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Short communication

Synthesis of dansyl labeled sphingosine kinase 1 inhibitor

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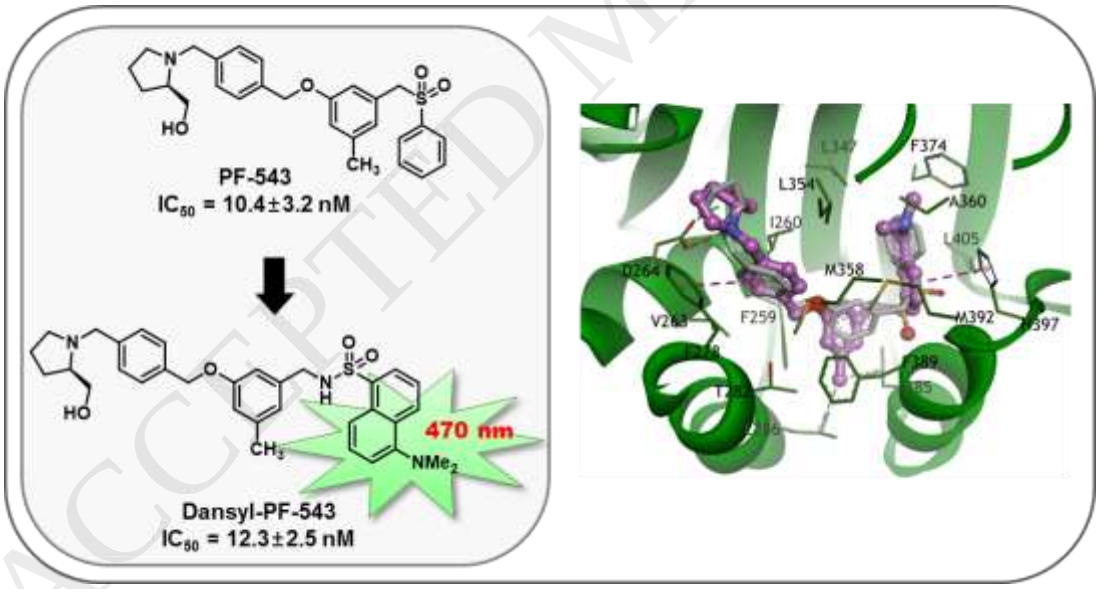
Graphical Abstract



Highlights

- We synthesized labeled PF-543 for biochemical studies involving SK1.
- A similar SK1 inhibition of dansyl-PF-543 and PF-543 demonstrated that the dansyl group can be biologically replaced by benzene sulfonyl group in PF-543.
- A docking study of SK1 confirmed the structural similarity between the synthetic dansyl-PF-543 and PF-543.

- Dansyl-PF-543 showed appropriate emission wavelength (470 nm) for biological study.

Abstract

PF-543 is a non-sphingosine analogue with inhibitory effect against SK1, based on a Ki of 4.3 nM and 130-fold selectivity for SK1 over SK2. Since the development of PF-543, animal studies demonstrated its valuable role in multiple sclerosis, myocardial infarction, and colorectal cancer. We synthesized labeled PF-543 for biochemical studies involving SK1. Overall, the 8-step synthetic route used 3,5-dimethylphenol as the starting material. A docking study of SK1 and SK1 inhibitory activity confirmed the structural similarity between the synthetic dansyl-PF-543 and PF-543. We also provide fluorescence spectra of dansyl-PF-543.

