Supplemental Information for

α-Synuclein as a Target for Metallo-Anti-Neurodegenerative Agents

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3. Supplemental References

1. Supplemental Experimental Procedures

Materials

Phospholipids, 3,3′-diaminobenzidine, apomorphine (APO) and chloral hydrate were purchased from Sigma-Aldrich (St. Louis, MO). NAMI-A was synthesized according to a literature method.[1] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), thioflavin T (ThT) and reactive oxygen species assay kit were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). BCA protein assay kit and FITC were bought from Beyotime (Shanghai, China). $^{15}NHCl_4$ and D_2O were purchased from Cambridge Isotope Laboratories. DMEM Medium, trypsin–EDTA and fetal bovine serum (FBS) were obtained from Biological Industries (Kibbutz Beit-Haemek, Israel). The model septapeptides from the N-terminus of α-syn (MDVFMKG, α-syn_7) and N-acetylated septapeptide (AcMDVFMKG, Ac-syn_7) were synthesized by GenScript (Nanjing, China). Anti-α-syn and anti-tyrosine hydroxylase antibodies (TH) were obtained from Abcam (Cambridge, US). All of these chemicals were used without further purification. Ultra-purified water was prepared using a Milli-Q Synthesis System (Millipore, Bedford, MA).

Protein Expression and Purification

The cDNA fragment encoding the full-length α-synuclein (α-syn) was obtained by gene synthesis and amplified by polymerase chain reaction (PCR). The PCR product was inserted into a pST-GB1 vector using the ligation-independent cloning (LIC) method. The pST-GB1 vector encodes an N-terminal His_6-tag followed by GB1, a solubility enhancement tag. A TEV cleavage site was inserted between the GB1-tag and the target protein. The plasmid was transformed into BL21 (DE3) competent cells for the overexpression of fusion protein. The uniformly ^{15}N isotopically-labeled protein was obtained from the growth of *E. coli* in the minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. ^{13}C-methyl selective labeling of α-syn methionines was achieved by growing *E. coli* in a M9 medium containing $^{13}CH_3$-labeled methionine. The protein was first purified using Ni-NTA affinity chromatography. The GB1-tag was cleaved by TEV protease, and was removed using Ni-NTA affinity chromatography. The protein was further purified through gel filtration on fast protein liquid chromatography (AKTA).

N-Terminal acetylated α-syn (Ac-syn) was over-expressed by co-transforming two plasmids that encode α-syn plasmid and NatB in BL21 *E. coli.* The NatB plasmid was the kind gift of Dr. Daniel Mulvihill. The *E. coli* were cultured in LB medium containing 35 μg/L chloramphenicol and 50 μg/L ampicillin at 37°C with shaking. Protein expression was induced by addition of 0.8 mM IPTG at OD_{600} of 0.6-0.8 and allowed to proceed at 20°C overnight. Cells were harvested by centrifugation. The Ac-syn protein was purified as reported.[2]

Preparation of α-syn oligomers and fibrils

Oligomeric α-syn species were prepared using the literature method.[3] Briefly, monomeric α-syn in Milli-Q water was lyophilized and subsequently resuspended in PBS (pH 7.4, contains 137 mM NaCl) at ca. 0.8 mM (12 mg/mL). The resulting solution was passed through a filter (0.22 μm cutoff) and then incubated at 37°C for 20-24 h under quiescent conditions. The excess monomeric protein and small oligomers were removed by multiple steps of ultrafiltration (100 kDa cutoff). The final concentration of oligomers was measured based on the absorbance at 275 nm using a molar extinction coefficient of 5,600 $M^{-1} \cdot cm^{-1}$.

The preformed α-syn fibrils (PFFs) were prepared by incubation of 0.36 mM (5 mg/mL) α-syn monomer in PBS buffer at 37°C in a shaker (1000 rpm). After 5 days incubation, the fibrils were collected by centrifugation (20,000 g) and resuspended in PBS buffer. The samples were stored at -80 °C. The formation of amyloid fibrils was confirmed by using thioflavin fluorimetry and TEM imaging.

Electrospray Ionization Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was carried out on an Exactive Plus (Thermo Fisher Scientific, CA, U.S.A.) mass spectrometer. For the measurement of product in the NAMI-A reactions, 50 μM α-syn was incubated NAMI-A in 50 mM ammonium acetate buffer at 37°C for 2 h. All samples were diluted to 10 μM protein before injection into the mass spectrometer. Positive

ion mode was used in the ESI-MS experiments. Data were processed using XCalibur software (version 2.0, Thermo Finnigan).

Fluorescence Measurements

The fluorescence measurements were performed on a RF-5301PC spectrofluorometer (Shimadzu) using a quartz cuvette with a path length of 5 mm. The excitation wavelength was set at 280 nm for measuring the intrinsic fluorescence of proteins, and the fluorescence spectra were recorded from 290 to 450 nm. The relative intensity of the fluorescence was calculated using the formula $(F - F_S)/(F_0 - F_S)$, where F_0 is the initial fluorescence, F_S is the final fluorescence of in titration, and F is the fluorescence at the given concentration of metal ions.

Circular Dichroism

Circular dichroism (CD) measurements were performed on a Jasco J-810 CD spectrometer flushed with high purity nitrogen. A 1.0 mm path length quartz cuvette was used in the measurements. All spectra were recorded from 280 to 190 nm with a scan speed of 100 $nm \cdot min^{-1}$ and data pitch of 1 nm. A band width of 1 nm was used with a detector response time of 1 s. Protein samples were prepared at a final concentration of 0.15 mg/mL in 10 mM phosphate buffer. The spectrum of buffer was recorded for the baseline corrections. All measurements were repeated three times.

Equilibrium Dialysis

The affinity of NAMI-A for α-syn was estimated via equilibrium dialysis. The situation is complicated by the complexity of the ligand substitution pathways involving release of chloride, DMSO and imidazole ligands and formation of species which are not all equally reactive towards α-syn. Also some equilibria are reached slowly and ligand substitution may be influenced by sequential interactions with protein binding sites.

α-Syn (50 μM) was incubated with 150 μM NAMI-A in 50 mM ammonium acetate buffer pH 7.0 at 37°C for 3 h. The unreacted ruthenium species were removed by ultrafiltration. The reaction product (Ru-α-syn) was dialyzed against NH_4Ac buffer (10 mM) at 25°C in the dark for 16 h; then the ruthenium concentrations in the dialysis tube and dialysis buffer were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Table S2). The dissociation constant was calculated from the concentrations of ruthenium bound to α-syn and unbound ruthenium in solution.

Preparation of DOPS liposomes

Liposomes were prepared using a literature method.[4] 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC, 95%) and cholesterol (5%) were dissolved in chloroform and transferred to a 10 mL round-bottom flask. The chloroform was removed by rotation evaporation. The dried lipid film deposited on the flask was hydrated by adding 10 mM potassium phosphate buffer (pH 7.0) containing 50 mM NaCl, and obtained solution was ultrasonicated at 50°C. The stock liposomes were prepared at a concentration of 40 mM.

NMR Spectroscopy

The NMR spectra were collected on Bruker 850 MHz or 600 MHz (for ^1H) NMR spectrometers equipped with cryogenic probes at 25°C. The ^1H-^{15}N heteronuclear single quantum coherence (HSQC) spectra were recorded on 0.2 mM ^{15}N-labeled α-syn in 50 mM phosphate buffer (pH 7.4) containing 100 mM NaCl and 10% D_2O (v/v). The ^1H-^{15}N signals of the protein were assigned according to the literature.[5] The 1D ^1H spectra of α-syn were recorded on 0.15 mM protein in PBS (pH 7.4) at 298 K with 256 transients into 16 k data points with a relaxation delay of 2.0 s between transients. DSS was used as an internal reference (0 ppm) for ^1H spectra. A WATERGATE pulse sequence was used to suppress the water signal for samples containing 10% D_2O, and a presaturation sequence was used to suppress HDO signal of samples in D_2O. ^1H-^{13}C HSQC spectra were recorded for 0.2 mM α-syn with ^{13}C-methionine selective labeling, and the protein samples were lyophilized and then dissolved in D_2O for NMR measurements. The reactions of ^{13}C-Met labeled α-syn were performed with NAMI-A in different concentrations (0.4, 0.8, 1.2 mM) in 20 mM phosphate buffer (pH 7.4). Peak assignments were achieved by site-mutations of the three

individual methionine residues. The spectra were recorded at 298K. The data were processed and analyzed using TopSpin software.

Transmission Electron Microscopy

End-point α-syn aggregates incubated for 40 h were collected, diluted with PBS and sonicated for 5 min. Five microliters of the sonicated samples were placed rapidly on a carbon-coated copper grid and incubated for 2 min. The grids were dried with a filter paper to withdraw the excess of sample and immediately washed twice with Milli-Q water. Finally, 5 μL of 2% (w/v) phosphotungstic acid was added to the top of the grid and incubated for 2 min. The excess of phosphotungstic acid was removed with a filter paper and grids were left to air-dry for 5 min. Images were recorded on a TEM Jeol 1400 (Peabody, MA, United States) operating at an accelerating voltage of 120 kV. A minimum of 10 fields were screened per sample in order to obtain representative images.

Cell membrane interaction

HeLa cells were seeded in confocal dishes at a density of 8×10^4 cells per well and cultured with DMEM containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 for 24 h. After replacing the medium with 1 mL of the FITC labeled α-syn (100 μg/mL), cells were further incubated for 4 h. For the inhibition analysis, the α-syn treated cells were further cultured with NAMI-A supplemented medium for 2 h. Then, the medium was replaced by 1.50 mL DMEM containing 10 μL Hoechst 33342 (0.4 mg/mL). After 15 min of incubation, the medium was removed and the cells were washed three times with PBS. The fluorescence imaging of cells was recorded by confocal laser scanning microscopy.

ROS detection in cells

ROS generation in cells was measured using an ROS assay Kit. SH-SY5Y cells were seeded in 6-well plates at a density of 3×10^5 cells per well and cultured with DMEM supplemented with 10% FBS. After incubation at 37°C in 5% CO_2 for 24 h, the cells were treated with 1 μM oligomers (with or without 2 – 5 μM NAMI-A) and incubated for another 1 h. Then, the medium was removed and the DMEM containing 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) and 10 μL Hoechst 33342 (0.4 mg/mL) was added. After 15 min of incubation, the medium was removed and the cells were washed three times with PBS. The imaging of cells was recorded with fluorescence microscopy.

Cytotoxicity assay

Cell viability was determined using the MTT assay. SH-SY5Y cells were seeded in 96-well plates at 8000 cells per well and cultured with 100 μL of DMEM containing 10% FBS for 12 h. After the treatment of α-syn oligomers in different concentrations, the cells were divided into two groups, NAMI-A was added to one and the other was used as a control. After further incubation for 24 h, the medium was replaced with fresh neutral culture medium and 20 μL MTT (5 mg/mL) was added. The cells were incubated for another 4 h to convert the yellow MTT into dark blue formazan crystals. Finally, the formazan product was dissolved in DMSO and quantified by the absorbance at 570 nm using a Bio-Rad 680 microplate reader.

Animals

Male Sprague-Dawley rats (200-250 g) were obtained from Department of Neurosurgery, Anhui Provincial Hospital Affiliated to University of Science and Technology of China. The animals received food and water ad libitum and were kept under strictly controlled environmental conditions (12 h light/dark cycle, with light on between 7:00 and 19:00 o'clock; room temperature 25°C). The animal experiments were approved by the Animal Research Ethics Committee of the First Affiliated Hospital of the University of Science and Technology of China (Accreditation number: 202107191146000372991).

Surgery and Stereotaxic Injections

Rats were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and placed in a Kopf stereotaxic apparatus (Narishige, Japan). An injection cannula (a 30-gauge stainless-steel cannula

connected to a Hamilton 10 μL syringe driven by a microinfusion pump) was slowly inserted through a hole drilled into the skull in the central part of the left striatum of each animal using the following coordinates (in millimeters): anteroposterior 0 mm; mediolateral ±2.5 mm; dorsoventral -5.0 mm.[6] The preformed α-syn fibrils (PFFs) were diluted into sterile PBS and sonicated briefly before intracerebral injection. PFFs were injected into the left striatum (n = 5 or 8, 8 for rats infused with PFFs, 5 for rats infused with PBS as a control), whereas the contralateral side was left intact. Infusions were performed during surgery; PFFs (10 mg/mL, equivalent to 0.7 mM in monomer) were configured in PBS and delivered at a flow rate of 0.5 μL/min for 4 min. The cannula was slowly removed after the infusion and the incision was tightly closed after 5 min.

