

Absolute Configuration of (–)-2-(4-Hydroxyphenyl)propionic acid: Stereochemistry of Soy Isoflavone Metabolism

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Biotransformation of natural products by human intestinal bacteria has recently drawn a significant interest, due to the emerging strong correlation between gut microbiota and human health.¹ Microbial metabolism of natural products by intestinal bacteria in small intestine and colon proceeds the phase I and II xenobiotic metabolisms in the liver. The metabolites were found to exhibit different biological activities,² and affect human etiology.³ For example, many beneficial effects of dietary polyphenols in human health are attributed to the microbial metabolites produced by intestinal bacteria⁴ and the modulation of gut microbiota composition.¹

Daidzein, genistein, glycitein, and their derivatives, are major isoflavones in soybean.⁵ Traditional fermentation processes change the compositions of isoflavones in the soybean products.⁶ However, biotransformation of soy isoflavones by intestinal bacteria is known to produce the more potent phytoestrogen,⁷ (–)-(*S*)-equol, which exhibits anticancer and anti-osteoporosis effects.⁸

Stereochemically, biosynthetic pathway of (–)-(*S*)-equol is of a great interest, because all biochemical reactions are stereospecific.⁹ We have recently elucidated the entire biosynthetic step, including the final steps of (–)-(*S*)-equol production.¹⁰ Genistein and glycitein also appeared to follow similar biotransformation pathway,^{11–13} but stereochemical information of the metabolites was not available. Regarding genistein metabolism, we reported (*R*)-dihydrogenistein is produced from genistein by MRG-1, similarly to daidzein metabolism.¹⁴

Wang *et al.*, first reported absolute stereochemistry of (–)-2-(4-hydroxyphenyl)propionic acid ((–)-2-HPPA) isolated from genistein biotransformation as *S*-configuration based on specific rotation.¹⁵ However, Niwa *et al.*, also recently reported stereochemistry of 2-HPPA produced by strain SY8519 as (*R*)-2-HPPA based on chiral separation without chiroptical properties.¹⁶ Furthermore, (–)-2-HPPA is recognized as (*R*)-2-HPPA from SciFinder.¹⁷ But we were not able to find any scientific evidences supporting such assignment. Because correct absolute configuration determination of metabolites is important to pharmacokinetic researches and other mechanistic studies, we set to determine the absolute configuration of (–)-2-HPPA produced from genistein metabolism by *Eubacterium ramulus* Julong 601 by means of

chiroptical spectroscopy. Here, we report the absolute configuration of (–)-2-HPPA produced by Julong 601,¹⁸ along with stereochemistry of isoflavone metabolism by human intestinal bacteria.

To determine the absolute configuration of (–)-2-HPPA produced from genistein metabolism by *E. ramulus* Julong 601, molecular structures of possible conformers of (*R*)-2-HPPA were generated by HyperChem (HyperChem 7.5 for Windows, Hypercube, Gainesville, FL, USA). Geometry optimization of the built models was performed to find the stable conformers using AM1 semi-empirical method without configuration interaction, and Polak–Ribiere was chosen as the minimization algorithm.¹⁹ Conformation of (*R*)-2-HPPA was solely determined by dihedral angles of carboxylic acid and 4-hydroxyl groups at the 2-*C* *sp*³ chiral carbon, and four structures were found as stable conformers (Fig. 1).

The energy values of the AM1 geometry-optimized four (*R*)-2-HPPA conformers were very close within 1 kcal/mol as listed at Table 1. Therefore, all the geometry-optimized conformers were optimized further by density functional theory (DFT) functional at the 6-31++G basis set level in the gas phase.²⁰ Gaussian geometry optimizations of the same conformers in ethanol were also calculated. To consider solvent effects, integral equation formalism (IEF) polarized continuum model (PCM) was implemented for all calculations.²¹ Gibbs free energy of each conformer was obtained with frequencies calculations (FREQ). Energy values obtain-

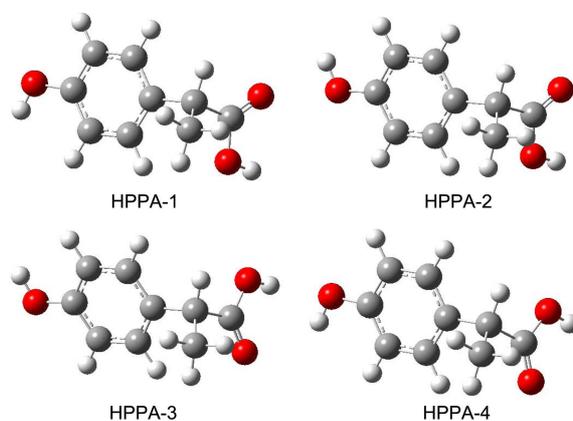


Figure 1. Structures of the geometry-optimized (*R*)-2-HPPA conformers.

