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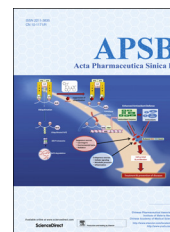


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ORIGINAL ARTICLE

4-Hydroxybenzyl-substituted amino acid derivatives from *Gastrodia elata*



Qinglan Guo, Yanan Wang, Sheng Lin, Chenggen Zhu, Minghua Chen,
Zhibo Jiang, Chengbo Xu, Dan Zhang, Huailing Wei, Jiangong Shi*

State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica,
Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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Abstract Seven new 4-hydroxybenzyl-substituted amino acid derivatives (**1–7**), together with 11 known compounds, were isolated from an aqueous extract of the rhizomes of *Gastrodia elata* Blume. Their structures were determined by spectroscopic and chemical methods. Compounds **1–3** are pyroglutamate derivatives containing 4-hydroxybenzyl units at the N atom and **4–7** are the first examples of natural products with the 4-hydroxybenzyl unit linked *via* a thioether bond to 2-hydroxy-3-mercaptopropanoic acid (**4–6**) and 2-hydroxy-4-mercaptoputanoic acid (**7**), which would be biogenetically derived from cysteine and homocysteine, respectively. The structures of **1** and **2** were verified by synthesis, while the absolute configurations of **4**, **5** and **7** were assigned using Mosher's method based on the MPA determination rule of $\Delta\delta_{RS}$ values. The known compound 4-(hydroxymethyl)-5-nitrobenzene-1,2-diol (**8**) exhibited activity against Fe^{2+} -cysteine induced rat liver microsomal lipid peroxidation with IC_{50} values of 9.99×10^{-6} mol/L.

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*Corresponding author. Tel.: +86 10 83154789; fax: +86 10 63037757.

E-mail address: shijg@imm.ac.cn (Jiangong Shi).

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1. Introduction

Gastrodia elata Blume is a holomycotrophic perennial plant of the Orchidaceae family, and is widely cultivated in several provinces of China to meet the demands of pharmaceutical and food industries¹. The steamed and dried rhizome of *G. elata*, known as “Tianma” in Chinese, is used for the treatment of neuralgic and nervous disorders, such as headaches, migraine, dizziness, tetanus, epilepsy, neuralgia and paralysis. It is also considered to have health benefits enhancing strength and virility and improving memory and blood circulation². Chemical and pharmacological studies indicated that 4-hydroxybenzyl analogs and 4-hydroxybenzyl-substituted metabolites were main active constituents of ethanol or methanol extracts of this medicine^{3–8}. As part of a program to assess the chemical and biological diversity of traditional Chinese medicines^{9–16}, a detailed chemical study was conducted on the aqueous extract of *G. elata* rhizomes, together with biological assays, since their decoctions are practically used in a variety of formulations. A fraction mainly contained parishin and parishins B and C (total content >50%), and at dosages of 10.00–0.25 mg/kg, the purified parishins improved the impaired memory in mice caused by scopolamine or cycloheximide¹⁷. In addition, 23 known compounds were characterized from the extract¹⁸, and a minor component *N*⁶-(4-hydroxybenzyl)-adenosine (NHBA)¹⁹ was isolated as the key sedative and hypnotic constituent of the extract, exhibiting significant activity at a dosage of 0.2 mg/kg (*i.p.*)²⁰. Therefore, we carried out further investigation on other minor components in the extract. This has resulted in isolation and characterization of seven new 4-hydroxybenzyl-substituted amino acid derivatives **1–7** (Fig. 1), along with 11 known compounds. Reported herein are the isolation, structure determination, and biological activity of these isolates.

2. Results and discussion

Compound **1** showed IR absorptions due to hydroxyl (3216 cm⁻¹), carboxyl (1734 cm⁻¹ and 1658 cm⁻¹), and aromatic ring (1616 cm⁻¹ and 1516 cm⁻¹) functionalities. Its molecular formula C₁₂H₁₃NO₄ was indicated by HR-ESI-MS at *m/z* 236.0925 [M+H]⁺ (Calcd. for C₁₂H₁₄NO₄ 236.0917) and the NMR spectral data (Table 1). The NMR spectral data of **1** showed that this

compound consisted of 4'-hydroxybenzyl and pyroglutamate moieties²¹. This was verified by the ¹H-¹H COSY correlations of H-2/H₂-3/H₂-4 and HMBC correlations from H-2 to C-1, from H₂-3 to C-1 and C-5, and from H₂-4 to C-5. In particular, the HMBC correlations from H₂-7' to C-2 and C-5 located the 4'-hydroxybenzyl unit at the N atom of the pyroglutamate moiety. Thus, the planar structure of **1** was determined as *N*-(4'-hydroxybenzyl)pyroglutamate. The absolute configuration of **1** was assigned by synthesis of enantiomers (+)-(*S*)- and (-)-(*R*)-[*N*-(4'-hydroxybenzyl)]pyroglutamates, starting with L- and D-glutamic acids, respectively. The CD and specific rotation data of **1** were consistent with those of (+)-(*S*)-[*N*-(4'-hydroxybenzyl)]pyroglutamate. Therefore, the structure of compound **1** was determined as shown.

Compound **2** has the molecular formula C₁₄H₁₇NO₄ as indicated by the HR-ESI-MS and NMR data (Table 1 and Section 4). Comparison of the NMR data of **2** and **1** indicated the presence of an ethoxy group [δ_{H} 4.11 (q, 2H, *J*=7.2 Hz) and δ_{H} 1.21 (t, 3H, *J*=7.2 Hz); δ_{C} 61.7 and δ_{C} 14.4] and shielded shifts of C-1 and C-5 by $\Delta\delta_{\text{C}}$ -0.7 ppm and -0.6 ppm, respectively in **2**. This revealed that **2** is the ethyl ester of **1**, which was confirmed by the HMBC correlation from OCH₂CH₃ to C-1 in the HMBC spectrum of **2**. The CD and specific rotation data of **2** were similar with those of **1**, indicating that the two compounds have the same configuration, which was further confirmed by synthesis of the enantiomers, ethyl (+)-(*S*)- and (-)-(*R*)-[*N*-(4'-hydroxybenzyl)]pyroglutamates. The CD and specific rotation data of **2** were in agreement with those of the former enantiomer. Thus, the structure of compound **2** was determined as shown.