In vivo Behavioral Analysis

After the injection PFFs, NAMI-A (2.1 mM or 3.5 mM) was configured in PBS and delivered at a flow rate of 0.5 μL/min for 4 min on the 30th day. On the 50th day, the rats were examined for spontaneous behavioral abnormalities by apomorphine (APO)-induced rotational asymmetry assessment. The number of rotations to the ipsilateral side was counted for 30 min starting from 10 min after an intraperitoneal administration of APO (0.5 mg/kg). All the testing was done repeatedly at the 50th day after unilateral administration of PFFs. PBS-injected animals were tested at the same time points as a control.

Histological Examination

The animals were anesthetized by an intravenous injection of chloral hydrate (300 mg/kg, i.p.) and perfused transcardially with 0.9% saline followed by 4% buffered formaldehyde. The brains were removed, blocked, immersed in the same fixative for 24 h, and then placed in saline with the addition of 30% sucrose until the block sank. They were then sectioned coronally with a cryostat (RM2015, Leica, Germany) at a thickness of 50 μm. Sections were collected in sequence; two series were collected onto gelatin-coated slides. The sections were deparaffinized and rehydrated. After treatment with 3% H_2O_2, the sections were washed with phosphate-buffered saline (PBS; ZLI-9062, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China) and incubated in citrate buffer (0.1 M, pH 5.8), then repeatedly washed with PBS for immunohistochemistry. Sections were immersed in a solution of 10% normal goat serum and 1% bovine serum albumin (prepared in PBS) for 1 h. Alternate sections were then incubated in anti-tyrosine hydroxylase (TH, 1:500) or rabbit polyclonal anti-α-syn antibody (1:200) overnight at 4 °C. Thereafter, the sections were incubated with biotinylated anti-rat for 15 min at room temperature. Finally, sections were incubated in the avidin-biotin-peroxidase complex for 15 min at room temperature. The bound peroxidase molecule was visualized using 3,3′-diaminobenzidine. The sections were washed several times with PBS between incubations. Each of the antibodies, together with the avidin-biotin-peroxidase complex, was diluted with PBS.

Sections were mounted on gelatin-coated slides, dried overnight, dehydrated in ascending alcohols, cleared in Histo-Clear and covers lipped with DPX. In control experiments, the antibodies were replaced by PBS and then reacted as above. Control sections were immunonegative. The number of dopaminergic cells was counted using TH immunoreactivity. This method was used to explore whether cell loss after PFFs treatment was due to loss of antigen expression (TH); in other words, whether there was a functional (TH) cell loss. This study did not endeavor to report on the total number of cells in the SNc of rats. Rather, the number of cells in the same nucleus of corresponding sections in different individual cases was compared. This method has been commonly used recently for examining damage of neuronal cells in PD models.

2. Supplementary Figures

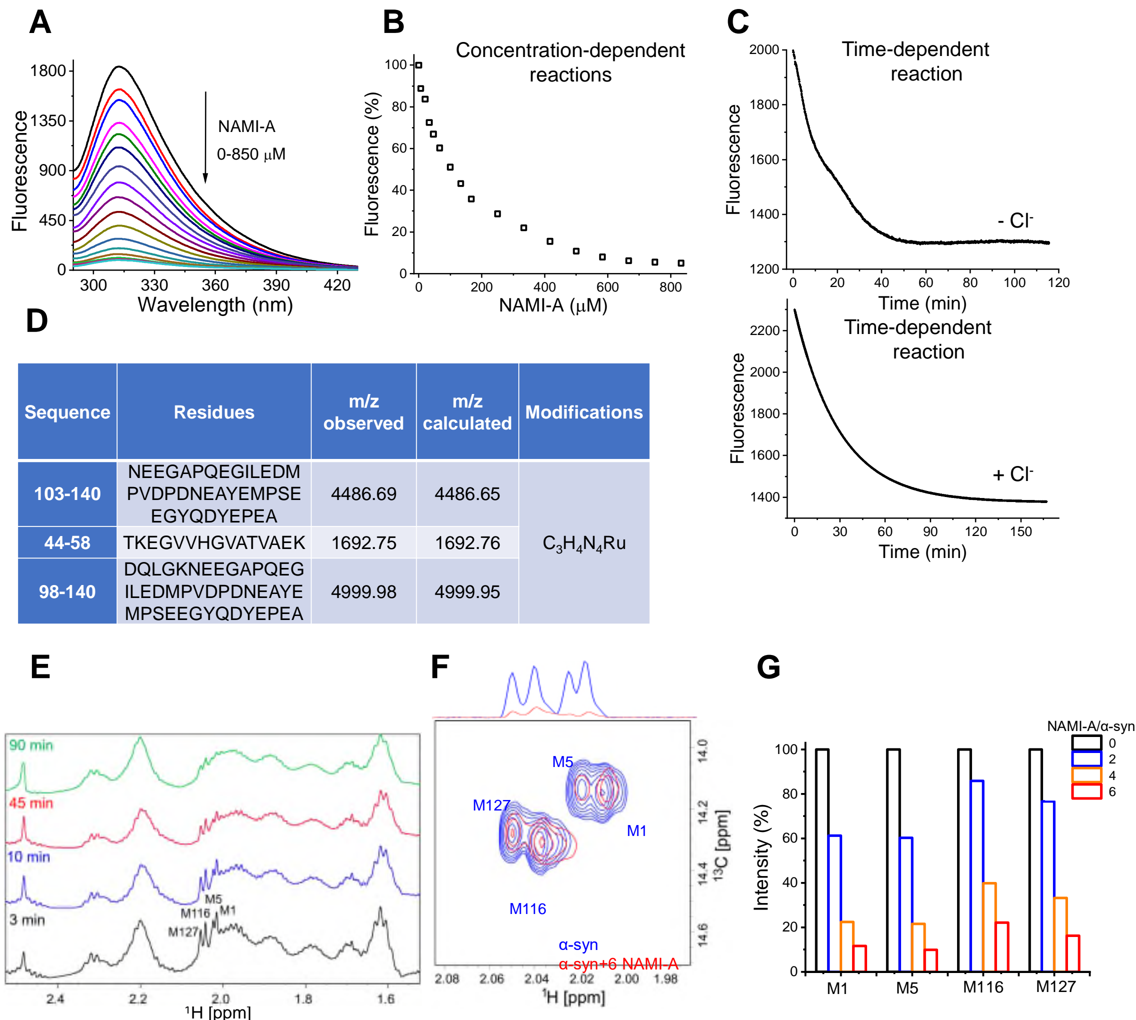


Figure S1. Studies of the reaction between NAMI-A and α-syn. (A) Fluorescence spectra of α-syn after incubation with NAMI-A. Spectra were recorded after incubation of 20 μM α-syn with different concentrations of NAMI-A (0 – 850 μM) for 2 h at 37°C in 20 mM phosphate buffer (pH 7.4). (B) The fluorescence at 310 nm decreases with increase in concentration of NAMI-A. (C) Time-dependent decrease in the fluorescence of α-syn (20 μM, 20 mM phosphate buffer) in the absence (upper) or presence (lower) of 135 mM NaCl at 37 °C, at 310 nm, during the reaction of NAMI-A (3 mol equiv). (D) ESI-MS analysis of the peptide fragments of α-syn that contain ruthenium after reaction with NAMI-A. The reaction mixture contained 10 μM protein and 50 μM NAMI-A in 50 mM NH_4HCO_3 (pH 7.4). After incubation at 37°C for 2 h, the products were subjected to tryptic digest for 16 h at 37°C prior to HPLC/ESI-MS analysis. (E) 1D ^{1}H-NMR spectra of α-syn (0.15 mM) incubated with 3 mol equiv NAMI-A (0.45 mM) in D_2O at pH 7.4 and 298K for the indicated times. (F) Superimposition of 2D ^{1}H-^{13}C HSQC spectra of 0.2 mM ^{13}C-Met labeled α-syn before (blue) and after (red) incubation with 6 mol equiv. of NAMI-A (1.2 mM) for 3 h at 298K in 20 mM phosphate buffer, pH 7.4). The 1D projections of 2D spectra along ^{13}C dimension are shown. (G) Integrated intensities of ^{13}C-Met peaks in HSQC spectra after reaction with different ratios of [NAMI-A]/[α-syn].

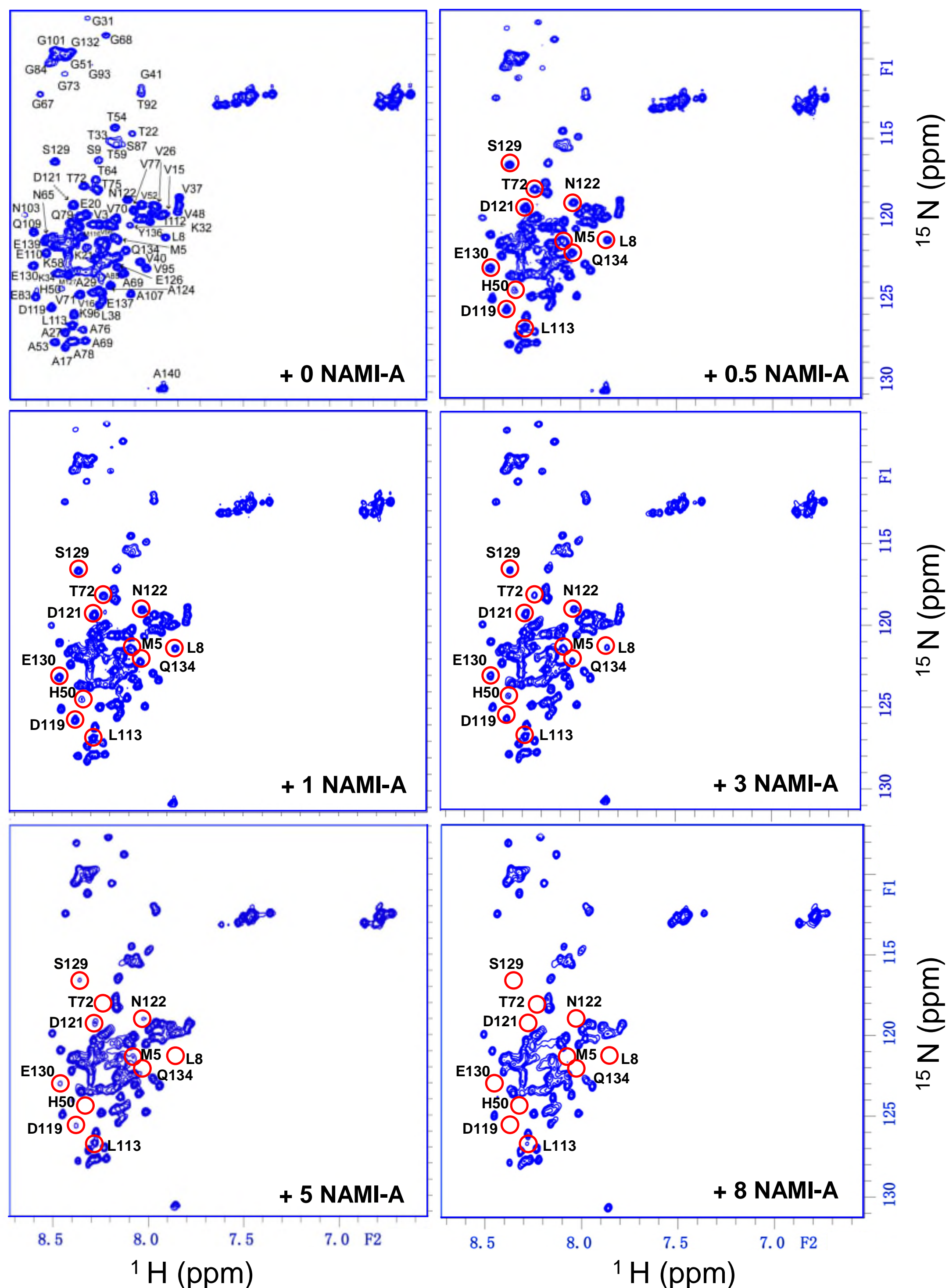


Figure S2. ^{1}H-^{15}N HSQC 2D NMR of ^{15}N-labeled α-syn and after reaction with NAMI-A. The reaction was carried out with 0.2 mM protein and various molar ratios of [NAMI-A]:[α-syn] (0, 0.5, 1, 3, 5, 8) at 310 K in 20 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. The spectra were recorded at 298 K after incubation for 2 h. Full peak assignments are given in the first spectrum before the reaction with NAMI-A. The 11 peaks which significantly decrease in intensity, are plotted in Figure 1E in main text, and are indicated by red circles in spectra for the NAMI-A reaction.

