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Separation and peroxisome proliferator-activated receptor-γ agonist activity evaluation of synthetic racemic bavachinin enantiomers

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ABSTRACT

Bavachinin, isolated from *Psoralea corylifolia* seeds, has been reported to demonstrate peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist activity. However, isolated bavachinin is actually a mixture of *S* and *R* configurations, with an enantiomeric excess value of approximately 24.3%. For further study on the structure–activity relationships of bavachinin, investigating the PPAR-γ agonist activity of the two enantiomers is crucial. Considering the limited availability, racemic bavachinin was prepared in this study using chemical synthesis. The enantiomers of racemic bavachinin were then separated using supercritical fluid chromatography. This concise strategy yielded (*S*)- and (*R*)-bavachinin in optical purity as high as ≥97.5%. The PPAR-γ agonist activity of the two enantiomers was evaluated using a time-resolved fluorescence resonance energy transfer-based competitive binding assay method; IC_{50} values of (*S*)- and (*R*)-bavachinin were 616.7 and 471.2 nM, respectively. The interaction between the compounds and PPAR-γ was further explored using a molecular docking method. This study suggests that (*S*)- and (*R*)-bavachinin demonstrate similar PPAR-γ agonist activities.

Flavanones are phenolic substances isolated from various vascular plants, with over 8000 known individual compounds. They exhibit antioxidant,[1] antineoplastic,[2] antihypertensive,[3] and antiinflammatory[4] activities. The chemical structure of all flavanones is based on the 2,3-dihydro-2-phenyl-benzopyran-4-one moiety. Nearly all flavanones have one chiral center at the C-2 position (Figure 1). Different spatial configurations may cause the enantiomers to have diverse bioactive, toxic, and environmental fates. Nevertheless, few studies have examined the activity, safety, efficacy, and toxicity of the individual enantiomers of chiral flavanones.

Bavachinin is a naturally occurring flavanone, which was isolated from *Psoralea corylifolia* seeds.[5] It has various biological activities, such as the ability to inhibit nitric oxide production and antiangiogenic, antitumor, antiallergic, and antibacterial activities.[6-10] Our previous study (WO2014/169800) showed that natural bavachinin exhibited potent peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist activity. Natural bavachinin is a mixture of *S* and *R* configurations (Figure 2), with an enantiomeric excess (ee) value of approximately 24.3% (Figure *S*1, Table *S*1). In this study, racemic bavachinin was synthesized. We then sought to separate the enantiomers of racemic bavachinin and evaluate their stereodependent biological activities.

Thus far, the chiral separation of flavanones on analytical or preparative scales has been achieved using mainly high-performance liquid chromatography (HPLC),[11] micellar electrokinetic chromatography,[12] and capillary electrophoresis.[13] Based on our research, few studies have investigated on the chiral separation of flavanones by using supercritical-fluid chromatography (SFC), which is becoming a widely accepted and used technique in both academic and commercial spheres.

SFC is a form of normal phase chromatography, which is performed on packed columns by using a mobile phase consisting of CO_2 and small proportions (2%-30%) of organic modifiers (mostly methanol or ethanol).[14] SFC is an alternative technique to HPLC and other techniques for routine applications in chiral and achiral separations, impurity quantification, and scaling up from analyses of small amounts to preparative-scale applications,[15] because SFC offers several advantages over HPLC, including improved resolution, faster separations, higher throughput, green mobile phases, lower disposal costs, and a lack of toxicity.[16]

According to our research, the proportion of (*R*)-bavachinin in plants is considerably lower than that of (*S*)-bavachinin. To obtain the two bavachinin enantiomers in high optical purity and quantity for additional bioassays and further chiral separation, racemic bavachinin **1** was prepared via the synthetic route (Scheme 1), which is a previously reported classical synthetic route of racemic bavachinin.[17] 4-Hydroxybenzaldehyde **2** was treated with chloromethyl methyl ether in DMF in the presence of N,N-diisopropylethylamine to obtain 4-(methoxymethoxy)benzaldehyde **3** in an 82.8% yield.[18] The 2′-hydroxyacetophenone **4** was treated with prenyl bromide in acetone with potassium carbonate by using Williamson ether synthesis to obtain **5**, which then yielded 56.1% (two steps) of **6** in N,N-diethylaniline through Claisen rearrangement.[19] The intermediate **6** was treated with **3** and potassium trimethylsilanolate in ethanol to obtain chalcone **7** by using base-catalyzed Claisen-Schmidt condensation; the obtained yield was 60.8%. Compound **7** was refluxed with potassium fluoride in methanol to obtain the corresponding methoxymethylated flavanone **8**, which was formed through cyclization of **7** in a 64.4% yield. The methoxymethylated flavanone was demethoxymethylated by using hydrogen chloride in methanol to obtain the desired racemic bavachinin **1** in a 79.1% yield.[20]

