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ENZYMATIC RESOLUTION AND PHARMACOLOGICAL ACTIVITY OF THE ENANTIOMERS OF 3,5-DIHYDROXYPHENYLGLYCINE, A METABOTROPIC GLUTAMATE RECEPTOR AGONIST

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Abstract: 3,5-Dihydroxyphenylglycine (3,5-DHPG) was resolved using an aminoacylase enzyme in solution, and the enantiomers characterised for ability to stimulate metabotropic glutamate receptor (mGluR) mediated phosphoinositide hydrolysis in rat hippocampus. The mGluR activity resided mainly in the (S) enantiomer, which should thus be a useful tool to further explore mGluR pharmacology and function.

Introduction

(R,S)-3,5-DHPG has been reported[1] to stimulate phosphoinositide hydrolysis in *Xenopus* oocytes in which cloned mGluR1α receptors of the excitatory neurotransmitter (S)-glutamic acid are expressed, whilst possessing no apparent activity on ionotropic glutamate receptors. Subsequently it has been shown[2] that this compound is a highly selective agonist for phosphoinositide - linked metabotropic glutamate receptors in the rat hippocampus.

(S)-3,5-DHPG is a component of vancomycin and related glycopeptide antibiotics. Williams *et al.*[3], while synthesising linear peptides modelled on vancomycin, reported the preparation of (S)-3,5-dimethoxyphenylglycine in >80% optical purity, but they were unable to cleave the side-chain methyl ethers to give the free amino acid.

A fermentation process for the manufacture of (D)-3,5-DHPG, using *Hansenula sp.*-mediated hydrolysis of a racemic phenylhydantoin precursor, has been patented[4]. Recently, Naruse *et al.*[5] have reported the presence of (D)-3,5-DHPG ($[\alpha]^{25}_D$ = -85°, 1M HCl, c=0.5) in acid hydrolysates of the antiviral antibiotics Kistamicins A and B. In neither of these cases were any data on the (S)-enantiomer of 3,5-DHPG reported.

We report here the first preparation of enantiomerically pure (ee>99%) (S)-3,5-DHPG ($[\alpha]^{26}_D$ = +98°,1M HCl, c=0.1) and demonstrate that the metabotropic glutamate receptor activity of 3,5-DHPG resides mainly in this enantiomer.

Results and Discussion

To overcome the tendency of racemisation associated with the benzylic hydrogen in 3,5-DHPG, and the problems involved with deprotection,[3] we sought to develop an enzymic method for this resolution which would furnish the free amino acid directly. An initial attempt to hydrolyse the aminonitrile precursor of 3,5-

DHPG using a nitrilase enzyme[6] gave only 3,5-dihydroxybenzaldehyde (retro-Strecker product). A hydantoinase[7] enzyme failed to hydrolyse the hydantoin precursor of 3,5-DHPG, and the use of a hog liver esterase[8] on the methyl ester of 3,5-DHPG gave material of only 44% ee. Finally, employment of an aminoacylase[9] enzyme, using the N-chloroacetyl derivative of 3,5-DHPG as substrate, gave the required (S)-3,5-DHPG[10] with ee >99%. (Figure 1).

