Cell-Free Biosynthesis of Fluoroacetate and 4-Fluorothreonine in *Streptomyces cattleya***

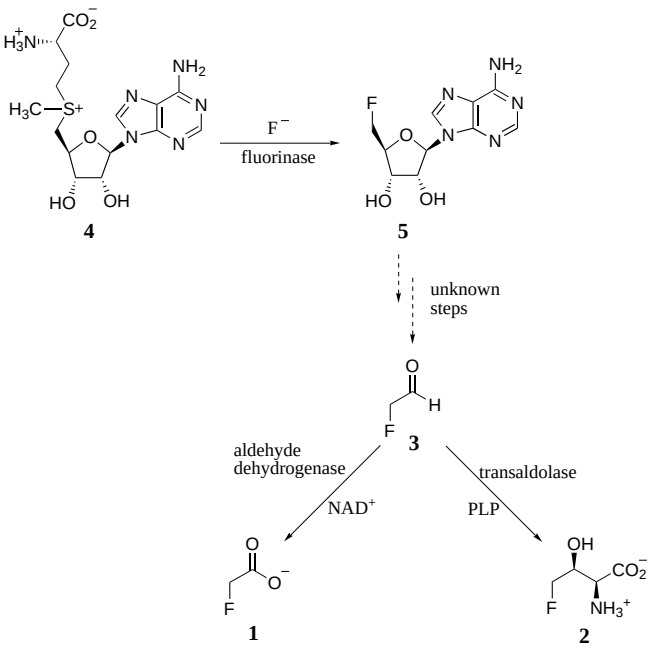
Christoph Schaffrath, Steven L. Cobb, and David O'Hagan*

Fluoroacetate **1** and 4-fluorothreonine **2** (Scheme 1) are secondary metabolites of the Actinomycete bacterium *Streptomyces cattleya*. These metabolites merit biosynthetic attention as they are among the very rare group of naturally occurring fluorine-containing compounds. It is perhaps surprising that Nature has hardly evolved the biochemistry of organofluorine metabolites, particularly as fluorine substitution can confer very particular properties such as toxicity, polarity, etc. to organic compounds. Accordingly, there are a huge number and range of commercially generated fluorinated compounds, but of course all of these are of anthropogenic origin. In this context, enzymatic syntheses of organofluorine compounds is interesting, not only at a mechanistic level but also since the prospects of biotransformation routes to this class of compound are attractive.

Fluoroacetate **1** is the most widely distributed fluorometabolite and is found as a toxin in a range of tropical and subtropical plants.[1] The identification of fluoroacetate **1** and also 4-fluorothreonine **2** from the bacterium *S. cattleya*[2,3] provided a more convenient investigative system than plants and could potentially provide further insight into fluorometabolite biosynthesis. We have recently identified the enzyme in *S. cattleya* that is responsible for C–F-bond formation.[4] This fluorination enzyme, the first of its class, mediates a reaction between an inorganic fluoride ion and (*S*)-adenosyl-L-methionine (SAM; **4**) to generate 5′-fluoro-5′-deoxyadenosine (**5**) (5′-FDA; Scheme 1). Also fluoroacetaldehyde (**3**) has been established as a late and common intermediate[5] in the formation of both fluoroacetate (**1**) and 4-fluorothreonine (**2**; Scheme 1) and each of the enzymes that converts **3** into **1** (an aldehyde dehydrogenase)[6] and **3** into **2** (a PLP transaldolase)[7] have been isolated and purified.

We report herein the cell-free biosynthesis of **1** from inorganic fluoride. The cell-free system has also been used to confirm 5′-FDA and fluoroacetaldehyde as genuine intermediates on the biosynthetic pathway to **1** and **2**.

