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Synthesis and steroid sulfatase inhibitory activities of *N*-alkanoyl tyramine phosphates and thiophosphates

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A series of phosphate and thiophosphate analogs based on the frameworks of *N*-alkanoyl tyramines have been synthesized and biologically evaluated. Their binding modes have been modeled using docking techniques. The inhibitory effects of the synthesized compounds were tested on STS isolated from the human placenta as well as the MCF-7, MDA-MB-231 and SkBr3 cancer cell lines. Most of the new STS inhibitors possessed potent activity against STS. In the course of our investigation, 4-(2-dodecanoylamino-ethyl)-phenyl dimethylphosphate **4a** demonstrated the greatest inhibitory effect, with IC_{50} values of 0.39 μM (IC_{50} value of 15.44 μM for the 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate used as a reference). The compound **4a** exhibited the highest potency against the MCF-7, MDA-MB-231 and SkBr3 cancer cell lines, with a GI_{50} values of 8.80, 6.48 and 5.76 μM, respectively. The structure-activity relationships of the synthesized phosphate- and thiophosphate-based tyramine derivatives with the STS enzyme are discussed.

Introduction

Approximately 1 in 9 women are affected by breast cancer during their lifetime, and this disease is a major cause of mortality of postmenopausal women. Estimates show that in the United States, more than 230,000 new cases of breast cancer were diagnosed and more than 40,000 deaths occurred from this disease in 2014 (according to National Cancer Institute data). Over the past decades, numerous reports have suggested the importance of biologically active hormone precursors in regulating the supply of estrogens to estrogen-dependent breast cancers. Sulfated steroids, including estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS), play an important role in the process of human steroidogenesis and are considered to be the key endocrine factor involved in the initiation and promotion of breast cancer.[1] There are three enzyme pathways (including aromatase, 17β-hydroxysteroid dehydrogenase and steroid sulfatase STS) that are responsible for the formation of active estrogens in the breast tissue of postmenopausal women. Within these pathways, STS plays a major role in the formation of biologically active estrogens or androgens and acts by hydrolyzing aryl and alkyl steroid sulfates (including E1S and DHEAS).[2] It is worth noting that STS expression is detected in 90% of breast tumors, with much higher activity than the aromatase complex.[3] The detailed studies have shown that estrone (E1) and dehydroepiandrosterone (DHEA) (products of the hydrolysis of E1S and DHEAS) can act as precursors for the formation of the estrogenic steroids estradiol (E2) and androstenediol (Adiol). In addition, a variety of scientific research has shown that estradiol (E2) and androstenediol (Adiol) are responsible for the stimulation and proliferation of breast cancer cells *in vitro* and play a pivotal role in breast cancer tumorigenesis.

Because STS is strongly implicated in estrogenic stimulation of hormone-dependent breast cancer, research work on the design and synthesis of new and more effective agents that inhibit the activity of STS is of particular importance and is a major challenge for modern medicinal chemistry. Significant progress has been made in the past two decades in discovering and synthesizing STS inhibitors for clinical development. The achievements of steroid sulfatase inhibitors were presented in a series of review articles.[3,4,5] Among the first inhibitors reported to possess good inhibitory activity were danazol,[6] 2-phenylindole sulfates,[7] steroid sulfonyl halides,[8] steroid sulfates[9] and phosphates.[10] The breakthrough in the design of effective STS inhibitors was the discovery of estrone-3-O-sulfamate (EMATE), which exhibited very high activity in MCF-7 cells, with an IC_{50} value of 65 pM. Although possessing potent inhibitory activity, surprisingly, EMATE exhibits estrogenic activity, rendering it unsuitable for use in the treatment of hormone-dependent breast cancer. For this reason, clinical trials for EMATE have been discontinued.

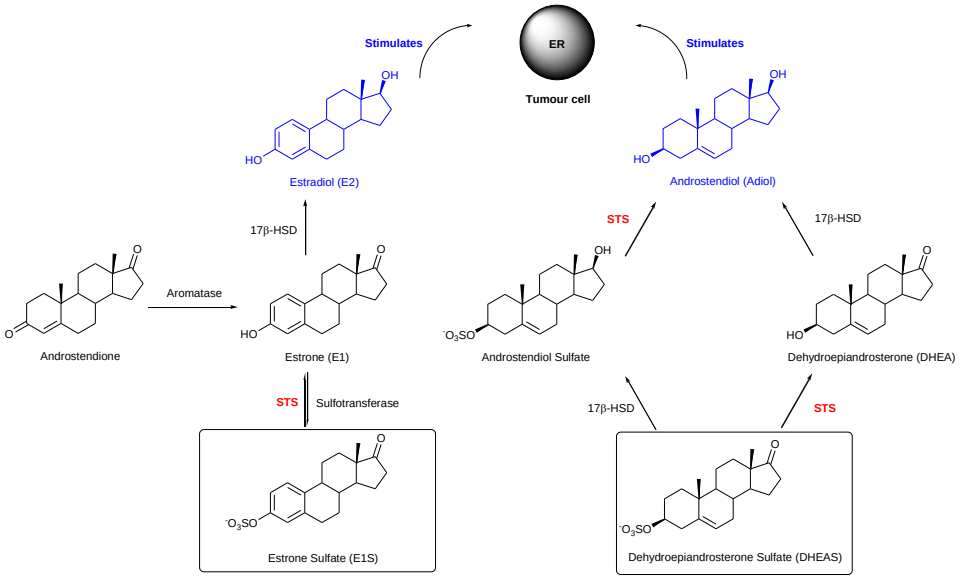


Fig. 1 The origin of estradiol (E2) and androstenediol (Adiol) in postmenopausal women with hormone-dependent breast cancer.

Alternative derivatives of EMATE have also been evaluated; in general, a number of compounds were found to be weaker inhibitors of STS than EMATE. These disadvantages of steroid-based STS inhibitors led to attempts to design and synthesize a series of compounds that would avoid the limitations of EMATE and its derivatives. A series of sulfamate-based chromenone derivatives was synthesized as part of this development process. One of these derivatives, 667 COUMATE (tricyclic coumarin sulfamate analog), was shown to be able to mimic the natural substrate, acting as a potent STS inhibitor and was found to possess an IC_{50} value of 8 nM in placental microsomes.[11] Furthermore, 667 COUMATE did not display any estrogenic activity in either *in vitro* or *in vivo* assays and currently is undergoing clinical trials. Other promising inhibitors that do not exhibit undesirable estrogenic effects included STX213 and STX1938.

The structure of STX213 is based on an *N*-substituted piperidine-2,6-dione ring, and this compound is classified as a potent steroid STS inhibitor. In the course of research on (MCF)-7STS xenografts in mice models, its higher activity than that of 667 COUMATE has been proven.[12] As a result of its high inhibitory activity combined with its increased stability *in vivo* and lack of estrogenicity, STX213 has recently entered into clinical trials. STX1938 displayed similar STS activity to STX213.[13] Parallel to the development of 667 COUMATE, a series of 17beta-(*N*-alkylcarbamoyl)-estra-1,3,5(10)trine-3-O-sulfamate derivatives have also been studied as inhibitors of STS.[14] Within the range of these derivatives, a new compound, KW-2581, was found to possess an IC_{50} of 4.0 nM, as evaluated on the crude enzymes isolated from recombinant Chinese hamster ovary cells expressing human arylsulfatases (ARSs). Currently, due to its high therapeutic potential, KW-2581 is a very promising candidate for clinical trials.[15]

The intention of our study is designing of the new STS inhibitors based on *N*-alkanoyltyramine scaffolds. Biogenic amines (BAs) (including tyramine) are formed *in vivo* by the decarboxylation of amino acids and are involved in many important biological functions including nervous transmission, the immune response, or cell growth and differentiation.[16] Due to their high biological activity and use in the design of new drugs, their synthesis has attracted the interest of many investigators. Some of them have been used as sulfamate based potent STS inhibitors exhibited very promising activities.[17] Our particular scientific interests are focused on the use of different phosphate or thiophosphate moieties in designing of novel STS inhibitors. As is widely recognized, phosphate and thiophosphate functional groups may undergo the nucleophilic substitution reaction on the phosphorus atom or create many interactions (e.g. hydrogen bonds) with a variety of amino acid residues found in the catalytic region of STS, what could have a significant impact for binding ability to STS.

