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# 3,4,2′-Trimethoxy-*trans*-stilbene – a potent CYP1B1 inhibitor†

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A novel series of methoxy-*trans*-stilbenes with 3,4-dimethoxy motifs was designed and synthesized. The inhibitory potency of 3,4-dimethoxystilbene derivatives against cytochrome P450 isozymes CYP1A1, CYP1B1 and CYP1A2 was evaluated. 3,4,2′-Trimethoxy-*trans*-stilbene (3,4,2′-TMS) exhibited extremely potent inhibitory action against CYP1B1 activity with an $IC_{50}$ of 0.004 μM. 3,4,2′-TMS exhibited 90-fold selectivity for CYP1B1 over CYP1A1 and 830-fold selectivity for CYP1B1 over CYP1A2. However, 3,4,2′,4′-tetramethoxy-*trans*-stilbene appeared to be the most selective inhibitor of both CYP1B1 and CYP1A1 showing very low affinity toward CYP1A2. Complementary experimental studies and computational methods were used to explain what structural determinants decide the specific affinity of stilbene derivatives to CYP1A2 and CYP1B1 binding sites.

## Introduction

*trans*-Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; Fig. 1) is the best known natural stilbene derivative that is found in grapes, peanuts, a variety of berries and medicinal plants. Numerous biological activities of resveratrol have been shown in studies *in vitro* and on animal models *in vivo*.[1,2] It is suggested that this polyphenol might play a role in the prevention and treatment of chronic diseases. This potentially chemopreventive and chemotherapeutic agent is currently under clinical trials.[3] Resveratrol analogues are designed and synthesized in order to obtain bioactive agents as promising as the parent compounds. In the last decade the inhibitory activity of resveratrol and its derivatives against cytochrome P450 enzymes has been extensively investigated.[4–13] Cytochromes P450 of family 1, cytochrome P450 1A1 (CYP1A1), cytochrome P450 1A2 (CYP1A2) and cytochrome P450 1B1 (CYP1B1), are involved in the activation of potential carcinogens. Recently, CYP1B1 has attracted increasing attention as a drug target. CYP1B1 is postulated to be involved in mammary carcinogenesis responsible for 17β-estradiol ($E_2$) metabolism to the highly carcinogenic 4-hydroxy-17β-estradiol (4-$OHE_2$).[14] CYP1B1 is found mainly in extrahepatic steroidogenic tissues such as the ovary, testis, and adrenal gland and in steroid-responsive tissues such as breast, uterus, and prostate. CYP1B1 demonstrates a higher expression level in premalignant and malignant tumors compared to normal tissues.[15] CYP1B1 up-regulation plays a crucial role in endometrial carcinogenesis by targeting multiple pathways.[16] A selective inhibition of CYP1B1 is recognized as beneficial for the prevention of hormone-related cancers.

Resveratrol and its natural analogues – pterostilbene (3,5-dimethoxy-4′-hydroxy-*trans*-stilbene), pinostilbene (3,4′-dihydroxy-5-methoxy-*trans*-stilbene), and desoxyrhapontigenin (3,5-dihydroxy-4′-methoxy-*trans*-stilbene) – inhibited CYP1B1 activity with an $IC_{50}$ of 1.4, 2.1, 0.8, and 2.6 μM, respectively.[4,11] Novel stilbene derivatives were designed and synthesized to obtain more potent and selective inhibitors of CYP1 enzymes. 3,2′,4′,5-Tetramethoxy-*trans*-stilbene (3,2′,4′,5-TMS; Fig. 1) was the first synthetic resveratrol analogue that was found to be an extremely potent inhibitor of CYP1B1 with an $IC_{50}$ of 2 nM (Chun *et al.*, 2001; Kim *et al.*, 2002).[6,10] Moreover, a strong cytotoxic activity of 3,2′,4′,5-TMS ($IC_{50}$; 0.8 μg $ml^{-1}$) in cultured human colon cancer cells and proapoptotic activity were shown.[17]

