

1        **Permeant Fluorescent Probes Visualize the Activation of SARM1 and**  
2                    **Uncover an Anti-neurodegenerative Drug Candidate**

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22

23 **Abstract:**

24 SARM1 regulates axonal degeneration through its NAD-metabolizing activity and is a  
25 drug target for neurodegenerative disorders. We designed and synthesized  
26 fluorescent conjugates of styryl derivative with pyridine to serve as substrates of  
27 SARM1, which exhibited large red-shifts after conversion. With the conjugates,  
28 SARM1 activation was visualized in live cells following elevation of endogenous NMN  
29 or treatment with a cell-permeant NMN-analog. In neurons, imaging documented  
30 SARM1 activation preceded vincristine-induced axonal degeneration by hours.  
31 Library screening identified a derivative of nisoldipine as a covalent inhibitor of  
32 SARM1 that reacted with Cys311 in its Armadillo-domain and blocked its  
33 NMN-activation, protecting axons from degeneration. CryoEM showed that SARM1  
34 was locked into an inactive conformation by the inhibitor, uncovering an unsuspected  
35 neuroprotective mechanism of dihydropyridines.

36

37 **Introduction**

38 Axon degeneration (AxD) occurs in most neurodegenerative disorders (Coleman  
39 et al., 2020). Sterile Alpha and TIR Motif-containing 1 (SARM1) acts as a main  
40 effector in this process (Osterloh et al., 2012) and its depletion significantly attenuates  
41 AxD (Geisler et al., 2016, Osterloh et al., 2012, Turkiew et al., 2017). SARM1 controls  
42 AxD through its enzymatic activity (Essuman et al., 2017). It is self-inhibitory and is  
43 activated by nicotinamide mononucleotide (NMN) (Zhao et al., 2019), resulting in  
44 depletion of the intracellular NAD-pool (Essuman et al., 2017, Zhao et al., 2019).

45 However, a recent study suggests that NAD may be an inhibitor of SARM1 activation  
46 and the decrease of NAD actually may activate SARM1 (Jiang et al., 2020). In any  
47 case, no direct evidence of SARM1 activation in live cells is available and no potent  
48 inhibitor has been identified. We thus aim to design and synthesize probes for  
49 visualizing SARM1-activation in live cells and to screen drug library for potent  
50 inhibitors.

51 We have documented that SARM1 is a multi-functional enzyme with  
52 properties similar to CD38, a universal signaling enzyme possessing not only NADase  
53 activity but also catalyzing both the cyclization of NAD to cyclic ADP-ribose (cADPR)  
54 and the exchange of nicotinamide in NADP with nicotinic acid to produce nicotinic acid  
55 adenine dinucleotide phosphate (NAADP) (Zhao et al., 2019). Both cADPR and  
56 NAADP are messengers regulating calcium mobilization in the endoplasmic reticulum  
57 and the endo-lysosomes, respectively (reviewed in(Galione, 1994, Lee, 2012, Lee et  
58 al., 2019)). The catalytic similarities and its ubiquitous presence in non-neuronal cells  
59 suggest that SARM1 may be a calcium signaling enzyme as well.

60 We focused on its base-exchange reaction for designing specific probes  
61 for SARM1 and had shown that pyridyl derivatives can readily serve as substrates  
62 (Graeff et al., 2006, Lee et al., 1997). We thus conjugated various styryl derivatives to  
63 pyridine to produce a series of conjugates (PCs) as fluorescent probes for SARM1  
64 activity (Fig. 1A).

65 **Results:**

66        **Probe design, synthesis, and characterization.** We reasoned that conjugating  
67 the electron-rich styryl derivative with pyridine should provide a donor- $\pi$ -acceptor  
68 framework (Pawlicki et al., 2009) (Fig. 1B). The positive charge of the pyridinium  
69 moiety of the product should delocalize over the conjugated  $\pi$ -system and lead to  
70 fluorescence changes (Fig. 1A). Pyridine conjugates (PC1-9, Figure 1—figure  
71 supplement 1, Figure 1—figure supplement 2, Figure 1—figure supplement 3A) were  
72 synthesized using the Pd-catalyzed cross-coupling strategy with yields ranging from  
73 33.5-85.0%. The synthesis details are in the Methods section and product  
74 characterizations are Figure 1—figure supplement 2.

75        The PC-probes were tested using a recombinant SARM1, SARM1-dN (Zhao et al.,  
76 2019) (described in Figure 1—figure supplement 3B), with NAD as the acceptor of  
77 base-exchange and NMN as an activator. As shown in Figure 1—figure supplement 4,  
78 significant shifts in UV-vis spectra after conversion were observed in the oxygenated  
79 derivatives (PC5-9, *O*-series), but not the nitrogenated derivatives (PC1-4, *N*-series).  
80 The emission spectra of the reactive *O*-series showed steady increase as the reaction  
81 progressed (Figure 1—figure supplement 5, spectra; Fig. 1D, kinetics; Figure  
82 1—figure supplement 3C, initial rate), with PC6, the chemical structure in Fig. 1C,  
83 exhibited the largest fluorescence increase (Fig. 1D).

84        The time course of the UV-spectra during conversion of PC6 showed decreases  
85 at 330 nm but increases at 400, with an isosbestic point at 350 nm (Figure 1—figure  
86 supplement 4 and Fig. 1E). Corresponding to the absorbance change was the  
87 red-shift in the fluorescence spectra, from the emission maximum at 430 nm of PC6 to

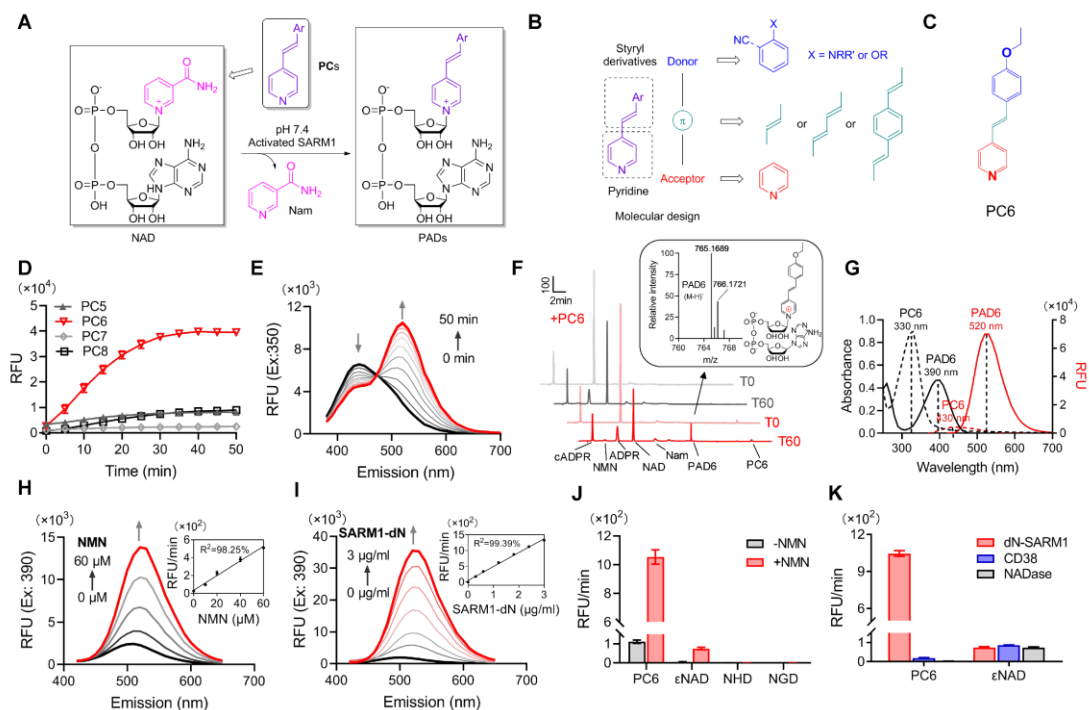


88 520 nm of PAD6 (Fig. 1E).

89 The conversion of PC6 to the exchange-product, PAD6, was verified by purifying  
90 it using HPLC and characterized by high resolution mass spectrometry (HRMS) (Fig.  
91 1F). The remarkably large spectral changes are anticipated from our design, as the  
92 pyridine ring becomes positively charged after its exchange into NAD (Fig. 1F, inlet), a  
93 much stronger electron acceptor in the D- $\pi$ -A structure, thereby increasing  
94 intramolecular charge transfer and shifting the emission maximum by over 100 nm.  
95 The conversion-induced spectral changes were consistent with the spectra of the  
96 HPLC-purified products, PAD6 (Fig. 1G).

97 The observed spectral changes showed a linear dependence on NMN, with as  
98 low as 10  $\mu$ M being effective (Fig. 1H), confirming that SARM1 is an auto-inhibitory  
99 enzyme activated by NMN(Zhao et al., 2019). The fluorescence increase was also  
100 proportional to the amount of NMN-activated SARM1 (Fig. 1I), with a detection limit of  
101 48 ng/ml. As an *in vitro* assay for SARM1, PC6 provides more than 100-fold higher  
102 sensitivity over other commonly used probes, such as  $\epsilon$ NAD, NGD or NHD (Fig. 1J).

103 In addition to sensitivity, PC6 also shows exquisite selectivity toward  
104 SARM1 versus CD38 and *N. crassa* NADase(Graeff et al., 1994). All three possess  
105 NADase activity as detected by  $\epsilon$ NAD (Fig. 1K), but only SARM1 could produce large  
106 fluorescence increases with PC6.



107

108 **Fig. 1. Design and characterization of PC probes.** (A) Strategy of fluorescent imaging

109 of the activated SARM1. (B) Designing based on pyridine and styryl derivatives with a

110 donor- $\pi$ -acceptor framework. (C) Structure of PC6. (D) The kinetics of the fluorescence

111 increase at the maximal absorbance wavelengths catalyzed by SARM1-dN, in the presence of

112 100  $\mu$ M NMN, 100  $\mu$ M NAD and 50  $\mu$ M PCs. (E) Time-dependent changes of the emission

113 spectra at the isosbestic point (350 nm). (F) HPLC of PC6 reaction. Red line: in the presence

114 of PC6, NMN and NAD; Gray line: without PC6. Inset: MS analysis and structure of PAD6. (G)

115 Absorbance and fluorescence spectra of 25  $\mu$ M PC6/PAD6. (H) Emission spectra with dose of

116 NMN in the presence of NAD, PC6 and SARM1-dN. Inset: the initial rates plotted to NMN

117 concentrations. (I) Emission spectra with dose of SARM1-dN in the presence of NMN, NAD

118 and PC6; Inset: the initial rate plotted to SARM1 concentration. (J) The reaction rates of 10  $\mu$ M

119 PC6 compared with NAD analogues (100  $\mu$ M) catalyzed by SARM1. (K) The reaction rates of

120 10  $\mu$ M PC6 catalyzed by SARM1, NADase and CD38.

121 The online version of this article includes the following figure supplements for figure 1:

122 **figure supplement 1.** Synthetic scheme of PC1-PC11

123 **figure supplement 2.** NMR spectra of PCs

124 (A)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC1** in  $\text{DMSO-}d_6$

125 (B)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC2** in  $\text{CDCl}_3$

126 (C)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC3** in  $\text{CDCl}_3$

127 (D)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC4** in  $\text{CDCl}_3$

128 (E)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC5** in  $\text{DMSO-}d_6$

129 (F)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC6** in  $\text{CDCl}_3$

130 (G)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC7** in  $\text{CDCl}_3$

131 (H)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC8** in  $\text{CDCl}_3$

132 (I)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC9** in  $\text{CDCl}_3$

133 **Figure supplement 3.** Structures of PC1-9 and activity screening.

134 (A) Chemical structures of PC1-9. (B) quantification of SARM1-dN. SARM1-dN was pulled

135 down by the BC2 nanobody(Zhao et al., 2019) conjugated beads, which efficiency was

136 close to 100%. The purified SARM1-dN proteins were supplied to SDS-PAGE and

137 Coomassie blue staining. The protein contents of SARM1-dN were calculated with a

138 standard curve of BSA after intensity scanning. (C) The initial rates of the fluorescence

139 increase at the maximal absorbance/emission wavelengths (PC5: 400nm/530nm; PC6:

140 390 nm/520 nm; PC7: 340 nm/445 nm; PC8: 375 nm/490 nm) catalyzed by SARM1-dN, in

141 the presence of NMN, NAD and PCs.

142 **Figure supplement 4.** UV-vis absorption spectra scanning of the reactants.

143 The reactants of 50  $\mu$ M PCs and 100  $\mu$ M NAD during the 50-min reactions catalyzed by  
144 the activated SARM1.

145 **Figure supplement 5.** Fluorescence spectra of the reactants.

146 The reactants of 50  $\mu$ M PCs and 100  $\mu$ M NAD during the 50-min reactions catalyzed by  
147 the activated SARM1.

148

149 **Imaging SARM1 activation in live cells.** PC6 was added to HEK293 cells  
150 overexpressing either wildtype SARM1 or the enzymatically inactive mutant,  
151 E642A(Essuman et al., 2017, Zhao et al., 2019) (Fig. 2A). Green fluorescence was  
152 clearly seen evenly distributed in the whole cells in the wildtype, but not in the mutant  
153 cells (Fig. 2B), indicating active SARM1 was required. Intracellular production of  
154 PAD6 was confirmed in extracts of wildtype but not the E642A cells (Fig. 2C). CZ-48,  
155 a cell-permeant mimetic of NMN and activator of SARM1(Zhao et al., 2019),  
156 dramatically increased the PAD6 fluorescence (Fig. 2B, right column) and none in  
157 E642A-cells. These results indicate that PC6 is cell permeant and can be exchanged  
158 into the cytosolic NAD by the activated SARM1 to produce PAD6 having a large  
159 red-shift in fluorescence. PAD6 was also cell impermeant because of its charged  
160 ADP-ribose moiety and accumulated in the cytosol, greatly increased its detection  
161 sensitivity in live cells.

162 PC6 also could detect the activity of SARM1 endogenously expressed in  
163 HEK293T cells (Zhao et al., 2019). CZ-48 activated the endogenous SARM1 and

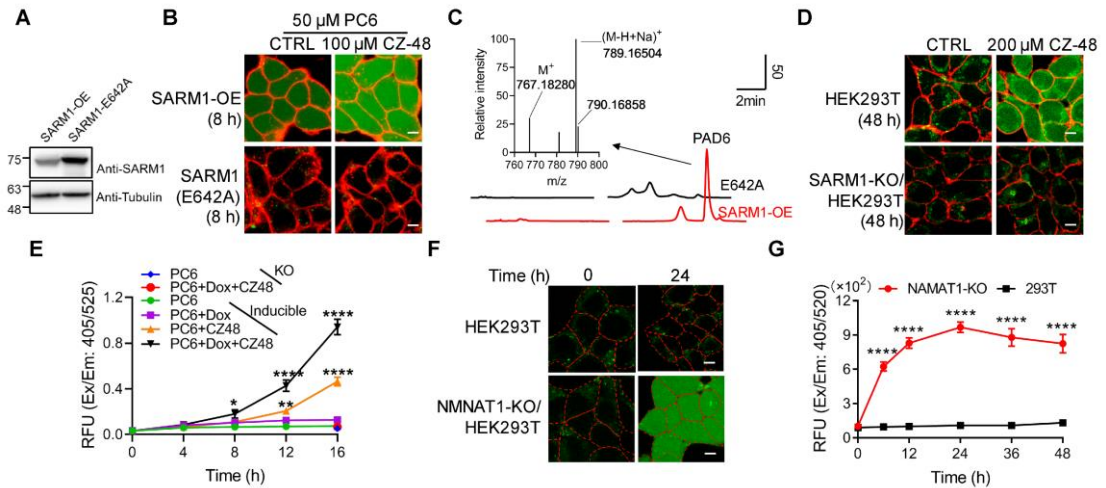
164 produced increase of cytosolic PAD6 signal (Fig. 2D, upper right), but none in the  
165 SARM1-knockout cells (Fig. 2D, right lower), confirming specificity.

166 HEK293-line carrying inducible SARM1 (Zhao et al., 2019) was used to further  
167 substantiate that the PAD6 fluorescence was derived from SARM1 activity. Without  
168 induction, only basal SARM1 (Figure 2—figure supplement 1A) with minimal activity  
169 was detected (Fig. 2E, green dots), which was activated by CZ-48, resulting in  
170 increase in PAD6-fluorescence (orange triangles). Induction of SARM1 (Figure  
171 2—figure supplement 1A) produced minimal signal also (Fig. 2E, purple squares),  
172 confirming SARM1 is auto-inhibitory. With CZ-48, both the basal and the induced  
173 SARM1 were activated, resulting in the largest signal (Fig. 2E, black triangles). In  
174 SARM1-knockout cells, no signal was detected (Fig. 2D, SARM1-KO; Fig. 2E, blue  
175 and red dots).

176 Endogenous NMN can be increased by ablating NMN-adenylyltransferase  
177 (NMNAT1)(Zhao et al., 2019), resulting in increased PAD6 fluorescence (Fig. 2F) in a  
178 time-dependent manner (Fig. 2G).

179 Consistent with the *in vitro* results, PC6 is highly selective for SARM1 over CD38  
180 in live cells. Cells expressing either wildtype or Type III mutant CD38(Liu et al., 2017,  
181 Zhao et al., 2012) did not show PAD6-signal after 48-hour incubation with PC6 (Figure  
182 2—figure supplement 1B), even though the expressed enzymes readily increased  
183 cellular cADPR (Figure 2—figure supplement 1C).

184



185

186 **Fig. 2. Live-cell imaging of SARM1 activation.** (A) Western blot of the overexpression of  
 187 SARM1 and inactive mutant, E642A in HEK293 cells. (B) Confocal fluorescence images of  
 188 cells in (A) after incubation with PC6 in presence or absence of CZ-48. Green: PAD4; Red:  
 189 ConA-Alex-647; (C) HPLC and MS analysis of PAD6 from SARM1-OE cells. The metabolites  
 190 were extracted by 0.6 M PCA from the cells in (A) after treating with 50  $\mu$ M PC6 for 24 h. Inset:  
 191 MS analysis. (D) Confocal images of wildtype, or SARM1-KO HEK293T cells with PC6 in  
 192 presence or absence of CZ-48. (E) The HEK293 cells carrying the inducible SARM1 were  
 193 incubated with 50  $\mu$ M PC6 in presence or absence of 0.5 mg/mL Dox and/or 100  $\mu$ M CZ-48.  
 194 The PAD6 fluorescence was analyzed by flow cytometry. (F) Confocal images of NMNAT1-KO/  
 195 HEK293T cells, incubated with PC6. Cell edges were marked according to the bright-field  
 196 images. (G) Quantification of the cell fluorescence in (F). All the above experiments were  
 197 repeated at least three times (means  $\pm$  SDs; n = 3; Student's *t*-test, \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , \*\*\*\*,  
 198  $p < 0.0001$ ). Scale bar: 10  $\mu$ m.

199 The online version of this article includes the following figure supplement for figure 2:

200 **Figure supplement 1.** The expression level of SARM1 for fig.2E and the activities of CD38.

201 (A) Western blots analysis of the expression of SARM1 in the inducible cell lines with or  
202 without treatment of 0.5 µg/ml Dox and 100 µM CZ-48 for the indicated time. (B) The  
203 HEK293T cells overexpressing wildtype and Type III mutant CD38 (mutCD38)(Liu et al.,  
204 2017, Zhao et al., 2012) were incubated with 50 µM PC6 for the indicated time and the  
205 fluorescence of PAD6 was analyzed in the flow cytometry; (C) The cellular cADPR levels  
206 in the cells (A) were measured by the cycling assay.(Graeff et al., 2002)

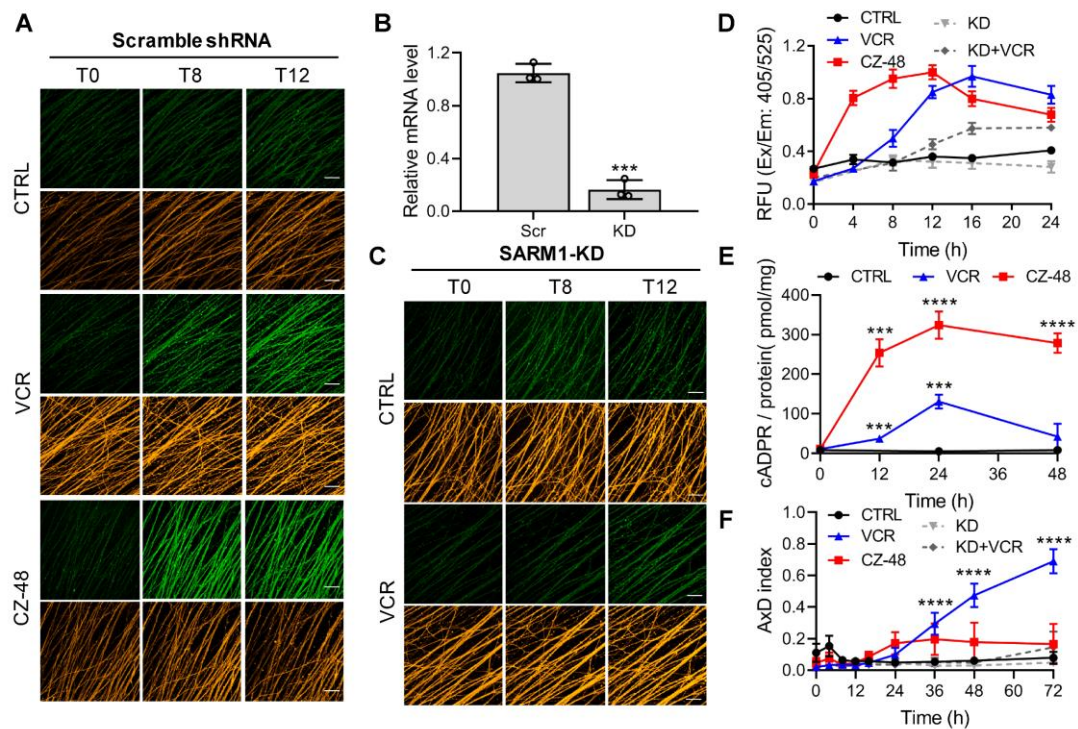
207

208 **Imaging SARM1 activation during AxD.** Vincristine (VCR)-induced AxD in  
209 peripheral neuropathy is a common side effect of chemotherapy (Essuman et al.,  
210 2017) and is thought to be due to SARM1-activation (Gerdtts et al., 2013). Mouse  
211 dorsal root ganglion (DRG) neurons were infected with lentivirus carrying TdTomato  
212 for visualizing the axons (Fig. 3A, 3C, orange), and with either a non-targeting (Fig.  
213 3A) or SARM1-specific shRNA (Fig. 3C). In the non-targeting group, VCR elevated  
214 PAD6-fluorescence (Fig. 3A, green), indicating activation of SARM1, by as early as  
215 4-8 hours and reaching a maximum by 16 hours (Fig. 3A and 3D, blue). AxD started at  
216 about 20 hours (Fig. 3F, blue; Figure 3—figure supplement 1A), temporally consistent  
217 with a causal role for SARM1. Another measure of SARM1 activation is the elevation  
218 of cellular cADPR (Zhao et al., 2019), which occurred (Fig. 3E, blue) by 12 hours,  
219 peaking at 24 hours. Neurons not treated with VCR showed neither SARM1-activation  
220 nor AxD (Fig. 3A, D, E, F and Figure 3—figure supplement 1A, CTRL).