Results and discussion

Molecular modeling

The X-ray crystal structure of human steroid sulfatase has been reported by Ghosh and coworkers.[18] To explore different binding modes, the X-ray structures of STS were obtained from the Protein Databank (Protein Data Bank accession code 1P49). After standard preparation procedures (including conversion of catalytic amino acid FGly75 (formylglycine) to the gem–diol form), docking analysis was conducted. Starting geometry of ligands was built with the program Portable HyperChem 8.0.7 Release (Hypercube Incorporation) and their energies were minimized using the MM+ force field and Polak – Ribiere conjugate gradient algorithm. Because of the large number of possible conformations (common for a long aliphatic chains) molecular modeling studies were performed 10 times for each investigated ligand structures using AutoDock Vina software. The best poses (illustrating the putative enzyme-ligand complexes) for a particular ligand were visually inspected. To identify possible ligand-enzyme interactions and to further SAR

analysis, the enzyme-ligand complexes with the lowest free binding energies were selected. The predicted free energy of binding (the scoring function of AutoDock Vina) was calculated from the intermolecular part of the lowest-scoring conformation.[19] The putative enzyme-ligand complex prior to the presumed inactivation of STS is shown in figure 2, with selected active site residues highlighted.

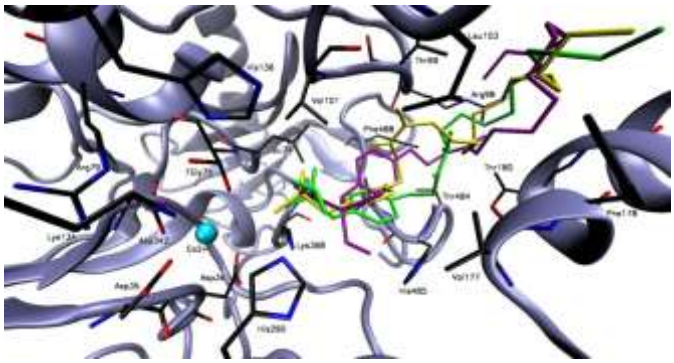


Fig. 2 Docked binding modes for compounds **4a'** (purple), **5c** (yellow) and 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate **1** (green).

Regarding the binding mode, docking experiments revealed that phosphate and thiophosphate analogs of *N*-alkanoyl tyramine might be able to bind STS, at least theoretically. As predicted, the newly designed STS inhibitors should be well accommodated in the STS active site by a variety of hydrophobic amino acid residues such as Leu74, Arg98, Thr99, Val101, Leu103, Val177, Phe178, Thr180, Thr484, His485, Phe488 and able to adopt a similar binding mode to 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate **1**, which was used as a reference. As observed in figure 2, phosphate and thiophosphate functional groups are directed to catalytic amino acid FGly75 (formylglycine) and are in close proximity to the proposed catalytic residues of Asp35, Asp36, Arg79, Lys134, His136, His290, Asp342, Lys368. Moreover, regarding the predicted free binding energies, we found that increasing the chain length of the *N*-alkanoyl tyramine scaffolds increases the binding ability to the active site of STS. On the other hand, the compounds lacking the hydrophobic long aliphatic *N*-alkanoyl chains exhibited a poor ability to bind STS and led to an increase in predicted free binding energies (for example, the value of the AutoDock Vina score for 4-(2-acetylamino-ethyl)-phenyl dimethylphosphate was -0.6 kcal mol^{-1}). Our experiments showed that the optimal numbers of carbons in the alkanoyl side chains were 12 to 14 (predicted free binding energies in the range of -5.6 to -6.1 kcal mol^{-1}). The docked free binding energies of these compounds were compared to a reference of 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate **1** (-5.0 kcal mol^{-1}). The docking of compounds with shorter or longer alkanoyl residues led to an increase in predicted free binding energies, which is associated with lower stability of the enzyme-inhibitor complex (for example, the values of the AutoDock Vina score for 4-(2-undecanoylamino-ethyl)-phenyl dimethylphosphate and 4-(2-pentadecanoylamino-ethyl)-phenyl dimethylphosphate were -5.2 and -4.8 kcal mol^{-1}, respectively). These outcomes may suggest that compounds with shorter alkanoyl residues undergo a weaker hydrophobic interactions with amino acid residues located in the STS pocket, while longer than 14 carbons seem to be too extensive.

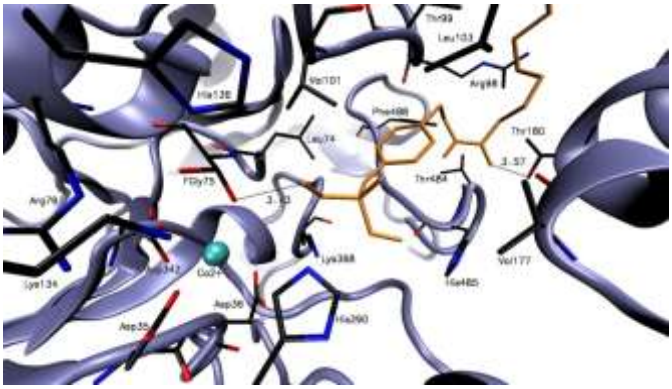


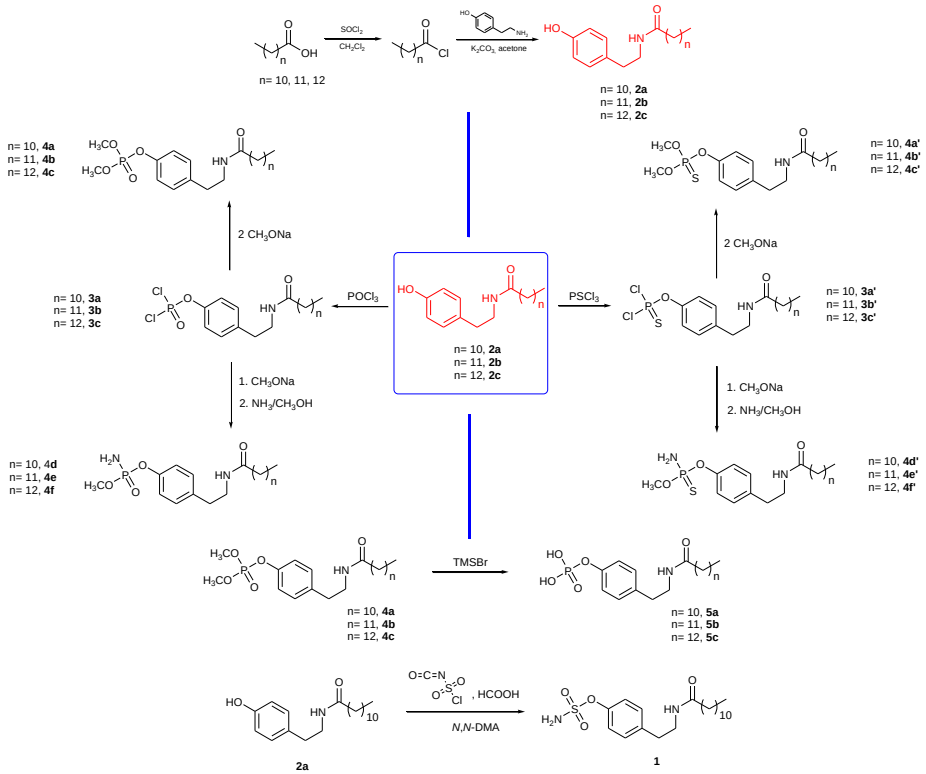
Fig. 3 Docked binding mode for compounds **4a** (orange) and distance to the catalytic amino acid FGly75.

