THE SYNTHESIS AND RESOLUTION OF TRITIATED 4-AMINO-3-PHENYLBUTANOIC ACID ([^{3}H]*R*- and [^{3}H]*S*-β-PHENYLGABA)

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SUMMARY

A synthesis of [^{3}H]*R*- and [^{3}H]*S*-4-amino-3-phenylbutanoic acid ([^{3}H]*R*- and [^{3}H]*S*-β-phenylGABA) from the unsaturated intermediate by catalytic hydrogenation using tritium gas in the presence of palladium on charcoal is reported. The [^{3}H]*R*- and [^{3}H]*S*-diastereoisomers of pantolactone ester of β-phenylGABA were separated by HPLC.

KEY WORDS

β-PhenylGABA, baclofen, $GABA_B$ receptors, resolution, HPLC

INTRODUCTION

The receptors for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) are differentiated on pharmacological grounds into $GABA_A$ and $GABA_B$ receptor subclasses. $GABA_A$ receptors are defined as those which are antagonised by the convulsant alkaloid bicuculline and activated by muscimol and isoguavacine. $GABA_B$ receptors are insensitive to bicuculline and activated by the agonist baclofen (β-(*p*-chlorophenyl) GABA) (1). While the multiplicity of $GABA_A$ receptors is well documented (2,3), the heterogeneity of $GABA_B$ receptors has yet to be well established. However, recent results from electrophysiological and neurochemical studies suggest the existence of $GABA_B$ receptor subtypes (4).

0362–4803/93/080767–09$09.50
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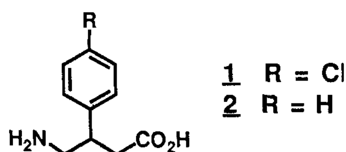
Received 15 March, 1993
Revised 28 March, 1993

The $GABA_B$ receptor agonists baclofen (4-amino-3-(4-chlorophenyl)butanoic acid) **1** and β-phenylGABA (4-amino-3-phenylbutanoic acid) **2**, differing in structure only by the *p*-chloro substituent, are used clinically for different purposes. Baclofen is used as a muscle relaxant and β-phenylGABA is used as a mood elevator and tranquilliser (5). On the isolated guinea-pig ileum preparation, β-phenylGABA and baclofen have been shown to have different actions, suggesting activation of separate populations of receptors which could explain the different therapeutic actions of these compounds (6).