Before the enantiomeric separation, an analytical SFC method was established. The racemic bavachinin solutions were separated on a Chiralpak OZ-H column by using isocratic elution. The racemic bavachinin was then purified using a customized preparative SFC system. The sample was separated on a preparative Chiralpak OZ-H column. Isocratic elution, similar to that used in the analytical method, was performed to obtain (*S*)- and (*R*)-bavachinin. The ee and optical rotation values of the two enantiomers are shown in Figure 3 and Table 1. According to a previous report, (-)-flavanones have *S*-chirality at the C-2 position, and the *S* configuration accounts for most natural bavachinin forms.[5] The optical rotation value of natural bavachinin was -10.4° ($[\alpha]_D^{30}$, $CHCl_3$),[5] which was consistent with the ee value of natural bavachinin that we investigated.

To evaluate the PPAR-γ agonist activity of bavachinin enantiomers, we used a time-resolved fluorescence resonance energy transfer[21] (TR-FRET)-based competitive binding assay to further validate PPAR-γ ligands according to manufacturer protocol. Competitive ligand binding to a nuclear receptor is detected on the basis of the ability of a test compound to displace labeled ligands from the receptor, which results in the loss of FRET signals (520 nm/495 nm).

The test results before the assays were performed revealed that the PerkinElmer EnVision Multilabel reader can detect changes in TR-FRET signals. IC_{50} values were calculated using GraphPad Prism 5.0. The inhibition constant (K_i) of the competing ligand was obtained from IC_{50} values by applying the Cheng-Prusoff equation. A commercially available PPAR-γ agonist, rosiglitazone, was used as a positive control for PPAR-γ activity. Natural bavachinin, (*S*)-bavachinin, and (*R*)-bavachinin were tested separately. The results suggested that (*S*)- and (*R*)-bavachinin demonstrated similar PPAR-γ agonist activities (Figure 4, Table 2).

To elucidate the interaction between bavachinin and PPAR-γ, we performed molecular docking of the two enantiomers onto the PPAR-γ receptor-binding domain. Before docking, the crystal structure of PPAR-γ (PDB ID: 1FM9)[22] was prepared using "Protein Preparation Wizard".[23-26] Protons were added, and bond orders and atomic charges were assigned. Protonation states for basic and acidic residues were assigned according to the optimization of hydrogen bonding patterns. Default parameters were adapted for the final minimization of the protein. Ligands were prepared using LigPrep[27] with Epik generating different protonation states at target pH values ranging from 5.0 to 9.0.

According to the docking result (Figure 5, Table 2), (*S*)- and (*R*)-bavachinin are located in the binding site of PPAR-γ, with similar orientations. Both enantiomers interact with the protein mainly through hydrophobic interactions with residues such as Ile281, Cys285, Leu330, Ile341, Met348, and Leu353 and a hydrogen-bond with the backbone of Ser342 (Figure 5). Because of the chirality, the phenolic hydroxyl group of (*R*)-bavachinin can form a hydrogen-bond with Glu259, whereas (*S*)-bavachinin cannot, which may explain the slightly higher affinity of (*R*)-bavachinin. The docking score achieved from molecular docking was consistent with the IC_{50} values obtained in the competitive binding assay experiment.

In conclusion, we established a concise and efficient method for rapid small-scale separation of bavachinin enantiomers in high optical purity by taking advantage of SFC. We also completed the initial evaluation of the PPAR-γ agonist activity of the two enantiomers. The results suggest that (*S*)- and (*R*)-bavachinin demonstrate similar PPAR-γ agonist activities. Further investigation on the structure-activity relationships of bavachinin and its synthetic analogs is ongoing in our laboratories to determine their PPAR-γ agonist activities.

