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Synthesis and biological evaluation of (*R*)- and (*S*)-2-(phosphonomethyl)pentanedioic acids as inhibitors of glutamate carboxypeptidase II

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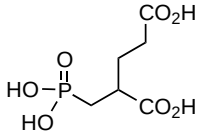
Abstract—Both enantiomers of 2-(phosphonomethyl)pentanedioic acid (2-PMPA), a potent and selective inhibitor of glutamate carboxypeptidase II (GCP II), were successfully prepared through the resolution of racemic 2-(hydroxyphosphinoylmethyl)pentanedioic acid dibenzyl ester with yohimbine and (*S*)-α-methylbenzylamine. The enantiomeric purity of the resolved phosphinic acid was measured by coupling it to (–)-menthol and analyzing the resulting ester by ^{31}P NMR. The absolute configuration of the 2-carbon atom in the resolved phosphinic acid was determined by X-ray crystallographic studies. Optically pure 2-PMPA was obtained by oxidation of the resolved phophinic acid to the phosphonic acid followed by catalytic hydrogenolysis. The biological activity of each enantiomer of 2-PMPA will also be described.

1. Introduction

The metalloprotease glutamate carboxypeptidase II, (GCP II, also known as *N*-acetylated alpha-linked acidic dipeptidase, NAALADase) cleaves *N*-acetyl-aspartyl-glutamate (NAAG) into *N*-acetyl-aspartate (NAA) and glutamate.[1] Since NAAG is believed to be a major source of glutamate in the nervous system, the enzyme is a target for the treatment of neurological disorders associated with excess glutamate such as stroke, spinal cord injury, amyotrophic lateral sclerosis (ALS), peripheral neuropathies, chronic pain, schizophrenia and epilepsy.[2]



2-PMPA, **1**

2-(Phosphonomethyl)pentanedioic acid (2-PMPA) **1** is the most potent known inhibitor of GCP II with a K_i value of 0.3 nM.[3] The high potency of 2-PMPA can be attributed to the strong chelation of the phosphonate group to an active site zinc atom as well as the interaction of the glutarate (pentanedioic acid) portion of the inhibitor with the glutamate recognition site of GCP II. 2-PMPA has been extensively utilized to study the mechanism and physiological role of GCP II as well as the potential therapeutic effects of GCP II inhibition. For example, 2-PMPA protects against ischemic injury both in a neuronal culture model of stroke, and in rats after transient middle cerebral artery occlusion.[4]

Despite the potential therapeutic value of 2-PMPA, this compound has never been synthesized in an enantiomerically pure form. Identification of the active enantiomer is important in understanding the enantiospecificity of the enzyme as well as the elimination of undesired pharmacological effects caused by the inactive enantiomer. In this paper, we report the synthesis and biological evaluation of both enantiomers of 2-PMPA as inhibitors of GCP II.

2. Results and discussion

Although a literature search on optically active phosphorous-containing compounds did not identify any

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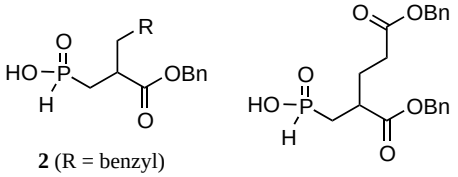
method applicable to the direct resolution of **1**, there were two cases where phosphinic acids **2**[5] and **3**[6] have been successfully resolved using (*S*)-α-methylbenzylamine. Based on these results, we predicted that a structurally analogous compound, 2-hydroxyphosphinoylmethyl-pentanedioic acid dibenzyl ester **4** could be resolved in a similar manner.

