The structural assembly switch of cell division protein FtsZ probed with fluorescent allosteric inhibitors

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Supplementary Information

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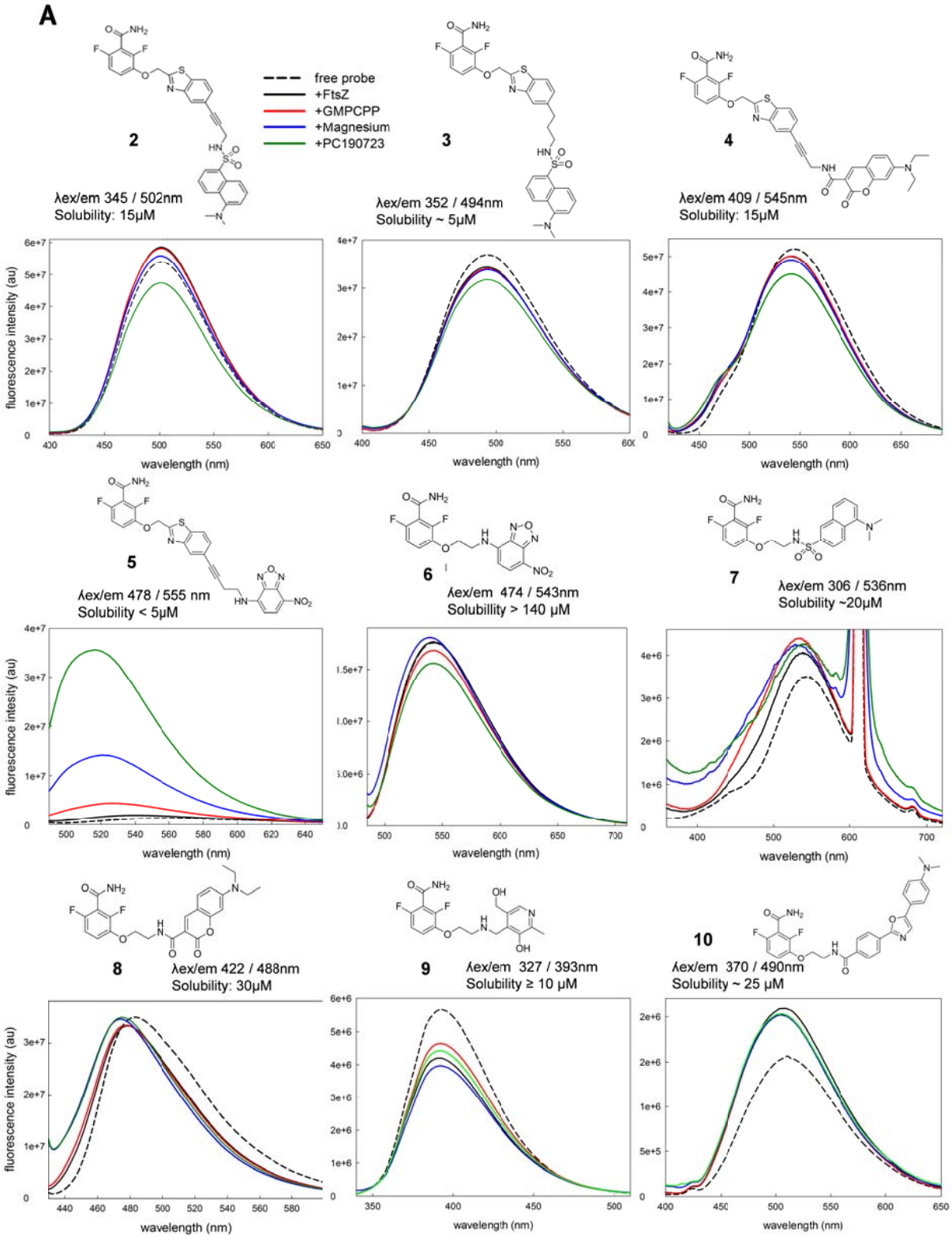
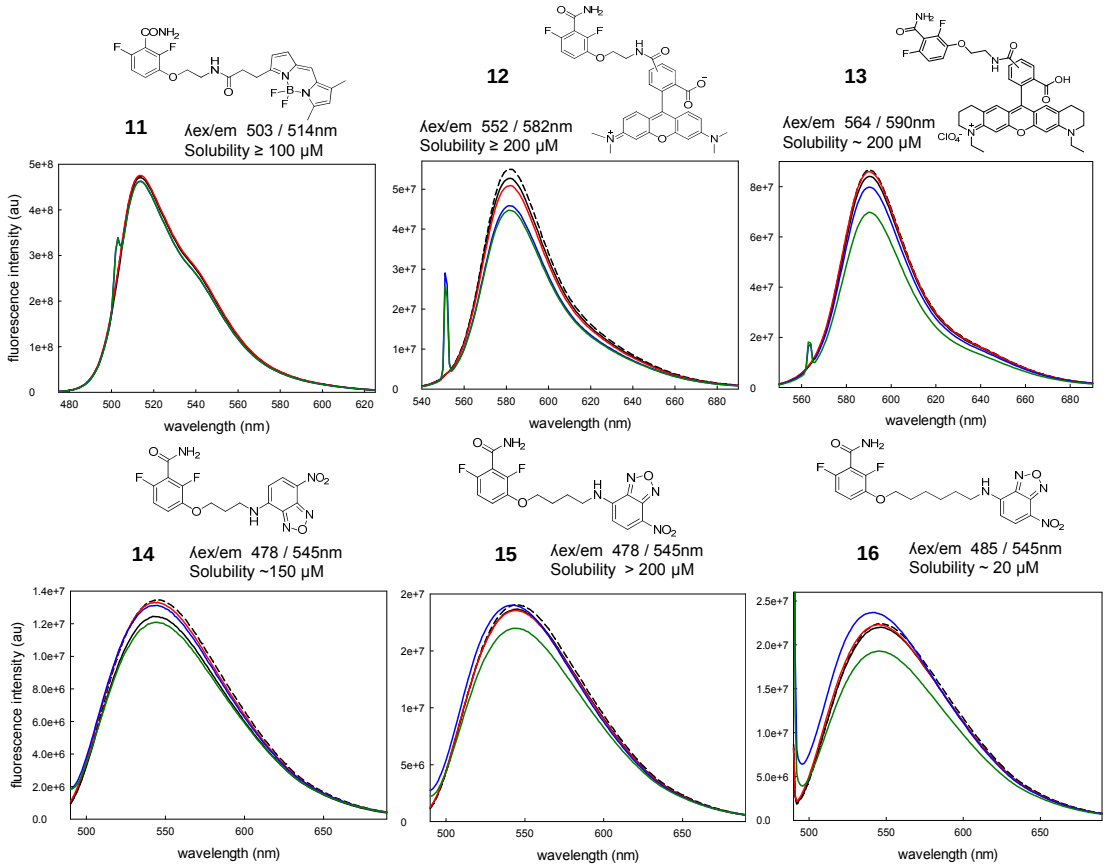
1. Supplementary Figures S1-S10





B

	ANISOTROPY (r)											
PROBE	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
probe	**0.083 0**	**.154 0.**	**017**	**0.130**	**0.026 0.**	**018**	**0.125**	**0.024 0.**	**213**	**0.0048**	**0.030**	**0.019**
+ BsFtsZ	0.085	0.164	0.017	0.203	0.034	0.026	0.133	0.023	0.212	0.0048	0.031	0.025
+ GMPCPP	0.086	0.171	0.016	0.246	0.035	0.029	0.139	0.022	0.240	0.0046	0.030	0.022
+ $MgCl_2$	**0.084 0**	**.188 0.**	**022**	**0.248**	**0.086 0.**	**020**	**0.149**	**0.028 0.**	**233**	**0.0080**	**0.032**	**0.025**
+ PC190723	0.079	0.158	0.032	0.328	0.036	0.035	0.106	0.025	0.228	0.0066	0.030	0.023
probe + GMPCPP	0.077	0.150	0.020	nd	0.026	0.018	0.100	0.022	0.234	0.0040	0.029	0.019
+ $MgCl_2$	0.077	0.170	0.040	nd	0.027	0.012	0.120	0.022	0.220	0.0037	0.030	0.019
+ PC190723	0.077	0.160	0.050	nd	0.026	0.023	0.140	0.025	0.212	0.0045	0.030	0.020

Figure S1. **Fluorescent benzamide derivatives studied in this work** . Related to Figure 1. **A**. Chemical structures, solubility in buffer, fluorescence excitation and emission maxima, and emission spectra of each compound alone (10 µM, dashed line), and following consecutive additions of 10 µM BsFtsZ (black line), 0.1 mM GMPCPP (red line), 10 mM $MgCl_2$ (blue line) and 10 µM PC190723 (green line). **B**. Corresponding fluorescence anisotropy values. The compounds collected in this table were initially excluded due to lack of specific binding to BsFtsZ in this assay, or to a high anisotropy value of the free compounds that was indicative of aggregation. The anisotropy values of selected compounds are in Figure 1C. All the measurements were performed in 50 mM Hepes-KOH, 50 mM KCl, 1 mM EDTA, pH 6.8, with 2% residual DMSO.

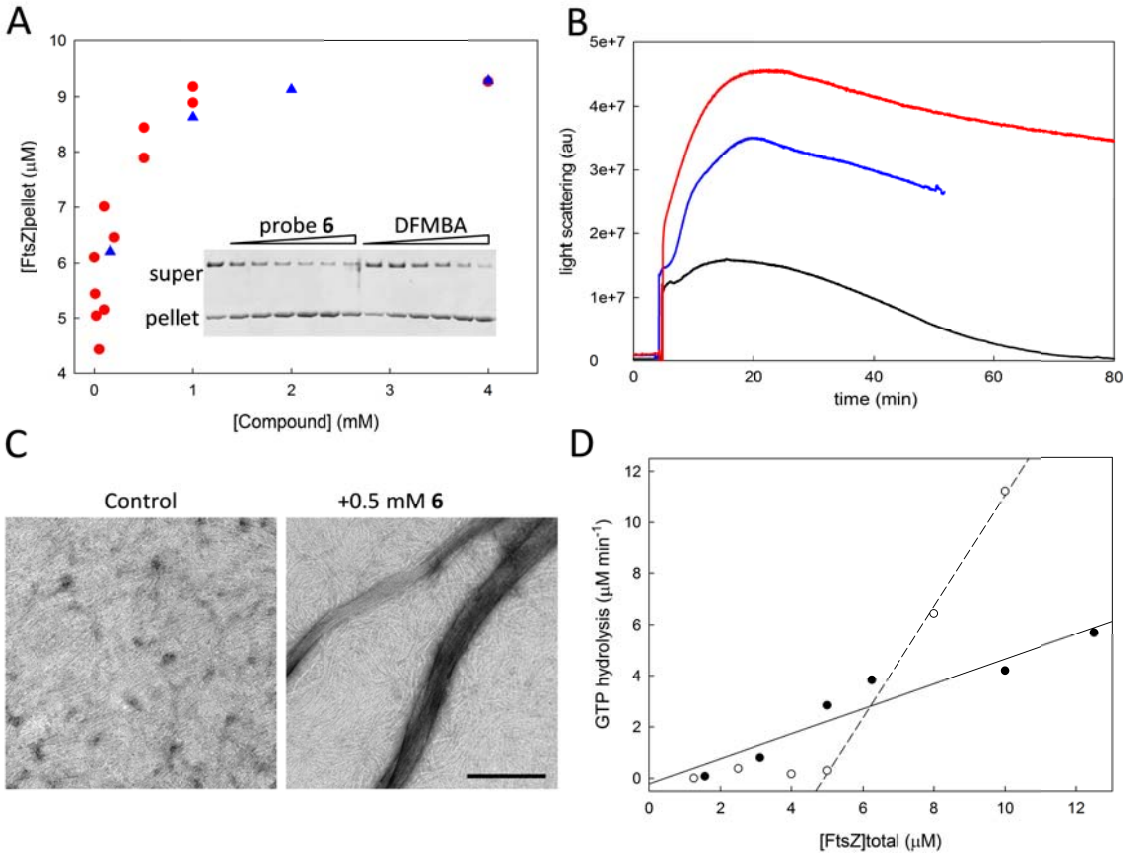


Figure S2. **Fluorescent probe 6 enhances BsFtsZ GTP-induced polymerization and inhibits the GTPase activity of BsFtsZ polymers.** Related to main text. **A**. Quantification of BsFtsZ polymerization in the presence of 2 mM GTP with increasing concentrations of DFMBA (blue triangles) or **6** (red circles), employing centrifugation and gel electrophoresis (inset). **B**. Light scattering time courses with 0.5 mM GTP in the presence of 0.5 mM DFMBA (blue line), 0.5 mM **6** (red line) and without compound (black line). **C**. Electron micrographs of BsFtsZ polymers in the absence and in the presence of 0.5 mM **6**. **D**. Rate of nucleotide hydrolysis in the presence of 2 mM GTP without compound (void circles) and in the presence of 0.2 mM **6** (black circles). Critical protein concentration values for polymerization measured under the same conditions were: **6** (0.2 mM), 3.04 ± 0.38 μM; DFMBA (4 mM), 0.96 ± 0.02 μM; control (no ligand), 4.08 ± 0.06 μM. Notice that the later value may be compared with Cr 2.8 ± 0.4 μM that we previously determined in Mes buffer (main text Ref 33), and is higher than ~1 μM Cr values in other studies under different solution conditions.

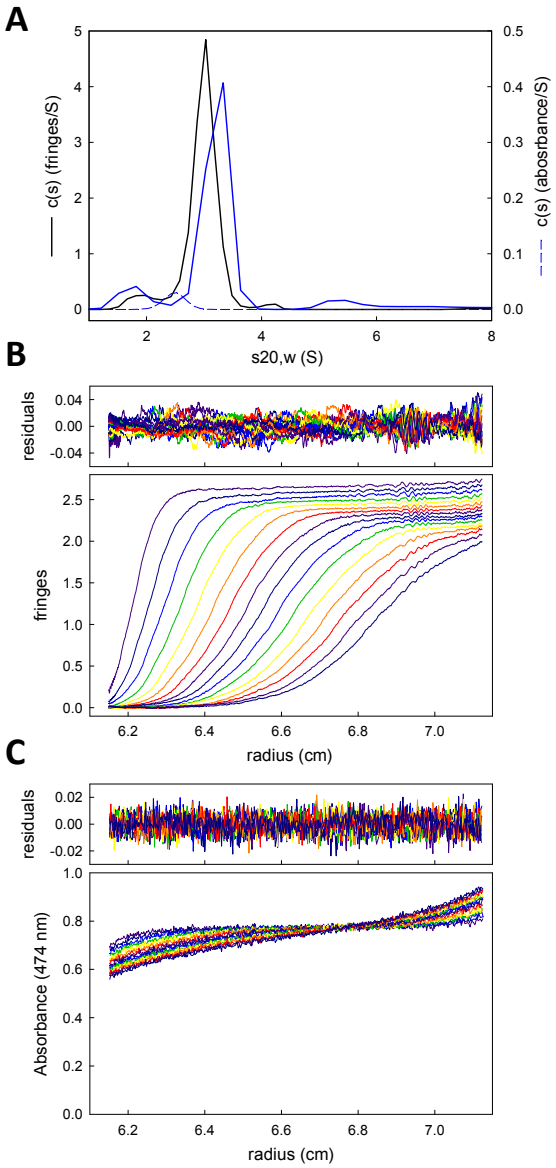


Figure S3. **BsFtsZ monomers do not bind the fluoresc ent probe 6.** Related to Figures 1 and 4. **A.** Sedimentation coefficient distribution c(s) of 20 µM FtsZ with 200 µM GDP (black line, peak average $s_{20,w}$ = 3.1 S) and with 20 µM **6** added (blue line, $s_{20,w}$ = 3.2 S). **6** followed by absorbance at 474 nm did not co-sediment with the protein (blue dashed line). The following panels show the analytical ultracentrifugation data from which the distributions in A was obtained with SEDFIT. The residuals of the fits are also shown. **B.** Successive radial interference scans showing the distribution of FtsZ in the presence of **6** during sedimentation. **C**. Corresponding radial absorbance scans showing a lack of co-sedimentation of **6** with FtsZ. These experiments with BsFtsZ and **6** were performed in 50 mM Hepes-KOH, 50 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 6.8, 2% DMSO at 25 ºC. For a positive control, see similar experiments showing co-sedimentation of ligands binding to the GTP site of BsFtsZ monomers (see Figure 3 in Ruiz-Avila *et al.*[1]).

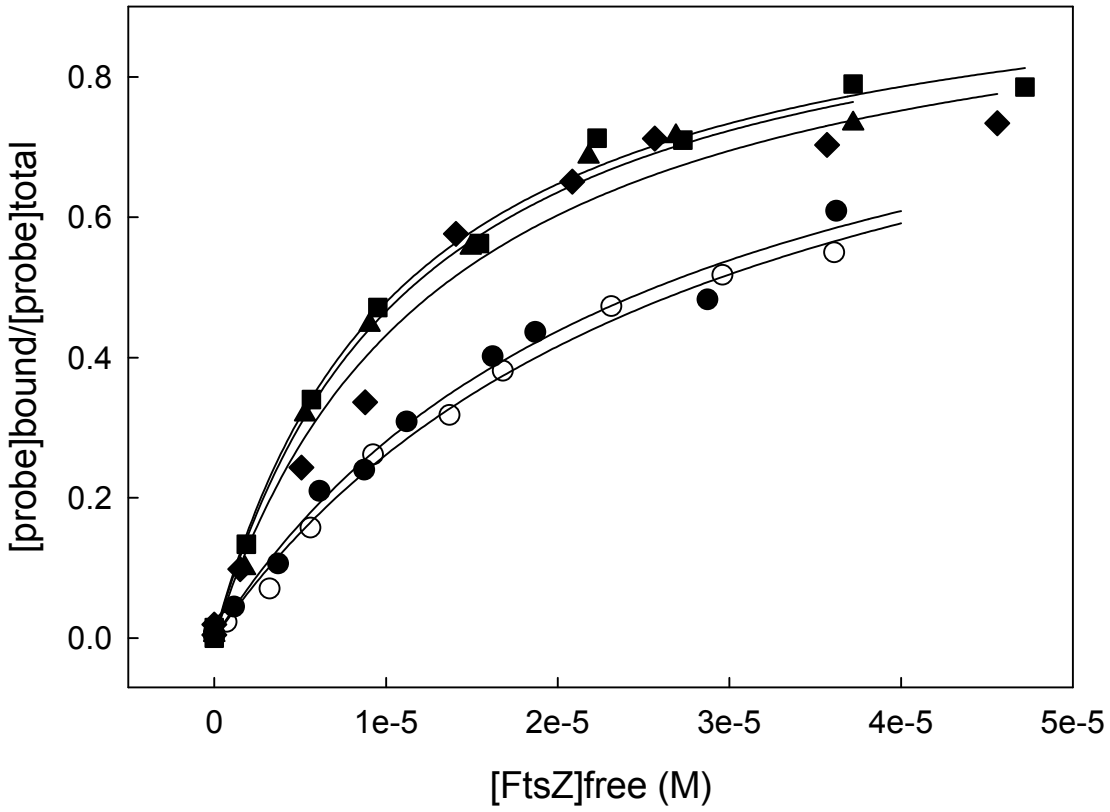


Figure S4. **Binding curves of fluorescent benzamide probes by BsFtsZ polymers.** Related to main text. Titration curves of 3 μM **6** (filled circles), **14** (diamonds), **15** (squares) and **16** (triangles) with BsFtsZ polymer binding sites formed with 0.1 mM GMPCPP, and titration of **6** with BsFtsZ polymers formed 2mM GTP (void circles). Binding was measured from the ligand fluorescence anisotropy change and the lines are best model fits to the data (see Methods).