More recently, a SAR study by Chun *et al.* made it possible to select a series of stilbenoids with 2,4-dimethoxy motifs as selective inhibitors of CYP1B1 with a lead compound 2,4,2′,6′-tetramethoxy-*trans*-stilbene (2,4,2′,6′-TMS; Fig. 1) showing remarkable potency with an $IC_{50}$ of 1.77 ± 0.14 nM.[8] Moreover, 2,4,2′,6′-TMS suppressed ethoxyresorufin-*O*-deethylase (EROD) activity and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced CYP1A1 or CYP1B1 gene expression in the human

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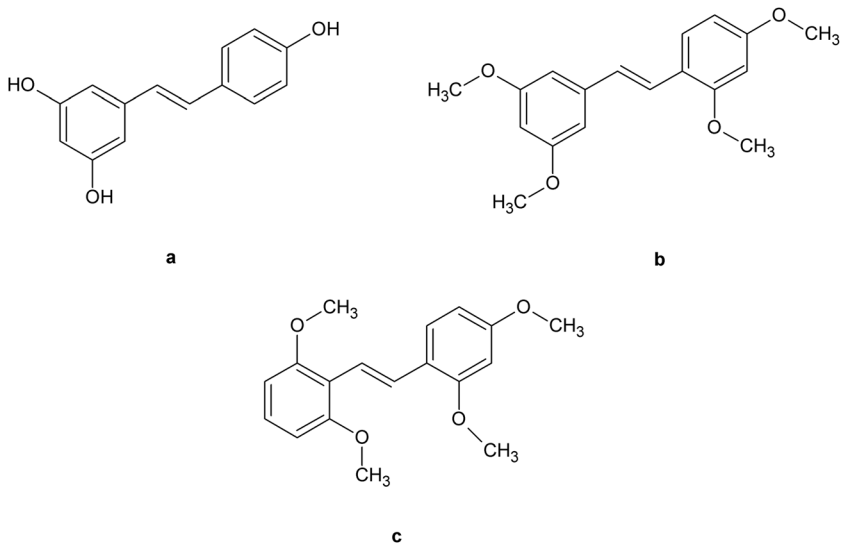



Fig. 1 Structures of selective CYP1B1 inhibitors: (a) *trans*-resveratrol; (b) 3,5,2′,4′-tetramethoxy-*trans*-stilbene; (c) 2,4,2′,6′-tetramethoxy-*trans*-stilbene.

hepatoma cells HepG2 and non-invasive epithelial cell line MCF-10A.[8] These studies have demonstrated the essential role of the 2-methoxy group linked to the stilbene scaffold; however, it does not seem to be the only determinant of the exceptionally strong inhibitory action of stilbene derivatives. Molecular modeling indicates that the whole pattern of substituents is an essential determinant of ligand affinity and it guarantees the unique fit of a ligand to the active site of the enzyme. By searching for the most effective arrangement of substituents, we designed and synthesized a series of stilbene derivatives with a 3,4-dimethoxyphenyl ring differing in the substitution pattern of the second ring, that have never been investigated previously. Their inhibitory effect on cytochrome P450 family 1 enzymes CYP1A1, CYP1A2 and CYP1B1 was investigated experimentally by means of EROD activity determination and *in silico* with the use of molecular docking methods.

