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Supplementary Information for

Ratiometric two-photon microscopy reveals attomolar copper buffering in normal and Menkes mutant cells

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This PDF file includes:

Figs. S1 to S12
Supplementary text (materials and methods)
NMR spectra of crisp-17, crisp-17ctrl, and synthetic intermediates (Figs. S13 to S26)
References for SI reference citations

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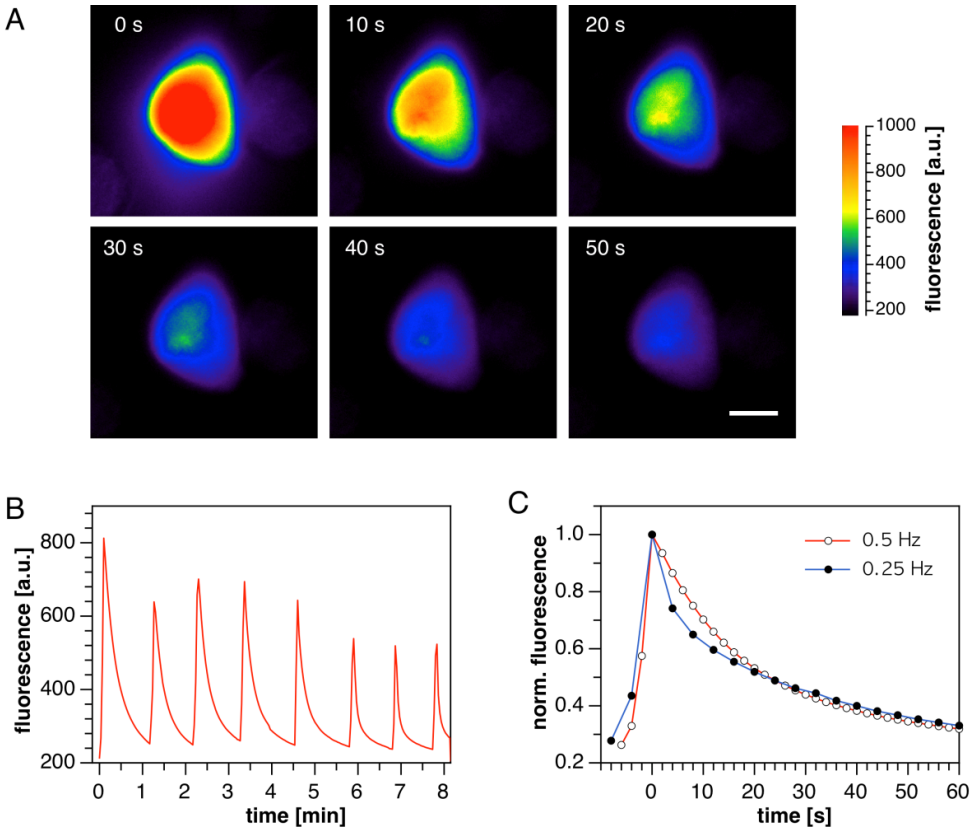


Figure S1: Competitive chelation of Cu(I) from CTAP-3 in BSC1 cells. (A) Fluorescence micrographs at indicated time points (in seconds) after microinjection of 0.8 mM CTAP-3-Cu(I) into live cells. Scale bar: 10 µm. (B) Time course of the average fluorescence intensity after repeated microinjections of CTAP-3-Cu(I) in the same cell. (C) Normalized fluorescence decay profiles at frame rates of 0.25 Hz and 0.5 Hz after microinjection of CTAP-3-Cu(I).

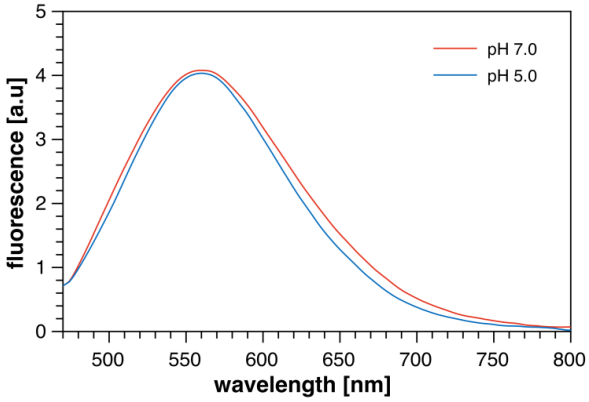


Figure S2: Effect of buffer pH on crisp-17 emission in lipid bilayers. Emission spectra (λ_{ex} = 450 nm) were recorded for a solution containing 2 µM crisp-17 in the presence of 4:1 DMPC-DMPG liposomes (100 µM total lipids) prepared in either pH 7.0 or pH 5.0 buffer (10 mM PIPES, 0.1 M KCl, 25°C).

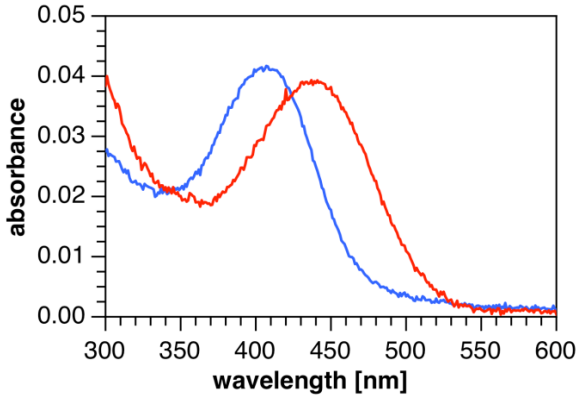


Figure S3: UV-vis absorption spectra of crisp-17 in lipid bilayers. Blue trace: 2 µM crisp-17 in the presence of 4:1 POPC-POPG liposomes (100 µM total lipids, 10 mM PIPES, pH 7.0, 0.1 M KCl, 25°C). Red trace: after addition of 2.5 µM Cu(I) as [Cu(I)MCL-2]PF_6. The scattering background of the liposome suspension has been subtracted from both spectra.

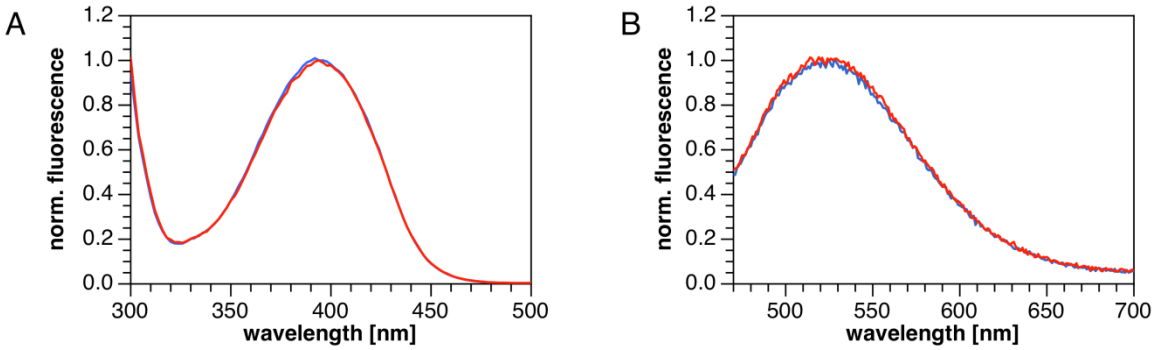


Figure S4: Fluorescence spectra of control fluorophore crisp-17ctrl in lipid bilayers. (A) Excitation spectra (λ_{em} = 525 nm) of 1 µM crisp-17ctrl in the presence of 4:1 DMPC-DMPG liposomes (100 µM total lipids, 10 mM PIPES, 0.1 M KCl, pH 7.0, 25°C) in the absence (blue) and presence (red) of 2 µM Cu(I) (provided as [Cu(I)MCL-2]PF_6). (B) Emission spectra (λ_{ex} = 450 nm) of the same solutions.

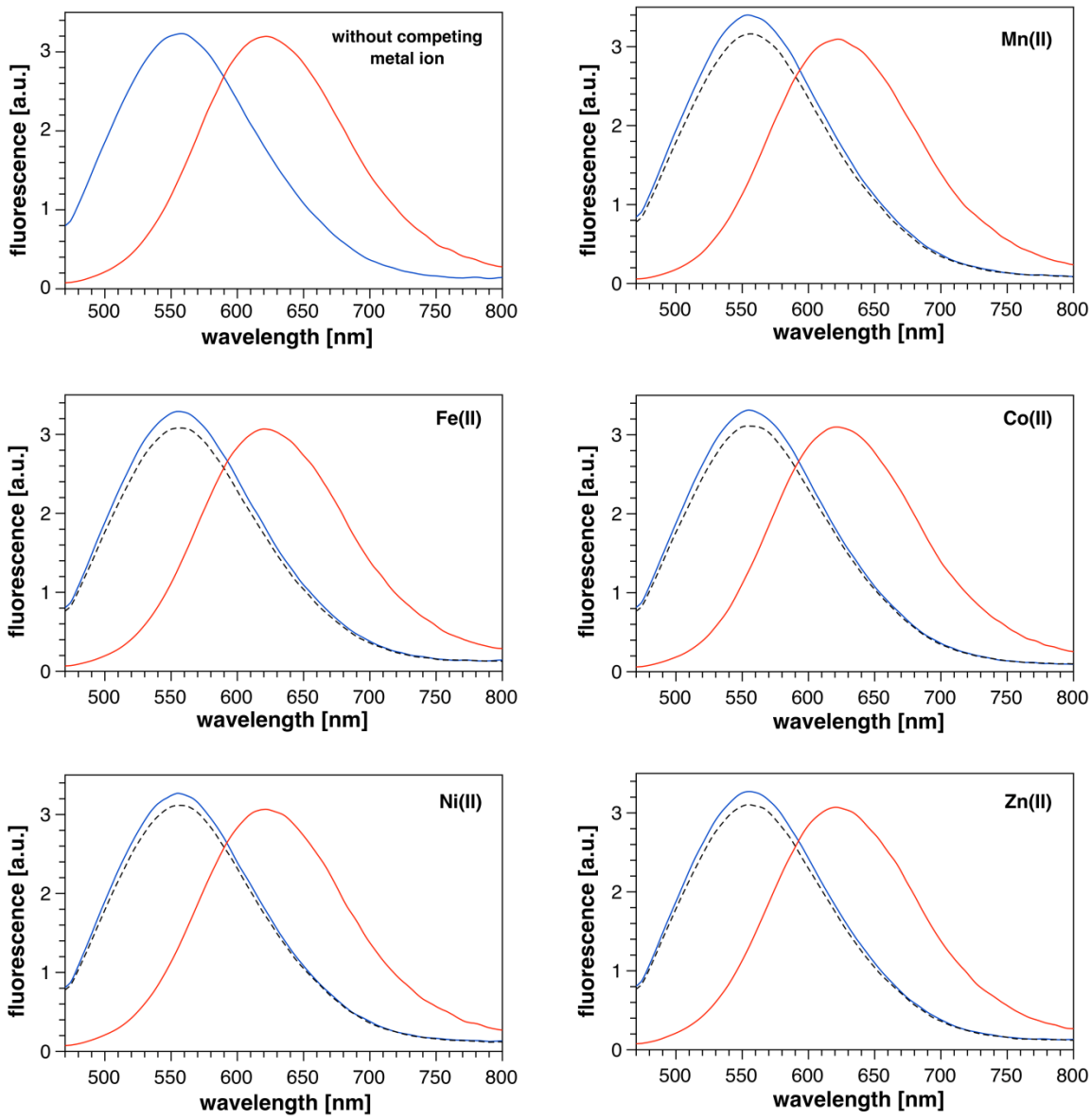


Figure S5: Fluorescence response of crisp-17 to Cu(I) in the presence of competing transition metal ions (10 µM). Blue traces: Emission spectra (λ_{ex} = 450 nm) of individual aliquots of crisp-17 (2 µM) equilibrated with liposome suspension (4:1 POPC-POPG, 100 µM total lipids, 10 mM PIPES, pH 7.0, 0.1 M KCl, 25°C). Dashed traces: spectra after addition of the indicated metal ion. Red traces: spectra after addition of 4 µM Cu(I) (as [Cu(I)MCL-2]PF_6) to the solution containing the competing metal ion.

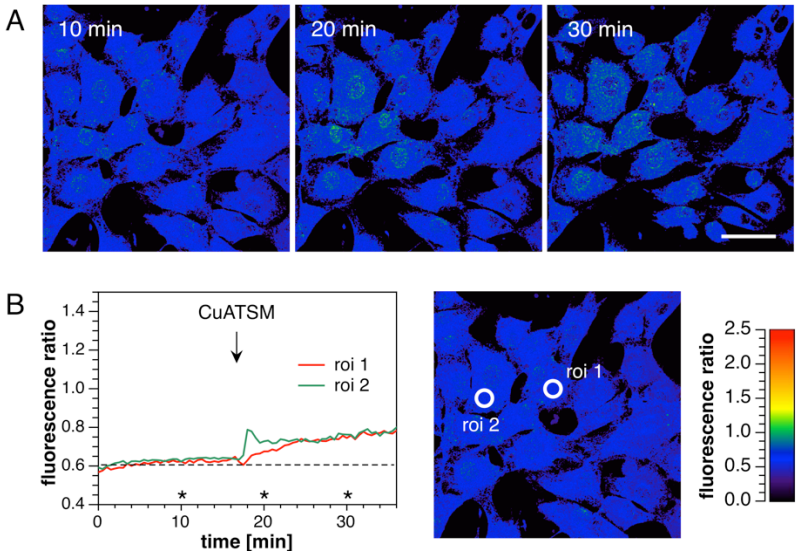


Figure S6: Ratiometric TPEM imaging (excitation at 880 nm) with crisp-17 (1 µM) of reductively released Cu(I) in live NIH 3T3 mouse fibroblasts upon exposure to CuATSM. The fluorescence intensity images were acquired with two emission channels set to 479 – 536 nm (BP1) and 611 – 750 nm (BP2). (A) Ratio images (BP2/BP1) at selected time points prior and after addition of 10 µM CuATSM. (B) Time course of the average intensity ratio for the ROIs indicated with a white circle in the right panel. The asterisks indicate time points for the respective ratio images shown in panel (A). Scale bar: 50 µm.

