Supporting Information

Staudinger reaction using 2,6-dichlorophenyl azide derivatives for robust aza-ylide formation applicable to bioconjugation in living cells

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Contents

General Remarks

All reactions were performed with dry glassware under atmosphere of argon, unless otherwise noted. Analytical thin-layer chromatography (TLC) was performed on precoated (0.25 mm) silica-gel plates (Merck Chemicals, Silica Gel 60 F254, Cat. No. 1.05715) or NH_2 silica-gel plates (Wako Pure Chemical Industries Ltd., NH_2 Silica Gel 60 F254 Plate-Wako, Cat. No.146-08631). Column chromatography was conducted using silica-gel (Kanto Chemical Co., Inc., Silica Gel 60, spherical, particle size 40–50 μm, Cat. No. 37562-85) or NH_2 silica-gel (Kanto Chemical Co., Inc., NH_2 Silica Gel 60, spherical, particle size 40–50 μm, Cat. No. 37567-79). Preparative thin-layer chromatography (PTLC) was performed on silica-gel (Wako Pure Chemical Industries Ltd., Wakogel® B-5F, Cat. No. 230-00043). Melting points (Mp) were measured on a YANACO MP-J3 instrument or with an OptiMelt MPA100 (Stanford Research Systems), and are uncorrected. ^{1}H NMR spectra were obtained with a Bruker AVANCE 500 spectrometer at 500 MHz. ^{13}C NMR spectra were obtained with a Bruker AVANCE 500 spectrometer at 126 MHz. ^{31}P NMR spectra was obtained with a Bruker AVANCE 400 spectrometer at 162 MHz. $CDCl_3$ (Kanto Chemical Co., Inc, Cat. No.07663-23), CD_3OD (Kanto Chemical Co., Inc, Cat. No.25221-33) or CD_3CN (Kanto Chemical Co., Inc, Cat. No.001055-96) was used as a solvent for obtaining NMR spectra. Chemical shifts (δ) are given in parts

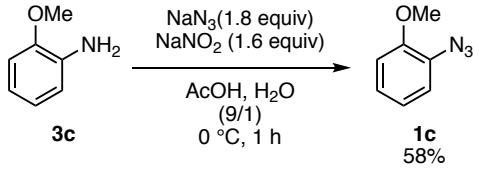
per million (ppm) downfield from $(CH_3)_4Si$ (δ 0.00 for ^1H NMR in $CDCl_3$) or the solvent peak (δ 77.0 for ^{13}C NMR in $CDCl_3$, δ 4.87 for ^1H NMR in residual CHD_2OD, δ 49.2 for ^{13}C NMR in CD_3OD and δ 1.94 for ^1H NMR in residual CHD_2CN) as an internal reference, and triphenylphosphine (δ –6.0 for ^{31}P NMR in $CDCl_3$) as an external standard with coupling constants (*J*) in hertz (Hz). The abbreviations s, d, t, sept, m and br signify singlet, doublet, triplet, septet, multiplet and broad, respectively. IR spectra were measured by diffuse reflectance method on a Shimadzu IRPrestige-21 spectrometer attached with DRS-8000A with the absorption band given in cm^{-1}. High-resolution mass spectra (HRMS) were measured on a Bruker micrOTOF mass spectrometer under positive electrospray ionization (ESI^+) conditions. Elemental analyses were carried out at A Rabbit Science Japan Co., Ltd.

1-Azido-2,6-diisopropylbenzene (**1a**),[S1] 1-azido-4-methoxybenzene (**1b**),[S2] 1-azido-4-chlorobenzene (**1e**),[S3] methyl 2-(diphenylphosphino)benzoate (**2e**),[S4] 4-(diphenylphosphino)benzoic acid (**2f**),[S5] *N*-succinimidyl 4-(diphenylphosphino)benzoate,[S5] (1*α*,8*α*,9*α*)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (**8**),[S6] 5,6-didehydro-11,12-dihydrodibenzo[*a*,*e*]cyclooctene (**10**),[S7] 2-(2-(6-chlorohexyloxy)ethoxy)ethyl-ammonium trifluoroacetate,[S8] lissamine rhodamine B sulfonyl chloride[S9] and TESRA-DBCO (**16**)[S8] were prepared according to the reported methods. Acetonitrile (Cat. No. 014-00386), dichloromethane (Cat. No. 130-02457), methanol (Cat. No. 131-01826), tetrahydrofuran (THF) (Cat. No. 204-08745), triethylamine (Cat. No. 202-02646), isopropylamine (Cat. No. 167-04866), oxalyl dichloride (Cat. No. 155-01642), *N*,*N*-dimethylformamide (DMF) (Cat. No. 045-02911), potassium fluoride (Cat. No. 169-03765), 4-(dimethylamino)pyridine (DMAP) (Cat. No. 042-19212), 4-amino-3,5-dichlorobenzoic acid (Cat. No. 321-53831), triphenylphosphine (**2a**) (Cat. No. 204-03061), tri(*o*-tolyl)phosphine (**2d**) (Cat. No. 323-67201), benzyl azide (**5**) (Cat. No. 355-40762) and dithiothreitol (Cat. No. 040-29223) were purchased from Wako Pure Chemical Industries Ltd. Copper(I) chloride (Cat. No. 07524-20) was purchased from Kanto Chemical Co. Inc. 2,6-Dimethoxyphenylboronic acid (Cat. No. 480096) and tris(4-methoxyphenyl)phosphine (**2c**) (Cat. No. 395102) were purchased from Sigma–Aldrich Japan. Trimethylsilyl azide (Cat. No. T0801), *tert*-butyl nitrite (Cat. No. N0357), 1,11-diamino-3,6,9-trioxaundecane (Cat. No. D3664), 11-azido-3,6,9-trioxa-undecan-1-amine (Cat. No. A2363) and *n*-dodecanethiol (Cat. No. D0970) were purchased from Tokyo Chemical Industry Co., Ltd. Tris(3,5-bis(trifluoromethyl)phenyl)phosphine (**2b**) (Cat. No. 15-9165) was purchased from Strem Chemicals, Inc. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (Cat. No. 344-03633) was purchased from Dojindo Molecular Technologies, Inc. All other chemical reagents used were commercial grade and used as received.

CAUTION! Azido-containing compounds are presumed to be potentially explosive. Although we have never experienced such an explosion with azido compounds used in this study, all manipulations should be carefully carried out behind a safety shield in a hood.

Chemical Experiments

*A typical procedure for the preparation of aromatic azide **1** from aniline*

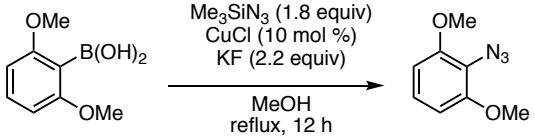


3c **1c** 58%

To a solution of 2-methoxyaniline (**3c**) (488 mg, 3.96 mmol) dissolved in AcOH (9.0 mL) and H_2O (1.0 mL) were added sodium azide (453 mg, 6.97 mmol) and sodium nitrite (438 mg, 6.35 mmol) at 0 °C. After stirring for 1 h at the same temperature, to the mixture was added a saturated aqueous solution of $NaHCO_3$ until the mixture became pH 10. The mixture was extracted with EtOAc (50 mL × 3), and the combined organic extract was washed with brine (50 mL), dried (Na_2SO_4), and after filtration, the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 30 g, *n*-hexane/EtOAc = 3/1) to give 2-methoxyphenyl azide (**1c**) (342 mg, 2.29 mmol, 57.8%) as a pale brown oil.

According to the procedure for the preparation of **1c**, 2,6-dichlorophenyl azide (**1g**) was prepared from 2,6-dichloroaniline (**3g**).

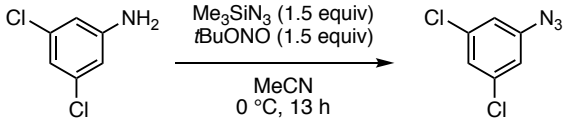
*Preparation of 2,6-dimethoxyphenyl azide (**1d**)*



1d 80%

To a solution of 2,6-dimethoxyphenylboronic acid (181 mg, 0.995 mmol) dissolved in MeOH (5.0 mL) were successively added trimethylsilyl azide (212 mg, 1.84 mmol), copper(I) chloride (10.5 mg, 0.106 mmol) and potassium fluoride (125 mg, 2.15 mmol) at room temperature. The mixture was heated at reflux with stirring for 12 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 20/1) to give 2,6-dimethoxyphenyl azide (**1d**) (143 mg, 0.798 mmol, 80.2%) as a pale brown oil.

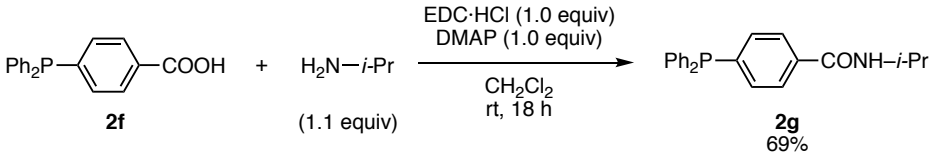
*Preparation of 3,5-dichlorophenyl azide (**1f**)*



3f **1f** 92%

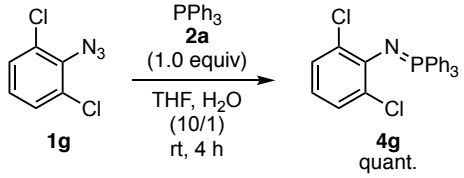
To a solution of 3,5-dichloroaniline (**3f**) (654 mg, 4.04 mmol) dissolved in MeCN (10 mL) were successively added trimethylsilyl azide (706 mg, 6.13 mmol) and *tert*-butyl nitrite (635 mg, 6.16 mmol) at 0 °C. After stirring for 13 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 60 g, *n*-hexane) to give 3,5-dichlorophenyl azide (**1f**) (695 mg, 3.70 mmol, 91.6%) as a pale brown solid.

*Preparation of 4-(diphenylphosphino)-N-isopropylbenzamide (**2g**)*



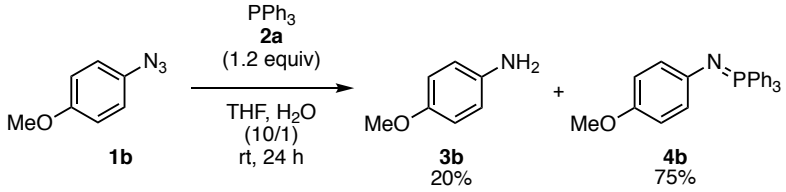
To a solution of 4-(diphenylphosphino)benzoic acid (**2f**) (308 mg, 1.01 mmol) dissolved in CH_2Cl_2 (5.0 mL) were successively added 2-aminopropane (66.7 mg, 1.13 mmol), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (194 mg, 1.01 mmol) and 4-(dimethylamino)pyridine (125 mg, 1.02 mmol) at room temperature. After stirring for 18 h at the same temperature, the mixture was concentrated under reduced pressure. After the addition of H_2O (20 mL), the mixture was extracted with EtOAc (30 mL × 3), and the combined organic extract was washed with brine (10 mL), dried (Na_2SO_4), and after filtration, the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 30 g, *n*-hexane/EtOAc = 2/1) to give 4-(diphenylphosphino)-*N*-isopropylbenzamide (**2g**) (241 mg, 0.694 mmol, 68.7%) as a colorless solid.

*Preparation of 2,6-dichloro-N-(triphenylphosphoranylidene)aniline (**4g**)*



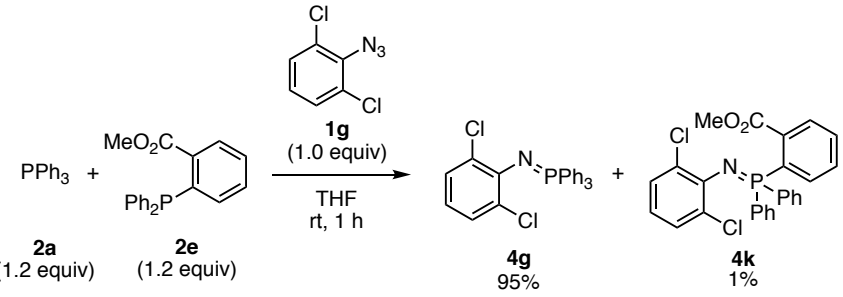
To a solution of 2,6-dichlorophenyl azide (**1g**) (471 mg, 2.51 mmol) dissolved in THF (10 mL) and H_2O (1.0 mL) was added triphenylphosphine (**2a**) (658 mg, 2.51 mmol) at room temperature. After stirring for 4 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 3/1) to give 2,6-dichloro-*N*-(triphenyl-phosphoranylidene)aniline (**4g**) (1.06 g, 2.51 mmol, quant.) as a colorless solid.

A typical procedure for the screening of aromatic azides and triarylphosphines to form stable aza-ylides



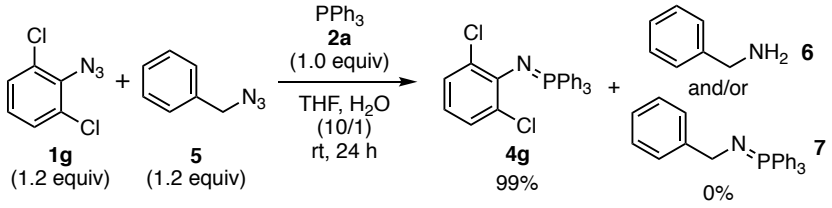
To a solution of 4-methoxyphenyl azide (**1b**) (15.9 mg, 0.107 mmol) dissolved in THF (1.0 mL) and H_2O (0.10 mL) was added triphenylphosphine (33.2 mg, 0.127 mmol) at room temperature. After stirring for 24 h at the same temperature, the mixture was concentrated under reduced pressure. To the residue was added 1,1,2,2-tetrachloroethane (10.5 mg, 62.6 μmol) as an internal standard and the mixture was dissolved in $CDCl_3$. The yields of **3b** and **4b** were determined by ^1H NMR analysis (400 MHz) to be 20.1% and 75.2% respectively, by comparing the relative values of integration for the peaks observed at 3.73 ppm (for **3b**) and 3.68 ppm (for **4b**) with that of 1,1,2,2-tetrachloroethane observed at 5.94 ppm.

*Competition experiment between triphenylphosphine (**2a**) and methyl 2-diphenylphosphinobenzoate (**2e**) with 2,6-dichlorophenyl azide (**1g**)*



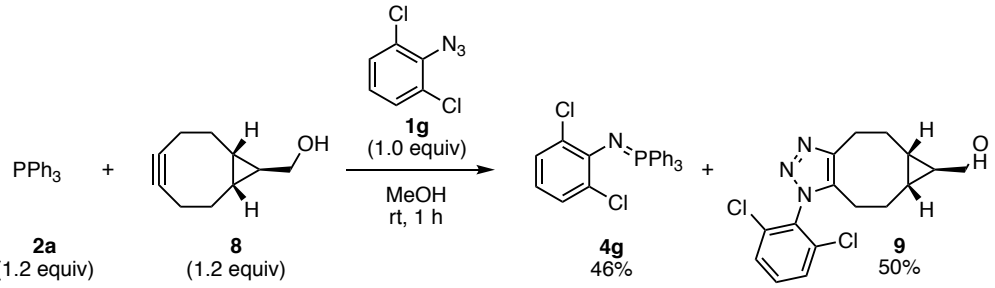
To a mixture of triphenylphosphine (**2a**) (31.9 mg, 0.122 mmol) and methyl 2-diphenylphosphinobenzoate (**2e**) (39.0 mg, 0.122 mmol) dissolved in THF (2.0 mL) was added a solution of 2,6-dichlorophenyl azide (**1g**) (19.4 mg, 0.103 mmol) dissolved in THF (1.0 mL) at room temperature. After stirring for 1 h at the same temperature, the mixture was concentrated under reduced pressure. To the residue was added 1,1,2,2-tetrachloroethane (13.4 mg, 79.8 μmol) as an internal standard and the mixture was dissolved in $CDCl_3$. The yields of **4g** and **4k** were determined by ^1H NMR analysis (400 MHz) to be 95% and 1% respectively, by comparing the relative values of integration for the peaks observed at 6.54 ppm (for **4g**) and 3.24 ppm (for **4k**) with that of 1,1,2,2-tetrachloroethane observed at 5.95 ppm.

*Competition experiment between 2,6-dichlorophenyl azide (**1g**) and benzyl azide (**5**) with triphenylphosphine (**2a**)*



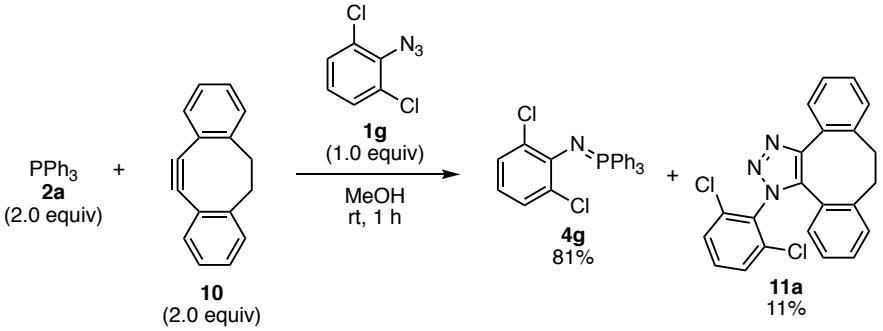
To a mixture of 2,6-dichlorophenyl azide (**1g**) (38.0 mg, 0.202 mmol) and benzyl azide (**5**) (28.3 mg, 0.213 mmol) dissolved in THF (1.0 mL) and H_2O (0.10 mL) was added triphenylphosphine (**2a**) (45.5 mg, 0.173 mmol) at room temperature. After stirring for 24 h at the same temperature, the mixture was concentrated under reduced pressure. To the residue was added 1,1,2,2-tetrachloroethane (21.4 mg, 0.128 mmol) as an internal standard and the mixture was dissolved in $CDCl_3$. The yields of **4g**, **6** and **7** were determined by ^1H NMR analysis (400 MHz) to be 99%, 0% and 0% by comparing the relative values of integration for the peaks observed at 6.53 ppm (for **4g**), 3.87 ppm (for **6**) and 4.28 ppm (for **7**) with that of 1,1,2,2-tetrachloroethane observed at 5.94 ppm.

*Competition experiment between triphenylphosphine (**2a**) and BCN derivative **8** with 2,6-dichlorophenyl azide (**1g**)*



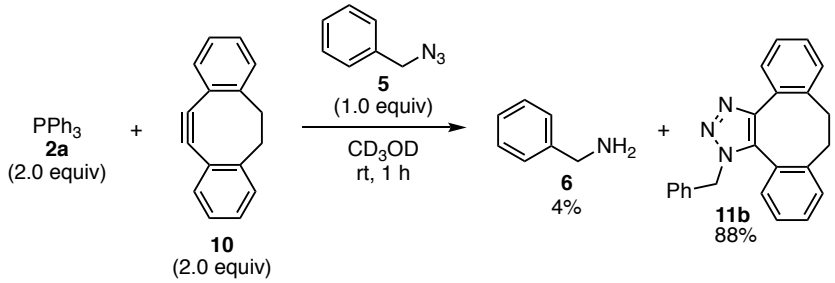
To a mixture of triphenylphosphine (**2a**) (23.6 mg, 90.0 μmol) and (1*α*,8*α*,9*α*)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (**8**) (13.5 mg, 89.9 μmol) dissolved in MeOH (3.0 mL) was added a solution of 2,6-dichlorophenyl azide (**1g**) (14.0 mg, 74.5 μmol) dissolved in MeOH (1.0 mL) at room temperature. After stirring for 1 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by preparative TLC (*n*-hexane/EtOAc = 3/1) to give 2,6-dichloro-*N*-(triphenylphosphoranylidene)aniline (**4g**) (14.5 mg, 34.3 μmol, 46.0%) as a pale yellow solid and ((5a*α*,6*α*,6a*α*)-1-(2,6-dichlorophenyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-*d*][1,2,3]triazol-6-yl)methanol (**9**) (12.6 mg, 37.3 μmol, 50.1%) as a colorless solid.

*Competition experiment between triphenylphosphine (**2a**) and 5,6-didehydro-11,12-dihydrodibenzo[a,e]cyclooctene (**10**) with 2,6-dichlorophenyl azide (**1g**)*



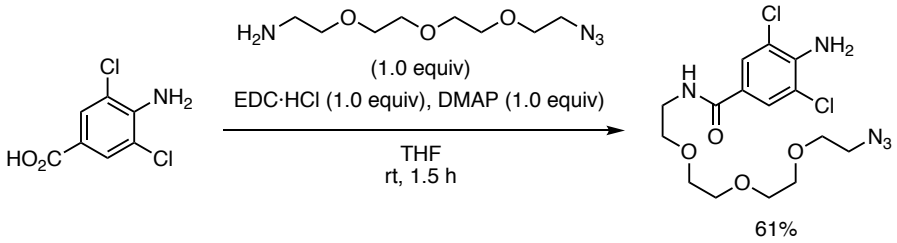
To a mixture of triphenylphosphine (**2a**) (39.9 mg, 0.152 mmol) and 5,6-didehydro-11,12-dihydrodibenzo[*a*,*e*]cyclooctene (**10**) (31.1 mg, 0.152 mmol) dissolved in MeOH (3.0 mL) was added a solution of 2,6-dichlorophenyl azide (**1g**) (14.2 mg, 75.5 μmol) dissolved in MeOH (1.0 mL) at room temperature. After stirring for 18 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 5 g, *n*-hexane/EtOAc = 1/0 to 3/1) to give 2,6-dichloro-*N*-(triphenylphosphoranylidene)aniline (**4g**) (25.7 mg, 60.9 μmol, 80.7%) as a pale yellow solid and 1-(2,6-dichlorophenyl)-8,9-dihydro-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazole (**11a**) (3.3 mg, 8.4 μmol, 11%) as a colorless solid.

*Competition experiment between triphenylphosphine (**2a**) and 5,6-didehydro-11,12-dihydrodibenzo[a,e]cyclooctene (**10**) with benzyl azide (**5**)*



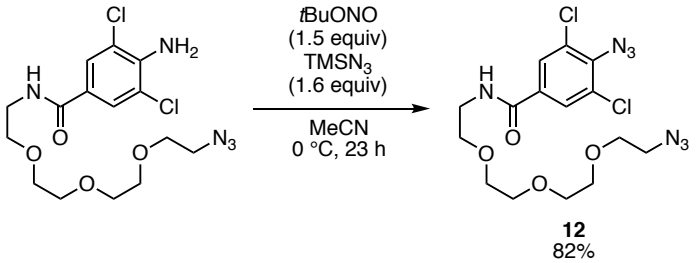
To a mixture of triphenylphosphine (**2a**) (39.0 mg, 0.149 mmol) and 5,6-didehydro-11,12-dihydrodibenzo[*a*,*e*]cyclooctene (**10**) (31.0 mg, 0.152 mmol) dissolved in CD_3OD (3.0 mL) was added a solution of benzyl azide (**5**) (10.1 mg, 75.9 μmol) dissolved in CD_3OD (1.0 mL) at room temperature. After stirring for 1 h at the same temperature, to the mixture was added 1,1,2,2-tetrachloroethane (11.5 mg, 68.5 μmol) as an internal standard. The yields of **6** and **11b** were determined by ^{1}H NMR analysis (400 MHz) to be 4% and 88% respectively, by comparing the relative values of integration for the peaks observed at 3.79 ppm (for **6**) and 5.64 ppm (for **11b**) with that of 1,1,2,2-tetrachloroethane observed at 5.95 ppm.

Preparation of 4-amino-N-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide



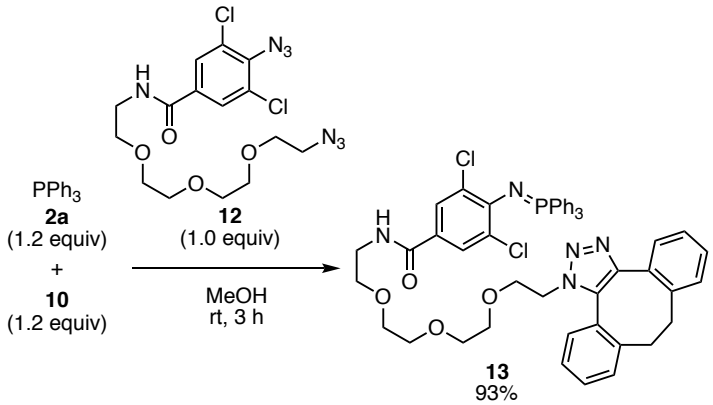
To a solution of 4-amino-3,5-dichlorobenzoic acid (416 mg, 2.02 mmol) dissolved in THF (3.0 mL) were successively added a solution of 11-azido-3,6,9-trioxaundecyl-1-amine (450 mg, 2.06 mmol) dissolved in THF (1.0 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (389 mg, 2.03 mmol) and 4-(dimethylamino)pyridine (250 mg, 2.05 mmol) at room temperature. After stirring for 1.5 h at the same temperature, the mixture was concentrated under reduced pressure. After the addition of H_2O (20 mL), the mixture was extracted with EtOAc (30 mL × 3), and the combined organic extract was washed with brine (10 mL), dried (Na_2SO_4), and after filtration, the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 45 g, *n*-hexane/EtOAc = 1/2) to give 4-amino-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide (504 mg, 1.24 mmol, 61.4%) as a colorless oil.