221 Reducing endogenous SARM1 using shRNA (Fig. 3B, D and F, KD) reduced the  
222 PAD6 fluorescence without altering its peaking at 16 hours (Fig. 3C; 3D, KD+VCR)



223 and reduced AxD (Fig. 3F, KD+VCR; Figure 3—figure supplement 1B), further  
 224 substantiating a causal role for SARM1. CZ-48 induced SARM1-activation more  
 225 rapidly (Fig. 3A and 3D, red) and elevated cADPR higher (Fig. 3E, red), confirming its  
 226 direct action. Intriguingly, CZ-48 did not induce massive AxD as VCR (Fig. 3F, CZ-48;  
 227 S5A). These results indicate SARM1-activation is a necessary and causal factor, but  
 228 not a sufficient one for AxD. Other critical factors might be downstream events leading  
 229 to microtubular dysfunction.



230  
 231 **Fig. 3. SARM1 activation in mouse DRG upon vincristine treatment.** (A,C) Confocal  
 232 imaging of SARM1 activation in DRG neuronal axons. The neurons were infected with virus  
 233 expressing TdTomato to provide easy imaging of the axons. Cells were additionally transfected  
 234 with either scramble (A) or SARM1-specific (C) shRNAs and treated with 50  $\mu$ M PC6, 200  $\mu$ M  
 235 CZ-48 or 50 nM Vincristine and imaged in the indicated time points. Green: PAD4; Orange:  
 236 TdTomato; scale bars: 50  $\mu$ m. (B) Knockdown efficiency of SARM1. Scr, scramble shRNA; KD,



237 SARM1-specific shRNA. (D) Quantification of the fluorescence intensity of PAD6 in DRGs. (E)

238 Intracellular cADPR contents. (F) Indices of AxD. All the above experiments were repeated at

239 least three times (means  $\pm$  SDs;  $n = 3$ ; Student's  $t$ -test, \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ).

240 The online version of this article includes the following figure supplement for figure 3:

241 **Figure supplement 1.** The integrity of axons visualized by the TdTomato fluorescence.

242 The DRG neurons, on Div6, were infected with the lentivirus expressing TdTomato and

243 scramble shRNA (A) or SARM1-specific shRNA (B) and 3 days later, incubated with 50

244 nM VCR or 200  $\mu$ M CZ-48 with 50  $\mu$ M PC6. The photos of axons with TdTomato

245 fluorescence were captured under the confocal microscope (Nikon, A1). Scale bars: 50

246  $\mu$ m.

247

248 **Dehydronitrosonisodipine (dHNN) is an inhibitor of SARM1 activation.**

249 Another prompt application of PC6 is library screening for inhibitors of SARM1. The

250 feasibility was verified by measuring the  $IC_{50}$  of a reported inhibitor of SARM1,

251 nicotinamide (Nam)(Essuman et al., 2017), to be around 140  $\mu$ M (Figure 4—figure

252 supplement 1A). NMN-activated SARM1 was incubated with drugs of the library (Fig.

253 4A) and its activity measured with PC6 in the presence of NAD (cf. Fig. 1). Out of

254 2015 drugs, 34 had more than 80% inhibition (Fig. 4B), which were further tested for

255 inhibition of the SARM1-NADase activity using HPLC. Fig. 4C shows the plot the

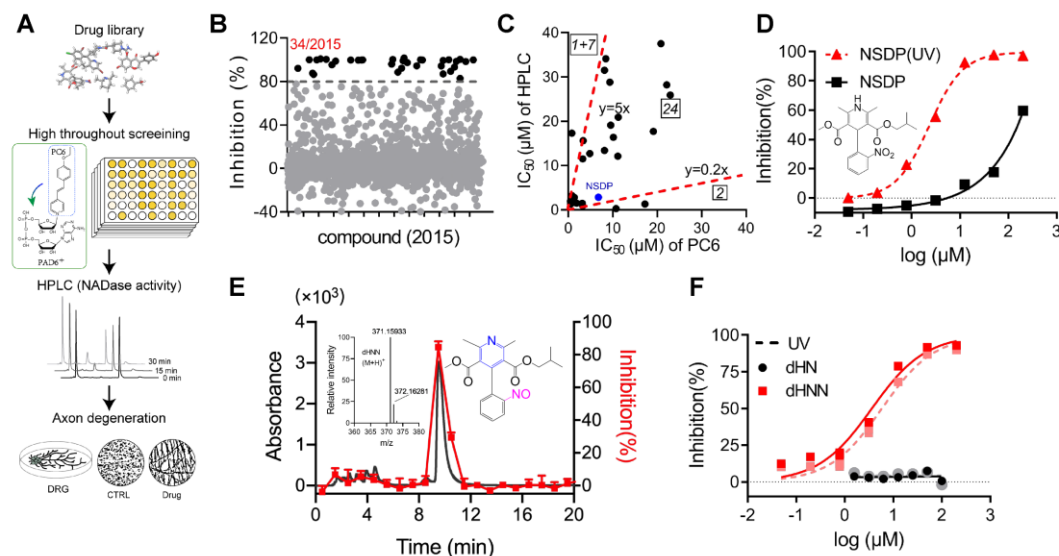
256  $IC_{50}$ -values of these drugs measured with both the PC6 and the NADase/HPLC

257 assays. Twenty-four drugs are in the middle sector, indicating they inhibited both

258 reactions similarly. Two inhibited the PC6 activity 5-fold less than the NADase (Fig.

259 4C, left sector), and eight in the right sector (7 have IC<sub>50</sub>s higher than 40 μM) inhibited  
260 NADase less than the base-exchange. These remarkable differences underscore the  
261 importance of using more than one assay for drug screening (see Discussion).

262 In the middle sector is Nisoldipine (NSDP), a calcium channel blocker having  
263 beneficial effects on neurodegenerative diseases. Peculiarly, its inhibition of SARM1  
264 varied widely among batches. Investigations indicated the active compound was not  
265 NSDP but its derivative. Fig. 4D shows fresh NSDP had an IC<sub>50</sub>-value of about 150  
266 μM (squares), but its potency increased 75-fold after exposure to UV (Fig. 4D,  
267 triangles, IC<sub>50</sub>=2 μM). Also, fresh NSDP had an HPLC-elution peak at 12.2 min  
268 (Figure 4—figure supplement 1B), but was completely converted by UV to a  
269 compound having a peak at 9.8 min that strongly inhibited of SARM1 (Fig. 4E, red).  
270 HRMS showed that the active compound had a mass of 370.15205 Da (Fig. 4E, inset)  
271 identical to a known derivative of NSDP, dehydronitrosonisoldipine  
272 (dHNN)(Marinkovic et al., 2003). The HPLC-elution profile of the active compound  
273 was also the same as dHNN (Figure 4—figure supplement 1B, purple line and green  
274 dash). Indeed, authentic dHNN was active and could not be further activated by UV  
275 (Fig. 4F, red line and dash). Another derivative of NSDP, dehydronisoldipine (dHN,  
276 elution peak at 8.7 min, Figure 4—figure supplement 1B), showed no inhibition before  
277 or after UV (Fig. 4F, black line and dash), indicating that the nitroso group is essential  
278 for the effect.



279

280 **Fig. 4. Identification of dHNN as an inhibitor of SARM1.** (A) Flowchart of the PC6-based

281 high-throughput screening. (B) Inhibitory effects of the 2,015 compounds (50  $\mu\text{M}$ ) from an

282 approval drug library. The activity of drug-treated SARM1-dN was determined with PC6 assay.

283 (C) Plot of  $\text{IC}_{50}$ s of the 27 most potent inhibitory compounds from high-throughput screening,

284 determined by PC6 (x axis) versus by HPLC (y axis) assays. See Method section. (D)

285 Inhibition curves of NSDP before (Black) and after (NSDP(UV), red) UV at 254 nm for 30 min.

286 (E) HPLC elution profile of dHNN. NSDP after 30 min UV treatment was analyzed using a C-18

287 column with a gradient of 0.1% TFA and ACN in 0.1% TFA. Fractions were assayed for

288 inhibition of SARM1-dN by PC6 assay. The derivative in the elution peak was identified by MS.

289 Black line: Absorbance at 275 nm; Red dots: inhibition activity. Insets: MS of the peak fraction

290 showing its mass was the same as dHNN and the chemical structure of dHNN. (F)

291 Concentration-inhibition curves of dHN (black solid line), UV-treated dHN (black dotted line),

292 dHNN (red solid line) and UV-treated dHNN (red dotted line), measured by PC6 assay.

293 The online version of this article includes the following figure supplement for figure 4:

294 **Figure supplement 1.** The inhibitory mechanism of dHNN against SARM1.

295 (A) The inhibition curves of nicotinamide to SARM1-dN measured with PC6-based  
296 reaction. (B) HPLC analysis of NSDP after treating by UV at 254 nm for 30 min. black:  
297 standard of NSDP; purple: NSDP after UV; red: standard of dHN; green: standard of  
298 dHNN; (C) The irreversibility of the inhibition of dHNN. SARM1-dN was pre-incubated with  
299 20  $\mu$ M dHNN or 5 mM Nam at RT for 20 min and washed using Centricon filter. Activity  
300 was analyzed afterward with PC6 assay. (D) Time dependent inhibition of dHNN to  
301 SARM1-dN. 10  $\mu$ M dHNN or 300  $\mu$ M of Nam was pre-incubated with SARM1-dN for the  
302 indicated time. Activity was analyzed afterward with PC6 assay (E) Western blots analysis  
303 of the expression of SARM1 with single cysteine mutation to alanine. C215A and C226A  
304 show much lower protein expression, indicating the mutations seems affect the stability of  
305 the whole protein, although the  $IC_{50}$ s of decreased to 30  $\mu$ M and 11  $\mu$ M, respectively,  
306 comparing to that of wildtype SARM1 (6  $\mu$ M).

307

308 **dHNN inhibits SARM1 and AxD by covalently modifying cysteines.** The  
309 dHNN-inhibition was irreversible by washing (Figure 4—figure supplement 1C, red  
310 bars), while that by Nam was reversible. Also, dHNN-inhibition was time-dependent,  
311 but not Nam (Figure 4—figure supplement 1D), strongly suggesting dHNN covalently  
312 reacted with SARM1.

313 To determine the target of dHNN, we truncated the inhibitory ARM-domain,  
314 producing a constitutively active SAM-TIR, which showed a right-shifted  
315 inhibition-curve comparing to the full-length form (Fig. 5A), with around 50-fold  
316 increase in the  $IC_{50}$ . dHNN decreased the cellular cADPR in cells expressing SARM1,

317 but not in those expressing SAM-TIR (Fig. 5B). These results suggest that dHNN is  
318 cell-permeant and acts mainly by blocking SARM1 activation and not its enzymatic  
319 activities.

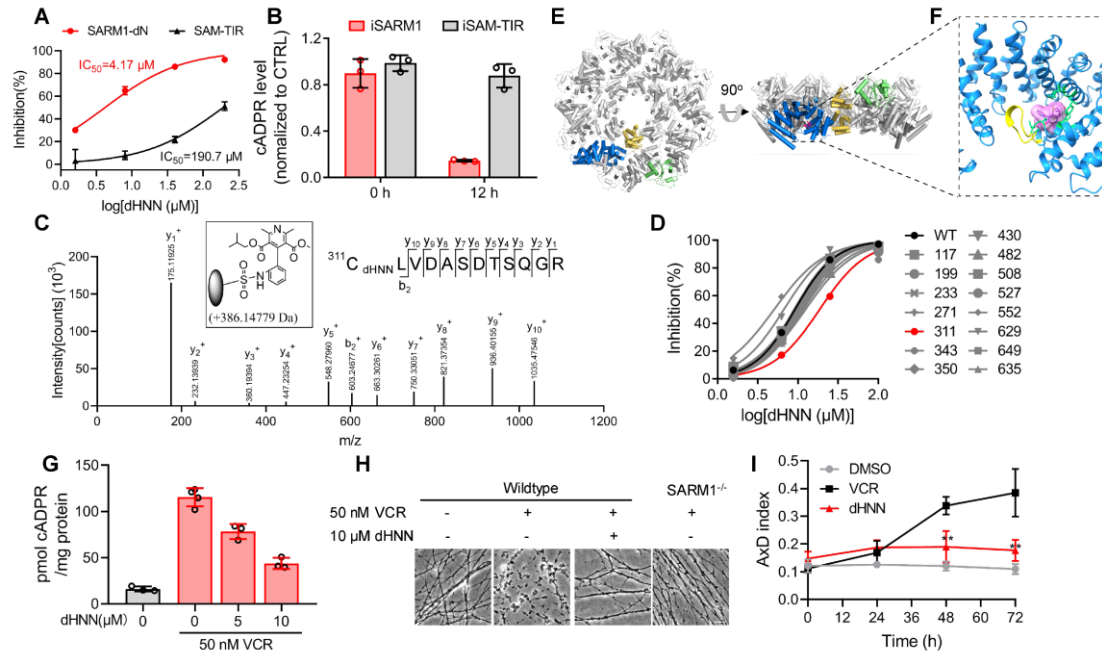
320 The nitro group of dHNN may covalently modify cysteines (Callan et al., 2009) in  
321 SARM1. Indeed, LC-MS/MS identified dHNN-modifications (+386.1483 Da, Fig. 5C,  
322 inlet) were mainly in Cys311 of the ARM domain (Fig. 5E, Figure 5—figure  
323 supplement 1A-L). Many peptides of other proteins with cysteines were also identified  
324 but none showed modification by dHNN (Figure 5—figure supplement 1M), indicating  
325 specificity of dHNN. Single mutation of all the cysteines to alanine showed that  
326 Cys311A significantly decreased the response to dHNN (Fig. 5D).

327 With Cryo-EM, we found that dHNN stabilized a similar inhibitory conformation of  
328 SARM1 as that induced by NAD (PDB: 7cm6)(Jiang et al., 2020). In 2D-classification  
329 of the untreated SARM1, most particles presented only the SAM octamer ring (Figure  
330 5—figure supplement 3A). For the dHNN-treated SARM1, larger octamer ring  
331 corresponding to both the SAM and ARM/TIR domains could be clearly observed  
332 (Figure 5—figure supplement 2, Supplementary file 1, Figure 5—figure supplement  
333 3B). Its 3D-structure was constructed at 2.4 Å resolution (Figure 5—figure supplement  
334 3C) with residues from 56 to 549 (ARM and SAM domains) and 561 to 702 (TIR  
335 domain) all fitted into the cryo-EM map (Fig. 5E and Figure 5—figure supplement 3D).  
336 Structural superimposition with the NAD-bound SARM1 (PDB: 7cm6)(Jiang et al.,  
337 2020) showed RMSD values of 0.91 (Figure 5—figure supplement 3E), suggesting  
338 that dHNN constrains SARM1 in an inactive conformation similar to that induced by

339 NAD. Extra electron density was only observed near residue Cys311 (Figure 5—figure  
340 supplement 4A), the dHNN-target (Fig. 5C, 5D), but not other cysteines, consistent  
341 with it being derived from dHNN (5F, purple). dHNN interacts with Leu265, Leu268,  
342 Phe308, Arg322 and Tyr348 (Fig. 5F and Figure 5—figure supplement 4A, green) in  
343 the ARM domain, pushing the insertion loop (Fig. 5F, yellow) towards ARM1 and  
344 stabilizes the domain. This is similar to that observed with NAD, which binds at the  
345 other side of the insertion loop (Figure 5—figure supplement 4B) and stabilizes the  
346 ARM domain possibly via ligating ARM1 and the insertion loop (Figure 5—figure  
347 supplement 4B).

348 By preventing SARM1 from activation, dHNN also inhibited the VCR-activated  
349 cADPR production (Fig. 5G) in neurons and blocked not only the VCR-induced AxD  
350 (Fig. 5H, 3<sup>rd</sup> picture; Fig. 5I, red line) but also AxD after axotomy (Figure 5—figure  
351 supplement 5A, 3<sup>rd</sup> column; B, red line) as effective as knocking out SARM1 (Figure  
352 5—figure supplement 5B, grey lines).

353



354

355 **Fig. 5. dHNN reduces AxD by inhibiting SARM1 through covalent modification of the**

356 **cysteine.** (A) Inhibition of SARM1-dN and SAM-TIR by dHNN *in vitro*. See Method. (B)

357 Inhibition of SARM1-dN and SAM-TIR by dHNN *in cellulo*. See Method. (C) MS of SARM1-dN

358 modification by dHNN. Peptide spectrum match shows that Cys311 was modified by dHNN,

359 increasing its mass to 386.1483 Da. (D) Each cysteine in SARM1-dN was mutated to alanine.

360 The dHNN- $IC_{50}$ s were measured by PC6 assay. (E) Top (left) and side (right) view of the

361 SARM1 octamer.  $\alpha$ -helices are shown as cylinders. dHNN modifications are shown as sticks.

362 One protomer is colored in blue for ARM, gold for SAM and green for TIR. The other protomers

363 are colored in grey. (F) Zoom-in view of the dHNN-modified pocket. dHNN: purple stick and

364 molecular surface; interacting residues: green; loop: yellow. (G) DRG neurons were treated

365 with dHNN for 16 h in the presence of VCR. The cellular cADPR contents were measured. (H)

366 Micrographs of AxD of DRG neurons after treatment of VCR in presence of dHNN for 72 h. (I)

367 Quantification of AxD indices after 0, 24, 48, 72 h treatment with VCR as in (H).

368 The online version of this article includes the following figure and file supplements for figure 5:

369 **Figure supplement 1.** The dHNN modifications on the peptides of SARM1 analyzed by  
370 LC-MSMS.

371 (A-L) The peptides of SARM1 containing the dHNN modifications characterized by  
372 LC-MSMS. (M) The statistical analysis of the peptides containing cysteines with or without  
373 the dHNN modifications characterized by LC-MS/MS.

374 **Figure supplement 2.** Data processing procedure for the SARM1-dHNN structure.

375 **Figure supplement 3.** The structure of SARM1 was stabled in inactive form after dHNN  
376 treatment.

377 (A-B) Representative 2D class averages of SARM1-dN in the absence (A) or presence (B)  
378 of 50  $\mu$ M dHNN in 100 mM Tris, 150 mM NaCl and 1 mM EDTA at pH 8.0. (C) Local  
379 resolution estimation calculated by RELION for the 3D reconstruction of SARM1-dHNN.  
380 Left, top view; middle, bottom view; right, side view. (D) Density fit to the 3D reconstruction.

381 (E) Superimposition of SARM1-dHNN onto SARM1-NAD (PBD codes 7cm6 and 7cm7)  
382 shows no obvious structural deviation. Left: octamer, blue for SARM1-dHNN, green for  
383 7cm6 and grey for 7cm7; right, stereo view for the superimposition of one protomer in  
384 SARM1-dHNN and SARM-NAD (7cm6).

385 **Figure supplement 4.** dHNN bound to residue C311 in the ARM domain of SARM1

386 (A) dHNN covalently links to residue C311 in ARM domain. ARM, SAM and TIR domains  
387 were shown as cartoon and colored in blue, yellow, and pink, respectively. The insertion  
388 loop between residues L312 and G323 was colored in yellow. The electron density  
389 corresponding to dHNN was shown as grey surface. dHNN and residues interacting with



390 dHNN were shown as stick models and were colored in violet and green, respectively. (B)  
391 Superposition of SARM1-dHNN onto SARM1-NAD (PDB code 7mc6). SARM1-dHNN and  
392 SARM1-NAD were shown as blue and grey cartoon, respectively. The insertion loop was  
393 in SARM1-dHNN was shown in red. NAD was shown as stick models and colored with  
394 gold carbons. Residues interacting with NAD, W103, R110 and R157, were shown as  
395 stick models with grey carbons. dHNN and residues interacting with dHNN were shown as  
396 in (A).

397 **Figure supplement 5.** dHNN attenuates the axotomy-induced AxD.

398 DRG neurons were pre-treated with 3  $\mu$ M dHNN for 0.5 h and axotomy performed to  
399 induce AxD. Images were capture at 0, 24, 48 h (A) and the AxD index was analyzed by  
400 ImageJ (B).

401 **Supplementary file 1.** Refinement statistics for the SAMR1-dHNN structure

402

## 403 **Discussion**

404 Visualizing the activity of a signaling enzyme in live cells provides clearer  
405 understanding of the spatial and temporal aspects of its mechanism and function, a  
406 goal sought by many. The PC-probes developed here are particularly advantageous.  
407 They are cell permeant, but the SARM1-catalyzed exchange products are  
408 impermeant and accumulates in the cytosol, enhancing its detection. The remarkably  
409 large red shift of the product fluorescence provides easy visualization away from the  
410 interference of auto-fluorescence. Our approach requires neither expression of  
411 construct nor cell manipulation. That it is applicable to any live cells was documented

412 using CZ-48, a cell permeant activator of SARM1. With the probes, we provided the  
413 first direct evidence in live DRG neurons that SARM1 activation precedes AxD by  
414 several hours and that it is a necessary but insufficient factor for AxD.

415 Screening library to identify compounds of interest is a straightforward strategy  
416 widely used. The case for SARM1 is more complicated, as it is not only a  
417 multi-domain protein but also an auto-regulated enzyme catalyzing multiple reactions.  
418 Compounds may target the regulatory ARM domain as shown here for dHNN, or the  
419 catalytic TIR domain as Nam thought to be. For SARM1, the substrates are different  
420 for the base-exchange and the NADase reactions and may thus be differentially  
421 affected by the inhibitor-induced conformational changes of the catalytic site.  
422 Although the exact reason remains to be determined, the compounds shown here that  
423 can selectively block one reaction much more than the other are of interest. Many  
424 believe that the NADase activity of SARM1, leading to cellular NAD depletion, is its  
425 dominant property for regulating AxD. But the two calcium messengers, cADPR and  
426 NAADP produced by its cyclase and base-exchange reactions may well have  
427 functional roles as well. Compounds with differential inhibition can thus be an  
428 important tool to resolve the issue.

