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Stereospecificity of *Pseudomonas fluorescens* Kynureninase for Diastereomers of β-methylkynurenine

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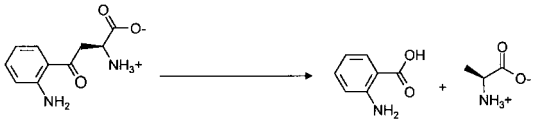
Abstract—The diastereomers of β-methyl-L-kynurenine were prepared by preparative ozonolysis of the respective diastereomers of β-methyl-L-tryptophan. A practical method for preparative enzymatic resolution of the diastereomers of β-methyltryptophan was developed using carboxypeptidase A digestion of the *N*-trifluoroacetyl derivatives. The stereochemical assignment was confirmed by X-ray crystal structure determination of (2*S*,3*R*)-*threo*-β-methyl-L-tryptophan. (2*S*,3*S*)-*erythro*-β-Methyl-L-kynurenine is a slow substrate for kynureninase from *Pseudomonas fluorescens* ($k_{cat}/K_m = 0.1\%$ that of L-kynurenine), producing anthranilic acid, while (2*S*,3*R*)-*threo*-L-kynurenine is about 390-fold less reactive than *erythro*. Rapid-scanning stopped-flow measurements show that β-methyl substitution affects the rate of α-deprotonation of the L-kynurenine-pyridoxal-5′-phosphate Schiff's base. This is consistent with the stereoelectronic requirements of the reaction. These results are the first demonstration that β-substituted kynurenines can be substrates for kynureninase, and may be useful in the design of mechanism-based inhibitors.

Introduction

L-Kynurenine is an intermediate in the catabolism of L-tryptophan in plants, animals, and in some species of bacteria, including *Pseudomonas fluorescens*.[1] In bacteria, the hydrolytic cleavage of L-kynurenine to give L-alanine and anthranilic acid is catalysed by kynureninase [EC 3.7.1.3] (eq (1)). In plants and animals, a similar enzyme preferentially cleaves 3-hydroxykynurenine in the biosynthetic pathway to NAD^+.[2] The kynurenine pathway in mammals ultimately results in the biosynthesis of quinolinic acid, a neurotoxic compound which has been implicated in the etiology of a number of neurodegenerative diseases, including Huntingdon's chorea and AIDS-related dementia.[3–10]



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Key words: Kynureninase; β-methyl-L-kynurenine; β-methyl-L-tryptophan; pyridoxal-5′-phosphate; carboxypeptidase.
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Previously, we examined the interaction of *P. fluorescens* kynureninase with the diastereomers of dihydrokynurenine.[11] We found that the (4*R*)-isomer of dihydrokynurenine is a substrate for a retro-aldol cleavage reaction, while the (4*S*)-isomer is a potent competitive inhibitor, with a K_i value of 0.3 μM. This potent inhibition was suggested to be due to the similarity of these compounds to a postulated *gem*-diolate intermediate. These results prompted us to prepare and evaluate a series of *S*-aryl-L-cysteine *S,S*-dioxides as kynureninase inhibitors, due to their similar structures to the proposed intermediate.[12] The most potent of these latter compounds, *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide, has a K_i value of 0.070 μM, providing further evidence in support of a *gem*-diolate intermediate. However, there have been no reports of the substrate or inhibitory activity of kynurenines with β-substituents with kynureninase. We have now prepared both *threo* and *erythro* diastereomers of β-methyl-L-kynurenine, and we have examined them as substrates and inhibitors of kynureninase. We find that the *erythro* isomer of β-methyl-L-kynurenine exhibits significantly greater substrate activity with kynureninase than does the *threo* isomer. This result is consistent with and provides additional support for our previously postulated kynureninase mechanism.[11–14] In the course of these studies, we developed a procedure for the enzymatic

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resolution of the *threo* and *erythro* diastereomers of β-methyl-L-tryptophan.