*Preparation of diazide **12***



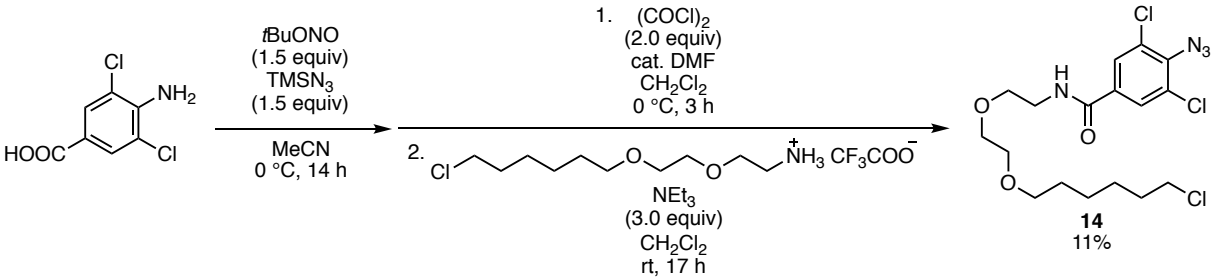
To a solution of 4-amino-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide (395 mg, 0.972 mmol) dissolved in MeCN (5.0 mL) were successively added *tert*-butyl nitrite (155 mg, 1.50 mmol) and trimethylsilyl azide (184 mg, 1.60 mmol) at 0 °C. After stirring for 23 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 20 g, CH_2Cl_2/EtOAc = 2/1) to give 4-azido-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide (**12**) (344 mg, 0.796 mmol, 81.9%) as a pale brown oil.

*Reaction of diazide **12** with an equimolar mixture of triphenylphosphine (**2a**) and 5,6-didehydro-11,12-dihydrodibenzo[a,e]cyclooctene (**10**)*



To a mixture of triphenylphosphine (**2a**) (26.0 mg, 99.1 μmol) and 5,6-didehydro-11,12-dihydrodibenzo[*a*,*e*]cyclooctene (**10**) (20.3 mg, 99.4 μmol) dissolved in MeOH (3.0 mL) was added a solution of 4-azido-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide (**12**) (35.7 mg, 82.6 μmol) dissolved in MeOH (1.0 mL) at room temperature. After stirring for 3 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 4 g, EtOAc) to give 1-(11-(3,5-dichloro-*N*-triphenylphosphoranylidene-4-aminophenyl)carbamoyl-3,6,9-trioxaundecyl)-8,9-dihydro-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazole (**13**) (66.9 mg, 76.8 μmol, 93.0%) as a colorless solid.

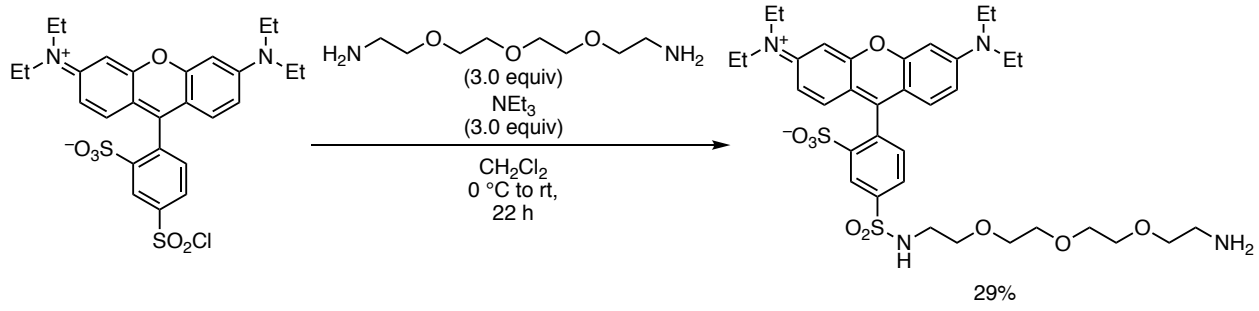
*Preparation of azido-HaloTag ligand (**14**)*



To a solution of 4-amino-3,5-dichlorobenzoic acid (828 mg, 4.02 mmol) dissolved in MeCN (10 mL) were successively added *tert*-butyl nitrite (620 mg, 6.01 mmol) and trimethylsilyl azide (706 mg, 6.13 mmol) at 0 °C. After stirring for 14 h at the same temperature, the reaction mixture was concentrated under reduced pressure. The residue that contained 4-azido-3,5-dichlorobenzoic acid (729 mg, 88% purity determined by ^{1}H NMR analysis, 2.80 mmol, 69.7%) was used in the next step without further purification.

An aliquot of the mixture containing 4-azido-3,5-dichlorobenzoic acid (122 mg, 88% purity, 0.470 mmol) was dissolved in CH_2Cl_2. To the solution were successively added one drop of DMF (ca. 3 mg, ca. 40 μmol) and oxalyl chloride (124 mg, 0.977 mmol) at 0 °C. After stirring for 2.5 h at the same temperature, the mixture was allowed to warm to room temperature. To the mixture were successively added triethylamine (0.207 mL, 1.49 mmol) and a solution of 2-(2-(6-chlorohexyloxy)ethoxy)ethylammonium trifluoroacetate (383 mg, 1.13 mmol) dissolved in CH_2Cl_2 (1.0 mL) at room temperature. After stirring for 17 h at the same temperature, the mixture was concentrated under reduced pressure. After the addition of H_2O (20 mL), the mixture was extracted with CH_2Cl_2 (30 mL × 3), and the combined organic extract was washed with brine (10 mL), dried (Na_2SO_4), and after filtration, the filtrate was concentrated under reduced pressure. The residue was purified by preparative TLC (*n*-hexane/EtOAc = 1/1) to give 4-azido-*N*-(2-(2-(6-chlorohexyl-oxy)ethoxy)ethyl)-3,5-dichlorobenzamide (azido-HaloTag ligand, **14**) (22.1 mg, 50.5 μmol, 10.7%) as a brown oil.

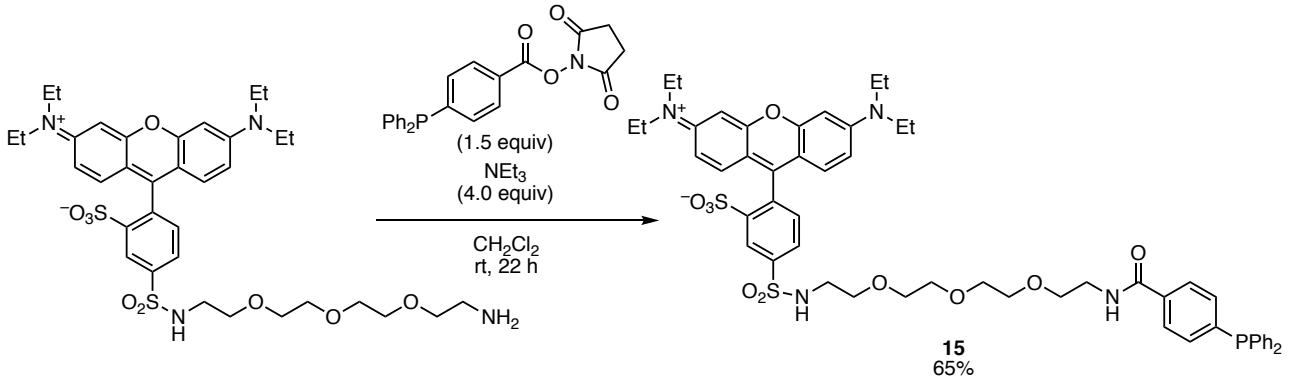
Preparation of N-(11-amino-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide



To a solution of 1,11-diamino-3,6,9-trioxaundecane (84.1 mg, 0.437 mmol) dissolved in CH_2Cl_2 (5.0 mL) were successively added triethylamine (60.5 μL, 0.434 mmol) and a solution of lissamine rhodamine B sulfonyl chloride (83.2 mg, 0.144 mmol) dissolved in CH_2Cl_2 (5.0 mL) at 0 °C. After gradually warming to room temperature, the mixture was stirred for 22 h at the same temperature. The mixture was concentrated under reduced pressure, and the residual solid was collected by filtration and then washed with EtOAc. The collected solid was purified by flash column chromatography (NH_2-silica-gel 15 g, CH_2Cl_2/MeOH = 10/1). The resulting residue was dissolved in CH_2Cl_2 (5.0 mL) and H_2O (5.0 mL). To the solution was added an aqueous solution of HCl (1 M) until the mixture became pH 2–3. After washing the solution with CH_2Cl_2 (30

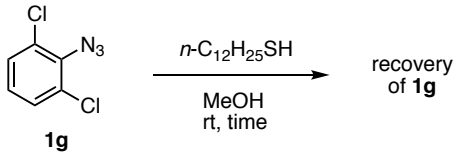
mL × 3), to the aqueous layer was added a saturated aqueous solution of $NaHCO_3$ until the mixture became pH 10. The mixture was extracted with CH_2Cl_2 (30 mL × 3), and the combined organic extract was washed with brine (10 mL), dried (Na_2SO_4), and after filtration, the filtrate was concentrated under reduced pressure. The residue solid was washed with EtOAc to give *N*-(11-amino-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide (30.5 mg, 41.6 μmol, 28.9%) as a purple solid.

Preparation of N-(11-(4-(diphenylphosphino)benzamido)-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide (TESRA-phosphine, ***15****)*



To a solution of *N*-(11-amino-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide (76.3 mg, 0.104 mmol) dissolved in CH_2Cl_2 (10 mL) were successively added triethylamine (57.7 μL, 0.414 mmol) and succinimidyl 4-(diphenylphosphino)benzoate (64.5 mg, 0.160 mmol) at room temperature. After stirring for 22 h at the same temperature, to the mixture was added H_2O (20 mL) and extracted with CH_2Cl_2 (30 mL × 3). The combined organic extract was washed with brine (10 mL), dried (Na_2SO_4), and after filtration, the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, CH_2Cl_2/MeOH = 10/1) to give TESRA-phosphine (**15**) (69.5 mg, 68.1 μmol, 65.5%) as a purple solid.

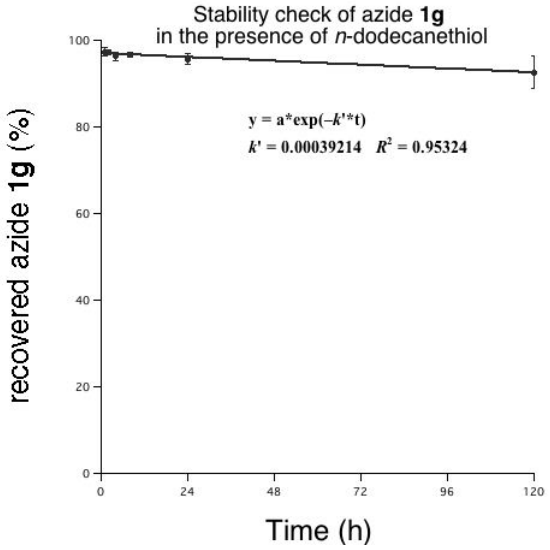
Stability check of azide ***1g*** *in the presence of an excess amount of n-dodecanethiol*



The rate measurement of the degradation of 2,6-dichlorophenyl azide (**1g**) in the presence of excess amount of *n*-dodecanethiol was monitored by HPLC analysis for 120 h at room temperature. To a solution of *n*-dodecanethiol (60.7 mg, 0.300 mmol) dissolved in MeOH (0.30 mL) were added di(*p*-tolyl) ether (30 μmol, 100 mM MeOH solution, 0.30 mL) as an internal standard and 2,6-dichlorophenyl azide (**1g**) (30 μmol, 100 mM MeOH solution, 0.30 mL). The consumption of **1g** was monitored by HPLC analysis. After stirring for 1, 2, 4, 8, 24, or 120 h, an aliquot (20 μL) of the mixture was diluted by MeOH to 2.0 mL in a volumetric flask. The amount of recovered **1g** was determined by analytical reverse phase HPLC [column: Shiseido CAPCELL PAK MG II (4.6 mm i.d. × 250 mm); mobile phase: CH_3CN:H_2O = 40:60 (0–5 min), linear gradient from 40:60 to 99:1 (5–25 min), 99:1 (25–35 min); flow rate: 1.00 mL/min; detection: UV at 254 nm] based on the calibration curve from the integration ratio between azide **1g** (R*t* = 24.4 min) and di(*p*-tolyl) ether (R*t* = 28.4 min). The analyses were repeated in triplicate. Error bars represent standard error from three replicate experiments.

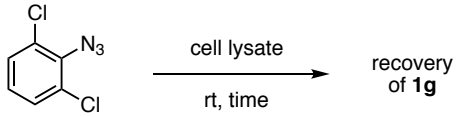
The recovered amount of azide **1g** was plotted versus time and fitted to a first order exponential decay curve. The pseudo-first-order rate constant (*k'*) was determined by least-squares fitting of the data to the following exponential equation using KaleidaGraph ver. 4.1.4.

*y = a*exp(–k'*t)*
*a: initial amount of 2,6-dichlorophenyl azide (**1g**)*
t: time (h) from the starting point of the observation



The half-life of azide **1g** (33 mM) in the presence of 0.33 M *n*-dodecanethiol in methanol at room temperature was found to be 74 ± 7 days.

*Stability check of azide **1g** in cell lysate*



1g

Preparation of cell lysate: HEK293 cells were suspended in PBS at 7.0×10^7 cells mL^{-1}, and then lysed using Q125 sonicator (QSonica, LLC, Newtown, CT, USA). The sonicated cell lysate was centrifuged at 15,000 rpm for 20 min to remove debris. The supernatant was used to examine stability of azido-moiety in the cell lysate.

The rate measurement of the degradation of 2,6-dichlorophenyl azide (**1g**) in cell lysate was monitored by HPLC analysis for 24 h at room temperature. To a DMSO solution (20 μL) of 2,6-dichlorophenyl azide (**1g**) (10 μmol) and di(*p*-tolyl) ether (10 μmol) as an internal standard was added cell lysate (80 μL) prepared as above. The consumption of **1g** was monitored by HPLC analysis. After stirring for 1, 2, 4, 8, and 24 h, an aliquot (ca. 2 μL) of the mixture was diluted by MeOH (ca. 1 mL) and passed through a RephiQuik PTFE syringe filter (RephiLe Bioscience, Ltd.; pore Size 0.2 μm, diameter 13 mm, Cat. No. RJF1322NH). The amount of recovered **1g** was determined by analytical reverse phase HPLC [column: Shiseido CAPCELL PAK MG II (4.6 mm i.d. × 250 mm); mobile phase: CH_3CN:H_2O = 40:60 (0–5 min), linear gradient from 40:60 to 99:1 (5–25 min), 99:1 (25–35 min); flow rate: 1.00 mL/min; detection: UV at 254 nm] based on the calibration curve from the integration ratio between azide **1g** (R*t* = 24.4 min) and di(*p*-tolyl) ether (R*t* = 28.4

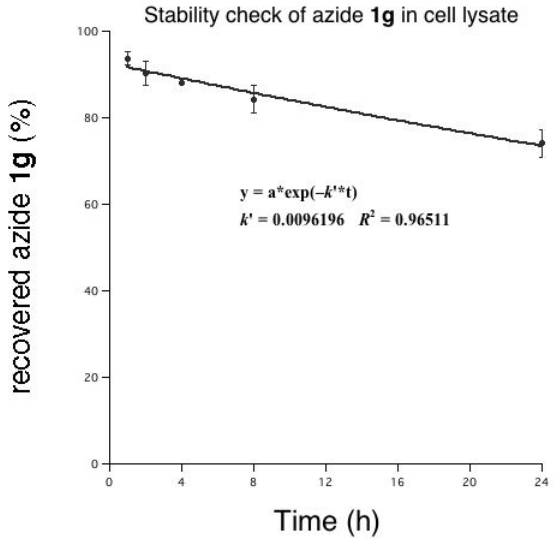
min). The analyses were repeated in quadruplicate. Error bars represent standard error from four replicate experiments.

The recovered amount of azide **1g** was plotted versus time and fitted to a first order exponential decay curve. The pseudo-first-order rate constant (*k*") was determined by least-squares fitting of the data to the following exponential equation using KaleidaGraph ver. 4.1.4.

*y = a*exp(–k'*t)*

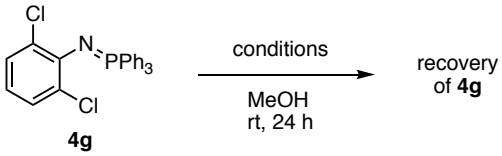
*a: initial amount of 2,6-dichlorophenyl azide (**1g**)*

t: time (h) from the starting point of the observation



The half-life of azide **1g** (100 mM) in cell lysate was found to be 72 ± 7 h.

*Stability check of aza-ylide **4g** under various conditions*



Entry	Conditions	Recovery (%)[a]
1	aq. HCl (1 M)	quant.
2	aq. $NaHCO_3$ (sat.)	quant.
3	L-cysteine (3.0 equiv)	quant.
4	L-lysine (3.0 equiv)	quant.
5	L-tyrosine (3.0 equiv)	99

[a]Determined by ^{1}H NMR analysis.

Entry 1: To a solution of 2,6-dichloro-*N*-(triphenylphosphoranylidene)aniline (**4g**) (41.4 mg, 98.0 μmol) dissolved in MeOH (1.0 mL) was added an aqueous solution of HCl (1 M, 1.0 mL) at room temperature. After stirring for 24 h at the same temperature, to the mixture was added a saturated aqueous solution of $NaHCO_3$ until the mixture became pH 10. The mixture was extracted with EtOAc (5 mL × 3), and the combined organic extract was dried (Na_2SO_4). After filtration, the filtrate was concentrated under reduced pressure. To the residue was added dibenzyl ether (12.8 mg, 64.6 μmol) as an internal standard. The

recovered amount of **4g** was determined by ^{1}H NMR analysis (400 MHz) to be quantitative by comparing the relative values of integration for the peak observed at 6.54 ppm (for **4g**) with that of dibenzyl ether observed at 4.56 ppm.

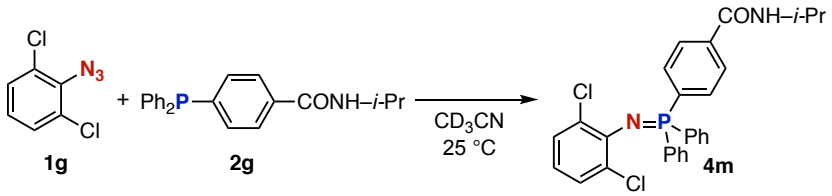
Entry 2: To a solution of 2,6-dichloro-*N*-(triphenylphosphoranylidene)aniline (**4g**) (42.6 mg, 0.101 mmol) in MeOH (1.0 mL) was added a saturated aqueous solution of $NaHCO_3$ (1.0 mL) at room temperature. After stirring for 24 h at the same temperature, the mixture was extracted with EtOAc (5 mL × 3), and the combined organic extract was dried (Na_2SO_4). After filtration, the filtrate was concentrated under reduced pressure. To the residue was added 1,1,2,2-tetrachloroethane (16.8 mg, 0.100 mmol) as an internal standard. The recovered amount of **4g** was determined by ^{1}H NMR analysis (400 MHz) to be quantitative by comparing the relative values of integration for the peak observed at 6.54 ppm (for **4g**) with that of 1,1,2,2-tetrachloroethane observed at 5.95 ppm.

Entry 3: To a solution of 2,6-dichloro-*N*-(triphenylphosphoranylidene)aniline (**4g**) (42.1 mg, 99.7 μmol) in MeOH (3.0 mL) was added L-cysteine (37.0 mg, 0.305 mmol) at room temperature. After stirring for 24 h at the same temperature, the mixture was concentrated under reduced pressure. To the residue was added 1,1,2,2-tetrachloroethane (15.0 mg, 89.4 μmol) as an internal standard. The recovered amount of **4g** was determined by ^{1}H NMR analysis (400 MHz) to be quantitative by comparing the relative values of integration for the peak observed at 6.55 ppm (for **4g**) with that of 1,1,2,2-tetrachloroethane observed at 5.96 ppm.

According to the procedure for entry 3, the stability of aza-ylide **4g** in the presence of L-lysine and L-tyrosine (entries 4 and 5) were similarly conducted.

Kinetic Study

*Experimental procedure for the kinetic study on the reaction of 2,6-dichlorophenyl azide (**1g**) with 4-(diphenylphosphino)-N-isopropylbenzamide (**2g**)*



The rate measurement of the reaction between equimolar amounts of 2,6-dichlorophenyl azide (**1g**) and 4-(diphenylphosphino)-*N*-isopropylbenzamide (**2g**) that afforded 2,6-dichloro-*N*-((4-isopropylcarbamoyl-phenyl)diphenylphosphoranylidene)aniline (**4m**) was performed by monitoring the reaction by ^{1}H NMR for 30 min at 25 °C.

To 0.30 mL of CD_3CN solution of 4-(diphenylphosphino)-*N*-isopropylbenzamide (**2g**) in five different concentrations (8.0, 10, 12, 14, or 16 mM; final concentration at 4, 5, 6, 7, and 8 mM, respectively) placed in an NMR tube (temperature kept at 25 °C) was added 0.30 mL of CD_3CN solution of 2,6-dichlorophenyl azide (**1g**) in the same concentration with **2g**. The formation of **4m** and consumption of phosphine **2g** were monitored by ^{1}H NMR analysis (400 MHz). Since aza-ylide **4m** was obtained quantitatively as the sole product, the amount of aza-ylide **4m** formed was equated to the decreased amount of phosphine **2g** determined by the integrations.

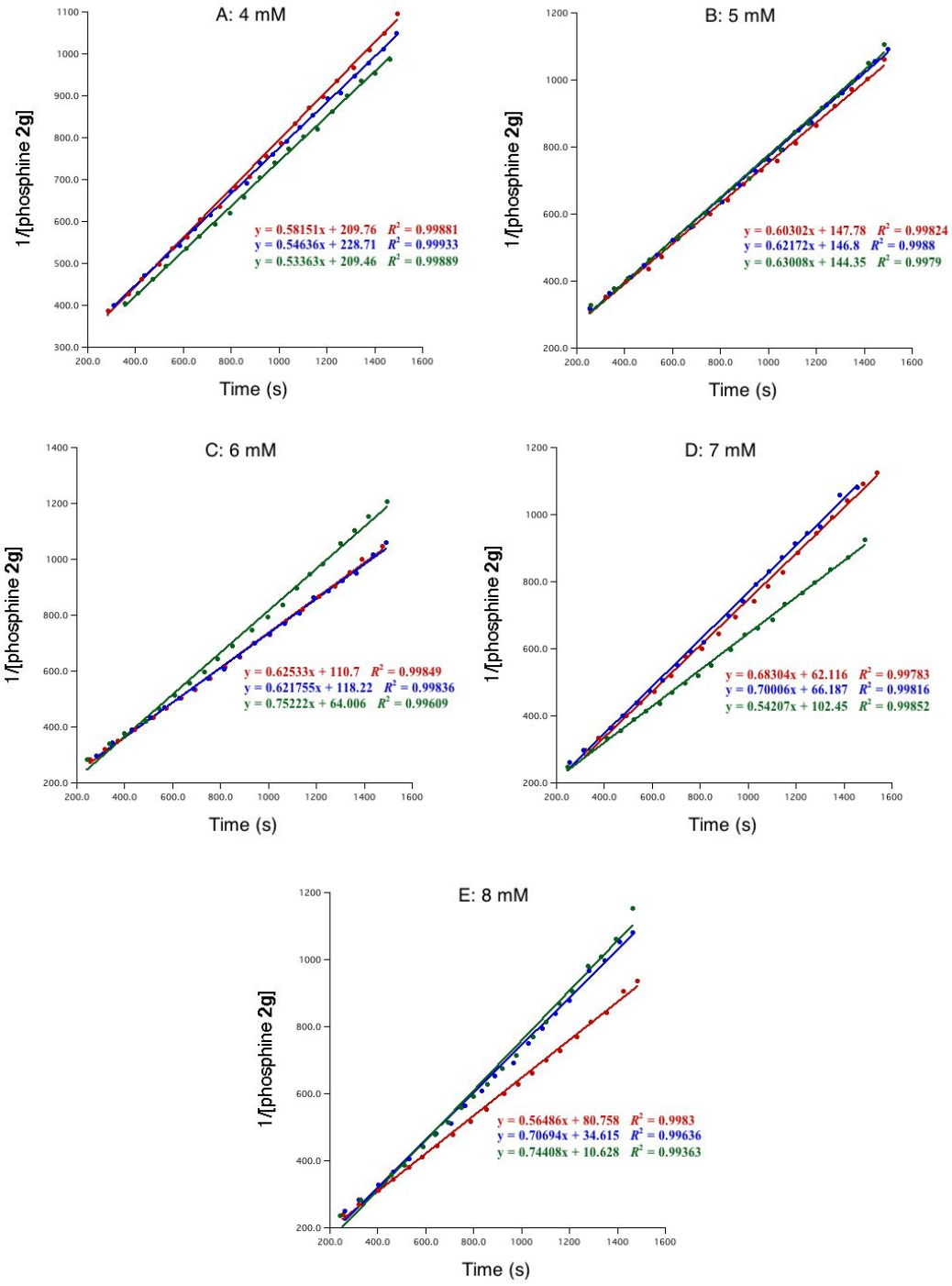
The ^{1}H NMR analysis was performed every 30–90 s for 1500 s. The second-order rate constant (k) was determined by plotting 1/[observed concentrations of phosphine **2g**] versus time. The points fitted to a linear

regression and the slope corresponds to the second-order rate constant (k), which was determined by linear regression analysis using KaleidaGraph ver. 4.1.4 (graphs A–E below).

