Bioorganic Chemistry 64 (2016) 37–41

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Exploration of a potential difluoromethyl-nucleoside substrate with the fluorinase enzyme

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ARTICLE INFO

Article history:
Received 7 October 2015
Revised 15 November 2015
Accepted 17 November 2015
Available online 18 November 2015

Keywords:
Fluorinase
Difluoromethyl
Isothermal titration calorimetry
Protein crystallography

ABSTRACT

The investigation of a difluoromethyl-bearing nucleoside with the fluorinase enzyme is described. 5′,5′-Difluoro-5′-deoxyadenosine **7** (F_2DA) was synthesised from adenosine, and found to bind to the fluorinase enzyme by isothermal titration calorimetry with similar affinity compared to 5′-fluoro-5′-deoxyadenosine **2** (FDA), the natural product of the enzymatic reaction. F_2DA **7** was found, however, not to undergo the enzyme catalysed reaction with L-selenomethionine, unlike FDA **2**, which undergoes reaction with L-selenomethionine to generate *Se*-adenosylselenomethionine. A co-crystal structure of the fluorinase and F_2DA **7** and tartrate was solved to 1.8 Å, and revealed that the difluoromethyl group bridges interactions known to be essential for activation of the single fluorine in FDA **2**. An unusual hydrogen bonding interaction between the hydrogen of the difluoromethyl group and one of the hydroxyl oxygens of the tartrate ligand was also observed. The bridging interactions, coupled with the inherently stronger C–F bond in the difluoromethyl group, offers an explanation for why no reaction is observed.

1. Introduction

The fluorinase enzyme, isolated originally from the soil bacterium *Streptomyces cattleya* [1] and recently identified in additional bacterial species [2], catalyses formation of a C–F bond from fluoride ion and *S*-adenosylmethionine **1** (SAM), generating 5′-fluoro-5′-deoxyadenosine **2** (FDA) (Scheme 1). The reaction has been shown to proceed by an inversion of configuration [3,4], and QM/MM calculations provide strong support for an S_N2 reaction mechanism [5]. The fluorination reaction is reversible, and the enzyme has the capacity to produce SAM **1** and fluoride ion when incubated with FDA **2** and L-methionine [6]. The fluorinase is of interest to biotechnology through its use as a biocatalyst for the introduction of fluorine into organic molecules, and this ability has been exploited using [^{18}F]fluoride for the synthesis of PET radiotracers [7–10]. The difluoromethyl and fluoromethyl groups remain comparatively unexplored in terms of their application to bioactive molecules [11]. Enzymatic synthesis of the fluoromethyl group (R–CH_2F) has been extensively explored [12], and we were interested in exploiting the ability of the fluorinase to activate fluoride ion in water, to extend fluorinase biocatalysis to the synthesis of a difluoromethyl group (R–CF_2H) from an appropriate substrate.

The difluoromethyl group is of interest for introduction into bioactive molecules as it bears a hydrogen atom which is available as a hydrogen bond donor, although a more lipophilic hydrogen bond donor than the more common hydrophilic O–H and N–H groups [13]. The lipophilic hydrogen bonding capacity of the difluoromethyl group has led to its development as a bio-isostere of alcohols [14], thiols [15] and hydroxamic acids [16]. Routes to access the difluoromethyl motif involve reaction of an aldehyde with sulfur tetrafluoride [17], reaction of nucleophilic "RCF_2^-" species with electrophiles [18–21], reaction of a nucleophilic C, S, N, or O nucleophiles with difluorocarbene [22–26], or transfer of a difluoromethyl radical to an appropriate substrate [27–29].