Acknowledgements

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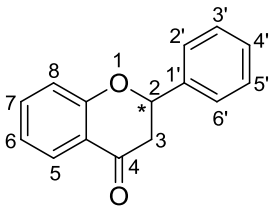
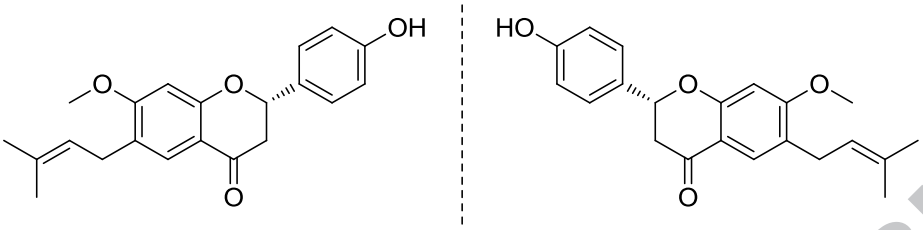
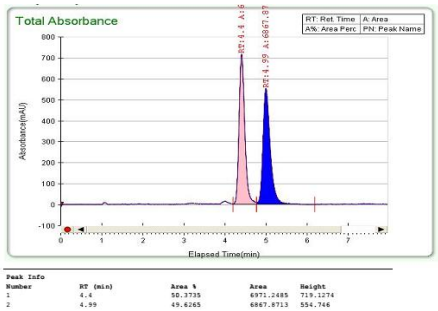


Figure 1. Chemical structures of flavanones.

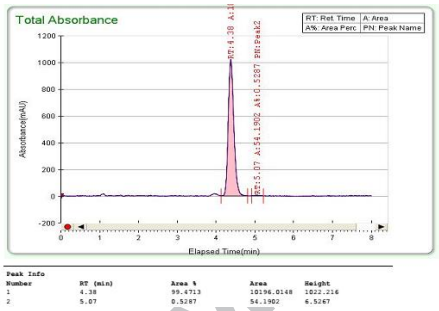


(*S*)-bavachinin (*R*)-bavachinin

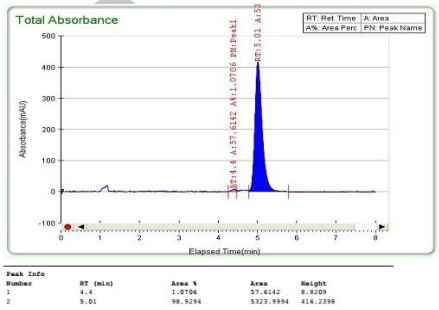
Figure 2. Chemical structures of (*S*)-bavachinin and (*R*)-bavachinin.



(a)



(b)



(c)

Figure 3. Chiral-SFC analysis of bavachinin enantiomers: (a) racemic bavachinin; (b) (*S*)-bavachinin; (c) (*R*)-bavachinin.

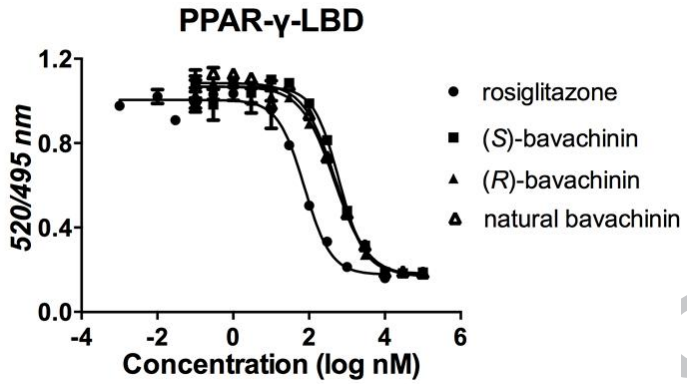


Figure 4. Binding of test compounds on LBD of PPAR-γ in a TR-FRET competitive binding assay.