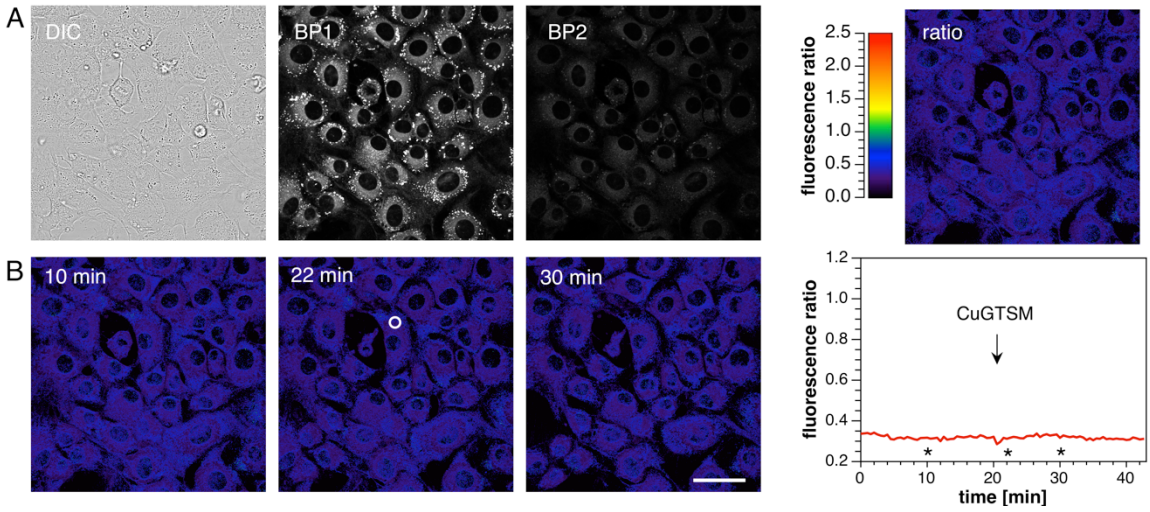


Figure S7: Two-photon ratiometric imaging of live mouse fibroblasts stained with crisp-17ctrl (1 µM). (A) Left: Phase contrast image (DIC) and fluorescence intensity images acquired with emission channels of 450-506 nm (BP1) and 581-720 nm (BP2) at 880 nm excitation. Right: Intensity ratio image with R = BP2/BP1. (B) Left: Ratio images before and after addition of 10 µM CuGTSM. Right: Time course of the average intensity ratio for the ROI indicated with a white circle in the left panel. The asterisks indicate time points for the respective ratio images shown to the left. Scale bar: 50 µm.

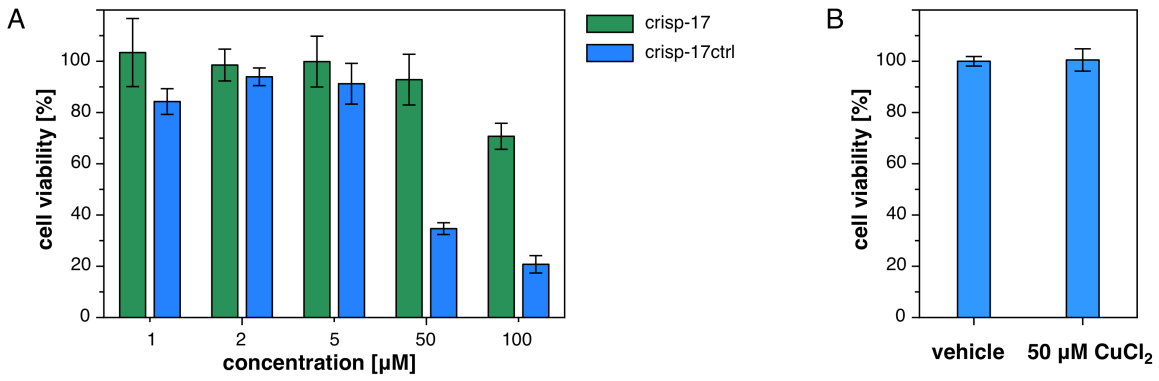


Figure S8: Cell viability of mouse 3T3 fibroblasts in response to (A) addition of crisp-17 (green) and crisp-17ctrl (blue) to the growth medium for 25 hours at the indicated concentrations or (B) supplementation of the growth medium with 50 µM $CuCl_2$ for one week. Cell viability was assessed relative to untreated cells based on the MTT assay.

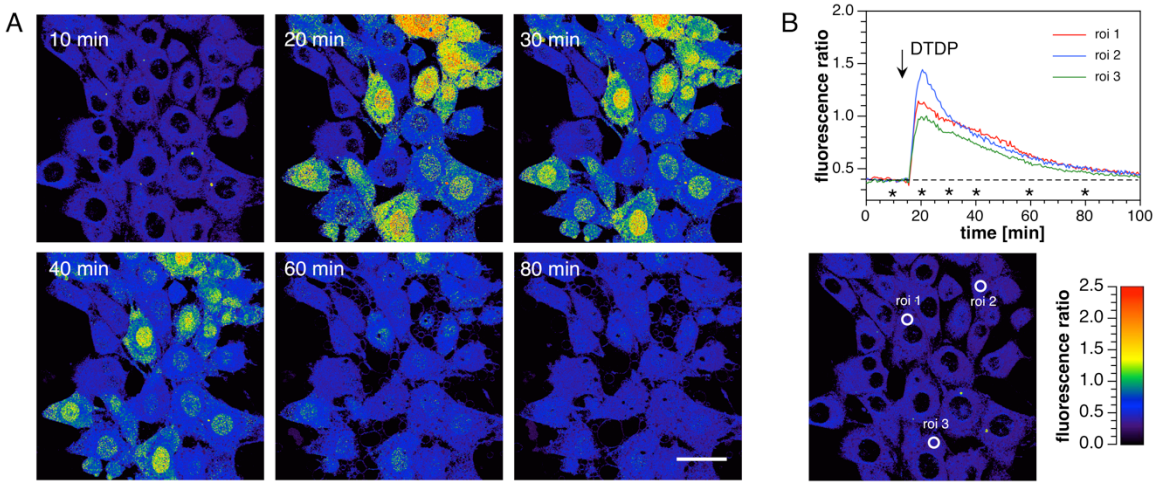


Figure S9: Ratiometric TPEM imaging (excitation at 880 nm) of labile copper dynamics in live NIH 3T3 mouse fibroblasts with crisp-17 (1 µM) after the addition of DTDP (1 mM) over an extended time period. The fluorescence intensity images were acquired with two channels, 479 – 536 nm (BP1) and 611 – 750 nm (BP2). (A) Ratio images (BP2/BP1) at selected time points prior and after addition of 1 mM DTDP. (B) Time course of the average intensity ratio change for the ROIs indicated with white circles in the bottom right panel. The asterisks indicate time points for the respective ratio images shown in (A). Scale bar: 50 µm.

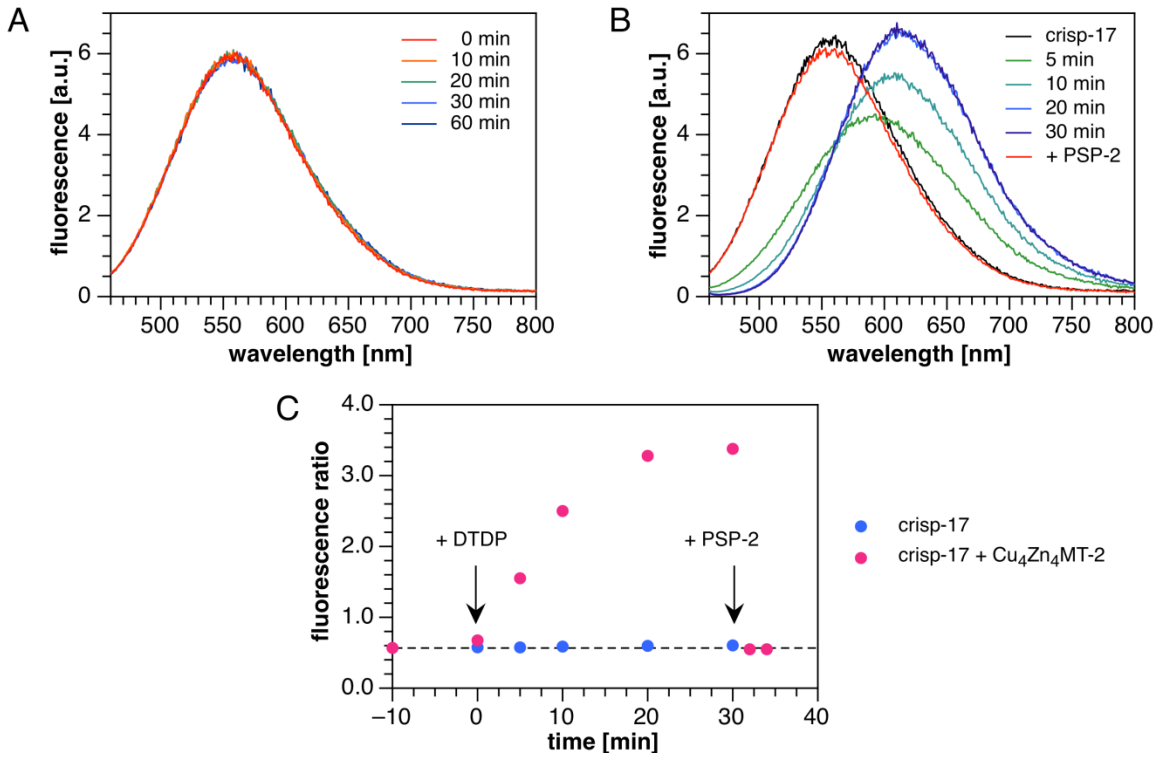


Figure S10: DTDP-induced Cu(I) release from Cu_4Zn_4-metallothionein-2. (A) Emission spectra (λ_{ex} = 450 nm) of crisp-17 (2 µM) in the presence of 4:1 DMPC-DMPG liposomes (100 µM total lipids, 10 mM PIPES, pH 7.0, 0.1 M KCl, 25°C) after incubation with 100 µM 2,2'-dithiodipyridine (DTDP) for the indicated time. The zero time spectrum was acquired immediately prior to DTDP addition. (B) Spectra acquired as in (A) but with 2 µM $Cu(I)_4Zn(II)_4$MT-2 added 10 minutes prior to DTDP. PSP-2 (10 µM) was subsequently added to confirm that the fluorescence response represents Cu(I) transfer. (C) Fluorescence ratio ($F_{590\text{-}750}/F_{480\text{-}580}$) versus DTDP exposure time for the experiments described in (A) and (B). The –10 min and 0 min measurements were acquired immediately prior to addition of metallothionein and DTDP, respectively.

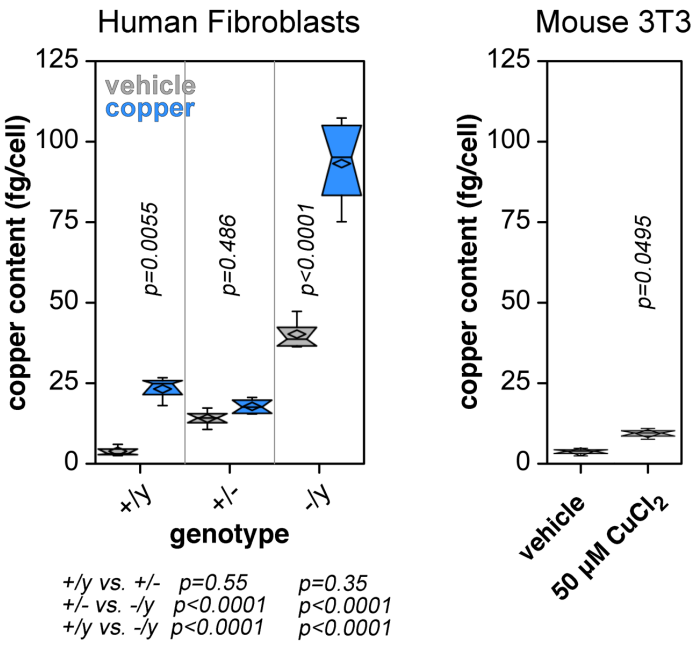


Figure S11: Cellular copper content measurements by total reflection X-ray fluorescence (TRXF). Human skin fibroblasts expressing ATP7A (+/y and –/+, GM1983), ATP7A nulls (–/y, GM1981), or mouse 3T3 cells were incubated with vehicle or 50 µM $CuCl_2$ in complete media. Human cells were incubated with $CuCl_2$ for 24 h and mouse cells for 7 days. Cells were washed in cold PBS and cell pellets analyzed by total reflection X-ray fluorescence. The effect of genotype and copper incubation in human fibroblasts was determined by Between Subjects ANOVA followed by Bonferroni-Dunn Test ($\alpha = 0.05$; adjusted $\alpha = 0.0167$). Mouse 3T3 cells were compared by Mann-Whitney U Test. n=3.

