THE PREPARATION OF TRITIATED *E*- and *Z*-4-AMINOBUT-2-ENOIC ACIDS, CONFORMATIONALLY RESTRICTED ANALOGUES OF THE INHIBITORY NEUROTRANSMITTER 4-AMINOBUTANOIC ACID (GABA)

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SUMMARY

A synthesis of [^{3}H]*E*- and [^{3}H]*Z*-4-aminobut-2-enoic acids from methyl 4-*N*-phthalimidobut-2-ynoate by catalytic hydrogenation using tritium gas in the presence of a homogeneous catalyst, *tris*(triphenylphosphine)rhodium(I) chloride, is reported. HPLC separation of the *E*- and *Z*-isomers, and the saturated analogue, 4-aminobutanoic acid (GABA), is also described.

KEY WORDS

CACA, GABA, TACA, homogeneous catalyst, HPLC

INTRODUCTION

4-Aminobutanoic acid, [γ-aminobutyric acid (GABA)] **1** acts as an inhibitory neurotransmitter at receptor sites in the mammalian central nervous system. There are two generally accepted classes of GABA receptors: $GABA_A$ receptors which are sensitive to bicuculline and insensitive to baclofen; and $GABA_B$ receptors, which are insensitive to bicuculline and sensitive to baclofen (1). Information regarding active conformations of GABA at these receptor sites has been derived from conformationally restricted analogues (2). Of the open chain analogues, *E*- and *Z*-4-aminobut-2-enoic acids **2** and **3**, which are commonly referred to as TACA (*trans*-aminocrotonic acid) and CACA (*cis*-

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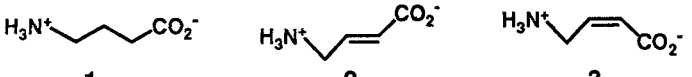
aminocrotonic acid), respectively, exhibit significant pharmacological activities (2-4). The *E*-isomer (TACA) **2** is potent at $GABA_A$ and active at $GABA_B$ receptors while the *Z*-isomer (CACA) **3** is much less active at $GABA_A$ and has no effect on $GABA_B$ receptors (4). It is therefore possible that CACA could be used as a selective agonist (5) for a new class of bicuculline- and baclofen-insensitive Non-$GABA_A$ Non-$GABA_B$ (NANB) (6) receptors. Consequently, a synthesis of [^{3}H]CACA was sought to aid in the characterisation of this putative receptor class.



1 **2** **3**

Both *E*- and *Z*-4-aminobut-2-enoic acids **2** and **3** have been synthesised by Allan *et al* (7,8). The synthesis via *N*-phthalimidobut-2-ynoic acid **4** is amenable to tritium labelling because the starting material for tritiation **4** carries only one labile hydrogen and esterification removes this acidic proton therefore eliminating isotopic dilution by exchange reaction. Moreover, this procedure also gave the less thermodynamically stable *Z*-isomer as a major product (8).

