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Design, synthesis and spectroscopic studies of resveratrol aliphatic acid ligands of human serum albumin

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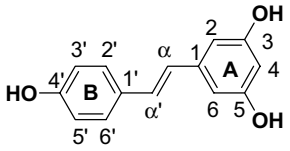
ABSTRACT

As one of the natural polyphenols, resveratrol possesses hydroxyl substituted *trans*-stilbene structure and exerts impact on health by inhibiting multiple human enzymes, such as cyclooxygenase, F1 ATP-ase, and tyrosinase. Resveratrol has to be bound by human serum albumin (HSA) to keep a high concentration in serum, since its solubility is low in water. To improve water solubility and bioavailability, two resveratrol aliphatic acids and their esters have been designed and synthesized. The solubilities of the resveratrol and its derivatives have been measured using a standard procedure. The two aliphatic acids showed better solubilities in pure water and phosphate buffer (pH 7). The binding affinities of resveratrol derivatives for HSA were also measured, and the drug–protein interaction mechanism was investigated using fluorescence, UV–vis, and NMR spectroscopies. Interestingly, resveratrol hexanoic acid (**5**) was found to be a much better ligand ($K_a = (6.70 \pm 0.10) \times 10^6\ M^{-1}$) for HSA than resveratrol ($K_a = (1.64 \pm 0.07) \times 10^5\ M^{-1}$), and there was 41-fold improvement for the binding affinity. It was the first time that the increase of fluorescence of resveratrol moiety was observed during the binding to HSA, suggesting that **5** should be bound tightly by HSA. The UV–vis absorption spectroscopy revealed a maximum absorption shift from 318 to 311 nm with decreasing intensity by 20% upon complexation, suggesting that the π–π conjugation of the stilbene structure was impaired during the binding. Although HSA was reported to have only one binding site for resveratrol, the Job's and molar ratio plots suggested that HSA should bind two molecules of **5**. NMR study suggested that phenyl group (B ring) in the center of the molecule of **5** should be involved in the π–π stacking interactions with HSA aromatic amino acid residues. Molecular geometry calculation of **5** with Spartan software showed that the stilbene structure had two conformers, orthogonal and planar ones. The former ($E = -1.432$ KJ/mol) was more stable than the latter ($E = -0.128$ KJ/mol), suggesting that the former should be the conformer of **5** in the complexation with HSA.

1. Introduction

Resveratrol is one of the natural polyphenols found in grapes and red wines.[1] It is a hydroxyl substituted stilbene (Scheme 1). Multiple beneficial effects on human health such as anti-inflammatory and anti-cancer activities have been reported.[2] It has also been revealed that the compound has potential inhibitory effects on cyclooxygenase,[2] rat liver mitochondrial ATPase,[3] human F1 ATP-ase,[4,5] and tyrosinase.[6] Resveratrol is currently in clinical phase II trials as an anti-cancer drug for treatment of human colon cancer.[7]

Since resveratrol is a therapeutic agent for cancer treatment, high concentration of resveratrol is required. However, resveratrol is not very soluble in water.[8] Human serum albumin was reported to bind resveratrol and maintain a high concentration in human serum.[9] However, the HSA binds resveratrol only when its concentration is high. Thus, the distribution of resveratrol in humans will be affected by its low solubility in water and binding affinity to human serum albumin.[9]



Scheme 1. Structural formula of resveratrol *trans*-3,4′,5-trihydroxystilbene (**1**). The numbering of the carbon atoms is according to IUPAC nomenclature. The numbering of the aromatic rings is also given.

Human serum albumin is the dominating plasma protein in man.[10] It is a 66 KDa monomer containing three homologous helical domains (I–III), and each is divided into A and B subdomains. The protein binds various ligands besides resveratrol, including fatty acids, salicylic acid, and many commonly used drugs, such

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as warfarin and ibuprofen. The improvement of the binding of the drugs to the protein will increase drug absorption, distribution, metabolism and elimination (ADME), and potency and stability.[11] The better binding of HSA will also improve the pharmacodynamic and pharmacokinetic properties for the drugs.[12–17]

Recently, some resveratrol derivatives have been synthesized for better bioactivity. The resveratrol fatty alcohols showed beneficial effects on the differentiation of neural stem cells and modulation of neuroinflammation.[18] Resveratrol analogues were used as inhibitors of tyrosinase, and they also showed anti-inflammatory activity.[6,19] Resveratrol derivatives, such as esters and ethers, were reported to have improved anti-cancer effects.[20] To have better solubility for resveratrol in water and better meditation effects for humans, hydroxypropyl β-cyclodextrin was used to form a complex with the resveratrol.[21]

To improve the binding affinity to HSA and solubility in water, resveratrol aliphatic acids were designed rationally based on known crystal structures of HSA and resveratrol,[5] and synthesized using direct alkylation of resveratrol using brominated aliphatic acid esters, followed by saponification and acidification. The ester derivatives of resveratrol aliphatic acids were also isolated and characterized. The binding affinities of resveratrol and the synthesized compounds were evaluated using HSA fluorescence quenching method (Method 1). The chemical binding mechanism of **5** with HSA was investigated using fluorescence, UV–vis absorption, and NMR spectroscopies.

2. Results and discussion

2.1. Design and synthesis of resveratrol aliphatic acids and esters

Resveratrol has a *trans*-stilbene structure (Scheme 1), and it has low water solubility.[8] Besides β-hydroxypropylcyclodextrin, ethanol was also used to increase the solubility of resveratrol in the form of liquors.[1,21] Because of the toxicity of ethanol, alcohol has multiple side effects as a solvent of drugs. Here, in order to increase the solubility in water at pH 7 without any additive, different carboxyl groups were introduced into resveratrol, individually, to make resveratrol aliphatic acids.

Human serum albumin was reported to carry resveratrol to the tissues and human organs after consumption.[9] Although resveratrol was a reported ligand for HSA, the co-crystal structure for the compound and HSA is not available.[9,22] The resveratrol did form a co-crystal with human protein F1 ATPase, which showed that it was sitting between two parallel α helices.[5] It was also surrounded by three β bends (Fig. 1A). Its 4′-OH group was facing the hydrophobic exterior. Human serum albumin was reported to have two binding sites for salicylic acid.[10] Both binding sites were adjacent to the binding pockets for myristates. One of them sat between two α helices and one β bend. In this domain of the complex structure of HSA, both phenol hydroxyl groups of salicylic acid and the carboxyl group of myristate were facing the exterior of the protein (Fig. 1B). Thus, to have better binding of resveratrol derivatives to human albumin, its 4′-OH should be the best group for derivatization. This is because monoalkylated ether at 4′ position showed the best results in the cell killing effects on the nasopharyngeal epidermoid tumor cell line KB.[20a] Thus, resveratrol aliphatic acids were designed, and synthesized from the alkylation of phenoxide at 4′ position (Scheme 1).[20]

The synthesis of compounds **2** and **3** was carried out using resveratrol as a starting material. A weak base potassium carbonate was used to abstract the proton from hydroxyl group at 4′ position, resulting in resveratrol phenoxide, which was alkylated with alkyl bromoacetate and bromohexanate, respectively (Scheme 2).[20] The resulting esters **2** and **3** were hydrolyzed to carboxylic acids **4** and **5**, respectively, using KOH in ethanol containing water (Scheme 2).[23a]

