Supporting Information (I)

Proteome profiling reveals potential cellular targets of staurosporine using a clickable cell-permeable probe

Haibin Shi,[a] Xiamin Cheng,[a] Siu Kwan Sze[b] and Shao Q. Yao*[a]

[a]*Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543.*
[b]*School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551.*

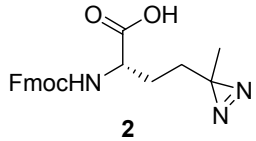
*To whom correspondence should be addressed (chmyaosq@nus.edu.sg).

1. General Information

All chemicals were purchased from commercial vendors and used without further purification, unless indicated otherwise. All reaction requiring anhydrous conditions were carried out under argon or nitrogen atmosphere using oven-dried glassware. HPLC-grade solvents were used for all reactions. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F_{254}, 0.25 μm) and spots were visualized by UV or iodine stain. Flash column chromatography was carried out using Merck 60 F_{254}, 0.040-0.063 μm silica gel. All NMR spectra (^{1}H-NMR, ^{13}C-NMR) were recorded on a Bruker 300/500 MHz NMR spectrometer. Chemical shifts are reported in parts per million referenced with respect to appropriate internal standards or residual solvent peaks ($CDCl_3$ = 7.26 ppm, CD_3OD = 3.31 ppm, DMSO-d_6 = 2.50 ppm). The following abbreviations were used in reporting spectra, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, br = broad. All analytical HPLC were carried out on Shimadzu LCMS (IT-TOF) system or Shimadzu LCMS-2010EV system equipped with an auto-sampler using reverse-phase Phenomenex Luna 5 μm $C_{18(2)}$ 100 Å 50 × 3.0 mm columns. Water with 0.1% TFA and acetonitrile with 0.1% TFA were used as eluents and the flow rate was 0.6 mL/min. For enzyme inhibition and IC_{50} measurements, Tecan microplate reader (Multimode Reader, Infinite®200) in luminescence mode with *i*-control™ software was used. Fluorescence scanning of the SDS-PAGE gels was carried out with Typhoon 9410 fluorescence gel scanner (Amersham Biosciences), and where applicable, the bands were quantified with ImageQuant 3.3 (Molecular Dynamics)

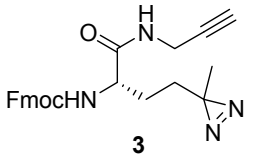
software installed on the scanner. All enzymes used were expressed in *E. coli* strain BL21-DE3 and purified as described previously.[1] Staurosporine (98%) were purchased from Invitrogen. Tris(2-carboxyethyl) phosphine (TCEP), and the click chemistry ligand, tris[(1-benzyl- 1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) were purchased from Sigma-Aldrich. Antibodies against PKA C-α (SC-903), c-Src (SC-8056), Rsk-4 (JS-31), PRPS2 (L-22), PFK-1 (G-11), Creatine kinase-B (N-20), ADK (F-5), PRK2 (C-6) and DTYMK (C-17) were purchased from Santa Cruz Biotechnology.

2. Synthesis of Staurosporine Probe (STS-1)



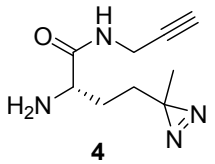
2

To a solution of diazirine free amino acid (100 mg, 0.7 mmol), prepared from previously reported procedures,[2] and 10% Na_2CO_3 (5.2 mL) in dioxane (3.9 mL), Fmoc-Cl (258 mg, 1 mmol) was added at r.t. After 20 h stirring, *p*H value of the reaction was adjusted to 2~3 using 1N HCl, and the mixture was extracted with ethyl acetate (5 mL × 3). The combined organic layer was dried with Na_2SO_4, concentrated, purified with flash chromatography to give the product **2** in 85% yield.



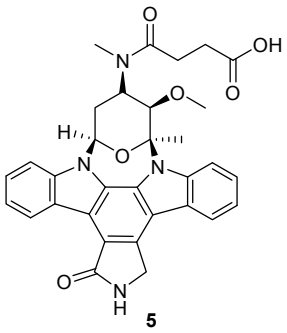
3

To a solution of of propargylamine (49 μL) in DMF (5 mL), coupling reagent HOBt (104 mg, 0.78 mmol), HBTU (288 mg, 0.78 mmol) and DIEA (200 mg, 1.56 mmol) were added. Compound **2** (190 mg, 0.5 mmol) was then added to the above solution and the reaction was stirred at r.t. overnight. Subsequently, the reaction was quenched with water and extracted with EA. The organic layer was washed with water (20 mL), sodium bicarbonate solution (20 mL), brine (20 mL). The organic layer was concentrated and purified by column chromatography to afford compound **3** in 93% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (d, J = 7.4 Hz, 2H), 7.56 (d, J = 7.4 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.34-7.29 (m, 2H), 6.41 (s, 1H), 5.37-5.34 (m, 1H), 4.44-4.42 (m, 2H), 4.22-4.17 (m, 1H), 4.15-4.11 (m, 1H), 4.00 (s, 2H), 2.17 (s, 1H), 1.49-1.42 (m, 2H), 1.32-1.21 (m, 2H), 1.01 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.62, 156.30, 143.54, 141.25, 127.74, 127.05, 124.89, 119.97, 78.80, 71.88, 67.00, 53.88, 47.05, 30.26, 29.17, 27.11, 19.54.



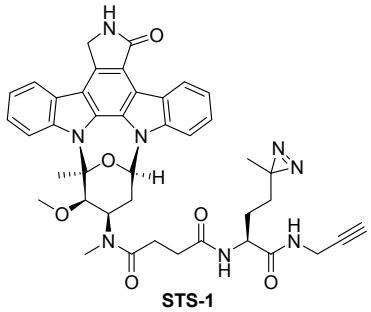
4

Compound **3** was dissolved into 20% piperidine in DMF, and stirred for 10 min at r.t. The reaction was concentrated and purified by column chromatography to give compound **4** in 98% yield. ^{1}H NMR (300 MHz, DMSO) δ 8.22 (s, 1H), 3.84 (s, 2H), 3.09-3.07 (m, 2H), 1.52-1.34 (m, 2H), 1.32-1.06 (m, 2H), 0.97 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 174.9, 81.6, 73.2, 54.2, 40.7, 40.4, 40.1, 39.9, 39.6, 39.3, 39.0, 30.5, 29.8, 28.1, 26.2, 19.8.



5

To a solution of staurosporine (**STS**) (5.0 mg, 10 μmol) in DMSO, succinic anhydride (1.5 mg, 15 μmol) and DMAP (61 μg, 20 μmol) were added under dark. After 30 h stirring, the mixture was precipitated with 0.1 % TFA in water, and the precipitate was triturated twice with 0.1 % TFA in water to afford compound **5** in 92% yield. ^{1}H NMR (300 MHz, DMSO-d6) δ 12.06 (s, 1H), 9.29 (d, J = 7.9 Hz, 1H), 8.59 (s, 1H), 8.05 (d, J = 7.7 Hz, 1H), 7.99 (d, J = 8.5 Hz, 1H), 7.67 (d, J = 8.2 Hz, 1H), 7.48 (t, J = 7.7 Hz, 2H), 7.35 (t, J = 7.5 Hz, 1H), 7.29 (t, J = 7.5 Hz, 1H), 7.03 (t, J = 7.5 Hz, 1H), 5.00 (s, 3H), 4.22 (s, 1H), 2.81 (s, 3H), 2.77 (s, 3H), 2.68 (d, J = 5.6 Hz, 1H), 2.60-2.56 (m, 2H), 2.33 (s, 3H), 2.28-2.17 (m, 1H). LCMS m/z calcd for $C_{32}H_{30}N_4O_6$ $[M+H]^+$ = 567.220, found 567.199 (IT-TOF).