The molecular formula C₂₁H₂₃NO₅ of compound **3** was determined from its HR-ESI-MS and NMR data (Table 1 and Section 4). Comparison of the NMR data of **3** and **2** suggests that **3** is an analog of **2** with an additional 4''-hydroxybenzyl unit substituted at C-3'. This was confirmed by the ¹H-¹H COSY correlations of H-2/H₂-3/H₂-4 and HMBC correlations of H-2/C-1 and C-5; H₂-3/C-1 and C-5; H₂-4/C-5; OCH₂CH₃/C-1; 4''-OH/C-3'', C-4'', and C-5''; H-7''/C-2', C-3', C-4', C-1'', C-2'', and C-6''; 4'-OH/C-3', C-4', and C-5'; H-7'/C-1', C-2, C-2', C-5, and C-6'. Similarity of the CD and specific rotation data between **3** and **2** suggested the same 2(*S*) configuration for the two compounds. Thus, compound **3** was determined as ethyl (+)-(*S*)-{*N*-[4'-hydroxy-3'-(4''-hydroxybenzyl)benzyl]}pyroglutamate.

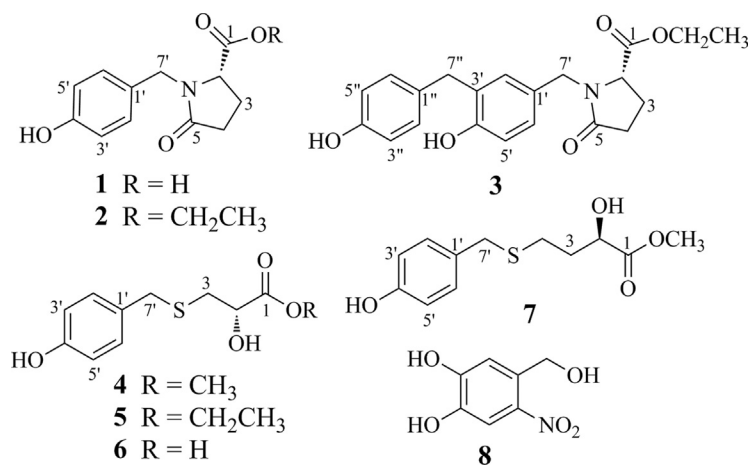


Figure 1 The structures of compounds **1–8**.

Table 1 ^1H NMR and ^{13}C NMR spectral data (δ) for compounds **1–7**.^a

No.	1		2		3^b		4		5		6		7	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		173.4		172.7		172.6		173.0		172.5		176.7		174.3
2	4.01 dd (9.0, 3.6)	58.9	3.97 dd (9.0, 3.0)	59.4	3.90 dd (8.4, 3.6)	59.2	4.19 m	70.4	4.16 m	70.4	4.26 brs	72.1	4.11 m	68.6
3a	2.33 m	23.4	2.28 m	23.4	2.22 m	23.3	2.67 dd (13.5, 5.5)	34.5	2.67 dd (14.0, 5.5)	34.5	2.83 brd (13.8)	36.3	1.82 m	33.7
3b	2.08 m		2.01 m		1.99 m		2.58 dd (13.5, 6.5)		2.57 dd (14.0, 6.0)		2.69 dd (13.8, 6.6)		1.76 m	
4a	2.43 m	30.2	2.38 m	30.3	2.37 m	30.5							2.41 m	26.3
4b	2.36 m		2.30 m		2.26 m									
5		175.5		174.9		174.9								
1'		127.9		128.1		127.9		128.3		128.3		130.4		128.5
2'	7.08 d (8.4)	130.5	7.05 d (8.4)	130.5	6.88 d (2.4)	131.3	7.09 d (8.0)	130.0	7.09 d (8.5)	130.0	7.15 d (8.4)	131.3	7.08 d (8.5)	129.9
3'	6.79 d (8.4)	116.1	6.78 d (8.4)	116.1		129.4	6.69 d (8.0)	115.1	6.69 d (8.5)	115.1	6.71 d (8.4)	116.1	6.68 d (8.5)	115.1
4'		157.7		157.7		155.3		156.2		156.2		157.5		156.2
5'	6.79 d (8.4)	116.1	6.78 d (8.4)	116.1	6.79 d (8.4)	116.0	6.69 d (8.0)	115.1	6.69 d (8.5)	115.1	7.15 d (8.4)	116.1	6.68 d (8.5)	115.1
6'	7.08 d (8.4)	130.5	7.05 d (8.4)	130.5	6.87 dd (8.4, 2.4)	128.0	7.09 d (8.0)	130.0	7.09 d (8.5)	130.0	6.71 d (8.4)	131.3	7.08 d (8.5)	129.9
7'a	4.94 d (15.0)	45.2	4.82 d (15.0)	45.3	4.80 d (14.4)	45.3	3.67 s	35.3	3.67 s	35.3	3.73 s	37.1	3.60 s	34.5
7'b	3.85 d (15.0)		3.87 d (15.0)		3.79 d (14.4)									
OCH ₃							3.63 s	51.6					3.62 s	51.5
OCH ₂ CH ₃			4.11 q (7.2)	61.7	4.08 q (7.2)	61.7			4.09 q (7.0)	60.2				
OCH ₂ CH ₃			1.21 t (7.2)	14.4	1.18 t (7.2)	14.4			1.19 t (7.0)	14.1				
2-OH							5.75 d (6.0)		5.71 d (6.0)				5.49 d (6.0)	
4'-OH			8.31 s		8.44 s		9.32 s		9.33 s				9.34 s	

^aNMR data (δ) were measured in Me₂CO-*d*₆ for **1–3** at 600 MHz for ^1H and at 150 MHz for ^{13}C , in DMSO-*d*₆ for **4**, **5**, and **7** at 500 MHz for ^1H and at 125 MHz for ^{13}C and in MeOH-*d*₄ for **6** at 600 MHz for ^1H and at 150 MHz for ^{13}C . Proton coupling constants (*J*) in Hz are given in parentheses. The assignments were based on DEPT, ^1H - ^1H COSY, HSQC, and HMBC experiments.

