Supplementary Information

Highly Sensitive Detection and Labelling of Proteins with a Molecular Rotor-based Fluorogenic probe

Na Chen,‡ Qingyi Wu,‡ Xia Yuan and Hexin Xie *

State Key Laboratory of Bioreactor Engineering, Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China.

E-mail: xiehexin@ecust.edu.cn

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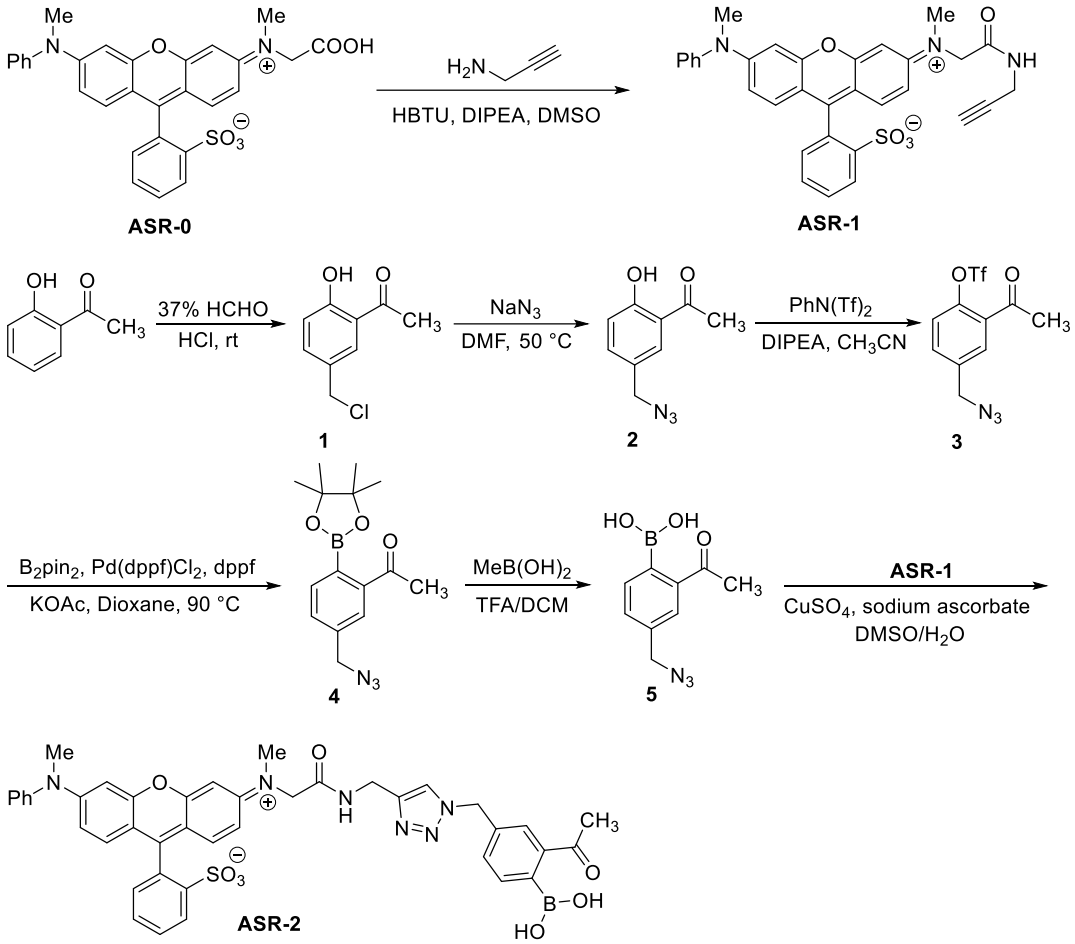


Scheme S1. Synthesis of fluorogenic probe ASR-2

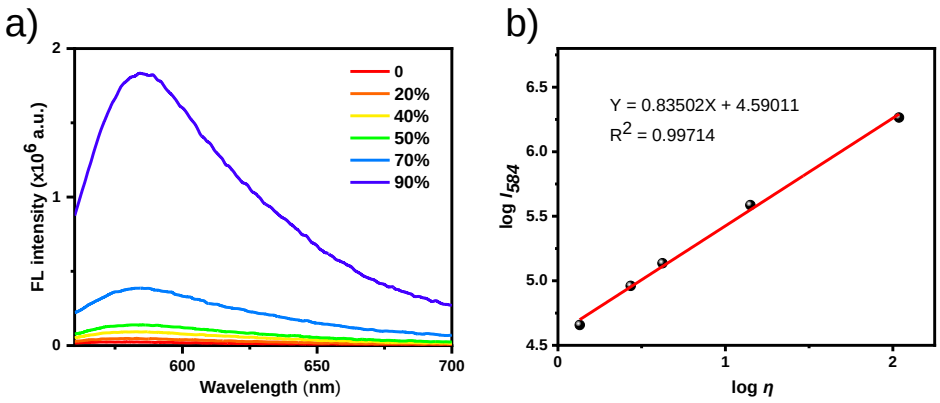


Figure S1. (a) Fluorescence spectra of **ASR-2** (5.0 μM) in water-glycerol mixtures with glycerol fraction varying from 0% to 90%. (b) Linear correlation between log I_{582} and log η at 30 °C. $\lambda_{ex/em}$ = 550/582 nm. η (viscosity, centipoises) of the mixture of water-glycerol at 30 °C.

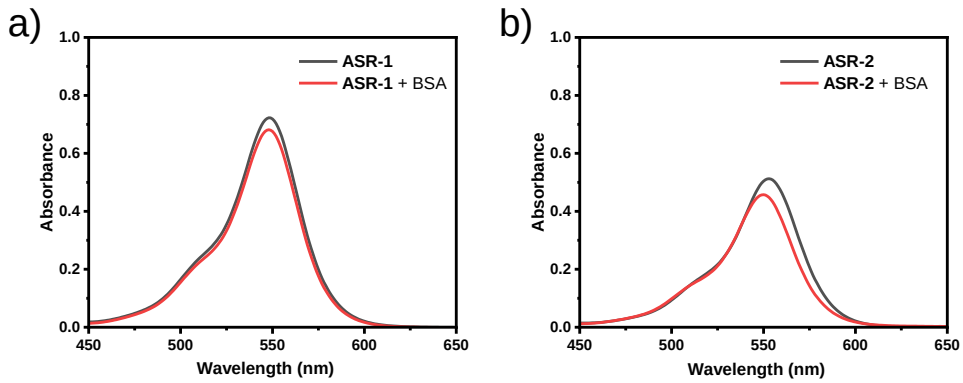


Figure S2. UV-Vis absorption spectra of **ASR-1** (10 μM, a) or **ASR-2** (10 μM, b) in PBS (pH 7.4) before and after incubation with BSA (1 μM) at 37 °C for 30 min.

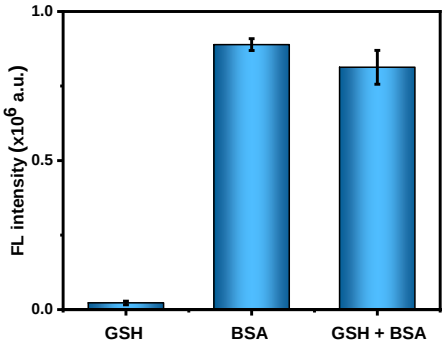


Figure S3. Fluorescence intensity of **ASR-2** (5 μM) in PBS with BSA (1 μM), GSH (50 μM), or both. GSH: glutathione. $\lambda_{ex/em}$ = 550/582 nm.

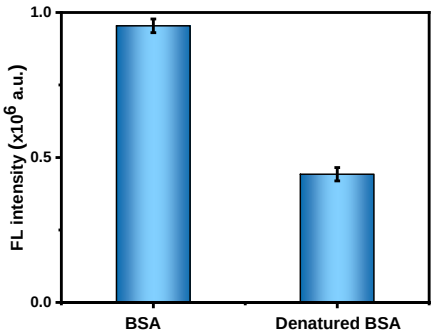


Figure S4. Fluorescence intensity of **ASR-2** (5 μM) incubated with BSA (1 μM) or denatured BSA (obtained by heated at 95 °C for 5 min) at 37 °C for 20 min. $\lambda_{ex/em}$ = 550/582 nm.

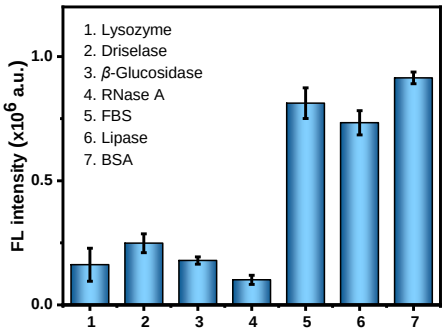


Figure S5. Fluorescence intensity of **ASR-2** (5 μM) incubated with indicated proteins (0.0664 mg/mL) at 37 °C for 20 min. $\lambda_{ex/em}$ = 550/582 nm; FBS: fetal bovine serum.