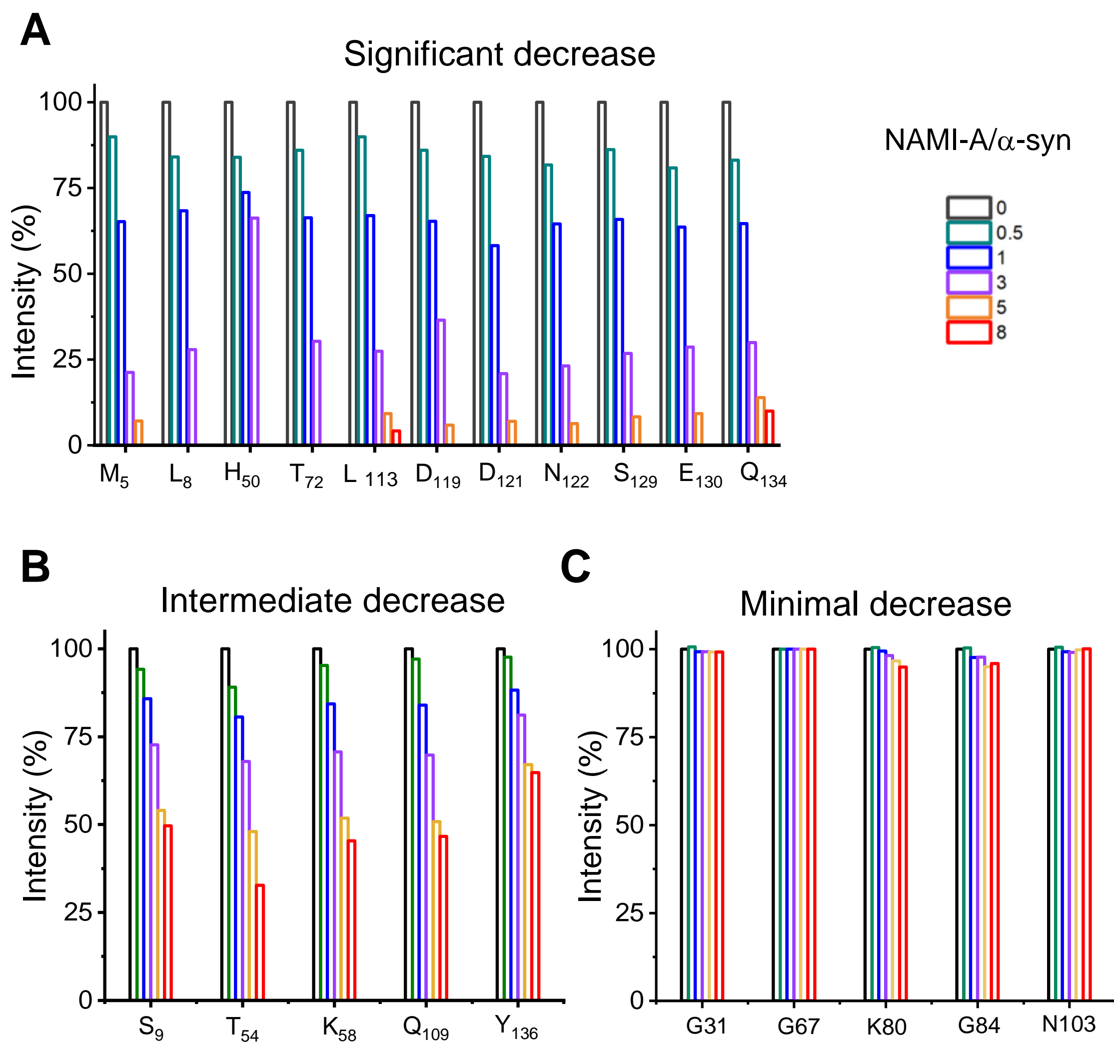


Figure S3. The effect of reactions with NAMI-A (0.5-8 mol equiv) on the intensities of 2D NMR ^{1}H-^{15}N cross-peaks of ^{15}N-labeled α-syn in **Figure S2**. (A) 11 most affected peaks, highlighted by red circles in **Figure S2**. (B) 5 selected signals that moderately decreased in intensity. (C) 5 selected signals that are not affected by the reaction. The intensities of peaks are relative to the unchanged peak G67, as determined by peak volume integration.

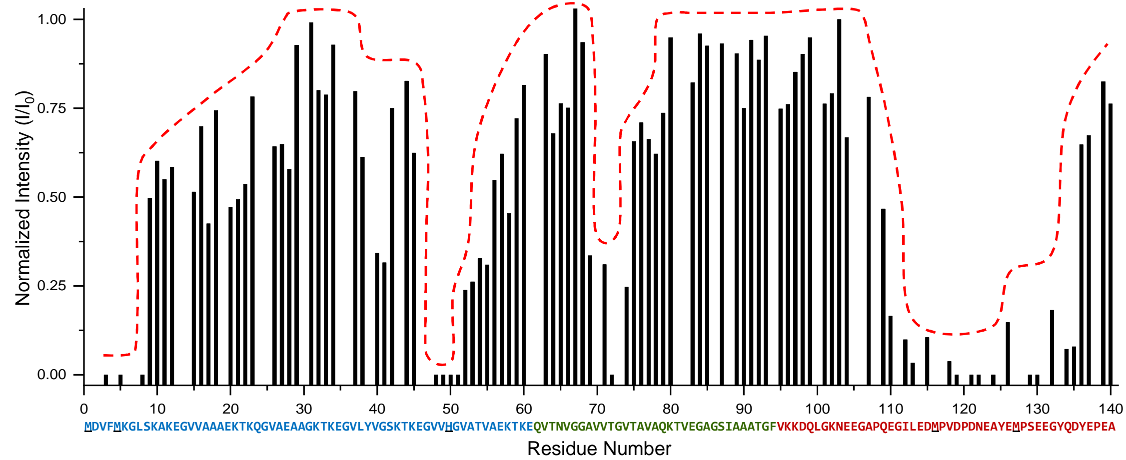


Figure S4. Dependence of intensity changes of 2D NMR ^{1}H-^{15}N cross-peaks of ^{15}N-labeled α-syn after the reaction with NAMI-A (8 eq.) for 5 h on the amino acid sequence. The relative intensities of peaks are with respect to the unchanged peak of G67, by integration of peak volumes.

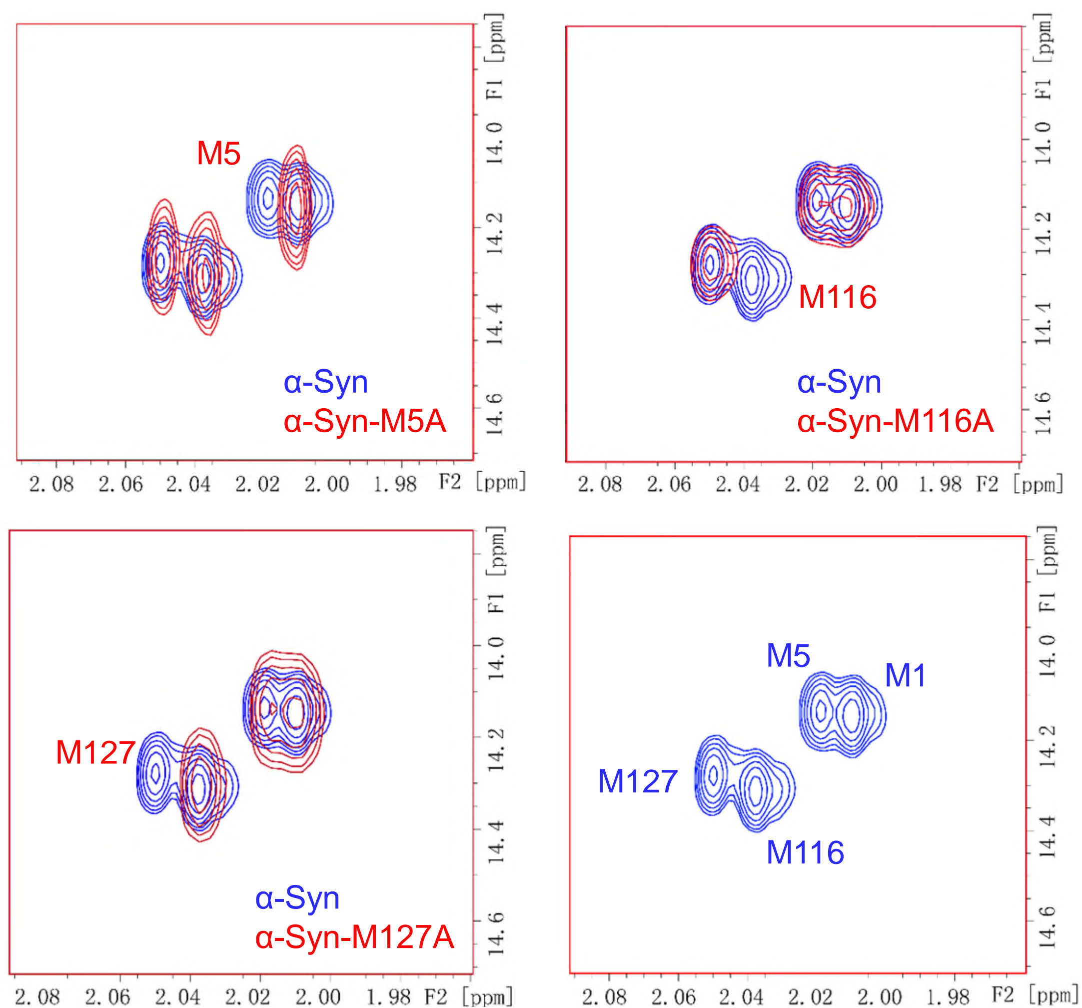


Figure S5. Superimposition of ^{1}H-^{13}C HSQC 2D NMR spectra recorded on 0.2 mM WT α-syn (blue) or Met mutants (red) in PBS at 298K, providing assignments of the four well-resolved cross-peaks for methionine $SC^{\varepsilon}H_3$ signals.

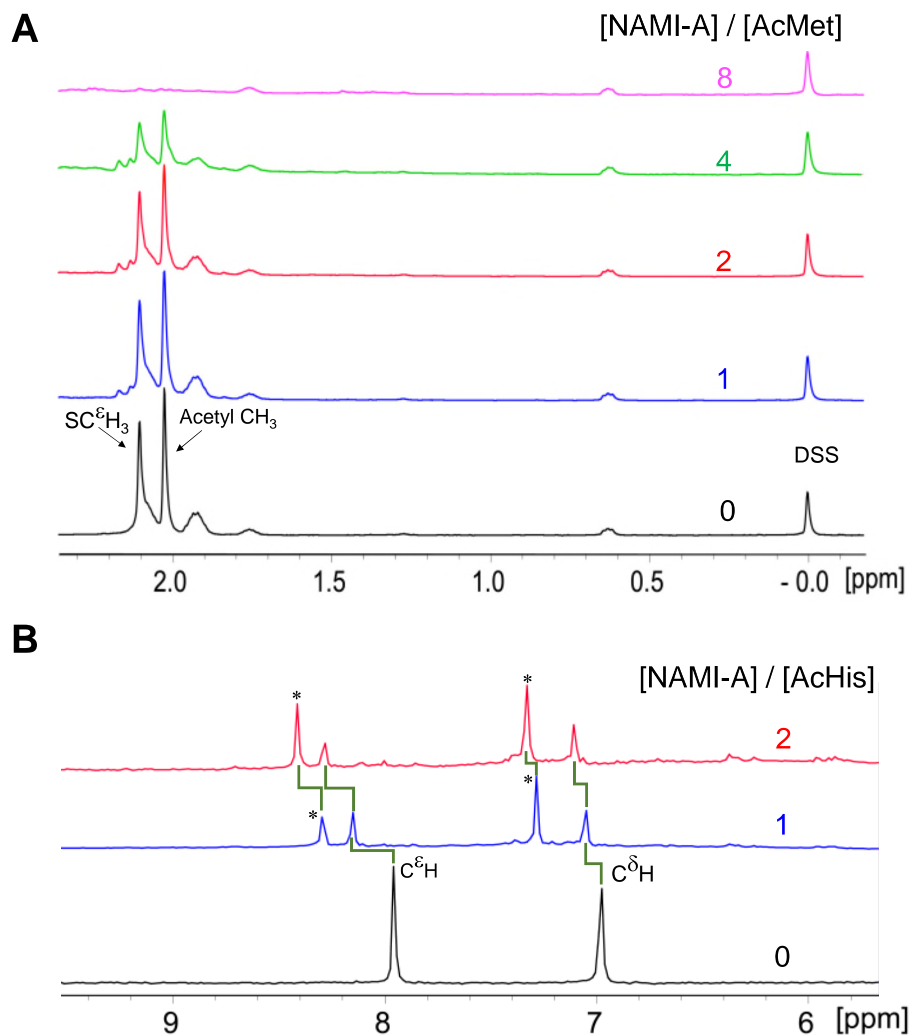


Figure S6. 1D ^{1}H NMR spectra of (A) AcMet or (B) AcHis before and after reaction with NAMI-A. The spectra were recorded on 1 mM acetylated amino acid with various concentrations of NAMI-A at 37°C for 8 h in 10 mM PBS (pH 7.4, contains 137 mM NaCl). The molar concentration ratios are marked in the figure. Asterisks (∗) indicate the signals of free imidazole from NAMI-A counter cation. The decrease in intensity of the methionine methyl signal and the shift of histidine imidazole signal are paralleled by similar changes for peaks of Met and His of α-syn in the reaction with NAMI-A.