## Results and discussion

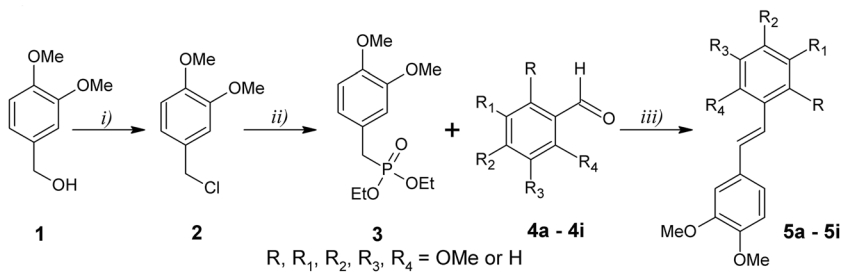
### Chemistry

Synthesis of a series of polymethoxystilbenes **5a–5i** was performed according to the procedure described above.[18] The three-step synthesis (Scheme 1) started from 3,4-dimethoxybenzyl chloride **2** preparation by converting 3,4-dimethoxybenzyl alcohol **1** which was next *via* Michaelis–Arbuzov reaction modified to phosphonate **3** (diethyl (3,4-dimethoxybenzyl)phosphonate). Compound **3** was further transformed in Horner–Wadsworth–Emmons reaction with proper aromatic aldehydes (**4a–4i**) into nine investigated *trans* polymethoxystilbene compounds. The reaction yield of compound **5a** to **5i** varies between 25 and 72%. The structure of the newly synthesized compounds was established by spectroscopic methods ($^1$H NMR, $^{13}$C NMR), elementary analysis and mass spectra.

The structure of synthesized products **5a–5i** was confirmed using 1D and 2D NMR techniques and each $^1$H and $^{13}$C signal for all compounds was annotated (ESI†). Fig. 3 shows the exemplary structure of **5b**.

### Crystallographic data

The identification of compounds **5a**, **5b**, **5f**, **5g**, and **5i** was confirmed by X-ray crystallography. For the most active CYP1B1 inhibitor **5b**, the double bond C7–C8 in the conjugated linkage is in the *trans* configuration [torsion angle C(4)–C(7)–C(8)–C(9), 177.67(13)°]. Furthermore, the value for the observed double




Scheme 1 Synthesis of compounds **5a–5i**. Reaction conditions: (i) $SOCl_2$, $CHCl_3$, 0–25 °C, 1 h; (ii) $P(OEt)_3$, 130 °C, 24 h; (iii) NaOMe, DMF, r.t., 1 h, next 100 °C, 1.5 h.

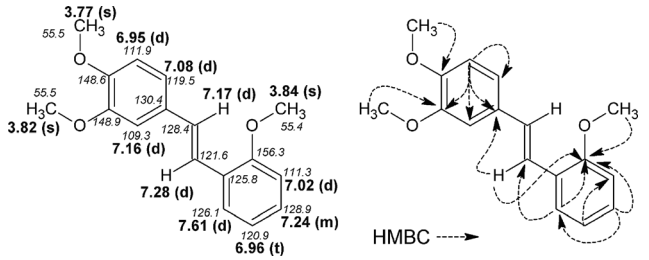



Fig. 2 Annotated signal $^{1}H$ and $^{13}C$ NMR spectra according to 2D experiments ($^{1}H-^{1}H$ COSY, HSQC and HMBC). The abbreviations s, d, t and m mean: singlet, doublet, triplet and multiplet.

bond [C7–C8, 1.334(2) Å] is longer than the normal value (1.32 Å) and the single bonds [C(4)–C(7) – 1.465(2) Å and C(8)–C(9) – 1.467(2) Å] are shorter than the normal values (1.51 Å),[19] indicating the formation of a weak conjugated π-electron system. These values for all the studied structures (**5a**, **5b**, **5f**, **5g**, and **5i**) are shown in the ESI.†

The aromatic rings do not deviate significantly from a coplanar arrangement, with a dihedral angle of 3.07(9)° between the planes. Among the three methoxy substituents on the aromatic ring, only the one at C2 is not exactly coplanar with the benzene ring (10.54°). The other two, at C1 and C10, are approximately coplanar with the benzene ring.[20] Only for structure **5g** the aromatic rings deviate significantly from a coplanar arrangement, with a dihedral angle of 51.53(5)° between the planes. This value for the other structures is between 4.95(11) and 6.99(9) degrees.