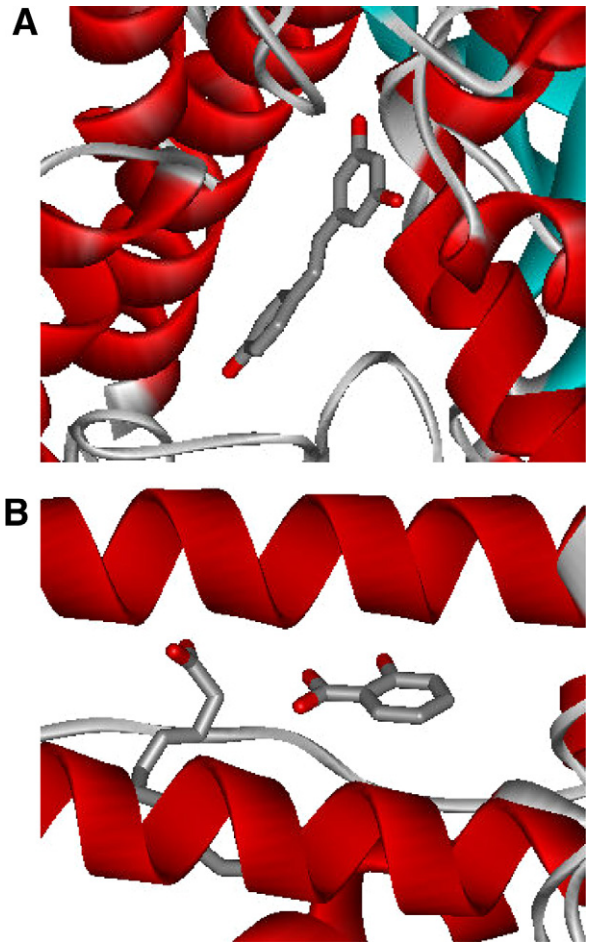
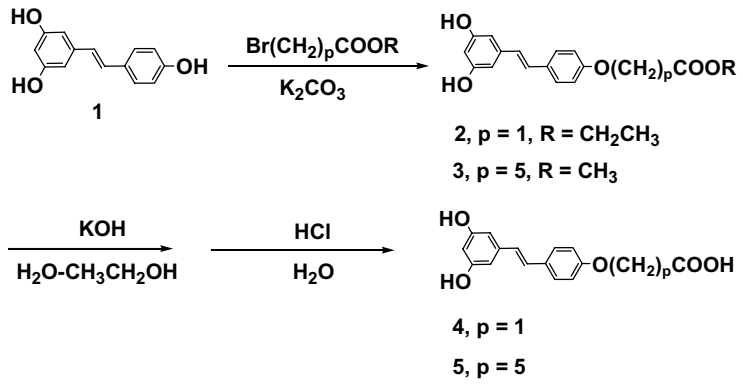


Figure 1. Binding pockets of F1 ATPase and HSA for phenoxy containing compounds resveratrol and salicylic acid individually. (A) The resveratrol with orthogonal conformation was bound by F1 ATPase between two α helices and three β bends, 4′-OH is pointing toward hydrophilic exterior (2jiz.pdb). (B) The salicylic acid was bound by HSA between two α helices and one β pleated strand. The hydroxyl group of salicylic acid and a neighboring carboxyl group of the bound myristic acid are also pointing toward hydrophilic exterior (2i2z.pdb).

2.2. Solubilities of resveratrol aliphatic acids and esters in water and phosphate buffer

The solubilities of resveratrol and its derivatives **1**–**5** have been measured in pure water and a phosphate buffer (50 mM, pH 7) at 295 K.[23b] In pure water and at 304 nm of UV absorbance, the extinction coefficient of resveratrol was measured to be 33,913 M^{-1} cm^{-1}, which was also used to determine the concentrations of its derivatives **2**–**5**. After calculations, the solubilities of the resveratrol and its derivatives are listed in Table 1. As expected, the formation of esters of resveratrol did not improve the solubilities of compounds **2** and **3** in either water or phosphate buffer at pH 7. After the hydrolysis of the resveratrol esters, the resveratrol aliphatic acids **4** and **5** had improved water solubility, compared to resveratrol, suggesting that the introduction of carboxyl groups did increase the water solubility. When the phosphate buffer (50 mM, pH 7) was used, the solubilities of compounds **4** and **5** were improved further. In the phosphate buffer the solubility of **4** was about 97-fold of resveratrol, and that of **5**



Scheme 2. Synthesis of compounds **2–5** from resveratrol **1**.

Table 1
Solubilities of resveratrol and its derivatives **1–5** in water and phosphate buffer[a]

Compound	Water (mg/100 g)	Phosphate buffer (mg/100 g)
1	3.24 (4.0)[b]	3.24
2	1.93	1.40
3	0.032	0.085
4	80.91	313.8 (867.8)[c]
5	4.52	10.54

[a] 295 K, 50 mM phosphate buffer, pH 7.
[b] Literature value.[8]
[c] Over-saturated solution, which was made by tapping a suspension of **4** in the phosphate buffer for 2 min.

Table 2
Binding parameters for HSA with resveratrol derivatives (298 K)

Compound	$K_a \times 10^5\ M^{-1}$		
	Method 1 (literature)[a]	Method 2[b]	Method 3[c]
1	1.64 ± 0.07 (2.56)[d]	nd	nd
2	1.14 ± 0.08	nd	nd
3	3.05 ± 0.10	nd	nd
4	2.95 ± 0.10	nd	nd
5	67.0 ± 1.0	31.2 ± 14.3	26.2 ± 12.5

[a] Based on HSA's fluorescence intensity at 350 nm.
[b] Based on resveratrol's fluorescence intensity at 388 nm.
[c] Based on resveratrol's UV–vis absorption intensity at 318 nm.
[d] Literature value.[22]

was about of 3.3-fold of resveratrol, indicating that compounds **4** and **5** were more soluble in the phosphate buffer (pH 7) than resveratrol.

2.3. Binding of resveratrol aliphatic acids and esters revealed by HSA fluorescence

The protein HSA is fluorescent because of a tryptophan residue, Trp 217, and there is maximum emission at 350 nm under the excitation at 280 nm (Fig. 2).[9,22] The fluorescence of the HSA was quenched during the binding of drugs (Fig. 2).[9,22] Thus, the binding of the resveratrol and derivatives to HSA was determined by the quenching of the fluorescence at 350 nm. The association constants were estimated using the modified Stern–Volmer equation (Table 2).[9,22] The association constant for resveratrol was determined to be $K_a = (1.64 \pm 0.07) \times 10^5\ M^{-1}$, which was close to a literature value of $K_a = (2.56 \pm 0.11) \times 10^5\ M^{-1}$.[22] The association constant for compound **5** was found to be $K_a = (6.7 \pm 0.1) \times 10^6\ M^{-1}$, which was 40.8-fold higher than that for resveratrol, suggesting that compound **5** was a much better ligand for HSA than resveratrol. This can be explained in that HSA had high affinities to resveratrol and aliphatic carboxylic acid individually,[20] and compound **5** was a derivative of both resveratrol and aliphatic carboxylic acid.

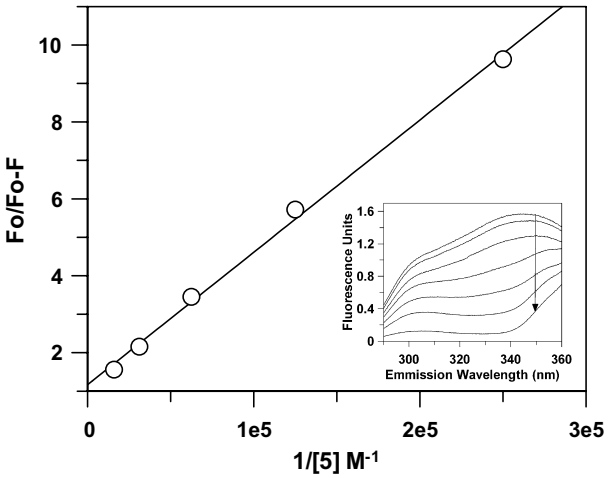


Figure 2. Plot of $F_o(F_o - F)$ as a function of 1/[**5**] (M^{-1}) in the determination of the association constant for **5** during binding with HSA (4 μM) using Method 1. There was a decrease of HSA tryptophan fluorescence intensity during the binding with **5** from 4.0 to 62.5 μM (298 K).

