Food Chemistry 135 (2012) 1239–1244

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Hypohalous acid-mediated halogenation of resveratrol and its role in antioxidant and antimicrobial activities

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ARTICLE INFO

Article history:
Received 29 February 2012
Received in revised form 19 April 2012
Accepted 8 May 2012
Available online 17 May 2012

Keywords:
Myeloperoxidase
Resveratrol
Hypohalous acids
Antioxidant
Antimicrobial
Cytotoxicity

ABSTRACT

The reactions of resveratrol with proinflammatory oxidants including hypochlorous and hypobromous acids in phosphate-buffered saline/methanol solution were carried out and eight halogenated resveratrol derivatives differing in the number and position of halogen atoms, and the configuration of double bond were obtained. Halogenation of resveratrol took place only at the aromatic A ring, and interestingly, the halogenation increased antioxidant activity of this parent molecule in the 2,2′-azobis(2-amidinopropane) hydrochloride-induced RBC haemolysis model. Additionally, antimicrobial activity of the derivatives against Gram-positive bacteria, Gram-negative bacteria and fungi were tested, and toward *Candida albicans*, 2-chloro-resveratrol and 2-bromo-resveratrol were more active than the unmodified form and the reference compound fluconazole.

1. Introduction

Myeloperoxidase (MPO), a haem enzyme released by activated phagocytes during inflammation, catalyses the reaction of hydrogen peroxide with physiological concentrations of chloride and bromide anions to produce hypochlorous acid (HOCl) and hypobromous acid (HOBr), respectively (Chapman, Skaff, Senthilmohan, Kettle, & Davies, 2009). HOCl and HOBr could function as potent antibacterial agents and play a pivotal role in the immune response (Thomas, 1979), but they are also oxidants and electrophiles which react easily with biological molecules such as proteins, lipids and DNA (Davies, Hawkins, Pattison, & Rees, 2008; Hawkins & Davies, 2002; Hawkins, Pattison, & Davies, 2003; Pattison & Davies, 2001, 2006). Thus, tissue damage resulted from excessive or misplaced production of hypohalous acids has been implicated in many inflammatory diseases including atherosclerosis, neurodegeneration and cancer (Nicholls & Hazen, 2005; Ohshima, Tatemichi, & Sawa, 2003; Yap, Whiteman, & Cheung, 2007). This has prompted interest in modulating hypohalous acid-mediated damage by using antioxidants (Folkes, Candeias, & Wardman, 1995; Pattison, Hawkins, & Davies, 2003; Skaff, Pattison, & Davies, 2007), and investigating hypohalous acid-mediated fate of antioxidants (Boersma et al., 1999; Ho, Soldevilla, Hook, & Southwell-Keely, 2000; Nguyen & Southwell-Keely, 2007). Interestingly, chlorination of quercetin and soy isoflavones (genistein and daidzein) mediated by HOCl and neutrophil MPO, respectively, leads to the enhanced antioxidant activity in comparison with their parent molecules. This suggests that inflammatory cell-specific metabolism could be an important pathway to generate novel pharmacophores for polyphenolics and modify their properties at the local site of inflammation (Binsack et al., 2001; Boersma et al., 2001, 2003).

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenolic phytoalexin present in grape and red wine, has attracted considerable attention because of its multiple pharmacological actions ranging from antibacterial and antifungal effects to antioxidative, anti-inflammatory, cardioprotective, neuroprotective and cancer chemopreventive activities (Aggarwal & Shishodia, 2006; Baur & Sinclair, 2006; Pezzuto, 2008). Studies have indicated that this compound shows significant dose-dependent inhibitory effect on the production of HOCl and nitric oxide by stimulated human neutrophils through preventing the release of MPO (Cavallaro, Ainis, Bottari, & Fimiani, 2003), and on the chlorination, oxidation and nitration activities of MPO by a direct interaction with the enzyme (Kohnen et al., 2007). However, the reaction products of resveratrol with hypohalous acids (HOCl and HOBr) and their biological activities, are yet to be determined. As part of our laboratory's continuous effort to find resveratrol-inspired antioxidants and cancer chemopreventive agents (Cao et al., 2012; Fan et al., 2009; Shang

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http://dx.doi.org/10.1016/j.foodchem.2012.05.043

et al., 2009; Tang et al., 2011; Yang et al., 2010), we initiated the current study on the reaction of resveratrol with hypohalous acids under physiological conditions to mimic MPO-mediated fate of this molecule and to investigate antioxidant, antimicrobial and antiproliferative activities of the related products.

