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ARTICLE

Synthesis and steroid sulfatase inhibitory activities of *N*-phosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates†

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In the present work, we report convenient methods for the synthesis and biological evaluation of *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate as potential steroid sulfatase (STS) inhibitors. Their binding modes have been modeled using docking techniques. The inhibitory effects of the synthesized compounds were tested on STS isolated from human placenta. All of the newly synthesised coumarin derivatives were powerful inhibitors of STS with IC_{50} values ranging between 0.19 and 0.78 μM. In particular, we found that the 3-[4-(diphenoxy-phosphorylamino)-phenyl]-coumarin-7-*O*-sulfamate **10e** and 3-[4-(dibenzyloxy-phosphorylamino)-phenyl]-coumarin-7-*O*-sulfamate **10f** produced the largest inhibitory effects, with IC_{50} values of 0.19 and 0.24 μM, respectively (IC_{50} values of 1.38 μM for the coumarin-7-*O*-sulfamate **2** and 1.03 μM for the coumate **3** used as references). The structure-activity relationships of the synthesized coumarin derivatives toward the STS enzyme have been discussed.

Introduction

Steroid sulfatase (STS) is a target enzyme of growing therapeutic importance. STS is responsible for the hydrolysis of steroid sulfates to their active forms (e.g., estrone sulfate to estrone), therefore, inhibition of this enzyme decreases the biosynthesis of active hormones- responsible for breast, endometrial or prostate cancer.[1] STS is widely distributed throughout the body, and its action is involved in physiological and pathological conditions.[2] Approaches to development of effective and potent STS inhibitors include three different categories of compounds: alternative substrates (including competitive reversible inhibitors), reversible inhibitors, and irreversible inhibitors.[3] Most of the STS inhibitors discovered to date act in an irreversible way. EMATE **1**, one of the first irreversible inhibitors, exhibited a very potent activity in MCF-7 cells with IC_{50} value of 65 pM.[4] Despite the exceptional potency of the EMATE **1**, clinical trials for this compound have been discontinued due to its estrogenic properties.[5] The attempts to synthesize nonsteroidal agents (devoid of undesirable adverse endocrine effects *in vivo*) have promoted the generation of the coumarin sulfamates. These compounds are also able to mimic the AB rings of the natural substrate and modulate several cancer specific enzymes such as aromatase or steroid sulfatase.[6] The first potent inhibitors based on the coumarin scaffold were coumarin-7-*O*-sulfamate **2** or 4-methylcoumarin-7-*O*-sulfamate (coumate) **3**, which exhibited good activity when evaluated against placental microsomes.[7] Further modifications of their structures led to a wide range of tricyclic coumarin derivatives, which showed more potent inhibitory activities than coumate **3**. For example, 667-coumate **4** (the first of steroid sulfatase inhibitors entered into clinical trials for patients with hormone-dependent breast cancer) and 6610- coumate **5** have demonstrated potent activity toward STS with IC_{50} values of 8 nM and 1 nM, respectively.[8]

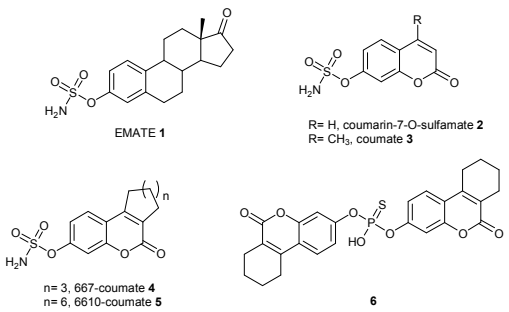

Fig.1 Chemical structures of the STS inhibitors **1-6**.