***Keywords**:* sphingosine kinase, inhibitor, PF-543, dansyl, cancer, label.

1. Introduction

Sphingosine kinase (SK) catalyzes sphingosine-1-phosphate (S1P) synthesis by phosphorylation of sphingosine. S1P controls the cell survival, proliferation, and migration through the five S1P-specific G-protein-coupled receptors (GPCRs). S1P is a drug discovery target because it is associated with a variety of diseases including cancer and Alzheimer's disease. SK has two isoforms: SK1 and SK2. SK1 inhibition reduces tumor growth (Pitman et al., 2016). In contrast, the function of SK2 is not fully established and, in some cases, it exhibits an anti-apoptotic effect (Hait et al., 2009). Therefore, the development of an inhibitor with SK1 or SK2 selectivity is required. Most SK inhibitors developed so far exhibit a sphingolipid structure with a long aliphatic chain (Figure 1). SK inhibitors such as SK1-I and *N*,*N*-dimethylsphingosine (DMS), which were developed in the early years of study, are derived from sphingosine (Paugh et al., 2008; Yatomi et al., 1996). These inhibitors have been synthesized by introducing various head groups into the structure of sphingosine or FTY720 (Fingolimod) (Brinkmann et al., 2010) leading to unique biological effects depending on their structure. For example, RB-005 and (*R*)-FTY720-OMe (ROME) selectively inhibit SK1 and SK2, respectively (Figure 1) (Baek et al., 2013; Lim et al., 2011). Pharmaceutical companies such as Merck, Pfizer, and Apogee have developed non-lipid SK inhibitors via library screening methodologies. The 4-pyridinemethyl 3-(4'-chlorophenyl)-adamantane-1-carboxamide (ABC294640), developed by Apogee Biotechnology, has Ki values of 10 μM for SK2 inhibition, for selective inhibition of SK2 and reduced formation of intracellular S1P in cancer cells (French et al., 2010). ABC294640 lowered the S1P levels in a first-in-human clinical trial of patients diagnosed with advanced solid tumors, and a stable disease was observed in patients with various solid tumors (Britten et al., 2017).

PF-543 is developed by Pfizer and the most potent SK1 inhibitor. PF-543 is a non-sphingosine analogue inhibitor targeting SK1, with a Ki of 4.3 nM and 130-fold selectivity for SK1 over SK2 (Schnute et al., 2012). Since the development of PF-543, animal studies of various diseases have shown its effectiveness in multiple sclerosis, myocardial infarction, and colorectal cancer (Pyne et al., 2016; Zhang et al., 2016; Ju et al., 2016). These results encouraged studies into PF-543, leading to the identification of the crystal structure of SK1 with PF-543 (Wang et al., 2014). Recently, Pfizer published the first study of PF-543 derivatives. The inhibitory effect of PF-543 derivatives was significantly reduced against SK1 and SK2, in which the phenylsulfonyl tail was modified with a methylsulfonyl group (Schnute, 2017). This result demonstrates the importance of phenylsulfonyl tail in the design of PF-543 derivatives. Studies involving PF-543 and its potential for medicinal application have prompted the investigation of the intracellular mechanisms of PF-543 inhibition of SK1.

2. Results and discussion

2.1. Synthesis of dansyl-PF-543 (2)

We synthesized labeled PF-543 for biochemical studies involving SK1. The fluorescent labels commonly used to investigate lipid metabolism include BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene), NBD (7-nitrobenz-3-diazol-4-yl), and dansyl (5-(dimethylamino)naphthalene-1-sulfonyl) groups (Schwarzmann et al., 2014; Nussbaumer, 2008; Wiegand et al., 2003). Based on the crystal structure of PF-543 and the activity of the derivatives, it was considered appropriate to label the tail portion of the PF-543 structure with a dansyl group containing an aromatic group similar to the sulfonyl group, with a similar mechanism of action. The synthesis of dansyl-PF-543 (**2**) is described in Scheme 1, as follows. Compound **3** was obtained by protecting the hydroxyl group of 3,5-dimethylphenol with an acetyl. Brominated compound **4** was obtained using NBS and microwave radiation. Addition of azide to compound **4** and hydrolysis of acetate **5** yielded compound **6**. Compound **8** was synthesized by the addition of *p*-xylylene dibromide and prolinol to compound **6**. Compound **8** was hydrogenated and reacted with the dansyl group to synthesize the final dansyl-PF-543 (**2**). Overall, the 8-step synthetic route (five purification steps) was used to make dansyl-PF-543 and we obtained 41 mg of final product **2** (overall yield, 27%). This pathway may be used for the synthesis of amine-added PF-543 derivatives based on compound **9** or the fluorescence-based assay of sphingosine kinases.

*2.2. SK1 inhibitory activity of PF-543 (**1**) and dansyl-PF-543 (**2**)*

To examine whether dansyl labeling of PF-543 affects SK1 inhibitory activity, we compared the IC_{50} of PF-543 (**1**) and dansyl-PF-543 (**2**). PF-543 and dansyl-PF-543 inhibited SK1 activity with an IC_{50} value

10.4 ± 3.2 nM and 12.3±2.5 nM, respectively (Figure 2). The similar SK1 inhibitory effect of dansyl-PF-543 and PF-543 demonstrated that the benzenesulfonyl group of PF-543 can be replaced by a dansyl group.