## Biological *in vitro* assay

**The inhibition of CYP1A1, CYP1A2 and CYP1B1 activities.** The inhibitory effect of synthesized 3,4-dimethoxy-*trans*-stilbene derivatives was determined using the *E. coli* membrane expressed human recombinant cytochromes CYP1A1, CYP1A2 and CYP1B1 with human NADPH-P450 reductase. The activities of EROD catalyzed by CYP1A1, CYP1A2 and CYP1B1 were measured spectrofluorimetrically according to the method described in the ESI.† The results concerning the inhibitory potency and selectivity of the studied stilbenes (Table 1) demonstrate variations highly dependent on the differences in the substitution pattern.

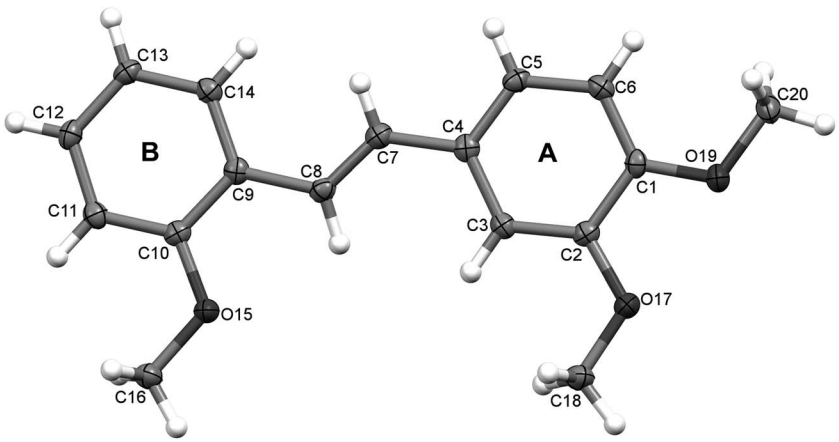



Fig. 3 A view of **5b**, showing the atom-labeling scheme. Displacement ellipsoids are drawn at the 50% probability level and H atoms are shown as small spheres of arbitrary radii.

Table 1 The effect of 3,4-dimethoxy-*trans*-stilbene derivatives on cytochrome P450 CYP1A1, CYP1A2 and CYP1B1 activities[a]

| | | $IC_{50}$[b] [μM] | | | | |
|---|---|---|---|---|---|---|
| Compound | R | CYP1A1 | CYP1A2 | CYP1B1 | CYP1A2/CYP1B1 | CYP1A1/CYP1B1 |
| **5a** | 3,4-Dimethoxy | 0.44 ± 0.12 | 6.04 ± 1.98 | 0.30 ± 0.04 | 20.1 | 1.5 |
| **5b** | 3,4,2′-Trimethoxy | 0.36 ± 0.12 | 3.32 ± 0.81 | 0.0040 ± 0.0006 | 830 | 90 |
| **5c** | 3,4,2′,3′-Tetramethoxy | 0.45 ± 0.10 | 16.02 ± 7.15 | 0.37 ± 0.05 | 43.3 | 1.2 |
| **5d** | 3,4,2′,4′-Tetramethoxy | 0.51 ± 0.27 | >100 | 0.21 ± 0.07 | >480 | 2.4 |
| **5e** | 3,4,2′,5′-Tetramethoxy | 0.50 ± 0.05 | >100 | 0.62 ± 0.07 | >160 | 0.8 |
| **5f** | 3,4,2′,6′-Tetramethoxy | 0.23 ± 0.04 | 2.31 ± 0.026 | 0.31 ± 0.07 | 7.5 | 0.74 |
| **5g** | 3,4,2′,3′,4′-Pentamethoxy | 5.18 ± 1.35 | >100 | 4.17 ± 1.44 | >24 | 1.2 |
| **5h** | 3,4,2′,4′,5′-Pentamethoxy | 1.78 ± 0.77 | >100 | 4.44 ± 0.86 | >22.5 | 0.40 |
| **5i** | 3,4,2′,4′,6′-Pentamethoxy | 0.27 ± 0.09 | >100 | 0.30 ± 0.05 | >333 | 0.9 |

[a] CYP1A1, CYP1A2 and CYP1B1 activities were determined by the use of 7-ethoxyresorufin-*O*-deethylation assay according to the method described in the ESI. [b] $IC_{50}$ values are the mean ± range of three separate experiments determined using nonlinear regression methods by GraphPad Prism software (San Diego, CA).