The biotransformation of fluoride ions (10 mM) into fluoroacetate was carried out in an NMR tube with cell-free extracts of *S. cattleya*, supplemented with **4** (0.4 mM). The progress of the biosynthesis was monitored in real time by recording ^{19}F NMR spectra at one-hour intervals over 17 h at 25 °C. No such reactions took place without added cell-free extract. Over the course of the experiment, signals for three



Scheme 1. Enzymatic formation of 5′-FDA (**5**) from SAM (**4**) leading to the formation of fluoroacetaldehyde (**3**), the common precursor for fluoroacetate (**1**) and 4-fluorothreonine (**2**).

organofluorine species are apparent (Figure 1). The peak at −215 ppm is assigned to fluoroacetate **3**. The presence of fluoroacetate in the final mixture was confirmed independently by derivatization and GC/MS analysis.[8] The remaining two ^{19}F NMR signals A ($\delta = -229.4$ ppm) and B ($\delta = -229.7$ ppm) have identical multiplicities (doublets of quartets, $J = 47$ Hz and $J = 29$ Hz), which indicates that they have similar fluorine environments.

It is clear that the cell-free extract contains all the enzymes in an active form, required to convert fluoride ion into **1**. The biosynthesis is efficient with fluoroacetate, emerging after 1 h.

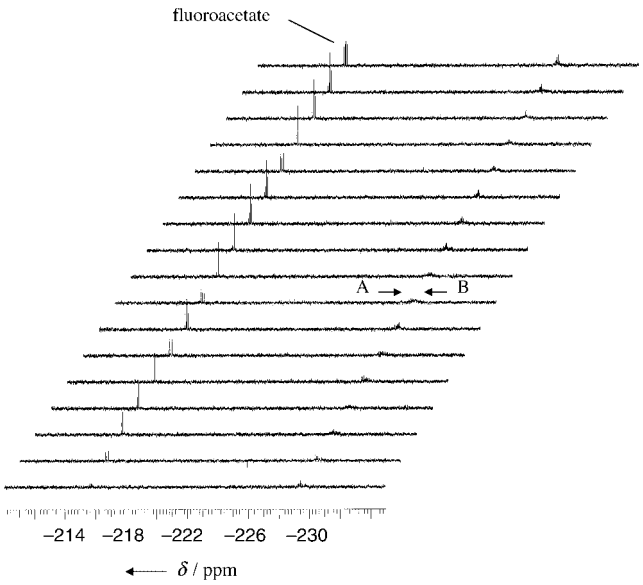


Figure 1. ^{19}F NMR spectra (recorded every hour) of fluorine-containing products generated in the *S. cattleya* cell-free extract when incubated with SAM (0.4 mM) and fluoride ion (10 mM).

[*] Prof. Dr. D. O'Hagan, C. Schaffrath, S. L. Cobb
Centre for Biomolecular Sciences
School of Chemistry, University of St. Andrews
North Haugh, St. Andrews, Fife, KY16 9ST (UK)
Fax: (+44) 1334-463-808
E-mail: do1@st-andrews.ac.uk

[**] This work was supported by the Engineering and Physical Science Research Council (SC) and the University of St. Andrews (CS)

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  0044-8249/02/4120-3913 $ 20.00+.50/0

The last step in fluoroacetate biosynthesis involves the action of an NAD^+-dependent aldehyde dehydrogenase, an enzyme that is inhibited by iodoacetamide.[6] When the cell-free experiments were carried out under a similar set of conditions but with added iodoacetamide (10 mM), fluoroacetate **1** no longer accumulated, which is consistent with the inhibition of the aldehyde dehydrogenase. The compounds that give rise to signals A and B accumulated as before, and signal A completely disappeared after 20 h, whereas signal B remained. The compound that gives rise to signal B was isolated by preparative HPLC and was analyzed both by ^1H and ^{19}F NMR spectroscopy as well as by MALDI-TOF mass spectrometry and GC-MS, after derivatization by *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). Based on these studies, signal B was assigned to 5′-fluoro-5′-deoxyinosine (**6**; 5′-FDI). 5′-FDI is presumably derived from a hydrolytic reaction of 5′-FDA (the product of the fluorination reaction) mediated by an adenosine deaminase.[9] The compound that gives rise to signal A could not be identified.