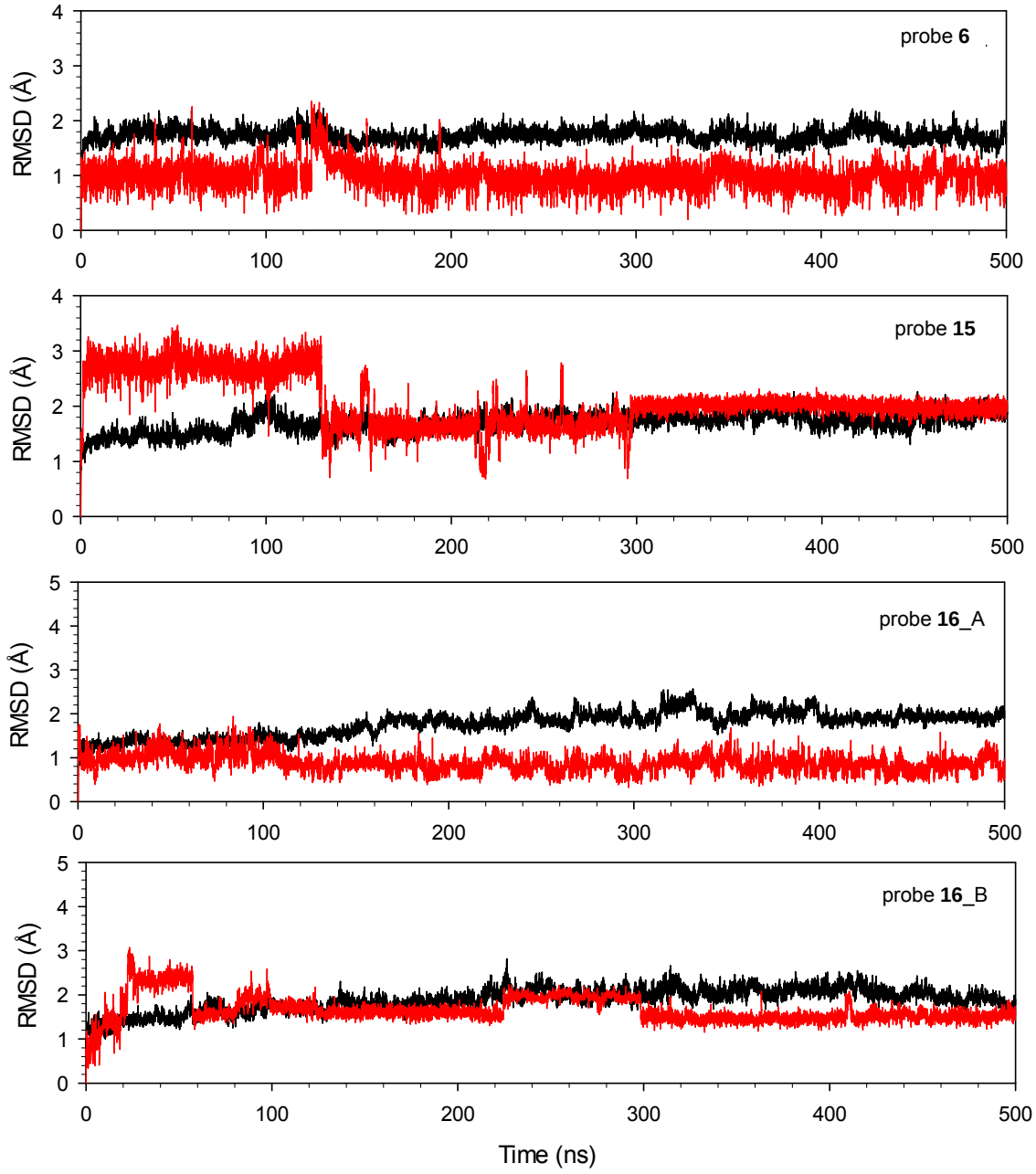


Figure S5 . MD trajectories of th e docked DFB A-NBD complexes. Related to Figure 2. RMSD evaluated along the trajectories for the SaFtsZ (black lines) and fluorescent probes (red lines). Notice how the simulations reached a steady state suggesting the stability of the binding model. However, there is an initial relaxing period (up to 130 ns) in where the docking poses usually accommodate the NBD moiety to the final equilibrium position. See also the corresponding supporting videos of the entire trajectories to appreciate the initial relaxation periods and the relative stability of the probes inside the active sites localized along the cleft.

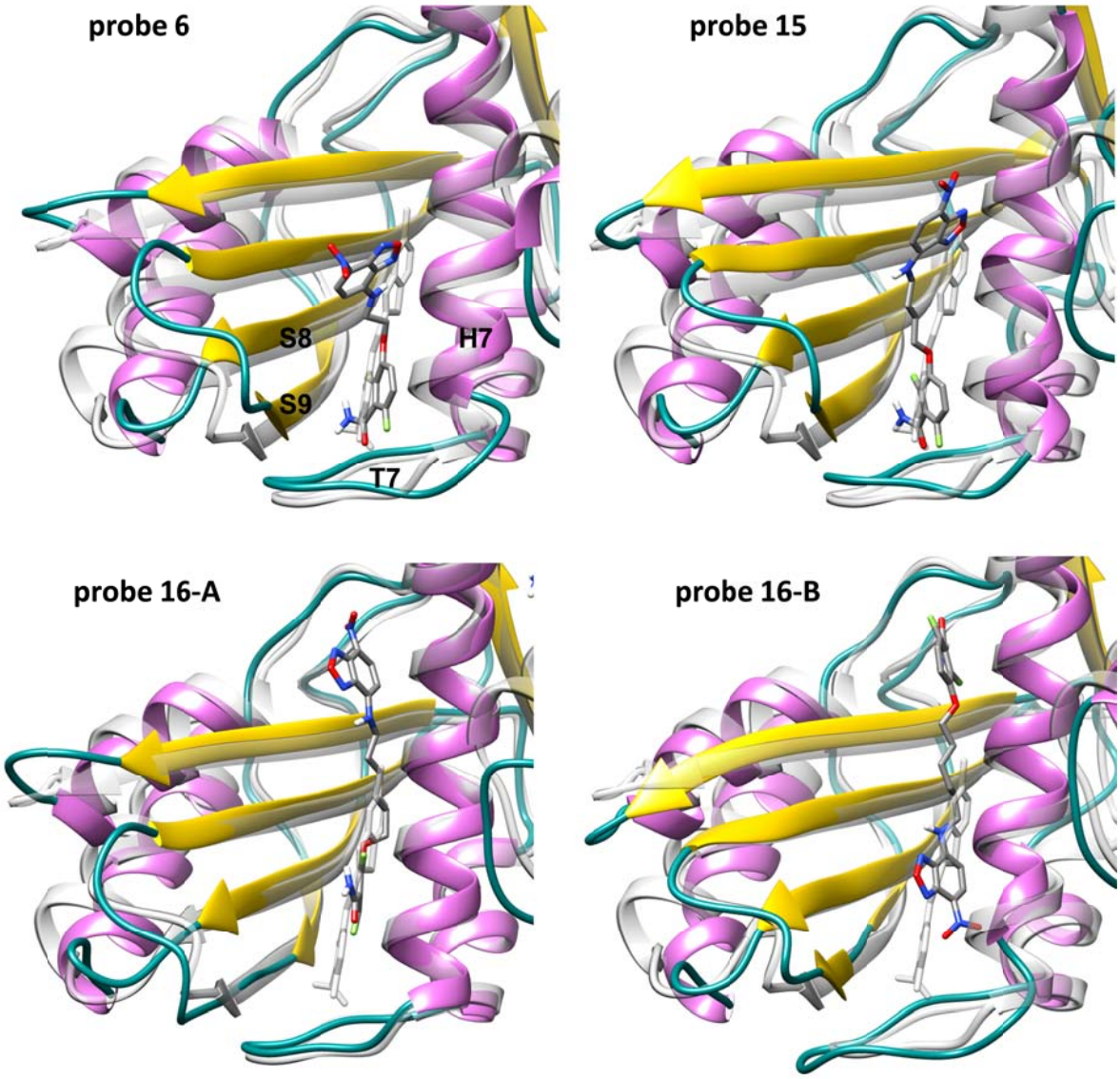


Figure S6. Binding modes of the fluorescent probes. Related to Figure 2. MD snapshots of the final equilibrated SaFtsZ complexes with **6**, **15** and **16** are superimposed with the crystallographic PC190723-bound conformation (light gray).

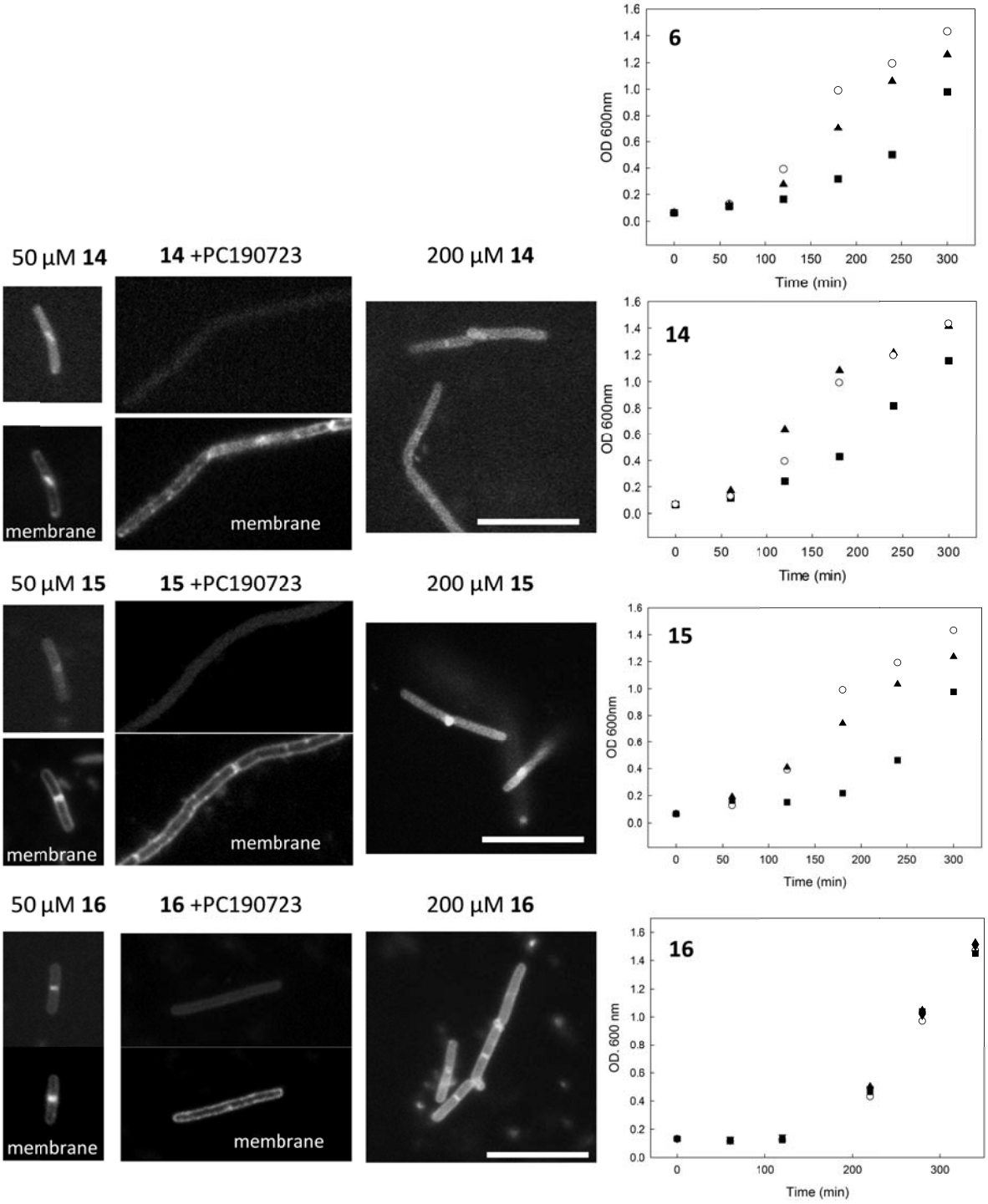


Figure S7. FtsZ polymer imaging in growing B. subtilis cells with NBD-benzamide probes. Related to main text. Representative images of *B. subtillis* 168 cells visualized by fluorescence microscopy after 1 h incubation with 50 µM NBD-benzamide probes, after addition of 25 µM PC190723 and 1 h more incubation, or with 200 µM fluorescent probe (2 h). Bars, 10 µm. Also shown are the growing curves of *B. subtillis* in the absence (circles) or presence of 50 µM (triangle up), 100 µM (triangle down) or 150 µM (square) of each probe.

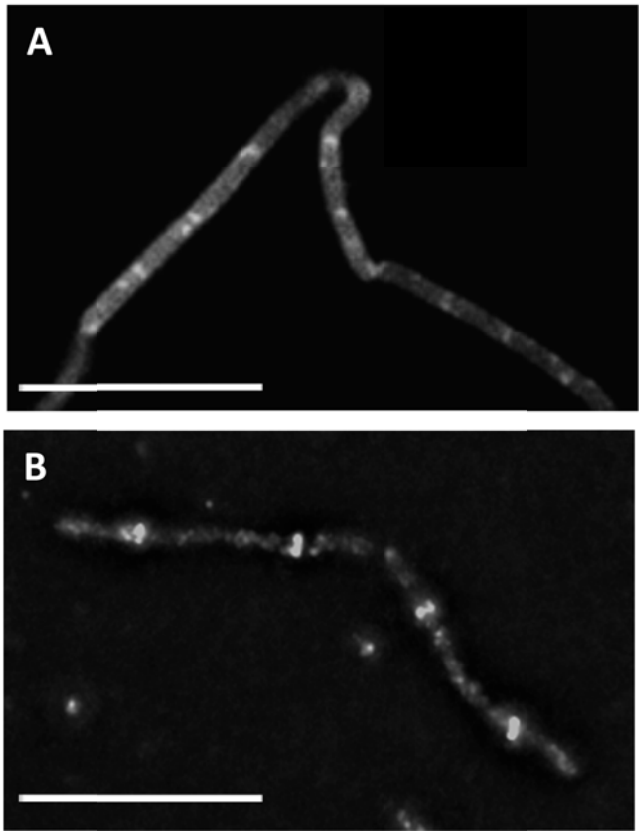


Figure S8. Effects of a small molecule inhibitor on cellular FtsZ localization imaged with probe 6. Related to main text. *B. subtillis* 168 were incubated with 4 μM compound **28**[2] for 150 min, then 50 μM probe **6** was added and incubated 30 min more, then cells were directly visualized by fluorescence microscopy (**A**) or by indirect immunofluorescence with anti-FtsZ antibodies (**B**). Bars, 10 μm.

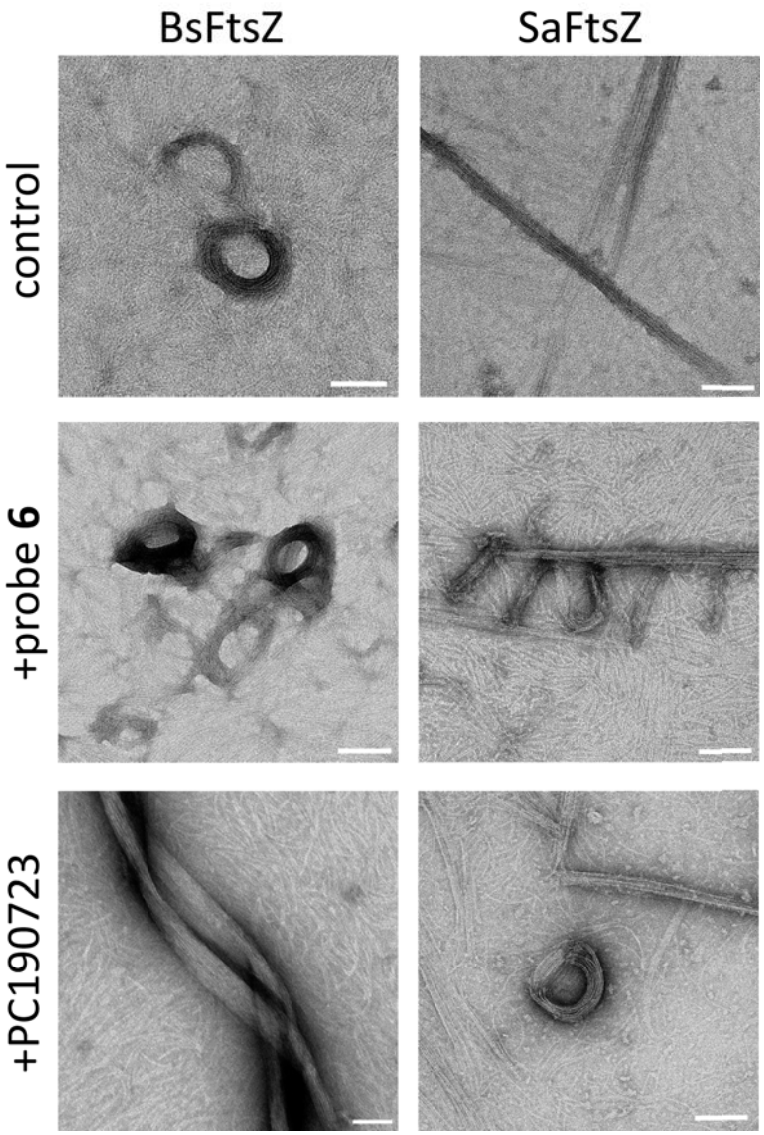


Figure S9. Electron micrographs of FtsZ assembly products with GMPCPP, probe 6 and PC190732. Related to Figure 4. BsFtsZ and SaFtsZ (10 μM) polymers formed in the presence of 0.1 mM GMPCPP without compound, with 10 μM **6** or with 10 μM PC190723. Experiments with BsFtsZ were performed in 50 mM Hepes-KOH, 50 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 6.8, 2% DMSO at 25 ºC. Experiments with SaFtsZ were performed in 50 mM Mes-KOH, 50 mM KCl, 1 mM EDTA, 6 mM $MgCl_2$, pH 6.5, 2% DMSO at 25 ºC. Bars, 100 nm.

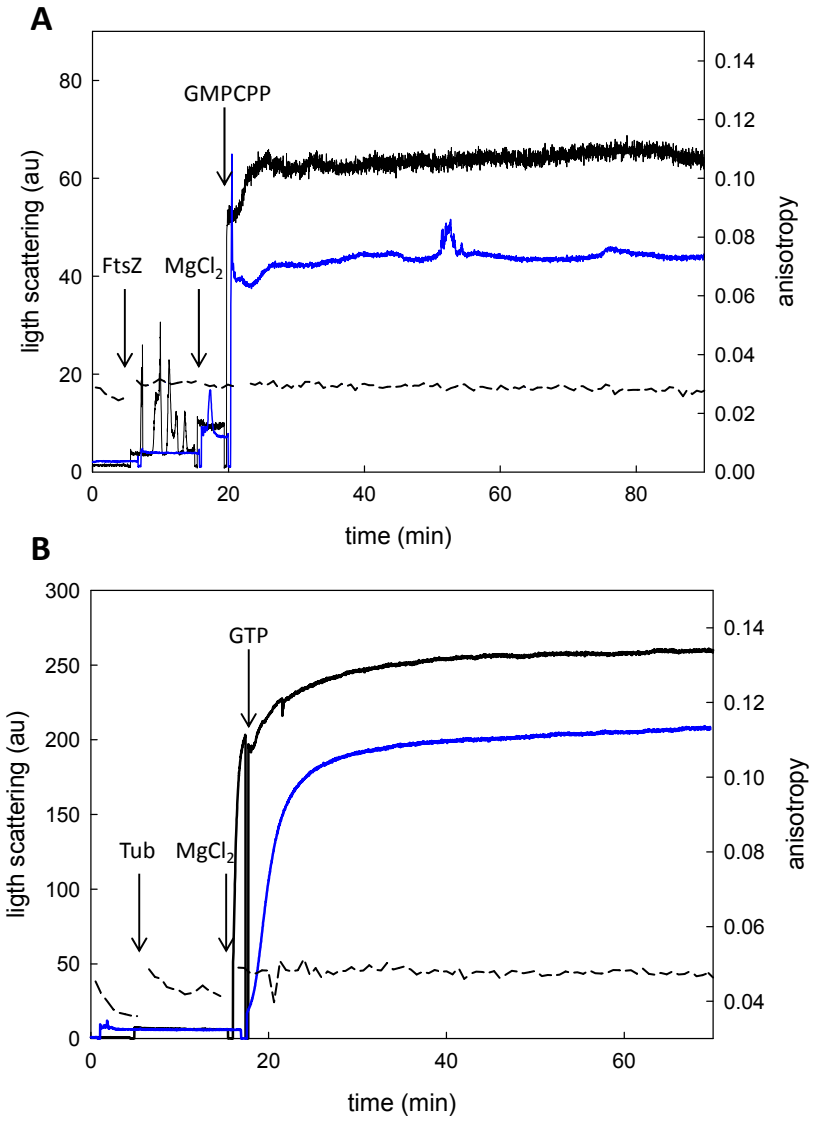


Figure S10. The fluore scence anisotropy of the ben zamide probe 6 is insensitive to assembly of non-suscep tible EcFts Z and of the eukaryotic homolog t ubulin. Controls related to Figure 4. 10 µM EcFtsZ (**A**) and 15 µM tubulin (**B**) assembly followed by light scattering in the absence (black line) and in the presence of 10 µM **6** (blue line). At the times indicated with arrows, protein, $MgCl_2$ and 0.9 mM GTP or 0.1 mM GMPCPP were added. The assay was exactly repeated and the anisotropy of **6** was recorded during the time course (dashed line). The experiments with EcFtsZ were performed in 50 mM Hepes-KOH, 50 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 6.8, 2% DMSO at 30 ºC. Experiments with tubulin were performed in GAB buffer, 6 mM $MgCl_2$, 2% DMSO at 37 ºC.

2. Materials and Methods

2.1. Chemistry

2.1.1. General Experimental Details

Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Abcr, Acros, ATTO-TEC, Lumiprobe, Scharlab, Sigma-Aldrich, or Thermo Fisher Scientific, and were used without further purification. All non-aqueous reactions were performed under an argon atmosphere in oven-dried glassware. Tetrahydrofuran (THF) and dichloromethane (DCM) were dried using a Pure SolvTM Micro 100 Liter solvent purification system. Triethylamine was dried over KOH and distilled before using. Reactions under microwave (MW) irradiation were performed in a Biotage Initiator 2.5 reactor. Reactions were monitored by analytical thin-layer chromatography (TLC) on plates supplied by Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light (254 nm) or 10% phosphomolybdic acid solution in EtOH. Flash chromatography was performed on a Varian 971-FP flash purification system using silica gel cartridges (Varian, particle size 50 μm).