^bData for 4'-hydroxybenzyl in **3**: δ_{H} 8.16 (s, 1H, OH-4'), 7.05 (d, 2H, *J* = 8.4 Hz, H-2''/6''), 6.71 (d, 2H, *J* = 8.4 Hz, H-3''/5''), 3.82 (s, 2H, H₂-7''); δ_{C} 132.7 (C-1''), 130.6 (C-2''/6''), 115.8 (C-3''/5''), 156.4 (C-4''), 35.4 (C-7'').

Compound **4** has the molecular formula C₁₁H₁₄O₄S as indicated by the HR-ESI-MS and NMR data (Table 1 and Section 4). Comparing the NMR data between **4** and the synthetic methyl *S*-(4'-hydroxybenzyl)-L-cysteinate, the chemical shift of C-2 (δ_{C} 70.4) and the presence of an exchangeable hydroxy proton [δ 5.75 (d, 1H, *J* = 6.0 Hz)] in the NMR spectra of **4** in DMSO-*d*₆ demonstrated the replacement of the amino group in the synthetic compound by a hydroxyl group in **4**. This was proved by the two- and three-bond correlations from H₂-3 to C-7'; from H₂-7' to C-1', C-2' (C-6'), and C-3; from OH to C-1, C-2, and C-3; and from OCH₃ to C-1 in the HMBC spectrum of **4**. The absolute configuration at C-2 in **4** was determined by the modified Mosher's method²². Esterification of **4** with (-)-(*R*)- and (+)-(*S*)- α -methoxyphenylacetic acid (MPA) afforded the corresponding derivatives, **4**-bis-(*R*)-MPA and **4**-bis-(*S*)-MPA. Since the MPA moiety at C-4' of the benzyl unit is away from the chiral center (C-2) in the bis-MPA esters, the chemical shift change of protons around C-2 is mainly induced by the MPA moiety at C-2. From the MPA determination rule based on the $\Delta\delta_{\text{RS}}$ values (Fig. 2), the configuration of **4** was assigned as 2*S*. Therefore, the structure of compound **4** was determined as shown.

Compound **5** (C₁₂H₁₆O₄S) is an analog of **4**, as indicated by its spectroscopic data (Table 1 and Section 4). Comparison of the NMR data of **5** and **4** indicated that the methoxyl group in **4** was substituted by an ethoxyl group [δ_{H} 4.09 (q, 2H, *J* = 7.0 Hz, OCH₂CH₃) and δ_{H} 1.19 (t, 3H, *J* = 7.0 Hz, OCH₂CH₃); and δ_{C} 60.2 and δ_{C} 14.1] in **5**. The HMBC correlations from OCH₂CH₃ to C-1 confirmed the ester linkage of the ethoxyl group. The *S*-configuration of **5** was verified by Mosher's method (Fig. 2). Thus, compound **5** was determined as ethyl (+)-(*S*)-2-hydroxy-3-[(4'-hydroxybenzyl)thio]propanoate.

The spectroscopic data of compound **6** indicated that it is the acid form of **4** and **5**. The NMR data of **6** demonstrated a deshielded shift of the C-1 resonance ($\Delta\delta_{\text{C}} > +3.0$ ppm), as compared with that of **4** or **5**, in addition to the absence of the methoxyl or ethoxyl group. An ethanol solution of **6** was treated with thionyl chloride (SOCl₂) to yield **5**. Therefore, compound **6** was determined as (+)-(*S*)-2-hydroxy-3-[(4'-hydroxybenzyl)thio]propanoic acid.

The spectroscopic data of compound **7** indicated that it is an isomer of **5**. Comparing the NMR data of these two compounds

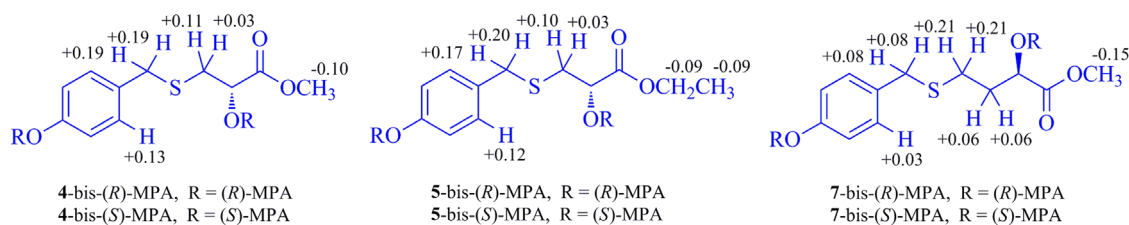


Figure 2 $\Delta\delta_{RS}$ values ($\delta_R - \delta_S$, black data in ppm) for bis-MPA esters of compounds **4**, **5**, and **7**.

demonstrated that instead of containing an ethyl 2-hydroxypropanoate moiety as in **5**, compound **7** contained a methyl 2-hydroxybutyrate moiety. The ^1H - ^1H COSY correlations of H-2/H₂-3/H₂-4 and HMBC correlations of H-2/C-1, C-3, and C-4; H₂-3/C-1, C-2, and C-4; H₂-4/C-2 and C-3, and OCH₃/C-1, along with their chemical shifts, confirmed the presence of the methyl 2-hydroxybutyrate moiety with the sulfur atom substituted at C-4 in **7**. In addition, the HMBC correlations from H₂-4 to C-7' and from H₂-7' to C-4 verified the 4'-hydroxybenzyl unit located at the sulfur atom. The 2*R* configuration of **7** was determined by using the same protocol as described for **4** and **5** (Fig. 2). Therefore, compound **7** was determined as methyl (–)-(*R*)-2-hydroxy-4-[(4'-hydroxybenzyl)thio]butyrate.