$1/[A] = 1/[A_0] + kt$

A: concentration of 4-(diphenylphosphino)-*N*-isopropylbenzamide (**2g**)

t: time (s) from the starting point of the observation



The experiment was triplicated for each concentration of azide **1g** and phosphine **2g** and the average of fifteen experiments indicates the second-order rate constant (k) to be 0.63 ± 0.02 $M^{-1}s^{-1}$ for the Staudinger reaction between azide **1g** and phosphine **2g**.

Biological Experiments

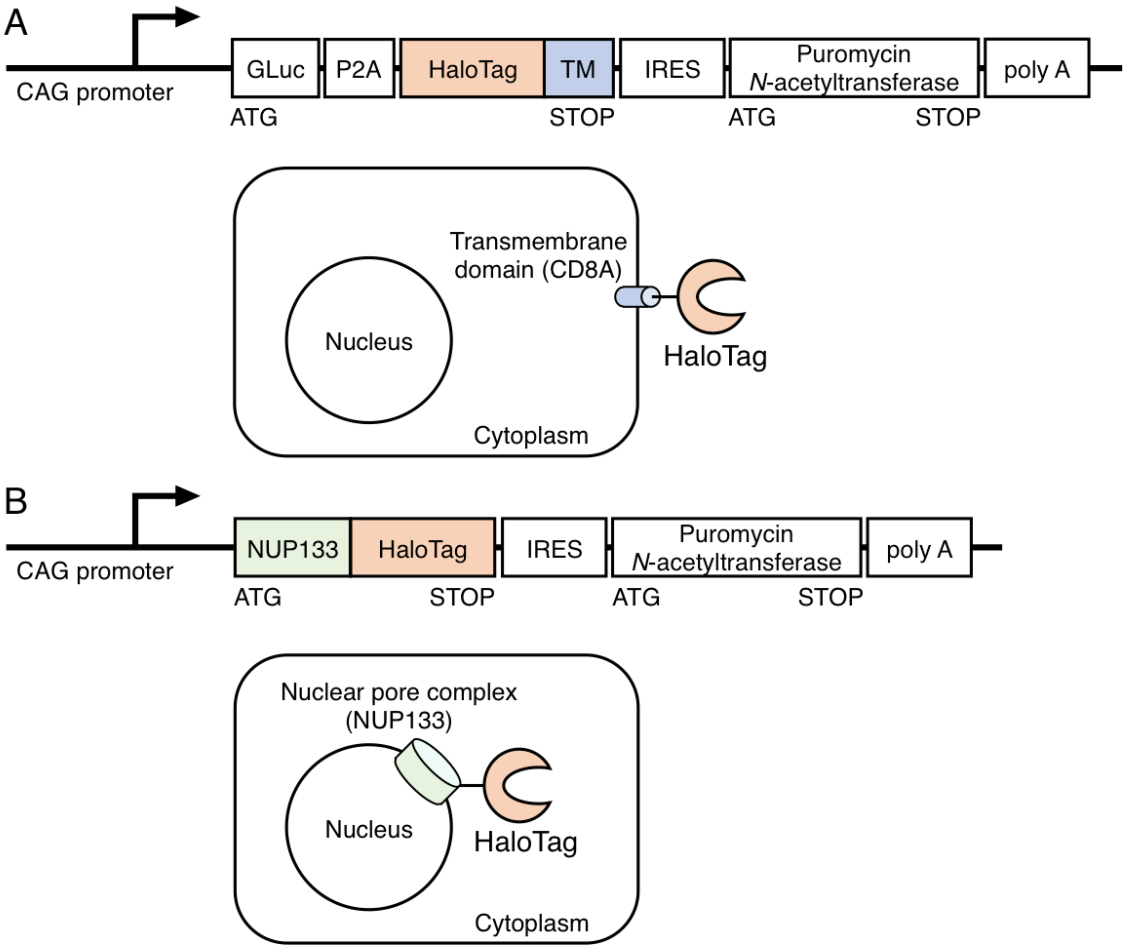
Plasmid construction

The vector of GST-HaloTag for the recombinant protein expression in *E. coli* cells was constructed previously.[S8]

To construct the expression vector of HaloTag fused with the nuclear pore complex protein NUP133, cDNA of NUP133 was obtained as FlexiClone pF1KB0166 (Kazusa DNA Research Institute, Chiba, Japan). A PCR-amplified fragment of NUP133 was digested with Xho I and Not I, and then subcloned into Xho I/Not I site of pCAGIPuro. A PCR-amplified fragment of HaloTag was inserted into the Not I site of pCAGIPuro harboring NUP133 by In-Fusion HD cloning kit (Clontech, TAKARA BIO Inc., Shiga, Japan), generating the expression vector of NUP133-HaloTag. Between NUP133 and HaloTag, Gly-Ser linker (15 a.a.) was inserted by designing PCR primers.

To construct the expression vector of HaloTag anchored on the cell surface, HaloTag was fused with transmembrane domain of human CD8A. cDNA of Gaussia luciferase harboring signal sequence (GLuc) was obtained from NanoLight Technologies (Arizona, USA). cDNAs of P2A, FLAG, Myc, HA, and PA peptides fused in-frame with transmembrane domain (TM) of human CD8A (P2A-FLAG-Myc-HA-PA-TM) was synthesized (GenScript Japan Inc., Tokyo, Japan). P2A is a ribosome skipping sequence to give two individual proteins from one mRNA.[S10] PCR-amplified fragments of GLuc and P2A-FLAG-Myc-HA-PA-TM were fused and inserted into Xho I/Not I site of pCAGIPuro by In-Fusion HD cloning kit, generating the expression vector of GLuc-P2A-FLAG-Myc-HA-PA-TM. A PCR-amplified fragment of HaloTag was inserted into Afl II site between PA and TM of the vector by In-Fusion HD cloning kit, resulting in establishment of the GLuc-P2A-FLAG-Myc-HA-PA-HaloTag-TM expression vector.

All of the PCR products were sequenced by using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3130 or ABI 3730 Genetic Analyzer (Applied Biosystems, California, USA). The reconstituted vector sequences are available upon request. The structural cartoons of these expression vectors and its coding proteins were shown below.



Structural cartoons of the expression vectors and the cellular localizations of its coding proteins.

(A) The upper cartoon shows the structure of the expression vector for HaloTag protein fused with the transmembrane domain (TM) of human CD8A. Its expression was driven by human CMV enhancer and chicken β-actin promoter (CAG promoter). IRES (internal ribosome entry site) allows bicistronic expression of GLuc-P2A-HaloTag-TM and puromycin *N*-acetyltransferase. Poly A indicates polyadenylation site. The lower cartoon shows the cellular localization of HaloTag-TM. HaloTag-TM is anchored on the cell surface, facing the extracellular milieu.

(B) The upper cartoon shows the structure of the expression vector for HaloTag protein fused with the nuclear pore complex protein NUP133. The lower cartoon shows the cellular localization of NUP133-HaloTag. NUP133 is integrated into the nuclear pore complex, resulting in the localization of HaloTag on the nucleus.

Production of recombinant GST-HaloTag protein in E. coli

E. coli strain Rosetta (DE3) pLysS cells (Novagen, Merck Chemicals Ltd., Nottingham, UK) were transformed with the pGEX6P-1-HaloTag vector,[S8] and cultured in LB media containing 50 mg L^{-1} Carbenicillin (Nacalai Tesque, Kyoto, Japan) and 34 mg L^{-1} chloramphenicol (Nacalai Tesque). Expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (final concentration at 1 mM) (Nacalai Tesque), when the culture had reached an OD600 of approximately 0.8. After induction for 16 h at 30 °C, the cells were collected by centrifugation at 4,500 g for 15 min, and frozen in liquid N_2. After thawing, the cells were suspended in cell lysis buffer containing 20 mM HEPES-KOH (pH 8.0), 200 mM NaCl, 2 mM tris(2-carboxyethyl)phosphine hydrochloride (Nacalai Tesque), 10% glycerol (Nacalai Tesque), and 1% Triton X-100, and then lysozyme (TCEP; Nacalai Tesque) was added to the cell lysate, which were

incubated on ice for 30 min. $MgCl_2$ (final concentration at 10 mM) and DNase I (final concentration of approximately 20 μg mL^{-1}) were added into the cell lysate, and incubation was continued for 1 h at 4 °C. Cell debris and larger particles were removed by centrifugation at 8,000 g for 20 min at 4 °C, and the supernatant was then filtered through a 0.45-μm filter. The supernatant of the cell lysate containing the GST-HaloTag protein was applied onto a GST-Accept COSMOGEL (Nacalai Tesque), which had been pre-equilibrated with cell lysis buffer. After excessive washing of the resin with PBS (TAKARA BIO Inc.), the bound GST-HaloTag protein was subjected to the chemical modification. To analyze the concentration of the GST-HaloTag protein, we performed SDS-PAGE. The protein sample was diluted by 1:1 with 2× SDS sample loading buffer (Nacalai Tesque), heated for 5 min at 98 °C, and then loaded onto the gel. The proteins were stained with Coomassie brilliant blue (CBB) rapid stain kit (Nacalai Tesque). The concentration of the recombinant proteins was determined by comparing with bovine serum albumin (Fraction V; Nacalai Tesque) as the standard.

Chemical modification of GST-HaloTag

GST-HaloTag (total 0.36 nmol in 200 μL of reaction mixture), bound on the GST-Accept resin (bed volume; 5 μL), was incubated with or without 100 μM of azido-HaloTag ligand (**14**) in PBS overnight at 4 °C. The azide-incorporated GST-HaloTag protein bound on the resin (azido-GST-HaloTag-resin) was extensively washed with PBS, and then mixed with 50 μM of TESRA-phosphine (**15**) in PBS. The tubes were incubated for 30 min at room temperature. The azido-GST-HaloTag-resin was extensively washed with PBS for the following SDS-PAGE. GST-HaloTag was eluted by incubation for 5 min at 98 °C with 1× SDS sample loading buffer. We used 50 μL bed volume of the labeled GST-HaloTag-resin for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. The labeled HaloTag was excised from GST by the addition of PreScission protease (GE Healthcare UK Ltd, Buckinghamshire, UK) in 50 mM Tris-HCl, containing 150 mM NaCl, 1 mM TCEP and 1 mM EDTA to the resin and incubation overnight at 4 °C. The eluates were subjected to MALDI-TOF-MS analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out under reducing conditions using a 5–20% polyacrylamide gel (ATTO, Tokyo, Japan). The gels were directly visualized by laser-scanning in a fluorescence imaging analyzer Typhoon 9410 (GE Healthcare). The gels were also stained with CBB rapid stain kit (Nacalai Tesque).

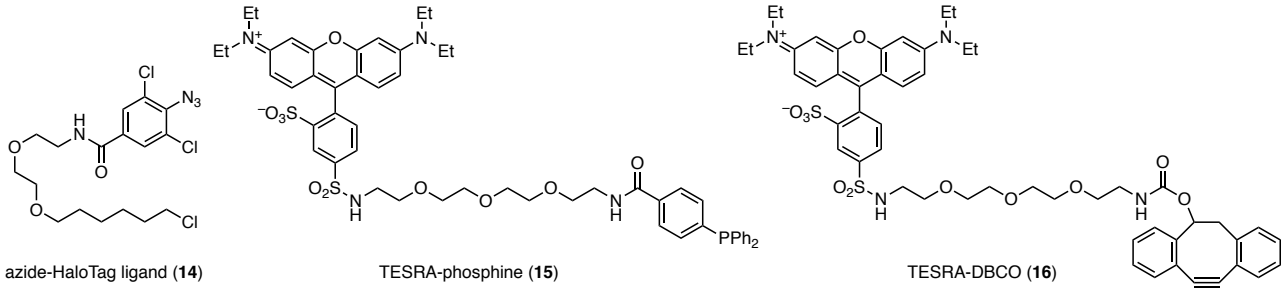
Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed on an ultrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics Inc., Massachusetts, USA). The accelerating voltage in the ion source was set to 20 kV. Data were acquired in the positive linear mode of operation. Time-to-mass conversion was achieved by external calibration using standards of trypsinogen (*m/z* 23982) and protein A (*m/z* 44613, 22307). The matrix for proteins was sinapic acid (SA, Mw = 224; Bruker Daltonics Inc.). Saturated SA matrix solution was prepared in a 30% (v/v) solution of acetonitrile in water containing 0.1% trifluoroacetic acid. The matrix (2 μL) was mixed with a solution (2 μL) of the labeled HaloTag protein (approximately 20 μg μL^{-1}), and 0.5 μL of the mixture was applied on a steel sample plate (MTP AnchorChip 384 BC target plate; Bruker Daltonics Inc.). The mixture was allowed to air dry before being introduced into the mass spectrometer.

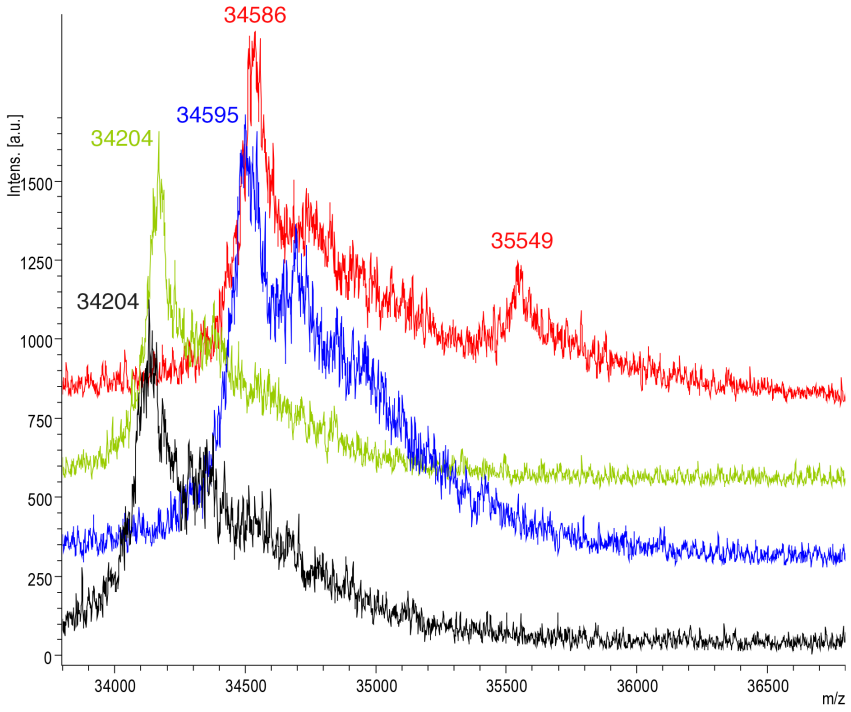
Fluorescence Labeling of the Living Cells

HEK293 cells were routinely maintained in a 5% CO_2, water-saturated atmosphere, and grown in low-glucose Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque) supplemented with 10% Fetal Bovine Serum (FBS; JRH Biosciences, Inc., Lenexa, KS), 100 U mL^{-1} of penicillin (Nacalai Tesque), and 100 μg mL^{-1} of streptomycin (Nacalai Tesque). The cells were cultured on 8-well chamber slides (Matsunami, Osaka, Japan), and transfected with the pCAGIPuro vectors harboring NUP133-HaloTag or HaloTag-TM using polyethylenimine MAX (Polysciences Inc., *Pennsylvania,* USA). The cells were washed with pre-warmed DMEM once, and then incubated with DMEM supplemented with 10 μM of azido-HaloTag ligand (**14**) for 30 min at 37 °C. After a wash with pre-warmed DMEM, the cells were incubated with 1 μM of TESRA-phosphine (**15**) or TESRA-DBCO (**16**) for 30 min at 37 °C. After incubation with pre-warmed fresh DMEM10%FBS for 30 min, the cells were fixed with 4% paraformaldehyde in phosphate buffer (Nacalai Tesque) for 10 min at room temperature. The fixed cells were incubated with PBS containing 0.1% Triton X-100 and Hoechst 33342 (1 μg mL^{-1}) for 1 h at room temperature to stain nuclei. After a wash with PBS followed by a wash with ultrapure water, the slides were mounted with ProLong Diamond (Thermo fisher Scientific, Massachusetts, USA). Fluorescence images were obtained with a confocal laser microscopy LSM800 (Carl Zeiss Microscopy, Oberkochen, Germany) equipped with 63× Plan-Apochromat NA 1.40 objective lens and imaging software (ZEN 2.3 system). Images were imported into Photoshop (Ver. CC 2017; Adobe) for cropping and linear contrast adjustment.

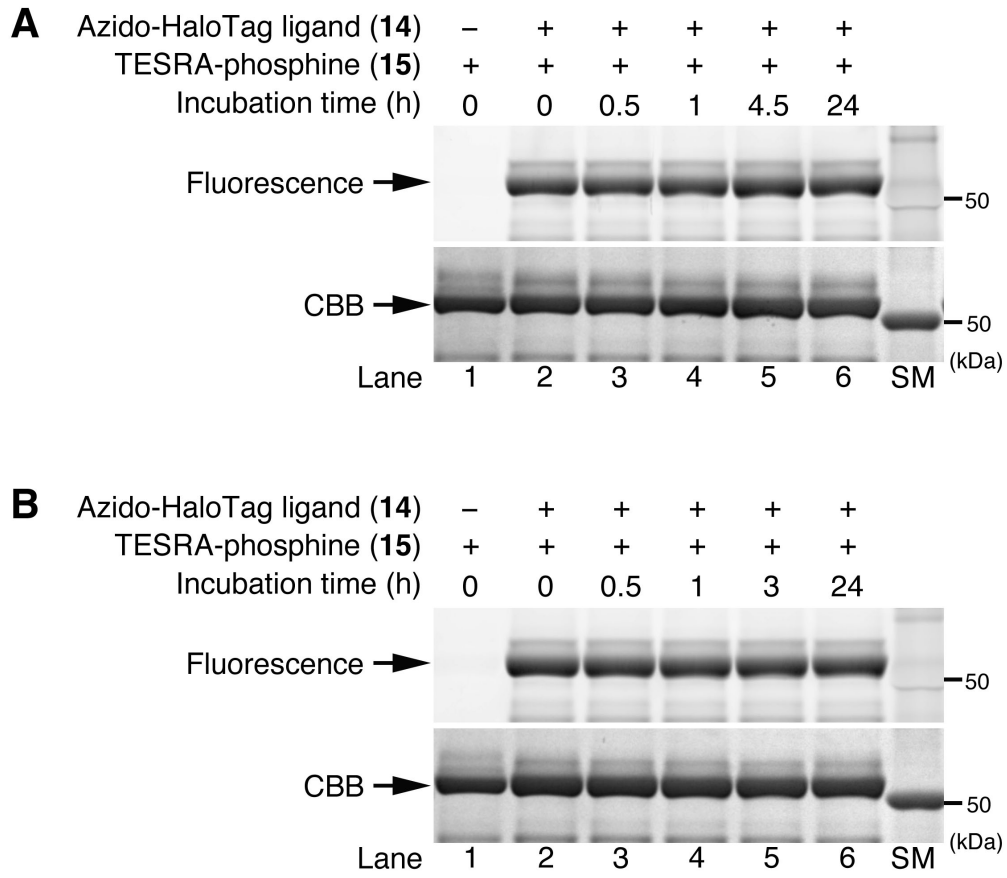
Supplemental Figures



azide-HaloTag ligand (**14**) TESRA-phosphine (**15**) TESRA-DBCO (**16**)



Mass spectra of the HaloTag proteins modified with azido-HaloTag ligand (**14**) and TESRA-phosphine (**15**). The each modified GST-HaloTag was digested with the PreScission protease overnight at 4 °C to elute the modified HaloTag from the resin. The eluted proteins were analyzed by MALDI-TOF-MS. Mass spectrum of the unlabeled HaloTag is shown as a black line; the HaloTag protein reacted with **14**, blue line; the HaloTag protein reacted with **15**, green line; the HaloTag protein reacted with **14** followed by with **15**, red line. The mass of the unlabeled HaloTag observed at *m/z* 34204 as a major peak matched with its calculated value of 34336 (black line). After incorporation of azido-HaloTag ligand (**14**, Mw = 438) into the HaloTag protein, the major peak shifted to a mass of 34595, which corresponds to the azido-HaloTag (Calcd. Mw = 34642) (blue line). Mass analysis after the reaction of the azido-HaloTag protein with TESRA-phosphine (**15**, Mw = 1021) showed two major peaks at *m/z* 34586 and 35549, which corresponds to the unreacted azido-HaloTag protein and the reacted product (Calcd. Mw = 35616), respectively (red line). Thus, these increases of mass at each step were in good agreement with the calculated mass values of the modified HaloTag proteins. In addition, the observed mass spectrum of the HaloTag protein incubated with TESRA-phosphine (**15**) without azido-HaloTag ligand (**14**) (green line) was almost the same with that of the unlabeled HaloTag protein (black line), indicating that TESRA-phosphine (**15**) did not react with amino acid residues of the HaloTag protein.

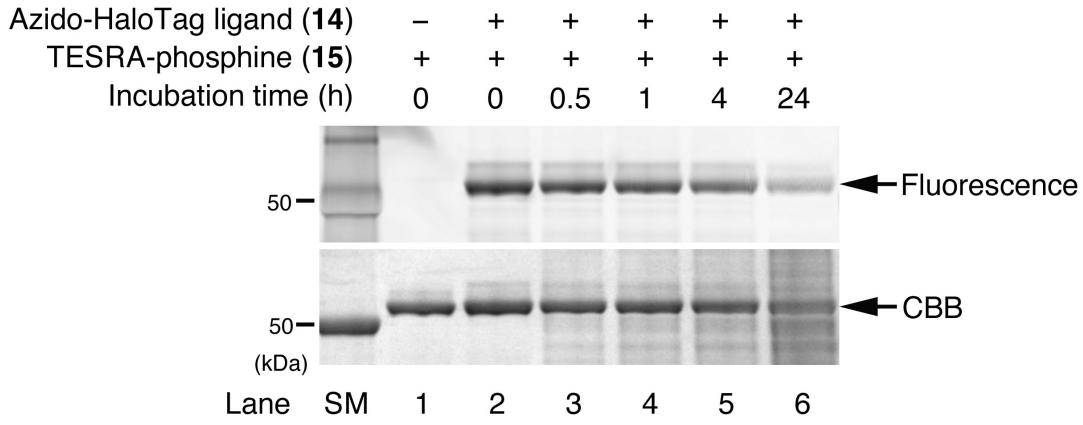


Stability analyses of azido- and aza-ylide moieties on the HaloTag proteins in cell culture condition.

(A) Stability of azido-moiety on the HaloTag proteins was examined in cell culture condition. Prior to the reaction with TESRA-phosphine (**15**), azido-HaloTag on the resin prepared using azido-HaloTag ligand (**14**) was incubated in cell culture medium (low-glucose DMEM10% FBS supplemented with penicillin and streptomycin) for the indicated time (0 to 24 h). Then, the incubated azido-HaloTag proteins were reacted with TESRA-phosphine (**15**) for 30 min in the medium. The labeled GST-HaloTag proteins eluted from the resin were separated by SDS-PAGE. The gel was scanned with a fluorescence image analyzer and then stained with Coomassie brilliant blue (CBB). SM indicates the size marker lane.

(B) Stability of aza-ylide moiety on the HaloTag proteins was examined in cell culture condition.