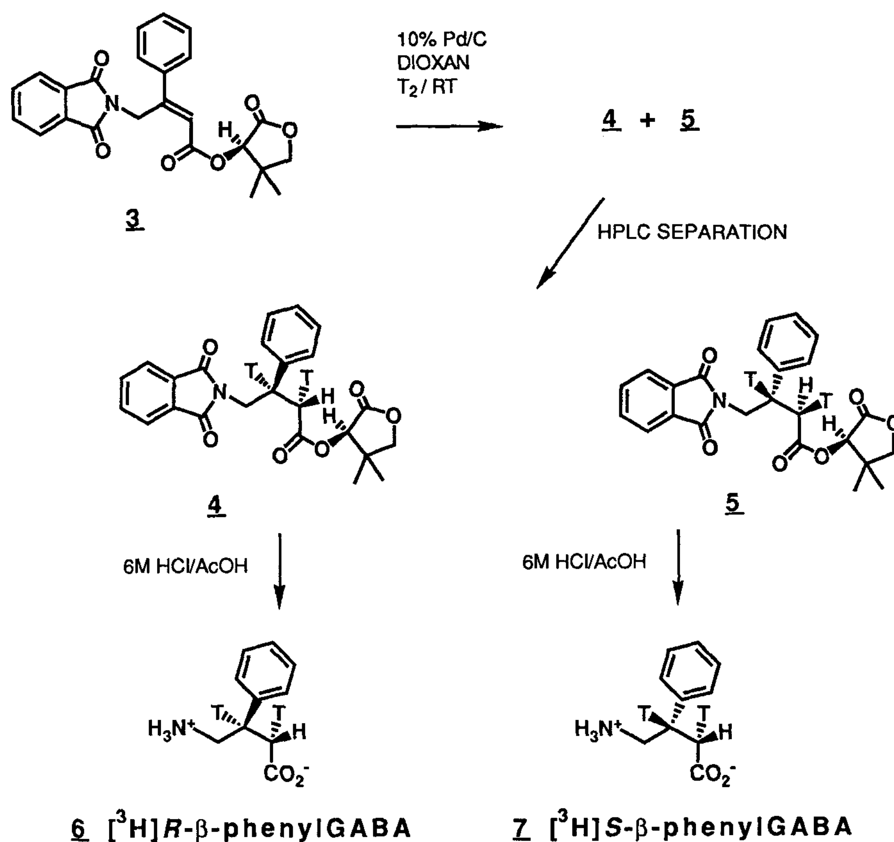


To provide further insight into different subpopulations of receptors, radiolabelled β-phenylGABA was required for binding and autoradiographical studies. [^{3}H]Baclofen has been prepared by catalytic bromine/tritium exchange on 4-(3-bromo-4-chlorophenyl) pyrrolidone (7) and by catalytic hydrogenation using tritium of 4-(4-chlorophenyl)-1,5-dihydro-2*H*-pyrrol-2-one (8). Both methods gave radiolabelled racemic baclofen which needed to be resolved as only the *R*-enantiomer of baclofen is pharmacologically active (9). Since the biological activity of β-phenylGABA also resides in the *R*-enantiomer (10,11), it is preferable that the optical purity of radiolabelled *R*-β-phenylGABA is high. Because of the small amount of material generally involved in the labelling process, resolution of radiolabelled compounds is normally carried out by chromatographic separation of the enantiomers or diastereoisomers. Enantiomeric separation of racemic [^{3}H]baclofen by HPLC using a chiral mobile phase has previously been successful, but this led to a much lower than expected recovery of radioactive product (12). We have recently shown that diastereoisomeric *(R)*-(-)-pantolactone esters (13) of *N*-phthalimido-β-phenylGABA could be separated by HPLC (11). We now report the use of the corresponding radiolabelled diastereoisomers, formed by catalytic reduction of the unsaturated intermediate **3** with tritium gas, as intermediates in the synthesis of [^{3}H]*R*- and [^{3}H]*S*-β-phenylGABA with high optical purity.

RESULTS AND DISCUSSION

Figure 1 outlines the preparation of tritiated resolved β-phenylGABA which involves the synthesis of tritiated diastereoisomers by the reduction of the olefinic bond of the unsaturated intermediate 3 by tritium gas. An advantage of this synthetic method is that the optical purity of the tritiated diastereoisomers 4 and 5 could be ascertained by analyses of the ^{1}H n.m.r. spectrum.

Figure 1.



The ^{3}H n.m.r. spectra of the diastereoisomeric mixture basically showed two resonances (both having small shoulders) at δ 2.59 and δ 3.89 assignable to 2-CT and 3-CT respectively in the diastereoisomers 4 and 5. Small shoulders associated with both of these peaks were accountable in terms of the couplings between two adjacent tritons. Integration suggested that the amount of the products with two tritons on adjacent

carbons was quite small, approximately 5-10 % of the total, indicating that extensive exchange reaction had occurred. The absence of significant levels of impurities in the ^{3}H n.m.r. spectrum indicated high radiochemical purity of the sample. After partial purification by short column vacuum chromatography (14) which removed some impurities as well as some starting material, as indicated by t.l.c. analysis, the mixture was subjected to further purification by HPLC. Within the limit of ^{1}H and ^{3}H n.m.r. analyses, each of the diastereoisomers was free from the other.

