Novel steroid sulfatase inhibitors based on *N*-thiophosphorylated 3-(4-aminophenyl)-coumarin-7-O-sulfamates

Mateusz Daśko,*[1] Sebastian Demkowicz,*[2] Karol Biernacki,[2] Amira Harrous,[2] Janusz Rachon,[2] Witold Kozak,[3] Aleksandra Martyna,[4] Maciej Masłyk,[4] Konrad Kubiński,[4] Anna Boguszewska-Czubara[5]

[1] *Department of Inorganic Chemistry, Faculty of Chemistry, Gdansk University of Technology, Narutowicza 11/12, 80-233 Gdansk, Poland, E-mail: mateusz.dasko@pg.edu.pl*

[2] *Department of Organic Chemistry, Faculty of Chemistry, Gdansk University of Technology, Narutowicza 11/12, 80-233 Gdansk, Poland, E-mail: sebdemko@pg.gda.pl.*

[3] *Department of Physical Chemistry, Faculty of Chemistry, University of Gdansk, Wita Stwosza 63, 80-308 Gdansk, Poland.*

[4] *Department of Molecular Biology, Faculty of Biotechnology and Environment Sciences, The John Paul II Catholic University of Lublin, Konstantynów 1i, 20-708 Lublin, Poland.*

[5] *Department of Medical Chemistry, Medical University of Lublin, Chodźki 4A, 20-093, Lublin, Poland*

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ABSTRACT

In the present work, we described convenient methods for the synthesis of *N*-thiophosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates as steroid sulfatase (STS) inhibitors. To design the structures of the potential STS inhibitors, molecular modeling techniques were used. A computational docking method was used to determine the binding modes of the synthesized inhibitors as well as to identify potential interactions between specified functional groups on the inhibitors and the amino acid residues present in the active site of the enzyme. The inhibitory activities of the synthesized compounds were tested in an enzymatic assay with STS isolated from a human placenta. Within the set of newly synthesized compounds, **9e** demonstrated the highest inhibitory activity in the enzymatic assay with an IC_{50} value of 0.201 μM (the IC_{50} value of **667-COUMATE** in the same test was 0.062 μM). Furthermore, we tried to verify if the obtained STS inhibitors are able to pass through the cellular membrane effectively in cell line experiments. In the course of our study, we determined the STS activity in the MCF-7 cell line after incubation in the presence of the inhibitors (at 100 nM concentration). For this evaluation, we included newly synthesized compounds **9a-g** and their *N*-phosphorylated analogs **6a-h**, whose synthesis has been previously described. We found that the lowest STS activities were measured in the presence of *N*-phosphorylated derivatives **6e** (0.1% of STS activity) and **6f** (0.2% of STS activity). The measured STS activity in the presence of **667-COUMATE** (used as a reference) was 0.1%. Moreover, at concentrations up to 1 μM, the most active compounds (**6e**, **6f**, **9b** and **9e**) did not exert any toxic effects on zebrafish embryos.

1. INTRODUCTION

The STS enzyme plays a crucial role in the hydrolysis of biologically inactive steroid sulfates of into their unsulfated derivatives with biological activity. The activity of STS is responsible for the production of steroidal hormones, which induce the proliferation of cancer cells in a number of hormone-dependent cancers [Shah et al., 2016]. Inhibition of the STS enzyme may be important in the development of a new treatment strategy for hormone-dependent cancers, e.g., breast cancer, which is the most common cancer and the main cause of cancer mortality in women worldwide [Ferlay et al., 2010]. One of the strategies for developing new STS inhibitors is the synthesis of sulfamated analogs based on steroidal cores. For example, the steroidal derivative EMATE **1** (**Fig. 1**) demonstrated strong inhibitory activity with an IC_{50} value of 65 pM in the MCF-7 cell line [Purohit et al., 1996]. The unique activity of this compound has spawned hundreds of such sulfamate-based STS inhibitors based around the aryl sulfamate pharmacophore [Potter, 2018; Thomas, M. P et al., 2015]. To avoid adverse side effects (estrogenic properties), several research groups have obtained new STS inhibitors based on nonsteroidal analogs, e.g., coumarins. One of the first potent inhibitors based on the coumarin core was **COUMATE 2**, which exhibited good activity with an IC_{50} value of 380 nM (when evaluated against placental microsomes) [Woo et al., 1998]. Further modification of its structure led to a wide range of bicyclic or tricyclic coumarin derivatives that mimic the AB or ABC rings of the natural substrate [Ganeshapillai et al., 2018]. For example, **667-COUMATE 3** demonstrated potent activity toward STS with an IC_{50} value of 8 nM [Malini et al., 2000].

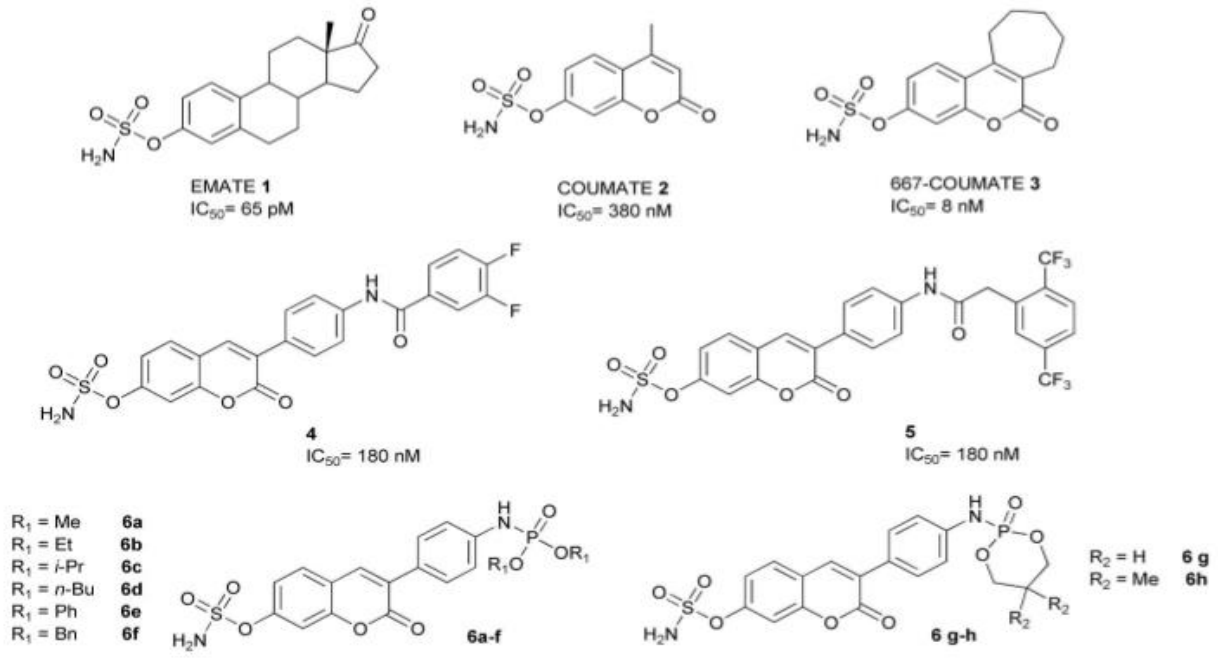


FIGURE 1 Chemical structures of STS inhibitors

Recently, extensive research on the preparation of many coumarin derivatives as STS inhibitors has been carried out. Inhibitory activity against STS was determined in the case of phosphate and thiophosphate derivatives of tricyclic coumarin [Kozak et al., 2014; Kozak et al., 2015], fluorinated derivatives of 3-phenylcoumarin-7-*O*-sulfamate [Demkowicz et al., 2016] and fluorinated *N*-benzoyl and *N*-phenylacetoyl derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate [Daśko et al., 2017]. For example, compounds **4** and **5** demonstrated inhibitory activity with an IC_{50} value of 180 nM (for both compounds) in an assay with isolated STS.