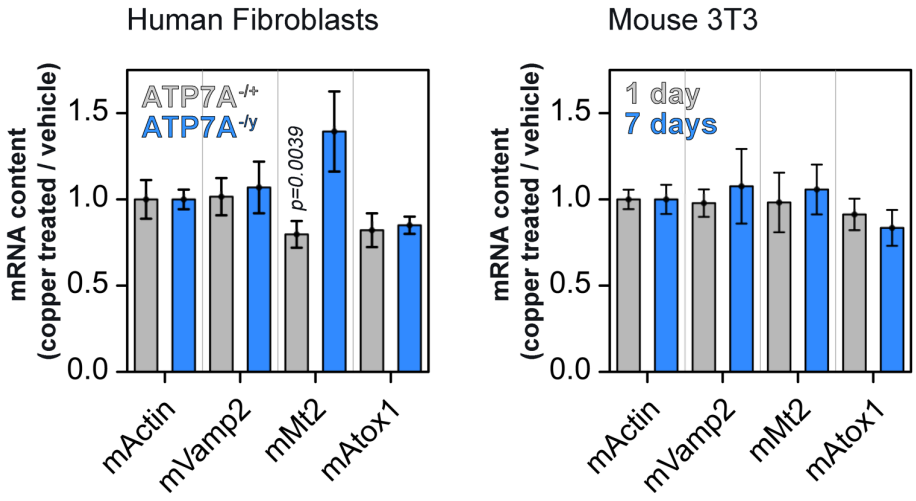


Figure S12: Metallothionein mRNA expression is increased in Menkes cells. Control human skin fibroblasts (+/y and –/+, GM1983), Menkes ATP7A nulls (–/y, GM1981), or mouse 3T3 cells were incubated with vehicle or 50 µM $CuCl_2$ in complete media. Human cells were incubated with $CuCl_2$ for 24 h and mouse cells for 1 and 7 days. mRNA expression was quantified by qRT-PCR and expressed as a fold of change between copper-treated and vehicle-treated. Actin and the membrane protein VAMP2 were used as housekeeping controls. Metallothionein 2 (Mt2) and Atox1 were used as genes whose expression is susceptible or required for copper buffering. Only Mt2 expression is significantly affected in Menkes cells. Kruskal-Wallis Test followed by pairwise comparisons with Mann-Whitney U Test. n=6. Average ± SD.

Materials and Methods

Optical absorption and fluorescence spectroscopy. All spectra were acquired at 25°C under stirring in Quantum Northwest temperature-controlled cuvette holders using 1 cm path length quartz cuvettes. Absorption spectra were recorded on a Varian Cary Bio50 spectrophotometer. Fluorescence spectra were recorded using a PTI fluorimeter equipped with a 75 W xenon arc lamp excitation source and model 814 photomultiplier detection system. Slit widths were set to 5 nm bandpass for both excitation and emission monochromators, and spectra were automatically corrected for the spectral sensitivity of the detection system and the spectral irradiance of the excitation source. All measurements were conducted in air-equilibrated solutions except for titration in the presence of GSH, which was conducted under anaerobic conditions to avoid possible superoxide formation (1). All working solutions of crisp-17 were prepared from 1-3 mM stock solutions in DMSO, which were divided into aliquots, stored at –20°C, and thawed on the day of use.

Molar-ratio titration of crisp-17 with Cu(I). A 5 μM solution of crisp-17 in 95% methanol-5% water was titrated with [Cu(I)MCL-2]PF_6 (2) (300 μM aqueous stock solution) in 0.5 μM steps up to a final concentration of 6.5 μM. Absorption (350-600 nm) and fluorescence emission spectra (470-750 nm; λ_{ex} = 460 nm) were recorded after a 1 minute mixing period following each aliquot. After the last aliquot, PSP-2 (20 μM) was added to test the reversibility of the response.

Preparation of liposomes. DMPG:DMPC (4:1) liposomes used for photophysical characterization were prepared as previously published (3) in Chelex-treated PIPES buffer (10 mM PIPES, pH 7.0, 0.1 M KCl). POPC-POPG (4:1) liposomes were prepared similarly using 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC, 24.3 mg) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho(1'-rac-glycerol) sodium salt (POPG, 6.2 mg) in 1:1 DCM-MeOH (4 mL). After rotary evaporation, the lipid film was hydrated in the same buffer as above (20 mL) and processed as previously described (3). All liposome preparations were extruded at 0.5-2 mM total lipid concentration and diluted with buffer to 100 μM immediately before use. The resulting suspensions were allowed to equilibrate under stirring at 25°C for 15 min or until light scattering intensity at 400 nm remained constant to ensure complete conversion of bilayers to the fluid phase prior to addition of the probe and subsequently equilibrated for 15 min or until fluorescence intensity at 580 nm remained constant to ensure complete uptake of the probe into the bilayers. As we previously detected traces of adventitious Cu(I) in buffers during fluorescent probe characterization, even when employing high purity reagents (4), PSP-2 was added where indicated to obtain spectra of fully demetallated crisp-17.

Fluorescence response and metal ion selectivity of crisp-17 in lipid bilayers. A 12-mL working solution of PSP-2 (0.5 μM) and crisp-17 (2 μM) in 4:1 POPC:POPG liposome suspension was equilibrated under stirring in a 25°C bath. The solution was divided into aliquots (1.5 mL) and each divalent metal cation (10 μM added from a 5 mM aqueous stock solution) was added separately. A fluorescence spectrum was acquired over the emission range of 470-800 nm with 450 nm excitation after a 1-minute mixing period. Each solution was supplemented with Cu(I) (4 μM, provided as [Cu(I)MCL-2]PF_6 (2) from a 3 mM stock solution in H_2O) and a second fluorescence spectrum was acquired after a further 1 minute equilibration. Co(II), Ni(II) were supplied as nitrate salts, and Mn(II), Fe(II), and Zn(II) were supplied as sulfates. To avoid aerial oxidation of Fe(II), the stock solution was prepared immediately before use.

Absorption spectra and fluorescence quantum yields in lipid bilayers. Absorption spectra (300-600 nm) of an equilibrated solution of crisp-17 (2 μM) in POPC-POPG liposome suspension were recorded before and after saturation with Cu(I) as [Cu(I)MCL-2]PF_6, revealing a crossover point at 422 nm. Absorption (300-600 nm) and fluorescence spectra (λ_{ex} = 422 nm, λ_{em} 440-800 nm) were acquired for a fresh aliquot of liposome suspension prior to addition of the probe, after equilibration with crisp-17, after subsequent addition of 2.5 μM [Cu(I)MCL-2]PF_6, and finally after addition of PSP-2 (20 μM) to confirm full reversibility of the probe response. The scattering backgrounds of the liposome suspension in both UV and fluorescence spectra were subtracted from the corresponding spectra of the free and Cu(I)-saturated probe in liposomes. A reference solution of Coumarin 153 in EtOH (Φ_F = 0.544) (5) was adjusted to similar absorbance at 422 nm relative to the free and Cu(I)-saturated probe after background subtraction. For all spectra, the absorption intensity at 422 nm was taken as the average from 421-423 nm to improve signal-to-noise ratio. Duplicate measurements (mean ± difference) yielded Φ_F = 0.53 ± 0.02 and 0.21 ± 0.01 for free and Cu(I)-saturated crisp-17, respectively.

Effect of pH. Two liposome suspensions, each containing 4:1 DMPC-DMPG (100 μM final lipid concentration) were prepared in parallel in 10 mM PIPES/0.1 M KCl buffer adjusted to either pH 7.0 or 5.0 and equilibrated at 25°C. Crisp-17 (2 μM from a 1 mM DMSO stock solution) was added to a 3 mL aliquot of each liposome suspension, and fluorescence spectra (λ_{ex} = 422 nm, λ_{em} 440-800 nm) were acquired after a 30 min equilibration period. The superimposed spectra (Fig. S4) revealed no significant differences in emission maximum or fluorescence intensity.

Cu(I) binding affinity of crisp-17. An equilibrated solution of crisp-17 (1 μM) in 4:1 DMPC-DMPG liposomes was metallated with [Cu(I)MCL-1]PF_6 (10 μM) in the presence of 100 μM sodium ascorbate. Emission spectra were recorded at λ_{ex} = 450 nm, and mixture was back-titrated with 10-640 μM MCL-1 over 7 steps. Nonlinear least-squares fitting over the emission wavelength range of 460-750 nm using the Specfit software package yielded an apparent affinity of log $K_{Cu(I)}$ = 17.0 ± 0.1. A second titration was conducted with crisp-17 (2 μM) which was metallated with [Cu(I)MCL-2]PF_6 (2.5 μM). Emission spectra were recorded at λ_{ex} = 440 nm, and the mixture was titrated with 1-300 μM MCL-1 over 17 steps. Nonlinear least squares yielded log $K_{Cu(I)}$ = 17.23 ±0.07.

Titration with Cu(I) in the presence of glutathione. A 5 mM solution of GSH in deoxygenated PIPES-KCl buffer was prepared and readjusted to pH 7.0 with standard 1 M KOH under a blanket of argon. A 2.4 mL aliquot of this solution and 600 μL of 500 μM extruded 4:1 DMPC-DMPG liposome concentrate (deoxygenated by repeated inversion mixing with argon) were mixed in an argon-flushed, septum-sealed cuvette to yield a final solution of 100 μM total lipids and 4 mM GSH. After equilibration with crisp-17 (2.0 μM) and PSP-2 (1.0 μM) the fluorescence spectrum (λ_{ex} = 450 nm) was recorded from 460-750 nm, and [Cu(I)MCL-2]PF_6 (1.0 μM from an aqueous stock solution) was subsequently added to saturate PSP-2. Additional Cu(I) was injected in 0.2 μM aliquots up to 3 μM followed by 1 μM aliquots up to 10 μM, and the fluorescence spectrum was recorded after each addition. To obtain a reference spectrum of the Cu(I)-saturated probe under these conditions, the calculated quantity of HCl was added to adjust the pH to 6.0, which lowers the apparent Cu(I)-affinity of GSH by 1.5 orders of magnitude (6). The apparent fractional saturation (f) of the probe was calculated from the integrated emission intensity from 500-550 nm according to the equation $f = (I_{free}-I)/(I_{free}-I_{sat})$, where I is the fluorescence intensity at each titration point, I_{free} is the fluorescence intensity before addition of Cu(I), and I_{sat} is the fluorescence intensity of the Cu(I)-saturated probe.

Expression, purification, and metal reconstitution of (Cu,Zn)-metallothionein-2 ($Cu(I)_4Zn(II)_4$MT-2). Recombinant human MT-2 was expressed in *Escherichia coli* BL21(DE3)pLys using a pET-3d plasmid (Novagen) encoding for the codon optimized sequence of *wt*MT-2. The protein was expressed and purified as Cd(II)-bound protein as reported by Faller et al. (7) by addition of 0.4 mM $Cd(II)SO_4$ 30 minutes after IPTG induction. The protein was purified by ethanol precipitation, size–exclusion chromatography, and anion exchange chromatography as described previously (8). The apoMT-2 was then generated by Cd(II) release through addition of HCl following the method of Vašák (9), and reconstituted to the Zn_7MT form by $ZnCl_2$ addition followed by pH adjustment using TRIS to pH 8.0. Cu_4Zn_4MT-2 was generated by reacting Zn_7MT-2 with 4 Cu^{2+} equivalents (as $CuCl_2$) for 1h at 25°C. Free and loosely-bound metals were removed using 10 mg/ml Chelex 100 resin, followed by centrifugation (14000 x g, 2.5 min) to remove the resin beads. Protein concentrations were determined photometrically (Agilent Cary 300 UV-Vis Spectrophotometer) in 0.1 mM HCl using $\varepsilon_{220} = 47500\ M^{-1}cm^{-1}$ for apoMT-2. Cysteine-to-protein ratios were determined by photometric quantification of sulfhydryl groups (CysSH) upon reaction with 2,2'-dithiodipyridine (DTDP) in 0.2 M sodium acetate/1 mM EDTA (pH 4.0) ($\varepsilon_{343} = 7\ 600\ M^{-1}cm^{-1}$). Metal-to-protein ratios were determined by ICP-MS (Agilent 7900) on samples digested in 1% HNO_3.