Encouraged by our previous research,[9] we decided to design and synthesize a series of *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate as compounds with potential STS inhibitory activity. We found that introduction of different phosphate or thiophosphate moieties

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into the structures of coumarin scaffolds can significantly increase the inhibitory properties of tested compounds (analog **6**, Fig.1.). Furthermore, as is widely recognized, phosphate or thiophosphate groups may undergo a nucleophilic substitution reactions on the phosphorus atom or create many electrostatic interactions (e.g., hydrogen bonds) with a variety of amino acid residues found within the active site of the STS. Generally, the predicted hydrogen bonds or the ability to create an electrostatic interactions could favor the binding and may have a significant impact on the enzyme-ligand complex stability.

Results and discussion

Chemistry

The synthesis of all newly designed STS inhibitors based on *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin scaffolds were achieved using the pathway shown in Scheme 1. In the first step, we synthesized 7-hydroxy-3-(4-nitrophenyl)-coumarin **7** starting from 4-nitrophenylacetyl chloride obtained *in situ* by the treatment of 4-nitrophenylacetic acid with thionyl chloride in dry dichloromethane. For the synthesis of 7-hydroxy-3-(4-nitrophenyl)-coumarin **7**, raw 4-nitrophenylacetyl chloride was refluxed with 2,4-dihydroxybenzaldehyde in the presence of potassium carbonate. Next, reduction of 7-hydroxy-3-(4-nitrophenyl)-coumarin **7** to 7-hydroxy-3-(4-aminophenyl)-coumarin **8** using sodium hydrosulfite ($Na_2S_2O_4$) was performed. The progress of reaction was monitored by TLC analysis, and after full conversion of starting material, the product **8** was isolated from the reaction mixture with satisfactory yield (58%). In the next step, 7-hydroxy-3-(4-aminophenyl)-coumarin **8** was *N*-phosphorylated with corresponding chlorophosphates in dry pyridine. Upon work-up and fractionation of the crude products by flash column chromatography, the desired coumarin derivatives **9a-h** were obtained in a good yields (60 - 78%). Finally, OH groups of the compounds **9a-h** were sulfamoylated. In these cases, the solutions of stable *N*-phosphorylated derivatives of 7-hydroxy-3-(4-aminophenyl)-coumarin **9a-h** in *N*,*N*-dimethylacetamide (DMA) were treated with H_2NSO_2Cl (previously generated by the reaction of chlorosulfonyl isocyanate and formic acid in the presence of a catalytic amount of *N*,*N*-dimethylacetamide). The yields of these reactions were high and reached 92%. After standard isolation procedure we obtained desired compounds **10a-h**.

Molecular modelling

To examine the possible interactions of *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate with amino acid residues within the active site of STS, these molecules were docked into the crystal structure of the human steroid sulfatase (STS). The X-ray structure of STS was retrieved from the Protein Databank (Protein Data Bank accession code 1P49) and prepared for docking using required procedure (see experimental section). The docking of the optimized inhibitors into the prepared structure of the human steroid sulfatase was performed using the Autodock Vina 1.1.2 software (The Molecular Graphic Laboratory, The Scripps Research Institute, La Jolla, CA, USA). Figure 2 shows a putative enzyme-ligand complex before the presumed inactivation of the STS and the superimposed best conformations of the three *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates **10d** (yellow), **10e** (CPK colored) and **10f** (red).