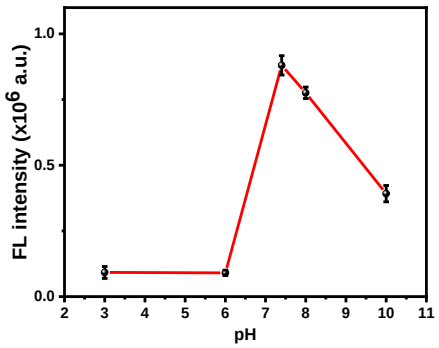


Figure S6. Fluorescence intensity of **ASR-2** (5 μM) after incubated with BSA (1 μM) at 37 °C for 20 min at indicated pH.

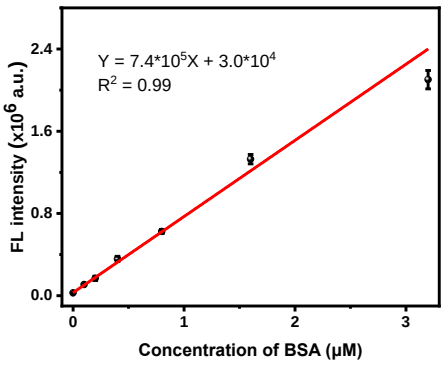


Figure S7. Fluorescence intensity of SYPRO Orange (1 x) in PBS upon incubation with BSA at a series of concentrations (LOD = 14 nM). $\lambda_{ex/em}$ = 470/569 nm.

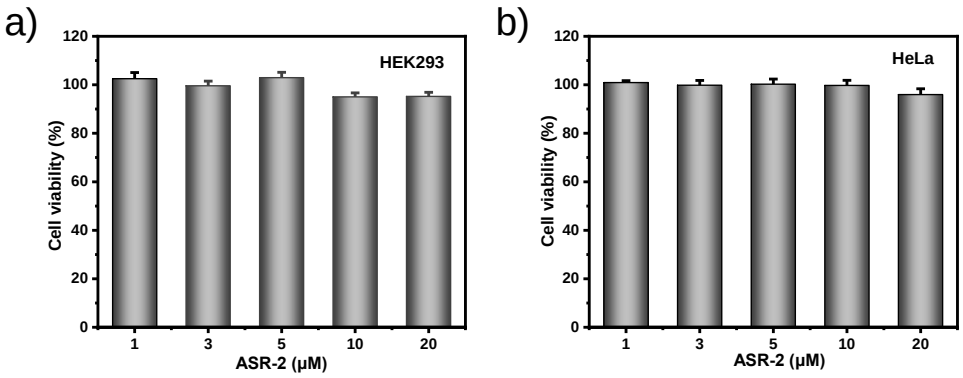


Figure S8. Test of cell viability with **ASR-2**. a) HEK293 or b) HeLa cells were incubated with **ASR-2** at 0, 1, 3, 5, 10, and 20 μM for 24 h, and the cell viability was determined by CCK-8 assay. Error bars mean ± s.d (n = 3).

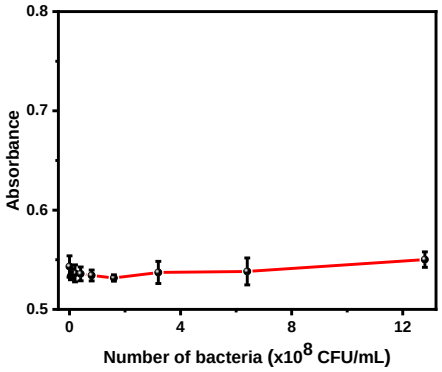


Figure S9. UV-Vis absorbance of the BCA test solution containing *E. coli* lysate correlated with varying cell counts.

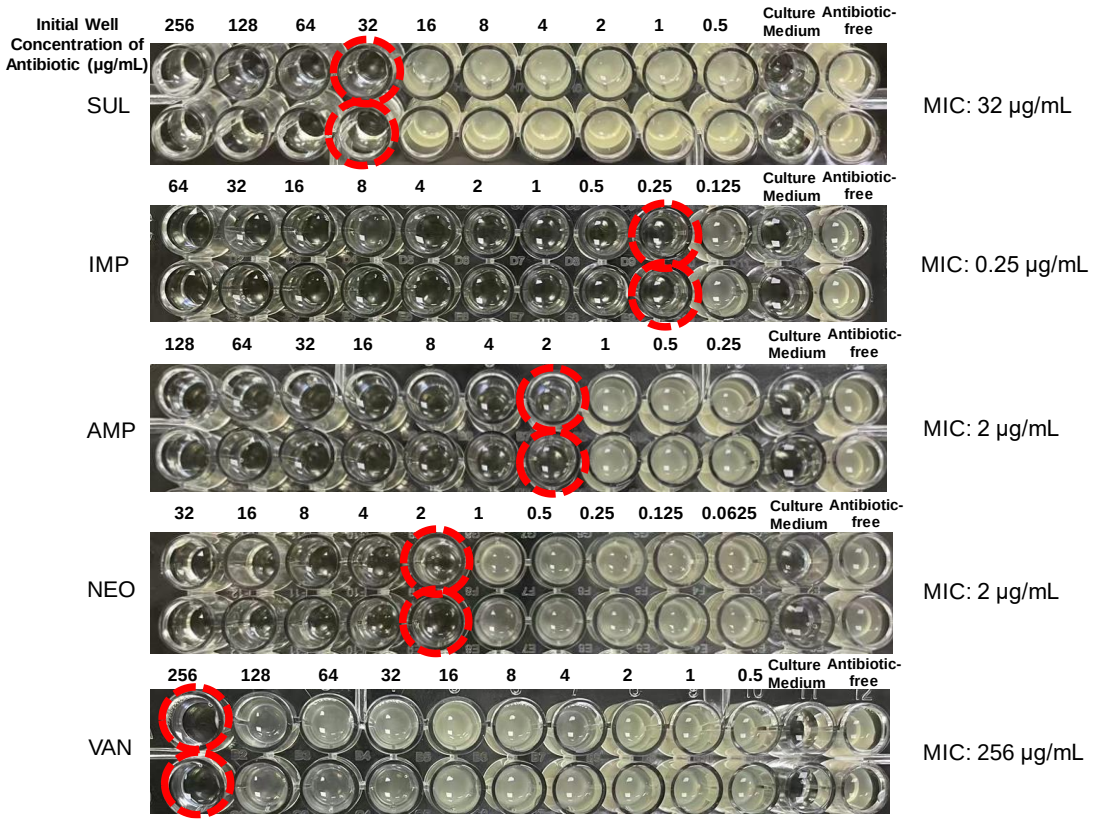


Figure S10. Determination of MIC of antibiotics against *E. coli* (ATCC 25922) using the OD_{600}-based method. IMP: Imipenem; SUL: Sulbactam; AMP: Ampicillin; NEO: Neomycin; VAN: Vancomycin.

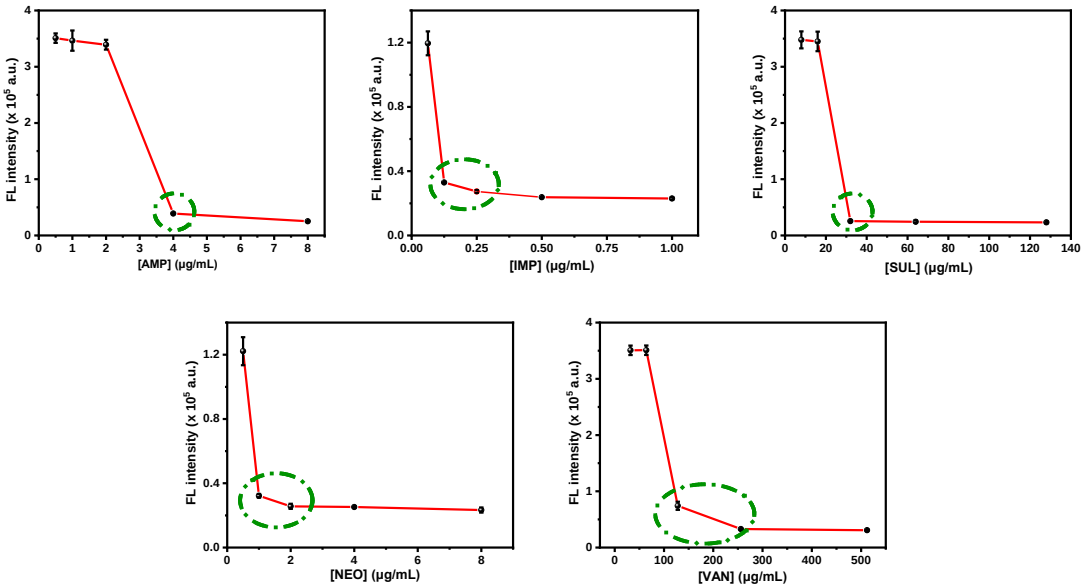


Figure S11. Determination of MIC of different antibiotics against *E. coli* (ATCC 25922) based on fluorescence intensity of **ASR-2**. The MIC was determined as the minimum concentration at which fluorescence intensity was not significantly

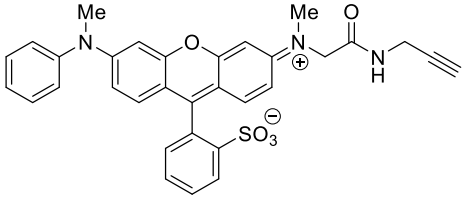
increased. AMP: Ampicillin; IMP: Imipenem; SUL: Sulbactam; NEO: Neomycin; VAN: Vancomycin.