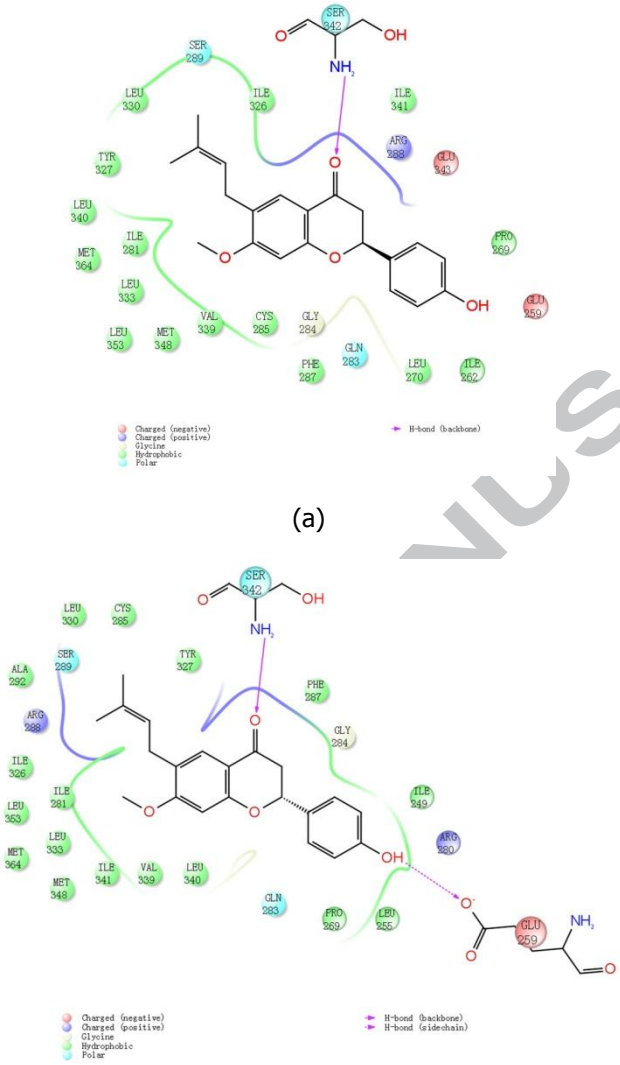
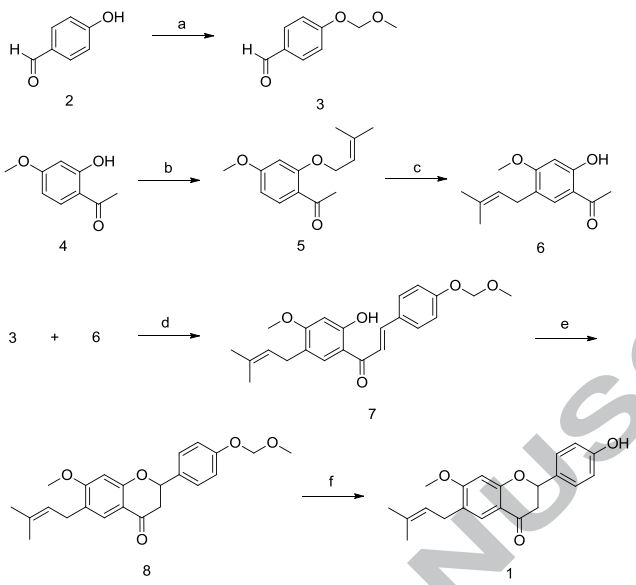


Figure 5. Two-dimensional schematic interactions of bavachinin enantiomers in the LBD of PPAR-γ: (a) (*S*)-bavachinin; (b) (*R*)-bavachinin.



Scheme 1. Reagents and conditions: (a) MOMCl, DIPEA, DMF, rt, 2 h; (b) prenyl bromide, K_2CO_3, acetone, reflux, 5 h; (c) $PhNEt_2$, reflux, 4 h; (d) $(CH_3)_3SiO^-K^+$, C_2H_5OH, reflux, 4 h; (e) KF, CH_3OH, reflux, 8 h; (f) HCl, CH_3OH, reflux, 10 min.

Table 1. Results of enantiomeric separation of racemic bavachinin

Compds	t_R (min)	e.e. (%)	$[\alpha]_D^{25}$ (*c*= 0.1, $CHCl_3$)
(*S*)-bavachinin	4.40	99.0	-22°[a]
(*R*)-bavachinin	4.99	97.9	+18°

[a] According to a previous report, (-)-flavanones have *S*-chirality at the C-2 position. The optical rotation value of natural bavachinin was -10.4° ($[\alpha]_D^{30}$, $CHCl_3$).[5]

Table 2. Inhibitory concentrations (IC_{50}, nM), binding affinity constants (K_i, nM) and docking score of test compounds for PPAR-γ

Compds	IC_{50}[a]	K_i	Docking Score[b]
natural bavachinin	488.8 ± 0.91	175.5 ± 0.33	——
(*S*)-bavachinin	616.7 ± 0.88	221.4 ± 0.32	-7.24
(*R*)-bavachinin	471.2 ± 0.90	169.1 ± 0.32	-7.76
rosiglitazone	75.52 ± 0.83	27.11 ± 0.30	-8.96

[a] The data is indicated as the mean ± SEM (n= 3).

[b] Docking score was calculated via molecular docking.