme mediates a reaction between

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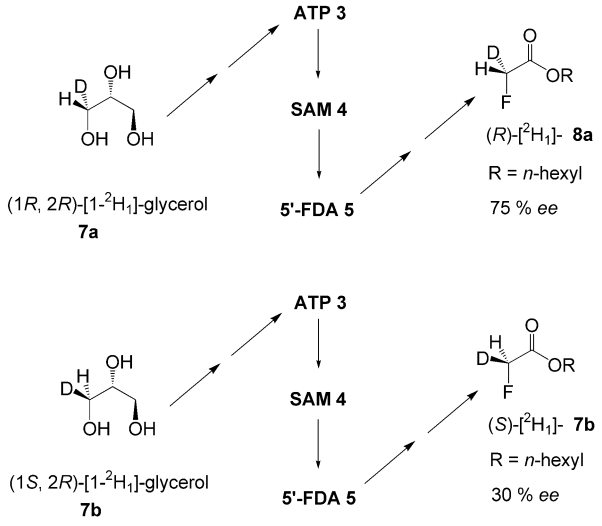
 DOI: 10.1002/cbic.200300839

S-adenosyl-L-methionine (SAM; **4**) and fluoride ion to generate 5′-fluoro-5′-deoxyadenosine (5′-FDA; **5**). This enzyme has recently been purified to homogeneity and it is the first native fluorination enzyme to have been isolated.[9] An amino acid sequence from the purified protein has allowed the gene to be amplified by the polymerase chain reaction (PCR), and the fluorination enzyme has now been cloned and over-expressed in *E. coli*.[10] Nature has hardly developed a biochemistry of organic fluorine compounds, and, as this enzymatic C–F bond formation is extremely rare, the enzyme is of mechanistic interest and has some biotechnological potential for the enzymatic synthesis of organofluorine compounds. In this context, 5′-FDAS has recently been used to prepare labelled [^{18}F]-5′-FDA from $^{18}F^-$ and has potential as a catalyst for C–^{18}F bond formation for positron emission tomography (PET) applications.[11]

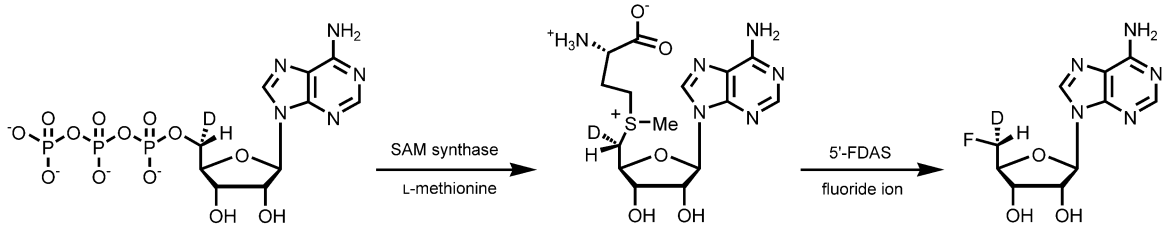
Mechanistic information can be gleaned from a knowledge of the stereochemistry of the reaction process, and exploring the stereochemical course of biological fluorination has been a recent objective in our laboratory. We have already gained some insight into the stereochemical course of biological fluorination in whole-cell incubations of *S. cattleya*.[12] These experiments involved adding glycerol that was chiral by virtue of deuterium-isotope labelling in its pro-*R* hydroxymethyl arm. (1*R*,2*R*)- and (1*S*,2*R*)-[1-^{2}H$_1$]-glycerols **7a** and **7b** were added in separate experiments to the growth medium of *S. cattleya* in whole-cell incubations, and, after several days, the biosynthesised fluoroacetate, which carried a deuterium atom on the fluoromethyl group, was recovered. Deuterium NMR in chiral liquid-crystalline solvent has proven to be an excellent method for the analytical determination of enantiomers of chiral [^{1}H,^{2}H,FC]-fluoromethyl groups and takes advantage of the quadrupolar nature of the deuterium nucleus.[12] By using this approach, the biosynthetically derived [2-^{2}H$_1$]-fluoroacetates were converted to their hexyl esters **8a** and **8b** and were analysed. The enantiomers were easily resolved by ^{2}H NMR in a chiral liquid-crystalline solvent. The stereochemical results, which are summarised in Scheme 2, indicated that the hydroxymethyl C–O bond of the pro-*R* arm of glycerol was converted to a C–F bond in the fluoroacetate with retention of configuration.[12] The enantiomeric excesses varied in the two complementary experiments, largely as the isotope content in the derivatised fluoroacetates was low and the signal to noise ratio was low for the analytical method. Taking into account the knowledge that SAM synthase operates with an inversion of