Results

Synthesis of β-methylkynurenines. The diastereomers of β-methyl-DL-tryptophan were synthesized by a modification of the procedure of Snyder and Matteson.[15] However, to our knowledge, an efficient procedure for the enzymatic resolution of both β-methyltryptophan diastereomers on a preparative scale has not been previously reported, although (2*S*,3*R*)-*threo*-β-methyltryptophan is a natural product. The racemic *threo* and *erythro* diastereomers of *N*-acetyl-β-methyltryptophan were separated by crystallization, then subjected to acid hydrolysis to obtain the respective racemic amino acids.[15] These were then converted to the *N*-trifluoroacetyl derivatives and resolved by carboxypeptidase A-catalysed hydrolysis, as we have utilized before for a number of other tryptophan analogues.[6–8] The asymmetric synthesis of the four isomers of β-methyltryptophan as the N^{In}-mesitylenesulfonyl protected derivatives was reported by Boteju et al.,[16] but the preparation of the free amino acids was not described. The preparation of (2*R*,3*S*)-*threo*-β-methyltryptophan by an asymmetric hydrogenation reaction has been recently reported by Hoerner et al.[17] Based on the rotation of $+27.1°$ reported by Hoerner for (2*R*,3*S*)-*threo*-β-methyltryptophan of 91% ee, our observed rotation for this product of $+29.8°$ indicates that our material has greater than 99% ee. The stereochemical assignment of the β-methyl-L-tryptophans was confirmed by X-ray crystal structure determination of (2*S*,3*R*)-*threo*-β-methyl-L-tryptophan (Fig. 1). Both diastereomers of β-methyl-L-kynurenine were then prepared by ozonolysis of the respective β-methyl-L-tryptophans in 1 M HCl. Under these conditions, the initial ozonolysis product, *N*-formyl-β-methyl-L-kynurenine, hydrolyzes rapidly, and the hydrochloride salt of β-methyl-L-kynurenine is obtained by low pressure reverse phase column chromatography and evaporation.

Reaction of β-methylkynurenines with kynureninase. Both isomers of β-methyl-L-kynurenine were found to be weak competitive inhibitors, with K_i values of about 5 mM. The reaction of kynureninase is conveniently monitored by following the absorbance decrease at 360 nm due to loss of the *ortho*-aminophenylketone chromophore. When we performed these measurements with the β-methylkynurenines as substrates, we found that (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine readily showed evidence for reaction (Fig. 2). There is an isosbestic point between the 364 nm peak of the β-methylkynurenine and the 310 nm peak of the anthranilic acid product, indicating that these are the only species in the reaction mixture (Fig. 2). However, the K_m value of kynureninase for (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine is about 4.8 mM, about 200-fold higher than that for L-kynurenine, which is 25 mM.[11] The k_{cat} for (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine, $0.9\,s^{-1}$, is about 15% that of L-kynurenine; thus, k_{cat}/K_m is $195\,M^{-1}\,s^{-1}$, compared to $2\times10^5\,M^{-1}\,s^{-1}$ for L-kynurenine.[13] Hence, the catalytic efficiency of kynureninase has been reduced about 1000-fold by the presence of the *erythro* β-methyl group. In contrast, (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine showed no change in UV spectrum under the reaction conditions of Fig. 2, even with larger amounts of enzyme and with longer periods of incubation. However, when reaction mixtures of (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine were analysed by HPLC with fluorescence detection, time-dependent formation of anthranilic acid was observed in the presence of kynureninase. At a concentration of 2 mM (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine, the apparent rate constant of formation of anthranilic acid is $0.001\,s^{-1}$. Assuming a K_m value of 5 mM, based on the K_i value of the *erythro* isomer as a competitive inhibitor, k_{cat}/K_m for *threo*-β-methyl-L-kynurenine can be estimated as $0.5\,M^{-1}\,s^{-1}$. Thus, the reaction of kynureninase with the β-methyl-L-kynurenines is highly diastereospecific, 390-fold greater for the *erythro* than the *threo* isomer.

Rapid scanning stopped-flow kinetic studies of kynurenine and β-methylkynurenines. When kynureninase is mixed with 1 mM L-kynurenine, a quinonoid intermediate with λ_{max} at 494 nm is completely formed within the dead

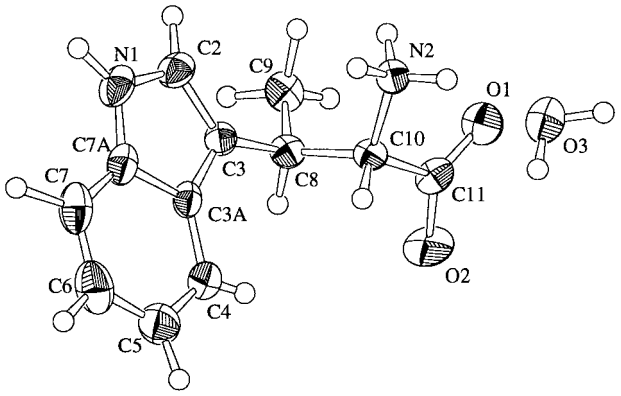


Figure 1. Structure of (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine hydrate.