STS-1

To a solution of compound **5** in DMSO (1 mL), coupling reagent DMAP (2.4 mg) and EDC (15.4 mg) were added, and the resulting mixture was stirred for 10 min at r.t. Compound **4** was subsequently added into the reaction at 0 ^{o}C. The mixture was then allowed to warm to r.t. After overnight stirring, the mixture was precipitated with 0.1% TFA in water to afford compound **STS-1** in 92% yield. ^{1}H NMR (300 MHz, DMSO) δ 9.29 (d, J = 7.7 Hz, 1H), 8.61 (s, 1H), 8.33-8.31 (m, 1H), 8.16-8.11 (m, 1H), 8.08-8.03 (m, 1H), 7.99-7.94 (d, J = 8.2 Hz, 1H), 7.66 (t, J = 7.1 Hz, 1H), 7.49 (t, J = 7.1 Hz, 2H), 7.33 (dt, J = 18.4 Hz, 2H), 7.04-6.97 (m, 1H), 5.00 (s, 3H), 4.21-4.19 (m, 2H), 3.92-3.86 (m, 2H), 3.19-3.15 (m, 1H), 2.82-2.74 (m, 6H), 2.69-2.57 (m, 3H), 2.45-2.37 (m, 2H), 2.36-2.33 (m, 3H), 2.26-2.17 (m, 1H), 1.45-1.38 (m, 3H), 1.22-1.20 (s, 1H), 1.02 (s, 3H); LCMS m/z calcd for $C_{41}H_{43}N_8O_6$ $[M+H]^+$ = 743.330,

found 743.322; $C_{41}H_{42}N_8O_6Na$ $[M+Na]^+$ = 765.3120, found 765.303 (IT-TOF).

3. Cell Culture and Western Blotting

Cell lines were obtained from the National Cancer Institute Developmental Therapeutics Program (NCI60). HepG2 was cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Thermo Scientific, Rockford, IL) and maintained in a humidified 37 °C incubator with 5% CO_2. To generate protein lysates, cells were washed twice with cold phosphate buffered saline (PBS), and harvested with 1× trypsin or a cell scraper, and collected by centrifugation. Cell pellets were then washed with PBS and lysed with HEPES buffer (25 mM HEPES, *p*H 7.5, 150 mM NaCl, 2 mM $MgCl_2$) containing 1% NP-40. Protein concentration was determined by Bradford protein assay (Bio-Rad USA).

For western blotting experiments, samples from HepG2 cells were resolved by SDS-PAGE gel, and transferred to polyvinylidene fluoride membranes. Membranes were then blocked with 3% BSA in TBST (0.05% Tween in TBS) for 1 h at r.t. After blocking, membranes were incubated with respective primary antibodies for 1 h at r.t. After incubation, membranes were washed with TBST for 4 times (10 min/time), and then incubated with appropriate secondary antibodies. After incubation, blots were washed again with TBST before the development with SuperSignal West Dura Kit (Thermo Scientific).

4. Determination of IC_{50} Values

Concentration-dependent experiments were performed to determine the inhibition potency and the binding affinity of the probe towards PKA. The inhibition assay was performed with ProFluor® PKA Assay Kit (Promega, USA) following the manufacturer's instructions. Briefly, recombinant PKA, Bisamide Rhodamine 110 peptide substrate (PKA R110 Substrate), ATP and **STS-1** were mixed in 1× reaction buffer provided by kits at a volume of 10 µL in a flat-bottom black 384-well plate. The incubation was allowed to continue for 20 min at r.t. and 5 µL of the protease reagent in 1× termination buffer were added into the reaction, mixed and incubated for 30 min at r.t. Finally, the reaction was quenched by the addition of 5 µL stabilizer reagent in 1× termination buffer, mixed and the fluorescence readouts from the wells were measured at 485/530 nm using Tecan microplate reader with *i*-control software. The fluorescence intensity from each well was measured and the inhibition potency was calculated using the following relation.

$$\text{Potency} = 1 - \text{Remaining activity}$$

Dose-dependent inhibition assays were performed by varying the concentration of the probe under optimized enzyme concentration of ~50 nM. The IC_{50} value of the probe was calculated from the percentage activity vs. log[concentration of probe] curves generated using GraphPad Prism software.

5. Cell Proliferation Assays

Cell viability was determined using XTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines. Briefly, cells were grown to 20-30% confluence (since they will reach ~90% confluence within 48 to 72 h in the absence of drugs) in 96-well plates under conditions described above. The medium was aspirated, and then washed with PBS, and treated in duplicate, with 0.1 mL of the medium containing different concentrations of **STS-1** (1-1000 nM) or staurosporine (1-1000 nM as a positive control). Compounds were applied from DMSO stocks whereby DMSO never exceeded 1% in the final solution. The same volume of DMSO was used as a negative control. Fresh mediums, along with the probe and drug, were added every 24 h. After treatments of 72 h, proliferations were assayed using the XTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines (read at 450 nm).

6. Cell Permeability Assays

The assay was carried out as previously described.[3] MDCK (Madin-Darby canine kidney) cells were used for testing the cell permeability. Cells were seeded with 600,000~700,000 cells per cm^2 (0.11 cm^2 per well insert, Millipore® #PSHT004R1) and cultured for 3 days before testing. Upon removal of media, the cells were rinsed with Hanks' Balanced Salt Solution (HBSS, Gibco® #14025). Transport assay donor solutions consisted of 100 μM of Staurosporine, **STS-1** and caffeine each in a transport medium containing 60 μM Lucifer Yellow (LY) and 1% DMSO. Transport assays were conducted using 75 μL of apical (AP) donor solution and 235 μL of basolateral (BL) acceptor solution (transport medium, *p*H 7.4, following manufacturer's protocol (Millipore® #PSHT004R1). Monolayers were incubated with donor and acceptor solutions for 60 min at 37 °C, 95% humidity. Subsequently, the donor and acceptor solutions of each compound were collected and quantified by HPLC. Lucifer Yellow (LY) was quantified using a fluorescence 96-well plate reader (BioTek® Synergy 4 fluorescence plate reader at λ_{ex} = 485 nm and λ_{em} = 539 nm). P_{app} (apparent permeability) values were calculated according to the following equation:

$$Papp = \left(\frac{dQ}{dt}\right) \times \frac{1}{Co} \times \frac{1}{A}$$

where dQ/dt is the permeability rate, C_0 is the initial concentration in the donor compartment, and *A* is the surface area of the filter. Permeability rates were calculated by plotting the percent of initial AP drug mass (peak area) found in the BL compartment versus time and determining the slope of the line. Lucifer yellow (LY) results were used as an internal control for each monolayer to verify tight junction integrity during the entire assay period. Accordingly, LY P*app* values were quantified from 60 min basolateral samples after background subtraction. As a quality control,

results from MDCK monolayer with LY P*app* > 30 nm/s were not used. The results are shown in Table S1, indicating both staurosporine and **STS-1** are cell-permeable.