Acid hydrolysis removed both the phthalimido and pantolactone ester groups to give the corresponding tritiated amino acid. After purification by cation exchange chromatography followed by reversed-phase HPLC, each amino acid was subjected to cation exchange chromatography once more, mainly to remove the salt content in the buffer. Both [^{3}H]*R*- **6** and [^{3}H]*S*-β-phenylGABA **7** coeluted on HPLC as well as cochromatographed on t.l.c. with the corresponding added unlabelled derivative. The assignment of the tritiated amino acid was confirmed by comparison of the ^{1}H n.m.r. spectra with that of unlabelled β-phenylGABA. Radiochemical purities of the tritiated amino acids **6** and **7** as determined by ^{3}H n.m.r. and t.l.c. are shown in Table 1.

Table 1. RADIOCHEMICAL PURITIES

Amino acid	^{3}H n.m.r. %	t.l.c. %
[^{3}H](-)-*R*- β-phenylGABA **6**	96	95
[^{3}H](+)-*S*- β-phenylGABA **7**	87	84

Apart from the variation due to the tritium isotope, the mass spectra of **6** and **7** were the same as those of the unlabelled compounds. The specific activities (Table 2) of the tritiated diastereoisomers **4** and **5**, and the tritiated amino acids **6** and **7** were calculated from radioactivities determined by liquid scintillation counting and the concentrations obtained from UV spectrophotometry at 220 nm (diastereoisomers) and 219 nm (amino acids).

Table 2. SPECIFIC ACTIVITIES

Diastereoisomer	Specific Activity (Ci/mmol)	Amino acid	Specific Activity (Ci/mmol)
[^{3}H]*R,R*-**4**	1.6	[^{3}H](-)-*R*-β-phenylGABA **6**	1.4
[^{3}H]*R,S*-**5**	1.9	[^{3}H](+)-*S*-β-phenylGABA **7**	1.8

These relatively low specific activities were due possibly to exchange between tritium gas and dioxan and / or by back exchange of the labelled compounds during the reduction step as indicated by the very high radioactivity in the solvent as compared to the radioactivity in the reduction product.

CONCLUSION

The method proved suitable and convenient for labelling and resolution of β-phenylGABA. If amino acid of considerably higher specific activity is required, modifications such as increasing the amount of tritium gas and utilisation of more reactive catalysts would have to be investigated.

EXPERIMENTAL

The radiochemical purity was determined by ^{3}H n.m.r. and by t.l.c. 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.

^{1}H n.m.r. spectra were measured at 300 MHz on a Bruker CXP-300 spectrometer. 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 broad-band 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 (15,16). Tritium chemical shifts were measured by ghost-referencing from internal non-tritiated TMS (17). The ^{1}H and ^{3}H n.m.r. spectra of the amino acids were measured in D_2O and solvent suppression was employed in the measurement of the ^{1}H n.m.r. spectra. The 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.

T.l.c. was carried out on silica gel $60F_{254}$ using UV detection. The solvent systems were light petroleum/ethyl acetate : 4/1 (diastereoisomers) and butanol/acetic acid/water : 4/1/1 (amino acids). Ninhydrin was also used in the visualisation of the amino acids.

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 254 nm. Chromatographic peaks were recorded on a Curken chart recorder Model 250-2.

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

R-*(-)-2-(3,3-Dimethylbutyro-1,4-lactonyl) Z-3-phenyl-4-phthalimidobut-2-enoate* ***3***

The pantolactone ester (PL ester) of the unsaturated precursor of β-phenylGABA **3** was prepared according to an established procedure (11). All spectral data were identical to those published.