The acid/ester pair of **1/2** and the ethyl ester **3** are considered as natural products because HPLC-ESI-MS analysis using the ion extraction method demonstrated their occurrence in the crude extract or an CH₃CN-eluted fraction without contacting with EtOH. In addition, methylation or ethylation of the acids and hydrolysis of the esters were unlikely to occur in the isolation procedure because refluxing the EtOH solution of L-[N-(4'-hydroxybenzyl)]glutamic acid only produced **1**, whereas **2** was obtained by subsequent addition of thionyl chloride (SOCl₂) in the solution. However, the esters **4**, **5**, and/or **7** may be artifacts because **6** was esterified by keeping the MeOH solution at room temperature for a month, producing the ester with the spectroscopic features identical to that of **4** (Figs. S125–127 in Supporting information).

The known compounds were identified by comparing their spectroscopic data with the reported data as cyclo[glycine-L-5-(4'-hydroxybenzyl)cysteine]²³, 2-[4-(β-D-glucopyranosyl)benzyl]citrate²⁴, 1-ethyl citrate²⁵, 6-ethyl citrate²⁶, parishin E, 4-(hydroxymethyl)-5-nitrobenzene-1,2-diol (**8**)²⁷, (–)-(6*R*)-6,7-dihydroxy-3,7-dimethyl-(2*E*)-octenoic acid²⁸, bis(4-hydroxybenzyl)sulfide²⁹, ethyl (+)-(2*S*)-2-hydroxy-3-(4-hydroxyphenyl)propanoate³⁰, 1-(4'-hydroxyphenyl)propan-1,2-dione³¹, and (–)-4-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyloxybenzyl alcohol³².

In the *in vitro* bioassays, compound **8** showed activity against Fe²⁺-cysteine induced rat liver microsomal lipid peroxidation, with IC₅₀ value of 9.99×10^{-6} mol/L (the positive control, glutathione, gave IC₅₀ 20.21×10^{-6} mol/L). All other compounds isolated in this experiment were inactive at the same concentration. In addition, these compounds were also evaluated for the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and their inhibitory activity against Fe²⁺/cysteine-induced liver microsomal lipid peroxidation, several human cancer cell lines, HIV-1 replication, and all of them were inactive at a concentration of 10^{-5} mol/L.

3. Conclusion

Seven new 4-hydroxybenzyl-substituted amino acid derivatives (**1–7**), together with 11 known compounds, were isolated from an aqueous extract of the rhizomes of *G. elata*. Blume. Compounds

1–3 are pyroglutamate derivatives containing 4-hydroxybenzyl units at the N atom and **4–7** are the first examples of natural products with the 4-hydroxybenzyl unit linked *via* a thioether bond to 2-hydroxy-3-mercaptopropanoic acid (**4–6**) and 2-hydroxy-4-mercaptopbutanoic acid (**7**), which would be biogenetically derived from cysteine and homocysteine, respectively. The enantiomers of compounds **1** and **2** were synthesized, and the absolute configurations of **4**, **5** and **7** were assigned using Mosher's method. These results, combined with our previous studies¹⁸, provide an important clue for further studies of chemical transformation, structural modification, and biosynthesis of the diverse 4-hydroxybenzyl-substituted amino acid derivatives from the rhizome of *G. elata*, as well as for evaluations on other pharmacological models though the new compounds were inactive in the assays carried out in this study.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured using a Rudolph Research Autopol III polarimeter. UV spectra were measured on a Cary 300 spectrometer. CD spectra were measured on a JASCO J-810 spectropolarimeter. IR spectra were recorded on a Nicolet 5700 FT-IR microscope spectrometer (FT-IR microscope transmission) by microscope transmission method. 1D- and 2D-NMR spectra were obtained on INOVA 400 MHz, 500 MHz, or SYS 600 MHz spectrometers (Varian), with solvent peaks serving as references (unless otherwise noted). ESI-MS data were measured with a Q-Trap LC/MS/MS (Turbo IonSpray source) spectrometer. HR-ESI-MS data were, in turn, measured on an AccuTOF-CS JMS-T100CS spectrometer (JEOL), and HR-EI-MS data were measured using a Micromass Autospec-Ultima ETOF spectrometer. Column chromatography was performed with silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), Toyopearl HW-40C and HW-40F (Tosoh Bioscience LLC, Tokyo, Japan), and MCI gel (CHP20P) (Mitsubishi Chemical Inc., Tokyo, Japan). HPLC separation was performed on an instrument with a Waters 600 controller, a Waters 600 pump, and a Waters 2487 dual λ absorbance detector (detecting wavelength: 230 nm) on a Grace (250 mm \times 10 mm, i.d.) semi-preparative column packed with C18 (5 μm), and an YMC-Pack (250 mm \times 10 mm, i.d.) column packed with Ph (5 μm). Glass precoated silica gel GF254 plates were used for TLC. Spots were visualized under UV light or by spraying with 5% H₂SO₄ in EtOH, followed by heating.

4.2. Plant material

The rhizomes of *G. elata* were collected at the plantation field of Xiaocao Ba, Yunnan province, China, in December 2009.

Plant identification was verified by Mr. Lin Ma (Institute of Materia Medica, Beijing 100050, China). A voucher specimen (No. ID-S-2384) was deposited at the herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Beijing 100050, China.

4.3. Extraction and isolation

The steamed and air-dried *G. elata* rhizomes (50 kg) were pulverized and ultrasonicated with H₂O (150 L × 3 × 1 h). The aqueous extracts were combined and evaporated under reduced pressure to yield a concentrated solution (50 L), which was loaded on a macroporous adsorbent resin (HPD-100, 30 kg) column (20 cm × 200 cm), and eluted successively with H₂O (50 L), 30% EtOH (150 L), 50% EtOH (120 L) and 95% EtOH (80 L) to yield four corresponding fractions A–D. After removing the solvent under reduced pressure, fraction C (1.9 kg) was chromatographed over MCI gel (CHP 20P, 10 L), with successive elution using H₂O (30 L), 30% EtOH (70 L), 50% EtOH (70 L), 95% EtOH (30 L) and Me₂CO (20 L), to afford fractions C1–C5.