2. Materials and methods

2.1. General experimental procedures

Resveratrol was purchased from Shanxi Sciphar Biotechnology Company, China and further purified before use. NaOCl and *N*-bromosuccinimide (NBS) were from Tianjin Guangfu Fine Chemical Research Institute, China. 2,2′-Azobis (2-amidinopropane hydrochloride) (AAPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich and used as received. RPMI 1640 medium were purchased from GIBCO. All ^{1}H, ^{13}C and 2D NMR spectra were measured using a Bruker Avance III 400 MHz NMR spectrometer with d_6-CH_3COCH_3 as solvent. High-resolution mass spectra (HR-MS) were obtained on an APEX II FT-ICR MS spectrometer.

2.2. Reaction of resveratrol with HOCl or HOBr

Phosphate-buffered saline (PBS, pH 7.3, 200 ml) was added to a solution of resveratrol (550 mg) in methanol (90 ml). The suspension was stirred vigorously for 10 min before the addition of 5% NaOCl (50 ml, 36.1 mmol). Stirring was continued for 7 h at room temperature under conditions of darkness. The mixture was extracted with ethyl acetate, and the organic phase was washed with water and brine, dried, and concentrated. The residue was separated by silica gel column chromatography using chloroform/methanol from 40:1 to 120:1 as elution phase, and the products were collected and recrystallized from light petroleum/acetone to give pure compounds **1**–**5**.

(E)-2-chloro-3, 5, 4′-trihydroxystilbene (**1**): Yield: 1%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.47 (d, J = 2.8 Hz, H-4), 6.79 (d, J = 2.8 Hz, H-6), 6.87 (d, J = 8.4 Hz, H-3′, H-5′), 7.06 (d, J = 16.0 Hz, H-8), 7.32 (d, J = 16.0 Hz, H-7), 7.47 (d, J = 8.4 Hz, H-2′, H-6′), 8.41 (brs, –OH), 8.49 (brs, –OH), 8.54 (brs, –OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 103.7 (1C, C-4), 105.1 (1C, C-6), 111.6 (1C, C-2), 116.6 (2C, C-3′, C-5′), 122.7 (1C, C-7), 129.2 (2C, C-2′, C-6′), 129.8 (1C, C-1′), 132.0 (1C, C-8), 138.1 (1C, C-1), 154.9 (1C, C-3), 157.6 (1C, C-5), 158.7 (1C, C-4′); HR-ESI-MS: *m/z*: calcd. for $C_{14}H_{11}ClO_3$ + H: 263.0469; found: 263.0476, error = 2.7 ppm.

(E)-2, 4-dichloro-3, 5, 4′-trihydroxystilbene (**2**): Yield: 1%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.86 (d, J = 8.8 Hz, H-3′, H-5′), 6.99 (s, H-6), 7.06 (d, J = 16.0 Hz, H-8), 7.26 (d, J = 16.0 Hz, H-7), 7.45 (d, J = 8.8 Hz, H-2′, H-6′), 8.74 (brs, –OH), 8.86 (brs, –OH), 9.29 (brs, –OH).

(E)-2, 6-dichloro-3, 5, 4′-trihydroxystilbene (**3**): Yield: 1%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.67 (s, H-4), 6.88 (d, J = 8.0 Hz, H-3′, H-5′), 6.96 (d, J = 16.4 Hz, H-7), 7.03 (d, J = 16.4 Hz, H-8), 7.48 (d, J = 8.0 Hz, H-2′, H-6′), 8.59 (s, 4′-OH), 8.74 (s, 3-OH, 5-OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 103.7 (1C, C-4), 112.4 (2C, C-2, C-6), 116.6 (2C, C-3′, C-5′), 121.2 (1C, C-7), 129.1 (2C, C-2′, C-6′), 129.5 (1C, C-1′), 136.9 (1C, C-1), 137.5 (1C, C-8), 153.5 (2C, C-3, C-5), 158.9 (1C, C-4′); HR-ESI-MS: *m/z*: calcd. for $C_{14}H_{10}Cl_2O_3$ – H: 294.9934; found: 294.9928, error = 2.0 ppm.