Scheme 2. *A summary of conclusions from a previous stereochemical study,[12] which involved the incorporation of stereospecifically [^{2}H]-labelled glycerols into fluoroacetate in whole cells of* S. cattleya. *This study indicated that the C–O bond of glycerol was replaced by the C–F bond of fluoroacetate with an overall retention of configuration. Because SAM synthetase, which converts ATP* ***3*** *to SAM* ***4****, proceeds with inversion of configuration this study suggested that the fluorination enzyme also proceeds with an inversion of configuration.*

configuration,[13] this observation implies that the fluorination event proceeds with an *inversion of configuration*, that is, two inversions is an overall retention. However that study relied on the correct metabolic and stereochemical interpretation of events as the isotope on the glycerol was assumed to enter the glycolytic pathway, and then pass into the pentose phosphate pathway prior to labelling ribose and becoming incorporated into the ribose ring at the C-5′ position of ATP and then SAM prior to fluorination. Further, the isotope was analysed in the fluoroacetate products and not in the immediate product of the enzymatic fluorination, and it was assumed that there was no configurational change during the latter stages of the pathway between 5′-FDA and fluoroacetate. These assumptions were a limitation of the whole-cell experiments. With the purified and over-expressed 5′-FDAS now available,[10] it appeared appropriate to re-examine the stereochemical course of the fluorination event on the enzyme directly.

We envisaged a preparation of (5′*R*)-[5′-^{2}H$_1$]-ATP **3a** followed by its incubation with SAM synthase to generate a sample of (5′*S*)-[5′-^{2}H$_1$]-SAM **4a** (Scheme 3). Treatment of this stereo- and



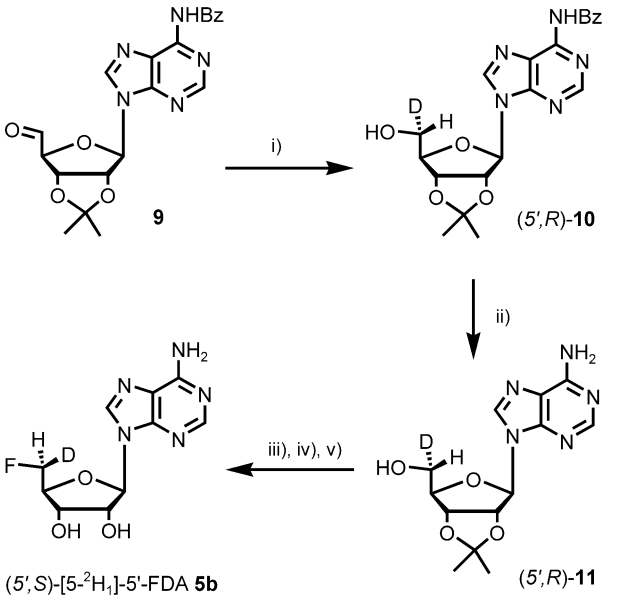
(5′*R*)-[5′-^{2}H]-ATP **3a** SAM **4a** (5′*R*)-[5′-^{2}H]- 5′-FDA **5a**

Scheme 3. *Treatment of stereospecifically labelled (5′*R*)-[5′-^{2}H]-ATP with SAM synthase and 5′-fluorodeoxyadenosine synthase generates (5′*R*)-[5′-^{2}H]-5′-FDA; this indicates that there are two sequential inversions of configuration resulting in an overall retention of configuration.*

isotopically labelled SAM with fluoride ion in the presence of the fluorination enzyme (5′-FDAS) should provide a sample of [5′-$^2H_1$]-5′-FDA **5a**, stereospecifically labelled at the C-5′ position. With a knowledge that SAM synthase mediates a configurational inversion,[13] the resultant configuration at C-5′ of [5′-$^2H_1$]-5′-FDA in the coupled enzyme assay will allow the stereochemical course of the fluorination reaction to be deduced. The entire strategy depends on a reliable assay for determining the absolute stereochemistry at C-5′ of [5′-$^2H_1$]-5′-FDA. In this paper we describe how we have used ^2H NMR in a chiral liquid-crystalline medium to determine the absolute stereochemistry of [5′-$^2H_1$]-5′-FDA.

