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The identification of (3*R*,4*S*)-5-fluoro-5-deoxy-D-ribulose-1-phosphate as an intermediate in fluorometabolite biosynthesis in *Streptomyces cattleya*

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Abstract

(3*R*,4*S*)-5-Fluoro-5-deoxy-D-ribulose-1-phosphate (5-FDRulP) has been identified as the third fluorinated intermediate on the biosynthetic pathway to fluoroacetate and 4-fluorothreonine in *Streptomyces cattleya*. 5-FDRulP is generated after formation of 5′-fluoro-5′-deoxyadenosine (5′-FDA) and then phosphorolysis of 5′-FDA to 5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FDRP) by the action of a purine nucleoside phosphorylase. An isomerase mediates the conversion of 5-FDRP to 5-FDRulP. The identity of the (3*R*,4*S*) diastereoisomer of 5-FDRulP was established by comparative $^{19}F\{^{1}H\}$ NMR studies whereby 5-FDRulP that accumulated in a cell free extract of *S. cattleya*, was treated with a phytase to generate the non-phosphorylated sugar, 5-fluoro-5-deoxy-D-ribulose (5-FDRul). This *S. cattleya* product was compared to the product of an *in-vitro* biotransformation where separately 5-fluoro-5-deoxy-D-ribose and 5-fluoro-5-deoxy-D-xylose were converted to 5-fluoro-5-deoxy-D-ribulose and 5-fluoro-5-deoxy-D-xylulose respectively by the action of glucose isomerase. It was demonstrated that 5-fluoro-5-deoxy-D-ribose gave the identical diastereoisomer to that observed from 5-FDRulP.
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1. Introduction

Streptomyces cattleya is a bacterium with the unusual capacity to elaborate organo-fluorine metabolites [1]. In 1986 it was noticed that supplementation of the fermentation medium with fluoride resulted in the simultaneous production of the toxin fluoroacetate (FAc)[1] **1** and the antibiotic 4-fluorothreonine (4-FT) **2** [2]. Due to the rare occurrence of fluorinated metabolites in Nature, but with a backdrop that fluorinated entities are a significant sector of the chemicals industry, it is of some interest to establish how this organism generates these metabolites, as this could reveal biotechnological prospects for fluorochemical production. In this regard we have been exploring fluorometabolite biosynthesis in *S. cattleya* and have been able to identify a number of enzymes on the pathway (see Scheme 1).

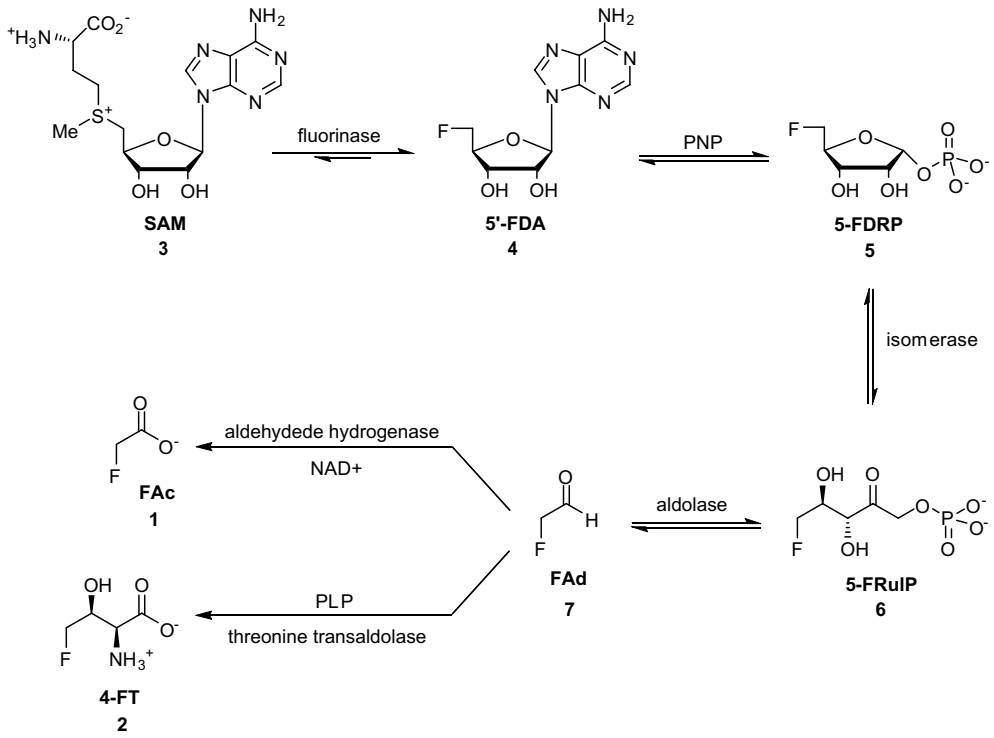
The first enzyme is 5′-fluoro-5′-deoxyadenosine synthase (fluorinase), the C–F bond forming enzyme which generates 5′-fluoro-5′-deoxyadenosine (5′-FDA) **4** from *S*-adenosyl-L-methionine (SAM) **3** and fluoride ion [3,4]. The product **4** is then acted upon by a purine nucleoside phosphorylase (PNP) which mediates a phosphorolytic displacement of the adenine base to generate 5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FDRP) **5** [5]. Further along the pathway, fluoroacetaldehyde (FAd) **7** has been identified as the last common intermediate to **1** and **2** [6]. An NAD^+ dependent fluoroacetaldehyde dehydrogenase has been purified and characterised [7] which oxidises FAd **7** to FAc **1**. Separately a pyridoxal phosphate (PLP) dependent enzyme mediates a transaldol reaction with L-threonine and **7** to generate 4-FT **2** and acetaldehyde, in a unique PLP dependent process [8]. With the early and later steps in fluorometabolite biosynthesis now relatively well understood, it became a focus to establish how 5-FDRP **5** is converted to fluoroacetaldehyde **7**. In this paper we describe the biotransformation of 5-FDRP **5** to 5-FDRulP **6**, which involves an isomerization. This suggests that the product of the isomerization, 5-FDRulP **6**, is converted to FAd **7** by the action of an aldolase enzyme, and then through to the fluorometabolites **1** and **2**.

