Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Nintedanib exerts anti-pulmonary fibrosis activity via inhibiting TANK-binding kinase 1 (TBK1) phosphorylation

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1. Supplementary Figures and Table

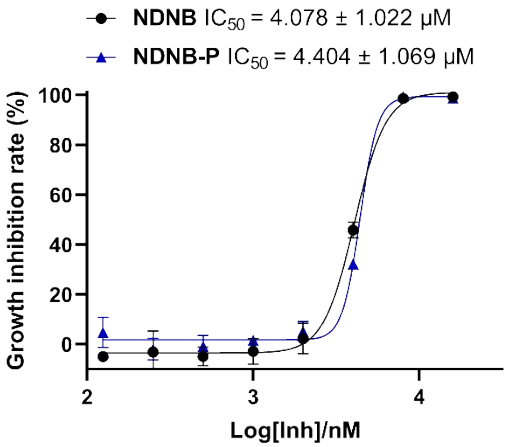


Fig. S1 Antiproliferative effects of NDNB and NDNB-P against HPF cells measured by CCK8 assay. Data are presented as mean ± SEM, n = 4 biological replicates per group. NDNB: nintedanib.

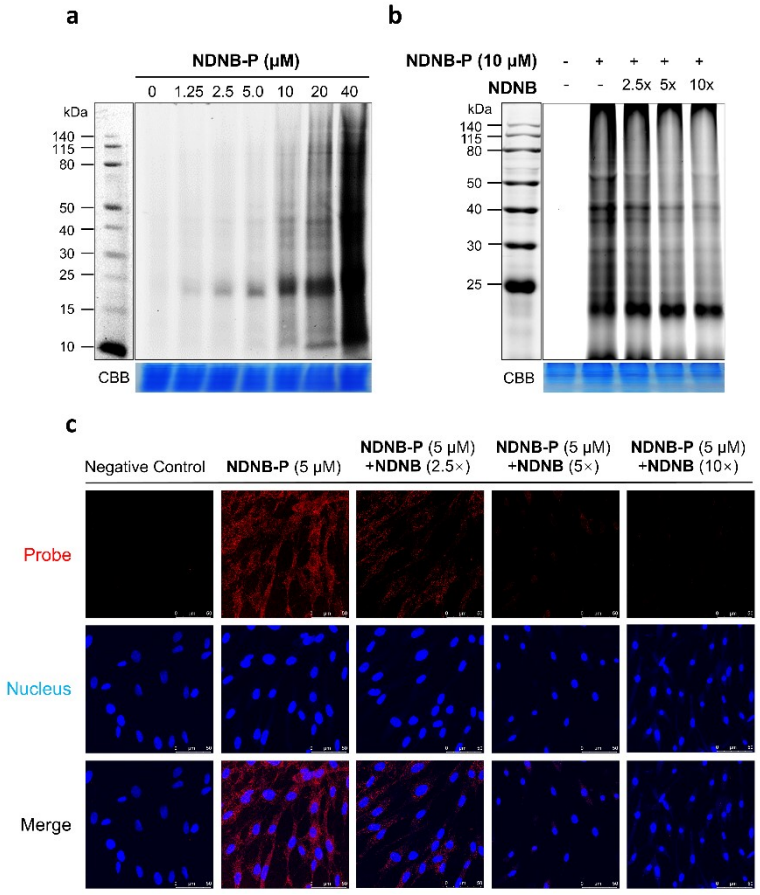


Fig. S2 (a) Photoaffinity labelling of potential targets in HPF cell lysates by increasing concentrations of NDNB-P. CBB = Coomassie staining. (b) In-gel fluorescence image and (c) confocal images of competitive labelling by NDNB-P and excessive NDNB. Blue: nuclear DAPI channel, red: TAMRA channel. Scale bar = 25 μm.

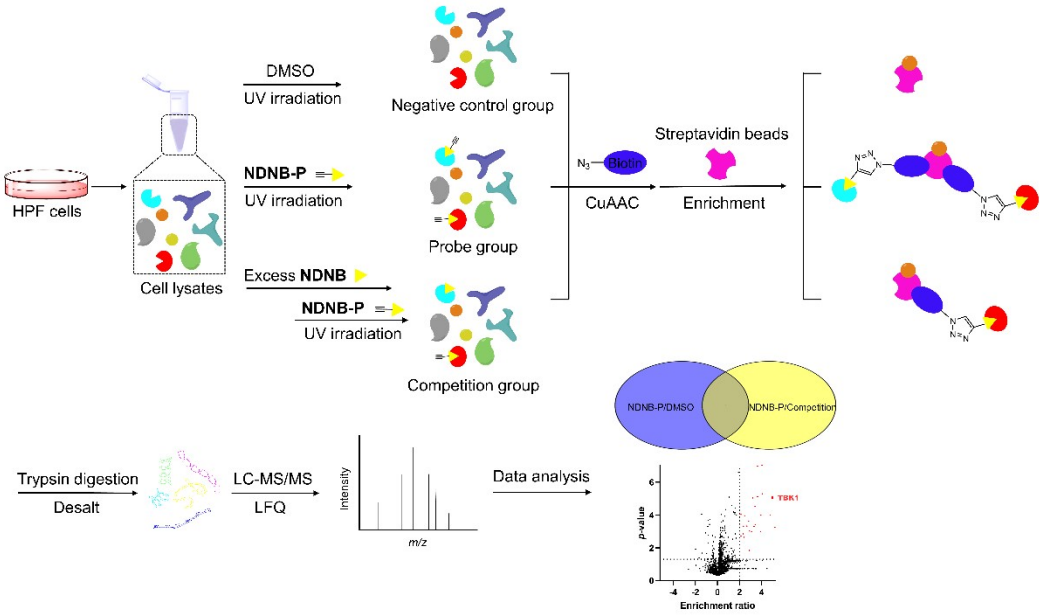


Fig. S3 Schematic workflow for target identification of NDNB with the competitive LC-MS/MS-based ABPP technology. For each group, three biological replicates were performed.

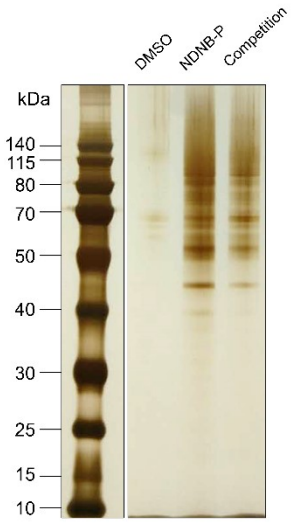


Fig. S4 Silver staining of binding proteins of **NDNB-P** (10 μM) after affinity enrichment in HPF cell lysates.