*2.3. Docking study of dansyl-PF-543 (**2**)*

A docking study was conducted to confirm the structural similarity between the dansyl-PF-543 and PF-543 (Figure 3). Hydroxymethyl pyrrolidine (containing OH and N of pyrrolidine) of dansyl-PF-543 contains a hydrogen bond with Asp264 and shows electrostatic interaction (protonated amine form). Pyrrolidine-linked phenyl groups are located between Phe278 and Ile260 and show hydrophobic interaction. The phenyl moiety in the methyl sulfonyl group shows hydrophobic interaction with Phe259, Leu286, Ile385, and Phe389. The terminal dimethyl-naphthalene-amine group of dansyl-PF-543 is located in the pocket formed by Leu347, Leu354, Ala360, Phe374, His397, and Leu405 via hydrophobic interaction. The results of this docking study show that synthetic dansyl-PF-543 binds similar to PF-543.

*2.4. UV–Vis and fluorescence spectroscopy of dansyl-PF-543 (**2**)*

The maximum absorption and emission wavelength of dansyl-PF-543 were 329 nm and 470 nm, respectively (Figure 4). The quantum yields of dansyl-PF-543 was 0.11 (the standard used Fluorescein in 0.1 N NaOH at 366 nm, Q.Y = 0.96). Maximum emission wavelengths of fluorescence labeled-sphingolipids BODIPY-FTY720 (Li and Bittman, 2007) and NBD-S1P (Billich and Ettmayer, 2004) were normally near 500 nm. Dansyl-PF-543 showed appropriate emission wavelength for the biological study. Therefore, it represents a useful tool for analysis of the biological action of sphingosine kinase 1.

3. Experimental procedures

3.1. General synthetic methods

All chemicals were reagent grade and used as purchased. Reactions were run under nitrogen and monitored by TLC using silica gel 60 F254 aluminum-backed plates. Flash column chromatography was performed with silica gel grade 60 (230−400 mesh). All solvents were of anhydrous quality and used as received. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance I spectrometer, and chemical shifts were reported in δ units relative to deuterated solvents, which served as internal references at 400 and 100 MHz, respectively. High-resolution mass spectra were recorded on an Agilent Technologies G6520A Q-TOF mass spectrometer using electrospray ionization (ESI). All compounds were ≥95% pure based on their HRMS and 1H NMR spectra.

3.2. 3,5-Dimethylphenyl acetate (**3**)

To a solution of 3,5-dimethylphenol (1 g, 0.0082 mol) in pyridine (15 mL) was added acetic anhydride (3.87 mL, 0.041 mol) at room temperature. After being stirred at room temperature for 12 h, the reaction mixture was evaporated, diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. Flash column chromatography with hexane/EtOAc (20:1) as the eluent gave compound **3** (Schnute et al., 2012) (1.3 g, 98%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (s, 1H), 6.69 (s, 2H), 2.31 (s, 6H), 2.27 (s, 3H).

3.3. 3-(Azidomethyl)-5-methylphenol (**6**)

To a solution of **3** (200 mg, 1.22 mmol) in EtOAc (10 mL) was added *N*-bromosuccinimide (217 mg, 1.22 mmol). The reactant was irradiated in a microwave oven at 100 °C, 250 W for 20 min. The reaction mixture was evaporated, diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. To a solution of crude compound **4** (350 mg, 1.44 mmol) mixture in DMF (10 mL) was added sodium azide (281 mg, 4.32 mmol). After the reaction mixture was heated at 80 °C for 12 h, saturated aqueous ammonium chloride solution was added, and the mixture was extracted with EtOAc. Crude compound **5** was dissolved in MeOH (10 mL), and aq. $NaHCO_3$ (1 mL) was added. After being stirred at room temperature for 2 h, the reaction mixture was evaporated, diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. Flash column chromatography with hexane/EtOAc (5:1) as the eluent gave compound **6** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.68 (s, 1H), 6.61 (s, 1H), 6.58 (s,

1H), 4.22 (s, 2H), 2.29 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.8, 140.4, 136.9, 121.5, 116.1, 112.2, 54.6, 21.3; ESI-HRMS $(M+H)^+$ m/z calcd for $C_8H_{10}N_3O$ 164.0824, found 164.0833.