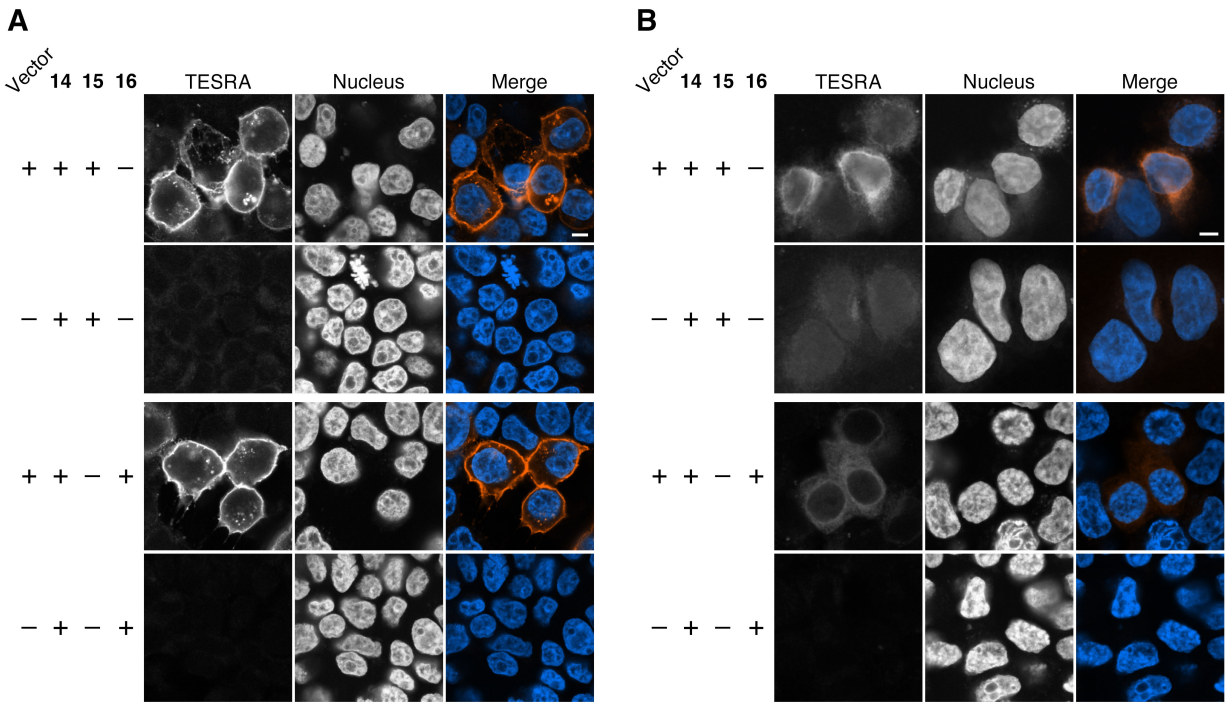
Azido-HaloTag on the resin was reacted with TESRA-phosphine (**15**) in the medium. The resultant fluorescent GST-HaloTag on the resin was incubated in the medium for the indicated time (0 to 24 h), and then analyzed.



Stability analysis of azido-moiety on the HaloTag proteins in cell lysate.

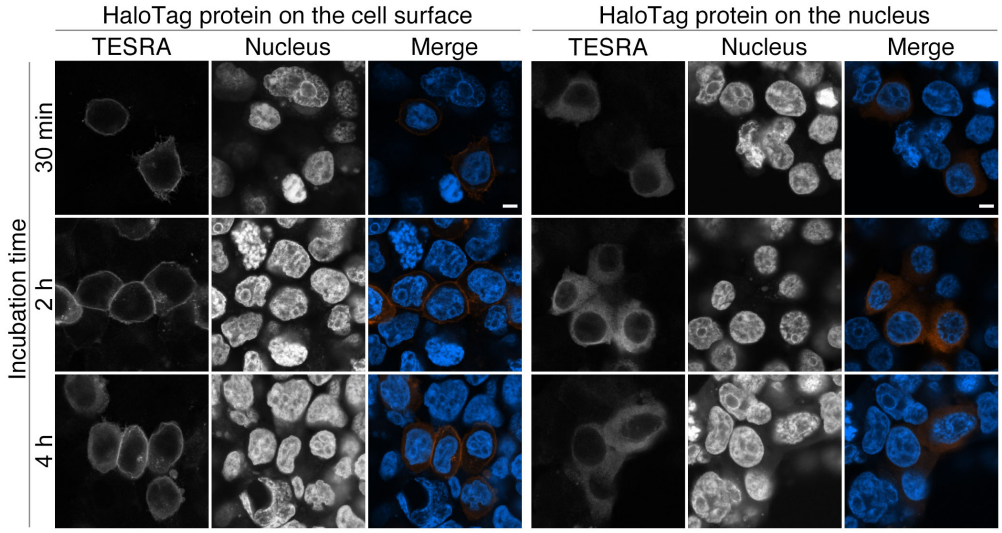
Stability of azido-moiety on the HaloTag proteins was examined in cell lysate, which were prepared by sonication of HEK293 cells in PBS (7.0×10^7 cells mL^{-1}). Prior to the reaction with TESRA-phosphine (**15**), azido-HaloTag on the resin prepared using azido-HaloTag ligand (**14**) was incubated in the cell lysate for the indicated time (0 to 24 h). Then, the incubated azido-HaloTag proteins were reacted with TESRA-phosphine (**15**) for 30 min in the cell lysate. The labeled GST-HaloTag proteins eluted from the resin were separated by SDS-PAGE. The gel was scanned with a fluorescence image analyzer and then stained with Coomassie brilliant blue (CBB). SM indicates the size marker lane.

CBB band intensities of the HaloTag proteins were decreased in a time-course dependent manner, indicating proteolytic degradation of the HaloTag proteins by proteases in the cell lysates. Fluorescence band intensities also decreased, which are consistent with the CBB bands. Thus, the labeling efficiency was slightly lowered during the incubation with the cell lysate.



Fluorescent labeling of living cells with TESRA-phosphine (**15**) or TESRA-DBCO (**16**).

(A) HEK293 cells with HaloTag protein on the cell surface outside the cells. (B) HEK293 cells with HaloTag protein on the nucleus inside the cells. HEK293 cells were transiently transfected with each expression vector for the HaloTag fusion proteins. The transfected cells were incubated with 10 μM of azido-HaloTag ligand (**14**) for 30 min at 37 °C. After a wash, the cells were incubated with 1 μM of TESRA-phosphine (**15**) or TESRA-DBCO (**16**) for 30 min at 37 °C. The data of the cells labeled with **14** and **15** are the same as those in **Fig. 5**. Vector (+) indicates the expression of the HaloTag fusion proteins, and (–) indicates no expression. Scale bar, 5 μm.



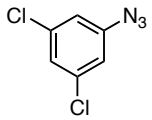
Stability analyses of fluorescent aza-ylide in living HEK293 cells.

HEK293 cells with HaloTag protein on the cell surface (left) or on the nucleus (right) were labeled with azido-HaloTag ligand (**14**) and TESRA-phosphine (**15**) as described above. The labeled living cells were incubated in the cell culture medium for the indicated time at 37 ºC, then fixed and observed. The fluorescent labels maintained for up to 4 h in the living cells. Scale bar, 5 μm.

Characterization Data of New Compounds

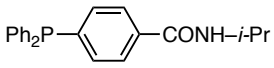
2-Methoxyphenyl azide (**1c**),[S11] 2,6-dimethoxyphenyl azide (**1d**)[S12] and 2,6-dichlorophenyl azide (**1g**)[S13] were identical in spectra data with those reported in the literatures.

3,5-Dichlorophenyl azide (**1f**)



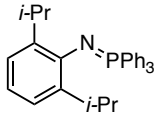
Pale yellow solid; Mp 30–31 °C; TLC R_f 0.56 (*n*-hexane); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.92 (d, 2H, J = 1.8 Hz, aromatic), 7.14 (t, 1H, J = 1.8 Hz, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 117.7 (2C), 125.1 (1C), 136.0 (2C), 142.5 (1C); IR (KBr, cm^{-1}) 804, 843, 1287, 1437, 1570, 1587, 2112; HRMS (ESI^+) *m*/*z* 187.9786 ($[M+H]^+$, $C_6H_4{}^{35}Cl_2N_3$ requires 187.9777).

4-(Diphenylphosphino)-*N*-isopropylbenzamide (**2g**)



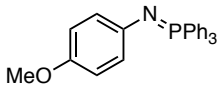
Colorless solid; Mp 128–130 °C; TLC R_f 0.58 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.25 (d, 6H, J = 6.6 Hz, isopropyl), 4.28 (dsept, 1H, J = 7.3, 6.6 Hz, isopropyl), 5.87 (br d, 1H, J = 7.3 Hz, NH), 7.28–7.39 (m, 12H, aromatic), 7.67–7.70 (m, 2H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 22.8 (2C), 41.9 (1C), 126.7 (d, 2C, $J_{C–P}$ = 6.6 Hz), 128.6 (d, 4C, $J_{C–P}$ = 7.2 Hz), 129.0 (2C), 133.5 (d, 2C, $J_{C–P}$ = 18.9 Hz), 133.8 (d, 4C, $J_{C–P}$ = 19.5 Hz), 135.0 (1C), 136.3 (d, 2C, $J_{C–P}$ = 10.8 Hz), 141.7 (d, 1C, $J_{C–P}$ = 13.2 Hz), 166.3 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –6.24 (t, J = 6.9 Hz); IR (KBr, cm^{-1}) 696, 743, 1433, 1537, 1632; HRMS (ESI^+) *m*/*z* 370.1328 ($[M+Na]^+$, $C_{22}H_{22}NNaOP^+$ requires 370.1331).

2,6-Diisopropyl-*N*-(triphenylphosphoranylidene)aniline (**4a**)



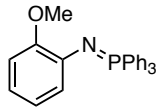
Colorless solid; Mp 109–111 °C; TLC R_f 0.59 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.80 (d, 12H, J = 6.8 Hz), 3.28 (sept, 2H, J = 6.8 Hz), 6.78–6.83 (m, 1H, aromatic), 6.91–6.94 (m, 2H, aromatic), 7.36–7.43 (m, 6H, aromatic), 7.45–7.58 (m, 9H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 23.5 (4C), 28.3 (2C), 119.3 (d, 1C, $J_{C–P}$ = 3.3 Hz), 122.6 (d, 2C, $J_{C–P}$ = 2.3 Hz), 128.3 (d, 6C, $J_{C–P}$ = 11.9 Hz), 131.2 (d, 3C, $J_{C–P}$ = 2.3 Hz), 132.4 (d, 6C, $J_{C–P}$ = 9.4 Hz), 132.7 (d, 3C, $J_{C–P}$ = 101.3 Hz), 143.2 (d, 2C, $J_{C–P}$ = 6.7 Hz), 144.0 (d, 1C, $J_{C–P}$ = 2.9 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –5.5 to –5.0 (m); IR (KBr, cm^{-1}) 521, 694, 712, 1107, 1356, 1433, 2959; HRMS (ESI^+) *m*/*z* 438.2344 ($[M+H]^+$, $C_{30}H_{33}NP^+$ requires 438.2345).

4-Methoxy-*N*-(triphenylphosphoranylidene)aniline (**4b**)



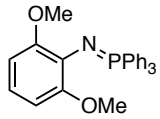
Pale brown oil; TLC R_f 0.33 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.69 (s, 3H, OCH_3), 6.58–6.63 (m, 2H, aromatic), 6.69–6.75 (m, 2H, aromatic), 7.41–7.47 (m, 6H, aromatic), 7.49–7.54 (m, 3H, aromatic), 7.70–7.77 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 55.5 (1C), 114.1 (2C), 123.8 (d, 2C, $J_{C–P}$ = 16.6 Hz), 128.5 (d, 6C, $J_{C–P}$ = 11.9 Hz), 131.3 (d, 3C, $J_{C–P}$ = 98.7 Hz), 131.6 (d, 3C, $J_{C–P}$ = 2.6 Hz), 132.6 (d, 6C, $J_{C–P}$ = 9.7 Hz), 144.3 (1C), 151.9 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 1.6–2.3 (m); IR (KBr, cm^{-1}) 525, 694, 714, 721, 1047, 1105, 1233, 1263, 1335, 1435, 1499; HRMS (ESI^+) *m*/*z* 384.1515 ($[M+H]^+$, $C_{25}H_{23}NOP^+$ requires 384.1512).

2-Methoxy-*N*-(triphenylphosphoranylidene)aniline (**4c**)



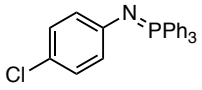
Pale yellow solid; Mp 130–132 °C; TLC R_f 0.27 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.49 (s, 3H, OCH_3), 6.62–6.68 (m, 2H, aromatic), 6.68–6.73 (m, 1H, aromatic), 6.80–6.85 (m, 1H, aromatic), 7.38–7.45 (m, 6H, aromatic), 7.45–7.51 (m, 3H, aromatic), 7.71–7.78 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 54.8 (1C), 110.9 (1C), 117.6 (1C), 120.8 (1C), 124.0 (d, 1C, $J_{C–P}$ = 16.7 Hz), 128.3 (d, 6C, $J_{C–P}$ = 11.9 Hz), 131.2 (d, 3C, $J_{C–P}$ = 2.7 Hz), 132.48 (d, 6C, $J_{C–P}$ = 9.4 Hz), 132.54 (d, 3C, $J_{C–P}$ = 100.4 Hz), 140.3 (d, 1C, $J_{C–P}$ = 2.1 Hz), 152.9 (d, 1C, $J_{C–P}$ = 13.2 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 3.0–3.6 (m); IR (KBr, cm^{-1}) 529, 694, 714, 1107, 1352, 1435, 1495; HRMS (ESI^+) *m*/*z* 384.1511 ($[M+H]^+$, $C_{25}H_{23}NOP^+$ requires 384.1512).

2,6-Dimethoxy-*N*-(triphenylphosphoranylidene)aniline (**4d**)



Pale yellow solid; Mp 133–135 °C; TLC R_f 0.31 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.47 (s, 6H, OCH_3), 6.46 (dd, 2H, *J* = 8.2 Hz, $J_{H–P}$ = 1.4 Hz, aromatic), 6.59–6.64 (m, 1H, aromatic), 7.35–7.41 (m, 6H, aromatic), 7.41–7.47 (m, 3H, aromatic), 7.69–7.76 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 55.2 (2C), 104.9 (2C), 116.3 (1C), 128.0 (d, 6C, $J_{C–P}$ = 12.1 Hz), 129.2 (d, 1C, $J_{C–P}$ = 3.2 Hz), 130.6 (d, 3C, $J_{C–P}$ = 2.7 Hz), 132.3 (d, 6C, $J_{C–P}$ = 9.3 Hz), 134.3 (d, 3C, $J_{C–P}$ = 102.4 Hz), 153.6 (d, 2C, $J_{C–P}$ = 11.9 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 2.9–3.4 (m); IR (KBr, cm^{-1}) 694, 713, 1109, 1238, 1354, 1437, 1470, 1491; HRMS (ESI^+) *m*/*z* 414.1621 ($[M+H]^+$, $C_{26}H_{25}NO_2P^+$ requires 414.1617).

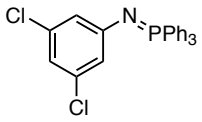
4-Chloro-*N*-(triphenylphosphoranylidene)aniline (**4e**)



Pale yellow solid; Mp 113–115 °C; TLC R_f 0.63 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.67–6.71 (m, 2H, aromatic), 6.91–6.96 (m, 2H, aromatic), 7.42–7.49 (m, 6H, aromatic), 7.51–7.56 (m, 3H, aromatic), 7.69–7.76 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 122.1 (1C), 124.4 (d, 2C, $J_{C–P}$ =17.5

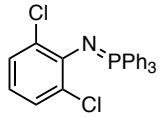
Hz), 128.4 (2C), 128.7 (d, 6C, $J_{C–P}$ = 12.0 Hz), 130.6 (d, 3C, $J_{C–P}$ = 99.2 Hz), 131.8 (d, 3C, $J_{C–P}$ = 2.8 Hz), 132.6 (d, 6C, $J_{C–P}$ = 9.4 Hz), 149.8 (d, 1C, $J_{C–P}$ = 2.5 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 3.1–3.6 (m); IR (KBr, cm^{-1}) 527, 692, 716, 1107, 1339, 1437, 1485, 1582; HRMS (ESI^+) *m*/*z* 388.1021 ($[M+H]^+$, $C_{24}H_{20}{}^{35}ClNOP^+$ requires 388.1016).

3,5-Dichloro-*N*-(triphenylphosphoranylidene)aniline (**4f**)



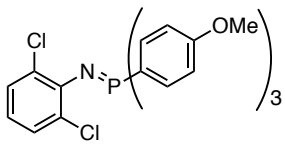
Pale yellow solid; Mp 115–116 °C; TLC R_f 0.69 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.60 (dd, 2H, *J* = 1.7 Hz, $J_{H–P}$ = 0.9 Hz, aromatic), 6.61 (dt, 1H, *J* = 1.7, 1.7 Hz, aromatic), 7.45–7.51 (m, 6H, aromatic), 7.53–7.59 (m, 3H, aromatic), 7.68–7.75 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 117.2 (1C), 121.5 (d, 2C, $J_{C–P}$ = 18.2 Hz), 128.8 (d, 6C, $J_{C–P}$ = 12.0 Hz), 129.9 (d, 3C, $J_{C–P}$ = 99.9 Hz), 132.1 (d, 3C, $J_{C–P}$ =2.7 Hz), 132.5 (d, 6C, $J_{C–P}$ = 9.9 Hz), 134.2 (d, 2C, $J_{C–P}$ = 2.2 Hz), 153.6 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 4.4–4.8 (m); IR (KBr, cm^{-1}) 525, 692, 718, 1109, 1333, 1437, 1449, 1541, 1568; HRMS (ESI^+) *m*/*z* 422.0627 ($[M+H]^+$, $C_{24}H_{19}{}^{35}Cl_2NP^+$ requires 422.0627).

2,6-Dichloro-*N*-(triphenylphosphoranylidene)aniline (**4g**)



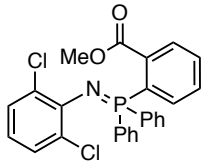
Pale yellow solid; Mp 122–124 °C; TLC R_f 0.72 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.52–6.56 (m, 1H, aromatic), 7.10–7.13 (m, 2H, aromatic), 7.38–7.45 (m, 6H, aromatic), 7.47–7.52 (m, 3H, aromatic), 7.71–7.79 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 118.8 (d, 1C, $J_{C–P}$ = 2.5 Hz), 127.7 (d, 2C, $J_{C–P}$ = 1.7 Hz), 128.2 (d, 6C, $J_{C–P}$ = 12.3 Hz), 131.4 (d, 3C, $J_{C–P}$ = 2.7 Hz), 131.5 (d, 2C, $J_{C–P}$ =8.9 Hz), 132.1 (d, 3C, $J_{C–P}$ = 103.7 Hz), 132.6 (d, 6C, $J_{C–P}$ = 10.0 Hz), 144.6 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 0.2–0.6 (m); IR (KBr, cm^{-1}) 529, 692, 714, 1111, 1435, 1454, 1467, 1483; HRMS (ESI^+) *m*/*z* 422.0627 ($[M+H]^+$, $C_{24}H_{19}{}^{35}Cl_2NP^+$ requires 422.0627).

2,6-Dichloro-*N*-(tris(4-methoxyphenyl)phosphoranylidene)aniline (**4i**)



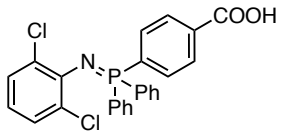
Colorless solid; Mp 131–133 °C; TLC R_f 0.67 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.82 (s, 9H, OCH_3), 6.51–6.55 (m, 1H, aromatic), 6.88–6.93 (m, 6H, aromatic), 7.10–7.14 (m, 2H, aromatic), 7.59–7.67 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 55.3 (3C), 113.7 (d, 6C, $J_{C–P}$ = 13.4 Hz), 118.6 (d, 1C, $J_{C–P}$ = 2.6 Hz), 123.9 (d, 3C, $J_{C–P}$ = 110.5 Hz), 127.7 (d, 3C, $J_{C–P}$ = 1.9 Hz), 131.7 (d, 2C, $J_{C–P}$ = 8.8 Hz), 134.4 (d, 6C, $J_{C–P}$ = 11.5 Hz), 145.2 (1C), 161.9 (d, 2C, $J_{C–P}$ = 2.9 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 6.21 (t, *J* = 11.2 Hz); IR (KBr, cm^{-1}) 800, 1028, 1113, 1179, 1254, 1292, 1456, 1501, 1595; HRMS (ESI^+) *m*/*z* 512.0923 ($[M+H]^+$, $C_{27}H_{25}{}^{35}Cl_2NO_3P^+$ requires 512.0944).

2,6-Dichloro-*N*-((2-methoxycarbonylphenyl)diphenylphosphoranylidene)aniline (**4k**)



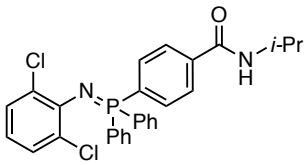
Colorless solid; Mp 150–152 °C; TLC R_f 0.74 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.24 (s, 3H, OMe), 6.50–6.54 (m, 1H, aromatic), 7.08–7.12 (m, 2H, aromatic), 7.38–7.46 (m, 4H, aromatic), 7.46–7.53 (m, 4H, aromatic), 7.56–7.61 (m, 1H, aromatic), 7.75–7.84 (m, 4H, aromatic), 7.91–7.96 (m, 1H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 52.0 (1C), 118.3 (d, 1C, $J_{C–P}$ = 3.2 Hz), 127.7 (d, 2C, $J_{C–P}$ = 1.9 Hz), 128.1 (d, 4C, $J_{C–P}$ = 13.1 Hz), 130.8 (d, 2C, $J_{C–P}$ = 7.9 Hz), 130.9 (d, 1C, $J_{C–P}$ = 8.5 Hz), 131.1 (d, 2C, $J_{C–P}$ = 2.8 Hz), 131.25 (d, 1C, $J_{C–P}$ = 2.5 Hz), 131.27 (d, 1C, $J_{C–P}$ = 11.2 Hz), 132.4 (d, 4C, $J_{C–P}$ = 10.2 Hz), 132.7 (d, 1C, $J_{C–P}$ = 92.6 Hz), 133.3 (d, 2C, $J_{C–P}$ = 115.5 Hz), 134.8 (d, 1C, $J_{C–P}$ = 10.1 Hz), 135.1 (d, 1C, $J_{C–P}$ = 5.8 Hz), 144.7 (1C), 167.2 (d, 1C, $J_{C–P}$ = 2.4 Hz); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 0.9–1.4 (m); IR (KBr, cm^{-1}) 723, 1115, 1292, 1435, 1470, 1483, 1728; HRMS (ESI^+) *m*/*z* 480.0666 ($[M+H]^+$, $C_{26}H_{21}{}^{35}Cl_2NO_2P^+$ requires 480.0681).

2,6-Dichloro-*N*-((4-carboxyphenyl)diphenylphosphoranylidene)aniline (**4l**)



Colorless solid; Mp 205–207 °C; TLC R_f 0.53 (EtOAc); ^{1}H NMR (CD_3OD, 500 MHz) δ 6.71–6.78 (m, 1H, aromatic), 7.16 (br d, 2H, *J* = 8.0 Hz, aromatic), 7.46–7.56 (m, 4H, aromatic), 7.60–7.67 (m, 2H, aromatic), 7.70–7.79 (m, 4H, aromatic), 7.79–7.87 (m, 2H, aromatic), 8.07–8.13 (m, 2H, aromatic); ^{13}C NMR (CD_3OD, 126 MHz) δ 123.1 (1C), 129.2 (d, 2C, $J_{C–P}$ = 2.5 Hz), 129.8 (d, 4C, $J_{C–P}$ = 12.6 Hz), 130.1 (d, 2C, $J_{C–P}$ = 103.4 Hz), 130.5 (d, 2C, $J_{C–P}$ = 12.4 Hz), 133.9 (d, 2C, $J_{C–P}$ = 2.6 Hz), 134.0 (d, 2C, $J_{C–P}$ = 10.0 Hz), 134.1 (d, 4C, $J_{C–P}$ = 10.0 Hz), 134.4 (d, 2C, $J_{C–P}$ = 7.6 Hz), 135.4 (d, 1C, $J_{C–P}$ = 100.5 Hz), 136.8 (1C), 143.7 (1C), 169.3 (1C); ^{31}P NMR (CD_3OD, 162 MHz) δ 9.8–10.4 (m); IR (KBr, cm^{-1}) 544, 692, 714, 775, 1111, 1248, 1263, 1393, 1437, 1470, 1714; HRMS (ESI^+) *m*/*z* 488.0348 ($[M+Na]^+$, $C_{25}H_{18}{}^{35}Cl_2NNaOP^+$ requires 488.0344).