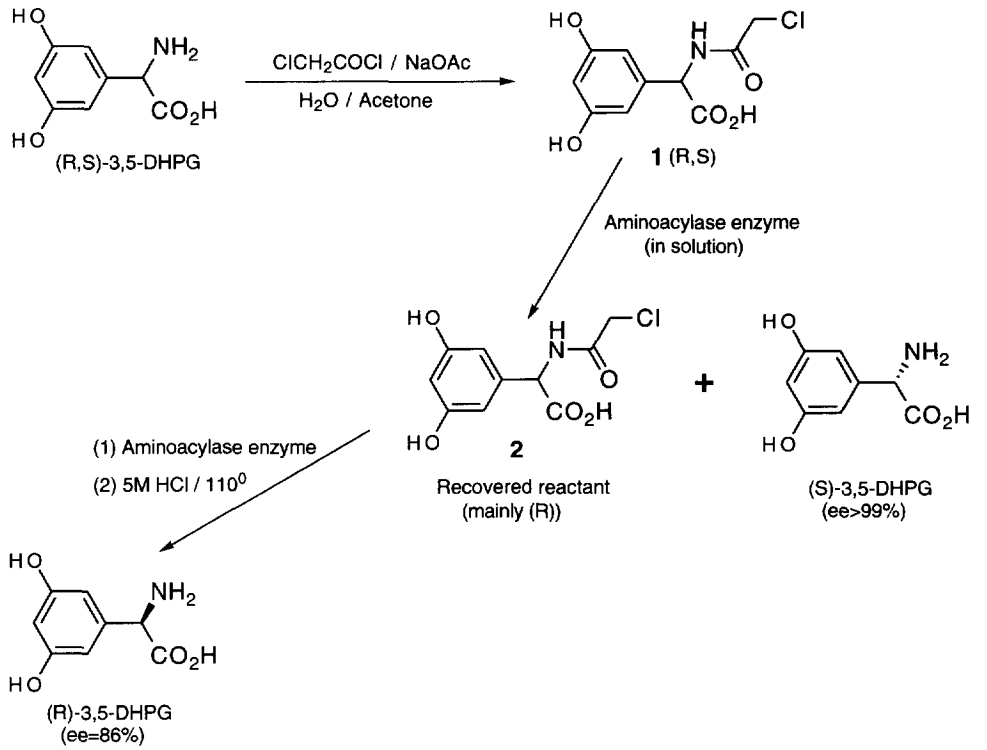

Figure 1 Resolution of 3,5-DHPG using aminoacylase enzyme.

Reaction of racemic 3,5-DHPG with chloroacetyl chloride furnished the N-chloroacetyl compound as substrate for the aminoacylase enzyme. The enzyme-catalysed hydrolysis was allowed to proceed until the ratio of product:starting material (**1**) was just less than 1:1. The (S)-3,5-DHPG product was then separated from unreacted starting amide (**2**) by ion-exchange chromatography. The recovered amide (**2**) (mainly R) was then subjected to a further aminoacylase-catalysed hydrolysis to remove residual (S) amide and purified by ion-exchange chromatography. A final acid-catalysed hydrolysis gave (R)-3,5-DHPG, (ee=86%). The lower enantiomeric purity of the (R) enantiomer probably reflected the harsher reaction conditions associated with its final chemical hydrolysis.

Because of the cheapness and ready availability of the aminoacylase enzyme used in this resolution, and the ease of separation of product amino acid from starting amide by ion-exchange chromatography, the process should be amenable to scale-up to multigram quantities.

The chiral purities of the enantiomers were determined by chiral capillary electrophoresis of their dansyl derivatives, using hydroxypropyl-β-cyclodextrin in the developing buffer.

The mGluR agonist activity of the 3,5-DHPG enantiomers was determined using hippocampal slices from neonatal (9 day-old) rats as detailed previously[2]. As shown in figure 2A, (S)-3,5-DHPG (EC_{50} = 10.9± 2.13 μM, n=4) was about 10 times more potent than (R)-3,5-DHPG (EC_{50} = 106 ± 4.28 μM, n=4) in activating phosphoinositide hydrolysis. It is worth noting however, that the apparent residual biological activity of (R)-3,5-DHPG may possibly be explained by its lower optical purity (86% ee, equivalent to the presence of *ca* 7% of the (S) enantiomer as contaminant). Stimulation of phosphoinositide hydrolysis by (S)-3,5-DHPG was not additive with stimulation by (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD], another agonist for phosphoinositide coupled mGluRs (figure 2B). This suggests that both compounds act on the same receptors or populations of mGluRs in the rat hippocampus. Previous studies[2] have shown (R,S)-3,5-DHPG to be selective for subtypes of mGluRs that are coupled to phosphoinositide hydrolysis, with no effect on subtypes linked to inhibition or stimulation of cAMP formation. Thus, (S)-3,5-DHPG should be a useful tool to further study the pharmacology and functions of *in situ* phosphoinositide-linked metabotropic glutamate receptors.