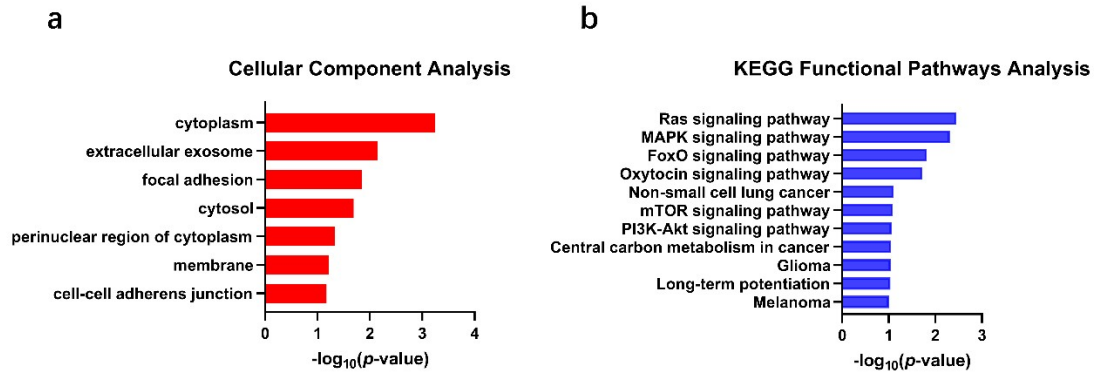


Fig. S5 (a) Gene ontology (GO) cellular component categorization and (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional pathway enrichment analysis of highly reliable NDNB-interacting proteins. The enriched pathways are ranked based on their *p*-values.

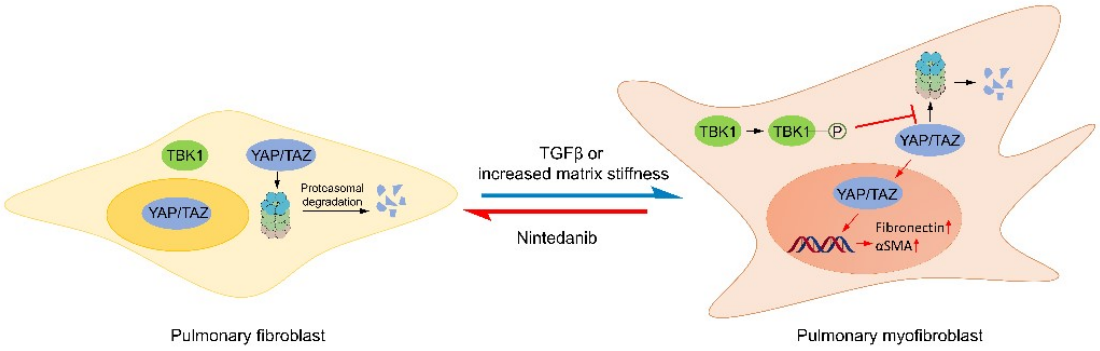


Fig. S6 Schematic representations of anti-pulmonary fibrosis mechanism of NDNB in fibroblasts.

Table S1 and **Table S2** are available in attached excel table.

Table S3. Related information of the 25 protein hits identified by pull-down/LC-MS/MS.

Protein names	Gene names	MW (kDa)	P/competition		P/DMSO	
			Fold change	*p*-value	Fold change	*p*-value
Discoidin domain-containing receptor 2	DDR2	96.7	36.66699	0.00058	80.10444	0.00054
Serine/threonine-protein kinase TBK1	TBK1	73.8	31.40978	0.00001	31.40978	0.00001
5-AMP-activated protein kinase catalytic subunit alpha-1	PRKAA1	64.0	27.26002	0.00010	27.26002	0.00010
Serine/threonine-protein kinase 4; Serine/threonine-protein kinase 4 37kDa	STK4	55.6	16.47388	0.00001	16.47388	0.00001
Cyclin-G-associated kinase	GAK	143.2	16.32325	0.00000	16.32325	0.00000

Protein names	Gene names	MW (kDa)	P/competition		P/DMSO	
			Fold change	*p*-value	Fold change	*p*-value
Serine/threonine-protein kinase 10	STK10	112.1	15.26322	0.00011	15.26322	0.00011
STE20-like serine/threonine-protein kinase	SLK	142.7	15.10847	0.00102	15.10847	0.00102
Mitogen-activated protein kinase kinase kinase kinase 5	MAP4K5	95.0	12.38308	0.00001	12.38308	0.00001
Ribosomal protein S6 kinase alpha-3	RPS6KA3	80.6	12.30435	0.00000	14.32041	0.00000
Non-specific protein-tyrosine kinase; Tyrosine-protein kinase Yes	YES1	61.4	10.62143	0.00040	10.62143	0.00040
Calcium/calmodulin-dependent protein kinase type 1	CAMK1	41.3	10.53791	0.00003	10.53791	0.00003
Ankycorbin	RAI14	110.0	9.96187	0.00050	9.96187	0.00050
Platelet-derived growth factor receptor alpha	PDGFRA; FIP1L1	122.7	9.02660	0.00001	9.02660	0.00001
Ribosomal L1 domain-containing protein 1	RSL1D1	48.2	8.52210	0.00103	8.52210	0.00103
Xaa-Pro aminopeptidase 1	XPNPEP1	62.1	7.46317	0.00095	7.46317	0.00095
Serine/threonine-protein kinase 24; Serine/threonine-protein kinase 24 36 kDa subunit; Serine/threonine-protein kinase 24 12 kDa subunit	STK24	45.8	7.40497	0.01451	16.88733	0.01074
Protein S100-A16	S100A16	11.8	7.08504	0.00025	7.08504	0.00025
Enhancer of rudimentary homolog	ERH	12.3	6.37386	0.00047	6.37386	0.00047
Myotrophin	MTPN	5.7	5.53942	0.00011	5.53942	0.00011
Protein CutA	CUTA	19.1	5.36371	0.00229	19.91779	0.00001
Pyrroline-5-carboxylate reductase 2; Pyrroline-5-carboxylate reductase	PYCR2	33.6	5.09790	0.00048	5.09790	0.00048
Protein C10	C12orf57	13.2	4.93661	0.00140	4.93661	0.00140
Phosphatidylserine synthase 2	PTDSS2	56.3	4.36881	0.00009	4.36881	0.00009
Dual specificity mitogen-activated protein kinase kinase 2	MAP2K2	44.4	4.36714	0.00193	56.93271	0.00055
Testis-expressed sequence 264 protein	TEX264	34.2	4.00336	0.00009	4.00336	0.00009

2. Supplementary Methods

2.1 General Information

Chemistry Materials

Solvents and reagents were purchased from commercial suppliers and used without further purification. **NDNB-COOH** was synthesized in-house as previously reported.