Our previous research indicated that *N*-phosphorylated coumarin derivatives **6a-h** are also potent STS inhibitors [Daśko et al., 2016]. The most active derivatives, **6e** and **6f**, inhibited the STS enzyme with IC_{50} values of 190 nM and 240 nM, respectively. In the course of our research, we found a strong correlation between measured IC_{50} values and calculated log*P* parameters of tested STS inhibitors, which suggests that an increase in the hydrophobic properties of *N*-phosphoryl moieties causes an increase in the inhibitory activity. The higher inhibitory activities of the more hydrophobic derivatives may be explained by the establishment of stronger hydrophobic interactions with the lipophilic amino acid residues lining the cavity of STS. Encouraged by our previous research, we decided to design and synthesize a series of *N*-thiophosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates as compounds with potential STS inhibitory activity. We expect that the enhancement in the hydrophobic properties through replacement of the *N*-phosphoryl group with an *N*-thiophosphoryl moiety could lead to stronger STS inhibitors.

2. MATERIALS AND METHODS

Thionyl chloride, 4-nitrophenylacetic acid, potassium carbonate, 2,4-dihydroxybenzaldehyde, sodium hydrosulfite, chlorosulfonyl isocyanate, *N*,*N*-dimethylacetamide, formic acid, dimethylchlorothiophosphate and diethylchlorothiophosphate were commercially available from Sigma-Aldrich. Diphenylchlorothiophosphate, di-*i*-propylchlorothiophosphate, di-*n*-butylchlorothiophosphate, dibenzylchlorothiophosphate and 2-chloro-[1,3,2]dioxaphosphinane 2-sulfide were obtained according to the literature procedures [Jacobson et al., 1999]. Pyridine, dichloromethane and acetone were dried and distilled using standard procedures. Melting points (uncorrected) were determined with a Stuart Scientific SMP30 apparatus. NMR spectra were recorded on a Bruker Avance III HD 400 MHz spectrometer. Chemical shifts are reported in ppm relative to the residual solvent peak (DMSO-d6 2.49 ppm for ^{1}H, 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. Elemental analysis was performed using a CHNS-Carlo Erba EA-1108. Mass spectra were recorded on a TripleTOF 5600+ SCIEX mass spectrometry

system. Column chromatography was performed using silica gel 60 (230-400 mesh, Merck). Preparative thin-layer chromatography was performed with Polygram SIL G/UV254 silica gel (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

2.1. General method for the synthesis of the *N*-thiophosphorylated derivatives of 3-(4-aminophenyl)-7-hydroxy-coumarin (**8a-g**)

The corresponding chlorothiophosphate (3.0 mmol) was added to a solution of 3-(4-aminophenyl)-7-hydroxy-coumarin **7** (380 mg, 1.5 mmol) in dry pyridine (4.5 mL). The reaction mixture was stirred under a nitrogen atmosphere for 24 h. Next, the solvent was evaporated, and the resulting residue was purified by column chromatography using CH_2Cl_2:MeOH 100:1 as an eluent to give the desired products **8a-g**.

*3-[4-(dimethoxy-thiophosphorylamino)-phenyl]-7-hydroxy-coumarin **8a**.* Yield 53%, mp 165-169 °C; ν_{max} (KBr)/cm^{-1} 3290, 1675, 1609, 1517, 1453, 1282, 1226, 1132, 1014, 938, 634; ^{1}H NMR δ_H (400 MHz, DMSO) 10.56 (1H, s, OH), 8.59 (1H, d, *J* 15.2 Hz, NH), 8.07 (1H, s, CH), 7.62-7.54 (3H, m, Ar-H), 7.13 (2H, d, *J* 8.7 Hz, Ar-H), 6.82 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 6.75 (1H, d, *J* 2.2 Hz, Ar-H), 3.67 (6H, d, *J* 14.0 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 161.4, 160.6, 155.1, 140.7, 140.3, 130.2, 129.5, 128.5, 122.4, 117.9 (d, J_{P-C} 7.5 Hz), 113.8, 112.6, 102.2, 53.5 (d, J_{P-C} 4.7 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 69.04. Anal. calcd for: $C_{17}H_{16}NO_5PS$: C, 54.11; H 4.27; N, 3.71; S, 8.50. Found: C, 54.02; H, 4.33; N, 3.80; S, 8.39. HRMS (*m/z*) [M-H]- calcd 376.0409, found 376.0616.

*3-[4-(diethoxy-thiophosphorylamino)-phenyl]-7-hydroxy-coumarin **8b**.* Yield 54%, mp 156-159 °C; ν_{max} (KBr)/cm^{-1} 3268, 1676, 1595, 1514, 1454, 1274, 1230, 1118, 1021, 927, 634; ^{1}H NMR δ_H (400 MHz, DMSO) 10.56 (1H, s, OH), 8.51 (1H, d, *J* 15.3 Hz, NH), 8.08 (1H, s, CH), 7.61-7.54 (3H, m, Ar-H), 7.14 (2H, d, *J* 8.8 Hz, Ar-H), 6.82 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 6.75 (1H, d, *J* 2.2 Hz, Ar-H), 4.15-3.95 (4H, m, CH_2), 1.25 (6H, t, *J* 7.1 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 161.4, 160.6, 155.1, 141.0, 140.2, 130.2, 129.4, 128.3, 122.4, 117.8 (d, J_{P-C} 7.7 Hz), 113.8, 112.6, 102.1, 63.0 (d, J_{P-C} 4.6 Hz), 16.1 (d, J_{P-C} 5.0 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 64.50. Anal. calcd for: $C_{19}H_{20}NO_5PS$: C, 56.29; H 4.97; N, 3.45; S, 7.91. Found: C, 56.21; H, 4.92; N, 3.55; S, 7.82. HRMS (*m/z*) [M-H]- calcd 404.0722, found 404.1158.

*3-[4-(diisopropoxy-thiophosphorylamino)-phenyl]-7-hydroxy-coumarin **8c**.* Yield 74%, mp 145-148 °C; ν_{max} (KBr)/cm^{-1} 3269, 1680, 1606, 1516, 1449, 1281, 1213, 1124, 970, 933, 633; ^{1}H NMR δ_H (400 MHz, DMSO) 10.56 (1H, s, OH), 8.43 (1H, d, *J* 16.1 Hz, NH), 8.08 (1H, s, CH), 7.62-7.55 (3H, m, Ar-H), 7.14 (2H, d, *J* 8.8 Hz, Ar-H), 6.81 (1H, dd, *J* 8.5, 2.2 Hz, Ar-H), 6.75 (1H, d, *J* 2.1 Hz, Ar-H), 4.73-4.60 (2H, m, CH), 1.32 (6H, d, *J* 6.2 Hz, CH_3), 1.18 (6H, d, *J* 6.2 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 161.4, 160.6, 155.1, 141.3, 140.1, 130.2, 129.1, 128.0, 122.3, 117.8 (d, J_{P-C} 7.7 Hz), 113.8, 112.6, 102.1, 72.1 (d, J_{P-C} 4.7 Hz), 23.8 (d, J_{P-C} 5.0 Hz), 23.5

(d, $J_{P\text{-}C}$ 4.6 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 61.31. Anal. calcd for: $C_{21}H_{24}NO_5PS$: C, 58.19; H 5.58; N, 3.23; S, 7.40. Found: C, 58.11; H, 5.51; N, 3.32; S, 7.48. HRMS (*m/z*) [M-H]- calcd 432.1035, found 432.1207.