Table S1. The results of cell permeability assay.

	Positive control	**Negative control**	**Staurosporine**	**STS-1**
Caffeine / 50 μM	+	-	-	-
Lucifer Yellow / 60 μM	+	+	+	+
Compound / 100 μM	-	-	+	+
DMSO	1%	1%	1%	1%
P*app*/nm.s^{-1}	762	n.d.	320	142

n.d.: not determined.

7. Labeling Experiments with Purified Kinases

Unless otherwise indicated, all labeling experiments were carried out following previous procedure with minor modification.[4] Briefly, the kinase (final concentration: 80-150 nM) and the probe (final concentration: 1 μM) in the reaction buffer (25 mM HEPES, *p*H 7.5, 150 mM NaCl, 2 mM $MgCl_2$) were incubated for 30 min at r.t., followed by UV irradiation for 20 min. The reaction mixtures were then subjected to click-chemistry with rhodamine-N_3 based on the following conditions: to each reaction was added 1 μL each of rhodamine-N_3 (final concentration: 50 μM), $CuSO_4$ (final concentration: 1 mM), TBTA (final concentration: 100 μM) and sodium ascorbate or TCEP (final concentration: 1 mM). The reaction was incubated for 2 h at r.t., then quenched by addition of 4 μL of 6×SDS loading buffer, followed by boiling at 95 °C for 10 min. Samples were resolved on a 10% SDS-PAGE gel and in-gel fluorescence scanning was done with a Typhoon 9410 gel scanner (GE Healthcare).

To investigate the labeling specificity of **STS-1**, labeling experiments were performed with different recombinant kinases (c-Src, CSK and PKA C-α). Briefly, different kinases (final concentration: 100 nM) were incubated with **STS-1** (final concentration: 1.0 μM**)**, respectively, in above reaction buffer for 30 min at r.t. followed by UV irradiation for 20 min. After click conjugation, SDS-PAGE gel was run and in-gel fluorescence was taken.

In order to determine the detection limit of the probe, dose-dependent experiments were carried out by varying the concentration of PKA in the reaction (Fig. S1). Briefly, to a solution of **STS-1** (1 μL, 20 μM stock solution in DMSO) in 18 μL of HEPES buffer (25 mM HEPES, *p*H 7.5, 150 mM NaCl, 2 mM $MgCl_2$), different concentration of PKA (1.0 μL) was added. The reactions were then incubated for 30 min at r.t. in the dark. Subsequently, the reaction mixtures were irradiated for 20 min. Staurosporine was used as competitor in the labeling reaction to determine the activity-based nature of labeling (Fig. S2). Briefly, labeling reactions with PKA (a final concentration of 100 nM), **STS-1** (a final concentration of 1 μM) in the reaction buffer (25 mM HEPES, *p*H 7.5, 150 mM NaCl, 2 mM $MgCl_2$), in the presence of staurosporine (0-1 μM), were incubated for 30 min at r.t. followed by UV irradiation for 20 min. To further optimize the amount of the probe to be used in a labeling reaction, we carried

out labeling experiments with different concentrations of **STS-1**. Briefly, PKA (100 nM), **STS-1** (0-10 μM) in the reaction buffer (25 mM HEPES, *p*H 7.5, 150 mM NaCl, 2 mM $MgCl_2$) were incubated for 30 min at r.t. followed by irradiation UV irradiation for 20 min (Fig. S3). All reaction mixtures were subjected to click-chemistry with a rhodamine-N_3 before SDS-PAGE analysis and in-gel fluorescence scanning.

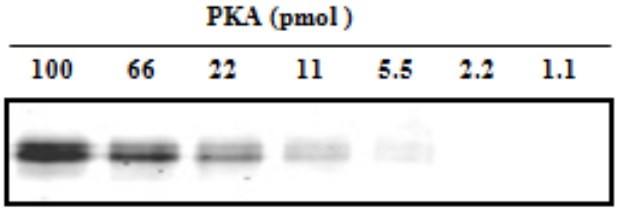


Fig. S1. Dose-dependent labeling of PKA with **STS-1** (1 μM).

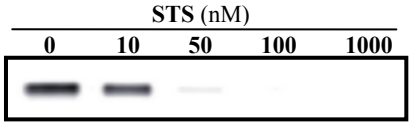


Fig. S2. Labeling of recombinant PKA with **STS-1** (1 μM) in the presence of Staurosporine.

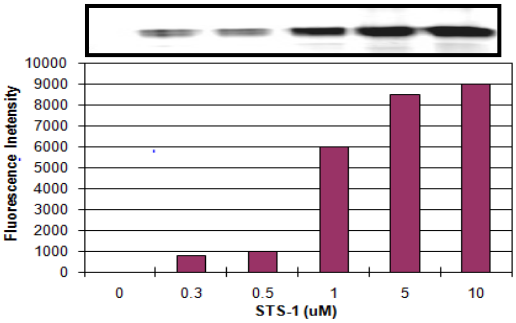


Fig. S3. Recombinant PKA (100 nM) labeled with increasing concentrations of **STS-1.**

8. Fluorescence Labeling of Overexpressed PKA in Bacterial Proteomes

To evaluate the performance of **STS-1** in a complex bacterial proteome, labeling experiments were carried out with **STS-1** and bacterial lysates. Bacterial lysates were prepared using previous reported procedures with minor modification.[5] The bacterial culture was grown at 37 ºC with shaking to reach OD_{600} = 0.8. Subsequently, IPTG (0.2 mM final concentration) was added to induce PKA expression, followed by further incubation for 6 h at 37 ºC with shaking (230 rpm). Portions of the culture were taken out before and after inducing, respectively, and harvested by centrifugation at 4000 rpm for 10 min at 4 ºC. The resulting pellets were resuspended in lysis buffer (25 mM HEPES, *p*H 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 50 μM PMSF), followed by centrifugation for 15 min (13,000 rpm at 4 ºC). Total protein concentrations of these lysates were quantified by Bio-Rad protein assay (Bio-Rad USA) and stored in -20 °C until use. To different amounts of above bacterial lysates, **STS-1 (**final 1 μM**)** was added, followed by incubation for 30 min at r.t. Subsequently,

the reaction was irradiated with UV light (~350 nm) for 20 min followed by click-chemistry with rhodamine-N_3, separated by SDS PAGE gel, and analyzed by in-gel fluorescence scanning.