To explore the enzymatic synthesis of a difluoromethyl group, an appropriate substrate was required. To investigate the putative transformation in the forward direction, an *S*-adenosylmethionine analogue bearing a fluorine atom at the 5′-position would be required, however such 5′-modified SAM derivatives have not been reported. Simple α-fluorosulfonium species, including (fluoromethyl)dimethylsulfonium tetrafluoroborate [30], and diaryl fluoromethylsulfonium salts [31] have been investigated as electrophiles, suggesting that the motif may be susceptible to attack by a suitably activated fluoride nucleophile. While the literature reports of the α-fluorosulfonium motif suggest that such a fluorinated SAM derivative may be thermodynamically stable, synthesis of this highly functionalised substrate would not be trivial.

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http://dx.doi.org/10.1016/j.bioorg.2015.11.003
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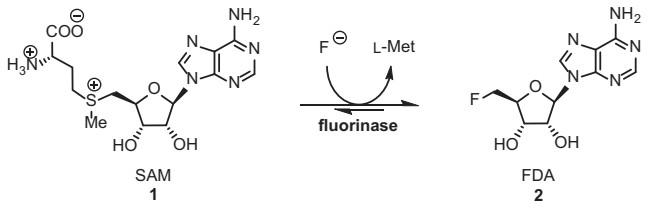


Scheme 1. The reversible synthesis of a C–F bond from fluoride and *S*-adenosylmethionine **1** (SAM) by the fluorinase enzyme.

We envisioned utilising the fluorinase-catalysed reaction in the reverse direction, where incubation of a difluoromethylated substrate (F_2DA **7**) was proposed to generate a fluoro–SeSAM intermediate upon reaction with L-selenomethionine [6] as the incoming nucleophile. In this paper, we describe a modified synthesis of F_2DA **7**, and an exploration of this putative substrate for selective substitution of one of the fluorine atoms of a difluoromethyl group, attempting to capitalise on the fluoride-activating properties of the fluorinase enzyme.

2. Results and discussion

2.1. Synthesis of F_2DA 7

The synthesis of 5′,5′-difluoro-5′-deoxyadenosine **7** has been previously reported by Jarvi et al. [32,33], where fluorination of *N*,*N*-dibenzoyl-2′,3′-*O*-isopropylidneadenosine-5′-aldehyde **5** with diethylaminosulfur trifluoride (DAST) was the key fluorination step. In a modified version of their synthesis, adenosine **3** was protected as its 2′,3′-acetonide with 2,2-dimethoxypropane in acetone, catalysed by perchloric acid, as shown in Scheme 2. The resultant acetonide was dibenzoylated in excellent yield with TMSCl, and an excess of benzoyl chloride to furnish **4**. Oxidation of **4** under Moffatt conditions, but substituting DCC for EDCI.HCl, gave excellent conversion of the alcohol to a mixture of aldehyde **5** and its hydrate, without the requirement for trapping as the aminal as reported by Jarvi et al. [32,33]. Azeotropic removal of water by repeated co-evaporation with toluene furnished aldehyde **5** in quantitative yield, which was used without further purification.