Reaction progress was monitored by silica gel plates GF254 (Yantai chemical research institute, China) with detection by UV (λ = 254 or 365 nm). Flash chromatography was performed on gel (90–150 mm; Qingdao Marine Chemical Inc.) with the indicated eluent. NMR spectra (^{1}H-NMR, ^{13}C-NMR) were recorded on Bruker AVANCE III HD 600 instrument at room temperature. Chemical shifts were reported in parts per million (ppm) referenced with respect to appropriate internal standards (TMS) or residual solvent peaks ($CDCl_3$ = 7.26 ppm for ^{1}H-NMR; $CDCl_3$ =77.00 ppm for ^{13}C-NMR). The following abbreviations were used in reporting spectra, s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublets), td (triplet of doublets) and coupling constant (Hz). High-resolution mass spectra (HRMS) were obtained on an Agilent Q-TOF 6550 mass spectrometer by electrospray ionization–time-off light (ESI-TOF).

Cell culture

Human pulmonary fibroblasts (HPF) cell line was obtained from ScienCell (#3300) and cultured in Fibroblast Medium (FM, ScienCell, #2301) containing 2% (v/v) fetal bovine serum (FBS, ScienCell), 1% (v/v) fibroblast growth supplement (FGS, ScienCell) and 1% (v/v) penicillin/streptomycin solution (P/S, ScienCell) at 37 °C with 5% (v/v) CO_2 in a water-saturated incubator.

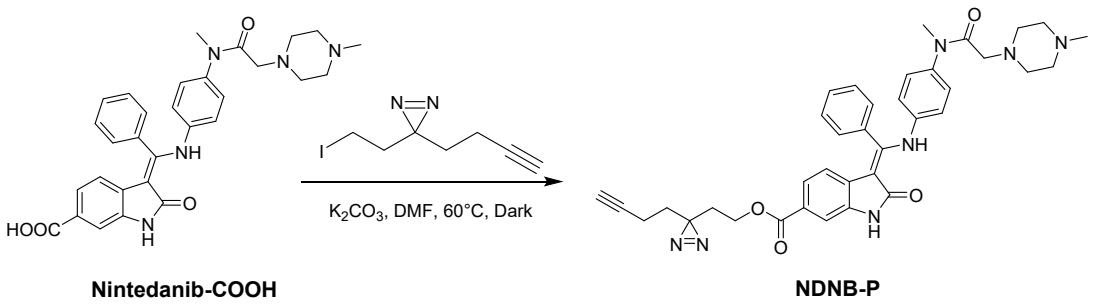
Western blotting

HPF cells were collected by scraping and homogenized with RIPA buffer (APPLYGEN, #C1053) containing protease (MedChemExpress, #HY-K0010) and phosphatase inhibitors (MedChemExpress, #HY-K0021, #HY-K0022). The supernatant of soluble proteins was obtained by centrifugation (16000 × g, 15 min, 4 °C). Protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific, #23225) and the concentration of each group was adjusted to the same level. Samples were added with 5× SDS-PAGE sample buffer (GenStar, #E153-01) and boiled at 100 °C for 10 min. Then samples with equal volume were loaded on Blot™ 4–12 % Bis-Tris Plus gel (Invitrogen, #NW04120BOX) and subsequently protein transfer was performed using semi-dry iBlot 2 Dry System (Invitrogen,

#IB21001) and iBlot 2 PVDF Regular Stacks (Invitrogen, #IB24001). Membranes were blocked with 5% (w/v) bovine serum albumin (BSA) (Sigma Aldrich, #V900933) / TBST (Cell Signaling Technology, #9997S) at room temperature for 1 h, then incubated at 4 °C overnight with the desired primary antibodies followed by washing three times with TBST. Membranes were incubated with corresponding secondary antibodies at room temperature for 1 h. Immunoblots were imaged following addition of ECL chemiluminescent substrate (Tanon, #180-506) using a Tanon-5200 Chemiluminescent Imaging System.

2.2 Synthetic Procedures

Synthesis of 2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethyl (Z)-3-(((4-(N-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-6-carboxylate (NDNB-P).



Scheme S1. Synthetic route of **NDNB-P**

Nintedanib-COOH was synthesized based on previously published procedures by our group.[1]

To a solution of **Nintedanib-COOH** (158 mg, 0.3 mmol, 1.0 equiv) in 3 mL anhydrous DMF was added anhydrous K_2CO_3 (63 mg, 0.45 mmol, 1.5 equiv), followed by addition of 3-(but-3-yn-1-yl)-3-(2-iodoethyl)-3*H*-diazirine (67.5 μL, 0.45 mmol, 1.5 equiv). The reaction mixture was stirred at 60 °C for 12 h in dark conditions. The resulting reaction mixture was subsequently poured into water (30 mL) and extracted with DCM (3 × 10 mL). The combined organic phase was separated, washed with water (3 × 20 mL) and saturated sodium chloride (20 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified

by flash column chromatography on silica (DCM: MeOH = 12:1) to give product **NDNB-P** (167 mg, 86% yield) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.21 (s, 1H), 9.66 (s, 1H), 7.65 (d, *J* = 1.2 Hz, 1H), 7.57 (m, 1H), 7.52 (m, 2H), 7.42 (d, *J* = 7.2 Hz, 2H), 7.40 (dd, *J* = 8.4, 1.2 Hz, 1H), 6.97 (d, *J* = 8.4 Hz, 2H), 6.80 (d, *J* = 8.4 Hz, 2H), 6.01 (d, *J* = 8.4 Hz, 1H), 4.16 (t, *J* = 6.0 Hz, 2H), 3.17 (s, 3H), 2.80 (s, 2H), 2.45 (m, 8H), 2.29 (s, 3H), 2.00 (td, *J* = 7.2, 2.4 Hz, 2H), 1.96 (t, *J* = 2.4 Hz, 1H), 1.82 (t, J = 6.0 Hz, 2H), 1.67 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1, 169.3, 166.5, 158.3, 139.8, 137.9, 135.7, 132.3, 130.5, 129.6 (ß2), 129.3, 128.5 (ß2), 127.8 (ß2), 124.8, 123.9 (ß2), 122.7, 118.2, 110.4, 98.4, 82.5, 69.3 (ß2), 59.4 (ß2), 54.7 (ß2), 52.9, 45.7, 37.3, 32.4, 32.1, 26.4, 13.2. ESI-HRMS (*m/z*): $[M+H]^+$ calculated for $C_{37}H_{40}N_7O_4$: 646.3142, found: 646.3132.