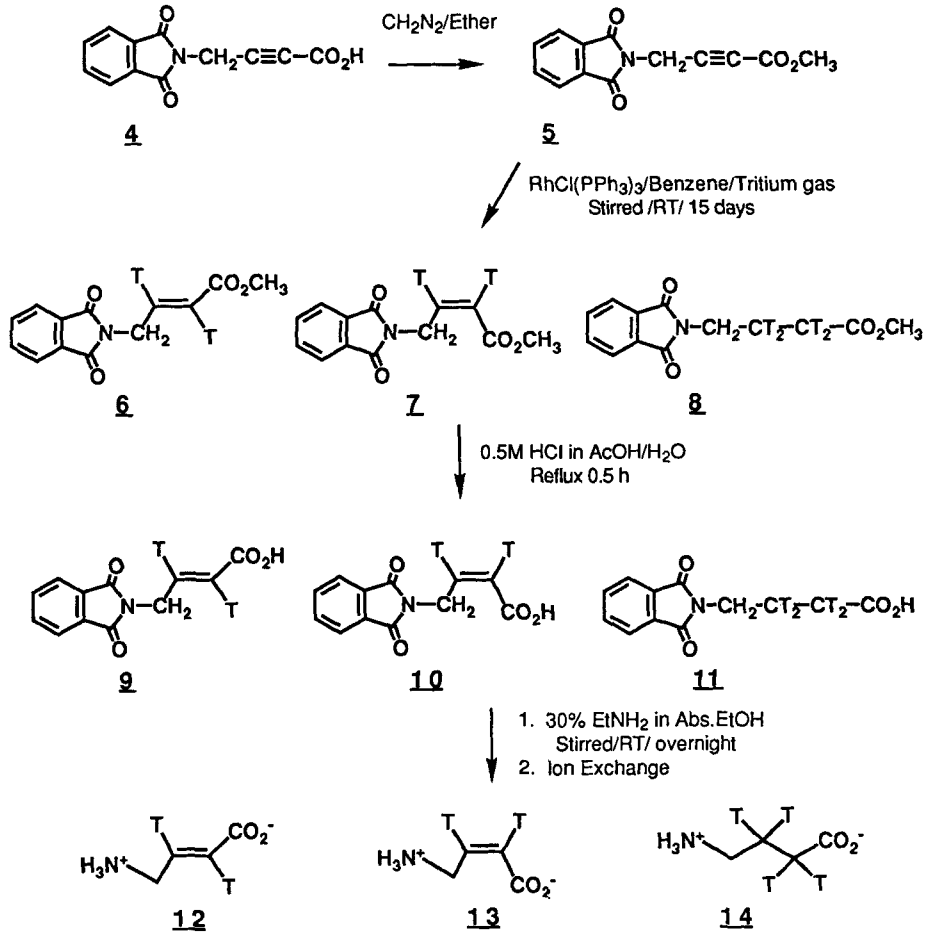
The use of *tris*(triphenylphosphine)rhodium(I) chloride in catalytic tritiation is well documented for steroids (9). It is a catalyst of choice for the reduction of acetylenic and olefinic bonds where specific labelling and low incidence of isotope scramblings are required (10,11). In addition, it provides uniform addition of tritium across the α,β-unsaturated carbons which heterogeneous catalyst fails to achieve (12). Moreover, the exchange rate with the solvent is very slow compared to the rate of substrate hydrogenation when *tris*(triphenylphosphine)rhodium(I) chloride is used as catalyst (13-15). We anticipated that its ability to catalyse the reduction of acetylenic bonds faster than the corresponding olefinic bonds (13,16) would enable the preparation of tritiated *E*-**2** and *Z*-4-aminobut-2-enoic acid **3** with minimal over-reduction.

RESULTS AND DISCUSSION

The outline of the synthesis of [^{3}H]*E*- and [^{3}H]*Z*-4-aminobut-2-enoic acids **12** and **13** is shown in Figure 1. As anticipated, the homogeneous catalyst *tris*(triphenylphosphine)-rhodium(I) chloride proved to be the catalyst of choice for this reduction. The

incorporation of tritium (determined by liquid scintillation counting) into benzene and into the crude residue following vacuum transfer of the solvent was 4.7 and 290 mCi respectively, suggesting that the reduction of the starting material **5** by tritium gas had occurred with very little exchange between tritium gas and benzene. About 15% of the tritium gas was incorporated into the reaction mixture.

Figure 1.



4 **5** **6** **7** **8** **9** **10** **11** **12** **13** **14**

Following preliminary short column vacuum chromatography (17) purification, the mass spectrum of the mixture contained peaks at m/z(%) 252(15.3), 250(50), 248(24.9), 246(6.9) and 244(7.3) indicating the addition of up to 4 tritium atoms (MH^+ 252) with the highest contribution coming from the ion at m/z 250 (3 tritium atoms). The ^3H n.m.r. spectrum of the mixture indicated that there were 3 tritiated products assignable to

compounds **6**, **7** and **8**; the assignments and the coupling constants are shown in Table 1. The coupling constants of 17 Hz in the first pair of doublets and 13 Hz in the second pair were consistent with the *E*- and *Z*- arrangements respectively of geminal tritons bonded to the sp^2 hybridised carbons in **6** and **7** while the coupling constant of 8.3 Hz was well within the range of couplings between geminal tritons bonded to the sp^3 hybridised carbons in **8**. The absence of significant levels of impurities in the ^3H n.m.r. spectrum exemplified the high degree of selectivity of the catalyst (13-16) and its low propensity to promote label scrambling (10,11).