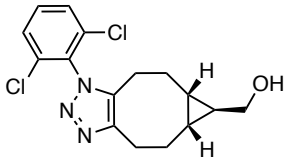
2,6-Dichloro-*N*-((4-isopropylcarbamoylphenyl)diphenylphosphoranylidene)aniline (**4m**)



Colorless solid; Mp 170–172 °C; TLC R_f 0.47 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.26 (d, 6H, *J* = 6.6 Hz, isopropyl), 4.28 (dsept, 1H, *J* = 7.8, 6.6 Hz, isopropyl), 5.92 (br d, 1H, *J* =7.8 Hz, NH), 6.54–6.59 (m, 1H, aromatic), 7.10–7.14 (m, 2H, aromatic), 7.40–7.46 (m, 4H, aromatic), 7.48–7.54 (m, 2H, aromatic), 7.69–7.80 (m, 6H, aromatic), 7.81–7.87 (m, 2H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 22.8 (2C), 42.1 (1C), 119.0 (d, 1C, $J_{C–P}$ = 2.7 Hz), 126.6 (d, 2C, $J_{C–P}$ = 12.3 Hz), 127.8 (d, 2C, $J_{C–P}$ = 1.7 Hz), 128.4 (d, 4C, $J_{C–P}$ = 12.2 Hz), 131.46 (d, 2C, $J_{C–P}$ = 104.1 Hz), 131.48 (d, 2C, $J_{C–P}$ = 8.7 Hz), 131.7 (d, 2C, $J_{C–P}$ = 2.8 Hz), 132.6 (d, 4C, $J_{C–P}$ = 10.1 Hz), 132.8 (d, 2C, $J_{C–P}$ = 10.2 Hz), 135.8 (d, 1C, $J_{C–P}$ = 102.5 Hz), 137.6 (d, 1C, $J_{C–P}$ = 2.9 Hz), 144.3 (1C), 166.0 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –0.8 to –0.2 (m); IR

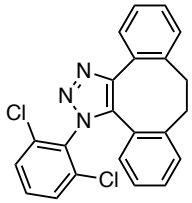
(KBr, cm^{-1}) 692, 718, 727, 1111, 1437, 1452, 1483, 1530, 1632; HRMS (ESI^+) *m*/*z* 507.1143 ($[M+H]^+$, $C_{28}H_{26}{}^{35}Cl_2N_2OP^+$ requires 507.1154).

((5a*R*,6*R*,6a*S*)-1-(2,6-Dichlorophenyl)-1,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-*d*][1,2,3]-triazol-6-yl)methanol (**9**)



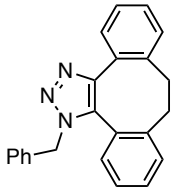
Colorless solid; Mp 118–120 °C; TLC R_f 0.55 (EtOAc); ^1H NMR ($CDCl_3$, 500 MHz) δ 0.72–0.78 (m, 1H), 0.81–0.89 (m, 1H), 0.89–0.96 (m, 1H), 1.37–1.52 (m, 2H), 2.27–2.35 (m, 1H), 2.44–2.58 (m, 3H), 2.93–3.02 (m, 1H), 3.21–3.30 (m, 1H), 3.43–3.55 (m, 2H), 7.42–7.47 (m, 1H, aromatic), 7.49–7.54 (m, 2H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 22.2 (1C), 22.3 (1C), 22.8 (1C), 26.0 (1C), 26.5 (1C), 27.1 (1C), 27.6 (1C), 66.4 (1C), 128.75 (1C), 128.78 (1C), 131.8 (1C), 132.6 (1C), 134.7 (1C), 134.8 (1C), 135.3 (1C), 144.4 (1C); IR (KBr, cm^{-1}) 733, 793, 1030, 1441, 1487, 2930, 3333, 3350, 3374; HRMS (ESI^+) *m*/*z* 360.0638 ($[M+Na]^+$, $C_{16}H_{17}{}^{35}Cl_2N_3NaO^+$ requires 360.0641).

1-(2,6-Dichlorophenyl)-8,9-dihydro-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazole (**11a**)



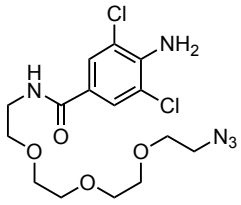
Colorless solid; Mp 265–267 °C; TLC R_f 0.76 (EtOAc); ^1H NMR ($CDCl_3$, 500 MHz) δ 2.86–3.68 (br, 4H), 6.91–6.95 (AA'B, 1H, aromatic), 6.96–7.02 (AA'B, 1H, aromatic), 7.19–7.32 (m, 5H, aromatic), 7.37 (dd, 1H, *J* = 8.0, 8.0 Hz, aromatic), 7.41–7.55 (br, 2H, aromatic), 7.64–7.70 (m, 1H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 33.5 (1C), 35.9 (1C), 125.3 (1C), 126.0 (1C), 126.1 (1C), 128.3 (1C), 128.7 (1C), 128.8 (2C+1C, two singles overlapped), 129.4 (1C), 129.6 (1C), 130.4 (1C), 130.6 (1C), 131.6 (1C), 131.7 (2C), 132.6 (1C), 135.8 (1C), 138.3 (1C), 141.3 (1C), 145.9 (1C); IR (KBr, cm^{-1}) 731, 762, 781, 1204, 1431, 1439, 1479, 1503; HRMS (ESI^+) *m*/*z* 414.0532 ($[M+Na]^+$, $C_{22}H_{15}{}^{35}Cl_2N_3Na^+$ requires 414.0535).

1-Benzyl-8,9-dihydro-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazole (**11b**)



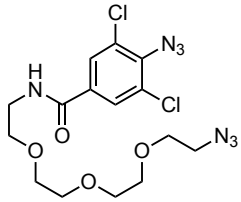
Colorless solid; Mp 135–136 °C; TLC R_f 0.52 (*n*-hexane/EtOAc = 1/1); ^1H NMR ($CDCl_3$, 500 MHz) δ 2.66–2.78 (m, 1H), 2.78–2.89 (m, 1H), 2.96–3.08 (m, 1H), 3.22–3.34 (m, 1H), 5.51–5.60 (m, 2H), 7.03–7.33 (m, 12H, aromatic), 7.51–7.56 (m, 1H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 32.8 (1C), 36.4 (1C), 52.2 (1C), 126.0 (1C), 126.26 (1C), 126.34 (1C), 127.5 (2C), 128.05 (1C), 128.11 (1C), 128.7 (2C), 129.0 (1C), 129.7 (1C), 129.8 (1C), 130.0 (1C), 130.8 (1C), 131.6 (1C), 134.0 (1C), 135.4 (1C), 137.7 (1C), 141.6 (1C), 147.0 (1C); IR (KBr, cm^{-1}) 692, 712, 727, 748, 762, 777, 1454; HRMS (ESI^+) *m*/*z* 360.1471 ($[M+Na]^+$, $C_{23}H_{19}N_3Na^+$ requires 360.1471).

4-Amino-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide



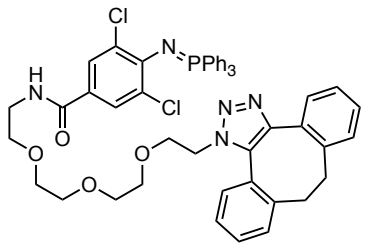
Colorless oil; TLC R_f 0.57 (EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.34 (t, 2H, J = 5.1 Hz), 3.59–3.72 (m, 14H), 4.80 (br s, 2H, NH_2), 6.75–6.82 (br, 1H, NH), 7.67 (s, 2H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 39.7 (1C), 50.5 (1C), 69.5 (1C), 69.9 (1C), 70.1 (1C), 70.4 (1C), 70.5 (1C), 70.6 (1C), 118.6 (2C), 124.1 (1C), 126.9 (2C), 142.6 (1C), 165.1 (1C); IR (KBr, cm^{-1}) 1103, 1119, 1298, 1487, 1549, 1614, 2106, 2922, 3343; HRMS (ESI^+) *m*/*z* 428.0864 ($[M+Na]^+$, $C_{15}H_{21}{}^{35}Cl_2N_5NaO_4^+$ requires 428.0863).

4-Azido-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide (**12**)



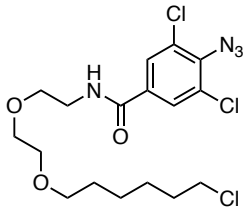
Pale brown oil; TLC R_f 0.62 (EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.35 (t, 2H, J = 5.0 Hz), 3.61–3.72 (m, 14H), 6.86–6.95 (br, 1H, NH), 7.76 (s, 2H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 40.0 (1C), 50.6 (1C), 69.4 (1C), 70.0 (1C), 70.2 (1C), 70.4 (1C), 70.6 (1C), 70.7 (1C), 127.9 (2C), 129.2 (2C), 132.6 (1C), 136.5 (1C), 164.1 (1C); IR (KBr, cm^{-1}) 820, 1123, 1304, 1454, 1545, 1647, 2122, 2870; HRMS (ESI^+) *m*/*z* 454.0763 ($[M+Na]^+$, $C_{15}H_{19}{}^{35}Cl_2N_7NaO_4^+$ requires 454.0768).

1-(11-(3,5-Dichloro-4-((triphenylphosphoranylidene)amino)phenyl)carbamoyl-3,6,9-trioxaundecyl)-8,9-dihydro-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazole (**13**)



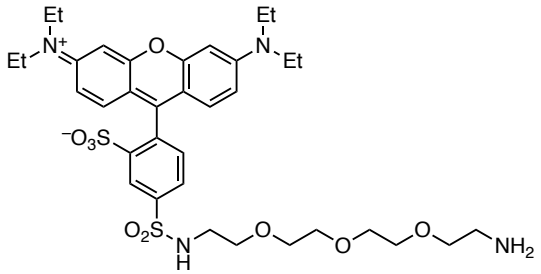
Colorless solid; Mp 71–73 °C; TLC R_f 0.55 (EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.82–2.93 (m, 1H), 3.05–3.18 (m, 2H), 3.36–3.62 (m, 13H), 3.90 (t, 2H, J = 5.6 Hz), 4.38–4.45 (m, 1H), 4.47–4.55 (m, 1H), 6.67–6.73 (br, 1H, NH), 7.10–7.35 (m, 8H, aromatic), 7.38–7.45 (m, 6H, aromatic), 7.47–7.53 (m, 3H, aromatic), 7.61 (d, 2H, J_{H-P} = 1.2 Hz), 7.69–7.78 (m, 6H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 32.8 (1C), 36.5 (1C), 39.6 (1C), 47.9 (1C), 69.4 (1C), 69.7 (1C), 70.1 (1C), 70.3 (1C), 70.4 (1C), 70.6 (1C), 124.5 (d, 1C, J_{C-P} = 2.4 Hz), 125.9 (1C), 126.35 (1C), 126.38 (1C), 126.8 (2C), 128.0 (1C), 128.3 (d, 6C, J_{C-P} = 12.6 Hz), 129.3 (1C), 129.6 (1C), 129.8 (1C), 129.9 (1C), 130.8 (1C), 130.9 (d, 2C, J_{C-P} = 9.1 Hz), 131.6 (d, 3C, J_{C-P} = 2.6 Hz), 131.7 (1C), 131.8 (d, 3C, J_{C-P} = 104.6 Hz), 132.5 (d, 6C, J_{C-P} = 10.1 Hz), 134.6 (1C), 137.6 (1C), 141.7 (1C), 146.4 (1C), 147.8 (1C), 165.7 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 0.9–1.7 (m); IR (KBr, cm^{-1}) 694, 716, 1113, 1294, 1437, 1483; HRMS (ESI^+) *m*/*z* 892.2526 ($[M+Na]^+$, $C_{49}H_{46}{}^{35}Cl_2N_5NaO_4P^+$ requires 892.2557).

4-Azido-*N*-(2-(2-(6-chlorohexyloxy)ethoxy)ethyl)-3,5-dichlorobenzamide (azido-HaloTag ligand, **14**)



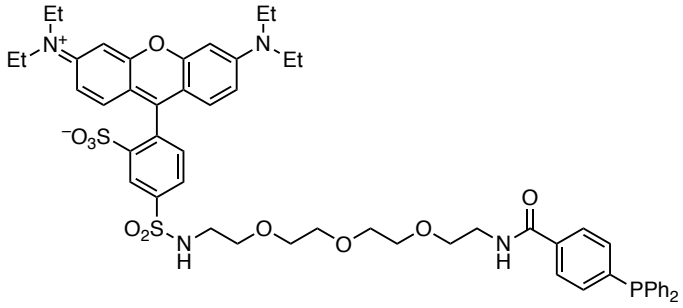
Brown oil; TLC R_f 0.38 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.29–1.38 (m, 2H), 1.38–1.47 (m, 2H), 1.58 (tt, 2H, *J* = 7.1, 7.1 Hz), 1.74 (tt, 2H, *J* = 7.1, 7.1 Hz), 3.48 (t, 2H, *J* = 6.7 Hz), 3.52 (t, 2H, *J* = 6.7 Hz), 3.58–3.71 (m, 8H), 6.72–6.81 (br, 1H, NH), 7.73 (s, 2H, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 25.3 (1C), 26.6 (1C), 29.3 (1C), 32.4 (1C), 39.9 (1C), 45.0 (1C), 69.3 (1C), 70.0 (1C), 70.2 (1C), 71.3 (1C), 127.9 (2C), 129.3 (2C), 132.6 (1C), 136.6 (1C), 164.1 (1C); IR (KBr, cm^{-1}) 1116, 1310, 1454, 1547, 1643, 2124, 2862, 2936; HRMS (ESI^+) *m*/*z* 459.0720 ($[M+Na]^+$, $C_{17}H_{23}{}^{35}Cl_3N_4NaO_3^+$ requires 459.0728).

N-(11-Amino-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide



Purple solid; Mp 252–254 °C; TLC (Amino) R_f 0.16 (CH_2Cl_2/MeOH = 10/1); ^{1}H NMR (CD_3OD, 500 MHz) δ 1.30 (t, 12H, *J* = 7.1 Hz), 3.08 (t, 2H, *J* = 4.7 Hz), 3.22 (t, 2H, *J* = 5.3 Hz), 3.33–3.37 (br, 2H), 3.59 (t, 2H, *J* = 5.3 Hz), 3.61–3.72 (m, 18H), 6.95 (d, 2H, *J* = 2.3 Hz, aromatic), 7.01 (dd, 2H, *J* = 9.5, 2.3 Hz, aromatic), 7.11 (d, 2H, *J* = 9.5 Hz, aromatic), 7.53 (d, 1H, *J* = 8.0 Hz, aromatic), 8.13 (dd, 1H, *J* = 8.0, 1.8 Hz, aromatic), 8.65 (d, 1H, *J* = 1.8 Hz, aromatic). The signal for the N–H proton was not observed; ^{13}C NMR ($CDCl_3$, 126 MHz) δ 12.8 (4C), 41.0 (1C), 44.1 (1C), 46.8 (4C), 68.5 (1C), 70.7 (1C), 71.1 (1C), 71.2 (1C), 71.5 (1C), 71.6 (1C), 97.0 (2C), 115.0 (2C), 115.3 (2C), 127.6 (1C), 129.3 (1C), 132.5 (1C), 133.6 (1C), 133.5 (2C), 144.0 (1C), 147.2 (1C), 157.2 (2C), 157.7 (1C), 159.4 (2C); IR (KBr, cm^{-1}) 1076, 1180, 1248, 1339, 1418, 1466, 1591; HRMS (ESI^+) *m*/*z* 755.2719 ($[M+Na]^+$, $C_{35}H_{48}N_4NaO_9S_2$ requires 755.2755).

N-(11-(4-(Diphenylphosphino)benzamido)-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide (TESRA-phosphine, **15**)



Purple solid; Mp 251–253 °C; TLC R_f 0.21 (CH_2Cl_2/MeOH = 10/1); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.27 (t, 12H, J = 7.1 Hz), 3.23 (dt, 2H, J = 5.7, 4.5 Hz), 3.44–3.56 (m, 8H), 3.58–3.68 (m, 8H), 3.70–3.78 (m, 6H), 6.40 (t, 1H, J = 5.8 Hz, NH), 6.62 (d, 2H, J = 2.4 Hz, aromatic), 6.75 (dd, 2H, J = 9.5, 2.4 Hz, aromatic), 7.17 (d, 1H, J = 7.9 Hz, aromatic), 7.19–7.36 (m, 14 H, aromatic), 7.78 (t, 1H, J = 5.7 Hz, NH), 7.83 (dd, 2H, J = 8.3 Hz, J_{H-P} = 1.3 Hz, aromatic), 7.97 (dd, 1H, J = 7.9, 1.8 Hz, aromatic), 8.91 (d, 1H, J = 1.8 Hz, aromatic); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 12.6 (4C), 39.6 (1C), 43.3 (1C), 45.8 (4C), 69.53 (1C), 69.54 (1C), 70.1 (1C+1C, two singles overlapped), 70.3 (1C), 70.4 (1C), 95.6 (2C), 113.5 (2C), 114.3 (2C), 127.0 (1C), 127.3 (1C), 127.4 (d, 2C, J_{C-P} = 6.6 Hz), 127.7 (1C), 128.6 (d, 4C, J_{C-P} = 7.0 Hz), 128.9 (2C), 129.6 (1C), 133.3 (d, 2C, J_{C-P} = 18.8 Hz), 133.4 (2C), 133.5 (1C), 133.8 (d, 4C, J_{C-P} = 19.9 Hz), 134.6 (1C), 136.6 (2C), 142.2 (1C), 148.2 (1C), 155.5 (2C), 157.9 (2C), 158.9 (1C), 167.1 (1C); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –0.63 to –0.61 (m); IR (KBr, cm^{-1}) 1076, 1134, 1180, 1246, 1275, 1339, 1416, 1481, 1591, 1647; HRMS (ESI$^+$) *m*/*z* 1043.3424 ([M+Na]$^+$, $C_{54}H_{61}N_4NaO_{10}PS_2$ requires 1043.3459).

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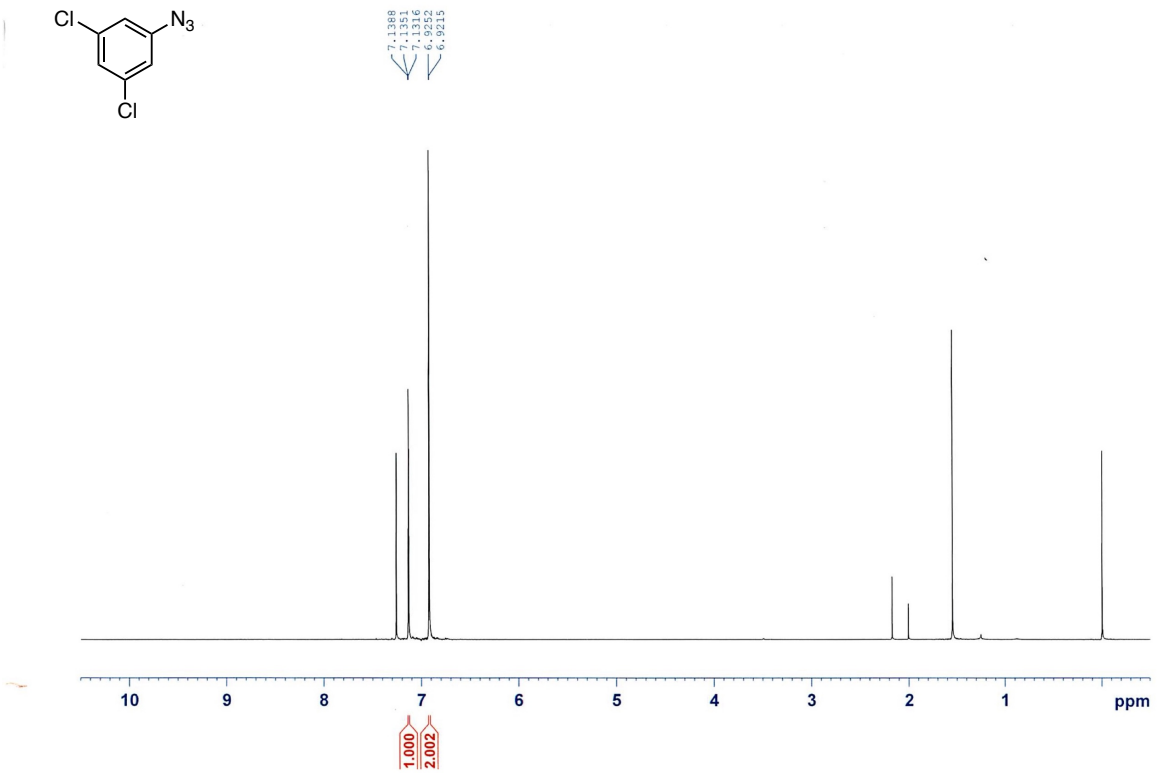
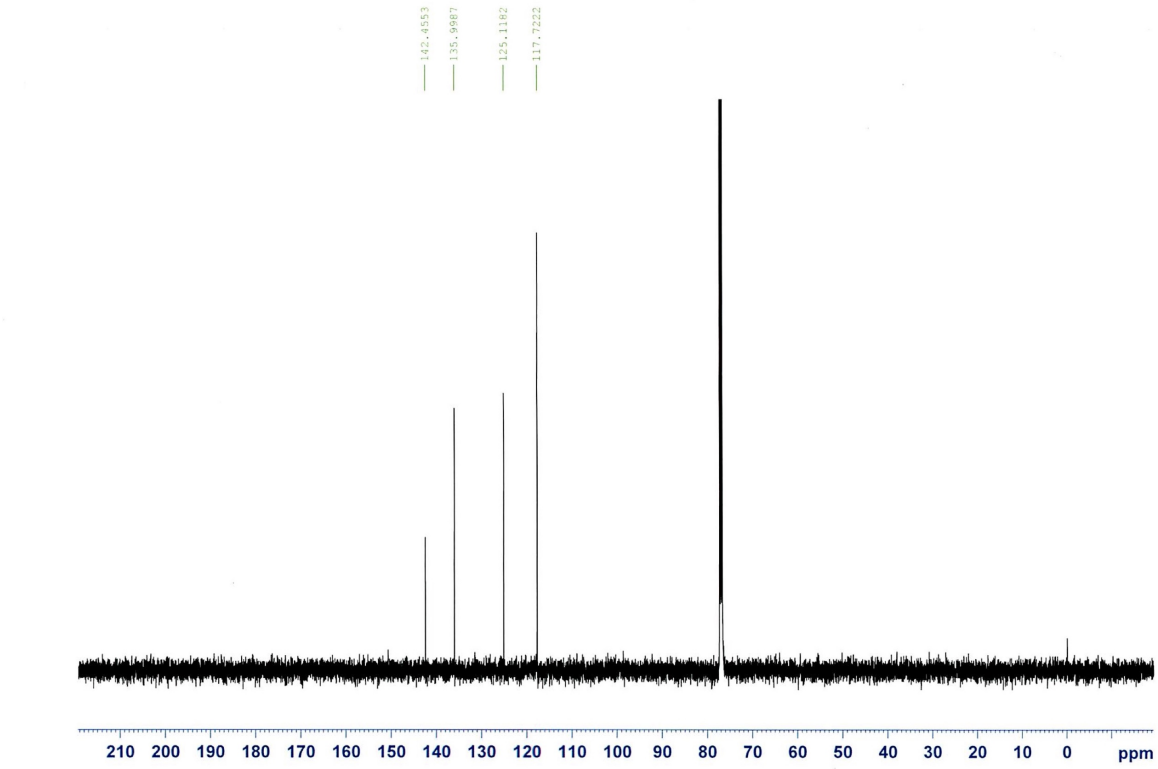
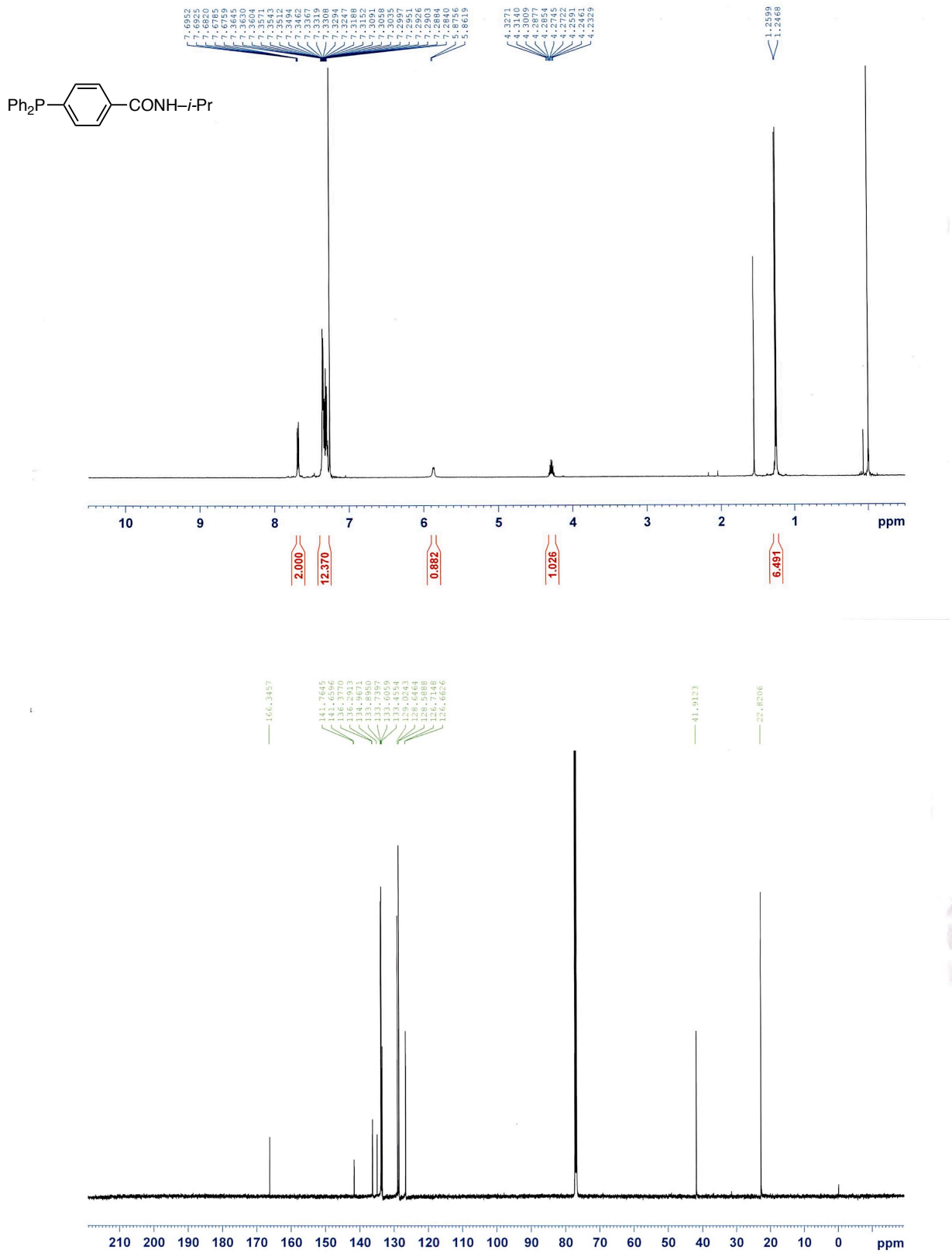
^{1}H and ^{13}C NMR Spectra of Compounds