9. *In vitro* and *In situ* Labeling in Crude Mammalian Proteomes

Both *in vitro* and *situ* proteome labeling experiments with **STS-1** were carried out with HepG2 cell lysates and live cells, respectively. Firstly, **STS-1** was added to HepG2 cell lysates (50 µg; prepared as previously described[4]) in 50 μL of HEPES buffer at a final concentration of 1-20 μM in the presence or absence of staurosporine (100 μM). Unless indicated otherwise, samples were incubated for 30 min at r.t., irradiated for 20 min under UV light (~350 nm) followed by click reaction as above described. For *in situ* labeling, cells were grown to 80-90% confluence in 24-well plates under the conditions described above. The medium was removed, and cells were washed twice with cold PBS, treated with 0.5 mL of growth medium containing **STS-1** (1-50 μM), with or without staurosporine (100 μM). **STS-1** was applied from DMSO stocks whereby DMSO never exceeded 1% in the final solution. After 5 h of incubation at 37 ºC/5% CO_2, the medium was aspirated, and cells were washed twice gently with PBS to remove the excessive probe followed by UV irradiation (~350 nm) for 20 min on ice. The cells were then trypsined and pelleted by centrifugation. Eventually, the cell pellets were resuspended in PBS (50 μL), homogenized by sonication, and diluted to 1 mg/mL with PBS. Both *in vitro* and *situ* labeled samples were subjected to click chemistry with rhodamine-N_3, SDS-PAGE analysis, and in-gel fluorescence scanning. In the *in situ* live cell experiments, we did observed some degree of cell death (<10%) under our optimized labeling conditions (with and without staurosporine). This is obviously due to the toxicity of **STS-1** and staurosporine. Prolonged incubation and higher concentrations of either STS-1 and/or staurosporine in the cells led to more severe cell death problems. Since our aim was to use **STS-1** for labeling of cellular targets in live cells, our optimized labeling/pull-down conditions were chosen based on minimal cell death (< 10%) in the assay. Furthermore, dead cells became detached from the cell plates, and were immediately washed away prior to the trypsinization step. Consequently, only live HepG2 cells labeled by **STS-1** were collected from our labeling/pull-down experiments.

10. Cell Imaging

HepG2 cells were seeded in 24-well plates containing sterile glass cover slips and grown until 70-80% confluence. Cells were then treated with 0.5 mL of DMEM with 20 μM of **STS-1** (with or without 100 µM of staurosporine), or DMSO (negative control). After 1 h incubation, the growth medium was removed, and cells were washed gently twice with PBS. Subsequently, cells were irradiated for 10 min under UV lamp on ice. Some dead cells (< 10%) were observed, but they were not chosen for subsequently imaging experiments. After fixation in 3.7% paraformaldehyde in PBS for 15 min at r.t., cells were washed twice with cold PBS again, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at r.t. followed by blocking

with 2% BSA in PBS for 30 min at r.t., and then washed twice with PBS. Cells were subsequently treated with a freshly premixed click chemistry reaction solution in a 200 µL volume at final concentrations of the following reagents: 1 mM of $CuSO_4$, 1 mM of TCEP, 100 µM of TBTA, and 10 µM of rhodamine-N_3 in PBS for 2 h at r.t. with vigorous shaking. Cells were washed with PBS three times, then imaged with a Leica TCS SP5X Confocal Microscope System equipped with Leica HCX PL APO 63x/1.20 W CORR CS, 405 nm Diode laser, White laser (470 nm to 670 nm, with 1 nm increments, with 8 channels AOTF for simultaneous control of 8 laser lines, each excitation wavelength provides 1.5mV), PMT detector range from 410 nm to 700 nm for steady state fluorescence. Images were processed with Leica Application Suite Advanced Fluorescence (LAS AF).

11. Molecular Modeling

Molecular docking studies were carried out using Autodock Vina software.[6,7] The PKA structure was obtained from the PDB databank (PDB ID 1STC[8]). Explicit hydrogen atoms were added, all water molecules were then deleted. The peptide ligand was removed and the protein structure was processed using AutoDock Tools. Compound **STS-1** was prepared for docking using AutoDock Tools to assign AD4 atom types, calculate Gasteiger charges, and set all rotatable bonds as active torsions. The ligand was docked into the protein using AutoDock Vina (version 1.11, The Scripps Research Institute). The exhaustiveness parameter was set to 100 (default = 8, linear scale); all other default settings were used. The macromolecule molecular surface and secondary structure were displayed by PyMol (version 0.99, DeLano Scientific LLC.

12. Pull-Down and LCMS

To identify *in vitro* and *in situ* targets of staurosporine in HepG2 cells, pull-down experiments were carried out followed by MS/MS protein identification, as previously described.[4] For *in vitro* experiments, fresh cell lysates were prepared as above previously. Protein concentrations were determined according to Bradford assay. Cell lysates (5 mg) were supplemented in 200 µL of 5×HEPES buffer (125 mM HEPES, *p*H 7.5, 750 mM NaCl, 10 mM $MgCl_2$). The reaction volume was later adjusted to 1 mL with milli-Q water. Subsequently, a solution of **STS-1** (10 µM; 10 µL of 1 mM stock solution) was added followed by incubation for 2 h at 4 ºC. The reaction mixture was then UV-irradiated for 20 min, followed by click chemistry with biotin-N_3 (100 µM) under conditions described above. Subsequently the sample was acetone-precipitated, and resolubilized in 0.1% SDS in PBS by brief sonication. The sample was then incubated with avidin-agarose beads (100 µL/mg protein) overnight at 4 ºC. After centrifugation, supernatant were removed and the beads were washed with 0.1% SDS once and PBS for 4 times. After washing, the beads were boiled in 1×SDS loading buffer (200 mM Tris *p*H 6.8, 400 mM DTT, 8% SDS) for 15 min. For *in situ*, live cell pull-down, **STS-1** (20 µM) was directly added to HepG2 cells followed by incubation for 1 h. DMSO should never exceed 1% in the final solution. After 1 h of incubation at 37 ºC/5% CO_2, the medium was aspirated, and cells were

washed twice gently with PBS to remove the excessive probe followed by UV irradiation for 20 min on ice. The cells were then trypsined and pelleted by centrifugation. Eventually, the cell pellets were resuspended in PBS (50 μL), homogenized by sonication, and diluted to 1 mg/mL with PBS. The labeled lysates were then subjected to click reaction with biotin-N_3 (100 μM). All subsequent steps followed the *in vitro* pull-down procedures described above. Samples were separated by SDS-PAGE gels and silver-stained (Fig. S4). Next, trypsin digestion was performed with In-Gel Trypsin Digestion Kit (Pierce) for respective visible protein bands.[4] Digested peptides were then extracted from the gel with 50% acetonitrile and 1% formic acid. Tryptic peptide extracts were evaporated by speedvac and reconstituted with 10 μL of 0.1% TFA. The peptides were separated and analyzed on a Shimadzu UFLC system (Shimadzu, Kyoto, Japan) coupled to an LTQ-FT Ultra (Thermo Electron, Germany). Mobile phase A (0.1% formic acid in H_2O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish the 60 min gradient comprised of 45 min of 5-35% B, 8 min of 35-50% B and 2 min of 80% B followed by re-equilibrating at 5% B for 5 min. Peptides were then analyzed on LTQ-FT with an ADVANCE™ CaptiveSpray™ Source (Michrom BioResources, USA) at an electrospray potential of 1.5 kV. A gas flow of 2 L/min, ion transfer tube temperature of 180 °C and collision gas pressure of 0.85 m Torr were used. The LTQ-FT was set to perform data acquisition in the positive ion mode as previously described except that the m/z range of 350-1600 was used in the full MS scan. The raw data were converted to mgf format. The database search was performed using an in-house Mascot server (version 2.2.07, Matrix Science, UK) with MS tolerance of 10 ppm and MS/MS tolerance of 0.8 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification, and oxidation (M) and phosphorylation (S, T and Y) were set as variable modifications.[4]