2.3 Biological Procedures

FGFR1 Enzyme Inhibition Assay

The FGFR1 inhibition assays were obtained using the HTRF® KinEASE TK kit (Cisbio, #62TK0PEC) and procedure. For this experiment, three groups were set up, including complete activity control (inhibitor-free, MAX), non-activity negative control (ATP-free, NEG) and sample (different concentrations of compound). The kinase reaction was performed in HTRF 96-well low volume plate with a reaction mixture of final volume of 10 μL in kinase buffer, containing 5 mM $MgCl_2$, 1 mM DTT, 0.0123 ng/μL FGFR1 (Thermo Fisher, #PV 3146), serially diluted compound in DMSO, 1 μM TK Substrate-biotin and 99.99 μM ATP. The final concentrations of NDNB and **NDNB-P** were in the range of 10 μM–3 nM. The reaction was initiated with ATP in and kept going for 50 min at 25 °C. The reaction was stopped by adding 5 μL Streptavidin-XL665 and 5 μL TK Antibody-Cryptate prepared with detection buffer. After incubation at 25 °C for 60 min, the emission signals of 665 nm and 620 nm for each individual well were detected with 330 nm excitation by BioTek Synergy H1 microplate reader, then the reader automatically calculates the ratio (665 nm / 620 nm * 10000). According to the formula, (RatioMAX-Ratiosample) / (RatioMAX-

RatioNEG) * 100, the inhibition rate (%) of kinase activity was calculated, followed by determination of IC_{50} values of NDNB and **NDNB-P** for FGFR1 using GraphPad Prism version 8.4.2.

TBK1 Enzyme Inhibition Assay

The TBK1 inhibition assay of NDNB was detected using ADP-Glo™ Kinase Assay kit (Promega, #V9102) and procedure. For this experiment, three groups were set up, including positive control (enzyme-free), negative control (inhibitor-free) and sample (different concentrations of compound). The kinase reaction was carried in 384-well plate with a reaction mixture of final volume of 4 μL in 40 mM Tris buffer, containing 20 mM $MgCl_2$, 0.1% BSA (w/v), 0.5 mM DTT, 1 nM TBK1 (Carna, #05-115), serially diluted compound in DMSO, 0.1 mg/mL MBP substrate and 40 μM ATP. The final concentrations of NDNB were in the range of 100 μM–1.53 nM. The reaction was initiated with enzyme/ATP mixture in and kept going for 60 min at 25 °C. The reaction was stopped by adding 4 μL ADP-Glo reagent. After incubation at 25 °C for 40 min to consume unreacted ATP, 8 μL kinase detection reagent was added and incubated for additional 40 minutes to convert ADP to ATP and generate luminescence signals. Relative luminescence unit signals were measured by BioTek Synergy 4 microplate reader. The inhibition rate (%) of kinase activity was calculated according to the formal, $[1-(Lum_{sample}-Lum_{positive}) / (Lum_{negtive} - Lum_{positive})]* 100$, IC_{50} value of NDNB for TBK1 was determined using GraphPad Prism version 8.4.2.

Cell proliferation inhibition assay

The cytotoxicity of NDNB and **NDNB-P** against HPF cells were assessed by CCK-8 (MedChemExpress, #HY-K0301) assay according to the manufacturers protocol. 12000 cells per well were plated in a 96-well plate and grown to 80% confluence after cultured at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 for 24 h. Then NDNB or **NDNB-P** were incubated with HPF cells at different final concentrations (0.125 μM to 16 μM) for 48 h. Considering that both NDNB and **NDNB-P** have bright color, the supernatants were removed to avoid the influence of compounds on absorbance. Subsequently, 100 μL CCK-8 solution (10 μL CCK-8: 100 μL medium)

(10%) was added to each well and the absorbance at 450 nm was measured by TECAN M200 microplate reader after incubation for 2 h. IC_{50} values were calculated using GraphPad Prism version 8.4.2.

Evaluation of anti-pulmonary fibrosis activity (*in cellulo*)

15000 cells per well were plated in a 6-well plate and grown to 80% confluence after cultured at 37 °C in a humidified atmosphere with 5% (v/v) CO2 for 24 h. Then HPF cells were treated with 10 ng/mL TGFβ and 2 μM NDNB or NDNB-P for 48 h before harvesting, followed by cell lysis, protein extraction, and western blotting analysis.

Gel-based Photoaffinity Proteome Labeling (*in vitro*)

For *in vitro* labeling, procedures were based on previously published protocols with the following optimizations.[2] HPF cells were seeded in T75 cell culture flask to 80% confluence and collected by digesting with 0.05% trypsin (Gibco, #25300054) and centrifuging (1000 × g, 5 min, 20 °C). Then the cells were resuspended in cell lysate (PBS with 0.2% (v/v) Triton X-100, protease and phosphatase inhibitors) and lysed by sonication (power 20%, 2s on/2s off, total time 1.0 min). Following centrifugation (16000 × g, 15 min, 4 °C), the supernatant was divided in different administration groups, then the mixture was incubated for 1 h at 37 °C and followed by UV-irradiated (365 nm) for 40 min. Proteins were precipitated by the addition of a 4-fold volume excess of pre-chilled acetone and incubated overnight at –20 °C. The precipitated proteins were subsequently collected by centrifugation (16000 × g, 15 min, 4 °C), and washed with 200μL pre-chilled methanol twice and air dried. This step is meant to remove excess compounds. Then 200 μL 0.1% (v/v) SDS/PBS was added to dissolve the protein by sonication (power 20%, 2s on/2s off, total time 1.0 min). After centrifugation (16000 × g, 15 min, 4 °C), the supernatant was incubated with a pre-mixture of click reagents (100 μM TAMRA-azide from 10 mM stock solution in DMSO, 1 mM TCEP from 100 mM stock solution in ddH_2O, 100 μM TBTA from 10 mM stock solution in DMSO, 1 mM $CuSO_4$ from 100 mM stock solution in ddH_2O) and gently rotated at room temperature for 1 h. Proteins were precipitated, centrifuged, and adjusted to the same concentration as above, 5× SDS-

PAGE sample buffer was added and then heated for 10 min at 100 °C. For gel-based analysis, 20 μL samples were loaded to a NuPAGE 4–12% Bis-Tris gel (Invitrogen, #NP0335BOX) and run at 120 V with MES running buffer (Invitrogen, #NP0002). The gels were scanned with a Typhoon FLA 9500 Biomolecular Imager (GE Healthcare) with 532 nm laser excitation for TAMRA and 635 nm (Cy5) for pre-stained marker. To stain the total proteins, the gel was subsequently incubated with Coomassie brilliant blue reagent (Invitrogen, #LC6065).