Fraction C3 (237 g) was subjected to column chromatography (CC) over silica gel, eluting with a gradient of increasing MeOH concentration (0–100%) in EtOAc followed by 30% EtOH, to yield fractions C3-1–C3-5 based on TLC analysis. Fraction C3-1 (27.3 g) was separated by silica gel CC (petroleum ether-ethyl acetate, 50:1 v/v to 1:1 v/v) to give C3-1-1–C3-1-6. Separation of C3-1-1 (780 mg) by RP flash CC (10%–70% MeOH in H₂O) afforded C3-1-1-1–C3-1-1-8. Purification of C3-1-1-1 (120 mg) by HPLC (C18 column, 2.0 mL/min, 45% MeOH in H₂O) gave **2** (34 mg, *t_R* = 28.7 min), and C3-1-1-4 (42.5 mg) by HPLC (45% MeCN in H₂O) gave **5** (22 mg, *t_R* = 18.2 min, C18 column, 2.0 mL/min). Separation of C3-1-3 (900 mg) by silica gel CC (CHCl₃–MeOH, 15:1 v/v) yielded subfractions C3-1-3-1–C3-1-3-7, of which C3-1-3-7 (234 mg) was further fractionated by CC over Sephadex LH-20 (MeOH–H₂O, 1:1 v/v) to obtain C3-1-3-7-1 and C3-1-3-7-2. Fraction C3-1-3-7-1 (27 mg) was purified by HPLC (Ph column, 52% MeOH in H₂O, 2.0 mL/min) to obtain **3** (2.7 mg, *t_R* = 34.5 min). Fraction C3-1-4 (1.25 g) was further separated by silica gel CC (CHCl₃–MeOH, 10:1 v/v to 1:1 v/v) to afford C3-1-4-1–C3-1-4-4, of which C3-1-4-4 (640 mg) was fractionated by CC over Sephadex LH-20 (MeOH–H₂O, 1:1 v/v) to yield C3-1-4-4-1–C3-1-4-4-7. Separation of C3-1-4-4-3 (74 mg) by CC over HW-40F (MeOH) gave C3-1-4-4-3-1 and C3-1-4-4-3-2, of which C3-1-4-4-3-2 (37 mg) was purified by RP HPLC (Ph column, 20% MeOH in H₂O containing 0.1% TFA, 1.5 mL/min) to yield **1** (19 mg, *t_R* = 14.3 min). Fraction C3-1-4-4-6 (46 mg) was chromatographed over HW-40F (MeOH), followed by RP HPLC (C18 column, 25% MeOH in H₂O containing 0.1% TFA, 2.0 mL/min), to afford **6** (9 mg, *t_R* = 45.3 min). Fraction C3-2 (120 g) was separated by silica gel CC (ethyl acetate–MeOH, 100:1 v/v to 1:1 v/v) to give C3-2-1–C3-2-7. Separation of C3-2-5 (47 g) by CC over Sephadex LH-20 (30% MeOH in H₂O) afforded subfractions C3-2-5-1–C3-2-5-21, of which C3-2-5-18 (240 mg) was purified by RP HPLC (C18 column, 55% MeOH in H₂O, 2.0 mL/min) to give **4** (19.7 mg, *t_R* = 16.5 min), and C3-2-5-6 by reduced pressure HPLC (C18 column, 50% MeOH in H₂O, 2.0 mL/min) gave **7** (11.1 mg, *t_R* = 21.2 min).

4.3.1. (+)-(S)-[N-(4'-Hydroxybenzyl)]pyroglutamate (**1**)

Colorless gum; [α]_D²⁰ +43.2 (*c* 0.11, MeOH); UV (MeOH) λ_{\max} (log ϵ): 203 (3.16), 226 (3.05), 277 (2.20) nm; CD (MeOH) 224

($\Delta\epsilon$ +16.3) nm; IR (Nujol): ν_{\max} 3216, 3020, 2952, 2719, 1735, 1659, 1616, 1516, 1452, 1420, 1359, 1232, 1174, 1108, 961, 851, 837 cm⁻¹; ¹H NMR (CD₃OD, 600 MHz) data, see Table 1; ¹³C NMR (CD₃OD, 150 MHz) data, see Table 1; (+)-ESI-MS *m/z* 236 [M+H]⁺, 258 [M+Na]⁺, 274 [M+K]⁺, 471 [2M+H]⁺, 493 [2M+Na]⁺; (+)-HR-ESI-MS *m/z* 236.0925 [M+H]⁺ (Calcd. for C₁₂H₁₄NO₄, 236.0917).

4.3.2. Ethyl (+)-(S)-[N-(4'-hydroxybenzyl)]pyroglutamate (**2**)

Colorless gum; [α]_D²⁰ +40.2 (*c* 0.09, MeOH); UV (MeOH) λ_{\max} (log ϵ): 203 (3.36), 226 (3.22), 277 (2.84) nm; CD (MeOH) 224 ($\Delta\epsilon$ +7.8) nm; IR (Nujol): ν_{\max} 3241, 2982, 2935, 1740, 1669, 1615, 1597, 1517, 1451, 1419, 1367, 1269, 1230, 1203, 1107, 1031, 959, 849, 838 cm⁻¹; ¹H NMR (DMSO-*d*₆, 600 MHz) data, see Table 1; ¹³C NMR (DMSO-*d*₆, 150 MHz) data, see Table 1; (+)-ESI-MS *m/z* 264 [M+H]⁺, 286 [M+Na]⁺, 527 [2M+H]⁺, 549 [2M+Na]⁺; (+)-HR-ESI-MS *m/z* 264.1235 [M+H]⁺ (Calcd. for C₁₄H₁₈NO₄, 264.1230), 286.1052 [M+Na]⁺ (Calcd. for C₁₄H₁₇NO₄Na, 286.1050).