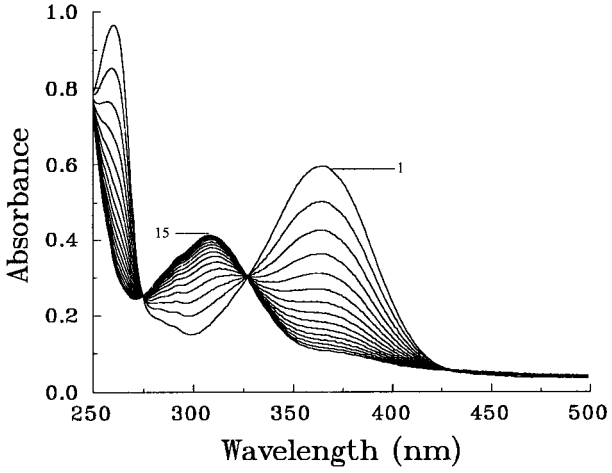


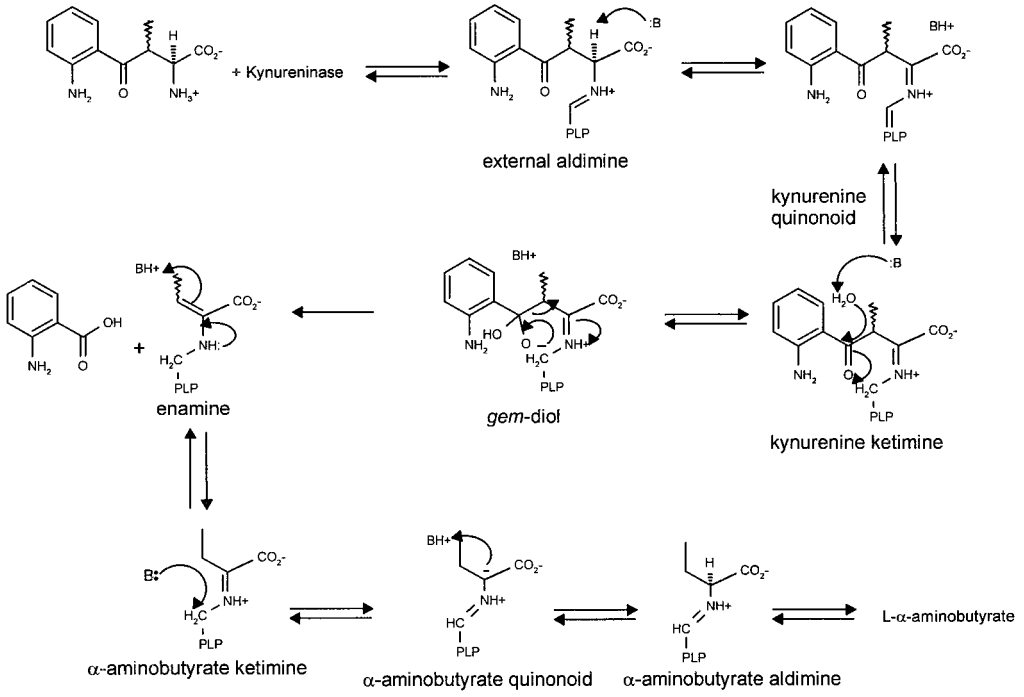
Figure 2. Reaction of (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine with kynureninase. The reaction mixture contained 0.15 mM (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine in 50 mM potassium phosphate, pH 7.8, and 2.8 mM kynureninase at 25°C. Scans were taken every 15 min.

time of the stopped-flow instrument (ca. 2 ms) (Scheme 1). Thus, the rate constant for deprotonation of the α-carbon of the L-kynurenine external aldimine must be greater than 1300 s^{-1}. This quinonoid intermediate then decays rapidly in a biphasic manner, with apparent rate constants of 750 and 20 s^{-1}.[14] The rate determining step in the reaction of L-kynurenine appears to be deprotonation of the pyruvate ketimine intermediate at C4′,[13,14] so there is very little of the quinonoid intermediate observeable in the steady-state of the reaction. In contrast, when 2 mm (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine is mixed with kynureninase, a weakly absorbing peak at 500 nm is formed at an apparent rate constant of 0.05 s^{-1} and persists in the steady-state (data not shown). Thus, the apparent α-deprotonation rate constant has been reduced more than 20,000-fold by substitution of a methyl on the β-carbon of kynurenine. Since the rate constant of quinonoid intermediate formation is comparable in magnitude to the value measured for k_{cat}, α-deprotonation is likely to be at least partly rate determining in the reaction of (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine. When the rapid-scanning stopped-flow experiment was performed in the presence of benzaldehyde, an intermediate with an absorbance peak forms at 496 nm, with a rate constant of 8.5×10^{-3} s^{-1} (Fig. 3). With L-kynurenine, a similar intermediate forms in the presence of benzaldehyde, with a rate constant of 67.4 s^{-1}.[14] Hence, the formation of this intermediate is about 8000-fold slower with (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine. In contrast, the reaction of (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine did not show any measureable absorbance increase at 500 nm over a period up to 2 min, consistent with the extremely slow k_{cat} value for this isomer. Additionally, no absorbance increase at 500 nm was seen for up to 4 h when benzaldehyde was included in incubations. Since there is no detectable formation of quinonoid intermediates absorbing at 500 nm for the *threo* isomer, it is possible that either α-deprotonation, external aldimine formation, or both, is rate-determining for the reaction of (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine.