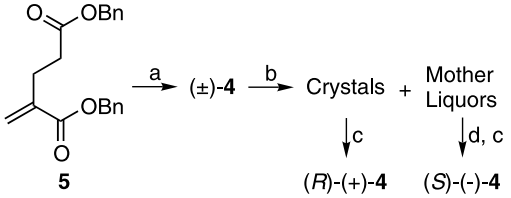


2 (R = benzyl)
3 (R = *i*-propyl)

4

Racemic phosphinic acid (±)-**4** was prepared by treating 2-methylene-pentanedioic acid dibenzyl ester **5**[3] with bis(trimethylsilyl)phosphonite (BTSP) generated in situ from ammonium hypophosphite, chlorotrimethylsilane and triethylamine. After extensive screening (e.g. quinine, quinidine, cinchonine, cinchonidine and bruicine in various solvent systems), we found that the phosphinic acid **4** can be resolved by preparation of the yohimbine salt in acetone/water followed by recrystallization from acetone/water (Scheme 1). Treatment of the salt with 10% sulfuric acid then liberates the phosphinic acid (*R*)-(+)-**4**. To obtain the other enantiomer (*S*)-(−)-**4**, the combined mother liquors from the yohimbine resolution were converted back to the free phosphinic acid, which was resolved by preparing the (*S*)-α-methyl benzylamine salt and recrystallizing from acetone/water. The free acid (*S*)-(−)-**4** was liberated from the salt by treatment with 10% sulfuric acid.

Reiter's group reported that neither direct quantitative measurement of the diastereomeric purity of **3**·(*S*)-α-methylbenzylamine salt by ^{1}H or ^{31}P NMR nor their attempts to determine the enantiomeric purity of free acid **3** by chiral HPLC, ^{1}H and ^{31}P NMR were successful.[6] This prompted us to develop an alternative method to measure the optical purity of the resolved phosphinic acid **4**. Thus, the phosphinic acid **4** was coupled to (−)-menthol with EDC using $CDCl_3$ as a solvent in a NMR tube (Scheme 2) and the resulting phosphinate ester **6** was subsequently analyzed by ^{31}P NMR spectroscopy.



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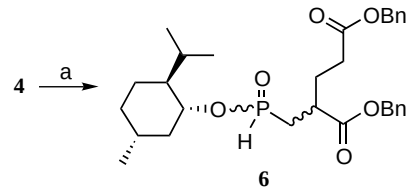
Scheme 1. *Reagents and conditions*: (a) BTSP, CH_2Cl_2. (b) Yohimbine, acetone/H_2O. (c) i. Recrystallization from acetone/H_2O; (ii) 10% H_2SO_4. (d) i. 10% H_2SO_4; ii. (*S*)-α-methylbenzylamine, acetone/H_2O.



6

Scheme 2. *Reagents and conditions*: (a) (−)-Menthol, EDC, $CDCl_3$.

The proton-decoupled ^{31}P NMR spectrum of the phosphinate ester **6** derived from (±)-**4** exhibits four peaks at 30.1, 31.3, 35.6 and 36.6 ppm in the ratio of 0.19:0.27:0.23:0.31 (Fig. 1A). The emergence of the multiple peaks is due to the creation of a new stereogenic center at the phosphorous atom that results in the formation of four diastereomers of compound **6**. The ratio of the two outer peaks to the two inner peaks turned out to be 1:1. In theory, each pair of peaks should correspond to two diastereomers derived from a single enantiomer of **4**. Indeed, the proton-decoupled ^{31}P NMR spectrum of phosphinate ester **6** derived from (*R*)-(+)-**4** (Fig. 1B) exhibits two inner peaks while that of (*S*)-(−)-**4** (Fig. 1C) only exhibits two outer peaks. These experiments clearly indicate that both of the resolved phosphinic acids (*R*)-(+)-**4** and (*S*)-(−)-**4** were obtained in enantiomerically pure form. This ^{31}P