429 Much effort is being invested in targeting SARM1-mediated NAD depletion for  
430 therapeutic protection from AxD. Chemical blockers may well be an ideal tool for  
431 turning off the NAD depletion. dHNN uncovered in this study is the first compound  
432 ever described that can block the activation of SARM1, revealing a druggable  
433 allosteric site and can thus usher in a new approach for therapeutic drug development.

434 Another point of interest is that dHNN is a derivative of NSDP. Metabolic conversion of  
435 NSDP to dHNN, leading to inhibition of SARM1, may well account for the neural  
436 protective effects of NSDP (Siddiqi et al., 2019). That NSDP is already a clinical drug  
437 makes the path of dHNN to therapeutic use smoother.

## 438 **Materials and Methods**

### 439 **Animals**

440 This study was carried out in strict accordance with animal use protocol approved by  
441 Peking University Shenzhen Graduate School Animal Care and Use Committee  
442 (#AP0015001). All animals (C57BL6/J), purchased from Guangdong Medical Laboratory  
443 Animal Center (China), were handled in accordance with the guidelines of the Committee  
444 on the Ethic of Animal Experiments. All surgery was performed after euthanasia and  
445 efforts were made to minimize suffering.

### 446 **Reagents**

447 NAD, NMN, Digitonin, Poly-L-lysine, 5-fluoro-2'-deoxyuridine and uridine,  $\text{KH}_2\text{PO}_4$ ,  
448  $(\text{NH}_4)_2\text{SO}_4$  and iodoacetic acid were purchased from Sigma-Aldrich. DMEM,  
449 Neurobasal™ Plus Medium, Trypsin-EDTA, penicillin/streptomycin solution, B27 plus,  
450 GlutaMax, Laminin, Lipofectamine 2000, ConA-Alex-647, formic acid, Acetonitrile were  
451 purchased from Thermo Fisher. NGF was purchased from Sino Biological. FBS was  
452 obtained from PAN Bitotech. Approval drug library (L1000) and Nisoldipine power (Cas #  
453 63675-72-9) were purchase from Targetmol. Dehydro Nisoldipine (Cas #103026-83-1)  
454 was obtain from TRC while dehydronitrosonisoldipine (Cas #87375-91-5) were purchase  
455 from Glpbio and TRC. Vincristine was purchase from Selleck. General chemicals for

456 probe synthesis were purchased from Dieckmann, Alfa, Energy or Sangon Biotech  
457 (Shanghai).

#### 458 **Synthesis and characterization of pyridine conjugates (PCs)**

459 All air and water sensitive reactions were carried out with anhydrous solvents in  
460 flame-dried flasks under argon atmosphere, unless otherwise specified. All the reagents  
461 were obtained commercially and used without further purification, unless otherwise  
462 specified. Anhydrous DMF was vacuum distilled from barium oxide, acetonitrile and  
463 dichloromethane was distilled from calcium hydride. Yields refer to isolated yields, unless  
464 otherwise specified. Reactions were monitored by thin-layer chromatography (TLC)  
465 carried out on 0.25 mm silica gel plates (60F-254) that were analyzed by UV light as  
466 visualizing method and by staining with anisaldehyde (450 mL of 95% EtOH, 25 mL of  
467 conc. H<sub>2</sub>SO<sub>4</sub>, 15 mL of acetic acid, and 25 mL of anisaldehyde) or KMnO<sub>4</sub> (200 mL H<sub>2</sub>O of  
468 1.5 g KMnO<sub>4</sub>, 10 g K<sub>2</sub>CO<sub>3</sub> and 1.25 mL of 10% aq. NaOH). Silica gel (200-300 mesh) was  
469 used for flash column chromatography. Nuclear magnetic resonance (NMR) spectra were  
470 recorded on either a 300 (<sup>1</sup>H: 300 MHz, <sup>13</sup>C: 75 MHz), 400 (<sup>1</sup>H: 400 MHz, <sup>13</sup>C: 100 MHz),  
471 or 500 (<sup>1</sup>H: 500 MHz, <sup>13</sup>C: 125 MHz) NMR spectrometer. The following abbreviations were  
472 used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd =  
473 doublet of doublets, m = multiplet, br = broad. High resolution mass spectra (HRMS) were  
474 obtained from a MALDI-TOF mass spectrometer.

475 To synthesize PC1, to a stirred solution of 4-vinylpyridine (210 mg, 2.0 mmol),  
476 4-iodoaniline (220 mg, 1.0 mmol), P(*o*-tol)<sub>3</sub> (61 mg, 20 mol%) and triethylamine (0.40 mL,  
477 2.9 mmol) in degassed CH<sub>3</sub>CN (15 mL) under argon was added Pd(OAc)<sub>2</sub> (23 mg, 10

478 mol%) quickly. The resulting mixture was stirred at 100 °C for 5 h. The mixture was then  
479 diluted with water (20 mL) and aqueous phase was extracted with ethyl acetate (15 mL ×  
480 3). The combined organic extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and  
481 evaporated under reduced pressure. Silica gel flash column chromatography (ethyl  
482 acetate/hexanes = 3:1) of the residue gave a pale-yellow solid (66 mg, 34%) as the  
483 product. PC1: mp = 272-273 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.47 (d, *J* = 5.3 Hz, 2H),  
484 7.46 (d, *J* = 6.0 Hz, 2H), 7.42 – 7.31 (m, 3H), 6.88 (d, *J* = 16.4 Hz, 1H), 6.59 (d, *J* = 8.5 Hz,  
485 2H), 5.51 (s, 2H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ 149.8, 145.4, 133.7, 128.5, 123.6,  
486 120.0, 120.0, 113.8. HRMS (+ESI) *m/z* calcd. for C<sub>13</sub>H<sub>12</sub>N<sub>2</sub> (M+H)<sup>+</sup> 197.1073, found  
487 197.1072.

488 To synthesize PC2, to a stirred solution of 4-iodoaniline (0.60 g, 2.7 mmol) in DMF  
489 (7.5 mL) was added ethyl bromide (0.25 mL, 3.35 mmol) and Na<sub>2</sub>CO<sub>3</sub> (0.50 g, 4.72 mmol)  
490 at rt. The resulting mixture was stirred at 70 °C for 6 h. The mixture was then diluted with  
491 water (20 mL) and the aqueous phase was extracted with ethyl acetate (15 mL × 3). The  
492 combined organic extracts were washed with water (15 mL × 3), dried over anhydrous  
493 Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure. Silica gel flash column  
494 chromatography (ethyl acetate/hexanes = 1:20) gave a brown solid (79 mg, 12%) as the  
495 product (**1**). Spectral data of **1** is consistent with those reported in the literature.<sup>(Ni et al., 2017)</sup>

496 To a stirred solution of 4-vinylpyridine (53 mg, 0.5 mmol), **1** (74 mg, 0.3 mmol), P(*o*-tol)<sub>3</sub> (30  
497 mg, 20 mol%) and triethylamine (0.40 mL, 2.9 mmol) in degassed CH<sub>3</sub>CN (5 mL) under  
498 argon was added Pd(OAc)<sub>2</sub> (11 mg, 10 mol%) quickly. The resulting mixture was stirred at  
499 100 °C for 12 h. The mixture was then diluted with water (20 mL) and aqueous phase was

500 extracted with ethyl acetate (15 mL × 3). The combined organic extracts were dried over  
501 anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure. Silica gel flash  
502 column chromatography (ethyl acetate/hexanes = 2:1) of the residue gave a pale-orange  
503 solid (51 mg, 76%) as the product. PC2: mp = 199-200 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ  
504 8.52 (d, *J* = 5.8 Hz, 2H), 7.39 (d, *J* = 8.5 Hz, 2H), 7.31 (dd, *J* = 4.7, 1.4 Hz, 2H), 7.23 (d, *J*  
505 = 16.3 Hz, 1H), 6.79 (d, *J* = 16.2 Hz, 1H), 6.60 (d, *J* = 8.6 Hz, 2H), 3.21 (q, *J* = 7.1 Hz, 2H),  
506 1.29 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 149.9, 149.0, 145.5, 133.4, 128.5,  
507 125.2, 121.2, 120.4, 112.6, 38.2, 14.8. HRMS (+ESI) *m/z* calcd. for C<sub>15</sub>H<sub>16</sub>N<sub>2</sub> (M+H)<sup>+</sup>  
508 224.1386, found 224.1382.

509 To synthesize PC3, to a stirred solution of 4-iodoaniline (1.2 g, 5.5 mmol) in DMF (15  
510 mL) was added ethyl bromide (2.0 mL, 27 mmol) and Na<sub>2</sub>CO<sub>3</sub> (1.0 g, 9.4 mmol) at rt. The  
511 resulting mixture was stirred at 70 °C for 6 h. The mixture was diluted with water (20  
512 mL) and the aqueous phase was extracted with ethyl acetate (15 mL × 3). The combined  
513 organic extracts were washed with water (15 mL × 3), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>,  
514 filtered and evaporated under reduced pressure. Silica gel flash column chromatography  
515 of the residue (ethyl acetate/hexanes = 1: 30) gave a brown oil (814 mg, 54%) as the  
516 product (**2**). Spectral data of **2** is consistent with those reported.<sup>(Kolvari et al., 2014)</sup> To a stirred  
517 solution of **2** (273 mg, 1.0 mmol), 4-vinylpyridine (210 mg, 2.0 mmol), P(*o*-tol)<sub>3</sub> (61 mg, 20  
518 mol%) and triethylamine (0.40 mL, 2.9 mmol) in degassed CH<sub>3</sub>CN (15 mL) under argon  
519 was added Pd(OAc)<sub>2</sub> (23 mg, 10 mol%) quickly. The resulting mixture was stirred at 100  
520 °C for 12 h. The mixture then was diluted with water (20 mL) and the aqueous phase  
521 extracted with ethyl acetate (15 mL × 3). The combined organic extracts were dried over

522 anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure. Silica gel flash  
523 column chromatography (ethyl acetate/hexanes = 1:1) of the residue gave a pale-yellow  
524 solid (138 mg, 55%) as the product. **PC3**: mp = 184-185 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ  
525 8.50 (d, *J* = 5.6 Hz, 2H), 7.47 – 7.38 (m, 2H), 7.30 (d, *J* = 6.1 Hz, 2H), 7.25 – 7.19 (m, 1H),  
526 6.74 (t, *J* = 16.9 Hz, 1H), 6.66 (t, *J* = 5.9 Hz, 2H), 3.47 – 3.18 (m, 4H), 1.31 – 1.02 (m, 6H).  
527 <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 149.9, 148.2, 145.7, 133.4, 128.6, 123.2, 120.4, 120.4,  
528 111.5, 44.5, 12.7. HRMS (+ESI) *m/z* calcd. for C<sub>17</sub>H<sub>20</sub>N<sub>2</sub> (M+H)<sup>+</sup> 253.1699, found  
529 253.1699.

530 To synthesize PC4, to a stirred solution of **2** (273 mg, 1.0 mmol),  
531 1-bromo-4-vinylbenzene (183 mg, 1.0 mmol), P(*o*-tol)<sub>3</sub> (61 mg, 20 mol%), triethylamine  
532 (0.40 mL, 2.9 mmol) in degassed CH<sub>3</sub>CN (15 mL) under argon was added Pd(OAc)<sub>2</sub> (23  
533 mg, 10 mol%) quickly. The resulting mixture was stirred at 100 °C for 12 h. The mixture  
534 was then diluted with water (20 mL) and the aqueous phase was extracted with ethyl  
535 acetate (15 mL × 3). The combined organic extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>,  
536 filtered and evaporated under reduced pressure. Silica flash column chromatography  
537 (ethyl acetate/hexanes = 1:30) gave a pale green solid (234 mg, 71%) as the product (**3**).  
538 Spectral data of **3** is consistent with those reported in the literature.<sup>(Lemercier et al., 2006)</sup> To a  
539 stirred solution of **3** (165 mg, 0.50 mmol), 4-vinlypyridine (105 mg, 1.0 mmol), P(*o*-tol)<sub>3</sub> (30  
540 mg, 20 mol%) and triethylamine (0.20 mL, 1.5 mmol) in degassed CH<sub>3</sub>CN (10 mL) under  
541 argon was added Pd(OAc)<sub>2</sub> (11 mg, 10%) quickly. The resulting mixture was stirred at 100  
542 °C for 12 h. The mixture was then diluted with water (20 mL) and the aqueous phase was  
543 extracted with ethyl acetate (15 mL × 3). The combined organic extracts were dried over

544 anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure. Silica gel flash  
545 column chromatography (ethyl acetate/hexanes = 3:1) of the residue gave a pale-yellow  
546 solid (128 mg, 72%) as the product. PC4: mp = 225-226 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ  
547 8.54 (d, *J* = 5.7 Hz, 2H), 7.54 – 7.44 (m, 4H), 7.42 – 7.38 (m, 2H), 7.36 (d, *J* = 6.0 Hz, 2H),  
548 7.29 (d, *J* = 16.3 Hz, 1H), 7.09 (d, *J* = 16.2 Hz, 1H), 6.99 (d, *J* = 16.3 Hz, 1H), 6.88 (d, *J* =  
549 16.2 Hz, 1H), 6.67 (d, *J* = 8.9 Hz, 2H), 3.39 (q, *J* = 7.0 Hz, 4H), 1.19 (t, *J* = 7.0 Hz, 6H). <sup>13</sup>C  
550 NMR (100 MHz, CDCl<sub>3</sub>) δ 150.1, 147.6, 144.9, 139.1, 134.2, 133.1, 129.7, 129.0, 128.6,  
551 128.0, 127.4, 126.3, 124.9, 124.4, 122.9, 120.8, 111.7, 44.4, 12.7. HRMS (+ESI) *m/z* calcd.  
552 for C<sub>25</sub>H<sub>26</sub>N<sub>2</sub> (M+H)<sup>+</sup> 355.2169, found 355.2167.

553 To synthesize PC5, to a stirred solution of 4-vinylpyridine (210 mg, 2.0 mmol),  
554 4-iodophenol (220 mg, 1.0 mmol), P(*o*-tol)<sub>3</sub> (61 mg, 20 mol%) and triethylamine (0.40 mL,  
555 2.9 mmol) in degassed CH<sub>3</sub>CN (15 mL) under argon was added Pd(OAc)<sub>2</sub> (23 mg, 10  
556 mol%) quickly. The resulting mixture was stirred at 100 °C for 12 h. The mixture was then  
557 diluted with water (20 mL). Upon addition of 5% HCl leads to partial precipitation of the  
558 product. The aqueous phase was extracted with ethyl acetate (15 mL × 3). The combined  
559 organic extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under  
560 reduced pressure. Silica gel flash column chromatography (ethyl acetate/hexanes = 3:1)  
561 of the residue gave an off-white solid (130 mg, 66%) as the product. **PC5**: mp = 281-282.  
562 <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.83 (s, 1H), 8.55 (d, *J* = 4.5 Hz, 2H), 7.67 – 7.42 (m, 5H),  
563 7.07 (d, *J* = 16.4 Hz, 1H), 6.85 (d, *J* = 9.3 Hz, 2H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>) δ 158.2,  
564 149.8, 144.8, 133.1, 128.7, 127.2, 122.4, 120.5, 115.7. HRMS (+ESI) *m/z* calcd. for  
565 C<sub>13</sub>H<sub>11</sub>NO (M+H)<sup>+</sup> 198.0913, found 198.0913.



566 To synthesize PC6, to a stirred solution of 4-vinylpyridine (631 mg, 6.0 mmol),  
567 1-ethoxy-4-iodobenzene (1.24 g, 5.0 mmol), P(*o*-tol)<sub>3</sub> (305 mg, 20 mol%) and triethylamine  
568 (2.0 mL, 15 mmol) in degassed CH<sub>3</sub>CN (15 mL) under argon was added Pd(OAc)<sub>2</sub> (112  
569 mg, 10 mol%) quickly. The resulting mixture was stirred at 100 °C for 12 h. The mixture  
570 was then diluted with water (20 mL) and the aqueous phase was extracted with ethyl  
571 acetate (15 mL × 3). The combined organic extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>,  
572 filtered and evaporated under reduced pressure. Silica gel flash column chromatography  
573 (ethyl acetate/hexanes = 3:1) of the residue gave an off-white solid (958 mg, 85%) as the  
574 product. PC6: mp = 146-147 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.55 (dd, *J* = 4.6, 1.5 Hz,  
575 2H), 7.51 – 7.42 (m, 2H), 7.33 (dd, *J* = 4.6, 1.5 Hz, 2H), 7.27 (t, *J* = 8.1 Hz, 1H), 6.97 –  
576 6.81 (m, 3H), 4.07 (q, *J* = 7.0 Hz, 2H), 1.44 (t, *J* = 7.0 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  
577 δ 159.6, 150.1, 145.0, 132.8, 128.7, 128.4, 123.6, 120.6, 114.8, 63.6, 14.8. HRMS (+ESI)  
578 *m/z* calcd. for C<sub>15</sub>H<sub>15</sub>NO (M+H)<sup>+</sup> 226.1226, found 226.1226.

579 To synthesize PC7, to a stirred solution of 4-iodophenol (1.09 g, 4.93 mmol) and  
580 triethylamine (749 mg, 7.40 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (25 mL) was added acetyl chloride (465 mg,  
581 5.92 mmol) at rt. The resulting mixture was stirred at 0 °C for 20 min and then rt for 2 h.  
582 The solution was then diluted with water (20 mL) and the aqueous phase was extracted  
583 with ethyl acetate (15 mL × 3). The combined extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>,  
584 filtered and evaporated under reduced pressure. The resulting pale brown oil (1.15 g,  
585 89 %) was obtained as the product (**4**) and was used for the next step without any further  
586 manipulation. Spectral data of **4** is consistent with those reported in the literature.<sup>(Flaherty et  
587 al., 2010)</sup> To a stirred solution of 4-vinylpyridine (210 mg, 2.0 mmol), **4** (240 mg, 1.0 mmol),

588 P(*o*-tol)<sub>3</sub> (61 mg, 20 mol%) and triethylamine (0.40 mL, 2.9 mmol) in degassed CH<sub>3</sub>CN (15  
589 mL) under argon was added Pd(OAc)<sub>2</sub> (23 mg, 10 mol%) quickly. The resulting mixture  
590 was stirred at 100 °C for 6 h. The mixture was then diluted with water (30 mL) and the  
591 aqueous phase was extracted with ethyl acetate (15 mL × 3). The combined organic  
592 extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced  
593 pressure. Silica gel flash column chromatography (ethyl acetate/hexanes = 3:1) of the  
594 residue gave a white solid (103 mg, 43%) as the product. PC7: mp = 152-153 °C. <sup>1</sup>H NMR  
595 (400 MHz, CDCl<sub>3</sub>) δ 8.58 (d, *J* = 5.9 Hz, 2H), 7.63 – 7.48 (m, 2H), 7.36 (dd, *J* = 4.7, 1.4 Hz,  
596 2H), 7.28 (d, *J* = 16.3 Hz, 1H), 7.15 – 7.09 (m, 2H), 6.97 (d, *J* = 16.3 Hz, 1H), 2.32 (s, 3H).  
597 <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 169.4, 150.9, 150.2, 144.5, 133.9, 132.2, 128.1, 126.2,  
598 122.1, 120.9, 21.2. HRMS (+ESI) *m/z* calcd. for C<sub>15</sub>H<sub>13</sub>NO<sub>2</sub> (M+H)<sup>+</sup> 240.1019, found  
599 240.1018.

600 To synthesize PC8, to a stirred solution of 5-bromo-2-hydroxy-benzonitrile (0.60 g, 30  
601 mmol) in DMF (7 mL) was added ethyl bromide (0.37 mL, 5.0 mmol), and K<sub>2</sub>CO<sub>3</sub> (1.0 g,  
602 9.4 mmol) at rt. The resulting mixture was stirred at 70 °C for 6 h. The mixture was then  
603 diluted with water (20 mL), and the aqueous phase was extracted with ethyl acetate (15  
604 mL × 3). The combined organic extracts were washed with water (15 mL × 3), dried over  
605 anhydrous Na<sub>2</sub>SO<sub>4</sub>, and evaporated under reduced pressure. A white solid was obtained  
606 as the product (**12**). To a stirred solution of the crude product (**12**), 4-vinylpyridine (315 mg,  
607 3.0 mmol), P(*o*-tol)<sub>3</sub> (183 mg, 20 mol%) and triethylamine (1.2 mL, 8.7 mmol) in degassed  
608 CH<sub>3</sub>CN (30 mL) under argon was added Pd(OAc)<sub>2</sub> (67mg, 10 mol%) quickly. The resulting  
609 mixture was stirred at 100 °C for 5 h. The mixture was then diluted with water (30 mL) and

610 the aqueous phase was extracted with ethyl acetate (15 mL × 3). The combined organic  
611 extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced  
612 pressure. Silica gel flash column chromatography (ethyl acetate/hexanes = 3:1) of the  
613 residue gave a pale-yellow solid (433 mg, 58%) as the product. **PC8**: mp = 114-115 °C. <sup>1</sup>H  
614 NMR (400 MHz, CDCl<sub>3</sub>) δ 8.59 (d, *J* = 5.9 Hz, 2H), 7.80 – 7.60 (m, 2H), 7.35 (d, *J* = 5.9 Hz,  
615 2H), 7.19 (d, *J* = 16.3 Hz, 1H), 6.99 (d, *J* = 8.8 Hz, 1H), 6.91 (d, *J* = 16.3 Hz, 1H), 4.19 (q, *J*  
616 = 7.0 Hz, 2H), 1.50 (t, *J* = 7.0 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 160.4, 150.0, 143.9,  
617 132.5, 131.8, 130.2, 128.9, 125.7, 120.6, 115.9, 112.3, 102.4, 64.8, 14.3. HRMS (+ESI)  
618 m/z calcd. for C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>O (M+H)<sup>+</sup> 251.1179, found 251.1178.