To confirm the role of 5′-FDA (**5**) as an intermediate in the biosynthetic pathway to fluoroacetate, it was necessary to prepare a sample of 5′-FDA for biotransformation studies. Accordingly, **5** was prepared in a three-step route starting from **7**. Mesylation[10] of the protected adenosine to generate **8** followed by treatment with tetrabutylammonium fluoride (TBAF) allowed fluorination at the 5′ position to give **9**. Deprotection under mild acidic conditions then gave the required 5′-FDA (**5**) as shown in Scheme 2**.**

The cell-free extract preparation of *S. cattleya* was then incubated with synthetic **5** (10 mM) and the in vitro biotransformation was monitored in real time by recording ^{19}F NMR spectra at one-hour intervals over 20 h at 25 °C (Figure 2).

The resultant ^{19}F NMR spectra shown in Figure 2 clearly indicates a number of organofluorine compounds derived from **5**. Fluoroacetate **1** (which gives rise to a triplet at $\delta = -215.8$ ppm) is obvious as before. Furthermore, the high-intensity signals between $\delta = -229$ ppm and -230 ppm correspond to added 5′-FDA **5** and its rapid conversion into 5′-FDI (**6**) by the action of an adenosine deaminase. Notably, 4-fluorothreonine (**2**) ($\delta = -230.1$ ppm) is evident, which indicates that all the activities and cofactors are present in the cell-free extract for its biosynthesis from 5′-FDA **5**. The presence of 4-fluorothreonine in the extract was independently confirmed by GC-MS analysis.[8] Perhaps the most surprising observation in this experiment is the presence of 2-fluoroethanol (**10**) ($\delta = -223$ ppm). The signal at $\delta = -234.3$ ppm in the ^{19}F NMR spectrum was assigned to a complex between **3** and tris(hydroxymethyl)aminoethane (**11**), a constituent of Tris buffer. Although the structure of this complex is ambiguous at present, control reactions with synthetic fluoroacetaldehyde at various pH values and with different buffers have confirmed that this ^{19}F NMR signal is identical to that observed when only fluoroacetaldehyde is dissolved in Tris buffer. In the cell-free extract, a complex between **3** and **11** accumulates and then disappears, thus indicating a reversible reaction, which presumably generates an imine or a cyclic aminal. Concomitantly, the concentration of fluoroethanol **10** steadily increases. It was previously established that fluoroethanol is not involved in the biosynthesis of the organofluorine compounds in *S. cattleya*.[11] It appears therefore that under these conditions **3** is generated from **5** and that there is an adventitious and irreversible bioconversion (alcohol dehydrogenase) of **3** into **10**, which shifts the equilibrium from the aldehyde–Tris complex to **10**.

There is another signal in the ^{19}F NMR spectrum at $\delta = -227$ ppm, which appears before the signal for **3** and accumulates for a short period (between 2–10 h), before disappearing. This unknown and transient compound may be an intermediate between 5′-FDA (**5**) and **3**.

In conclusion, we have described a successful bioconversion of fluoride ion and SAM (**4**) in a cell-free extract of *S. cattleya* to generate fluoroacetate **1**, which indicates that all the