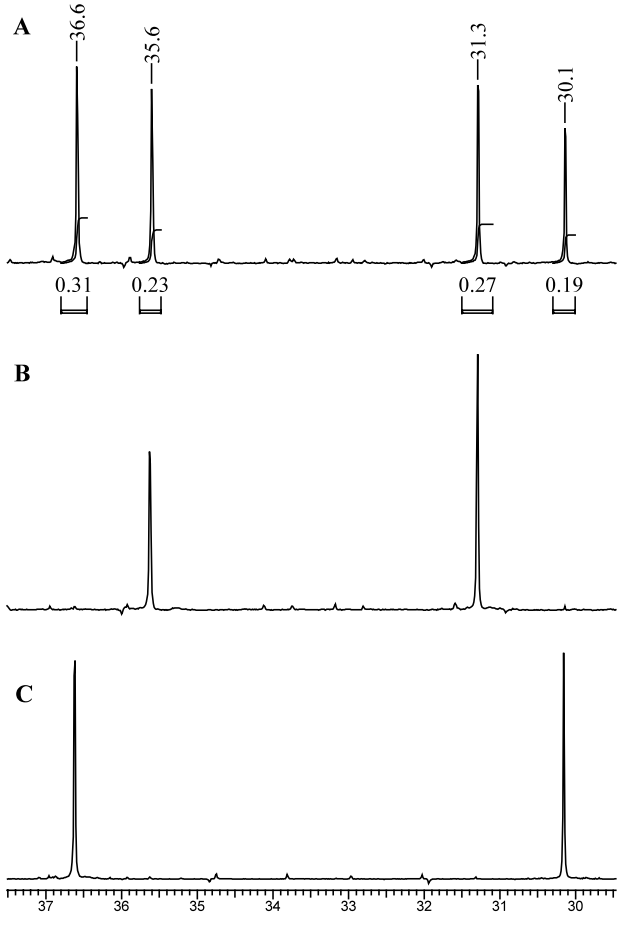


Figure 1. Proton-decoupled ^{31}P NMR spectra of (−)-menthol ester **6**. (A) Ester **6** derived from (±)-**4**. (B) Ester **6** derived from (*R*)-(+)-**4**. (C) Ester **6** derived from (*S*)-(−)-**4**.

NMR-based approach served as an accurate, reliable, and convenient method for measuring the enantiomeric purity of **4** and may be applicable to a wide range of optically active phosphinic acids.

The stereochemistry at C-2 of phosphinic acids **4** was established by X-ray structure analysis of (–)-**4**·1-adamantanamine salt[7] as shown in Fig. 2. The X-ray analysis revealed that (–)-**4** is the (*S*)-enantiomer of the phosphinic acid.

The enantiomers of 2-PMPA, (*R*)-**1** and (*S*)-**1**, were obtained by oxidation of the resolved phosphinic acids (*R*)-**4** and (*S*)-**4** with sodium periodate followed by catalytic hydrogenolysis (Scheme 3).

The biological activity of (*RS*)-**1**, (*R*)-**1**, and (*S*)-**1** are summarized in Table 1. GCP II inhibitory assay revealed that (*S*)-**1** is slightly more potent ($IC_{50}=0.1$ nM) than (*RS*)-**1** ($IC_{50}=0.3$ nM) while (*R*)-**1** has 100-fold lower potency ($IC_{50}=30$ nM). These biological results demonstrate that the potent inhibition of GCP II by 2-PMPA is enantiospecific for the (*S*)-enantiomer, which has stereochemistry corresponding to L-glutamate of the endogenous substrate NAAG. Serval's group reported that GCP II-catalyzed hydrolysis of NAAG is specific to *N*-acetyl-aspartyl-L-glutamate and that *N*-acetyl-aspartyl-D-glutamate is neither substrate nor inhibitor of GCP II.[8] Their finding is in a good agreement with our results with the two enantiomers of 2-PMPA. We also assessed the protective effects of (*RS*)-**1**, (*R*)-**1**, and (*S*)-**1** in a tissue culture model of cerebral ischemia. As shown in Table 1, (*RS*)-**1** and (*S*)-**1** exhibited a strong neuroprotection with median effective concentration (EC_{50}) values of 1.2 and 0.2 nM, respectively. Compound (*R*)-**1** exhibited much weaker potency in this model with an EC_{50} value of 70 nM. The good correlation between GCP II inhibitory and neuroprotective potencies of (*R*)-**1** (less potent in both assays) and (*S*)-**1** (more potent in both assays) provides further experimental evidence that the neuroprotective effect of these compounds is due to their ability to inhibit GCP II.