Table 1. TRITIUM NMR DATA

Compound number	Resonances δ(ppm), multiplicities, J(Hz)	Assignment	Integration	% Tritiated product[#1]
6	δ 5.91, d, J = 17	3-CT	7.9[#2]	15
	δ 6.93, d, J = 17	2-CT	1.7[#3]	
7	δ 5.94, d, J = 13	3-CT	7.9[#2]	50
	δ 6.16, d, J = 13	2-CT	5.3[#3]	
8	δ 1.94, t, J = 8.3	3-CT_2	7.9[#4]	35
	δ 2.19, t, J = 8.3	2-CT_2	8.4[#4]	

[#1]Calculated from integration, [#2]Overlapping resonances at δ 5.91.and δ 5.94 unsuitable for percentage calculation, [#3] Values used in calculation, [#4]Averaged values used in calculation

The ^1H n.m.r. spectrum showed no absorptions in the olefinic regions suggesting that the olefins in the mixture were predominantly olefins doubly labelled with tritium (ca.98%). Table 2. shows the assignments of the resonances in the allylic and propargylic region. The presence of an *E*- double bond in **6** and a *Z*- double bond in **7** was confirmed by the appearance of the respective allylic resonances.

Table 2. PROTON NMR DATA (Allylic and propargylic region)

Compound number	Resonances δ(ppm), multiplicities	Assignment	Integration	Ratio of compounds **5**, **6**, **7**
5	δ 4.40-4.42, brd	C≡C-CH_2	3.8	26
6	δ 4.17-4.19, m	CT=CT-CH_2	2.8	19
7	δ 4.90-4.93, m	CT=CT-CH_2	8.2	55

To minimise concomitant *Z*- to *E*- isomerisation of the double bond, hydrolysis of the ester function was initially carried out under alkaline conditions. However, alkaline hydrolysis also caused partial cleavage of the phthalimido group which complicated the subsequent reaction in that the partly opened phthalimido ring was not removed by ethylamine. This complication was not encountered when the ester group was hydrolysed under mild acid conditions. The products from mild acid hydrolysis, without further purification other than removal of HCl, were reacted with ethylamine for the removal of the phthalimido protecting group to give a mixture of products containing free amino acids.

The mixture was fractionated on cationic exchange resin (Dowex 50) to give a mixture (36 mCi) containing neutral amino acids. Lipophilic impurities were removed from the mixture by short column vacuum chromatography (17) using reversed-phase silica. The mixture containing amino acids was eluted first with water and lipophilic impurities were eluted with increasing concentrations of acetonitrile. The first and second water fractions which contained the majority of materials (34 mCi), as assessed from the radioactivity determined by liquid scintillation counting, were combined as the major fraction, as were the third and fourth fractions (minor fraction) which contained a minor amount (1.5 mCi). Model experiments with unlabelled 4-aminobutanoic acid **1** and *E*- and *Z*-4-aminobut-2-enoic acids **2** and **3** indicated that these amino acids eluted mainly in the first two fractions.

Table 3. HPLC ANALYSES

FRACTIONS FROM REVERSED-PHASE SHORT COLUMN VACUUM CHROMATOGRAPHY

Compounds (Order of elution)	Retention time (10 μm EXSIL) (min.)	% in major fraction	Retention time (5 μm EXSIL) (min.)	% in minor fraction
Impurities	5.5, 9, 12, 16	14	6,12	55
[^{3}H]*Z*-4-Aminobut--2-enoic acid **13**	21	5	29	19
[^{3}H]4-Aminobutanoic acid **14**	26	74	36	10
[^{3}H]*E*-4-Aminobut -2-enoic acid **12**	37.5	7	47	16

HPLC analyses (Table 3.) showed that both major and minor fractions contained all the expected amino acids, with a variation in the amount of [^{3}H]4-aminobutanoic acid **14** present, being highly elevated in the major fraction (74%) and much depleted in the minor fraction (10%) indicating partial separation of [^{3}H]4-aminobutanoic acid **14** from its [^{3}H]unsaturated analogues **12** and **13**. While the major fraction contained fewer and lesser amounts of radiochemical impurities formed during the deprotection steps (14% in relation to the amount of amino acids present), the minor fraction contained considerably more (55%). This result showed that reversed-phase short column vacuum chromatography not only removed the less polar impurities but also resulted in partial separation of the amino acids.

