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Gram scale synthesis of 3-fluoro-1-hydroxyacetone phosphate: a novel donor substrate in rabbit muscle aldolase-catalyzed aldol reactions†

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An efficient gram scale synthesis of 3-fluoro-1-hydroxyacetone phosphate (FHAP) has been developed. As a close analog to dihydroxyacetone phosphate, FHAP was used as a novel donor substrate for rabbit muscle aldolase catalyzed reactions. The different binding affinities of the *gem*-diol and keto form of FHAP were studied by ^{19}F-NMR.

Rabbit muscle aldolase (RAMA) catalyses the stereospecific aldol addition of dihydroxyacetone phosphate (DHAP) to an aldehyde, thus forming a C–C bond with D-*threo* configuration.[1] The enzyme tolerates a wide variety of functionalized aldehydes (>100) as acceptor substrates,[2] reflected by its applications in numerous syntheses of ketoses, aldoses, iminosugars and cyclitols.[1,3] In contrast to its versatile acceptor tolerance, RAMA is very restrictive concerning the nucleophilic donor DHAP, as summarized in Fig. 1. Variations at position C3 of the donor phosphate are not tolerated.[2a,4,5] The hitherto investigated 3-halo analogs of DHAP are either competitive (chloride: pH = 7) or irreversible (iodide; bromide and chloride: pH = 10) inhibitors of RAMA, acting by oxidizing sulfhydryl groups to disulfides or by forming stable covalent derivatives with sulfhydryl groups.[5] At present, only three analogs of DHAP are established substrates for RAMA-catalyzed reactions (Fig. 1).[2a,6–8] A substitution of the free hydroxyl group of DHAP by fluorine constitutes the smallest possible bioisosteric change at the C3 position,[9] while simultaneously offering a direct access to biologically important fluorinated sugars and sugar-derived compounds.[10] 3-Fluoro-1-hydroxyacetone phosphate (FHAP) was previously synthesized in a laborious sequence by Silverman *et al.* starting from epichlorohydrin in low overall yield (~1.3%) and purity.[11] Previous studies on the substrate specificity of fructose-1,6-bisphosphate aldolase suggested that FHAP may not act as a substrate for the enzyme,[12] which is in contrast to our recent observations. Additionally, neither experimental details of the preparation of FHAP and inhibitor studies nor spectroscopical proof of identity were given.[12]

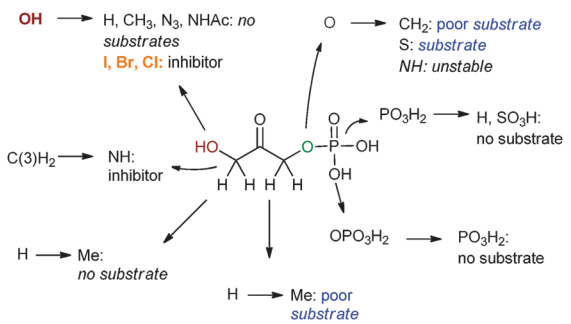
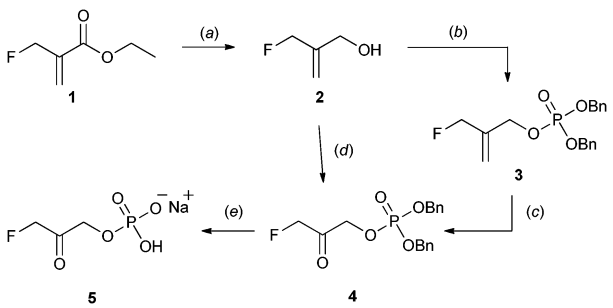


Fig. 1 Donor substrate variations of DHAP.[2a,4–8]

Herein, we report on a short and efficient gram scale synthesis of 1-fluoro-3-hydroxyacetone phosphate and its application in RAMA-catalyzed aldol reactions. The stereochemistry of the aldol condensation product **8** was proven by an independent chemical synthesis. Furthermore, a fast RAMA-catalyzed proton–deuterium exchange of FHAP with D_2O and the different binding affinities of the *gem*-diol and keto form of FHAP were studied by ^{19}F-NMR.

The synthetic sequence leading to FHAP (**5**) is outlined in Scheme 1. Starting from easily accessible ethyl α-fluoromethylacrylate (**1**),[13] allylic fluoride **2** was synthesized by reduction of the ester moiety with diisobutylaluminium hydride (DIBAL-H).[14] The phosphate functionality of FHAP was introduced *via* dibenzyl *N*,*N*-diisopropylphosphoramidite, followed by a



Scheme 1 Synthesis of FHAP (**5**): (a) DIBAL-H, CH_2Cl_2, −78 °C → 0 °C, 63%; (b) $iPr_2NP(OBn)_2$, 1H-tetrazole, CH_3CN; then 30% H_2O_2, 74%; (c) O_3, CH_2Cl_2, −78 °C; $(CH_3)_2S$, 95%; (d) $iPr_2NP(OBn)_2$, 1H-tetrazole, CH_3CN; then O_3, CH_2Cl_2, −78 °C; $(CH_3)_2S$, 52%; (e) H_2, 10% Pd/C, *t*BuOH; 1 N NaOH, lyophilized, 95%.