2. Materials and methods

2.1. General

Streptomyces cattleya NRRL8057 was obtained from the Northen Utilization Research and Development Division, US Department of Agriculture, Peoria, Illinois.

[1] *Abbreviations*: FAc, fluoroacetate; 4-FT, 4-fluorothreonine; SAM, *S*-adenosyl-L-methionine; 5′-FDA, 5′-fluoro-5′-deoxyadenosine; 5-FDRP, 5-fluoro-5-deoxy-D-ribose-1-phosphate; 5-FDRulP, 5-fluoro-5-deoxy-D-ribulose-1-phosphate; FAd, fluoroacetaldehyde; PNP, purine nucleoside phosphorilase; NAD(H), oxidased or reduced nicotinamide adenine dinucleotide; PLP, pyridoxal phosphate; DHAP, dihydroxyacetone phosphate; 5′-FDI, 5′-fluoro-5′-deoxyinosine; CFE, cell free extract; EDTA, ethylenediaminetetraacetic acid; 5-FDR, 5-fluoro-5-deoxy-D-ribose; MSTFA, *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide; 5-FDRul, 5-fluoro-5-deoxyribulose; 5-FDX, 5-fluoro-5-deoxy-D-xylose; 5-FDXul, 5-fluoro-5-deoxy-D-xylulose.



Scheme 1. Biosynthetic pathway to fluorometabolites **1** and **2** in *Streptomyces cattleya*.

All microbiological work was carried out under sterile conditions in a Gallenkamp flowhood. Glassware, media and consumables were sterilised by autoclaving. Cultures were incubated in a temperature controlled Gallenkamp orbital incubator and resting cells on a temperature controlled Innova 2000 platform shaker. Centrifugation was carried out on a Beckman Avanti J-25 centrifuge at 14,000 rpm. For microcentrifugation (50–1500 μl), Eppendorf 5415 C centrifuge was used. Sonication was performed Sonics & Materials Inc., Vibra Cell. Lyophilisation was carried out on an FTS, flexi-dry freeze dryer.

All work described involving cells, cell-free extract or protein solutions was performed at 4 °C if not otherwise stated.

Reagents and enzymes were purchased from either Sigma, Fluka or Acros. The details for the enzymes are as follows: purine nucleoside phosphorylase (PNP, EC 2.4.2.1, unknown bacterial source, N-8264, 15.6 units/mg), 5′-adenylic acid deaminase (EC 3.5.4.6, from *Aspergillus* species, A 1907, 0.11 units/mg), phytase (EC 3.1.3.8, from *Aspergillus ficuum*, P 9792, 3.5 units/mg), and glucose isomerase (Sweetzyme T, from *Streptomyces murinus*, G-4166, 350 units/mg).

NMR analyses were performed on a Bruker AC 300, Bruker Avance 500 MHz or a Varian Inova 500 MHz spectrometers. NMR spectra were recorded in $CDCl_3$ or D_2O for preparative sample analyses and in D_2O (~10%) for biological analyses. Chemical shifts are given in parts per million (ppm) and coupling constants J are given in Hertz (Hz).