3.4. 1-(Azidomethyl)-3-((4-(bromomethyl)benzyl)oxy)-5-methylbenzene (**7**)

To a solution of compound **6** (73 mg, 0.45 mol) in DMF (10 mL) was added sodium hydride (54 mg, 1.34 mmol) and *p*-xylylene dibromide (590 mg, 2.24 mmol) at room temperature. After being stirred at room temperature for 4 h, the reaction mixture was evaporated, diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. Flash column chromatography with hexane/EtOAc (20:1) as the eluent gave compound **7** (127 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (s, 4H), 6.75 (s, 1H), 6.72 (s, 2H), 5.03 (s, 2H), 4.49 (s, 2H), 4.25 (s, 2H), 2.32 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.8, 140.1, 137.4, 137.2, 136.6, 129.4, 127.9, 121.6, 115.5, 111.6, 69.6, 54.9, 33.1, 21.4; ESI-HRMS $(M+H)^+$ m/z calcd for $C_{16}H_{17}BrN_3O$ 346.0555, found 346.0569.

3.5. (R)-(1-(4-((3-(Azidomethyl)-5-methylphenoxy)methyl)benzyl)pyrrolidin-2-yl)methanol (**8**)

To a solution of **7** (19 mg, 0.055 mmol) in acetonitrile (3 mL) was added (*R*)-(-)-prolinol (17 mg, 0.16 mmol). The reaction mixture was stirred at 50 °C for 12 h and concentrated. Purification by silica gel chromatography, eluting with CH_2Cl_2/MeOH (10:1), gave 16 mg (78%) of compound **8** as a slightly yellow waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (dd, *J* = 8.4, 12.0 Hz, 4H), 6.75 (s, 1H), 6.71 (s, 2H), 5.02 (s, 2H), 4.24 (s, 2H), 4.05 (d, *J* = 13.1 Hz, 1H), 3.67 (dd, *J* = 11.2, 3.2 Hz, 1H), 3.57–3.49 (m, 2H), 3.10–3.06 (m, 1H), 2.90–2.88 (m, 1H), 2.40 (dd, *J* = 17.3, 8.7 Hz, 1H), 2.31 (s, 3H), 2.01–1.61 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.1, 140.1, 136.6, 136.3, 129.6, 127.8, 121.6, 115.5, 111.5, 69.6, 65.1, 61.7, 58.4, 54.8, 54.3, 27.5, 23.4, 21.5; ESI-HRMS $(M+H)^+$ m/z calcd for $C_{21}H_{27}N_4O_2$ 367.2134, found 367.2192.

3.6. (R)-5-(Dimethylamino)-N-(3-((4-((2-(hydroxymethyl)pyrrolidin-1-yl)methyl)benzyl)oxy)-5-methylbenzyl)-naphthalene-1-sulfonamide (**2**)

To a solution of compound **8** (37 mg, 0.10 mmol) in benzene/H_2O (10/1, 5 mL) was added triphenylphosphine (41 mg, 0.20 mmol). The reaction mixture was heated at 60 °C for 12 h and concentrated. To a solution of the reaction mixture **9** (0.10 mmol) in acetonitrile (4 mL) was added triethylamine (0.5 mL) and dansyl chloride (54 mg, 0.20 mmol). The reaction mixture was stirred at 50 °C for 12 h and concentrated. Purification by silica gel chromatography, eluting with CH_2Cl_2/MeOH (10:1), gave 41 mg (72%) of compound **2** as a slightly yellow waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, *J* = 8.6 Hz, 1H), 8.29 (d, *J* = 8.6 Hz, 1H), 8.24 (dd, *J* = 7.3, 1.0 Hz, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.49 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 2H), 7.29 (d, *J* = 8.1 Hz, 2H), 7.16 (d, *J* = 7.5 Hz, 1H), 6.58 (s, 1H), 6.43 (s, 2H), 5.10 (br, OH), 4.77 (s, 2H), 4.20 (d, *J* = 13.2 Hz, 1H), 4.01 (d, *J* = 5.3 Hz, 2H), 3.79 (d, *J* = 12.9 Hz, 1H), 3.75–3.60 (m, 2H), 3.27 (dd, *J* = 17.9, 7.1 Hz, 1H), 3.15 (dd, *J* = 19.8, 9.3 Hz, 1H), 2.85 (s, 6H), 2.71–2.58 (m, 1H), 2.15 (s, 3H), 2.03–1.94 (m, 1H), 1.93–1.75 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.6, 152.0, 139.8, 137.7, 134.7, 130.6, 130.5, 129.9, 129.8, 129.7, 129.6 (2C), 128.5 (2C), 127.8 (2C), 123.2, 121.2, 118.8, 115.6, 115.1, 110.6, 69.3, 61.5, 60.4, 58.7, 54.4, 53.5, 47.3, 45.4, 29.7, 23.2, 21.2; ESI-HRMS $(M+H)^+$ m/z calcd for $C_{33}H_{40}N_3O_4S$ 574.2740, found 574.2724.