619 To synthesize PC9, to a stirred solution of 1-ethoxy-4-iodobenzene (1.24g, 5.0 mmol)  
620 and 3,3-diethoxyprop-1-ene (1.03 g, 7.9 mmol), P(*o*-tol) (305 mg, 20 mol%), Cs<sub>2</sub>CO<sub>3</sub>  
621 (2.28g, 7.0 mmol) and KCl (370 mg, 5mmol) in DMF (30 mL) under argon was added  
622 Pd(OAc)<sub>2</sub> (115 mg, 10 mol%) quickly. The resulting mixture was stirred at 90 °C for 5 h  
623 and then treated with 5% HCl (10 mL) and stirred at rt for 10 min. The mixture was then  
624 diluted with water (20 mL) and the aqueous phase was extracted with ethyl acetate (15  
625 mL × 3). The combined organic extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and  
626 evaporated under reduced pressure. Silica gel flash column chromatography (ethyl  
627 acetate/hexanes = 1:5) gave a pale-yellow solid (443 mg, 50 %) as the product (**6**).  
628 Spectral data of **6** is consistent with those reported in the literature.<sup>(Lator et al., 2018)</sup> To a  
629 stirred solution of **6** (88 mg, 0.50 mmol), 4-methylpyridine (93 mg, 1.0 mmol) in Ac<sub>2</sub>O (2  
630 cmL) was added NaOAc (272 mg, 2.0 mmol) at rt. The resulting mixture was heated under  
631 reflux for 21 h. Then the mixture was cooled to rt and diluted with CH<sub>2</sub>Cl<sub>2</sub>, washed with

632 H<sub>2</sub>O, 5% HCl, H<sub>2</sub>O and saturated aqueous NaHCO<sub>3</sub>. The aqueous phase was extracted  
633 with ethyl acetate (15 mL × 3). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>,  
634 filtered and evaporated under reduced pressure. Silica gel flash column chromatography  
635 (ethyl acetate/hexanes = 3:1) gave a pale-yellow solid (47 mg, 37%) as the product. **PC9**:  
636 mp = 132-133 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.58 (d, *J* = 5.9 Hz, 2H), 7.44 (d, *J* = 8.7  
637 Hz, 2H), 7.36 – 7.29 (m, 2H), 7.17 (dd, *J* = 15.5, 10.2 Hz, 1H), 6.99 – 6.74 (m, 4H), 6.57 (d,  
638 *J* = 15.5 Hz, 1H), 4.11 (d, *J* = 7.0 Hz, 2H), 1.48 (t, *J* = 7.0 Hz, 3H). <sup>13</sup>C NMR (101 MHz,  
639 CDCl<sub>3</sub>) δ 159.0, 149.8, 144.7, 135.4, 133.8, 129.2, 128.2, 127.9, 125.8, 120.3, 114.6, 63.3,  
640 14.6. HRMS (+ESI) *m/z* calcd. for C<sub>17</sub>H<sub>18</sub>NO (M+H)<sup>+</sup> 252.1383, found 252.1384.

#### 641 **Preparation and quantification of the enzymes**

642 A truncated form of SARM1, SARM1-dN, was prepared as described.(Zhao et al.,  
643 2019) In brief, the recombinant protein was expressed in HEK293T cells and released by  
644 100 μM digitonin in PBS with protease inhibitor cocktail (Roche). The cell lysate prepared  
645 with the same method from HEK293T was used as the negative control.

646 To quantify the protein concentration, SARM1-dN was pulled down by BC2  
647 nanobody(Bruce et al., 2017) conjugated beads which were prepared by conjugating BC2  
648 nanobody to NHS-beads (GE Healthcare). The purified SARM1-dN, named as SARM1-IP,  
649 together with the certain amounts of standard protein BSA, was applied to SDS-PAGE,  
650 which was stained by Coomassie blue. The protein contents of SARM1-dN was calculated  
651 with a standard curve of BSA after intensity scanning by Image J.

652 Recombinant CD38 and *N. crassa* NADase were prepared as described  
653 previously.(Graeff et al., 1994, Munshi et al., 1997)

654 ***In vitro* fluorescence assays**

655 To analyze the activity of SARM1 with PCs *in vitro*, reactions were started by  
656 incubating the enzyme with the reaction mixture, 50  $\mu\text{M}$  PC, 100  $\mu\text{M}$  NAD and 100  $\mu\text{M}$   
657 NMN in PBS. The absorbance and fluorescence were measured in a quartz cuvette or  
658 black 96-well plates (Corning), respectively, with an Infinite M200 PRO microplate reader  
659 (Tecan). For the assays with  $\epsilon\text{NAD}$ , NHD or NGD as the substrate, 100  $\mu\text{M}$  the  
660 compounds were incubated with the enzymes and fluorescence were monitored at  $\lambda_{\text{ex}} =$   
661 300 nm,  $\lambda_{\text{em}} = 410$  nm.

662 **HPLC analysis of the base-exchange reaction of PC6 catalyzed by SARM1**

663 The reactions were prepared by mixing SARM1-IP (SARM1 binding on BC2-beads,  
664 around 2.5  $\mu\text{g}/\text{ml}$ ) with 100  $\mu\text{M}$  NAD, 50  $\mu\text{M}$  PC6, 100  $\mu\text{M}$  NMN and 0.1 mg/ml BSA in  
665 PBS and incubated for 60 min at 37  $^{\circ}\text{C}$ . SARM1-IP was removed by centrifugation at  
666 4,500 rpm for 1 min. The cleaned mixtures were applied to a C-18 reverse phase column  
667 equipped on an HPLC (Agilent 1260) with a gradient of 0.1 M  $\text{KH}_2\text{PO}_4$  (pH 6.0) and 0.1 M  
668  $\text{KH}_2\text{PO}_4$  (pH 6.0) with MeOH (7:3) to elute NMN, cAPPR, ADPR, NAD and a gradient of  
669 ACN from 30% to 70% to elute PAD6 and PC6. The fractions of PAD6 were collected and  
670 lyophilized for absorption spectra and fluorescence spectra scanning.

671 To analyze the PAD6 in cells, the metabolites were extracted from the cells treated  
672 with 50  $\mu\text{M}$  PC6 by 0.6 M perchloric acid, followed by neutralization with Chloroform:  
673 Tri-n-octylamine (3:1). The extracts were applied to a C-18 column and eluted with water  
674 and acetonitrile by 2% acetonitrile for 8 min then 30% acetonitrile for 8 min.

675 **Confocal imaging of PAD6 in living cells**

676 HEK293 cells, overexpressing wildtype or the enzymatically dead form (E642A) of  
677 SARM1 or HEK293T Knocking out NAMAT1 were constructed as before.(Zhao et al.,  
678 2019) Cells, grown on 0.05 mg/ml poly-L-lysine coated Chambered coverglass (Thermo  
679 fisher, #155411) overnight were treated with 50  $\mu$ M of PC6 in the presence or absence of  
680 100  $\mu$ M CZ-48 for 8 h (SARM1-OE cells) and 200  $\mu$ M CZ-48 for 48 h (original cell lines),  
681 respectively. To demonstrate the edges of the cells, they were stained with 50  $\mu$ g/ml  
682 Concanavalin A, Alexa Fluor™ 647 Conjugate (Thermo fisher) at 4°C for 10 min before  
683 imaging. The fluorescence signals (Ex/Em: 405/520 nm for PAD6; Ex/Em: 561/590 for  
684 ConA) were captured under a confocal microscope (Nikon A1).

#### 685 **Analysis of PAD6 signals in living cells by flow cytometry**

686 HEK293 cells carrying an inducible expression cassette of SARM1 were constructed  
687 as previous describe.(Zhao et al., 2019) The cells were treated with 50  $\mu$ M PC6, 100  $\mu$ M  
688 CZ-48 or 0.5 mg/mL Dox for 4, 8, 12 and 16 h. The cells were trypsinized and the  
689 fluorescence of PAD6 (Ex/Em: 405/525 nm) was analyzed by flow cytometry (CytoFlex,  
690 Beckman).

#### 691 **DRG culture and imaging**

692 Mouse DRG culture was performed as described.(Sasaki et al., 2016) Briefly, DRGs  
693 were dissected from the embryos at Day 12.5 to 14.5 (E12.5-E14.5) and digested by 0.05%  
694 Trypsin solution containing 0.02% EDTA (Gibco). The dispersed cells were seeded in  
695 Neurobasal™ Plus Medium supplemented with 2% B27 plus, 1 mM GlutaMax, 1%  
696 penicillin/streptavidin solution and 37.5 ng/ml NGF on the Chambered coverglass  
697 pre-coated with (0.1 mg/ml) poly-L-Lysine, (0.02 mg/ml) laminin and 5% FBS. Every 3 day,

698 50 % of the culture media was replaced by fresh media with the addition of 5  $\mu$ M  
699 5-fluoro-2'-deoxyuridine and 5  $\mu$ M uridine.

700 On Div6 the neurons were infected with lentivirus. Three days later, the cells were  
701 treated with 50  $\mu$ M PC6 in the absence or presence of 200  $\mu$ M CZ-48 or 50 nM vincristine.  
702 The fluorescence images (Ex/Em: 405/520 nm for PAD6; Ex/Em: 561/590 for TdTomato)  
703 were captured under a confocal microscope (Nikon A1) with a 60x object. The mean  
704 fluorescence intensity was quantified by NIS-Elements AR analysis (Nikon). Axon  
705 degeneration was quantified base on axon morphology using ImageJ. The TdTomato  
706 fluorescence images were binarized and measured the total axon area (size = 20-infinity  
707 pixels) and the degenerated axon (size = 20-4,000,000 pixels) with particle analyzer  
708 module of ImageJ. Axon degeneration index was calculated as the ratio of the  
709 degeneration axon over total axon area.

#### 710 **Lentivirus preparation and infection of DRG neurons**

711 pLKO.1-shRNA-mSARM1 plasmids were constructed as described previously.(Zhao  
712 et al., 2019) Briefly, shRNA targeting mSARM1 (5'-  
713 CCGGCTGGTTTCTTACTCTACGAATCTCGAGATTCGTAGAGTAAGAAACCAGTTTTTTG  
714 -3') or the scrambled shRNA (5'-  
715 CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTT  
716 G-3') were inserted to pLKO.1-puro (Addgene, #8453) with EcoRI and AgeI, followed by  
717 replacement of the puromycin resistance gene with a fluorescent protein, TdTomato  
718 (GenBank: LC311026.1) with KpnI/BamHI. The lentiviral particles were prepared by  
719 transfecting HEK293T cells with the corresponding lentivectors, pMD2.G and

720 psPAX26,(Liu et al., 2017) followed by concentration of the virus with Lenti-Concentin  
721 Virus Precipitation Solution (ExCell Bio), which was resuspended in Neurobasal™ Plus  
722 Medium. Determine the virus titer by series infection of HEK293T cells. For infection of  
723 DRG neurons, added the same MOI of virus to infect the cells on Div 6 and carried out  
724 further experiment 72 hours after infection.

### 725 **Imaging and quantification of AxD after axotomy and vincristine treatment.**

726 For axotomy, one DRG was seeding into a 24-well plate, and 5  $\mu$ M  
727 5-fluoro-2'-deoxyuridine and 5  $\mu$ M uridine was added the other day. On div5, axons were  
728 pre-incubated with drugs for 0.5 h and severed near the soma with a 3 mm flat blade  
729 under microscope guidance removing the cell bodies. For vincristine treatment, DRGs  
730 was digested with 0.05% Trypsin and seeding into 24-well plate. DRGs on Div9-13 were  
731 incubated 50 nM vincristine in the presence or absence of the drug.

732 9-12 brightfield images per treatment of the axon were acquired with a 20x object at  
733 the indicated time points using invert optical microscope (Olympus). Axon degeneration  
734 was quantified base on axon morphology using ImageJ. For each treatment, 60 random  
735 grid-squares with 147x147 pixels were cropped, binarized and measured the total axon  
736 area (size = 16-infinity pixels) and the degenerated axon (size = 16-10,000 pixels) with  
737 particle analyzer module of ImageJ. Axon degeneration index was calculated as the ratio  
738 of the degeneration axon over total axon area.

### 739 **Measuring cADPR level of DRG treating with vincristine**

740 DRG neurons were treated with 50 nM Vincristine or 200  $\mu$ M CZ-48 for 0, 12, 24, 48 h  
741 on Div6. After incubation, DRG was washed with cold PBS and lysed with 0.6 M perchloric



742 acid. cADPR was extracted and analyzed as described previously.(Graeff et al., 2002)

#### 743 **Q-RT-PCR**

744 After infection of virus for 48 h, total RNAs were extracted from DRG neurons with  
745 RNA extraction kit (OMEGA) and transcribed with Transcript II One-step gDNA Removal  
746 and cDNA synthesis Supermix (Sangon Biotech). The mRNA level of SARM1 relative to  
747 GAPDH was quantified with by Q-RT-PCR using TransStart Tip Green qPCR SuperMix  
748 (TransGen Biotech) on CFX Connect Real-Time PCR Detection System (Bio-Rad). The  
749 following primer pairs were used: SARM1 sense, 5'-CTTTCTCCAAGGAGGACGAGC-3',  
750 antisense, 5'-CTTGTGTCACCTGGCATCCACC-3'; GAPDH sense, 5'-  
751 TGGCCTTCCGTGTTCCCTAC-3', antisense, 5'-GAGTTGCTGTTGAAGTCGCA-3'.

#### 752 **PC6 assay**

753 For high-throughput screening, inhibition of 1.5 µg/ml SARM1-dN *in vitro* determined  
754 by 20 µM PC6 with 50 µM of 2046 compound from a drug approval library (Targetmol,  
755 L1000) in the presence of 50 µM NAD and 50 µM NMN. Thirty-four compounds with high  
756 fluorescence overlapped with PC6 assay were removed.

757 For IC<sub>50</sub> measurement, 0.4 µg/ml SARM1-dN was pre-incubated with dose of  
758 compounds *in vitro* for 10 min, and started the reaction by adding 50 µM NAD, 50 µM  
759 NMN and 50 µM DA4. Calculation of IC<sub>50</sub> by plotting the initial rate to dose of compounds.

#### 760 **HPLC analyze SARM1 NADase activity**

761 1 µg/ml of SARM1-dN was pre-incubated with compounds for 15 min at room  
762 temperature, and started the reaction by adding 100 µM NAD and 100 µM NMN. The  
763 reactions were stopped by removing the enzyme with MultiScreen® Filter Plates

764 ( Millipore ) after 0, 15 and 30 min incubation at 37 °C and analyzed by a C-18 column  
765 (Aligent, 20 RBAX SB-C18) with a gradient of 0.1 M KH<sub>2</sub>PO<sub>4</sub> (pH 6.0) and 0.1 M KH<sub>2</sub>PO<sub>4</sub>  
766 (pH 6.0) with MeOH (7:3) to elute NMN, cAPPR, ADPR, NAD, Nam. The amount of ADPR  
767 were used to calculate the initial rate. IC<sub>50</sub> of NADase activity was calculated by plotting  
768 dose of compounds to the initial rate.

#### 769 **HPLC to analysis nisoldipine and its derivatives**

770 10 mM NSDP was prepared freshly in DMSO and treated with UV at 254 nm for 30  
771 min. 2.5 nmole NSDP-UV and standards were applied to a C-18 reverse phase column  
772 (ZORBAX SB-C18) equipped on a HPLC (Aligent 1260) and eluted with 50% of 0.1%TFA  
773 and 50% of 0.1%TFA with 99% ACN. 0.3 μmole of the product after UV treatment was  
774 collected and purified by HPLC described above. The inhibitory activity of these fractions  
775 was determined by PC6 assay after neutralized with 100 mM Tris (pH7.5), and the main  
776 peak was characterized by HRMS (thermo, Q Exactive Focus).

#### 777 **Measure the inhibitory activity of dHNN *in vitro* and *in cellulo***

778 To determine whether dHNN inhibits activation or enzymatic activity of SARM1 *in vitro*,  
779 SARM1-dN, the autoinhibited form, and SAM-TIR, the constitutively active form, were  
780 pre-incubated with different concentrations of dHNN at RT for 10 min, after which the activity  
781 was measured with PC6 assay and the inhibition rate was calculated.

782 To test the same effect *in cellulo*, HEK293 cells overexpressing inducible SARM1  
783 (iSARM1) or SAM-TIR (iSAM-TIR) were pre-incubated with 20 μM dHNN for 1.5 h and then  
784 treated with 100 μM CZ-48 or 0.5 μg/ml doxycycline for the indicated time. Control cells were  
785 similarly induced but pre-incubated with vehicle DMSO. Cellular cADPR of the sample cells

786 was measured and divided by that of the corresponding control cells to determine the percent  
787 reduction of cADPR content.

#### 788 **Modification analysis of SARM1 by dHNN**

789 dtSARM1-dN that had the N-terminal targeting signal removed, was tagged with strep  
790 tag II and flag for purification, constructed into pENTR1A-GFP-N2 and cloned into  
791 Plenti-CMV-puro-Dest (Invitrogene) by LR clonase II enzyme according to the  
792 manufacturer's instructions. HEK293F cells were infected by lentivirus carrying  
793 dtSARM1-dN produced by HEK293T cells with lipo2000 transfection and selected with 1  
794 µg/ml puromycin. The cells were harvested by PBS and the pellet were stocked at -80°C  
795 before using. Protein extracted with 200 µM digitoinin were performed immunoprecipitated  
796 with StrepTactin beads overnight. The beads were washed with buffer W (pH7.5)  
797 containing 100 mM Tris, 150 mM NaCl and 1 mM EDTA for four times and eluted on ice  
798 with 2 mM biotin in buffer W. 0.17 mg/ml dtSARM1-dN pre-incubated with 50 µM dHNN at  
799 RT for 30 min were boiled in SDS loading buffer and applied to SDS-PAGE gel. The gel  
800 was stained by simplyBlue™ SafeStain (Thermo fisher) for 1 h and destained by water for  
801 2 h with water changing for three times. dtSARM1-dN were dissected form the gel,  
802 dehydrated with 100% ACN, and performed alkylation by incubated with 10 mM DTT at  
803 55°C for 30 min and 22.5 mM IAA for another 30 min in dark. Then the gel were destained  
804 with 50% ACN in 25 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, dehydrated with ACN and protein were digested in  
805 gel with Trypsin at 37°C overnight. Peptide were extracted from the gel with 5% FA and 50%  
806 ACN in water, then lyophilized and resuspended with 0.1% FA in water for mass  
807 spectrometry identification (Thermo, Q Exactive HF-X). The modification of dHNN on

808 cysteine were analyzed by protein discoverer (Thermo fisher).

### 809 **Cysteine mutants**

810 The mutants of cysteine to alanine in dtSARM1-dN, described above, were cloned by  
811 overlapping PCR with the primer below by PrimeStar HS polymerase with high GC buffer.  
812 The genes were constructed into pCDH-EF1-MCS-IRES-neo by Xba I and Not I, and  
813 transfected into HEK293 cells by lipofectamine 2000 or Polyethylenimine according to the  
814 manufacturer's instructions. Proteins were extracted 48-72 h after transfection and  
815 determined the IC<sub>50</sub> with dHNN by PC6 assay *in vitro*.

816 C117A-F: 5'-GTAGCCCAGGGTCTGGCC GACGCCATCCGC-3'

817 C117A-R: 5'-GCGGATGGCGTCGGCCAGACCCTGGGCTAC-3'

818 C199A-F: 5'-CATTCGGAGGAGACAGCC CAGAGGCTGGTG-3'

819 C199A-R: 5'-CACCAGCCTCTGGGCTGTCTCCTCCGAATG-3'

820 C215A-F: 5'-GCGGTGCTGTATTGGGCACGCCGCACGGAC-3'

821 C215A-R: 5'-GTCCGTGCGGCGTGCCCAATACAGCACCGC-3'

822 C226A-F: 5'-GCGCTGCTGCGCCACGCAGCGCTGGCGCTG-3'

823 C226A-R: 5'-CAGCGCCAGCGCTGCGTGGCGCAGCAGCGC-3'

824 C233A-F: 5'-CTGGCGCTGGGCAACGCAGCGCTGCACGGG-3'

825 C233A-R: 5'-CCCGTGCAGCGCTGCGTTGCCAGCGCCAG-3'

826 C271A-F: 5'-CTTCGGCTGCACGCCGCACTCGCAGTAGCG-3'

827 C271A-R: 5'-CGCTACTGCGAGTGCGGCGTGCAGCCGAAG-3'

828 C311A-F: 5'-GGCCGCTTCGCCCGCGCC CTGGTGGACGCC-3'

829 C311A-R: 5'-GGCGTCCACCAGGGCGCGGGCGAAGCGGCC-3'

830 C343A-F: 5'-CGCTTGGAGGCGCAGGCAATCGGGGCTTTC-3'

831 C343A-R: 5'-GAAAGCCCCGATTGCCTGCGCCTCCAAGCG-3'

832 C350A-F: 5'-GGGGCTTTCTACCTCGCAGCCGAGGCTGCC-3'

833 C350A-R: 5'-GGCAGCCTCGGCTGCGAGGTAGAAAGCCCC-3'

834 C430A-F: 5'-GGTTTCTCCAAGTACGCAGAGAGCTTCCGG-3'

835 C430A-R: 5'-CCGGAAGCTCTCTGCGTACTTGGAGAAACC-3'

836 C482A-F: 5'-GCCAACTATTCTACGGCC GACCGCAGCAAC-3'

837 C482A-R: 5'-GTTGCTGCGGTGCGCCGTAGAATAGTTGGC-3'

838 C508A-F: 5'-TACGGCCTGGTCAGCGCAGGCCTGGACCGC-3'

839 C508A-R: 5'-GCGGTCCAGGCCTGCGCTGACCAGGCCGTA-3'

840 C527A-F: 5'-CAGCTGCTGGAAGACGCAGGCATCCACCTG-3'

841 C527A-R: 5'-CAGGTGGATGCCTGCGTCTTCCAGCAGCTG-3'

842 C552A-F: 5'-CACTCCCCGCTGCCCGCAACTGGTGGCAAAC-3'

843 C552A-R: 5'-GTTTGCCACCAGTTGCGGGCAGCGGGGAGTG-3'

844 C629A-F: 5'-GGAGCACTGGACAAGGCAATGCAAGACCAT-3'

845 C629A-R: 5'-ATGGTCTTGCATTGCCTTGTCCAGTGCTCC-3'

846 C635A-F: 5'-ATGCAAGACCATGACGCAAAGGATTGGGTG-3'

847 C635A-R: 5'-CACCCAATCCTTTGCGTCATGGTCTTGCAT-3'

848 C649A-F: 5'-GTGACTGCTTTAAGCGCC GGCAAGAACATT-3'

849 C649A-R: 5'-AATGTTCTTGCCGGCGCTTAAAGCAGTCAC-3'

850 dtSARM1-dN-F: 5'-CAGTCTAGAATGGACTACAAGGATGACGATG-3'

851 dtSARM1-dN-R: 5'-ATAGCGGCCGCTTAGGTTGGACCCA-3'

852 **Western blots**

853 Cells was harvested and lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1  
854 mM EDTA and 0.05% triton, pH 7.4). Each sample was loaded onto 10-12% SDS-PAGE  
855 gels and transferred to PVDF membranes after electrophoresis. The membranes were  
856 blocked with 5% Milk and blotted with anti-SARM1 (produced as described previously)  
857 and anti-Tubulin antibody (TransGen Biotech) to control the loading.