Microscopy-based imaging assay (*in cellulo*)

Referring to the previously reported procedures.[3,4] HPF cells were seeded in 24-well plates containing cell slide (solarbio, #YA0350) and grown to 70% confluence after 24 h. For the competitive photoaffinity labeling assay, cells were preincubated with 0.5 mL of FM containing DMSO, 25, 50, 100 μM NDNB for 1 h, respectively, then treated with DMSO or 10 μM **NDNB-P** for another 1 h. The medium was discarded and the cells were gently washed twice with PBS (0.5 mL), followed by UV irradiation (365 nm) with ice cooling for 5 min. Subsequently the cells were fixed for 30 min at room temperature with 4% (v/v) formaldehyde in PBS, washed three times with cold PBS, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min. For click chemistry, click reagents (100 μM TAMRA-azide from 10 mM stock solution in DMSO, 1 mM TCEP from 100 mM stock solution in ddH_2O, 100 μM TBTA from 10 mM stock solution in DMSO, 1 mM $CuSO_4$ from 100 mM stock solution in ddH2O) were added to the cells in a 200 μL volume and the cells were gently rotated at room temperature for 2 h. Then cells were washed with PBS three times, followed by once with PBST (0.1% (v/v) Tween-20). Finally, nuclear were stained with DAPI (ZSGB-BIO, # ZLI-9557) prior to imaging. For co-localization assay, cells were blocked with 5% (w/v) BSA in TBS for 1 h before click chemistry and incubated with anti-TBK1 antibody (1:200, Cell Signaling Technology, #38066S), after an additional incubation for 2 h at 37°C, the plates were stored overnight at 4°C. Then washed three times with TBST, and incubated with 1% (w/v) BSA/TBS containing Alexa Fluor 488 goat anti-rabbit IgG H&L (1:500, ZSGB-BIO, #ZF-0511)

at 37°C for 2 h, followed by washing again. For immunostaining assay, HPF cells were seeded in 24-well plates containing cell slide and grown to 70% confluence after 24 h. In negative control group, 0.1% (v/v) DMSO was added. In model group, 10 ng/mL TGFβ was added. In NDNB group, 10 ng/mL TGFβ and 2 μM NDNB was added. After incubating for 48 h, immunostaining steps with anti-YAP1 (proteintech, #13584-1-AP) antibody and anti-TAZ antibody (proteintech, #23306-1-AP) were identical to those described above. Imaging was done with the Leica TCS SP8 confocal microscope.

Pull-down experiments

To identify and validate the direct interacting targets of NDNB, pull-down experiments were carried out and followed by LC-MS/MS or western blotting. The general procedures were similar to previously published protocols.[1] HPF cells were seeded in T75 cell culture flask to 80% confluence and collected by digesting with 0.05% trypsin and centrifuging (1000 × g, 5 min, 20 °C). Then the cells were resuspended in cell lysate (PBS with 0.2% (v/v) Triton X-100, protease and phosphatase inhibitors) and lysed by sonication (power 20%, 2s on/2s off, total time 1.0 min). Following centrifugation (16000 × g, 15 min, 4 °C), the supernatant was divided in three groups: negative control group, probe group and competition group, and treated with different compounds, followed by UV-irradiated (365 nm) for 40 min. Proteins were precipitated by the addition of a 4-fold volume excess of pre-chilled acetone and incubated overnight at –20 °C. The precipitated proteins were subsequently collected by centrifugation (16000 × g, 15 min, 4 °C), and washed with 200 μL pre-chilled methanol twice and air dried. This step is meant to remove excess compounds. Then 200 μL 0.1% (v/v) SDS/PBS was added to dissolve the protein by sonication (2s on/4s off, 1 min). After Centrifugation (16000 × g, 15 min, 4 °C), the supernatant was incubated with a pre-mixture of click reagents (100 μM biotin-azide from 10 mM stock solution in DMSO, 1 mM TCEP from 100 mM stock solution in ddH2O, 100 μM TBTA from 10 mM stock solution in DMSO, 1 mM $CuSO_4$ from 100 mM stock solution in ddH2O) and gently rotated at room temperature for 1 h.

Proteins were precipitated and centrifuged as above. For enrichment, the pellet was dissolved in 1.0 mL of PBS with 0.2 % (v/v) SDS by sonication (power 20%, 2s on/2s off, total time 1.0 min). The protein solution was centrifuged (16000 × g, 15 min, 4 °C) and the supernatant was transferred to 1.5 mL protein LoBind microcentrifuge tubes (Eppendorf, #0030108442). The streptavidin agarose beads (Thermo Scientific, #20349) were pre-washed with PBS (3 × 1.0 mL) and centrifugation was performed at 3000 × g for 3 min each time. 100 μL beads (50% slurry) were added to each sample and incubated under gentle shaking at 20 °C for 4 h. The beads were pelleted by centrifugation (3000 × g, 3 min), washed successively with 1% (v/v) SDS in PBS (3 × 1 mL), 6M Urea in PBS (3 × 1 mL) and PBS (3 × 1 mL). For the first of each set of washes, the beads were rotated for 10 min at room temperature. Finally, the beads were pelleted by centrifugation (3000 × g, 3 min), prepared for subsequent on-bead digestion. To evaluate the efficiency of enrichment, 50 μL 2× SDS-PAGE sample buffer was added and heated for 5 min at 100 °C. The enriched proteins from beads described above were separated by SDS-PAGE and then silver stain (Invitrogen, #24612) according to the manufacturer's instructions. To validate the identified target, part of protein supernatant (80 μL) was not incubated with beads and served as an input. Add 50 μL 2× SDS-PAGE sample buffer and heated for 5 min at 100 °C, samples were analyzed by western blotting by anti-TBK1 antibody.