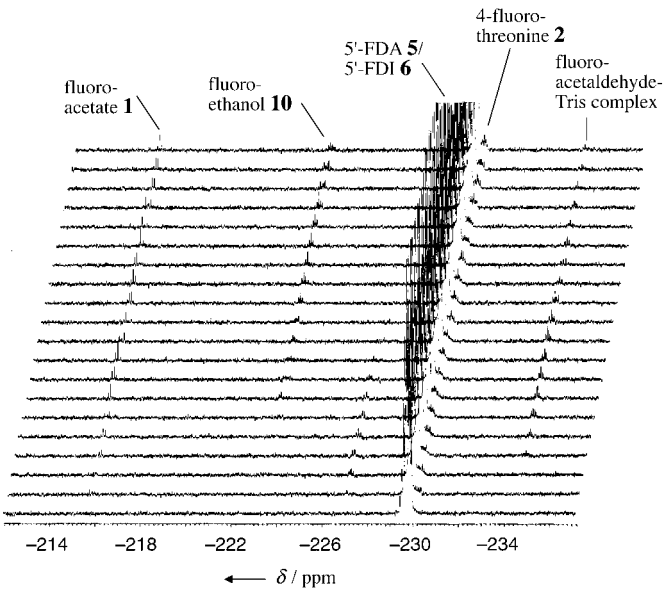
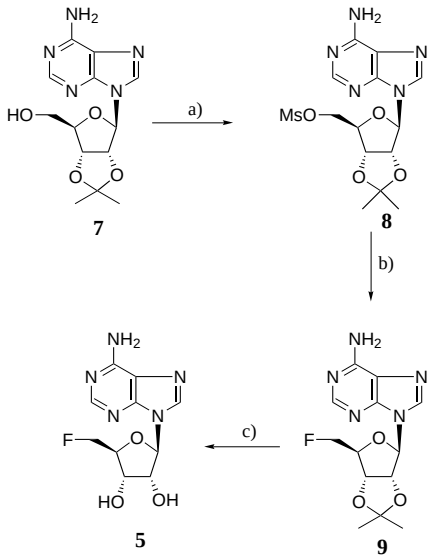


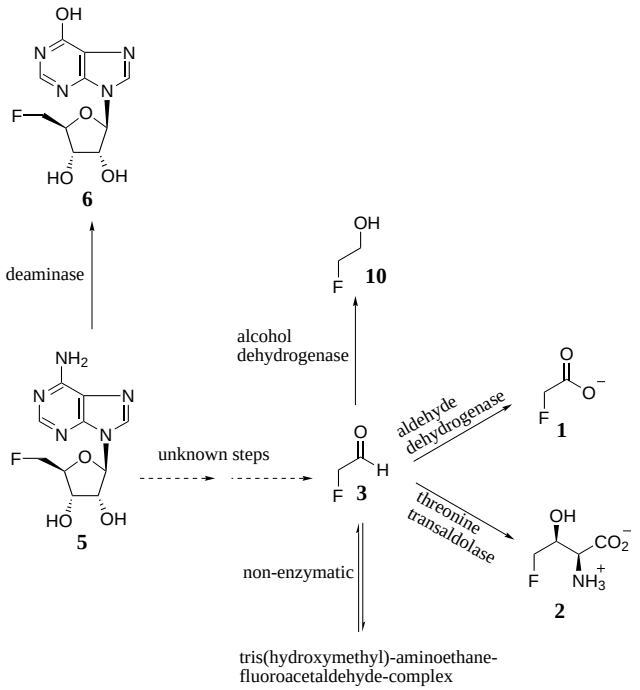
Figure 2. ^{19}F NMR spectra (recorded every hour) of fluorine-containing products generated in the *S. cattleya* cell-free extract when incubated with 5′-FDA (10 mM).



Scheme 2. Reagents and conditions: a) MsCl, pyridine, 25 °C, 20 h, 73%; b) TBAF (2.5 equiv), MeCN, reflux, 24 h, 46%; c) dilute H_2SO_4, 25 °C, 4 h, 70%.

  0044-8249/02/4120-3914 $ 20.00+.50/0 *Angew. Chem. Int. Ed.* **2002**, *41*, No. 20

enzymatic activities are present within the extract to mediate the entire biosynthesis. Incubation of a synthetic sample of 5′-FDA (**5**), the initial product of enzymatic fluorination, with the cell-free extract resulted in the accumulation of **3** (and **10**), thus confirming the role of **5** as a biosynthetic intermediate of **1** and **2**. An overview of this biotransformation in shown in Scheme 3. The accumulation of **3** in this case appears to have promoted the biosynthesis of **2** as well as **1**. A transient signal ($\delta = -227$ ppm) was also observed in the ^{19}F NMR spectrum in this experiment, which is presumably a metabolic intermediate between **5** and **3**. This compound has not yet been identified.