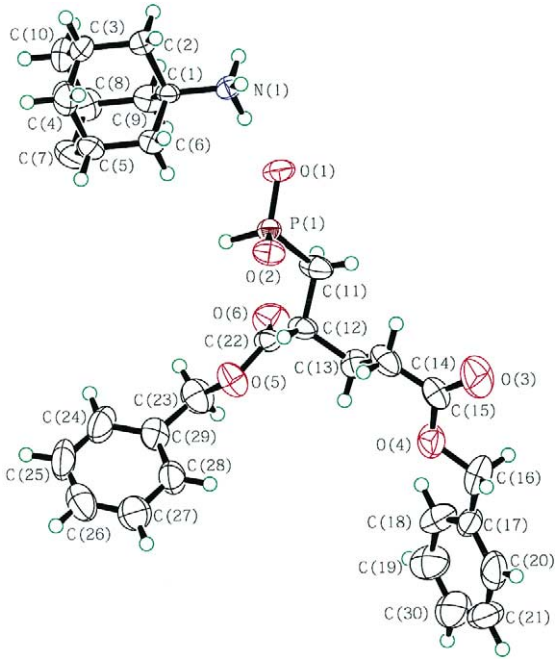
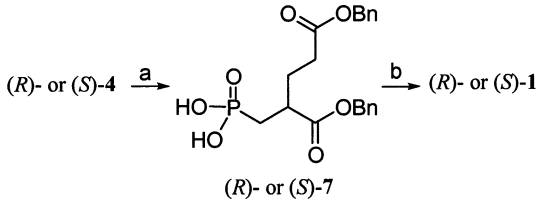


Figure 2. Crystal structure of (*S*)-(–)-**4**·1-adamantanamine salt.



Scheme 3. *Reagents and conditions*: (a) $NaIO_4$, CH_3CN/H_2O. (b) H_2, Pd/C, H_2O.

Table 1. Biological activity of (*RS*)-**1**, (*R*)-**1**, and (*S*)-**1**

Compound	GCP II inhibition	Cell culture ischemia assay
	IC_{50} (nM)	EC_{50} (nM)
(*RS*)-**1**	0.3	1.2
(*R*)-**1**	30	70
(*S*)-**1**	0.1	0.2

3. Conclusions

Both enantiomers of 2-hydroxyphosphinoylmethyl-pentanedioic acid dibenzyl ester (*R*)-(+)-**4** and (*S*)-(–)-**4** have been successfully obtained by fractional recrystallization of the corresponding yohimbine and (*S*)-α-methylbenzylamine salts. A facile method to measure the optical purity of **4** allowed us to demonstrate that both enantiomers were obtained in optically pure forms. Both enantiomers of 2-PMPA have been successfully synthesized from (*R*)-(+)-**4** and (*S*)-(–)-**4** and evaluated in GCP II assay. The active enantiomer (*S*)-**1** should serve as a more appropriate probe in understanding the physiological role of GCP II as well as the full therapeutic potential of GCP II inhibition.

4. Experimental

4.1. General

^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker DRX-400 instrument at 400, 100, and 162 MHz, respectively. Seeds used in crystallization of yohimbine and (*S*)-α-methylbenzylamine salts were obtained from preliminary small-scale experiments. Elemental analyses were obtained from Atlantic Microlabs, Norcross, GA and optical rotations were obtained from Robertson Microlit Laboratories, Inc., Madison, NJ. *N* - Acetyl - L - aspartyl - L - [3,4 - ^3H]glutamate (NAA[^{3}H]G) was obtained from Perkin–Elmer Life Sciences Inc., Boston, MA.