Table 1. Total energy values of four (*R*)-2-HPPA conformers obtained at the different levels of calculations

Conformer	AM1 (kcal/mol) ^a	Gas phase		In ethanol	
		DFT (Hartree) ^b	Distribution (%) ^c	DFT (Hartree)	Distribution (%)
HPPA-1	-51932.56	-574.385839	11	-574.421189	22
HPPA-2	-51932.56	-574.385870	12	-574.421566	33
HPPA-3	-51933.09	-574.386932	36	-574.421108	20
HPPA-4	-51933.20	-574.387076	41	-574.421289	25

^aFrom semi-empirical AM1 calculations. ^bSum of electronic and thermal free energies (Gibbs free energy) obtained from frequencies calculations (FREQ) with DFT functional at the level of B3LYP/6-31++G. ^cDistribution of conformers was obtained from $K = \exp(-\Delta G/RT)$ at 300 K.

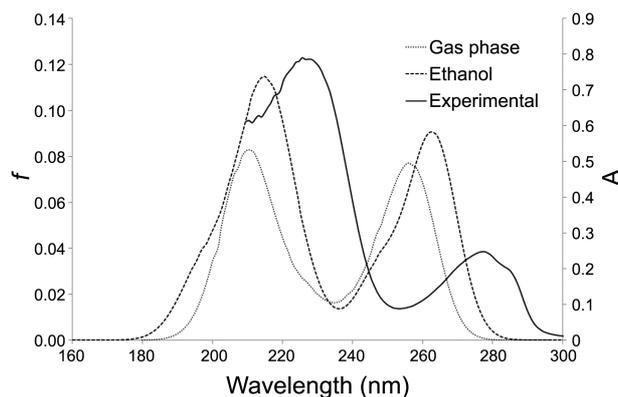
Table 2. Predicted theoretical specific rotation values of (*R*)-2-HPPA by Polar = OptRot

Conformer in the gas phase	$[\alpha]_D$	Conformer in ethanol	$[\alpha]_D$
HPPA-1	+94.69	HPPA-1	+127.19
HPPA-2	+80.15	HPPA-2	+88.63
HPPA-3	-150.28	HPPA-3	-260.78
HPPA-4	-136.56	HPPA-4	-259.91
Total	-89.92	Total	-59.82

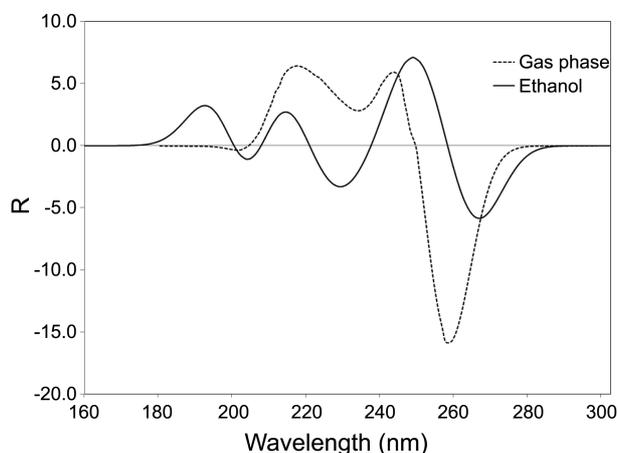
ed in Hartree (1 Hartree = 627.509 kcal/mol) were further converted to Gibbs free energy to estimate thermodynamic distribution of the conformers in the gas phase and ethanol (Table 1). Whereas the most stable conformer in the gas was conformer HPPA-4, the conformer HPPA-2 was most stable in ethanol probably due to the solvent effects.

Specific optical rotation of each conformer was obtained by Polar method with OptRot option in the framework of DFT using the B3LYP exchange correlation functional at the 6-31++G basis set level. As shown at Table 2, conformers of HPPA-1 and HPPA-2 showed positive specific rotations and the other HPPA-3 and HPPA-4 showed negative specific rotations. It seems orientation of carboxylic acid group in 2-HPPA is the key factor of the sign of specific optical rotation. Specific rotation values of the (*R*)-2-HPPA conformers in ethanol were found much higher than those in the gas phase. Considering thermodynamic distribution of conformers, theoretical specific optical rotation of (*R*)-2-HPPA was obtained as $[\alpha]_D = -89.92^\circ$ and -59.60° for (*R*)-2-HPPA in the gas phase and ethanol, respectively, after Boltzmann weighting of four specific optical rotation values. The reported specific rotation values of (*-*)-2-HPPA in ethanol were -38.6° with 98% *ee*¹⁵ and -72° with 97% *ee*.²² The theoretical specific rotation value was reasonably close to the experimental values, and therefore the absolute stereochemistry of (*-*)-2-HPPA was assigned as (*R*)-2-HPPA.