3-[4-(di-n-butoxy-thiophosphorylamino)-phenyl]-7-hydroxy-coumarin ***8d****.* Yield 46%, mp 135-136 °C; ν_{max} (KBr)/cm^{-1} 3277, 1680, 1595, 1513, 1452, 1270, 1230, 1123, 1019, 935, 631; ^{1}H NMR δ_H (400 MHz, DMSO); 10.56 (1H, s, OH), 8.51 (1H, d, *J* 15.5 Hz, NH), 8.08 (1H, s, CH), 7.61-7.54 (3H, m, Ar-H), 7.13 (2H, d, *J* 8.7 Hz, Ar-H), 6.82 (1H, dd, *J* 8.5, 2.2 Hz, Ar-H), 6.75 (1H, d, *J* 2.1 Hz, Ar-H), 4.11-3.85 (4H, m, CH_2), 1.59 (4H, quint, *J* 6.5 Hz, CH_2), 1.34 (4H, sext, *J* 6.7 Hz, CH_2), 0.86 (6H, t, *J* 7.4 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO); 161.4, 160.6, 155.1, 141.0, 140.1, 130.2, 129.3, 128.3, 122.3, 117.7 (d, $J_{P\text{-}C}$ 7.6 Hz), 113.8, 112.6, 102.1, 66.5 (d, $J_{P\text{-}C}$ 4.9 Hz), 31.9 (d, $J_{P\text{-}C}$ 8.0 Hz), 18.8, 13.9; ^{31}P NMR δ_C (162 MHz, DMSO); 65.09. Anal. calcd for: $C_{23}H_{28}NO_5PS$: C, 59.86; H 6.12; N, 3.03; S, 6.95. Found: C, 59.94; H, 6.03; N, 2.98; S, 7.09. HRMS (*m/z*) [M-H]- calcd 460.1348, found 460.1775.

3-[4-(diphenoxy-thiophosphorylamino)-phenyl]-7-hydroxy-coumarin ***8e****.* Yield 70%, mp 215-216 °C; ν_{max} (KBr)/cm^{-1} 3219, 1685, 1592, 1513, 1452, 1277, 1209, 1122, 1023, 934, 627; ^{1}H NMR δ_H (400 MHz, DMSO) 10.59 (1H, s, OH), 9.33 (1H, d, *J* 17.5 Hz, NH), 8.13 (1H, s, CH), 7.70 (2H, d, *J* 8.7 Hz, Ar-H), 7.58 (1H, d, *J* 8.6 Hz, Ar-H), 7.47-7.35 (6H, m, Ar-H), 7.29-7.20 (6H, m, Ar-H), 6.82 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 6.76 (1H, d, *J* 2.2 Hz, Ar-H); ^{13}C NMR δ_C (101 MHz, DMSO) 161.5, 160.6, 155.2, 150.5 (d, $J_{P\text{-}C}$ 6.4 Hz), 140.6, 140.3 (d, $J_{P\text{-}C}$ 3.9 Hz), 130.4, 130.3, 129.8, 129.3, 126.0, 122.2, 121.4 (d, $J_{P\text{-}C}$ 4.8 Hz), 118.5 (d, $J_{P\text{-}C}$ 7.8 Hz), 113.8, 112.6, 102.2; ^{31}P NMR δ_C (162 MHz, DMSO) 56.23. Anal. calcd for: $C_{27}H_{20}NO_5PS$: C, 64.67; H 4.02; N, 2.79; S, 6.39. Found: C, 64.55; H, 3.96; N, 2.70; S, 6.52. HRMS (*m/z*) [M-H]- calcd 500.0722, found 500.1005.

3-[4-(dibenzyloxy-thiophosphorylamino)-phenyl]-7-hydroxy-coumarin ***8f****.* Yield 10%, mp oil; ν_{max} (KBr)/cm^{-1} 3282, 1676, 1609, 1516, 1453, 1291, 1211, 1130, 992, 951, 629; ^{1}H NMR δ_H (400 MHz, DMSO) 10.58 (1H, s, OH), 8.78 (1H, d, *J* 15.1 Hz, NH), 8.07 (1H, s, CH), 7.60-7.54 (3H, m, Ar-H), 7.42-7.28 (10H, m, Ar-H), 7.17 (2H, d, *J* 8.7 Hz, Ar-H), 6.82 (1H, dd, *J* 8.5, 2.2 Hz, Ar-H), 6.75 (1H, d, *J* 2.2 Hz, Ar-H), 5.19-4.99 (4H, m, CH_2); ^{13}C NMR δ_C (101 MHz, DMSO) 161.4, 160.6, 155.1, 140.2, 136.4 (d, $J_{P\text{-}C}$ 9.0 Hz), 130.2, 129.4, 129.0, 128.9, 128.7, 128.3, 126.9, 122.3, 118.1 (d, $J_{P\text{-}C}$ 7.6 Hz), 113.8, 112.6, 102.1, 68.4 (d, $J_{P\text{-}C}$ 4.2 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 65.65. Anal. calcd for: $C_{29}H_{24}NO_5PS$: C, 65.78; H 4.57; N, 2.65; S, 6.06. Found: C, 65.91; H, 4.49; N, 2.72; S, 6.00. HRMS (*m/z*) [M-H]- calcd 528.1035, found 528.1502.

3-{4-(2-thioxo-2λ^5-[1,3,2]dioxaphosphinan-2-ylamino)-phenyl}-7-hydroxy-coumarin ***8g****.* Yield 35%, mp >300 °C (with decomposition); ν_{max} (KBr)/cm^{-1} 3255, 1705, 1606, 1515, 1451, 1284, 1234, 1133, 1020, 921, 636; ^{1}H NMR δ_H (400 MHz, DMSO) 10.57 (1H, s, OH), 8.70 (1H, d, *J* 13.4 Hz, NH), 8.08 (1H, s, CH), 7.64-7.54 (3H, m, Ar-H), 7.18 (2H, d, *J* 8.7 Hz, Ar-H), 6.82 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 6.75 (1H, d, *J* 2.2 Hz, Ar-H), 4.55-4.33 (4H, m, CH_2),

2.18-1.88 (2H, m, CH_2); ^{13}C NMR δ_C (101 MHz, DMSO) 161.4, 160.6, 155.1, 140.6, 140.3, 130.2, 129.4, 128.7, 122.4, 118.4 (d, $J_{P\text{-}C}$ 7.5 Hz), 113.8, 112.6, 102.2, 68.0 (d, $J_{P\text{-}C}$ 6.6 Hz), 26.3 (d, $J_{P\text{-}C}$ 6.6 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 61.26. Anal. calcd for: $C_{18}H_{16}NO_5PS$: C, 55.52; H 4.14; N, 3.60; S, 8.24. Found: C, 55.39; H, 4.21; N, 3.71; S, 8.32. HRMS (*m/z*) [M-H]- calcd 388.0409, found 388.0772.

