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Synthesis of 2S-[2-^{2}H]-Kynurenine and Use in Kinetic Isotope Effect Studies with Kynureninase[1]

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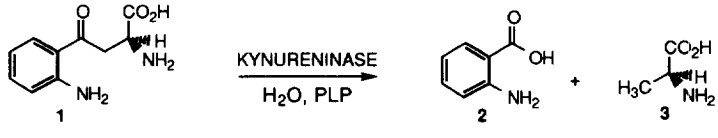
Abstract: 2S-[2-^{2}H]-Kynurenine has been synthesised by two different routes. Diacetylation/racemisation of racemic kynurenine in deuterium oxide followed by acylase catalysed resolution provides the most direct method. The alternative is to prepare 2S-[2-^{2}H]-tryptophan by a similar procedure and then convert this through to 2S-[2-^{2}H]-kynurenine *via* ozonolysis. Preliminary results on measurements of the primary deuterium isotope effect, and solvent isotope effect, are described.

Kynureninase (EC 3.7.1.3) is a pyridoxal 5'-phosphate (PLP) dependent enzyme which catalyses the β,γ-cleavage of kynurenine **1** to give anthranilic acid **2** and L-alanine **3** (Scheme 1).[2] The enzyme plays a key regulatory role on the neurologically important tryptophan metabolic pathway.[3,4] Quinolinic acid, which is a potent neurotoxin, is one of the biosynthetic products of the pathway and has been implicated as an important etiological factor in various neurodegenerative disorders including epilepsy, Huntington's disease and AIDS-related dementia.[5] As part of our mechanistic studies on this enzyme we required 2S-[2-^{2}H]-kynurenine in order to determine the primary deuterium isotope effect.



Scheme 1

The synthesis of 2S-[2-^{2}H]-kynurenine **4** had not been previously reported. Acetylation and racemisation of amino acids in deuterium oxide affords a simple method of introducing deuterium into the C-2 position. This procedure was carried out using commercial R,S-kynurenine (Scheme 2). Reaction with acetic anhydride and sodium deuteroxide in deuterium oxide resulted in acetylation of both of the amino groups along with exchange at the 2-position, *via* the azlactone intermediate, to give racemic *N,N'*-diacetyl RS-[2-^{2}H]-kynurenine **5**, in 98% yield. Resolution was then achieved using the acylase from *Aspergillus meleus*, which

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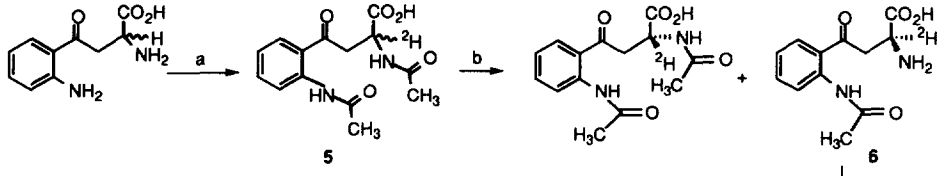
has been reported to be more effective with aromatic amino acids than pig liver acylase.[6] This was the first time diacetyl kynurenine had been employed as a substrate for this acylase. The reaction was monitored by NMR and appeared to proceed smoothly. Interestingly the enzyme did not hydrolyse the aromatic acetyl group and therefore gave *N'*-acetyl-2S-[2-^{2}H]-kynurenine **6** as the product in 63% isolated yield. Separation was carried out using ion-exchange (Dowex 1), the acetyl group was removed using 2M HCl and the free amino acid isolated using propylene oxide to trap excess HCl. Chiral hplc showed an enantiomeric excess of 98% (see Figure 1) and confirmed that the product was indeed the 2S-[2-^{2}H]-kynurenine, demonstrating that the enzyme showed its normal specificity for the 2S-amino acid.[7]



a) NaO^2H, Ac_2O, ^2H_2O, 98%; b) Acylase from *Aspergillus meleus*, 63%;
c) Separation by Ion Exchange, using Dowex 1
d) 2M HCl, 1 hour then IPA, propylene oxide, 26%.

Scheme 2

The only disadvantage of this procedure is the high cost of kynurenine, 50% of which is by necessity not used during the reaction. Therefore an alternative was also examined. The most convenient synthetic route to 2S-kynurenine is *via* the ozonolysis of protected 2S-tryptophan, which cleaves the double bond of the pyrrole

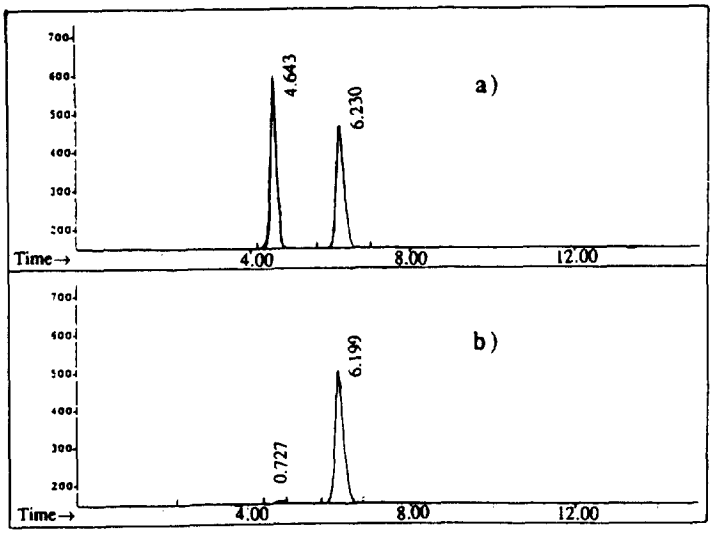


Figure 1: HPLC analysis of 2S-[2-2H]-Kynurenine. Conditions; Crownpak CR(+) (150 mm x 4 mm id, 5 μM) using 2.5% methanol in aq. HClO4 at pH 2 (premixed) at 1 ml min-1 and 50 °C.
a) Commercial R,S-kynurenine (Sigma Chemical Co. Ltd.);
b) 2S-[2-2H]-kynurenine. Ratio of peak areas gives ee at 97.4%.