The results of molecular calculations predicted the best binding ability to STS for dihydrogenphosphate and dimethylphosphate analogs of *N*-alkanoyl tyramine in terms of free docking energies. Among them, 4-(2-dodecanoylamino-ethyl)-phenyl dimethyl phosphate **4a** led to the best value of -6.1 kcal mol^{-1}. When studying inhibitor-enzyme interactions in atomic detail, it can be observed that complex of compound **4a** in the STS active site could be stabilized by a weak electrostatic hydrogen bond between the acyl residue of **4a** and the hydroxyl group of Thr180 (3.57 Å). Furthermore, the dimethyl phosphate residue is in close proximity to the hydroxyl group of catalytic amino acid FGly75 (3.43 Å). In general, phosphoric acid alkyl esters are widely used as alkylating agents. For this reason, we suggest that methylation of FGly75 by the dimethylphosphate derivative **4a** may play a significant role in the inhibition mechanism.

Chemistry

There are many synthetic and enzymatic methods for the preparation of tyramine and its derivatives, including the Curtius rearrangement of β-(*p*-hydroxyphenyl)propionyl azide,[20] the hydrogenation of (α-chloro-α-oximino)acetophenone by transition metal catalyst,[21] the decarboxylation of tyrosine,[22] enzymatic decarboxylation using the enzyme tyrosine decarboxylase,[23] and the reduction of β-nitrostyrenes.[24]

Encouraged by our previous research and the high biological activities of biogenic amines reported in the literature, we decided to synthesize a series of phosphate and thiophosphate analogs of *N*-alkanoyl tyramines as compounds with potential STS inhibitory activity. We previously reported a series of tricyclic coumarin-based STS inhibitors with various phosphate functional groups introduced at the 3 or 7 positions of coumarin scaffolds.[25] *N*-alkanoyl tyramine frameworks **2a**-**2c** have been synthesized from dodecanoyl, tridecanoyl and tetradecanoyl chlorides (obtained *in situ* from the corresponding carboxylic



Scheme 1 Synthesis of phosphate, thiophosphate *N*-alkanoyl tyramine derivatives and the reference compound **1**.

acids by reaction with thionyl chloride) and commercially available 4-hydroxyphenethylamine (tyramine) in the presence of potassium carbonate. This step of synthesis has proved to be highly efficient and leads to obtaining products in very good yield and high purity. Next, direct functionalization of the hydroxyl group of *N*-alkanoyl tyramine scaffolds was conducted. For this purpose, [2-(4-hydroxy-phenyl)-ethyl]-dodecanoic acid amide **2a**, [2-(4-hydroxy-phenyl)-ethyl]-tridecanoic acid amide **2b** and [2-(4-hydroxy-phenyl)-ethyl]-tetradecanoic acid amide **2c** were treated with phosphoryl or thiophosphoryl chloride in the presence of triethylamine at 0°C to yield phosphoryl or thiophosphoryl dichloride derivatives **3a-c** and **3a'-3c'**. The progress of the reactions was monitored by ^{31}P NMR spectra. As expected, functionalization of the hydroxyl group using $PSCl_3$ requires a longer reaction time, and the complete disappearance of the starting material was observed only after 3 h. After removal of the precipitated triethylamine hydrochloride, the phosphoryl or thiophosphoryl dichlorides **3a-c** and **3a'-3c'** were treated at 0°C with nucleophilic agents including MeONa or NH_3/MeOH in different stoichiometric ratios to obtain the corresponding phosphate and thiophosphate *N*-alkanoyl tyramine derivatives in good yield (Scheme 1). Finally, 4-(2-dodecanoylamino-ethyl)-phenyl, 4-(2-tridecanoylamino-ethyl)-phenyl and 4-(2-tetradecanoylamino-ethyl)-phenyl dimethyl phosphates **4a-c** were transformed into the corresponding dihydrogenphosphate analogs **5a-c** by treatment with bromotrimethylsilane (TMSBr). For comparision of phosphate or thiophosphate *N*-alkanoyl tyramine analogs to sulfamate based inhibitors we synthesized 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate **1** (reference compound) as shown in Scheme 1. In this case, [2-(4-hydroxy-phenyl)-ethyl]-dodecanoic acid amide **2a** was treated with H_2NSO_2Cl, obtained in reaction of chlorosulfonyl isocyanate and formic acid in the presence of catalytic amount of *N*,*N*-dimethylacetamide (*N*,*N*-DMA).[26]

STS enzyme assays

The screening tests for the inhibition of steroid sulfatase by synthesized compounds (**4a-5c**) were performed using STS enzyme extracted from human placenta and purified by 3-step chromatography protocol as previously described.[27] The affinity of all synthesized compounds for STS was determined using an *in vitro* STS assay, following reported methods.[28,29] A summary of the results is presented in Table 1.

No.	IC_{50}[μM]
4a	0.39±0.04
4b	>>1000
4c	234.40±21.3
4d	1.31±0.25
4e	218.70±18.7
4f	103.20±9.3
4a'	76.90±6.2
4b'	245.70±24.3
4c'	84.75±7.1
4d'	15.10±2.4
4e'	215.70±19.6
4f'	111.90±18.5
5a	68.84±5.9
5b	23.31±2.1
5c	11.17±1.4
Reference 1	15.44±2.1

Table 1 Activities of the synthesized compounds and a reference inhibitor in STS enzyme assays

The enzyme assay results showed that the highest efficiency was exhibited by compounds containing a hydrophobic dodecanoyl carbon chain in the structure of the *N*-alkanoyl tyramine scaffold. These results may suggest that a framework based on [2-(4-hydroxy-phenyl)-ethyl]-dodecanoic acid amide **2a** is more suitable for anchoring the enzyme active site. However, for dihydrogenphosphate analogs (compounds **5a**-**5c**), increasing the length of the hydrophobic carbon chain significantly improved the biological activity. In these cases, the highest inhibition was observed for 4-(2-tetradecanoylamino-ethyl)-phenyl dihydrogen phosphate **5c** with an IC_{50} value of 11.17 μM. These results indicate that for compounds (**5a**-**5c**), increasing the hydrophobic properties of *N*-alkanoyl tyramine scaffold could favor the binding through the creation of stronger hydrophobic interactions with the cavity delimited by lipophilic amino acids in the STS enzyme pocket.

When studying the influence of the phosphate or thiophosphate functional groups on the STS activity two effects are noticeable. The first is associated with the alkylating properties of tested compounds suggesting the possible methylation of FGly75. The second effect is related to the possibilities of occurrence an electrostatic interactions (e.g. hydrogen bonds) with amino acids located in the catalytic region of STS. In the course of our investigation, we found that the most promising candidate exhibiting the highest inhibitory property towards STS was 4-(2-dodecanoylamino-ethyl)-phenyl dimethyl phosphate **4a** (IC_{50} values of 0.39 μM; 15.44 μM for the reference **1**). As is widely known, phosphoric acid methyl esters are much stronger alkylating agents than their thiophosphate counterparts, what could explain a higher activity of **4a** compared to 4-(2-dodecanoylamino-ethyl)-phenyl dimethyl thiophosphate **4a'** (IC_{50} = 76.90 μM). In addition, phosphate group (in contrast to thiophosphate) may participate in the formation of electrostatic interactions with catalytic amino acid residues as hydrogen bond acceptor (P=O). Introducing the NH_2 moiety into the phosphorus atom decreases alkylating ability of tested compounds resulting in the lower STS activity of 4-(2-dodecanoylamino-ethyl)-phenyl methyl phosphoroamidate **4d** (IC_{50} values of 1.31 μM) compared to **4a**. However, in the case of 4-(2-dodecanoylamino-ethyl)-phenyl methyl thiophosphoroamidate **4d'** (IC_{50} = 15.10 μM), lower alkylating ability could be overwhelmed by electrostatic interactions of amino group in the catalytic region of STS and cause an increase of its inhibitory potency compared to **4a'**.