HPLC separation of [^{3}H]4-aminobutanoic acid **14** and [^{3}H]*E*- and [^{3}H]*Z*-4-aminobut-2-enoic acids **12** and **13** were carried out using aminopropyl silica columns which have weak ion-exchange properties. Conditions for the HPLC separation of **14**, **12** and **13** were established using unlabelled amino acids. The unsaturated amino acids **2** and **3** were detectable at 230 nm, while the saturated analogue **1**, almost UV transparent, was detected by derivatisation with *o*-phthalaldehyde then analysed fluorometrically (18). There were no significant differences in the elution profiles between the columns packed with HYPERSIL Amino and EXSIL Amino. However, changing particle size from 10 μm to 5 μm caused some increase in retention times with no appreciable improvement in the separation between the amino acids.

All three tritiated amino acids eluted within the the established retention time ranges, while radioactive material in fractions containing [^{3}H]*E*- and [^{3}H]*Z*-4-aminobut-2-enoic acids **12** and **13**, coeluted with the added unlabelled amino acids **2** and **3** respectively, and each corresponded with the appearance of an absorption at 230 nm. As expected, fractions containing [^{3}H]4-aminobutanoic acid **14** were transparent at this wavelength. Each tritiated amino acid cochromatographed with the corresponding unlabelled derivative on t.l.c. After HPLC separation, tritiated amino acids were further purified by ion exchange chromatography to remove substances still detectable in the mass spectrum of the amino acid. Much of the products were lost in the removal of the protecting groups and during the various purification procedures. It is estimated that

quantities of the purified amino acids were as follows: [^{3}H]TACA **12** 4.8 mCi, [^{3}H]CACA **13** 3.4 mCi, and [^{3}H]GABA **14** 49.8 mCi.

Specific activities (Table 4) of the amino acids **12**, **13** and **14** were determined by mass spectrometry. The mass spectra of both [^{3}H]TACA **12** and [^{3}H]CACA **13** contained two principal peaks; both isomers showed a base peak at m/z 89(100%), (MH^+-OH) with the second peak at m/z 88(25%,TACA), (27%, CACA), (MH^+-H_2O). These peaks are 4 mass units higher than the corresponding peaks for the unlabelled compounds and thus indicate high incorporation of tritium at both positions (C-2 and C-3). The specific activities were calculated from the measurement of the relative abundances of m/z 89, 87 and 85 ions. In contrast, in the case of [^{3}H]GABA **14**, three large peaks corresponding to MH^+-H_2O were observed at m/z 94(100%), 92(73), and 90(55), indicating incorporation of 4, 3 and 2 tritium atoms respectively, at C-2 and C-3. The specific activity was calculated from the measurement of the relative abundances of m/z 94, 92, 90, 88 and 86 ions.

Table 4. SPECIFIC ACTIVITY AND RADIOCHEMICAL PURITY

	Specific activity (Ci/mmol)	Radiochemical purity t.l.c.
[^{3}H]*E*-4-Aminobut-2-enoic acid ([^{3}H]TACA) **12**	52.6	91
[^{3}H]*Z*-4-Aminobut-2-enoic acid ([^{3}H]CACA) **13**	55.8	88
[^{3}H]4-Aminobutanoic acid ([^{3}H]GABA) **14**	83.3	48

During the HPLC purification of tritiated amino acids, unexpected burning sensations to the face, eyes and hands were experienced by personnel handling the samples. The "burning" sensations were accompanied by physical symptoms of redness, tautness and soreness so that the general appearance of the affected areas was similar to a moderate dose of sunburn. The reason for the "burns" has not been established. However, the reaction may involve a sensitisation to unknown agents, possibly tritiated amino-acrylamides (*E*- and *Z*-*N*-ethylacetamido-4-aminobut-2-enoate) and *N*-ethylacetamido-butanoate, in the HPLC fractions so that the person becomes extremely sensitive to light, heat and chemicals during and following the exposure. As contact with the tritiated amino acids decreased, the effect diminished and the degree of sensitisation appeared to be related to the degree of contact. There has, to date, been

no prolonged period without contact so it is not known for how long this condition will persist.

CONCLUSION

The method proved successful for the preparation of the tritiated amino acids with high specific activities but lengthy purification procedures are required and recovery of tritiated material is low. Modification should also be made to avoid the presence of the amino-amides. The products, **9**-**11**, from hydrolysis should be purified initially with an anion exchange resin such as Dowex 1 before reacting with ethylamine. Because of sensitisation of the workers, this procedure should be adopted only with great care.