ring to directly give the N'-formyl kynurenine.[8,9] Firstly, 2S-[2-^{2}H]-tryptophan **7** was prepared from 2S-tryptophan using the racemisation/acetylation procedure followed by resolution with acylase, as above, in 42% overall yield. This material was also analysed by chiral hplc and shown to have an enantiomeric excess of 99% (Figure 2).

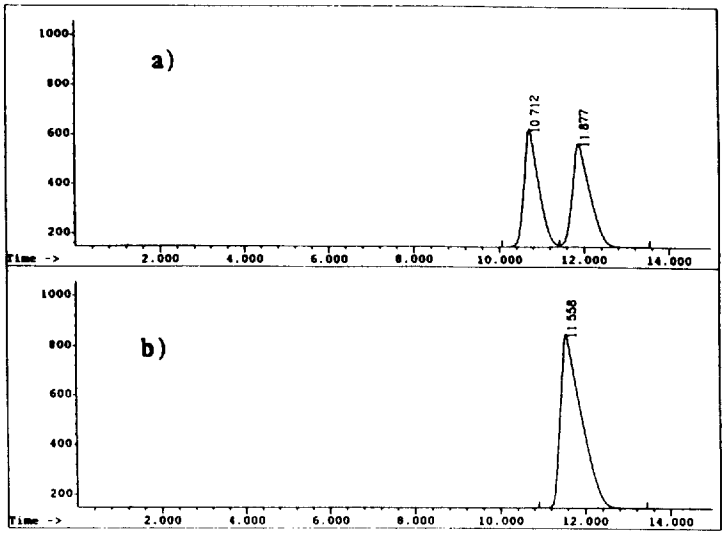
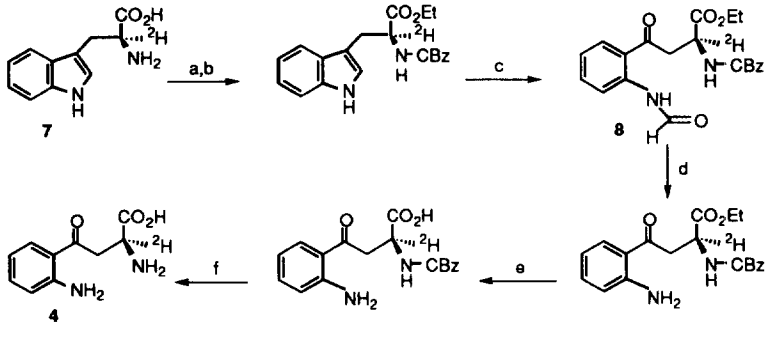


Figure 2: HPLC analysis of 2S-[2-2H]-Tryptophan. Conditions; Crownpak CR(+) (150 mm x 4 mM id, 5 μm) using 2.5% methanol in aq. HClO4 at pH 2 (premixed) at 1 ml min-1 and 50 °C.
a) Commercial R,S-tryptophan (Sigma Chemical Co. Ltd.);
b) 2S-[2-2H]-tryptophan. Ratio of peak areas gives ee at 99%.

The carboxylic acid and amino groups of the 2S-[2-^{2}H]-tryptophan were then protected as the ethyl ester and *N*-carbobenzyloxy derivatives, respectively, in 60% overall yield. Although the procedure for conversion of this material through to kynurenine has been reported, there are very few experimental details[10]



a) EtOH, $SOCl_2$, 72%; b) $PhCH_2OCOCl$, H_2O, Na_2CO_3, 82%; c) O_3, MeOH, -78 °C, 33%;
d) HCl/Dioxan,98%; e) LiOH, H_2O/THF, 95%; f) H_2, Pd-C, 45%,

Scheme 3

and so it was necessary to optimise the reaction conditions. The ozonolysis was performed in methanol solution at -78 °C for one hour to give the protected *N'*-formyl 2S-[2-^{2}H]-kynurenine **8** in 33% isolated yield. The three

protecting groups could then be removed sequentially. The *N*-formyl group was removed under acid conditions and the ethyl ester hydrolysed using lithium hydroxide in a 1:1 mixture of THF and water. Finally the amino group was deprotected by hydrogenation over palladium on charcoal to afford the 2S-[2-^{2}H]-kynurenine **4**. The material was identical in all respects to that obtained from the first method, with an enantiomeric excess of 98%.