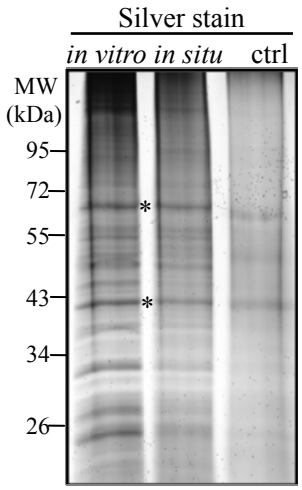


Fig. S4. Pull-down/LCMS experiments. Silver-stained gels of *in vitro* and *in situ* pull-down samples from HepG2 cells after labeling with **STS-1**, The asterisks mark the positions of endogenous PKA and c-Src, respectively. Ctrl, avidin beads without **STS-1**.

LCMS results obtained from above experiments (in vitro, in situ and negative control with DMSO) were processed as shown below and results were summarized in

Table S2 and in SI_2. As in the case of most large-scale LCMS experiments,[9] a large number of proteins were identified from each LCMS run, many of which were "sticky" and/or highly abundant proteins. They were automatically removed. "False" hits which appeared in control pull-down/LCMS experiments (DMSO was used in place of **STS-1**) were further eliminated, giving the final full list of proteins (SI_2). From this list, we placed our focus on those proteins that might be related to kinase activities/interactions (kinases, potential kinase interacting partners, kinase-like protein, etc). They were further identified and listed in Table S2.

What was interesting from the list shown in Table S2, was PKA C-α, which appeared in both of our *in vitro* and *in situ* pull-down experiments (Figure 3D). However, in our subsequent pull-down/LCMS experiments, this protein was identified ONLY in the *in situ* but not in the *in vitro* experiment (Table S2). The exact reason for this remains unknown, although we speculated that it might have been due to the pull-down/LCM sample prep procedures, as well as differences arisen form detection sensitivity of the mass spectrometer from different runs.[9b] Therefore it is important to note that LCMS results only give preliminary "hits". Further confirmation will always be needed for target validation before any biological conclusions should be made.

13. Immunoblotting and Validation Experiments with Select Hits.

For immunoblotting analysis/validation of select hits, pulled-down samples were prepared first according to previous procedure. In brief, for *in vitro* pull-down experiment, fresh cellular lysates (5 mg) were supplemented with 200 μL 5× HEPEs buffer (125 mM HEPES at *p*H 7.5, 750 mM NaCl, 10 mM $MgCl_2$), the reaction volume will be adjusted to 1 mL with milli-Q water. Subsequently, a solution of probe **STS-1** (10 μM, 10 μL of a 1 mM stock soloution) was added, and equilibration was carried out for 2 h at 4 ^oC. The reaction mixture was then irradiated for 20 min on ice using a 4W UV lamp. Subsequently, the reaction was reacted with biotin-azide (100 μM) by click chemistry under the conditions described before. After 12 h incubation, the reactions were acetone precipitated, and resolubilized in 0.1% SDS in PBS with brief sonication. This resuspended sample was then incubated with avidin-agarose beads (100 μL/mg protein) overnight at 4 ^oC. After centrifugation, supernatant were removed and the beads were washed with 0.1% SDS once and PBS for 4 times. After washing, the beads were boiled in 1× SDS loading buffer (200 mM Tris *p*H 6.8, 400 mM DTT, 8% SDS) for 15 min. After filtration, the supernatant was kept in 4^oC for use.

For *in situ* pull-down, briefly, probe **STS-1** (20 μM) was directly added in live cells followed by incubation for 1 h at 37 ^oC/5% CO_2. DMSO should never exceed 1% in the final solution. After 1 h of incubation, the medium was aspirated, and cells were washed twice gently with PBS to remove the excessive probe followed by UV irradiation (~350 nm) for 20 min on ice. The cells were then trypsined and pelleted by centrifugation. Eventually, the cell pellet was resuspended in PBS (50 μL), homogenized by sonication, and diluted to 1 mg/mL with PBS. The labeled lysates were then subjected to click reaction with biotin azide (100 μM), and all the subsequent experiments just follow above *in vitro* pull-down procedure.

In order to validate the potential protein hits, western blotting experiments were carried out. Briefly, samples from above *in vitro* and *situ* pull-down experiments were resolved by SDS-PAGE gel together with pull-down sample from DMSO-treated, unlabeled lysates (negative controls). After SDS-PAGE gel separation, proteins were then transferred to a PVDF membrane and subsequently blocked with 3% (w/v) BSA/TBST (0.05% Tween in TBS) for 1 h at r.t. After blocking, Membranes were incubated with the respective antibodies (i.e., *anti*-PKA, *anti*-c-Src, *anti*-PKN2, *anti*-RPS6KA6, *anti*-PFKM, *anti*-CKB, *anti*-PRPS2 and *anti*-ADK) for 1 h at r.t. After four times of washing with TBST, blots were further incubated with the appropriate secondary antibody for 1 h at r.t. After incubation, the blot was washed again with TBST for 4 times and SuperSignal West Dura Kit (Thermo Scientific) was used to develop the blot (Fig. S5 and Fig. 4c).

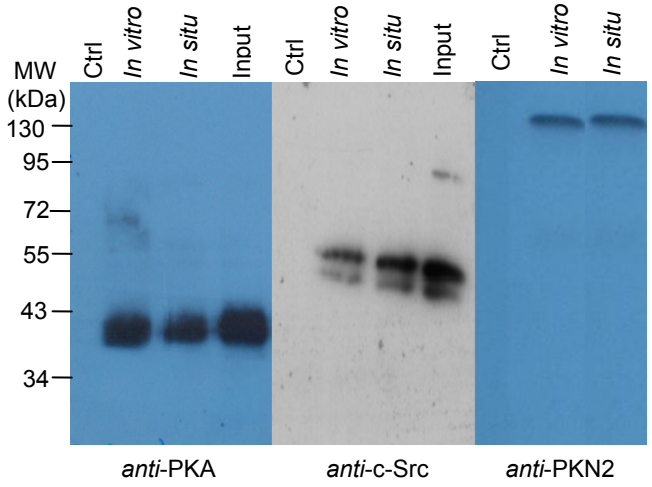


Fig. S5. Western blotting of PKA, c-Src and PKN2 from both *in vitro* and *in situ* pull-down experiments. Results indicated all three proteins were successfully labeled by **STS-1** both *in vitro* and in live HepG2 cells.