EXPERIMENTAL

^{1}H n.m.r. spectra were measured at 300 MHz on either a Varian Gemini broadband spectrometer or a Bruker CXP-300 spectrometer. The ^{13}C n.m.r. spectrum was measured on a Varian Gemini broadband spectrometer operating at 75.46 MHz with broadband proton decoupling. Chemical shift values are given in ppm relative to internal tetramethylsilane (TMS). ^{3}H n.m.r. spectra were obtained on a Bruker CXP-300 operating at 320 MHz with broadband proton decoupling as well as off-resonance proton decoupling. Samples were sealed in 5 mm n.m.r. tubes and doubly encapsulated in 10 mm n.m.r. tubes for safety reasons (19,20). Tritium chemical shifts were measured by ghost-referencing from internal non-tritiated TMS (21). Melting points (uncorrected) were determined on a Reichert hot-stage apparatus. Methane (CH_5^+) chemical ionisation mass spectra (CIMS) were obtained on a Finnigan-Mat TSQ46 system. The microanalysis was carried out by the Australian Microanalytical Service.

The radiochemical purity was determined by t.l.c. (Silica gel 60 F_{254}, butanol / acetic acid / water : 3 / 1 / 1, ninhydrin) followed by liquid scintillation counting. The tritiated amino acids were identified by comparison of t.l.c. (Rf values) and HPLC (retention times) with the corresponding unlabelled authentic sample and by mass spectral analysis.

High Performance Liquid Chromatography (HPLC) was carried out on a Varian Model 5000 Liquid Chromatograph coupled to a Kortec K95 variable wavelength HPLC

UV detector (ICI Instruments) set at 230 nm. Chromatographic peaks were recorded on a Curken chart recorder Model 250-2. For HPLC, a 10 μm GoldPak HYPERSIL Amino column (25 x 4.6 mm i. d.), 5 μm and 10 μm GoldPak EXSIL Amino columns (25 x 4.6 mm i. d.) were used with isocratic elution with 80% acetonitrile and a flow rate of 1 ml/min.

Fluorescence was measured at λ_{Ex} = 340 nm and λ_{Em} = 455 nm on Perkin-Elmer Model MPF 44 B Fluorescence Spectrophotometer.

UV Absorbance was measured at 230 nm on Hitachi U-2000 UV Spectrophotometer.

Liquid scintillation counting with Emulsifier Safe™ Scintillator 299TM Packard Scintillator in a Packard Model 300 CD Liquid Scintillation Spectrometer was used for radioactivity measurements.

4-N-Phthalimidobut-2-ynoic acid **4**

The acid **4** was prepared by the oxidation of the corresponding phthaloyl-protected acetylenic amino alcohol according to an established procedure (22), m.p.172-173°C, [lit m.p. 174-176°C]. m/z (%) 230(28), MH^+; 212(100), MH^+-H_2O; 186(52), MH^+-CO_2. ^1H n.m.r. (δCD_3OD) 4.60, s, CH_2; 7.80, s, 7.81, s, 7.818, s, 7.821, s, 7.83, s, *m*-ArH; 7.866, s, 7.875, s, 7.878, s, 7.884, s, 7.895, s, *o*-ArH. ^{13}C n.m.r. (δCD_3OD) 27.7, CH_2; 75.9, C-2; 82.0, C-3; 124.5, ArC-4 and ArC-5; 133.1, ArC-1 and ArC-2; 135.7, ArC-3 and ArC-6; 155.5, 2 x PhthCO; 168.1, CO_2H.

Methyl 4-N-phthalimidobut-2-ynoate **5**

A solution of diazomethane (50 mmol) in diethyl ether (100 ml) was added dropwise rapidly to a well stirred solution of 4-*N*-phthalimidobut-2-ynoic acid **4** (5.62 g, 24.5 mmol) in methanol (50 ml) cooled to 0°C; stirring was continued overnight at room temperature (ca. 25 °C). Solid which precipitated was filtered off and the filtrate evaporated under reduced pressure to dryness then taken up in dichloromethane. The mixture was filtered and the filtrate evaporated under reduced pressure to dryness to give an oily residue (1.5 g). The residue was fractionated using vacuum chromatography (17) (silica gel H 60, light petroleum 60°-80°C / ethyl acetate : 3/1) to give the required product as a white