Two complementary routes are thus presented for the synthesis of 2S-[2-^{2}H]-kynurenine, both of which give material of comparable purity and enantiomeric excess. The most appropriate for a particular application will depend on the availability of kynurenine and whether further elaboration is required. Direct synthesis from racemic kynurenine is the shorter route, giving an overall yield of 8%, but the starting material is expensive. The synthesis of kynurenine from tryptophan is longer but begins from the much cheaper tryptophan. The overall yield is 8% from 2S-[2-^{2}H]-tryptophan, but 4% from 2S-tryptophan. It also offers the additional advantage that it produces a protected compound from which the three protecting groups can be selectively removed.

The 2S-[2-^{2}H]-kynurenine was then employed to determine the primary deuterium kinetic isotope effect for the enzyme catalysed reaction using a preparation of kynureninase isolated from *Pseudomonas fluorescens* (Strain ATCC 11250).[11] The reaction was followed spectrophotometrically, monitoring the decrease in the absorbance due to kynurenine at 360 nm. The 2S-[2-^{2}H]-kynurenine exhibited an extinction coefficient (4516 mol dm^{-3} cm^{-1}) identical to that of the unlabelled material within experimental error, confirming its purity. Reactions were carried out under the same conditions employed for the standard kynureninase assay[11] (0.04 M potassium phosphate buffer at pH 7.0, in the presence of 40 mM PLP at 25 °C). The kinetic parameters are summarised in Table 1.

Table 1: Summary of Kinetic Data

	2S-Kynurenine	**2S-[2-^{2}H]-Kynurenine**	**2S-Kynurenine[a]**
K_m (μM)	25.5 ± 0.6[c]	93.6 ± 5.3	26.5 ± 0.6
V_{max} [b] (10^{-3} mol dm^{-3} min^{-1})	1.07 ± 0.01	1.09 ± 0.02	0.24 ± 0.01

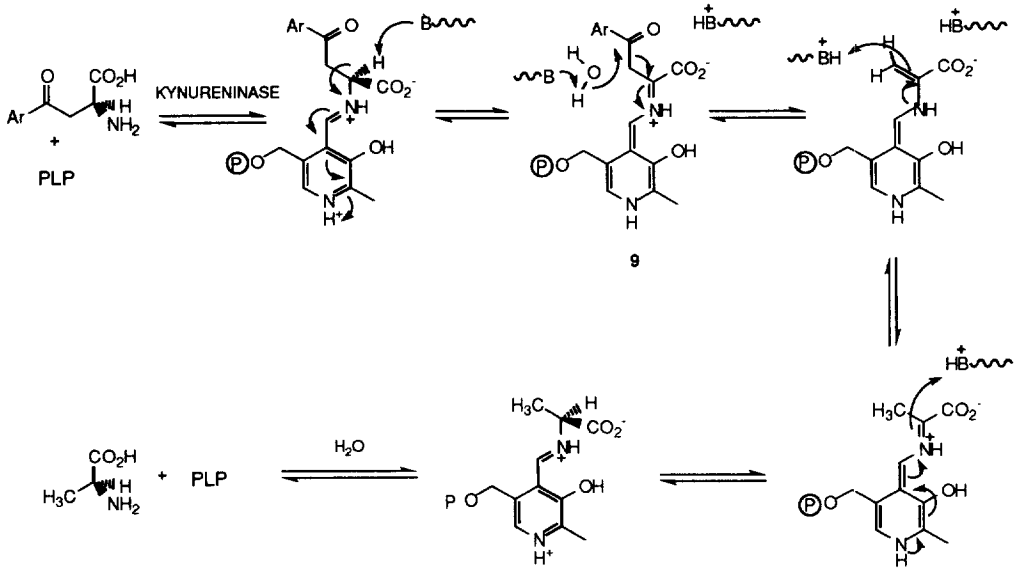
a - Reaction carried out in deuterium oxide

b - Corrected for One unit of enzyme under standard assay conditions.

c - The K_m value for kynurenine is in good agreement with literature values e.g. Ref. 12

The kinetic data for 2S-[2-^{2}H]-kynurenine corresponds to a primary deuterium kinetic isotope effect of 3.6 on V/K, but only 0.98 on V. This implies that cleavage of the C-H bond to form the Schiff's base **8** (Scheme 4) is partially rate-limiting for the reaction catalysed by kynureninase. However, as the isotope effect is expressed only on V/K and not on V, the isotope effect must be being suppressed by a subsequent slow step in the mechanism. This is in contrast to the related PLP dependent enzyme, tryptophan synthase,[12] where an

isotope effect of 4.0 is observed for both V/K and V. In this case cleavage of the C-H bond is cleanly rate limiting. The solvent deuterium isotope effect was also determined by measuring the kinetic parameters for 2S-kynurenine in deuterium oxide. The results (See Table 1) gave a solvent isotope effect of 4.4 on V/K and 4.6 on V. This demonstrates that a proton transfer step is also partially rate limiting and as the isotope effect on V is not suppressed, there are no subsequent slow steps insensitive to changes in solvent. It can be seen from the putative mechanism in Scheme 4 that there are a number of proton transfer steps that could be rate limiting, including the general base catalysed attack of water at the carbonyl group. Attack by a water molecule under general base catalysis from the enzyme is thought to be the most likely mechanism consistent with current evidence,[4] although the reaction could also be mediated by an enzymic nucleophile and occur *via* a covalent intermediate. Further experiments are underway to examine these isotope effects in more detail. These include an examination of the variation of the isotope effects with changes in pH and also the interdependence of the two isotope effects.