*[^{3}H]*R-*(-)-2-(3,3-Dimethylbutyro-1,4-lactonyl) 3-phenyl-4-phthalimidobutanoate* ***4*** *and* ***5***

To a solution of unsaturated pantolactone ester **3** (6.9 mg, 0.016 mmol) in dioxan (freshly distilled from sodium/benzophenone) (2 ml) was added 10 % palladium on charcoal (9.7 mg). The resulting mixture was frozen, and 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) 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 10 days. Dioxan was removed by vacuum line transfer and the residue redissolved in freshly distilled dioxan. The incorporations of tritium (determined by liquid scintillation counting) into dioxan and residue were 1550 and 58.5 mCi respectively. The residue was partially purified using short column vacuum chromatography (14), (silica gel H60 (4.5 x 3.5 cm i.d.), and eluting with 25% ethyl acetate in light petroleum 60-80°C) to give a fraction (48.1 mCi) which contained a mixture of compounds **3**, **4** and **5** as indicated by t.l.c. The mixture was evaporated to

dryness under reduced pressure, dissolved in light-petroleum 60-70°C (ca. 30 ml) and stored at 4°C. ^3H n.m.r. δ(C_6D_6) 2.53, brsh, 2.59, brs, $CHTCO_2PL$; 3.86, brsh, 3.89, brs, PhCT.

*HPLC Separation of the tritiated diastereoisomers **4** and **5***

Conditions for the HPLC separation of the starting material **3**, and the tritiated diastereoisomers **4** and **5** were established with unlabelled compounds using Spheri-5, silica column, (5 μm, 250 mm x 4.6 mm i.d.), isocratic elution with 25% ethyl acetate in light-petroleum at a standard 1 ml/min flow rate. Fractions (1 ml) were collected from HPLC, the presence of **3**, **4** and **5** was determined by UV absorbance measurement at 254 nm. The retention times for the staring material **3**, and diastereoisomers **4** and **5** were found to be 20.0, 21.8 and 25.4 minutes, respectively. The tritiated mixture was fractionated by HPLC and appropriate diastereoisomeric fractions (determined by radioactivity and retention times) were pooled, concentrated under reduced pressure and stored at 4°C. [^3H]*R,R*-Diastereoisomer **4** (23.3 mCi) : ^1H n.m.r. δ(C_6D_6) 0.42, s, 0.45, s, $2xCH_3$; 0.77-0.91, brm, 1.01-1.40, brm, 1.42-1.88, brm, grease contamination; δ_A 2.72, δ_B 2.68, J_{AB} = 16.5 Hz, J_{AX} = 7.6 Hz, J_{BX} = 7.2 Hz, CH_2CO_2PL; δ_A 3.16, δ_B 2.95, J_{AB} = 8.8 Hz, CH_2OCO; δ_A 3.76, δ_B 3.69, J_{AB} = 13.5 Hz, J_{AX} = 6.4 Hz, J_{BX} = 8.3 Hz, $PhthNCH_2$; δ_X (X common to both ABX subsets), 3.82-3.91, m (apparent quintet centred at 3.87), PhCH; 4.97, s, OCHCO; 6.79-6.82, m, 6.91-6.97, m, 7.02-7.08, m, 7.34-7.37, m, ArH. ^3H n.m.r. δ(C_6D_6) 2.66, dd, $^{1,2}J$ = 17.3 Hz, $^{1,3}J$ = 8.1 Hz, 1T, $CHTCO_2PL$; 3.78-3.89, m (apparent triplet centred at 3.84), 1T, PhCT. [^3H]*R,S*-Diastereoisomer **5** (16.3 mCi) : ^1H n.m.r. δ(C_6D_6) 0.31, s, 0.49, brs, $2xCH_3$; 0.71-0.97, brm, 1.01-1.47, brm, 1.49-1.96, brm, grease contamination; δ_A 2.58, δ_B 2.55, J_{AB} = 15.3 Hz, J_{AX} = 8.6 Hz, J_{BX} = 6.8 Hz, CH_2CO_2PL; δ_A 3.14, δ_B 2.96, J_{AB} = 8.8 Hz, CH_2OCO; δ_A 3.73, δ_B 3.68, J_{AB} = 13.6 Hz, J_{AX} = 7.4 Hz, J_{BX} = 8.5 Hz, $PhthNCH_2$; δ_X (X common to both ABX subsets), 3.87-3.97, m (apparent quintet centred at 3.92), PhCH; 4.99, s, OCHCO; 6.78-6.80, m, 6.97-6.99, m, 6.97-7.00, m, 7.02-7.08, m, 7.36-7.39, m, ArH. ^3H n.m.r. δ(C_6D_6) 2.56, dd, $^{1,2}J$ = 16.0 Hz, $^{1,3}J$ = 10.3 Hz, 1T, $CHTCO_2PL$; 3.85-3.95, m (apparent quintet centred at 3.90), 1T, PhCT.