3.7. Sphingosine kinase activity assay

Sphingosine kinase 1 inhibitory effect was measured with (0.40 μM PF-543 (**1**) and dansyl-PF-543 (**2**) using 100 μM sphingosine, 10 μM ATP and 0.5 ng/μl of recombinant sphingosine kinase 1. SK1 activity was detected with an Echelon's Sphingosine Kinase Activity Assay kit (Echelon Biosciences Incorporated) according to the manufacturer's protocol. The IC_{50} was calculated using Sigma plot software.

3.8. Molecular modeling of dansyl-PF-543 against SK1

Molecular modeling of dansyl-PF-543 against SK1 was performed using Induced Fit Docking (IFD) protocol as implemented in Schrödinger Suite 2017-2 (Schrödinger, LLC, http://www.schrodinger.com). The PF-543-bound crystal structure of SK1 obtained from the Protein Data Bank (http://www.rcsb.org/pdb) showed that PDB code was 4V2414 (Wang et al., 2014). The grid box of receptor was set using the bound PF-543 and all constraints were set to default value. The Glide SP (Standard Precision) mode docking process was used for the identification of initial docking pose, and 20 ligand poses were generated. A van der Waals scaling of 0.7 and 0.5 were used for the protein and ligand non-polar atoms, respectively. The trim side chains were set automatically. In addition, only the residues within 5.0 Å of the ligand were refined using Prime Refinement. The refined complexes were ranked by Prime energy, and the receptors within 30 kcal/mol of the minimum energy structure were selected for a Glide Redocking step. The binding pose with the lowest IFD score structure (-770.542 kcal/mol) was selected and the docking pose was determined using Discovery Studio 2016 (http://www.biovia.com).

3.9. UV–Vis and fluorescence spectroscopy

UV–Vis spectra were recorded at 25 °C in a 10-mm path quartz cell using a Cary 100 UV–Vis spectrophotometer (Agilent, Santa Clara, CA, USA) with pure solvent as a reference. Fluorescence spectra were recorded at 25 °C using a Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA, USA) (cell path length: 1 cm). The fluorescence quantum yields (ΦF) were determined using a 0.1-M aqueous NaOH solution of fluorescein as a standard (Brouwer, 2011).

Conflict of interest: None

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Figure captions

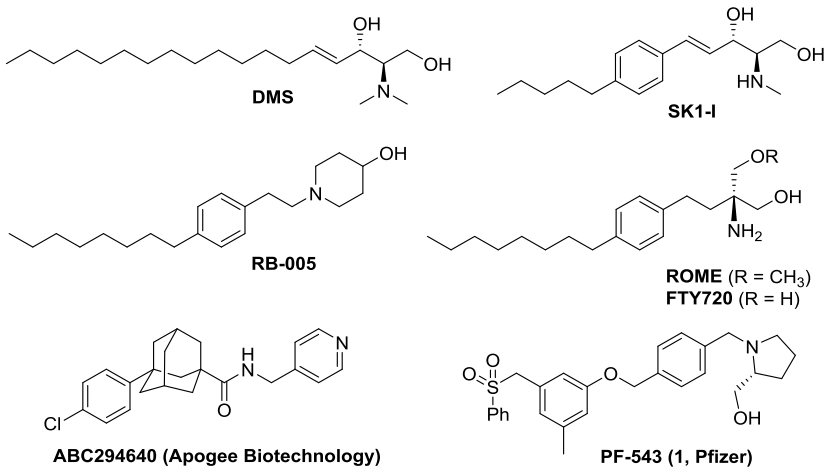


Figure 1. Known sphingosine kinase inhibitors.