858 **CryoEM sample preparation, data collection and processing**

859 SARM1-dN tagged with strep-tag II and flag-tag was immunoprecipitated with  
860 StrepTactin resin (GE healthcare), washed with buffer W (100 mM Tris-HCl pH8.0, 150  
861 mM NaCl and 1 mM EDTA) for four times and eluted with 2 mM biotin in buffer W. The  
862 eluent was concentrated to 3 mg/ml and pre-incubated with 50  $\mu$ M dHNN at RT for 10 min.

863 SARM1-dHNN protein was applied to glow-discharged gold grid, blotted in FEI  
864 Vitrobot Mark IV (Thermo Fisher Scientific) before frozen by liquid ethane and stored in  
865 liquid nitrogen. The sample without inhibitor was examined at the Cryo-EM center of  
866 Chinese University Hong Kong (Shenzhen) on a 300kV Titan Krios (Thermo Fisher  
867 Scientific) equipped with Gatan K3 direct electron detector under magnification of  
868 105,000x, with the corresponding pixel size of 0.85 $\text{\AA}$ . The dose rate was set to 17.6  
869 e/pix/s and exposure time was set to 2.5s to obtain 50 frames, which led to an  
870 accumulated dose of 61 electrons per  $\text{\AA}^2$ . The total dataset consists of 2,692 raw movies  
871 with a defocus value range of -1.0 to -2.0  $\mu$ m. Motion correction and CTF parameter  
872 estimation were performed with cryoSPARC (Punjani et al., 2017). 2,012,198 particles  
873 were autopicked. After several rounds of 2D classification, 712,139 particles were

874 selected for generation of the final 2D average results.

875 The dHNN-treated sample was examined at the Cryo-EM center of Southern  
876 University of Science and Technology on a Titan Krios G3 (Thermo Fisher Scientific) with  
877 Gatan K2 summit detector with a nominal magnification of 130,000X and corresponding  
878 pixel size of 1.076 Å. A total accumulative dose of 50 e<sup>-</sup>/Å<sup>2</sup> was set for each exposure and  
879 split into 39 frames during data acquisition. The defocus range was set between -0.8 to  
880 -2.0 μm. In total, 2,890 images were collected. Motion correction and CTF parameter  
881 estimation were performed with MotionCor2 and CTFFind4 built within Relion  
882 3.1(Fernandez-Leiro et al., 2017). After CTF estimation, images with thick ice, obvious  
883 shift or cleft were removed, which left 2673 images for further processing. 2,655,835  
884 particles were autopicked from these images. After several rounds of 2D classification,  
885 700,472 particles were selected and exported for generation of the final 2D average  
886 results with CryoSparc and 3D refinement with CisTEM beta-1.0.0 (Grant et al., 2018).  
887 The particle stack was subject for 10 rounds of 3D auto-refinement among 6 classes using  
888 6WPK as initial model. Four classes with higher estimated resolution were selected and  
889 combined for 20 more rounds of 3D manual global refinement and one class with the  
890 highest occupation (62.5%) and best resolution was chosen for several more rounds of 2D  
891 and 3D classification with Relion 3.1 and CisTEM beta-1.0.0. The resolution for the final  
892 map was around 2.4 Å..

893 The previously reported structures of the SARM1 SAM domain (PDB: 6O0S) and TIR  
894 domain (PDB: 6O0Q) were used as model templates during initial model building. The  
895 initial model of ARM domain was built de novo in Coot (Emsley et al., 2010). The three

896 domains of SARM1 were connected in Coot and docked into density maps using Dock in  
897 Map module of Phenix 1.16 (Adams et al., 2010) with C8 symmetry and then subjected to  
898 multiple rounds of Real-space refinement in Phenix. The dHNN molecule was built and  
899 fitted into the density around Cys311 initial model in Coot. The final models were validated  
900 with Comprehensive Validation module of Phenix and the refinement statistics were listed  
901 in Supplementary file 1. The model and EM map have been deposited in Protein Data  
902 Bank with accession codes of PDB ID 7DJT and EMD-30700.

### 903 **Data analysis.**

904 All experiments contained at least three biological replicates. Data shown in each  
905 figure are all means  $\pm$  SD. The unpaired Student's t-test was used to determine statistical  
906 significance of differences between means (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001,  
907 \*\*\*\*P<0.0001). GraphPad Prism 7 was used for data analysis.

908

### 909 **ACKNOWLEDGMENTS**

910 We would like to thank the Cryo-EM center of Southern University of Science and  
911 Technology for Cryo-EM data collection and the HPC-Service Station in Cryo-EM  
912 center of Southern University of Science and Technology for data processing. We  
913 acknowledge Beijing Artivila Biopharma Co. Ltd for the supports.

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### 916 **References**

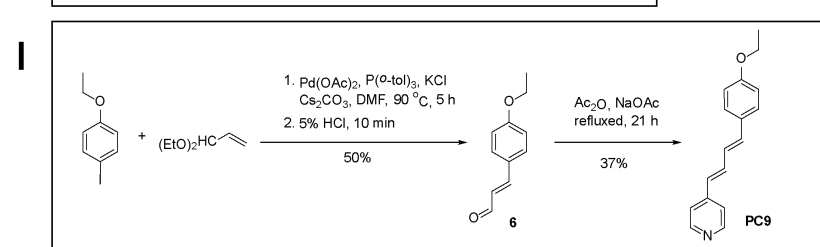
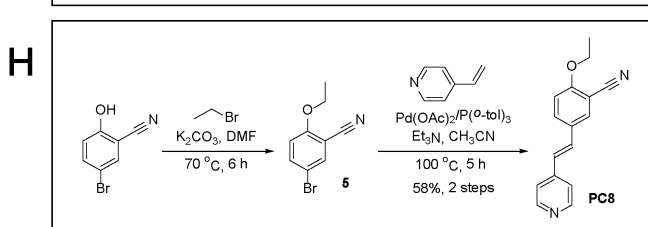
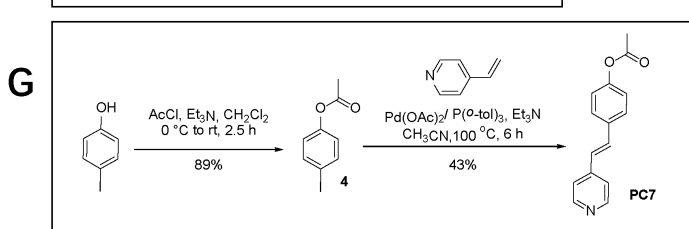
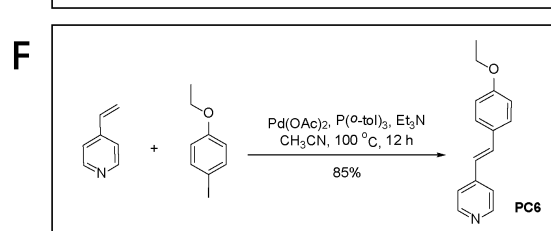
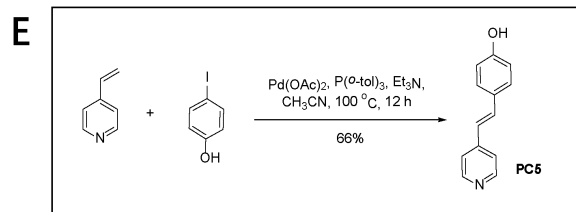
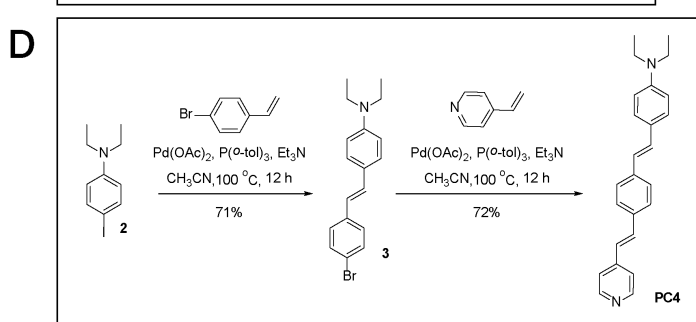
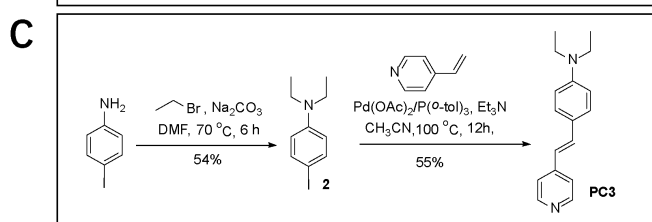
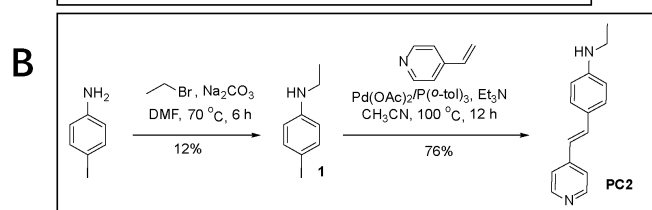
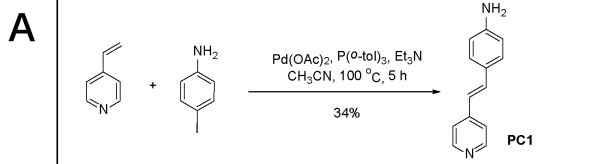
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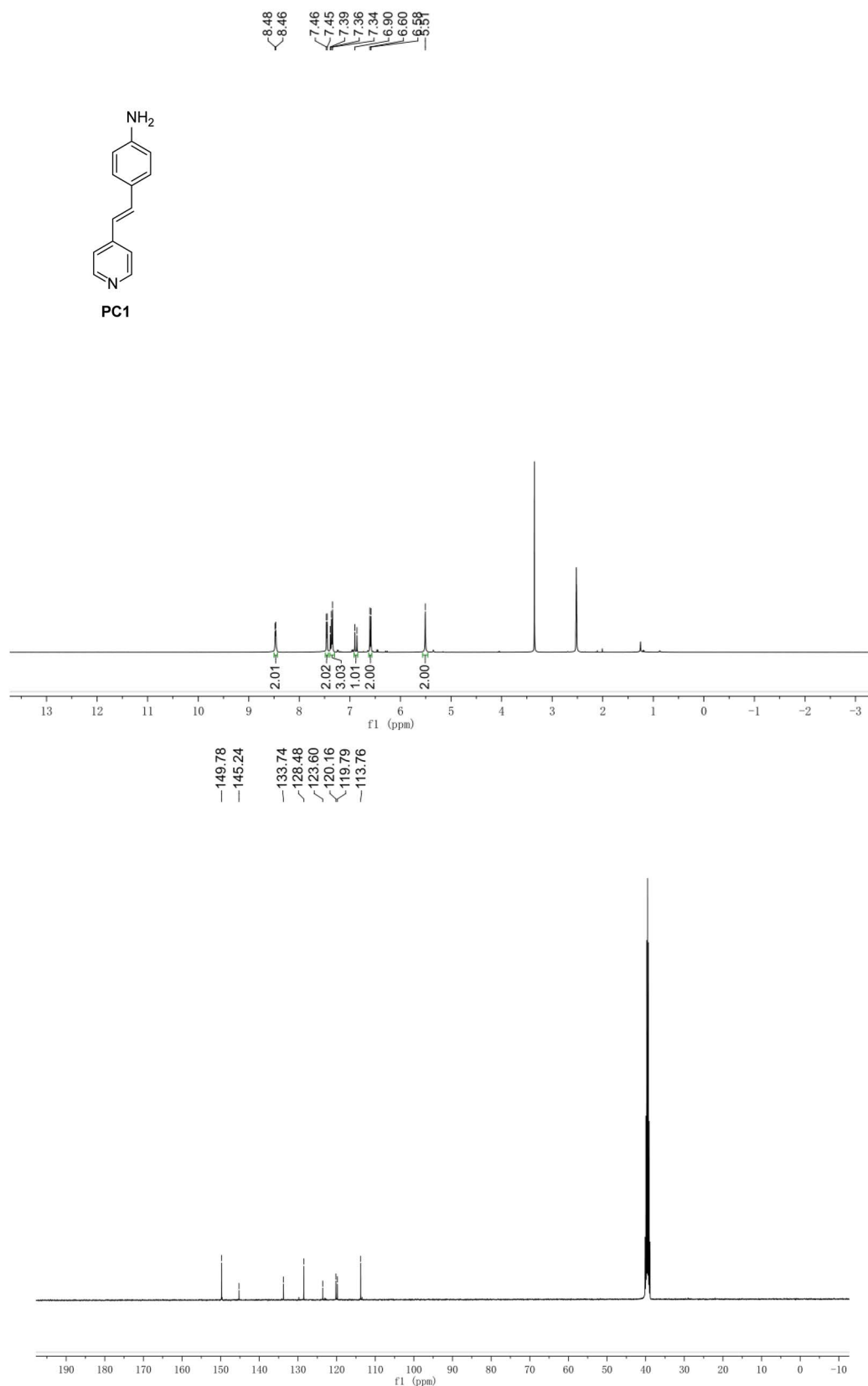
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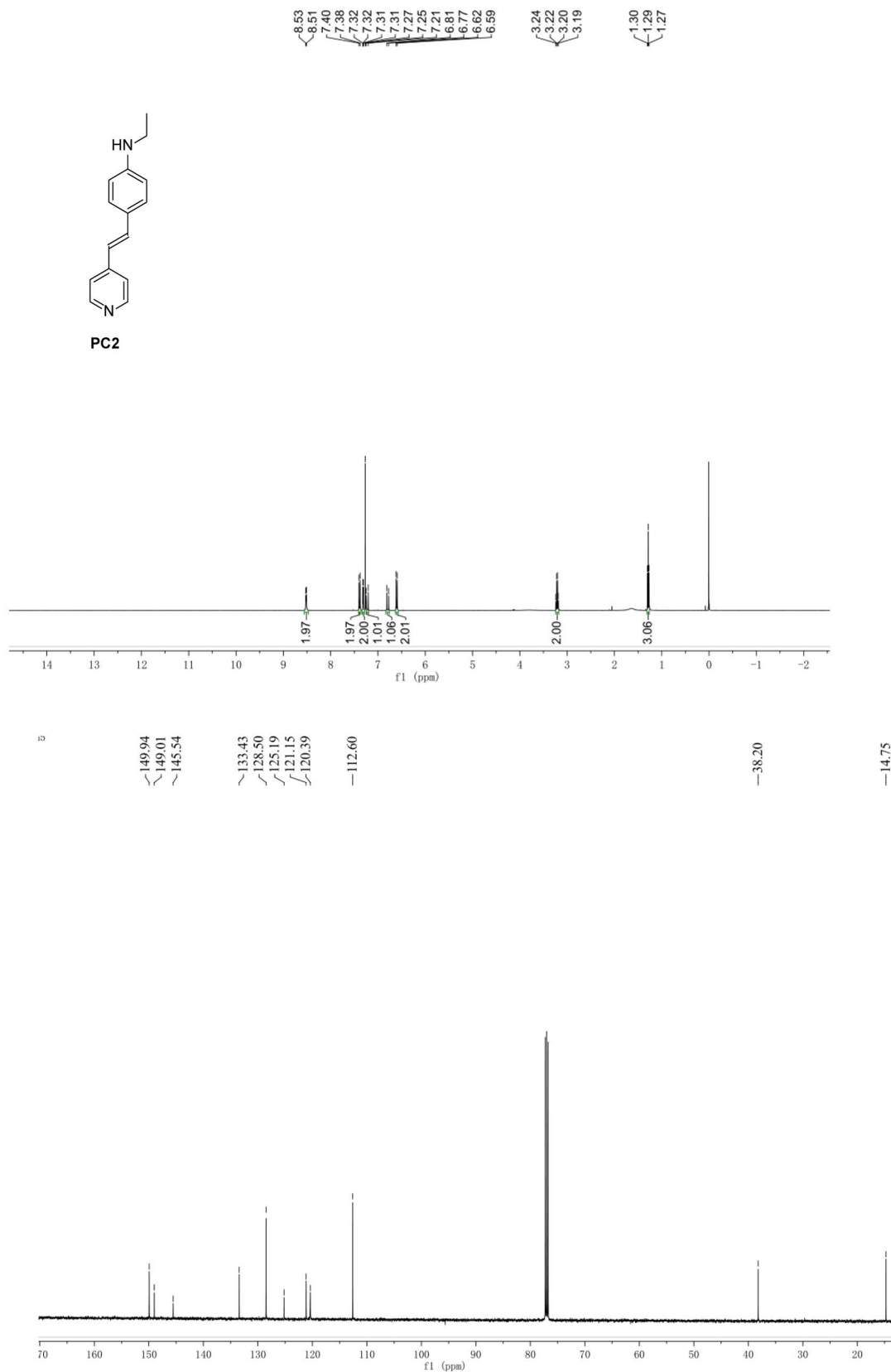
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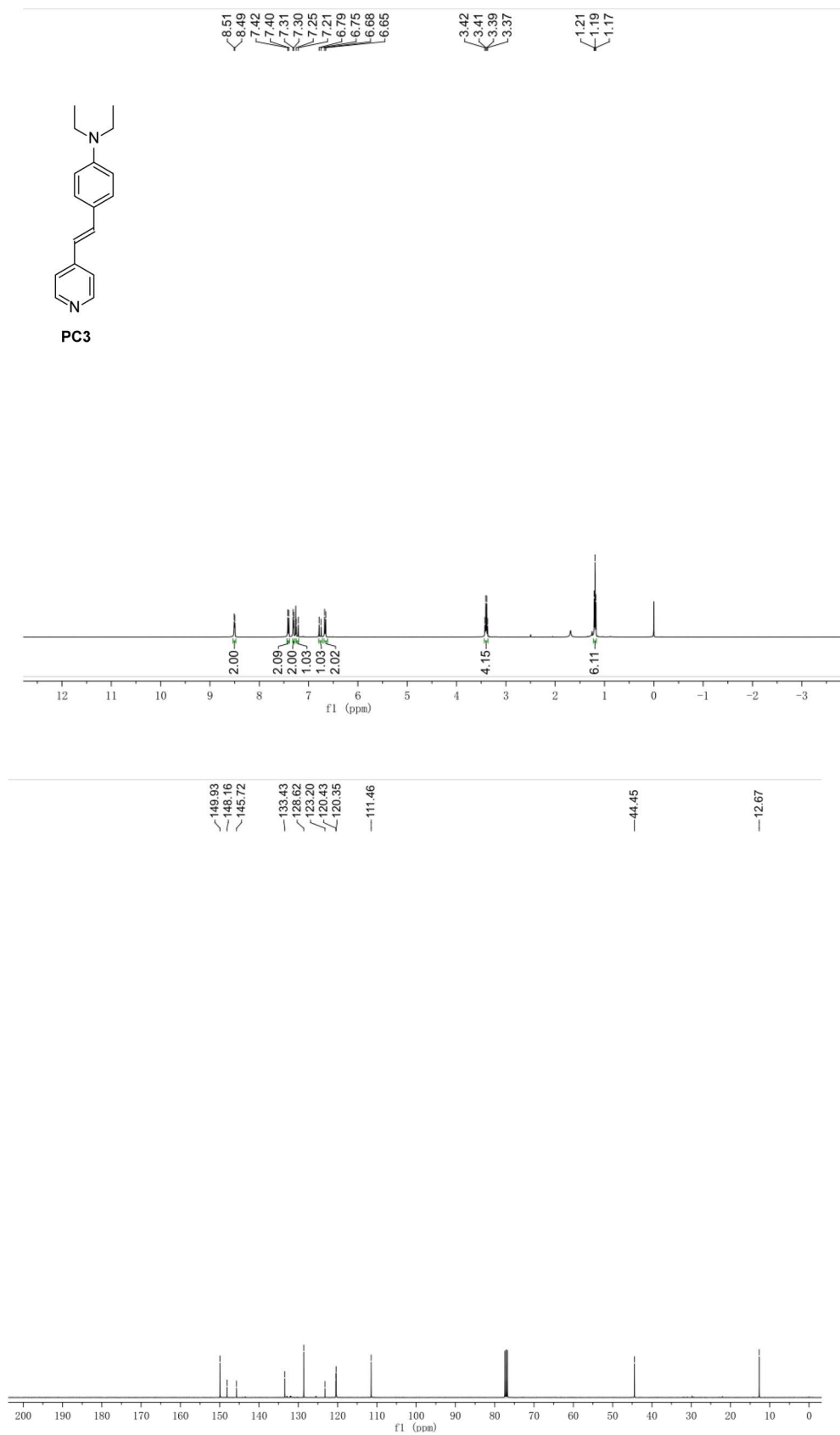
## NMR spectra of PCs



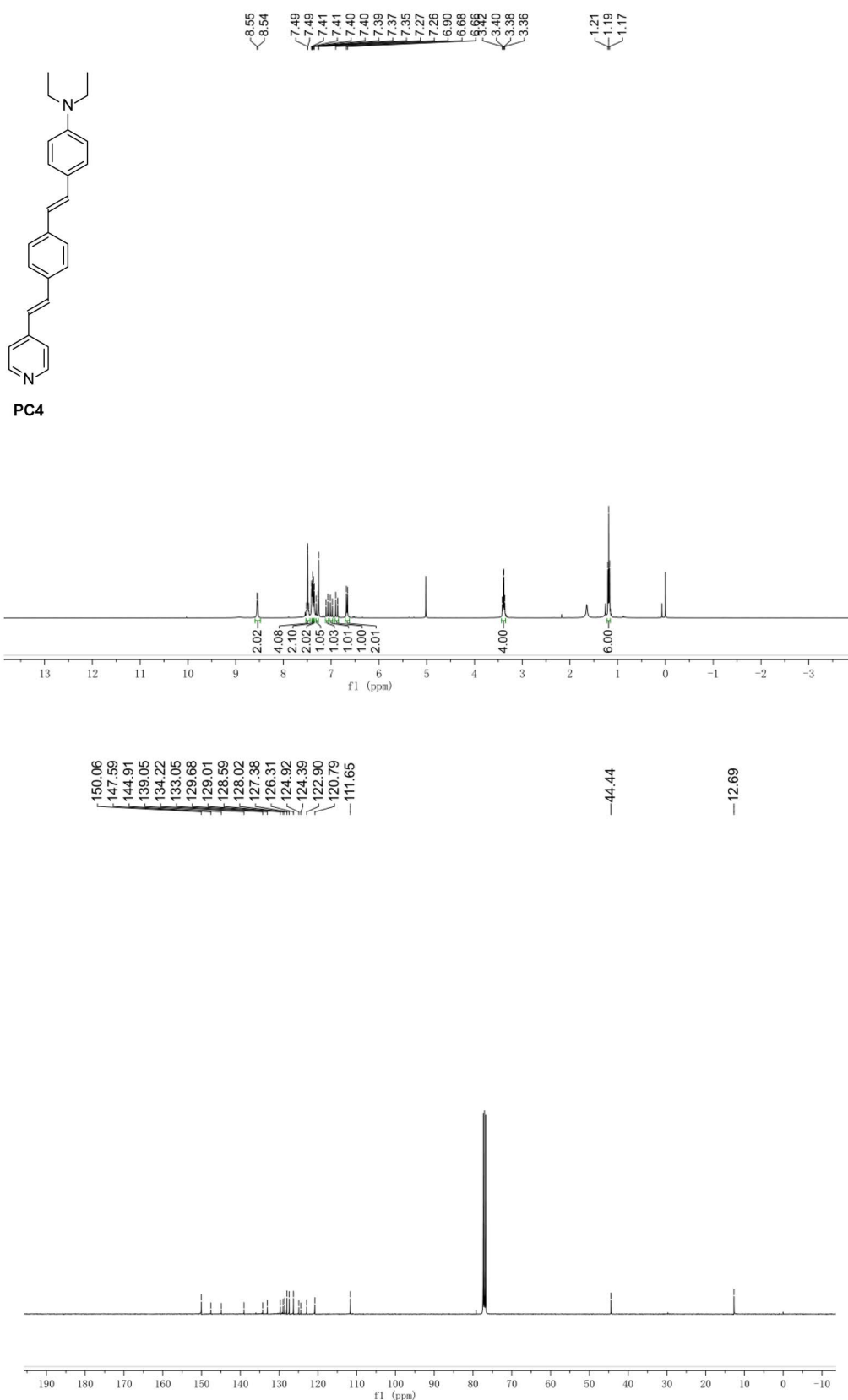
(A) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of PC1 in DMSO-*d*<sub>6</sub>