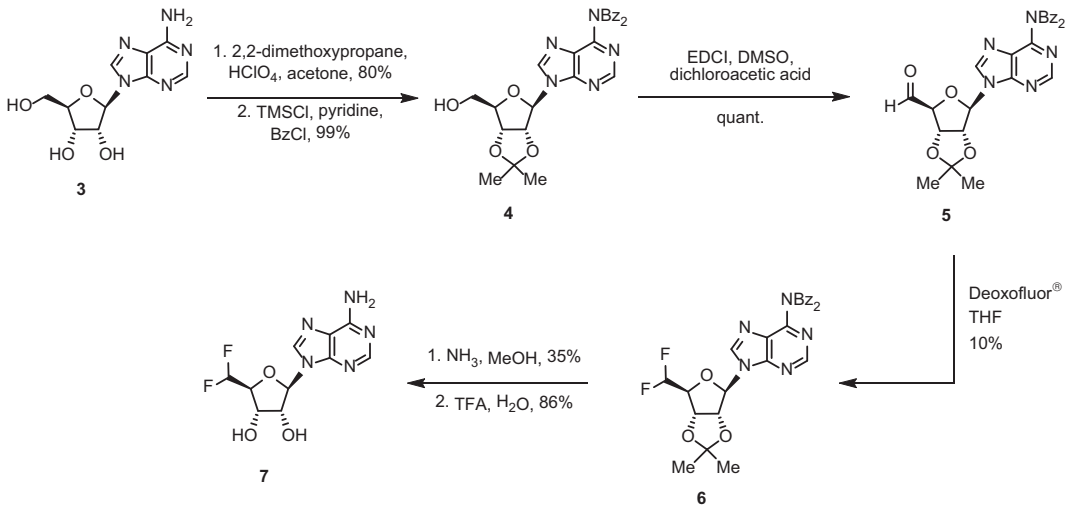
Jarvi et al. [32,33] report a low yield for the fluorination of **5** (18%) using DAST in DCM, however, we found that use of the reported conditions led to the formation of multiple fluorinated by-products. After optimisation of the reaction conditions, fluorination of aldehyde **5** with Deoxofluor® in THF furnished **6** with fewer fluorinated by-products, ultimately offering **6** in 10% yield after purification. With protected F_2DA **7** in hand, the benzoyl groups were cleaved by reaction of **6** with a freshly saturated solution of ammonia in methanol, in a sealed tube at 60 °C for 16 h. After isolation, the resultant acetonide was hydrolysed in a mixture of TFA and water, to provide F_2DA **7** as a colourless powder, in good yield. With a sample of F_2DA **7** in hand, we set out to explore its interaction with the fluorinase enzyme.

2.2. Isothermal titration calorimetry

The binding of F_2DA **7** to the fluorinase was investigated using isothermal titration calorimetry (ITC). Accordingly, a solution of F_2DA **7** in a phosphate buffer was titrated into a solution of the fluorinase in the same buffer. The binding curve obtained is illustrated below in Fig. 1A, and the data was fitted to a 1:1 isotherm assuming a single binding site. A similar experiment was conducted by titrating FDA **2**, the natural substrate, into the fluorinase for comparison, and the resultant titration curve is illustrated in Fig. 1B. The titration curve revealed that F_2DA **7** exhibited strong binding to the fluorinase. The association constant (K_a) for this interaction was calculated to be $27.1 \pm 2.01 \times 10^5\ M^{-1}$. Comparison of the association constant with that of FDA **2** revealed that F_2DA **7** bound with slightly higher affinity for the fluorinase, by a factor of nearly two. Both compounds show favourable enthalpic contributions, but F_2DA **7** shows a greater exotherm upon binding, likely due to increased lipophilicity of the difluoromethyl group compared to the fluoromethyl group. The greater enthalpic contribution for F_2DA **7** is compensated for by a larger entropic penalty, which results in the similar nett free energy change to that observed for FDA **2**. The presence of the second fluorine substituent at C-5′ appears to increase the affinity of the nucleoside for the enzyme.

2.3. Incubation of F_2DA 7 with the fluorinase

Confident that the candidate substrate, F_2DA **7**, binds to the enzyme, attention turned to investigating whether the fluorinase was able to catalyse substitution of one of the fluorine atoms of with L-selenomethionine (L-SeMet) rather than L-methionine. Selenium (Se) is a better nucleophile [34] than sulfur due to the presence of a higher energy HOMO on the selenium atom, and



Scheme 2. Synthesis of F_2DA **7**.