Melting points (mp, uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Infrared (IR) spectra were measured on a Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200-650 cm^{-1} transmission range; frequencies (ν) are expressed in cm^{-1}. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance III 700 MHz (^{1}H, 700 MHz; ^{13}C, 175 MHz), Bruker Avance 500 MHz (^{1}H, 500 MHz; ^{13}C, 125 MHz), or Bruker DPX 300 MHz (^{1}H, 300 MHz; ^{13}C, 75 MHz) instruments at the Universidad Complutense de Madrid (UCM) NMR core facilities. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), qt (quintet), m (multiplet), br (broad), app (apparent). 2D NMR experiments (HMQC and HMBC) of representative compounds were carried out to assign protons and carbons of the new structures. Mass spectrometry (MS) was carried out on a Bruker LC-Esquire spectrometer in electrospray ionization (ESI) mode at the UCM's mass spectrometry core facility. Spectroscopic data of all described compounds were consistent with the proposed structures.

High performance liquid chromatography coupled to mass spectrometry (HPLC-MS) analysis was performed using an Agilent 1200LC-MSD VL instrument. LC separation was achieved with a Zorbax Eclipse XDB-C18 column (5 μm, 4.6 mm x 150 mm) or a

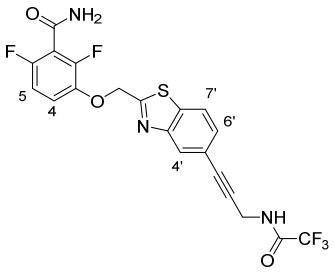
Zorbax SB-C3 column (5 µm, 2.1 mm x 50 mm), both together with a guard column (5 µm, 4.6 mm x 12.5 mm). The gradient mobile phases consisted of A (95:5 water/MeOH) and B (5:95 water/MeOH) with 0.1% ammonium hydroxide and 0.1% formic acid as the solvent modifiers. MS analysis was performed with an ESI source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set at 72 eV. The drying gas temperature was 350 ºC, the drying gas flow was 10 L/min, and the nebulizer pressure was 20 psi. Spectra were acquired in positive or negative ionization mode from 100 to 1200 m/z and in UV-mode at four different wavelengths (210, 230, 254, and 280 nm).

Elemental analyses (C, H, N or C, H, N, S) of final compounds were obtained on a LECO CHNS-932 apparatus at the UCM or at Universidad Autónoma de Madrid analysis services and were within 0.4% of the calculated values.

N-Methyl-7-nitro-2,1,3-benzoxadiazol-4-amine (**17**),[3] 3-[(5-bromo-1,3-benzothiazol-2-yl)methoxy]-2,6-difluorobenzamide (**18**),[4] and 3-[(6-chloro[1,3]thiazolo[5,4-b]pyridin-2-yl)methoxy]-2,6-difluorobenzamide (PC190723)[4] were synthesized following procedures previously described and their spectroscopic data are in agreement with those previously reported.

2.1.2. Synthesis and Characterization Data of Compounds 1-4

2,6-Difluoro-3-[(5-{3-[(trifluoroacetyl)amino]prop-1-ynyl}-1,3-benzothiazol-2-yl) methoxy]benz amide (19). A suspension of bromo derivative **18** (300 mg, 0.75 mmol), 2,2,2-trifluoro-*N*-prop-2-ynylacetamide (341 mg, 2.3 mmol), triethylamine (0.16 mL, 1.1 mmol), CuI (74 mg, 0.075 mmol) and $Pd(PPh_3)_4$ (87 mg, 0.075 mmol) in anhydrous DMF (15 mL) was heated in the MW at 100 ºC for 45 min. Then, the mixture was concentrated under reduced pressure and the residue was purified by chromatography (from hexane to ethyl acetate) to afford intermediate **19** (303 mg, 86%).

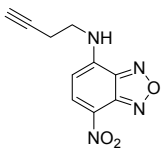


Rf (hexane/ethyl acetate, 1:1) 0.16; mp 187-189 ºC. IR (ATR) ν 3301, 1716, 1679, 1553, 1489, 1439; ^{1}H NMR (300 MHz, CD_3SOCD_3) δ 4.32 (d, J = 5.6, 2H, CH_2N), 5.70 (s, 2H, CH_2O), 7.11 (td, J = 8.9, 1.8, 1H, H^5), 7.39 (td, J = 9.3, 5.2, 1H, H^4), 7.51 (dd, J = 8.4, 1.5, 1H, $H^{6'}$), 7.88 (br s, 1H, NH), 8.08 (d, J = 1.0, 1H, $H^{4'}$), 8.16-8.18 (m, 2H, $H^{7'}$,

NH), 10.10 (t, J = 5.3, 1H, NH); ^{13C} NMR (75 MHz, CD_3SOCD_3) δ 29.4 (CH_2N), 68.6 (CH_2O), 82.1, 85.3 (C≡C), 111.1 (dd, $J_{C\text{-}F}$ = 23.2, 4.0, C^5), 116.2 (d, $J_{C\text{-}F}$ = 9.3, C^4), 116.6-117.0 (m, C^1, CF_3), 119.9 ($C^{5'}$), 123.1 ($C^{7'}$), 125.5 ($C^{4'}$), 128.3 ($C^{6'}$), 135.1 ($C^{7'a}$), 141.9 (dd, $J_{C\text{-}F}$ = 11.0, 3.2, C^3), 148.3 (dd, $J_{C\text{-}F}$ = 248.7, 8.4, C^2/C^6), 152.3 (dd, $J_{C\text{-}F}$ = 242.2, 7.1, C^2/C^6), 152.7 ($C^{3'a}$), 156.2 (d, $J_{C\text{-}F}$ = 37.0, COCF$_3$), 161.1 ($C^{2'}$), 169.3 ($CONH_2$); ESI-MS 469.6 $(M+H)^+$.

3-{[5-(3-Aminoprop-1-yn-1-yl)-1,3-benzothiazol-2-yl]methoxy}-2,6-difluorobenzamide (20). To a solution of intermediate **19** (70 mg, 0.15 mmol) in MeOH (15 mL), NH_3 (28% aqueous, 10 mL, 282 mmol) was added and the reaction was stirred at room temperature overnight. Then, the solvent was evaporated under reduced pressure and the crude was triturated with Et_2O, filtered and dried under high vacuum to afford amine **20** (55 mg, 99%), which was used in the next step without further purification. ESI-MS 374.1 $(M+H)^+$.

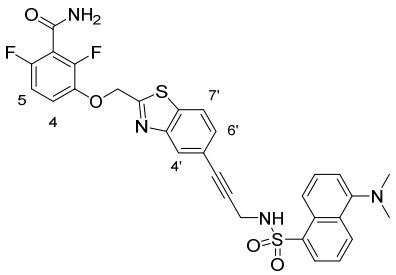
***N*-But-3-yn-1-yl-7-nitro-2,1,3-benzoxadiazol-4-amine (21).** To a solution of Cl-NBD (1.10 g, 5.3 mmol) and DIPEA (4.8 mL, 27 mmol) in MeOH (50 mL), a solution of 1-amino-3-butyne (0.64 mL, 5.3 mmol) in MeOH (50 mL) was added and the reaction was stirred for 15 h at room temperature. Then, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (from hexane to DCM) to afford compound **21** (948 mg, 77% yield).



Rf (DCM) 0.59; mp 134-136 ºC; IR (ATR) ν 3286, 1582, 1496, 1299; ^{1}H NMR (300 MHz, CD_3COCD_3) δ 2.50 (t, J = 2.6, 1H, C≡CH), 2.74 (td, J = 7.0, 2.7, 2H, CH_2), 3.83-3.86 (m, CH_2N), 6.54 (d, J = 8.8, 1H CH_{NBD}), 8.28 (s, 1H, NH), 8.51 (d, J = 8.8, 1H, CH_{NBD}); ^{13}C NMR (75 MHz, CD_3COCD_3) δ 18.9 (CH_2), 43.4 (CH_2N), 71.9 (CH≡C), 81.6 (CH≡C), 100.2 (br s, CH_{NBD}), 123.9 (C_{NBD}), 137.6 (CH_{NBD}), 145.1(C_{NBD}), 145.5 ($2C_{NBD}$); ESI-MS 233.0 $(M+H)^+$.

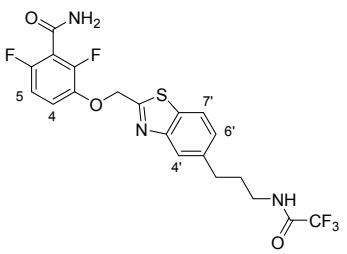
3-({5-[3-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)prop-1-ynyl]-1,3-benzothiazol-2-yl}methoxy)-2,6-difluorobenzamide (2). To a solution of the amine **20** (55 mg, 0.15 mmol) and triethylamine (62 μL, 0.44 mmol) in a 2:1 mixture of anhydrous DCM and DMF (3 mL), a solution of dansyl chloride (60 mg, 0.22 mmol) in anhydrous DCM (1 mL) was added. The reaction was stirred at room temperature for 24 h. Afterward, the solvent was evaporated under reduced pressure and the crude was

purified by chromatography (from DCM to ethyl acetate) to afford final compound **2** (25 mg, 28%).



Rf (ethyl acetate) 0.65; mp 194-196 ºC; IR (ATR) ν 1739, 1489, 1461; ^{1}H NMR (700 MHz, CD_3SOCD_3) δ 2.68 (s, 6H, $2CH_3$), 4.04 (s, 2H, CH_2N), 5.68 (s, 2H, CH_2O), 6.79 (dd, J = 8.3, 1.5, 1H, CH_{Ds}), 7.12 (t, J = 9.5, 1H, H^5), 7.22 (d, J = 7.5, 1H, CH_{Ds}), 7.27 (s, 1H, $H^{4'}$), 7.39 (td, J = 9.3, 5.1, 1H, H^4), 7.59-7.64 (m, 2H, $H^{6'}$, CH_{Ds}), 7.90 (br s, 1H, $CONH_2$), 7.96 (d, J = 8.3, 1H, $H^{7'}$), 8.17 (br s, 1H, $CONH_2$), 8.22 (dd, J = 7.2, 1.1, 1H, CH_{Ds}), 8.32 (d, J = 8.6, 1H, CH_{Ds}), 8.36 (d, J = 8.6, 1H, CH_{Ds}), 8.53 (br s, 1H, $NHSO_2$); ^{13}C NMR (175 MHz, CD_3SOCD_3) δ 32.4 (CH_2N), 44.9 ($2CH_3$), 68.5 (CH_2O), 82.2, 86.3 (C≡C), 111.2 (dd, $J_{C\text{-}F}$ = 25.0, 2.9, H^5), 115.0 (CH_{Ds}), 116.2 (d, $J_{C\text{-}F}$ = 8.7, C^4), 116.9 (dd, $J_{C\text{-}F}$ = 25.5, 20.0, C^1), 119.2 (CH_{Ds}), 119.6 ($C^{5'}$), 122.6 ($C^{7'}$), 123.6 ($C^{4'}$), 124.9 ($C^{6'}$), 127.7, 127.8, 129.0 ($3CH_{Ds}$), 129.4 (C_{Ds}), 129.8 (CH_{Ds}), 132.1 ($C^{7'a}$), 134.7, 136.0 ($2C_{Ds}$), 142.3 (dd, $J_{C\text{-}F}$ = 10.8, 2.4, C^3), 148.0 (dd, $J_{C\text{-}F}$ = 248.8, 8.4, CF), 151.4 (C_{Ds}), 152.1 ($C^{3'a}$), 152.5 (dd, $J_{C\text{-}F}$ = 243.1, 6.7, CF), 161.1 ($C^{2'}$), 169.0 (CO); ESI-MS 607.1 $(M+H)^+$; elemental analysis (calcd., found for $C_{30}H_{24}F_2N_4O_4S_2$): C (59.39, 59.57), H, (3.99, 4.27), N (9.27, 8.93), S (10.57, 10.77).

3-({5-[3-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)propyl]-1,3-benzothiazol-2-yl}methoxy)-2,6-difluorobenzamide (3). To a suspension of alkyne **19** (250 mg, 0.53 mmol) in a mixture of anhydrous THF (20 mL) and MeOH (40 mL), Raney-Ni (1 mL, slurry in water) was added. The reaction was stirred under hydrogen atmosphere (1 bar) at room temperature for 3 h. Then, the mixture was filtered through celite and concentrated under reduced pressure. The crude was purified by chromatography (from hexane to ethyl acetate) to afford 2,6-difluoro-3-[(5-{3-[(trifluoroacetyl)amino]propyl}-1,3-benzothiazol-2-yl)methoxy]benzamide (55 mg, 22%).

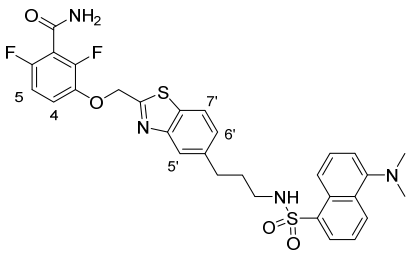


R_f (hexane/ethyl acetate, 1:1) 0.16; mp 102-104 ºC; IR (ATR) ν 3303, 1710, 1680, 1489; ^{1}H NMR (300 MHz, CD_3SOCD_3) δ 1.96 (qt, J = 7.5, 2H, $CH_2CH_2CH_2$), 2.82 (t, J = 7.7, 2H, CH_2Ar), 3.34 (t, J = 7.2, 2H, CH_2N), 5.57 (s, 2H, CH_2O), 6.97 (td, J = 8.9, 1.8, 1H, H^5), 7.32 (td, J = 9.2, 5.0, 1H, H^4), 7.35 (dd, J = 8.3, 1.3, 1H, $H^{6'}$), 7.54-7.64 (m, 3H, 3NH), 7.84 (s, 1H, $H^{4'}$),

7.92 (d, J = 8.3, 1H, $H^{7'}$); ^{13}C NMR (75 MHz, CD_3SOCD_3) δ 31.7, 33.9 (CH_2CH_2Ar), 40.3 (CH_2N), 70.4 (CH_2O), 112.1 (dd, $J_{C\text{-}F}$ = 23.7, 3.7, C^5), 117.0-117.2 (m, C^1), 117.6 (q, $J_{C\text{-}F}$ = 286.0, CF_3) 118.7 (d, J = 9.2, C^4), 123.2 ($C^{7'}$), 127.8 ($C^{4'}$), 130.0 ($C^{6'}$), 133.9 ($C^{5'}$), 141.7 ($C^{7'a}$), 143.8 (dd, $J_{C\text{-}F}$ = 11.2, 2.4, C^3), 150.7 (dd, $J_{C\text{-}F}$ = 252.3, 8.2, CF), 154.2 ($C^{3'a}$), 154.9 (dd, $J_{C\text{-}F}$ = 244.5, 6.6, CF), 159.0 (d, $J_{C\text{-}F}$ = 36.8, COCF$_3$), 165.0 ($C^{2'}$), 169.7 (CO); ESI-MS 473.7 $(M+H)^+$.

To a solution of 2,6-difluoro-3-[(5-{3-[(trifluoroacetyl)amino]propyl}-1,3-benzothiazol-2-yl)methoxy]benzamide (60 mg, 0.13 mmol) in MeOH (20 mL), NH_3 (28% aqueous, 7.8 mL) was added and the reaction was stirred at room temperature overnight. Then, the solvent was evaporated under reduced pressure to afford 3-{[5-(3-aminopropyl)-1,3-benzothiazol-2-yl]methoxy}-2,6-difluorobenzamide (49 mg, 99%), which was used in the next step without purification. ESI-MS 378.1 $(M+H)^+$.

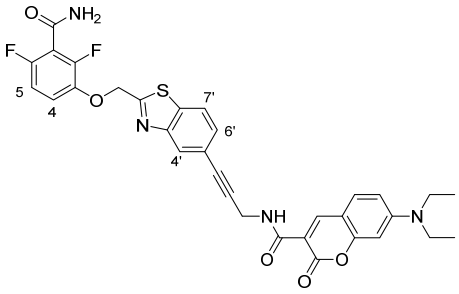
To a solution of 3-{[5-(3-aminopropyl)-1,3-benzothiazol-2-yl]methoxy}-2,6-difluorobenzamide (50 mg, 0.13 mmol) and triethylamine (58 μL, 0.41 mmol) in a 2:1 mixture of anhydrous DCM and DMF (3 mL), a solution of dansyl chloride (56 mg, 0.21 mmol) in anhydrous DCM (1 mL) was added. The reaction was stirred at room temperature for 24 h. Afterward, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (from DCM to ethyl acetate) to afford final compound **3** (21 mg, 26%).



R_f (DCM/ethyl acetate, 1:4) 0.64; mp 185-186 ºC; IR (ATR) ν 3435, 1670, 1604, 1486, 1450; ^{1}H NMR (700 MHz, CD_3SOCD_3) δ 1.58 (qt, J = 7.2, 2H, $CH_2CH_2CH_2$), 2.55 (t, J = 7.7, 2H, CH_2Ar), 2.82 (s, 6H, $2CH_3$), 2.83 (t, J = 7.3, 2H, CH_2N), 5.66 (s, 2H, CH_2O), 7.02 (dd, J = 8.2, 1.5, 1H, CH_{Ds}), 7.10 (t, J = 9.0, 1H, H^5), 7.26 (d, J = 7.3, 1H, CH_{Ds}), 7.38 (td, J = 9.2, 5.1, 1H, H^4), 7.57-7.62 (m, 3H, CH_{Ds}, $H^{4'}$, $H^{6'}$), 7.89 (br s, 1H, $CONH_2$), 7.92 (d, J = 8.2, 1H, $H^{7'}$), 8.01 (t, J = 5.9, 1H, $NHSO_2$), 8.07 (dd, J = 7.1, 1.1, 1H, CH_{Ds}), 8.17 (br s, 1H, $CONH_2$), 8.33 (d, J = 8.7, 1H, CH_{Ds}), 8.45 (d, J = 8.4, 1H, CH_{Ds}); ^{13}C NMR (175 MHz, CD_3SOCD_3) δ 31.1, 31.8 (CH_2CH_2Ar), 41.8 (CH_2N), 45.1 ($2CH_3$), 68.5 (CH_2O), 111.1 (d, $J_{C\text{-}F}$ = 21.6, C^5), 115.1 (CH_{Ds}), 116.2 (d, $J_{C\text{-}F}$ = 8.5, C^4), 116.8 (t, $J_{C\text{-}F}$ = 23.4, C^1), 119.1 (CH_{Ds}), 122.0 ($C^{7'}$), 123.6 ($C^{4'}$), 126.2 ($C^{6'}$), 127.9 ($2CH_{Ds}$), 128.3 (CH_{Ds}), 129.1 (C_{Ds}, $C^{5'}$), 129.4 (CH_{Ds}), 131.9 ($C^{7'a}$), 136.1, 140.1 ($2C_{Ds}$), 142.0 (d, $J_{C\text{-}F}$ = 9.0, C^3), 148.0 (dd, $J_{C\text{-}F}$ =

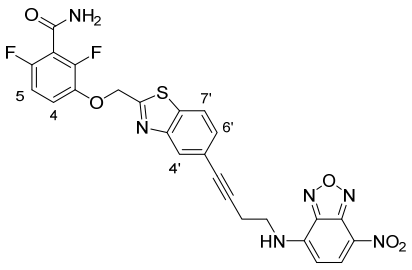
249.5, 7.7, CF), 151.4 (C_{Ds}), 152.4 (d, J_{C-F} = 240.4, CF), 152.9 ($C^{3a'}$), 161.1 ($C^{2'}$), 167.4 (CO); ESI-MS 611.2 $(M+H)^+$; elemental analysis (calcd., found for $C_{30}H_{28}F_2N_4O_4S_2$): C (59.00, 58.71), H, (4.62, 4.63), N (9.17, 9.17), S (10.50, 10.19).

***N*-(3-(2-((3-Carbamoyl-2,4-difluorophenoxy)methyl)benzo[d]thiazol-5-yl)prop-2-ynyl)-7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamide (4).** To a solution of amine **20** (119 mg, 0.32 mmol), PyBroP (268 mg, 0.57 mmol) and DIPEA (111 μL, 0.64 mmol) in anhydrous DMF (5 mL), a solution of 7-diethylaminocoumarin-3-carboxylic acid (100 mg, 0.38 mmol) in anhydrous DMF (1 mL) was added. The reaction was stirred at room temperature for 4 h. Afterward, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (from DCM to ethyl acetate) to afford final compound **4** (30 mg, 15%).