In addition to the optical rotation study of (*R*)-2-HPPA, ECD spectrum simulation by time-dependent density functional theory (TD-DFT) was also carried out using the B3LYP exchange correlation functional at the 6-311++G basis set level with *n* = 10 states option. TD-DFT calculations in the gas phase and ethanol produced theoretical UV spectra of all conformers (Supporting information). All the UV spectra of 2-HPPA conformers in the gas phase were similar

**Figure 2.** Predicted UV spectra of (*R*)-HPPA obtained from TD-DFT calculations in the gas phase and in ethanol, along with experimental spectrum. UV data was presented with oscillator strengths *f*.

each other with absorption bands at 210 and 256 nm. It appeared that electronic transitions of four conformers were very similar due to the fact that the orientations of the hydroxyl and carboxylic acid groups didn't perturb electronic configuration of the molecule. In ethanol, UV spectra of 2-HPPA conformers exhibited hyperchromic effect and bathochromic shifts and the absorption bands were found at 215 and 264 nm. The measured UV spectrum of 2-HPPA showed absorptions at 227 nm ($16,300 \text{ cm}^{-1}\text{M}^{-1}$) and 277 nm ($5,300 \text{ cm}^{-1}\text{M}^{-1}$) (Fig. 2).

**Figure 3.** Predicted ECD spectra of (*R*)-HPPA obtained from TD-DFT calculations in the gas phase and in ethanol. ECD spectra were presented with rotatory strengths *R* in 10^{-40} cgs units.

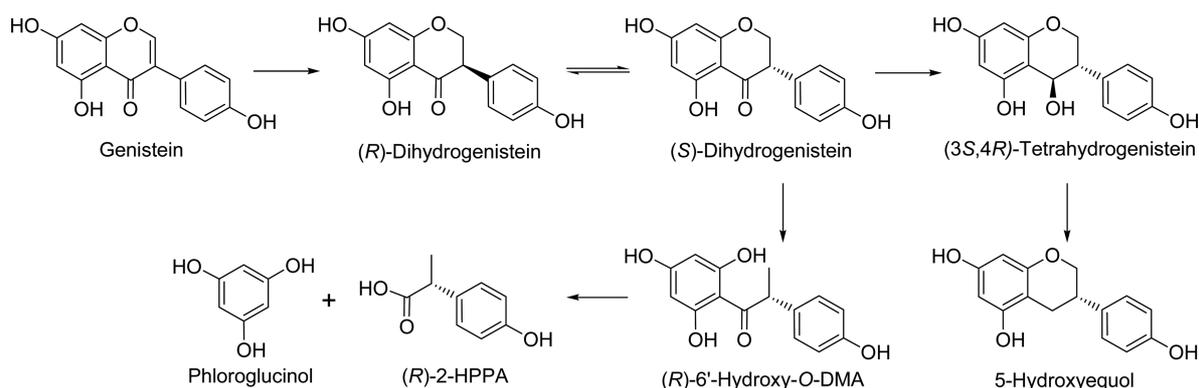


Figure 4. Proposed genistein metabolism in human intestine.

In the gas phase, the conformers HPPA-1 and HPPA-2 showed positive Cotton effects at the region of 200 and 257 nm and a negative Cotton effect at around 229 nm (Supporting information). The ECD spectra of HPPA-3 and HPPA-4 were almost mirror image of those of HPPA-1 and HPPA-2, but rotatory strengths at 229 nm were significantly smaller than the other conformers. In ethanol, all the ECD transitions showed red shift and some minor changes on the spectrum were observed. However, general pattern of ECD transitions in ethanol was not significantly different from those in the gas phase.

When four ECD spectra were combined according to Boltzmann weighting, theoretical ECD spectra of (*R*)-HPPA in both states were generated (Fig. 3). When compared to the reported ECD spectrum of (–)-2-HPPA,¹⁵ theoretical ECD spectrum of (*R*)-2-HPPA in ethanol was close to the reported spectrum. Negative Cotton effects at 210 and 230 nm were matched with the simulated spectrum. Therefore, the absolute stereochemistry of (–)-2-HPPA was confirmed as (*R*)-2-HPPA.

From the result obtained from this study, genistein metabolism has been suggested as shown in Figure 4. In details, genistein is stereospecifically reduced to (*R*)-dihydrogenistein, which is further isomerized to (*S*)-dihydrogenistein by enzymatic or abiological keto-enol tautomerization, similarly to daidzein metabolism.^{14,23} Further sequential reduction of (*S*)-dihydrogenistein, *via* (3*S*,4*R*)-tetrahydrogenistein, is believed to produce (*S*)-5-hydroxyequol. Alternative metabolism of (*S*)-dihydrogenistein is known to produce 6'-hydroxy-*O*-DMA and which further metabolized to phloroglucinol and (*R*)-2-HPPA.²⁴

In this study, we have elucidated stereochemistry of (–)-2-HPPA. Determination of (*R*)-2-HPPA stereochemistry also provided stereochemical information of genistein metabolism. Considering the stereochemistry of 2-HPPA, the precursor of (*R*)-2-HPPA should be (*R*)-6'-hydroxy-*O*-DMA. Besides, it is clear that only (*S*)-dihydrogenistein is the possible precursor of (*R*)-6'-hydroxy-*O*-DMA.²⁵ Therefore, genistein metabolism is suggested to follow the same stereochemical pathway like daidzein.

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