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β-Carboline Derivatives and Diphenols from Soy Sauce Are in Vitro Quinone Reductase (QR) Inducers

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ABSTRACT: A murine hepatoma (Hepa 1c1c7) cellular bioassay was used to guide the isolation of phase II enzyme inducers from fermented soy sauce, using quinone reductase (QR) as a biomarker. A crude ethyl acetate extract, accounting for 8.7% of nonsalt soluble solids of soy sauce, was found to double relative QR specific activity at 25 μg/mL (concentration required to double was defined as a "CD value"). Further silica gel column fractionation yielded 17 fractions, 16 of which exhibited CD values for QR induction of <100 μg/mL. The four most potent fractions were subfractionated by column and preparative thin layer chromatography, leading to the isolation and identification of two phenolic compounds (catechol and daidzein) and two β-carbolines (flazin and perlolyrin), with respective CD values of 8, 35, 42, and 2 μM. Western blots confirmed that the increases in QR activity corresponded to dose-dependent increases in cellular levels of NAD[P]H:quinone oxidoreductase 1 protein by these four QR inducers. To the authors' knowledge, this is the first report on the ability of β-carboline-derived alkaloids to induce phase II enzymes.

KEYWORDS: soy sauce, phase II enzyme, quinone reductase, catechol, daidzein, flazin, perlolyrin, β-carboline alkaloids, cancer chemoprevention

INTRODUCTION

Cancer continues to be one of the major causes of death worldwide.[1] Modest progress has been made over the past few decades in reducing the morbidity and mortality associated with cancer, and the most desirable way of reducing the impact of cancer in humans is by prevention. Cancer chemoprevention has been defined as the use of pharmacological and dietary interventions that prevent, inhibit, and/or reverse the process of carcinogenesis before malignancy occurs.[2]

Cancer chemopreventive action is generally related to antioxidant function of dietary components or can be more specifically related to the ability of these agents to interfere with stages of neoplasia known as initiation, promotion, and progression.[3,4] Cancer-suppressing agents are effective in the latter two stages of neoplasia and act by interfering in cell cycle progression, transcriptional regulation, or signal transduction or by inducing apoptosis. Cancer-blocking agents impede initiation events by reducing carcinogen uptake, scavenging reactive oxygen species, and metabolizing or detoxifying (pro)-carcinogens. One type of food that has been intensively studied for the ability to reduce the risk of cancer and other diseases is soybean and the myriad products prepared from it.[5,6] Soy foods contain some unique and fairly well characterized components, such as phytoestrogens, saponins and other sterols, protease inhibitors, and isoflavones, the latter of which are particularly implicated to have potential cancer chemopreventive effects.[3] Fermented soy foods are considered to potentially expand health-promoting effects because microbial transformation may increase the biological availabilities of bioactive compounds, as in the case of phytoestrogens.[7] There is some evidence for soy sauce, one of the most widely consumed fermented soy products,[8] to provide health benefits derived from anticarcinogenic, anticataract, antiplatelet, antioxidant, and histidine decarboxylase inhibition effects.[9]

One of the most effective means by which dietary components may help reduce cancer risk as blocking agents is by the up-regulation of the antioxidant response element (ARE), which codes for phase II enzymes, heat shock proteins and other proteins that help cells defend against stress in general. Phase II enzyme-inducing agents, which mediate the detoxification and excretion of (pro)carcinogens, have been identified from a diversity of dietary sources.[3,10−12] Whereas soybean, soy protein isolates, and particularly the isoflavones have been widely studied in this regard, there has been limited study of soy sauce. A previous study noted an ethyl acetate extract of soy sauce to induce glutathione *S*-transferase (GST) activity in mouse liver and forestomach, but the isolated flavorants and antioxidants furaneol, homofuronol, and norfuraneol were not associated with this effect.[13] Aside from isoflavones, the compounds in soy sauce that could be responsible for the induction of the phase II enzymes have not been identified. Moreover, whether the fermentation of soybean has any effects on the types of phase II enzyme inducers that evolve remains unknown. Therefore, the present study focused on the evaluation of quinone reductase (QR) induction (a biomarker for phase II enzymes) in murine hepatoma cells by isolates prepared from soy sauce. A bioassay-guided approach was conducted with the objective to identify the most potent QR inducers that occur in soy sauce at sufficient concentrations to permit their isolation.

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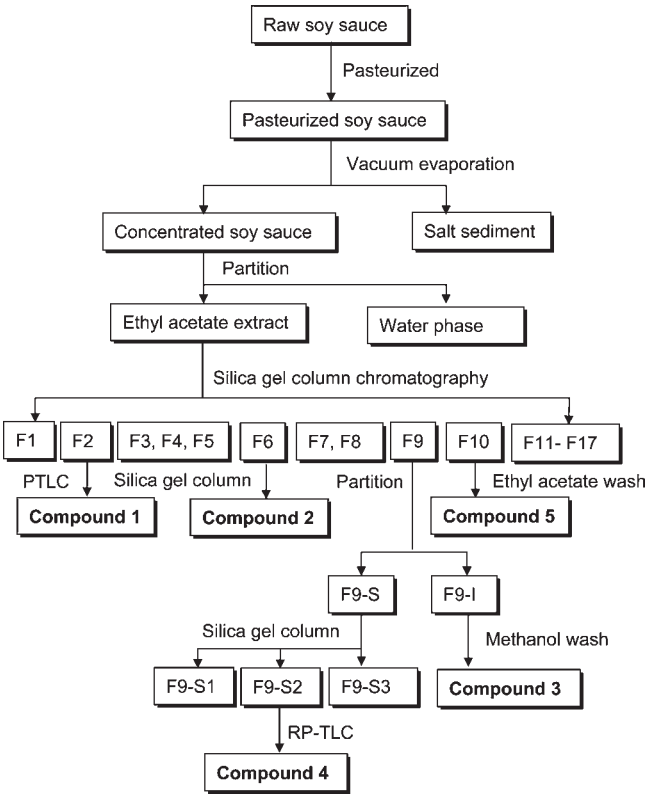

Figure 1. Fractionation scheme for identifying quinone reductase (QR) inducers from soy sauce.