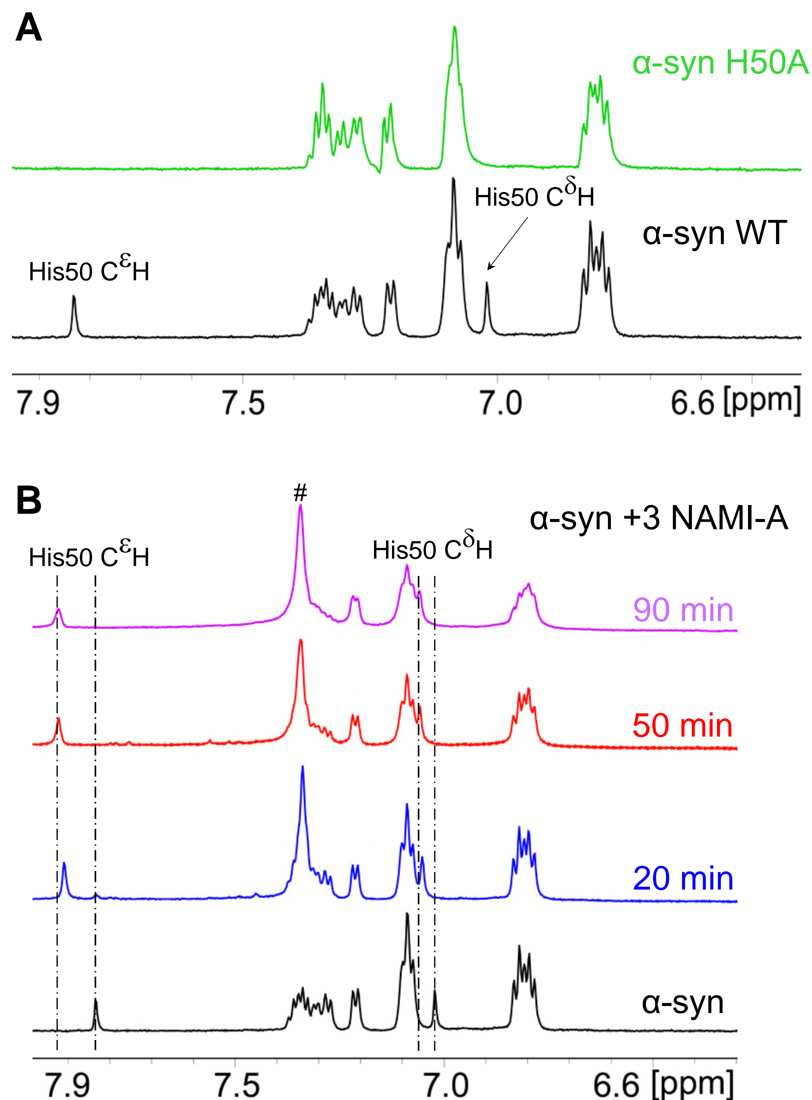


Figure S7. 1D ^{1}H NMR spectra of α-syn before and after reaction with NAMI-A. (A) The NMR spectra of wild-type (WT) α-syn and its H50A mutant in PBS in D_2O. It is clearly seen that the imidazole signal of His50 disappears in the spectra of the H50A mutant. (B) The NMR spectra of 0.15 mM α-syn before or after incubation of 0.45 mM NAMI-A in PBS in D_2O (pH 7.4) at 298K. The incubation time is labeled on each spectrum. # Indicates the signal from the free imidazole of NAMI-A. The dashed lines show the positions of the His50 imidazole signals (C$^{\delta}$H and C$^{\varepsilon}$H) of α-syn before and after reaction with NAMI-A.

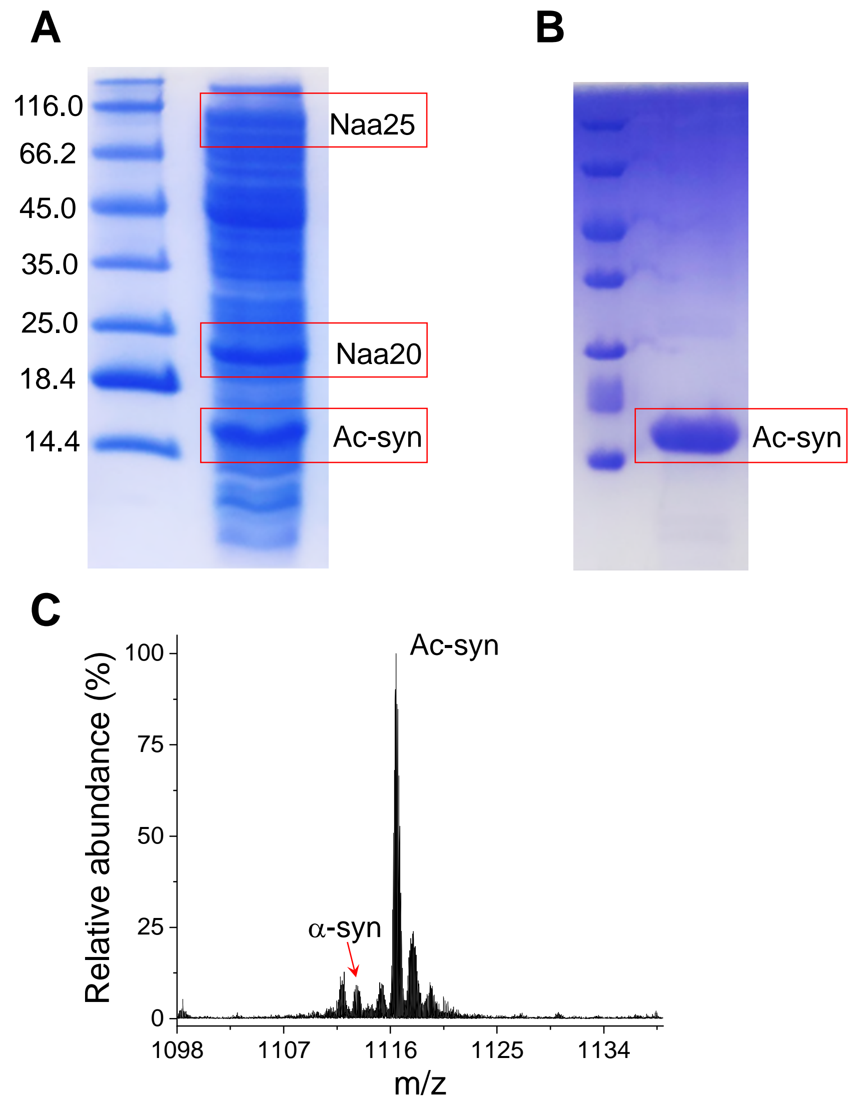
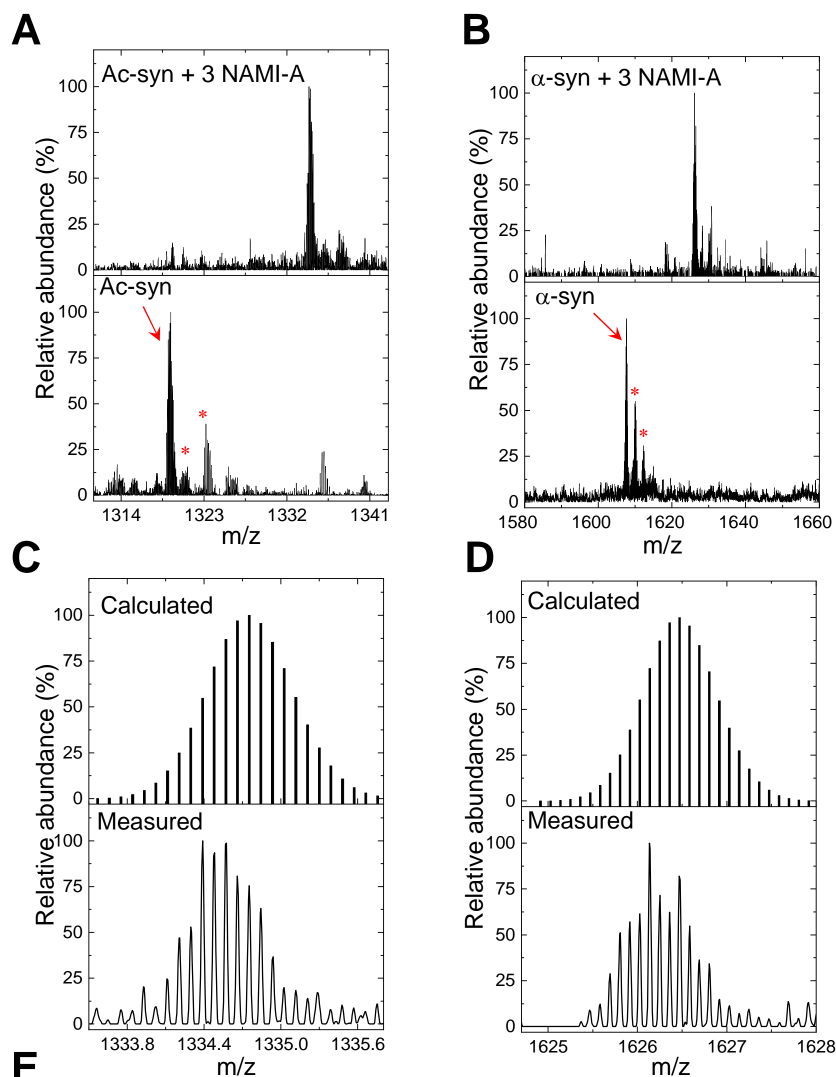


Figure S8. Expression and characterization of Ac-syn. (A) SDS-PAGE analysis of the cell lysate of *E. coli* for expression and N-acetylation of α-syn. The bands of two acetyltransferases (Naa25 and Naa20) and Ac-syn are labeled. (B) SDS-PAGE analysis of the purified Ac-syn. (C) ESI-MS analysis of the purified Ac-syn in 50 mM NH_4Ac. The minor peak for un-acetylated α-syn is also indicated.



Composition	Formula	m/z observed	m/z calculated
α-syn	$C_{627}H_{1012}N_{166}O_{216}S_4$	1607.70 z=9	1607.69 z=9
α-syn + $[Ru(Im)]^{3+}$	$C_{630}H_{1016}N_{168}O_{216}S_4Ru$	1626.14 z=9	1626.48 z=9
Ac-syn	$C_{629}H_{1014}N_{166}O_{217}S_4$	1319.30 z=11	1319.39 z=11
Ac-syn + $[Ru(Im)]^{3+}$	$C_{632}H_{1018}N_{168}O_{217}S_4Ru$	1334.57 z=11	1334.76 z=11

Figure S9. ESI-MS spectra of the products from reactions of Ac-syn and α-syn with NAMI-A. (A) Ac-syn; (B) α-syn. (C-D) Expanded observed ESI-MS peaks and their theoretical isotopic patterns. (E) Peak assignments. The asterisk (∗) indicates peaks with addition of Na^+ ions. The reactions were performed on proteins (10 μM) with NAMI-A (30 μM) at 37°C for 3 h in 50 mM NH_4Ac. The spectra were recorded in positive-ion mode.