GC–MS analysis was performed on a Hewlett Packard 5890 gas chromatograph linked to an HP 5970B mass selective detector controlled by an HP300 series computer. The gas

chromatograph was fitted with an autosampler and equipped with an Ultra 1 fused-silica wall-coated open tubular capillary column (12×0.2 mm $\times$ 0.33 μm) with 5% biphenyl, 95% dimethyl polysiloxane as the bonded phase.

2.2. Chemical syntheses

2.2.1. Preparation of 5′-FDA (**4**), 5-FDR (**14**) and 5-FDX (**16**)

Detailed synthetic routes to the compounds name above have been reported earlier [9–11].

2.3. Biochemical transformations

2.3.1. Preparation of 5′-FDI (**13**) and 5-FDRP (**5**)

Detailed procedures to **13** and **5** have been previously described by our group [5].

2.3.2. Preparation of 5-FDRul (**15**)

Immobilised glucose isomerase (30 mg) was added to an aqueous solution of 5-FDR **14** (1 ml, 5 mg/ml KH_2PO_4 buffer, 50 mM, pH 6.8). Samples were incubated at 37 °C for 6 h, following the reaction by ^{19}F NMR every hour. Samples (100 μl) of the reaction mixture were taken every hour and the protein was denatured by heating (100 °C, 3 min) and removed by centrifugation (14,000 rpm, 15 min). The supernatant was then analysed by ^{19}F NMR to reveal the production of 5-FDRul **15**. δ_F (470 MHz, D_2O): −231.22 (dt, $^2J_{F-H}$ 46.9 and $^3J_{F-H}$ 20.5).

2.3.3. Preparation of 5-FDXul (**17**)

Immobilised glucose isomerase (30 mg) was added to an aqueous solution of 5-FDX **16** (1 ml, 5 mg/ml KH_2PO_4 buffer, 50 mM, pH 6.8). Samples were incubated at 37 °C for 3 h, following the reaction by ^{19}F NMR every hour. Samples (100 μl) of the reaction mixture were taken every hour and the protein was denatured by heating (100 °C, 3 min) and removed by centrifugation (14,000 rpm, 15 min). The supernatant was then analysed by ^{19}F NMR to reveal the production of 5-FDXul **17**. δ_F (470 MHz, D_2O): −228.55 (dt, $^2J_{F-H}$ 46.1 and $^3J_{F-H}$ 15.4).

2.4. Preparation of S. cattleya cell free extract

Culture medium and growth conditions for the bacterium, as well as the procedure to prepare a cell free extract, were followed as previously described [5].

2.5. Incubations in cell free extract of S. cattleya

2.5.1. Preparation of 5-fluoro-5-deoxy-D-ribulose-1-phosphate (**6**)

A stock solution of 5-FDRP **5** was prepared by incubating 5′-FDI **13** (5 mg/ml) with an immobilised PNP (30 mg) enzyme until the best conversion was observed (approx. 40% conversion to 5-FDRP **5**) [5].

Samples were prepared using the following protocol: *S. cattleya* CFE (400 μl, 0.1 g cells/ml) was pre-incubated with EDTA (30 mM) for 30 min. 5-FDRP **5** stock solution (100 μl) was then added and the incubation continued for 16 h at 37 °C. The protein was denatured by heating (100 °C, 3 min) and removed by centrifugation (14,000 rpm,

15 min). The supernatant was then analysed by ^{19}F NMR, and after lyophilisation and derivatisation with MSTFA was also analysed by GC–MS. Selected spectroscopic data for **6**: δ_F (470 MHz, D_2O): −231.34 (dt, $^2J_{F-H}$ 47.0 and $^3J_{F-H}$ 20.9). GC–MS data for per-trimethylsilyl derivative of **6**: *m/z* (%): 521 ($M+H^-$, 100), 506 ($M^- - CH_3$, 52).

2.5.2. Preparation of 5-fluoro-5-deoxy-D-ribulose (*15*)

Phytase (100 μl, 10 mg/ml) was added to a sample of 5-FDRulP **6** (500 μl) prepared using the protocol described above. The sample was incubated at 37 °C for 16 h. The protein was then denatured by heating (100 °C, 3 min) and removed by centrifugation (14,000 rpm, 15 min). The supernatant was then analysed by ^{19}F NMR. Selected spectroscopic data for **15**: δ_F (470 MHz, D_2O): −231.22 (dt, $^2J_{F-H}$ 46.9 and $^3J_{F-H}$ 20.2).