DTDP-induced release of Cu(I) from metallothionein (Cu_4Zn_4(MT-2)). Crisp-17 (2 µM) was equilibrated in 3 mL of 4:1 DMPC-DMPG liposome suspension at 25°C and the fluorescence spectrum (λ_{ex} = 450 nm) was recorded from 460-800 nm. Cu_4Zn_4MT-2 (2 µM) was added as a 50 µM stock solution in TRIS buffer, and the fluorescence spectrum was recorded after 10 min. DTDP (100 µM) was added from a 50 mM stock solution in DMSO, and fluorescence spectra were recorded after 5, 10, 20, and 30 min. PSP-2 (10 µM) was added and spectra were recorded after 1 and 3 min to confirm reversibility of the crisp-17 response. To further verify that the response does not involve a direct interaction of the oxidant DTDP with crisp-17, a control run was conducted as described above but without addition of Cu_4Zn_4MT-2 or PSP-2 and with an additional spectrum acquired at 60 min.

Acute toxicity (MTT Assay). Mouse fibroblast 3T3 cells were plated at a concentration of 10^5 cells/ml in 96-well plate and incubated overnight at 37°C in 5% CO_2. Crisp-17 or crisp-17ctrl were added to the wells at various concentrations ranging from 1-100 µM diluted from a 15 mM DMSO stock solution in complete DMEM. The cells were incubated for 24 hours at 37°C in 5% CO_2, compounds were removed, 20 µL of 5 mg/ml MTT (Thiazolyl Blue Tetrazolium Bromide) was added to each well and the plates were further incubated in the dark for 4 hours. The formazan crystals formed were dissolved in 150 µL of DMSO and absorbance of the colored product was measured at 570 nm using a plate reader. The experiment was performed in triplicate and the average absorbance value was calculated for each ligand incubation concentration.

Western blot of cell lysate from Menkes and control fibroblasts. Cells were obtained from Coriell cell repository (GM01981 male –/y, and GM01983 female –/+) and Rutgers Cell Repository (MH0162676 male +/y). All fibroblasts were cultured at 37°C, 5% CO_2 in DMEM media (Corning 10-013-CV) supplemented with 15% fetal bovine serum (Atlanta Biologicals S12450), 100 mg/ml penicillin and streptomycin (Hyclone SV30010), and 50 µM $CuCl_2$ (Sigma 203149) for 24 hours prior to cell lysis. Cells were washed twice with phosphate buffered saline (PBS Corning 21-040-CV) containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ and then scraped up in lysis buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, 0.5% Triton X-100) containing Complete (Roche 11-836-145-001) protease inhibitor. The cell lysate was incubated at 4°C with periodic vortexing for 30 min, and spun at 16,100 x g for 15 min at 4°C. The cleared lysate was collected for protein concentration and Western blot analysis.

Protein concentrations were determined using the Bradford Assay (BioRad 5000006). For Western blot, the cell lysate was reduced and denatured in sample buffer containing SDS and 2-mercaptoethanol and heated for 5 min at 75°C. Equal concentrations of sample were loaded into Criterion gels (BioRad 5671094) for electrophoresis and transferred to PVDF (Millipore IPFL00010) using the semi-dry transfer method. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Triton X-100 (TBST), rinsed, and incubated overnight with primary antibody diluted in antibody base solution (PBS with 3% bovine serum albumin, 0.2% sodium azide). Primary antibodies include polyclonal rabbit anti-ATP7A (Bethly Laboratories against SEPDKHSLLVGDFREDDDTT, (10)) and monoclonal mouse anti-β-actin (Sigma-Aldrich Cat#A5441). Membranes were then washed in TBST and incubated with HRP-conjugated secondary antibody diluted 1:5000 in the blocking solution above. Secondary antibodies were against mouse or rabbit (Thermo Fisher Scientific A10668 and G21234). Washed membranes were then exposed to Amersham Hyperfilm ECL (GE Healthcare 28906839) with Western Lightning Plus ECL reagent (Perkin Elmer NEL105001EA). Densitometry was performed and quantified with Fiji Image J 1.51n software.

Quantitative RT-PCR. Cell pellets were processed for RNA extraction following the TRIzol Reagent manufacturer's protocol (Cat # 15596026 Invitrogen). RNA quantity and purity were measured with the NanoDrop One^{C} (Thermo Scientific). The SuperScript III First Strand Synthesis System kit (Cat # 18080051 Invitrogen) was used for cDNA synthesis. Manufacturer's protocol was followed with 5 µg RNA in each reaction using the supplied random hexamer primer option. The LightCycler 480 SYBR Green I Master (Cat # 04707516001 Roche) system was utilized for relative quantity reverse transcription PCR. Fast thermal cycling 96-well format plates (Cat # 4346907 Invitrogen) were run on a QuantStudio 6 Flex System (Invitrogen) using the following program: Denaturing Stage: Temperature was held at 55°C for 2 min and ramped up to a 5 minute hold 95°C at a rate of 3.4°C/s. Amplification Stage: 5 second hold at 95°C with 2.2°C/s ramp from 95°C to 55°C. Temperature was held at 55°C for 10 seconds and ramped to 72°C at 3.4°C/s. Temperature was held at 72°C for 20 seconds whereby a data acquisition point was recorded. The temperature was ramped back to 95°C at 1.6°C/s. After 40 cycles of PCR a melting curve was produced with 1 minute hold at 65°C and ramp to 97°C at 0.1°C/s. For analysis, relative standard curves were generated for each primer set. Standard curves were applied for each transcript and quantities were normalized to the endogenous control, beta-Actin. A ratio of 50 µM $CuCl_2$ treatment to normalized vehicle treatment is reported. Primers used were designed using the Roche Universal Probe Library Assay Design Center or the IDT Real Time qPCR Assay Entry websites. Primers were purchased from either Sigma or IDT custom DNA oligo synthesis services.

The following primers were employed:

Transcript	Forward	Reverse	Species
ACTB	ACCTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	Mus musculus
VAMP2	CATCTTGGGAGTGATCTGCG	GAAAGATATGGCTGAGAGGTGG	Mus musculus
ATOX1	GCGAGTCCTTCCGGATATAAAC	TGAGCAGTTGGTGTTCCAG	Mus musculus
MT2	GCTCCTAGAACTCTTCAAACCG	CAGGAAGTACATTTGCATTGTTTG	Mus musculus
ACTB	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG	Homo sapiens
VAMP2	CCCTCCAAACCTCACCAGTA	CCAGCTCCGACAGCTTCT	Homo sapiens
ATOX1	AAGACTGTTTCCTACCTTGGC	CAGGAACACCATCACCCG	Homo sapiens
MT2a	CTCCAAGTCCCAGCGAAC	GAGCAGTTGGGATCCATGG	Homo sapiens

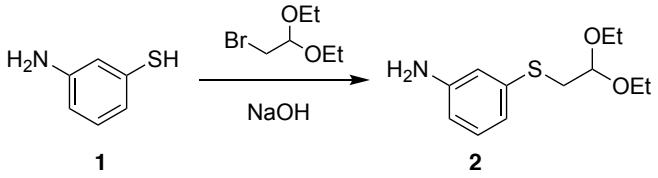
Determination of cellular copper levels. Copper determinations by inductively coupled plasma mass spectrometry (ICP-MS) were made at the University of Georgia Center for Applied Isotype Studies. Samples were prepared from human fibroblasts cultured in 10% FBS culture media with or without a 24 hour pulse of 50 μM $CuCl_2$. Cells were then washed with PBS, lifted in PBS supplemented with 10 mM EDTA and pelleted, suspended to wash in plain PBS, pelleted and sent for digestion and analysis or washed in PBS, lysed as above, and sent for analysis. For copper measurements by total reflection X-ray fluorescence (TXRF), cells were seeded in 10 cm diameter plates and grown to 80% confluency either in regular media or media supplemented with 50 μM $CuCl_2$. Cells were washed with PBS and harvested after trypsinization in 1 mL trypsin-EDTA. Cells were pelleted by centrifugation at 5000 rpm at 4°C for 5 min. For the determination of total content of copper, the cell pellets were treated with 200 μL of concentrated nitric acid and digested for 48 h at room temperature. The analysis by TXRF was performed using a Bruker S2 PICOFOX spectrometer and the digested samples were standardized using 1 μg/mL of As.

Live cell microscopy imaging and microinjection of [Cu(I)CTAP-3]. CTAP-3-Cu(I) complex was prepared by adding 100 μL of a 1.5 mM aqueous stock solution of CTAP-3 ammonium salt (4) and a 2.5 mM acetonitrile stock solution of $[(CH_3CN)_4Cu(I)]PF_6$ (48 μL) to methanol (1 mL). The mixture was concentrated to dryness under a stream of argon, re-dissolved in methanol (1 mL), and concentrated to dryness again to yield a thin film. The film was dissolved in 0.1 M aqueous KCl and diluted to 150 μL to yield final concentrations of 1 mM CTAP-3 and 800 μM Cu(I) for injection. BSC1 green monkey kidney cells were grown on glass bottom culture dishes (MatTek) to 70% confluency using Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 200 μM L-glutamine, and 100 μM sodium pyruvate at 37°C in 5% CO_2. The cell culture medium was replaced with pre-warmed PBS (control), and CTAP-3-Cu(I) solution was loaded into an injection capillary (Eppendorf Femtotip, 0.5 μm ±0.2 μm), which was mounted on a micromanipulator (Eppendorf InjectMan NI 2). Microinjections were performed with an Eppendorf Femtojet apparatus set to an injection pressure of 650 hPa, injection time 1.5 s, and compensation pressure 275 hPa. Fluorescence micrographs were acquired at 1 or 4 second intervals with a Zeiss Axiovert 200 fluorescence microscope equipped with a 63x oil-immersion objective and DAPI filter set. Changes of the average cellular fluorescence intensity were analyzed with ImageJ (11).

Emission-ratiometric two-photon excitation microscopy with crisp-17. Cells were grown on glass bottom culture dishes (MatTek) to 70% confluency in DMEM supplemented with 10% FBS and Penicillin/Streptomycin. Fresh growth medium supplemented with 50 μM $CuCl_2$ was supplied 21-24 hours prior to imaging. Before imaging, the media was replaced with pre-warmed DMEM without phenol red supplemented with 10% FBS, penicillin/streptomycin, and 100 mM sodium pyruvate containing 1 μM of crisp-17 and incubated at 37˚C in 5% CO_2 for 20 minutes. Cells were imaged at 37°C under a humidified 5% CO_2 atmosphere using a Zeiss LSM confocal NLO 710 microscope equipped with a femtosecond pulsed Ti:sapphire laser. Scanning fluorescence micrographs were acquired with excitation at 880 nm and emission simultaneously collected over two channels with bandpass ranges of 479-536 and 611-750 nm. After imaging under basal conditions, oxidative stress response was stimulated by exposure of cells to a buffer solution containing 100 μM 2,2′-dithiodipyridine (DTDP). The quantitative image analysis software package, Image J, was used to analyze the change in the fluorescence emission ratio and fractional saturation of crisp-17 over time as described previously for chromis-1 (3). The emission ratio for the region of interest was averaged at each time point.

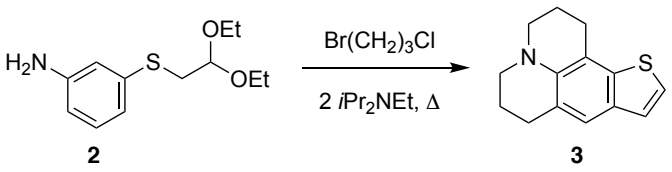
Synthesis of Crisp-17 and Crisp-17ctrl

Materials and reagents. Trimethylphosphine sulfide was prepared as previously described (12). Diethyl chlorophosphite, methyl dichlorophosphite, and 3-aminothiophenol were purchased from Alfa Aesar and 2,3,5-tribromothiophene from Oakwood Products. Diethyl ether and tetrahydrofuran (DriSolv anhydrous grade with BHT stabilizer) were purchased from EMD. All other reagents were from standard commercial sources, and all commercial reagents and solvents were used as received. Column chromatography: flash chromatography on general purpose silica gel (60 Å pore size, 250 mesh, Sorbent Technologies). NMR: ^{1}H spectra were recorded at 400 MHz and referenced to internal TMS. ^{13}C spectra were acquired at 100 MHz and referenced to the known chemical shift of the solvent peak ($CDCl_3$: 77.0 ppm). ^{31}P NMR spectra were recorded at 162 MHz and referenced to a sealed capillary of 85% D_3PO_4. Mass spectra were recorded by the Georgia Tech Mass Spectrometry Facility.