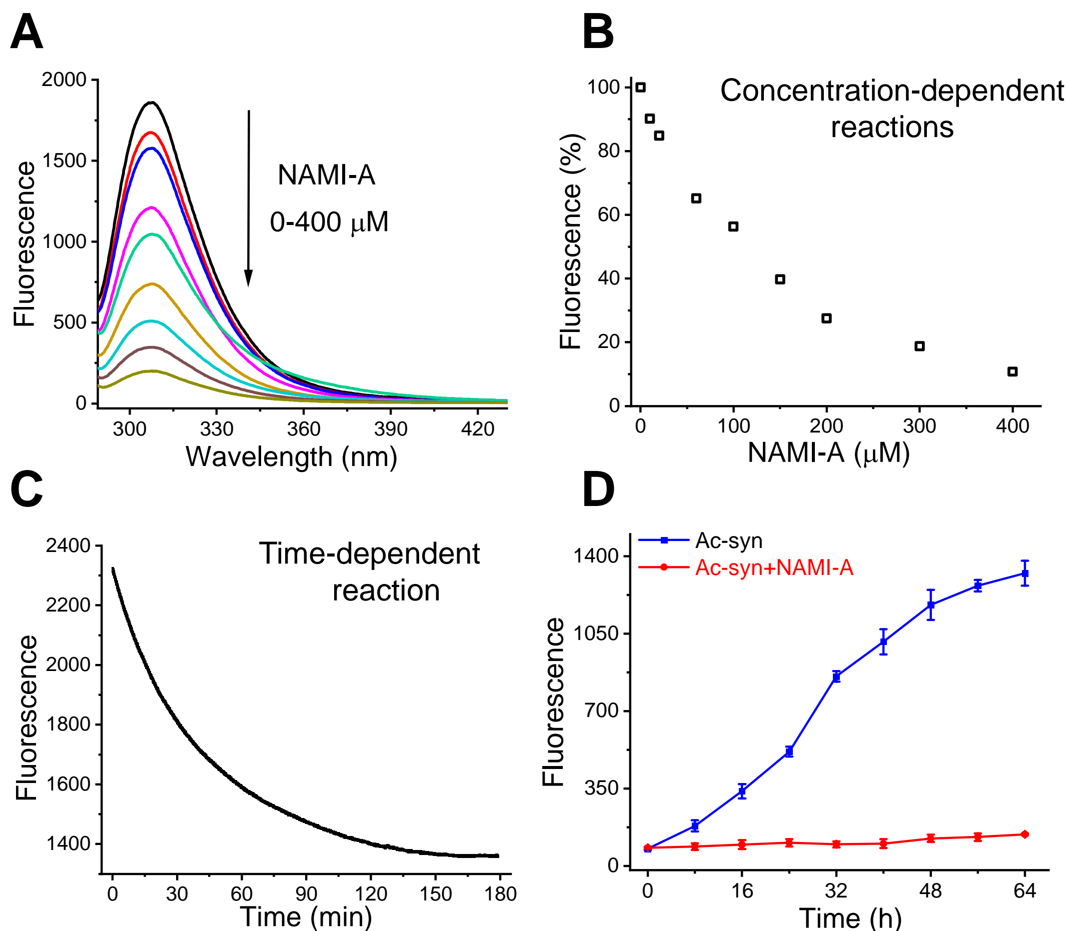


Figure S10. Fluorescence of Ac-syn in the reaction with NAMI-A. (A) Fluorescence spectra of Ac-syn (20 μM) after incubation with various concentrations of Ac-syn of NAMI-A (0 – 400 μM) for 2 h at 37°C in 20 mM phosphate buffer (pH 7.4). (B) Variation of intensity of fluorescence at 310 nm with NAMI-A concentration. (C) Time-dependent decrease of the fluorescence of Ac-syn at 310 nm during the reaction of NAMI-A. The reaction was performed using 20 μM protein with 3 mol equiv NAMI-A in 20 mM phosphate buffer with 135 mM NaCl at 37°C. (D) ThT assay of the inhibitory effect of NAMI-A on the filamentous aggregation of Ac-syn. Ac-syn (70 μM) was incubated with ThT (20 μM), and NAMI-A (8 mol. equiv.) was added at start time. The fluorescence was recorded at 495 nm with excitation at 446 nm.

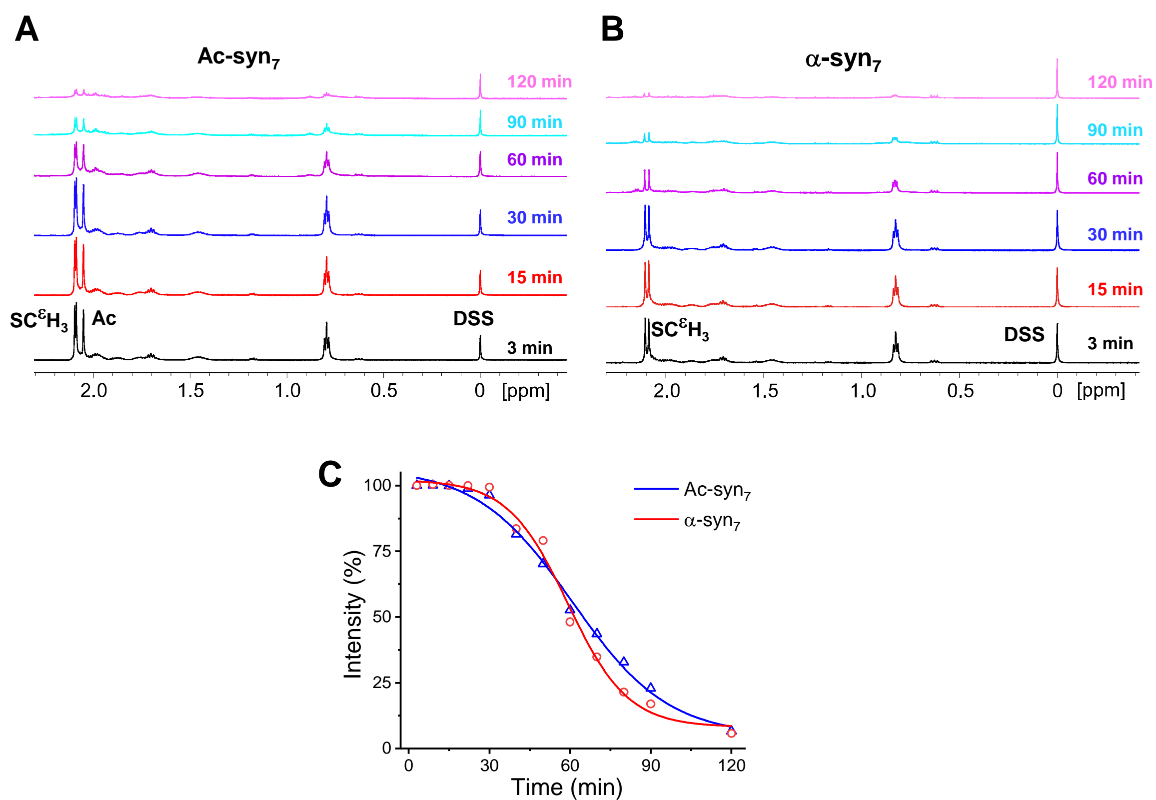
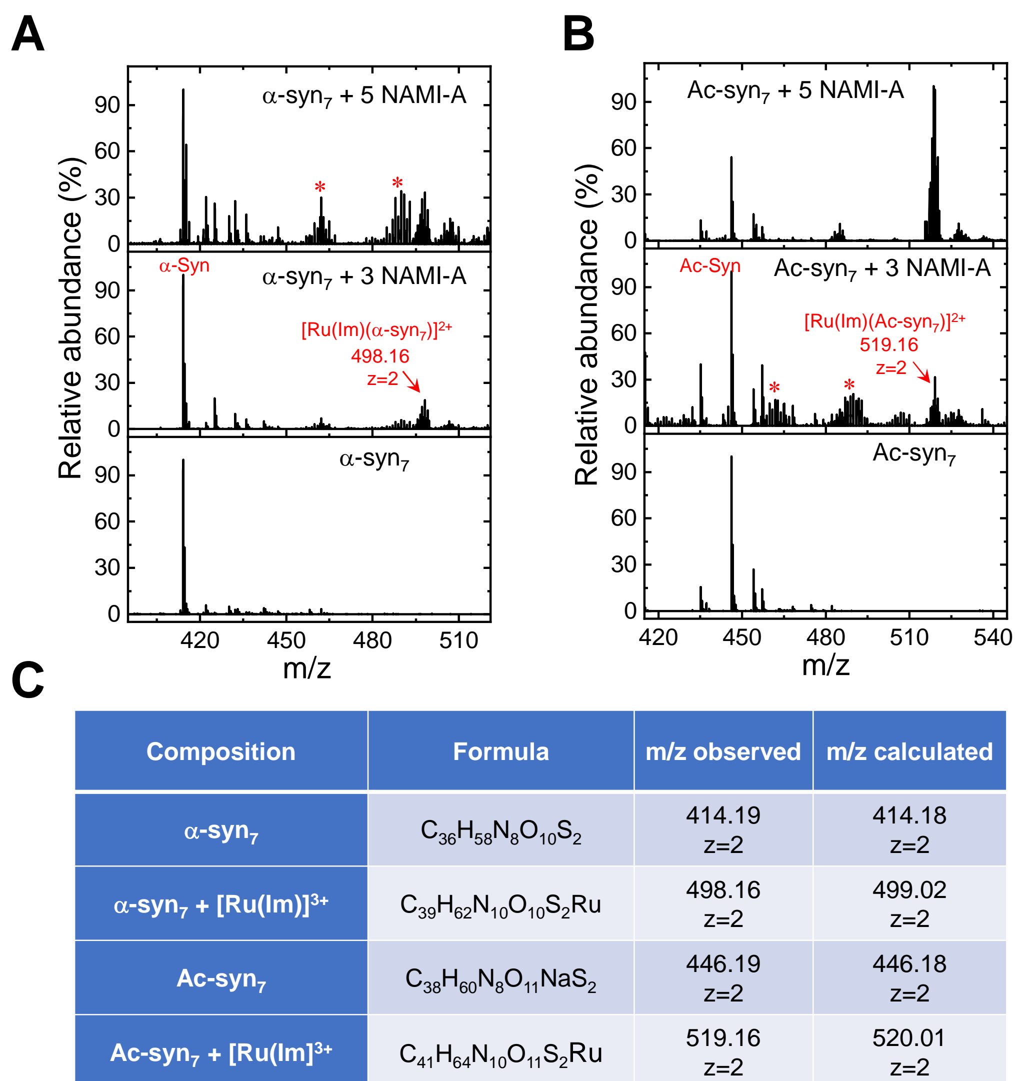


Figure S11. Effect of N-acetylation on the reaction of model septapeptide α-syn$_7$ (MDVFMKG) with NAMI-A. ^{1}H NMR spectra were recorded after reaction for different times. (A) ^{1}H NMR spectra of the acetylated peptide Ac-syn$_7$. (B) ^{1}H NMR spectra of non-acetylated peptide α-syn$_7$. Peaks of SC$^\varepsilon$H$_3$ (Met1, Met5) and acetyl CH$_3$ of Met are labeled. (C) Time-dependent intensity (integrations) of peaks of SC$^\varepsilon$H$_3$ of peptides in the reactions of NAMI-A. The reactions were performed on 0.5 mM peptides with 2.5 mM NAMI-A in PBS (prepared in D$_2$O) at pH 7.4 and 310 K. DSS (0.15 mM) was used as an internal reference (0 ppm and consistent intensity). The incubation time is labeled on each spectrum.



Composition	Formula	m/z observed	m/z calculated
α-syn$_7$	$C_{36}H_{58}N_8O_{10}S_2$	414.19 z=2	414.18 z=2
α-syn$_7$ + $[Ru(Im)]^{3+}$	$C_{39}H_{62}N_{10}O_{10}S_2Ru$	498.16 z=2	499.02 z=2
Ac-syn$_7$	$C_{38}H_{60}N_8O_{11}NaS_2$	446.19 z=2	446.18 z=2
Ac-syn$_7$ + $[Ru(Im)]^{3+}$	$C_{41}H_{64}N_{10}O_{11}S_2Ru$	519.16 z=2	520.01 z=2

Figure S12. ESI-MS spectra of the products from reactions of model peptides with NAMI-A. (A) α-syn$_7$; (B) Ac-syn$_7$. (C) Assignments of peaks. The reactions were performed on peptides (10 μM) and NAMI-A (30 μM or 50 μM) at 37°C for 3 h in 50 mM NH_4Ac. ESI-MS spectra were recorded in positive-ion mode. The asterisks (∗) indicate a hydrolysis product from NAMI-A.