Discussion

Implications of diastereospecificity for the reaction mechanism

In the present study, we have found that kynureninase is highly stereospecific for reaction with diastereomers of β-methyl-L-kynurenine. Recent pH dependence results suggest that only a single base is involved in the mechanism of kynureninase,[13] in agreement with the results of Palcic et al.[18] Thus, all proton transfers must occur from the same face of the PLP complex. A mechanism for the reaction of β-methyl-L-kynurenines with kynureninase is shown in Scheme 1. After removal of the α-proton from the external aldimine to form the quinonoid intermediate, C-4′ is protonated by the conjugate acid to give a ketime intermediate (Scheme 1). The hydrolytic cleavage of the β–γ C−C bond then takes place. β-Protonation of the enamine intermediate subsequently forms a pyruvate ketimine. In the presence of benzaldehyde, the enamine intermediate can be trapped to form a stable quinonoid intermediate with $\lambda_{max}=496$ nm.[14] The deprotonation of the pyruvate ketimine intermediate at C-4′appears to be rate-determining in the reaction of L-kynurenine.[14,15] The major kinetic effect of the addition of the β-methyl group appears to be on the rate of formation of the quinonoid intermediate. The β-γ carbon–carbon bond must be oriented perpendicular to the plane of the PLP-π-system,



Scheme 1.

according to Dunathan's postulate,[19] for the cleavage reaction to proceed. Thus, there must be a rotation around the α–β bond concomitant with α-deprotonation to orient the aryl group for the subsequent hydration and cleavage. In the case of (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine, this rotation will eclipse the methyl and the carboxylate of the substrate in the transition state, resulting in a large increase in the activation energy for quinonoid intermediate formation (Scheme 2). Based on the differences in k_{cat}/K_m for L-kynurenine and (2*S*,3*R*)-*threo*-β-methyl-L-kynurenine, there is a difference in catalytic efficiency of about 2×10^5. This corresponds to an activation energy difference of 7.2 kcal/mol at 25°C. In a simple model, 2-ethylacrylic acid, the conformation in which the methyl group is eclipsed with the carboxylic acid is 5.3 kcal/mol higher in energy than the gauche conformation, based on molecular mechanics calculations. For (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine, there are no strongly unfavorable eclipsing interactions in the transition state for α-deprotonation. However, the transition state strain due to the presence of the *gauche* methyl group still dramatically reduces the rate of α-deprotonation. This could be due to steric restriction of the enzyme active site.

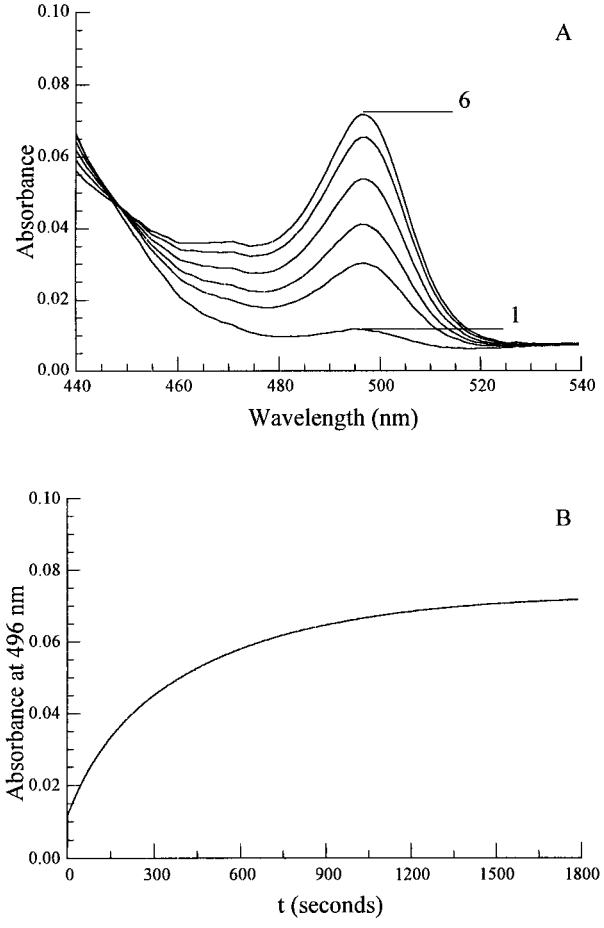


Figure 3. (A) Rapid-scanning stopped-flow spectra of the reaction of 1 mM (2*S*,3*S*)-*erythro*-β-methyl-L-kynurenine and 10 mM benzaldehyde with 7 mM kynureninase in 50 mM potassium phosphate, pH 7.8. Scans were collected at 1 s intervals. The scans shown were collected at (1) 3 s, (2) 120 s, (3) 240 s, (4) 480 s, (5) 960 s, and (6) 1800 s. (B) Time course of the reaction at 496 nm.