MATERIALS AND METHODS

Materials. 1,2-Dihydroxybenzene (catechol), crystal violet, sodium dodecyl sulfate (SDS), digitonin, flavin adenine dinucleotide, menadione, acetonitrile, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Costar 96-well microtiter plates were obtained from Corning Inc. (Corning, NY). Fetal bovine serum (FBS), antibiotics, and α-minimum essential medium (MEM) were from Invitrogen (Carlsbad, CA). Vanillic acid, formic acid, ethyl acetate, hexane, acetone, methanol, silica gel for column chromatography (60 Å, 230−400 mesh), and Whatman precoated TLC silica gel F_{254} plates (60 Å, 250 μm thickness) were acquired from Fisher Scientific (Chicago, IL). Daidzein was purchased from Indofine Chemical Co. (Hillsborough, NJ).

Fermented raw soy sauce was produced by Haday Co. (Foshan, China). Generally, a blend of soybeans, bran, and wheat was inoculated with a pure *Aspergillus oryzae* culture to form *koji*, which was further fermented in saline (target NaCl content of 18%) for 3 months to yield raw soy sauce.[8] Ten liters of the raw soy sauce sample was obtained from the 30×10^3 kg fermentation tank at the end of the liquid fermentation stage. The sample was then pasteurized at 85 °C for 30 min and stored at 4 °C until further use. The total soluble solids content of soy sauce was determined according to AOAC methods.[14] The salt content was determined by volumetric titration with $AgNO_3$ using Mohr's method.[15]

Fractionation and Isolation of Soy Sauce Components. *Liquid−Liquid Partitioning.* The first step in the fractionation (Figure 1) was to concentrate soy sauce in a 5L rotary evaporator under vacuum at 40−50 °C for 3 h to yield about one-third of the initial volume. After holding at 4 °C overnight, the dark liquid concentrate was collected, and the crystallized salt in the bottom of the vessel was discarded. The concentrate was then extracted six times with ethyl acetate (1:1, v/v), and the aqueous phase was discarded. The pooled ethyl acetate extracts were subjected to solvent rotary evaporation under vacuum at 40 °C to obtain the residue. The resulting ethyl acetate extract (EAE) was stored at −20 °C until use. Nine liters of pasteurized soy sauce yielded 14 g of ethyl acetate extractable solids.

Fractionation of Bioactive Agents from the EAE. Twelve grams of EAE was redissolved in a minimum volume of ethyl acetate and mixed with silica gel. The mixture was subjected to vacuum rotary evaporation to remove solvent, and the extract-laden silica gel was loaded onto a 4.5 cm × 33 cm normal phase silica gel column. The column was eluted successively with 10% step gradients of hexane/acetone (from 9:1, 8:2, 7:3, 6:4 ... to 0:10, v/v; 2 L for each of 10 steps) and two steps with acetone/methanol (from 9:1 and 8:2, v/v; 1 L each step). Portions of 100 mL of eluant were collected in glass tubes and subjected to thin-layer chromatography (TLC; 20 cm × 10 cm) using hexane/acetone at 7:3 (v/v) for the initial eluates and progressing through step gradients of the same solvents (6:4, 5:5, 3:7) to 2.5:7.5 (v/v) with successive eluates (all solvent mixes were supplemented with 0.042 volume formic acid). Spots were detected by brief ultraviolet illumination (254 and 365 nm) and exposure to iodine vapors. Eluted material from successive tubes showing qualitatively similar TLC patterns was pooled as a single fraction, and 17 fractions (F1−F17) in total were obtained. Solvent from each fraction was removed by vacuum rotary evaporation at 30 °C, and reconstituted dry matter was tested in the QR induction bioassay (described later). The fractions representing the greatest potency and/or enrichment of QR-inducing activity were F2, F6, and F9 (see Results), and these fractions were subjected to further isolation and purification steps.

Subfractionation of the Most Potent QR-Inducing Fractions. F2 was further developed with CH_2Cl_2/acetone/formic acid (8.25:1.75:0.6, v/v/v) on a preparative silica gel F_{254} plate (10 cm × 20 cm). Silica gel bands absorbing UV light were scraped, and material was desorbed with developing solvent. Residues corresponding to subfractions of F2-1−F2-4 (respective R_f = 0.92, 0.69, 0.58, and 0.50) were recovered by solvent evaporation and subjected to further QR induction analyses. Compound **1** was obtained as the material recovered from the R_f = 0.58 band.

A portion of F6 (260 mg) was applied to a normal phase silica gel column (5.5 cm × 27 cm) and resolved using the following solvent elution steps: chloroform/ethyl acetate (1:1, v/v, 500 mL), chloroform/ethyl acetate (1:4, v/v, 500 mL), and ethyl acetate/acetone (1:1, v/v, 500 mL), to yield four subfractions, F6-1 (15 mg), F6-2 (76 mg), F6-3 (39 mg), and F6-4 (26 mg). The QR bioassay indicated F6-1 as the most active isolate, and F6-1 was loaded onto another silica gel column (1.8 cm × 24 cm) and eluted by chloroform/ethyl acetate (2:3, v/v, for 120 mL and then 1:4, v/v, for 300 mL). Eluates of 7 mL each were collected and pooled to yield isolates F6-1-1 and F6-1-2 according to TLC patterns. F6-1-1 was developed by preparative TLC (20 × 20 cm, chloroform/ethyl acetate, 9:1, v/v) to yield four fractions. None of the TLC isolates derived from F6-1-1 were obtained in sufficient quantity to proceed further. F6-1-2 showed a single spot in different solvent systems by TLC, and this isolate (compound **2**) was directly subjected to spectroscopic analyses and the QR bioassay.

Resuspension of F9 in ethyl acetate was used to separate this isolate into soluble (F9-S) and insoluble portions (F9-I). Compound (**3**) was obtained from F9-I as crystals yielded after washing with methanol. About 200 mg of F9-S was loaded onto a silica gel column (3.5 cm × 15 cm) and resolved by hexane/ethyl acetate (2:3, v/v) into three subfractions (F9-S1, F9-S2, and F9-S3). F9-S2 was further purified by reverse-phase TLC (methanol/water, 4:1, v/v) to yield compound **4** by scraping the silica band exhibiting an intense fluorescence under 365 nm light and redissolving in developing solvent.

Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS analyses were performed on a model 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) with a mass spectrometer operating in the electron ionization (EI) mode. Helium was used as a carrier gas with a flow rate of 1 mL/min. A 1 μL sample was injected onto an Rtx-5MS (5% diphenyl−95% dimethyl polysiloxane) capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Restek, Bellefonte, PA) in splitless mode at an oven temperature of 40 °C. The GC oven temperature was programmed at 40 °C for 5 min, increased to 220 °C at 10 °C/min, and then held for 4 min at 220 °C. The mass selective

detector was operated in positive EI mode with a mass scan range of m/z 29−350. The NIST/EPA/NIH version 2.0d database was used to determine compound structures and compare with authentic standards.

^{1}H Nuclear Magnetic Resonance (NMR). ^{1}H NMR analyses were collected using a Varian Unity-Inova 400 MHz NMR spectrometer at the Analytical Instrumentation Center (AIC) in the School of Pharmacy, University of Wisconsin—Madison. Purified subfractions were dissolved in corresponding deuterated solvent and transferred to solvent-matched 5 mm glass tubes (Shigemi, Inc., Allison Park, PA) shortly before analysis. Tests were carried out at ∼20 °C.

Mass Spectrometry (MS) Analysis. MS of purified components was conducted using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) using instruments housed at the AIC at the University of Wisconsin—Madison. APCI was performed for both positive and negative mode on an Agilent 1100 LC-MSD quadrupole instrument. High-resolution ESI mass spectrometric (HR-ESI-MS) analyses were obtained on an Agilent LC/MSD TOF. Purine (m/z 121.050873) and HP-0921 (m/z 922.009798) were used to internally calibrate masses.

QR Bioassay. The QR induction assay was conducted with murine Hepa 1c1c7 cells (ATCC, Rockville, MD).[11,16] Cells were cultured as previously described[17] in 96-well microtiter plates, using MEM supplemented with 10% FBS and antibiotics. Twenty four hours after initial cell seeding, test samples were dissolved in the growth medium containing 0.5% (v/v) DMSO or methanol and added to the wells in 2-fold serial dilutions to generate a dose range. The treated cells were incubated for an additional 48 h before analysis.

QR activity and cell protein (viability) assays were conducted as previously described.[17] Relative specific activity was calculated as the ratio $(\text{QR activity/protein})_{\text{sample}}/(\text{QR activity/protein})_{\text{control}}$, with control cells assigned a value of 1.0. A CD value was defined as the concentration required to double QR specific activity, and an IC_{50} value was defined as the concentration causing a reduction in protein by 50%; these values were interpolated from semilog plots of the corresponding dose-dependent responses. A chemopreventive index (CI) was calculated as the ratio of the IC_{50}/CD values to compare the relative margin between negative effects on viability and positive biological response of cells to the isolates.

Immunoblotting of NAD[P]H:Quinone Oxidoreductase 1 (NQO1). Hepa 1c1c7 cells were seeded at 10^6/well in 6-well plates, incubated at 37 °C in 5% CO_2 for 24 h, and then treated with isolates for another 48 h. Harvested cells were washed with ice-cold sodium phosphate-buffered saline (PBS) and lysed with protease inhibitor cocktail (Calbiochem-Novabiochem Inc., La Jolla, CA) on ice for 30 min. The homogenates were centrifuged at 13000 rpm using an Eppendorf (Hamburg, Germany) model 5424 microfuge for 10 min at 4 °C, and the supernatants were collected for protein analysis using a Bio-Rad *Dc* Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as calibrating standard. Samples containing 50 μg of protein were mixed with bromphenol blue, heated at 65 °C for 10 min, cooled on ice for 2 min, and microcentrifuged. Protein samples were resolved on 10% Tris−glycine SDS−polyacrylamide gels at 100 V (Mini-Protein 3 cell, Bio-Rad) and blotted onto nitrocellulose membranes (0.45 μm, Bio-Rad) for 2 h at 110 V using a Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad). The resulting blots were blocked with Tris-buffered saline with Tween (TTBS) containing 5% skim milk powder at ∼20 °C overnight and exposed to primary rabbit NQO1 antibody (1:1000 dilution in blocking buffer; Epitomics, Burlingam, CA) for 2 h at ∼20 °C. After a washing with TTBS, a secondary antibody of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:1000 dilution in TTBS, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was mixed with the membrane for 1 h at ∼20 °C and then rinsed with TTBS. Finally, the immunoreactive signals were visualized by soaking in SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and exposed using CL-XPosure Film (Pierce, Rockford, IL), which was developed by radiographic film processors (X-OMAT 2000A, Eastman Kodak Co., Rochester, NY). β-Actin was immunoblotted on the same membrane using monoclonal anti-β-actin antibody from mouse (Sigma-Aldrich) and mouse monoclonal immunoglobulin G (Santa Cruz Biotechnology Inc.) as primary and secondary antibodies, respectively. The intensity of bands was processed by Quantity One software (Bio-Rad), and the intensities of β-actin were used to normalize sample loading.

Statistical Analysis. Bioassays were performed at least three times each with duplicate samples, unless otherwise noted, and all values are represented as the mean ± SD. One-way analysis of variance (ANOVA) with a significance level of $\alpha = 0.05$ was employed to compare differences among treatments using the SPSS 13.0 statistical software package for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Fractionation of Soy Sauce Solids. Soy sauces generally have total soluble solid contents in the range of 24.8−57.9 g/100 mL, with salt contents of 11.09−22.18 g/100 mL.[18] The soy sauce used in this study contained 38.6 g/100 mL total soluble solids, with salt accounting for 51% of the solids. The nonsalt soluble solid content was 18.9 g/100 mL. Ethyl acetate was used as a liquid−liquid partitioning solvent with an intent to extract bioactive compounds with intermediate polarities from the concentrated soy sauce (Figure 1). The ethyl acetate extract (EAE) accounted for 8.7% of nonsalt soluble solids of soy sauce and exhibited QR-inducing activity with a CD value of 24.9 ± 3.2 μg/mL and a CI value of >10 ($n = 10$).