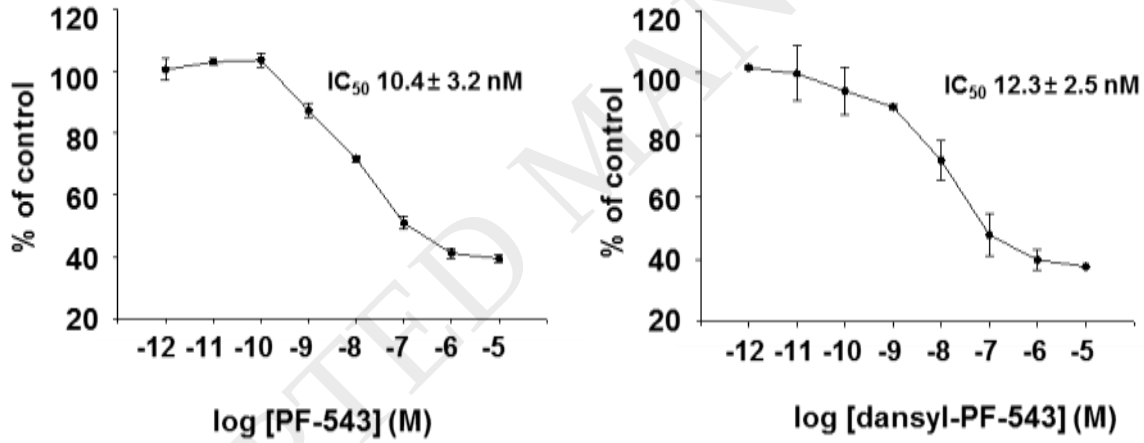


Figure 2. Effect of PF-543 (**1**) and dansyl-PF-543 (**2**) on SK1 activity (n = 2-3 for each compound, results are expressed as % of control ± S.D.).

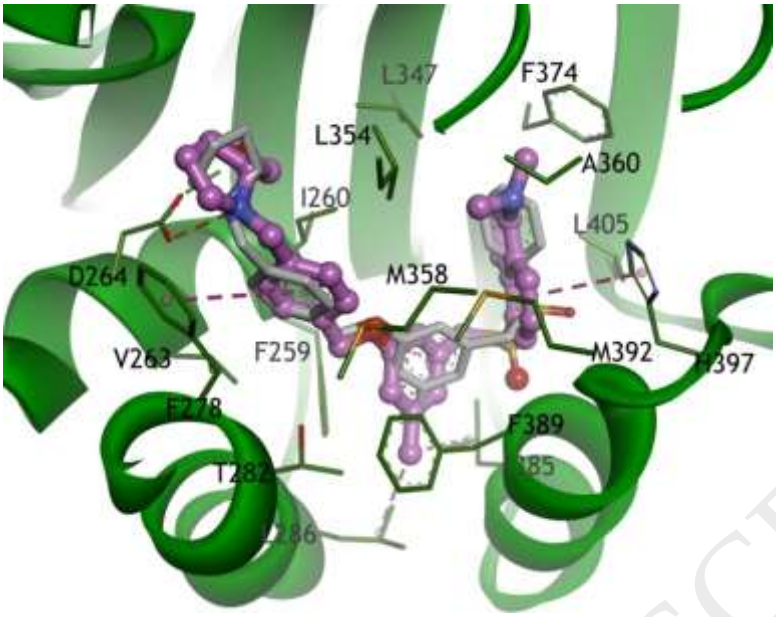


Figure 3. Predicted binding model of dansyl-PF-543 (pink, ball and stick model) superimposed with x-ray binding conformation of PF-543 (gray, stick model) to SK1 (green ribbon model). The SK1 crystal structure in complexed with PF-543 was obtained from the Protein Data Bank (PDB code 4V24). The hydrogen bonds are shown as a green dashed line and electrostatic interactions are displayed as an orange dashed line. Also the hydrophobic interactions are shown as a pink dashed line. For clarity, only the key residues are visible in stick model and are labeled using the 1-letter amino acid code.