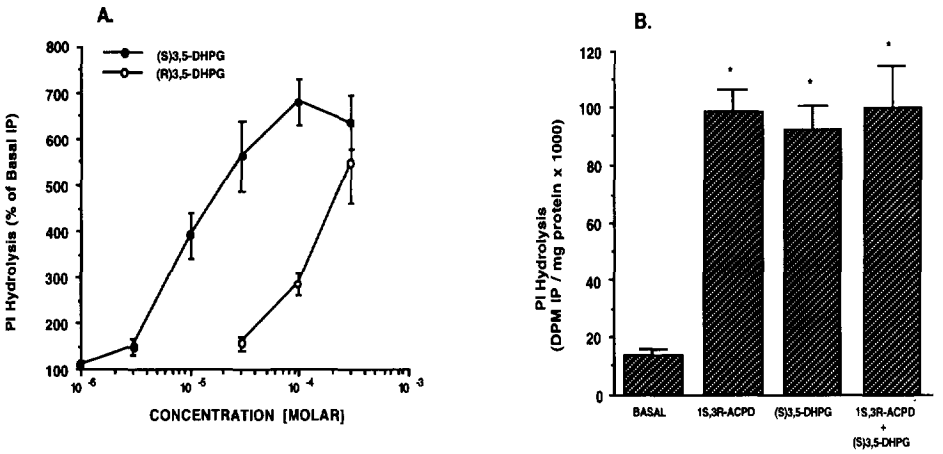

Figure 2. Activation of phosphoinositide hydrolysis in the rat hippocampus by the enantiomers of 3,5-DHPG. Panel A shows the relative potency of (S)- versus (R)- 3,5-DHPG. Panel B demonstrates that (S)-3,5-DHPG (100 μM) and (1S,3R)-ACPD (100 μM) are equally effective and non-additive mGluR agonists in this preparation ($^*p < 0.05$ when compared with basal).

Experimental section

(1) Synthesis of N-(chloroacetyl)-3,5-dihydroxyphenylglycine.

To a solution of racemic 3,5-dihydroxyphenylglycine[11] (1g, 5.5mmol) and sodium acetate (1.49g, 11mmol) in a 2:1 mixture of acetone:water (20ml) was added chloroacetyl chloride (0.52mL, 6.5mmol).

After stirring at room temperature for 16h the reaction mixture was evaporated to dryness *in vacuo,* water was added and then the mixture extracted with ethyl acetate (5x). The combined organic extracts were dried over magnesium sulphate, filtered and evaporated *in vacuo* to give a yellow gum (680mg, 56%). ^{1}H NMR confirmed the structure[12].

(2) Synthesis of (S)-3,5-dihydroxyphenylglycine (enzyme catalysed hydrolysis).

N-(chloroacetyl)-3,5-dihydroxyphenylglycine (660mg, 2.5mmol) was dissolved in 0.1 molar phosphate buffer (pH 7; from 0.1 M K_2HPO_4 + 0.1M KH_2PO_4) (30mL) and an aqueous solution of cobalt (II) chloride hexahydrate (2.5mM, 9mL) added. The pH was then re-adjusted to 7 using 1M potassium hydroxide solution, and an aqueous solution of sodium azide (154mM, 0.2mL) added. A solution of aminoacylase enzyme in phosphate buffer[13] (25mg/mL, 4mL) was then added and the reaction mixture stood at room temperature, while the progress of the reaction was followed by reverse phase HPLC[14]. When the ratio of starting material to product was approximately 55:45 (about three hours), the reaction was stopped by acidifying to pH 4 with glacial acetic acid.

The reaction mixture was diluted with four volumes of ice-cold ethanol and stored at 4°C for 16h. The resulting fine precipitate of enzyme-related protein was removed by filtration and the filtrate evaporated *in vacuo*. The residual gum was redissolved in a small volume of water and purified by cation-exchange chromatography (Dowex 50X8-100; column eluted sequentially with H_2O, H_2O:THF 1:1 and H_2O again. The amino acid was finally eluted with H_2O:pyridine 9:1). The pyridine was removed *in vacuo* and the residual solid redissolved in water and freeze-dried to give a fluffy off-white solid (152mg, 65%). Mp >250°C. ^{1}H NMR data confirms 3,5-dihydroxyphenylglycine structure[15]. $[\alpha]^{26}_D$ = +98° (1M HCl, c = 0.1); $[\alpha]^{29}_D$ = +70° (H_2O, c = 0.1). ee = >99%.[16]

(3) Synthesis of (R)-3,5-dihydroxyphenylglycine (Recovery and chemical hydrolysis of unreacted N-(chloroacetyl)-3,5-dihydroxyphenylglycine from (2).)

Evaporation of the 1:1 H_2O:THF washings of the ion-exchange column in **(2)** gave an amber glass (75mg).(^{1}H NMR confirms N-(chloroacetyl)-3,5-DHPG)[12]. To hydrolyse any residual (S)-enantiomer in the recovered starting material it was again subjected to enzymatic hydrolysis as above. The unreacted starting material from this second enzymic hydrolysis was again recovered by ion-exchange chromotography as before to give a light-brown gum (50mg).