General Information

Unless otherwise mentioned, all chemicals were obtained from commercial sources (e.g., Adamas-Beta, Energy Chemical and TCI China) and used without further purification. Analytical thin layer chromatography was performed on 0.20 mm Qingdao Haiyang silica gel plates and visualized by ultraviolet light. HPLC was performed on a Shimadzu HPLC System equipped with a LC-20AT gradient pump and an inline diode array UV-Vis detector. A reversed-phase C18 column (phenomenex, 5 μm, 4.6 x 250 mm for analysis or 21.2 x 250 mm for preparation) was used with a MeCN/H_2O gradient mobile phase containing 0.1% trifluoroacetic acid at a flow of 1 or 12 mL/min for the analysis or purification. The ^{1}H and ^{13}C NMR spectra were taken on Bruker nuclear magnetic resonance spectrometer (400 MHz or 600 MHz for ^{1}H NMR; 100 MHz or 150 MHz for ^{13}C NMR). Chemical shifts are reported as δ in units of parts per million (ppm) relative to internal standard (^{1}H NMR: $SiMe_4$ = 0.00 ppm) or residual solvent peaks (^{1}H NMR: 7.26 for $CDCl_3$, 2.50 for DMSO-*d*$_6$; ^{13}C NMR: 77.0 for $CDCl_3$, 39.52 for DMSO-*d*$_6$; Data for ^{1}H NMR spectra are reported as follows: chemical shifts are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (δ = 0, s); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as a J value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. High-resolution mass spectra (HRMS) were recorded on a Bruker micro-TOF-QII time of flight mass spectrometer with electrospray ionization. Absorbance spectrum was determined by a UV1800 Series UV-Vis spectrophotometer (Shimadzu, Japan). Fluorescence spectra were obtained on a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon). Absorbance for CCK-8 assay was determined in a microplate reader (Molecular Devices, SpectraMax *i*3). Fluorescence images of live cells were taken using a DMI 3000B

fluorescence microscope (non-confocal, Leica, Germany) or a TCS SP8 confocal microscopy (Leica, Germany). Image processing was made on image J software (National Institutes of Health, USA). HeLa cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

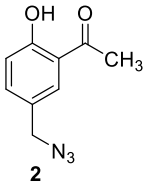
Synthesis and Characterization



ASR-1

(*E*)-2-(3-(methyl(2-oxo-2-(prop-2-yn-1-ylamino)ethyl)iminio)-6-(methyl(phenyl)amino)-3H-xanthen-9-yl)benzenesulfonate (ASR-1)

A solution of **ASR-0**[1] (89.3 mg, 0.17 mmol), HBTU (128.0 mg, 0.33 mmol), propargylamine (23 μL, 0.34 mmol) and DIPEA (56 μL, 0.34 mmol) in DMSO (2.5 mL) was stirred at room temperature for 1 h. Upon completion of the reaction (monitored by TLC), DCM (10 mL) was added and the precipitate was washed with brine (10 mL x 3). The residue was purified by flash chromatography on a silica gel column (DCM:MeOH = 9:1 to 7:1) to afford the title compound as a purple black powder (85.6 mg, 89%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ^{1}H NMR (400 MHz, DMSO) δ 8.72 (t, *J* = 5.6 Hz, 1H), 7.98 (d, *J* = 7.9 Hz, 1H), 7.63 (t, *J* = 7.6 Hz, 1H), 7.60 - 7.50 (m, 3H), 7.43 (dd, *J* = 17.9, 7.6 Hz, 3H), 7.19 (d, *J* = 7.5 Hz, 1H), 7.09 - 7.02 (m, 3H), 6.94 (s, 1H), 6.85 (d, *J* = 8.6 Hz, 2H), 4.37 (s, 2H), 3.90 (d, *J* = 5.5 Hz, 1H), 3.55 (s, 3H), 3.25 (s, 3H), 3.14 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 167.46, 162.08, 158.00, 157.65, 156.96, 147.36, 145.11, 133.19, 130.95, 130.13, 130.06, 129.55, 129.20, 129.15, 128.49, 128.09, 127.13, 115.32, 115.24, 115.12, 114.85, 97.53, 96.69, 81.19, 73.86, 41.67, 40.86, 40.50, 28.60. HRMS (ESI) m/z calcd $C_{32}H_{26}N_3O_5S$ $[M-H]^-$ 564.1593, found 564.1591.

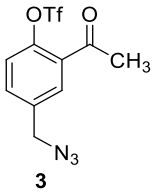


2

1-(5-(azidomethyl)-2-hydroxyphenyl)ethan-1-one (2)

A solution of 1-(2-hydroxyphenyl)ethan-1-one (3.33 g, 24.45 mmol), HCHO (37% in H_2O, 7.5 mL, 100.72 mmol), and conc. hydrochloric acid (22 mL, 26.32 mmol) were stirred at room temperature for 17 h. Upon completion of reaction (monitored by TLC), the precipitate was collected by filtration, w9hich was dissolved in DCM and washed with water (30 mL x 1). Upon drying over $NaSO_4$, filtration, and evaporated in vacuum, compound **1** (2.86 g) was obtained as a crude product, which was used in the next step without further purification.

A solution of **1** (2.45 g, 13.27 mmol) and NaN_3 (1.53 g, 23.50 mmol) in DMF (8 mL) were stirred at room temperature for 3 h. Upon completion of reaction (monitored by TLC), the reaction mixture was diluted with EA (20 mL) and the resulting solution was washed with brine (30 mL x 3). Upon drying over $NaSO_4$, filtration, and evaporated in vacuum, the residue was purified by flash chromatography on a silica gel column (PE: EA = 16:1) to give the title compound as a white solid (1.5193 g, 38% over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.29 (s, 1H), 7.67 (d, *J* = 1.8 Hz, 1H), 7.43 (dd, *J* = 8.6, 2.0 Hz, 1H), 7.01 (d, *J* = 8.6 Hz, 1H), 4.32 (s, 2H), 2.66 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 204.39, 162.51, 136.55, 130.56, 126.04, 119.66, 119.27, 54.24, 26.83. HRMS (ESI) m/z calcd $C_9H_8N_3O_2$ $[M-H]^-$ 190.0617, found 190.0615.

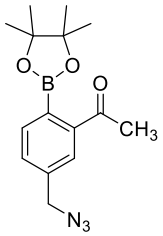


3

2-acetyl-4-(azidomethyl)phenyl trifluoromethanesulfonate (3)

A solution of **2** (225.3 mg, 1.18 mmol) and $PhN(Tf)_2$ (530.3 mg, 1.48 mmol) in CH_3CN (5 mL) was added DIPEA (253 μL, 1.30 mmol). The mixture was stirred at room temperature for 17 h. Upon completion of the reaction (monitored by TLC), the solvent was removed under reduced pressure and the residue was purified by flash

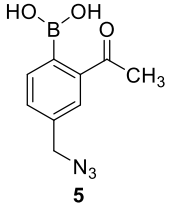
chromatography on a silica gel column (PE:EA = 25:1) to afford the title compound as a white solid (359.6 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76-7.73 (d, 1H), 7.55 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.36 (d, *J* = 8.5 Hz, 1H), 4.48 (s, 2H), 2.65 (s, 3H).