(B) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of **PC2** in CDCl<sub>3</sub>

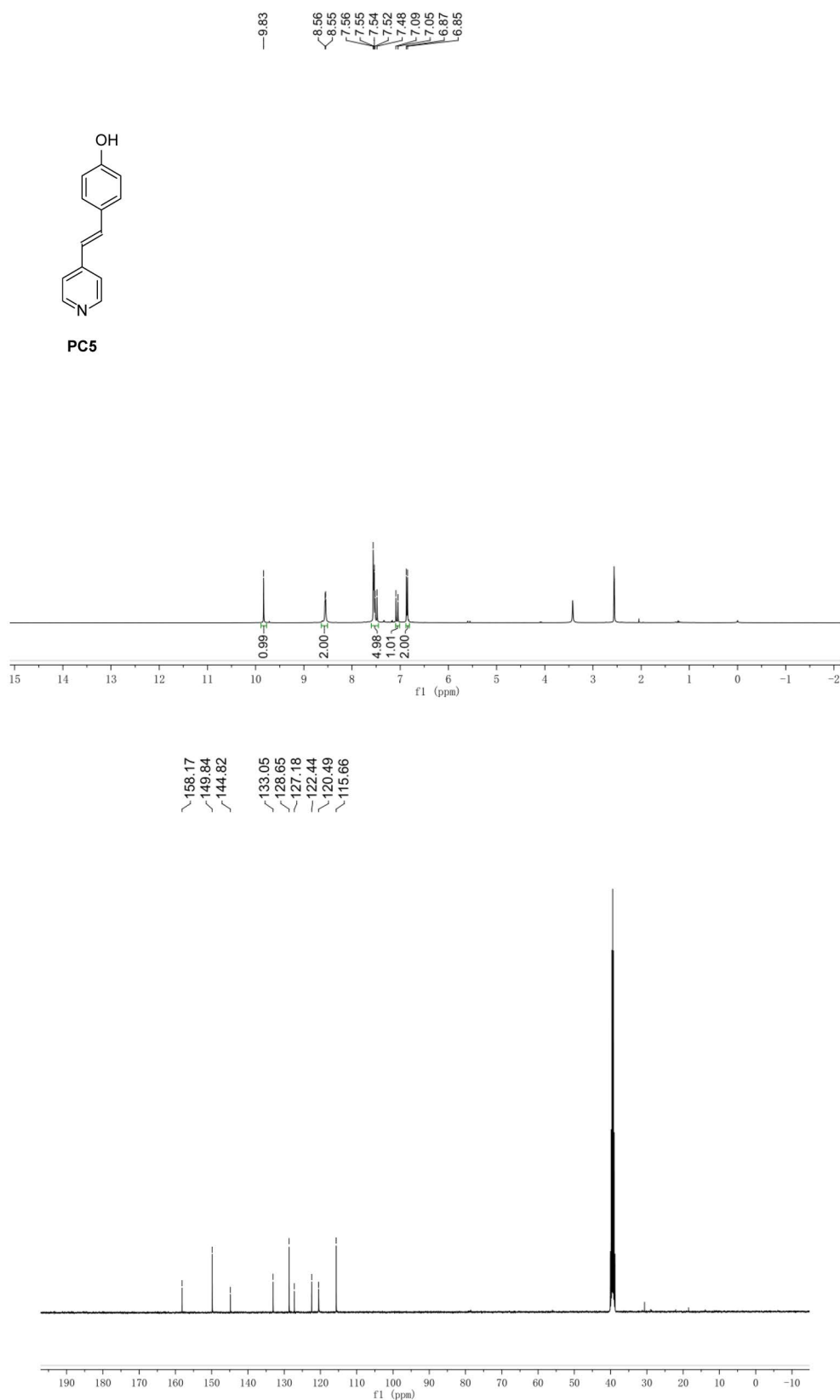


(C) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of **PC3** in CDCl<sub>3</sub>

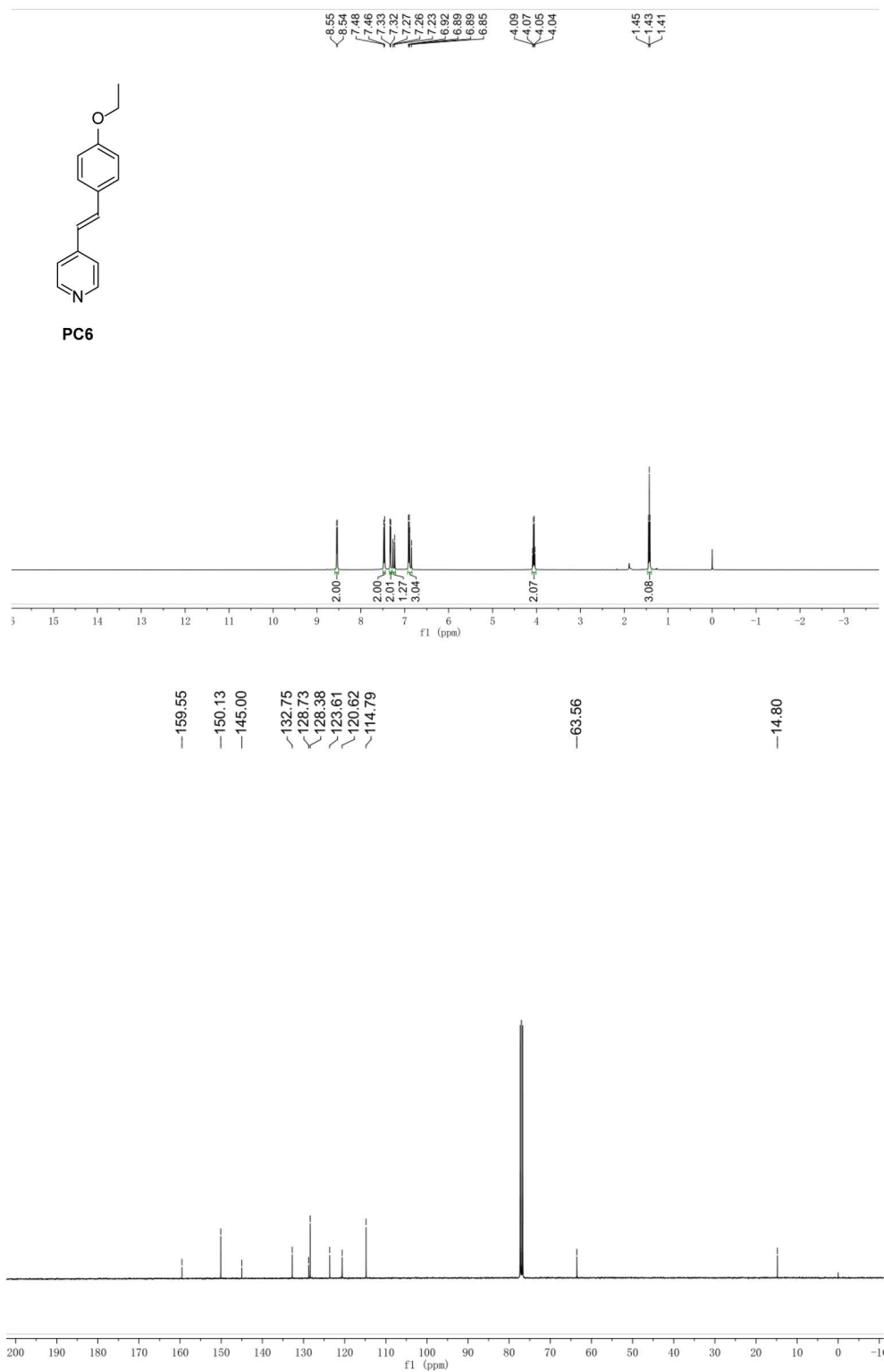


(D) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of **PC4** in CDCl<sub>3</sub>

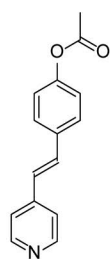




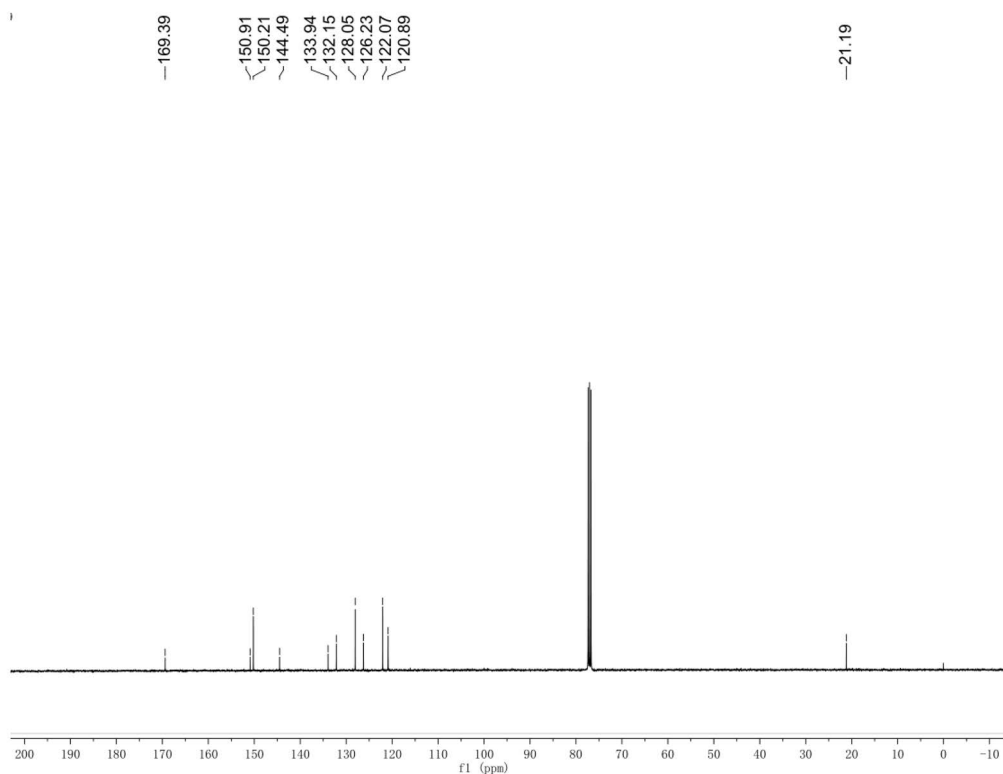
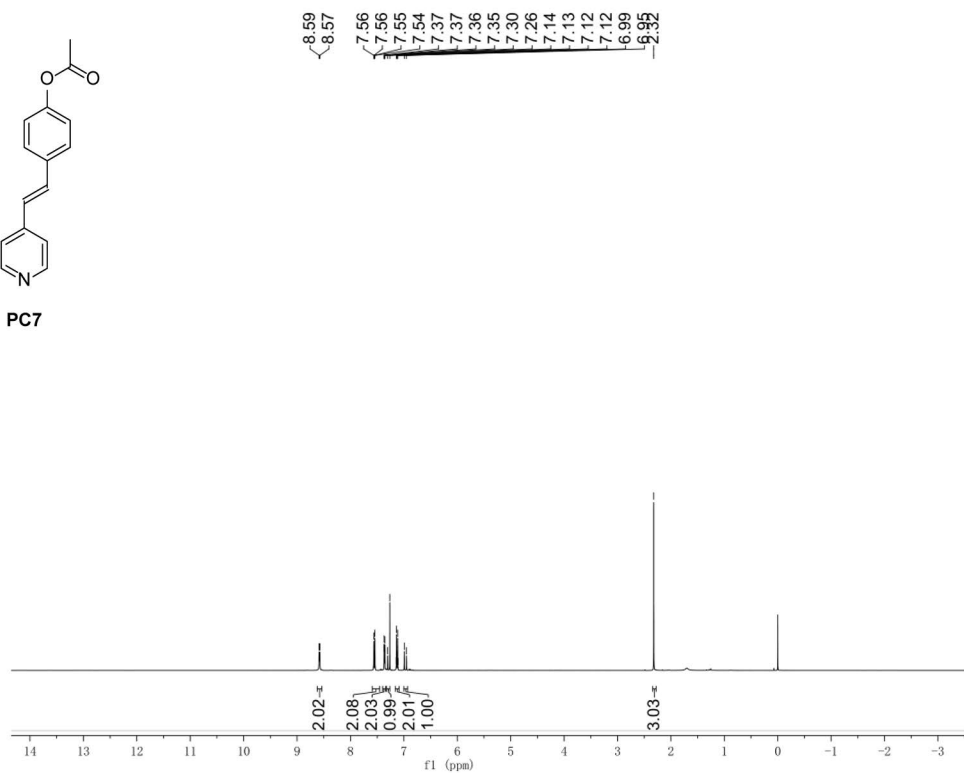
(E) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of **PC5** in DMSO-*d*<sub>6</sub>



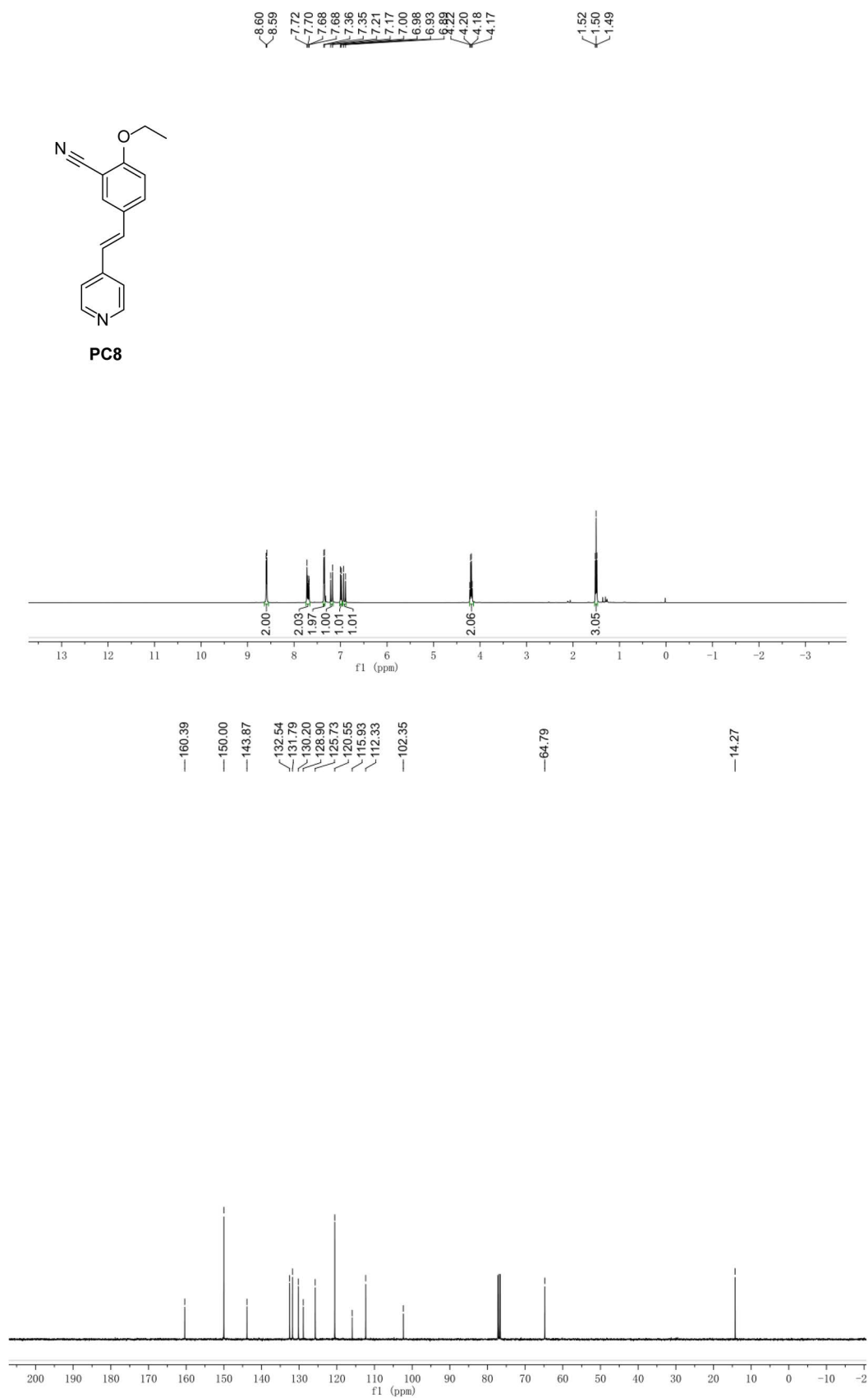
(F)  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of **PC6** in  $\text{CDCl}_3$