Implications for the design of kynureninase inhibitors

Kynureninase is a potential target for the design of drugs for the treatment of neurological disorders, due to its role in the biosynthesis of quinolinic acid, a known neurotoxin.[3–10] Our results demonstrate for the first time that β-substituted kynurenines, especially those with the *erythro* configuration, can be substrates for kynureninase. Replacement of the β-methyl group with other groups that can be activated during catalysis may thus result in specific 'suicide substrates' of kynureninase.

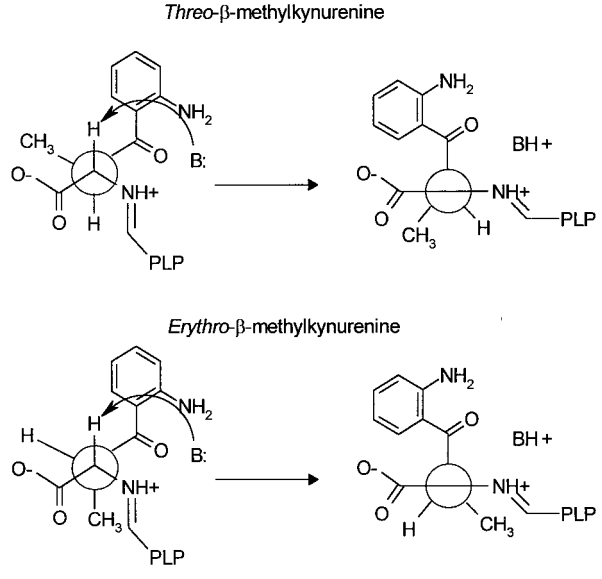
Experimental

General

Melting points are uncorrected. Column chromatography was performed on silica gel (70–230 mesh). All reactions with air and moisture sensitive compounds were conducted in oven dried glassware under an atmosphere of dry nitrogen.

Instruments

^{1}H NMR spectra were recorded at 300 MHz with TMS as an internal standard, and ^{13}C NMR spectra were recorded at 75 MHz with $CDCl_3$ as an internal standard, using a Bruker AC-300 spectrometer. Optical rotations were measured with an Autopol IV from Rudolph Research. UV/visible spectra and enzyme assays were performed with a Cary 1 spectrophotometer. Rapid-scanning stopped-flow studies were performed on an RSM instrument from OLIS, Inc. equipped with a stopped-flow mixer, as described elsewhere.[14] High pressure liquid chromatography (HPLC) was performed on an instrument with two Gilson 502 pumps and a Gilson filter fluorometer detector, using



Scheme 2.

Gilson Unipoint software. The excitation filter had an upper cut-off of 350 nm and the emission filter had a bandpass of 390–600 nm. Molecular mechanics calculations were performed using SPARTAN (Wavefunction, Inc.) running on the Origin2000 in the Research Computing Resource of the University of Georgia. X-ray diffraction data were collected on an Enraf–Nonius CAD4 diffractometer with graphite monochromated Cu-Kα radiation.

Enzymes

Kynureninase was isolated from cells of *Pseudomonas fluorescens* grown on 0.2% L-tryptophan as described previously,[11] or from *E. coli* DH5α containing the plasmid pTZKYN.[20] Carboxypeptidase A was obtained from Sigma as a crystalline slurry in H_2O.

Kinetic measurements

The kynureninase activity was determined by following the decrease in absorbance at 360 nm ($\Delta\varepsilon = -4500\ M^{-1}\,cm^{-1}$) as previously described.[11,13] For *erythro*-β-methylkynurenine, activity was determined at 400 nm due to the high K_m value which led to absorbances greater than 3 at 360 nm. For the stopped-flow measurements, kynureninase was preincubated for 30 min with excess PLP, then passed through a small desalting column (Pharmacia, PD-10) equilbrated with 0.05 M potassium phosphate, pH 7.8 to remove excess PLP. The stopped-flow reactions were then performed in 0.05 M potassium phosphate, pH 7.8 with the syringes in a water bath held at 25°C.