(E)-2, 4, 6-trichloro-3, 5, 4′-trihydroxystilbene (**4**): Yield: 6.51%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.89 (d, J = 8.4 Hz, H-3′, H-5′), 6.94 (d, J = 16.4 Hz, H-7), 7.05 (d, J = 16.4 Hz, H-8), 7.49 (d, J = 8.4 Hz, H-2′, H-6′), 8.57 (s, 4′-OH), 8.81 (s, 3-OH, 5-OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 109.7 (1C, C-4), 113.2 (2C, C-2, C-6), 116.5 (1C, C-3′), 116.6 (1C, C-5′), 120.4 (1C, C-7), 129.0 (1C, C-1′), 129.1 (2C, C-2′,C-6′), 134.9 (1C, C-1), 138.0 (1C, C-8), 149.9 (2C, C-3, C-5), 159.1 (1C, C-4′); HR-ESI-MS: *m/z* calcd. for $C_{14}H_9Cl_3O_3$ + H: 330.9690; found: 330.9692, error = 0.6 ppm.

(Z)-2, 4, 6-trichloro-3, 5, 4′-trihydroxystilbene (**5**): Yield: 0.14%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.20 (d, J = 12.4 Hz, H-7), 6.66 (d, J = 8.8 Hz, H-3′, H-5′), 6.71 (d, J = 12.4 Hz, H-8), 6.93 (d, J = 8.8 Hz, H-2′, H-6′); HR-ESI-MS: *m/z* calcd. for $C_{14}H_9Cl_3O_3$–H: 328.9545; found: 328.9550, error = 1.5 ppm.

To a solution of resveratrol (550 mg) in methanol (90 ml) was slowly added PBS (200 ml). After being stirred for 10 min, a solution of 5% self-prepared NaOBr (50 ml, 22.1 mmol) was added dropwise over 1 h and the mixture stirred for 7 h at room temperature under conditions of darkness. The mixture was extracted with ethyl acetate, and the organic layer was washed with water and brine, and dried over $MgSO_4$ followed by evaporation *in vacuo*. The residue was separated by silica gel column chromatography using chloroform/methanol from 40:1 to 80:1 as elution phase, and the products were collected and recrystallized from light petroleum/acetone to give pure compounds **6**–**8**.

(E)-2-bromo-3, 5, 4′-trihydroxystilbene (**6**): Yield: 3.72%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.48 (d, J = 2.8 Hz, H-4), 6.79 (d, J = 2.8 Hz, H-6), 6.87 (d, J = 8.4 Hz, H-3′, H-5′), 7.01 (d, J = 16.4 Hz, H-8), 7.32 (d, J = 16.4 Hz, H-7), 7.47 (d, J = 8.4 Hz, H-2′, H-6′), 8.43 (brs, 4′-OH), 8.54 (brs, 3-OH, 5-OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 102.6 (1C, C-2), 103.5 (1C, C-4), 105.7 (1C, C-6), 116.6 (2C, C-3′, C-5′), 125.6 (1C, C-7), 129.2 (2C, C-2′, C-6′), 129.8 (1C, C-1′), 132.1 (1C, C-8), 139.9 (1C, C-1), 155.9 (1C, C-3), 158.4 (1C, C-5), 158.7 (1C, C-4′); HR-ESI-MS: *m/z* calcd. for $C_{14}H_{11}BrO_3$+H: 306.9964; found: 306.9957, error = 1.3 ppm.

(E)-2, 4-dibromo-3, 5, 4′-trihydroxystilbene (**7**): Yield: 1%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.88 (d, J = 8.4 Hz, H-3′, H-5′), 7.01(s, H-6), 7.03 (d, J = 16.0 Hz, H-8), 7.26 (d, J = 16.0 Hz, H-7), 7.47 (d, J = 8.4 Hz, H-2′, H-6′), 8.22 (brs, 3-OH), 8.57 (brs, 4′-OH), 8.93 (brs, 5-OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 98.9 (1C, C-4), 103.0 (1C, C-2), 105.7 (1C, C-6), 116.7 (2C, C-3′, C-5′), 124.8 (1C, C-7), 129.3 (2C, C-2′, C-6′), 129.5 (1C, C-1′), 132.8 (1C, C-8), 138.5 (1C, C-1), 152.6 (1C, C-3), 155.1 (1C, C-5), 158.9 (1C, C-4′); HR-ESI-MS: *m/z* calcd. for $C_{14}H_{10}Br_2O_3$ + H: 384.9069; found: 384.9073, error = 1.0 ppm.