QR-Inducing Activities of Chromatographic Fractions. Gradient elution using normal phase chromatography was employed for the separation of EAE (Figure 1). Guided by qualitatively similar profiles in TLC patterns, eluants were pooled to obtain 17 fractions (F1−F17). Collectively, the 17 fractions comprised a recovery of 8.0 g of dry matter (67% of the loaded material) (Table 1). All fractions were subjected to QR-inducing activity evaluation, and 16 of them were able to double QR activity at concentrations below 100 μg/mL. Of these active fractions, F2, F6, and F9 exhibited QR-inducing potencies greater (lower CD values) than that of the original EAE, indicating an enrichment in the bioactive principles. These fractions doubled QR-specific activity in the range of 2.5−24 μg/mL and collectively comprised 11.5% of the recovered mass. Among them, F2 was the most potent QR inducer (CD value of 2.5 μg/mL) and also exhibited the greatest antiproliferative activity among all of the fractions collected, with an IC_{50} of 6.8 μg/mL. Five other fractions (F4, F7, F10, F11, and F16) exhibited slightly less potent QR-inducing properties (CD values of 30−36 μg/mL). Of these, F10 was also subjected to further isolation of QR inducers because of the abundance of this fraction and limited adverse effect on cell viability.

Isolation of QR-Inducing Agent from Fraction F2. Although F2 was among the least abundant fractions recovered (Table 1), it was further resolved using preparative silica gel (F_{254}) chromatography to yield four subfractions (F2-1−F2-4). The QR bioassay was used to survey the relative potencies among the resulting subfractions, and isolate F2-3 ($R_f = 0.58$, 8 mg) exhibited the most potent QR-inducing activity (CD value of 1.3 ± 0.5 μg/mL) relative to the other isolates (F2-1, F2-2, F2-4; CD values >30 μg/mL) ($p < 0.05$) (Table 2). Isolate F2-3 was repurified on TLC plates and subjected to structure identification. GC-MS showed that this subfraction contained >77% catechol (1,2-dihydroxybenzene; **1**, see Figure 2) (t_R = 22.295 min) with EI-MS signals of m/z 110 and fragments matching the NIST/EPA/NIH version 2.0d database with a probability of >94%. Furthermore, the dominant ^{1}H NMR signals obtained from this subfraction confirmed the identity of catechol (400 MHz, $CDCl_3$): δ 6.85 (2H, m, 3-H and 6-H), 6.79 (2-H, m, 4-H and 5-H). The splitting patterns and chemical shifts were consistent with the ^{1}H NMR spectrum contained in the Human Metabolome Database (HMDB)[19] as well as a commercial standard. Standard catechol was then tested for QR-inducing activity (Figure 3;

Table 1. Recoveries and QR-Inducing Potency Effects on Cell Viability of Fractions Obtained from an Ethyl Acetate Extract of Soy Sauce[a]

fraction	recovery (mg)	CD[b] (μg/mL)	$CD_{fraction}/CD_{EAE}$	IC_{50}[c] (μg/mL)	CI[d] (IC_{50}/CD)
F1	216.2	86.2	3.46	>250	>2.9
F2	41.9	2.5	0.10	6.8	2.7
F3	386.8	63.3	2.54	>250	>3.9
F4	338.2	30.4	1.22	162	5.3
F5	316.9	62.8	2.52	>250	>4.0
F6	603.1	21.5	0.86	>250	>11.6
F7	464.9	30.5	1.22	217	7.1
F8	292.1	43.4	1.74	228	5.3
F9	289.7	24.0	0.96	>250	>10.4
F10	742.9	36.3	1.46	>250	>6.9
F11	266.0	34.8	1.40	>250	7.1
F12	1597.7	95.1	3.82	>250	>2.6
F13	39.9	>250	NA[e]	>250	NA
F14	594.9	77.5	3.11	>250	>3.2
F15	431.9	70.5	2.83	>250	>3.5
F16	390.7	33.7	1.35	>250	>7.4
F17	1099.5	73.2	2.93	>250	>3.4

[a] Results represent the mean ± SD from ≥3 replicate experiments, each with duplicate samples. [b] CD value is the level of isolate required to double QR-specific activity. [c] IC_{50} is the level of isolate required to reduce cell viability (protein) by 50%. [d] CI is the chemopreventive index, defined as IC_{50}/CD. [e] NA means no activity or not applicable.

Table 2. Further Subfractionation and QR-Inducing Activities of Soy Sauce Fractions[a]

fraction	subfraction	recovery (mg)	CD[b] (μg/mL)	IC_{50}[c] (μg/mL)	CI[d] (IC_{50}/CD)
F2	F2-1	3.5	74.1	>250	>3.4
	F2-2	8.0	84.0	>250	>3.0
	F2-3	6.6	1.3	4.9	3.8
	F2-4	1.5	34.5	>250	>7.2
F6	F6-1	15.2	18.1	>250	>34
	F6-2	76.8	163	>250	>1.5
	F6-3	39.1	106	>250	>2.4
	F6-4	26.0	>250	>250	NA[e]
	F6-1-2	4.9	17.4	>42	>2.5
F9	F9-S	25.6	23.5	>250	>11
	F9-I	210.5	8.9	24.5	2.8
	F9-S1	35.3	6.5	85.7	14
	F9-S2	2.0	1.5	48.2	32.2
	F9-S3	58.3	54.2	252	4.65
	F9-S2-RP	1.5	0.51	4.8	9.6
F10	yellow precipitate	116.8	12.7	>50	>3.9

[a] Results represent the mean ± SD from ≥3 replicate experiments, each with duplicate samples. [b] CD value is the level of isolate required to double QR specific activity. [c] IC_{50} is the level of isolate required to reduce cell viability (protein) by 50%. [d] CI is the chemopreventive index, defined as IC_{50}/CD. [e] NA means no activity or not applicable.