Table S2. The list of kinases identified from the pull-down/LCMS experiments. Unique peptides are the number of all unique peptides observed for a particular protein. Protein kinases are shaded in blue , other kinases are labeled in white. The unique kinases identified from *in situ* pulldown were shaded yellow (also in Fig. 4a). "-": no data.

No	Kinase Name	ID	MS (kDa)	In Vitro			In Situ		
				Peptides Matched	Score	emPAI	Peptides Matched	Score	emPAI
1	Isoform 2 Proto-oncogene tyrosine-protein kinase Src	IPI00328867	61.06	2	39	0.05	-	-	-
2	Proto-oncogene tyrosine-protein kinase Yes	IPI00013981	61.28	2	39	0.05	-	-	-
3	Tyrosine-protein kinase CSK	IPI00013212	51.24	38	60	0.07	-	-	-
4	Tyrosine-protein kinase BTK	IPI00029132	76.92	41	704	1.18	-	-	-
5	Isoform 2 of Serine/threonine-protein kinase PCTAIRE-3 (PCTK3)	IPI00394661	54.73	3	46	0.14	-	-	-
6	Isoform 1 of DNA-dependent protein kinase catalytic subunit (DNA-PKc)	IPI00296337	47.37	236	4475	0.64	41	644	0.16
7	Serine/threonine-protein kinase 25 (STK25)	IPI00012093	48.31	1	37	0.07	-	-	-
8	Ribosomal protein S6 kinase alpha-3 (p90-RSK2)	IPI00020898	84.03	3	45	0.08	1	55	0.04
9	Ribosomal protein S6 kinase alpha-6	IPI00007123	84.39	-			1	56	0.04
10	Interferon-induced, double-stranded RNA-activated protein kinase (eIF-2 α)	IPI00019463	62.51	2	84	0.05	2	72	0.05
11	Isoform 2 of cAMP-dependent protein kinase, alpha-catalytic subunit (PKA C-α)	IPI00217960	39.91	-			2	47	0.08
12	serine/threonine protein kinase MST4 isoform	IPI00182383	39.86	1	37	0.07	-	-	-

13	Cell division protein kinase 2 (CDK2)	IPI00031681	34.08	5	120	0.1	-	-	-
14	Serine/threonine-protein kinase N2 (PKN2)	IPI00002804	131.9	2	55	0.06	-	-	-
15	Isoform 1 of Dephospho-CoA kinase domain-containing protein	IPI00291417	26.65	3	43	0.43	-	-	-
16	Myristoylated alanine-rich C-kinase substrate	IPI00219301	31.71	2	95	0.11	-	-	-
17	Isoform 1 of Nucleoside diphosphate kinase B	IPI00026260	17.4	3	44	0.43	-	-	-
18	Isoform 1 of Acylglycerol kinase, mitochondrial precursor	IPI00019353	47.56	2	45	0.07	1	50	0.14
19	Isoform M2 of Pyruvate kinase isozymes M1/M2	IPI00479186	58.54	154	3549	3.38	18	357	0.64
20	Phosphoglycerate kinase 1	IPI00169383	44.98	67	1591	2.11	10	163	0.53
21	Isoform 1 of Pyridoxal kinase	IPI00013004	35.31	35	881	2.21	2	45	0.2
22	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	IPI00294380	71.45	7	157	0.2	-	-	-
23	6-phosphofructokinase type C	IPI00009790	86.45	2	145	0.08	3	69	0.8
24	Isoform 1 of 6-phosphofructokinase, liver type	IPI00332371	85.76	4	114	0.16	2	54	0.04
25	Isoform 2 of 6-phosphofructokinase, muscle type	IPI00219585	82.6	-	-	-	3	48	0.04
26	Phosphofructokinase, platelet	IPI00639981	26.4	-	-	-	1	70	0.13
27	Isoform 1 of Adenylate kinase 2, mitochondrial	IPI00215901	26.69	12	273	0.8	1	53	0.42
28	Ketosamine-3-kinase	IPI00099986	34.62	1	38	0.1	-	-	-
29	Isoform 1 of Nucleoside diphosphate kinase A	IPI00012048	17.31	1	44	0.43	1	61	0.2
30	Creatine kinase B-type	IPI00022977	42.90	-			17	95	0.16
31	Ribose-phosphate pyrophosphokinase 1	IPI00219616	35.33	15	100	0.31	13	254	0.71
32	Isoform 2 of ADP-dependent glucokinase	IPI00329593	54.27	1	56	0.05	-	-	-
33	Ribose-phosphate pyrophosphokinase 2	IPI00219617	35.33	-			11	254	0.57
34	Isoform Short of Adenosine kinase	IPI00234368	39.08	-			1	-	-
35	Isoform 1 of Hexokinase-1	IPI00018246	103.6	20	70	0.09	-	-	-
36	LOC727761 Thymidylate kinase	IPI00013862	23.98	-			1	42	0.08

37	Isoform 2 of SH3-containing GRB2-like protein 3	IPI00216201	39.28	48	159	1.12	-	-	-
38	Isoform 1 of Uridine-cytidine kinase 2	IPI00065671	29.45	1	45	0.11	-	-	-
39	Isoform 1 of Inhibitor of Bruton tyrosine kinase	IPI00792333	15.06	-	-	-	1	41	0.02
40	Isoform R-type of Pyruvate kinase isozymes R/L	IPI00027165	62.19	1	41	0.05	-	-	-
41	FN3KRP Ketosamine-3-kinase	IPI00099986	34.6	1	38	0.1	-	-	-
42	Thymidine kinase 1, soluble	IPI00299214	30.56	2	78	0.07	-	-	-
43	Isoform 1 of S-phase kinase-associated protein 1A	IPI00301364	18.82	1	39	0.04	-	-	-