4.3.3. Ethyl (+)-(S)-[N-(4'-hydroxy-3'-(4'-hydroxybenzyl)benzyl)]pyroglutamate (**3**)

Colorless gum; [α]_D²⁰ +3.1 (*c* 0.19, MeOH); UV (MeOH) λ_{\max} (log ϵ): 204 (3.39), 227 (2.82), 279 (2.16) nm; CD (MeOH) 222 ($\Delta\epsilon$ +1.7) nm; IR (Nujol): ν_{\max} 3334, 3017, 2981, 2930, 1739, 1668, 1612, 1513, 1444, 1368, 1265, 1209, 1111, 1017, 963, 913, 827 cm⁻¹; ¹H NMR (Me₂CO-*d*₆, 600 MHz) data, see Table 1; ¹³C NMR (Me₂CO-*d*₆, 150 MHz) data, see Table 1; (+)-ESI-MS *m/z* 370 [M+H]⁺, 392 [M+Na]⁺, 408 [M+K]⁺; (+)-HR-ESI-MS *m/z* 370.1661 [M+H]⁺ (Calcd. for C₂₁H₂₄NO₅, 370.1649), 392.1487 [M+Na]⁺ (Calcd. for C₂₁H₂₃NO₅Na, 392.1468).

4.3.4. Methyl (+)-(S)-2-hydroxy-3-[(4'-hydroxybenzyl)thio]propanoate (**4**)

White amorphous powder; [α]_D²⁰ +68.7 (*c* 0.02, MeOH); UV (MeOH) λ_{\max} (log ϵ): 226 (4.32), 279 (2.73) nm; IR (Nujol): ν_{\max} 3366, 3021, 2955, 2924, 1893, 1738, 1677, 1612, 1514, 1443, 1225, 1143, 1098, 1012, 970, 837 cm⁻¹; ¹H NMR (DMSO-*d*₆, 500 MHz) data, see Table 1; ¹³C NMR (DMSO-*d*₆, 125 MHz) data, see Table 1; (+)-HR-ESI-MS *m/z* 265.0508 [M+Na]⁺ (Calcd. for C₁₁H₁₄O₄SNa, 265.0505).

4.3.5. Ethyl (+)-(S)-2-hydroxy-3-[(4'-hydroxybenzyl)thio]propanoate (**5**)

White powder; [α]_D²⁰ +34.2 (*c* 0.04, MeOH); UV (MeOH) λ_{\max} (log ϵ): 227 (4.28), 279 (2.83) nm; IR (Nujol): ν_{\max} 3372, 2983, 2927, 1890, 1733, 1679, 1612, 1596, 1514, 1446, 1370, 1224, 1097, 1024, 837 cm⁻¹; ¹H NMR (DMSO-*d*₆, 500 MHz) data, see Table 1; ¹³C NMR (DMSO-*d*₆, 125 MHz) data, see Table 1; (+)-HR-ESI-MS *m/z* 257.0837 [M+H]⁺ (Calcd. for C₁₂H₁₇O₄S, 257.0842), 279.0659 [M+Na]⁺ (Calcd. for C₁₂H₁₆O₄SNa, 279.0662).

4.3.6. (+)-(S)-2-Hydroxy-3-[(4'-hydroxybenzyl)thio]propanoate (**6**)

Colorless gum; [α]_D²⁰ +8.51 (*c* 0.80, MeOH); UV (MeOH) λ_{\max} (log ϵ): 204 (3.43), 227 (3.31), 280 (2.49) nm; IR (Nujol): ν_{\max} 3290, 3020, 2921, 1894, 1729, 1612, 1597, 1514, 1445, 1368, 1235, 1097, 1044, 1022, 980, 835 cm⁻¹; ¹H NMR (MeOH-*d*₄, 600 MHz) data, see Table 1; ¹³C NMR (MeOH-*d*₄, 150 MHz) data, see Table 1; (–)-ESI-MS *m/z* 227 [M–H][–], 455 [2M–H][–]; (+)-HR-ESI-MS *m/z*

251.0349 [M+Na]⁺ (Calcd. for C₁₀H₁₂O₄SNa, 251.0349), 267.0080 [M+K]⁺ (Calcd. for C₁₀H₁₂O₄SK, 267.0088).

4.3.7. (–)-(R)-2-Hydroxy-4-[(4'-hydroxybenzyl)thio]butyrate (7) White powder; [α]_D²⁰ –61.7 (c 0.01, MeOH); UV (MeOH) λ_{max} (logε): 222 (4.14), 278 (2.77) nm; IR (Nujol): ν_{max} 3352, 2956, 2919, 1891, 1733, 1680, 1613, 1597, 1514, 1443, 1364, 1305, 1234, 1206, 1142, 1097, 1024, 925, 838, 802 cm⁻¹; ¹H NMR (DMSO-*d*₆, 500 MHz) data, see Table 1; ¹³C NMR (DMSO-*d*₆, 125 MHz) data, see Table 1; (+)-HR-ESI-MS *m/z* 279.0662 [M+Na]⁺ (Calcd. for C₁₂H₁₆O₄SNa, 279.0662).

4.4. Synthesis of 1 and 2

To a solution of L- or D-glutamic acid (2 g) in MeOH (30 mL), 4-hydroxybenzaldehyde (3 g) and anhydrous Na₂CO₃ (3 g) were added. The mixture was stirred at r.t. for 4 h, cooled to 0 °C, and NaBH₄ (1 g) was slowly added by keeping the temperature at 0–5 °C. The mixture was stirred at r.t. for 40 min, and acidified with 2 mol/L HCl to pH 3 at 0–5 °C to produce precipitate, which was collected by filtration, washed with cold water, and dried to afford L- or D-[N-(4-hydroxybenzyl)]glutamic acid (~1.4 g)³³.