Results and Discussion

To succeed in this approach it was necessary to prepare a sample of [5′-$^2H_1$]-5′-FDA of known stereochemical configuration at C-5′ as a reference for the ^2H NMR assay in chiral-liquid crystalline media. Our approach to the synthesis of the (5′*S*) isomer of [5′-$^2H_1$]-5′-FDA **5b**, summarised in Scheme 4, was initiated following the previously reported asymmetric reduction of aldehyde **9**.[14,15] $LiAlD_4$ treatment with added 2-methylbutan-2-ol and lithium iodide gave alcohol **10** in a 4:1 *ratio* (60% *de*) of diastereoisomeric products. It has previously been established that the major product is the (5′*R*) diastereoisomer.[15] The (5′*S*) isomer of [5′-$^2H_1$]-5′-FDA was prepared from **11** after mesylation of (5′*R*)-**10** and then TBAF treatment. It is assumed that the substitution of the mesylate by the fluoride ion proceeds with a stereochemical inversion of configuration. Acetonide deprotection then gave (5′*S*)-[5′-$^2H_1$]-5′-FDA. The synthetic sample of (5′*S*)-[5′-$^2H_1$]-5′-FDA was analysed by ^2H NMR in a chiral liquid crystalline medium generated by dissolving poly-γ-benzyl-L-glutamate (PBLG) in DMF.[16,17] The resultant spectrum is shown in Figure 1a. It exhibits both the ^2H NMR spectrum of our mixture of diastereoismers and that of the solvent (DMF) at natural abundance level. The latter is made of three quadrupolar doublets centred on the chemical shift of the three possible isotopomers of DMF, that is, the isotopomer in which the deuterium is at the aldehydic position and the two isotopomers in which the deuterium is on the nonequivalent methyl groups. These six lines are labelled with an asterisk. The other doublets are attributed to our diastereoisomers (4:1), which are clearly resolved by the technique. The predominant (5′*S*) isomer has a smaller quadrupolar splitting than the minor (5′*R*) isomer. The signal of the (5′*S*) compound is split into two components. This is due to the total spin–spin coupling, T_{DF}, between the deuterium and the fluorine nuclei with $T_{DF} = 2D_{DF} + J_{DF}$. In this expression, J_{DF} is the scalar coupling and D_{DF} is the dipolar coupling. The (5′*R*) compound does not show such fluorine deuterium coupling. This is due to the fact that, here, the scalar and the dipolar couplings are of opposite signs yielding, fortuitously, a null or almost null splitting.



Scheme 4. *i) LiAlD4 + 2-methylbutan-2-ol/LiI, −78 °C in THF for 3 h (36%); ii) NH_4OH/MeOH (1:1), 18 h (95%); iii) MsCl/pyr, 20 °C, 3 h (73%); iv) TBAF/CH_3CN, reflux 3 h (35%); v) TFA (90%), 30 min (81%).*

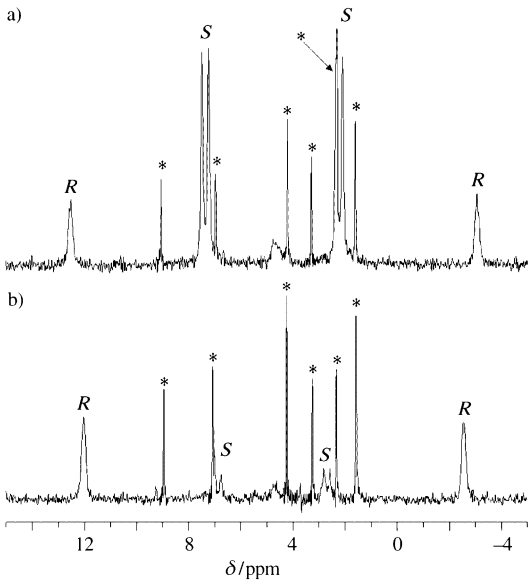
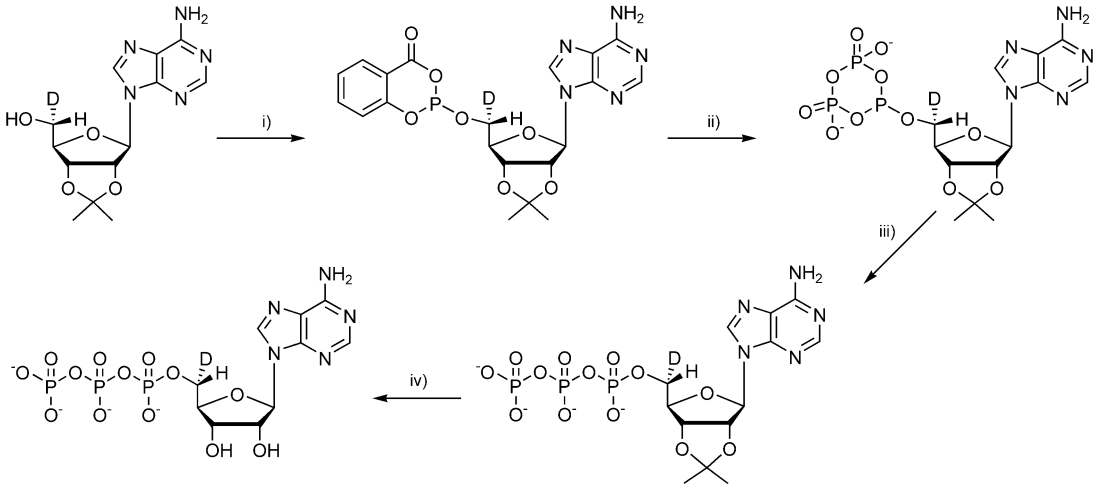