(E)-2, 6-dibromo-3, 5, 4′-trihydroxystilbene (**8**): Yield: 0.65%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.71 (s, H-4), 6.80 (d, J = 16.4 Hz, H-7), 6.89 (d, J = 16.4 Hz, H-8), 6.89 (d, J = 8.8 Hz, H-3′, H-5′), 7.47 (d, J = 8.8 Hz, H-2′, H-6′), 8.56 (brs, 4′-OH), 8.81 (brs, 3-OH, 5-OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 102.7 (1C, C-4), 103.2 (2C, C-2, C-6), 116.6 (2C, C-3′, C-5′), 125.7 (1C, C-7), 129.0 (2C, C-2′, C-6′), 129.3 (1C, C-1′), 137.2 (1C, C-8), 140.6 (1C, C-1), 155.1 (2C, C-3, C-5), 158.9 (1C, C-4′); HR-ESI-MS: *m/z* calcd. for $C_{14}H_{10}Br_2O_3$–H: 382.8924; found: 382.8934, error = 2.6 ppm.

2.3. Synthesis of (E)-2, 4, 6-tribromo-3, 5, 4′-trihydroxystilbene

Dissolved resveratrol (545 mg) in the mixture of anhydrous acetonitrile (25 ml) and methanol (2 ml), and NBS (1.27 g) was slowly added to the solution. After being stirred for 24 h at room temperature, the solvent was removed *in vacuo*, giving a tan oil. The oil was disolved in ethyl acetate, and washed with water and brine before dried over $MgSO_4$. Removal of the solvent *in vacuo* again yielded a brown oil, which was separated by silica gel column chromatography using chloroform/methanol (120/1, v/v) as eluting solvent. The crude product was collected and recrystallized from light petroleum/acetone to give pure compound **9**.

(E)-2, 4, 6-tribromo-3, 5, 4′-trihydroxystilbene (**9**): Yield: 29.3%; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ (ppm) = 6.81 (d, J = 16.4 Hz, H-7), 6.87 (d, J = 16.4 Hz, H-8), 6.89 (d, J = 8.4 Hz, H-3′, H-5′), 7.48 (d,

J = 8.4 Hz, H-2′, H-6′), 8.50 (s, 3-OH, 5-OH), 8.55 (s, 4′-OH); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ (ppm) = 99.0 (1C, C-4), 103.3 (2C, C-2, C-6), 116.6 (2C, C-3′, C-5′), 125.2 (1C, C-7), 128.9 (1C, C-1′), 129.2 (2C, C-2′, C-6′), 137.7 (1C, C-8), 139.4 (1C, C-1), 152.0 (2C, C-3, C-5), 159.0 (1C, C-4′); HR-ESI-MS: m/z calcd. for $C_{14}H_9Br_3O_3-H$: 460.8029; found: 460.8023, error = 1.3 ppm.

2.4. Assay for anti-haemolysis activity

Human red blood cells (RBCs) were obtained from the Red Cross Centre for Blood (Gansu, China). The procedures for determining the extent of haemolysis have been described previously (Qian et al., 2011).

2.5. Assay for antimicrobial activity

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albican* were provided by the Clinic Microbial Test Centre of Gansu Provincial People's Hospital.

A broth microdilution method (Carson, Hammer, & Riley, 1995) was used to determine the minimal inhibitory concentration (MIC), which is defined as the lowest concentration of a sample at which the microorganism does not demonstrate visible growth. All tests were performed in nutrient broth. Overnight broth cultures of each strain were prepared and the final concentration of each well was adjusted to 2×10^6 CFU/ml. Each tested compound was dissolved in 5% dimethylsulphoxide (DMSO) and then diluted with the highest concentration (2 mg/ml) to prepare serial twofold dilutions in a 96-well microtiter plate with the range of 0.0039–2 mg/ml. Standards and controls were made as follows: wells containing nutrient broth only; each type of bacteria but with no compounds; nutrient broth containing compounds. Levofloxacin and fluconazole at the concentration range of 0.0039∼2 mg/ml were prepared in nutrient broth, and served as the positive controls. The plate was covered with a sterile plate sealer. Contents of each well were mixed on a plate shaker for 20 s, and plates were incubated at 37 °C for 24 h for bacteria and at 28 °C for 48 h for the yeast. Each test in this study was repeated at least twice.