Table 3), and the results (CD value of 0.93 μg/mL or 8.4 μM and IC_{50} of 30 μM) were consistent with those of F2-3 (Table 2). The minor discrepancy between standard catechol and subfraction F2-3 is likely conferred by impurities in this isolate, although catechol appears to largely account for the behavior of this subfraction. A similar CD value of 4.5 μM for catechol in Hepa 1c1c7 cells was reported previously.[20]

Catechol occurs naturally in fruits and vegetables, and the isolation of catechol from soy sauce was previously reported.[21] Catechol in soy sauce could arise from components intrinsic to the soybean and wheat raw materials and/or fungal metabolites of these intrinsic components. Soybean contains catechol at levels of 22 mg/kg protein.[22] In addition, the main fungus used in soy sauce fermentation, *A. oryzae*, is capable of transforming tryptophan to kynurenine and then

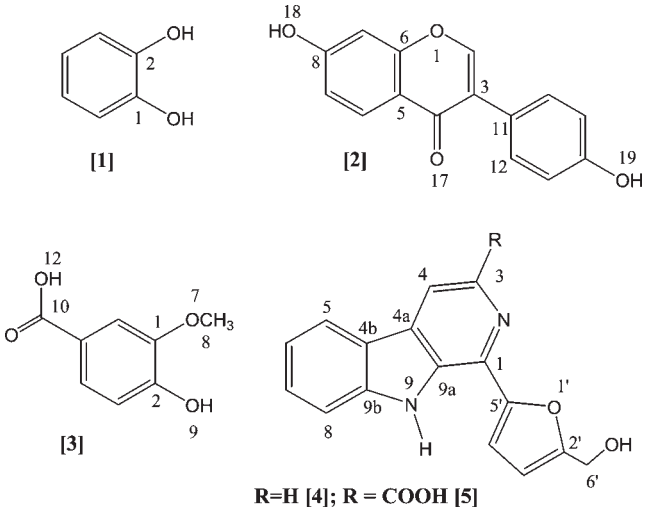

Figure 2. Chemical structures of catechol [**1**], daidzein [**2**], vanillic acid [**3**], perlolyrin [**4**], and flazin [**5**].

anthranilic acid, to ultimately form catechol.[23] The biological functionality of catechol is founded on the *o*-diphenolic structure, which like 1,4-diphenolic species, is capable of reversible redox cycling to quinones, a feature essential for diphenols to be effective QR inducers.[20] Catechol is a monofunctional inducer of NQO_1 and upregulates the expression of the antioxidant response element (ARE) without requiring activation by cytochrome P450.[24]

Isolation of QR-Inducing Agent from Fraction F6. The dry matter recovered as F6 comprised about 5% of the original 12 g of EAE loaded on the silica gel column (Table 1). The second silica gel column separation of 260 mg of F6 resulted in the recovery of about 60% of the loaded material as four subfractions, F6-1 (15 mg), F6-2 (76 mg), F6-3 (39 mg), and F6-4 (26 mg). Isolate F6-1 was the most active component ($p < 0.05$) with a CD value of 18.1 μg/mL (Table 2). It was further separated into isolates F6-1-1 (5.1 mg) and F6-1-2 (4.9 mg) using a smaller column under the same conditions. F6-1-1 was then resolved further by preparative TLC to afford four subfractions, but insufficient yield of material precluded subsequent tests of biological activity and purification. A single band at $R_f = 0.51$ was detected by silica gel TLC resolution of isolate F6-1-2 using a developing system of chloroform/ethyl acetate (1:9, v/v). Material recovered from this band was a white solid estimated to contain >75% vanillic acid (4-hydroxy-3-methoxybenzoic acid; **2**). HR-ESI-MS $[(M - H)]^-$, m/z 167.0352 ($C_8H_7O_4$). ^{1}H NMR (400 MHz, acetone-d_4) δ 7.59 (1H, dd, J = 8.0 Hz, 2.0 Hz, 4-H), 7.56 (1H, d, J = 2.0 Hz, 6-H), 6.92 (1H, d, J = 8.0 Hz, 3-H), 3.90 (3H, s, -OCH_3). ^{1}H NMR and mass spectra agreed with the standard compound and results from a previous study.[25] The CD value for QR induction of F6-1-2 was 17.4 ± 1.0 μg/mL (n = 4) (Table 2). However, a commercial vanillic acid standard could induce QR only 1.5-fold ($p < 0.05$) at the concentration of 1000 μg/mL in this bioassay. Thus, although vanillic acid was the major component of F6-1-2, it had limited contribution to QR-inducing activity of this subfraction. While there may be some potent phase II enzyme inducers in this isolate remaining to be identified, the small quantity (4.9 mg) prevented further evaluation. Compounds structurally related to vanillic acid include vanillin and ferulic acid, both of which have limited capacity for inducing QR.[11,26] On the other hand, the methyl ferulate derivative provides for a better Michael reaction acceptor group than ferulic acid and confers greater QR-inducing activity.

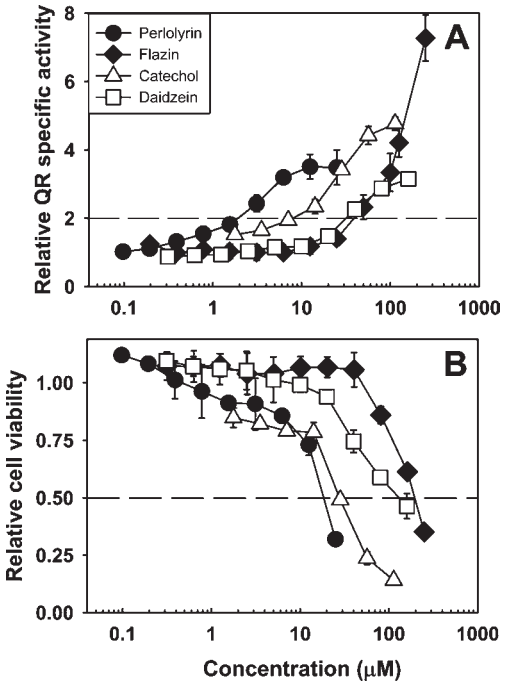

Figure 3. Relative QR-specific activities (A) and cell viabilities (B) of Hepa cells treated with perlolyrin, catechol, flazin, and daidzein. Results are expressed as the mean ± SD from three separate experiments, each with two replicates.