Scheme 4

EXPERIMENTAL

General. Optical rotations were measured at room temperature using an Optical Activity Ltd. AA 1000 polarimeter. Infrared spectra were recorded on a Perkin-Elmer series 1420 IR spectrometer. NMR spectra were recorded on a Bruker AM-300 f.t. spectrometer (^{1}H, 300 MHz; ^{13}C, 74.76 MHz) and a Varian Gemini f.t. spectrometer (^{1}H, 200 MHz; ^{13}C, 50.31 MHz). ^{1}H NMR spectra were referenced on chloroform, TMS, methanol or d^6-DMSO, ^{13}C NMR spectra were referenced on chloroform, methanol or d^6-DMSO. Mass spectra and accurate mass measurements were recorded on a Kratos MS50, obtained on an EPSRC service basis at the University of Swansea using a VG ZAB E and at Merck Sharp and Dohme Research Laboratories using a VG

Quattro. Elemental analyses were carried out in the departmental microanalytical laboratory. Flash chromatography was performed according to the procedure of Still[13] using Sorbisil C60 (40-60 mm) silica gel and Kieselgel 60. Ozonolysis was carried out using a Fischer Ozon-generator 500. Assays were carried out using a UVICON 932 spectrophotometer. Solvents were dried and purified according to the methods of Perrin and Armarego.[14] *Pseudomonas fluorescens* (Strain ATCC 11250) was obtained from NCIB in Aberdeen.

N, N'-Diacetyl-[2-^{2}H]-kynurenine (5).

(2*RS*)-Kynurenine (1 g, 4.8 mmol) was dissolved in deuterium oxide (10 ml). Sodium (0.46 g, 0.02 mol) was added carefully after washing in petroleum ether and ethanol. Acetic anhydride (3 ml, 31.8 mmol) was added slowly, and the solution was shaken vigorously. The solution was then stirred at room temperature for three hours. Sulphuric acid (1 M, 10 ml) was added and the solution concentrated at reduced pressure. The residue was extracted with dichloromethane (4 x 50 ml). The combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give a pale yellow solid (1.17 g, 98%), m.p. 194 °C (Lit. (for non-deuterated compound)[15] 198 °C), υ_{max} (nujol)/cm^{-1} 3320 (OH), 1675 (CO acid), 1645 (CO amide); δ_H (200 MHz; d^6-DMSO) 1.85 (3H, s, NHOC$\underline{H}_3$), 2.15 (3H, s, NHOC$\underline{H}_3$ aromatic), 4.70 (1H, d, *J* 7.5 Hz, N$\underline{H}$, aromatic), 7.25 (1H, t, *J* 10 Hz, 4'$\underline{H}$), 7.62 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 8.00 (1H, d, *J* 10 Hz, 6'$\underline{H}$), 8.21 (1H, d, *J* 7.5 Hz, 3'$\underline{H}$), 8.28 (1H, s, N$\underline{H}$); δ_C (50.31 MHz; d^6-DMSO) 22.64 (s, NHCO$\underline{C}$H$_3$), 24.96 (s, NHCO$\underline{C}$H$_3$ aromatic), 41.57 (s, β-$\underline{C}$), 48.27 (s, α-$\underline{C}$), 121.26, (s, 3'$\underline{C}$), 123.42 (s, 5'$\underline{C}$), 124.96 (s, 1'$\underline{C}$), 130.78 (s, 6'$\underline{C}$), 134.28 (s, 4'$\underline{C}$), 138.85 (s, 2'$\underline{C}$), 169.08, 169.42 (NH$\underline{C}$OCH$_3$), 173.20 (s, $\underline{C}$O$_2$H); *m/z* (CI) 310 ([*M*+NH4]$^+$, 17%), 293 (100, [*M*+H]$^+$), 275 (24, [*M*+H-H$_2$O]$^+$), 162 (5, [*M*+H-CH$_2$C(CO$_2$H)(^{2}H)NHCOCH$_3$]$^+$).

N'-Acetyl-(2*S*)-[2-^{2}H]-kynurenine (6).

N, N''-Diacetyl-[2-^{2}H]-(2*RS*)-kynurenine (0.2 g, 0.68 mmol) was added to water (10 ml) and solution was effected by adjusting the pH to 7.5 with concentrated NH_4OH. Acylase from *Aspergillus meleus* (100 mg, 0.47 units/mg) was added and the solution incubated at 38 °C for eighteen hours. Examination by tlc (cellulose, IPA: conc. aq NH_3: H_2O; 3: 6: 10) gave no indication if the reaction was complete. A further portion of acylase (100 mg) was added and the reaction incubated for eighteen hours. As the two tlcs were comparable an NMR was run which showed that the reaction had occurred to completion. The acylase and other protein fragments were filtered off and the product evaporated to approximately one ml. Ion exchange column chromatography was carried out using strongly basic anion exchanger (DOWEX 1). The product was obtained as a pale yellow solid (0.045 g, 62.5%); m.p. 156 °C; *m/z* (Found: [*M*+H]$^+$ 252.1102. $C_{12}H_{13}O_4N_2{}^2H$ requires 252.1095); υ_{max} (nujol)/cm^{-1} 3320 (OH), 1680 (CO acid), 1650 (CO amide); δ_H (200 MHz; ^2H_2O) 2.05 (3H, s, NHCOC$\underline{H}$3), 3.55 (2H, s, β-C$\underline{H}_2$), 7.18 (1H, t, *J* 7.5 Hz, 4'$\underline{H}$), 7.45 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 7.70 (2H, 2 x d, *J* 10 Hz, 6'3'$\underline{H}$); δ_C (50.31 MHz; ^2H_2O) 26.42 (s, NHCO$\underline{C}$H$_3$ aromatic), 42.91 (s, β-$\underline{C}$), 53.21 (s, α-$\underline{C}$), 125.93 (s, 3'$\underline{C}$), 127.91 (s, 5'$\underline{C}$), 128.94 (s, 1'$\underline{C}$), 133.10 (s,6'$\underline{C}$), 137.44 (s, 4'$\underline{C}$), 139.29 (s, 2'$\underline{C}$), 175.66 (s, $\underline{C}$O$_2$H); *m/z* (FAB) 252 ([*M*+H]$^+$, 100%), 274 (50.5, [*M*+Na]$^+$).