1 **2**

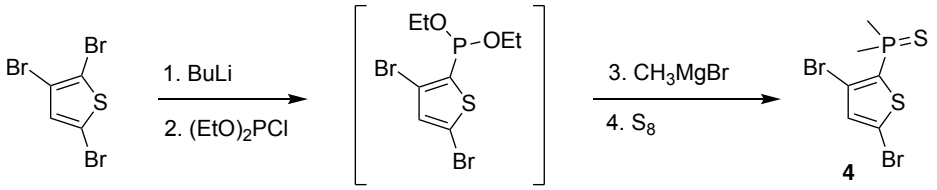
A 100 mL 2-necked flask containing a stir bar was connected to an argon inlet via the side neck, and 3-aminothiophenol **1** (5.13 g, 41.0 mmol) was added through the top neck against a gentle flow of argon. Ethanol (25 mL, deoxygenated by bubbling with argon) was added, followed by NaOH pellets (1.97 g, 1.2 equiv.) and the flask was sealed with a rubber septum vented to a bubbler. Bromoacetaldehyde diethyl acetal (7.4 mL, 1.2 equiv.) was added by syringe, and the mixture was stirred overnight at room temperature. As a TLC (silica gel, 1:2 hexane-CH_2Cl_2) indicated incomplete consumption of 3-aminothiophenol, the septum was replaced with a reflux condenser connected to an argon line and bubbler via a T-adapter, and the mixture was heated to reflux for 1 hour. After cooling, the mixture was partitioned between toluene (150 mL) and water (60 mL). The organic layer was washed with a further 60 mL of water, dried with Na_2SO_4, and concentrated. The residue was purified by column chromatography (hexane-MTBE gradient) to give the product **2** as a pale yellow oil. Yield 8.62 g (35.7 mmol, 87%). ^{1}H NMR ($CDCl_3$) δ 1.21 (t, J = 7.1 Hz, 6H), 3.11 (d, J = 5.6 Hz, 2H), 3.55 (dq, J = 9.3 7.1 Hz, 2H), 3.66 (br. s, 2H), 3.67 (dq, J = 9.3, 7.1 Hz, 2H), 4.65 (t, J = 5.6 Hz, 1H), 6.49 (ddd, J = 8.0, 2.3, 0.9 Hz, 1H), 6.70 (dd, J = 2.2, 1.8 Hz, 1H), 6.75 (ddd, J = 7.7, 1.8, 0.9 Hz, 1H), 7.05 (dd, J = 8.0, 7.8 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 15.2, 37.0, 62.1, 101.6, 112.9, 115.3, 119.0, 129.6, 137.2, 146.8. EI-MS m/z 241 (M^+, 40), 196 (15), 150 (50), 103 (100), 75 (70). EI-HRMS calcd for M^+ $C_{12}H_{19}NO_2S$ 241.1131, found 241.1133.



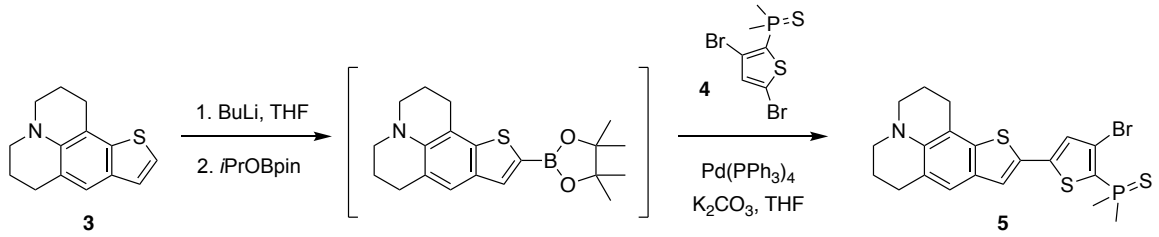
2 **3**

A mixture of acetal **2** (2.00 g, 8.29 mmol), *N*,*N*-diisopropylethylamine (2.9 mL, 2.0 equiv.) and 1-bromo-3-chloropropane (10 mL, 12 equiv.) was heated to reflux under argon. The initially yellow solution abruptly turned black after 1 hour. After 20 hours a precipitate had formed and the mixture was allowed to cool. The resulting gray paste was transferred to an Erlenmeyer flask using

methanol (25 mL), diluted with water (100 mL), made basic with NaOH, and stirred vigorously with cyclohexane (200 mL) until the mixture readily separated into two clear liquid layers with a thin boundary of tarry material. The cyclohexane layer was separated, dried with Na_2SO_4, and concentrated. The product **3** was isolated by column chromatography (hexane-MTBE) and further purified by recrystallization from methanol to give colorless crystals, which quickly turned brown upon storage in air. Yield 863 mg (3.76 mmol, 45%). ^{1}H NMR ($CDCl_3$) δ 1.99-2.05 (m, 2H), 2.08-2.14 (m, 2H), 2.85 (t, J = 6.7 Hz, 2H), 2.90 (t, J = 6.6 Hz, 2H), 3.15-3.19 (m, 4H), 7.05 (d, J = 5.4 Hz, 1H), 7.11 (d, J = 5.4 Hz, 1H), 7.27 (unresolved coupling, 1H). ^{13}C NMR ($CDCl_3$) δ 21.8, 22.3, 26.1, 28.2, 50.2, 50.5, 113.4, 120.9, 121.1, 121.3, 123.8, 129.9, 138.9, 140.4. EI-MS m/z 229 (M^+, 100), 228 (90), 200 (35). EI-HRMS calcd for M^+ $C_{14}H_{15}NS$ 229.0920, found 229.0919. The mother liquor was concentrated to dryness and taken up in 9:1 diethyl ether-ethanol. Chlorotrimethylsilane was added dropwise under stirring, and the resulting precipitate was recrystallized from methanol-isopropanol to give the hydrochloride salt as a cream-colored, air-stable crystalline powder. Yield 105 mg (395 µmol, 5%). Mp 191-193°C. A portion of the HCl salt was converted to the free base by partitioning between 5% NaOH and $CDCl_3$ and verified as the pure product by ^{1}H NMR. Total yield 4.15 mmol (50%).

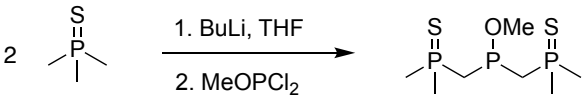


A solution of 2,3,5-tribromothiophene (7.15 g, 22.3 mmol) in Et_2O (50 mL) was cooled to –78°C under argon. A solution of *n*-BuLi in hexanes (2.5 M, 8.9 mL, 1 molar equiv.) was added dropwise under stirring. After 15 min, diethyl chlorophosphite (3.2 mL, 1 equiv.) was added, and the cooling bath was removed. After warming to room temperature, the mixture was diluted with toluene (100 mL) containing triethylamine (1.5 mL), filtered through a short plug of basic alumina, and concentrated under reduced pressure to give the phosphonite intermediate as a light brown oil, which was used without further purification. ^{1}H and ^{31}P NMR in $CDCl_3$ were consistent with a 7:2 mixture of the desired diethyl (3,5-dibromothiophene-2-yl)phosphonite (^{31}P δ 141.8 ppm, $J_{P\text{-}C\text{-}C\text{-}C\text{-}H}$ = 1.6 Hz) and the undesired 4,5-dibromo isomer (^{31}P δ 142.6 ppm, $J_{P\text{-}C\text{-}C\text{-}H}$ = 3.1 Hz). The intermediate was transferred with toluene (25 mL) to a 2-necked flask containing a stir bar, and the flask was flushed with argon and sealed with a rubber septum vented to a bubbler. A solution of methylmagnesium bromide in Et_2O (3 M, 19 mL, 2.5 equiv.) was added, and the mixture was heated in a 40°C bath for 2 hours. The resulting slurry was cooled in an ice bath and carefully quenched by dropwise addition of ethanol (2 mL) followed by a solution of citric acid (4.3 g, 1 equiv.) in water (25 mL) under stirring. The septum was removed, and crystalline sulfur (786 mg, 1.1 equiv.) was added against a gentle back-flow of argon. After 30 min, the mixture was partitioned between toluene and water, and the organic layer was dried with Na_2SO_4 and concentrated. The residue was separated by column chromatography (hexane-MTBE) to give the product **4** as a light yellow oil, which solidified on standing. Yield 3.75 g (50%). ^{1}H NMR δ 2.16 (d, J = 13.7 Hz, 6H), 7.12 (d, J = 1.8 Hz, 1H). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 28.9 (s). EI-MS m/z 336 (40), 334 (80), 332 (M^+, 40), 321 (15), 319 (30), 317 (15), 255 (100), 253 (95). EI-HRMS calcd for M^+ $C_6H_7S_2Br_2P$ 331.8088, found 331.8087.



3 **5**

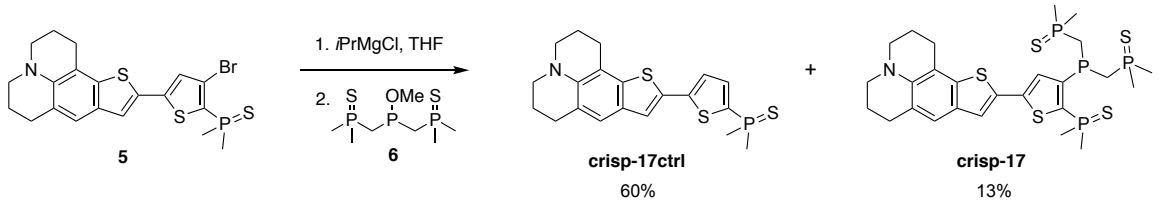
Thienojulolidine **3** (352 mg, 1.54 mmol) was dissolved in dry THF (5 mL) under argon. Diisopropylamine (43 µL, 0.2 equiv.) was added, and the mixture was cooled to –78°C. A solution of *n*-BuLi in hexanes (737 µL, 1.2 equiv.) was added dropwise, and a yellow precipitate slowly formed. The mixture was warmed briefly to 0°C before cooling back to –78°C, and 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (376 µL, 1.2 equiv.) was added. After warming to room temperature, the resulting homogeneous solution was quenched with 1 M aqueous disodium citrate (1.8 mL, 1.2 equiv.) and quickly diluted with toluene (15 mL) under stirring. The aqueous layer was allowed to settle, removed with a pipette, and extracted with an equal volume of toluene. The combined organic layers were filtered through a plug of Na_2SO_4 and concentrated into a 25 mL rb flask. Dibromide **4** (508 mg, 1.54 mmol), $Pd(PPh_3)_4$ (44 mg, 2.5 mol%), K_2CO_3 (630 mg, 3 equiv.) THF (8 mL) and water (6 mL) were added, and the mixture was refluxed under argon for 15 hours. The mixture was partitioned between toluene and water, and the organic layer was collected, dried with Na_2SO_4, and concentrated. The residue was purified by column chromatography using a gradient of increasing MTBE in 3:1 hexane-CH_2Cl_2 followed by crystallization from boiling CH_2Cl_2-MTBE to give compound **5** as orange crystalline powder. Yield 610 mg (1.26 mmol, 83%). Mp >200°C (dec). ^{1}H NMR ($CDCl_3$) δ 1.97-2.03 (m, 2H), 2.07-2.13 (m, 2H), 2.19 (d *J* = 13.7 Hz, 6H), 2.79 (t, *J* = 6.6 Hz, 2H), 2.86 (t, *J* = 6.4 Hz, 2H), 3.19-3.23 (m, 4H), 7.17 (d, *J* = 1.8 Hz, 1H), 7.20 (unresolved coupling, 1H), 7.30 (s, 1H). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 28.8 (s). EI-MS *m*/*z* 483 (100), 481 (M^+, 90), 422 (35), 420 (30), 61 (35). EI-HRMS calcd for M^+ $C_{20}H_{21}NPS_3Br$ 480.9752, found 480.9742.



6

A 250 mL 2-necked flask equipped with a thermometer, argon inlet-thermometer adapter, and stir bar was charged with trimethylphosphine sulfide (6.04 g, 55.9 mmol), flushed with argon, sealed with a rubber septum, and vented to an oil bubbler. Diethyl ether (80 g by mass loss of bottle, 112 mL) was added via cannula, and THF (8.75 mL, 108 mmol) was added via syringe. The mixture was cooled to –78°C, and *n*-BuLi solution (22.4 mL, 2.5 M in hexane, 55.9 mmol) was added under stirring. The granular Me_3PS mostly dissolved and a fluffy white precipitate abruptly formed. After complete addition, the mixture was allowed to warm to the point of complete dissolution (–5°C) and the resulting clear solution was cooled in an ethanol-liquid nitrogen bath (–116°C). The precipitate reappeared when the internal temperature fell below –35°C. When the temperature reached –100°C, methyl dichlorophosphite (2.60 mL, 3.59 g, 27.0 mmol) was injected gradually below the surface of the slurry. The mixture was allowed to warm, reaching –70°C after 30 min. Triethylamine (2 mL) and methanol (1 mL) were added before opening the flask to the atmosphere, and the mixture was concentrated to dryness. The residue was taken up in dichloromethane (200 mL) containing a few drops of triethylamine, pressure-filtered through a short column of sand to remove LiCl, and concentrated to dryness. The residue was recrystallized from methanol containing

a few drops of triethylamine, and the resulting colorless crystalline product **6** was collected by filtration under argon pressure and dried under vacuum. Yield 4.77 g (17.3 mmol, 64%). Mp 194-196°C. ^{1}H NMR ($CDCl_3$) δ 1.86 (d, J = 13.0 Hz, 6H), 1.89 (d, J = 12.9 Hz, 6H), 2.53 (dd, J = 14.1, 11.9 Hz, 2H), 2.74 (m, 2H), 3.65 (d, J = 14.0 Hz, 2H). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 32.5 (d, J = 58.6 Hz, 2P), 119.0 (t, J = 58.6 Hz, 1P). EI-MS m/z 276 (M^+, 20), 261 (30), 169 (100), 93 (40), 75 (85), 61 (30). EI-HRMS calcd for M^+ $C_7H_{19}OP_3S_2$ 276.0091, found 276.0090.