Scheme 3. Schematic representation of the bioconversions observed after incubating 5′-FDA with the *S. cattleya* cell-free extract.

Experimental Section

Streptomyces cattleya NRRL 8057 was grown in 500-mL flasks in a medium of the composition previously described.[3] After 6 days of incubation, the cells were harvested and washed with Tris buffer (50 mM, pH 7.8) and resuspended in the same buffer (0.1 g wet-cell weight mL^{-1}). Ultrasonication and centrifugation gave a supernatant that was used directly as the cell-free extract. Biotransformations were initiated by supplementation of SAM (0.4 mM) and NaF (10 mM) or by the addition of 5′-FDA (10 mM) to the cell-free extract, and the reactions were incubated at 28 °C for up to 20 h. For real-time analysis by ^{19}F NMR spectroscopy, the sample was prepared in the same manner but D_2O was added (100 μL). ^{19}F NMR spectra were recorded on a Varian Inova 500-MHz NMR spectrometer. GC-MS analyses were performed on an Agilent 6890 gas chromatograph connected to an Agilent 5973 mass-selective detector. The methods used for analysis were similar to those previously described.[8] Fluoroacetate was analyzed by GC-MS in the scan mode after preparation of its *p*-phenylphenacyl derivative.[8] MS (EI+): *m*/*z* (%): 272 (9.5), 182 (13.7), 181 (100), 152 (65.9). 4-Fluorothreonine (**2**) was analyzed as its per(trimethylsilylated) derivative (obtained by treatment with MSTFA) by GC-MS operated in the scan mode. MS (EI+): *m*/*z* (%): 236 (53.4), 218 (100), 147 (15.6), 128 (14.2), 100 (11.2).

9: White powder; m.p. 159–160 °C; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): $\delta =$ 1.40 (s, 3H; CH_3), 1.64 (s, 3H; CH_3), 4.52 (dm, 1H; 4′-H), 4.62 (ddd, $J =$ 46.7, 14.5, 10.4 Hz, 1H; 5′-H_a/5′-H_b), 4.63 (ddd, $J = 46.7$, 13.5, 10.4 Hz, 1H; 5′-H_a/5′-H_b), 5.10 (dd, $J(3'H,2'H) = 6.3$ Hz, $J(3'H,4'H) = 3.8$ Hz, 1H; 3′-H), 5.37 (dm, $J(2'H,3'H) = 6.3$ Hz, 1H; 2′-H), 5.68 (br s, 2H; NH_2), 6.19 (d, $J(1'H,2'H) = 1.93$ Hz, 1H; 1′-H), 7.93 (s, 1H; 2-H/8-H), 8.36 ppm (s, 1H; 2-H/8-H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): $\delta = 25.7$ (CH_3), 27.5 (CH_3), 81.1 (d, J(C3′, F) = 6.8 Hz; C3′), 83.3 (d, J(C5′,F) = 171.8 Hz; C5′), 84.9 (C′2), 85.9 (d, J(C4′,F) = 19.5 Hz; C4′), 91.3 (C′1), 115.0, 120.5, 139.7 (C2/C8), 149.8, 153.7 (C2/C8), 156.0 ppm; ^{19}F NMR (282.2 MHz, $CDCl_3$, 25 °C): $\delta =$ −228.86 ppm (dt, $J(F,4'H) = 23.6$ Hz, J(F, 5′H) = 46.7 Hz); MS (CI, CH_4): *m*/*z* (%): 310 (100) [M^++H], 290 (10) [−HF], 251 (8), 207 (5), 175 (7), 75 (40); HRMS (CI): calcd for $C_{13}H_{17}FN_5O_3$ [M+H$^+$]: 310.1315, found: 310.1321.