All candidates expressed satisfactory predicted free docking energies (in the range of -6.2 to -8.3 kcal/mol) and exhibited significantly lower values of the AutoDock Vina score compared to a reference compounds (for example, the value of predicted free docking energy for coumate was -4.1 kcal/mol). The best docking result was obtained for the 3-[4-(dibenzyloxy-phosphorylamino)-phenyl]-coumarin-7-*O*-sulfamate **10f**, which led to value within the lowest energy of -8.3 kcal/mol. Furthermore, we found that introduction of *N*-phosphoryl moieties in *para* positions of the coumarin's phenyl rings was the most preferred, leading to the lowest free docking energies of the enzyme-inhibitor complexes. Analysis of the docking studies for the newly designed STS inhibitor candidates showed that these compounds could adopt substrate-like poses in active site of STS in a similar manner to the mode of the reported STS inhibitor (coumate). We found that sulfamate functional groups are directed to catalytic amino acid FGly75 coordinated to Ca2+, and they are surrounded by the proposed catalytic residues of Asp35, Asp36, Arg79, Lys134, His136 and Lys368. Furthermore, the coumarin scaffolds are well accommodated to the cavity delimited by lipophilic amino acids in the enzyme pocket (Arg98, Leu103, Phe104, Leu167, Val177, Phe178, Phe182, Leu185, Phe230, Phe233, Phe237, Thr484, Val486, Phe488, Trp550 and Phe553). Molecular modelling studies suggest that increasing the hydrophobic properties of coumarin frameworks (by introducing more hydrophobic esters of phosphorus acid) could favor binding by the establishment of hydrophobic interactions with lipophilic amino acids in the enzyme pocket. Furthermore, as shown in Figure 2, and exemplified by compounds **10d**, **10e** and **10f**, the phosphoryl groups of these compounds are within hydrogen bonding distance from the backbone NH group of Arg98. This additional interaction may be a contributing factor that further assists the binding of these molecules to the enzyme active site.

STS enzyme assay

The ability of the compounds synthesized (**10a-h**) to inhibit steroid sulfatase activity was tested using an *in vitro* STS assay, according to the methods reported previously.[10,11] The screening tests were performed using STS enzyme extracted from human placenta and purified by 3-step chromatography. After the purification, the fractions were used directly as an enzyme source. Table 1 shows a summary of the results. The enzyme assay results showed that the highest efficiency was exhibited by compounds containing a hydrophobic diphenoxy-phosphorylamino and dibenzyloxy-phosphorylamino groups in the structure of the coumarin scaffold (compounds **10e** and **10f**) which is in agreement with the data of molecular modelling studies.