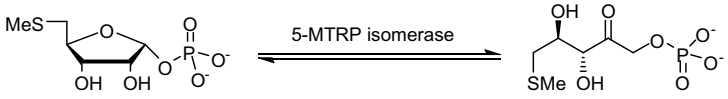
2.6. GC–MS Determination of the persilylated derivative of 5-fluoro-5-deoxy-D-ribulose-1-phosphate (*6*)

Using the method described by Hamilton et al. [12], lyophilised supernatant (1 ml) of 5-FDRulP **6** was derivatised by the addition of *N*-trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA) (1 ml) and then heating to 100 °C for 1 h. After cooling to room temperature, the samples were passed through a Pasteur pipette containing a cotton wool plug and analysed by GC–MS under the following conditions. The oven was programmed to start at 100 °C for 1 min and then ramped at 10 °C/min to 300 °C. The injector port temperature was set at 250 °C and the sample (1 μl) injected splitless. The mass spectrometer was operated in the full scan electron impact mode measuring ion currents between 30 and 550 amu to obtain the mass spectra of fluororibulose phosphate. The molecular weight of the persilylated 5-FDRulP was confirmed using chemical ionization (−ve ion mode) mass spectrometry with methane as the reagent gas.

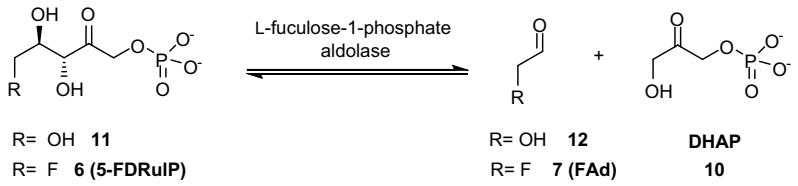
3. Results and discussion

The intermediate 5-FDRP **5** is already established as the second fluorinated intermediate on the fluorometabolite biosynthetic pathway [5]. During the L-methionine salvage pathway in SAM **3** metabolism, Abeles and co-workers have shown that 5-*S*-methyl-5-thio-D-ribose-1-phosphate **8** undergoes an isomerization to 5-methylthioribulose phosphate **9** in humans [13,14] (Scheme 2).

A number of 5-*S*-methyl-5-thioribose-1-phosphate (5-MTRP) isomerases have since been purified from yeast and bacteria [15,16] after gene cloning and the yeast structure has been solved [15]. However, the mechanism of this reaction remains to be elucidated. This isomerase offered a model in metabolism as to what might be occurring during the



Scheme 2. Conversion of 5-MTRP **8** to 5-MTRulP **9** by 5-methylthio-D-ribose-1-phosphate isomerase.



Scheme 3. A L-Fuculose-1-phosphate aldolase could catalyse the retro-aldol reaction generating FAd **7** and DHAP **10** from 5-FDRulP **6**.

fluorometabolite pathway in *S. cattleya*, where F replaces the MeS substituent at C-5. An analogous transformation on 5-FDRP **5** would generate 5-FDRulP **6**. Such a transformation would offer a convenient route to fluoroacetaldehyde **7**, as the ribulose phosphates are classical products/substrates of aldolase enzymes, acting on dihydroxyacetone phosphate (DHAP) **10** and various aldehydes [17]. Thus the reverse reaction would generate DHAP **10** and FAd **7**, which is established as a biosynthetic intermediate on this pathway [6] (Scheme 3).

In order to test this hypothesis and establish how 5-FDRP **5** is metabolised in *S. cattleya*, it was necessary to prepare a sample of this intermediate for incubation studies with cell free extracts of the bacterium. This was achieved by incubation of 5′-fluoro-5′-deoxyinosine (5′-FDI) **13**, itself prepared by adenosine deaminase treatment of 5′-FDA **4** [9] and