5: White powder, m.p 203–204 °C [lit.[12] 205–206 °C]; ^{1}H NMR (300 MHz, [D_6]DMSO, 25 °C): $\delta = 4.11$ (dm, $J(F,4'H) = 23.8$, 1H; 4′-H), 4.26 (m, 1H; 3′-H), 4.60 (m, 1H; 2′-H), 4.64 (dm, J(F, 5′CH_2) = 47.8, 2H; CH_2), 5.46 (br s, 1H; OH), 5.65 (br s, 1H; OH), 5.94 (d, $J(1'H,2'H) = 4.86$ Hz, 1H; 1′-H), 7.33 (br s, 2H; NH_2), 8.16 (s, 1H; 2-H/8-H), 8.27 ppm (s, 1H; 2-H/8-H); ^{13}C NMR (75 MHz, [D_6]DMSO, 25 °C); $\delta = 69.7$ (d, J(C3′,F) = 6.0 Hz), 73.4 (C′2), 82.6 (d, J(C4′,F) = 18.0 Hz), 83.3 (d, J(C5′,F) = 171.8 Hz), 88.0 (C1′), 119.4, 139.7, (C2/C8), 149.7, 153.1 (C2/C8), 156.4 ppm; ^{19}F NMR (282.2 MHz, [D_6]DMSO, 25 °C); $\delta = -230.0$ ppm (dt, $J(F,4'H) = 23.8$ Hz, $J(F,5'H) = 47.8$ Hz); MS (ES): *m*/*z* (%): 269.98 (100) [M^+].

Received: August 5, 2002 [Z19887]

[1] D. B. Harper, D. O'Hagan, *Nat. Prod. Rep.* **1994**, 123–133.
[2] M. Sanada, T. Miyano, S. Iwadare, J. M. Williamson, B. H. Arison, J. L. Smith, A. W. Douglas, J. M. Liesch, E. Inamine, *J. Antibiot.* **1984**, *39*, 259–265.
[3] K. A. Reid, J. T. G. Hamilton, R. D. Bowden, D. O'Hagan, L. Dasaradhi, M. R. Amin, D. B. Harper, *Microbiology* **1995**, *141*, 1385–1393.
[4] D. O'Hagan, C. Schaffrath, S. Cobb, J. T. G. Hamilton, C. D. Murphy, *Nature* **2002**, *416*, 279–279.
[5] S. J. Moss, C. D. Murphy, J. T. G. Hamilton, W. C. McRoberts, D. O'Hagan, C. Schaffrath, D. B. Harper, *Chem. Commun.* **2000**, 2281–2282.
[6] C. D. Murphy, S. J. Moss, D. O'Hagan, *Appl. Environ. Microbiol.* **2001**, *67*, 4919–4921.
[7] C. D. Murphy, D. O'Hagan, C. Schaffrath, *Angew. Chem.* **2001**, *113*, 4611–4613; *Angew. Chem. Int. Ed.* **2001**, *40*, 4479–4481.
[8] J. T. G. Hamilton, C. D. Murphy, M. R. Amin, D. O'Hagan, D. B. Harper, *J. Chem. Soc. Perkin Trans. 1* **1998**, 759–767.
[9] Z. Wang, F. A. Quiocho, *Biochemistry* **1998**, *37*, 8314–8324.
[10] D. Gani, A. W. Johnson, *J. Chem. Soc. Perkin Trans. 1* **1982**, 1197–1204.
[11] S. J. Moss, PhD thesis, University of Durham, **1999**.
[12] H. M. Kissman, M. J. Weiss, *J. Am Chem. Soc.* **1958**, *80*, 5559–5564.

  0044-8249/02/4120-3915 $ 20.00+.50/0