4.2. Synthesis of (±)-2-hydroxyphosphinoylmethyl-pentanedioic acid dibenzyl ester (±)-4

To a suspension of ammonium hypophosphite (229 g, 2.76 mol) in dichloromethane (4400 mL) were added chlorotrimethylsilane (791 g, 7.28 mol) and triethylamine (671 g, 6.63 mol) maintaining the temperature below 10°C. The reaction mixture was allowed to stir at 0–10°C for 30 min. A solution of **5**[3] (179 g, 0.55 mol) in dichloromethane (200 mL) was added keeping the temperature below 10°C. The reaction mixture was allowed to warm to 17–22°C and stirred for 18–20 h, and then quenched by careful addition of 3N HCl (2000 mL) ensuring that the temperature did not exceed 25°C. The organic layer was washed with 3N HCl (4×1000 mL) and water (2×1000 mL). The organic layer was concentrated on a rotary evaporator at 40°C to give (±)-**4** as a viscous colorless oil (173 g, 80%): ^{1}H NMR ($CDCl_3$) δ 1.8–1.9 (m, 1H), 2.0–2.1 (m, 2H), 2.1–2.3 (m, 1H), 2.3–2.4 (m, 2H), 2.8–3.0 (m, 1H), 5.07 (s, 2H), 5.11 (s, 2H), 6.62 (brs, 1H), 7.12 (d, J=565 Hz, 1H), 7.1–7.3 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 28.5 (d, J=12.1 Hz) 31.6 (d, J=94.4 Hz), 31.7, 38.5 (d, J=1.8 Hz), 66.8, 67.4, 128.7 (2 C), 128.7, 128.7 (2 C), 128.8, 129.0 (2 C), 129.0 (2 C), 135.8, 136.2, 172.6, 173.9 (d, J=6.2 Hz): ^{31}P NMR ($CDCl_3$) δ 36.0 (dm, J=578 Hz). Anal. calcd for $C_{20}H_{23}O_6P$: C, 61.54; H, 5.94. Found: C, 61.72; H, 5.98%.

4.3. Preparation of (*R*)-(+)-4

4.3.1. Formation of yohimbine salt. A mixture of (±)-**4** (213 g, 0.55 mol) and yohimbine (193 g, 0.55 mol) in acetone (1070 mL) was heated to reflux (55–57°C) and water (120 mL) was added at reflux until a solution was obtained. The resulting solution was cooled to 45°C and a seed of enantiomerically pure yohimbine salt was added. The mixture was cooled to 25°C over one hour and acetone (3200 mL) was added. The mixture was cooled to 5°C and held for 2 h. The solids were isolated by filtration, washed with cold acetone (426 mL×2), and dried under vacuum at 17–22°C to give the yohimbine salt as a white crystalline solid (169 g, 41%).

4.3.2. Recrystallization of yohimbine salt. The yohimbine salt (136 g, 0.18 mol) was heated in acetone (679 mL) to reflux (55–57°C) and water (140 mL) was added, maintaining reflux until a solution was attained. The resulting solution was cooled to 45°C and a seed of enantiomerically pure yohimbine salt was added. The mixture was cooled to 25°C over 1 h and acetone (2000 mL) was added. The mixture was cooled to 0–5°C and held for 2 h. The solids were isolated by filtration, washed with cold acetone (2×270 mL), dried under vacuum at 17–22°C to give the yohimbine salt as a white crystalline solid (122 g, 89%).

4.3.3. Liberation of (*R*)-(+)-4 from the yohimbine salt. The recrystallized yohimbine salt (123 g, 0.17 mol) in dichloromethane (1200 mL) and 10% H_2SO_4 (615 mL) was stirred for 15 min. The organic layer was washed with 10% H_2SO_4 (2×615 mL) and water (2×615 mL). The organic layer was concentrated by rotary evaporation at 40°C to give (*R*)-(+)-**4** as a thick, pale yellow oil (56.9 g, 88%): ^{1}H and ^{31}P NMR spectral data were identical to those of (±)-**4**; $[\alpha]_D^{20}$=+2.9 (*c* 1.0, $CHCl_3$). Anal. calcd for $C_{20}H_{23}O_6P{\cdot}0.1H_2O$: C, 61.25; H, 5.96. Found: C, 60.93; H, 5.99%.

4.4. Preparation of (*S*)-(−)-4

4.4.1. Recovery of 4 from the mother liquors. The mother liquors and washings from Sections 4.3.1 and 4.3.2 were combined and concentrated to dryness by rotary evaporation at 40°C to give 241 g of the yohimbine salt. The residue was dissolved in dichloromethane (3000 mL) and the solution was washed with 10% H_2SO_4 (3×1000 mL) and water (2×1000 mL). The organic layer was concentrated to dryness by rotary evaporation at 40°C to give the free phosphinic acid **4** as a viscous orange oil (116 g, 92% recovery).