2.2. General method for the synthesis of the *N*-thiophosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate (**9a-g**)

A mixture of formic acid (96 mg, 1.54 mmol) and *N*,*N*-dimethylacetamide (1.4 mg, 0.016 mmol) was added to a stirred solution of chlorosulfonyl isocyanate (212 mg, 1.50 mmol) in dry dichloromethane (0.5 mL) at 40 °C over a period of 3.5 h. Then, a stirred solution of the appropriate *N*-thiophosphorylated derivative of 7-hydroxy-3-phenylcoumarin **8a-g** (253 mg, 1.00 mmol) in *N*,*N*-dimethylacetamide (3.4 mL) was added to the mixture. The resulting mixture was stirred at ambient temperature overnight and then poured into water (10 mL). Eventually, a white precipitate formed. The suspension stirred at ambient temperature for another 2 h. The resulting precipitate was filtered, washed with water and recrystallized from acetonitrile or CH_2Cl_2 to give the desired product **9a-g**.

3-[4-(dimethoxy-thiophosphorylamino)-phenyl]-coumarin-7-O-sulfamate ***9a***. Yield 32%, mp 184-186 °C; ν_{max} (KBr)/cm^{-1} 3284, 1687, 1614, 1516, 1456, 1392, 1194, 938, 774, 655; ^{1}H NMR δ_H (400 MHz, DMSO) 8.65 (1H, d, *J* 15.1 Hz, NH), 8.25 (2H, s, NH_2), 8.22 (1H, s, CH), 7.84 (1H, d, *J* 8.6 Hz, Ar-H), 7.64 (2H, d, *J* 8.6 Hz, Ar-H), 7.36 (1H, d, *J* 2.2 Hz, Ar-H), 7.30 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 7.16 (2H, d, *J* 8.7 Hz, Ar-H), 3.68 (6H, d, *J* 14.0 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 160.0, 153.6, 152.3, 141.4, 139.0, 130.0, 129.8, 127.8, 126.5, 119.1, 118.5, 117.9 (d, $J_{P\text{-}C}$ 7.5 Hz), 53.6 (d, $J_{P\text{-}C}$ 4.7 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 68.95. Anal. calcd for: $C_{17}H_{17}N_2O_7PS_2$: C, 44.73; H 3.75; N, 6.14; S, 14.05. Found: C, 44.81; H, 3.79; N, 6.21; S, 13.96. HRMS (*m/z*) [M-H]- calcd 455.0137, found 455.0344.

3-[4-(diethoxy-thiophosphorylamino)-phenyl]-coumarin-7-O-sulfamate ***9b***. Yield 58%, mp 196-198 °C (with decomposition); ν_{max} (KBr)/cm^{-1} 3280, 1685, 1613, 1516, 1461, 1389, 1195, 929, 772, 649; ^{1}H NMR δ_H (400 MHz, DMSO) 8.58 (1H, d, *J* 15.2 Hz, NH), 8.24 (2H, s, NH_2), 8.23 (1H, s, CH), 7.84 (1H, d, *J* 8.6 Hz, Ar-H), 7.64 (2H, d, *J* 8.6 Hz, Ar-H), 7.36 (1H, d, *J* 2.0 Hz, Ar-H), 7.30 (1H, dd, *J* 8.5, 2.2 Hz, Ar-H), 7.17 (2H, d, *J* 8.7 Hz, Ar-H), 4.16-3.96 (4H, m, CH_2), 1.25 (6H, t, *J* 7.0 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 160.0, 153.6, 152.2, 141.6, 138.9, 130.0, 129.7, 127.5, 126.5, 119.1, 118.5, 117.8, (d, $J_{P\text{-}C}$ 7.6 Hz), 109.9, 63.1 (d, $J_{P\text{-}C}$ 4.7 Hz), 16.1 (d, $J_{P\text{-}C}$ 7.7 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 64.40. Anal. calcd for: $C_{19}H_{21}N_2O_7PS_2$: C, 47.10; H 4.37; N, 5.78; S, 13.24. Found: C, 47.15; H, 4.31; N, 5.90; S, 13.17. HRMS (*m/z*) [M-H]- calcd 483.0450, found 483.0821.

3-[4-(diisopropoxy-thiophosphorylamino)-phenyl]-coumarin-7-O-sulfamate ***9c****.* Yield 32%, mp 190-191 °C; ν_{max} (KBr)/cm^{-1} 3285, 1685, 1614, 1516, 1459, 1386, 1195, 933, 768, 643; ^{1}H NMR δ_H (400 MHz, DMSO) 8.50 (1H, d, *J* 16.1 Hz, NH), 8.26-8.22 (3H, m, CH, NH_2), 7.84 (1H, d, *J* 8.6 Hz, Ar-H), 7.64 (2H, d, *J* 8.7 Hz, Ar-H), 7.35 (1H, d, *J* 2.2 Hz, Ar-H), 7.29 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 7.17 (2H, d, *J* 8.8 Hz, Ar-H), 4.74-4.61 (2H, m, CH), 1.32 (6H, d, *J* 6.2 Hz, CH_3), 1.18 (6H, d, *J* 6.2 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 160.0, 153.6, 152.2, 142.0, 138.8, 130.0, 129.4, 127.2, 126.4, 119.1, 118.5, 117.9 (d, $J_{P\text{-}C}$ 7.6 Hz), 109.9, 72.1 (d, $J_{P\text{-}C}$ 4.8 Hz), 23.8 (d, $J_{P\text{-}C}$ 5.0 Hz), 23.5 (d, $J_{P\text{-}C}$ 4.7 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 61.20. Anal. calcd for: $C_{21}H_{25}N_2O_7PS_2$: C, 49.21; H 4.92; N, 5.47; S, 12.51. Found: C, 49.33; H, 4.87; N, 5.41; S, 12.44. HRMS (*m/z*) [M+H]+ calcd 513.0919, found 513.3477.

3-[4-(di-n-butoxy-thiophosphorylamino)-phenyl]-coumarin-7-O-sulfamate ***9d****.* Yield 48%, mp 175-176 °C; ν_{max} (KBr)/cm^{-1} 3285, 1693, 1614, 1515, 1454, 1375, 1193, 936, 769, 653; ^{1}H NMR δ_H (400 MHz, DMSO) 8.58 (1H, d, *J* 15.5 Hz, NH), 8.24 (2H, s, NH_2), 8.23 (1H, s, CH), 7.85 (1H, d, *J* 8.6 Hz, Ar-H), 7.64 (2H, d, *J* 8.6 Hz, Ar-H), 7.36 (1H, d, *J* 2.1 Hz, Ar-H), 7.29 (1H, dd, *J* 8.5, 2.2 Hz, Ar-H), 7.16 (2H, d, *J* 8.7 Hz, Ar-H), 4.12-3.89 (4H, m, CH_2), 1.60 (4H, quint, *J* 7.8 Hz, CH_2), 1.34 (4H, sext, *J* 7.4 Hz, CH_2), 0.86 (6H, t, *J* 7.4 Hz, CH_3); ^{13}C NMR δ_C (101 MHz, DMSO) 160.0, 153.6, 152.2, 141.6, 138.9, 130.0, 129.6, 127.5, 126.4, 119.1, 118.5, 117.7 (d, $J_{P\text{-}C}$ 7.5 Hz), 109.9, 66.6 (d, $J_{P\text{-}C}$ 5.0 Hz), 31.9 (d, $J_{P\text{-}C}$ 7.9 Hz), 18.8, 13.9; ^{31}P NMR δ_C (162 MHz, DMSO) 65.00. Anal. calcd for: $C_{23}H_{29}N_2O_7PS_2$: C, 51.10; H 5.41; N, 5.18; S, 11.86. Found: C, 51.02; H, 5.49; N, 5.29; S, 11.73. HRMS (*m/z*) [M+H]+ calcd 541.1232, found 541.3912.