In the present study, 3,4,2′-trimethoxy-*trans*-stilbene (**5b**) inhibited CYP1B1 activity at the nanomolar level with an $IC_{50}$ of 4.0 nM. This compound was shown to be a particularly selective inhibitor of CYP1B1, exhibiting a 90-fold selectivity for CYP1B1 over CYP1A1 and an 830-fold selectivity for CYP1B1 over CYP1A2. The other studied compounds (listed in Table 1) possessing additional 3′-, 4′-, 5′- or 6′-methoxy substituents displayed a potent inhibitory action on CYP1B1, which was, however, not so significant. For example, pentamethoxy derivatives (**5g** and **5h**) were 1000-fold weaker CYP1B1 inhibitors compared to **5b**. It is worth noting that among pentamethoxy derivatives only **5i** with methoxy groups at 2′ and 6′ positions was a potent inhibitor of CYP1A1 and CYP1B1 exhibiting an $IC_{50}$ < 1 μM.

All 3,4-dimethoxystilbene derivatives potently inhibited CYP1A1 activity. With the exception of pentamethoxystilbenes **5g** and **5h**, for the other studied compounds, the $IC_{50}$ values were less than 1 μM, and significant differences in $IC_{50}$ were not observed. In an earlier study of a stilbene derivative, 2,3,4-trimethoxy-4′-methylthio-*trans*-stilbene was reported to be a potent and selective inhibitor of the isozymes CYP1A1 and CYP1B1, displaying an extremely low affinity towards CYP1A2.[21] Interestingly, **5g** was a significantly weaker inhibitor of CYP1A1 activity in comparison with 2,3,4-trimethoxy-4′-methylthio-*trans*-stilbene. It may be concluded that an additional 3-methoxy substituent in the stilbene with the 2,3,4-trimethoxy motif influences the localization and interaction of **5g** in the CYP1A1 cavity.

The affinity of stilbenes to the CYP1A2 binding site is determined substantially by the size and shape of the molecule as a binding pocket of this enzyme is recognized to be more flat and tight than those of CYP1A1 and CYP1B1.[22] The relatively small and compact molecules of stilbenes **5b** and **5f** were the most active against CYP1A2 with an $IC_{50}$ of 3.32 and 2.31 μM, respectively.

The inhibitory activity of moderately strong inhibitors with $IC_{50}$ at the micromolar level may be explained and predicted on the basis of their structure. However, in the case of very potent inhibitors, the subtle differences in affinities to CYP1 binding sites ($IC_{50}$ < 0.1 μM) could be possibly elucidated only with computational studies comprising molecular modeling and docking with a detailed description of molecular interactions.

## Computational study

We used a molecular docking approach to elucidate the strong and selective affinity of 3,4,2′-TMS to the CYP1B1 binding site. The analyzed molecules were docked to the active sites of CYP1A2 (PDB: 2HI4)[23] and CYP1B1 (PDB: 3PM0)[24] with the use of Accelrys Discovery Studio 3.5[25] by the LigandFit procedure.[26] The binding energy, interaction energy and strain energy were also estimated (values for the best poses are presented in the ESI†).

Molecular docking to the CYP1A2 cavity was performed for all synthesized compounds. The highest values of the DockScore function were obtained for **5a**, **5b** and **5f** (52.6, 53.0 and 42.2, respectively). The ligands were preferably oriented with ring B (phenyl with the changing pattern of substituents) toward heme (Fig. 4a). Other tetramethoxy ligands – **5c**, **5d** and **5e** – had significantly lower scores (values ranging from 24.0 to 34.5). The LigandFit procedure failed to dock pentamethoxy derivatives into the CYP1A2 binding site with only one exception of **5i**, which was characterized by a low score and a high strain energy value.