(2*S*)-[2-^{2}H]-Kynurenine (4).

*N'*Acetyl-(2S)-[2-^{2}H]-kynurenine (0.14 g, 0.56 mmol) was dissolved in 2 M HCl (15 ml) The solution was heated under reflux for one hour then evaporated to dryness under reduced pressure. Isopropanol (1.25 ml) was added containing a small amount of *RS*-dithiothreitol. The dark orange solid which resulted was neutralised with

propylene oxide (0.15 g, 2.75 mmol). Diethyl ether was added and a pale yellow solid precipitated out of solution. After filtration more solid was seen to precipitate out of the diethyl ether. On filtering this became a yellow oil and was taken up in water and the resulting pale yellow solution was freeze dried to give (0.05 g, 42%) of a yellow solid; m.p. 188 °C; *m/z* (Found: $[M+H]^+$ 210.0986. $C_{10}H_{11}N_2O_3{}^2H$ requires 210.0989); $[\alpha]^{20}_D$ +25.5° (c 0.5 in H_2O); υ_{max} (nujol)/cm^{-1} 3320 (OH), 1730 (CO), 1650 (CO acid); δ_H (200 MHz; ^2H_2O) 3.75 (2H, s, β–C$\underline{H}_2$), 7.30 (1H, d, *J* 7.5 Hz, 3'$\underline{H}$), 7.40 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 7.62 (1H, t, *J* 7.5 Hz, 4'$\underline{H}$), 8.02 (1H, d, *J* 7.5 Hz, 6'$\underline{H}$); δ_C (74.76 MHz; ^2H_2O), 36.95 (s, β–$\underline{C}$), 47.98 (s, α-$\underline{C}$), 117.78 (s, 3'$\underline{C}$), 117.89 (s, 5'$\underline{C}$), 129.53 (s, 6'$\underline{C}$), 133.80 (s, 4'$\underline{C}$), 171.07 (s, $\underline{C}O_2H$), 197.73 (s, $\underline{C}OCH_2$); *m/z* (FAB) 210 ($[M+H]^+$, 100%), 232 (35, $[M+Na]^+$). Chiral hplc showed an enantiomeric excess of 97.4%.

_N_-Acetyl (2*SR*)-[2-^{2}H]-tryptophan.

Tryptophan (5 g, 24.5 mmol) was added to ^2H_2O (50 ml) with stirring. Sodium (2.34 g, 105.6 mmol) was added carefully after washing in petroleum ether and ethanol. Acetic anhydride (15 ml) was added to the solution in 5 ml portions, shaking after each addition. The resulting dark orange solution was left stirring for two hours, after which time a white solid had precipitated from solution. A further amount of ^2H_2O (5 ml) was added and the solution was left to stir overnight. The resulting solution was cooled in the refrigerator for several hours and then the crystals were filtered off and washed with cold water. The residue was suspended in 0.2 M HCl, cooled, filtered and washed with cold water until free of the chloride ion. The pale pink crystals obtained (4.55 g, 75%) were dried, m.p. 208 °C (Lit.(for non-deuterated compound)[16] 206-207 °C); υ_{max} (nujol) /cm^{-1} 3380 (indole NH), 1710 (CO ester), 1610 (CO amide); δ_H (200 MHz; d^6-DMSO) 1.85 (3H, s, NHCOC$\underline{H}_3$), 3.05 (2H, dd, *J* 15 Hz, β-C$\underline{H}_2$), 6.95 (2H, m, 5'$\underline{H}$, 6'$\underline{H}$), 7.15 (1H, s, 2'$\underline{H}$), 7.30 (1H, d, *J* 7.5 Hz, 7'$\underline{H}$), 7.50 (1H, d, *J* 7.5Hz, 4'$\underline{H}$), 8.25 (1H, s, indole N$\underline{H}$); δ_C (50.31 MHz; d^6-DMSO) 22.62 (s, $\underline{C}H_3$), 27.27 (s, β-$\underline{C}$), 52.94 (t, α–$\underline{C}$), 110.15 (s, 3'$\underline{C}$), 111.65 (s, 7'$\underline{C}$), 118.37 (s, 5'$\underline{C}$), 118.65 (s, 6'$\underline{C}$), 121.20 (s, 4'$\underline{C}$), 123.78 (s, 2'$\underline{C}$), 127.40 (s, 7a'$\underline{C}$), 136.32 (s, 3a'$\underline{C}$), 169.70 (s, $CH_3\underline{C}O$), 173.81 (s, $\underline{C}O_2H$); *m/z* (CI) 265 ($[M+NH_4]^+$,12%), 248 (100, $[M+H]^+$), 230 (53, $[M+H\text{-}H_2O]^+$), 130 (100, $[CH_2$ ind$]^+$).