A 25 mL two-necked flask equipped with a stopcock-gas inlet adapter and stir bar was charged with bromide **5** (583 mg, 1.2 mmol), sealed with a rubber septum, evacuated, back-filled with argon, and vented through the septum to an oil bubbler. THF (5 mL) was added, followed after 5 min by isopropylmagnesium chloride (2 M in THF, 725 µL, 1.2 equiv). Upon addition of the Grignard reagent, the orange starting material rapidly dissolved, and a yellow crystalline precipitate began to form within 15 min. The argon flow rate was increased to evaporate most of the solvent over 1 hour, leaving a yellow paste. The septum was removed and the solid methoxyphosphine **6** (467 mg, 1.4 equiv.) was added against a current of argon. The septum was replaced, and THF (4 mL) was added. After 30 min the flask was placed in a 45°C bath. The stopcock-gas inlet adapter was closed and a very slow current of argon was instead introduced via a 90°-bent needle through the septum to minimize solvent evaporation. After 18 hours, the mixture was allowed to cool, quenched with 1 M aqueous disodium citrate (prepared from trisodium citrate and citric acid, 5 mL), and extracted with CH_2Cl_2 (25 mL). The organic layer was dried with Na_2SO_4 and concentrated to dryness, and the residue was separated by column chromatography, sequentially eluting a trace of unconsumed **5**, crisp-17ctrl, and crisp-17 with 15:15:1, 5:5:1, and 2:2:1 hexane-CH_2Cl_2-MTBE. Both products were crystallized by bubbling argon through solutions in CH_2Cl_2-CH_3OH at 45°C followed by cooling. Crisp-17: orange prisms, yield 98.5 mg (152 µmol, 13%). Mp > 200°C (dec). ^{1}H NMR (CD_2Cl_2) δ 1.79 (d, J = 12.9 Hz, 6H), 1.84 (d, J = 12.9 Hz, 6H), 1.95-2.01 (m, 2H), 2.05-2.12 (m, 2H), 2.28 (d, J = 13.5 Hz, 6H), 2.57-2.63 (m, 2H), 2.79 (d, J = 6.6 Hz, 2H) 2.85 (t, J = 6.5 Hz, 2H), 3.11-3.18 (m, 2H), 3.19-3.24 (m, 4H), 7.20 (unresolved coupling, 1H), 7.32 (s, 1H), 7.48 (dd, J = 2.1, 1.0 Hz, 1H). ^{31}P{^{1}H} NMR (CD_2Cl_2) δ -60.6 (dt, J = 65.4, 11.5 Hz, 1P), 27.4 (d, J = 11.5 Hz, 1P), 35.6 (d, J = 65.4 Hz, 2P). ESI-HRMS calcd for M^+ $C_{26}H_{37}NP_4S_5$ 647.0475, found 647.0473. Crisp-17ctrl: yellow prisms, Yield 295 mg (730 µmol, 60%). Mp 183-184°C. ^{1}H NMR ($CDCl_3$) δ 1.98-2.04 (m, 2H), 2.05 (d, J = 13.4 Hz, 6H), 2.06-2.13 (m, 2H), 2.80 (t, J = 6.6 Hz, 2H), 2.87 (t, J = 6.5 Hz, 2H), 3.19-3.23 (m, 4 H), 7.19 (dd, J = 3.8, 1.7 Hz, 2H), 7.20 (unresolved coupling, 1H), 7.27 (s, 1H), 7.50 (dd, J = 8.7, 3.8 Hz, 1H). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 26.0. EI-MS m/z 403 (M^+, 100),342 (25), 69 (30). EI-HRMS calcd for M^+ $C_{20}H_{22}NPS_3$ 403.0646, found 403.0645.

NMR Spectra of Synthetic Intermediates and Crisp-17

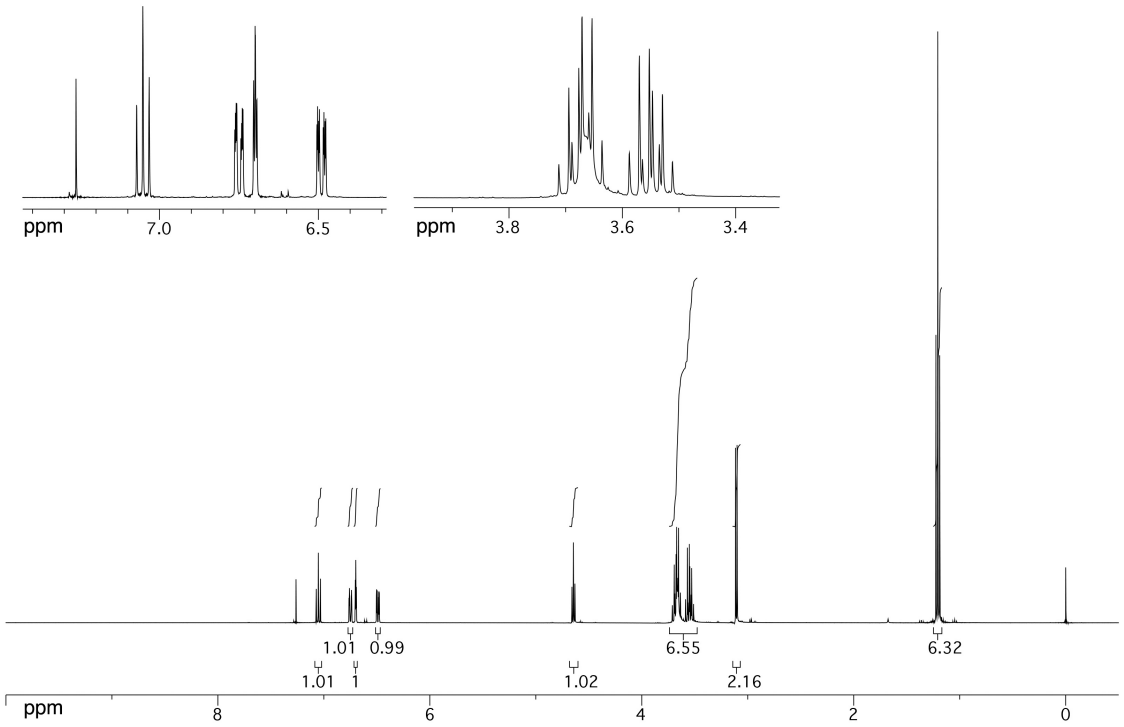


Figure S13: ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 25°C) of compound **2**.

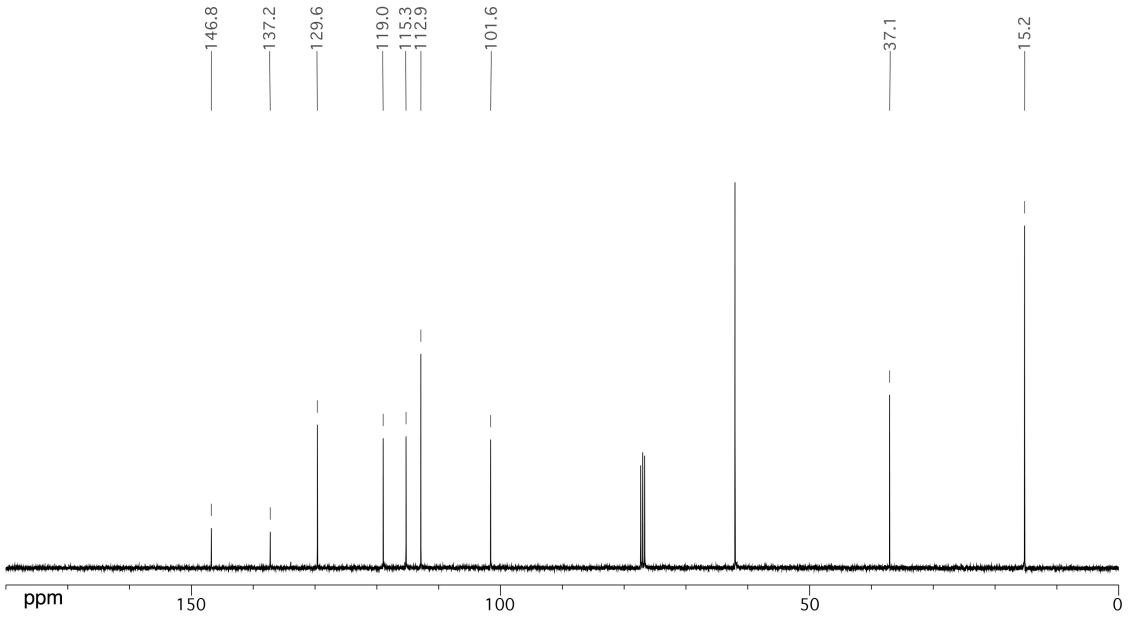


Figure S14: ^{13}C NMR spectrum (100 MHz, $CDCl_3$, 25°C) **2**.

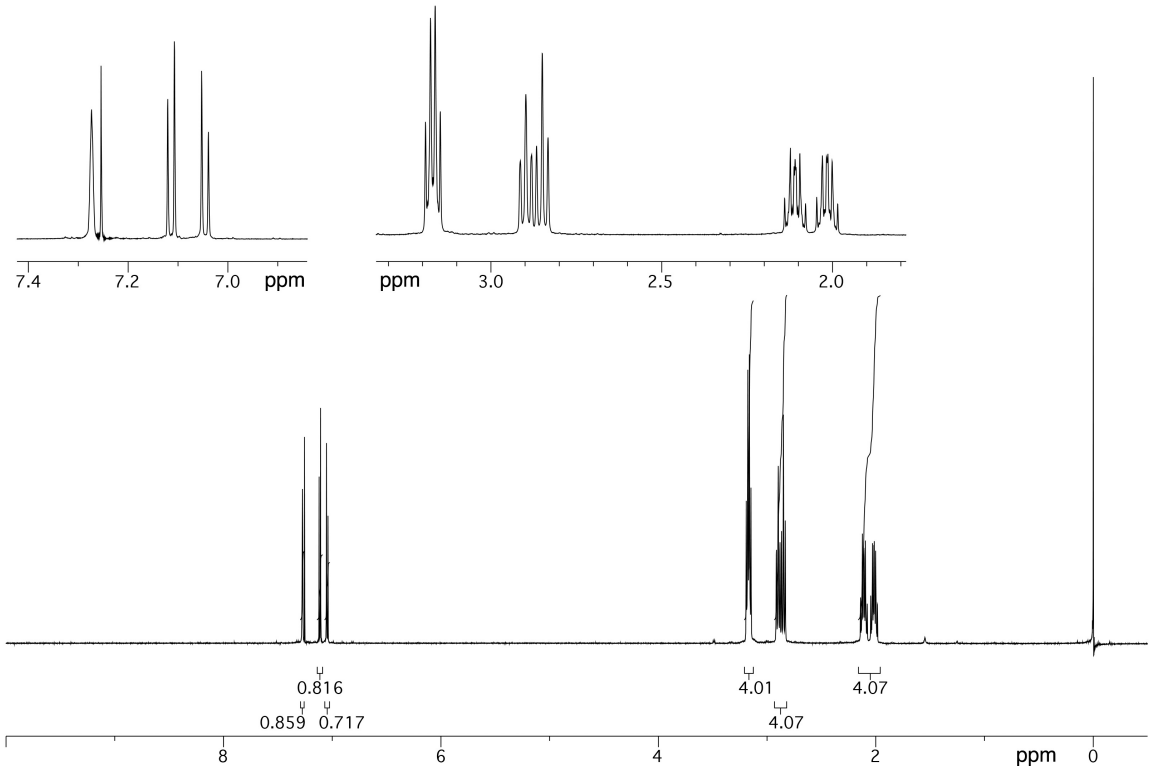


Figure S15: ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 25°C) of compound **3**.

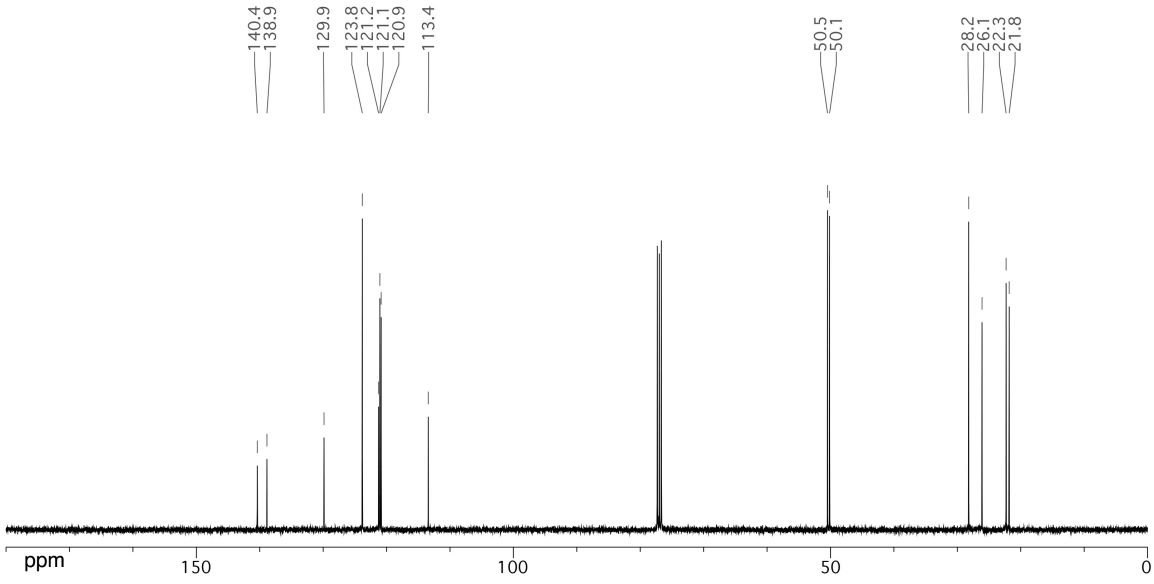


Figure S16: ^{13}C NMR spectrum (100 MHz, $CDCl_3$, 25°C) of compound **3**.