4.4.2. Formation of (*S*)-α-methylbenzylamine salt. A solution of the above phosphinic acid **4** (107 g, 0.27 mol) and (*S*)-α-methylbenzylamine (34.9 mL, 0.27 mol) in acetone (267 mL) was heated to reflux then was cooled to 40°C and seeded with enantiomerically pure (*S*)-α-methylbenzylamine salt. Cooling was resumed to 25°C and acetone (800 mL) was added. The reaction mixture was cooled to 0–5°C and held for 1–2 h. The solid product was washed with cold acetone (2×214 mL) and dried under vacuum at 17–22°C to give the (*S*)-α-methylbenzylamine salt as an off-white solid (124 g, 89%).

4.4.3. Recrystallization of the (*S*)-α-methylbenzylamine salt. The above (*S*)-α-methylbenzylamine salt (76.0 g, 0.15 mol) was heated in acetone (380 mL) and water (28 mL) was added to reflux, maintaining reflux to achieve dissolution. After cooling to 40–45°C, the solution was seeded with enantiomerically pure (*S*)-α-methylbenzylamine salt. Cooling was resumed to 25°C and acetone (1100 mL) was added. The mixture was cooled to 0–5°C and held for 2 h. The product was filtered, washed with acetone (2×152 mL), dried under vacuum at 17–22°C to give the (*S*)-α-methylbenzylamine salt as a white crystalline solid (63.2 g, 83%).

4.4.4. Liberation of (*S*)-(−)-4 from the (*S*)-α-methylbenzylamine salt. A mixture of the (*S*)-α-methylbenzylamine salt (62.0 g, 0.12 mol), dichloromethane (620 mL), and 2N HCl (300 mL) was stirred for 15 min. The organic layer was washed with 2N HCl (300 mL×2) and water (300 mL×2). The organic layer was concentrated to dryness on a rotary evaporator at 40°C to give (*S*)-(−)-**4** as a viscous pale yellow oil (47.6 g, 100%): ^{1}H and ^{31}P NMR spectral data were identical to those of (±)-**4**; $[\alpha]_D^{20}$=−2.3 (*c* 1.0, $CHCl_3$). Anal. calcd for $C_{20}H_{23}O_6P0.1H_2O$: C, 61.25; H 5.96. Found: C, 60.97; H, 5.99%.

4.5. Determination of enantiomeric purity of (*R*)-(+)-4 and (*S*)-(−)-4. Preparation and proton-decoupled ^{31}P NMR analysis of (−)-menthol ester 6

An NMR tube was charged with **4** (3.1 mg, 0.008 mmol), $CDCl_3$ (0.6 mL), (−)-menthol (0.21 g, 1.3 mmol) and EDC (4.0 mg, 0.02 mmol). After 3–5 h, the sample was analyzed by proton-decoupled ^{31}P NMR (see Fig. 1).

4.6. Preparation of 1-adamantanamine salt of (*S*)-(−)-4[7]

Phosphinic acid (*S*)-(−)-**4** (1.17 g, 3.0 mmol) from Section 4.4.4 was dissolved in diethyl ether (10 mL) and treated with a solution of 1-adamantylamine (0.454 g, 3.0 mmol) in diethyl ether (10 mL). The resulting precipitate was collected, washed with ether and dried under vacuum to give (*S*)-(−)-**4**·1-adamantanamine salt as a white solid (1.50 g, 92%): mp 174–176°C; $[\alpha]_D^{20} = -0.6$ (*c* 1.0, MeOH). Anal. calcd for $C_{30}H_{40}NO_6P$-$0.2H_2O$: C, 66.09; H 7.47; N, 2.57. Found: C, 65.73; H, 7.49; N, 2.53%. The salt was recrystallized from ethanol/water prior to the X-ray analysis.