A suspension of L- or D-[N-(4-hydroxybenzyl)]glutamic acid (200 mg) in ethanol (15 mL) was refluxed for 5 h. The resulting solution was filtrated, followed by evaporation of the filtrate, to afford (+)-(S)-[N-(4-hydroxybenzyl)]pyroglutamate (126 mg) from L-[N-(4-hydroxybenzyl)]glutamic acid or (–)-(R)-[N-(4-hydroxybenzyl)]pyroglutamate (138 mg) from D-[N-(4-hydroxybenzyl)]glutamic acid. (+)-(S)-[N-(4-Hydroxybenzyl)]pyroglutamate: colorless gum; [α]_D²⁰ +49.8 (c 1.6, MeOH); CD (MeOH) 223 (Δε +11.9) nm; ¹H NMR (400 MHz, CD₃COCD₃): δ 7.06 (d, 2H, *J*=7.6 Hz, H-2'/6'), 6.78 (d, 2H, *J*=7.6 Hz, H-3'/5'), 4.93 (d, 1H, *J*=14.8 Hz, H-7'a), 3.97 (d, 1H, *J*=8.4 Hz, H-2), 3.82 (d, 1H, *J*=14.8 Hz, H-7'b), 2.32–2.41 (m, 3H, H-3a/4a/4b), 2.08 (m, 1H, H-3b); ¹³C NMR (100 MHz, CD₃COCD₃): δ 175.6 (C-5), 173.4 (C-1), 157.7 (C-4'), 130.5 (C-2'/6'), 128.0 (C-1'), 116.2 (C-3'/5'), 59.0 (C-2), 45.3 (C-7'), 30.2 (C-4), 23.5 (C-5); (+)-ESI-MS *m/z* 236 [M+H]⁺, 258 [M+Na]⁺. (–)-(R)-[N-(4-Hydroxybenzyl)]pyroglutamate: colorless gum; [α]_D²⁰ –46.5 (c 1.1, MeOH); CD (MeOH) 223 (Δε –31.3) nm; ¹H NMR (400 MHz, CD₃COCD₃): δ 7.07 (d, 2H, *J*=8.0 Hz, H-2'/6'), 6.78 (d, 2H, *J*=8.0 Hz, H-3'/5'), 4.94 (d, 1H, *J*=14.8 Hz, H-7'a), 4.00 (d, 1H, *J*=8.0 Hz, H-2), 3.84 (d, 1H, *J*=14.8 Hz, H-7'b), 2.30–2.45 (m, 3H, H-3a/4a/4b), 2.07 (m, 1H, H-3b); ¹³C NMR (100 MHz, CD₃COCD₃): δ 175.9 (C-5), 173.4 (C-1), 157.8 (C-4'), 130.6 (C-2'/6'), 127.8 (C-1'), 116.2 (C-3'/5'), 59.0 (C-2), 45.3 (C-7'), 30.4 (C-4), 23.5 (C-5); (+)-ESI-MS *m/z* 236 [M+H]⁺, 258 [M+Na]⁺. The NMR data of the synthetic compounds (Figs. S16–18 and S21–23 in Supporting information) were consistent with those of 1.

To the suspension of L-[N-(4-hydroxybenzyl)]glutamic acid (200 mg) in ethanol (15 mL), SOCl₂ (5 mL) was slowly added at 0 °C, and stirred for 1 h. Then, the mixture was refluxed for 5 h. The resulting solution was filtrated, followed by evaporation of the filtrate, to afford ethyl (+)-(S)-[N-(4-hydroxybenzyl)]pyroglutamate (167 mg). By changing the starting material to D-[N-(4-hydroxybenzyl)]glutamic acid, ethyl (–)-(R)-[N-(4-hydroxybenzyl)]pyroglutamate (124 mg) was obtained. Ethyl (+)-(S)-[N-(4-hydroxybenzyl)]pyroglutamate: colorless gum; [α]_D²⁰ +47.5 (c 1.7, MeOH); CD (MeOH) 222 (Δε +10.0) nm; ¹H NMR (400 MHz, CD₃COCD₃): δ 8.31 (s, 1H, OH-4'), 7.05 (d, 2H, *J*=7.6 Hz, H-2'/6'), 6.78 (d, 2H, *J*=7.6 Hz, H-3'/5'), 4.82 (d,

1H, *J*=14.8 Hz, H-7'a), 4.11 (q, 2H, *J*=7.2 Hz, OCH₂CH₃), 3.97 (d, 1H, *J*=6.0 Hz, H-2), 3.87 (d, 1H, *J*=14.8 Hz, H-7'b), 2.39 (m, 1H, H-4a), 2.27 (m, 2H, H-3a/4b), 2.00 (m, 1H, H-3b), 1.21 (t, 3H, *J*=7.2 Hz, OCH₂CH₃); ¹³C NMR (150 MHz, CD₃COCD₃): δ 174.9 (C-5), 172.7 (C-1), 157.7 (C-4'), 130.5 (C-2'/6'), 128.1 (C-1'), 116.1 (C-3'/5'), 61.7 (OCH₂CH₃), 59.3 (C-2), 45.2 (C-7'), 30.2 (C-4), 23.4 (C-5), 14.4 (OCH₂CH₃); (+)-ESI-MS *m/z* 263 [M+H]⁺, 286 [M+Na]⁺, 527 [2M+H]⁺, 549 [2M+Na]⁺. Ethyl (–)-(R)-[N-(4-hydroxybenzyl)]pyroglutamate: colorless gum; [α]_D²⁰ –45.7 (1.5, MeOH); CD (MeOH) 222 (Δε –17.0) nm; ¹H NMR (600 MHz, CD₃COCD₃): δ 8.45 (s, 1H, OH-4'), 7.07 (d, 2H, *J*=8.0 Hz, H-2'/6'), 6.80 (d, 2H, *J*=8.0 Hz, H-3'/5'), 4.85 (d, 1H, *J*=14.4 Hz, H-7'a), 4.14 (q, 2H, *J*=7.2 Hz, OCH₂CH₃), 4.00 (dd, 1H, *J*=8.4, 2.4 Hz, H-2), 3.91 (d, 1H, *J*=14.4 Hz, H-7'b), 2.43 (m, 1H, H-4a), 2.32 (m, 1H, H-4b), 3.20 (m, 1H, H-3a), 2.05 (m, 1H, H-3b), 1.23 (t, 3H, *J*=7.2 Hz, OCH₂CH₃); ¹³C NMR (150 MHz, CD₃COCD₃): δ 174.8 (C-5), 172.6 (C-1), 157.6 (C-4'), 130.4 (C-2'/6'), 128.0 (C-1'), 116.0 (C-3'/5'), 61.6 (OCH₂CH₃), 59.3 (C-2), 45.1 (C-7'), 30.3 (C-4), 23.3 (C-5), 14.3 (OCH₂CH₃); (+)-ESI-MS *m/z* 263 [M+H]⁺, 286 [M+Na]⁺, 527 [2M+H]⁺, 549 [2M+Na]⁺. The NMR data of the synthetic compounds (Figs. S39–41 and S44–46 in Supporting information) were identical with those of 2.