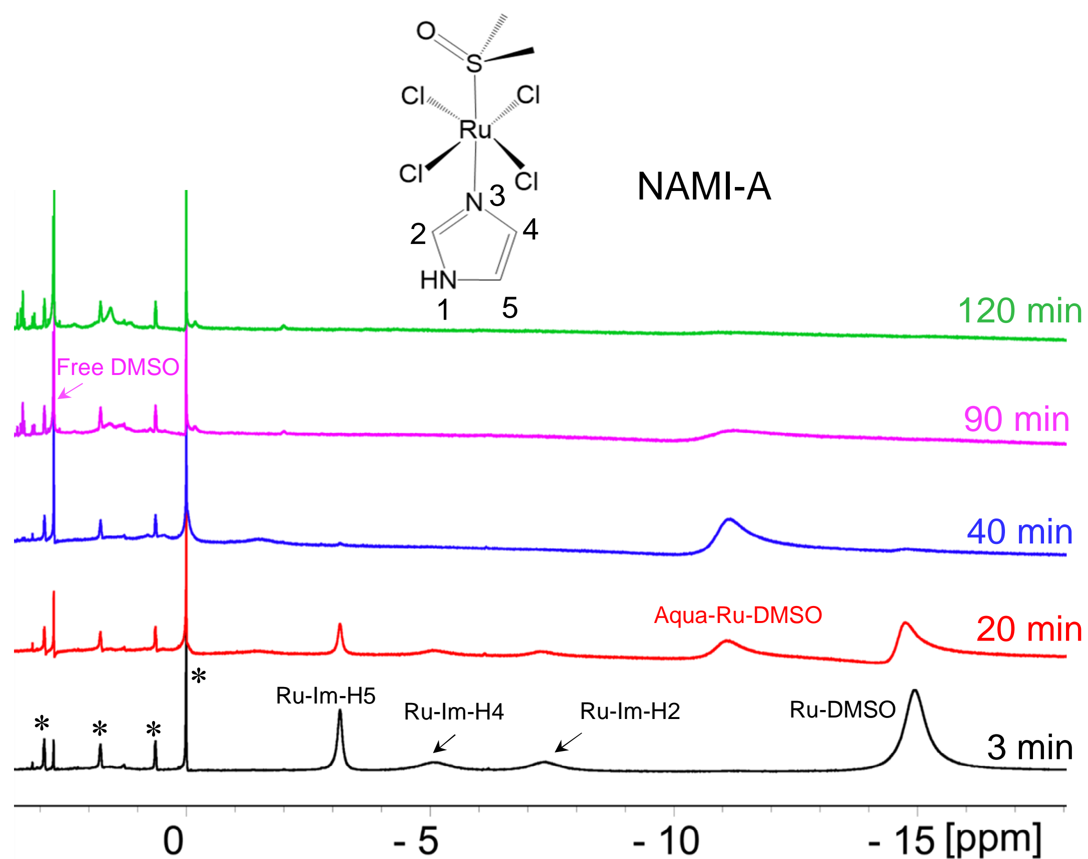
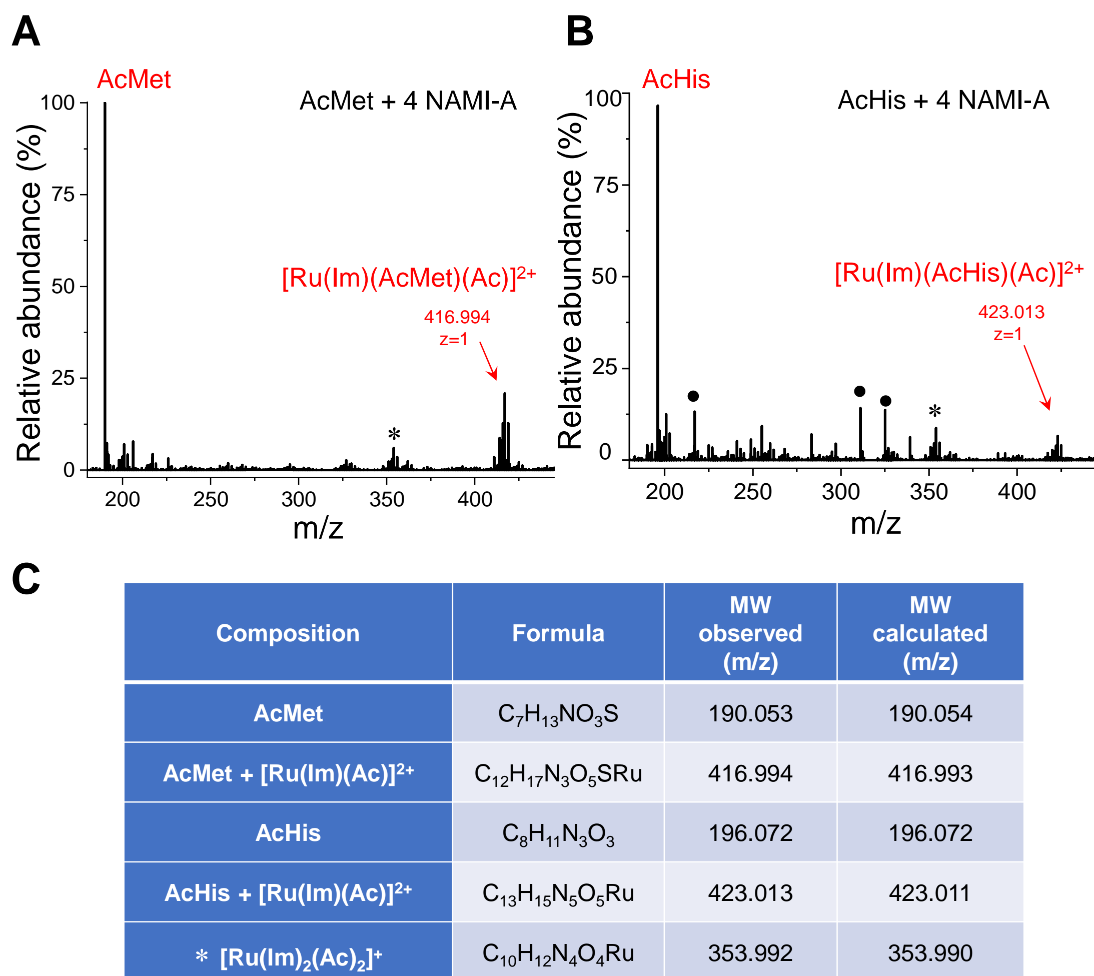


Figure S13. 1D ^{1}H NMR spectra of NAMI-A (1 mM) recorded during the hydrolysis in PBS at 37°C. Selected regions of the spectra show the paramagnetically-shifted signals of NAMI-A at different hydrolysis times. NMR signals were assigned according to the literature.[7] The asterisks (*) indicate the peaks of reference DSS. The structure of NAMI-A and the numbering of imidazole atoms are shown above the spectra.



Composition	Formula	MW observed (m/z)	MW calculated (m/z)
AcMet	$C_7H_{13}NO_3S$	190.053	190.054
AcMet + $[Ru(Im)(Ac)]^{2+}$	$C_{12}H_{17}N_3O_5SRu$	416.994	416.993
AcHis	$C_8H_{11}N_3O_3$	196.072	196.072
AcHis + $[Ru(Im)(Ac)]^{2+}$	$C_{13}H_{15}N_5O_5Ru$	423.013	423.011
* $[Ru(Im)_2(Ac)_2]^+$	$C_{10}H_{12}N_4O_4Ru$	353.992	353.990

Figure S14. ESI-MS spectra for the reactions of NAMI-A with (A) AcMet, and (B) AcHis. The compositions of the adducts are listed in the table underneath the spectra. The reactions were performed on amino acid (10 μM) and NAMI-A (40 μM), 37°C for 3 h in 50 mM NH_4Ac. ESI-MS spectra were recorded in negative mode. The asterisk (*) indicates a hydrolysis product of NAMI-A containing a second imidazole (Im) bound to Ru(III); the black circles (•) indicate impurities in the amino acid. (C) Assignments of peaks in the ESI-MS spectra.

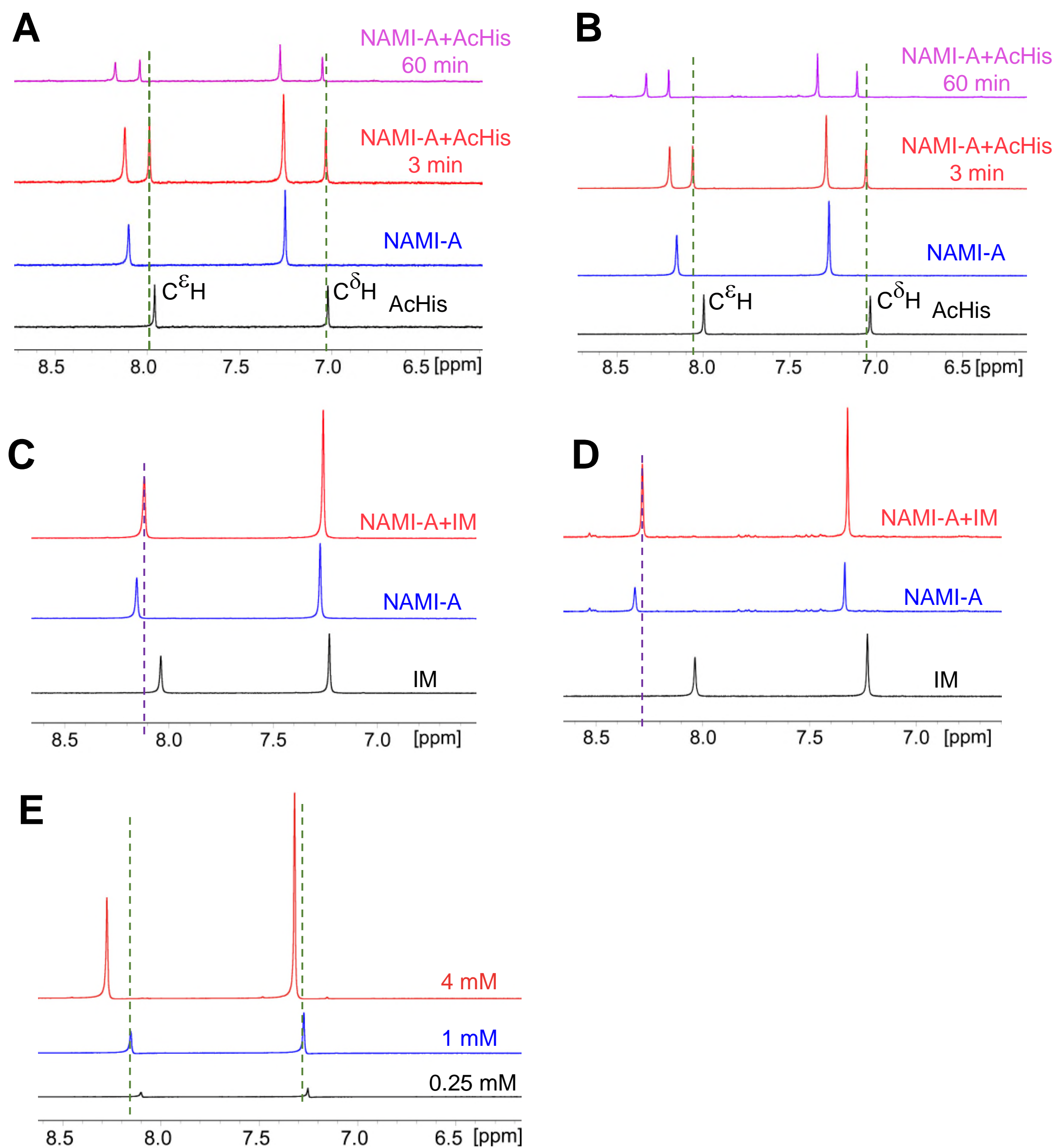


Figure S15. 1D ^{1}H NMR spectra for the reaction of AcHis with NAMI-A. The spectra were recorded on (A) 0.25 mM, and (B) 1 mM AcHis with equal molar ratio of NAMI-A in 10 mM PBS in D_2O (pH 7.4). (C-D) 1D ^{1}H NMR spectra of imidazole in the reaction with NAMI-A. The spectra were recorded on 1 mM imidazole with equal molar ratio of NAMI-A in 10 mM PBS in D_2O (pH 7.4). (C) Addition of imidazole to freshly prepared NAMI-A. (D) After 60 min hydrolysis of NAMI-A, imidazole was added. (E) 1D ^{1}H NMR spectra for various concentrations of NAMI-A in 10 mM PBS in D_2O (pH 7.4). DSS (0.15 mM) was used as an internal reference (0 ppm).