3-[4-(diphenoxy-thiophosphorylamino)-phenyl]-coumarin-7-O-sulfamate ***9e****.* Yield 43%, mp 208-210 °C (with decomposition); ν_{max} (KBr)/cm^{-1} 3236, 1691, 1616, 1517, 1487, 1392, 1181, 916, 771, 672; ^{1}H NMR δ_H (400 MHz, DMSO) 9.40 (1H, d, *J* 17.4 Hz, NH), 8.28 (1H, s, CH), 8.25 (2H, s, NH_2), 7.85 (1H, d, *J* 8.6 Hz, Ar-H), 7.75 (2H, d, *J* 8.7 Hz, Ar-H), 7.47-7.38 (6H, m, Ar-H), 7.37 (1H, d, *J* 2.2 Hz, Ar-H), 7.32-7.20 (7H, m, Ar-H); ^{13}C NMR δ_C (101 MHz, DMSO) 160.0, 153.7, 152.3, 150.5 (d, $J_{P\text{-}C}$ 6.5 Hz), 140.9 (d, $J_{P\text{-}C}$ 3.6 Hz), 139.3, 130.4, 130.1, 130.0, 128.5, 126.3, 126.0, 121.4 (d, $J_{P\text{-}C}$ 4.8 Hz), 119.2, 118.5 (d, $J_{P\text{-}C}$ 7.8 Hz), 118.4, 109.9; ^{31}P NMR δ_C (162 MHz, DMSO) 56.15. Anal. calcd for: $C_{27}H_{21}N_2O_7PS_2$: C, 55.86; H 3.65; N, 4.83; S, 11.05. Found: C, 55.71; H, 3.71; N, 4.91; S, 10.94. HRMS (*m/z*) [M+H]+ calcd 581.0606, found 581.4347.

3-[4-(dibenzyloxy-thiophosphorylamino)-phenyl]-coumarin-7-O-sulfamate ***9f****.* Yield 38%, mp 148-149 °C; ν_{max} (KBr)/cm^{-1} 3246, 1693, 1612, 1513, 1456, 1372, 1191, 933, 772, 656; ^{1}H NMR δ_H (400 MHz, DMSO) 8.85 (1H, d, *J* 15.0 Hz, NH), 8.24 (2H, s, NH_2), 8.22 (1H, s, CH), 7.84 (1H, d, *J* 8.6 Hz, Ar-H), 7.63 (2H, d, *J* 8.7 Hz, Ar-H), 7.39-7.32 (11H, m, Ar-H), 7.29 (1H, dd, *J* 8.5, 2.3 Hz, Ar-H), 7.20 (2H, d, *J* 8.8 Hz, Ar-H), 5.17-4.99 (4H, m, CH_2);

^{13}C NMR δ_C (101 MHz, DMSO) 159.9, 153.6, 152.2, 141.4, 139.0, 136.4 (d, $J_{P\text{-}C}$ 9.0 Hz), 130.1, 129.7, 128.9, 128.7, 128.3, 127.8, 126.4, 119.1, 118.5, 118.1 (d, $J_{P\text{-}C}$ 7.7 Hz), 109.9, 68.42 (d, $J_{P\text{-}C}$ 4.3 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 65.55. Anal. calcd for: $C_{29}H_{25}N_2O_7PS_2$: C, 57.23; H 4.14; N, 4.60; S, 10.54. Found: C, 57.13; H, 4.21; N, 4.49; S, 10.61. HRMS (*m/z*) [M-H]- calcd 607.0763, found 607.1429.

3-{4-(2-thioxo-2λ^5-[1,3,2]dioxaphosphinan-2-ylamino)-phenyl}-coumarin-7-O-sulfamate ***9g***. Yield 40%, mp 267-270 °C (with decomposition); ν_{max} (KBr)/cm^{-1} 3239, 1685, 1616, 1517, 1456, 1391, 1189, 927, 763, 651; ^{1}H NMR δ_H (400 MHz, DMSO) 8.76 (1H, d, *J* 13.3 Hz, NH), 8.25 (2H, s, NH_2), 8.23 (1H, s, CH), 7.85 (1H, d, *J* 8.6 Hz, Ar-H), 7.66 (2H, d, *J* 8.6 Hz, Ar-H), 7.36 (1H, d, *J* 2.1 Hz, Ar-H), 7.30 (1H, dd, *J* 8.5, 2.2 Hz, Ar-H), 7.22 (2H, d, *J* 8.7 Hz, Ar-H), 4.56-4.34 (4H, m, CH_2), 2.20-1.91 (2H, m, CH_2); ^{13}C NMR δ_C (101 MHz, DMSO) 160.0, 153.6, 152.3, 141.3, 139.0, 130.1, 129.7, 128.0, 126.5, 119.1, 118.5, 118.4 (d, $J_{P\text{-}C}$ 7.6 Hz), 109.9, 68.0 (d, $J_{P\text{-}C}$ 6.7 Hz), 26.3 (d, $J_{P\text{-}C}$ 6.9 Hz); ^{31}P NMR δ_C (162 MHz, DMSO) 61.14. Anal. calcd for: $C_{18}H_{17}N_2O_7PS_2$: C, 46.15; H 3.66; N, 5.98; S, 13.69. Found: C, 46.24; H, 3.73; N, 6.05; S, 13.55. HRMS (*m/z*) [M+H]+ calcd 469.0293, found 469.2661.

2.3. Computational studies

2.3.1. Ligand preparation

Prior to the docking procedures, the potential inhibitors were prepared using Portable HyperChem Release 8.0.7 (Hypercube, Inc., Gainesville, FL, USA). Each ligand was optimized using an MM+ force field and the Polak–Ribierè conjugate gradient algorithm (terminating at a gradient of 0.05 kcal mol^{-1} Å^{-1}).

2.3.2. Protein preparation

For docking, the X-ray structure of human STS (obtained from Protein Data Bank – accession code 1P49) was prepared using the standard procedure. First, the water molecules from crystallization were removed from the structure, and the catalytic amino acid fGly75 was converted to the *gem*-diol form using the Protein Preparation Wizard module, delivered by Maestro (Schrödinger, LLC, New York, NY, USA). Next, the hydrogen atoms were added onto the structure, and the prepared model of the enzyme was optimized using the OPLS-AA force field.

2.3.3. Molecular docking

The docking of the optimized ligands to the prepared structure of human STS was carried out with AutoDock Vina 1.1.2 software (The Molecular Graphic Laboratory, The Scripps Research Institute, La Jolla, CA, USA) [Trott et al., 2010]. For all docking studies, a grid box of size 30 Å x 30 Å x 30 Å centered on the Cβ atom of amino acid 75 was used. The best poses for a particular ligand were inspected visually. Illustrations of the 3D models were generated using VMD 1.9 (University of Illinois at Urbana – Champaign, Urbana, IL, USA).

2.3.4. Log*P* calculations

Log*P* parameters were calculated using ChemDraw Ultra 7.0 (CambridgeSoft Corporation, Cambridge, MA, USA).

2.4. Biological assays

2.4.1. Enzyme purification

STS was extracted from a human placenta and purified to homogeneity following a multistep chromatography protocol as previously described [Hernandez-Guzman et al., 2001].

2.4.2. *In vitro* activity in an enzymatic assay

The reaction mixture, at a final volume of 100 μL, 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 a purified enzyme (1 U is the amount of an enzyme that hydrolyzes 100 μM 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 Biotek ELx800 Microplate Reader (BioTek Instruments, Inc. Winooski, VT, USA). The IC_{50} values were calculated using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). All measurements were performed in triplicate.