All ligands were successfully docked to the binding site of CYP1B1; however, pentamethoxy derivatives had a very low score. When comparing the calculated parameters for both enzymes, it must be emphasized that all DockScore values for the studied stilbenes docked to CYP1B1 were higher than the

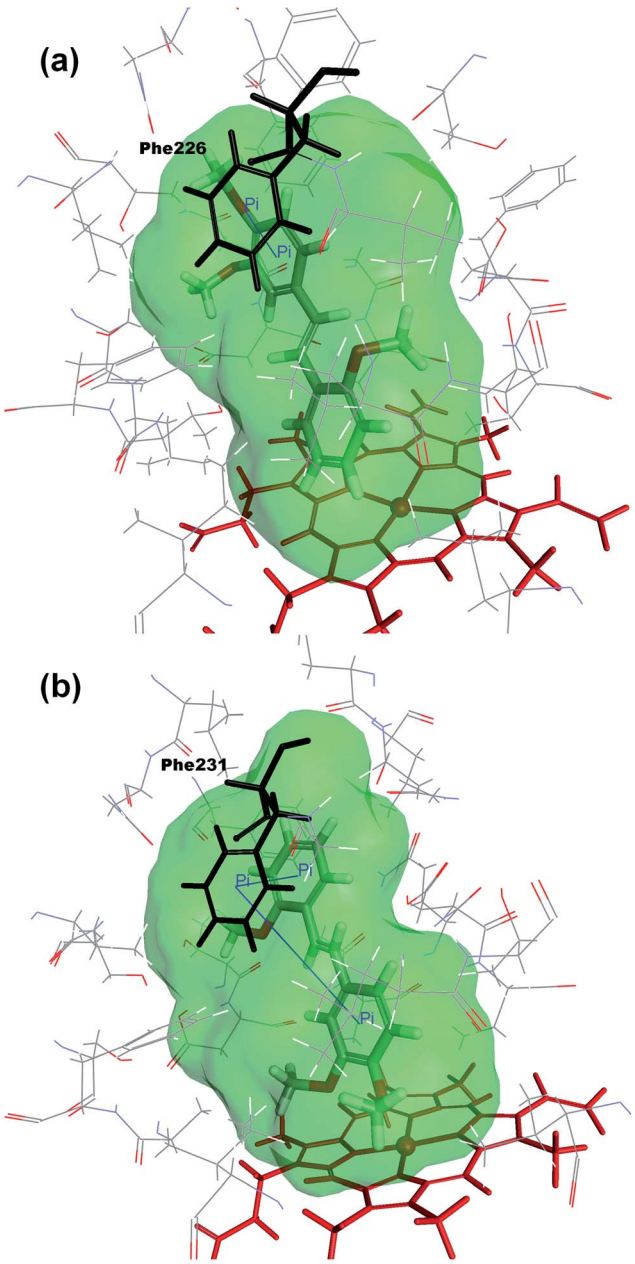



Fig. 4 The highest scored pose of **5b** in (a) CYP1A2 and (b) CYP1B1 binding sites. Solid blue lines represent π–π interactions; heme is represented as a stick model in red.

