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Identification of a PLP-Dependent Threonine Transaldolase: A Novel Enzyme Involved in 4-Fluorothreonine Biosynthesis in *Streptomyces cattleya***

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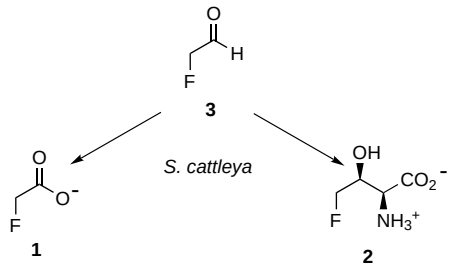
Fluorinated natural products are extremely rare in nature. Fluoroacetate (**1**) is the most widely distributed of this group of natural products and has been identified as a toxin in a range of tropical and sub-tropical plants[1] and in the actinomycete *Streptomyces cattleya*.[2, 3] In this bacterium fluoroacetate is co-produced with 4-fluorothreonine (**2**), the only naturally occurring fluorinated amino acid which has been identified to date. The biosynthetic pathways leading to fluoroacetate and 4-fluorothreonine have been investigated by using ^{13}C- and ^{2}H- enriched precursors.[4–6] These experiments have revealed that the carbon substrate for fluorination is closely related to an intermediate of the glycolytic pathway between glycerol and pyruvate. Furthermore, the labeling studies demonstrated that fluoroacetate and the C-3 and C-4 atoms of 4-fluorothreonine have the same biosynthetic origin and that there is a single fluorination enzyme. Most recently[7] fluoroacetaldehyde (**3**) has been identified as the common precursor of both fluorometabolites (Scheme 1) and the aldehyde dehydrogenase responsible for the oxidation of fluoroacetaldehyde to fluoroacetate has been isolated and purified.[8] Herein we describe the nature of the biotransformation of fluoroacetaldehyde to 4-fluorothreonine. Sanada et al.,[2] who first reported fluorometabolite production by *S. cattleya*, suggested that 4-fluorothreonine may arise by the direct condensation of fluoroacetaldehyde with glycine in a classical aldolase reaction. However, when [2-^{13}C]glycine was incubated with resting cell cultures of the bacterium,[6] the 4-fluorothreonine produced was doubly labeled in the C-1 and C-2 positions. Thus glycine does not directly contribute to 4-fluorothreonine biosynthesis, but is probably metabolized by serine hydroxymethyl transferase. We now report that L-threonine is the amino acid that condenses with fluoroacetaldehyde in *S. cattleya* to generate 4-fluorothreonine in a transaldolase-mediated reaction.



Scheme 1. Fluoroacetaldehyde is the common precursor of fluoroacetate and 4-fluorothreonine.

Upon incubation of a cell-free extract of *S. cattleya* with L-threonine, pyridoxal 5'-phosphate (PLP), and fluoroacetaldehyde, 4-fluorothreonine (ca. 0.25 mM) was detected by ^{19}F NMR spectroscopy. When the assay was conducted in the absence of PLP or L-threonine, or with boiled cell-free extract, there was no production of 4-fluorothreonine. When [1-^{2}H]fluoroacetaldehyde was used, a high level (61%) of isotopic label was detected in the (C-2–C-4) fragment of 4-fluorothreonine by GC-MS analysis, which indicates the direct incorporation of this unit into 4-fluorothreonine generated in vitro. This result is consistent with our previous observation in whole cells.[7] The enzyme was partially purified by ammonium sulfate precipitation and assayed with a range of amino acids (glycine, L-serine, L-cysteine, L-aspartate, L-alanine, and L-*allo*-threonine) instead of threonine. In none of the assay mixtures was 4-fluorothreonine production detected. The key observation that glycine is not a substrate for this enzyme is consistent with whole cell studies with [2-^{13}C]glycine.[6] The requirement for PLP and L-threonine suggests that a novel threonine transaldolase activity is responsible for 4-fluorothreonine production in the bacterium (Scheme 2).