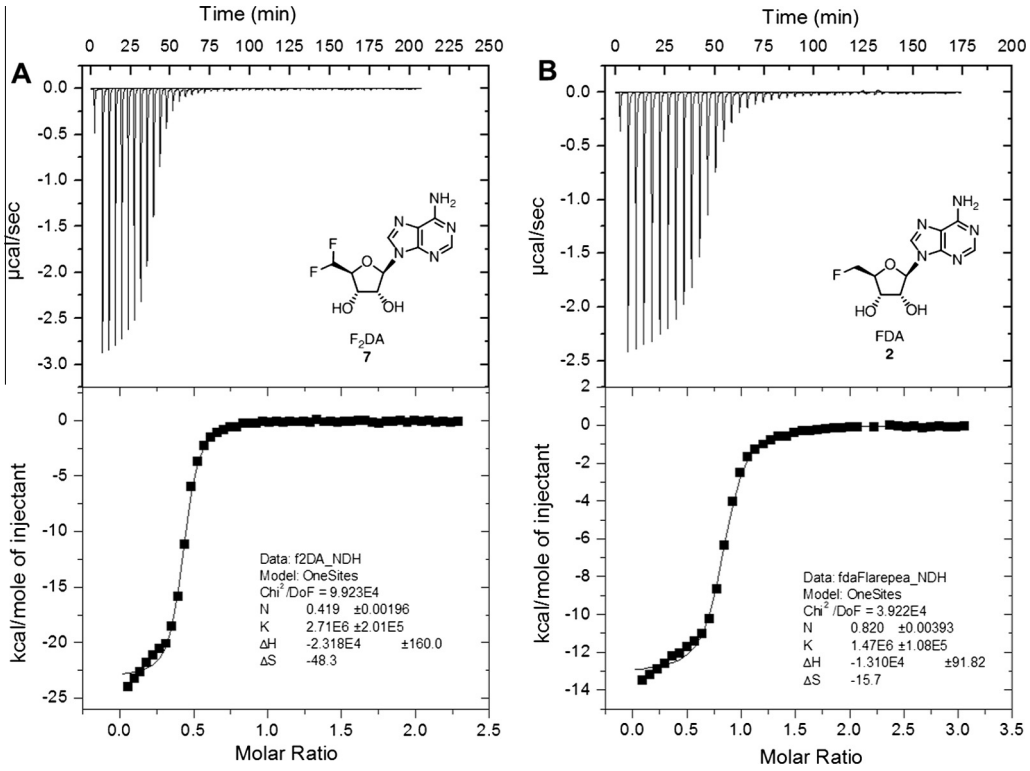
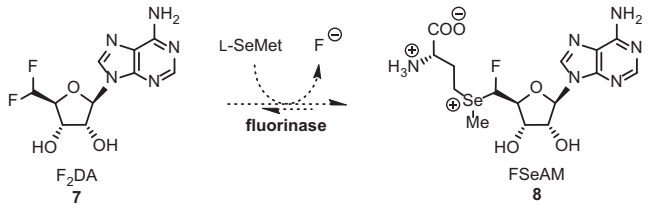


Fig. 1. Isothermal titration calorimetric determination of the binding affinity of A. F_2DA **7** (0.72 mM) into the fluorinase (60.3 µM) and B. FDA **2** (1.03 mM) into the fluorinase (53.6 µM).

the increased polarizability of Se over S [35]. In addition, it has previously been demonstrated that, in the reverse direction, the fluorinase catalysed reaction between FDA **2** and L-SeMet occurs 6-fold faster [6] than with the L-Met, suggesting that assays with L-SeMet are better suited for investigating putative substrates. The anticipated transformation of F2DA **7** to FSeAM **8** is illustrated above in Scheme 3.

F_2DA **7** was incubated with L-SeMet (0.6 mM) and the fluorinase enzyme in phosphate buffer. Reaction progress was monitored by HPLC at $t = 0$, 1, 2, and 19 h, and the results are illustrated in Fig. 2. The HPLC trace did not reveal any change in the concentration of F_2DA **7** over 19 h, and there were no significant new peaks evident in the chromatogram. A similar experiment, substituting L-Met for L-SeMet did not result in any conversion to new products. This data suggested that the fluorinase is not capable of catalysing the proposed substitution.