Another observation for binding between **5** and HSA was that the tryptophan fluorescence of HSA was quenched severely (Fig. 2). The quenching of the fluorescence also showed the change of conformation during the binding with resveratrol moiety of the compound.

Furthermore, the association constant for **3** was found to be $(3.05 \pm 0.10) \times 10^5\ M^{-1}$, which was about 1.9-fold higher than that of resveratrol, suggesting that the introduction of a long chain carboxylate ester group to resveratrol will increase the binding to HSA. The compound **4** also had better binding affinity to HSA than resveratrol, suggesting that the additional carboxyl group should assist the binding to HSA. The introduction of ester group in compound **2** had little additive effect on the binding affinity. Overall, the introduction of a long chain aliphatic acid to resveratrol greatly increased its binding affinity to HSA.

2.4. Binding of resveratrol aliphatic acids with HSA revealed by resveratrol fluorescence

Resveratrol is a fluorescent molecule with maximum emission at 385 nm (Fig. 3A). Resveratrol has a binding affinity of $K_D = 1/K_a = 6.1$ μM; however, the binding of the resveratrol (4 μM) with HSA (4 μM) resulted in little change of resveratrol fluorescence

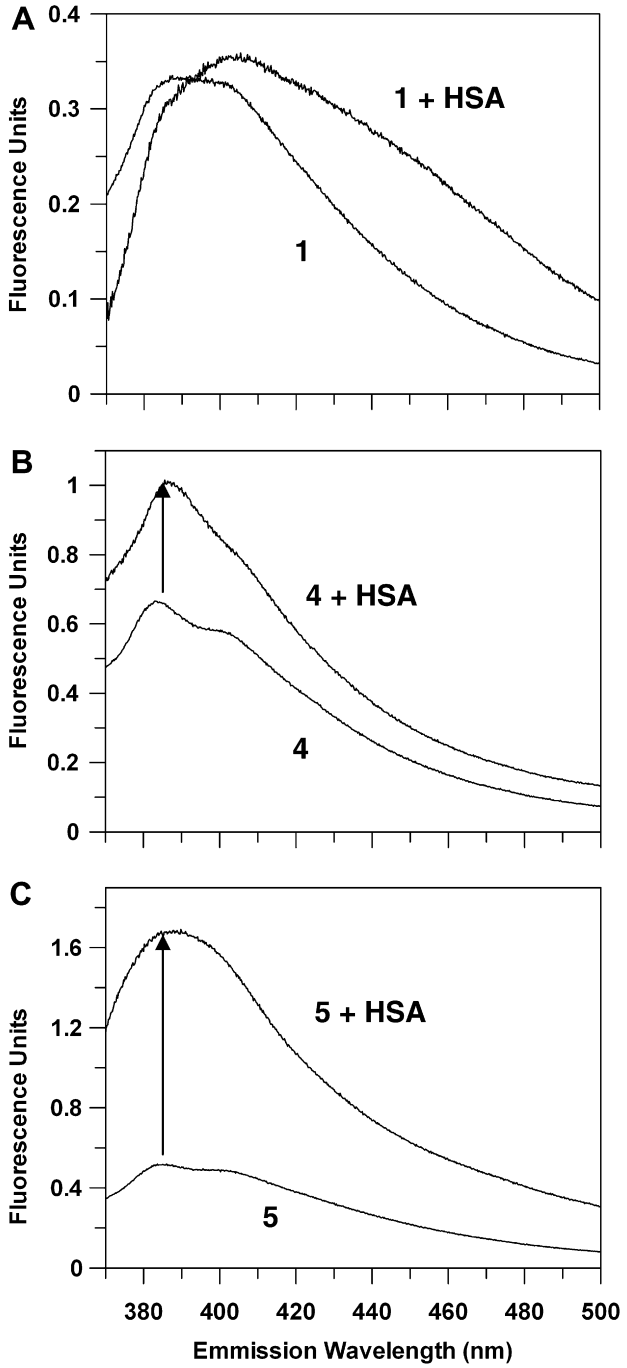


Figure 3. Fluorescence spectra. (A) Free resveratrol (4 μM) and resveratrol complex (4 μM resveratrol + 4 μM HSA). (B) Free **4** (4 μM) and **4**'s complex (4 μM **4** + 4 μM HSA). (C) Free **5** (4 μM) and **5**'s complex (4 μM **5** + 4 μM HSA).

intensity (Fig. 3A). Interestingly, the binding of compound **4** (4 μM) with HSA (4 μM) resulted in 50% increase of fluorescence intensity (Fig. 3B), and the binding of compound **5** (4 μM) with HSA (4 μM) resulted in a 3-fold increase of the fluorescence intensity at 385 nm, suggesting that compounds **4** and **5** were tightly bound by HSA (Fig. 3C). These results also confirmed that compound **4** was a better ligand for HSA than resveratrol, and compound **5** was a much better ligand for HSA (Fig. 3). It was the first time that the increase of fluorescence of resveratrol moiety was observed during the binding to the protein HSA without an increased concentration of resveratrol or its derivatives.

Since fluorescence intensity of compound **5** will increase during binding, the binding affinity to HSA was also estimated using res-

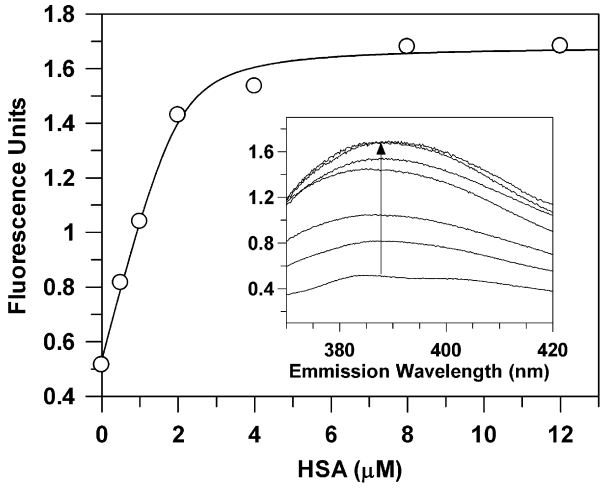


Figure 4. Plot of the relative fluorescence units as a function of [**HSA**] in the determination of the association constant for **5** (4 μM) during binding with HSA using Method 2. There was an increase of resveratrol fluorescence intensity during the binding (298 K).

veratrol moiety fluorescence by fixing the concentration of compound **5**, but increasing the concentrations of HSA (Method 2) (Fig. 4). The binding affinity of compound **5** to HSA was calculated using non-linear-fit[24] (Grafit 6), which gave $K_a = (3.12 \pm 1.43) \times 10^6\ M^{-1}$, which was close to the value ($(6.70 \pm 0.10) \times 10^6\ M^{-1}$) from Method 1. The results also indicated that compound **5** can be bound to HSA very tightly.