2.4.3. *In vitro* activity in a cell line experiment

The inhibition of sulfatase activity in MCF-7 breast cancer cells was measured using a method described earlier with some modifications. MCF-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and cultured in the above medium until 80% confluence. For experiments, cells were seeded in 24-well microplates (Nest Biotechnology) at a density of 1×10^5 cells/well. Cell number was determined using a *Bürker* Counting *Chamber.* Incubation of cells was conducted for 20 h at 37 °C in a 5% CO_2 humidified incubator in serum-free medium (0.5 mL) with the addition of [^{3}H]E1S (4×10^5 cpm, 3 nM) with or without inhibitor (100 nM). After incubation, medium (0.45 mL) was collected from each well, and the product formed by STS was isolated from the mixture by extraction with toluene (4 mL). Sulfatase activity was measured using the Radioluminometer MicroBeta (Perkin Elmer). Assays involving MCF-7 cells were carried out in triplicate.

2.4.4. Influence of the STS inhibitors on zebrafish embryo development

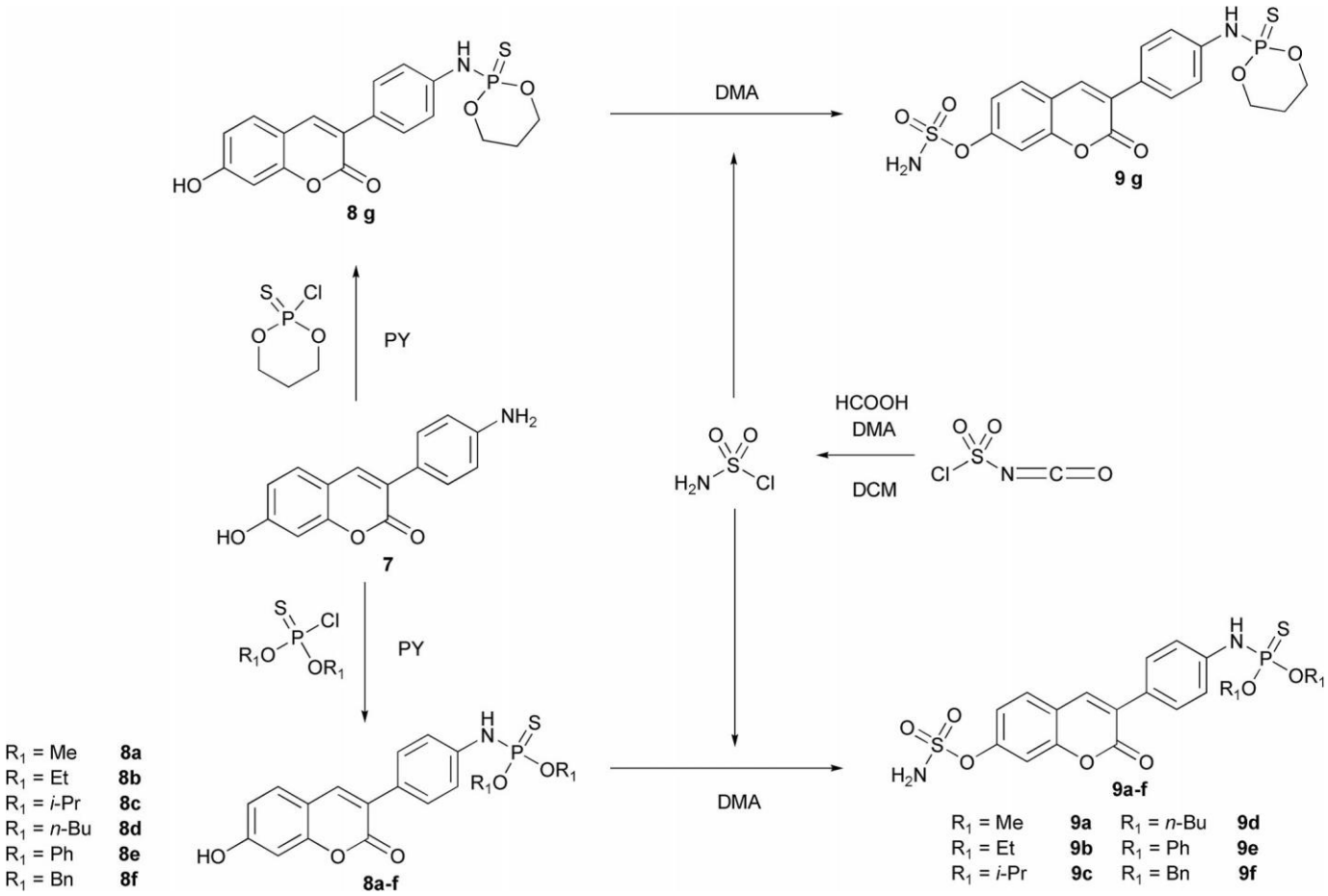
The collected zebrafish embryos were transferred to a Petri dish with E3 medium (5 mM NaCl, 0.33 mM $MgCl_2$, 0.33 mM $CaCl_2$, 0.17 mM KCl; pH 7.2) and then placed in 6-well plates with 10 embryos in each well. Stock solutions of **6e**, **6f**, **9b** and **9e** were prepared in DMSO. In the present experiments, 0.1, 0.5, and 1 μM solutions of the compounds were employed, which were freshly prepared each time by dissolving the stock solutions in E3

medium directly before their addition to the wells. The solutions were changed once daily, and the embryos were maintained in an incubator at 28.5 °C. Zebrafish embryo development and viability were evaluated for up to 5 days using bright-field microscopy (Zeiss Axio Vert, ZEISS, Germany). Image analysis to determine the percent of dead and malformed embryos over time was performed using Zeiss software. The same embryos (n = 10) were followed throughout the whole study.

3. RESULTS AND DISCUSSION

3.1. Chemistry

In the course of our research, we synthesized all of the newly designed STS inhibitors, which are *N*-thiophosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate (**9a-g**), using the pathway shown in **Scheme 1**. In the first step, we synthesized 7-hydroxy-3-(4-aminophenyl)-coumarin **7** according the procedure that we previously described [Daśko et al., 2017]. Next, coumarin derivative **7** was *N*-thiophosphorylated with the corresponding chlorothiophosphate in anhydrous pyridine. The isolation of the crude products of the thiophosphorylation reaction was performed by flash column chromatography to give coumarin derivatives **8a-g**. Finally, the OH groups of compounds **8a-g** were sulfamoylated. In this cases, the solutions of stable *N*-thiophosphorylated derivatives **8a-g** in *N*,*N*-dimethylacetamide (DMA) were treated with H_2NSO_2Cl that was previously generated in the reaction of chlorosulfonyl isocyanate and formic acid in the presence of a catalytic amount of DMA. After the standard isolation procedure, we obtained the desired compounds **9a-g**. Furthermore, to perform the additional biological evaluation, we decided to resynthesize *N*-phosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates **6a-h** according to the procedure that we previously described [Daśko et al., 2016].



Scheme 1 Synthesis of *N*-thiophosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate **9a-g**.

3.2. Molecular modeling

To determine the binding modes of the designed *N*-thiophosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates **9a-g**, a computational docking method was used. The docking studies also involved in identifying potential interactions between specified functional groups on the inhibitors and amino acid residues present in the active site of STS. The X-ray structure of STS was retrieved from the Protein Data Bank (Protein Data Bank accession code 1P49) and properly prepared for docking calculations. The docking procedure of the optimized ligands was performed using AutoDock Vina 1.1.2 software (Molecular Graphics Laboratory, The Scripps Research Institute, La Jolla, CA, USA).