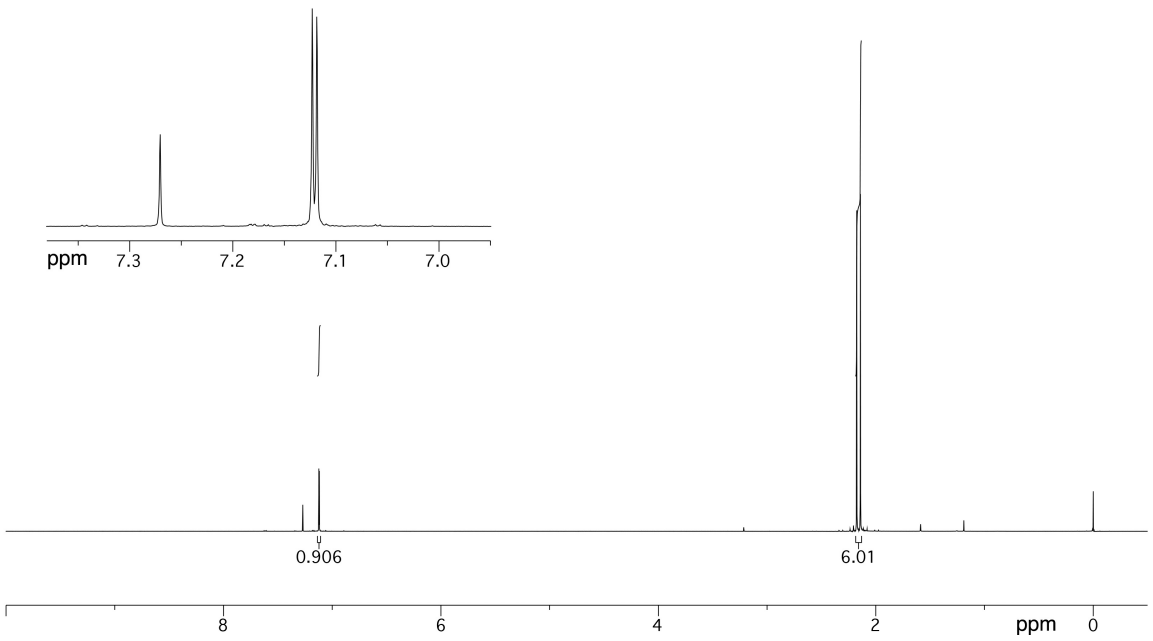


Figure S17: ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 25°C) of compound **4**.

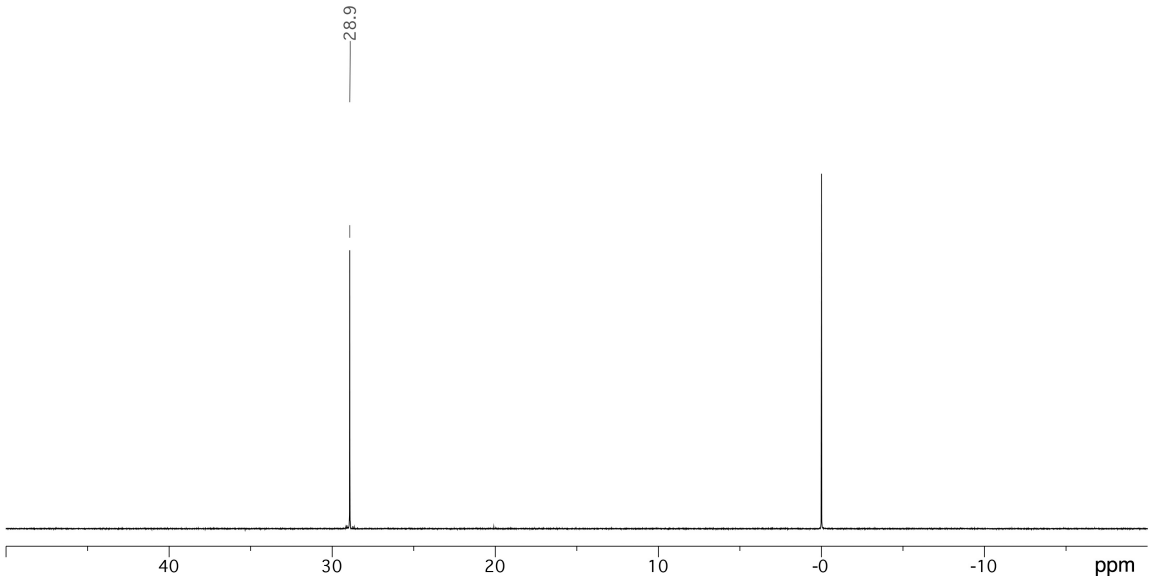


Figure S18: ^{31}P NMR spectrum (162 MHz, $CDCl_3$, 25°C) of compound **4**.

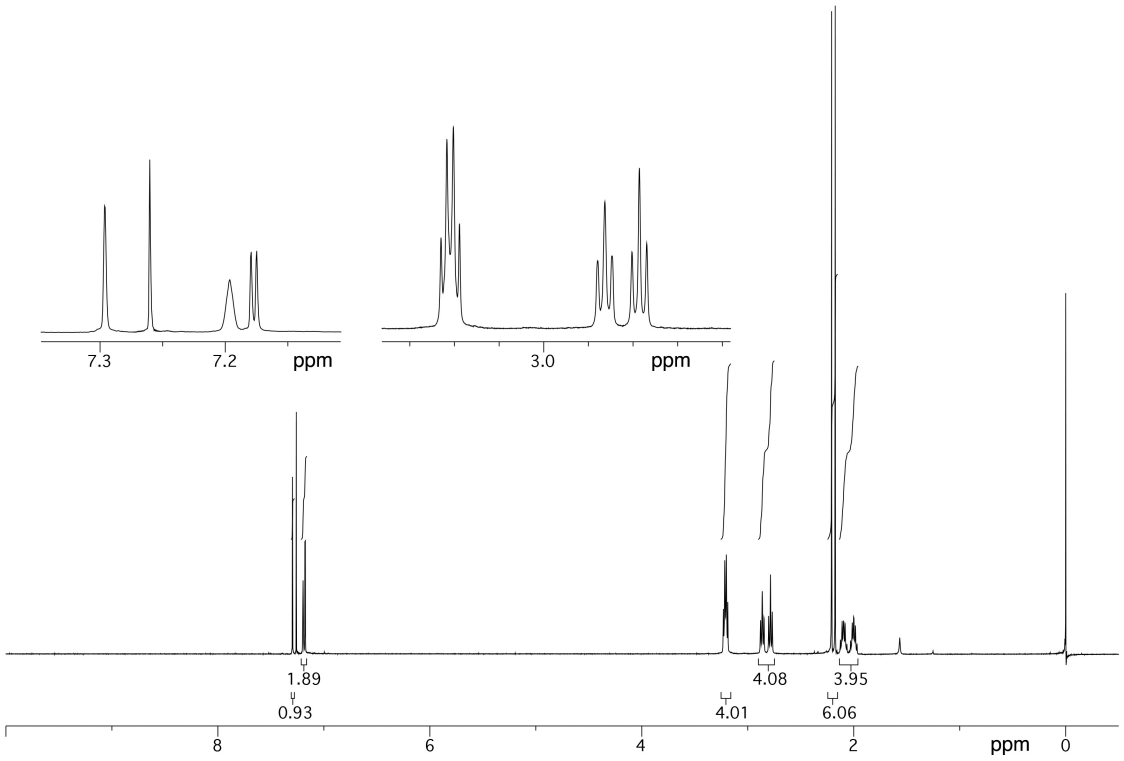


Figure S19: ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 25°C) of compound **5**.

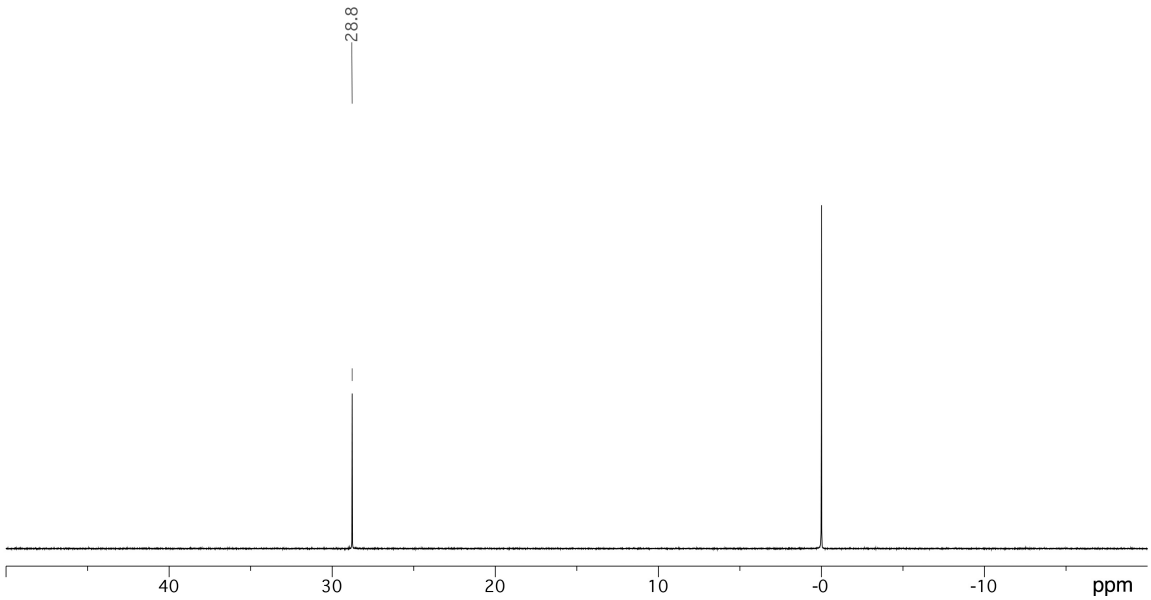


Figure S20: ^{31}P NMR spectrum (162 MHz, $CDCl_3$, 25°C) of compound **5**.

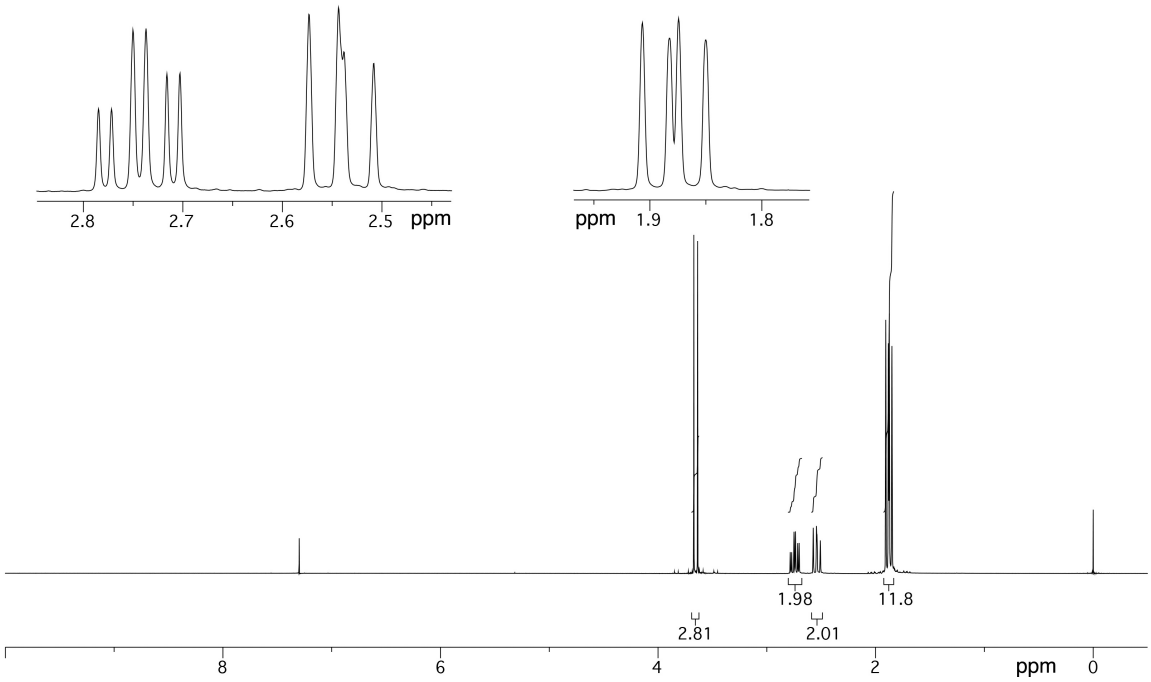


Figure S21: ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 25°C) of compound **6**.