^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **1f** ($CDCl_3$)

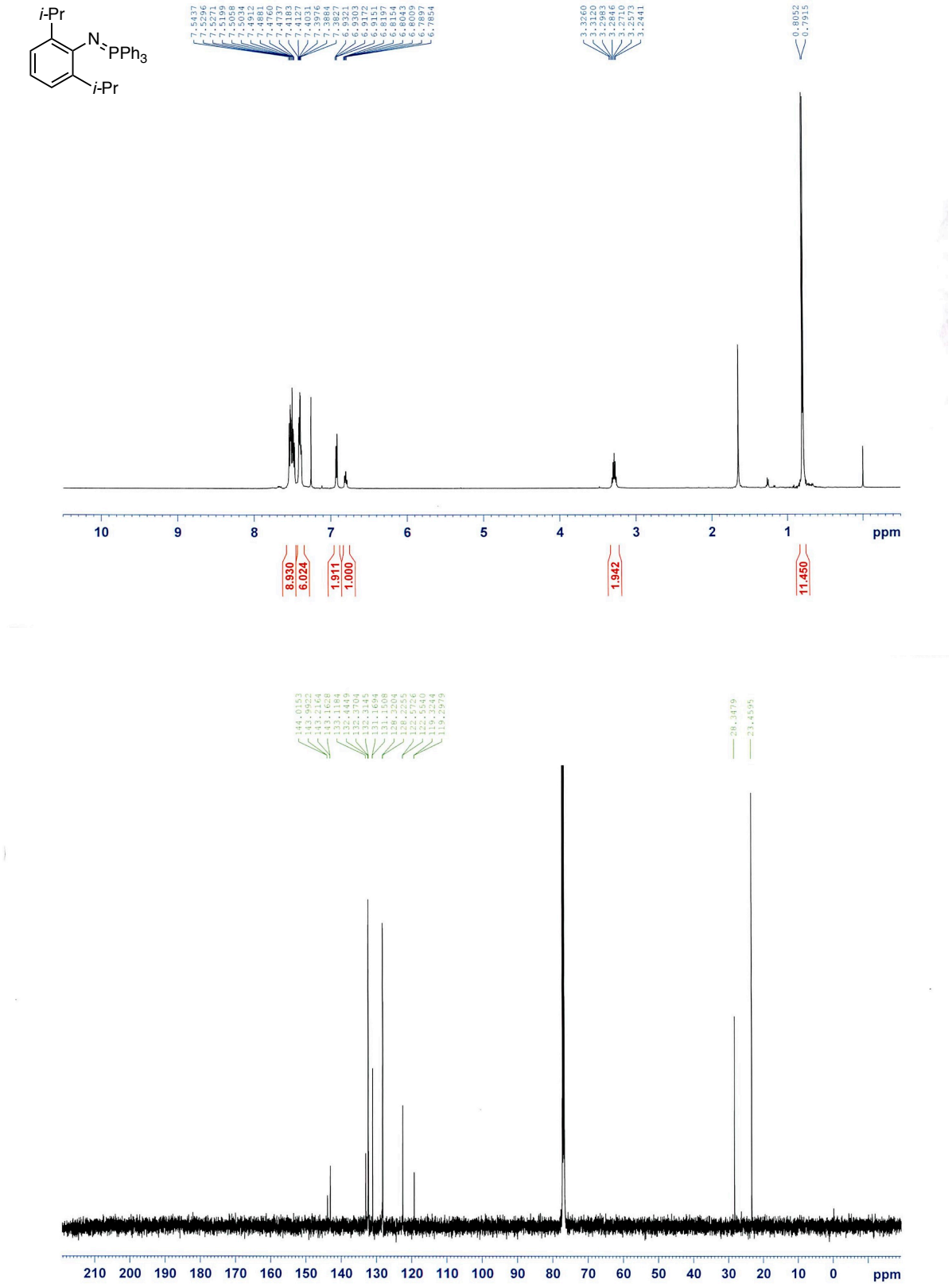




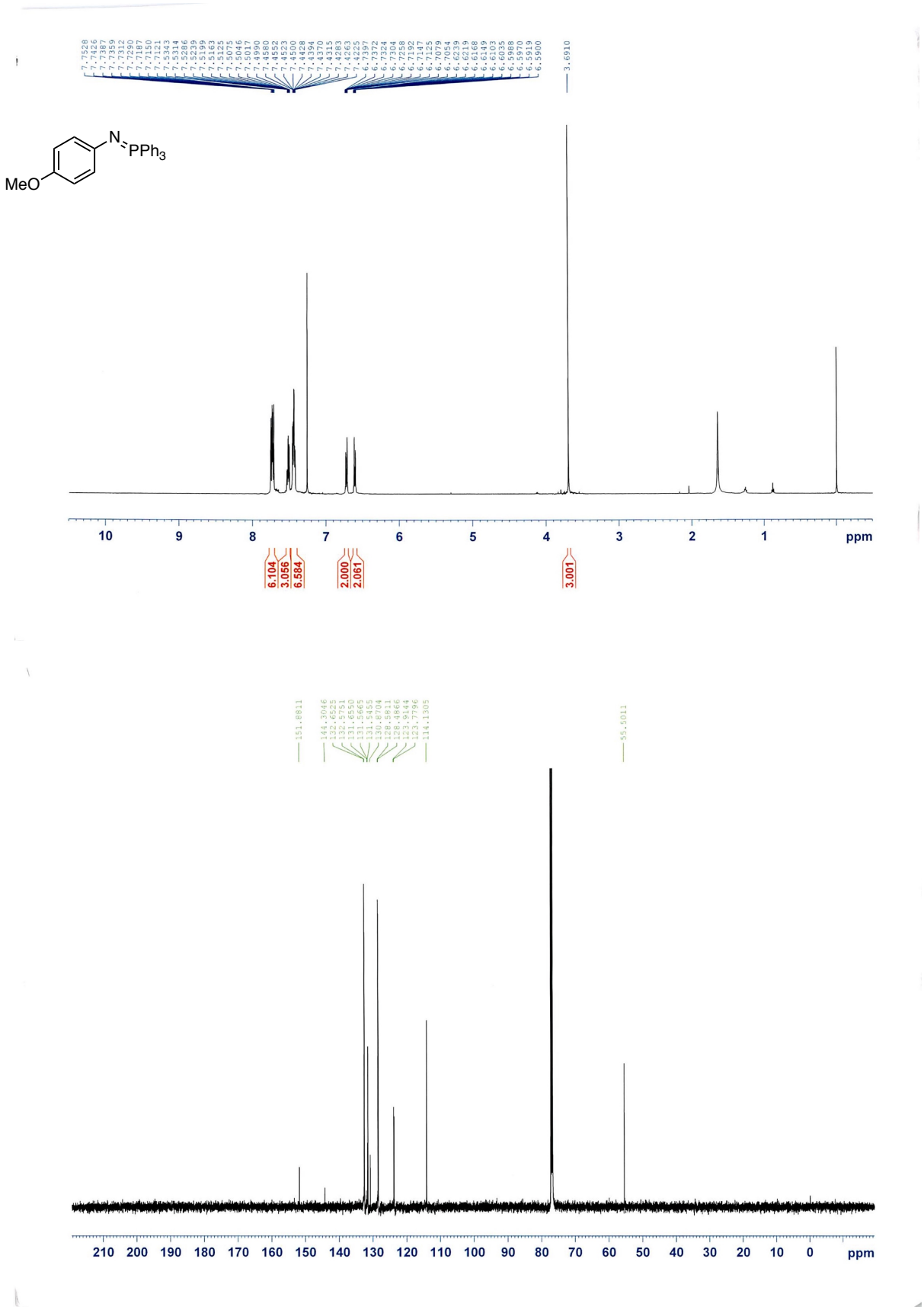
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **2g** ($CDCl_3$)



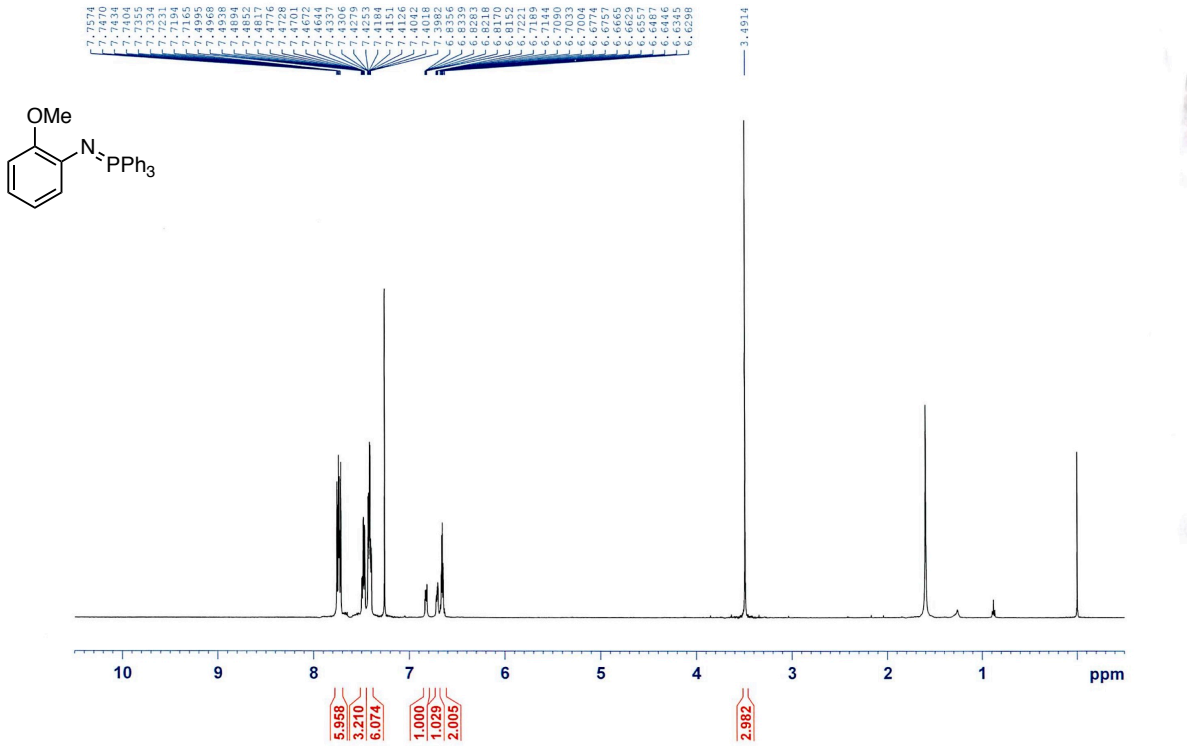
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4a** ($CDCl_3$)

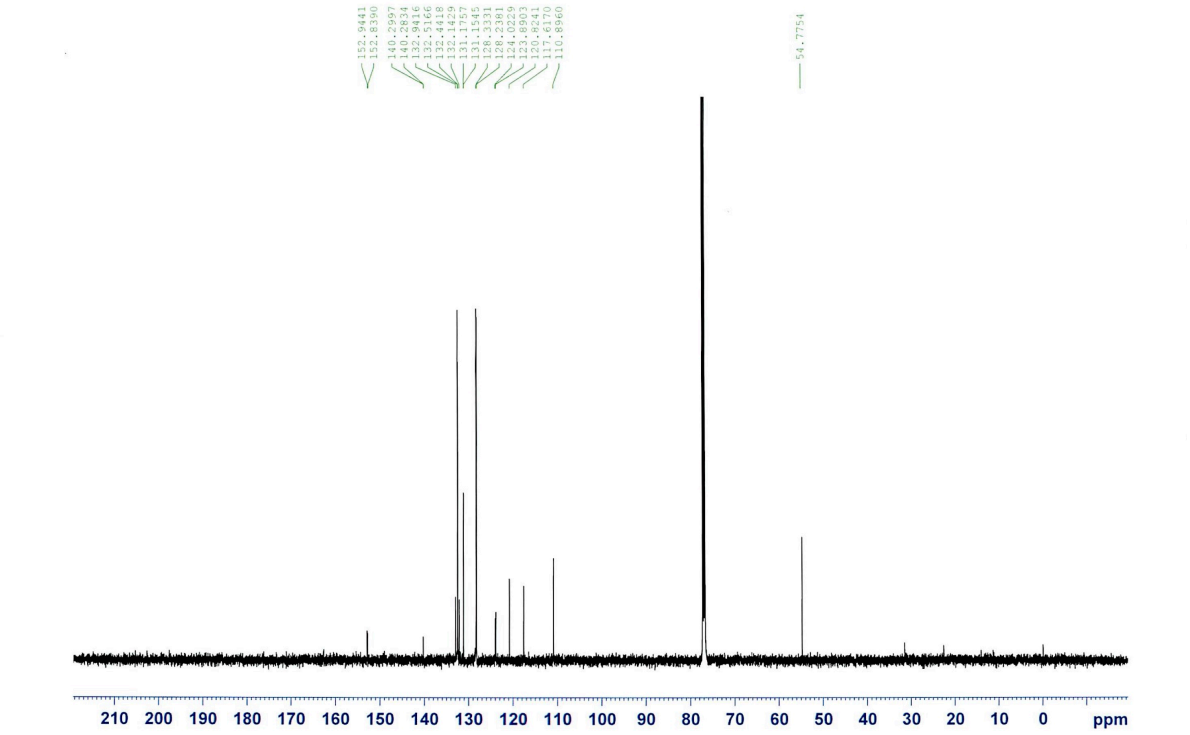


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4b** ($CDCl_3$)

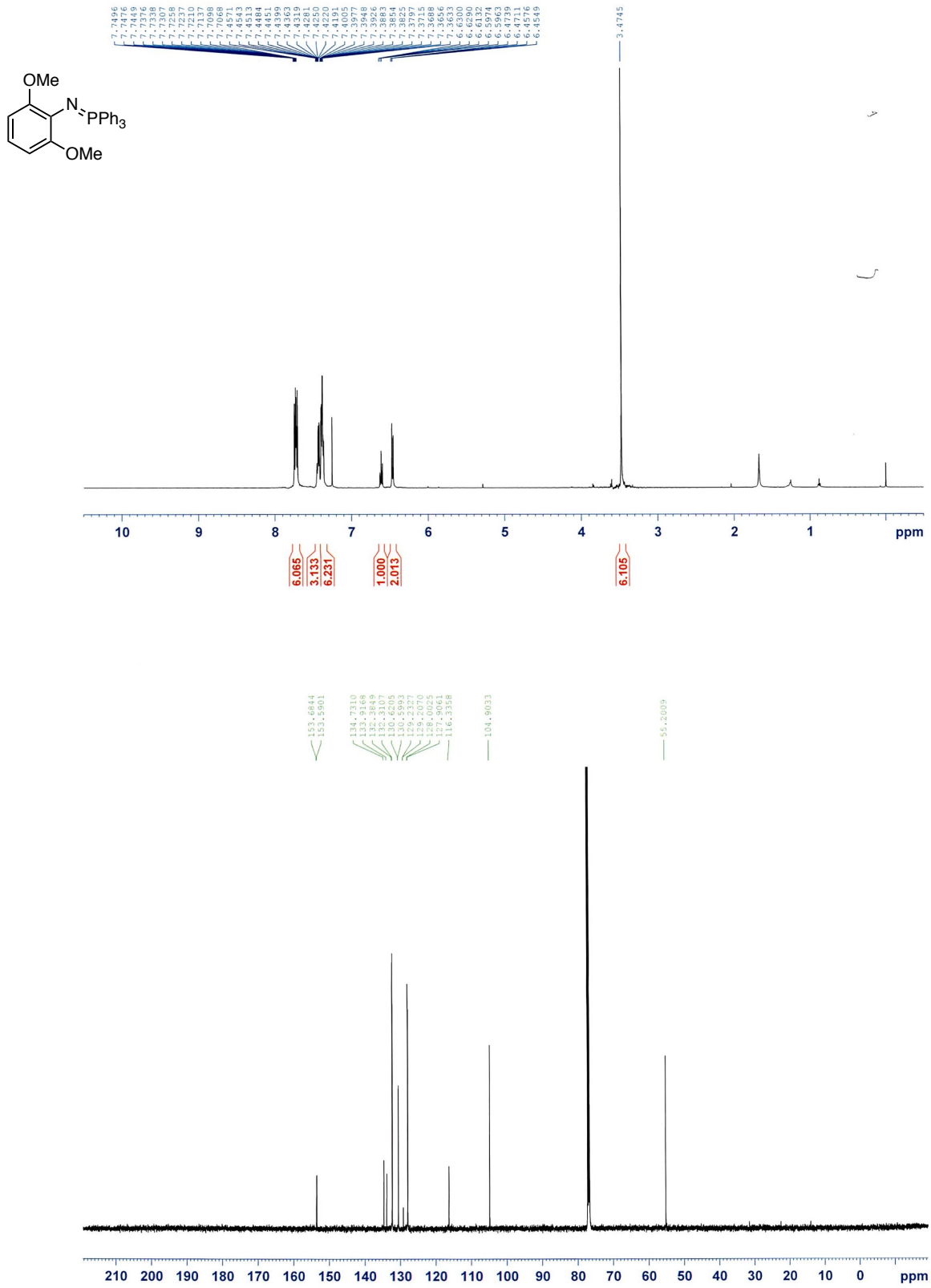


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4c** ($CDCl_3$)

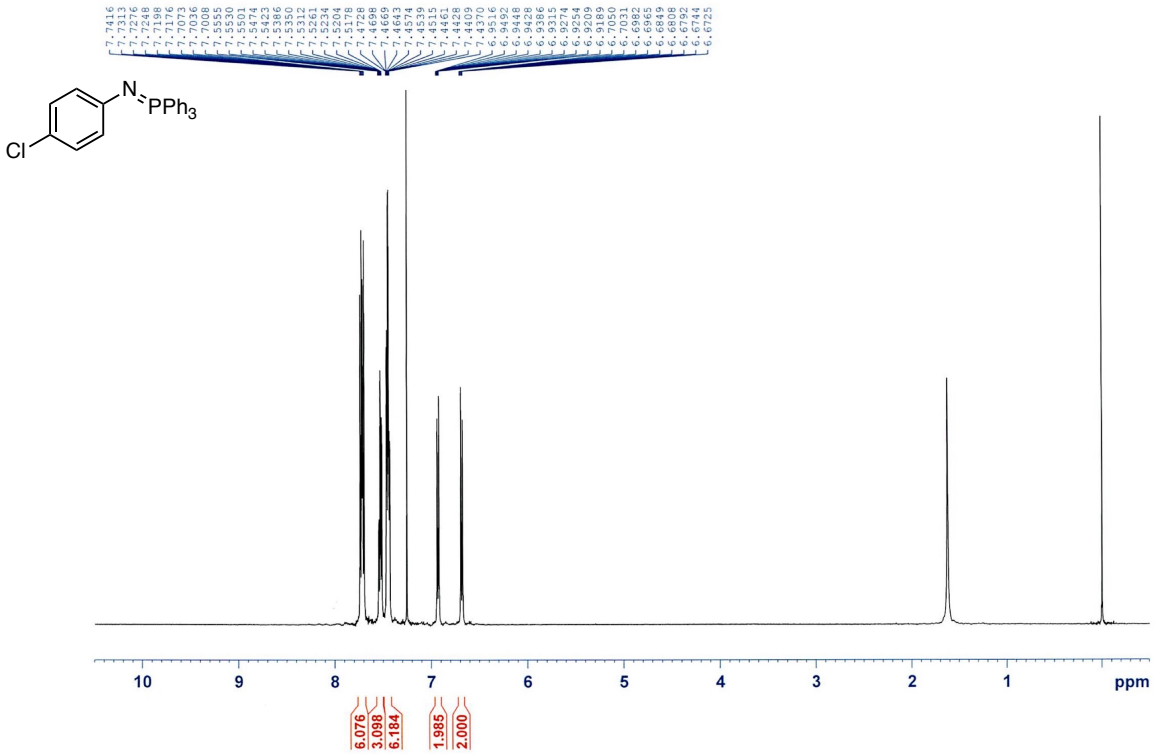


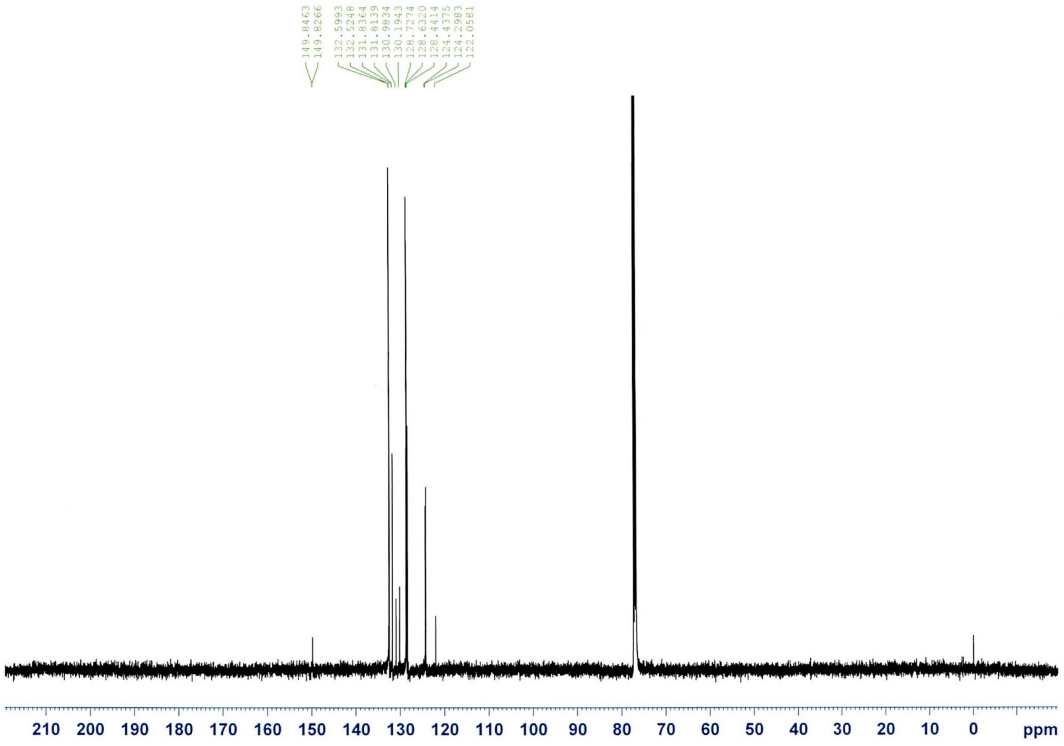


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4d** ($CDCl_3$)