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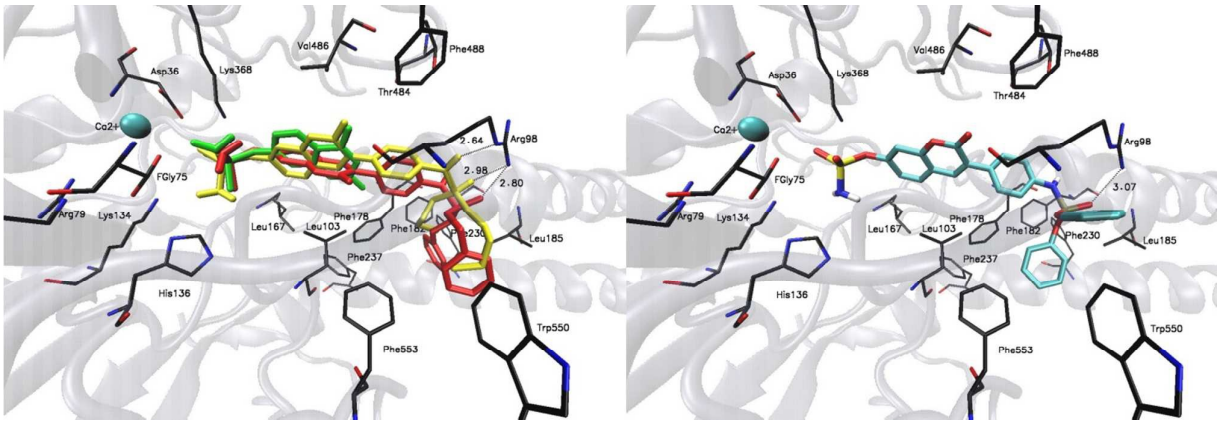
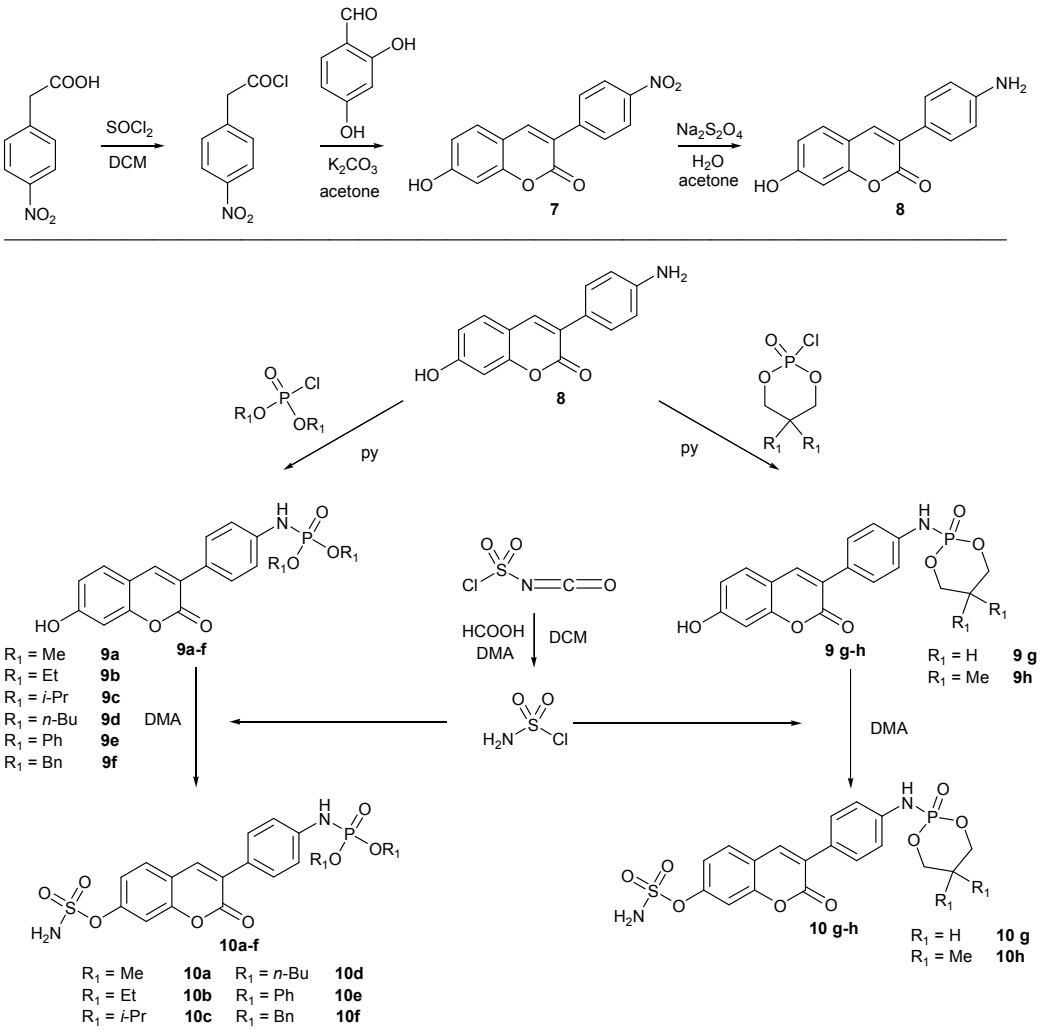


Fig. 2 Docked binding modes and distances to Arg98 for compounds **10d** (yellow), **10e** (CPK colored), **10f** (red) and coumate (green).



Scheme 1 Synthesis of *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate **10a-h**.

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These results may suggest that the frameworks based on 3-[4-(diphenoxy-phosphorylamino)-phenyl]-7-hydroxy-coumarin and 3-[4-(dibenzyloxy-phosphorylamino)-phenyl]-7-hydroxy-coumarin are more suitable for anchoring the enzyme active site and provided the best potency. In the course of investigations, the highest inhibition was observed for 3-[4-(diphenoxy-phosphorylamino)-phenyl]-coumarin-7-*O*-sulfamate **10e** with IC_{50} value of 0.19 μM (IC_{50} values of 1.38 and 1.03 μM for the coumarin-7-*O*-sulfamate and coumate used as references). Furthermore, we found a strong correlation between the IC_{50} and calculated LogP values of tested STS inhibitors, which suggests that increase the hydrophobic properties of *N*-phosphoryl moieties favor binding by the establishment of stronger hydrophobic interactions with lipophilic amino acids lining the cavity of STS. However, our preliminary molecular docking studies suggest that P=O·····H interactions between the phosphoryl groups and hydrogen bond donor residue Arg98 might also contribute to the high potency observed for tested compounds.