4

1-(5-(azidomethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one (4)

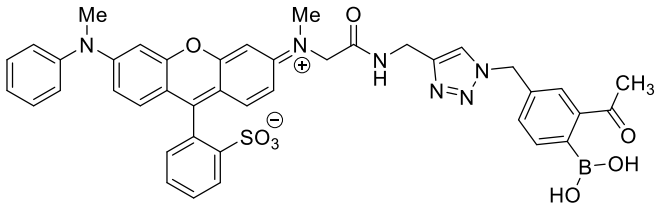
Under N_2 atmosphere, a mixture of **3** (415.0 mg, 1.28 mmol), B_2pin_2 (815.1 mg, 3.21 mmol), $Pd(dppf)Cl_2$ (47.3 mg, 0.064 mmol), dppf (35.7 mg, 0.064 mmol), AcOK (377.7 mg, 3.82 mmol) in 1,4-dioxane (8 mL, anhydrous) as bubble by N_2 for 20 minutes before being heated to 90 ℃ for 70 minutes. After disappearance of starting material (monitored by HPLC), the reaction mixture was cooled to room temperature and diluted by ethyl acetate (20 mL). The resulting mixture was washed with brine (20 mL x 3). Upon drying over $NaSO_4$, filtration, and evaporated in vacuum, the residue was purified by flash chromatography on a silica gel column (PE:EA = 10:1) gave the title compound as a colorless oil (182.7 mg, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 1.6 Hz, 1H), 7.54 (d, *J* = 7.4 Hz, 1H), 7.49 (dd, *J* = 7.4, 1.6 Hz, 1H), 4.41 (s, 2H), 2.63 (s, 3H), 1.44 (s, 12H).



5

(2-acetyl-4-(azidomethyl)phenyl)boronic acid (5)

A solution of **4** (8.3 mg, 0.0276 mmol), methylboronic acid (16.8 mg, 0.281 mmol) in a mixture of DCM/TFA (10/1, 0.143 mL) was stirred at room temperature for 24 h. Upon disappearance of starting material (monitored by TLC), the title compound was purified by flash chromatography on a silica gel column (DCM:MeOH = 10:1) as a white solid (2.7 mg, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (d, *J* = 7.4 Hz, 1H), 7.71 (d, *J* = 1.4 Hz, 1H), 7.55 (dd, *J* = 7.6, 1.5 Hz, 1H), 4.37 (s, 2H), 2.75 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.33, 135.70, 134.37, 131.59, 126.99, 54.38, 23.60. HRMS (ESI) m/z calcd $C_9H_9BN_3O_3$ $[M-H]^-$ 218.0737, found 218.0735.



ASR-2

(*E*)-2-(3-((2-(((1-(3-acetyl-4-boronobenzyl)-1H-1,2,3-triazol-4-yl)methyl)amino)-2-oxoethyl)(methyl)iminio)-6-(methyl(phenyl)amino)-3H-xanthen-9-yl)benzenesulfonate (ASR-2)

A solution of **ASR-1** (8.3 mg, 0.0157 mmol), **5** (6.4 mg, 0.029 mmol), $CuSO_4$ (0.2 mg, 00015 mmol), and sodium ascorbate (11.4 mg, 0.065 mmol) in DMSO/H_2O (120/70 μL) were stirred at room temperature for 1 h. After complete of the reaction (monitored by HPLC), the title compound was obtained as a purple-red solid after purification by a preparative RP-HPLC on a C18 column with H_2O and CH_3CN (with 0.1% TFA) as eluent. The purity of compound was confirmed by HPLC analysis. HRMS (ESI) m/z calcd $C_{41}H_{36}BN_6O_8S$ $[M-H]^-$ 783.2408, found 783.2406.

In Vitro characterization

Linear correlation analysis between fluorescence intensity and solution viscosity

The relationship between the fluorescence intensity of **ASR-2** and the solution viscosity was determined by the following the Förster-Hoffmann equation:[2, 3]

$$\log I = C + x \log \eta$$

where η is the viscosity of solution, I represents the fluorescence intensity of **ASR-2** at 582 nm, C is a constant and x represents the sensitivity of the fluorophore to the solution viscosity.

The fluorescence intensity of **ASR-2** (5 μM) in water with 0%, 20%, 40%, 50%, 70%, 90% of glycerol (by weight) was measured at 30 °C and the solution viscosity (η, centipoises) was obtained from reported data. (https://www.cleaninginstitute.org/industry-priorities/science/research/physical-properties-glycerine-and-its-solutions)

Absorption spectrum

Absorption spectra of samples (10 μM in PBS (pH 7.4)) were recorded in a quartz cuvette using a wavelength-calibrated UV1800 Series UV-Vis spectrophotometer (Shimadzu, Japan) with wavelength range of 400-700 nm.

Fluorescence spectrum

The fluorescence spectra were obtained on a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon) with **ASR-2** (5 μM in PBS) alone or **ASR-2** (5 μM in PBS) pre-incubated with BSA (1 μM) at 37 °C for 20 min (λ_{ex} = 550 nm). For the BSA pre-incubated with acetic anhydride, BSA (1 μM) was incubated with acetic anhydride (10 mM) in PBS for 30 min followed by the addition of NaOH (1 M) to adjust the pH of the solution back to 7.4.

For the test in the presence of glycine, **ASR-2** (5 μM) in PBS (pH 7.4) was incubated with glycine (50 μM) at 37 ºC for 30 min (or followed by the incubation

with BSA (1 μM) for 20 min) before the fluorescence spectrum was collected as aforementioned.

For the test in the presence of GSH, **ASR-2** (5 μM) in PBS (pH 7.4) was incubated with GSH (50 μM) at 37 ºC for 30 min (or followed by the incubation with BSA (1 μM) for 20 min) before the fluorescence intensity was measured on a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 550/582 nm).

Experiments for testing of reversibility of iminoborate bonding

ASR-2 (5 μM) was incubated with BSA (1 μM) in PBS (pH 7.4) at 37 ºC for 30 minutes before measuring the fluorescence intensity. Glycine or GSH (50 mM) was then added and incubated for 20 minutes before measuring the fluorescence intensity. ($\lambda_{ex/em}$ = 550/582 nm).

Determination of the limit of detection

ASR-2 (5 μM) in PBS (pH 7.4) was incubation with BSA at a series of concentrations (ranging from 0 to 0.4 μM) in a 96-well plate at 37 ºC for 20 min, and the fluorescence intensity was measured on a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 550/582 nm). The slope (*k*) was obtained from linear fitting between fluorescent intensity and concentration of BSA. The LOD was calculated as LOD = 3δ/*k* (δ is the standard deviation of a blank sample). All experiments were performed in triplicate.

Similarly, SYPRO Orange (1 x) in PBS (pH 7.4) was incubated with BSA at a series of concentrations (ranging from 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μM) in a 96-well plate at 37 ºC for 20 min, and the fluorescence intensity was measured on a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 470/569 nm). The slope (*k*) was obtained from linear fitting between fluorescent intensity and concentration of BSA. The LOD was calculated as LOD = 3δ/*k* (δ is the standard deviation of a blank sample). All experiments were performed in triplicate.

For the test on bacteria, *E. coli* (ATCC 25922) was cultured in Mueller-Hinton (MH) Broth medium until OD_{600} reached 1 (approximately 8 x 10^8 CFU/mL), and the bacteria was harvested by centrifugation at 5500 rpm/min for 3 min. After washing with PBS and centrifugation, bacteria was lysed following the reported procedure[4] with minor modifications. In brief, the bacterial pellet in PBS (100 μL, pH 7.4) was added NaOH (10 M, 1 μL) and incubated at 37 ºC for 15 minutes, followed by the addition of HCl (aq., 10 M, 1 μL). The bacterial lysis was diluted with PBS to different concentrations (OD_{600} = 0.2, 0.1, 0.05, 0.025, 0.0125) and added to a black microplate. **ASR-2** (final concentration: 1 μM) was then added and the mixture were incubated at 37 ºC for 15 minutes before measuring on a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 550/582 nm). All experiments were performed in triplicate.

Briefly, the above bacterial lysates were diluted with PBS to different concentrations (OD_{600} = 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625, 0) and then added to a transparent microplate. BCA working solution was then added and absorbance at 595 nm was measured using a microplate reader (Molecular Devices, SpectraMax *i*3). All experiments were performed in triplicate.

Photostability test

A solution of **ASR-2** (5 μM) in phenol red-free DMEM was placed on a fluorescence microscope (Leica DMI 3000B) and irradiated continuously at 515-560 nm using the microscope's standard imaging configuration for 60 minutes. Fluorescence intensity of this sample was recorded at indicated time points using a microplate reader (Molecular Devices, SpectraMax *i*3). Each experiment was triplicated.

Cell-based experiments

Cell culture conditions

HeLa cells or HEK293 cells were cultured in high-glucose DMEM (Gibco) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) in 5% CO_2 humidified atmosphere (95%) at 37 ℃.