Figure 1. *$^2H\{^1H\}$ NMR spectra of a) synthetic (5′S)-[5′-^2H]-5′-FDA (4:1 diastereoisomeric ratio) and b) (5′R)-[5′-^2H]-5′-FDA (4:1 diastereoisomeric ratio) prepared by using enzymatic fluorination. The natural abundance signals from DMF (three doublets centred at 8.01, 2.90 and 2.79 ppm) are labelled with an asterisk. In these spectra, the chemical shift of the aldehydic deuterium of DMF (8.01 ppm) was used as an internal reference.*

In order to explore the stereochemistry of the enzymatic process it was necessary to prepare a sample of stereospecifically enriched [5′-$^2H_1$]-ATP. The (5′*R*) isomer of [5′-$^2H_1$]-ATP was prepared from alcohol (5′*R*)-**11** by a modification of a previously described procedure.[18,19] The route is illustrated in Scheme 5 and proved relatively straightforward to execute. This route utilised a sample of **11** prepared as described above as a 4:1 mixture (60% *de*) of (5′*R*) and (5′*S*) diastereoisomers. As there is no stereochemical inversion at C-5′ during the conversion of **10** to ATP, the corresponding [5′-$^2H_1$]-ATP is predom-



*Scheme 5. i) 2-chloro-4*H*-1,2,3-benzodioxaphosphorin-4-one in THF/pyr, 15 min; ii) tributylammonium pyrophosphate in DMF/Et_3N, 30 min; iii) I_2 (1%) in pyr/H_2O (98:2), 20 min; iv) TFA (25%), 30 min.*

inant in the (5′*R*) diastereoisomer, also in a 4:1 ratio. With this material in hand it was necessary to catalyse its conversion from ATP to [5′-$^2H_1$]-SAM by SAM synthase. This enzyme was partially purified from bakers' yeast (*Saccharomyces cerevisae*).[20] The key experiment was carried out by treatment of (5′*R*)-ATP with L-methionine, Mg^{2+}, K^+ and fluoride ion in a coupled enzyme reaction with SAM synthetase and the fluorination enzyme.[8,9] The resultant [5′-$^2H_1$]-5′-FDA was purified by preparative HPLC and was analysed by ^{2}H NMR with identical conditions to those described for the reference sample. The ^{2}H NMR spectrum is shown in Figure 1b. Although the amount of material analysed from the enzyme study was relatively small (~0.40 mg), it is clear from the analysis that the predominant diastereoisomer has the (5′*R*) configuration, again with an approximate ratio of 4:1; this is consistent with the stereochemical purity of the original starting material. (5′*R*)-[5′-$^2H_1$]-ATP provides (5′*S*)-[5′-$^2H_1$]-SAM after treatment with SAM synthase, therefore the observation that the product of the fluorination reaction gave (5′*R*)-[5′-$^2H_1$]-5′-FDA indicates that there have been two configurational inversions within the coupled enzyme assay. These experiments reinforce the earlier conclusion and show that the fluoride ion acts on the C5′–S bond of SAM to mediate a configurational inversion during C–F bond formation. The result is consistent with an S_N2 reaction mechanism operating during biological fluorination in *S. cattleya* and reinforces the conclusion made after the earlier whole-cell experiments with *S. cattleya*. An S_N2 mechanism is also consistent with the recent X-ray structure of the fluorination enzyme.[10]

These stereochemical conclusions have been drawn through the use of deuterium NMR in a chiral liquid-crystalline solvent. In such a solvent, enantiotopic or diastereotopic groups do not exhibit the same ordering properties due to the chirally orientated field. This differential ordering of enantiomers or diastereoisomers results in a difference that is often very large in the order-sensitive NMR interactions, such as quadrupolar splitting. The technique offers a powerful tool for examining the stereochemical course of enzymatic reactions with appropriately labelled substrates.