2.6. Calculation of ClogP

The octanol–water partition coefficient P, a descriptor of lipophilicity, is defined as the ratio of the concentrations of a compound in two immiscible phases (octanol and water) under equilibrium conditions, and is used usually in its logarithmic form, log$P$. The calculated log$P$ (Clog$P$) values of the halogenated resveratrol derivatives were obtained by Bio-Loom software (Biobyte Corp. version 5) (Hansch & Leo, 1995; Selassie, Kapur, Verma, & Rosario, 2005).

2.7. Cell culture

Human promyelocytic leukemia (HL-60) and human lung adenocarcinoma epithelial (A549) cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, penicillin (100 kU/L) and streptomycin (100 kU/L) at 37 °C in a CO_2 (5%) incubator. Exponentially growing cells were used throughout these experiments.

2.8. Assessment of antiproliferative activity

Antiproliferative activity of the halogenated resveratrol derivatives was assessed by the MTT colourimetric assay which is based on the reduction of MTT by the mitochondrial succinate dehydrogenase of intact cells to a purple formazan product (Hussain, Nouri, & Oliver, 1993). HL-60 cells (5×10^4/ml) were seeded in 96-well microtiter plates and incubated with various concentrations of compounds for 48 h at 37 °C, then 10 μl MTT solution (5 mg/ml in PBS) was added to each well and the cells were incubated for 4 h at 37 °C in the CO_2 (5%) incubator. One hundred ten micro litres extraction buffer (10% SDS-5% isobutanol-0.1 M HCl) was added and the incubation was continued overnight at 37 °C. A549 cells (5×10^4/ml) were seeded in 96-well microtiter plates and cultured for 24 h, then the medium was replaced by fresh medium before the cells were treatment with various concentrations of compounds. After 48 h, the medium was replaced by fresh medium which contained 0.5 mg/ml MTT and the cells were incubated for 4 h. Afterwards, the medium was removed and 100 μl DMSO was added. The absorbance was measured at 570 nm using a Bio-Rad M680 ELISA microplate reader.

3. Results and discussion

3.1. Halogenation of resveratrol

The reaction of resveratrol with HOCl or HOBr formed from NThe reaction of resveratrol with HOCl or HOBr formed from NaOCl or NaOBr (Nguyen & Southwell-Keely, 2007) was conducted in PBS/methanol solution. Under physiological conditions, HOCl and HOBr are in equilibrium with the corresponding conjugated base $^-$OCl and $^-$OBr, respectively. Thus, the used terms of HOCl and HOBr are representative of the mixture of these species (Morris, 1966; Skaff et al., 2007). Eight products (Fig. 1) were isolated and identified as (E)-2-chloro-3, 5, 4′-trihydroxystilbene (**1**), (E)-2, 4-dichloro-3, 5, 4′-trihydroxystilbene (**2**), (E)-2, 6-dichloro-3, 5, 4′-trihydroxystilbene (**3**), (E)-2, 4, 6-trichloro-3, 5, 4′-trihydroxystilbene (**4**), (Z)-2, 4, 6-trichloro-3, 5, 4′-trihydroxystilbene (**5**), (E)-2-bromo-3, 5, 4′-trihydroxystilbene (**6**), (E)-2, 4-dibromo-3, 5, 4′-trihydroxystilbene (**7**) and (E)-2, 6-dibromo-3, 5, 4′-trihydroxystilbene (**8**) by HR-ESI-MS and ^{1}H, ^{13}C and 2D NMR spectrum (see the Supporting Information). It should be pointed out that the yields of halogenation of resveratrol in the presence of HOCl or HOBr are quite low and the synthesis is not valuable as a preparative method, but the reaction research helps to clarify the MPO-mediated the fate of resveratrol and understand the reaction details. Additionally, the products could be more efficiently obtained by MPO in neutrophils than *in vitro*. To facilitate the structure–activity study in the following experiments, (E)-2, 4, 6-tribromo-3, 5, 4′-trihydroxystilbene (**9**) was also synthesized by the reaction of resveratrol (1 eq.) with *N*-bromosuccinimide (3 eq.).