Table 3. QR-Inducing Potency of β-Carboline Derivatives and Diphenols Isolated from Soy Sauce[a]

compound	CD[b] (μM)	IC_{50}[c] (μM)	CI[d] (IC_{50}/CD)	CD (μg/mL)
perlolyrin	1.7	19.7	12	0.45
flazin	42.3	180	4.26	13.1
catechol	8.4	29.5	3.5	0.93
daidzein	34.8	127	3.65	8.9

[a] Results represent the mean ± SD from two experiments for catechol (n = 2) and three experiments with the other compounds, each with duplicate samples (n = 6). [b] CD value is the concentration of compound required to double QR specific activity. [c] IC_{50} is the concentration of compound required to reduce cell viability (protein) by 50%. [d] CI is the chemopreventive index, defined as IC_{50}/CD.

Vanillic acid is a known flavor compound in soy sauce that may inhibit off-flavor production by yeasts during the fermentation.[27] Fungi and yeasts, such as white rot fungi, *Aspergillus niger*, and *Rhodotorula rubra*, can metabolize ferulic acid into vanillic acid.[28] In terms of biological activity, considerable interest exists in the antioxidant, nitric oxide scavenging, and adipogenesis-inhibiting potential of vanillic acid.[29−31]

Isolation of QR-Inducing Agent from Fraction F9. The material recovered as F9 accounted for 2.4% of the 12 g of crude EAE loaded onto the silica gel column (Table 1). Extraction of F9 with ethyl acetate yielded soluble (F9-S) and insoluble (F9-I) portions. F9-I was washed with methanol to yield 210 mg of white needle-like crystals (R_f = 0.61 on silica gel TLC using hexane/acetone, 3:7, v/v), and this isolate exhibited a CD value of 8.9 ± 0.7 μg/mL (n = 4) in the QR bioassay (Table 2). This isolate was analyzed by ^{1}H NMR and mass spectroscopy and identified as daidzein (7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one; **3**). APCI mass: m/z 254; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (1H, s, 2-H), 7.96 (1H, d, J = 8.8 Hz, 10-H), 7.37

(2H, d, J = 8.8 Hz, 12-H and 16-H), 6.93 (1H, dd, J = 8.8 Hz, 2.4 Hz, 9-H), 6.86 (1H, d, J = 2.4 Hz, 7-H), 6.80 (2H, d, J = 8.8 Hz, 13-H and 15-H). Mass spectra and ^{1}H NMR were consistent those previously reported.[19,32] QR-inducing activity and the IC_{50} value of the methanol-washed F9-I isolate (Table 2) were also comparable to those of standard daidzein, which exhibited a CD value of 34.8 ± 2.6 μM (n = 4) (Figure 3; Table 3). Other reports of QR induction by daidzein in the Hepa 1c1c7 cell line imply the importance of vehicle or carrier: 1.5-fold induction at 40 μM in culture medium alone[33] and 2-fold at 386 μM with a hydroxypropyl-β-cyclodextrin carrier.[26] Daidzein exhibits mainly bifunctional QR-inducing action in Hepa 1c1c7 cells, with some monofunctional behavior.[33] Moreover, daidzein was also reported to modulate phase II enzymes in the liver, kidney, and small intestine of mice during short-term feeding.[34]

Daidzein is a commonly found isoflavone, along with genistein and glycitein.[8] They predominately exist as malonylated or acetylated glucoconjugates and account for 72% total phenolics in defatted soy flakes.[35] During soy sauce processing, the collective steps of water soaking, steam cooking, and fermentation by *A. oryzae* in saline are associated with the transformation of the glucoconjugates to aglycons.[36] Hydrolytic enzymes from both soybean and koji mold may act during the above procedures in turns[37] and, consequently, soy sauce is rich in isoflavone aglycons.[38] In vivo metabolism of daidzein to *o*-, *m*-, and *p*-catechols indicates that daidzein may be a reservoir for the generation of more potent phase II enzyme inducers.[39]

The F9-S fraction had a CD value for QR induction similar to that of the parent F9 fraction (compare Tables 1 and 2). F9-S was resolved by silica gel column chromatography using hexane/acetone (2:3, v/v) as elution solvent to obtain three subfractions (F9-S1, F9-S2, and F9-S3). The main band of F9-S1 observed by TLC was of the same R_f as daidzein (same conditions as in previous paragraph), indicating that residual daidzein was the main component in this subfraction. The similarity between CD values for QR induction between isolates F9-S1 and F9-I (Table 2) also implicates daidzein as the common and predominate bioactive agent in these isolates, although F9-S1 exhibited a greater IC_{50} value (p < 0.05). TLC analysis of F9-S1 and F9-S2 indicated there was a minor band in the former that was prevalent in the latter. Because F9-S2 had the lowest CD value (p < 0.05) of the F9-S isolates at 1.5 ± 0.5 μg/mL (n = 6) (Table 2), the minor component in F9-S1 was probably responsible for the CD value for QR induction of the daidzein-rich F9-S1 isolate being less (and more potent) than that of pure daidzein. Therefore, focus was placed on identifying the active agent(s) in F9-S2.

F9-S2 exhibited a blue band when illuminated on the TLC plate by low-UV light at 254 nm and bright blue fluorescence under 365 nm light. Normal phase TLC could not completely resolve F9-S2, so this subfraction was resolved by reverse-phase TLC to obtain a band (R_f = 0.26; MeOH/H_2O, 8:2, v/v) that yielded 1.5 mg of yellow powder (F9-S2-RP). The CD value for QR induction by this isolate was 0.51 ± 0.24 μg/mL (n = 4) (Table 2). This compound was identified as perlolyrin ([5-(9*H*-pyrido[3,4-*b*]indol-1-yl)furan-2-yl]-methanol; **4**). APCI mass, m/z 264; HR-ESI-MS, $[M + H]^+$ m/z 265.0982, calcd 265.0971 for $C_{16}H_{13}N_2O_2$; $[M - H]^-$ m/z 263.0831, calcd 263.0826 for $C_{16}H_{11}N_2O_2$. ^{1}H NMR (400 MHz, CD_3OD): δ 8.27(1H, d, J = 5.2 Hz, 3-H), 8.17 (1H, d, J = 8.0 Hz, 5-H), 8.00 (1H, d, J = 5.2 Hz, 4-H), 7.68 (1H, d, J = 8.0 Hz, 8-H), 7.55 (1H, td, J = 7.2 Hz, 1.2 Hz, 7-H), 7.26 (1H, td, J = 7.2 Hz, 1.2 Hz, 6-H), 7.20 (1H, d, J = 3.6 Hz, 3′-H), 6.57 (1H, d, J = 3.6 Hz, 4′-H), 4.77 (2H, s, OCH_2). Mass spectra and ^{1}H NMR signals closely agreewith those of previous analyses.[40,41] The CD value for QR induction by purified perlolyrin was 0.45 ± 0.21 μg/mL (n = 6) (Table 3).