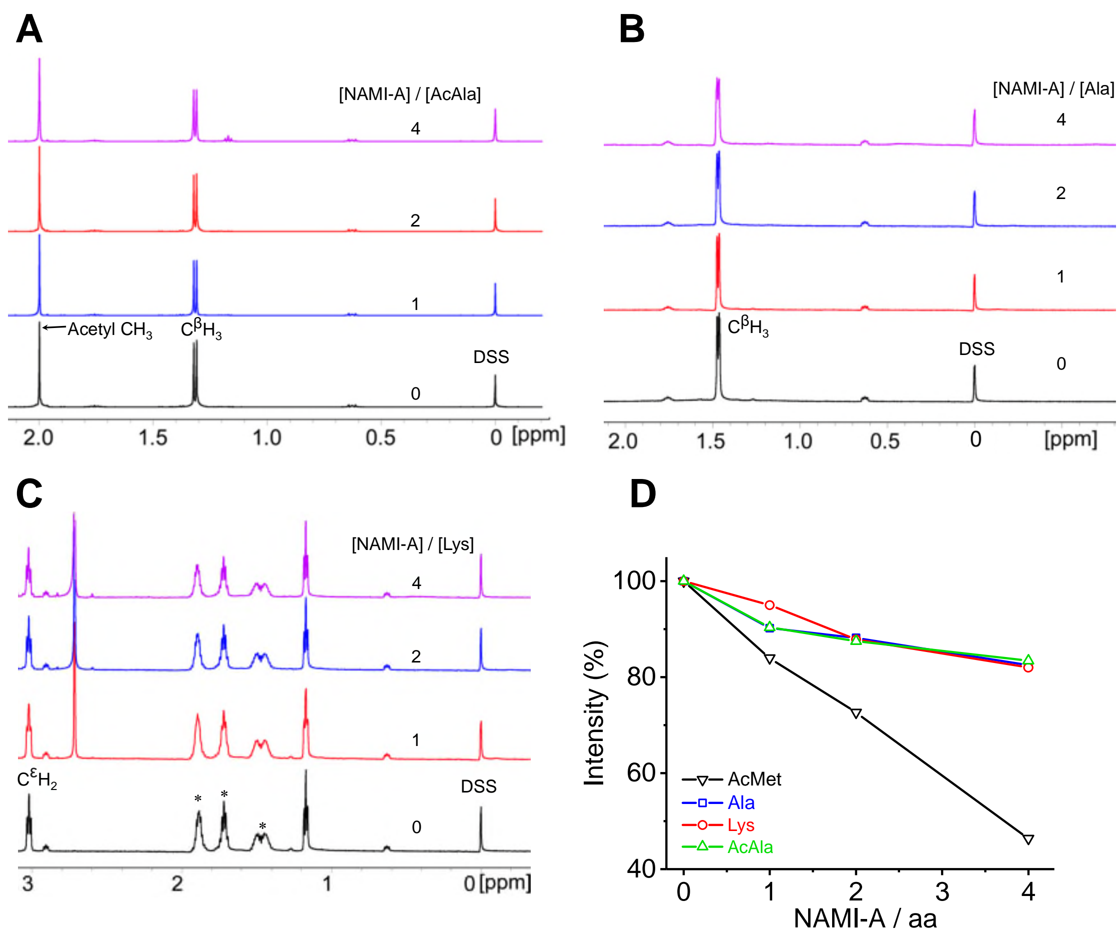


Figure S16. 1D ^{1}H NMR spectra for the reactions of NAMI-A with model amino acids. (A) AcAla; (B) Ala; (C) Lys. The spectra were recorded on 1 mM amino acid with different molar ratios of NAMI-A at 37°C for 8 h in PBS (pH 7.4). The asterisks (*) indicate the signals of $C^{\beta}H_2$, $C^{\gamma}H_2$, $C^{\delta}H_2$ of Lys. (D) The intensities of $C^{\beta}H_3$ or $C^{\varepsilon}H_2$ peaks after the incubation of different ratios of [NAMI-A]/[aa]. DSS (0.15 mM) were used as an internal reference (0 ppm).

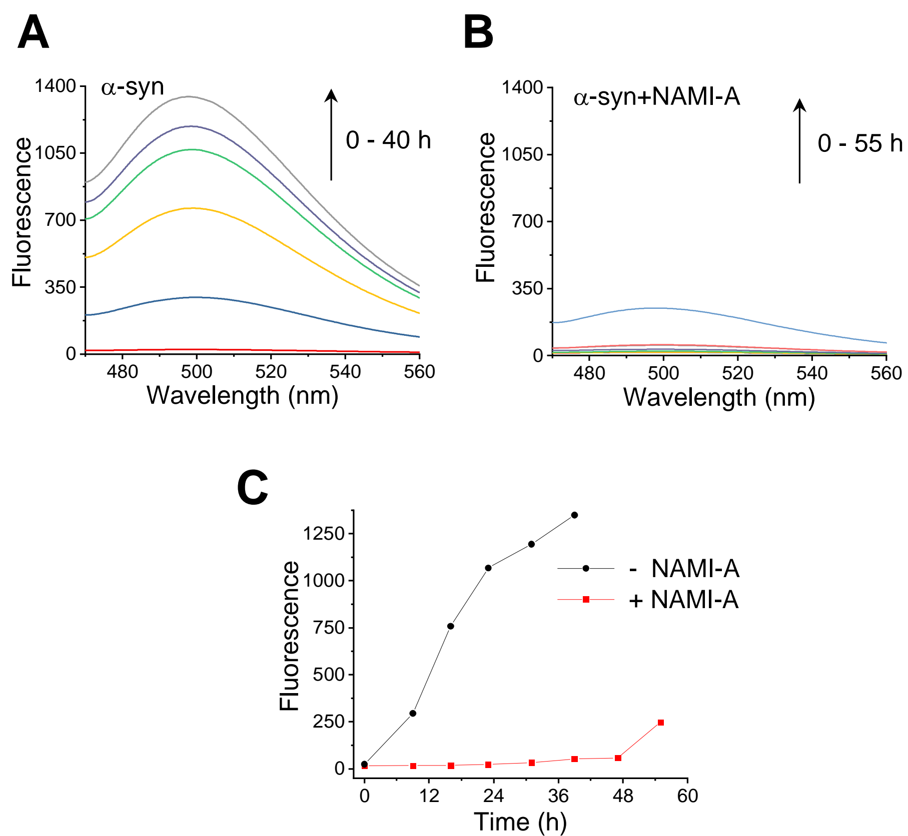


Figure S17. ThT assay of the effect NAMI-A on the filamentous aggregation of α-syn. 70 μM α-syn was incubated with 20 μM ThT (A) in the absence, or (B) presence of 350 μM NAMI-A at 37°C for various times. The fluorescence spectra were recorded with the excitation at 446 nm. (C) The fluorescence intensity at 495 nm during the assay. The color denotes the fluorescence in the absence (black) or presence (red) of NAMI-A.

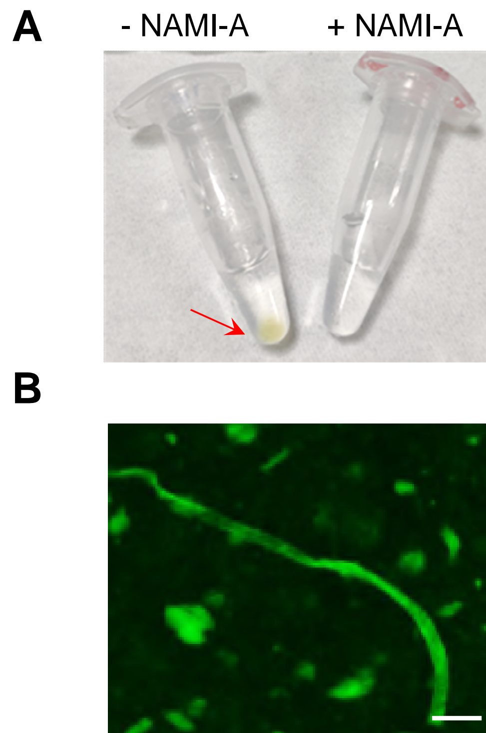


Figure S18. Visualization of α-syn fibril formation in the presence of ThT. (A) Fibrils formation in the absence (left) or presence (right) of NAMI-A. The samples were centrifuged after incubation of α-syn with ThT for 72 h. The arrow indicates yellow precipitate of the α-syn fibril. (B) Fluorescent microscopy image of α-syn fibrils observed by ThT fluorescence. Scale bar = 50 μm.

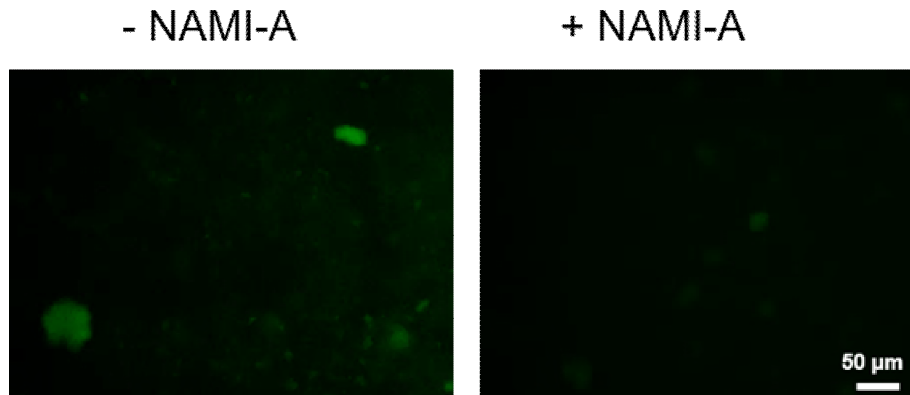


Figure S19. NAMI-A inhibits liposome interactions of α-syn. Fluorescence microscopy images of FITC labeled α-syn and DOPS liposomes in the absence (left) and presence (right) of NAMI-A. DOPS liposomes were pre-incubated with 10 µM FITC-labeled α-syn at 37°C for 30 min. Then samples were incubated with 50 µM NAMI-A for 2 h at 37°C. The images were obtained with excitation at 488 nm. The result shows the interaction of FITC-labeled α-syn with liposomes (left); the presence of NAMI-A inhibits this interaction (right).