Cancer cell viability assay

We sought to determine whether compounds that displayed high activity toward the isolated sulfatase would also inhibit the growth of breast cancer cells. To determine the antiproliferative activity, we selected five compounds (**4a**, **4a'**, **4d**, **4d'**, **5c**) and the following cell lines: MCF7, an estrogen (ER) and progesterone (PR) receptor positive luminal breast cancer, and two ER and PR negative metastatic breast cancer lines: MDA-MB-231 and SkBr3. The results obtained from this screening are presented in Table 2.

Compounds **4d** and **5c** did not significantly inhibit the growth of any of the cells at concentrations up to 100 μM. Analogs **4a**, **4a'** and **4d'** displayed cytotoxicity in the low micromolar range, 4-(2-dodecanoylamino-ethyl)-phenyl dimethyl phosphate **4a** being the most active with a GI_{50} value of 8.80±2.42 μM for MCF7 cells. However, **4a** and **4d'** activity toward all three cell types was comparable. 4-(2-Dodecanoylamino-ethyl)-phenyl dimethyl thiophosphate **4a'** was the only compound to preferentially inhibit (1.7-fold) the growth of ER+ MCF7 cells compared to ER- MDA-MB-231.

In contrast, thiophosphate derivatives **4a'** and **4d'** efficiently inhibited the growth of the aforementioned cancer cells even though they were only moderately active against the enzyme. This result can be attributed to the lower reactivity of the thiophosphate group than its phosphate analog and, in consequence, decreased off-target effects. In contrast, 4-(2-dodecanoylamino-ethyl)-phenyl methyl phosphoroamidate **4d** and 4-(2-tetradecanoylamino-ethyl)-phenyl dihydrogen phosphate **5c** showed poor antiproliferative activity despite their activity against the enzyme, suggesting that the alkylating properties of *N*-alkanoyl tyramine dimethyl phosphates or thiophosphates may be critical for the inhibition of cell growth.

No.	GI_{50} [μM]		
	MCF-7	**MDA-MB-231**	**SkBr3**
	ER+; PR+; HER2+	ER-; PR-; HER2-	ER-; PR-; HER2+
4a	8.80±2.42	6.48±0.24	5.76±0.41
4a'	12.71±0.67	21.77±1.55	11.94±0.55
4d	>100	>100	>100
4d'	15.37±0.50	12.01±1.26	9.36±1.46
5c	>100	>100	>100

ER, estrogen receptor; PR, progesterone receptor; HER2 epithelial growth factor receptor 2

Table 2 Antiproliferative activities of selected compounds

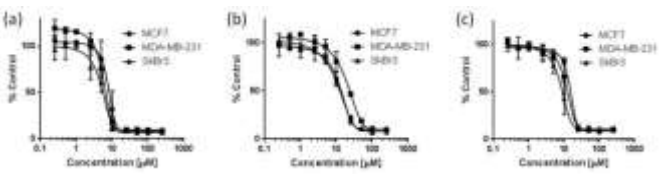


Fig. 4 Concentration response curves of proliferation of (●) MCF-7 (ER+), (■) MDA-MB-231 (ER-) and (▲)SkBr3 (ER-) treated with the following compounds: (a) **4a** (b) **4a'** and (c) **4d'**.

Conclusions

A series of phosphate and thiophosphate *N*-alkanoyl tyramines have been synthesized and biologically evaluated using STS

and MTT proliferation cytotoxicity assays. This study has determined that 4-(2-dodecanoylamino-ethyl)-phenyl dimethyl phosphate **4a** exhibits good affinity for STS and the highest inhibition potency, with IC_{50} values of 0.39 µM (the IC_{50} value of 15.44 µM for the 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate was used as a reference). In the course of investigation, we demonstrated its high ability to inhibit the proliferation of the MCF-7, MDA-MB-231 and SkBr3 cancer cell lines *in vitro*. Although the mechanism of action for compound **4a** is unknown, molecular modeling and STS enzyme assay studies suggest that two effects may be critical for its activity. The first is associated with the alkylating properties of dimethyl phosphates suggesting the possible methylation of FGly75, the second effect is related to the possibilities of occurrence an electrostatic interactions (e.g. hydrogen bond) with catalytic amino acid residues. Furthermore, stabilization of the enzyme-ligand complex by establishing of a weak electrostatic hydrogen bond between the acyl residue of **4a** and hydroxyl group of Thr180 may influence for the increase of its inhibitory potency.

Experimental

Materials and methods

Dodecanoic, tridecanoic and tetradecanoic acids, thionyl chloride, 4-hydroxyphenethylamine, potassium carbonate, phosphoryl chloride, thiophosphoryl chloride, triethylamine and bromotrimethylsilane are commercially available from Aldrich. Tetrahydrofuran and methanol were dried and distilled using standard procedures. Melting points (uncorrected) were determined with a Stuart Scientific SMP30 apparatus. NMR spectra were recorded on a Varian Gemini 500 MHz spectrometer. Chemical shifts are reported in ppm relative to the residual solvent peak ($CDCl_3$ = 7.26 ppm for ^1H, 77.0 ppm for ^{13}C, DMSO-d_6 2.49 ppm for ^1H, 39.5 ppm for ^{13}C) or to an external standard (85% H_3PO_4 = 0 for ^{31}P). Coupling constants are given in Hertz. IR spectra were measured on a Nicolet 8700. CHNS elemental analysis was performed on a Carlo Erba EA-1108. Column chromatography was performed using silica gel 60 (230-400 mesh, Merck). Preparative thin-layer chromatography was performed with Polygram SIL G/UV_{254} silica gel (Macherey-Nagel).

General method for the synthesis of 4-(2-dodecanoylamino-ethyl)-phenyl sulfamate 1 (reference 1)

A mixture of formic acid (1.54 mmol) and *N*,*N*-dimethyl acetamide (0.016 mmol) was added to a stirred solution of chlorosulfonyl isocyanate (1.50 mmol) in dry dichloromethane (0.5 mL) at 40 ºC within a period of 3.5 h. Then, to the resulting mixture a stirred solution of [2-(4-hydroxy-phenyl)-ethyl]-dodecanoic acid amide **2a** (0.319 g, 1.00 mmol) in *N*,*N*-dimethyl acetamide (3.5 mL) was added. The mixture was stirred at ambient temperature for 24 h, and then poured onto water (10 mL). The suspension was stirred at ambient temperature for 2 h, and filtered. The crude product was purified by column chromatography using AcOEt : $CHCl_3$ 1:30 as an eluent to give the product **1**.

4-(2-dodecanoylamino-ethyl)-phenyl sulfamate 1. Yield 91 %, mp 112-115 °C; ν_{max} (KBr)/cm^{-1} 3378, 3278, 1635, 1543, 1504, 1467, 1346, 1151, 879, 722; ^1H NMR δ_H (200 MHz, DMSO) 0.83 (3H, t, *J* 6.8, CH_3), 1.10-1.30 (16H, m, CH_2), 1.40-1.50 (2H, m, CH_2), 2.00 (2H, t, *J* 7.3, CH_2), 2.69 (2H, t, *J* 7.5, CH_2), 3.23 (2H, q, *J* 6.8, CH_2), 7.16 (2H, d, *J* 8.3, Ar-H), 7.25 (2H, d, *J* 8.3, Ar-H), 7.86 (1H, t, *J* 5.1, NH), 7.94 (2H, s, NH_2); ^{13}C NMR δ_C (125 MHz, DMSO) 172.8, 149.2, 138.6, 130.5, 122.7, 36.1, 35.2, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 26.0, 22.8, 14.6; Anal. Calcd for: $C_{20}H_{34}N_2O_4S$: C, 60.27; H, 8.60; N, 7.03; S, 8.05. Found: C, 60.31; H, 8.64; N, 7.11; S, 8.01.