4.7. X-Ray structure analysis of (*S*)-(−)-4·1-adamantanamine salt

Measurement of the crystal structure of (*S*)-(−)-**4**·1-adamantanamine salt was performed on a Rigaku RAXIS-RAPID diffractometer with graphite-monochromated Cu Kα ($\lambda = 1.54182$ Å) irradiation. Crystallographic data (excluding structure factors) for the structure in this paper has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 179558. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

4.8. Synthesis of (*R*)-2-(phosphonomethyl)pentanedioic acid (*R*)-1

To a solution of (*R*)-**4** (2.06 g, 5.12 mmol) in CH_3CN–H_2O (12 mL, 1:1 by volume) was added sodium periodate (1.37 g, 6.41 mmol) at rt and the mixture was stirred at 50°C for 3 h. The reaction mixture was then concentrated in vacuo. The residual material was dissolved in EtOAc (100 mL) and washed with saturated aq. $KHSO_4$ (75 mL), 0.2 M $Na_2S_2O_3$ (100 mL), H_2O (75 mL), and brine (75 mL), respectively. The organic layer was dried over $MgSO_4$ and the solvent was removed in vacuo. The residue was purified by column chromatography (hexanes/EtOAc/AcOH/MeOH, 1/1/0.01/0.01) to afford (*R*)-**7** as a colorless oil (1.14 g, 57%): ^{1}H NMR (CD_3OD) δ 1.72–2.21 (m, 4H), 2.28–2.38 (m, 2H), 2.76–2.93 (m, 1H), 5.02–5.14 (m, 4H), 7.24–7.39 (m, 10H). A solution of (*R*)-**7** (0.455g, 1.12 mmol) in H_2O (50 mL) was shaken under hydrogen (30 psi) in the presence of 10% Pd/C (80 mg) for 20 h. The catalyst was removed by filtration and the filtrate was concentrated to afford (*R*)-**1** as a hygroscopic white solid (0.201 g, 80%): ^{1}H NMR (D_2O) δ 1.75-1.95 (m, 3H), 2.08 (dt, $J = 9.1$, 16.3 Hz, 1H), 2.36 (t, $J = 7.3$ Hz, 2H), 2.60-2.75 (m, 1H); ^{13}C NMR (D_2O) δ 28.3 (d, $J = 13.8$ Hz), 29.4 (d, $J = 136.8$ Hz), 31.4, 40.0 (d, $J = 2.3$ Hz), 177.7, 179.1; ^{31}P NMR (D_2O) δ 26.8; $[\alpha]_D^{20} = -5.9$ (*c* 1.0, water). Anal. calcd for $C_6H_{11}O_7P0.6H_2O$: C, 30.42; H, 5.19. Found: C, 30.11; H, 4.90%.

4.9. Synthesis of (*S*)-2-(phosphonomethyl)pentanedioic acid (*S*)-1

The (*S*)-enantiomer (*S*)-**1** was synthesized as described above for (*R*)-**1** except starting with (*S*)-**4**: ^{1}H, ^{13}C and ^{31}P NMR spectral data were identical to those of (*R*)-**1**; $[\alpha]_D^{20} = +6.1$ (*c* 1.0, water). Anal. calcd for $C_6H_{11}O_7P0.6H_2O$: C, 30.42; H, 5.19. Found: C, 30.13; H, 4.88%.

4.10. Biological evaluation of (*RS*)-1, (*R*)-1 and (*S*)-1

The GCP II assay was carried out as outlined previously[9] with modifications. Radiolabeled NAA[^{3}H]G (30 nM, 15 Ci/mmol) and inhibitors (10 pM to 100 μM) were incubated with purified recombinant GCP II[10] (20 pM) in Tris buffer (pH 7.6, 50 mM) containing $CoCl_2$ (1 mM) in a total volume of 50 μL at 37°C for 15 min. The reaction was terminated with phosphate buffer (0.1 M, pH 7.4, 50 μL) and the material was applied to a strong anion exchange mini column (AG 1-X8 anion exchange resin, 200–400 mesh; formate form). [^{3}H]Glutamate was eluted with formate (1.0 M) while unreacted NAA[^{3}H]G remained bound to the column. The eluate was transferred to a solid scintillator coated plate and dried. The radioactivity was measured with a top scintillation counter. Tissue culture model of cerebral ischemia was performed as previously described.[4]

Acknowledgements

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