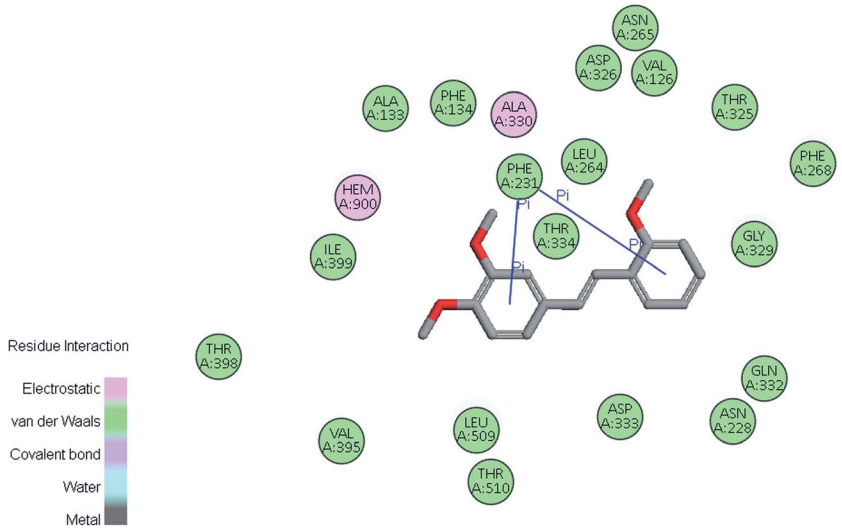



Fig. 5 The amino acid residues surrounding **5b** in the CYP1B1 binding site. Residues involved in van der Waals and polar interactions are colored in green and magenta, respectively. Solid blue lines represent π–π interactions.

respective values in the case of CYP1A2. Strain energies, with the exceptions of **5a** and **5e**, were significantly lower for stilbenes in the CYP1B1 binding site. Moreover, binding free energies for all compounds docked to CYP1B1 are considerably higher than that of CYP1A2; it may be suggested that complexes of CYP1B1 with ligands are much more stable in comparison with complexes of CYP1A2.

The extremely strong affinity of **5b** to CYP1B1 was reflected in the highest value of binding free energy, −443.1 kcal mol$^{-1}$, when ring A (3,4-dimethoxyphenyl) was directed to the heme (Fig. 4b). This pose seems to be more favorable because 17 of the 20 poses analyzed for **5b** docked to CYP1B1 were directed with 3,4-dimethoxyphenyl to the heme. For the opposite orientation the binding energy was significantly lower (−403.0 kcal mol$^{-1}$). Additionally, the interaction energy for the orientation of ring A or B towards heme differs considerably, with values of −59.0 and −47.2 kcal mol$^{-1}$, respectively. It is worth adding that according to the finding of Chun,[6] the more energetically favorable orientation enables demethylation of 3,4-dimethoxy stilbene derivatives.

Interestingly, in the energetically favorable orientation of **5b** docked to the CYP1B1 pocket, there were no hydrogen bonds formed. Most interactions were attributed to van der Waals forces and only π–π stacking interaction occurred for Phe231 (Fig. 5), whereas in the opposite orientation of **5b** (with a 2′-methoxyphenyl ring directed to the heme), hydrogen bonding between a 3-methoxy group and an amino acid residue Gln332 occurred. Hydrogen bonds were not observed, either, when the tested ligands were docked to the CYP1A2 active site. The binding specificity of polymethoxystilbenes was supposed to be based on the hydrophobic interactions between the methoxy groups and the specific CYP1 residues (Fig. 5), as it was observed when methoxyflavonoids were docked to CYP1A2 and CYP1B1.[27] However, it seems that in the case of the studied series of stilbenes spatial fitting plays an important role besides molecular interactions between the ligand and the binding site environment.

## Conclusions

CYP1B1 inhibition is extremely sensitive to the substitution pattern of *trans*-stilbene. In the present study, **5b** inhibited CYP1B1 activity in the nanomolar range with an $IC_{50}$ value of 4.0 nM. Moreover, **5b** displayed a 90-fold selectivity for CYP1B1 over CYP1A1 and an 830-fold selectivity for CYP1B1 over CYP1A2. With the use of *in silico* methods, the favorite pose of ligands with 3,4-dimethoxyphenyl directed toward heme in the CYP1B1 binding site was identified. The methoxy group in the 2′ position appeared to be a crucial substituent; however, other determinants of **5b** inhibitory potency toward CYP1B1 remain to be identified on the molecular level. Experimental studies of the inhibitory activity of stilbene derivatives supported by computational screening may be helpful in the design of molecules targeting the enzymes involved in the biotransformation of potential carcinogens. However, further improvements of analyzing functions would be expected to better estimate ligand-binding site interactions. On the other hand, finding a new very selective CYP1B1 inhibitor opens up an opportunity for further studies on 17beta-estradiol metabolism pathways in terms of inhibition of carcinogenic 4-$OHE_2$ generation.

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