General method for the synthesis of *N*-alkanoyl tyramine derivatives 2a-2c

$SOCl_2$ (12.79 mL) was added to a solution of the corresponding dodecanoic, tridecanoic or tetradecanoic acid (15 mmol) in dry CH_2Cl_2 (15 mL), and the suspension was refluxed for 2.5 h under a nitrogen atmosphere. The resulting solution was evaporated and to the residue was added 4-hydroxyphenethylamine (10 mmol) and anhydrous potassium carbonate (4.15 g, 30 mmol) in dry acetone (20 mL). The reaction mixture was refluxed under a nitrogen atmosphere for 3 h. After cooling to room temperature, the solution was filtered and the solvent was evaporated. The resulting residue was purified by column chromatography using CH_2Cl_2 : AcOEt 9:1 as an eluent to give the desired products.

[2-(4-hydroxy-phenyl)-ethyl]-dodecanoic acid amide 2a. Yield 65 %, mp 88-91 °C; ν_{max} (KBr)/cm^{-1} 3301, 1635, 1547, 1516, 1463, 1242, 832, 721; ^1H NMR δ_H (200 MHz, $CDCl_3$) 0.86 (3H, m, CH_3), 1.15-1.38 (16H, m, CH_2), 1.45-1.65 (2H, m, CH_2), 2.13 (2H, t, *J* 7.1, CH_2), 2.74 (2H, t, *J* 6.6, CH_2), 3.52 (2H, q, *J* 6.2, CH_2), 5.35-6.50 (1H, br s, NH), 6.80-7.08 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 174.4, 155.4, 130.0, 129.9, 115.9, 41.3, 36.9, 34.9, 32.2, 29.9, 29.7, 29.6, 29.6, 29.5, 26.1, 22.9, 14.4.

[2-(4-hydroxy-phenyl)-ethyl]-tridecanoic acid amide 2b. Yield 60 %, mp 89-92 °C; ν_{max} (KBr)/cm^{-1} 3305, 1635, 1548, 1516, 1463, 1240, 834, 725; ^1H NMR δ_H (200 MHz, $CDCl_3$) 0.89 (3H, t, *J* 6.7, CH_3), 1.15-1.40 (18H, m, CH_2), 1.45-1.70 (2H, m, CH_2), 2.13 (2H, t, *J* 7.9, CH_2), 2.75 (2H, t, *J* 7.0, CH_2), 3.49 (2H, q, *J* 6.2, CH_2), 5.30-6.50 (1H, br s, NH), 6.77-7.08 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 174.4, 155.3, 130.1, 130.0, 115.9, 41.3, 36.9, 34.9, 32.2, 29.9, 29.9, 29.7, 29.6, 29.6, 29.5, 29.3, 26.1, 25.0, 23.0, 14.4.

[2-(4-hydroxy-phenyl)-ethyl]-tetradecanoic acid amide 2c. Yield 72 %, mp 95-98 °C; ν_{max} (KBr)/cm^{-1} 3295, 1635, 1546, 1516, 1464, 1246, 825, 728; ^1H NMR δ_H (200 MHz, $CDCl_3$) 0.88 (3H, t, *J* 5.8, CH_3), 1.10-1.45 (20H, m, CH_2), 1.48-1.70 (2H, m, CH_2), 2.13 (2H, t, *J* 7.4, CH_2), 2.74 (2H, t, *J* 7.0, CH_2), 3.49 (2H, q, *J* 6.3, CH_2), 5.30-5.70 (1H, br s, NH), 6.77-7.06 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 174.4, 155.1, 130.3, 130.0, 115.8, 41.3, 36.8, 34.9, 34.0, 32.2, 29.9, 29.7, 29.6, 29.6, 29.5, 29.3, 26.1, 25.0, 22.9, 14.4.

General method for the synthesis of dimethyl phosphate derivatives 4a-4c

To an ice-cooled solution of phosphoryl chloride (92 mg, 0.6 mmol) in dry THF (10 mL) was added dropwise a solution of the corresponding *N*-alkanoyl tyramine derivatives (0.6 mmol) in THF, followed by triethylamine (61 mg, 0.6 mmol). The reaction mixture was stirred under a nitrogen atmosphere for 1 h. The triethylamine hydrochloride precipitate was removed by filtration, and sodium methoxide (1.2 mmol) (freshly prepared by the addition of absolute methanol to 60% NaH dispersed in mineral oil) was added. The reaction mixture was stirred for 1 h, and a precipitate of NaCl formed. The solution was filtered, and the solvent was evaporated. The resulting residue was purified by column chromatography using CH_2Cl_2 : AcOEt 1:1 as an eluent to give the desired products.

4-(2-dodecanoylamino-ethyl)-phenyl dimethyl phosphate 4a. Yield 67 %, mp 44-46 °C; ν_{max} (KBr)/cm^{-1} 3301, 1635, 1549, 1508, 1464, 1222, 1034, 954, 719; ^{1}H NMR δ_H (200 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.6, CH_3), 1.05-1.25 (16H, m, CH_2), 1.45-1.70 (2H, m, CH_2), 2.16 (2H, t, *J* 7.9, CH_2), 2.80 (2H, t, *J* 6.6, CH_2), 3.49 (2H, d, *J* 5.8, CH_2), 3.88 (6H, d, *J* 11.2, CH_3), 5.70-5.90 (1H, br s, NH), 7.10-7.25 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.8, 149.4, 136.1, 130.3, 120.2 (d, $J_{P\text{-}C}$ 4.9), 55.2 (d, $J_{P\text{-}C}$ 6.1), 40,9, 36.9, 35.5, 32.1, 29.6, 29.6, 29.5, 26.1, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) -2.89. Anal. Calcd for: $C_{22}H_{38}NO_5P$: C, 61.81; H, 8.96; N, 3.28. Found: C, 61.93; H, 9.01; N, 3.21.

4-(2-tridecanoylamino-ethyl)-phenyl dimethyl phosphate 4b. Yield 67 %, mp 50-52 °C; ν_{max} (KBr)/cm^{-1} 3305, 1637, 1549, 1506, 1464, 1217, 1028, 945, 719; ^{1}H NMR δ_H (500 MHz, $CDCl_3$) 0.85-0.90 (3H, m, CH_3), 1.20-1.35 (18H, m, CH_2), 1.50-1.70 (2H, m, CH_2), 2.12 (2H, t, *J* 7.8, CH_2), 2.79 (2H, t, *J* 6.9, CH_2), 3.49 (2H, d, *J* 6.4, CH_2), 3.87 (6H, d, *J* 11.2, CH_3), 5.35-5.50 (1H, br s, NH), 7.15-7.22 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.5, 149.4 (d $J_{P\text{-}C}$ 6.6), 136.2, 130.3, 120.2 (d, $J_{P\text{-}C}$ 4.8), 55.1 (d, $J_{P\text{-}C}$ 5.7), 40.7, 37.0, 35.2, 32.1, 29.9, 29.7, 29.6, 29,5, 26.0, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) -2.87. Anal. Calcd for: $C_{23}H_{40}NO_5P$: C, 62.56; H, 9.13; N, 3.17. Found: C, 62.70; H, 9.21; N, 3.23.

4-(2-tetradecanoylamino-ethyl)-phenyl dimethyl phosphate 4c. Yield 48 %, mp 55-57 °C; ν_{max} (KBr)/cm^{-1} 3305, 1637, 1548, 1508, 1464, 1221, 1035, 954, 719; ^{1}H NMR δ_H (200 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.6 CH_3), 1.15-1.40 (20H, m, CH_2), 1.45-1.75 (2H, m, CH_2), 2.13 (2H, t, *J* 7.1, CH_2), 2.79 (2H, t, *J* 6.7, CH_2), 3.49 (2H, q, *J* 6.2, CH_2), 3.87 (6H, d, *J* 11.7, CH_3), 5.30-5.55 (1H, br s, NH), 7.10-7.25 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.2, 149.2, 135.9, 130.0, 119.9 (d, $J_{P\text{-}C}$ 4.8), 58.8, 54.9 (d, $J_{P\text{-}C}$ 6.1), 40.5, 36.8, 35.0, 31.9, 29.6, 29.5, 29.3, 25.7, 22.7, 14.1; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) -2.87. Anal. Calcd for: $C_{24}H_{42}NO_5P$: C, 63.27; H, 9.29; N, 3.07. Found: C, 63.38; H, 9.34; N, 3.02.

