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SYNTHESIS OF (2*S*,3*R*)-β-METHYLTYROSINE CATALYZED BY TYROSINE PHENOL-LYASE

Kyonghee Kim and Philip A. Cole*

Laboratory of Bioorganic Chemistry, Rockefeller University New York, NY 10021, U.S.A.

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Abstract: A one-step enzymatic synthesis of the conformationally restrained tyrosine analog (2*S*,3*R*)-β-methyltyrosine is reported. This synthesis extends the preparative chemistry associated with tyrosine phenol-lyase. This β-methyltyrosine derivative was shown to be an efficient protein tyrosine kinase substrate, suggesting that conformational restraint may ultimately be used to enhance tyrosine kinase recognition of substrates.

The use of unnatural amino acids in the study of peptide and protein function and in the development of therapeutic agents[1] has prompted wide interest in the development of new methods for their stereoselective syntheses.[2] In particular, novel analogs of L-tyrosine (**1**) have proved to be powerful in the study of protein folding and in mechanistic and inhibitory studies on protein tyrosine kinases and phosphatases, SH2 domains, and G-protein coupled receptors.[3] β-methyltyrosine (**2**) and related amino acids are conformationally constrained analogs which have provided novel insights in the study of peptide ligand-receptor interactions.[4] However, β-methyltyrosine is not straightforward to prepare. The most efficient reported stereoselective syntheses of the various diastereomers require nine steps.[5]

The enzyme tyrosine phenol-lyase (TPL) from *Citrobacter freundii* has proved useful for the facile generation of ring-substituted tyrosine analogs.[6] TPL is a pyridoxal-linked enzyme which catalyzes the condensation of phenol along with pyruvate and ammonia to afford L-tyrosine (**1**). Its mechanism has been intensively studied and an X-ray structure has provided insight into TPL's active site architecture.[7] In principle, TPL could be used to generate β-substituted tyrosine derivatives (**2**) by employing alternative α-keto acid precursors to pyruvate. However, no such application of TPL has been reported to date. Tolerance toward even α-ketobutyrate in place of pyruvate could be difficult for TPL because the enzyme would not only have to facilitate deprotonation at a secondary rather than a primary carbon center but it would have to promote conjugate addition of phenol to the more sterically hindered substituted enamine (Figure). We set out to examine the feasibility of this reaction.

Substitution of α-ketobutyrate for pyruvate under standard conditions[3c] failed to afford significant product. However, by using increased pyridoxal 5'-phosphate, increased TPL, omitting 2-mercaptoethanol and running the reaction at pH 9, a ninhydrin positive spot on TLC, migrating with a similar *Rf* to tyrosine, could be detected.[8] Workup after 7 days and purification by cation exchange chromatography followed by recrystallization from water afforded (2*S*, 3*R*)-β-methyltyrosine (**2a**) (0.7 g from a 500 mL reaction).[8]

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That the reaction outcome appears stereospecific is not unexpected given the nature of most enzymatic reactions. Attack of the phenol must take place on the *re* face of C-3 of the enamine (Figure). A reasonable speculation that accounts for this facial selectivity derives from examination of the X-ray crystal structure of TPL in complex with 3-(4-hydroxyphenyl)-propionic acid.[9] The 3-pro-*R* hydrogen is in close proximity to the face of the aromatic side chain of tyrosine-71 whereas the 3-pro-*S* hydrogen is near two charged groups (a guanidinium nitrogen of arginine-355 and a pyridoxal phosphate oxygen). Thus a transition state hydrophobic interaction between the methyl group and tyrosine-71 may be favored over a transition state interaction between the methyl and the charged groups. Site-directed mutagenesis could ultimately be used to test this model. Elegant stereochemical studies on the tyrosine phenol-lyase reaction by Floss and coworkers showed that replacement of hydrogen by hydroxyphenyl in the substrate pyruvate methyl occurs with retention of configuration.[10] Moreover, isotopic transfer experiments indicate that addition of proton and hydroxyphenyl proceeds with *syn* addition to the enamine.[10] Extrapolating from this work, it can be surmised that in the α-ketobutyrate reaction of the current study, the pro-*S* hydrogen is removed and the (*Z*)-enamine is formed. Interestingly, tyrosine phenol-lyase can catalyze L-threonine formation from α-ketobutyrate,[11] which presumably involves hydroxy attack on the *re* face of the (*Z*)-enamine, analogous to (2*S*,3*R*)-β-methyltyrosine formation.