Only the chlorinated and brominated resveratrol derivatives were obtained in the reactions and the halogenation took place only at the aromatic A ring, in support of an electrophilic mechanism of hypohalous acids. In contrast to the aromatic B ring, A ring bears two electron-donating hydroxyl groups at positions 3 and 5 and has a relatively high electron density, hence facilitating occurrence of the electrophilic reactions. By analysing the products obtained, it is clear that position 2 on the aromatic A ring of resveratrol is the preferred site of electrophilic attack. It has been revealed that Cl^+ itself is not the actual species involved in chlorination by HOCl (Swain & Crist, 1972). However, the actual chlorinating species derived from NaOCl is unclear (Ho et al., 2000; Nguyen & Southwell-Keely, 2007). On the basis of the reference deals with the reaction of γ-tocopherol model compound and HOCl (Ho et al., 2000), the probable mechanism for formation of **1** are showed in Fig. 2.

Noticeably, a *cis*-configuration product **5** was obtained in the reaction mediated by HOCl. This can be rationalised by isomerization of product **4** under the condition of light or usage of solvents with low dielectric constant during the post-processing stage of

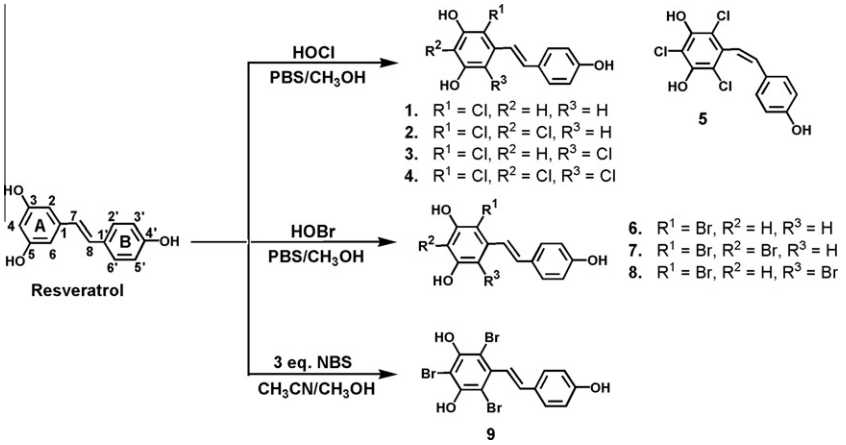

Fig. 1. The products formed in the reaction of resveratrol with HOCl, HOBr or NBS.

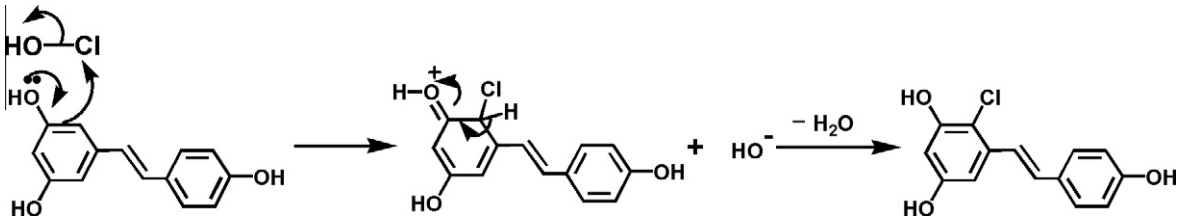

Fig. 2. Probable mechanism for formation of **1** by the reaction of resveratrol and HOCl.

reaction. It has been previously reported that the *cis*-resveratrol can be easily transformed from the *trans* form under intense UV light (Trela & Waterhouse, 1996), and the formation rate of *cis*-diethylstilbestrol from its *trans* form appears to be facilitated by solvents with low dielectric constant such as ethylacetate, chloroform, heptane and benzene (Winkler, Nyman, & Egan, 1971).