Cell viability assay

The cytotoxicity of **ASR-2** to HeLa cells or HEK293 cells, were evaluated by Cell Counting Kit-8 (CCK-8) following manufacture's protocol. In brief, cells (5×10^3 cells/well) in DMEM (supplemented with 10% FBS and 1% penicillin/streptomycin, 100 μL) were seeded in 96-well plate and incubated at 37 ℃ with 5% CO_2 and 95% humidity overnight. The culture medium was replaced by fresh DMEM (supplemented with 10% FBS) containing **ASR-2** at a serial of concentrations (1, 3, 5, 10, 20 μM) and culture at 37 ℃ for 24 h with 5% CO_2 and 95% humidity. 10 μL of CCK-8 solution was added to each well and incubate at 37 ℃ for 1-3 h. Absorbance at 450 nm was measured with microplate reader and cell viability was calculated relative to the untreated sample. Each experiment was triplicated.

Fluorescence imaging of cells

Cells were dispersed onto a glass-bottom dish (5×10^5 cells) (Cellvis, D35-20-1-N, 35 mm Dish with 20 mm bottom Well) and allowed to grow overnight. The culture medium was placed by FBS- and phenol red-free DMEM containing **ASR-1** or **ASR-2** (5 μM) and incubated at 37 ºC for 20 min before imaging with confocal fluorescence microscope (Leica, Germany) with excitation/emission at Cy5.5 channel. For the inhibition experiments, cells were pretreated with **5** (1 mM) for 30 min before incubation with **ASR-2** (5 μM) for 20 min. $\lambda_{ex/em}$ = 561/575-625 nm.

Broth Microdilution MIC Assay

E. coli (ATCC 25922) (1 x 10^5 CFU/mL) in Mueller-Hinton (MH) Broth medium containing antibiotics at serial of concentration were incubated at 37 ºC for

18 to 22 h. The MIC was determined as the lowest concentration of antibiotic without visible bacterial growth.

Fluorescence-based Broth Microdilution MIC Assay

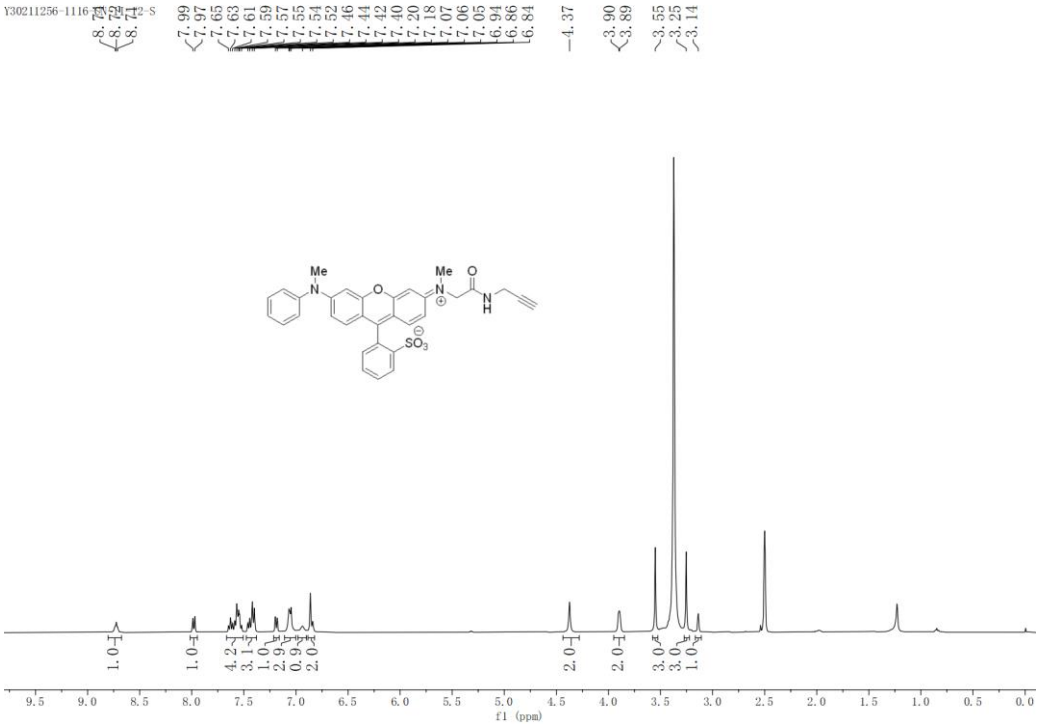
E. coli (ATCC 25922) (1 x 10^6 CFU/mL) in Mueller-Hinton (MH) Broth medium containing antibiotics at serial of concentration were incubated at 37 ºC for 3.5 h and the bacteria was lysed as aforementioned. The resulting bacterial lysate was incubated with **ASR-2** (1 μM) in PBS (pH 7.4) for 15 minutes and the fluorescence intensity was measured using a microplate reader (Molecular Devices, SpectraMax *i*3, $\lambda_{ex/em}$ = 550/582 nm). The MIC was determined as the minimum concentration at which fluorescence intensity was not significantly increased. Each experiment was triplicated.

References

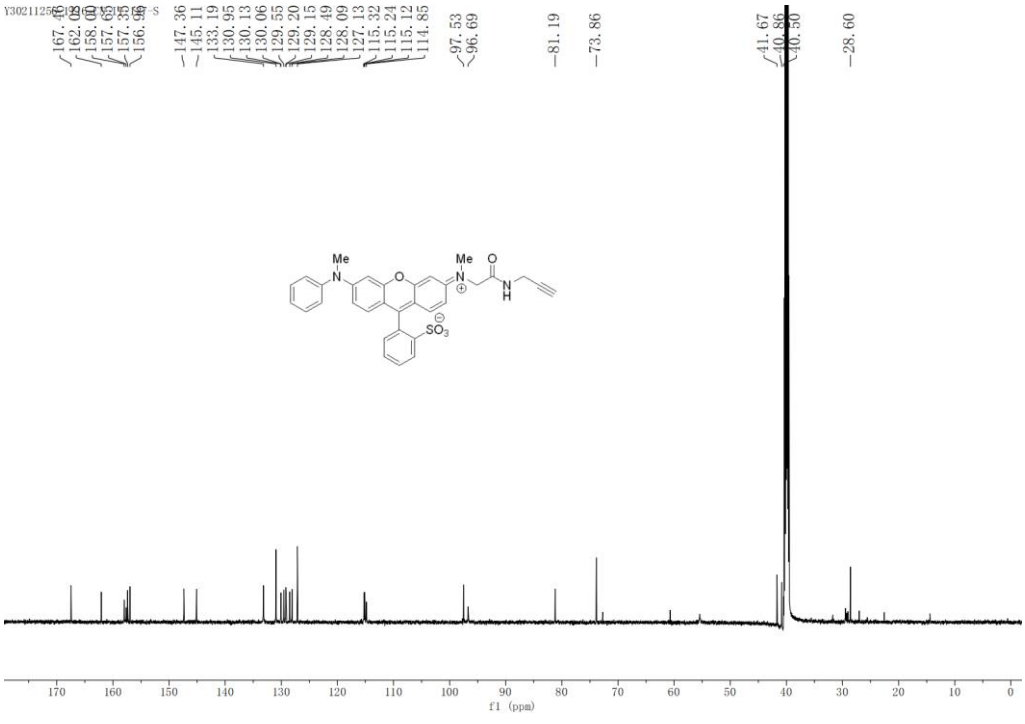
1. H. Tang, X. Yuan, Y. Chen, Y. Li, X. Xu and H. Xie, *Anal. Sens.*, 2024, **4**, e202300037.
2. T. Förster and G. Hoffmann, *Z. fur Phys. Chem.*, 1971, **75**, 63-76.
3. L. Xu, J. Zhao, F. Xiong, Y. Huang and Y. Sui, *Anal. Methods*, 2022, **14**, 2204-2211.
4. S. Lv, C. Wang, K. Xue, J. Wang, M. Xiao, Z. Sun, L. Han, L. Shi and C. Zhu, *Proc. Natl. Acad. Sci. U.S.A.*, 2023, **120**, e2302367120.