R_f (ethyl acetate) 0.70; mp 248-249 ºC; ^{1}H NMR (700 MHz, CD_3SOCD_3) δ 1.14 (t, *J* = 7.0, 6H, $2CH_3$), 3.49 (q, *J* = 7.0, 4H, $2CH_2$), 4.41 (d, *J* = 7.0, 2H, CH_2NH), 5.70 (s, 2H, CH_2O), 6.63 (d, *J* = 1.9, 1H, CH_{coum}), 6.82 (dd, *J* = 9.0, 2.1, 1H, CH_{coum}), 7.11 (t, *J* = 8.6, 1H, H^5), 7.39 (td, *J* = 9.2, 5.5, 1H, H^4), 7.51 (dd, *J* = 8.3, 1.1, 1H, $H^{6'}$), 7.71 (d, *J* = 9.0, 1H, CH_{coum}), 7.89 (s, 1H, $CONH_2$), 8.07 (s, 1H, $H^{4'}$), 8.15 (d, *J* = 8.4, 1H, $H^{7'}$), 8.17 (s, 1H, $CONH_2$), 8.71 (s, 1H, CH_{coum}), 8.99 (t, *J* = 5.6, 1H, CONH); ^{13}C NMR (175 MHz, CD_3SOCD_3) δ 12.3 ($2CH_3$), 29.2 (CH_2NH), 44.4 ($2CH_2$), 68.8 (CH_2O), 81.1, 87.5 (2C≡C), 95.9 (CH_{coum}), 107.7, 108.8 ($2C_{coum}$), 110.2 (CH_{coum}), 111.1 (dd, J_{C-F} = 24.3, 4.2, C^5), 116.2 (d, *J* = 9.9, C^4), 117.1-117.0 (m, C^1), 120.4 ($C^{5'}$), 123.0 ($C^{7'}$), 125.4 ($C^{4'}$), 128.4 (CH_{coum}), 132.0 ($C^{6'}$), 134.8 ($C^{7'a}$), 141.8-142.1 (m, C^3), 148.2 (CH_{coum}), 149.2 (d, J_{C-F} = 265.5, CF), 152.4 (d, J_{C-F} = 242.0, CF), 152.5 ($C^{3'a}$), 152.6, 157.4 ($2C_{coum}$), 161.1 (CONH), 161.7 ($C^{2'}$), 162.2 (CO_{coum}), 169.2 ($CONH_2$); ESI-MS 617.2 $(M+H)^+$; elemental analysis (calcd., found for $C_{32}H_{26}F_2N_4O_5S\cdot 3H_2O$): C (57.31, 57.21), H, (4.81, 4.55), N (8.35, 7.99), S (4.78, 4.58).

2,6-Difluoro-3-[(5-{4-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]but-1-yn-1-yl}-1,3-benzothiazol-2-yl)methoxy]benzamide (5). A suspension of bromo derivative **18** (150 mg, 0.38 mmol), alkyne **21** (262 mg, 1.13 mmol), $Pd(PPh_3)_4$ (37 mg, 0.02 mmol), CuI (43 mg, 0.04 mmol) and triethylamine (79 μL, 0.56 mmol) in anhydrous DMF (8 mL) was heated in the MW at 100 ºC for 1 h. Then, the mixture was concentrated under reduced pressure and the residue was purified by chromatography (from hexane to ethyl acetate) to afford final compound **5** (86 mg, 41%).

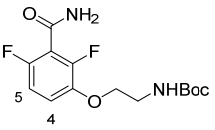


R_f (hexane/ethyl acetate, 1:1) 0.29; mp 189-192 ºC; IR (ATR) ν 3405, 1684, 1623, 1584, 1489; ^{1}H NMR (500 MHz, CD_3SOCD_3) δ 2.91 (t, J = 6.9, 2H, CH_2), 3.79 (br s, 2H, CH_2N), 5.69 (s, 2H, CH_2O), 6.61 (d, J = 8.9, 1H, CH_{NBD}), 7.11 (td, J = 9.1, 1.7, 1H, H^5), 7.36-7.46 (m, 2H, H^4, $H^{6'}$), 7.88 (br s, 1H, $CONH_2$), 7.97 (s, 1H, $H^{4'}$), 8.11 (d, J = 8.4, 1H, $H^{7'}$), 8.16 (br s, 1H, $CONH_2$), 8.54 (d, J = 8.9, 1H, CH_{NBD}), 9.62 (br s, 1H, NH); ^{13}C NMR (175 MHz, CD_3SOCD_3) δ 19.0 (CH_2), 42.2 (CH_2N), 68.6 (CH_2O), 81.5, 88.1 (C≡C), 99.7 (CH_{NBD}), 111.1 (dd, J_{C-F} = 23.0, 3.8, C^5), 116.2 (d, J_{C-F} = 9.4, C^4), 116.6 (dd, J_{C-F} = 25.3, 20.3, C^1), 120.9 ($C^{5'}$), 122.9 ($C^{7'}$), 125.3 ($C^{4'}$), 128.2 ($C^{6'}$), 128.7 (C_{NBD}), 134.5 ($C^{7'a}$), 137.7 (CH_{NBD}), 141.9 (dd, J_{C-F} = 11.1, 3.2, C^3), 144.1, 144.3, 144.4 ($3C_{NBD}$), 148.0 (dd, J_{C-F} = 248.6, 8.2, CF), 152.4 ($C^{3'a}$), 152.5 (dd, J_{C-F} = 242.6, 7.7, CF), 161.1 ($C^{2'}$), 169.0 (CO); ESI-MS 551.1 $(M+H)^+$; elemental analysis (calcd., found for $C_{25}H_{16}F_2N_6O_5S$): C (54.55, 54.45), H, (2.93, 3.12), N (15.27, 14.98), S (5.82, 5.71).

2.1.3. Synthesis and Characterization Data of Compounds 6-16

General Procedure for the Synthesis of 22, 24-26. To a solution of 2,6-difluoro-3-hydroxybenzamide (1 equiv), K_2CO_3 (3 equiv) and NaI (0.2 equiv) in anhydrous DMF (10 mL/mmol benzamide), the corresponding commercial *N*-protected-ω-bromoalkylamino derivative (1 equiv) was added dropwise. The reaction mixture was stirred at room temperature overnight. Then, the reaction was concentrated under reduced pressure and the residue was dissolved in ethyl acetate and washed with a saturated aqueous solution of $NaHCO_3$ and with water. The organic layer was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The crude was purified by chromatography (from hexane to ethyl acetate) to afford the corresponding compounds **22, 24-26**.

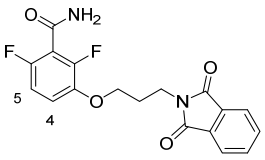
***tert*-Butyl {2-[3-(aminocarbonyl)-2,4-d ifluorophenoxy]ethyl}carbamate (22).** Obtained from 2,6-difluoro-3-hydroxybenzamide (574 mg, 3.3 mmol) and *N*-Boc-2-bromoethyl-amine (743 mg, 3.3 mmol) in 62% yield (645 mg).



R_f (ethyl acetate/hexane, 2:1) 0.35; mp 111-113 ºC; IR (ATR) ν 3331, 1675, 1629, 1520, 1491; ^{1}H NMR (300 MHz, CD_3OD) δ 1.44 (s, 9H, $3CH_3$), 3.43 (t, J = 5.5, 2H, CH_2N), 4.07 (t, J = 5.5, 2H, CH_2O), 6.95

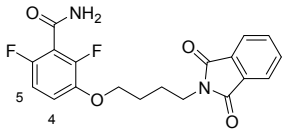
(td, J = 9.0, 2.0, 1H, H^5), 7.18 (td, J = 9.0, 5.0, 1H, H^4); ^{13}C NMR (75 MHz, CD_3OD) δ 28.7 ($3CH_3$), 40.9 (CH_2N), 70.2 (CH_2O), 80.3 ($C(CH_3)_3$), 111.9 (dd, $J_{C\text{-}F}$ = 23.0, 4.0, C^5), 117.0 (t, $J_{C\text{-}F}$ = 18.5, C^1), 117.9 (dd, $J_{C\text{-}F}$ = 9.0, 2.5, C^4), 144.9 (dd, $J_{C\text{-}F}$ = 11.0, 3.5, C^3), 150.5 (dd, $J_{C\text{-}F}$ = 251.5, 8.5, CF), 154.2 (dd, $J_{C\text{-}F}$ = 244.0, 6.0, CF), 158.5 (NCOO), 165.4 ($CONH_2$); ESI-MS 339.0 $(M+Na)^+$.

3-[3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)propoxy]-2,6-difluorobenzamide (24). Obtained from 2,6-difluoro-3-hydroxybenzamide (250 mg, 1.4 mmol) and *N*-(3-bromopropyl)phthalimide (387 mg, 1.4 mmol) in 57% yield (288 mg).



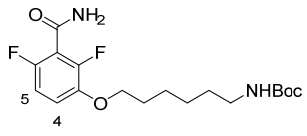
R_f (hexane/ethyl acetate, 1:3) 0.54; mp 200-203 ºC; IR (ATR) ν 3404, 1707, 1679, 1611, 1494; ^{1}H NMR (300 MHz, CD_3COCD_3) δ 1.62 (m, 2H, CH_2), 3.31 (t, J = 6.7, 2H, CH_2N), 3.63 (t, J = 5.9, 2H, CH_2O), 6.59 (td, J = 9.0, 1.8, 1H, H^5), 6.71 (td, J = 9.3, 5.3, 1H, H^4), 7.34-7.43 (m, 4H, $4CH_{pht}$), 7.50 (br s, 1H, $CONH_2$), 7.65 (br s, 1H, $CONH_2$); ^{13}C NMR (75 MHz, CD_3COCD_3) δ 27.7 (CH_2), 34.8 (CH_2N), 67.4 (CH_2O), 110.8 (dd, $J_{C\text{-}F}$ = 24.2, 5.4, C^5), 115.3 (d, $J_{C\text{-}F}$ = 9.2, C^4), 117.9 (br s, C^1), 122.9 ($2CH_{pht}$), 131.8 ($2C_{pht}$), 134.3 ($2CH_{pht}$), 143.0 (dd, $J_{C\text{-}F}$ = 13.9, 3.3, C^3), 148.0 (d, $J_{C\text{-}F}$ = 231.6, CF), 153.7 (d, $J_{C\text{-}F}$ = 224.7, CF), 161.3 ($CONH_2$), 168.1 ($2CO_{pht}$); ESI-MS 361.0 $(M+H)^+$.

3-[4-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)butoxy]-2,6-difluorobenzamide (25). Obtained from 2,6-difluoro-3-hydroxybenzamide (221 mg, 1.2 mmol) and *N*-(4-bromobutyl)phthalimide (344 mg, 1.2 mmol) in 87% yield (391 mg).



R_f (hexane/ethyl acetate, 1:1) 0.29; mp 155-157 ºC; IR (ATR) ν 3397, 1710, 1654, 1492; ^{1}H NMR (300 MHz, CD_3COCD_3) δ 1.84-1.88 (m, 4H, $2CH_2$), 3.74 (t, J = 6.7, 2H, CH_2N), 4.14 (t, J = 5.9, 2H, CH_2O), 6.93 (td, J = 9.0, 2.0, 1H, H^5), 7.14-7.22 (m, 2H, $CONH_2$, H^4), 7.41 (br s, 1H, $CONH_2$), 7.83-7.87 (m, 4H, $4CH_{pht}$); ^{13}C NMR (75 MHz, CD_3COCD_3) δ 25.8, 27.3 ($2CH_2$), 38.1 (CH_2N), 70.2 (CH_2O), 111.5 (dd, $J_{C\text{-}F}$ = 23.2, 4.1, C^5), 116.6 (dd, $J_{C\text{-}F}$ = 9.3, 2.9, C^4), 117.4 (br s, C^1), 123.7 ($2CH_{pht}$), 133.1 ($2C_{pht}$), 135.0 ($2CH_{pht}$), 144.6 (dd, $J_{C\text{-}F}$ = 11.0, 3.1, C^3), 147.6 (d, $J_{C\text{-}F}$ = 261.3, CF), 153.5 (dd, $J_{C\text{-}F}$ = 243.2, CF), 162.2 ($CONH_2$), 168.9 ($2CO_{pht}$); ESI-MS 375.0 $(M+H)^+$.

***tert*-Butyl {6-[3-(aminocarbonyl)-2,4-d ifluorophenoxy]hexyl}carbamate (26).** Obtained from 2,6-difluoro-3-hydroxybenzamide (250 mg, 1.4 mmol) and 6-(Boc-amino)hexyl bromide (405 mg, 1.4 mmol) as an oil in 77% yield (401 mg).



R_f (hexane/ethyl acetate, 1:3) 0.66; IR (ATR) ν 3326, 3193, 1669, 1490, 1366; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37-1.52 (m, 15H, $3CH_2$, $3CH_3$), 1.79 (qt, J = 6.8, 2H, CH_2), 3.10 (m, 2H, CH_2N), 3.99 (t, J = 6.4, 2H, CH_2O), 4.61 (br s, 1H, NH), 6.25 (br s, 1H, $CONH_2$), 6.48 (br s, 1H, $CONH_2$), 6.85 (td, J = 9.1, 1.8, 1H, H^5), 6.97 (td, J = 9.1, 5.2, 1H, H^4); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.6, 26.5 ($2CH_2$), 28.5 ($3CH_3$), 29.1, 30.1 ($2CH_2$), 40.5 (CH_2N), 70.4 (CH_2O), 79.2 ($C(CH_3)_3$), 111.1 (dd, $J_{C\text{-}F}$ = 23.5, 4.3, C^5), 114.2 (dd, $J_{C\text{-}F}$ = 20.4, 16.8, C^1), 117.0 (dd, $J_{C\text{-}F}$ = 9.6, 3.5, C^4), 144.0 (dd, $J_{C\text{-}F}$ = 11.4, 3.3, C^3), 150.1 (dd, $J_{C\text{-}F}$ = 254.1, 6.8, CF), 153.4 (dd, $J_{C\text{-}F}$ = 246.1, 5.8, CF), 156.1 (NCOO), 162.5 ($CONH_2$); ESI-MS 395.2 $(M+Na)^+$.

General Procedure for the Synthesis of Amines 23 and 29. To a solution of Boc derivative **22** or **26** (1 equiv) in anhydrous DCM (10 mL/mmol), trifluoroacetic acid (TFA) (20 equiv) was added dropwise and the reaction mixture was stirred at room temperature for 1 h. Then, the mixture was concentrated under reduced pressure and the residue was redissolved in DCM and neutralized with a saturated aqueous solution of $NaHCO_3$. The aqueous phase was extracted with DCM and the organic layer was dried (Na_2SO_4) and concentrated under reduced pressure to afford amine **23** or **29** in quantitative yield, that was used in the next step without further purification.

3-(2-Aminoethoxy)-2,6-difluorobenzamide (23). Obtained from **22** (60 mg, 0.19 mmol) in 99% yield (41 mg). ESI-MS 216.9 $(M+H)^+$.

3-[(6-Aminohexyl)oxy]-2,6-difluorobenzamide (29). Obtained from **26** (350 mg, 0.94 mmol) in 99% yield (253 mg). ESI-MS 273.1 $(M+H)^+$.

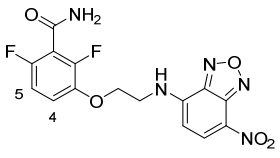
General Procedure for the Synthesis of Amines 27 and 28. To a solution of phthalimide **24** or **25** (1 equiv) in EtOH (15 mL/mmol), hydrazine monohydrate (2 equiv) was added dropwise and the reaction mixture was refluxed for 2 h. Then, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (from ethyl acetate to ethyl acetate/MeOH, 95:5) to afford the corresponding amine **27** or **28**.

3-(3-Aminopropoxy)-2,6-difluorobenzamide (27). Obtained from **24** (290 mg, 0.81 mmol) in 64% yield (119 mg). ESI-MS 231.1 $(M+H)^+$.

3-(4-Aminobutoxy)-2,6-difluorobenzamide (28). Obtained from **25** (350 mg, 0.93 mmol) in 55% yield (125 mg). ESI-MS 245.1 $(M+H)^+$.

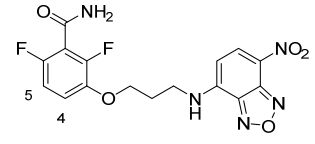
General P rocedure for the Synthesis of 6, 14-16. To a solution of the corresponding amine **23**, **27-29** (1 equiv), and *N,N*-diisopropylethylamine (DIPEA, 1.2 equiv) in anhydrous DMF (15 mL/mmol), a solution of Cl-NBD (1.2 equiv) in anhydrous DMF (2 mL/mmol) was added and the reaction was stirred at room temperature overnight. Then, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (from hexane to ethyl acetate) to afford final compounds **6**, **14-16**.

2,6-Difluoro-3-{2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethoxy}benzamide (6). Obtained from amine **23** (101 mg, 0.47 mmol) and Cl-NBD (98 mg, 0.56 mmol) in 30% yield (12 mg).



R_f (DCM/ethyl acetate, 8:2) 0.16; mp 169-172 ºC; IR (ATR) ν 3354, 1676, 1586, 1491; ^{1}H NMR (700 MHz, CD_3COCD_3) δ 4.15 (m, 2H, CH_2N), 4.52 (t, J = 5.2, 2H, CH_2O), 6.65 (d, J = 8.8, 1H, CH_{NBD}), 6.96 (td, J = 9.0, 1.9, 1H, H^5), 7.16 (br s, 1H, $CONH_2$), 7.24 (td, J = 9.2, 5.1, 1H, H^4), 7.40 (br s, 1H, $CONH_2$), 8.45 (br s, 1H, NH), 8.56 (d, J = 8.7, 1H, CH_{NBD}); ^{13}C NMR (175 MHz, CD_3COCD_3) δ 44.1 (br s, CH_2N), 68.8 (CH_2O), 100.2 (br s, CH_{NBD}), 111.6 (dd, J_{C-F} = 20.6, 4.1, C^5), 116.7 (d, J_{C-F} = 8.8, C^4), 117.5 (dd, J_{C-F} = 23.9, 20.3, C^1), 123.9 (C_{NBD}), 137.7 (CH_{NBD}), 144.1 (d, J_{C-F} = 9.3, C^3), 145.1, 145.6, 145.8 ($3C_{NBD}$), 149.7 (dd, J_{C-F} = 250.2, 8.0, CF), 153.7 (dd, J_{C-F} = 242.4, 6.0, CF), 162.0 (CO); ESI-MS 379.9 $(M+H)^+$; elemental analysis (calcd., found for $C_{15}H_{11}F_2N_5O_5$): C (47.50, 47.40), H, (2.92, 3.29), N (18.47, 18.13).

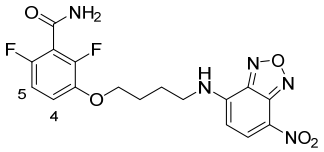
2,6-Difluoro-3-{3-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]propoxy}-benzamide (14). Obtained from amine **27** (184 mg, 0.51 mmol) and Cl-NBD (123 mg, 0.62 mmol) as an oil in 23% yield (46 mg).



R_f (DCM/ethyl acetate, 95:5) 0.29; IR (ATR) ν 3343, 1701, 1378, 1364; ^{1}H NMR (500 MHz, CD_3COCD_3) δ 2.36 (q, J = 6.4, 2H, CH_2), 3.91 (br s, 2H, CH_2N), 4.30 (t, J = 5.9, 2H, CH_2O), 6.54 (d, J = 8.8, 1H, CH_{NBD}), 6.96 (td, J = 8.9, 2.0, 1H, H^5), 7.18 (br s, 1H, $CONH_2$), 7.20 (td, J = 9.2, 5.2, 1H, H^4), 7.40 (br s, 1H, $CONH_2$), 8.36 (br s, 1H, NH), 8.54 (d, J = 8.8, 1H, CH_{NBD}); ^{13}C NMR (175 MHz, CD_3COCD_3) δ 32.6 (CH_2), 39.7(CH_2N), 68.8 (CH_2O), 99.6 (br s, CH_{NBD}), 111.5 (dd, J_{C-F} = 22.8, 4.1, C^5), 116.5 (dd, J_{C-F} = 9.4, 2.7, C^4), 117.4 (dd, J_{C-F} = 24.9, 19.5, C^1), 127.7 (C_{NBD}), 135.7 (br s, CH_{NBD}), 144.4 (dd, J_{C-F} = 10.7, 2.7, C^3), 145.1, 145.6, 147.0 ($3C_{NBD}$), 149.8 (dd, J_{C-F} =

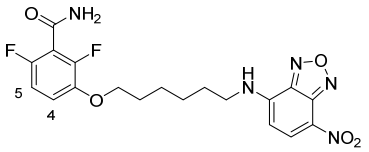
248.8, 7.9, CF), 153.5 (dd, $J_{C\text{-}F}$ = 242.2, 6.5, CF), 162.4 (CO); ESI-HRMS (calcd., found for $C_{16}H_{13}F_2NaN_5O_5$ $[M+Na]^+$): 416.0782, 416.0777.

2,6-Difluoro-3-{4-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]butoxy}benzamide (15). Obtained from amine **28** (188 mg, 0.50 mmol) and Cl-NBD (121 mg, 0.60 mmol) as an oil in 24% yield (49 mg).



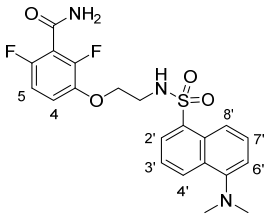
R_f (DCM/ethyl acetate, 95:5) 0.34; IR (ATR) ν 3431 (NH_2), 1701 (CO), 1364 (Ar); ^{1}H NMR (500 MHz, CD_3COCD_3) δ 1.99-2.05 (m, 4H, $2CH_2$), 3.78 (br s, 2H, CH_2N), 4.18 (t, J = 6.0, 2H, CH_2O), 6.51 (d, J = 8.8, 1H, CH_{NBD}), 6.95 (td, J = 9.0, 2.0, 1H, H^5), 7.16 (br s, 1H, $CONH_2$), 7.19 (td, J = 9.2, 5.2, 1H, H^4), 7.39 (br s, 1H, $CONH_2$), 8.34 (br s, 1H, NH), 8.54 (d, J = 8.8, 1H, CH_{NBD}); ^{13}C NMR (175 MHz, CD_3COCD_3) δ 27.3, 30.2 ($2CH_2$), 44.1 (br s, CH_2N), 70.3 (CH_2O), 99.5 (br s, CH_{NBD}), 111.5 (dd, $J_{C\text{-}F}$ = 23.2, 4.0, C^5), 116.6 (br d, $J_{C\text{-}F}$ = 8.7, C^4), 117.3 (dd, $J_{C\text{-}F}$ = 24.3, 20.0, C^1), 126.1 (C_{NBD}), 137.9 (CH_{NBD}), 144.5 (dd, $J_{C\text{-}F}$ = 11.1, 3.2, C^3), 145.2 (C_{NBD}), 145.5 ($2C_{NBD}$), 149.8 (dd, $J_{C\text{-}F}$ = 248.7, 8.0, CF), 153.5 (dd, $J_{C\text{-}F}$ = 242.2, 6.4, CF), 162.8 (CO); ESI-HRMS (calcd., found for $C_{17}H_{15}F_2NaN_5O_5$ $[M+Na]^+$): 430.0939, 430.0934.