(2*S*)-[2-^{2}H]-Tryptophan (7).

N-Acetyl (2*SR*)-2-[^{2}H]-tryptophan (5.14 g, 20.8 mmol) was added to water (200 ml) and the pH adjusted to 7.5 using concentrated ammonia. Acylase from *Aspergillus meleus* (520 mg, 0.47 units/mg) was added and the solution incubated at 38 °C. After a few days it was seen to have reacted to completion by ^{1}H NMR. The acylase was filtered off and the solution reduced in volume. Ion exchange chromatography (DOWEX 1) was carried out and the product fractions freeze dried to give a white solid (2.08 g, 97%), m.p. 232 °C; (Found: C, 64.60; H, 5.5.72; N, 13.61. Calc. for $C_{11}H_{11}N_2O_2{}^2H$: C, 64.375; H, 5.89; N, 13.65%); $[\alpha]^{20}_D$ -10.4° (c 0.5 in H_2O) (lit.,[204] -33.4° (c 1 in EtOH); υ_{max} (nujol) /cm^{-1} 3370 (indole NH), 1645 (CO ester); δ_H (200 MHz; ^2H_2O) 3.20 (2H, dd, *J* 15 Hz, β-C$\underline{H}_2$), 7.00 (1H, t, *J* 7.5 Hz, 5'$\underline{H}$), 7.15 (2H, m, 2'$\underline{H}$, 6'$\underline{H}$), 7.40 (1H, d, *J* 7.5 Hz, 7'$\underline{H}$), 7.60 (1H, d, *J* 7.5 Hz, 4'$\underline{H}$); δ_C (74.76 MHz; d^6-DMSO) 24.40 (s, β-$\underline{C}$), 53.19 (s, α-$\underline{C}$), 105.62 (s, 3'$\underline{C}$), 110.03 (s, 7'$\underline{C}$), 116.54 (s, 5'$\underline{C}$), 117.55 (s, 6'$\underline{C}$), 120.22 (s, 4'$\underline{C}$), 123.10 (s, 2'$\underline{C}$), 124.76 (s, 7a'$\underline{C}$), 134.43 (s, 3a'$\underline{C}$), 172.59 (s, $\underline{C}O_2H$); *m/z* (FAB) 433 ($[2M+Na]^+$, 2%), 411 (9, $[2M+H]^+$), 228 (14, $[M+Na]^+$), 206 (100, $[M+H]^+$), 160 (16, $[M\text{-}CO_2]^+$), 130 (53, $[CH_2$-indole$]^+$).

(2*S*)-[2-^{2}H]-Tryptophan ethyl ester hydrochloride.

(2*S*)-[2-^{2}H]-Tryptophan (3.12 g, 15.16 mmol) was dissolved in dry redistilled ethanol (60 ml) cooled in ice. Redistilled thionyl chloride (1.2 ml, 1.1 eq) was added and the solution was heated to reflux for one hour at approximately 100 °C. The solvent was concentrated at reduced pressure and recrystallised from ethanol to give an off white solid (2.53 g 72%), m.p. 224 °C (Lit. (for non-deuterated compound)[17] 225-226 °C); $[\alpha]^{20}_D$ +12.25° (c 1 in MeOH); υ_{max} (nujol)/cm^{-1} 3250 (indole NH) and 1725 (CO ester); δ_H (200 MHz; d^6–DMSO) 1.10 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.20 (2H, m, β–$C\underline{H}_2$), 4.10 (2H,q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 7.10 (3H, m, 2'H, 5'H, 6'H), 7.60 (1H, d, *J* 7 Hz, 7'H), 7.80 (1H, d, *J* 7 Hz, 4'H), 8.70 (1H, s, indole NH); δ_C (50.31 MHz; d^6–DMSO) 13.96 (s, $CO_2CH_2\underline{C}H_3$), 26.32 (s, β–C), 61.85 (s, $CO_2\underline{C}H_2CH_3$), 106.69 (s, 3'C), 111.79 (s, 7'C), 118.28 (s, 5'C), 118.81 (s, 6'C), 121.39 (s, 4'C), 125.18 (s, 2'C), 127.20 (s, 7a'C), 136.44 (s, 3a'C), 169.56 (s, $\underline{C}O_2Et$); *m/z* (EI) 233 ([*M*+H]$^+$, 10%), 130 (100, [CH_2-indole]$^+$) 103 (13, [*M*+H-CH_2-indole]$^+$).