The lack of any obvious reaction with either L-Met or L-SeMet suggested that the inherent properties of F_2DA **7**, possibly steric or electronic, or a combination thereof, prevent substitution of one of the fluorine atoms at C-5′. As the fluorinase reaction is known to be reversible, the possibility also exists that the equilibrium for this transformation lies far in favour of the difluorinated product. Without access to synthetic samples of a 5′-fluoro-SAM or its selenium analogue, distinguishing between equilibrium effects or inherent lack of reactivity is not straightforward.



Scheme 3. Exploration of the enzymatic transformation of F_2DA **7** to its corresponding FSeAM analogue **8**.

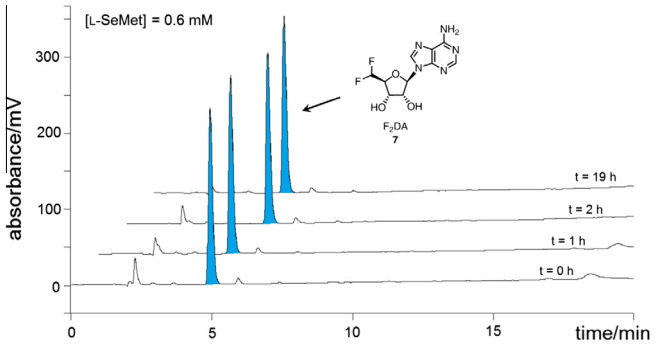


Fig. 2. HPLC time course of incubation of F_2DA **7** with L-SeMet (0.6 mM) and the fluorinase, with samples taken at $t = 0$, 1, 2 and 19 h. The chromatograms show no change in concentration of F_2DA **7**, and no new products were evident, suggesting that F_2DA **7** is not a substrate for the fluorinase enzyme.

2.4. Crystallography of F_2DA **7** with the fluorinase

To further probe the binding of F_2DA **7** to the enzyme and to try to understand the lack of reactivity, conditions were explored for the preparation of a fluorinase-F_2DA **7** co-crystal. A co-crystal suitable for diffraction was obtained and a structure solved to a resolution of 1.8 Å (PDB code: 5FIU) by molecular replacement using the original fluorinase crystal structure (PDB code: 1RQR) [36]. For direct comparison, the co-crystal structure of F_2DA **7** was overlaid with that of FDA **2** and L-Met bound in the active site of the fluorinase [36]. The structures are shown above in Fig. 3, with the bright coloured structure and ligand with grey C-atoms belonging to the F_2DA **7** structure, while pastel coloured structure and ligands with yellow C-atoms belonging to the FDA-L-Met structure.