The calculated free binding energies of the newly designed candidates **9a-g** were in the range of -6.5 to -9.0 kcal mol^{-1} and were significantly lower than the free binding energy value of the reference inhibitor **667-COUMATE** (-5.4 kcal mol^{-1}). The best docking result was determined for coumarin derivative **9e** (with a calculated free binding energy value of -9.0 kcal mol^{-1}). The free binding energies for resynthesized compounds **6e** and **6f** were -6.7 and -8.3 kcal mol^{-1}, respectively.

Our docking analysis for the newly designed STS inhibitor candidates **9a-g** showed that these compounds could associate well with the active site of STS in a similar manner to the mode of the reported STS inhibitor **667-COUMATE**. **Fig. 2** shows the structures of **9e** and **6e** and the reference inhibitor **667-COUMATE** docked into the active site of the STS enzyme. We found that the sulfamate functional groups are located very close to the catalytic amino acid residue fGly75 (~3.0 Å), coordinated to the Ca^{2+} ion, and surrounded by the catalytically essential residues (Asp35, Asp36, Arg79, Lys134, His136 and Lys368). The coumarin cores, with *N*-thiophosphoryl moieties in the case of the designed candidates **9a-g**, are well accommodated in the lipophilic cavity delimited by the lipophilic amino acids in the enzyme binding region (Arg98, Leu103, Phe104, Leu167, Val 177, Phe178, Phe182, Leu185, Phe230, Phe233, Phe237, Thr484, Val486, Phe488, Trp550 and Phe553). Furthermore, in the course of our previous research with *N*-phosphorylated compounds **6a-h**, we noticed that the *N*-phosphoryl groups of compounds **6a**-**h** are within hydrogen bonding distance from the backbone NH group of Arg98 [Daśko et al., 2016]. We suggested that this additional interaction may improve the stability of the enzyme-inhibitor complex and may influence the inhibitory activities of compounds **6a-h**. As shown in **Fig. 2**, we detected that the *N*-thiophosphoryl groups of the *N*-thiophosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates occupied the same region of the STS active site as the *N*-phosphoryl groups of *N*-phosphorylated 3-(4-aminophenyl)-coumarin-7-*O*-sulfamates. However, it should be noted that the sulfur atom in an *N*-thiophosphoryl group is a much weaker H-bond acceptor than the oxygen atom in an *N*-phosphoryl group.

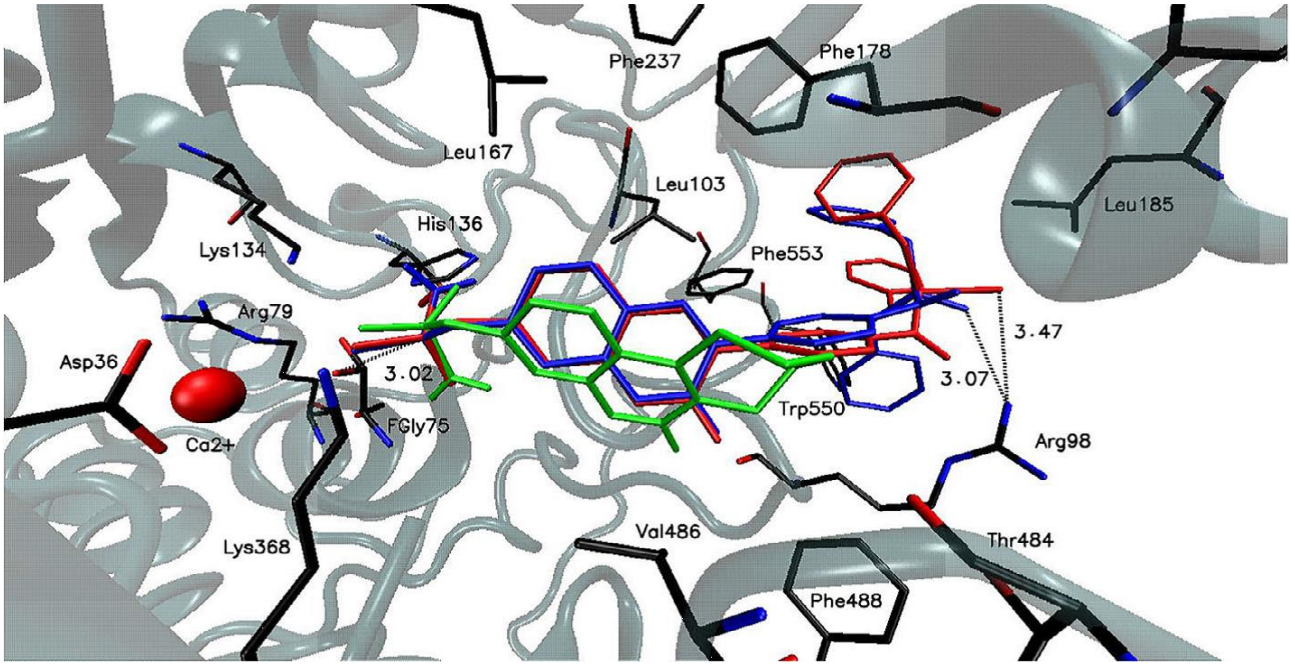


Figure 2 Docked binding modes and distances to fGly75 and Arg98 for compounds **6e** (blue), **9e** (red) and the reference inhibitor **667-COUMATE** (green)

3.3. Biological assays

Initially, the STS inhibitory activities of synthesized compounds **9a-g** were evaluated in an enzymatic assay according to previously reported methods [Vaccaro et al., 1987; Woo et al., 2010]. Screening tests were performed using the STS enzyme isolated from human placenta and purified by a 3-step chromatography procedure. After purification, the obtained fraction was used directly as the enzyme source.

The summarized results of the enzymatic assay used for newly synthesized *N*-thiophosphorylated coumarin derivatives **9a-g** and the reference compounds **6e**, **6f** and **667-COUMATE** are presented in **Table 1**. The received data showed that all of the newly synthesized inhibitors **9a-g** are able to effectively inhibit the actions of STS. In the course of our investigations, the highest inhibitory activity among the newly synthesized inhibitors **9a-g** was exhibited by compound **9e** with an IC_{50} value of 0.201 μM. This result is in agreement with the data from the molecular modeling studies. However, the *N*-phosphorylated derivative **6f** demonstrated a higher STS inhibitory effect (IC_{50} value of 0.083 μM) than all of the newly designed compounds **9a-g**. This observation indicated that the possibility of the creation of stronger hydrogen bonds between the *N*-phosphoryl group of compound **6f** and the Arg98 residue compared to the *N*-thiophosphoryl group may be significant for inhibitory properties. Furthermore, the inhibitory activity of compound **6f** was similar to that of **667-COUMATE** (IC_{50} value of 0.062 μM).

TABLE 1 Inhibitory activities and free binding energies calculated for the synthesized compounds **9a-g** and reference inhibitors (**6e**, **6f**, and **667-COUMATE**) in the STS enzyme assays

No.	Free binding energy (kcal Mol^{-1})	IC_{50} (μM)
9a	−6.6	0.768 ± 0.04
9b	−6.5	0.399 ± 0.05
9c	−6.7	0.783 ± 0.03
9d	−6.7	0.665 ± 0.02
9e	−9.0	0.201 ± 0.02
9f	−7.9	0.802 ± 0.06
9g	−7.6	0.572 ± 0.04
6e	−6.7	0.273 ± 0.02
6f	−8.3	0.083 ± 0.007
667-COUMATE	−5.4	0.062 ± 0.006

Table 1 Inhibitory activities and free binding energies calculated for the synthesized compounds **9a-g** and reference inhibitors (**6e**, **6f** and **667-COUMATE**) in the STS enzymatic assay.