Experimental Section

General methods: Air- and moisture-sensitive reactions were carried out under an inert atmosphere with oven-dried glassware (200 °C). All reagents of synthetic grade were used as supplied, if further purification was required, the procedures are detailed ref. [22]. Column chromatography was performed by using Merck Kieselgel 60 silica gel (230–400 nm mesh). NMR spectra were obtained by using a Varian Unity Plus 500, a Bruker Av-300 or a Varian Unity Plus 300. Melting points were determined by using a Gallenkamp Griffin MPA350.BM2.5 melting-point apparatus. Cation-exchange procedures were carried out on Dowex 50W (X8) resin with 50–100 mesh particles. High- and low-resolution mass spectrometry (HRMS/LRMS) were performed with a Micromass LCT spectrometer (Manchester, UK). HPLC analysis was carried out by using a Varian series 9012 pump/9050 UV lamp; analytical HPLC was performed on a Hypersil ODS C-18 column, 5 μm (250× 4.6 mm id); semipreparative HPLC was performed by using a Phenomenex Hypersil C-18 column, 5 μm (250×10 mm id).

Liquid crystalline NMR sample preparation: PBLG (151 mg, M_W 70 000–150 000 purchased from Sigma-Aldrich) was weighted into a 5 mm o.d. NMR tube. DMF (240 μL) was added, and the mixture was heated to 80–100 °C until complete dissolution of the polymer occurred. Chloroform (30 μL) and a solution of 5′-FDA (0.3 to 2.0 mg) in DMF (40 μL) were introduced in the NMR tube. In order to homogenize the viscous mixture, the NMR tube was repeatedly centrifuged (20×) on a low speed (600 rpm) bench-top centrifuge, with the tube being turned upside-down between each centrifugation.

Deuterium NMR measurements in PBLG/DMF liquid crystal: $^2H\{^1H\}$ NMR spectra were recorded at 61.4 MHz on a Bruker DRX-400 spectrometer equipped with a selective deuterium probe. The temperature was held at 330 K by a BVT3000 variable temperature unit. Proton broad-band decoupling was achieved by using the WALTZ-16 composite pulse sequence.

[(5′*R*)-$^2H_1$]-N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine (10):[15] A solution of N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine-5′-aldehyde (0.90 g, 2.2 mmol) and LiI (3.6 g, 26.3 mmol) in THF (20 mL) was stirred at ambient temperature for 10 min and then at −78 °C for 50 min. 2-Methylbutan-2-ol (2.9 mL, 26.6 mmol) was added to a suspension containing $LiAlD_4$ (0.37 g; 8.9 mmol) in THF (50 mL),

and the mixture was stirred at ambient temperature for 20 min and then at −78 °C for an additional 20 min. This solution was then added to the adenosine solution by syringe, and the reaction mixture was stirred at −78 °C over 3 h. After 15 min, NaOH (2 N, 3 mL) was added, and the resultant mixture was stirred for an additional 20 min. The precipitate was filtered, and the solution was concentrated under reduced pressure. The residue was partitioned between water and CH_2Cl_2, and the organic phase was dried over $MgSO_4$ and then concentrated. The residue was purified over silica gel with CH_2Cl_2/isopropanol/acetone (86:4:10) to give **10** (0.32 g, 35%) as an amorphous white solid. M.p. 148–150 °C (lit. 151–153 °C for the corresponding unlabeled compound[21]); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.36, 1.61 (2 s, 2×3 H; CMe_2), 3.79 (d, $J_{5'a,4'}$ = 1.79 Hz, 0.2 H; $C_{5'}H_a$), 3.97 (d, $J_{5'b,4'}$ = 1.28 Hz 0.8 H; $C_{5'}H_b$), 4.55 (m, 1 H; $C_{4'}H$), 5.04 (dd, $J_{2',3'}$ = 5.89 Hz, $J_{3',4'}$ = 1.28 Hz, 1 H; $C_{3'}H$), 5.23 (dd, $J_{2',1'}$ = 4.86 Hz, $J_{2',3'}$ = 5.89 Hz, 1 H; $C_{2'}H$), 5.80 (br s, 1 H; $C_{5'}OH$), 5.96 (d, $J_{1',2'}$ = 4.86 Hz, 1 H; $C_{1'}H$), 7.5, 8.0 (m, 5 H; arom.), 8.08, 8.70 (2 s, 2× 1 H; C_2H and C_8H), 9.20 ppm (br s, 1 H; N*H*COAr); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.7 and 27.9 (CMe_2), 63.0 (br s, C-5′), 81.9, 83.7, 86.7, 94.2 (C-1′, C-2′, C-3′, C-4′), 114.6 (CMe_2), 128.3, 129.1, 133.2, 133.8 (arom.), 124.5, 150.7, 151.0 (C-4, C-5, C-6), 142.9 and 152.7 (C-2, C-8), 165.3 ppm (NH*C*OAr).