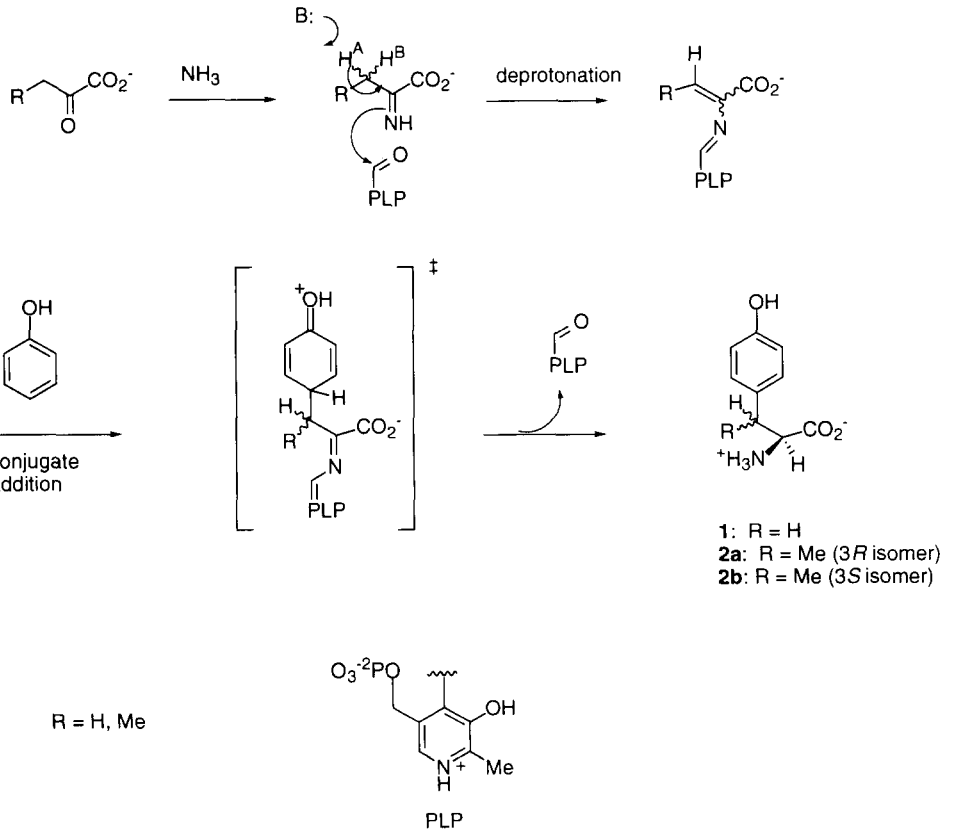

Figure. TPL (Tyrosine Phenol-Lyase) Catalyzed Tyrosine (**1**) and β-methyltyrosine (**2**) Formation

With β-methyltyrosine (**2a**) in hand, we prepared the corresponding Fmoc derivative of **2a** and then employed this material in automated solid phase peptide synthesis to generate the heptapeptide NH_2-EDNE**X**TA-CO_2H (**X = 2a**).[3c] It had previously been shown that this peptide sequence (**X** = Tyr) is a substrate for the protein tyrosine kinase Csk in which the chemical step is likely rate-determining for this reaction.[3b, 3c] We could therefore directly compare the potential effects of conformational restraint of the tyrosine residue within the peptide on catalytic processing by a protein tyrosine kinase. In the experiment, it was found that the peptide containing **2a** was capable of undergoing phosphorylation catalyzed by protein tyrosine kinase Csk. The phosphorylated peptide was purified by HPLC and its identitiy confirmed using electrospray MS. Using an HPLC based assay, a steady-state analysis of the peptide with **2** showed that it was identical in catalytic efficiency as a protein tyrosine kinase substrate (k_{cat}/K_m = 35 $M^{-1}s^{-1}$) compared to the standard tyrosine containing peptide (k_{cat}/K_m = 35 $M^{-1}s^{-1}$). These results suggest that neither the decreased conformational mobility of **2a** nor its tendency to favor specific rotamers[4] are deleterious to recognition and enzymatic phosphorylation by a protein tyrosine kinase. It will be interesting to test whether further rotameric restriction by increased ring substitution[4] may actually enhance kinase recognition, setting potential geometric constraints for substrate recognition and inhibitor design.

The stereospecific synthesis of (2*S*, 3*R*)-β-methyltyrosine (**2a**) in a single, environmentally friendly step from achiral starting materials step is a significant improvement over existing approaches. That TPL is capable of processing α-ketobutyrate suggests that TPL may be useful for the synthesis of a variety of β-substituted tyrosine analogs. Molecular diversity may further be achieved by using a combination of substituted phenols and α-ketoacids. A current limitation compared to the non-enzymatic synthesis of such analogs is the inability of obtaining products with varied stereochemistry at the α and β-carbons because of the enforced geometry associated with the wild-type enzyme. It is conceivable that site-directed mutants of TPL in residues lining the active site will not only allow altered stereochemical outcomes but increase tolerance of substituent types in the enzymatic reaction.

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8. A 500 mL aqueous solution containing phenol (1.9 g, 20 mmol), 2-ketobutyric acid (4.9 g, 48 mmol), pyridoxal-5'-phosphate (50 mg, 0.2 mmol), ammonium sulfate (2.6 g, 20 mmol), ammonium chloride (2.6 g, 50 mmol) was adjusted to pH 9 with 10% aqueous ammonium hydroxide and treated with purified recombinant tyrosine phenol-lyase (75 units)[3c] and left standing in the dark. After 4 days, an additional aliquot of tyrosine phenol-lyase (40 units) and pyridoxal-5'-phosphate (50 mg, 0. 2 mmol) was added and the solution was allowed to stand 3 more days before being acidified with acetic acid to pH 3, extracted with ethyl acetate (2x 300 mL) to remove unreacted phenol, and concentrated in vacuo to remove the organic solvent. The solution was loaded onto a pre-washed (6 N HCl, 6 N NaOH, water) Dowex cation exchange column (20 g), washed with 1-2 column volumes of water follwed by elution with 10% NH_4OH. Ninhydrin positive fractions were combined and lyophilized. The resulting mixture was recrystallized from water to yield **1** as a white solid (0.7 g, 18% based on phenol). Spectroscopic data (^1H NMR, ^{13}C NMR, MS, and $[\alpha]^{25}_D = +13.5°$ (c 0.1, aqueous 0.1 N HCl)) were essentially identical to those previously reported ($[\alpha]^{25}_D = +12.5°$ (c 1, aqueous 0.1 N HCl))[5] for authentic **2a** and ^1H NMR suggested that there was less than 5% of a diastereomeric impurity. The assignment of 2*S* stereochemistry is further supported by the known TPL stereospecificity at this site and the fact that the tyrosine kinase Csk fails to process the corresponding peptide with D-tyrosine in place of L-tyrosine.

9. Atomic coordinates of the TPL X-ray structure[6b] were downloaded from the Protein Data Bank and analyzed with the McImdad software package.

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