2,6-Difluoro-3-({6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexyl}oxy)-benzamide (16). Obtained from amine **29** (99 mg, 0.26 mmol) and Cl-NBD (63 mg, 0.33 mmol) as an oil in 26% yield (29 mg).



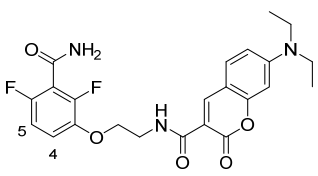
R_f (ethyl acetate) 0.73; IR (ATR) ν 3335, 1678, 1491, 1375, 1300; ^{1}H NMR (700 MHz, CD_3COCD_3) δ 1.58 (qt, J = 3.6, 4H, $2CH_2$), 1.82 (qt, J = 6.8, 2H, CH_2), 1.89 (qt, J = 6.9, 2H, CH_2), 3.67 (br s, 2H, CH_2N), 4.08 (t, J = 6.4, 2H, CH_2O), 6.48 (d, J = 8.8, 1H, CH_{NBD}), 6.94 (td, J = 9.0, 2.0, 1H, H^5), 7.14-7.17 (m, 2H, $CONH_2$, H^4), 7.41 (br s, 1H, $CONH_2$), 8.32 (br s, 1H, NH), 8.52 (d, J = 8.7, 1H, CH_{NBD}); ^{13}C NMR (175 MHz, CD_3COCD_3) δ 26.3, 27.3, 29.5, 30.2 ($4CH_2$), 44.5 (br s, CH_2N), 70.5 (CH_2O), 99.4 (br s, CH_{NBD}), 111.4 (dd, $J_{C\text{-}F}$ = 23.2, 3.9, C^5), 116.4 (dd, $J_{C\text{-}F}$ = 9.3, 2.5, C^4), 117.4 (dd, $J_{C\text{-}F}$ = 24.4, 19.9, C^1), 123.3 (br s, C_{NBD}), 138.0 (CH_{NBD}), 144.7 (dd, $J_{C\text{-}F}$ = 11.1, 3.1, C^3), 145.2, 145.6, 145.8 ($3C_{NBD}$), 149.8 (dd, $J_{C\text{-}F}$ = 249.8, 8.0, CF), 153.4 (dd, $J_{C\text{-}F}$ = 241.5, 6.6, CF), 162.3 (CO); ESI-HRMS (calcd., found for $C_{19}H_{18}F_2N_5O_5$ $[M-H]^-$): 434.1289, 434,1276.

Synthesis of 3-[2-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)ethoxy]-2,6-difluorobenzamide (7). To a solution of amine **23** (98 mg, 0.45 mmol) and triethylamine (0.19 mL, 1.4 mmol) in a 6:1 mixture of anhydrous DCM and DMF (3.5 mL), a solution of dansyl chloride (183 mg, 0.68 mmol) in anhydrous DCM (1 mL) was added. The reaction was stirred at room temperature for 24 h. Afterward, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (DCM) to afford final compound **7** in 28% yield (55 mg).



R_f (DCM/ethyl acetate, 95:5) 0.67; mp 130-132 ºC; IR (ATR) ν 3316, 1744, 1581, 1502, 1459; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.88 (s, 6H, $2CH_3$), 3.38 (app q, J = 5.5, 2H, CH_2N), 3.85 (t, J = 5.0, 2H, CH_2O), 5.25 (t, J = 6.0, 1H, NH), 6.78-6.85 (m, 2H, H^4, H^5), 7.17 (d, J = 7.2, 1H, $H^{6'}$), 7.52 (t, J = 7.9, 1H, $H^{3'}/H^{7'}$), 7.57 (t, J = 8.1, 1H, $H^{3'}/H^{7'}$), 8.24-8.27 (m, 2H, C_4H, $H^{8'}$), 8.54 (br d, J = 7.3, 1H, $H^{2'}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 43.2 (CH_2N), 45.6 ($2CH_3$), 68.9 (CH_2O), 111.3 (dd, $J_{C\text{-}F}$ = 20.6, 4.5, C^5), 115.5 (CH_{Ds}), 118.6 (br s, C^1), 120.4 (dd, $J_{C\text{-}F}$ = 9.1, 3.5, C^4), 128.8 ($2CH_{Ds}$), 129.5 ($2C_{Ds}$), 129.6 ($2CH_{Ds}$), 129.1 (C_{Ds}), 130.8 (CH_{Ds}), 135.1 (C_{Ds}), 142.9 (dd, $J_{C\text{-}F}$ = 9.3, 3.5, C^3), 153.0 (dd, $J_{C\text{-}F}$ = 262.9, 4.6, CF), 156.7 (dd, $J_{C\text{-}F}$ = 255.0, 2.9, CF), 164.7 (CO); ESI-MS 432.1 $(M+H)^+$; elemental analysis (calcd., found for $C_{21}H_{21}F_2N_3O_4S$): C (56.12, 56.17), H, (4.71, 5.16), N (9.35, 9.29), S (7.13, 6.83).

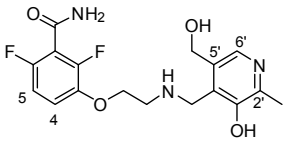
Synthesis of *N*-{2-[3-(aminocarbonyl)-2,4-difluorophenoxy]ethyl}-7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamide (8). To a solution of amine **23** (80 mg, 0.37 mmol), PyBroP® (311 mg, 0.66 mmol) and DIPEA (0.13 mL, 0.74 mmol) in anhydrous DMF (4 mL), a solution of 7-diethylaminocoumarin-3-carboxylic acid (100 mg, 0.38 mmol) in anhydrous DMF (1 mL) was added. The reaction was stirred at room temperature for 4 h. Afterward, the solvent was evaporated under reduced pressure and the crude was purified by chromatography (from DCM to DCM/ethyl acetate, 1:1) to afford final compound **8** in 27% yield (46 mg).



R_f (ethyl acetate) 0.4; mp 155-158 ºC; IR (ATR) ν 3366, 1709, 1663, 1618, 1583, 1537; ^{1}H NMR (700 MHz, $CD_3SO_2CD_3$) δ 1.14 (t, J = 7.0, 6H, $2CH_3$), 3.48 (q, J = 7.0, 4H, $2CH_2N$), 3.70 (app q, J = 5.7, 2H, CH_2NH), 4.18 (t, J = 5.7, 2H, CH_2O), 6.61 (d, J = 2.0, 1H, CH_{coum}), 6.81 (dd, J = 9.0, 2.2, 1H, CH_{coum}), 7.07 (t, J = 8.8, 1H, H^5), 7.29 (td, J = 9.3, 5.3, 1H, H^4), 7.69 (d, J = 9.0, 1H, CH_{coum}), 7.85 (br

s, 1H, $CONH_2$), 8.13 (br s, 1H, $CONH_2$), 8.69 (s, 1H, CH_{coum}), 8.93 (t, J = 5.7, 1H, NH); ^{13}C NMR (175 MHz, CD_3SOCD_3) δ 12.3 ($2CH_3$), 38.3 (CH_2NH), 44.3 ($2CH_2N$), 68.3 (CH_2O), 95.9 (CH_{coum}), 107.7, 109.0 ($2C_{coum}$), 110.2 (CH_{coum}), 111.0 (dd, $J_{C\text{-}F}$ = 22.6, 4.0, C^5), 115.6 (d, $J_{C\text{-}F}$ = 9.4, C^4), 116.7 (dd, $J_{C\text{-}F}$ = 25.1, 20.3, C^1), 131.7 (CH_{coum}), 142.8 (dd, $J_{C\text{-}F}$ = 10.6, 2.7, C^3), 147.9 (CH_{coum}), 148.0 (dd, $J_{C\text{-}F}$ = 248.5, 9.0, CF), 152.0 (dd, $J_{C\text{-}F}$ = 241.7, 7.2, CF), 152.5, 157.3 ($2C_{coum}$), 161.3, 161.8, 162.6 (3CO); ESI-MS 459.6 $(M+H)^+$; elemental analysis (calcd., found for $C_{23}H_{23}F_2N_3O_5$): C (60.13, 60.23), H, (5.05, 5.41), N (9.15, 9.44).

Synthesis of 2,6-difluoro-3-[(3'-hydroxy- 5'-hydroxymethyl-2'-methylpyridin-4'-yl)methylamino]ethyloxybenzamide (9). To a solution of the amine **23** (40 mg, 0.19 mmol) and $NaHCO_3$ (40 mg, 0.48 mmol) in EtOH/water (2:1, 3 mL), a solution of pyridoxal hydrochloride (38 mg, 0.19 mmol) in water (0.5 mL) was added. The reaction mixture was stirred at room temperature for 2 h, after which $NaBH_3CN$ (35 mg, 0.56 mmol) was added. After 3 h, the reaction mixture was concentrated under reduced pressure and the residue partitioned between ethyl acetate (15 mL) and water (15 mL). The aqueous layer was extracted with ethyl acetate and the combined organic layers were dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude was purified by chromatography (from ethyl acetate to ethyl acetate/MeOH, 9:1) to afford final compound **9** as an oil (12 mg, 18%).

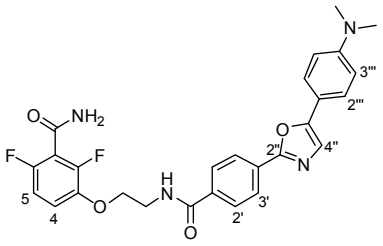


R_f (ethyl acetate/MeOH, 9:1) 0.15; IR (ATR) ν 3378, 1679, 1534, 1492; ^{1}H NMR (700 MHz, CD_3OD) δ 2.36 (s, 3H, CH_3), 3.08 (t, J = 5.0, 2H, CH_2N), 4.20 (t, J = 5.0, 2H, CH_2O), 4.22 (s, 2H, CH_2N), 4.59 (s, 2H, CH_2OH), 6.95 (td, J = 9.0, J = 2.0, 1H, H^5), 7.19 (td, J = 9.0, 5.0, 1H, H^4), 7.78 (s, 1H, $H^{6'}$); ^{13}C NMR (175 MHz, CD_3OD) δ 18.2 (CH_3), 47.7, 48.5 ($2CH_2N$), 60.8 (CH_2OH), 70.1 (CH_2O), 111.9 (dd, $J_{C\text{-}F}$ = 23.0, 4.0, C^5), 116.8 (dd, $J_{C\text{-}F}$ = 23.5, 19.0, C^1), 118.0 (d, $J_{C\text{-}F}$ = 8.5, C^4), 130.8 ($C^{5'}$), 134.4 ($C^{4'}$), 138.5 ($C^{6'}$), 144.7 (d, $J_{C\text{-}F}$ = 11.5, C^3), 147.6 ($C^{2'}$), 150.5 (dd, $J_{C\text{-}F}$ = 251.0, 7.5, CF), 154.3 (dd, $J_{C\text{-}F}$ = 244.0, 5.5, CF), 155.1 ($C^{3'}$), 165.3 (CO); ESI-MS 368.1 $(M+H)^+$; HRMS (ESI): calcd. for $C_{17}H_{20}F_2N_3O_4$ $(M+H)^+$: 368.1416; found: 368.1420.

General Procedure for the Synthesis of 10-13. To a stirred solution of the amine **23** (1.5 equiv) in DMF (50 mL/mmol), triethylamine (4.5 equiv) and the corresponding NHS ester (1 equiv) were added and the reaction mixture was stirred for 4-16 h at room

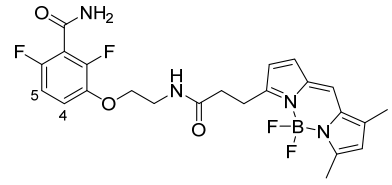
temperature. The solvent was removed under reduced pressure and the residue was purified by chromatography to afford the desired compounds **10**-**13**.

2,6-Difluoro-3-[2-(4-(5-(4-(dimethylamino)phenyl)oxazol-2-yl)benzamido)-ethoxy]benzamide (10). Obtained from amine **23** (8 mg, 37 μmol) and dapoxyl carboxylic acid succinimidyl ester (10 mg, 25 μmol) in 98% yield (12 mg). Chromatography (DCM/ethyl acetate 1:4 to 1:6).



R_f (EtOAc) 0.30; mp 189-192 ºC; IR (ATR) ν 3422, 3345, 1740, 1675, 1613, 1517, 1498; ^{1}H NMR (700 MHz, CD_3OD) δ 3.02 (s, 6H, $2CH_3$), 3.81 (t, J = 5.5, 2H, CH_2N), 4.27 (t, J = 5.5, 2H, CH_2O), 6.84 (d, J = 9.0, 2H, $H^{2'''}$), 6.96 (td, J = 9.0, J = 1.5, 1H, H^5), 7.24 (td, J = 9.0, J = 5.0, 1H, H^4), 7.41 (s, 1H, $H^{4''}$), 7.66 (d, J = 9.0, 2H, $H^{3'''}$), 7.98 (d, J = 9.0, 2H, $H^{2'}$), 8.14 (d, J = 9.0, 2H, $H^{3'}$); ^{13}C NMR (175 MHz, CD_3OD) δ 40.4 (CH_3), 40.7 (CH_2N), 69.7 (CH_2O), 111.9 (dd, $J_{C\text{-}F}$ = 23.0, J = 3.5, C^5), 113.5 ($C^{2'''}$), 116.6 (C^1), 118.0 (d, $J_{C\text{-}F}$ = 8.5, C^4), 121.3 ($C^{4''}$), 126.7 ($C^{3'''}$), 126.9 ($C^{3'}$), 129.1 ($C^{2'}$), 131.2 ($C^{1'}$), 136.7 ($C^{4'}$), 144.9 (d, $J_{C\text{-}F}$ = 12.0, C^3), 150.5 (dd, $J_{C\text{-}F}$ = 251.0, 7.5, CF), 152.5 ($C^{5''}$), 154.3 (dd, $J_{C\text{-}F}$ = 243.0, 5.0, CF), 154.9 ($C^{1'''}$), 160.3 ($C^{2''}$), 165.4, 169.6 (2CO); ESI-MS 507.2 $(M+H)^+$; HRMS (ESI): calcd. for $C_{27}H_{25}F_2N_4O_4$ $(M+H)^+$: 507.1838; found: 507.1845.

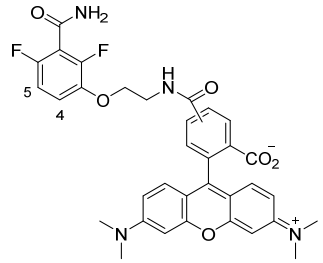
Synthesis of 3-{2-[3-(5,5-difluoro-7,9-dimethyl-5H-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanamido]ethoxy}-2,6-difluorobenzamide (11). Obtained from amine **23** (6 mg, 0.03 mmol) and bodipy-FL succinimidyl ester (10 mg, 0.02 mmol) in 91% yield (12 mg). Chromatography (hexane/ethyl acetate 2:6).



R_f (EtOAc) 0.48; IR (ATR) ν 3422, 3345, 1659, 1609, 1515, 1499; ^{1}H NMR (500 MHz, CD_3OD) δ 2.28 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 2.64 (t, J = 7.5, 2H, CH_2CO), 3.23 (t, J = 7.5, 2H, CH_2CH_2CO), 3.57 (t, J = 5.4, 2H, CH_2N), 4.08 (t, J = 5.4, 2H, CH_2O) 6.21 (s, 1H, CH_{bdpy}), 6.31 (d, J = 4.0, 1H, CH_{bdpy}), 6.90 (d, J = 3.9, 1H, CH_{bdpy}), 6.93 (td, J = 8.9, 1.9, 1H, H^5), 7.14 (td, J = 9.2, 5.1, 1H, H^4), 7.39 (s, 1H, CH_{bdpy}); ^{13}C NMR (175 MHz, CD_3OD) δ 11.2, 14.8 ($2CH_3$), 26.3 (CH_2CH_2CO), 35.9 (CH_2CO), 40.0 (CH_2N), 69.7 (CH_2O), 111.8 (dd, $J_{C\text{-}F}$ = 22.6, 4.0, C^5), 116.7 (dd, $J_{C\text{-}F}$ = 25.1, 20.3, C^1), 116.9 (d, $J_{C\text{-}F}$ = 9.3, C^4), 117.7, 121.3, 125.8, 129.6 ($4CH_{bdpy}$), 134.9, 136.5 ($2C_{bdpy}$), 144.8 (dd, $J_{C\text{-}F}$ = 10.6, 2.8, C^3), 147.8 (dd, $J_{C\text{-}F}$ =

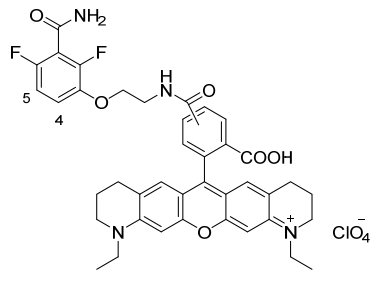
243.6, 8.7, CF), 153.1 (dd, $J_{C\text{-}F}$ = 240.9, 7.0, CF), 158.3, 161.8 ($2C_{bdpy}$), 165.3, 175.0 (2CO), 175.1 (C_{bdpy}); HRMS (ESI): calcd. for $C_{23}H_{22}BF_4N_4O_3$ $(M\text{-}H)^-$: 489.1727; found: 489.1719.

Mixture of 4- and 5-[({2-[3-(aminocarbonyl)-2,4-difluorophenoxy]ethyl}amino)carbonyl]-2-[6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl]benzoate (12). Obtained from amine **23** (4 mg, 19 μmol) and 4- and 5-TAMRA succinimidyl ester (mixed isomers, 10 mg, 19 μmol) in 99% yield (12 mg) as a 1:0.5 mixture of 4- and 5-TAMRA regioisomers.



R_f (DCM/MeOH/NH_3, 8:2:0.01) 0.29; IR (ATR) ν 3321, 1596, 1365, 1348; ^{1}H NMR (500 MHz, CD_3OD) δ 3.28 (s, 12H, $4CH_3$), 3.77 and 3.85 (t, J = 5.5, 2H, $C\underline{H}_2NH$), 4.23 and 4.30 (t, J = 5.5, 2H, CH_2O), 6.89-6.93 (m, 3H, $2CH_{Ar}$, H^5 regioisomer), 6.97 (td, J = 9.0, 1.9, 1H, H^5 regioisomer), 7.01 (dd, J = 9.5, 2.4, 2H, $2CH_{Ar}$), 7.19 (td, J = 9.2, 5.1, 1H, H^4 regioisomer), 7.24-7.28 (m, 3H, $2CH_{Ar}$, H^4 regioisomer), 7.36 (d, J = 7.9, 1H, CH_{Ar} regioisomer), 7.71 (d, J = 1.6, 1H, CH_{Ar} regioisomer), 8.05 (dd, J = 7.9, 1.9, 1H, CH_{Ar} regioisomer), 8.09 (dd, J = 8.1, 1.8, 1H, CH_{Ar} regioisomer), 8.14 (d, J = 8.2, 1H, CH_{Ar} regioisomer), 8.51 (d, J = 1.7, 1H, CH_{Ar} regioisomer). ESI-HRMS (calcd., found for $C_{34}H_{31}F_2N_4O_6$ $[M+H]^+$): 629.2206, 629.2185.

Mixture of 6-{4- and 6-{5-[({2-[3-(aminocarbonyl)-2,4-difluorophenoxy]ethyl}amino) carb onyl]-2-carboxyphenyl}-1,11-diethyl-1,2,3,4,9,10-hexahydro-8*H*-pyrano[3,2-*g*:5,6-*g*']diquinolin-11-ium perchlorate (13). Obtained from amine **23** (3.5 mg, 16 μmol) and 4- and 5-ATTO565 succinimidyl ester (mixed isomers, 10 mg, 16 μmol) in 66% yield (9 mg) as a 7:3 mixture of 4- and 5-ATTO565 regioisomers.