General method for the synthesis of dimethyl thiophosphate derivatives 4a'-4c'

To an ice-cooled solution of thiophosphoryl chloride (102 mg, 0.6 mmol) in dry THF (10 mL) was added dropwise a solution of the corresponding *N*-alkanoyl tyramine derivatives (0.6 mmol) in THF, followed by triethylamine (61 mg, 0.6 mmol). The reaction mixture was stirred under a nitrogen atmosphere for 3 h. The triethylamine hydrochloride precipitate was removed by filtration, and sodium methoxide (1.2 mmol) (freshly prepared by the addition of absolute methanol to 60% NaH dispersed in mineral oil) was added. The reaction mixture was stirred for 2 h. The solution was filtered, and the solvent was evaporated. The resulting residue was purified by column chromatography using CH_2Cl_2 : AcOEt 10:1 as an eluent to give the desired products.

4-(2-dodecanoylamino-ethyl)-phenyl dimethyl thiophosphate 4a'. Yield 73 %, mp 41-42 °C; ν_{max} (KBr)/cm^{-1} 3301, 1637, 1548, 1508, 1464, 1217, 1026, 947, 825, 719, 613; ^{1}H NMR δ_H (200 MHz, $CDCl_3$) 0.88 (3H, t, *J* 7.0, CH_3), 1.05-1.40 (16H, m, CH_2), 1.45-1.70 (2H, m, CH_2), 2.16 (2H, t, *J* 7.5, CH_2), 2.81 (2H, t, *J* 6.7, CH_2), 3.52 (2H, d, *J* 5.8, CH_2), 3.86 (6H, d, *J* 13.7, CH_3), 5.50-6.70 (1H, br s, NH), 7.05-7.20 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.7, 149.5, 136.2, 130.2, 121.2 (d, $J_{P\text{-}C}$ 4.5), 55.4 (d, $J_{P\text{-}C}$ 5.7), 40.9, 37.1, 35.3, 32.1, 29.9, 29.7, 29.6, 26.1, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) 67.71. Anal. Calcd for: $C_{22}H_{38}NO_4PS$: C, 59.57; H, 8.63; N, 3.16; S, 7.23. Found: C, 59.50; H, 8.59; N, 3.21; S, 7.19.

4-(2-tridecanoylamino-ethyl)-phenyl dimethyl thiophosphate 4b'. Yield 79 %, mp 48-50 °C; ν_{max} (KBr)/cm^{-1} 3305, 1637, 1548, 1506, 1464, 1217, 1027, 945, 827, 719, 612; ^{1}H NMR δ_H (500 MHz, $CDCl_3$) 0.88 (3H, m, CH_3), 1.20-1.35 (18H, m, CH_2), 1.55-1.65 (2H, m, CH_2), 2.12 (2H, t, *J* 7.3, CH_2), 2.80 (2H, t, *J* 3.3, CH_2), 3.50 (2H, q, *J* 6.9, CH_2), 3.87 (6H, d, *J* 13.0, CH_3), 5.42 (1H, s, NH), 7.10-7.20 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.4, 149.5 (d, $J_{P\text{-}C}$ 7.4), 136.3, 130.1, 121.2 (d, $J_{P\text{-}C}$ 4.4), 55.4 (d, $J_{P\text{-}C}$ 5.7), 40.7, 37.1, 35.3, 32.1, 29.9, 29.9, 29.7, 29.6, 29.5, 26.0, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) 67.71. Anal. Calcd for: $C_{23}H_{40}NO_4PS$: C, 60.37; H, 8.81; N, 3.06; S, 7.01. Found: C, 60.49; H, 8.92; N, 3.12; S, 7.10.

4-(2-tetradecanoylamino-ethyl)-phenyl dimethyl thiophosphate 4c'. Yield 70 %, mp 52-55 °C; ν_{max} (KBr)/cm^{-1} 3305, 1637, 1548, 1506, 1464, 1217, 1028, 945, 827, 719, 611; ^{1}H NMR δ_H (500 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.8 CH_3), 1.20-1.35 (20H, m, CH_2), 1.55-1.65 (2H, m, CH_2), 2.12 (2H, t, *J* 7.3, CH_2), 2.80 (2H, t, *J* 6.8, CH_2), 3.50 (2H, q, *J* 6.4, CH_2), 3.86 (6H, d, *J* 13.6, CH_3), 5.43 (1H, s, NH), 7.10-7.20 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.4, 149.5, 136.3, 130.1, 121.2 (d, $J_{P\text{-}C}$ 4.8), 55.4 (d, $J_{P\text{-}C}$ 5.7), 40.7, 37.1, 35.3, 32.2, 29.9, 29.9, 29.7, 29.6, 29.5, 26.0, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) 67.71. Anal. Calcd for: $C_{24}H_{42}NO_4PS$: C, 61.12; H, 8.98; N, 2.97; S, 6.80. Found: C, 61.21; H, 9.05; N, 2.89; S, 6.85.

General method for the synthesis of methyl phosphoroamidate derivatives 4d-4f

To an ice-cooled solution of phosphoryl chloride (184 mg, 1.2 mmol) in dry THF (20 mL) was added dropwise a solution of the corresponding *N*-alkanoyl tyramine derivatives (1.2 mmol) in THF, followed by triethylamine (122 mg, 1.2 mmol). The reaction mixture was stirred under a nitrogen atmosphere for 1 h. The triethylamine hydrochloride precipitate was removed by filtration, and sodium methoxide (1.2 mmol) (freshly prepared by the addition of absolute methanol to 60% NaH dispersed in mineral oil) was added. The reaction mixture was stirred for 1 h, and then 3 mL of NH_3 solution in methanol was added. After 30 min, the solvent was evaporated, and the crude product was purified by column chromatography using AcOEt: MeOH 20:1 as an eluent.

4-(2-dodecanoylamino-ethyl)-phenyl methyl phosphoroamidate 4d. Yield 54 %, mp 134-136 °C; ν_{max} (KBr)/cm^{-1} 3322, 3239, 3132, 1646, 1537, 1508, 1470, 1224, 1047, 997, 928, 799, 718; ^{1}H NMR δ_H (500 MHz, DMSO) 0.83 (3H, t, *J* 7.3, CH_3), 1.15-1.30 (16H, m, CH_2), 1.43 (2H, t, *J* 6.8, CH_2), 2.00 (2H, t, *J* 7.3, CH_2), 2.64 (2H, t, *J* 6.3, CH_2), 3.20 (2H, q, *J* 6.8, CH_2), 3.61 (3H, d, *J* 11.2, CH_3), 5.00 (2H, d, $J_{P\text{-}N}$ 6.9, NH_2), 7.00-7.20 (4H, m, Ar-H), 7.85 (1H, t, *J* 5.4, NH); ^{13}C NMR δ_C (125 MHz, DMSO) 172.7, 150.1, 136.1, 120.8 (d, $J_{P\text{-}C}$ 4.3), 53.3 (d, $J_{P\text{-}C}$ 5.7), 40.8, 36.1, 35.1, 32.0, 29.7, 29.5, 29.4, 26.0, 22.8, 14.7; ^{31}P NMR δ_P (202 MHz, DMSO) 9.41. Anal. Calcd for: $C_{21}H_{37}N_2O_4P$: C, 61.14; H, 9.04; N, 6.79. Found: C, 61.07; H, 9.16; N, 6.91.