2.5. Binding of resveratrol aliphatic acids with HSA revealed via UV–vis spectroscopy

Resveratrol hexanoic acid (**5**) was found to have the maximum UV absorbance at 318 nM. Surprisingly, the binding of **5** by HSA resulted in a decrease of UV absorbance by 20% (Fig. 5). The maximum absorbance shifted from 318 to 311 nm. The results suggested that the π–π conjugation of stilbene structure of **5** should be impaired during the binding. The planar geometry should be altered.[25–28]

Molecular energy calculations were carried out using Spartan software (Trident). Two stable conformers of **5**, orthogonal

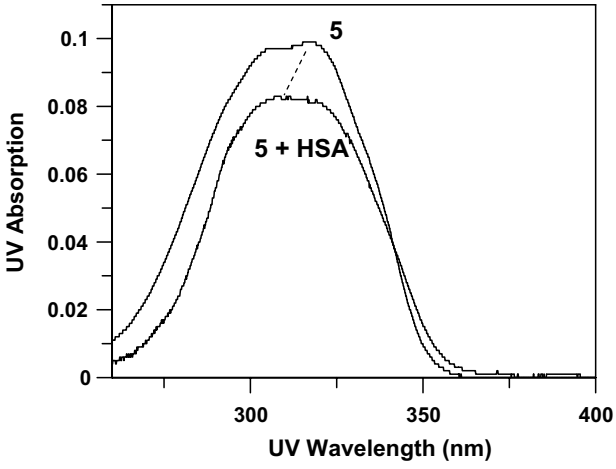


Figure 5. UV–vis spectra, free **5** (4 μM) and **5**'s complex (4 μM **5** + 4 μM HSA). There was blue shift for UV maximum absorption for **5** from 318 nm to 311 nm during the binding with HSA.

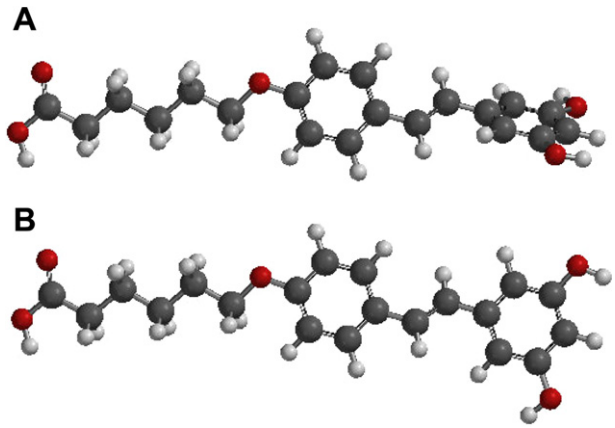


Figure 6. Conformers of **5**. (A) Orthogonal conformer ($E = -1.432$ KJ/mol) and (B) planar conformer ($E = -0.128$ KJ/mol), based on calculations using Spartan (Trident) software.

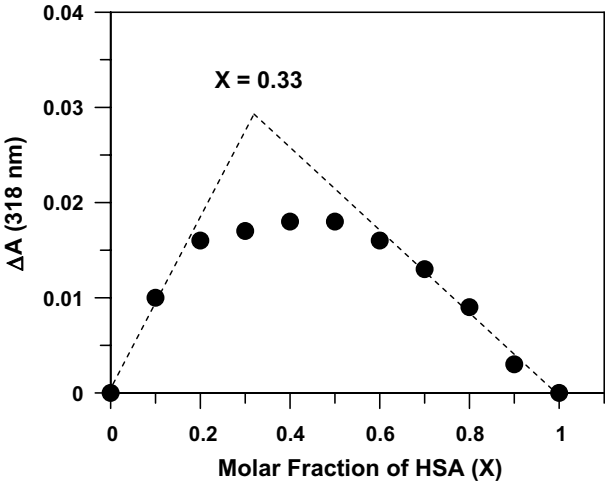


Figure 7. Job's plot for the complex between HSA and **5**.

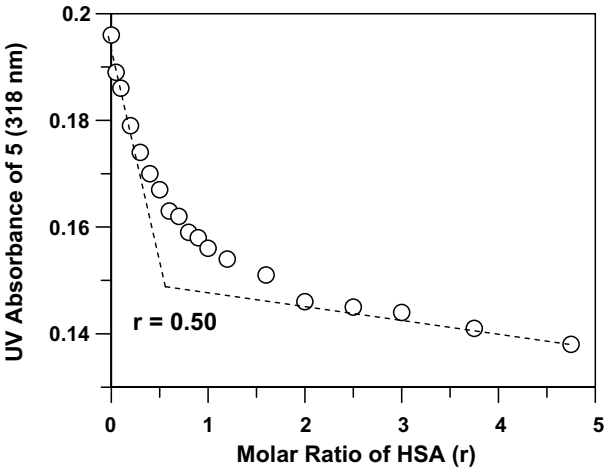


Figure 8. Molar ratio plot for the complex between HSA and **5**.

(Fig. 6A) and planar (Fig. 6B) ones, were found. The former was found to have an energy level of −1.432 KJ/mol, whereas the latter had an energy level of −0.128 KJ/mol, suggesting that the former should be more stable than the latter. Therefore, the orthogonal stilbene structure should be the conformer in the complexation with HSA. There are three pieces of evidence for this structure; one is the increase of fluorescence of stilbene structure of **5** during the binding to HSA. The increase of fluorescence indicates that the structure is changing from conjugated to unconjugated, and from a flexible structure to a rigid one.[29] The second evidence is the decrease of UV absorption. The third evidence is the shift of the maximum UV absorbance from 318 to 311 nm. This is because the impaired π–π conjugation results in a shorter wavelength of UV absorbance and lower intensity.[25–28] This structure should be consistent with the conformer structures of stilbene in the complexes of resveratrol with F1 ATPase (Fig. 1A),[5] and chalcone synthase (1CGZ), respectively,[30] in which resveratrol sat in the interior of the proteins. The proteins have hydrophobic interior and the hydrophobic stilbene structure should adopt orthogonal geometry with maximum surface contact areas with the proteins.[31] Resveratrol alone tended to adopt planar geometry in aqueous solution for minimum hydrophobic surface areas according to a study with NMR.[32] The resveratrol also tended to adopt planar geometry when it was in the exterior of proteins such as in the complex with transthyretin (1DVS) for the same reason.[33]

How many molecules of **5** can be bound by HSA? Based on a report, there was one binding site for resveratrol in HSA.[9] To test whether there is one binding site in HSA for compound **5**, Job's plot was carried out by preparing 11 solutions covering the whole range of molar fractions for **5** and HSA, keeping the total concentration constant (8 μM) (Fig. 7).[34] The UV absorbance at 318 nm for each solution was measured before and after the addition of a designed amount of HSA. A plot of the molar fraction of HSA versus the difference in UV absorbance was then carried out. Thus, Job's plot generated a curve, where two straight lines drawn through initial and final points generated a crossed point, $X = 0.33$. The stoichiometry of the complex was then calculated based on Eq. 1, giving $m = 1$ and $n = 2$, respectively.

$$mHSA + n\mathbf{5} \rightleftharpoons HSA_m\mathbf{5}_n$$
$$X = m/(m+n) \quad (1)$$

To confirm the result from Job's plot, a molar ratio plot was also carried out (Fig. 8).[35] A titration was done starting with a fixed concentration of **5** (8 μM) and increasing concentrations of HSA. All the UV absorbances were measured. A plot of the molar fraction of HSA relative to the concentration of **5** versus the UV absorbance was then carried out. Thus, molar ratio plot generated a curve, where two straight lines drawn through initial and final points generated a crossed point, $r = 0.5$. The stoichiometry of the complex was then calculated based on $r = m/n$. If m equals 1, n should be 2. Therefore, both Job's and molar ratio plots showed that HSA should bind two molecules of **5**. This was consistent with the crystal structure, which showed that HSA can accommodate two salicylic acid molecules.[10]

Based on the significant decrease of UV absorption of **5** during the binding of HSA, the binding affinity was also calculated (Fig. 9). The K_D was found to be (0.382 ± 0.182) μM. The K_a was then calculated to be $(26.2 \pm 12.5) \times 10^5$ M^{-1}, which was consistent with the results from two other measurements using fluorescence (Table 2). The results also suggested that the binding of **5** with HSA was much more significant than the binding with **4** or **1**, suggesting that **5** was a greater ligand for HSA.