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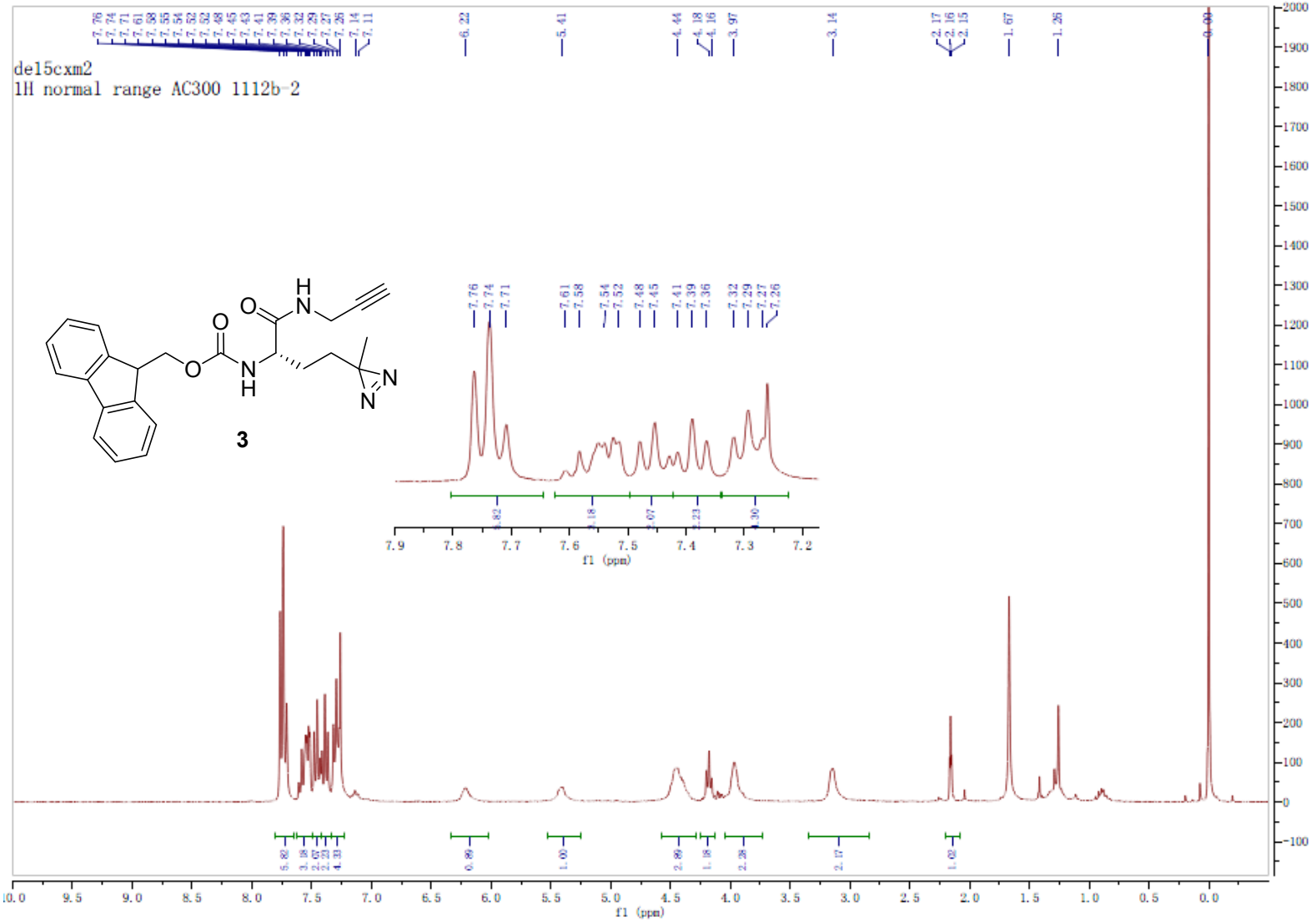
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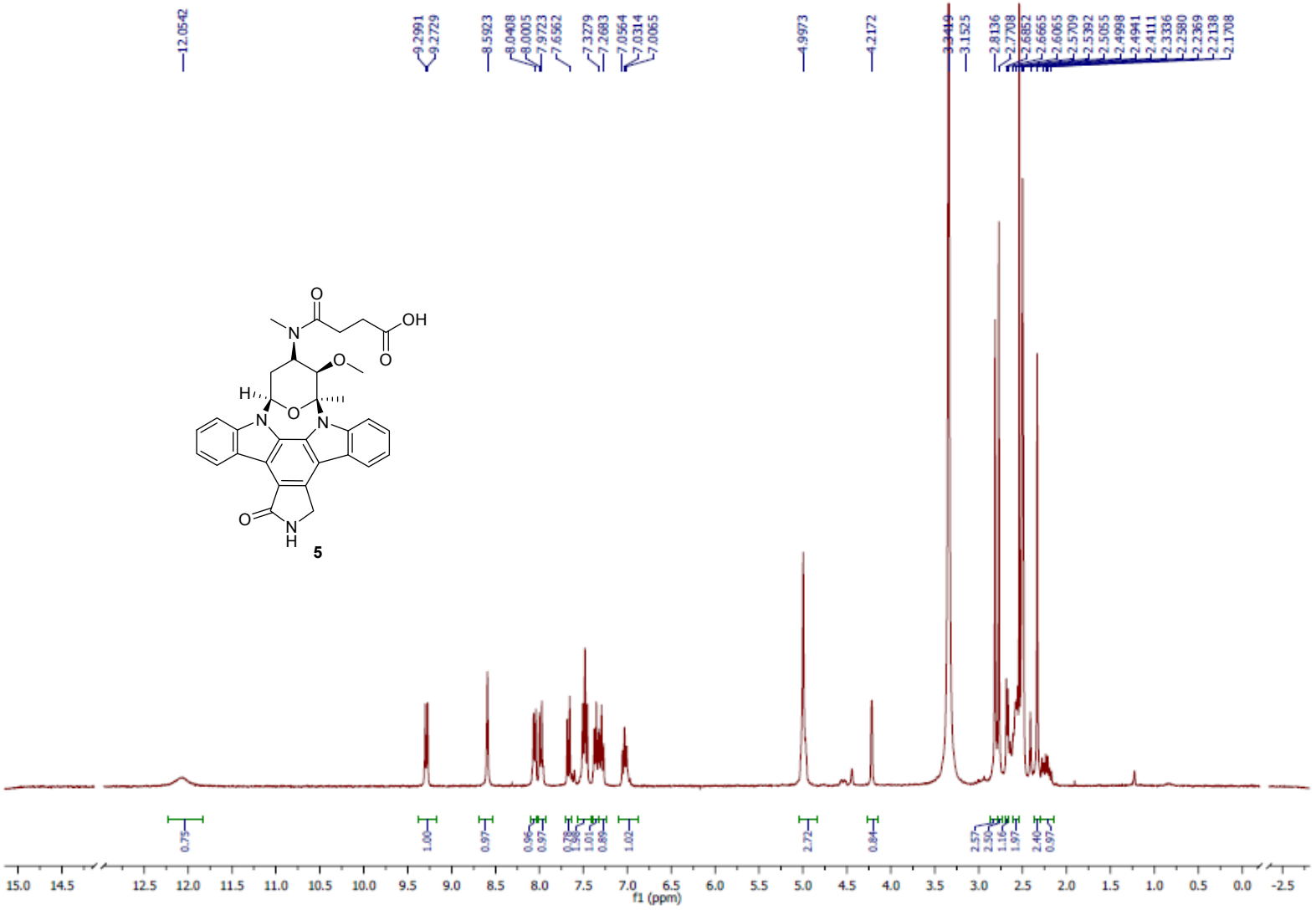
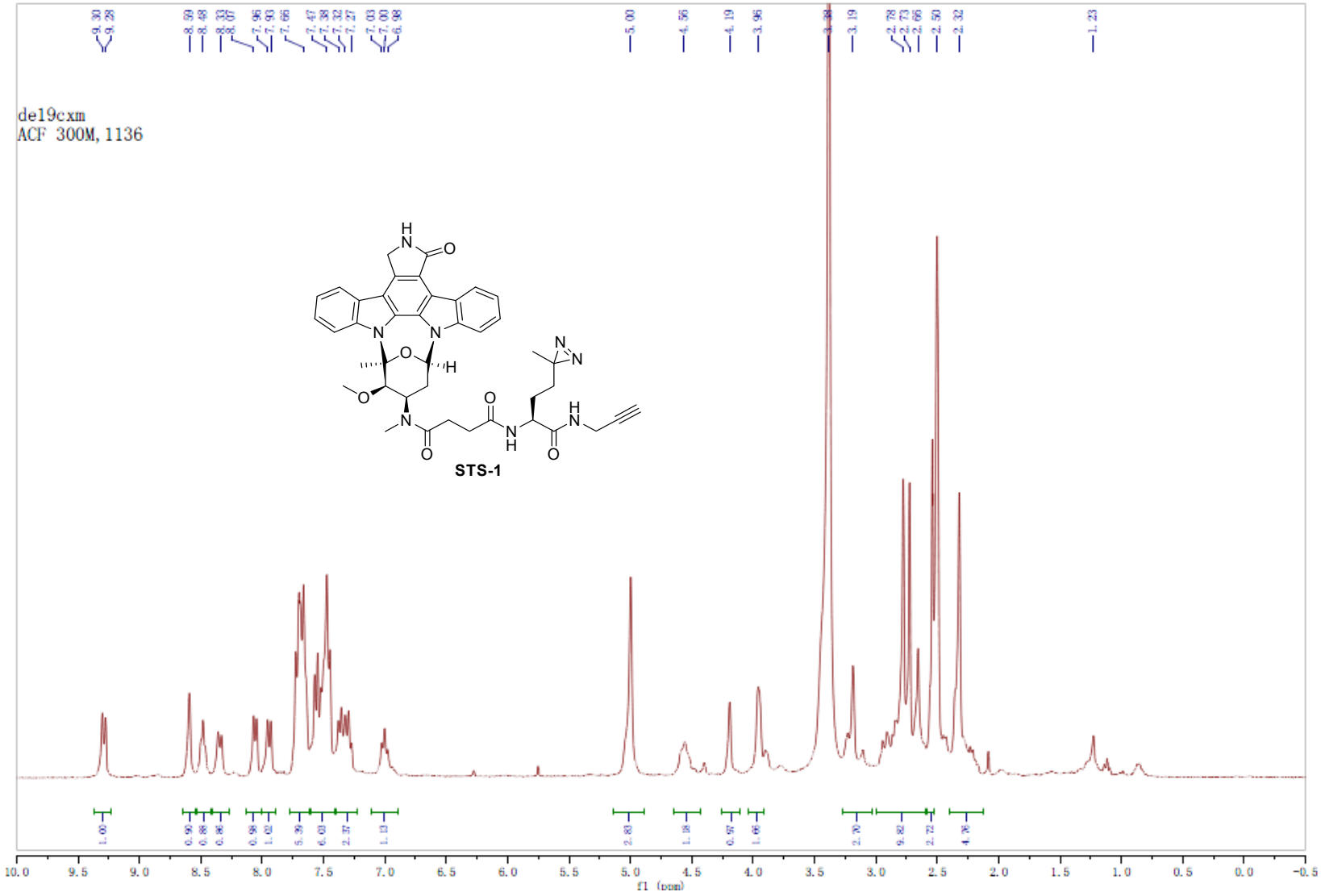