2-Trifluoroacetamido-3-(3-indolyl)-butanoic acid (*N*-trifluoroacetyl-β-methyltryptophan, isomer A). 3.5 g (16.1 mmol) β-methyl-DL-tryptophan (A-isomer),[15] was dissolved in dry methanol (15 mL) in a 50 mL round bottomed flask. Then, triethylamine (2.3 g, 22.6 mmol) and ethyl trifluoroacetate (2.5 g, 18.1 mmol) were simultaneously added. The reaction mixture was stirred overnight at ambient temperature. The solution was poured into water and acidified to pH 1 with concentrated HCl at 0°C. The product was extracted with ethyl acetate and dried over anhydrous $MgSO_4$. The solution was filtered and concentrated to obtain 4.2 g (85%) of crude product (mp 160–163°C). IR (KBr pellet) ν 3395, 31114, 3070, 2973, 1745, 1719, 1689, 1543, 1458, 1414, 1281, 1222, 1168, 1105, 1008, 839, 748, 674. ^{1}H NMR ($CDCl_3$-DMSO) δ 0.81–0.83 (d, 3H, J = 6.86 Hz), 2.47–3.19 (m, 1H), 4.13–4.19 (m, 1H), 6.38–6.51 (m, 3H), 6.74–6.77 (d, 1H, J = 7.92 Hz), 6.97–6.99 (d, 1H, J = 7.58 Hz), 8.37–8.40 (d, 1H, J = 8.51 Hz), 9.96 (s, 1H); ^{13}C NMR ($CDCl_3$-DMSO) δ 170.6, q (156.0, 155.5, 154.8), 152.2, 125.4, 121.2, 120.2, 117.6, 117.3, 114.1, 110.6, 56.7, 31.5, 15.9.

2-Trifluoroacetamido-3-(3-indolyl)-butanoic acid (*N*-trifluoroacetyl-β-methyltryptophan, isomer B). 3.5 g (16.1 mmol) β-methyl-DL-tryptophan (B-isomer)[15] was dissolved in dry methanol (15 mL) in a 50 mL round bottomed flask. Then, triethylamine (2.3 g, 22.6 mmol) and ethyl trifluoroacetate (2.5 g, 18.1 mmol) were added simultaneously. The reaction mixture was stirred overnight at ambient temperature. The solution was then poured into water and acidified to pH 1 with concentrated HCl at 0°C. The product was extracted with ethyl acetate and dried over anhydrous $MgSO_4$. The solution was filtered and concentrated to obtain 4.6 g (91%) of crude product (mp 166–168°C). IR (KBr pellet) ν 3415, 3399, 3306, 2974, 1700, 1552, 1459, 1433, 1198, 1168, 741, 676. ^{1}H NMR ($CDCl_3$-DMSO) δ 1.15–1.18 (d, J = 7.23 Hz), 3.55–3.65 (m, 1H), 4.49–4.54 (dd, J = 8.66, 4.59, 1H), 6.71–6.85 (m, 3H), 7.06–7.09 (d, J = 8.04 Hz, 2H), 7.36–7.39 (d, J = 7.73 Hz), 10.2 (s, 1H); ^{13}C NMR ($CDCl_3$-DMSO) δ 170.5, q (155.9, 155.4), 135.3, 125.3, 121.2, 120.1, 117.7, 117.6, 113.2, 110.5, 56.2, 31.9, 17.2.

(2*S*,3*R*)-2-Amino-3-(3-indolyl)-butanoic acid (β-methyl-L-tryptophan, isomer A). *N*-Trifluoroacetyl-β-methyl-DL-tryptophan (4.9 g, 15.6 mmol) was suspended in water (25 mL) and the pH of the solution was adjusted to 7.5 with triethylamine. Carboxypeptidase A (1.2 mL) was then added and the reaction was stirred for 16 h. The pH of the reaction mixture was then adjusted to 1 with concentrated H_2SO_4. The unreacted *N*-trifluoroacetyl-β-methyl-D-tryptophan was extracted with three 20 mL portions of ethyl acetate. The organic portion was dried, filtered and concentrated to provide 2.2 g of crude material. The water layer was also concentrated to dryness and redissolved in a minimum volume of water. The pH was then adjusted to 4.8–5. The β-methyl-L-tryptophan was obtained as white crystals, 1.37 g (81%), mp 266–268°C. IR (KBr pellet) ν 3436, 3334, 3096, 2975, 2025, 1633, 1585, 1485, 1450, 1388, 1349, 1220, 1159, 1085, 968, 906, 749. ^{1}H NMR (D_2O + DCl) δ 0.47–0.5 (d, 3H, J = 7.44 Hz), 2.86–2.94 (m, 1H), 3.45–3.47 (d, 1H, J = 4.31 Hz), 6.21–6.34 (m, 2H), 6.36 (s, 1H), 6.56–6.59 (d, 1H, J = 8.14 Hz), 6.69–6.72 (d, 1H, J = 7.73 Hz); ^{13}C NMR (D_2O + DCl) δ 170.6, 136.1, 124.9, 123.8, 121.9, 119.1, 117.9, 111.9, 111.8, 56.8, 31.0, 13.4. $[\alpha]_D^{24}$ −30.6° (*c* 1.69, 0.1 M HCl). Anal. calcd for $C_{12}H_{14}N_2O_2.H_2O$: C, 61.0, H, 6.83, N, 11.86. Found: C, 60.89, H, 6.86, N, 11.78.