2.6. Binding of resveratrol aliphatic acids by HSA revealed by NMR spectroscopy

A ^{1}H NMR study was carried out to investigate how **5** was bound to HSA in phosphate buffer in D_2O. Higher concentration of **5** (1.25 mM) and lower concentration of HSA (50 μM) were used

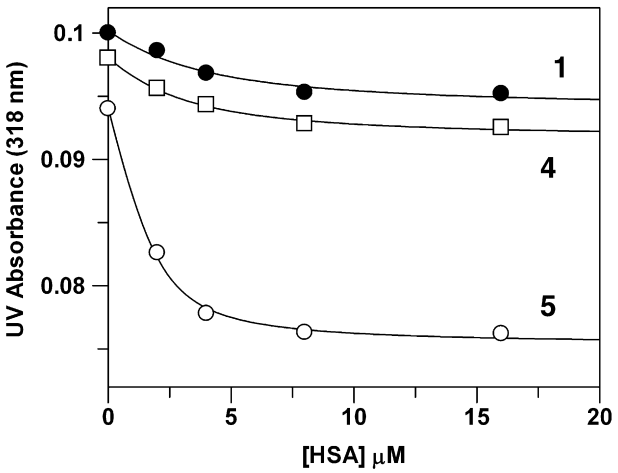


Figure 9. Plot of the UV absorbance of **5** as a function of [**HSA**] in the determination of the association constant for **5** during binding with HSA using Method 3. There was a decrease of resveratrol UV absorption intensity during the binding (298 K).

for better signals of **5**.[36] The differences of chemical shifts for aromatic and vinylic hydrogens were calculated based on spectra for **5** in the absence and presence of HSA (Fig. 10). The results showed that the chemical shifts of hydrogens 2′/6′ and 3′/5′ moved upfield by 0.058 and 0.062 ppm, respectively, upon the addition of HSA, suggesting that the ring B in the center of **5** should have significant π–π stacking interactions with aromatic residues of HSA.[37] The results also showed that the chemical shifts of hydrogens 2/6 and 4 moved upfield by 0.049 and 0.002 ppm, respectively, suggesting that the ring A should have less significant π–π stacking interactions with aromatic residues of HSA. The NMR data showed that the hydrophobic linkage of two molecules of **5** should be in the hydrophobic cavities, and their hydrophilic groups, including hydroxyl and carboxyl groups, should be exposed to the hydrophilic exterior of the protein. The two molecules of **5** were likely to be in the two salicylic acid binding pockets in HSA.[10]

3. Conclusions

The synthesized new resveratrol aliphatic acids **4** and **5** showed much better water solubility than resveratrol in phosphate buffer at pH 7, effectively solving the water solubility problem of resveratrol. More importantly, they showed much better binding affinity to HSA than resveratrol, as well. The chemical binding mechanism of **5** with HSA was revealed using fluorescence, UV–vis, and NMR spectroscopies. The binding of **5** with HSA resulted in a decrease of HSA fluorescence but increase of resveratrol moiety fluorescence, suggesting that **5** was tightly bound by the HSA, and the structure became very rigid. It was the first time that an increase in fluorescence of resveratrol moiety was observed during the measurement. It was also the first time that a decrease of UV absorption of resveratrol was observed with blue shift of UV absorption, suggesting the impairment of the π–π conjugation during the binding of **5** to HSA. According to molecular calculations with Spartan software, the most stable conformer for resveratrol moiety should be orthogonal. It should adopt this conformation in the binding site of HSA. To test whether HSA just has one binding site for resveratrol, Job's and molar ratio plots were carried out. The results suggested that there were two binding pockets for compound **5**.

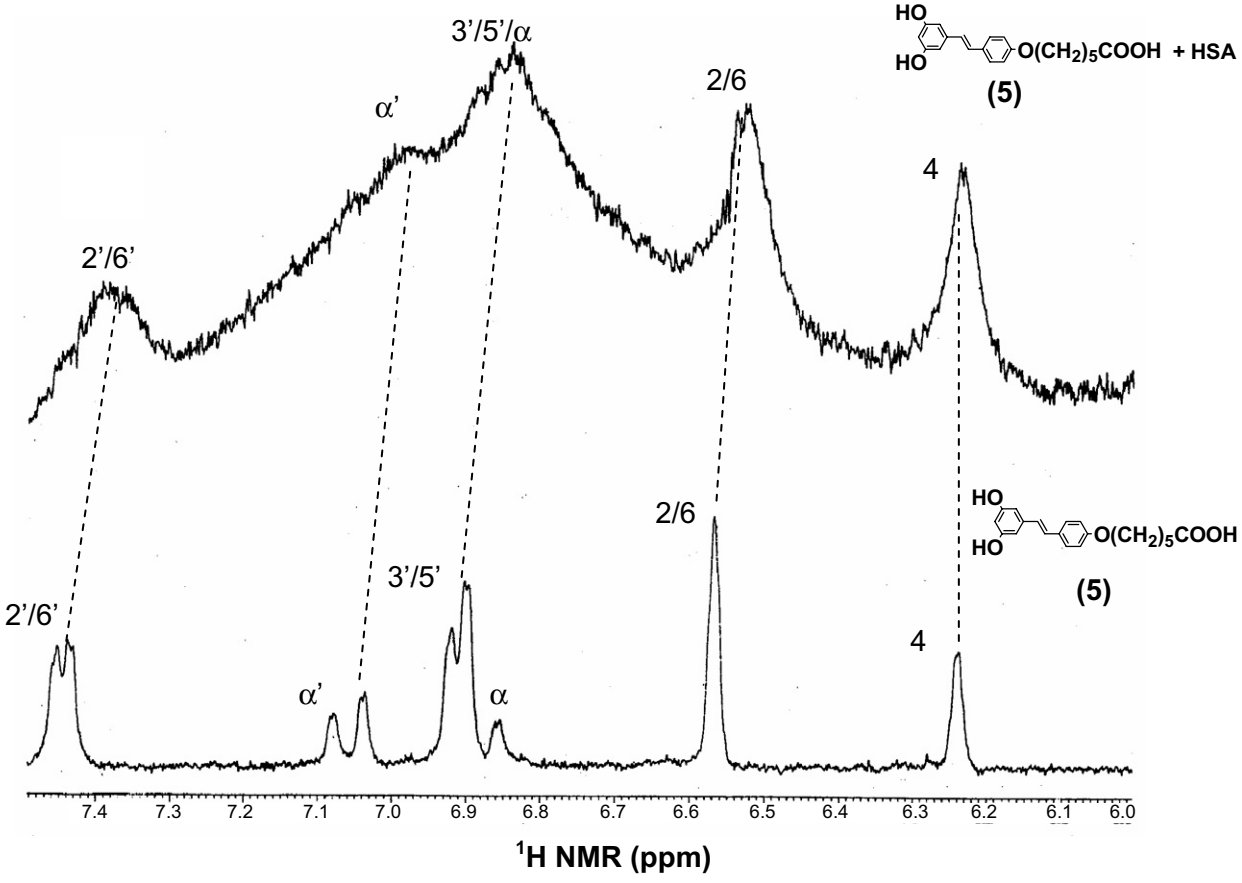


Figure 10. NMR spectra, free **5** (1.25 mM), and **5**'s complex (1.25 mM **5** + 50 μM HSA).