***N*-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-tryptophan ethyl ester.**

(2*S*)-Tryptophan ethyl ester hydrochloride (3.54 g, 15.2 mmol) was dissolved in water (80 ml). Sodium carbonate (2.2 eq, 33.40 mmol, 3.52 g) was added followed by benzylchloroformate (1.2 eq, 18.24 mmol, 2.6 g, 2.7 ml) The solution was left to stir overnight at room temperature. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether 3: 7). A pale brown precipitate was seen and the solid was filtered off to give (4.54 g, 82%) of an off white solid, m.p. 76-78 °C (Lit.(for non-deuterated compound)[18] 84-85 °C); $[\alpha]^{20}_D$ -9.0° (c 1 in EtOH); υ_{max} (nujol)/cm^{-1} 3300 (indole NH), 1720 (ester CO) and 1680 (amide CO); δ_H (200M Hz; d^6–DMSO) 1.10 (3H, m, $CO_2CH_2C\underline{H}_3$), 3.10 (2H, m, β–$C\underline{H}_2$), 4.05 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 5.05 (2H, s, $C\underline{H}_2Ph$), 7.05 (2H, m 5'H, 6'H), 7.15 (1H, s, 2'H), 7.35 (6H, m, 7'H, aromatic), 7.55 (1H, d, *J* 7 Hz, 4'H) 7.80 (1H, s, NH indole); δ_C (50.31 MHz; d^6–DMSO) 14.20 (s, $CO_2CH_2\underline{C}H_3$), 27.11 (s, β–C), 60.72 (s, $CO_2\underline{C}H_2CH_3$), 65.66 (s, $\underline{C}H_2Ph$), 109.84 (s, 3'C), 111.71 (s, 7'C), 118.24 (s, 5'C), 118.68 (s, 6'C), 121.23 (s, 4'C), 124.11 (s, 2'C), 127.30 (s, 7a'C), 127.88, 128.05, 128.60 (3x s, aromatic), 136.37 (s, quat. aromatic), 137.19 (s, 3a'C), 157.22 (s, $COCH_2Ph$), 172.49 (s, $\underline{C}O_2Et$); *m/z* (EI) 367 (*M*$^+$, 28%), 216 (10, [*M*-$PhCH_2OCONH_2$]$^+$), 130 (100, [CH_2-indole]$^+$), 77 (15, [Ph]$^+$).

***N*'Formyl, *N*-(benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine ethyl ester (8).**

N-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine ethyl ester (2.2 g, 2.0 mmol) was dissolved in dry distilled methanol (100 ml). The solution was cooled to -78 °C with stirring and then treated with ozone for one hour, the reaction was then quenched with dimethyl sulphide (10 ml). The reaction was checked by tlc (silica, ethyl acetate: dichloromethane, 1: 9) and left to stir for a further hour at -78 °C. The solution was then evaporated to a dark orange oil. The product was dissolved in diethyl ether (50 ml) and washed with brine (2 x 100 ml) and water (2 x 100 ml). The solution was then dried ($MgSO_4$) and evaporated under reduced pressure to an orange oil. Purification was carried out by column chromatography (silica, ethyl acetate: petroleum ether, 1: 2) giving the product (786 mg, 33%), m.p. 111-112 °C; (Found: C, 63.35; H, 5.81; N, 6.96; [*M*+H]$^+$ 400.1619. $C_{21}H_{21}N_2O_6{}^2H$ requires C, 63.15; H, 5.55; N, 7.01%; [*M*+H]$^+$ 400.1613); $[\alpha]^{20}_D$ +3.2° (c 1 in MeOH); υ_{max} (nujol)/cm^{-1} 3305 (indole NH), 1725 (ester CO), and 1680 (amide CO); δ_H (200 MHz; d^6–DMSO) 1.15 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.45 (2H, s, β–$C\underline{H}_2$), 4.10 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 5.05 (2H, s, $C\underline{H}_2Ph$), 7.25 (1H,t, *J* 7 Hz, 5'H); 7.35 (5H, m, aromatic), 7.65 (1H,t, *J* 7 Hz, 4'H), 7.75 (1H, s, NHCOH), 7.80 (1H, d, *J*

7 Hz, 3'H), 8.00 (1H, d, *J* 7 Hz, 6'H), 8.45 (1H, m, NHCOH); δ_C (74.76 MHz; d^6–DMSO) 14.03 (s, $CO_2CH_2\underline{C}H_3$), 41.16 (s, β–C), 60.91 (s, $CO_2\underline{C}H_2CH_3$), 65.64 (s, $\underline{C}H_2Ph$), 121.25 (s, 3'C), 123.45 (s, 5'C), 123.87 (s, 1'C), 127.82, 127.94, 128.43 (3x s, aromatic), 130.94 (s, 6'C), 134.37 (s, 4'C), 136.93 (s, quat. aromatic), 137.99 (s, 2'C), 155.92 (s, $PhCH_2O\underline{C}O$), 161.34 (s, NH$\underline{C}$OH), 171.57 (s, $\underline{C}O_2Et$), 200.07 (s, $Ph\underline{C}OCH_2$); *m/z* (CI) 417 ([*M*+NH_4]$^+$, 100%), 400 (55, [*M*+H]$^+$), 372 (6, [*M*+H-CO]$^+$), 356 (10, [*M*-NHCO]$^+$), 309 (32, [*M*+H-CH_2Ph]$^+$), 292 (12, [*M*-O_2CH_2Ph]$^+$), 108 (19, [$PhCH_2OH$]$^+$).