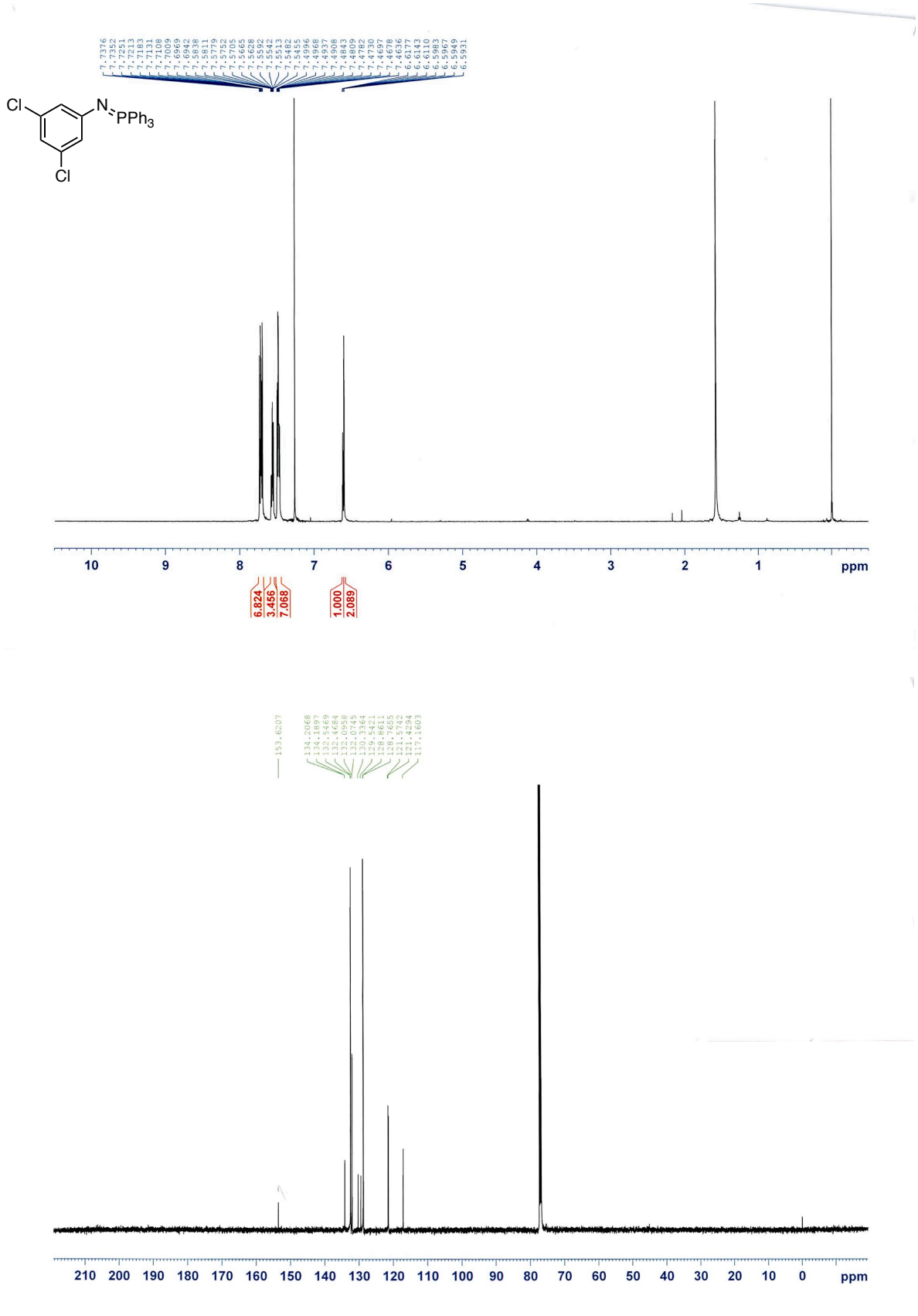


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4e** ($CDCl_3$)

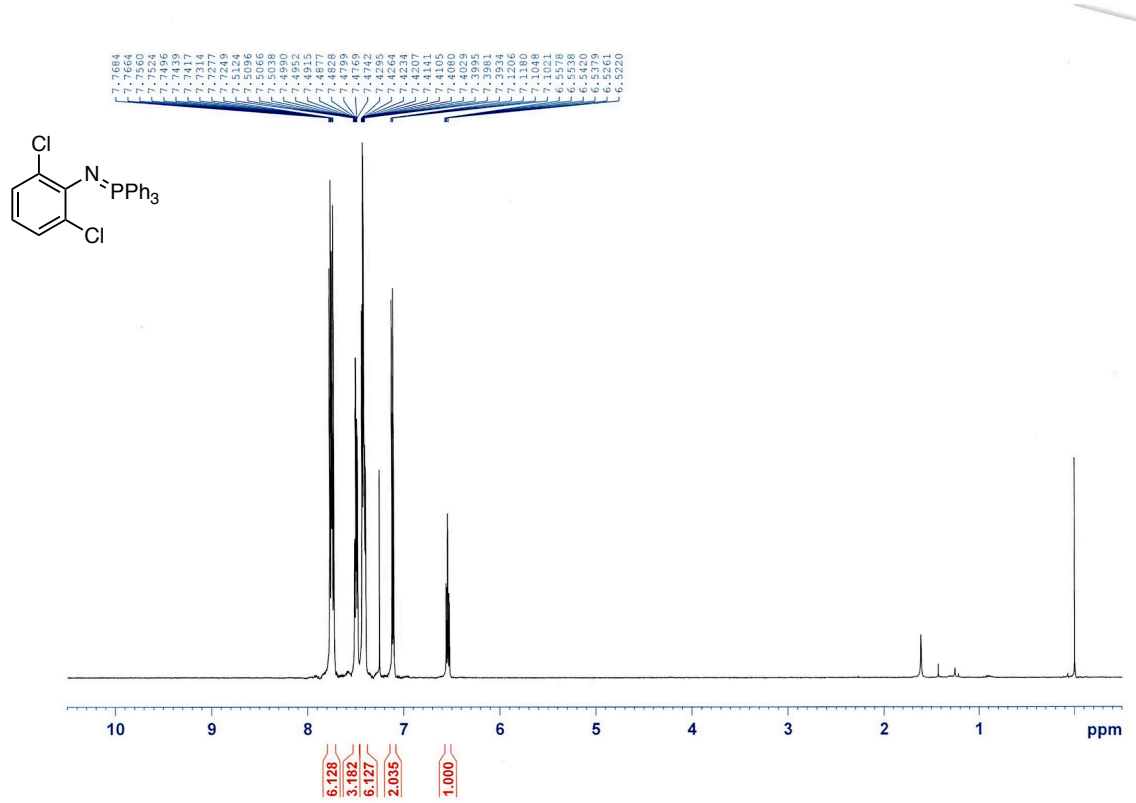


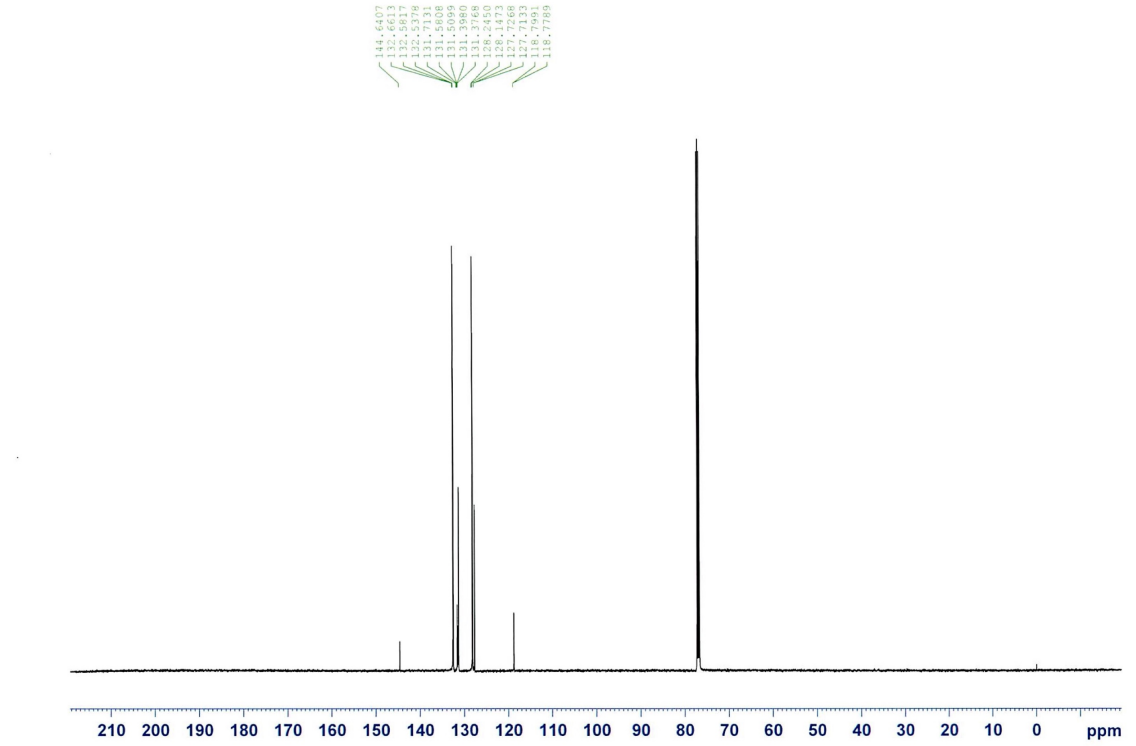


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4f** ($CDCl_3$)

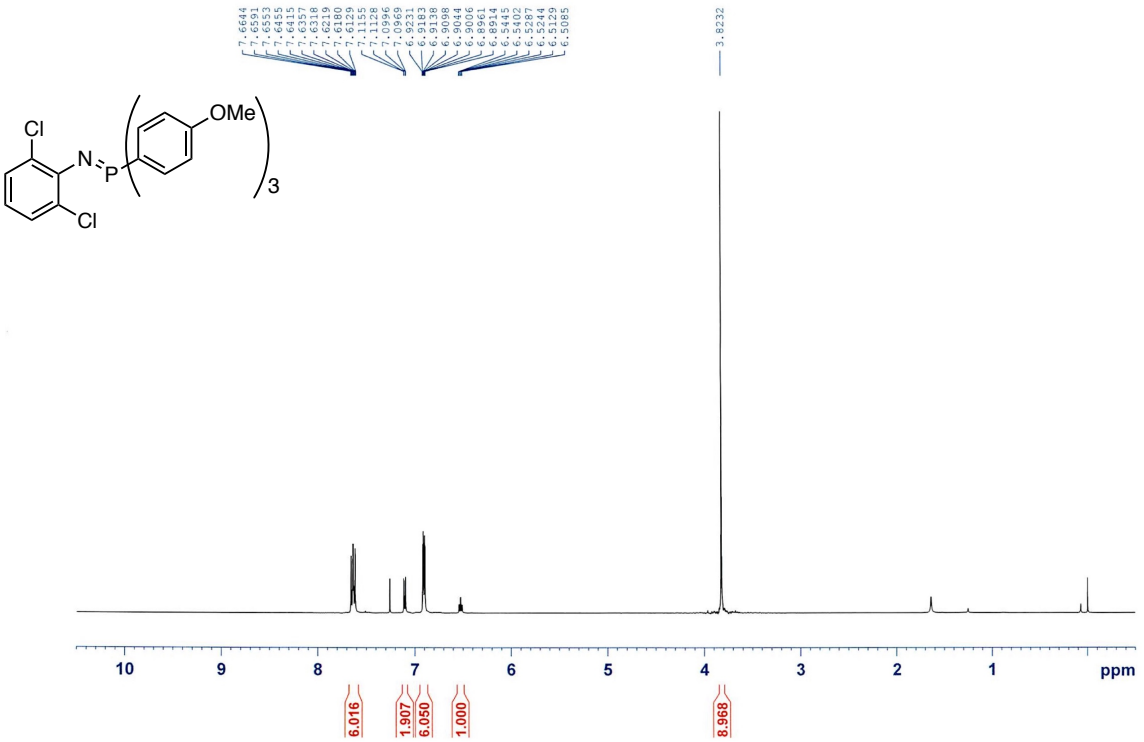


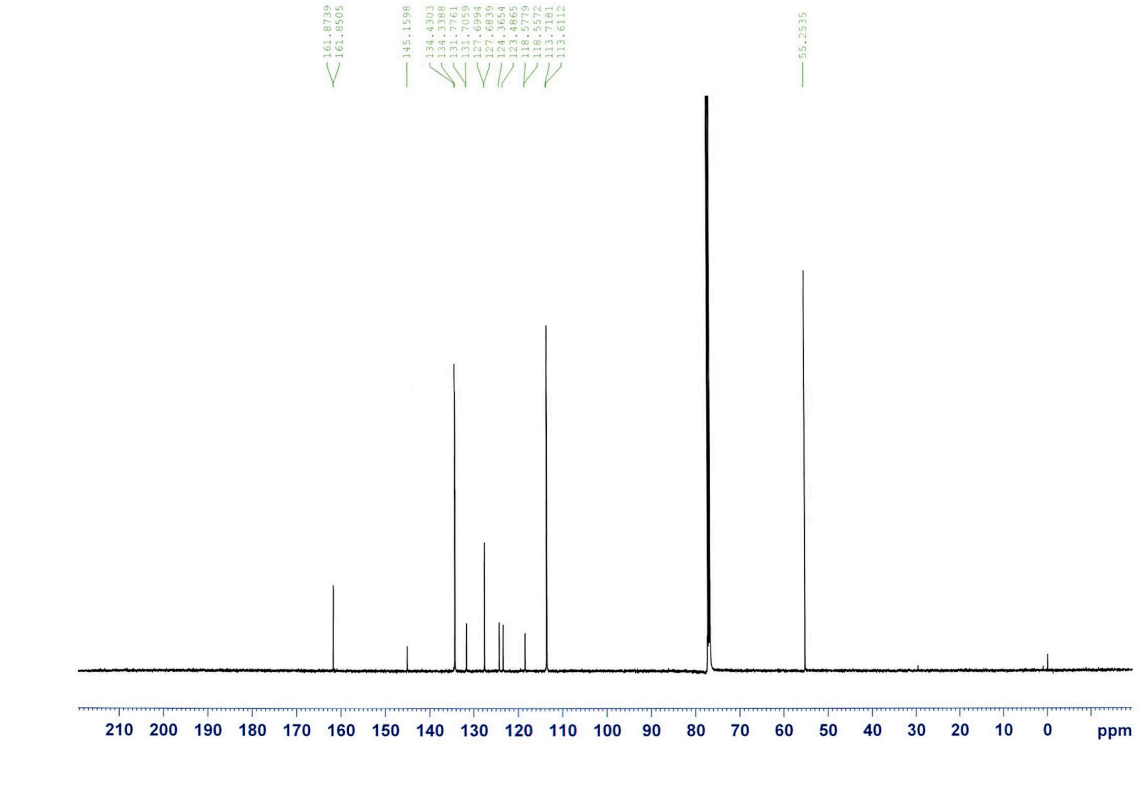
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4g** ($CDCl_3$)



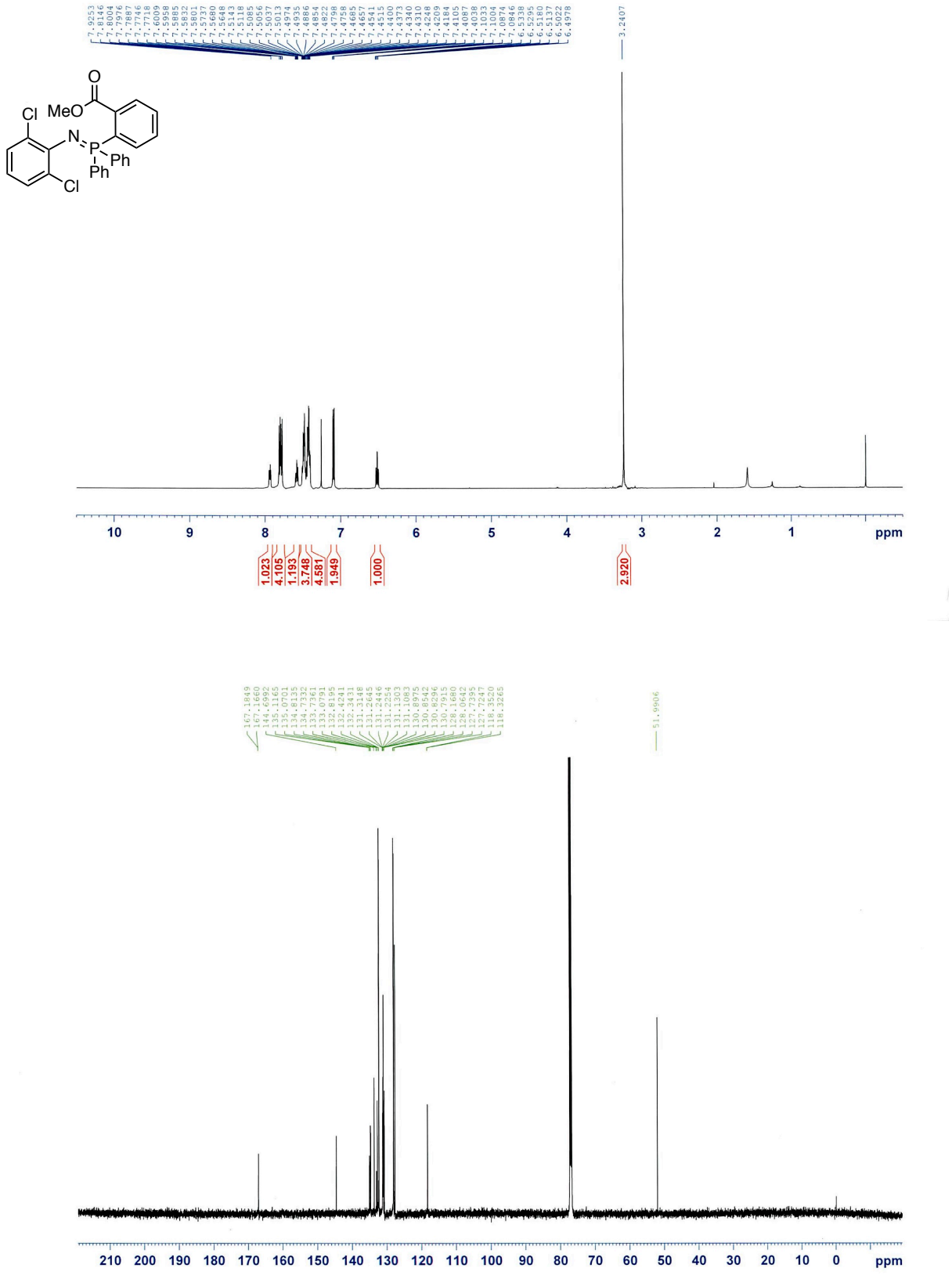


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4i** ($CDCl_3$)

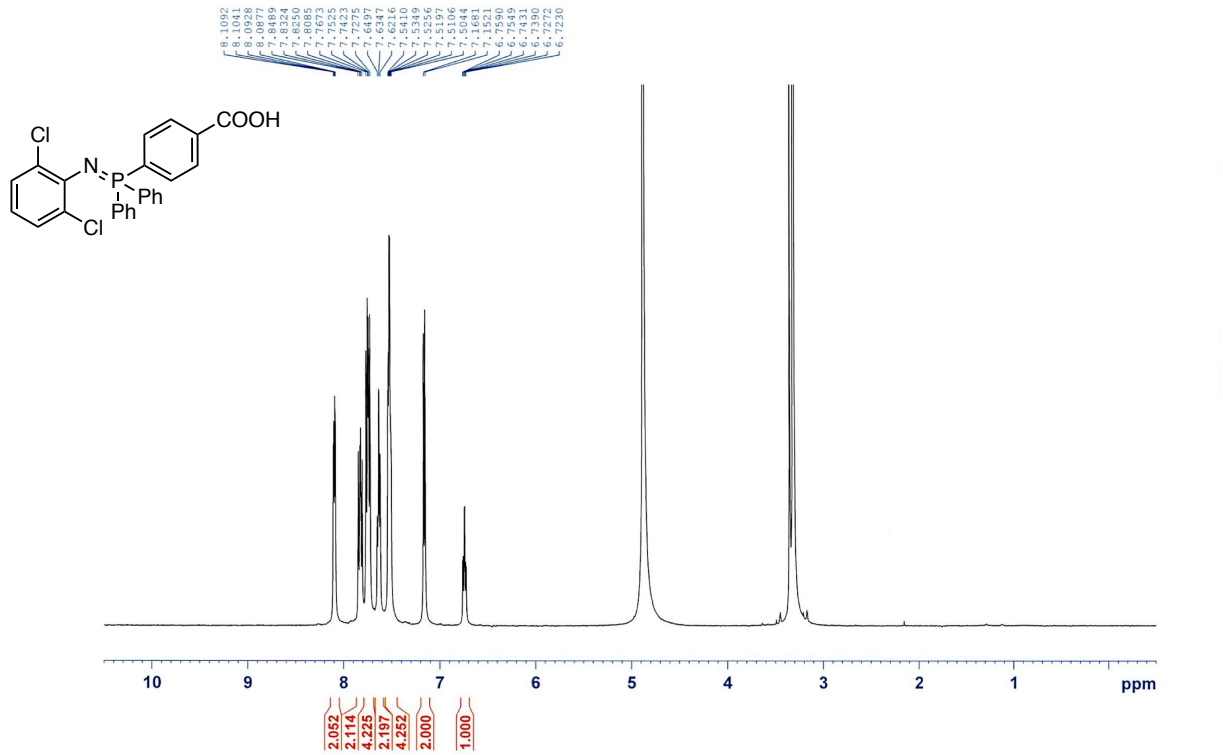


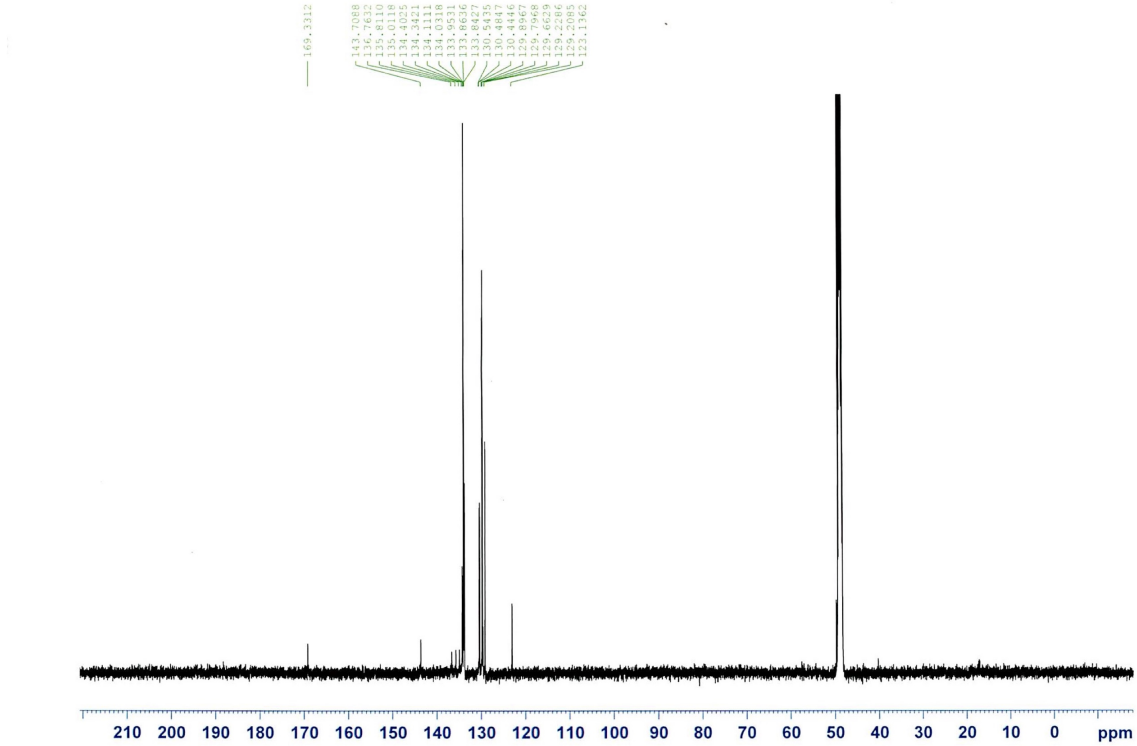


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4k** ($CDCl_3$)