4.5. Synthesis of bis-(R)-MPA and bis-(S)-MPA esters of 4, 5, and 7

R- or S-MPA (~10 mg) was added to solutions of 4, 5, or 7 (~0.5 mg), EDCI (~10 mg), and DMAP (~5 mg) in freshly distilled methylene chloride (3 mL), and kept at r.t. overnight. The reaction mixtures were separated by preparative TLC (mobile phase: petroleum ether/Me₂CO= 2:1 v/v) to yield 4-bis-(R)-MPA or 4-bis-(S)-MPA from 4, 5-bis-(R)-MPA or 5-bis-(S)-MPA from 5, and 7-bis-(R)-MPA or 7-bis-(S)-MPA from 7. 4-Bis-(R)-MPA: ¹H NMR (400 MHz, CDCl₃): δ 7.23 (d, 2H, *J*=8.4 Hz, H-2'/6'), 6.92 (d, 2H, *J*=8.4 Hz, H-3'/5'), 5.28 (dd, 1H, *J*=7.6, 4.0 Hz, H-2), 3.69 (s, 2H, H₂-7'), 3.61 (s, 3H, OCH₃), 2.84 (dd, 1H, *J*=14.4, 4.0 Hz, H-3a), 2.76 (dd, 1H, *J*=14.4, 7.6 Hz, H-3b). 4-Bis-(S)-MPA: ¹H NMR (400 MHz, CDCl₃): δ 7.10 (d, 2H, *J*=8.8 Hz, H-2'/6'), 6.88 (d, 2H, *J*=8.8 Hz, H-3'/5'), 5.28 (dd, 1H, *J*=6.8, 4.8 Hz, H-2), 3.71 (s, 3H, –OCH₃), 3.50 (s, 2H, H₂-7'), 2.73 (m, 2H, H₂-3). 5-Bis-(R)-MPA: ¹H NMR (400 MHz, CDCl₃): δ 7.23 (d, 2H, *J*=8.8 Hz, H-2'/6'), 6.92 (d, 2H, *J*=8.8 Hz, H-3'/5'), 5.26 (dd, 1H, *J*=7.2, 3.6 Hz, H-2), 4.08 (q, 2H, *J*=7.2 Hz, OCH₂CH₃), 3.70 (s, 2H, H₂-7'), 2.84 (dd, 1H, *J*=14.8, 3.6 Hz, H-3a), 2.77 (dd, 1H, *J*=14.8, 7.2 Hz, H-3b), 1.12 (t, 3H, *J*=7.2 Hz, OCH₂CH₃). 5-Bis-(S)-MPA: ¹H NMR (400 MHz, CDCl₃): δ 7.11 (d, 2H, *J*=8.4 Hz, H-2'/6'), 6.88 (d, 2H, *J*=8.4 Hz, H-3'/5'), 5.25 (dd, 1H, *J*=6.8, 5.2 Hz, H-2), 4.17 (q, 2H, *J*=7.2 Hz, OCH₂CH₃), 3.53 (d, 1H, *J*=13.2, H-7'a'), 3.50 (d, 1H, *J*=13.2, H-7'b'), 2.74 (m, 2H, H₂-3), 1.21 (t, 3H, *J*=7.2 Hz, OCH₂CH₃). 6-Bis-(R)-MPA: ¹H NMR (400 MHz, CDCl₃): δ 7.23 (d, 2H, *J*=8.4 Hz, H-2'/6'), 6.93 (d, 2H, *J*=8.4 Hz, H-3'/5'), 5.18 (dd, 1H, *J*=6.4, 6.0 Hz, H-2), 3.60 (s, 2H, H₂-7'), 3.56 (s, 3H, OCH₃), 2.36 (m, 2H, H₂-4), 2.06 (m, 2H, H₂-3). 6-Bis-(S)-MPA: ¹H NMR (400 MHz, CDCl₃): δ 7.20 (d, 2H, *J*=8.4 Hz, H-2'/6'), 6.94 (d, 2H, *J*=8.4 Hz, H-3'/5'), 5.16 (dd, 1H, *J*=7.6, 4.8 Hz, H-2), 3.71 (s, 3H, OCH₃), 3.52 (s, 2H, H₂-7'), 2.15 (m, 2H, H₂-4), 2.00 (m, 2H, H₂-3).

4.6. Synthesis of methyl S-(4-hydroxybenzyl)-L-cysteinate

Methyl L-cysteinate (10 mg) and 4-hydroxybenzylalcohol (13 mg) were added to 5 mL of 2 mol/L HCl. The mixture was stirred at r.t.

for 20 min, and extracted with EtOAc (5 × 3 mL). The organic layer was evaporated under reduced pressure. The residue was chromatographed over Toyopearl HW-40F, using H₂O as the mobile phase to afford methyl S-(4-hydroxybenzyl)-L-cysteinate (14 mg): white amorphous powder, $[\alpha]_D^{20} +37.6$ (c 2.43, MeOH); ¹H NMR (DMSO-*d*₆, 400 MHz): δ 2.94 (m, 2H, H₂-3), 3.70 (s, 2H, H₂-7'), 3.73 (s, 3H, OCH₃), 4.23 (brt, 1H, *J*=6.0 Hz, H-2), 6.73 (d, 2H, *J*=8.4 Hz, H-3'/5'), 7.12 (d, 2H, *J*=8.4 Hz, H-2'/6'), 8.85 (s, 3H, OH and NH₂). ¹³C NMR (DMSO-*d*₆, 100 MHz): δ 30.7 (C-3), 35.1 (C-7'), 51.8 (OCH₃), 52.9 (C-2), 115.3 (C-3'/5'), 127.5 (C-1'), 130.1 (C-2'/6'), 156.6 (C-4'), 168.7 (C-1). (+)-ESI-MS *m/z* 242 [M+H]⁺, 242 [M+Na]⁺.

4.7. Antioxidant activity assay against Fe²⁺/cysteine-induced liver microsomal lipid peroxidation

See Ref. 34.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.apsb.2015.02.002>.

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