4-(2-tridecanoylamino-ethyl)-phenyl methyl phosphoroamidate 4e. Yield 46 %, mp 130-133 °C; ν_{max} (KBr)/cm^{-1} 3319, 3238, 3130, 1646, 1537, 1508, 1469, 1228, 1043, 997, 937, 801, 719; ^{1}H NMR δ_H (500 MHz, DMSO) 0.84 (3H, t, *J* 6.8, CH_3), 1.15-1.30 (18H, m, CH_2), 1.44 (2H, t, *J* 6.4, CH_2), 2.00 (2H, t, *J* 7.8, CH_2), 2.64 (2H, t, *J* 7.4, CH_2), 3.21 (2H, q, *J* 6.3, CH_2), 3.62 (3H, d, *J* 11.8, CH_3), 5.00 (2H, d, $J_{P\text{-}N}$ 6.9, NH_2), 7.05-7.20 (4H, m, Ar-H), 7.84 (1H, t, *J* 6.9, NH); ^{13}C NMR δ_C (125 MHz, DMSO) 172.7, 150.1 (d, $J_{P\text{-}C}$ 6.2), 136.1, 130.3, 120.8 (d, $J_{P\text{-}C}$ 4.8), 53.7 (d, $J_{P\text{-}C}$ 5.7), 40.8, 36.1, 35.2, 32.0, 29.7, 29.6, 29.5, 29.4, 29.4, 26.0, 22.8, 14.7; ^{31}P NMR δ_P (202 MHz, DMSO) 9.40. Anal. Calcd for: $C_{22}H_{39}N_2O_4P$: C, 61.95; H, 9.22; N, 6.57. Found: C, 62.09; H, 9.31; N, 6.69.

4-(2-tetradecanoylamino-ethyl)-phenyl methyl phosphoroamidate 4f. Yield 51 %, mp 136-139 °C; ν_{max} (KBr)/cm^{-1} 3325, 3239, 3127, 1646, 1537, 1508, 1471, 1232, 1045, 997, 928, 800, 718; ^{1}H NMR δ_H (500 MHz, DMSO) 0.84 (3H, t, *J* 7.3, CH_3), 1.15-1.30 (20H, m, CH_2), 1.44 (2H, t, *J* 5.9, CH_2), 2.00 (2H, t, *J* 7.4, CH_2), 2.64 (2H, t, *J* 7.3, CH_2), 3.21 (2H, q, *J* 7.3, CH_2), 3.62 (3H, d, *J* 11.2, CH_3), 5.00 (2H, d, $J_{P\text{-}N}$ 6.8, NH_2), 7.05-7.20 (4H, m, Ar-H), 7.84 (1H, t, *J* 5.4, NH); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 172.7, 150.1, 136.1, 130.3, 120.8 (d, $J_{P\text{-}C}$ 4.8), 53.3 (d, $J_{P\text{-}C}$ 5.7), 40.8, 36.1, 35.2, 32.0, 29.8, 29.7, 29.6, 29.5, 29.4, 29.4, 25.6, 22.8, 14.7; ^{31}P NMR δ_P (202 MHz, DMSO) 9.40. Anal. Calcd for: $C_{23}H_{41}N_2O_4P$: C, 62.70; H, 9.38; N, 6.36. Found: C, 62.83; H, 9.44; N, 6.49.

General method for the synthesis of methyl thiophosphoroamidate derivatives 4d'-4f'

To an ice-cooled solution of thiophosphoryl chloride (204 mg, 1.2 mmol) in dry THF (20 mL) was added dropwise a solution of the corresponding *N*-alkanoyl tyramine derivatives (1.2 mmol) in THF, followed by triethylamine (122 mg, 1.2 mmol). The reaction mixture was stirred under a nitrogen atmosphere for 3 h. The triethylamine hydrochloride precipitate was removed by filtration, and sodium methoxide (1.2 mmol) (freshly prepared by the addition of absolute methanol to 60% NaH dispersed in mineral oil) was added. The reaction mixture was stirred for 2 h, and then 3 mL of NH_3 solution in methanol was added. After 30 min, the solvent was evaporated, and the crude product was purified by column chromatography using CH_2Cl_2 : AcOEt 5:1 as an eluent.

4-(2-dodecanoylamino-ethyl)-phenyl methyl thiophosphoroamidate 4d'. Yield 83 %, mp 88-90 °C; ν_{max} (KBr)/cm^{-1} 3357, 3282, 3100, 1620, 1552, 1506, 1469, 1209, 1037, 975, 907, 842, 812, 742, 696, 619; ^{1}H NMR δ_H (200 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.6, CH_3), 1.10-1.45 (16H, m, CH_2), 1.50-1.70 (2H, m, CH_2), 2.16 (2H, t, *J* 7.9, CH_2), 2.20-3.20 (2H, br s, NH_2), 2.82 (2H, t, *J* 7.1, CH_2), 3.52 (2H, d, *J* 5.6, CH_2), 3.40-3.60 (3H, m, CH_3), 5.80-6.00 (1H, br s, NH), 7.10-7.25 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.9, 149.7, 135.9, 130.0, 121.5 (d, $J_{P\text{-}C}$ 4.8), 54.3, 40.9, 36.9, 35.2, 32.1, 29.8, 29.7, 29.6, 29.5, 26.1, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) 71.28. Anal. Calcd for: $C_{21}H_{37}N_2O_3PS$: C, 58.85; H, 8.70; N, 6.54; S, 7.48. Found: C, 58.92; H, 8.76; N, 6.60; S, 7.52.

4-(2-tridecanoylamino-ethyl)-phenyl methyl thiophosphoroamidate 4e'. Yield 82 %, mp 91-95 °C; ν_{max} (KBr)/cm^{-1} 3350, 3299, 3100, 1622, 1548, 1506, 1466, 1209, 1043, 974, 909, 842, 812, 740, 694, 617; ^{1}H NMR δ_H (500 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.9, CH_3), 1.20-1.35 (18H, m, CH_2), 1.59 (2H, t, *J* 7.4 CH_2), 2.13 (2H, t, *J* 7.8, CH_2), 2.80 (2H, t, *J* 6.9, CH_2), 3.38 (2H, s, NH_2), 3.50 (2H, q, *J* 5.3, CH_2), 3.83 (3H, d, *J* 14.2, CH_3), 5.44 (1H, s, NH), 7.15-7.20 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.5, 149.7, 136.0, 130.0, 121,5 (d, $J_{P\text{-}C}$ 4.9), 54.3 (d $J_{P\text{-}C}$ 5.2), 40.7, 37.1, 35.3, 32.2, 29.9, 29.9, 29.7, 29.6, 29.5, 26.0, 22.3, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) 71.24. Anal. Calcd for: $C_{22}H_{39}N_2O_3PS$: C, 59.70; H, 8.88; N, 6.33; S, 7.24. Found: C, 59.64; H, 8.93; N, 6.40; S, 7.31.

4-(2-tetradecanoylamino-ethyl)-phenyl methyl thiophosphoroamidate 4f'. Yield 76 %, mp 93-95 °C; ν_{max} (KBr)/cm^{-1} 3347, 3302, 3100, 1622, 1549, 1506, 1468, 1209, 1043, 974, 908, 842, 812, 740, 694, 617; ^{1}H NMR δ_H (500 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.8, CH_3), 1.20-1.35 (20H, m, CH_2), 1.55-1.70 (2H, m, CH_2), 2.13 (2H, t, *J* 7.3, CH_2), 2.80 (2H, t, *J* 6.8, CH_2), 3.37 (2H, s, NH_2), 3.50 (2H, q, *J* 6.4, CH_2), 3.83 (3H, d, *J* 14.2, CH_3), 5.42 (1H, s, NH), 7.15-7.20 (4H, m, Ar-H); ^{13}C NMR δ_C (125 MHz, $CDCl_3$) 173.5, 149.7 (d, $J_{P\text{-}C}$ 7.9), 136.0, 130.0, 121.5 (d, $J_{P\text{-}C}$ 4.9), 54.3 (d, $J_{P\text{-}C}$ 5.3), 40.7, 37.1, 35.3, 32.2, 29.9, 29.9, 29.7, 29.6, 29.5, 26.0, 22.9, 14.4; ^{31}P NMR δ_P (202 MHz, $CDCl_3$) 71.25. Anal. Calcd for: $C_{23}H_{41}N_2O_3PS$: C, 60.50; H, 9.05; N, 6.13; S, 7.02. Found: C, 60.59; H, 9.12; N, 6.21; S, 7.09.