***N*-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine ethyl ester.**

The protected kynurenine (362 mg, 0.91 mmol) was dissolved in methanol (45 ml). 4 M HCl (4 ml, 3.44 mmol) and dioxane (15 ml) were added to the solution. The solution was stirred at room temperature for one hour. It was poured into saturated $NaHCO_3$ (120 ml) and extracted into ethyl acetate (150 ml). The organic extracts were then washed with water (2 x 50ml). They were dried ($MgSO_4$), filtered and evaporated at reduced pressure to obtain a yellow oil (33 mg, 98%) which crystallised on standing, m.p. 60 °C; (Found: [*M*+H]$^+$ 372.1673. $C_{20}H_{22}N_2O_5D$ requires 372.1670); υ_{max} (nujol)/cm^{-1} 1725 (ester CO) and 1680 (carbamate CO); δ_H (300 MHz; d^6–DMSO) 1.15 (3H, t, *J* 7 Hz, $CO_2CH_2C\underline{H}_3$), 3.40 (2H, m, β–C$\underline{H}_2$), 4.10 (2H, q, *J* 7 Hz, $CO_2C\underline{H}_2CH_3$), 5.10 (2H, s, C$\underline{H}_2$Ph), 6.65 (1H, t, *J* 7 Hz, 5'H), 6.80 (1H, d, *J* 7 Hz, 6'H), 7.15 (2H, s, N$\underline{H}_2$), 7.30 (1H, t, *J* 7 Hz, 4'H), 7.35 (5H, m, aromatic), 7.55 (1H, s, NH) 7.70 (1H, d, *J* 7 Hz, 3'H); δ_C (74.76 MHz; d^6–DMSO) 13.91 (s, $CO_2CH_2\underline{C}H_3$), 60.63 (s, $CO_2\underline{C}H_2CH_3$), 65.50 (s, $\underline{C}H_2Ph$), 114.46 (s, 3'C), 116.30 (s, 1'C), 116.98 (s, 5'C), 127.62, 127.76, 128.30 (3x s, aromatic), 131.09 (s, 6'C), 134.33 (s, 4'C), 136.95 (s, quat. aromatic), 151.22 (s, 2'C), 155.83 (s, $PhCH_2O\underline{C}O$), 171.77 (s, $\underline{C}O_2Et$), 197.75 ($Ph\underline{C}OCH_2$); *m/z* (CI) 372 ([*M*+H]$^+$, 100%), 264 (57, [*M*-$PhCH_2O$]$^+$), 108 (15, [$PhCH_2OH$]$^+$).

***N*-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine.**

N-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine ethyl ester (291 mg, 0.78 mmol), was dissolved in a 1: 1 mixture of THF and water (14 ml) and treated with lithium hydroxide monohydrate (91 mg, 2.18 mmol). The solution was warmed at 70 °C for four hours. The reaction was followed by tlc (silica, ethyl acetate: petroleum ether 1: 2). The solution was poured into 1 M HCl (7 ml) and extracted into ethyl acetate (125 ml). It was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow oil. This was then dissolved in ethanol and concentrated under reduced pressure to give a bright yellow solid (267 mg, 95%); m.p. 169-170 °C; (Found: [*M*+H]$^+$ 344.1365. $C_{18}H_{18}N_2O_5D$ requires 344.1357); $[\alpha]^{20}_D$ + 44.8° (c 0.13 in MeOH); υ_{max} (nujol)/ cm^{-1} 1725 (ester CO) and 1680 (amide CO); δ_H (300 MHz; d^6–DMSO) 3.40 (2H, m, β–C$\underline{H}_2$), 5.05 (2H, s, C$\underline{H}_2$Ph), 6.55 (1H, t, *J* 7 Hz, 5'H), 6.75 (1H, d, *J* 7 Hz, 6'H), 7.10 (2H, br s, N$\underline{H}_2$), 7.25 (1H, t, *J* 7 Hz, 4'H), 7.35 (5H, m, aromatic), 7.40 (1H, m, NH), 7.70 (1H, d, *J* 7 Hz, 3'H); δ_C (74.76 MHz; d^6–DMSO) 65.58 (s, $\underline{C}H_2$Ph), 114.65 (s, 3'C), 116.51 (s, 1'C), 117.11 (s, 5'C), 127.73, 127,87, 128.43 (3x s, aromatic), 131 22 (s, 6'C), 134.42 (s, 4'C), 137.06 (s, quat. aromatic), 151.28 (s, 2'C), 156.01 (s, $PhCH_2O\underline{C}O$), 173.40 (s, $\underline{C}O_2H$), 198.21 ($Ph\underline{C}OCH_2$), *m/z* (EI) 343 (*M*$^+$, 5%), 235 (5, [*M*-$PhCH_2OH$]).

(2*S*)-[2-^{2}H]-Kynurenine (4).

N-(Benzyloxycarbonyl)-(2*S*)-[2-^{2}H]-kynurenine (235 mg, 0.69 mmol) was dissolved in methanol (15 ml) and 5% Pd/C (235 mg) was added. This was hydrogenated overnight at atmospheric pressure and filtered through a celite bed. The methanol was evaporated under reduced pressure and isopropanol (1.50 ml) was added

containing a small amount of *RS*-dithiothreitol. The dark orange solid which resulted was neutralised with propylene oxide (0.18 g, 3.39 mmol). Diethyl ether was added and a pale yellow solid precipitated out of solution. After filtration more solid was seen to precipitate out of the diethyl ether. On filtering this became a yellow oil and was taken up in water and the resulting pale yellow solution was freeze dried to give (0.05 g, 42%) of a yellow solid (65 mg, 45%). The material was identical in all respects to that obtained from the first method, with an enantiomeric excess of 98%.

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