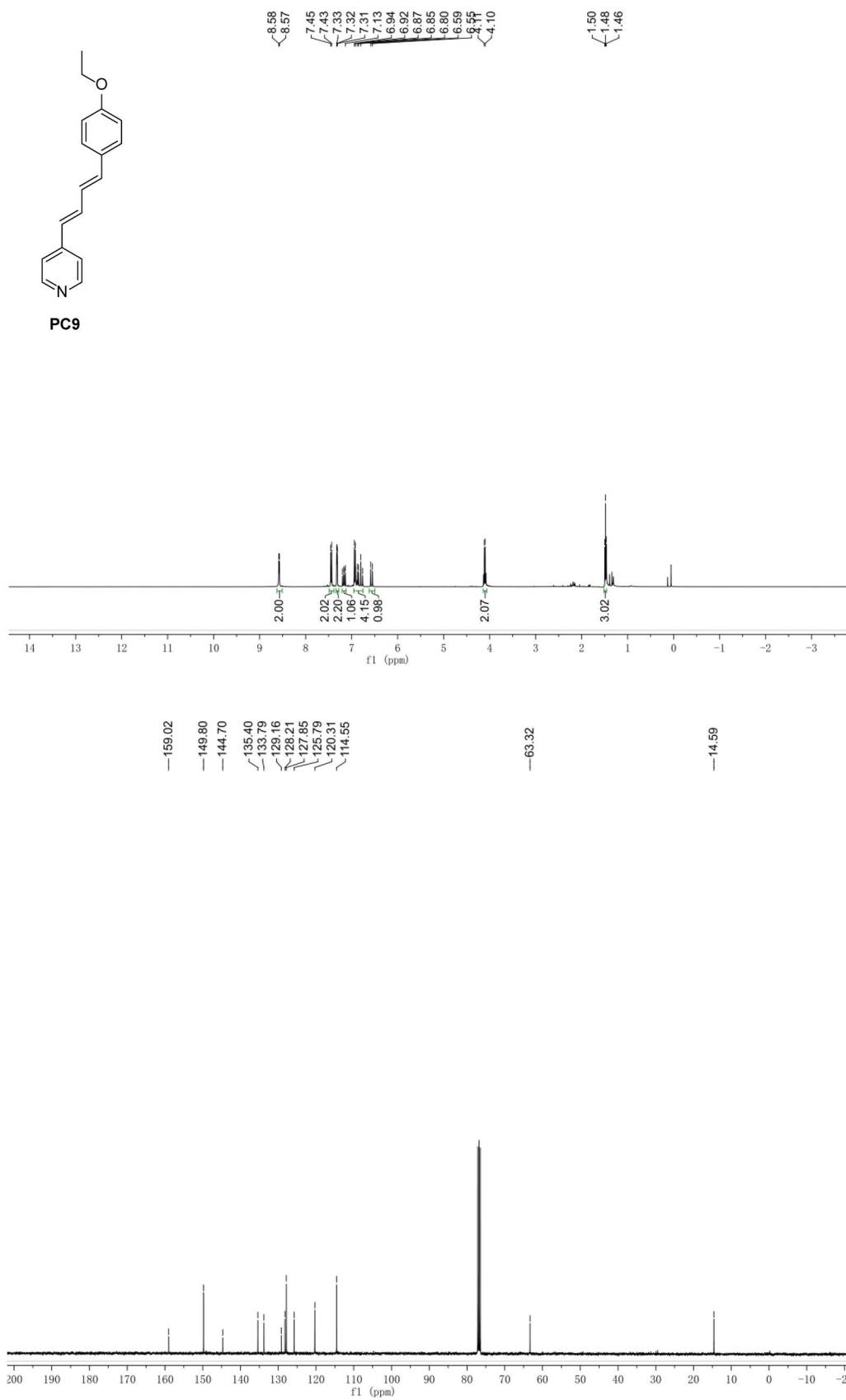
PC7



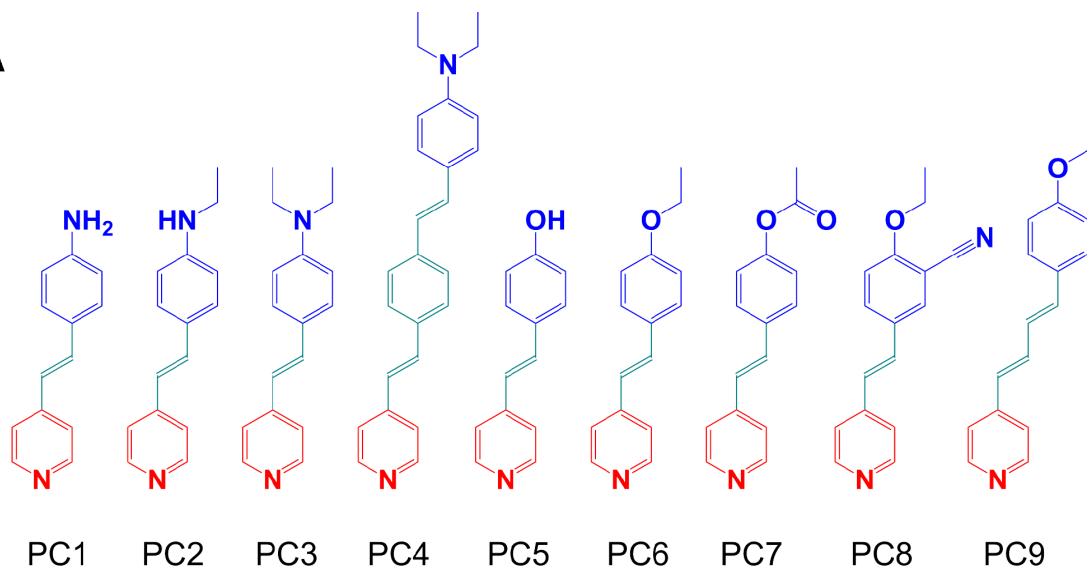
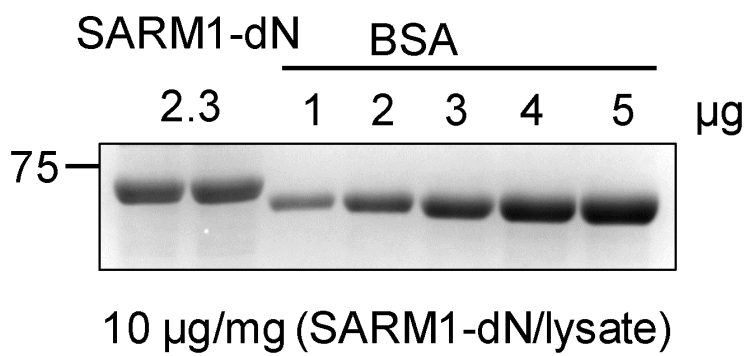
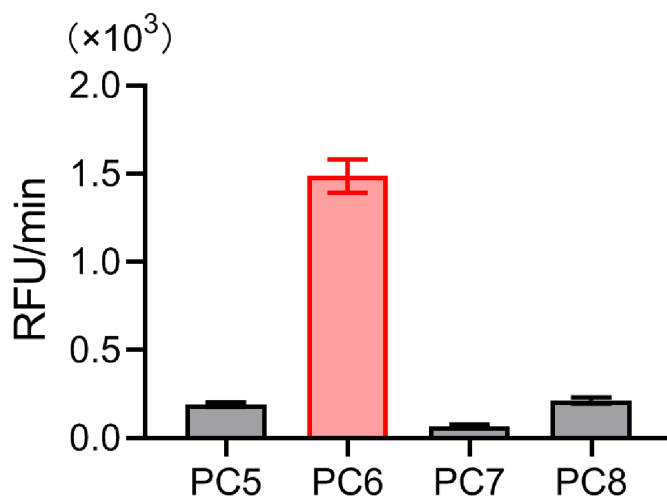
(G) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of PC7 in CDCl<sub>3</sub>

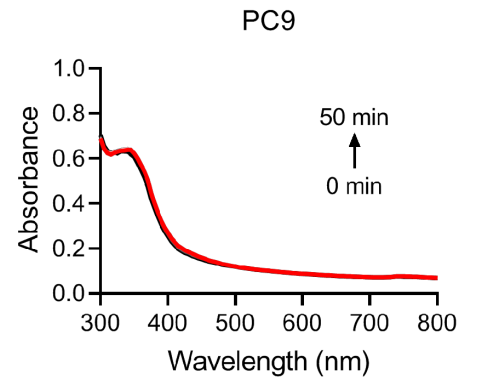
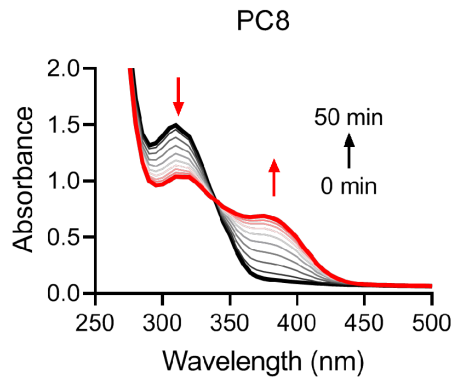
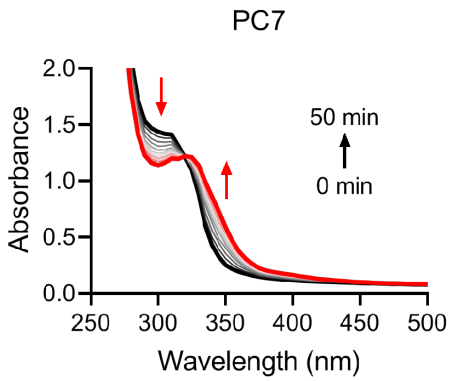
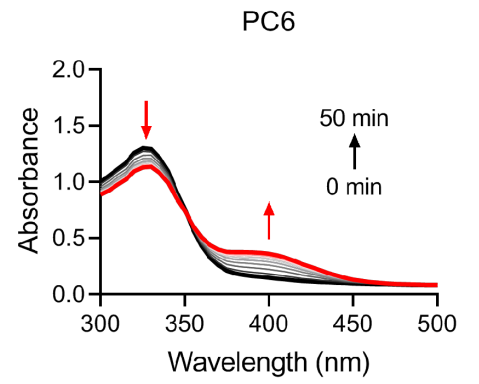
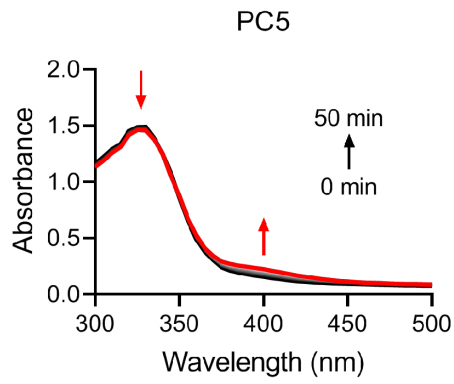
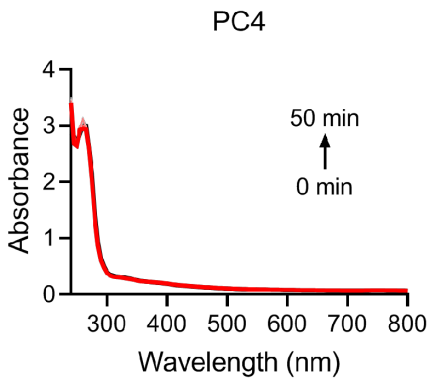
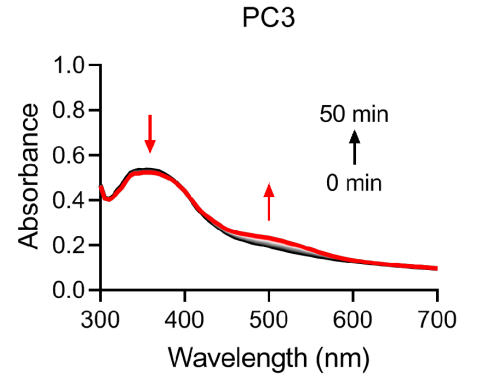
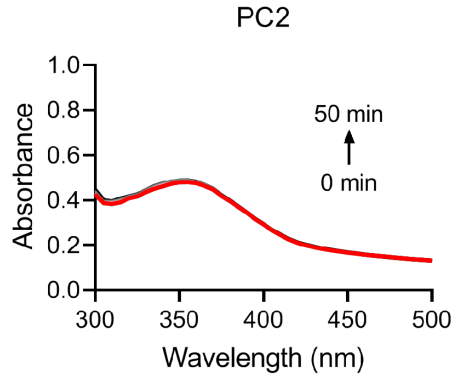
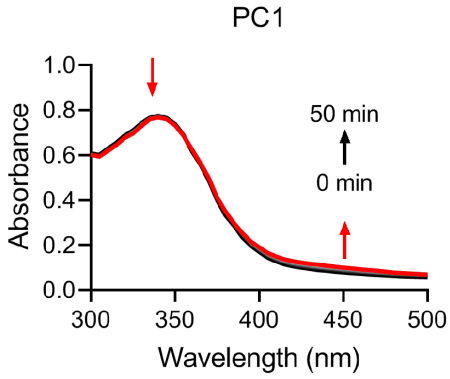


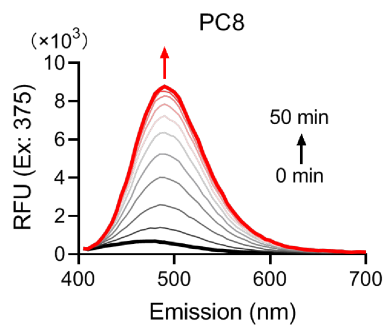
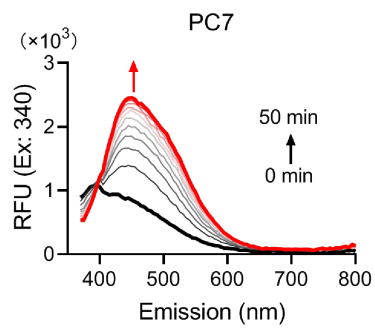
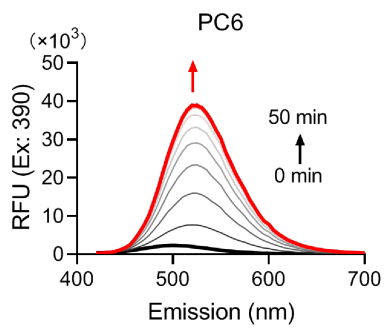
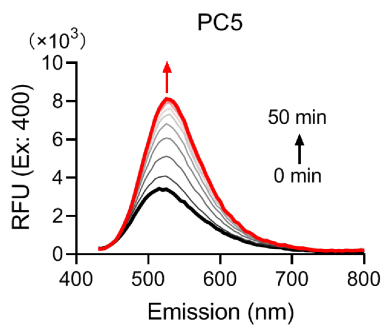
(H) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of **PC8** in CDCl<sub>3</sub>



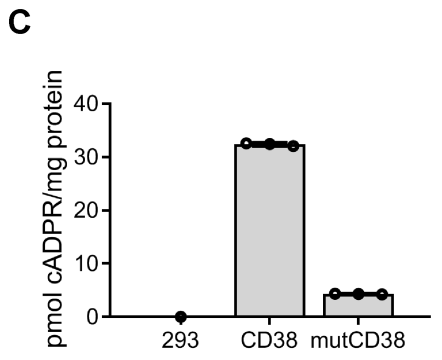
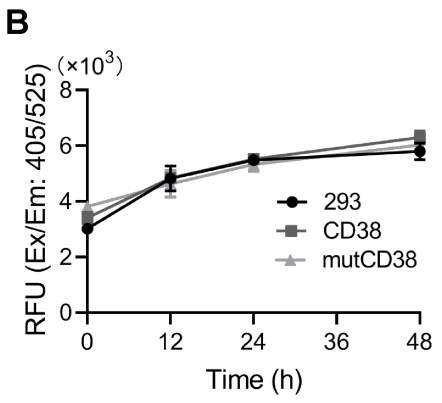
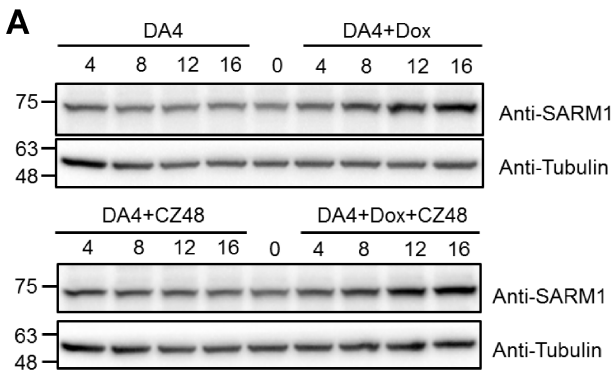
(I) <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of **PC9** in CDCl<sub>3</sub>

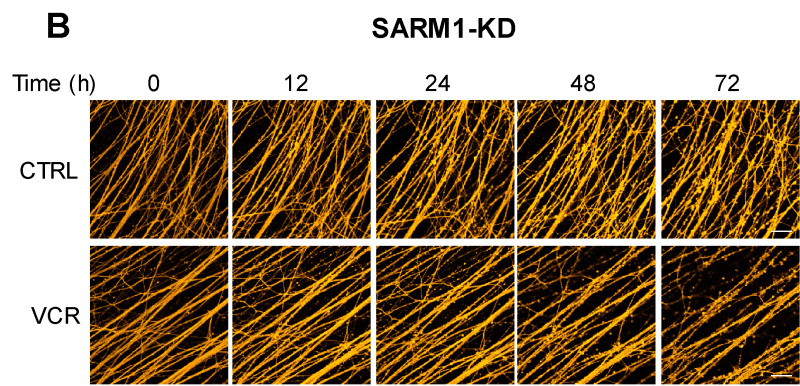
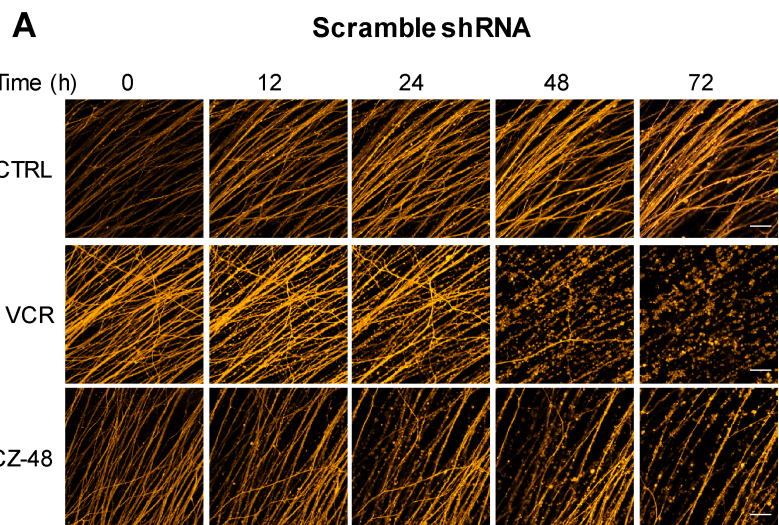
**A****B****C**

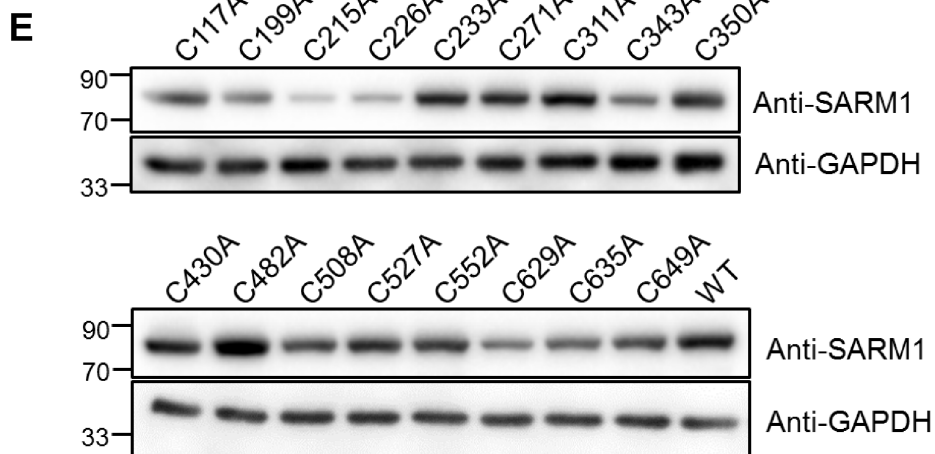
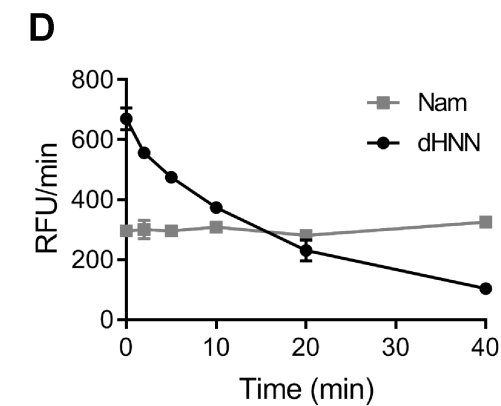
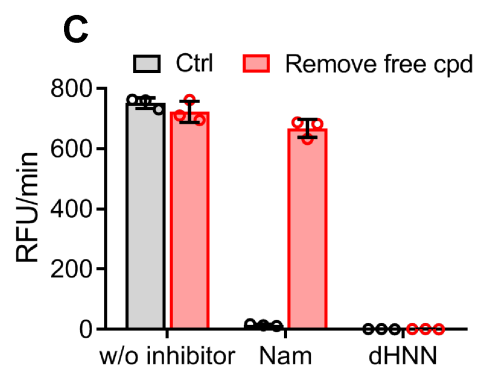
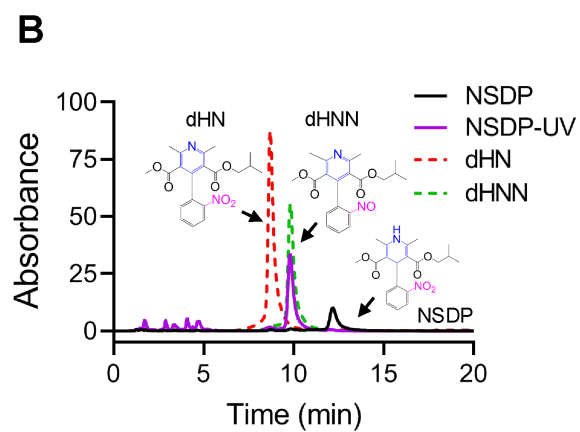
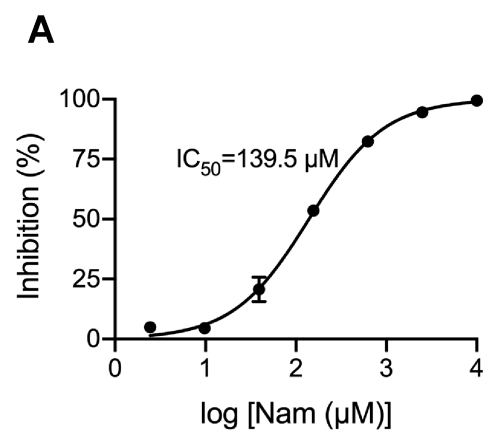


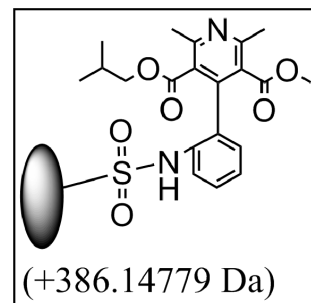
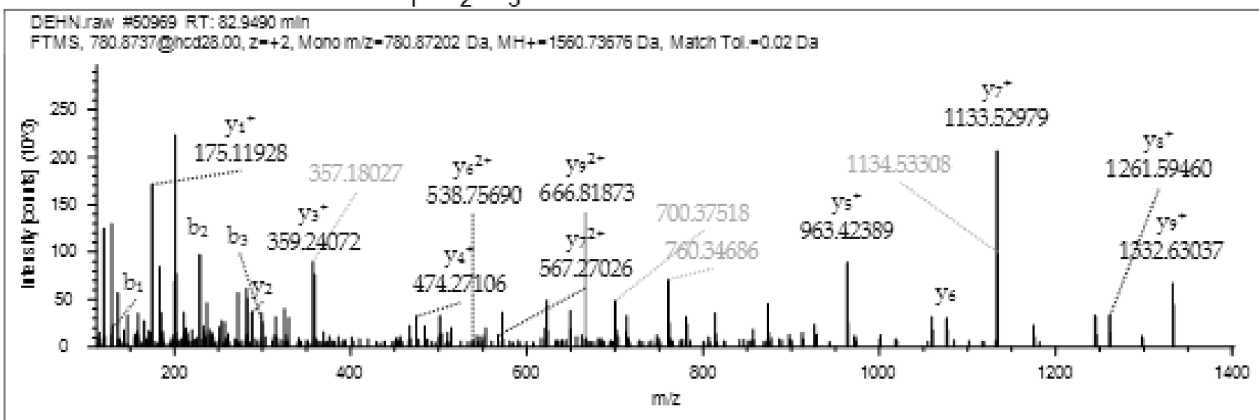
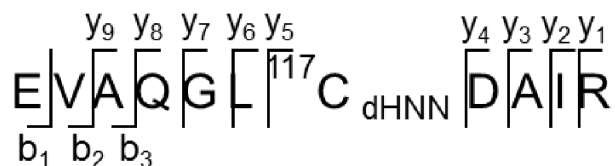
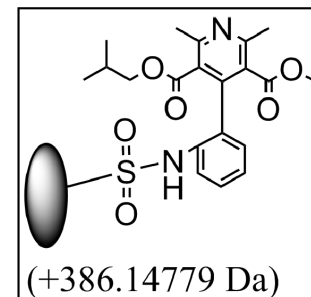
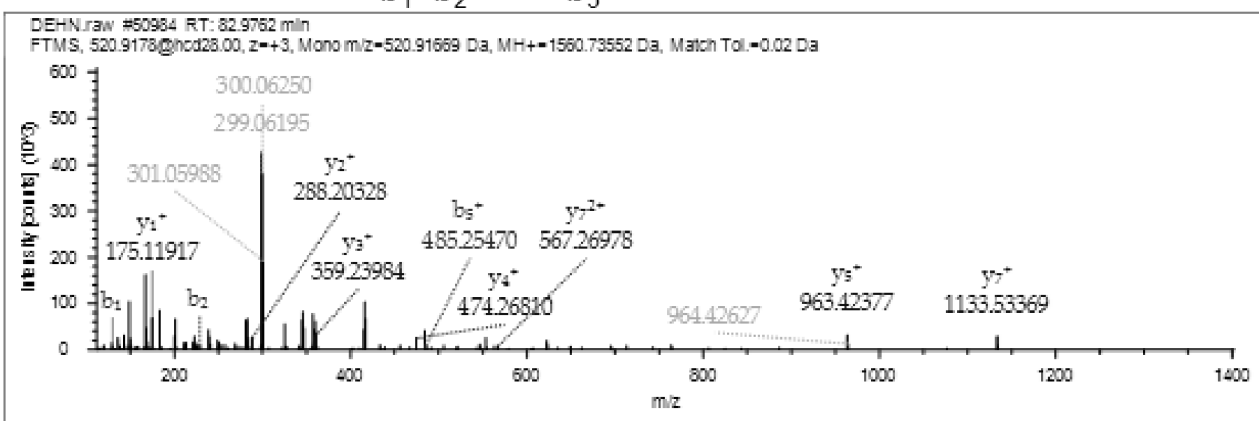
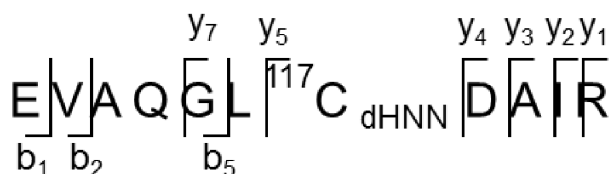
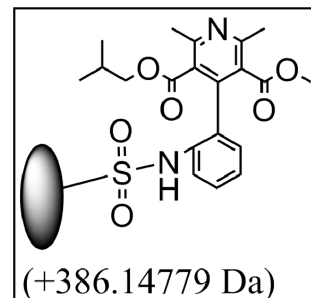
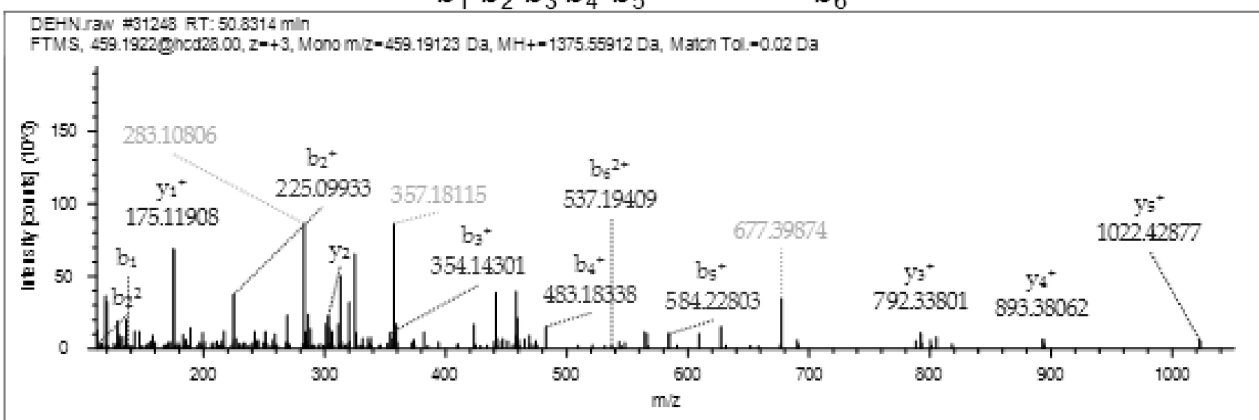
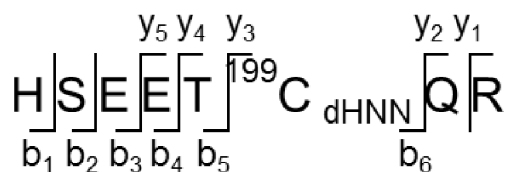