crystalline solid. Recrystallisation from cyclohexane-ethyl acetate gave methyl 4-*N*-phthalimidobut-2-ynoate **5** as colourless needles (m.p. 129-131°C, subl, 1.17 g, 19.6 %). Anal. Calcd. for $C_{13}H_9NO_4$: C, 64.20; H, 3.73; N, 5.76. Found C, 64.3; H, 3.7; N, 5.8 %. m/z (%) 272(28), $MC_2H_5^+$; 244(100), MH^+; 212(80), MH^+-CH_3OH. ^1H n.m.r. ($\delta CDCl_3$) 3.76, s, CH_3; 4.60, s, CH_2; 7.75, s, 7.76, s, 7.77, s, 7.78, s, *m*-ArH; 7.880, s, 7.889, s, 7.897, s, 7.907, s, *o*-ArH. ^{13}C n.m.r. ($\delta CDCl_3$) 27.1, CH_2; 53.0, CH_3; 74.7, C-2; 81.0, C-3; 123.7, ArC-4 and ArC-5; 131.8, ArC-1 and ArC-2; 134.4, ArC-3 and ArC-6; 153.2, 2 x PhthCO; 166.5, $\mathbf{C}O_2CH_3$.

CAUTION : All of the following experiments must be carried out in a well ventilated fumehood

[^{3}H]Methyl E- *and* Z-*4*- N-*phthalimidobut-2-enoate* **6** *and* **7** *and [^{3}H]methyl 4*-N-*phthalimidobutanoate* **8**

A solution of methyl 4-*N*-phthalimidobut-2-ynoate **5** (8.8 mg, 0.036 mmol) and *tris*(triphenylphosphine)rhodium(I) chloride (6.8 mg, 0.007 mmol) in anhydrous benzene (freshly distilled from sodium/benzophenone) (3 ml) was frozen, air and adsorbed gases were removed by a vacuum system, using three successive cycles of freezing, evacuation and thawing of the isolated reaction mixture. The resulting mixture (air and absorbed gases removed) was refrozen, then tritium gas (2 Ci, 0.3 atm) was transferred to the reaction flask with a Toepler pump. The flask was isolated, allowed to warm to room temperature (ca. 25°C) and the mixture was stirred for 15 days. Benzene was removed by vacuum line transfer and the residue redissolved in freshly distilled benzene. The residue was partially purified using vacuum chromatography (17), silica gel H60 (4.5 x 3.5 cm i.d.), and stepwise elution using light petroleum 60-80°C from 80% to 30% in ethyl acetate in 10% steps to give a fraction (245 mCi) containing a mixture of products **6**, **7** and **8**. The mixture was evaporated to dryness under reduced pressure, dissolved in light-petroleum 60-70°C (ca. 10 ml) and stored at 4°C. ^1H n.m.r. ($\delta CDCl_3$) 0.7-2.8, mixture of brm, mainly grease contamination; 3.62, s, 3.69, s, 3.71, s, 3.73, s, 3.76, s, a mixture of CO_2CH_3; 4.17-4.19, q, *E*-CH_2CT=CT; 4.40-4.42, brd, $CH_2C{\equiv}C$; 4.90-4.93, q, *Z*-CH_2CT=CT; 7.49-7.52, m, 7.66-7.74, m, 7.81-7.85, m, ArH. ^3H n.m.r. ($\delta CDCl_3$) 1.94, t, J = 8.26 Hz, CH_2CT_2; 2.29, t, J = 8.26 Hz, $CT_2CO_2CH_3$; 5.91, d, J = 17 Hz,

E-$CH_2CT{=}CTCO_2CH_3$; 5.94, d, J = 13 Hz, *Z*-$CH_2CT{=}CTCO_2CH_3$; 6.16, d, J = 13 Hz, *Z*-$CH_2\mathbf{C}T{=}CTCO_2CH_3$; 6.93, d, J = 17 Hz, *E*-$CH_2\mathbf{C}T{=}CTCO_2CH_3$.

*[^{3}H]E-and [^{3}H]Z-4-aminobut-2-enoic acids **12** and **13** and [^{3}H]4-aminobutanoic acid **14***

(i). Hydrolysis of the esters

The partially purified mixture of esters (153.5 mCi) was evaporated to dryness under reduced pressure then glacial acetic acid (15 ml) was added. The resulting homogeneous mixture was stirred at room temperature for 36h. To the solution was added 0.5 M HCl (15 ml) resulting in a slightly cloudy appearance which clarified when the mixture was brought to reflux with stirring for 30 min, during which time the colour gradually changed from colourless to very light brown. The mixture was cooled, then evaporated to dryness under reduced pressure. Residual acetic acid and HCl were removed by three successive cycles of addition of water (ca.10 ml) followed by evaporation under reduced pressure to dryness, giving a brown residue.