To test how **5** was bound by HSA, NMR investigation was also carried out. The results showed that the phenyl ring in the center of the molecule was quenched severely by HSA's aromatic residues, suggesting that the linkage of **5** should be in the hydrophobic cavity of HSA, but the hydrophilic groups of **5**, such as hydroxyl and carboxyl groups, should be in the exterior of the protein. Given the surprisingly high binding affinity of **5** to HSA with multiple readings of structural information from this investigation, **5** offers an exceptional example for designing, synthesizing and evaluating strong ligands and tight binding inhibitors for human proteins using multiple spectroscopies.[9]

4. Materials and methods

4.1. Materials and instruments

Resveratrol and human serum albumin (HSA) were purchased from Sigma (St. Louis, MO, USA). Other chemicals for the synthesis and analysis were also from Sigma. The purity of HSA (molecular weight 66,500 Da) was 99% according to the manufacturer. The phosphate buffer (50 mM, pH 7) for assay contained 20 mM of NaH_2PO_4 and 30 mM of Na_2HPO_4. During the preparation of the buffer for NMR experiments, the NaH_2PO_4 and Na_2HPO_4 were exchanged to NaD_2PO_4 and Na_2DPO_4 with D_2O (99.5%) three times under lyopholyzer. Fluorescence spectroscopy measurements were performed on FluoroMax-3 from HORIBA JOBIN YNON. Melting points were determined using MEL-TEMP. IR spectra were recorded on Genesis II FTIR. UV–vis absorption spectra were recorded on UV-1700 UV–vis spectrophotometer from Shimadzu and NMR spectra were recorded on Varian 400 (400 MHz).

4.2. Synthesis of ethyl 2-{4-[(1*E*)-2-(3,5-dihydroxyphenyl)ethenyl]phenoxy}acetate (2) and 2-{4-[(1*E*)-2-(3,5-dihydroxyphenyl)ethenyl]phenoxy}acetic acid (4)

To a one-neck round-bottomed flask were added resveratrol (0.50 g, 2.2 mmol), acetone (10 mL), potassium carbonate (0.607 g, 4.4 mmol), and ethyl 6-bromoacetate (244 μL, 2.2 mmol). The mixture was stirred at 50–60 °C for 12 h in reflux under N_2 atmosphere. The resulting mixture was submitted for column chromatography using a mixed solvent (3–30% of ethyl acetate in hexane in volume) to give compound **2** (0.1 g, 14%). The compound was recrystallized from ethyl acetate/hexane (1:1 volume). R_f value, 0.47 (EtOAc/hexane, 50:50, v/v). Mp 140–141 °C (lit.[20a] 117–119 °C). ^{1}H NMR (CD_3COCD_3, ppm) δ 1.24 (t, 3H, J = 7.2 Hz, CH_3), 4.19 (q, 2 H, J = 6.8 Hz, CH_2), 4.60 (s, 2H, CH_2), 6.23 (t, 1H, J = 2.2 Hz, Ar–H), 6.47 (d, 2H, J = 2.2 Hz, Ar–H), 6.84 (m, 4H, Ar–H), 7.38 (d, 2H, J = 8.8 Hz, Ar–H), 7.61 (s, 2H, OH). UV_{max} in water, 304 nm.

To a 5 mL round-bottomed flask were added compound **2** (0.070 g, 0.22 mmol), methanol (3 mL), water (0.10 g), and potassium hydroxide (0.6 g, 10.7 mmol). The resulting mixture was stirred for one day at room temperature.[23] The mixture was acidified to pH 2 using 6 N HCl. The resulting mixture was extracted with ethyl acetate (2× 10 mL). After removal of the solvent, the residue was purified with column chromatography using a mixed solvent (0.2–0.4% of acetic acid in ethyl acetate in volume) to give compound **4** (0.042 g, 67%). R_f value, 0.74 (HOAc/EtOAc, 2:98, v/v). Mp 223–224 °C. ^{1}H NMR (CD_3OD, ppm) δ 4.64 (s, 2H, CH_2), 6.18 (s, 1H, Ar–H), 6.46 (d, 2H, J = 2.2 Hz, Ar–H), 6.90 (m, 4H, Ar–H), 7.52 (d, 2H, J = 8.4 Hz, Ar–H). ^{13}C NMR (CD_3OD, ppm) δ 64.56, 126.91, 127.43, 127.59, 131.04, 138.69, 139.76, 157.67, 158.32, 171.46. IR (film, cm^{-1}) 3239, 2918, 1731, 1597, 1511, 1355, 1261, 1228, 1161, 1972, 1008, 964, 841. UV_{max} in water, 318 nm. ESI-TOE-high-acc (M+H) Calcd for $C_{16}H_{15}O_5$: 287.0914. Found: 287.0914.

4.3. Synthesis of methyl 6-{4-[(1*E*)-2-(3,5-dihydroxyphenyl)-ethenyl]phenoxy}hexanate (3) and 6-{4-[(1*E*)-2-(3,5-dihydroxyphenyl)ethenyl]phenoxy}hexanoic acid (5)

To a one-neck round-bottomed flask were added resveratrol (0.684 g, 3 mmol), acetone (20 mL), potassium carbonate (0.828 g, 6 mmol), and methyl 6-bromohexanoate (0.627 g, 3 mmol). The mixture was stirred at 50–60 °C for 16 h in reflux under N_2 atmosphere. The resulting mixture was submitted for column chromatography using a mixed solvent (3–30% of ethyl acetate in hexane in volume) to give a compound **3** (0.10 g, 9%). The compound was recrystallized from ethyl acetate/hexane (1:1 volume). R_f value, 0.71 (acetone/hexane, 50:50, v/v). Mp 182–183 °C. ^{1}H NMR (DMSO-d_6, ppm) δ 1.42 (m, 2H, CH_2); 1.59 (m, 2H, CH_2); 1.71 (m, 2H, CH_2); 2.33 (t, 3H, J = 7.2 Hz, CH_3), 3.62 (s, 3H, CH_3); 3.96 (t, 2H, J = 6.4 Hz, CH_2), 6.12 (t, 1H, J = 2.2 Hz, Ar–H), 6.40 (s, 2H, Ar–H), 6.92 (m, 4H, Ar–H), 7.56 (d, 2H, J = 8.4 Hz, Ar–H), 9.23 (s, 2H, OH). ^{13}C NMR (DMSO-d_6, ppm) δ 24.78, 25.42, 28.93, 33.78, 51.77, 67.84, 102.36, 102.45, 104.84, 104.94, 115.15, 127.12, 128.05, 128.32, 130.08, 139.66, 158.85, 158.93, 159.07, 173.90. IR (film, cm^{-1}) 3354, 3239, 2945, 2900, 2870, 1708, 1608, 1515, 1451, 1355, 1265, 1243, 1165, 1049, 1012, 960, 956, 729, 684. UV_{max} in water, 310 nm.