General method for the synthesis of phosphoric acid derivatives 5a-5c

To an ice-cooled solution of derivative 4a, 4b or 4c (1 mmol) in dry DCM (10 mL), TMSBr (4 mmol) was added dropwise. The reaction mixture was stirred under a nitrogen atmosphere for 1.5 h. After concentration under vacuum, 5 mL of methanol was added. All solvents were then evaporated, and the crude product was washed with ethyl acetate to give the desired product.

4-(2-dodecanoylamino-ethyl)-phenyl dihydrogen phosphate 5a. Yield 78 %, mp 102 °C (with decomposition); ν_{max} (KBr)/cm^{-1}; ^{1}H NMR δ_H (200 MHz, DMSO) 0.83 (3H, m, CH_3), 1.10-1.35 (16H, m, CH_2), 1.40-1.60 (2H, m, CH_2), 2.02 (2H, t, *J* 7.1, CH_2), 2.65 (2H, t, *J* 6.9, CH_2), 3.22 (2H, d, *J* 5.8, CH_2), 2.80-4.80 (2H, br s, OH), 7.00-7.22 (4H, m, Ar-H), 7.85 (1H, m, NH); ^{13}C NMR δ_C (125 MHz, DMSO) 172.8, 150.5 (d, J_{P-C} 6.6), 135.8, 130.5, 130.2, 120.6 (d, J_{P-C} 4.4), 40.9, 36.1, 35.1, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 26.0, 22.8, 14.7; ^{31}P NMR δ_P (202 MHz, DMSO) -5.06. Anal. Calcd for: $C_{20}H_{34}NO_5P$: C, 60.13; H, 8.58; N, 3.51. Found: C, 60.24; H, 8.53; N, 3.69.

4-(2-tridecanoylamino-ethyl)-phenyl dihydrogen phosphate 5b. Yield 76 %, mp 126 °C (with decomposition); ν_{max} (KBr)/cm^{-1} ; ^{1}H NMR δ_H (500 MHz, DMSO) 0.83 (3H, t, *J* 6.9, CH_3), 1.15-1.30 (18H, m, CH_2), 1.40-1.50 (2H, m, CH_2), 2.00 (2H, t, *J* 7.4, CH_2), 2.64 (2H, t, *J* 7.3, CH_2), 3.20 (2H, q, *J* 6.4, CH_2), 4.40-6.30 (2H, br s, OH), 7.00-7.20 (4H, m, Ar-H), 7.85 (1H, t, *J* 5.4, NH); ^{13}C NMR δ_C (125 MHz, DMSO) 172.8, 150.5 (d, J_{P-C} 6.6), 135.8, 130.2, 120.6 (d, J_{P-C} 4.8), 40.9, 36.1, 35.1, 32.0, 29.7, 29.6, 29.5, 29.4, 29.3, 26.0, 22.8, 14.7; ^{31}P NMR δ_P (202 MHz, DMSO) -5.06. Anal. Calcd for: $C_{21}H_{36}NO_5P$: C, 61.00; H, 8.78; N, 3.39. Found: C, 61.13; H, 8.87; N, 3.46.

4-(2-tetradecanoylamino-ethyl)-phenyl dihydrogen phosphate 5c. Yield 64 %, mp 124 °C (with decomposition); ν_{max} (KBr)/cm^{-1}; ^{1}H NMR δ_H (500 MHz, DMSO) 0.84 (3H, t, *J* 6.9, CH_3), 1.15-1.30 (20H, m, CH_2), 1.44 (2H, t, *J* 6.8, CH_2), 2.00 (2H, t, *J* 7.4, CH_2), 2.64 (2H, t, *J* 7.8, CH_2), 3.20 (2H, q, *J* 6.9, CH_2), 5.00-6.00) (2H, br s, OH), 7.00-7.20 (4H, m, Ar-H), 7.85 (1H, m, NH); ^{13}C NMR δ_C (125 MHz, DMSO) 172.8, 150.5, 135.8, 130.2, 120.6 (d, J_{P-C} 4.4), 40.9, 36.0, 35.1, 32.0, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 26.0, 22.8, 14.6; ^{31}P NMR δ_P (202 MHz, DMSO) -5.07. Anal. Calcd for: $C_{22}H_{38}NO_5P$: C, 61.81; H, 8.96; N, 3.28. Found: C, 61.96; H, 9.04; N, 3.39.

Molecular modeling

All the molecular structures of the ligands were built with the program Portable HyperChem 8.0.7 Release (Hypercube Incorporation) and were energy minimized using the MM+ force field and Polak – Ribiere conjugate gradient algorithm. The iteration procedure was continued until energy gradients became less than 0.05 kcal/mol/Å. The X-ray structures of human steroid sulfatase were taken from the Protein Databank (Protein Data Bank accession code 1P49). Water of crystallization were removed from the structure. The catalytic amino acid FGly75 (formylglycine) was converted to the gem – diol form using the Protein Preparation Wizard module, delivered with Maestro (Schrödinger, LLC, New York, NY). Hydrogen atoms were added to the structure, and the whole molecule was subjected to minimization using the OPLS-AA force field. The docking analysis was carried out with the AutoDock Vina 1.1.2 (The Molecular Graphics Laboratory, Scripps Research Institute) software. For all the docking studies, a grid box size of 30 Å x 30 Å x 30 Å, centered at coordinates 72.135 (x), -1.720 (y), and 28.464 (z) of the minimized PDB structure of steroid sulfatase (Cβ atom of amino acid 75), was used. The predicted free energy of binding was calculated from the intermolecular part of the lowest-scoring conformation. Graphic visualizations of the 3D model were generated using VMD 1.9 (University of Illinois at Urbana – Champaign).

Biological assays

Enzyme purification

STS was extracted from human placenta and purified to homogeneity following a previously described multi-step chromatography protocol (F. G. Hernandez-Guzman, T. Higashiyama, Y. Osawa and D. Ghosh, J. Steroid. Biochem. Mol. Biol., 2001, 78, 441.)

***In vitro* activity assay**

The reaction mixture, at a final volume of 100 µL, contained 20 µM Tris-HCl pH 7.4, 3 mM p-nitrophenyl sulphate (NPS), varied concentrations of an inhibitor (0.1-500 µM) and 5 U of purified enzyme (1 U is the amount of enzyme that hydrolyzes 100 µM of NPS in 1 h at 37°C). The reaction was performed at 37°C for 15 min and halted by the addition of 100 µL of 1 M NaOH. The absorbance of the released p-nitrophenol was measured at 405 nm using a Microplate Reader Biotek ELx800 (SERVA). IC_{50} values were calculated using GraphPad Prism software. All measurements were performed in triplicate.

Cell culture and viability assay

MCF7 (ATCC® HTB-22™), MDA-MB-231 (ATCC® HTB-26™) and SkBr3 (ATCC® HTB-30™) cells were cultured in phenol red-free DMEM high glucose medium supplemented with 10% FBS and antibiotics: penicillin (100 units/ml) and streptomycin (100 µg/ml). All cells were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. To determine cytotoxicity, exponentially growing cells were exposed to the indicated concentrations of the studied compounds for 7 days, and the viability was determined using the MTT assay. Dose-response curves were plotted using Prism 6 (GraphPad Software). All experiments were performed in triplicate.

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