The enzyme was purified further by anion-exchange chromatography. Experiments were conducted by using [1,2,2,2-$^{2}H_4$]acetaldehyde and DL-[4,4,4-$^{2}H_3$]threonine to explore the PLP-transaldolase aspect of the enzyme. After incubation of the enzyme with [1,2,2,2-$^{2}H_4$]acetaldehyde, PLP, and L-threonine, GC-MS analysis revealed a 20% incorporation [M+4] of isotope into the threonine pool. No labeled threonine was detected in a control experiment in which threonine was substituted with glycine. Thus the enzyme–PLP complex accepted acetaldehyde as a substrate and generated a new molecule of L-threonine in a neutral

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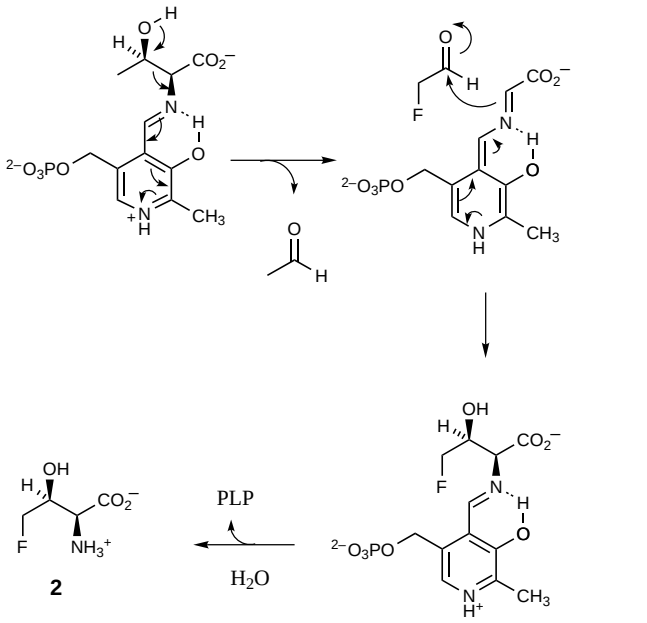
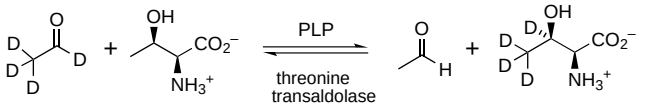


Scheme 2. Proposed mechanism for the enzymatic formation of 4-fluorothreonine by threonine transaldolase.

crossover reaction (Scheme 3). A proposed mechanism for the formation of 4-fluorothreonine from fluoroacetaldehyde and threonine is shown in Scheme 2. This mechanism was further supported by the detection of [2-$^2H_3$]acetate after



Scheme 3. The threonine transaldolase mediates a neutral crossover reaction with acetaldehyde as a substrate.

incubation of the enzyme with DL-[4,4,4-$^2H_3$]threonine, PLP, and fluoroacetaldehyde. The labeled acetate was detected after the [2-$^2H_3$]acetaldehyde generated in the assay was oxidized in situ by yeast aldehyde dehydrogenase and NAD^+. Derivitization using 4-(bromoacetyl)biphenyl followed by GC-MS analysis revealed 40% isotope incorporation [M+3] into the resultant acetate. A comparatively small amount of labeled acetate (ca. 8%) was detected in control experiments with no fluoroacetaldehyde, which suggests that a small amount of threonine cleavage had occurred in the absence of added aldehyde.

When the enzyme was incubated with L-threonine, PLP, and chloroacetaldehyde instead of fluoroacetaldehyde, 4-chlorothreonine was detected by GC-MS. 4-Chlorothreonine has been identified as a metabolite of several bacteria, either as a free amino acid[9] or as part of a larger peptide structure.[10, 11] It is possible that an enzyme similar to that described above catalyzes the formation of 4-chlorothreonine in these other microorganisms. Consistent with this suggestion is the observation that a radiolabel from [U-^{14}C]threonine is incorporated into the 4-chlorothreonine moiety of the lipodepsipeptide, syringomycin, in *Pseudomonas syringae*.[10]