The next step of the biological evaluation was to verify whether the obtained STS inhibitors are able to pass the cellular membrane effectively and inhibit STS inside the cell. In the course of our study, we determined the STS activity in the MCF-7 cell line after incubation in the presence of inhibitor at 100 nM concentration. For this evaluation, we included the newly synthesized compounds **9a-g** and their *N*-phosphorylated analogs **6a-h**, for which the synthesis had been previously described [Daśko et al., 2016]. The summarized results are presented in **Table 2**.

TABLE 2 Remained STS activities in MCF-7 cell line at 100 nM concentration and Log*P* parameters of the synthesized compounds **9a-g**, resynthesized compounds **6a-h** and reference inhibitor **667-COUMATE**

No.	STS activity (%)	Log*P* (–)
9a	64.8	2.75
9b	1.0	3.43
9c	11.1	4.26
9d	1.0	5.16
9e	2.9	6.11
9f	2.3	6.30
9g	82.5	2.62
6a	85.9	2.01
6b	79.8	2.70
6c	56.1	3.52
6d	1.2	4.43
6e	0.1	5.37
6f	0.2	5.56
6g	55.1	1.89
6h	21.6	2.99
667-COUMATE	0.1	2.04

Table 2 Remaining STS activity in the MCF-7 cell line at 100 nM concentration and Log*P* parameters of the synthesized compounds **9a-g**, resynthesized compounds **6a-h** and reference inhibitor **667 COUMATE**.

In the course of our research, we found that the lowest STS activities were measured in the presence of *N*-phosphorylated derivatives **6e** (0.1% of STS activity) and **6f** (0.2% of STS activity). The measured STS activity in the presence of **667-COUMATE** (used as a reference) was 0.1%. The inhibitory activities of *N*-phosphorylated compounds **6e** and **6f** were higher than their *N*-thiophosphorylated analogs **9e** and **9f** (despite the initial hypothesis that more lipophilic derivatives should more effectively inhibit STS activity as confirmed by molecular modeling studies). It suggests that the possibility of creating stronger hydrogen

bonds between the *N*-phosphoryl groups of compounds **6e** and **6f** (log*P* values of 5.37 and 5.56, respectively) and the Arg98 residue in the active site of STS is more critical than increasing the hydrophobicity, as with analogs **9e** and **9f** (log*P* values of 6.11 and 6.30, respectively). The above observations confirm that the theoretical results of molecular modeling studies as well as predicted log*P* values, cannot be interpreted uncritically.

Finally, an *in vivo* toxicity assay of four of the most potent STS inhibitors was performed. Because zebrafish develop rapidly, this a promising model of whole-organism toxicology screening, and therefore, the morphological changes of zebrafish embryos induced by the most active STS inhibitors (**6e**, **6f**, **9b** and **9e**) were evaluated. The experiment revealed no morphological changes in the zebrafish embryos or toxic effects of the compounds used at doses of 0.1–1 μM during 5 days of observation (Fig. 3).

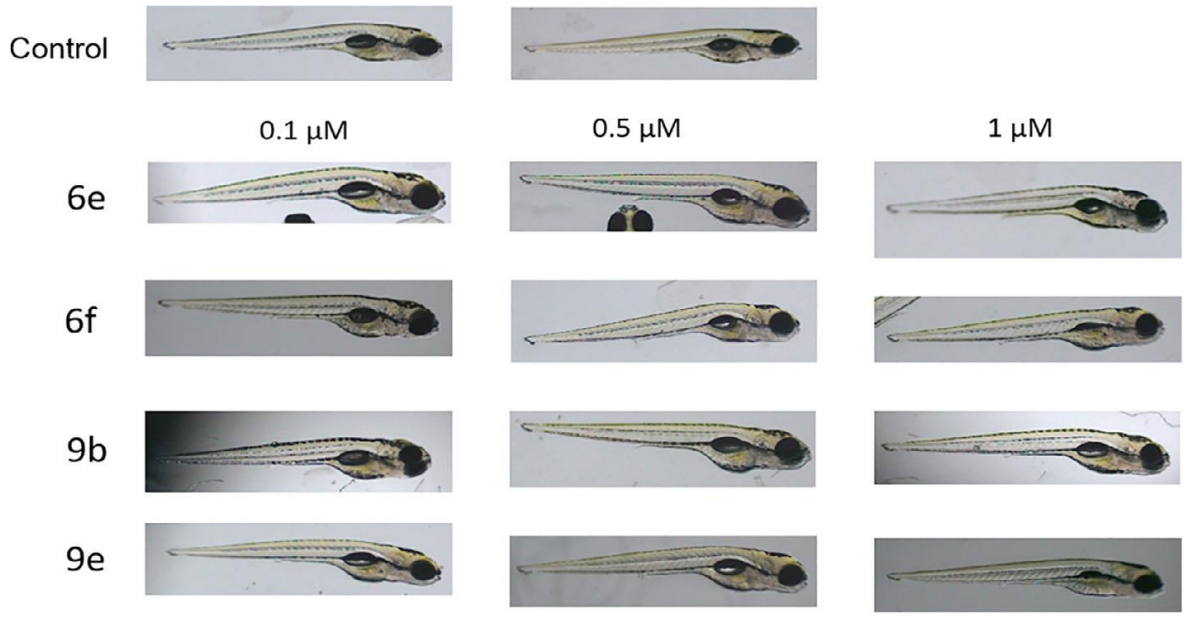


Figure 3 The influence of **6e**, **6f**, **9b** and **9e** on zebrafish embryos on the 5th day versus the control group.

4. CONCLUSIONS

In the present work, we described our research on molecular modeling, synthesis and biological evaluation of *N*-thiophosphorylated derivatives of 3-(4-aminophenyl)-coumarin-7-*O*-sulfamate (**9a-g**) as new STS inhibitors. The data of the enzymatic assay indicated that all of the newly synthesized inhibitors **9a-g** are able to effectively inhibit the actions of STS. Among them, the highest inhibitory activity was

exhibited by compound **9e** with an IC_{50} value of 0.201 μM. However, resynthesized phosphate derivative **6f** demonstrated a higher STS inhibitory effect (an IC_{50} value of 0.083 μM) than all of the newly designed compounds **9a-g**. The STS inhibitory properties of compound **6f** in the enzymatic assay were similar to **667-COUMATE** (an IC_{50} value of 0.062 μM) used as a reference. In the course of the cell line experiment, we observed the highest inhibition of STS in the presence of *N*-phosphorylated derivatives **6e** (0.1% of STS activity) and **6f** (0.2% of STS activity). The measured STS activity in the presence of **667-COUMATE** was 0.1%. The results of enzymatic and cell line experiments suggest that the possibility of creating stronger hydrogen bonds between the phosphoryl groups of compounds **6e** and **6f** (log*P* values of 5.37 and 5.56, respectively) and the Arg98 residue in the active site of STS is more critical for inhibitory effects than increasing the hydrophobicity, as with analogs **9e** and **9f** (log*P* values of 6.11 and 6.30, respectively). Toxicology analysis revealed that the tested compounds are safe for zebrafish embryos at concentrations 12 times greater than the IC_{50} values.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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