3.2. Anti-haemolysis activity of the halogenated resveratrol derivatives

An initiating radical produced by thermal decomposition of AAPH in the aqueous dispersion of human red blood cells (RBCs) could induce membrane lipid peroxidation, resulting in the occurrence of haemolysis, which is easily detected by measuring the absorbance of the haemolysate at 540 nm (Qian et al., 2011). Thus, the AAPH-induced RBC haemolysis model was used to evaluate antioxidant activity of the six selected compounds representative of mono-, di- and tri-chlorinated and brominated resveratrol derivatives.

Fig. 3 shows time courses for AAPH-dependent RBC haemolysis in the absence and presence of resveratrol and its halogenated derivatives. A typical time course for haemolysis consists of an initial inhibition phase followed by a propagation phase and finally termination. Addition of 50 mM AAPH caused fast haemolysis after an inhibition time (t_{inh}) of 95 min depending on the used sample (line a), which stems from the presence of the endogenous antioxidant such as vitamin E and/or ubiquinol-10 in the RBC membrane (Esterbauer & Ramos, 1995). Twenty micro molar resveratrol and its halogenated derivatives inhibited effectively AAPH-dependent RBC haemolysis, as judged from an increase in the intrinsic inhibition time of haemolysis (lines b–h). The inhibition times of resveratrol and compounds (**1**, **3**, **4**, **6**, **7** and **9**) were 120, 127, 145, 150, 135, 160 and 136 min, respectively, which correspond to the additional, or effective, inhibition time, t_{eff}, being 25, 32, 50, 55, 40, 65 and 41 min, respectively (Table 1). Anti-haemolysis activity of resveratrol and its halogenated derivatives decreased in the order of **7** > **4** > **3** > **9** ~ **6** > **1** > resveratrol by comparing the t_{eff} values.

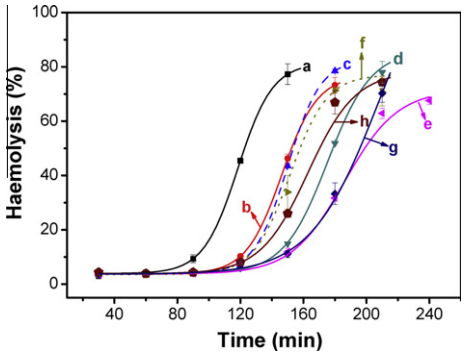

Fig. 3. Effects of resveratrol and its halogenated derivatives (20 μM) on 50 mM AAPH-induced haemolysis of 5% human RBCs in 0.15 M PBS (pH 7.4) under air atmosphere at 37 °C. (a) AAPH; (b) AAPH + resveratrol; (c) AAPH + **1**; (d) AAPH + **3**; (e) AAPH + **4**; (f) AAPH + **6**; (g) AAPH + **7**; (h) AAPH + **9**. Data are expressed as the mean of three determinations.

Interestingly, all of the used halogenated derivatives were more effective at inhibiting RBC haemolysis than this parent molecule (resveratrol). Given that RBCs are heterogeneous media, such a change in anti-haemolysis activity could be related to the lipophilicity differences among the compounds. The lipophilicity of compounds can be characterised by the octanol–water partition coefficient, *P*. Consequently, the calculation of the log*P* values for resveratrol and its halogenated derivatives was performed with Bio-Loom software (Hansch & Leo, 1995; Selassie et al., 2005), and the values are listed in Table 1. It can be seen from Table 1 that lipophilicity of compounds augments with the number of chlorine or bromine atoms attached to the aromatic A ring, and their anti-haemolysis activity first increases and then reduces with a maximum for compound **7** (t_{eff} = 65 min; Clog*P* = 4.240) as the lipophilicity is augmented. In contrast to the situation in homogeneous solutions, the antioxidant efficacy in heterogeneous media

Table 1
Anti-haemolysis, antimicrobial and antiproliferative activities of resveratrol and its halogenated derivatives.