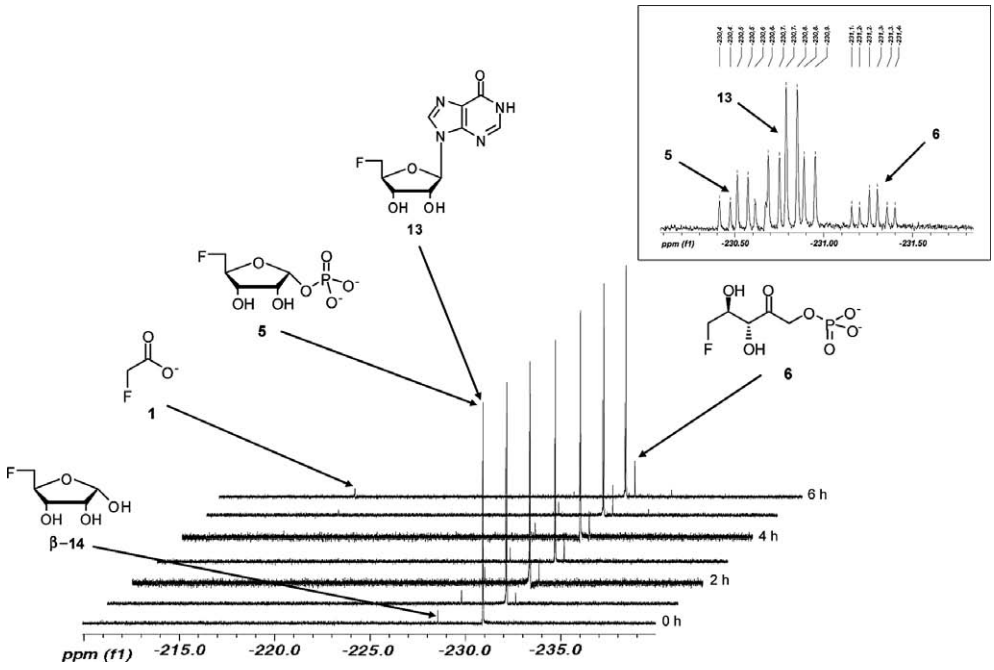


Fig. 1. $^{19}F\{^{1}H\}$ NMR time course of a CFE of *S. cattleya* incubated with 5-FDRP **5**/5′-FDI **13** (5-fluoro-5-deoxy-D-ribose (5-FDR) **14** residual in control sample due to phytase activity present in commercial PNP. However, **14** does not support fluorometabolite biosynthesis in *S. cattleya*, as previously reported [5].).

then a purine nucleoside phosphorylase (EC 2.4.2.1), as previously described [5]. This biotransformation generated a sample of 5-FDRP **5**, contaminated with residual 5′-FDI **13**. It is already established that **13** does not support fluorometabolite biosynthesis in cell free incubations of *S. cattleya* [18]. Incubation of the 5-FDRP **5**/5′-FDI **13** product mixture with the *S. cattleya* cell free extract could be followed by $^{19}F\{^{1}H\}$ NMR and a time course over 6 h is shown in Fig. 1. Fluoroacetate **1** was generated, consistent with the intermediacy of 5-FDRP **5** as previously reported [5].

However when EDTA (30 mM) and the 5-FDRP **5**/5′-FDI **13** mix were added to the cell free extract in an otherwise identical experiment, the production of FAc **1** was arrested and this led to the accumulation of a new fluorometabolite clearly evident by $^{19}F\{^{1}H\}$ NMR (−231.34 ppm, dt, $^{2}J_{F-H}$ 47.0 Hz and $^{3}J_{F-H}$ 20.9 Hz) as shown in Fig. 2. EDTA is a well known chelator of metal ions such as Zn^{2+} and it appears to inhibit a zinc dependent aldolase in the cell free extract, operating later on in the pathway.

This product mixture was analysed by GC–MS after derivatisation with MSTFA following the method described by Hamilton et al. [12] to generate the persilylated ribulose phosphate derivative. Control experiments with GC–MS (CI −ve ion mode) were carried out on the substrate and the product mixtures (as their persilylated derivates, see Fig. 3), and despite the identical molecular masses for these isomers, the product 5-FDRulP **6** was clearly distinguished from the incubated 5-FDRP **5** by very different retention times. A molecular ion on 521 amu was the predominant ion and a mass ion at 283 amu indicated the location of the phosphate at the C-1 position (Fig. 3).