Table 1 Activities and calculated LogP parameters of the synthesized compounds **10a-h** and reference inhibitors (coumate and coumarin-7-*O*-sulfamate) in the STS enzyme assays

No.	IC_{50} [μM]	LogP [-]
10a	0.78 ± 0.07	2.01
10b	0.51 ± 0.02	2.70
10c	0.47 ± 0.03	3.52
10d	0.31 ± 0.03	4.43
10e	0.19 ± 0.02	5.37
10f	0.24 ± 0.05	5.56
10 g	0.70 ± 0.05	1.89
10h	0.45 ± 0.03	2.99
coumate	1.03 ± 0.23	0.94
coumarin-7-*O*-sulfamate	1.38 ± 0.13	0.76

Experimental

Computational studies

Molecular modelling. All the molecular structures of the ligands were built with the program Portable HyperChem 8.0.7 Release (Hypercube, Inc., Gainesville, FL, USA) 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 structure of human steroid sulfatase used for molecular modelling study were taken from the Protein Databank (Protein Data Bank accession code 1P49). After standard preparation procedures (including removal of water molecules, conversion of catalytic amino acid FGly75 to the gem – diol form, addition hydrogen atoms and optimization of prepared structure of human STS) docking analysis was carried out. A docking studies was carried out using Autodock Vina 1.1.2 software (The Molecular Graphic Laboratory, The Scripps Research Institute, La Jolla, CA, USA).[12] For all the docking studies, a grid box size of 30 Å x 30 Å x 30 Å, centered on C_β atom of FGly75, was used. Graphic visualizations of the 3D model were generated using VMD 1.9 (University of Illinois at Urbana – Champaign, Urbana, IL, USA).

LogP calculations. LogP parameters were calculated using ChemDraw Ultra 7.0 (CambridgeSoft Corporation, Cambridge, MA, USA).

Biological assays

Enzyme purification. STS was extracted from human placenta and purified to homogeneity following a multi-step chromatography protocol as previously described.[13]

***In vitro* activity assay.** The reaction mixture, at a final volume of 100 mL, contained 20 mM Tris–HCl pH 7.4, 3 mM *p*-nitrophenyl sulfate (NPS), varied concentrations of an inhibitor (0.01–100 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 mL of 1 M NaOH. The absorbance of the released *p*-nitrophenol was measured at 405 nm using a Microplate Reader Biotek ELx800 (BioTek Instruments, Inc. Winooski, VT, USA). IC_{50} values were calculated using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). All measurements were performed in triplicate.

Conclusions

A series of *N*-phosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate have been synthesized and biologically evaluated using *in vitro* STS assay. This study has determined that 3-[4-(diphenoxy-phosphorylamino)-phenyl]-coumarin-7-*O*-sulfamate **10e** exhibits good affinity for STS and the highest inhibition potency, with IC_{50} value of 0.19 μM (IC_{50} values of 1.38 and 1.03 μM for the coumarin-7-*O*-sulfamate and coumate used as references). In the course of our research we found a strong correlation between the IC_{50} and calculated LogP values of tested STS inhibitors, which suggest the substantial participation of hydrophobic interactions for the enzyme-inhibitor complex stability. Furthermore, occurrence of hydrogen bonding between the phosphoryl residue of tested compounds and NH group of Arg98 may influence for the increase of their inhibitory potency. Further structural modifications for these compounds

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will be conducted in order to increase the activities of the new STS inhibitors.

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