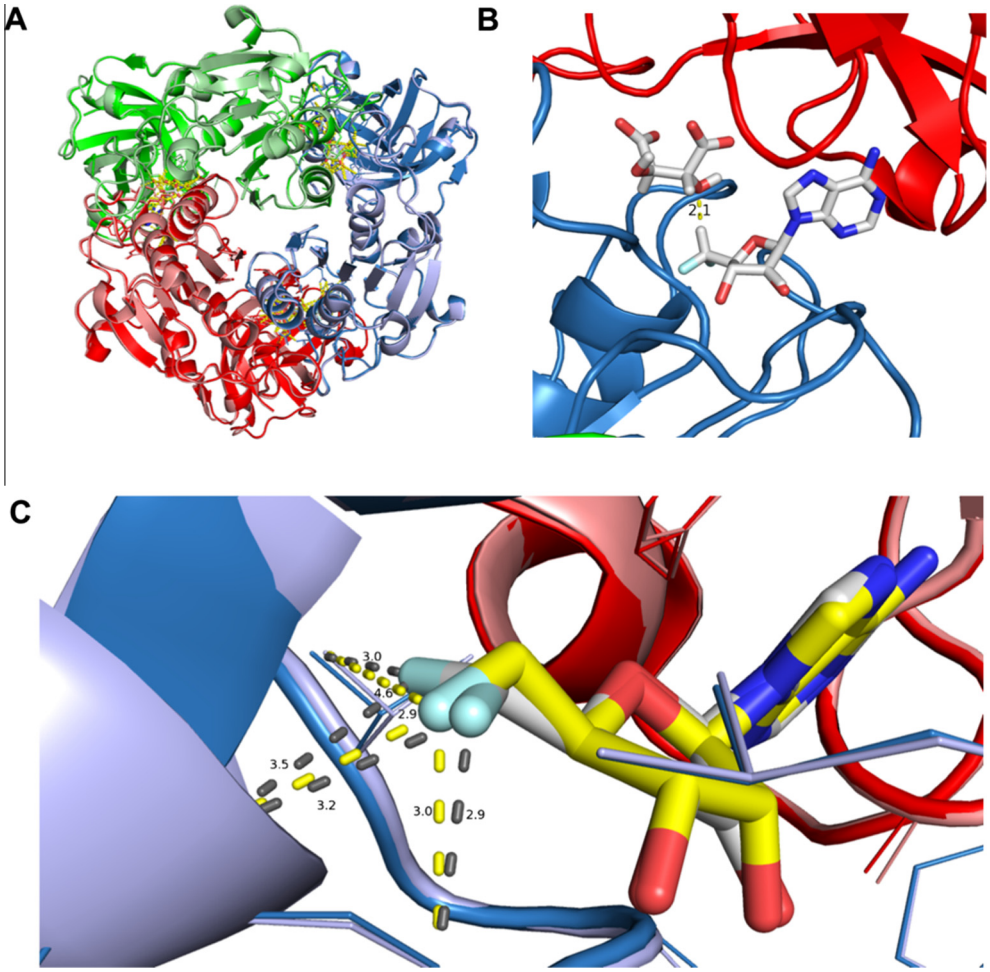


Fig. 3. A. Overlay of the structure of fluorinase bound to FDA **2** (pastel colours) and F_2DA **7** (bright colours) showing no gross conformational change when bound to F_2DA **7**. B. F_2DA **7** in the active site of the fluorinase, showing a hydrogen bonding contact between the hydrogen of the difluoromethyl group and one of the tartrate hydroxyl oxygens. The two monomers of the fluorinase shown in blue and red. The structure was obtained with a molecule of tartrate bound in the active site. C. Close up of the active site showing orientation of the fluorine atoms of F2DA **7** (grey carbon skeleton) compared to the orientation of the C–F bond of FDA **2** (yellow carbon skeleton). Distances (Å) from the fluorine atoms to key residues are shown for FDA **2** (yellow dashes) and F_2DA **7** (grey dashes).

Binding of F_2DA **7** does not result in any gross conformational change of the structure of the fluorinase, as shown in Fig. 3A. F_2DA **7** was located in the active site, along with a molecule of tartrate (from the crystallisation buffer), as shown in Fig. 3B. Examination of the structure revealed that F_2DA **7** engaged in numerous contacts to active site residues, as had been observed with other nucleosides co-crystallised with the enzyme. The adenine base was found to participate in π–π stacking interactions with Phe-254 and Trp-50, while the endo- and exo-cyclic nitrogen atoms formed hydrogen bonding interactions to Ala-279 and Asn-215. The 2′,3′-diol system of the ribose ring was found to participate in a hydrogen bonding interaction with Asp-16. Tartrate occupies the L-Met binding site, adopting a similar conformation to L-Met, where one of the carboxylates engages in polar interactions similar to those observed for carboxylate group of L-Met.

Closer examination of the difluoromethyl group revealed that the fluorine atoms were located in the hydrophobic fluoride binding pocket. The interactions previously identified [36] as important for reaction appeared conserved. One of the fluorine atoms lies 3.0 Å from the OH group of Thr-80, while the second fluorine makes contacts with the amide NH and side chain OH of Ser-150 (3.2 Å and 2.9 Å respectively). The two fluorine atoms appear to "share" the contacts usually observed between the protein and the fluorine atom of FDA **2**. The hydrogen atom of the difluoromethyl group appears to engage in a weak hydrogen bonding interaction with one of the hydroxyl oxygen atoms of the co-bound tartrate (CF_2H–O distance = 2.1 Å), and does not form close contacts to any amino acid residues in the active site.