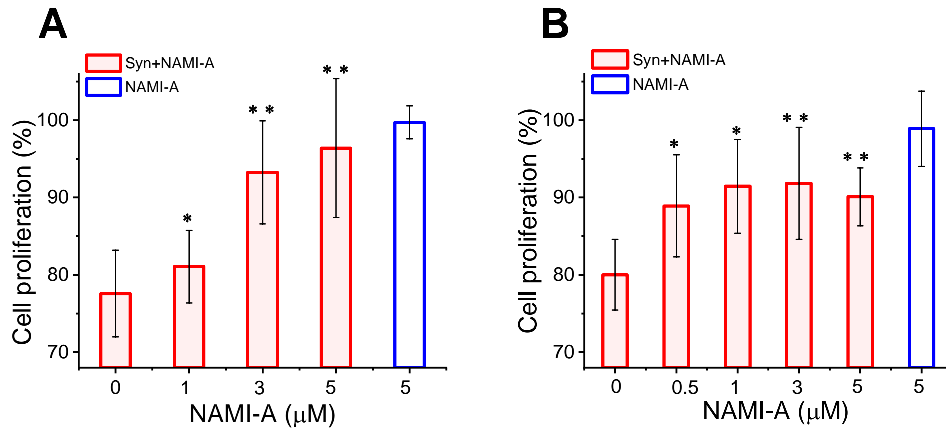


Figure S20. NAMI-A suppresses the cytotoxicity of α-syn oligomers in human neuroblastoma cells. Cell proliferation was determined using the MTT assay. (A) SH-SY5Y cells; (B) PC12 cells. Cells were treated with different concentrations of NAMI-A (0 μM, 1 μM, 3 μM, 5 μM) for 24 h at 37°C. The cells were also treated with NAMI-A in the absence of α-syn oligomers for the cytotoxicity analysis of NAMI-A (blue bars). The data show the mean and standard deviation of three replicates. The statistically significant difference between cells treated with and without NAMI-A is indicated by asterisks. (∗∗ $p \leq 0.01$, ∗ $p \leq 0.05$)

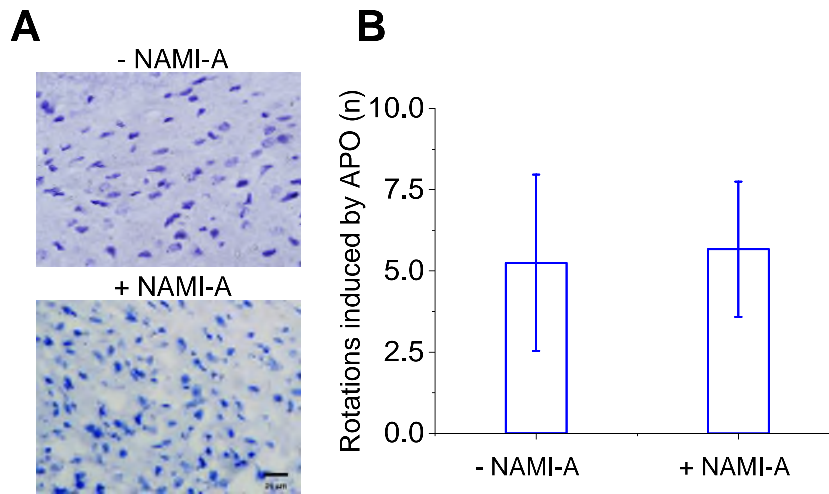
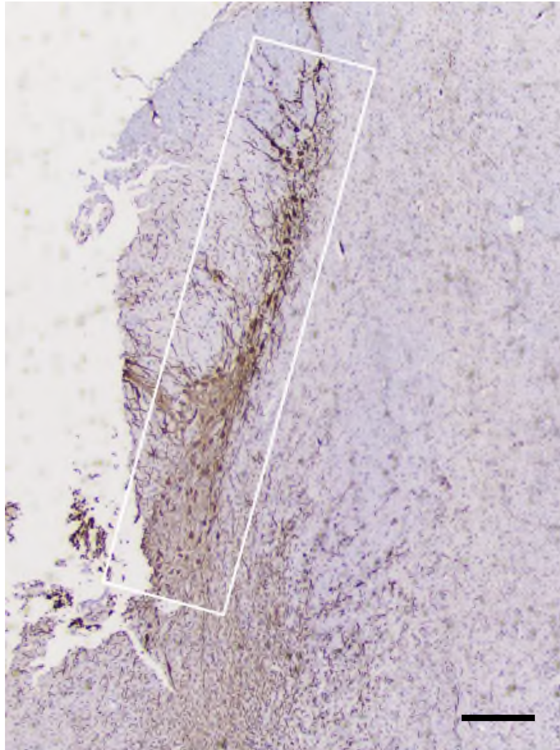
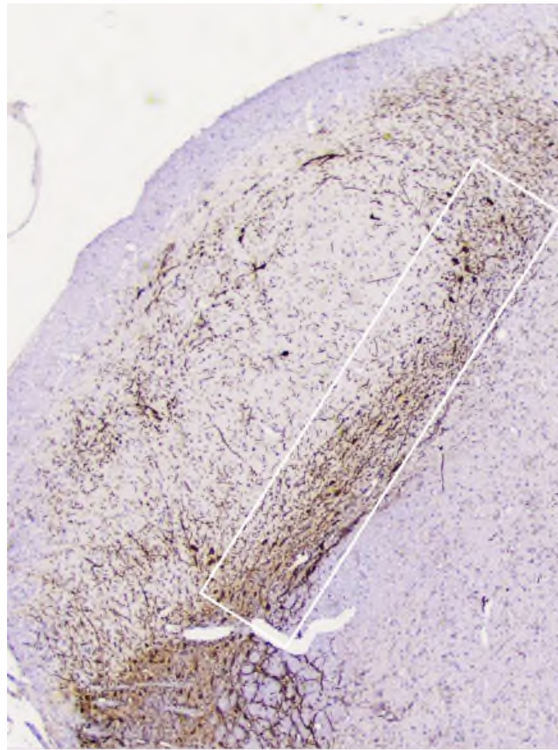


Figure S21. Effect of NAMI-A treatment on substantia nigra and movement of rats. (A) Representative IHC images of pSyn in substantia nigra. (Scale bar: 20 μm). (B) Apomorphine-induced rotation of rats. The rats were injected with 7 nmol NAMI-A. No detectable changes of substantia nigra in the brain or movement were observed after 30 days.





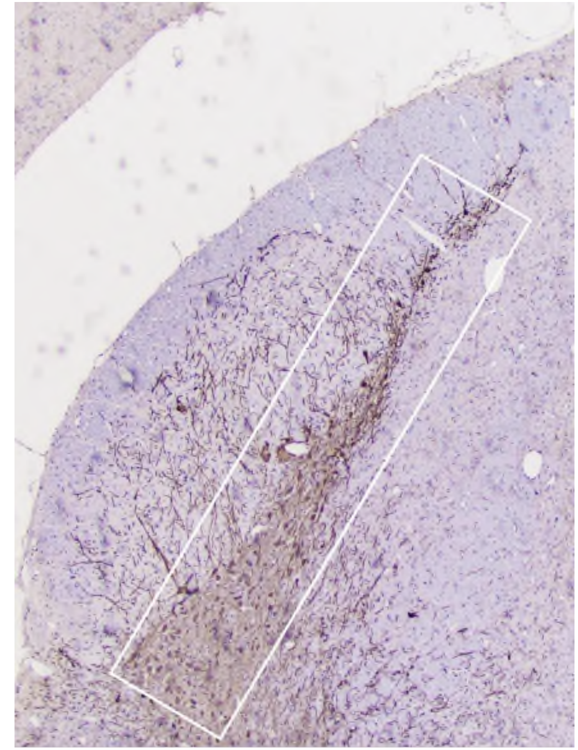


Figure S22. Enlarged images of IHC in TH-positive neurons after treatment with PFFs and NAMI-A injected in striatum (scale bar, 250 μm). The rats were administered 7 nmol NAMI-A on the 30th day after treatment with PFFs (1.4 nmol in monomer).

Control **PFFs induced** **NAMI-A treatment**

Figure S23. Representative photographs of the rats during the rotation test. (A) Rats treated with PBS; (B) Rats treated with PFFs; (C) Rats treated with PFFs and NAMI-A. The rotation test was performed by using apomorphine (APO) induction on the 50th day after the administration of α-syn.

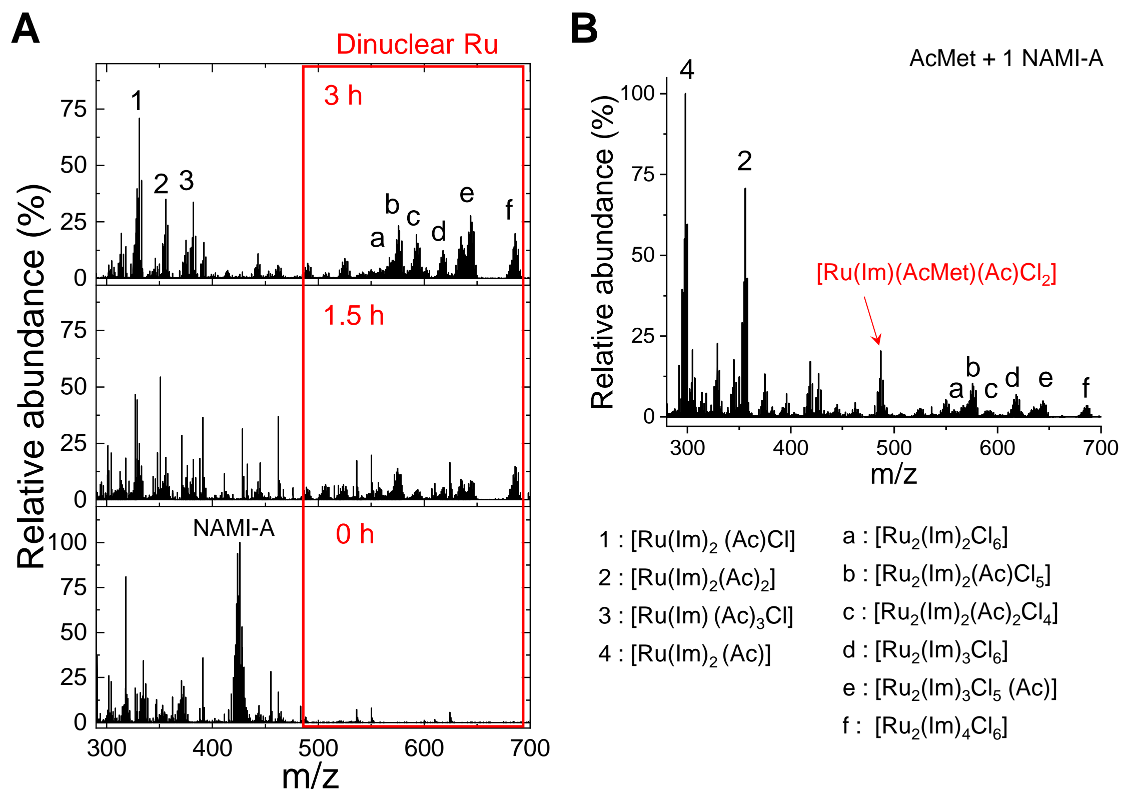


Figure 24. ESI-MS analysis of the speciation of NAMI-A. ESI-MS spectra for solutions of NAMI-A (0.2 mM) after incubation in 50 mM NH_4Ac at 37°C for different times, showing mononuclear (1, 2, 3) and dinuclear (a-f)) species, and (B) ESI-MS analysis of the adducts in the reaction of 50 μM AcMet with 50 μM NAMI-A in 50 mM NH_4Ac at 37°C for 3 h.

3. Supplementary Tables

Table S1. Analysis of the fragments of NAMI-A bound to α-syn based on ESI-MS peaks shown in Figure 1C*.

Composition	Formula	m/z (charge)	MW observed	MW calculated
α-syn	$C_{637}H_{1028}N_{170}O_{221}S_4$	1637.93 (+9) 1842.54 (+8)	14732.35	14732.42
α-syn + $[Ru(Im)]^{3+}$	$C_{640}H_{1032}N_{172}O_{221}S_4Ru$	1656.36 (+9) 1863.28 (+8)	14898.24	14898.62
α-syn + 2$[Ru(Im)]^{3+}$	$C_{643}H_{1036}N_{174}O_{221}S_4Ru_2$	1674.91 (+9) 1884.15 (+8)	15065.20	15064.76
α-syn + $[Ru(Im)(H_2O)]^{3+}$	$C_{640}H_{1034}N_{172}O_{222}S_4Ru$	1658.36 (+9) 1865.53 (+8)	14916.24	14916.63
α-syn + $[Ru(Im)]^{3+}$ + $[Ru(Im)(H_2O)]^{3+}$	$C_{643}H_{1038}N_{174}O_{222}S_4Ru_2$	1676.91 (+9) 1886.40 (+8)	15083.20	15082.78
α-syn + 2$[Ru(Im)(H_2O)]^{3+}$	$C_{643}H_{1040}N_{174}O_{223}S_4Ru_2$	1678.91 (+9) 1888.65 (+8)	15101.20	15100.79

* The reaction was carried out by incubation of α-syn with 5 mol equiv of NAMI-A for 3 h at 37°C, the sample was infused directly after dilution with 50 mM NH_4Ac solution.

Table S2. Ruthenium content of Ru-α-syn determined by ICP-MS after equilibrium dialysis

	Control[a]	Bound Ru[b]	Free Ru
Ru concentration **(μM)**	0.0041	45.57 ± 4.45	3.97 ± 0.29

[a] The final filtrate from ultrafiltration was used as the control.

[b] The Ru-α-syn sample, obtained by reaction of α-syn (50 μM) with 3 ml equiv NAMI-A in 50 mM ammonium acetate buffer pH 7.0 at 37°C for 3 h, was subjected to ultrafiltration to remove unbound Ru, and then equilibrium dialysis for 16 h. The free and bound Ru concentrations were then determined.

4. Supplemental References

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