On-bead digestion

The on-bead proteins were reduced with 20 mM dithiothreitol (DTT) in 200 μL ammonium bicarbonate (ABC) at 56 °C for 1 h under condition of gentle vortex. The alkylated reaction was completed by addition of 50 mM iodoacetamide (IAA) at room temperature for 45 min in the dark. Once completion of the reaction, the beads were pelleted by centrifugation (3000 × g, 3 min) and washed with 6M urea (1 × 200 μL) and ABC (2 × 200 μL). The on-bead proteins were resuspended in 100 μL ABC, vortexed for 1 min. Sequencing grade modified trypsin (Promega, #V5111) was added at 1:50 enzyme/substrate ratio and the digestion was firstly performed at 37 °C for 12 h, followed by 4 h with addition of trypsin (1:100). The digestion was stopped

by addition of 0.1% (v/v) formic acid (FA). The beads were vortexed and centrifuged (13000 × g, 4 °C, 5 min) to collect the supernatant, followed by wash with 200 μL ABC three times and combination of flow-through with the previous fraction. The digested peptides were transferred to new LoBind tubes, for desalting, the Oasis HLB 3 cc Vac cartridges (Waters, #WAT094226) was pre-treated with 3 mL MeOH once, 3 mL 0.1% (v/v) FA-H_2O twice prior to sample loading. The solution was loaded to the cartridges and washed with 3 mL 0.1% (v/v) FA-H_2O three times and 3 mL 0.1% (v/v) FA-2% (v/v) ACN/H_2O twice. The peptides were eluted with 1mL 80% (v/v) ACN/0.1% (v/v) FA, dried in the speed vacuum system and stored at –80 °C until LC-MS/MS analysis.

Nano LC-MS/MS Analysis

Before analysis, the peptides were reconstituted in 10 μL of 0.1% (v/v) formic acid. LC-MS/MS was performed on an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Scientific) coupled with EASY-nLC 1100 System. For each sample，2 μL of volume was loaded onto C18 PepMap100 trapcolumn (300 μm×5 mm) and eluted on a Thermo Acclaim PepMap RPLC analytical column (150 μm×15 cm). A procedure of 78 min gradient for each single-shot analysis was performed as followed: 6–9% B in 8 min, 9–14% B in 16 min, 14–30% B in 36 min, 30–40% B in 15 min, 40–95% B in 3 min (A = 0.1% (v/v) formic acid in water，B = 0.1% (v/v) formic acid in 90% (v/v) acetonitrile) .The flow rate was 0.3 μL/min. Data-dependent mode was operated for the mass spectrometer, with a full MS scan (300–1400 *m/z*) and 3 s cycle time was set. The MS spectra were acquired at a resolution of 60,000 with an automatic gain control (AGC) target value of 5×10^5 ions or a maximum integration time of 50 ms. High energy collision dissociation (HCD) with the energy set at 35 NCE was used to perform peptide fragmentation. The MS/MS spectra were acquired in the top 15 or 20 most intense precursors at a resolution of 15,000 with an AGC target value of 1×10^4 ions or a maximum integration time of 35 ms.

MS Data Analysis

The raw MS files were processed and searched against uniprot-Homo sapiens

database based on the species of the samples using MaxQuant (1.6.2.10). The mass tolerance was set to 20 ppm and 20 ppm for the precursor and the fragment ion respectively, with up to two missed cleavages allowed. Carbamidomethyl (+57.021 Da) was used as a fixed modification and oxidation and (M) was used as a variable modification. The results of protein identification were filtered with the criteria of mass tolerance less than 10 ppm for peptides and false positive rate less than 1% at the protein level. Label-free quantification was used to quantify the difference of protein abundances between different groups. Only proteins identified by at least two unique peptides were retained for further quantitative analysis. Protein ratios were calculated as the median of all peptide hits belonging to a protein and *p*-value obtained from the *t*-test analysis (one-tailed distribution, two-sample, equal variance) over three biological replicates.

Analysis of NDNB-interacting proteins in human proteome

Gene ontology and KEGG pathway analysis were performed using DAVID bioinformatics resources portal (https://david.ncifcrf.gov/).[5]

Cytoplasmic and Nuclear Extraction

Minute™ cytoplasmic and nuclear extraction kit (Invent Biotechnologies, #SC-003) was used for separating the nuclear and cytoplasmic cellular fractions by enrichment, as per the manufacturer's protocol. Briefly, HPF cells were seeded in 10 cm plate and grown to 80% confluence after 24 h. In the negative control group, 0.1% (v/v) DMSO was added. In the model group, 10 ng/mL TGFβ was added. In the NDNB group, 10 ng/mL TGFβ and 2 μM NDNB were added. After 48 h incubation, wash the cells twice in the cell culture plates using cold PBS, then harvested cells by cell scratcher and low-speed centrifugation (1000 × g, 5 min, 20 °C). Aspirate the supernatant completely, add 50 μL cytoplasmic extraction buffer containing protease and phosphatase inhibitors to cell pellets, vortex the tube vigorously for 1 min, incubate on ice for 20 min, and vortex briefly to minimize the contamination in the nuclear fraction. Then centrifuge the tube (18000 × g, 5 min, 4 °C) and transfer the supernatant to a fresh pre-chilled 1.5 mL tube to obtain the cytosol fraction. Wash the

pellet with 0.5 mL cold PBS to reduce contamination of cytosolic proteins, then add 25 μL nuclear extraction buffer containing protease and phosphatase inhibitors to the pellet, vortex vigorously for 30 s, incubate the tube on ice for 5 min, repeat this step 4 times. Immediately transfer the nuclear extract to a pre-chilled filter cartridge with a collection tube and centrifuge (18000 × g, 30 s, 4 °C). The filtrate was collected as the nuclear fraction.

Cellular Thermal Shift Assay (CETSA)

The CETSA experiments were performed similarly as published protocols.[6,7] For the temperature-dependent assay, HPF cells were seeded in 10 cm cell culture dishes and grown to 80% confluence. Then the cells were treated with NDNB (10 μM in fresh medium) or DMSO control (0.1%) for 1 h, after washing with PBS twice, harvested with a cell scraper. The cell suspensions were centrifuged (1000 × g, 5 min, 20 °C) and the supernatant was removed to yield cell pellets. The pellets were resuspended in 250 μL PBS containing protease inhibitor and phosphatase inhibitors, and distributed into each 0.2 mL PCR tubes with 20 μL of cell suspensions. The tubes were heated individually at different temperatures from 38 to 56 °C for 3 min, followed by cooling for another 3 min at room temperature. To extract the total proteins, cells were freeze-thawing a total of 3 cycles (frozen in liquid nitrogen for 2 min and thawed in a 37 °C water bath for 5 min). After centrifugation (15000 × g, 10 min, 4 °C), the soluble fractions were isolated. 20 μL of supernatant were mixed with 5 μL of 5× loading buffer and the samples were subjected to western blotting analysis of TBK1 as described above.