^{1}H, ^{13}C NMR and HRMS spectra

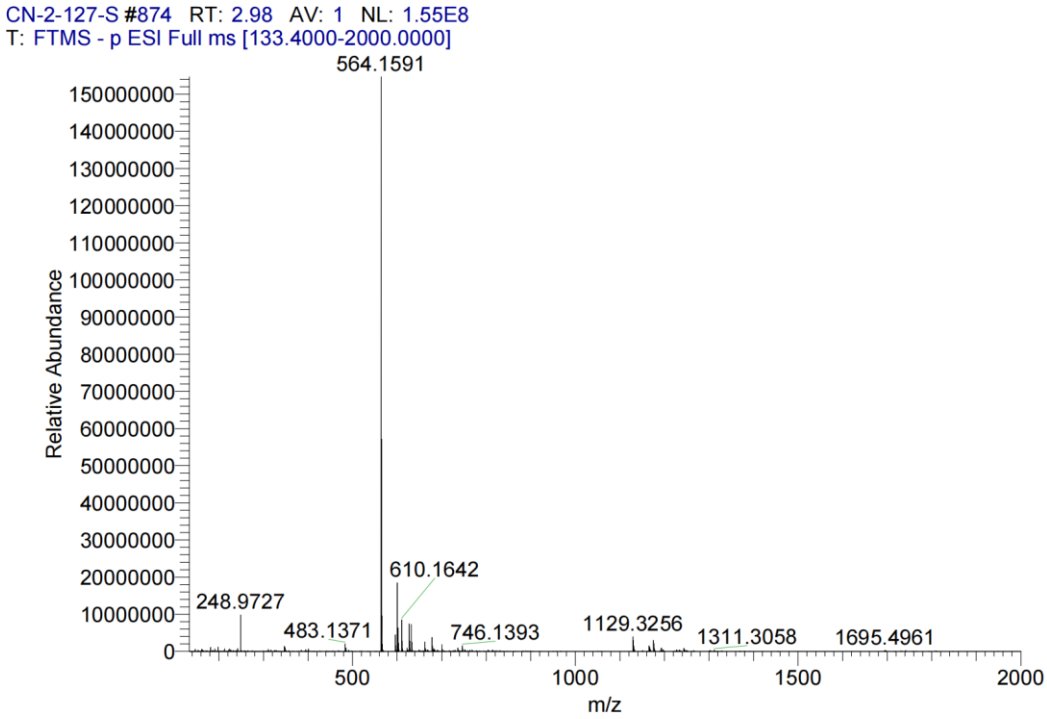
^{1}H NMR of **ASR-1**



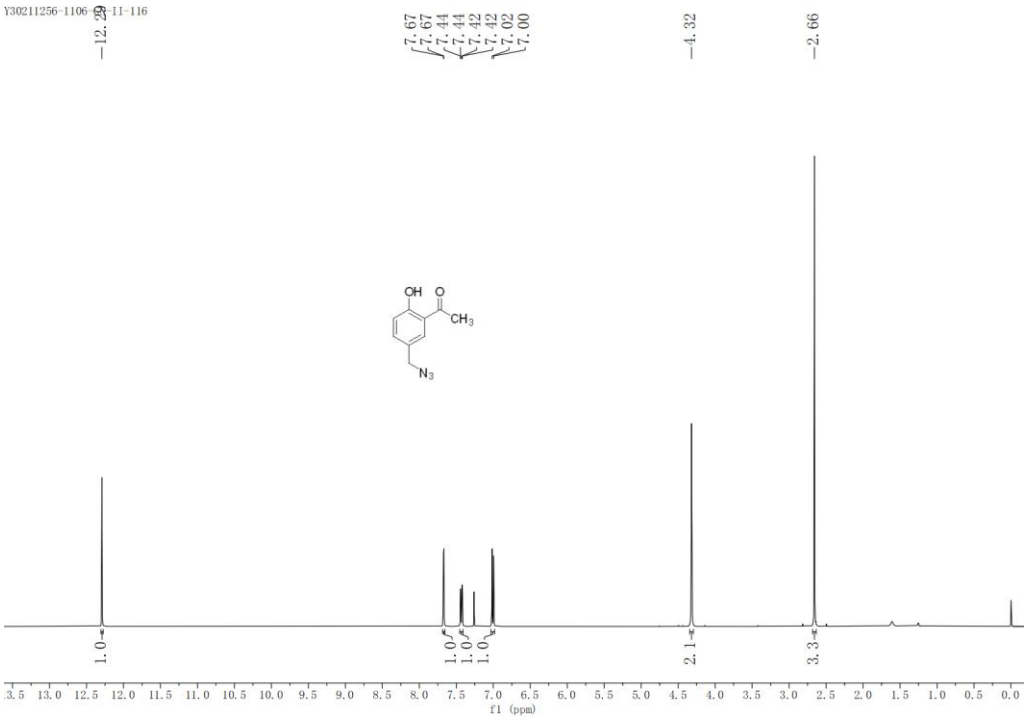
^{13}C NMR of **ASR-1**



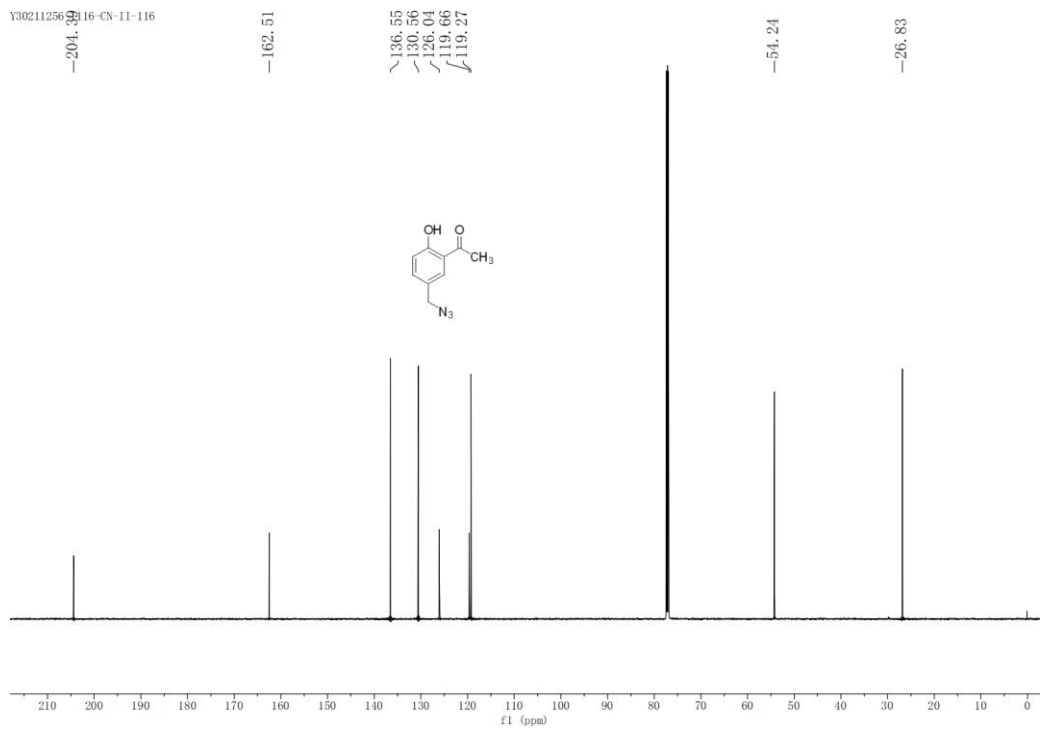
HRMS spectra of **ASR-1**



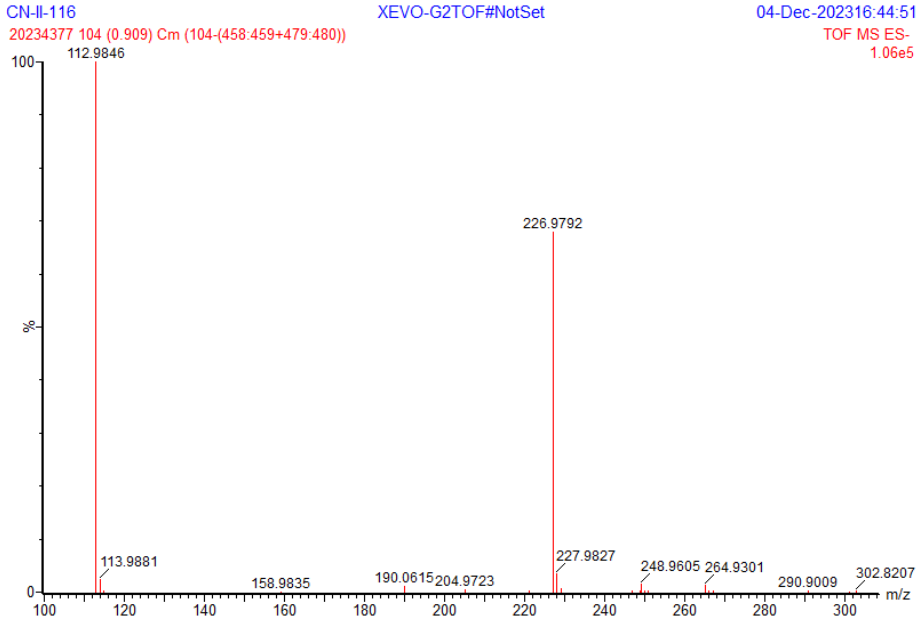