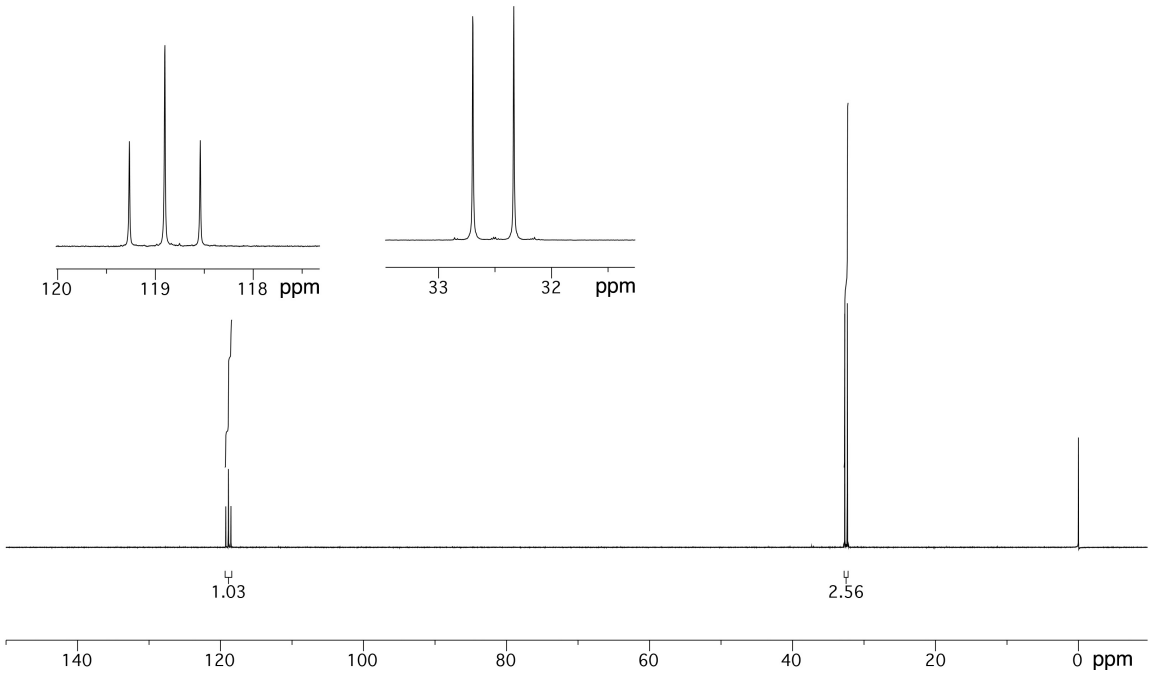


Figure S22: ^{31}P NMR spectrum (162 MHz, $CDCl_3$, 25°C) of compound **6**.

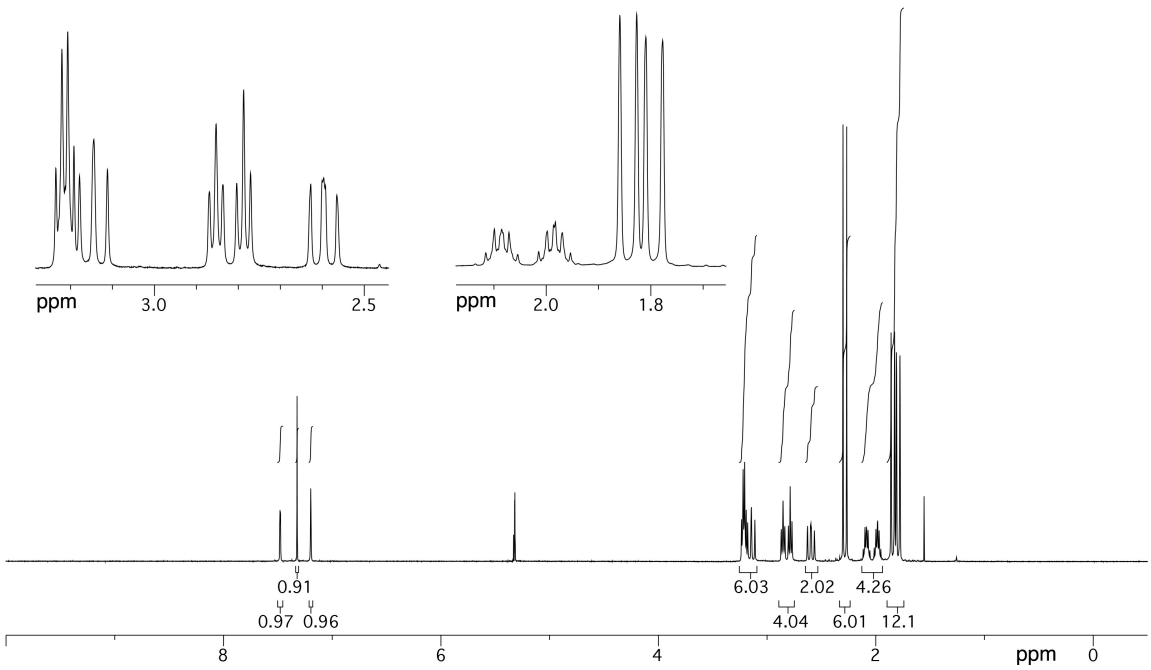


Figure S23: ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 25°C) of compound crisp-17.

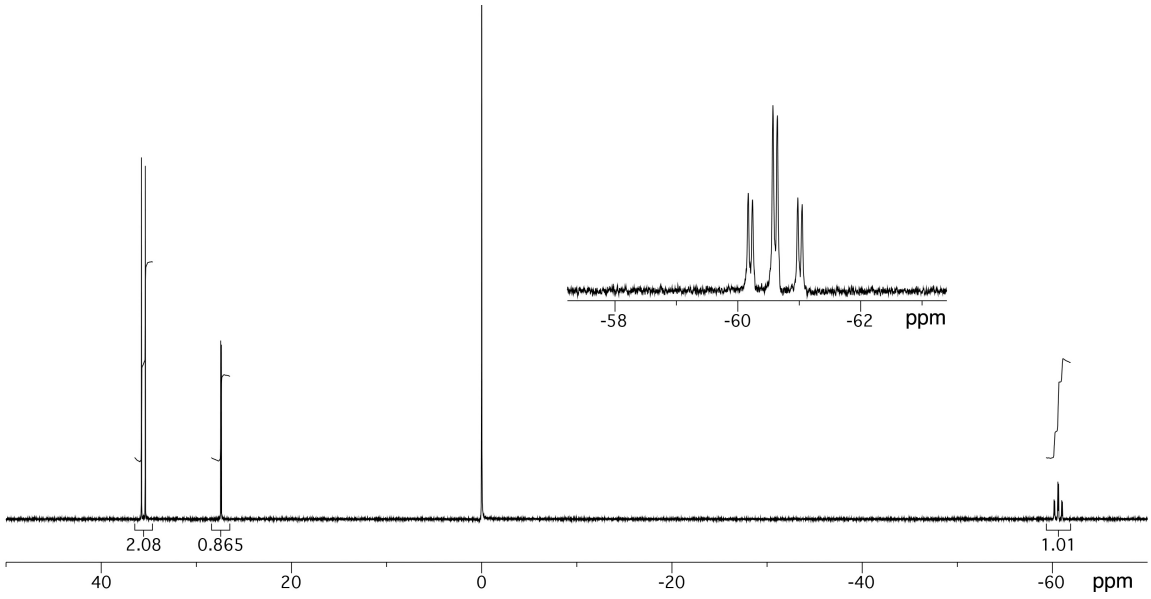


Figure S24: ^{31}P NMR spectrum (162 MHz, $CDCl_3$, 25°C) of compound crisp-17.

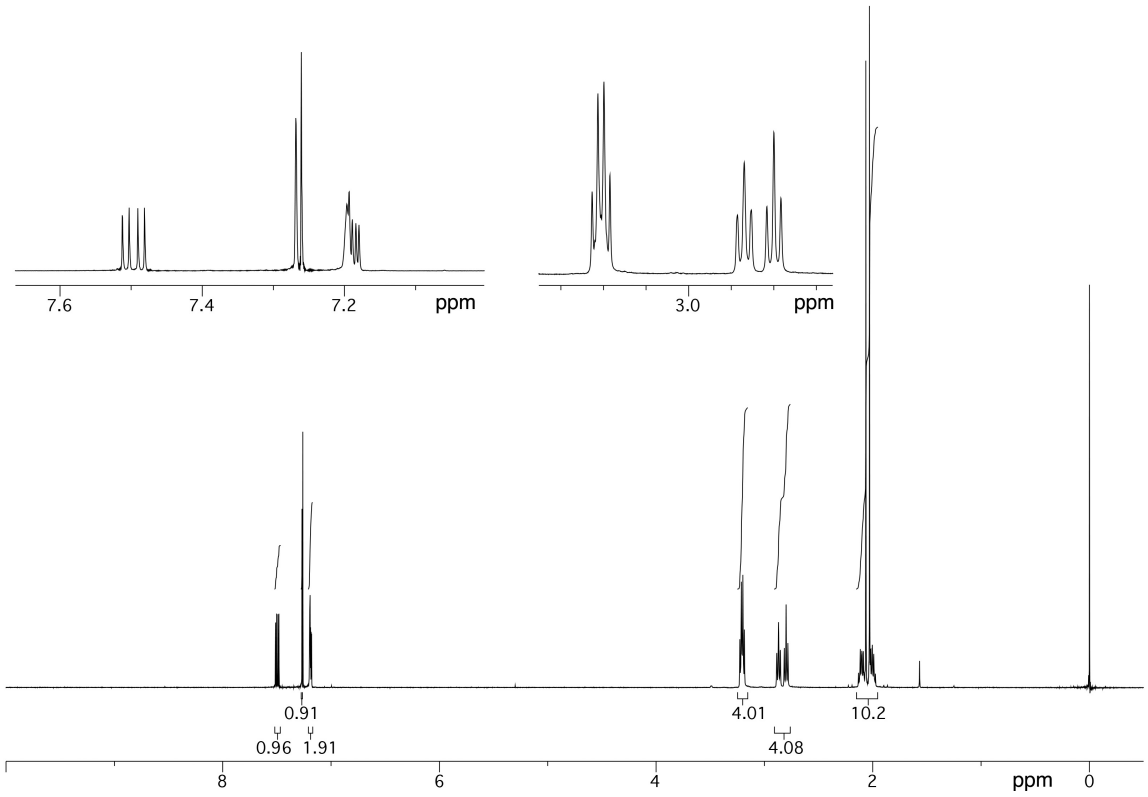


Figure S25: ^{1}H NMR spectrum of crisp-17ctrl in $CDCl_3$.

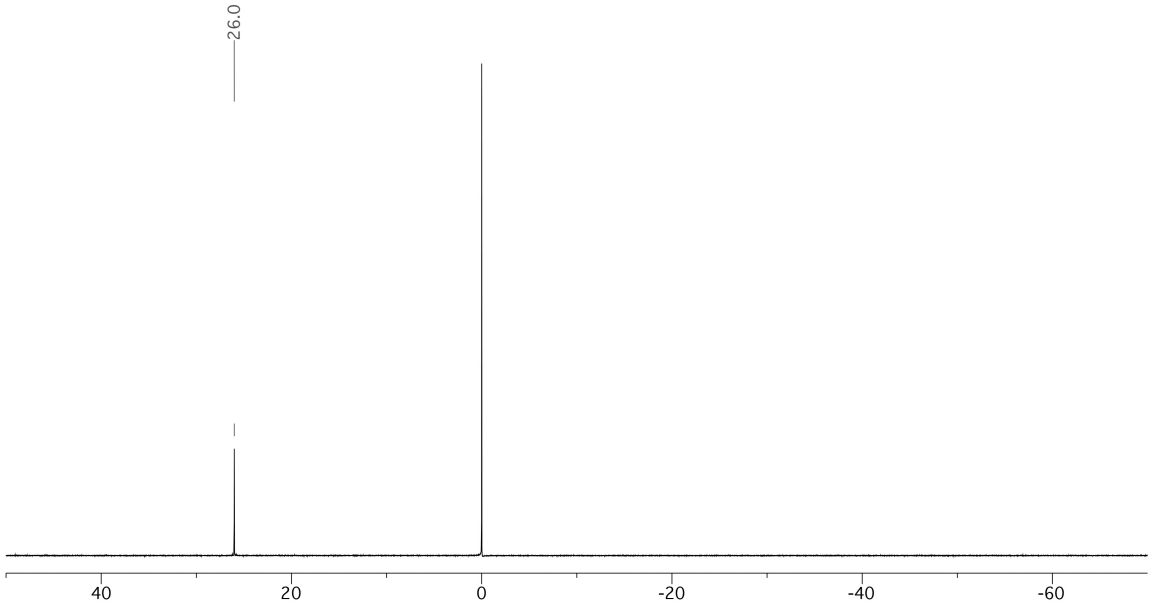


Figure S26: ^{31}P NMR spectrum (162 MHz, $CDCl_3$, 25°C) of crisp-17-ctrl

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