The PLP-dependent threonine transaldolase has been purified[12] and a monomer of approximately 60000 kDa was detected (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)). Passage of the native enzyme through a gel filtration column (Hiload 16/60 Superdex 200) indicated a molecular weight of 120 kDa, thus the enzyme is probably a dimer. In conclusion, we have identified a threonine transaldolase in *S. cattleya* that is distinct from more classical threonine aldolases in that it does not use glycine as a substrate. The enzyme uses fluoroacetaldehyde and threonine to generate 4-fluorothreonine and acetaldehyde. It can convert chloroacetaldehyde into 4-chlorothreonine, which is suggestive of the mode of biosynthesis of this metabolite in other organisms. Clearly this enzyme is important in the biosynthesis of 4-fluorothreonine in *S. cattleya*. However, it is less clear if the reversible interconversion of L-threonine and acetaldehyde has any metabolic significance to the organism's amino acid biochemistry.

Experimental Section

Streptomyces cattleya NRRL 8057 was grown in 500-mL flasks containing medium (90 mL) of the composition described in reference [3]. After 6 days incubation the cells were harvested and washed with potassium phosphate buffer (50 mM, pH 7) and resuspended in the same buffer (0.5 g wet cell weight per mL). Cell-free extract was prepared by sonication and centrifugation. Threonine transaldolase activity was assayed in a final volume of 1 mL by incubating cell-free extract (910 μL) with L-threonine (2 mM), PLP (0.1 mM), and fluoroacetaldehyde (2 mM) for 16 h at 26 °C. ^{19}F NMR spectra were recorded using a Varian Inova 500 MHz NMR spectrometer (^{19}F, 470.551 MHz); $\delta(D_2O) = -230.4$ (dt, $J = 47$, 25 Hz).

The enzyme activity was partially purified by adding solid $(NH_4)_2SO_4$ to the cell-free extract to 25% saturation and removing the precipitate by centrifugation. The supernatant was adjusted to 45% saturation with solid $(NH_4)_2SO_4$ and the precipitate collected after centrifugation. The pellet was resuspended in tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM, pH 8) and eluted from a Hi-Trap desalting column attached to an AKTA Prime system (Pharmacia). The enzyme was then applied to a Hi-Trap Q XL anion-exchange column (Pharmacia) and the activity eluted with a linear gradient of 0 to 1M KCl in Tris buffer over 25 mL.[12]

DL-[4,4,4-$^2H_3$]Threonine was prepared according to the method described in reference; [13] by GC-MS the label was about 89% [M+3] and 7% [M+2]. GC-MS analyses of isotopic label incorporation were performed by using an Agilent 6890 gas chromatograph connected to an Agilent 5973 mass-selective detector. The methods used for analysis were similar to those described in reference [6]. 4-Fluorothreonine, threonine, and 4-chlorothreonine were analyzed as the per-trimethylsilyl derivatives. The mass spectrometer was operated in the selected ion monitoring (SIM) mode for determination of the isotopic label in 4-fluorothreonine and threonine. Ion currents m/z 236 [M] and 237 [M+1] were monitored for isotope incorporation into (C-2–C-4) of 4-fluorothreonine and ion currents m/z 320 [M], 321 [M+1], 322 [M+2], 323 [M+3], and 324 [M+4] were monitored for incorporation of ^2H into threonine. The mass spectrometer was operated in the scan mode for identification of 4-chlorothreonine and a compound was detected that had the same mass spectrum as previously reported [9].

Acetate was analyzed by GC-MS as the phenylphenacyl derivative,[6] with the mass spectrometer operated in the SIM mode and at ion currents m/z 254 [M], 255 [M+1], 256 [M+2], and 257 [M+3].