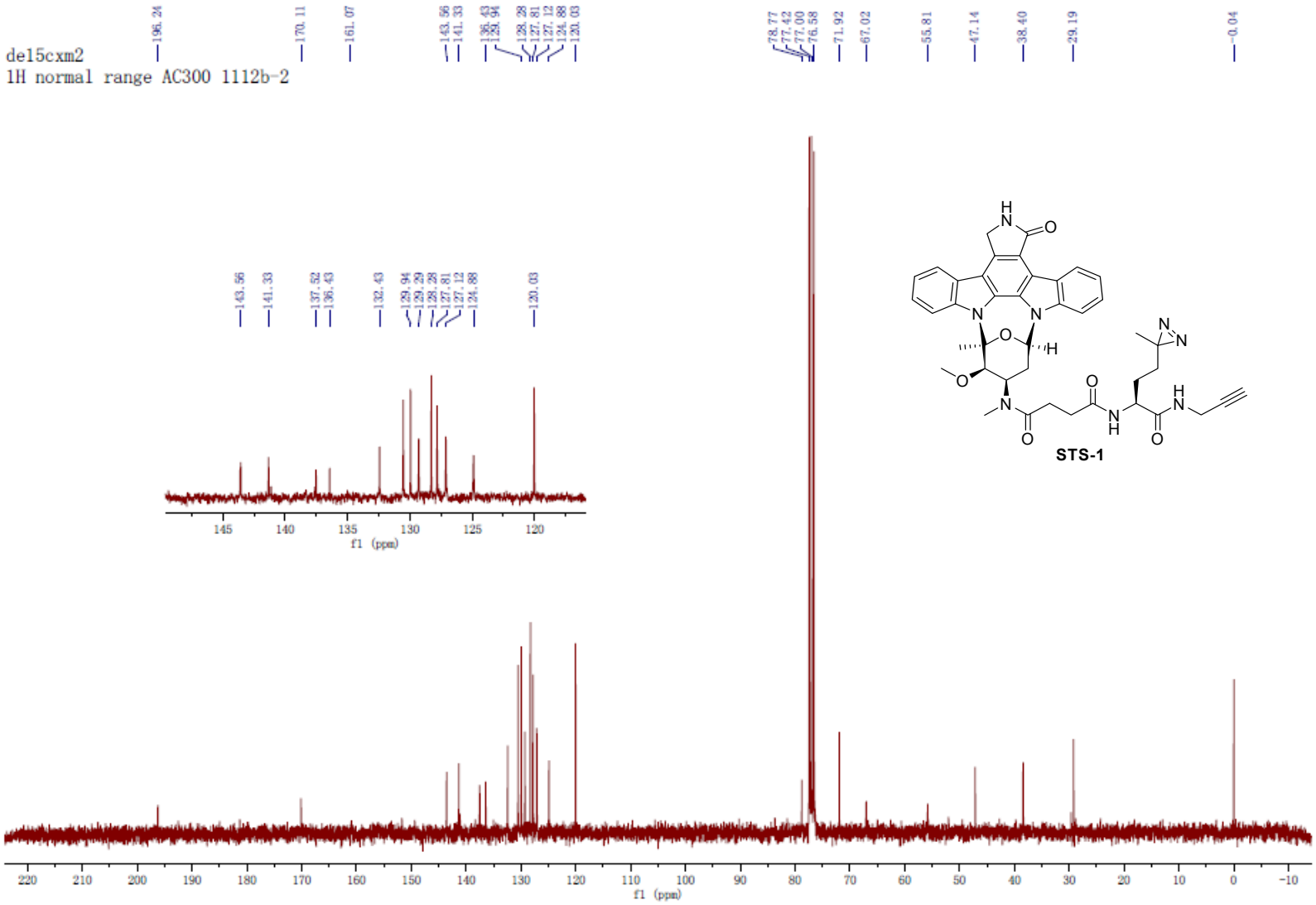
3
f1 (ppm)



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127.12
124.88
120.03
78.77
77.42
77.00
76.58
71.92
67.02
55.81
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29.19
-0.04
f1 (ppm)
STS-1