R_f (DCM/MeOH/NH_3 1:1:0.01) 0.18; IR (ATR) ν 3358, 1602, 1499; ^{1}H NMR (500 MHz, CD_3OD) δ 1.32 (t, J = 7.1, 6H, $2CH_3$), 1.92-1.94 (m, 4H, $2CH_2$), 2.67-2.71 (m, 4H, $2CH_2$), 3.53-3.57 (m, 4H, $2CH_2C\underline{H}_2N$), 3.62-3.67 (m, 4H, $2CH_3C\underline{H}_2N$), 3.78 (t, J = 5.5, 2H, $C\underline{H}_2NHCO$), 4.24 (t, J = 5.5, 2H, CH_2O), 6.87-6.89 (m, 4H, $4CH_{Ar}$),

6.93 (td, J = 8.9, 1.9, 1H, H^5), 7.20 (td, J = 9.2, 5.1, 1H, H^4), 7.66 and 7.71 (d, J = 1.4, 1H, CH_{Ar}), 8.06 (dd, J = 8.1, 1.7, 1H, CH_{Ar}), 8.08-8.10 (m, 1H, CH_{Ar}). ESI-HRMS (calcd., found for $C_{40}H_{40}F_2N_4O_6$ $[M+H]^+$): 710.2910, 710.2826.

2.1.4. Compounds solubility . Stock solutions of fluorescent compounds **2-16** were prepared in DMSO at stored at -20ºC. The solubility of each compound in experimental buffer was determined by ultracentrifugation and spectrophotometric measurements as previously described.[1] We note that apparent solubility increased in the presence of protein.

2.2. Molecular Modeling

Docking. The ICM package[5] was employed to give computational insights of the potential binding modes of the fluorescent probes **6**, **15** and **16**. The X-ray crystal structure of SaFtsZ (PDB ID 4DXD) was regularized using ICM standard protocol for the docking experiments. The boundaries of the docking box were defined including the whole cleft formed between the C-terminal domain and helix H7. For each fluorescent ligand, 3D atomic coordinates, tautomeric forms, stereochemistry, hydrogen atoms, and protonation states were assigned using standard procedures. Ligand molecules were prepared for docking by rotational search followed by a Cartesian minimization using MMFF[6] force field in the absence of the receptor. These free molecules were optimized with global energy in the internal coordinate space and the lowest-energy conformations were used for further studies. The ICM methodology utilizes ligand torsional or roto-translational variables to optimize flexible ligands in a grid-based receptor field. Energies are computed using MMFF partial charges with the ECEPP/3[7] force field. The ligand docking was also complemented by local energy minimization using a Biased Probability Monte Carlo method.[8] The best docking solutions of each probe were retained and subsequently used as starting configurations in the following molecular dynamics simulation protocol.

Molecular Dynamics Simulations . Molecular dynamics was used to assess the stability of the highest scored complex conformations obtained in the previous docking step. SaFtsZ and the probes were prepared as previously described.[1] All MD simulations and analysis were performed with GROMACS 4.6[9] using the AMBER ff99SB[10] force field. Prepared structures were immersed in dodecahedron boxes of TIP3P water molecules.[11] Counter ions were also added to maintain electro-neutrality.

Preparatory simulations: The docking starting structures were energy minimized and subsequently the solvent was equilibrated in three phases. For the first phase of equilibration, an NVT ensemble was applied during 5 ns. Position restraints using harmonic potentials with a force constant of 1000 kj mol^{-1} nm^{-2} were applied to all protein and ligands atoms. Two additional NVT equilibration phases of 5 ns were applied. In the first equilibration all protein atoms were restrained, whereas in second, the position restraints were limited to backbone atoms only. The pressure of the simulation box was kept at an average of 1 bar using the isotropic Berendsen barostat[12] with a time constant of 1 ps and a compressibility of 4.5 x 10^{-5} bar^{-1}. During the equilibration a 2 fs integrations timestep was used and the neighbor list were updated

every 10th time steps. Electrostatic interactions were evaluated using the particle-mesh Ewald (PME) method[13] with Van der Waals interactions truncated at 14 Å. The long range Lennard-Jones interactions were analytically corrected for in the calculations of the pressure and the energy. The solvent and the protein were coupled separately to an external heat bath at 298 K with the velocity-rescaling thermostat[14] using a time constant of 0.5 ps. Water molecules were constrained using the SETTLE algorithm[15] and the covalent bonds in the protein were constrained using the LINCS algorithm.[16] Boundaries were treated periodically.

Production simulations: Production MD simulations were carried out for 500 ns using NPT ensemble, in the absence of any restraints. The isotropic Parrinello-Rahman barostat[17] was used to keep the average pressure at 1 bar with a time constant of 1 ps. All other simulation parameters were the same as during the equilibration. The trajectories were sampled every 40 ps for analysis.

2.3. Biochemical and Biological Methods

Proteins p urification and assembly conditions . Full length FtsZ from *Bacillus subtilis* (BsFtsZ) was overproduced and purified as described.[1] Full-length untagged BsFtsZ point mutants, G196A and V307R, were constructed by site-directed mutagenesis using the pHis17-BsFtsZ plasmid[18] with appropriate pairs of complementary oligonucleotides carrying the mutation, mutated *ftsZ* genes were confirmed by complete open reading frame sequencing and the mutants were purified following the same procedure as for wild type BsFtsZ. The molecular weight of each protein was confirmed by mass spectroscopy (MS) at the CIB Proteomics Facility with a MALDI-TOF-TOF Autoflex III (Bruker Daltonics, Bremen, Germany): BsFtsZ, 40395 (calculated 40395); BsFtsZ-G196A, 40409 (calc. 40409); BsFtsZ-V307R, 40452 (calc. 40452)**.** These proteins were assembled in Hepes buffer pH 6.8 (50 mM Hepes, 50 mM KCl, 1 mM EDTA, pH 6.8) plus 10 mM $MgCl_2$ and 1 mM GTP or 0.1 mM GMPCPP at 25 °C. FtsZ from *Escherichia coli* (EcFtsZ) was purified as described[19] and assembled in Hepes Buffer pH 6.8 as above.

Full-length *Staphylococcus aureus* FtsZ (SaFtsZ) was cloned and purified using the IMPACT affinity system (NEB). The gene was cloned into the NdeI and SapI sites of pTXB1 and the protein was expressed at 16ºC overnight in *E. coli* BL21 (DE3) cells by the addition of 0.4mM IPTG. Cells were lysed in buffer A (20mM Hepes pH8.5, 0.5M NaCl, 1mM EDTA) by two French Press passes. The soluble fraction was loaded on a

chitin column (NEB), which was then thoroughly washed with buffer A and finally with 3 column volumes of buffer B (A + 50mM DTT) and left overnight at 4ºC to cleave off the intein tag. The untagged protein was eluted with buffer B and pooled fractions were concentrated and subjected to size exclusion chromatography (Superdex 75) in buffer C (20mM Tris-HCl pH7.5, 50mM KCl, 1mM EDTA, 10% glycerol). Peak fractions were concentrated and stored at -80ºC. SaFtsZ MS Mr was 41039 (calc. 41037). Due to the lack of aromatic residues, SaFtsZ concentration was measured with the BioRad protein assay using spectrophotometrically calibrated BsFtsZ standards. SaFtsZ was assembled in Mes buffer pH 6.5 (50 mM Mes, 50 mM KCl, 1 mM EDTA, pH 6.5) plus 10 mM $MgCl_2$ and 1 mM GTP or 0.1 mM GMPCPP.

Tubulin was purified from calf brain.[20] Prior to use, tubulin was equilibrated by gel chromatography in cold GAB buffer (3.4 M glycerol, 10 mM sodium phosphate, 1 mM EGTA and 0.1 mM GTP, pH 6.8) and assembled at with of 6 mM $MgCl_2$ and 1 mM GTP 37 °C.

FtsZ polymers pelleting . Samples (0.1 mL) were prepared in polycarbonate ultracentrifuge tubes in a thermostat at 25 ºC. Polymerization was initiated by the addition of nucleotide and then centrifuged for 20 min at 100,000 rpm and 25 ºC in a TLA-100 rotor employing a TLX ultracentrifuge. Pellets were resuspended in the same volume of buffer and, as the supernatant, diluted with sample solution. Aliquots of pellets and supernatants were loaded with an electrophoretic shift between them into the same lanes of 12%-polyacrylamide gels with 0.1% SDS. Gels were stained with Coomassie blue, scanned with a CS-800 calibrated densitometer (BioRad) and analyzed with Quantity One software (BioRad).

Monitoring proteins assembly w ith light scattering . Time course of protein polymerization was monitored with right angle light scattering from 0.5 mL samples placed in a 10 x 2 mm cell, in a Fluoromax-4 spectrofluorometer at 350 nm (with 0.5 nm excitation and emission band-pass). The light scattered by the sample was divided by the beam intensity (current of the internal reference detector, µA) to correct for any lamp fluctuations. Solution conditions and temperature were adjusted for each protein and the reaction was started by consecutive additions of magnesium and guanine nucleotide.

Electron microscopy. 20 µL of polymer solutions were adsorbed to carbon-coated EM grids, negatively stained with 2% uranyl acetate and examined with a Jeol 1230

electron microscope equipped with a 19 M pixel CMOS TVIPS Temcam-F416 camera and operated at 100 kV.

GTPase activity. GTP hydrolysis in FtsZ solutions at 25ºC was measured from the released inorganic phosphate employing the malachite green method.[21]

Fluorescence and anisotropy meas urements. The emission fluorescence and the anisotropy of the different probes were acquired in a Fluoromax-4 (Horiba Jobin Yvon) photon-counting spectrofluorometer using 2x10mm cells at 25ºC, employing the maximum excitation and emission wavelength for each probe with 2- and 5-nm bandwidths, respectively.

Stoichiometry and affinity of binding of the fluores cent probe to BsFtsZ polymers. The stoichiometry of binding of **6** to BsFtsZ polymers was measured using a centrifugation assay (adapted from Huecas *et al*.[22]). 10 μM BsFtsZ was polymerized at 25 ºC in Hepes buffer, 10 mM $MgCl_2$, 2% DMSO, 0.1 mM GMPCPP or 1 mM GTP at 25 ºC in the presence of different known concentrations of **6** in a final volume of 0.1 mL. Samples were then centrifuged as above. After centrifugation the supernatant was carefully withdrawn and the pellets were resuspended in the same volume of buffer. The concentration of free **6** was determined spectrophotometrically in the supernatant, employing the extinction coefficient ε_{474} = 20994 $M^{-1}cm^{-1}$ and the concentration of **6** bound to FtsZ was calculated as the difference of the known total concentration of nucleotide minus the free concentration. To calculate the FtsZ concentration, 5 μL of the samples were separated and applied to SDS-12% polyacrylamide gels, stained with coomassie blue, scanned and the protein bands quantified as above.

Binding of the NBD-derivatives probes to BsFtsZ was measured by the increase in anisotropy of the probe (adapted from Huecas *et al*.[22]). Fixed concentration of the probe (3 μM) were titrated with different BsFtsZ polymer concentrations (0-40 μM) in Hepes buffer with Mg^{2+}, to obtain the anisotropy increment, Δr_{max}, corresponding to all of the probe bound. The increase in anisotropy was plotted against BsFtsZ polymer concentration (calculated by subtracting to the total protein concentration, the critical concentration for polymerization under the same conditions) and iteratively least-squares fitted with an isotherm of binding to one site. The estimated values of Δr_{max} were used to approximate the free FtsZ concentrations, and these new values were employed again, until an unchanging Δr_{max} value was obtained. The convergent data were used to calculate the binding constant of FtsZ polymers to the fluorescent probe.

Analytical ultracentrifugation. Sedimentation velocity experiments were made in a Beckman Optima XL-I analytical ultracentrifuge equipped with interference and absorbance optics, using an An50/Ti rotor with 12-mm double-sector centerpieces at 45000 rpm, and at 25 ºC. Differential sedimentation coefficient distributions, c(s), were calculated with SEDFIT (ref). The weight average sedimentation coefficient values measured in buffer at 25 ºC were corrected to H_2O at 20 ºC, $s_{20,w}$.The interference optics was employed to measure BsFtsZ sedimentation and radial absorbance data at 474 nm were acquired to measure the ligand **6**.

Antibacterial activity. MIC value against *B. subtilis* 168 was determined in cation-adjusted Mueller-Hinton II broth (CAMHB) at 37 ºC with shaking, by the broth macrodilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).

Growth curves. *B subtilis* 168 was grown at 37 ºC in CAMHB with shaking to an optical density of 0.1-0.2, and then DMSO or compound at the desired concentration was added. The incubation of the culture was continued and the optical density at 600 nm was followed over time.

Phase-contrast light microscopy a nd Fluorescence microscopy . *B. subtilis* 168 cells were grown in CAMHB and *S. aureus* Mu50 cells were grown in TSB (Trypticasein Soy Broth) both with shaking at 37 ºC to an absorbance of 0.1-0.2 at 600 nm, then incubated either with DMSO or with the compounds and small aliquots were harvested at appropriate time intervals. The bacterial division phenotype was observed by phase-contrast light microscopy. The Z-ring was visualized with the fluorescent probes through a 100X PlanApochromatic objective with a Zeiss Axioplan fluorescence microscope equipped with a Hamamatsu CCD camera C11440. Membranes were stained with FM4-64 (1 μg/mL), added to the cells prior to visualization.

Immunofluorescence. Cells of *B s ubtilis* 168 were treated with compound **28** and FtsZ observed by immunofluorescence with anti-FtsZ monospecific antibodies as previously described.[2]

3. References

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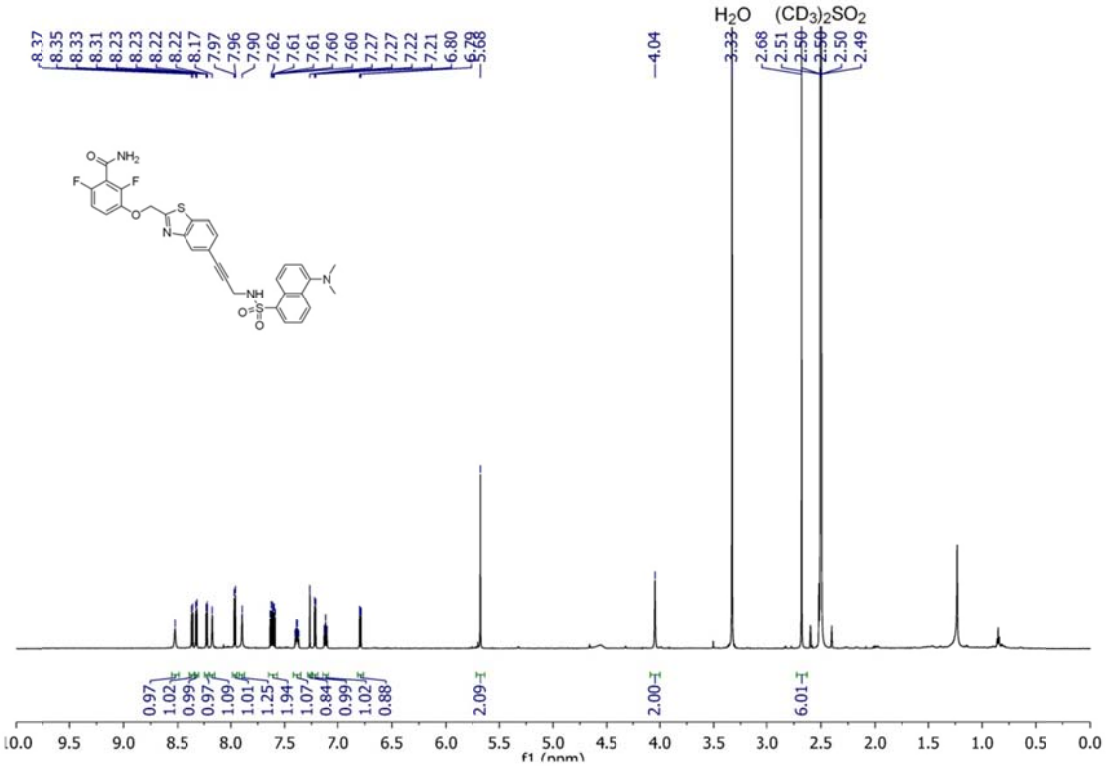
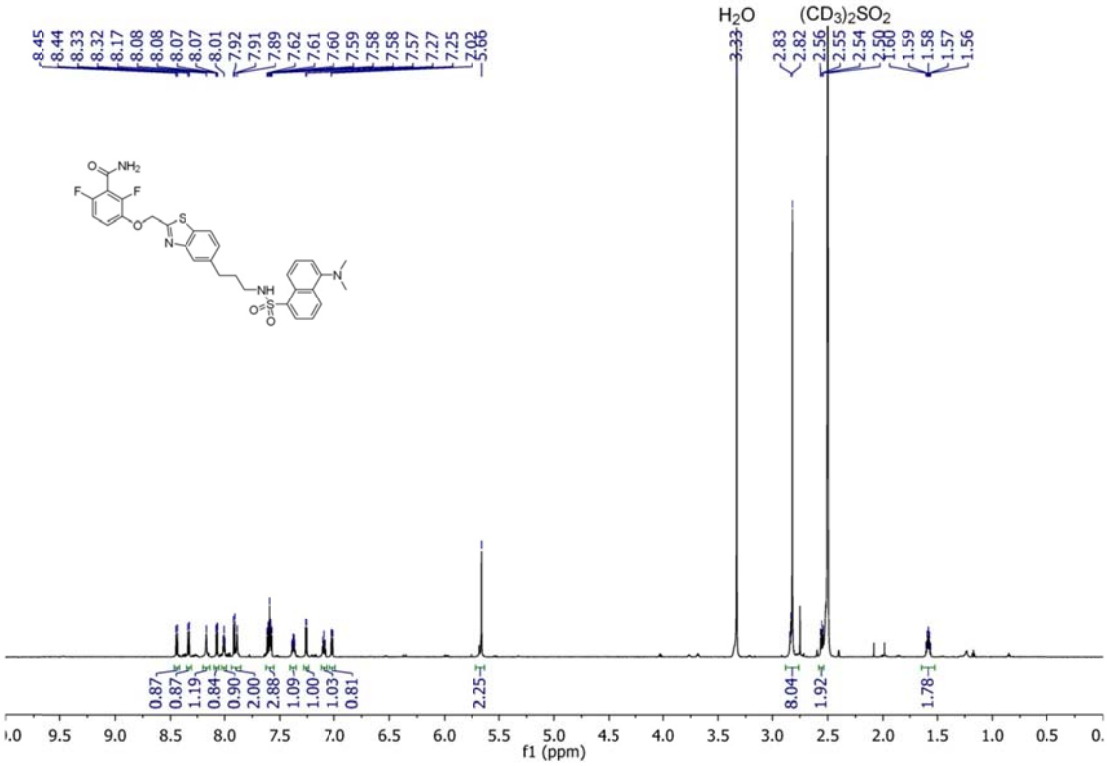
4. NMR Spectra

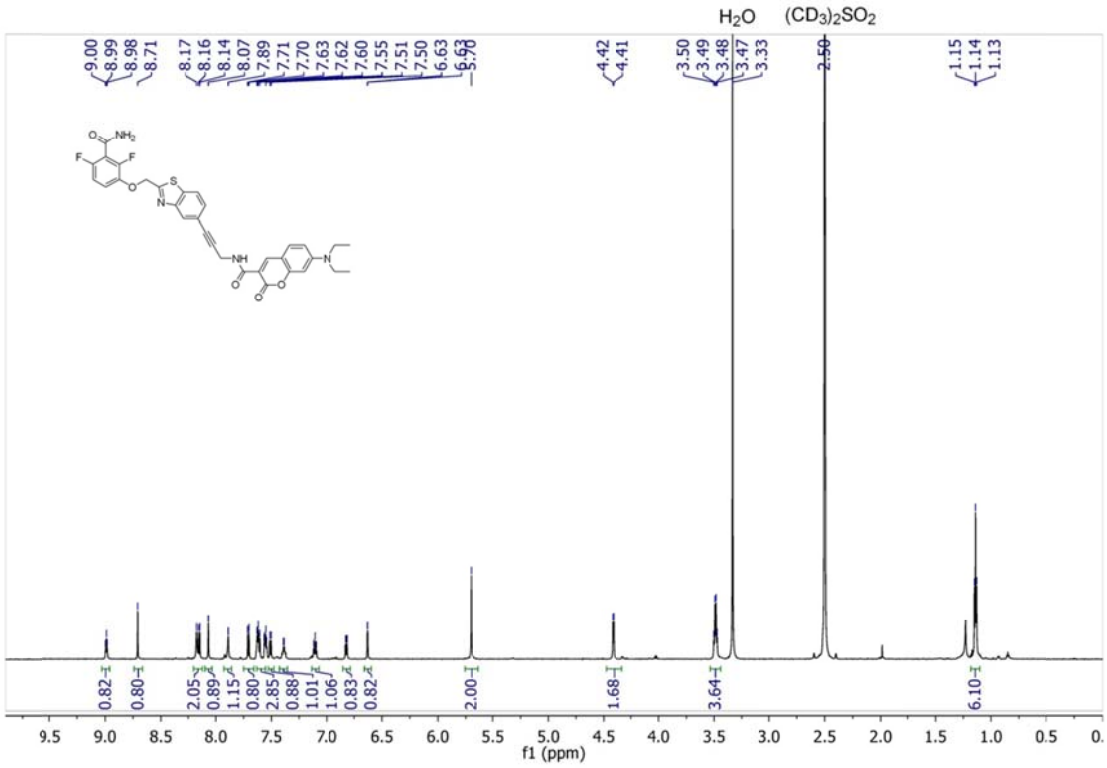
^{1}H NMR spectrum for **2** ($CD_3SO_2CD_3$, 700 MHz)