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[(Indenyl)Ru(biphop-F)]$^+$: A Lewis Acid Catalyst That Controls both the Diene and the Dienophile Facial Selectivity in Diels – Alder Reactions**

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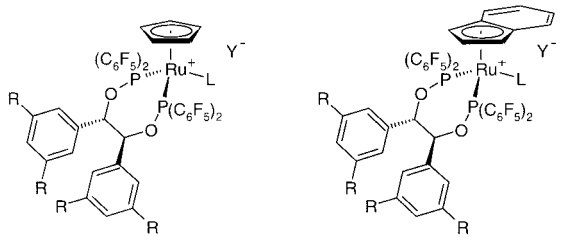
Cationic arene and cyclopentadienyl complexes of iron and ruthenium incorporating chiral bidentate ligands have been shown to be efficient Lewis acid catalysts for the asymmetric Diels – Alder reaction between enals and dienes.[1–5] Our research in this area has focused on cyclopentadienyliron and -ruthenium complexes, the former giving higher rates and enantiomeric excesses and the latter being more stable and easily recycled.[2] Enantioselectivity in the Ru complexes was strongly increased on changing the aryl groups in the ligand backbone, that is by using **3** rather than **2** as precatalysts.[2a]

The structurally characterized complex [CpRu(biphop-F)-(methacrolein)][SbF_6] (**1d**; biphop-F = 1,2-bis[bis(pentafluoro-



1 R = H, L = $CH_2C(Me)CHO$
2 R = H, L = Me_2CO
3 R = Me, L = Me_2CO

4 R = H, L = $CH_2C(Me)CHO$
5 R = H, L = Me_2CO
6 R = Me, L = Me_2CO

Y = OTf (**a**), BF_4 (**b**), PF_6 (**c**), SbF_6 (**d**), TFPB (**e**)

phenyl)phosphanyloxy]-1,2-diphenylethane)has provided a detailed picture of the catalyst/substrate interaction and of the influence of the anion on the rate of catalysis.[2b] Evidence from the solid-state structure of **1d** and from F/H NMR correlation spectra in solutions of the BF_4^- and the PF_6^- analogues (**1b** and **1c**, respectively) point to ion pairs in which the anion interacts with both the bound substrate and the cationic catalyst.[2a, b] We concluded that the anion slows down product/substrate exchange at the catalyst site. Consequently, complex **1e**, which incorporates the tetrakis[3,5-bis(trifluoromethyl)phenyl] borate anion (TFPB) whose bulk renders this proximity impossible, showed the highest activity. Another way to bring about a larger separation of the anion from the enal group in the catalyst site is to increase the size of the catalyst's capping ligand, for example by changing from the cyclopentadienyl to the indenyl complexes. With an indenyl ligand the question of preferred rotamers arises. Moreover, the propensity of the indenyl ligand to undergo a slip/fold rearrangement[6] risks the occurrence of a different mode of enal binding and reactivity than the desired single coordination site Lewis acid.

The results described herein provide answers to these questions. The properties of the indenyl complexes are indeed significantly altered from those of the cyclopentadienyl analogues, and diastereoselectivities not previously encountered in the Diels – Alder reaction of enals with dienes are realized. We also note that despite the detailed attention that indenyl complexes of Group 8 metals have received, asymmetric catalytic reactions with this family of compounds have not been reported.[7]

Given that the reaction of [$Ru_3(CO)_{12}$] with indene affords a low yield of the resultant complex,[8] we opted for the thermal substitution of the two PPh_3 ligands in [IndRu-(PPh_3)$_2$Cl][9](**7**) by the bidentate biphop-F[2c, 10](**8**) and Me_4biphop-F (**9**)[2a] ligands to give the Ru complexes **10** and **11** respectively (Scheme 1).

This reaction was complete in 1.5 h, whereas with the cyclopentadienyl analogue the reaction with the very similar ligand 1,2-bis[bis(pentafluorophenyl)phosphanyloxy]cyclopentane (cyclop-F)[2d] took one week to complete. In analogy to the mechanism established in reactions of [$IndRh(CO)_2$], the higher reactivity of the indenyl complex could be ascribed to a reversible η^5/η^3 slippage of the ligand in an associative mechanism (indenyl effect).[11] However, phosphane substitu-

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[**] This work was supported by the Swiss National Science Foundation (FNS grant 20-59374.99) and The Ministère des Affaires Etrangères from France (Lavoisier grant to V.A.). biphop-F = 1,2-bis[bis(pentafluorophenyl)phosphanyloxy]-1,2-diphenylethane.