(2*R*,3*S*)-2-Amino-3-(3-indolyl)-butanoic acid (β-methyl-D-tryptophan, isomer A). The unreacted *N*-trifluoroacetyl derivative (2.2 g, 7 mmol) obtained from the above reaction was suspended in 10 mL of water. Sodium hydroxide was then added (1.2 equiv, 8.4 mmol, 0.336 g). The reaction was stirred for 18 h. The reaction was stopped by adjusting the pH 4.6–5.0 with 4 N sulfuric acid. The product which crystallized from the solution was filtered and dried to provide 1.3 g (85%), mp 246–248°C. IR (KBr pellet) ν 3618, 3417, 3073, 2970, 2612, 2438, 1602, 1534, 1399, 1172, 1093, 921, 799, 751; ^{1}H NMR (D_2O + DCl) δ 0.94–0.96 (d, 3H, J = 6.92 Hz), 3.38–3.48 (m, 1H), 3.90–3.92 (d, 1H, J = 4.37 Hz), 6.61–6.76 (m, 2H), 6.81 (s, 1H), 6.98–7.01(d, 1H, J = 8.32 Hz), 7.16–7.19 (d, 1H, J = 7.67 Hz); ^{13}C NMR (D_2O) δ 170.9, 136.4, 125.2, 124.0, 122.2, 119.4, 118.2, 122.2, 112.0, 56.9, 31.3, 13.6. $[\alpha]_D^{24}$ +29.9° (*c* 1.66, 0.1 M HCl). Anal. calcd for $C_{12}H_{14}N_2O_2.H_2O$: C, 61.0, H, 6.83, N, 11.86. Found: C, 60.91, H, 6.83, N, 11.86.

(2*S*,3*S*)-2-Amino-3-(3-indolyl)-butanoic acid (β-methyl-L-tryptophan isomer B). *N*-Trifluoroacetyl-β-methyl-DL-tryptophan, isomer B, (6.8 g, 21.7 mmol) was suspended in water (30 mL) and the pH of the solution was adjusted to 7.5 with triethylamine. Carboxypeptidase A (1.5 mL) was then added and the reaction was stirred for 16 h. The pH of the reaction solution was then adjusted to 1 with concentrated H_2SO_4. The unreacted *N*-trifluoroacetyl-β-methyl-D-tryptophan was extracted with three 20 mL portions of ethyl acetate. The organic portion was dried, filtered and concentrated to provide 3.2 g of crude material. The water layer was then concentrated down to dryness and redissolved in a minimum volume of water. The pH was then adjusted to 4.8–5.0. The β-methyl-L-tryptophan was obtained as white crystals, 1.27 g (66%), mp 244–246°C. IR (KBr pellet) ν 3400, 3053, 1617, 1458, 1408, 1321, 1246, 1227, 1186, 1098, 1014, 818, 764, 741. ^{1}H NMR (D_2O + DCl) δ 0.64–0.61 (d, 3H, J = 7.34 Hz), 3.011–2.90 (m, 1H), 3.42–3.39 (d, 1H, J = 4.95 Hz), 6.43–6.25 (m, 2H), 6.46 (s, 1H), 6.66–6.63 (d, 1H, J = 8.60 Hz), 6.78–6.75 (d, 1H, J = 7.96 Hz); ^{13}C NMR (D_2O + DCl) δ 170.7, 135.8, 125.1, 123.8, 121.7, 119.0, 118.1, 111.6, 111.2, 57.1, 31.5, 16.8. $[\alpha]_D^{24}$ +47.6° (*c* 1.70, 0.1 M HCl). Anal. calcd for $C_{12}H_{14}N_2O_2$ 2/5H_2O:C, 63.93, H, 6.62, N, 12.42 Found: C, 63.95, H, 6.59, N, 12.47.