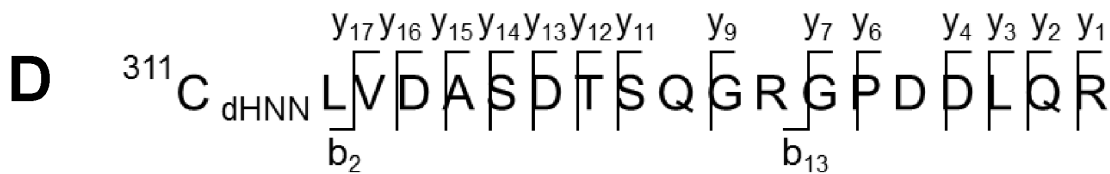




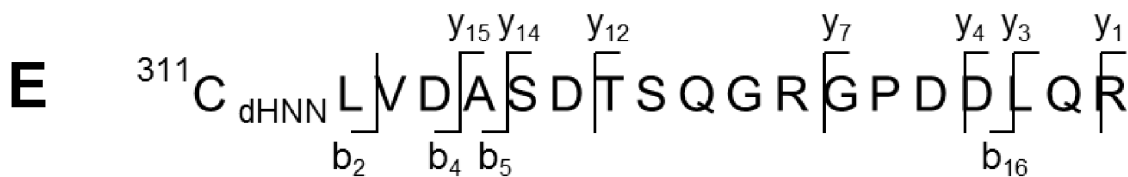
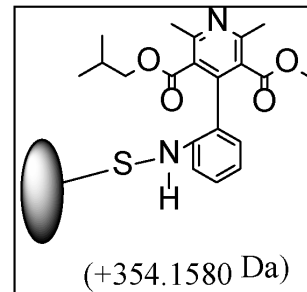
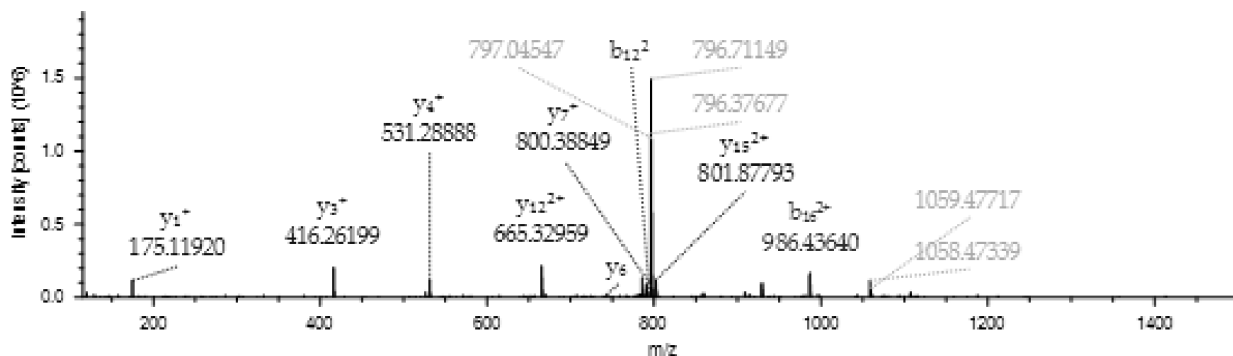




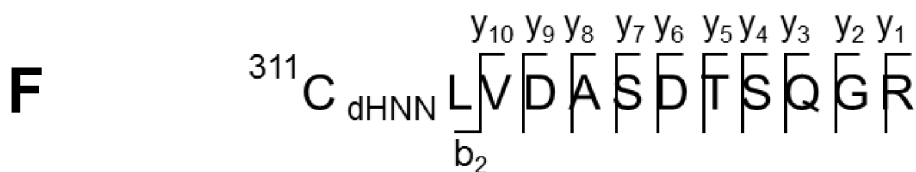
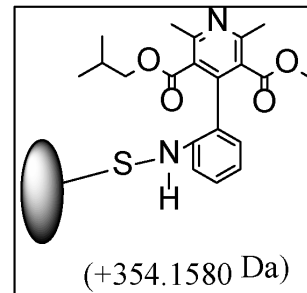
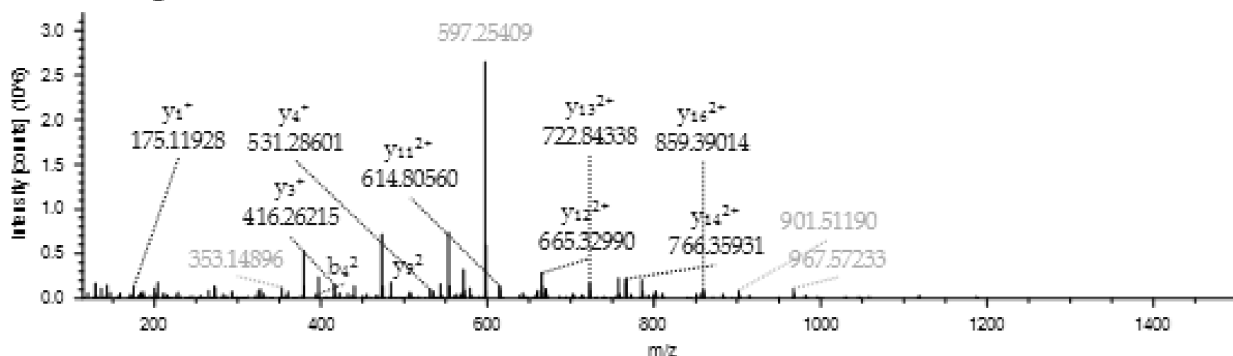
**A****B****C**



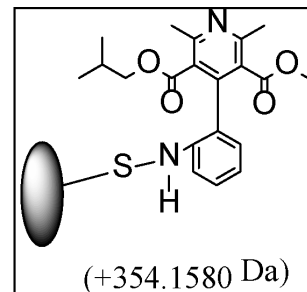
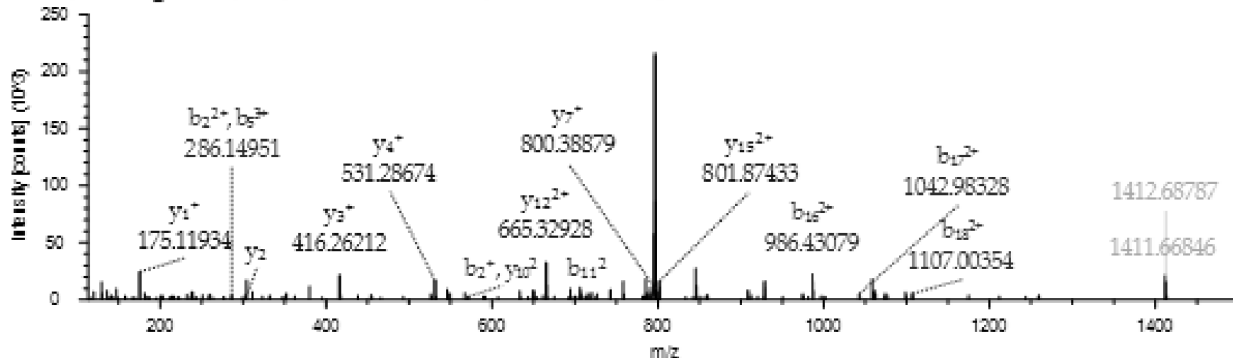
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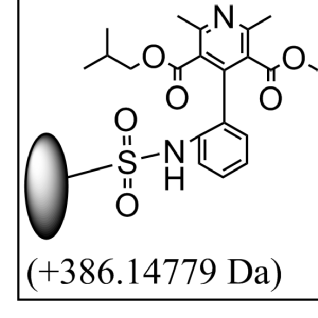
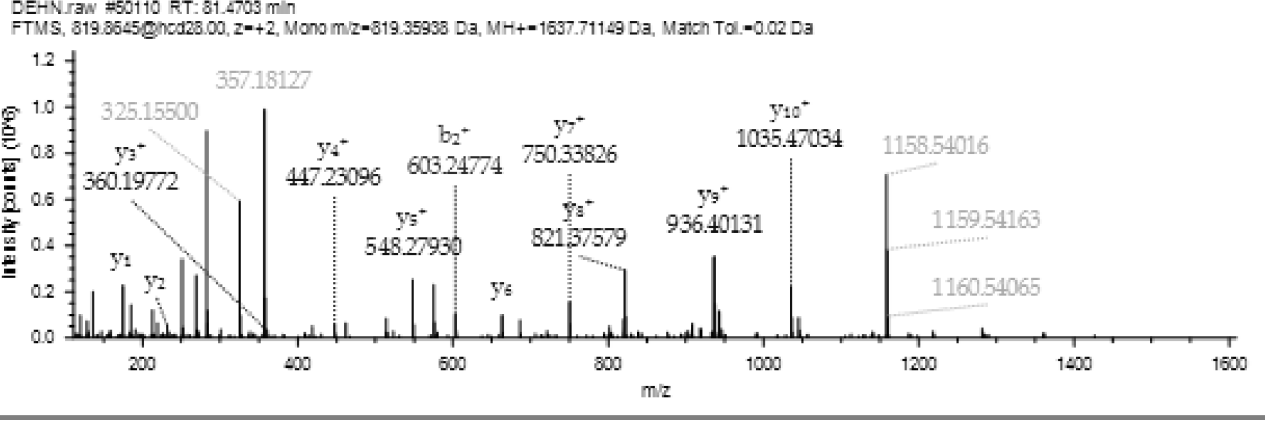
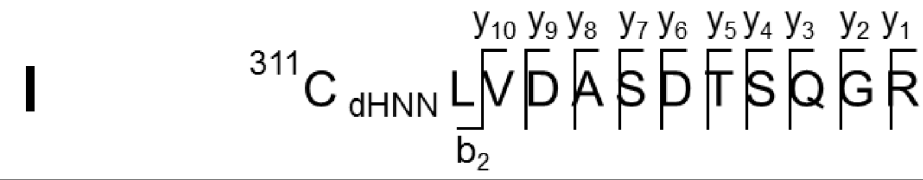
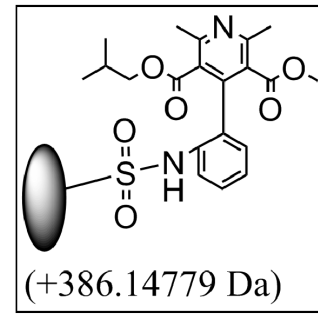
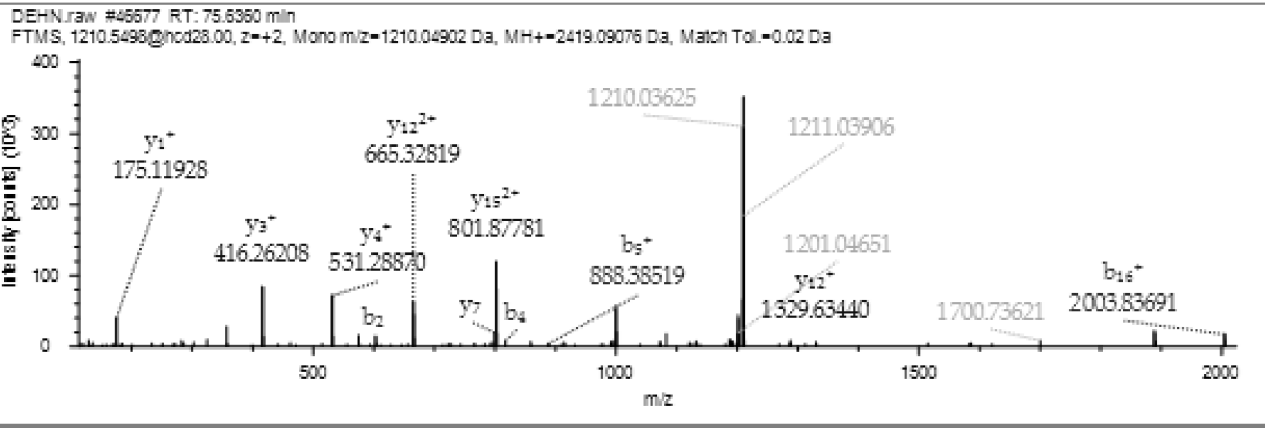
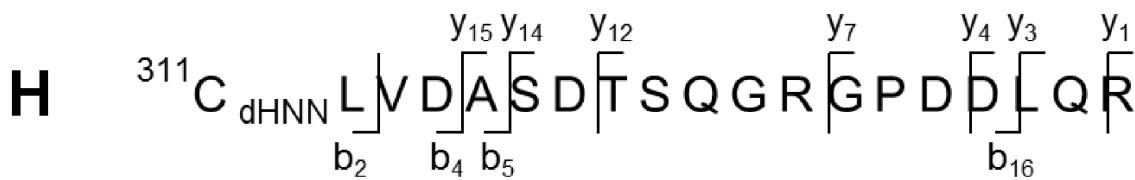
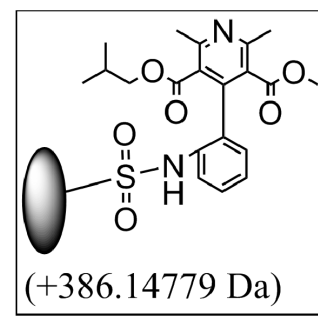
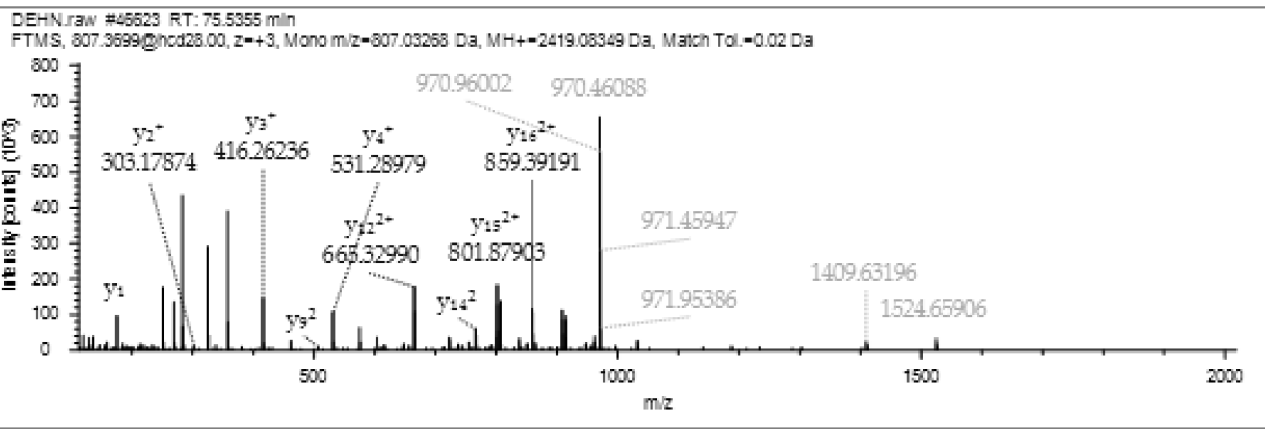
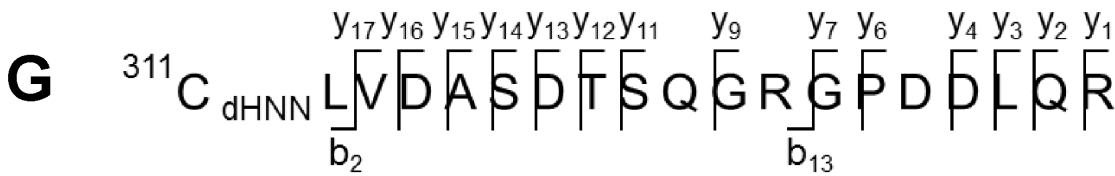


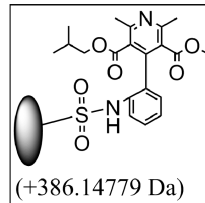
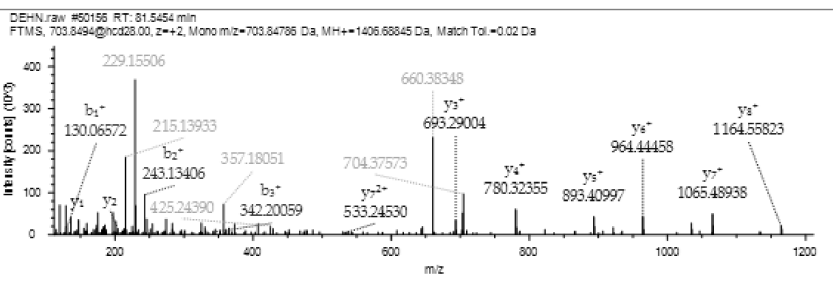
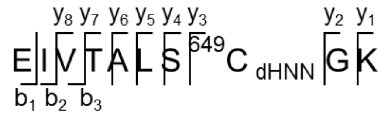
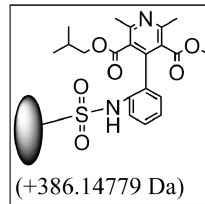
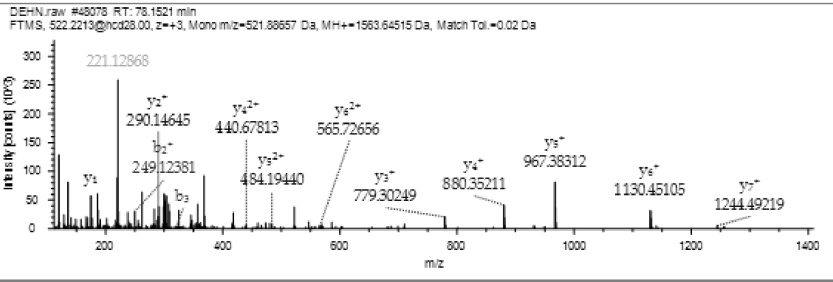
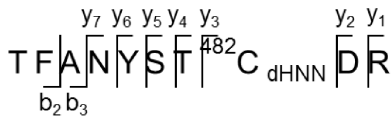
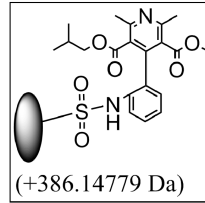
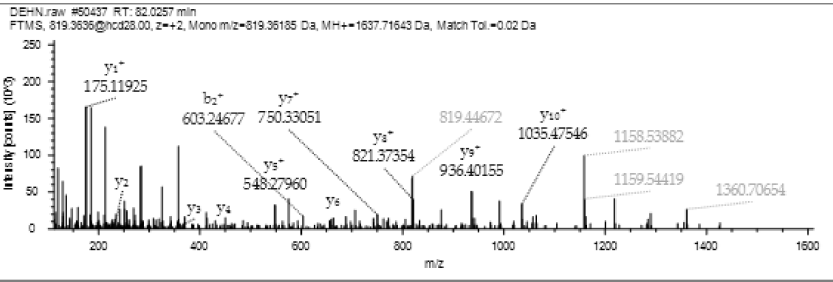
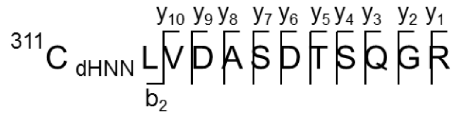
DEHN.raw #23105 RT: 38.3185 min  
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DMSO.raw #21631 RT: 38.3447 min  
FTMS, 796.3718@hcd28.00, z=+3, Mono m/z=796.37058 Da, MH+=2387.09720 Da, Match Tol.=0.02 Da







**M**

Peptide containing cysteine