For the concentration-dependent assay, HPF cells were seeded in 6 well-plate and grown to 80% confluence. Then the cells were treated with different concentrations of NDNB (0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 μM in fresh medium) or DMSO control (0.1%) for 1 h. The cells were harvested, lysed and analyzed in the same manner as for temperature-dependent assay except the heated temperature is 50 °C.

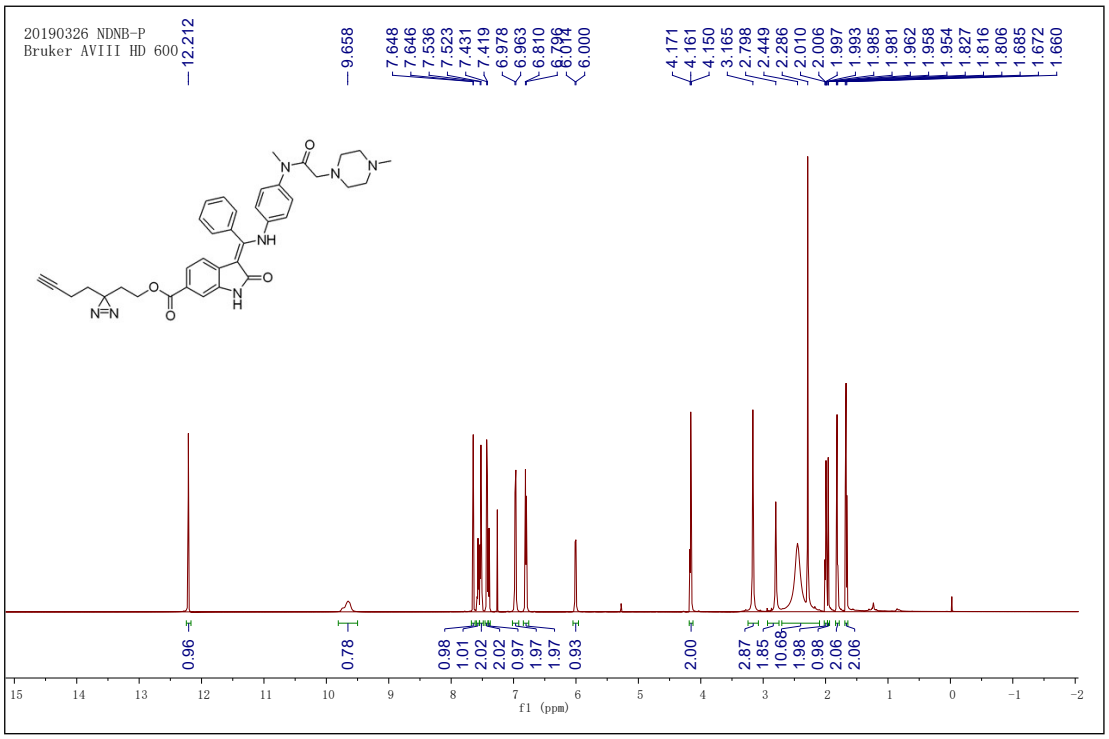
TBK1 hyperphosphorylation assay

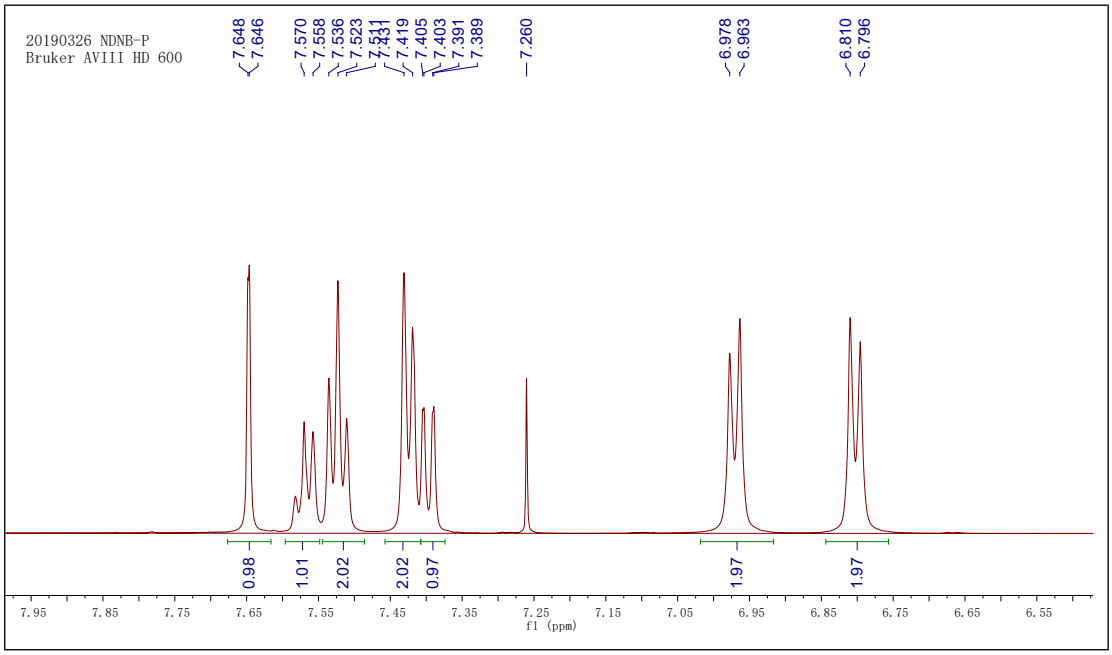
cGAS/STING/TBK1 signaling pathway activator 2’3’-cGAMP (Invivogen, #tlrl-