During removal of solvent from F10, a yellow precipitate gradually formed. This insoluble material was filtered and then washed with ethyl acetate to yield a yellow powder essentially pure (97% judged by HPLC) in a compound of MW 308 (117 mg, $[M + H]^+$ m/z 309.0860, $[M - H]^-$ m/z 307.0830) and matched to the molecular formula of $C_{17}H_{12}N_2O_4$. This component was identified as flazin (1-(5-(hydroxymethyl)furan-2-yl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid; **5**): HR-ESI-MS, $[M + H]^+$ m/z 309.0860, calcd 309.0869 for $C_{17}H_{13}N_2O_4$; $[M - H]^-$ m/z 307.0830 calcd 307.0724 for $C_{17}H_{11}N_2O_4$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.58 (2H, s, NH and OH), 8.84 (1H, s, 4-H), 8.42 (1H, d, J = 8.0 Hz, 5-H), 7.82 (1H, d, J = 8.4 Hz, 8-H), 7.62 (1H, t, J = 8.0 Hz, 7-H), 7.42 (1H, d, J = 3.6 Hz, 4′-H), 7.35 (1H, t, J = 7.2 Hz, 6-H), 6.62 (1H, d, J = 3.6 Hz, 3′-H), 5.49 (1H, s br, OH), 4.69 (2H, s, 2′-CH_2). Mass spectra and ^{1}H NMR were consistent with those previously reported.[19,40] The CD value for QR induction of this compound was 12.7 ± 0.4 μg/mL (n = 4) (Table 2).

Flazin and perlolyrin are fluorescent compounds previously identified in soy sauce.[41] These β-carbolines are likely to be formed by two processes. β-Carbolines are metabolic products of *Pediococcus halophilus*, a lactic acid bacterium vital to soy sauce fermentation.[42] Chemically, β-carbolines are formed by the Pictet−Spengler reaction involving the condensation of a β-arylethylamine, such as tryptophan or tryptamine, with an aldehyde or aldehyde surrogate to yield a Schiff base (as in the Maillard reaction), which spontaneously cyclizes to yield the tetrahydro β-carboline.[43,44] Subsequent oxidation and oxidative decarboxylation steps give rise to aromatic derivatives. Acidic media (pH 2−5) facilitate β-carboline formation through protonation of the aldehyde and imine (Schiff base), enhancing the electrophilicity of each and respectively hastening the condensation and cyclization steps. Flazin may be a source of perlolyrin via decarboxylation during the aging of soy sauce.[42] The natural existence of flazin was also reported in Japanese rice wine[45] and the fruiting body of *Suillus granulatus*,[46] and it is produced by the marine bacteria *Streptomyces* spp.[40] Perlolyrin is also found in other fermented soy products,[47] rye grass,[48] and *Codonopsis pilosula* (traditional Chinese medicine plant).[49]

Western Blots of NQO1 by Potent Compounds. The β-carbolines isolated from soy sauce, flazin and perlolyrin, caused concentration-dependent increases in NQO_1 protein in Hepa 1c1c7 cells after 48 h of exposure (Figure 4A,B). The relative levels of NQO_1 protein ranged from 1.3- to 2.2-fold greater than control cells for flazin at 5−100 μM (Figure 4A). Exposure to perlolyrin at concentrations of 0.1−5 μM resulted in a progressive increased in NQO_1 protein to levels 1.3−4.0-fold those of control cells (Figure 4B). Up-regulation of NQO_1 protein levels was also observed for daidzein at 10 and 20 μM and for catechol at 1 and 2 μM (Figure 4C). These increases in NQO_1 protein levels generally corresponded to the relative extent and potency of QR activity increases observed in Hepa cells under similar conditions (Figure 3A; Table 3).

Prospective Role of β-Carbolines as Anticancer Agents and Phase II Enzyme Inducers. Of the four effective phase II enzyme inducers isolated from soy sauce, the β-carbolines flazin and perlolyrin have not previously been shown to have this biological effect. β-Carbolines (pyrido(3,4-*b*)indoles) are found in some foods at levels up to 700 ppm, although more typically their concentrations in smoked, cooked, and fermented foods range from a few parts per million to 1−2 orders of magnitude less.[43] Despite the rather low levels in foods, β-carbolines can accumulate in some human tissues, including the brain.[50,51] β-Carbolines are readily absorbed from the diet and are also generated in vivo through physiological and metabolic processes, contributing to endogenous tissue

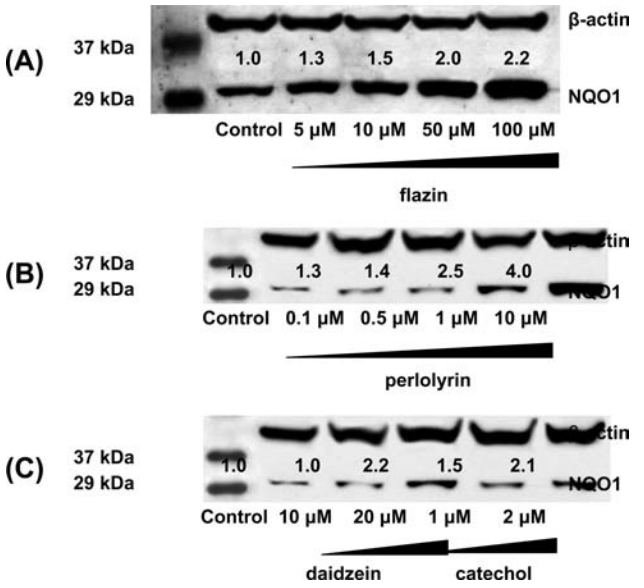

Figure 4. Induction of NAD(P)H:quinone oxidoreductase 1 protein expression in Hepa 1c1c7 cells by compounds isolated from soy sauce. Cells were plated at 10^6 cells/well in six-well plates exposed to either 0.5% DMSO medium (control) or flazin, perlolyrin, daidzein, or catechol at various concentrations for 48 h. Blots were also probed with anti-β-actin antibody to normalize loading. Prestained molecular weight markers were used.