[(5′*R*)-^{2}H$_1$]-2′,3′-*O*-isopropylideneadenosine (11):[23] A solution of [(5′*R*)-^{2}H$_1$]-*N*^{6}-benzoyl-2′,3′-*O*-isopropylideneadenosine (**10**; 0.125 g; 0.30 mmol) in methanolic ammonia (50%,15 mL) was kept at ambient temperature for 24 h and then evaporated to dryness. The residue was purified over silica gel with CH_2Cl_2/isopropanol/acetone (84:6:10) as eluent affording **11** (0.080 g; 95%) as a white amorphous solid. M.p. 220–222 °C (lit. 220–221 °C[23]); ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.31, 1.53 (2 s, 2×3 H; CMe_2), 3.50 (m, 1 H; $C_{5'a}H\,C^{-}_{5'b}H$), 4.20 (dd, $J_{4',3'}$ = 2.30 Hz, $J_{4',5'}$ = 4.86 Hz, 1 H; $C_{4'}H$), 4.95 (dd, $J_{2',3'}$ = 6.14, $J_{3',4'}$ = 2.30 Hz, 1 H; $C_{3'}H$), 5.35 (dd, $J_{2',1'}$ = 3.07, $J_{2',3'}$ = 6.14 Hz, 1 H; $C_{2'}H$), 6.11 (d, $J_{1',2'}$ = 3.07 Hz, 1 H; $C_{1'}H$), 7.36 (br s, 2 H; NH_2), 8.14, 8.33 ppm (2 s, 2×1 H; C_2H, C_8H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 25.5, 27.4 (CMe_2), 61.4 (br s, C-5′), 81.6, 83.5, 86.6, 89.9 (C-1′, C-2′, C-3′, C-4′), 113.4 (CMe_2), 140.1, 153.0 (C-2, C-8), 119.3, 149.1, 156.3 ppm (C-4, C-5, C-6).

[(5′*R*)-^{2}H$_1$]-2′,3′-*O*-isopropylidene-5′-mesyl-adenosine:[24] Methanesulfonyl chloride (0.19 mL, 2.44 mmol, 2.0 equiv) was added dropwise to a solution of [(5′*R*)-^{2}H$_1$]-2′,3′-*O*-isopropylideneadenosine (0.375 g; 1.22 mmol) in pyridine (20 mL) at 25 °C. The reaction mixture was cooled to 0 °C, then left to warm to ambient temperature over 3 h. Cold water (20 mL) was added, and the reaction mixture was concentrated and partitioned between dichloromethane and water. The organic layer was washed with water, dried over $MgSO_4$ and concentrated. Purification over silica gel eluting with CH_2Cl_2/IPA/acetone (84:6:10) afforded the mesylate (0.344 g; 73%) as a white amorphous solid. M.p. 140 °C (dec.); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.41, 1.63 (2 s, 2×3 H; CMe_2), 2.92 (s, 3 H; SO_2Me_3), 4.41 (m, 2 H; $C_{4'}H$, $C_{5'}H_a\,C^{-}_{5'}H_b$), 5.16 (dd, $J_{3',4'}$ = 2.9, $J_{3',2'}$ = 6.2 Hz, 1 H; $C_{3'}H$), 5.47 (dd, $J_{2',1'}$ = 2.0, $J_{2',3'}$ = 6.2 Hz, 1 H; $C_{2'}H$), 5.87 (br s, 2 H; NH_2), 6.13 (d, $J_{1',2'}$ = 2.0 Hz, 1 H; $C_{1'}H$), 7.92, 8.36 ppm (2 s, 2×1 H; C_2H, C_8H); HRMS: $C_{14}H_{18}DN_5O_6S$ calcd: 387.1204; found = 387.1197.