[^{3}H]R-6 and [^{3}H]S-β-PhenylGABA 7

A solution of the more polar diastereoisomer [^{3}H]*(R,R)*-4 (23.3 mCi) in glacial acetic acid (1 ml) and 6M HCl (2 ml) was heated under reflux with stirring for 3 h. Additional 6M HCl (3 ml) was added and stirring and refluxing continued for 18 h. The mixture was evaporated to dryness and resuspended in water (3 ml). The solid was filtered off and the filtrate purified on an ion exchange column (Dowex 50, 3 ml). Fractions [H_2O (5x10 ml) followed by 1M pyridine (5x10 ml)] were collected and analysed for radioactivity by liquid scintillation counting. Pyridine fractions 1 and 2 containing [^{3}H]*R*-β-phenylGABA 6 (19.2 mCi) were combined and evaporated to dryness under reduced pressure then stored at 4°C in water. The same procedure was carried out with the less polar diastereoisomer [^{3}H]*(R,S)*-5 (16.3 mCi) to give the fraction (10.3 mCi) containing [^{3}H]*S*-β-phenylGABA 7.

Reversed-phase HPLC purification of the tritiated amino acids 6 and 7

Conditions for HPLC purification of tritiated amino acids 6 and 7 were established with unlabelled β-phenylGABA using a reversed-phase MicroPak MCH-10 C18 column (10 μm, 30 cm x 4 mm i.d.), isocratic elution with 5% acetonitrile in 0.01 M ammonium acetate, flow rate 1 ml/min. The retention time for β-phenylGABA was 4.0 minutes. Tritiated *R*-6 and *S*-7 amino acids, both eluted at 4 minutes as well as coeluting with unlabelled β-phenylGABA, and accounted for 94 and 95 % of the total radioactivity by liquid scintillation counting respectively. The product was subjected to further purification by cation exchange chromatography (Dowex AG50). [^{3}H](-)-*R*-β-PhenylGABA 6 (9.2 mCi) : m/z (%) 182(13), 181(13), 180(100), 164(15), 163(47), 162(89). ^{1}H n.m.r. δ(D_2O, 4.80) 2.66-2.72, m, $CH_2CO_2^-$, 3.37-3.45, m, $CH_2NH_3^+$, PhCH; 7.45-7.57, m, ArH. ^{3}H n.m.r. δ(D_2O, 4.80) 2.52, s, 2.60 sh, $CHTCO_2^-$; 3.30, brs, PhCT. The tritiated *S*-amino acid 7 was purified using the same method as the tritiated *R*-enantiomer. [^{3}H](+)-*S*-β-PhenylGABA 7 (5.3 mCi) : m/z (%) 182(11), 181(12), 180(100), 164(14), 163(44), 162(90). ^{1}H n.m.r. δ(D_2O, 4.80) 2.67-2.77, m, $CH_2CO_2^-$, 3.34-3.46, m, $CH_2NH_3^+$, PhCH; 7.45-7.57, m, ArH. ^{3}H n.m.r. δ(D_2O, 4.80) 2.52, s, 2.60 sh, $CHTCO_2^-$; 3.30, brs, PhCT.

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ACKNOWLEDGMENTS

We thank the Department of Pharmacy, University of Sydney, for mass spectral measurements, Dr. Tahany Ghazy and Mr. Sadeh Rabbani for the measurement of ^{1}H and ^{3}H n.m.r. spectra. Support of this work by a National Health and Medical Research Council Grant is gratefully acknowledged.