levels.[50−52] Although the reported levels of flazin and perlolyrin in commercial soy sauce may be considered limited at 24 and 2.8 ppm, respectively,[47] the potential to accumulate in specific tissues could render them as physiologically significant factors. Myriad beneficial and detrimental biological effects have been established for the diverse group of β-carbolines derived from foods and other natural sources and as potential pharmaceuticals, and even subtle differences in structure are important in conferring these effects.[42,53] Some β-carbolines are potentially (co)mutagenic, genotoxic, and neurotoxic and may contribute to alcohol addiction.[43,50−52] The potential beneficial effects of β-carbolines include anticancer and antioxidant activities, and they may also reduce the negative impact of amine metabolism by inhibiting monoamine oxidase activities.[51,54−57]

Recently, β-carboline derivatives were extensively studied for their antitumor effects.[51] Some of the anticancer effects of β-carbolines appear to be related to their planar structure and ability to noncovalently interact with DNA by intercalation or minor groove binding.[58,59] In several cancer cell lines, this was associated with induction of DNA double-strand breaks by Mana-Hox (a β-carboline dimeric derivative), leading to tubulin depolymerization, mitotic arrest, and induction of apoptosis.[58] Flazin can inhibit 7,12-dimethylbenz-[α]anthracene-induced preneoplastic lesion formation in a mouse mammary organ culture model.[55] Antioxidant activity has been associated with β-carbolines, and especially the tetrahydro derivatives (reduced pyridine moiety).[51,54,57] Tetrahydro β-carbolines act as antioxidants by quenching singlet oxygen, superoxide, and hydroxyl radicals and donating H/electrons to radical species.[51]

Phase II enzyme inducers fall into at least nine chemical categories (chemotypes) and are typified by being oxidants/antioxidants, by possessing a Michael reaction acceptor group, and/or by being reactive with thiols.[11] The aromatic β-carbolines flazin and perlolyrin do not possess or exhibit these properties, and their QR-inducing activity cannot be explained on a conventional basis. However, the indole aromatic moiety of β-carbolines is subject to hydroxylation via reaction with reactive oxygen species or though metabolism by peroxidases and cytochrome P450s.[50,52] Preferred sites of hydroxylation are at C6 and secondarily at C3 sites of the β-carboline structure (cf. Figure 2). The resulting aromatic C6−OH group provides for antioxidant power and may become oxidized to the corresponding quinone. The pyrido-C3−OH moiety may isomerize with the neighboring N atom to yield the C3-quinone derivative.[50] β-Carboline "activated" in any of these manners may yield electrophilic centers, affording reactivity with glutathione,[52] and these properties are hallmarks of components capable of up-regulating phase II enzymes through the antioxidant response element.[11] Thus, it is likely that cellular metabolism or redox activation of flazin and perlolyrin is a requisite for their QR-inducing properties observed in the present study. Relative to perlolyrin, the free carboxyl group at C3 for flazin (Figure 2) may reduce potency of QR induction by conferring less lipophilicity (and correspondingly less cellular uptake) and/or block a potential site (C3) of metabolic activation. A similar dependence of relative QR-inducing potency on lipophilicity and the absence of a free carboxyl group was found for crotonic acid versus methyl acrylate and for cinnamic acid versus methyl cinnamate.[12]

Because the structures of flazin and perlolyrin are conjugates of 9*H*-pyrido[3,4-*b*]indole (norharman) and 2-furanmethanol moieties (Figure 2), it is possible that the furyl moieties may contribute to the QR-inducing activity observed. 5-Hydroxymethylfurfural can induce QR but fails to double QR specific activity at levels up to 1000 μg/mL.[60] Also, a previous study observed the anticarcinogenic action in mice of a diet supplemented with soy sauce ethyl acetate extract, where focus was placed on furanone flavoring agents.[13] However, 4-hydroxy-2,5, dimethyl-3(2*H*)-furanone did not induce GST activity in mouse liver and forestomach, although the ethyl acetate extracts of soy sauce did. It was suggested that the anticarcinogenic activity of the furyl components may be founded on antioxidant effects related to scavenging H_2O_2 and superoxide anion radicals. Thus, we conclude that it is principally the β-carboline nucleus and not the substituent furyl group that functions as a QR inducer.

In conclusion, the present work revealed potent phase II enzyme inducing agents in an ethyl acetate extract of soy sauce using a bioassay directed isolation approach. Two commonly known phenolic compounds and two β-carbolines were isolated by this approach. The levels of the two β-carbolines in soy sauce are in the upper ranges normally founds in foods.[43,47] Because β-carbolines are evolved through Maillard reactions in foods, there are likely to be β-carbolines remaining to be identified. These results extend previous knowledge regarding the potential cancer chemopreventive benefit of soy foods in general and soy sauce in particular. There are likely more phase II enzyme inducers to be isolated as the active agents in F6-1 could not be pursued for lack of material. Because this is the first known report of phase II enzyme inducing activity of β-carbolines, further studies are warranted on surveying structure−activity relationships of β-carbolines among other foods, as well as developing an understanding of the mechanism of action of β-carbolines and their cellular metabolites.

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ABBREVIATIONS USED

QR, quinone reductase; CD, concentration required to double relative QR specific activity; IC_{50}, concentration required to reduce cell viability by 50%; EAE, ethyl acetate extract; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; PTLC, preparative thin-layer chromatography; RP-TLC, reverse phase thin-layer chromatography; MS, mass spectrometry; ESI, electrospray ionization; HR-ESI-MS, high-resolution electrospray ionization−mass spectrometry; EtOAc, ethyl acetate; SDS, sodium dodecyl sulfate; TTBS, Tris-buffered saline with Tween; NQO_1, NAD[P]H:quinone oxidoreductase 1; RP-HPLC, reversed phase high-performance liquid chromatography; GC, gas chromatography; t_R, retention time; DMSO, dimethyl sulfoxide; GST, glutathione *S*-transferase.

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