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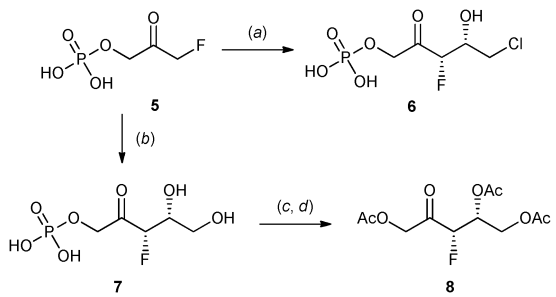
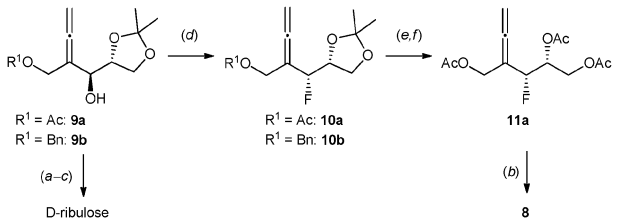
stepwise oxidation of the intermediately formed phosphite with 30% H_2O_2 to allylic phosphate **3** and ozonolysis of the double bond in a subsequent step to yield 2-oxo phosphate **4** in excellent yield. Alternatively a direct oxidation of the double bond and the phosphite with ozone gave the corresponding β-keto phosphate **4**. The dibenzyl protecting groups were cleaved by hydrogenolysis over 10% Pd/C in *t*BuOH,[15] thereby preventing a hemiketal formation which was observed when MeOH was used.[11] After conversion into the monosodium salt, lyophilized FHAP (**5**) was isolated in an overall yield of 42% over 4 steps (or alternatively in 31% yield over 3 steps) starting from ethyl α-fluoromethylacrylate (**1**). FHAP (**5**) is stable for several months at −20 °C. Suitable crystals for X-ray diffraction were obtained from the biscyclohexylammonium salt of FHAP, featuring the keto functionality in its *gem*-diol (hydrated) form (see ESI†). At neutral pD at 25 °C in D_2O,[16] FHAP (**5**) exists 91% in the *gem*-diol form (determined *via* ^{19}F-NMR), compared to 45% for DHAP.[17]

In the subsequent enzymatic reactions the ^{19}F nucleus was used to monitor the reaction progress by ^{19}F-NMR. For all conversions, spectroscopically pure FHAP (**5**) (^{1}H- and ^{19}F-NMR) was essential, since no product formation was detected with slightly impure substrates. Additionally, the acceptor substrates investigated showed the highly preferred formation of one product diastereomer, as judged by ^{19}F-NMR. Our initial studies focused on the RAMA-catalyzed condensation of FHAP (**5**) and chloroacetaldehyde in BIS–TRIS (2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol) buffer (Scheme 2). A slow conversion of FHAP (**5**) to deoxysugar **6** was observed by NMR (see ESI†). However, all attempts towards product isolations either as the barium salt of phosphate **6** or as the dephosphorylated sugar failed due to the instability of the products. Therefore we used glycolaldehyde as the acceptor substrate. Good conversion rates forming derivative **7** were obtained by repeated addition of RAMA over 3 days. Dephosphorylation with acid phosphatase and acetylation with acetic anhydride to simplify isolation and characterization yielded 1,4,5-tri-*O*-acetyl-3-deoxy-3-fluoro-D-*threo*-pent-2-ulose (**8**) (Scheme 2). The stereochemistry of compound **8** was assigned by an independent chemical synthesis (Scheme 3). As a prerequisite, the absolute configuration of allene **9a**[18] was confirmed by transformation into D-ribulose (see ESI†). Subsequently, the hydroxyl



Scheme 2 RAMA-catalyzed aldol condensation with chloroacetaldehyde or glycolaldehyde: (a) RAMA, $ClCH_2CHO$ solution (~50 wt. % in H_2O), BIS–TRIS buffer, pH = 6.8; (b) RAMA, $HOCH_2CHO$, BIS–TRIS buffer, pH = 6.8; (c) acid phosphatase, pH = 5.1; (d) Ac_2O, DMAP, pyridine, rt (7% over 3 steps).