^{1}H NMR of **2**



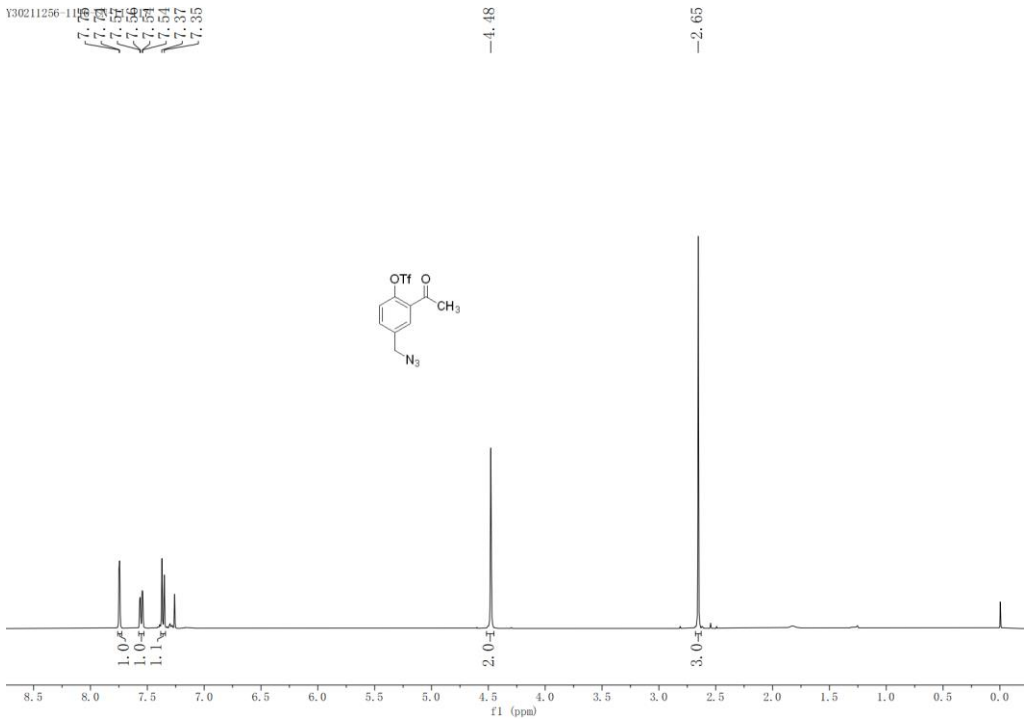
^{13}C NMR of **2**



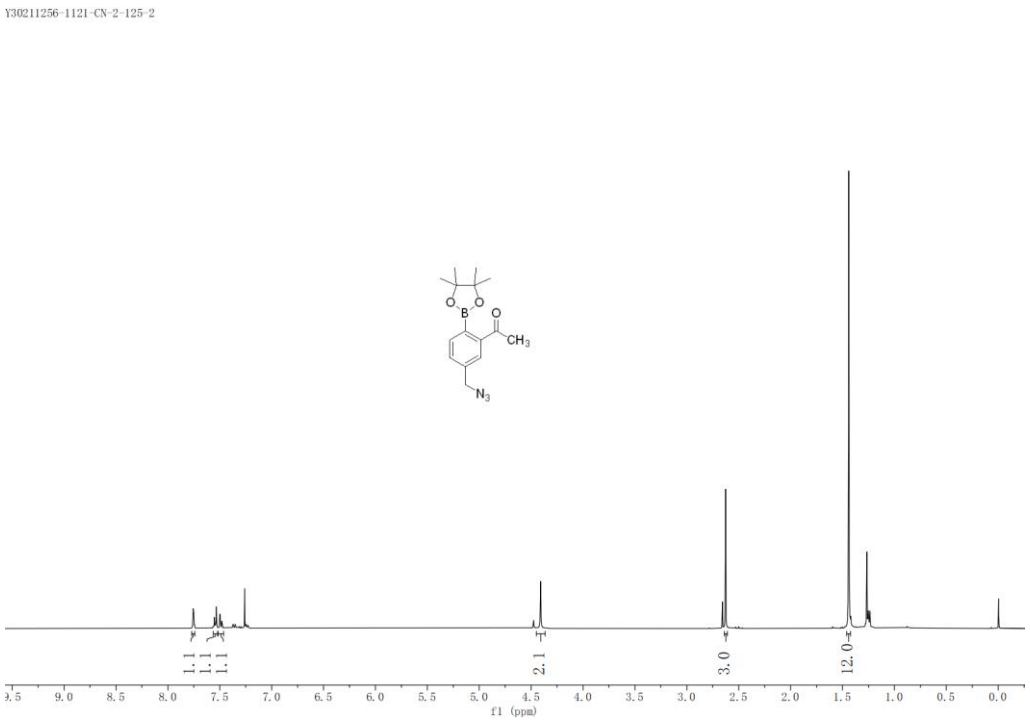
HRMS spectra of **2**



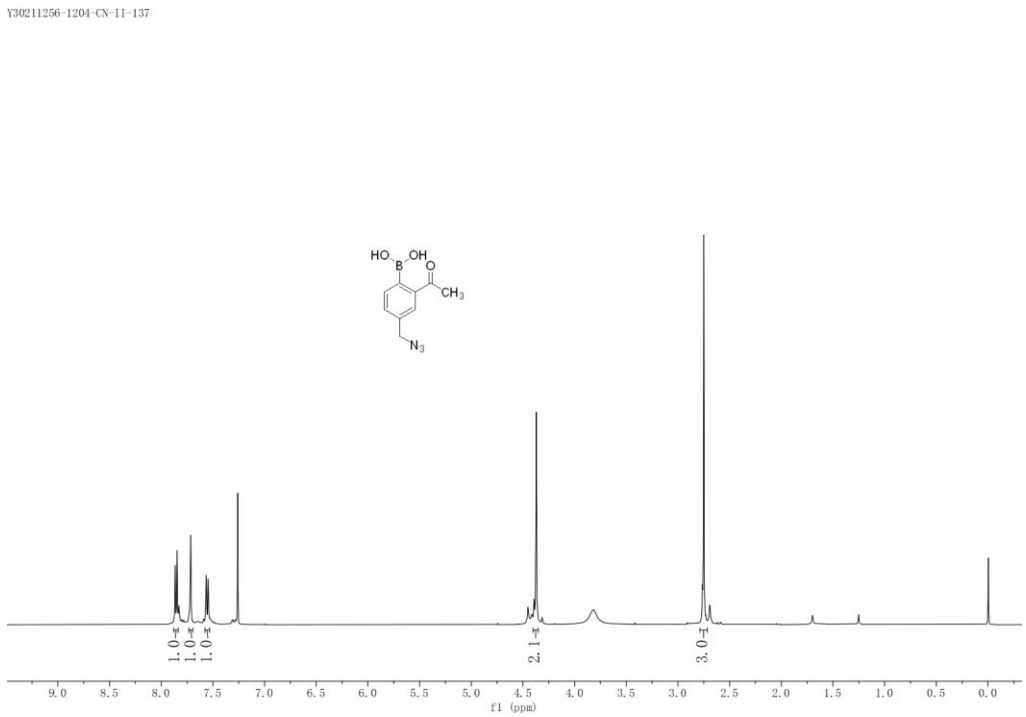
^{1}H NMR of **3**



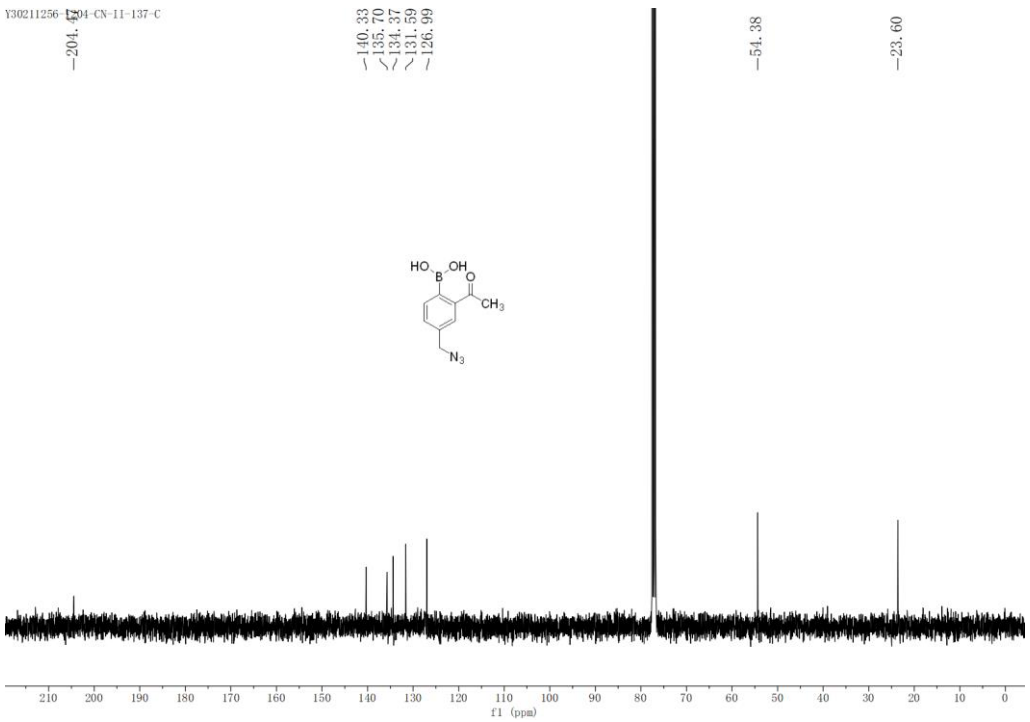
^{1}H NMR of **4**