Compounds	t_{inh}[a] (min)	t_{eff} (min)	ClogP	MICs (μg/ml)			IC_{50} (μM)[a]	
				E. coli	*S. aureus*	*C. albican*	HL-60	A549
Resveratrol	120 ± 4	25	2.833	250	3.90	125	36.3 ± 1.8[b]	104 ± 2
1	127 ± 2	32	3.602	31.3	31.3	3.90	77.7 ± 0.5	118 ± 6
3	145 ± 4	50	4.120	125	62.5	250	41.6 ± 1.9	95.2 ± 1.4
4	150 ± 2	55	4.550	–[c]	–[c]	–[c]	71.3 ± 0.7	90.9 ± 3.7
6	135 ± 4	40	3.802	62.5	31.3	3.90	39.7 ± 0.4	135 ± 1
7	160 ± 7	65	4.240	15.6	3.90	62.5	>150	159 ± 2
9	136 ± 4	41	5.150	31.3	7.81	125	>150	164 ± 2
Fluconazole						12.5		
Levofloxacin				0.156	0.156			

[a] Data are expressed as the mean ± SD for three determinations.
[b] Cited from Ref. Fan et al. (2009).
[c] No determination.

depends more on the lipophilicity, localisation and mobility than the chemical reactivity (Niki & Noguchi, 2004). On the one hand, halogenation of resveratrol improves its lipophilicity and facilitates its penetration into the membrane and subsequent reaction with the propagating lipid peroxyl radicals ($LOO^{\cdot}$) within the membranes. On the other hand, the excessive increase of lipophilicity could lead to the improper localisation of antioxidants in the membranes and thus decreases their $LOO^{\cdot}$-scavenging efficiency, as exemplified by compound **9**, which possesses the highest Clog*P* value (5.150) among the compounds investigated but is not the most effective in the anti-haemolysis experiment.

3.3. Antimicrobial activity of the halogenated resveratrol derivatives

In vitro antimicrobial activity of resveratrol and its halogenated derivatives were assessed against Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*) and yeast (*C. albicans*). The minimum inhibitory concentrations (MICs) of the compounds are displayed in Table 1. Among the compounds tested, resveratrol and di-brominated compound **7** turned out to be the most potent against *S. aureus*. However, resveratrol was less active against *E. coli* and *C. albicans*. Noticeably, 2-chloro-resveratrol (**1**) and 2-bromo-resveratrol (**6**) were most active against *C. albicans* among the compounds tested, and exhibited roughly 30- and 3-fold lower MIC values against *C. albicans* than this parent molecule (resveratrol) and the reference compound (fluconazole), respectively. Therefore, they could be employed for further development of effective antifungal drugs. The above results demonstrate that the introduction of chlorine or bromine atoms on the aromatic A ring of resveratrol can modify its antimicrobial profile. Similar results have been also found in the case of the halogenated bis(hydroxyphenyl)methanes (Oh et al., 2009).

3.4. Antiproliferative activity of the halogenated resveratrol derivatives

Antiproliferative activity of compounds **1**, **3**, **4**, **6**, **7** and **9** was finally investigated in human promyelocytic leukemia HL-60 and human lung adenocarcinoma epithelial A549 cells with resveratrol as a control using MTT method. Their concentrations to inhibit 50% cell viability (IC_{50}) are summarised in Table 1. As a whole, resveratrol and its halogenated derivatives showed moderate and weak activity against HL-60 and A549 cells. In HL-60 cells, resveratrol (IC_{50} = 36.3 μM) exhibited the highest antiproliferative activity among the compounds investigated, and 2-bromo-resveratrol (**6**) (IC_{50} = 39.7 μM) matched this parent molecule in this activity. All of the compounds displayed weak activity against A549 cells, but compounds **3** and **4** exhibited slightly higher activity than resveratrol.

4. Conclusion

This study describes the products formed by the reaction of resveratrol with HOCl or HOBr under physiological conditions and their antioxidant and antimicrobial activities, with the aim to investigate the fate of this bioactive stilbene in inflammatory cell-specific metabolism, and to find this phytoalexin-inspired antioxidants and antimicrobials. Eight chlorinated and brominated resveratrol derivatives were formed in the reactions. Interestingly, all of the investigated derivatives displayed the increased anti-haemolysis activity as compared to this parent molecule. Additionally, 2-chloro-resveratrol and 2-bromo-resveratrol surfaced as the important lead compounds, exhibiting more effective activity against *C. albican* than the unmodified form and the reference compound fluconazole.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 20972063), the 111 Project, Program for New Century Excellent Talents in University (NCET-06-0906) and the Fundamental Research Funds for the Central Universities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.05.043.

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