To a 5 mL round-bottomed flask were added compound **3** (0.070 g, 0.20 mmol), methanol (3 mL), water (0.10 g), and potassium hydroxide (0.6 g, 10.7 mmol). The resulting mixture was stirred for one day at room temperature.[23] The mixture was acidified to pH 2 using 6 N HCl. The resulting mixture was extracted with ethyl acetate (2× 10 mL). After removal of the solvent, the residue was purified with column chromatography using a mixed solvent (15–60% of acetone in hexane in volume) to give compound **5** (0.042 g, 62%). R_f value, 0.82 (HOAc/EtOAc, 0.4:99.6, v/v). Mp 196–197 °C. ^{1}H NMR (CD_3OD, ppm) δ 1.48 (m, 2H, CH_2); 1.65 (m, 2H, CH_2); 1.75 (m, 2H, CH_2); 2.30 (t, 3H, J = 7.2 Hz, CH_3), 3.92 (t, 2H, J = 6.2 Hz, CH_2), 6.17 (t, 1H, J = 2.2 Hz, Ar–H), 6.46 (d, 2H, J = 2.2 Hz, Ar–H), 6.83 (m, 3H, Ar–H), 6.94 (d, 2H, J = 16.1 Hz, Ar–H), 7.40 (d, 2H, J = 8.8 Hz, Ar–H). ^{1}H NMR (CD_3OD, ppm) δ 24.56, 25.45, 28.79, 33.56, 67.52, 101.46, 104.56, 114.38, 126.32, 127.41, 127.85, 130.04, 139.87, 158.35, 158.90, 176.35. IR (film, cm^{-1}) 3433, 3309, 2948, 2866, 1708, 1604, 1518, 1451, 1355, 1306, 1276, 1250, 1176, 1157, 997, 960, 841, 815, 733, 681. UV_{max} in water, 318 nm. ESI-TOE-high-acc (M+H) Calcd for $C_{20}H_{23}O_5$: 343.1540. Found: 343.1540.

4.4. Measurement of the solubilities of resveratrol and its derivatives in water and phosphate buffer

Mixing of resveratrol or its derivatives with a solvent, either pure water or phosphate buffer (50 mM, pH 7), resulted in a mixture. The mixture was added in the internal sealed dual-wall flask. Between the outer and inner walls of the flask, water at constant temperature (295 K) was circulated.[23b] The mixture was stirred for 1 h, transferred to a 1.5 mL Eppendorf tube, and centrifuged for 2 min. The clear solution was transferred to a new 1.5 mL Eppendorf tube and centrifuged again for 2 min. The resveratrol, or its derivative content in the new clear solution was then measured using UV–vis spectrophotometer. The concentrations and solubilities of resveratrol and its derivatives were then calculated using the extinct coefficient 33,913 M^{-1} cm^{-1}.

4.5. Fluorescence quenching measurements (Method 1)

The excitation wavelength of 280 nm was used, and the emission spectra were recorded from 290 to 360 nm at 298 K. Protein solution (4 μM) in phosphate buffer was used only once. Buffers without a resveratrol derivative and with increasing

concentrations of the resveratrol derivative were used as blanks for the corresponding measurements. All the solutions were mixed thoroughly and used 2 min after mixing for the measurements.[22]

The association constant (K_a) for binding of all the resveratrol derivatives to HSA indicated in Table 2 was determined with fixed concentrations of the HSA and with increasing amounts of the compounds. The binding data were fitted to the modified Stern–Volmer equation 2. The fluorescence intensity (F) at 350 nm was plotted against [resveratrol] to obtain the slope from Eq. 2 using Grafit 6 linear fit. K_a was then calculated based on the slopes and intercepts of the fittings, K_a = intercept/slope.[22]

$$F_o/(F_o - F) = 1/fK_a[\text{resveratrol}] + 1/f \quad (2)$$

4.6. Fluorescence enhancing measurements (Method 2)

The excitation wavelength of 300 nm was used, and the emission spectra were recorded from 370 to 420 nm at 298 K. Any resveratrol derivative (4 μM) in phosphate buffer was used only once. Buffers without HSA and with increasing concentrations of HSA were used as blanks for the corresponding measurements. All the solutions were mixed thoroughly and used 2 min after mixing for the measurements.

The association constant (K_a) and dissociation constants ($K_D = 1/K_a$) for binding of **5** to the HSA indicated in Table 2 were determined with fixed concentrations of **5** and with increasing amounts of HSA. The binding data were fitted to Eq. 3. The fluorescence intensity (F) at 385 nm was plotted against $[HSA]_{tot}$ to obtain the K_D from Eq. 3 using Grafit 6. This equation is based on the fact that HSA will bind two molecules of **5**.[24c] K_a was then calculated based on $K_a = 1/K_D$.[24]

$$F = F_o - \{(F_o - F_f)[\mathbf{5}]_{tot}/2\}\{b - (b^2 - 8[HSA]_{tot}[\mathbf{5}]_{tot})^{1/2}\}$$
$$b = K_D + 2[HSA]_{tot} + [\mathbf{5}]_{tot} \quad (3)$$

4.7. UV–vis measurements (Method 3)

The UV–vis measurements of a resveratrol derivative in the absence or presence of HSA were made in the range of 315–340 nm at 298 K. The resveratrol concentrations were fixed at 4 μM, while the HSA's concentrations were either fixed to 4 μM or increased from 2 to 16 μM. The phosphate buffer was used as a blank for all the measurements.

The association constant (K_a) and dissociation constants ($K_D = 1/K_a$) for binding of **5** to the HSA indicated in Table 2 were also determined with fixed concentrations of **5** and with increasing amounts of HSA. The binding data were fitted to Eq. 4, which is derived from Eq. 3. The UV absorbance (A) at 318 nm was plotted against $[HSA]_{tot}$ to obtain the K_D from Eq. 4 using Grafit 6. This equation is based on the fact that HSA will bind two molecules of **5**.[24c] K_a was then calculated based on $K_a = 1/K_D$.

$$A = A_o - \{(A_o - A_f)[\mathbf{5}]_{tot}/2\}\{b - (b^2 - 8[HSA]_{tot}[\mathbf{5}]_{tot})^{1/2}\}$$
$$b = K_D + 2[HSA]_{tot} + [\mathbf{5}]_{tot} \quad (4)$$

4.8. Job plots

Eleven solutions covering the whole range of molar fractions for **5** and HSA, keeping the total concentration constant (8 μM), were prepared. The UV absorbance at 318 nm for each solution was measured before and after the addition of a designated amount of HSA. A plot of the molar fraction of HSA versus the difference in UV absorbance was then carried out. Thus, Job's plot generated a curve, where two straight lines drawn through initial and final points generated a crossed point, which indicated the stoichiometry of the complex.[34]

4.9. Molar ratio plot

A titration was carried out starting with a fixed concentration of **5** (8 μM) and increasing concentrations of HSA from 0, 0.4, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6, 6.4, 7.2, 8.0, 9.6, 12.8, 16, 20, 24, 30 and 38 μM. All the UV absorptions were measured. A plot of the molar fraction of HSA relative to the concentration of **5** versus the UV absorbance was then carried out. Thus, molar ratio plot generated a curve, where two straight lines drawn through initial and final points generated a crossed point, which indicated the stoichiometry of the complex.[35]

4.10. NMR measurements

The phosphate buffer was prepared using D_2O as a solvent and deuteriumated NaD_2PO_4 and Na_2DPO_4 as buffer components. Compound **5**'s concentrations were fixed to 1.25 mM. The solutions from NMR study were prepared with or without the HSA (50 μM). All the NMR studies were carried out at 298 K.[36]