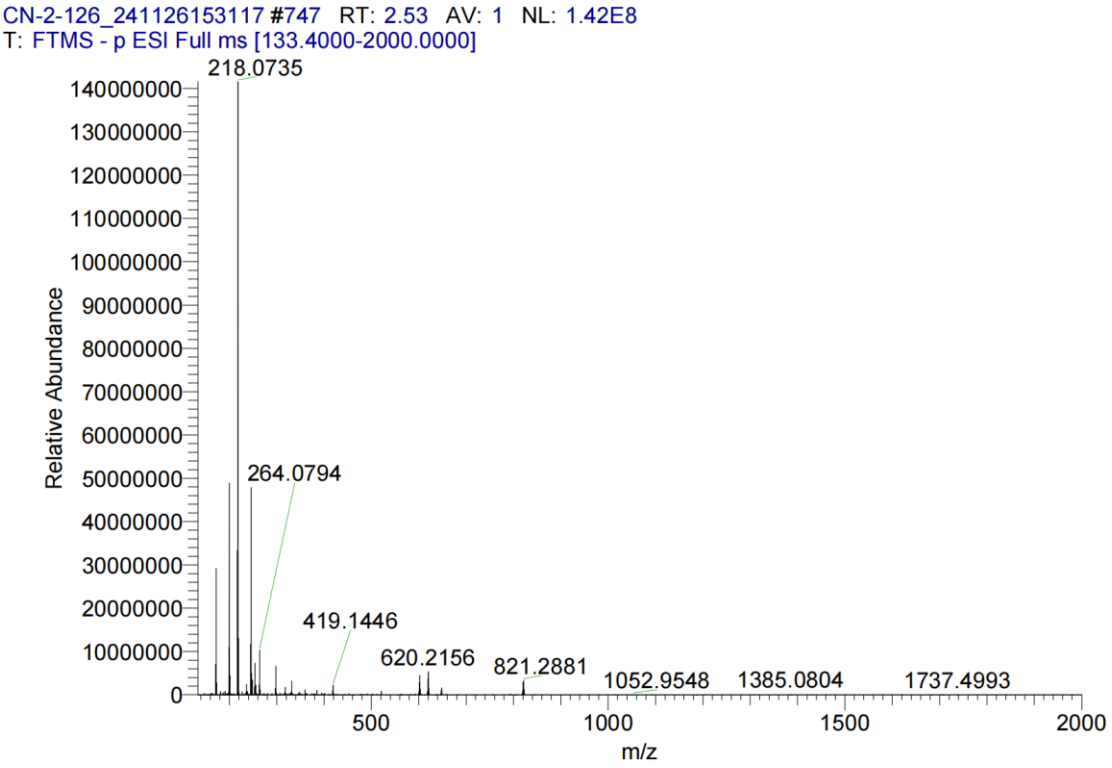
^{1}H NMR of **5**



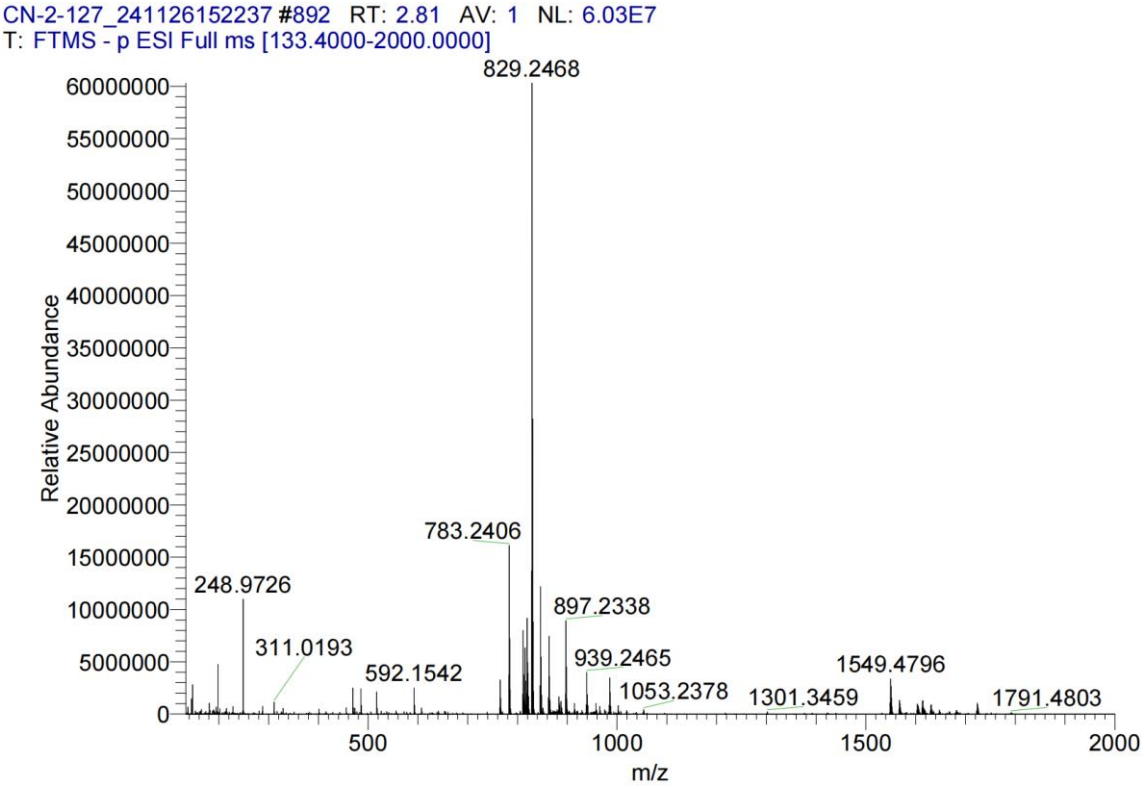
^{13}C NMR of **5**



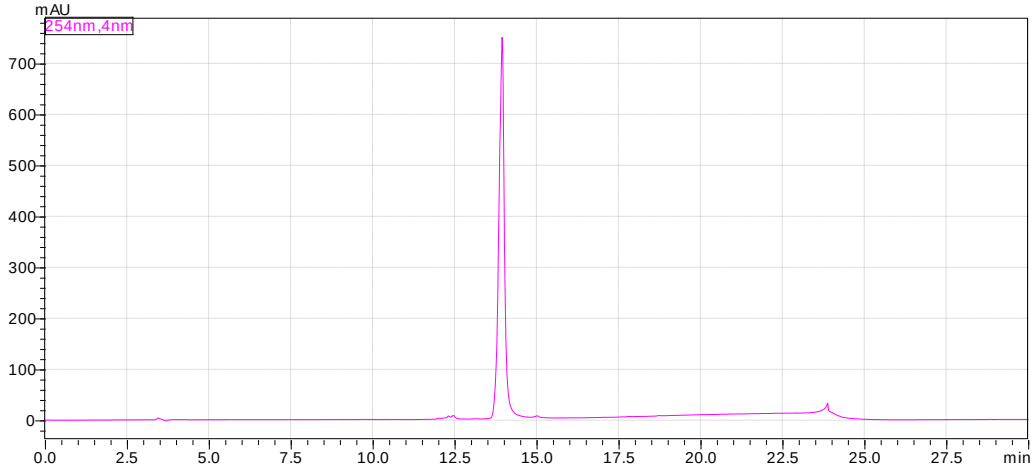
HRMS spectra of **5**



HRMS spectra of **ASR-2**



HPLC trace of **ASR-2** (at 254 nm)



HPLC trace of **ASR-2** (at 550 nm)