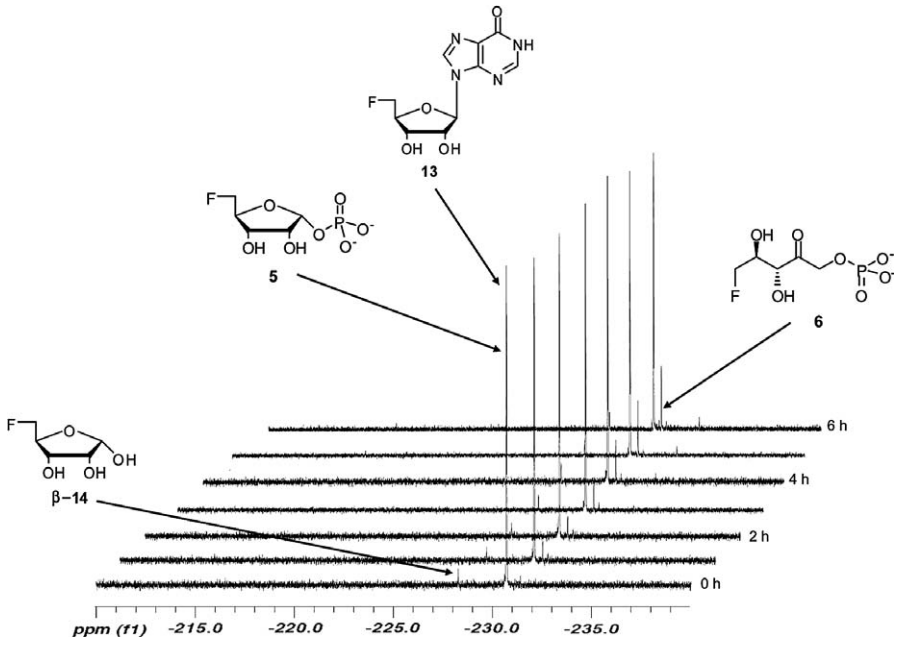


Fig. 2. $^{19}F\{^{1}H\}$ NMR time course of a cell free extract of *S. cattleya* incubated with 5-FDRP **5** and EDTA (30 mM) at 37 °C for 6 h (5-fluoro-5-deoxy-D-ribose (5-FDR) **14** residual in control sample due to phytase activity present in commercial PNP. However, **14** does not support fluorometabolite biosynthesis in *S. cattleya*, as previously reported [5].).

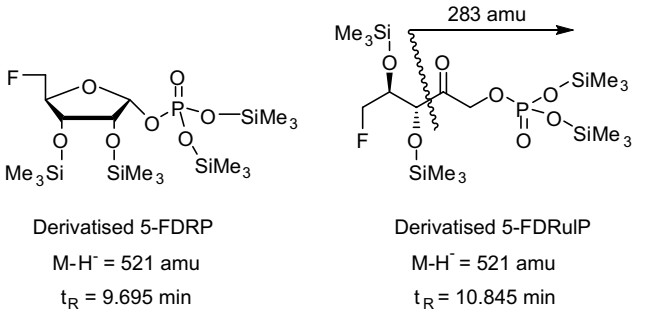
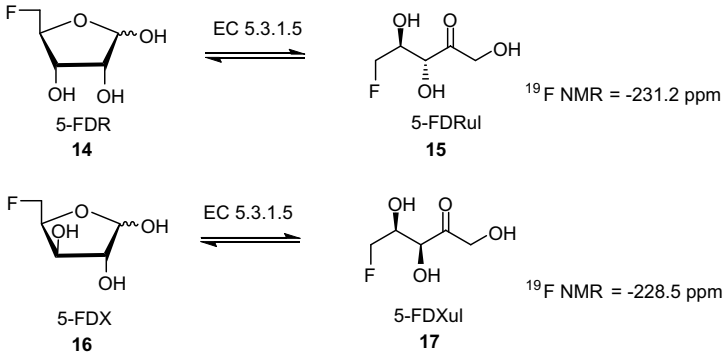


Fig. 3. Silyl derivatives analysed by GC–MS (CI) of 5-FDRP **5** and 5-FDRulP **6** after treatment with MSTFA.



Scheme 4. Immobilised glucose isomerase (Sweetzyme T, EC 5.3.1.5) can convert D-furanoses modified at C-5 into the corresponding open-chain 2-ketoses.

To further secure the identity of 5-FDRulP **6** as a product, a commercial phosphatase (EC 3.1.3.8, from *A. ficuum*) was added at the end of the cell free extract incubation. This resulted in an immediate change of the ^{19}F NMR chemical shift of the fluorinated metabolite **6** from −231.34 ppm to −231.22 ppm, indicating that a chemical transformation had occurred, consistent with phosphate cleavage. Thus this set of experiments supported the conversion of 5-FDRP **5** to 5-FDRulP **6**, and then to the free sugar 5-fluoro-5-deoxy-D-ribulose (5-FDRul) **15** by the action of the added phosphatase. For further confirmation, a sample of 5-fluoro-5-deoxy-D-ribose (FDR) **14** was prepared independently as a reference compound to cross reference the product from the sequence of biotransformations described above. Open-chain 2-keto sugars have been synthesised from glucose isomerase catalysed isomerisation of the corresponding D-ribo- and D-xylofuranoses as shown in Scheme 4 [19,20].

Therefore, a sterochemically pure sample of 5-FDR **14** was prepared by synthesis as previously described [10] and this compound was incubated with immobilised glucose isomerase (EC 5.3.1.5, Sweetzyme T from *S. murinus*). The reaction was monitored by ^{19}F{^{1}H} NMR and a time course is illustrated in Fig. 4.