[(5′*S*)-^{2}H$_1$]-2′,3′-*O*-isopropylidene-5′-fluorodeoxyadenosine: A solution of TBAF in THF (1 M, 1.2 mL, 1.2 mmol, 2.6 equiv) was added to a solution of [(5′*R*)-^{2}H$_1$]-2′,3′-*O*-isopropylidene-5′-*O*-mesyladenosine (0.18 g, 0.46 mmol) in acetonitrile (20 mL) at ambient temperature, and the resulting solution was heated under reflux for 3 h. The reaction was cooled to ambient temperature and concentrated, and the resultant oil was partitioned between chloroform and water. The organic layer was washed with water and dried over $MgSO_4$ and the product was purified over silica gel by eluting with CH_2Cl_2/IPA/acetone (84:6:10) to afford the title product (0.050 g; 35%) as a light brown powder. M.p. 157–159 °C (lit. 159–160 °C for the corresponding unlabeled compound[4]); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.40, 1.64 (2 s, 2×3 H; CMe_2), 4.55 (m, 2 H; $C_{4'}H$, $C_{5'}H_a\,C^{-}_{5'}H_b$), 5.10 (dd, $J_{3',2'}$ = 6.3, $J_{3',4'}$ = 3.8 Hz, 1 H; $C_{3'}H$), 5.37 (m, 1 H; $C_{2'}H$), 5.68 (br s, 2 H; NH_2), 6.19 (d, $J_{1',2'}$ = 1.93 Hz, 1 H; $C_{1'}H$), 7.93, 8.36 ppm (2 s, 2×1 H, C_2H, C_8H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.7, 27.5 (CMe_2), 81.1 (d, $J_{C3',F}$ = 6.8 Hz, C-3′), 82.5 (dt, $J_{C5',F}$ = 170, $J_{C5',D}$ = 23 Hz; C-5′), 84.9 (C-2′), 85.9 (d, $J_{C4',F}$ = 19.5 Hz; C-4′), 91.3 (C-1′), 115.0 (CMe_2), 120.5, 149.8, 153.7 (C-4, C-5, C-6), 139.7, 156.0 ppm (C-2, C-8); ^{19}F NMR, F-H decoupling (500 MHz, $CDCl_3$): δ = −229.31 (t, J = 6.73 Hz, 0.8 F; $F_{5'}D_{5'b}$), −229.27 ppm (t, J = 7.29 Hz, 0.2 F; $F_{5'}D_{5'a}$); HRMS: $C_{13}H_{15}DN_5O_3F$ calcd: 311.1380; found = 311.1378.

[(5′*S*)-[^{2}H$_1$]-FDA (5a):[23] A solution of [(5′*S*)-^{2}H$_1$]-2′,3′-*O*-isopropylidene-5′-fluorodeoxyadenosine (0.047 g; 0.15 mmol) in trifluoroacetic acid/water (9:1; 7 mL) was stirred at ambient temperature for 20 min and then evaporated to dryness. The residue was co-evaporated with ethanol (3×), then triturated with ether and finally dried under reduced pressure to give **5** as a colourless oil (0.033 g; 81%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 4.10 (dt, $J_{4',5'}$ = $J_{4',3'}$ = 5.12, $J_{4',5'F}$ = 23.81 Hz, 1 H; $C_{4'}H$), 4.27 (t, $J_{3',2'}$ = $J_{3',4'}$ = 5.12 Hz, 1 H; $C_{3'}H$), 4.57 (t, $J_{2',3'}$ = $J_{2',1'}$ = 5.12, 1 H; $C_{2'}H$), 4.60 (dd, $J_{5'a,4'}$ = 5.37, $J_{5'F,5'a}$ = 47.36 Hz, 0.8 H; $C_{5'}H_a$), 4.65 (dd, $J_{5'b,4'}$ = 3.07, $J_{5'F,5'b}$ = 47.60 Hz, 0.2 H; $C_{5'}H_b$), 5.95 (d, $J_{1',2'}$ = 5.12 Hz, 1 H; $C_{1'}H$), 7.54 (br s, 2 H; NH_2), 8.20, 8.32 ppm (s, 1 H; C_2H, C_8H); ^{19}F NMR (300 MHz; DMSO): δ = −228.035 ppm (m, 1 F; $C_{5'}F$); HRMS: $C_{10}H_{11}DN_5O_3F$ calcd: 271.1065; found = 271.1062 [M+H]$^+$.

[(5′*R*)-^{2}H$_1$]-ATP (3): A solution of 2-chloro-4*H*-1,2,3-benzodioxaphosphorin-4-one in dioxane (1 M, 0.52 mL, 0.52 mmol) was added to a solution of 2′,3′-*O*-isopropylideneadenosine in pyridine (0.126 g, 0.47 mmol, 0.4 mL) and dioxane (1.2 mL). After 20 min, a solution of tributylammonium pyrophosphate[19] in DMF (0.5 M, 1.28 mL, 0.64 mmol) and triethylamine (0.52 mL) was added. The reaction mixture was stirred for 30 min, then a solution of 1% iodine (8 mL, 0.31 mmol) in pyridine/water (98:2, 0.8 mL) was added. After 20 min, the excess iodine was destroyed by addition of 5% aqueous $NaHSO_3$. The resultant residue was dissolved in water, and the aqueous layer was washed with CH_2Cl_2. After evaporation of the organic solvent, the residue was dissolved in water (37.5 mL), and an aqueous solution of 50% TFA (10 ml) was added. The mixture was stirred for 30 min, adjusted to pH 8.5 by addition of NaOH (0.1 N, aq), and the solvent was evaporated to dryness. The residue was then washed with ethyl acetate, and the crude product was dried under reduced pressure. ^{31}P NMR (121.5 MHz, D_2O): δ = −8.9 (d, J = 21.16 Hz; Pγ), −9.4 (d, J = 18.4 Hz; Pα), −19.5 ppm (formal t; Pβ); MS (electrospray, negative ion mode) m/z: 551.3 [M+2 Na−H]$^-$; (unlabelled 550).