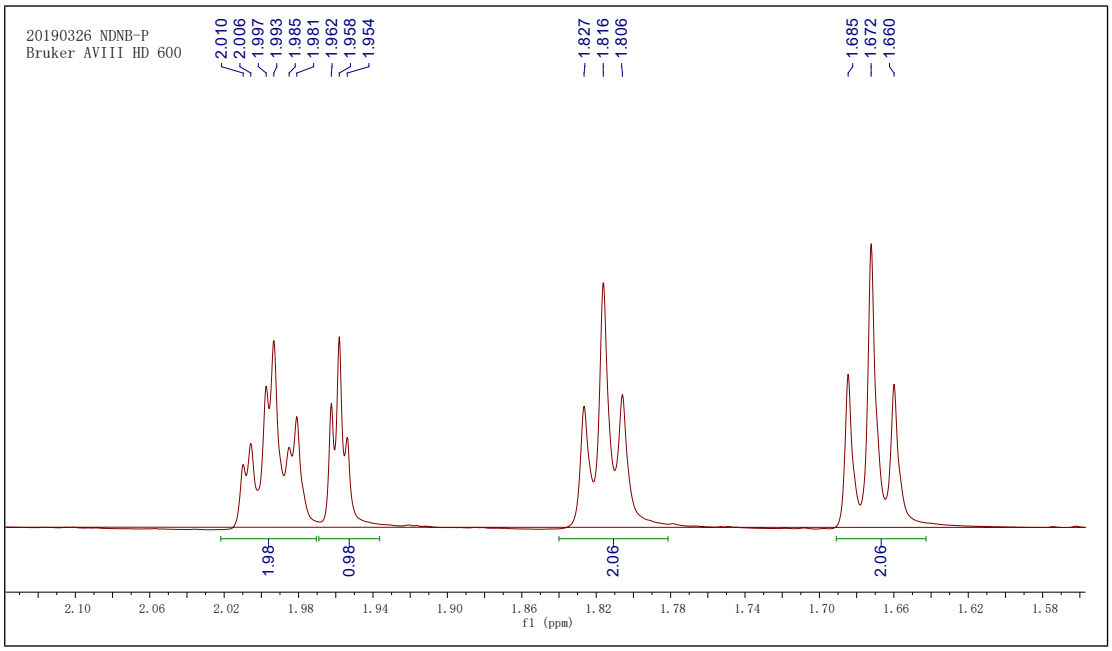
nacga23-1) and RIG-1/MAVS/TBK1 signaling pathway activator Poly (dA:dT)/LyoVec (Invivogen, #tlrl-patc) were respectively used for activating TBK1 phosphorylation according to the manufacturer's protocol. Briefly, HPF cells were seeded in 12 well-plate and grown to 80% confluence after 24 h. 70 μg/mL 2'3'-cGAMP or 2 μg/mL Poly (dA:dT)/LyoVec was added to stimulate cells for 24 h. Then the cells were treated with 10 ng/mL TGFβ and 2 μM NDNB for another 24 h before harvesting, followed by cell lysis, protein extraction, and western blotting analysis.

3. Related NMR Spectra

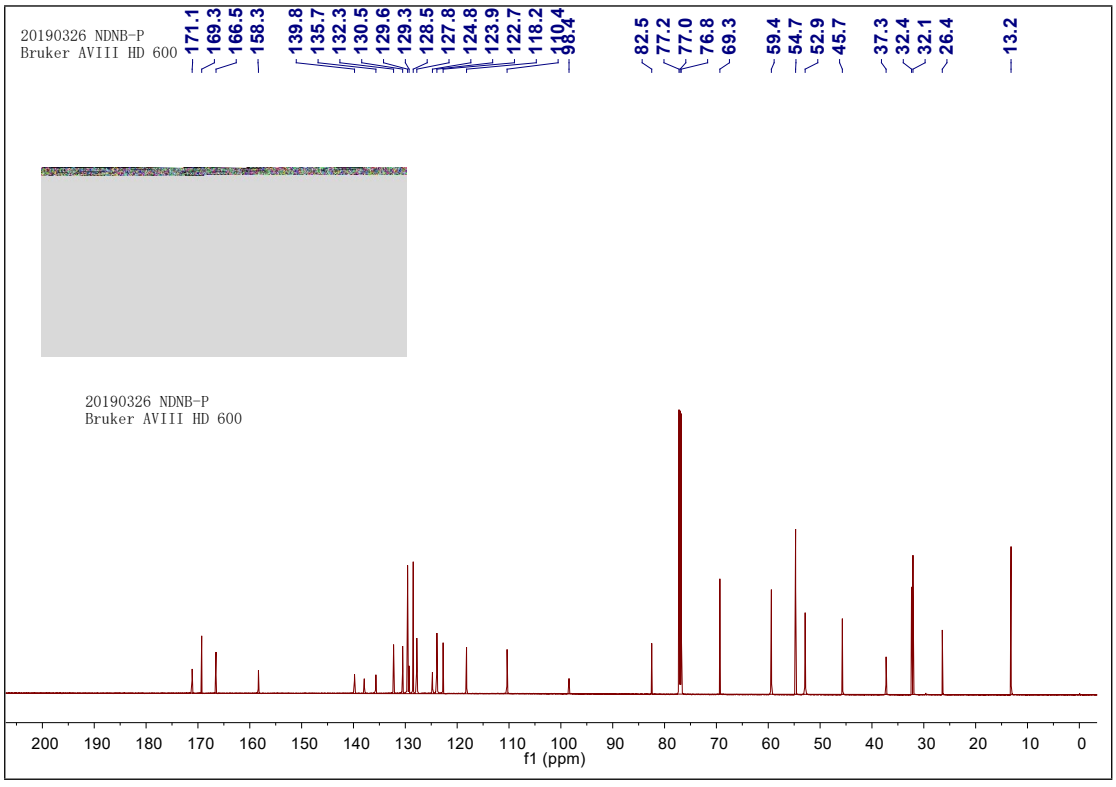
The ^{1}H NMR of NDNB-P in $CDCl_3$







The ^{13}C NMR of NDNB-P in $CDCl_3$



4. References

1 X. Chen, M. Li, M. Li, D. Wang and J. Zhang, *Chem Commun*, 2021, **57**, 3139-

3142.
2 D. Zhu, H. Guo, Y. Chang, Y. Ni, L. Li, Z. M. Zhang, P. Hao, Y. Xu, K. Ding and Z. Li, *Angew. Chem. Int. Ed*, 2018, **57**, 9284-9289.
3 N. Ma, Z. M. Zhang, J. S. Lee, K. Cheng, L. Lin, D. M. Zhang, P. Hao, K. Ding, W. C. Ye and Z. Li, *ACS Chem Biol*, 2019, **14**, 2546-2552.
4 H. Shi, C. J. Zhang, G. Y. Chen and S. Q. Yao, *J Am Chem Soc*, 2012, **134**, 3001-3014.
5 W. Huang da, B. T. Sherman and R. A. Lempicki, *Nat Protoc*, 2009, **4**, 44-57.
6 C. L. Gao, G. G. Hou, J. Liu, T. Ru, Y. Z. Xu, S. Y. Zhao, H. Ye, L. Y. Zhang, K. X. Chen, Y. W. Guo, T. Pang and X. W. Li, *Angew. Chem. Int. Ed*, 2020, **59**, 2429-2439.
7 R. Jafari, H. Almqvist, H. Axelsson, M. Ignatushchenko, T. Lundback, P. Nordlund and D. Martinez Molina, *Nat Protoc*, 2014, **9**, 2100-2122.