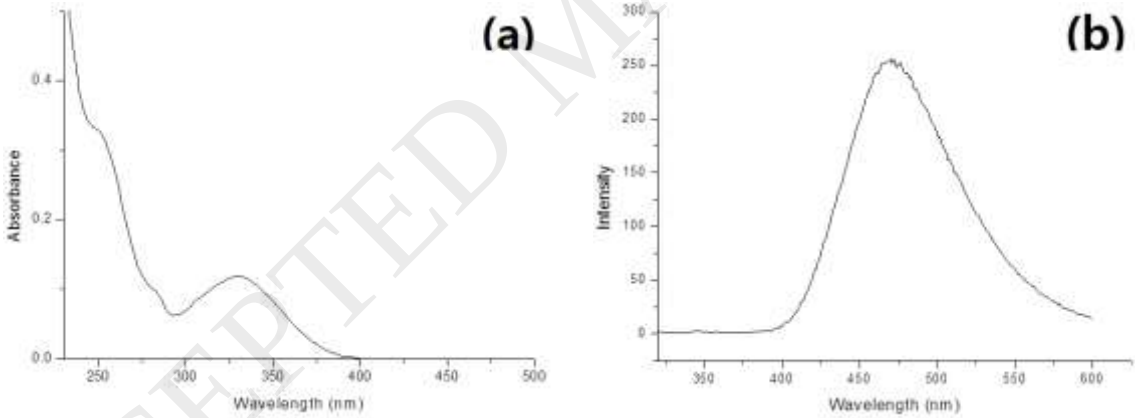
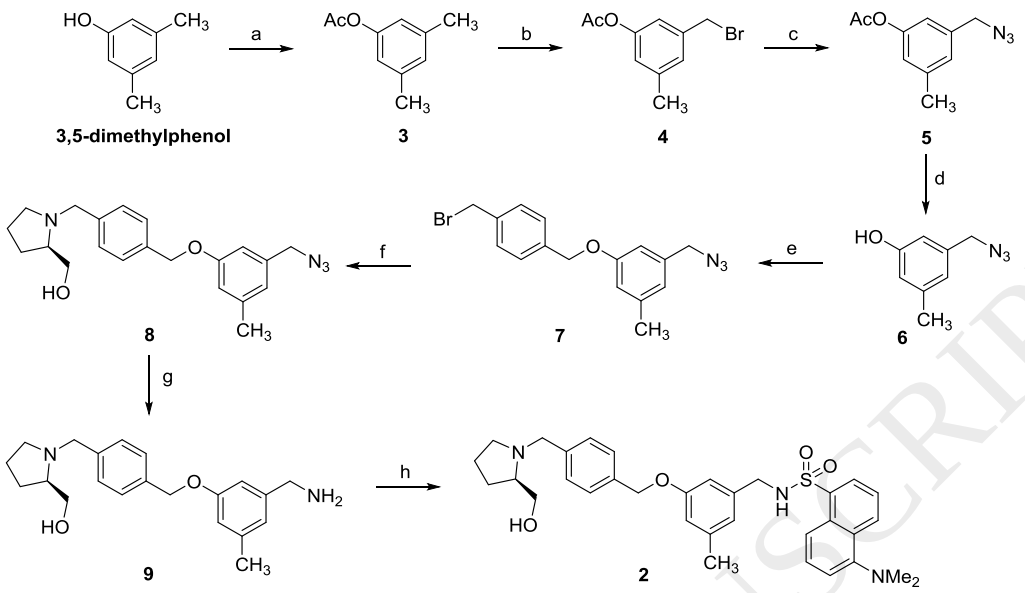


Figure 4. (a) Absorption spectra of dansyl-PF-543 (50 μM) in MeOH; (b) Emission spectra of dansyl-PF-543 (50 μM).

Scheme captions



Scheme 1. Synthesis of Dansy-PF-543 (**2**). Reagents and conditions: a) Ac_2O, pyridine, rt, 12 h; b) NBS, EtOAc, MW., 150 ºC, 250 W, 20 min.; c) NaN_3, DMF, 80 ºC, 12 h; d) aq. $NaHCO_3$, MeOH, rt, 2 h; e) *p*-xylylene dibromide, NaH, DMF, rt, 4 h; f) (*R*)-(-)-prolinol, MeCN, 50 ºC, 12 h; g) PPh_3, benzene/H_2O, 60 ºC, 12 h; h) dansyl chloride, NEt_3, MeCN, 50 ºC, 12 h.