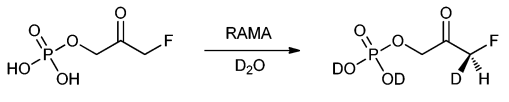


Scheme 3 Chemical synthesis of 1,4,5-tri-*O*-acetyl-3-deoxy-3-fluoro-xylulose (**8**): (a) NaOMe, MeOH, rt; (b) O_3, CH_2Cl_2, −78 °C; PPh_3; (c) Amberlyst 15 H^+, THF/H_2O (77% over 3 steps); (d) DAST, Collidine, CH_2Cl_2, −78 °C, 35%; (e) Dowex 50 H^+, THF/H_2O, rt, 80%; (f) Ac_2O, Pyridine, DMAP, rt, 95%.

functionality of **9a** was transformed to the allenylic fluoro derivative **10a** with (diethylamino)sulfur trifluoride (DAST) at −78 °C under inversion of the configuration at C3.[19] A potential 1,3-neighbouring group participation by the acetate moiety was ruled out, since using the corresponding benzyl ether **9b**[18] instead led to the formation of the same diastereomer **10b** (see ESI†). The *syn*-relationship was reflected in analogy to literature data[20] by the vicinal coupling constants (**10a**: $^3J_{H2-H3}$ = 7.8 Hz; **10b**: $^3J_{H2-H3}$ = 8.0 Hz). Cleavage of the isopropylidene protecting group and subsequent acetylation was performed under standard conditions to yield allene **11a**. After ozonolysis of the allene moiety of **11a** at −78 °C in CH_2Cl_2, pentulose **8** was isolated. All spectroscopical data were identical with the product of the enzymatic synthesis. As a consequence, the RAMA-catalyzed condensation of FHAP (**5**) with glycolaldehyde led to the identical stereochemical configuration (D-*threo*) as the one using the natural donor substrate DHAP.

In concordance with NMR studies of DHAP and RAMA in D_2O,[21] we observed a rapid proton–deuterium exchange for one of the two enantiotopic protons at the C3 position of FHAP (**5**), yielding deuterated FHAP **12** (Scheme 4 and ESI†). The rate constant determined for the deuteration of **5** (0.03 μmol min^{-1} U^{-1}) was significantly smaller than the one reported for the phosphonate analog of DHAP (0.18 μmol min^{-1} U^{-1}) and DHAP (1.2 μmol min^{-1} U^{-1}).[22] The second C3 proton of FHAP (**5**) displayed a much slower exchange rate and a further hydrogen–deuterium exchange at C1 could only be observed after several hours.[23] This finding is in contrast to the natural substrate DHAP, where the second exchange takes place at C1 at a slow rate, followed by an even slower exchange of the second C3 proton.[21] Without the enzyme FHAP does not exchange its protons for several months in D_2O at pD = 6.9.

Since line width and/or relaxation rates of ^{19}F-NMR resonances are sensitive to changes in molecular weight (molecular tumbling), reversible ligand binding processes can easily be analyzed by changes in signal broadening (ESI†). Such a significant change was only observed for the keto form of FHAP, thus indicating a specific binding to RAMA. In contrast, the signal for the *gem*-diol



Scheme 4 Deuterium incorporation into FHAP (**5**).

form of FHAP remained nearly unchanged after the first addition of enzyme.[24] These observations are in agreement with the proposed enzymatic reaction mechanism for the natural substrate (DHAP) with RAMA,[25] since only the keto form can lead to a covalent linked Schiff base intermediate with the enzyme. In analogy to the stereospecificity of RAMA with DHAP as a substrate,[26] the fast proton–deuterium exchange of the *pro-S* hydrogen of FHAP can be assumed.

The low overall yield of the enzymatic aldol addition requires some comment. Since product formation correlates with the accessibility of the carbonyl moiety,[17a] the low isolated yield may mainly be attributed to the high degree of hydration (91%) of the keto functionality and to the general difficulties in acetylation of pentuloses.[27] Furthermore, the enamine intermediates of the natural substrates DHAP and fructose-1,6-bisphosphate are stabilized by a hydrogen bond between the carboxyl functionality of Asp-33 of the enzyme and the hydroxyl group at C3 of the substrates.[28] The importance of this crucial interaction was demonstrated by mutations of Asp-33 into alanine, serine, glutamate and asparagines, which lowered V_{max} of the enzyme dramatically.[29] Our entire findings point to a similar positioning of FHAP in the active site of RAMA to that of DHAP. In contrast to DHAP, FHAP cannot form this seminal interaction with Asp-33, due to fluorine's property of lacking hydrogen bond donor capacities and functioning solely as a hydrogen bond acceptor.

In summary, we have developed an efficient gram scale synthesis of FHAP (**5**). In contrast to earlier reports[12] FHAP could be identified as a novel donor substrate for RAMA-catalyzed aldol condensations. This fact was demonstrated in the synthesis of 1,4,5-tri-O-acetyl-3-deoxy-3-fluoro-D-*threo*-pent-2-ulose **8**. FHAP is the first analog of DHAP with a modification at C3 accepted as a donor substrate by RAMA. The stereochemical configuration at C3 and C4 (D-*threo*) of the aldol condensation product has proven to be identical as for the natural substrate DHAP, which was confirmed by an independent chemical synthesis of pentulose **8**. Furthermore, a fast proton–deuterium exchange and the different binding preferences of the *gem*-diol and keto form of FHAP were analyzed by ^{19}F-NMR. A direct evolution of aldolases[30] might result in highly efficient catalysts for the aldol condensation of FHAP with aldehydes, giving access towards a great variety of fluoro containing carbohydrates with biological and medicinal relevance.[10]

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