The two anomers of the 5-FDR **14** starting material, (β-anomer: −228.53 ppm, dt, $^{2}J_{F–H}$46.5 Hz and $^{3}J_{F–H}$ 26.0 Hz; α-anomer: −230.83 ppm, dt, $^{2}J_{F–H}$ 46.5 Hz and $^{3}J_{F–H}$ 27.9 Hz) dominated at the outset and over time the 5-FDRul **15** product signal at

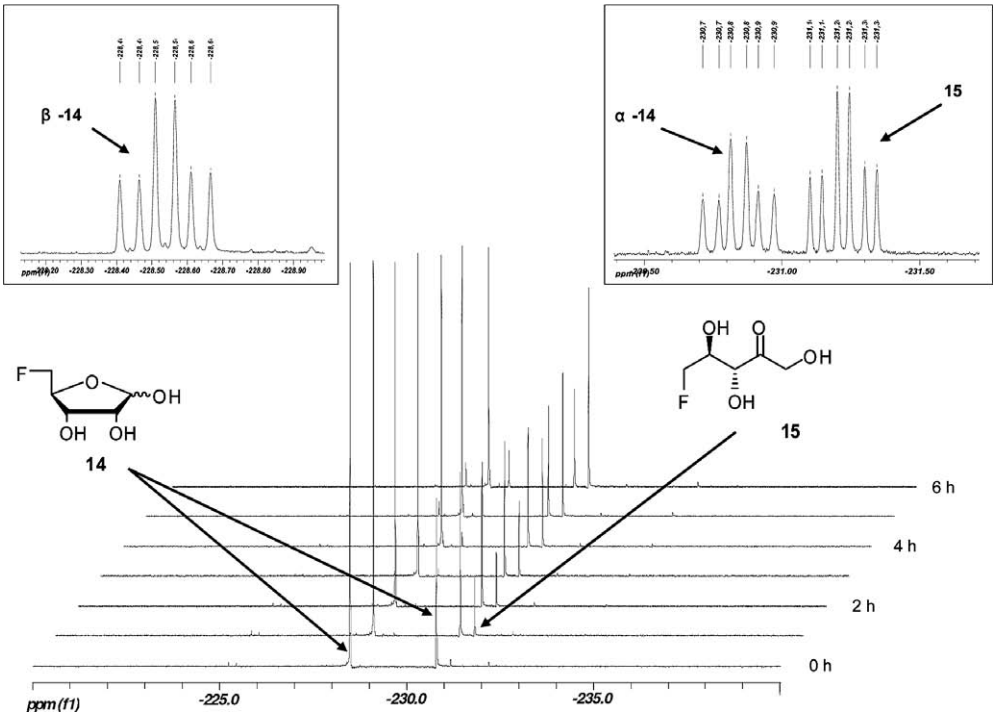


Fig. 4. $^{19}F\{^{1}H\}$ NMR time course of 5-FDR **14** (4.7 mM, 37 °C) incubated with a commercial immobilised glucose isomerase. Insets are ^{19}F NMR expansions of the product peaks without $\{^{1}H\}$ decoupling (Traces of product 5-FDRul **15** are already detected by $^{19}F\{^{1}H\}$ NMR in the 0 h sample due to the running time of the experiment (approx. 30 min).).

−231.22 ppm (dt, $^{2}J_{F-H}$ 46.9 Hz and $^{3}J_{F-H}$ 20.5 Hz) accumulates. Incubation times longer than 6 h did not result in further accumulation of product due to the reversible reaction reaching an equilibrium.

Notably the ^{19}F NMR product peak at −231.22 ppm for **15** has an identical chemical shift and coupling pattern to the product observed after incubating 5-FDRP **5** and EDTA with the cell free extract of *S. cattleya*, followed by phosphatase treatment.

Co-addition of the glucose isomerase reaction product to that of the EDTA-cell free extract/phosphatase reaction gave identical products as judged by ^{19}F NMR as shown in Fig. 5.

For comparison, a sample of 5-fluoro-5-deoxy-D-xylose **16** (5-FDX) was also prepared by synthesis in a steroespecific manner as previously described [11,20]. Incubation of this furanose isomer with glucose isomerase gave a different product as analysed by $^{19}F\{^{1}H\}$ NMR (−228.55 ppm), which does not correlate with the product of the cell free extract plus phytase treatment. Thus we conclude that the product of the cell free extract is indeed diastereoisomer **15**, as expected, and not diastereoisomer **17**.