This material was then dissolved in 5M HCl (2mL) and heated in a sealed vessel at 110°C for 16 hours. After cooling, the reaction mixture was evaporated *in vacuo* and the residue redissolved in H_2O and again subjected to ion-exchange chromatography, using the above conditions. Elution with H_2O : pyridine 9:1 and *in vacuo* removal of pyridine followed by solution in H_2O and freeze drying gave a fluffy light-brown solid (12mg,34%). Mp. >250°C. ^{1}H NMR data confirms 3,5-dihydroxyphenylglycine structure[15]. $[\alpha]^{28}_D$ = -58° (H_2O, c = 0.1). ee = 86%.[16]

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6 Cohen, M.A.; Sawden, J.;Turner, N.J. *Tet. Letts.* **1990,** 31(49), 7223. Enzyme kindly supplied by Nicholas J. Turner, Department of Chemistry,University of Exeter, Stocker Road, Exeter, EX4 4QD.

7 Enzyme and reaction conditions obtained from Boehringer Mannheim Biochemica, Am Nonnenwald 2, D-8122 Penzberg, Germany.

8 Enzyme obtained from Sigma Chemical Company, Fancy Road, Poole, Dorset BH17 7NH.

9 Enzyme obtained from Amano International Enzyme Company, Inc., P.O. Box 1000, Troy, Virginia, VA 22974 U.S.A.

10 The absolute configuration of the enantiomers was empirically assigned from the well documented stereoselectivity of this enzyme (Greenstein, J.P.; Winitz, M. *Chemistry of the Amino Acids,* John Wiley and Sons: New York, 1961; pp. 737-742 and 1753-1767) and from the reported optical rotation of (D)-3,5-DHPG (reference 5).

11 Synthesised from 3,5-dihydroxybenzaldehyde (purchased from the Aldrich Chemical Company, The Old Brickyard, New Road, Gillingham, Dorset SP8 4JL) by reaction with potassium cyanide and ammonium carbonate (Christensen, H.N.; Handlogten, M.E.; Vadgama, J.V.; De La Cuesta, E.; Ballesteros, P.; Trigo, G.C.; Avendo, C. *J. Med. Chem.* **1983,** 26(10), 1374), followed by hydrolysis of the resulting hydantoin in 2M sodium hydroxide at reflux under nitrogen.

12 ^{1}H NMR (300MHz, MeOH-d4, ∂ ppm) : 4.1 (2H, s, CH_2Cl), 5.22 (1H, s, methine), 6.22 (1H, t, 4-phenyl), 6.34 (2H, d, 2-phenyl).

13 The enzyme is supplied as a powder adsorbed onto Celite. A 25mg/mL suspension of this powder in 0.1 molar phosphate buffer is centrifuged (1500 rpm for 20 seconds) to remove the Celite, and the supernatant used in the enzyme-catalysed reactions.

14 Using a Waters 600E/484 instrument with a YMC AQ 5 micron ODS column; eluted with 70% water / 30%methanol containing 0.1% acetic acid. UV detection (λ =254nm.). With a flow rate of 1mL/minute the retention time of the N-(chloroacetyl)-3,5-dihydroxyphenylglycine starting material was 1.2 minutes and that of the 3,5-dihydroxyphenylglycine product was 1.0 minutes.

15 ^{1}H NMR (300MHz, D_2O, ∂ ppm) : 4.56 (1H, s, methine), 6.38 (1H, s, 4-phenyl), 6.42 (2H, s, 2-phenyl).

16 The optical purities were determined by the following method: (A) The N-dansyl derivatives of the enantiomers were prepared by the following procedure: A solution of the amino acid (2mg, 0.011mmol) in 0.1 molar sodium bicarbonate (0.22mL, 0.022mmol) was added to a solution of dansyl chloride (3.2mg, 0.014mmol) in acetone (0.4mL) and the reaction mixture stirred at room temperature for 16 h. After dilution with water the reaction mixture was acidified with concentrated hydrochloric acid and

extracted with ethyl acetate (5x). The combined organic extracts were dried over magnesium sulphate, filtered and evaporated *in vacuo* to give the dansyl derivative as a yellow solid (2mg).
(B) Chiral capillary electrophoresis of the dansyl derivatives was carried out on a ABI model 270A instrument, using a standard 72cm x 50 micron I.D. capillary with a path length of 50 cm to the detector and an applied voltage of 15KV. The running buffer was 15mM hydroxypropyl - ß - cyclodextrin in 100mM pH9 borax, with detection @ 200nm and 30°C. Migration time (S) enantiomer = 19.1 minutes. Migration time (R) enantiomer = 19.4 minutes.

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