(2*R*,3*R*)-2-Amino-3-(3-indolyl)-butanoic acid (β-methyl-D-tryptophan, isomer B). The unreacted *N*-trifluroacetyl derivative (3.2 g, 10.2 mmol) obtained from the above reaction was suspended in 20 mL of water followed by the addition of 1.2 equiv (12.2 mmol, 0.489 g) of sodium hydroxide. The reaction was stirred for 18 h. The reaction was stopped by adjusting the pH 4.6–5 with 4 N H_2SO_4. The product crystallised out upon cooling to give 1.9 g (85%), mp 241–243°C. IR (KBr pellet) ν 3396, 3052, 2936, 2495, 2032, 1615, 1458, 1409, 1320, 1227, 1186, 1098, 1021, 922, 818, 742; ^{1}H NMR (D_2O + DCl) δ 0.89–0.92 (d, 3H, J = 7.33 Hz), 3.20–3.31 (m, 1H), 3.67–3.69 (d, 1H, J = 5.14 Hz), 6.49–6.65 (m, 2H), 6.72 (s, 1H), 6.87–6.91 (d, 1H, J = 8.29 Hz), 7.02–7.05 (d, 1H, J = 7.95 Hz); ^{13}C NMR (D_2O-DCL) δ 171.1, 136.1, 125.4, 124.1, 122.1, 119.3, 118.4, 111.9, 111.6, 57.4, 31.9, 17.1. $[\alpha]_D^{24}$ −49.0° (*c* 1.65, 0.1 M HCl). Anal. calcd for $C_{12}H_{14}N_2O_2$.1/2.H_2O:C, 63.42, H, 6.65, N, 12.33. Found: C, 63.46, H, 6.37, N, 12.36.

(3*R*,2*S*)-*threo*-β-Methyl-L-kynurenine hydrochloride (A isomer). The β-methyl-L-tryptophan A-isomer (0.3 g, 1.38 mmol) was dissolved in 10 mL of 1 M HCl, and ozone was passed through the solution in a slow stream. The reaction was followed using UV-spectroscopy, monitoring the indole absorbance at 280 nm. When the reaction was complete, the reaction mixture was evaporated to dryness. The crude product was chromatographed on a low pressure C18 reverse-phase silica gel column in aqueous methanol to provide 0.19 g (53%) of light-yellow solid after evaporation. IR (KBr pellet) ν 3436, 3334, 3096, 2974, 2025, 1633, 1585, 1485, 1450, 1388, 1349, 1220, 1159, 1085, 968.5, 906.6, 749.8. ^{1}H NMR (D_2O + DCl) δ 1.02–1.05 (d, J = 7.59 Hz, 3H), 3.89–3.91 (d, J = 3.43 Hz, 1H), 4.02–4.11 (m, 1H), 6.57–6.64 (m, 1H), 6.66–6.69 (d, J = 8.3 Hz, 1H), 7.17–7.24 (m, 1H), 7.64–7.67 (d, J = 8.27 Hz, 1H); ^{13}C NMR (DMSO) δ 205.9, 173.0, 153.9, 136.2, 132.2, 118.7, 116.3, 115.9, 57.4, 35.9, 12.9. MS (ESI) *m*/*z* 223 ($M + H^+$). UV (H_2O), λ_{max} 227 nm (log ε = 4.29), 290 nm (log ε = 3.82), 364 nm (log ε = 3.61).

(3*S*,2*S*)-*erythro*-β-Methyl-L-kynurenine hydrochloride (B isomer). The β-methyl-L-tryptophan B-isomer (0.3 g, 1.38 mmol) was dissolved in 10 mL of 1 M HCl, and ozone was passed through the solution in a slow stream. The reaction was followed using UV-spectroscopy monitoring the decrease in indole absorbance at 280 nM. When the reaction was complete, the solution was evaporated to dryness. The crude product was chromatographed on a low pressure C18 reverse-phase silica gel column in aqueous methanol and evaporated to give a light-yellow solid (0.19 g, 53%). IR (KBr pellet) ν 3486, 3464, 3366, 3340, 3162, 2853, 2572, 2019, 1652, 1576, 1484, 1388, 1553, 1216, 1159, 979, 744. ^{1}H NMR (D_2O) δ 7.56–7.59 (d, 1H, J = 7.78 Hz), 7.12–7.17 (m, 1H), 7.01–7.06 (m, 1H), 6.89–6.92 (d, 1H, J = 8 Hz), 3.84–3.91 (m, 1H), 3.77–3.79 (d, 1H, J = 4.6 Hz), 0.75–0.77 (d, 3H, J = 7.5 Hz); ^{13}C NMR (D_2O) δ 203.8, 170.1, 135.6, 131.7, 130.0, 129.8, 126.3, 125.5, 54.0, 41.5, 13.4. MS (ESI) *m*/*z* 223 ($M + H^+$). UV (H_2O), λ_{max} 227 nm (log ε = 4.19), 290 nm (log ε = 3.72), 364 nm (log ε = 3.58).

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