Taken together this set of complementary biotransformations secures 5-FDRulP **6** as an intermediate formed immediately after 5-FDRP **5** on the pathway to the secondary fluorometabolites FAc **1** and 4-FT **2** in *S. cattleya*. The reaction involves an isomerization, which finds precedent in the isomeration of the methylthiosugars shown in

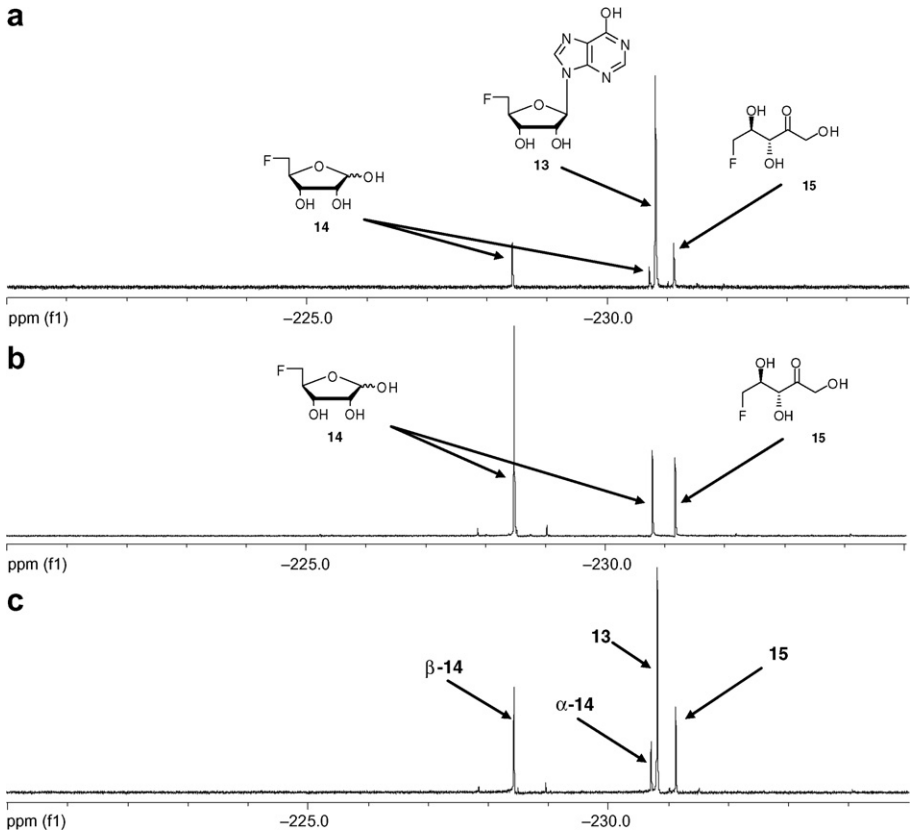


Fig. 5. $^{19}F\{^{1}H\}$ NMR spectra of : (a) *S. cattleya* cell free extract incubation of 5-FDRP **5**/5′-FDI **13** and subsequent treatment with phosphatase. (b) 5-FDRul **15** generated by incubation of synthetic 5-FDR **14** with glucose isomerase. (c) Co-addition of these samples indicating that the $^{19}F\{^{1}H\}$ NMR signal for **15** perfectly overlaps.

Scheme 2, an enzyme of the methionine salvage pathway. Recently a 10 kb region of the genes directly adjacent to the fluorinase gene on the *S. cattleya* genome have been sequenced, and approximately 10 genes were identified [21]. It is interesting that the fluorinase gene is sitting adjacent to the gene expressing the PNP enzyme, the second enzyme on the pathway that generates 5-FDRP **5**. Although a fluoroacetate resistance gene and some other fluorometabolite management genes were identified, no other biosynthetic genes are obvious in the cluster. There is no candidate isomerase gene. The absence of an obvious isomerase gene in this cluster leaves open the possibility that 5-FDRP **5** is converted to 5-FDRulP **6** by an enzyme of primary metabolism, and it is perhaps a substrate for the *S. cattleya* methylthioribose isomerase. This prospect is currently under investigation.

4. Conclusions

This study has identified 5-FDRulP **6** as an intermediate in fluorometabolite biosynthesis in *S. cattley*a, and that it is generated directly from 5-FDRP **5** by the action of an

isomerase. This was demonstrated by ^{19}F NMR and mass spectrometry, and the structure was further confirmed, after dephosphorylation by cross reference to 5-fluoro-5-deoxyribulose **15**. It was also demonstrated that 5-FDRulP **6** can support fluorometabolite biosynthesis in a cell free extract of *S. cattleya*, and that this process is arrested by the addition of EDTA. This latter observation is consistent with a pathway whereby 5-FDRulP **6** is converted to fluoroacetaldehyde **7** by the action of a metal dependent (Zn^{2+}) aldolase to generate **7** and DHAP **10**.

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