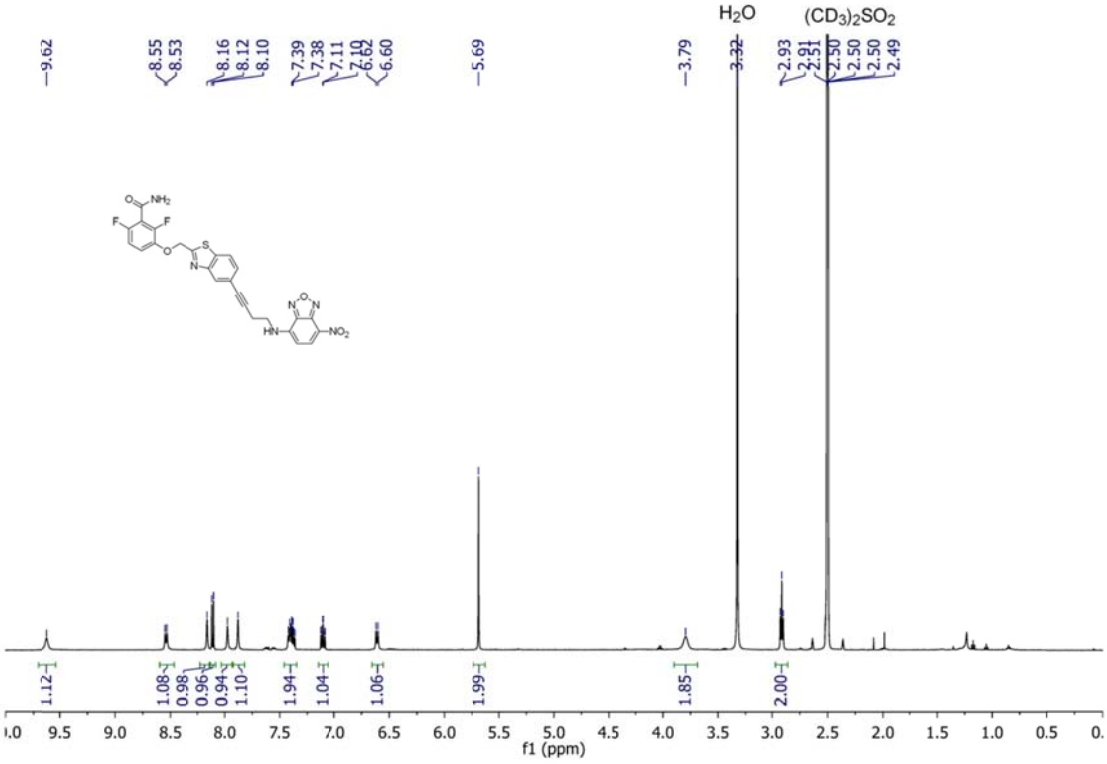
^{1}H NMR spectrum for **3** ($CD_3SO_2CD_3$, 700 MHz)



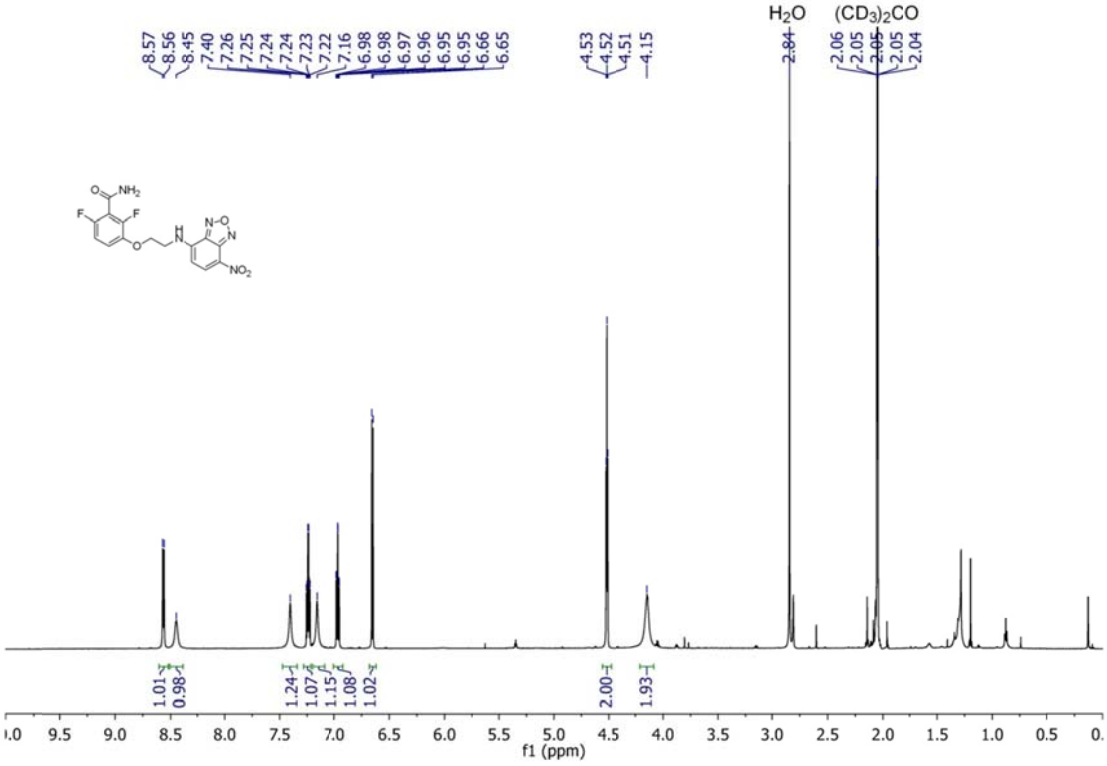
^{1}H NMR spectrum for **4** ($CD_3SO_2CD_3$, 700 MHz)



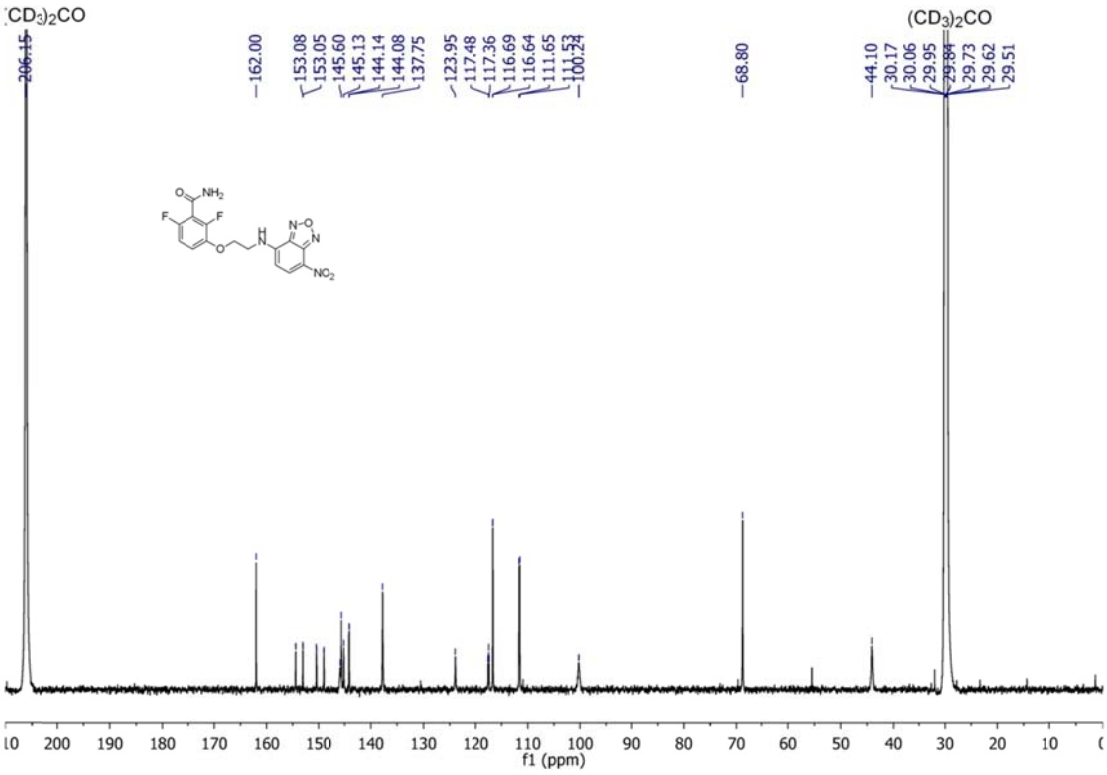
^{1}H NMR spectrum for **5** ($CD_3SO_2CD_3$, 500 MHz)



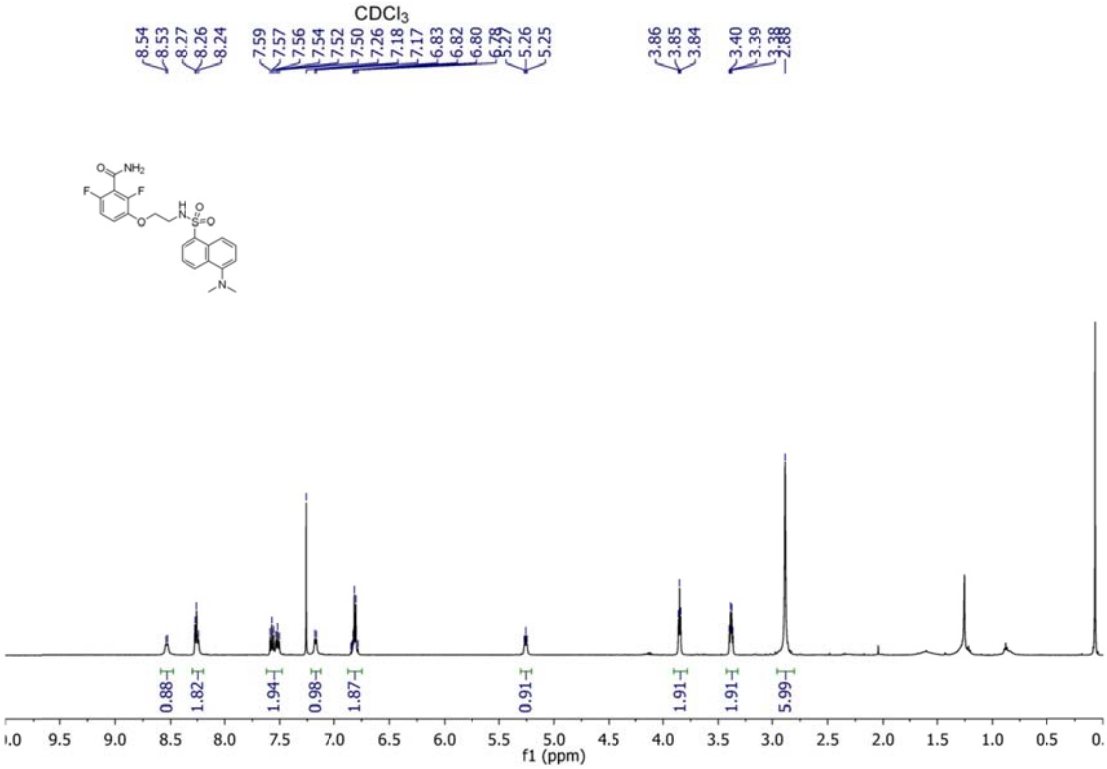
^{1}H NMR spectrum for **6** (CD_3COCD_3, 700 MHz)



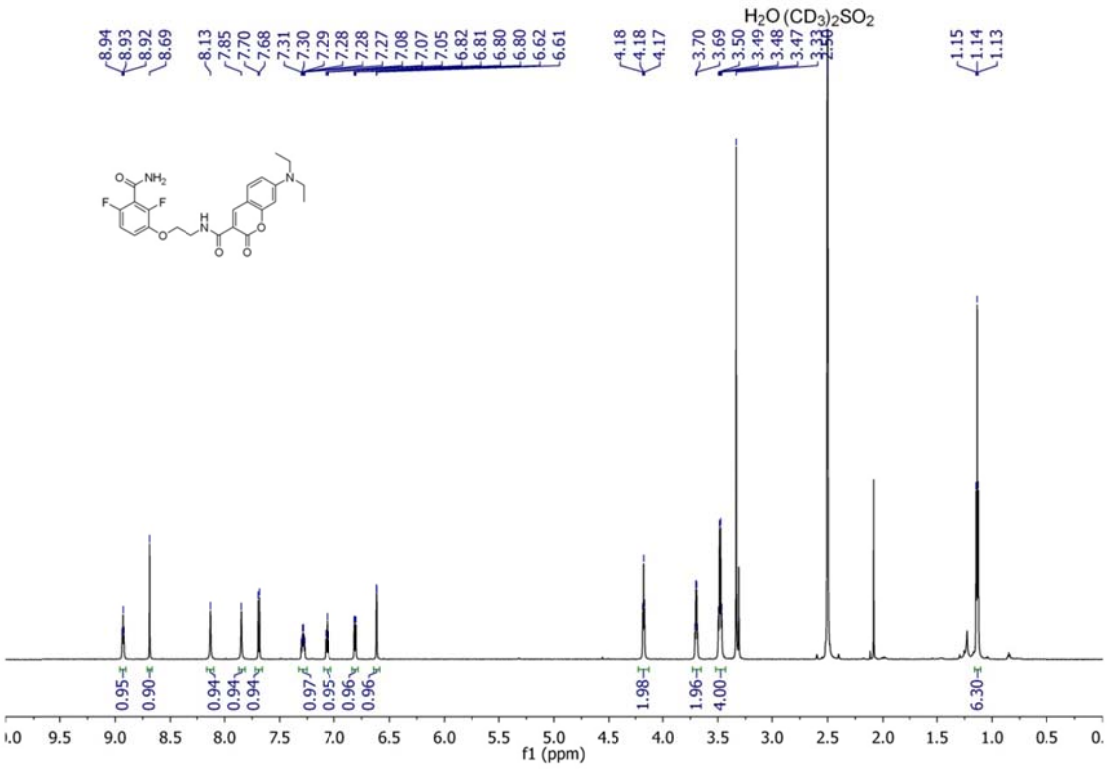
^{13}C NMR spectrum for **6** (CD_3COCD_3, 175 MHz)



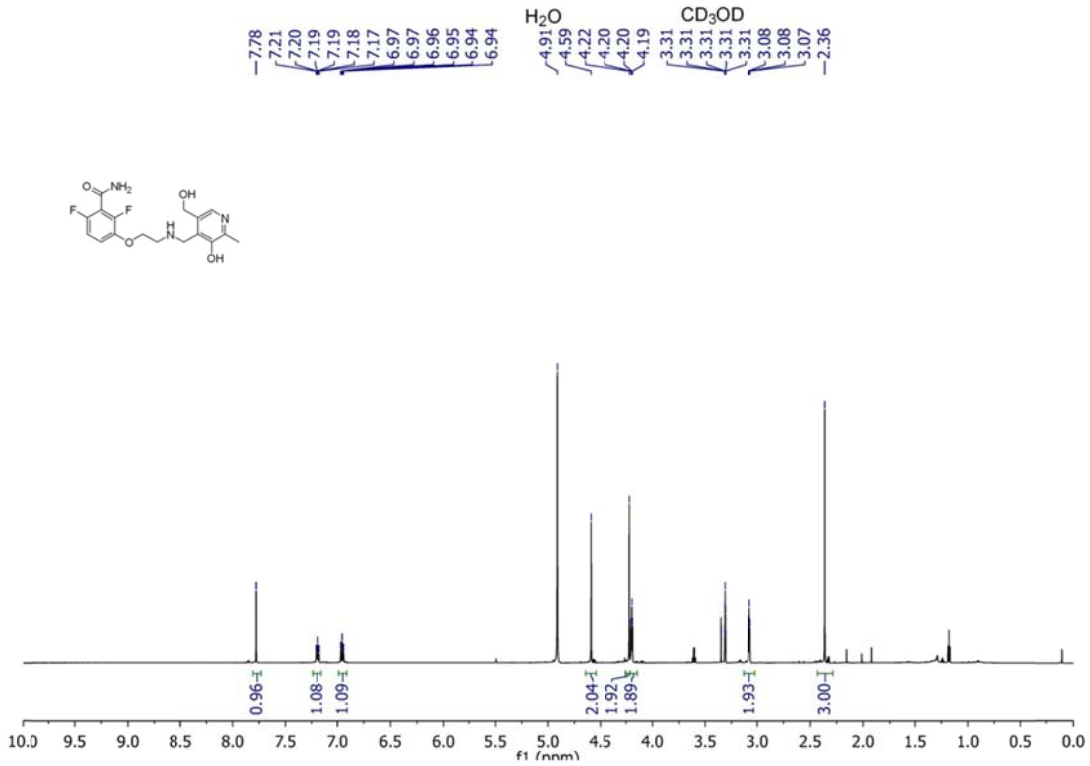
^{1}H NMR spectrum for **7** ($CDCl_3$, 300 MHz)



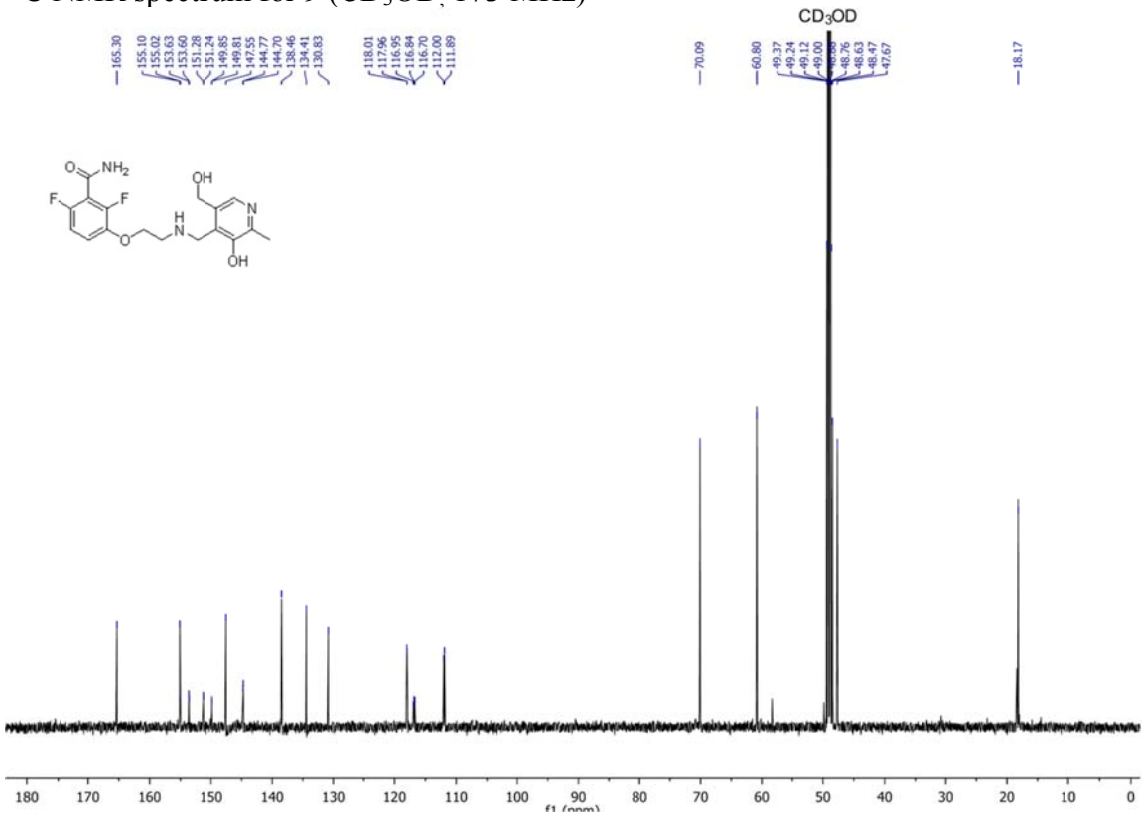
^{1}H NMR spectrum for **8** ($CD_3SO_2CD_3$, 700 MHz)



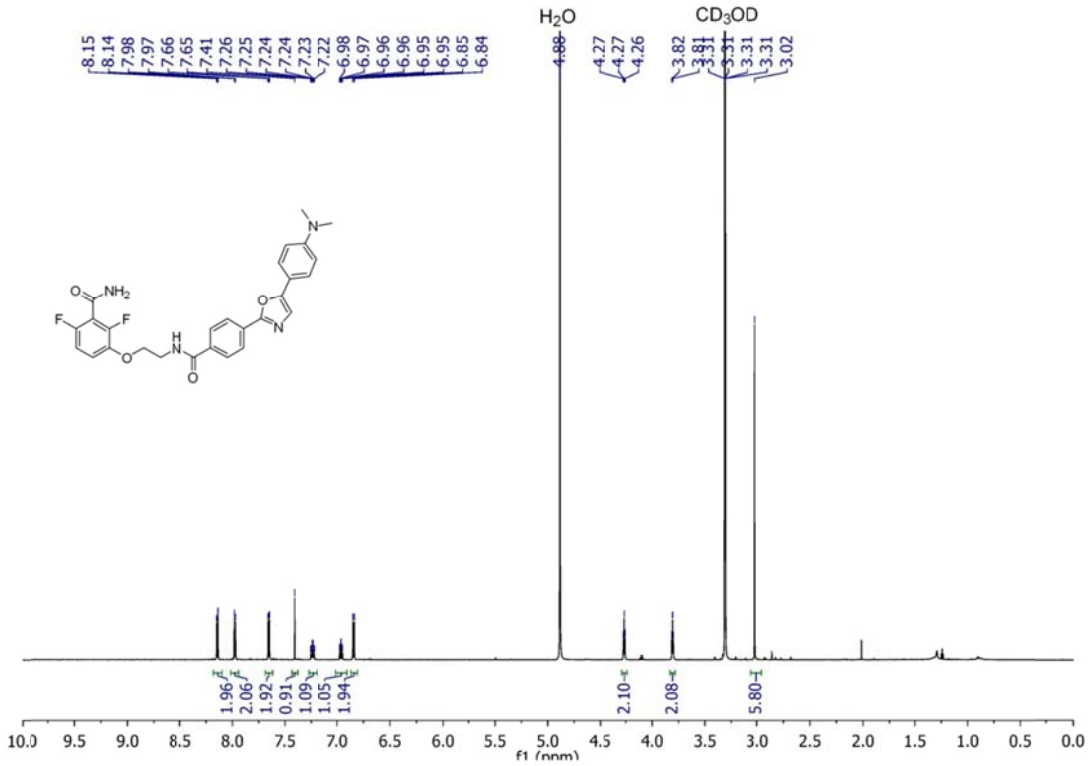
^{1}H NMR spectrum for **9** (CD_3OD, 700 MHz)