Purification of SAM Synthase: Bakers' yeast (*Saccharomyces cerevisiae*) was grown in a conical flask (2 L) containing a defined medium (500 mL) consisting of yeast extract (0.9%), peptone (1.8%) and D-glucose (2%). After incubation (37 °C, 200 rpm) for 12 h , the cells were harvested, washed with Tris-HCl buffer (50 mM, pH 7.8) and then resuspended in the same buffer. A cell-free extract was prepared by sonication (5×1 min, 60% duty cycle) and centrifugation of the cell debris (20 000 *g* for 20 min at 4 °C). Typically the protein concentration was 3.5 mg mL^{-1}. The enzyme activity was partially purified by adding solid $(NH_4)_2SO_4$ to the cell-free extract at 4 °C until 60% saturation, followed by centrifugation. The supernatant was adjusted to 80% saturation with solid $(NH_4)_2SO_4$, and the precipitate was collected after centrifugation. The pellet was dialysed in Tris-HCl buffer (50 mM, pH 7.8) (12 h,

4 °C), and the protein was eluted from an anion exchange column (Bio-RAD Macro-prep High Q) at 2 mL min^{-1} by using an AKTA Prime FPLC system (Amersham Pharmacia Biotech), initially with Tris-HCl buffer (40 mL) and then a linear gradient of 0 to 0.35 M KCl in Tris-HCl buffer (50 mM, pH 7.8) to a final volume of 80 mL. The fractions containing SAM synthase were concentrated to 3 mL by using a 10 kDa Macrosep (Pall Folton) centrifugal concentrator.

HPLC conditions for the SAM synthase/5′-FDAS assay and purification for 5′-fluoro-5′-deoxyadenosine (5): Partially purified SAM synthetase fractions (150 μL) were incubated at 37 °C for 6 h with ATP (20 mM, 50 μL), L-methionine (100 mM, 25 μL), $MgSO_4$ (1 M, 50 μL) and KCl (1 M, 25 μL) in final volume of 0.2 mL. The over-expressed 5′-FDAS solution (3 mg mL^{-1}, 500 μL) and KF (2 M, 200 μL) were then added, and the coupled enzyme reaction left at 37 °C for 12 h. Aliquots for HPLC analyses were treated at 95 °C (3 min), and the precipitated protein was removed by centrifugation. An aliquot (20 μL) of the clear supernatant was injected onto a Hypersil 5 μM C-18 column (250×4.6 mm, Macherey–Nagel) equilibrated with KH_2PO_4 (50 mM) and acetonitrile (95:5 *v/v*). Runs were monitored by UV detection at 260 nm by gradient elution starting from KH_2PO_4 (50 mM) and acetonitrile (95:5 *v/v*) to a final mobile phase consisting of KH_2PO_4 (50 mM) and acetonitrile (80:20 *v/v*). Samples were introduced through a high-pressure injector fitted with a 100 μL loop, and the flow rate was maintained at 1.0 mL min^{-1} with a total elution time of 20 min.

The isotopically labelled sample of FDA derived from the enzymatic reactions was purified by semipreparative HPLC on a C-18 column (Phenomenex Hypersil 5, 250×10.00 mm, 5 μm) under the same conditions as described for analytical HPLC. Samples were introduced through a high-pressure injector fitted with a 1 mL loop, and the flow rate was maintained at 5.0 mL min^{-1} with a total elution time of 30 min.

Acknowledgements

We thank the BBSRC for a research grant and the EPSRC and the CNRS for joint funding through the CERC-3 programme on "Topological Chirality". We also thank Drs. Jonathan Spencer and Fung Lu Huang of Cambridge University Chemical Laboratory for cloning and over-expression of 5′-FDAS.

Keywords: bioorganic chemistry · fluorine · NMR spectroscopy · stereochemistry

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Received: November 28, 2003 [F 839]