The adenine bases overlay well, as does the ribose ring. It is clear in Fig. 3C that the fluorine atom of FDA **2**, the natural product, lies between the two fluorine atoms of the difluoromethyl group of F_2DA **7**. The location of the two fluorine atoms and their suggested interactions with Thr-80 and Ser-158, as shown above, support the suggestion that the fluoromethyl group bridges the interactions usually engaged by the single fluorine atom of FDA **2**.

The C–F bond is the strongest single bond observed in organic molecules, having a bond dissociation energy, on average, of 105.4 kJ mol^{-1} [37]. In aliphatic systems, fluoride is well characterised as a poor leaving group [38] a consequence of the strength of the C–F bond. The fluorinase enzyme has, however, uniquely evolved the ability to catalyse the cleavage of the C–F, bond with L-Met as an incoming nucleophile. The analogous transformation has also been reported, using a range of Lewis acid or hydrogen bond catalysts to activate fluoride as a leaving group [39]. Substitution of both fluorine atoms in difluoromethylene and difluoromethyl groups is an even more challenging transformation. The addition of a second electronegative fluorine atom is expected to increase the strength of each of the C–F bonds, due to increased positive character at carbon [40]. The non-bonding electrons of the second fluorine atom are also expected to hinder approach of

an incoming nucleophile [41]. Despite this, substitution of such motifs has been reported using potent nucleophiles, such as $LiAlH_4$ [42], $AlCl_3$ and $AlMe_3$ [43].

Together, these observations suggest a possible reason why F_2DA **7** is not a substrate for the fluorinase. The "sharing" of key interactions between the two fluorine atoms as observed in the X-ray co-crystal structure may no longer sufficiently activate one of the fluorines for substitution by L-Met or L-SeMet. A second consequence of this "sharing" of interactions is that neither of the fluorine atoms is aligned in the optimal conformation for the S_N2-type substitution catalysed by the enzyme [5,6,36] with the incoming nucleophile, increasing the kinetic barrier to reaction. These two factors originating from the enzyme-substrate interactions, coupled to the inherently stronger C–F bond and more shielded C-5′ centre may all contribute to a lack of observed reactivity for the difluoromethyl nucleoside F_2DA **7**.

3. Conclusions

The difluoromethyl group plays an important role in a range of pharmaceuticals and agrochemicals in light of its unique physicochemical properties. In this context, we were interested in applying the fluorinase enzyme to the synthesis of difluoromethyl groups. F_2DA **7**, a difluormethylated nucleoside, was found to bind to the fluorinase with a marginally larger K_a compared to that of the natural substrate, FDA **2**. Assays were conducted where F_2DA **7** was incubated with both L-Met, and the more potent nucleophile, L-SeMet. No reaction was observed in HPLC assays for either set of conditions, and increasing the concentration of the nucleophile did not result in a reaction. A co-crystal structure of the fluorinase bound to F_2DA **7** was obtained and suggested that the two fluorine atoms were "sharing" interactions with Ser-158 and Thr-80, the two residues in the active site responsible for activating the fluorine atom for substitution. As a result, the conformation of neither of the C–F bonds matched that observed for the C–F bond in FDA **2**, suggesting that the geometry of the bond was no longer optimal for S_N2 attack by sulfur or selenium. These observations, coupled to the inherently stronger C–F bond observed in $-CF_2H$ compared to $-CFH_2$ motifs, most probably contributes to a prohibitive reaction barrier.

Acknowledgments

We thank EPSRC and the Scottish Imaging Network (SINAPSE) for grants. DO'H thanks the Royal Society for a Wolfson Research Merit Award and ST is grateful to the John and Kathleen Watson Scholarship for financial support.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2015.11.003.

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