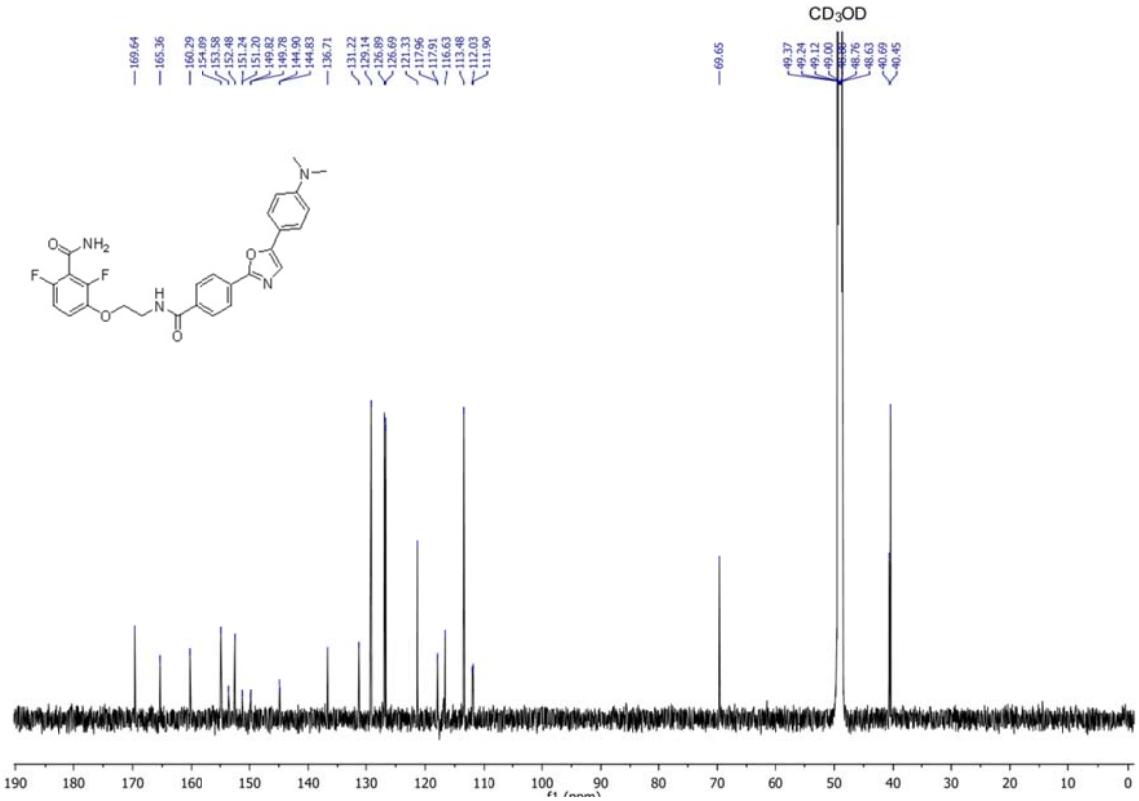
^{13}C NMR spectrum for **9** (CD_3OD, 175 MHz)



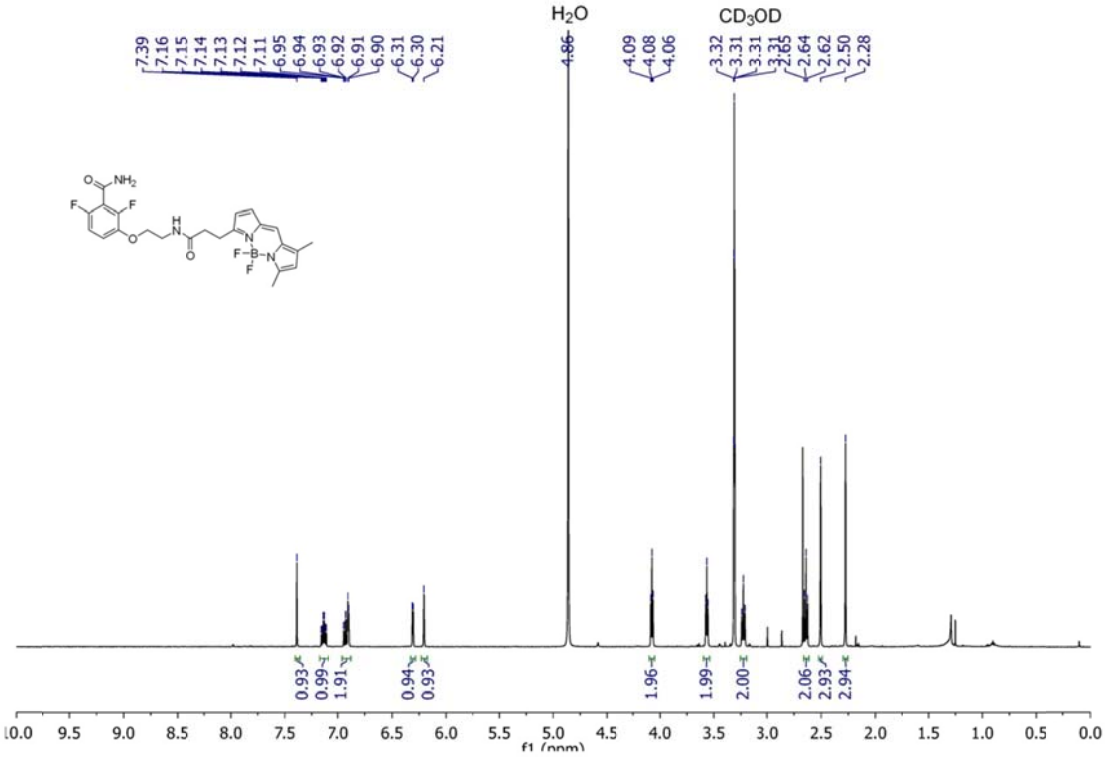
^{1}H NMR spectrum for **10** (CD_3OD, 700 MHz)



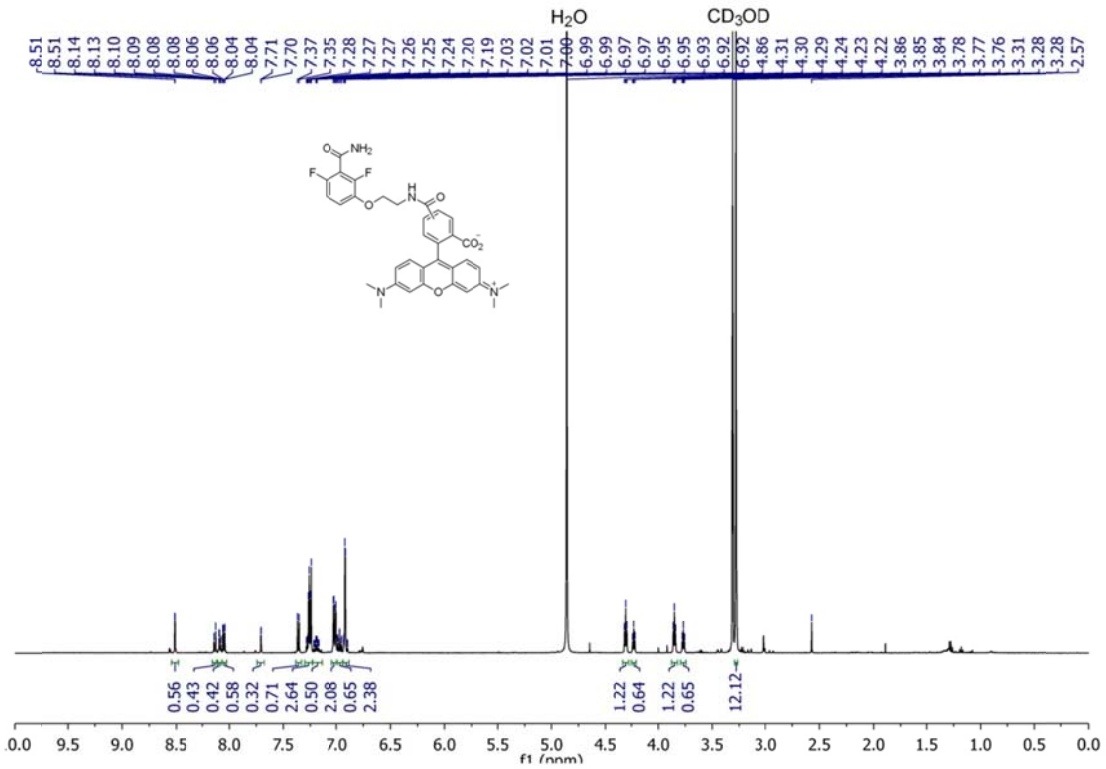
^{13}C NMR spectrum for **10** (CD_3OD, 175 MHz)



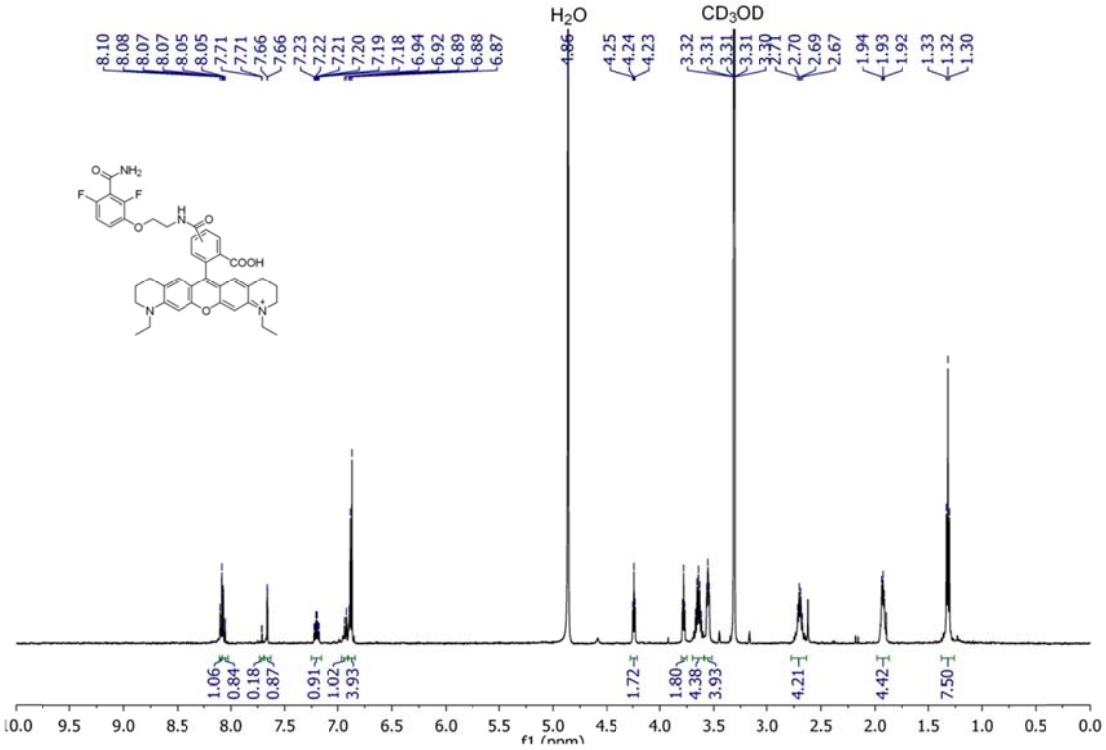
^{1}H NMR spectrum for **11** (CD_3OD, 500 MHz)



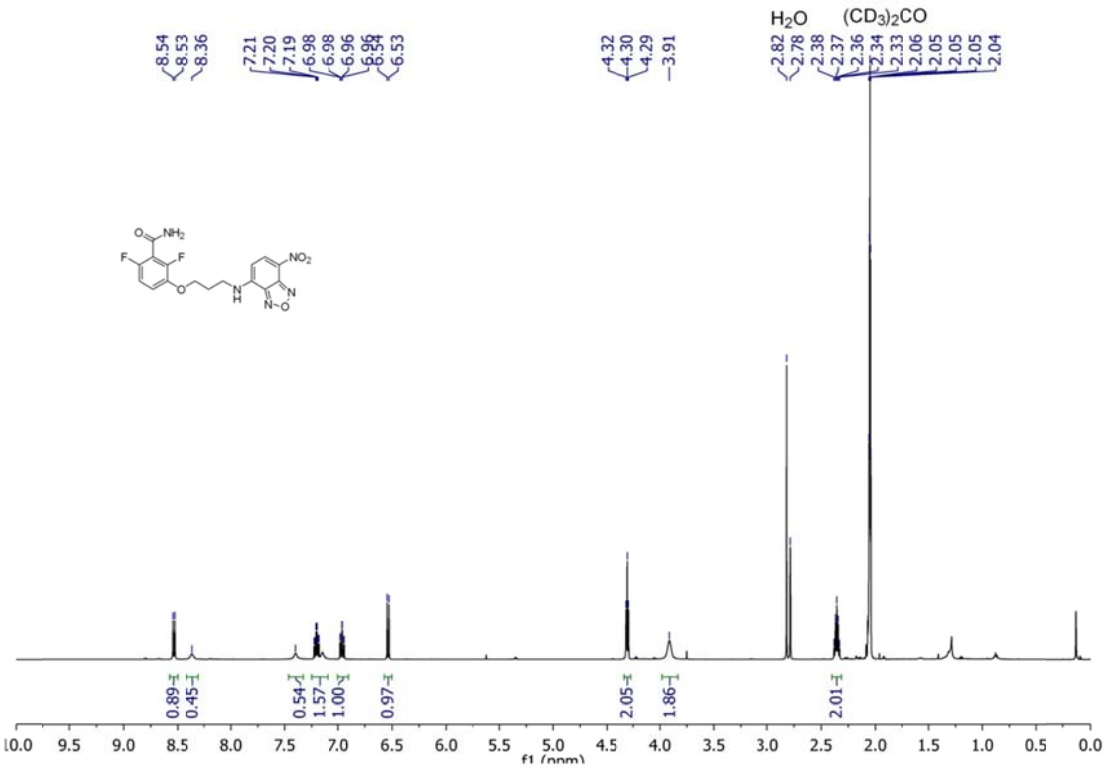
^{1}H NMR spectrum for **12** (CD_3OD, 500 MHz)



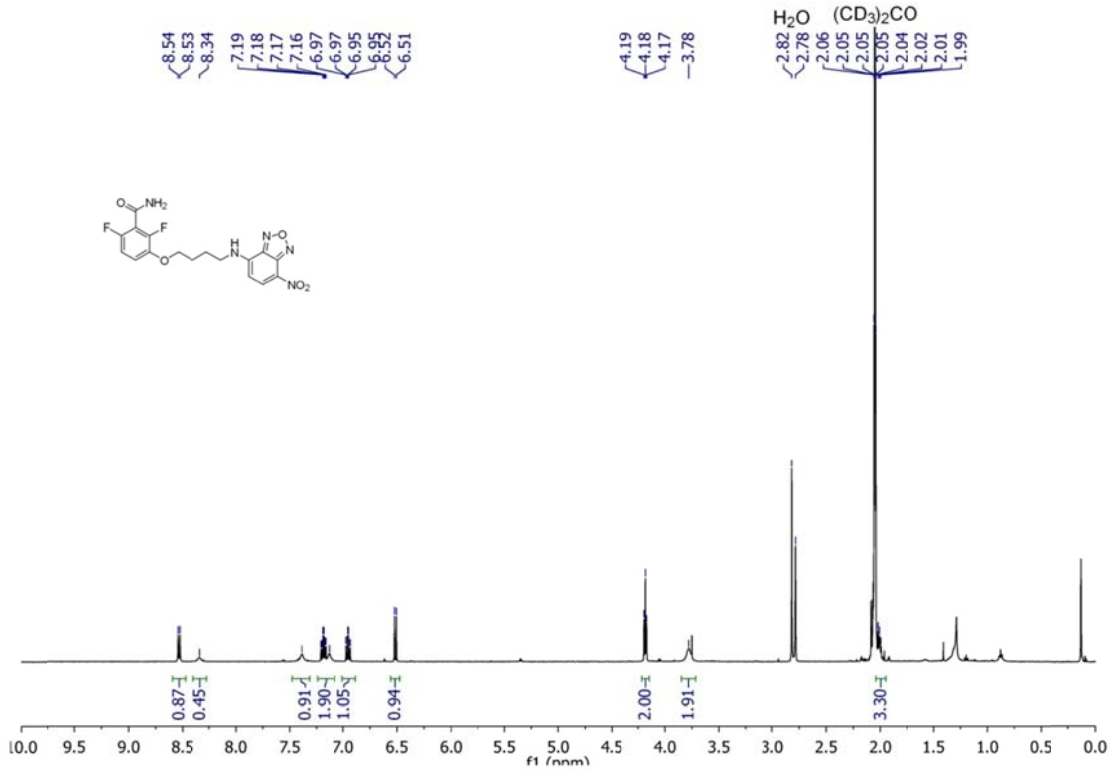
^{1}H NMR spectrum for **13** (CD_3OD, 500 MHz)



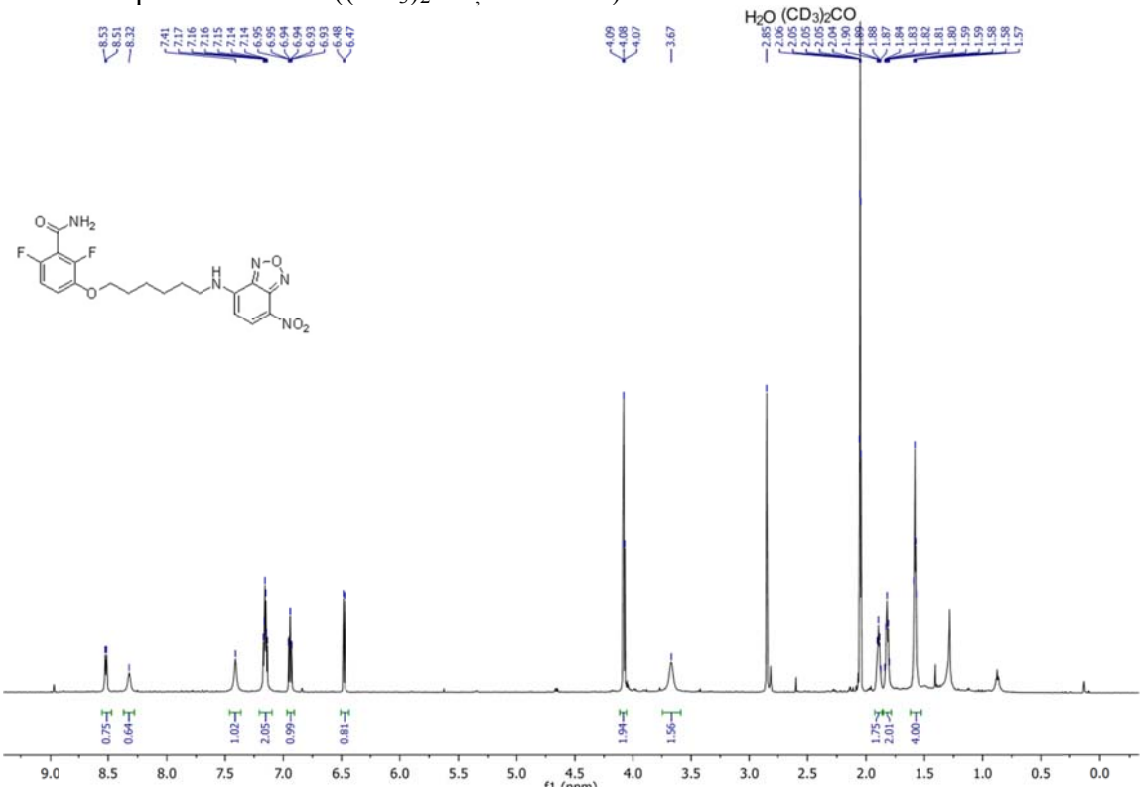
^{1}H NMR spectrum for **14** ($(CD_3)_2CO$, 500 MHz)



^{1}H NMR spectrum for **15** ($(CD_3)_2CO$, 500 MHz)



^{1}H NMR spectrum for **16** ($(CD_3)_2CO$, 700 MHz)



^{13}C NMR spectrum for **16** ($(CD_3)_2CO$, 175 MHz)