(ii). Removal of the phthalimido protecting group

The residue was taken up in 33% ethylamine in ethanol (2 ml) followed by the addition of absolute ethanol (18 ml) giving a clear yellowish brown solution which was stirred at room temperature (ca. 20°C) for 14h. The mixture was evaporated to dryness, water (10 ml) was added and the white precipitate filtered off giving a light brown filtrate.

(iii). Purification of the products

(a). Ion Exchange Chromatography

The filtrate was fractionated by two cycles on Dowex 50 cationic ion exchange resin (14 x 1 cm i.d.), eluting with water (10 x 10 ml), followed by 1M pyridine (20 x 5 ml). Fractions were analysed for radioactivity by liquid scintillation counting. Pyridine fractions 4-7 were combined and evaporated to dryness under reduced pressure. The second ion exchange cycle almost eliminated the radioactivity in the earlier water fraction. Pyridine fractions 5-7 from the second cycle containing neutral amino acids were again combined and evaporated under reduced pressure to dryness. Residual pyridine was removed by repeated addition of water (2 x 15 ml) followed by evaporation

to dryness under reduced pressure, and the residue containing neutral amino acids (36 mCi) was dissolved in water (6 ml) and stored at 4°C.

(b). Reversed-phase short column vacuum chromatography

The partly purified mixture from above (36 mCi) was further fractionated on reversed-phase short column vacuum chromatography [silanized silica gel H60 (25 g, 4.8 x 4.0 cm i. d.)]; eluting in order with water (4 x 50 ml) , 5% acetonitrile in water (50 ml), followed by stepwise elution with acetonitrile in water from 10% to 100% acetonitrile in 10% steps (50 ml fractions). Fractions were analysed for radioactivity by liquid scintillation counting. Water fractions and 5-20% acetonitrile fractions were combined, concentrated under reduced pressure, then refractionated under the same conditions. A second reversed-phase short column vacuum chromatography fractionation further removed small amounts of less polar impurities. Water fractions 1 (28.4 mCi) and 2 (5.3 mCi), 3 (0.9 mCi) and 4 (0.6 mCi) were separately combined and concentrated under reduced pressure for further fractionation by HPLC.

(c). High Performance Liquid Chromatography (HPLC)

Conditions for HPLC separation of tritiated amino acids were established using unlabelled amino acids at a standard 1 ml/min flow rate. Fractions were collected from HPLC, the presence of *E*- **2** and *Z*-4-aminobut-2-enoic acid **3** was determined by UV absorbance measurement at 230 nm, and the presence of 4-aminobutanoic acid **1** by derivatisation with *o*-phthalaldehyde followed by fluorometric analyses (18). The retention times (10 µm HYPERSIL Amino and 10 µm EXSIL Amino) of *Z*-4-aminobut-2-enoic acid **3**, 4-aminobutanoic acid **1** and *E*-4-aminobut-2-enoic acid **2** were found to range between 16-22, 25-32 and 35-45 min, respectively. After each injection, the columns became less efficient, resulting in decreased retention times. When the retention times began to approach the upper acceptable limits, the columns were reactivated by the injection of 300-500 µl of 0.15 M ammonia in methanol (prepared by diluting 1 ml of 15 M aqueous ammonia in 100 ml with methanol) and equilibrated until a stable base line was obtained.

The tritiated mixture was fractionated by HPLC with 1 ml fractions being collected.

Appropriate amino acid fractions (determined by radioactivity and retention times) were pooled, concentrated under reduced pressure and purified again by HPLC under the same conditions then subjected to a final purification by cation exchange chromatography as in *(a)*. The resulting tritiated amino acids were stored in water at 4°C. [^{3}H]*E*-4-Aminobut-2-enoic acid (TACA) **12**, m/z (%) 89(100), 88(25.4), 87(6.4), 86(4.4), 85(7.8), 84(8.4). [^{3}H]*Z*-4-Aminobut-2-enoic acid (CACA) **13**, m/z (%) 89(100), 88(26.6), 87(2.3), 86(1.2), 85(3.2), 84(1.3). [^{3}H]4-Aminobutanoic acid (GABA) **14**, m/z (%) 95(12.4), 94(100), 93(11.8), 92(73.2), 91(18.8), 90(55.4), 88(16.7), 87(12.4), 86(15.9).

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