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Supplementary data

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References and notes

1. Howitz, K. T.; Bitterman, K. J.; Cohen, H. Y.; Lamming, D. W.; Lavu, S.; Wood, J. G.; Zipkin, R. E.; Chung, P.; Kisielewski, A.; Zhang, L.-L.; Scherer, B.; Sinclair, D. A. *Nature* **2003**, *425*, 191.
2. Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218.
3. Kipp, J. L.; Ramirez, V. D. *Endocrine* **2001**, *15*, 165.
4. Wu, C.-P.; Calcagno, A. M.; Hladky, S. B.; Ambudkar, S. V.; Barrand, M. A. *FEBS J.* **2005**, *272*, 4725.
5. Gledhill, J. R.; Montgomery, M. G.; Leslie, A. G. W.; Walker, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 13632.
6. Song, S.; Lee, H.; Jin, Y.; Ha, Y. M.; Bae, S.; Chung, H. Y.; Suh, H. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 461.
7. Schwedhelm, E.; Maas, R.; Troost, R.; Boeger, R. H. *Clin. Pharmacokinet.* **2003**, *42*, 437.
8. Filip, V.; Plockova, M.; Smidrkal, J.; Spickova, Z.; Melzoch, K.; Schmidt, S. *Food Chem.* **2003**, *83*, 585.
9. Lu, Z.; Zhang, Y.; Liu, H.; Yuan, J.; Zheng, Z.; Zou, G. *J. Fluoresc.* **2007**, *17*, 580.
10. Yang, F.; Bian, C.; Zhu, L.; Zhao, G.; Huang, Z.; Huang, M. *J. Struct. Biol.* **2007**, *157*, 348.
11. Wosikowski, K.; Biedermann, E.; Rattel, B.; Breiter, N.; Jank, P.; Loeser, R.; Jansen, G.; Peters, G. J. *Clin. Cancer Res.* **2003**, *9*, 1917.
12. Quevedo, M. A.; Moroni, G. N.; Brinon, M. C. *Biochem. Biophys. Res. Commun.* **2001**, *288*, 954.
13. Yamashita, S.; Nadai, T.; Sumi, M.; Suda, Y. *Int. J. Pharm.* **1994**, *108*, 241.
14. Wang, H.-P.; Chuang, Y.-H.; Lee, C.-Y.; Wang, C.-L.; Hsieh, W.-H. *Yaowu Shipin Fenxi* **2006**, *14*, 225; Wang, H.-P.; Chuang, Y.-H.; Lee, C.-Y.; Wang, C.-L.; Hsieh, W.-H. . W.-H. . *Chem. Abstr.* **2007**, *146*, 134625.
15. Kurono, M.; Fujii, A.; Murata, M.; Fujitani, B.; Negoro, T. *Biochem. Pharmacol.* **2006**, *71*, 338.
16. Bi, S.-Y.; Ding, L.; Song, D.-Q.; Tian, Y.; Zhang, H.-Q. *Huaxue Xuebao* **2005**, *63*, 2169; Bi, S.-Y.; Ding, L.; Song, D.-Q.; Tian, Y.; Zhang, H.-Q. *Chem. Abstr.* **2007**, *144*, 120876.
17. Stehle, G.; Sinn, H.; Wunder, A.; Schrenk, H. H.; Schutt, S.; Maier-Borst, W.; Heene, D. L. *Anti-Cancer Drugs* **1997**, *8*, 677.
18. Hauss, F.; Liu, J.; Michelucci, A.; Coowar, D.; Morga, E.; Heuschling, P.; Luu, B. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4218.

19. Chen, G.; Shan, W.; Wu, Y.; Ren, L.; Dong, J.; Ji, Z. *Chem. Pharm. Bull.* **2005**, *53*, 1587.
20. (a) Ruan, B.-F.; Huang, X.-F.; Ding, H.; Xu, C.; Ge, H.-M.; Zhu, H.-L.; Tan, R.-X. *Chem. Biodivers.* **2006**, *3*, 975; (b) Sun, H.; Nikolovska-Coleska, Z.; Yang, C.-Y.; Xu, L.; Liu, M.; Tomita, Y.; Pan, H.; Yoshioka, Y.; Krajewski, K.; Roller, P. P.; Wang, S. *J. Am. Chem. Soc.* **2004**, *126*, 16686; (c) Mao, H.; Hajduk, P. J.; Craig, R.; Bell, R.; Borre, T.; Fesik, S. W. *J. Am. Chem. Soc.* **2001**, *123*, 10429; (d) Green, N. S.; Palaninathan, S. K.; Sacchettini, J. C.; Kelly, J. W. *J. Am. Chem. Soc.* **2003**, *125*, 13404; (e) Park, C.-M.; Oie, T.; Petros, A. M.; Zhang, H.; Nimmer, P. M.; Henry, R. F.; Elmore, S. W. *J. Am. Chem. Soc.* **2006**, *128*, 16206.
21. Dong, Y.; Ai, L.; Zhang, N. CN 1500479. *Chem. Abstr.* **2004**, *142*, 469331.
22. N'soukpoe-Kossi, C. N.; St-Louis, C.; Beauregard, M.; Subirade, M.; Carpentier, R.; Hotchandani, S.; Tajmir-Riahi, H. A. *J. Biomol. Struct. Dyn.* **2006**, *24*, 277.
23. (a) Mirk, D.; Waldvogel, S. R. *Tetrahedron Lett.* **2004**, *45*, 7911; (b) Oliveira, A. C.; Coelho, M. G.; Pires, R. F.; Franco, M. R., Jr. *J. Chem. Eng. Data* **2007**, *52*, 298.
24. (a) Jiang, Y. L.; Kwon, K.; Stivers, J. T. *J. Biol. Chem.* **2001**, *276*, 42347; (b) Jiang, Y. L.; Krosky, D. J.; Seiple, L.; Stivers, J. T. *J. Am. Chem. Soc.* **2005**, *127*, 17412; (c) Ozer, I.; Tacal, O. *Anal. Biochem.* **2001**, *294*, 1.
25. Hohlneicher, G.; Mueller, M.; Demmer, M.; Lex, J.; Penn, J. H.; Gan, L. X.; Loesel, P. D. *J. Am. Chem. Soc.* **1988**, *110*, 4483.
26. Suzuki, H. *Bull. Chem. Soc. Jpn.* **1960**, *33*, 406.
27. Lee, S. H.; Park, J. R.; Jeong, M.-Y.; Kim, H. M.; Li, S.; Song, J.; Ham, S.; Jeon, S.-J.; Cho, B. R. *ChemPhysChem* **2006**, *7*, 206.
28. Gano, J. E.; Kirschbaum, K.; Luzik, E. D., Jr.; Sekher, P. *Tetrahedron Lett.* **1998**, *39*, 6641.
29. Yang, J.-S.; Liau, K.-L.; Li, C.-Y.; Chen, M.-Y. *J. Am. Chem. Soc.* **2007**, *129*, 13183.
30. Ferrer, J. L.; Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nat. Struct. Biol* **1999**, *6*, 775.
31. Adekoya, O. A.; Willassen, N.-P.; Sylte, I. *J. Struct. Biol.* **1988**, *153*, 129.
32. Commodari, F.; Khiat, A.; Ibrahimi, S.; Brizius, A. R.; Kalkstein, N. *Magn. Reson. Chem.* **2005**, *43*, 567.
33. Klabunde, T.; Petrassi, H. M.; Oza, V. B.; Raman, P.; Kelly, J. W.; Sacchettini, J. C. *Nat. Struct. Biol.* **2007**, *7*, 312.
34. Herranz, F.; Santa Maria, M. D.; Claramunt, R. M. *J. Org. Chem.* **2006**, *71*, 2944.
35. Gonzalez-Gaitano, G.; Sainz-Rozas, P. R.; Isasi, J. R.; Guerrero-Martinez, A.; Tardajos, G. *J. Phys. Chem. B* **2004**, *108*, 14154.
36. Ji, Z.; Yuan, H.; Liu, M.; Hu, J. *J. Pharm. Biomed. Anal.* **2002**, *30*, 151.
37. Robledo-Luiggi, C.; Vera, M.; Cobo, L.; Jaime, E.; Martinez, C.; Gonzalez, J. L. *Biospectroscopy* **1999**, *5*, 313.