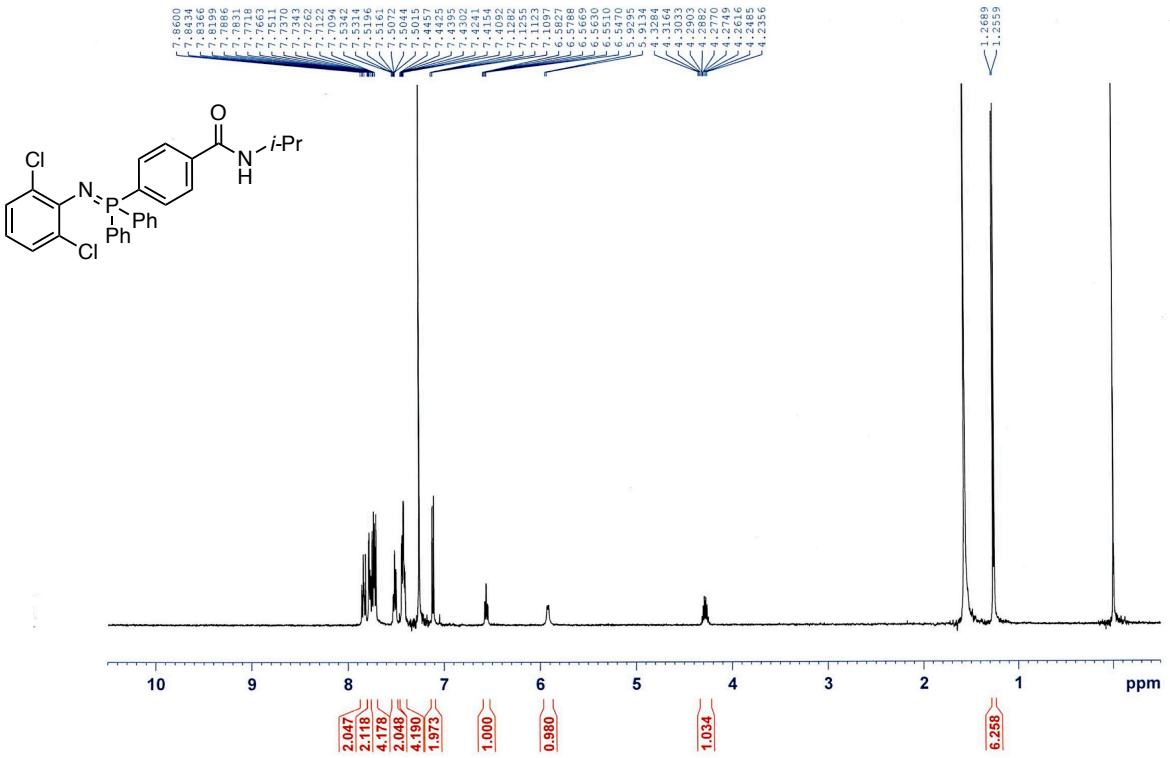


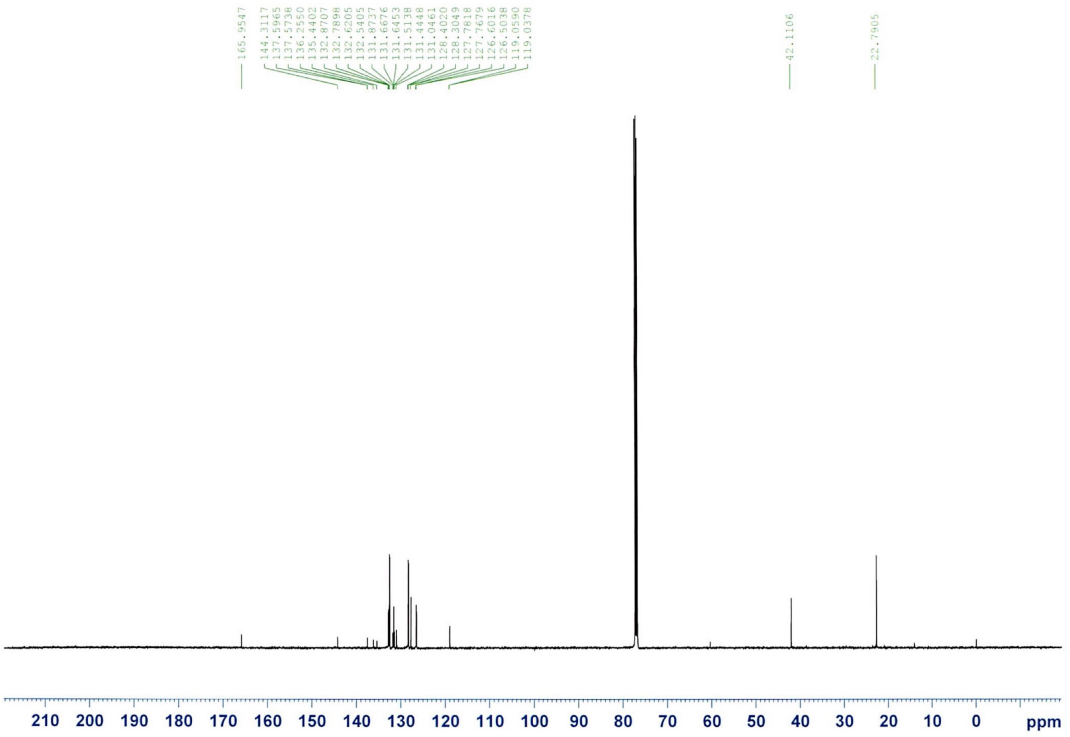
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4l** (CD_3OD)



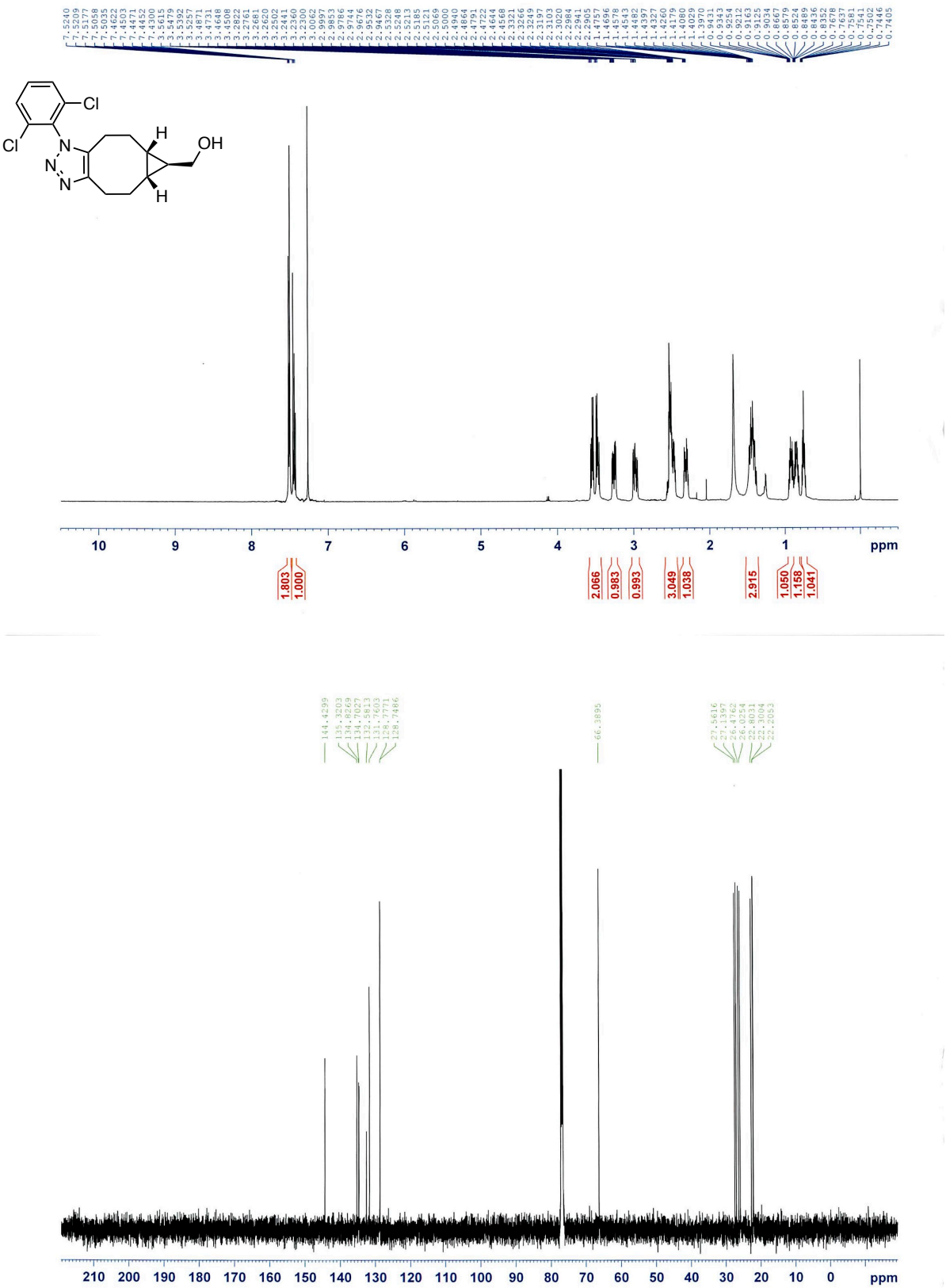


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **4m** ($CDCl_3$)

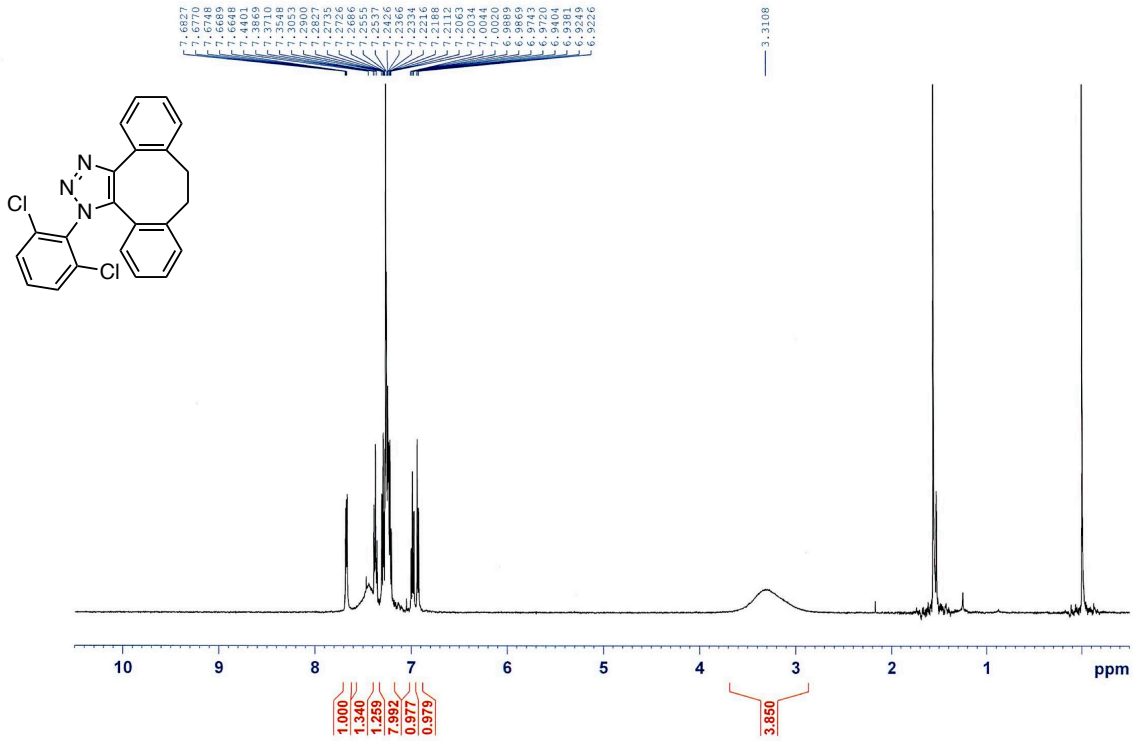


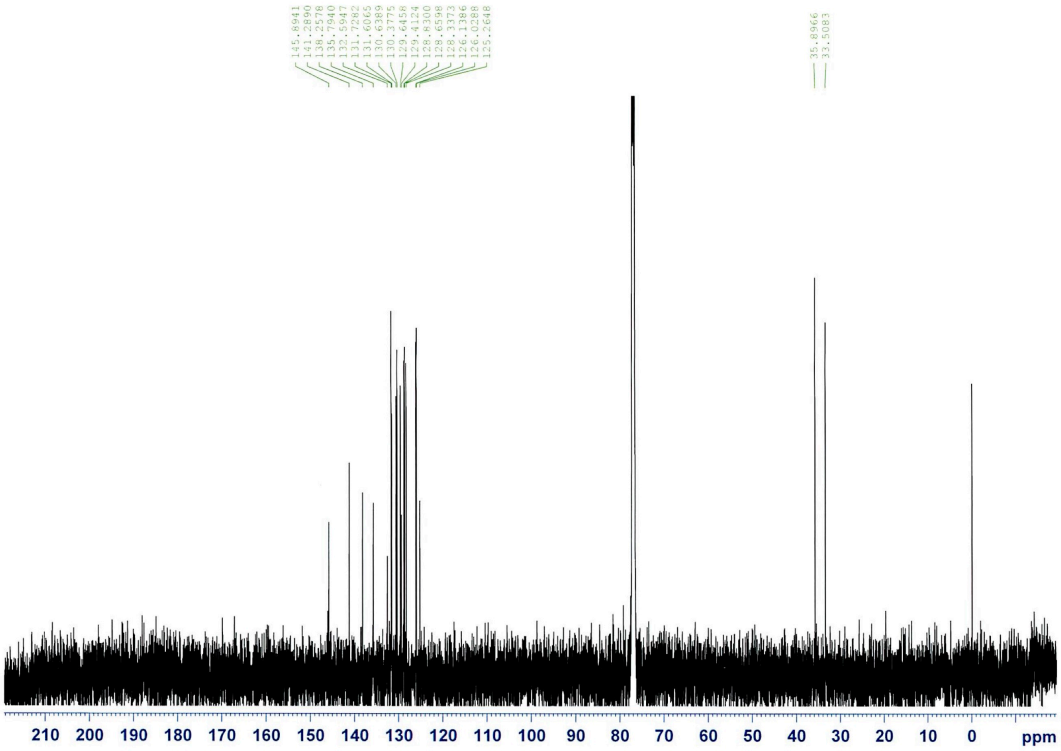


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **9** ($CDCl_3$)

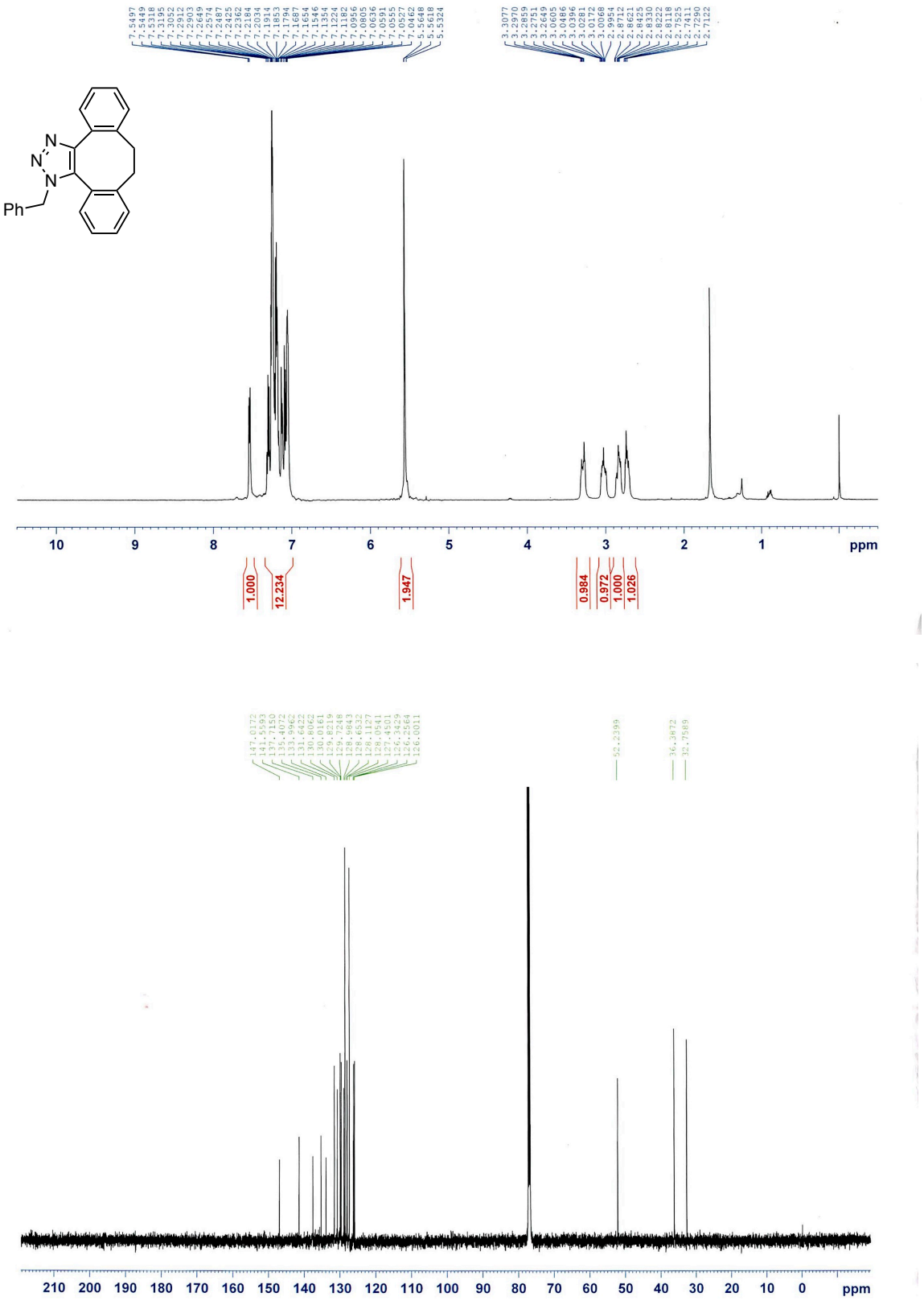


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **11a** ($CDCl_3$)

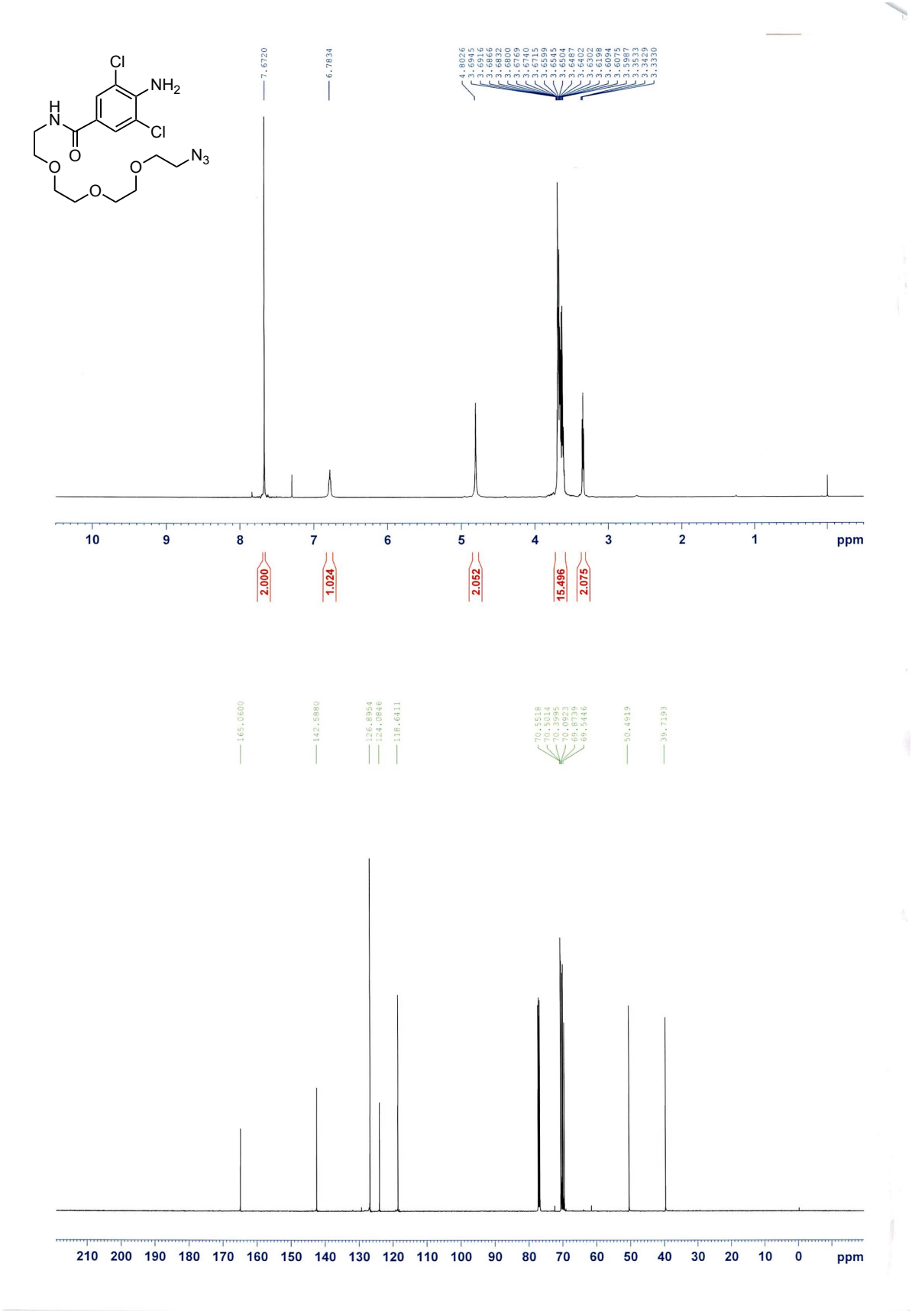




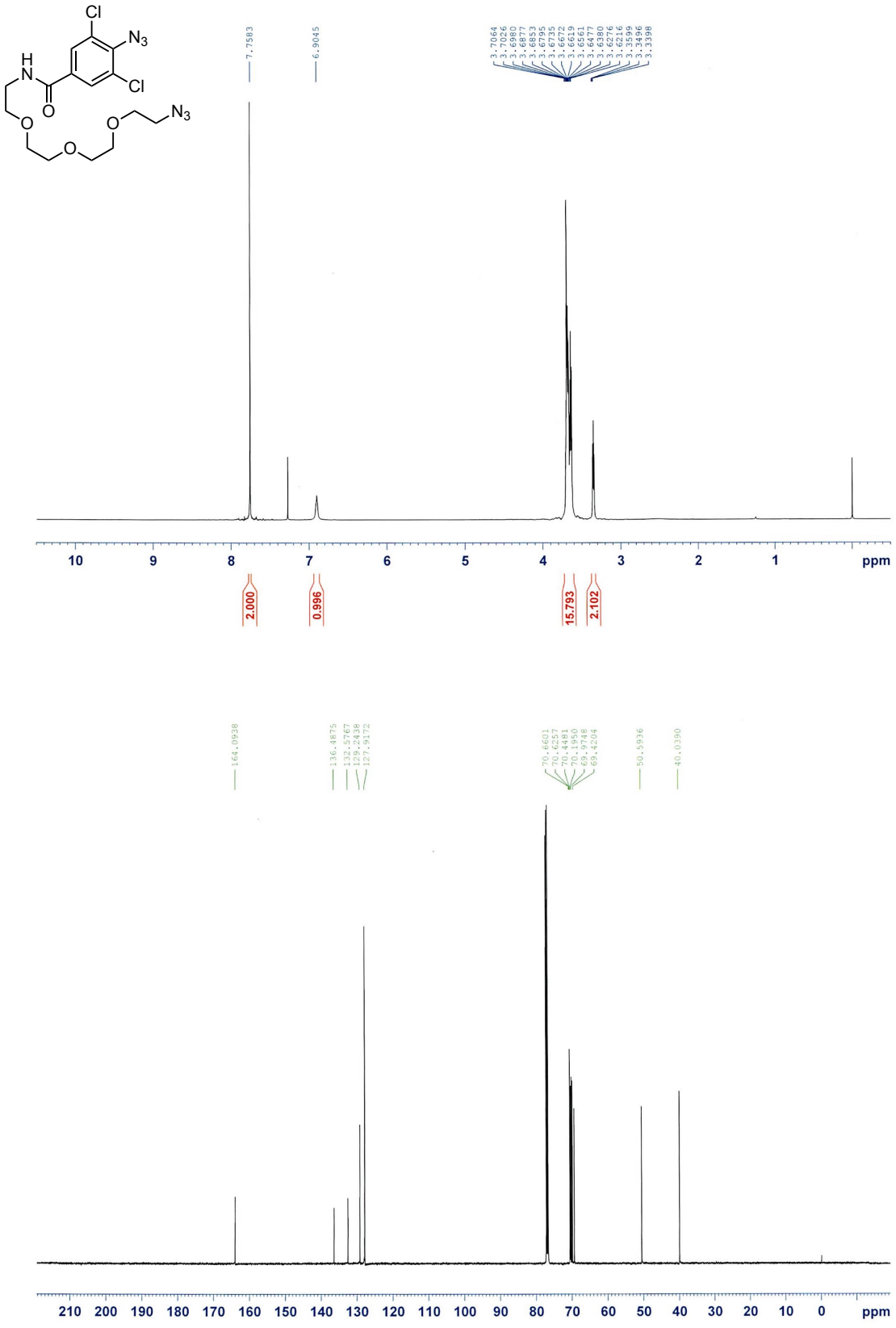
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **11b** ($CDCl_3$)



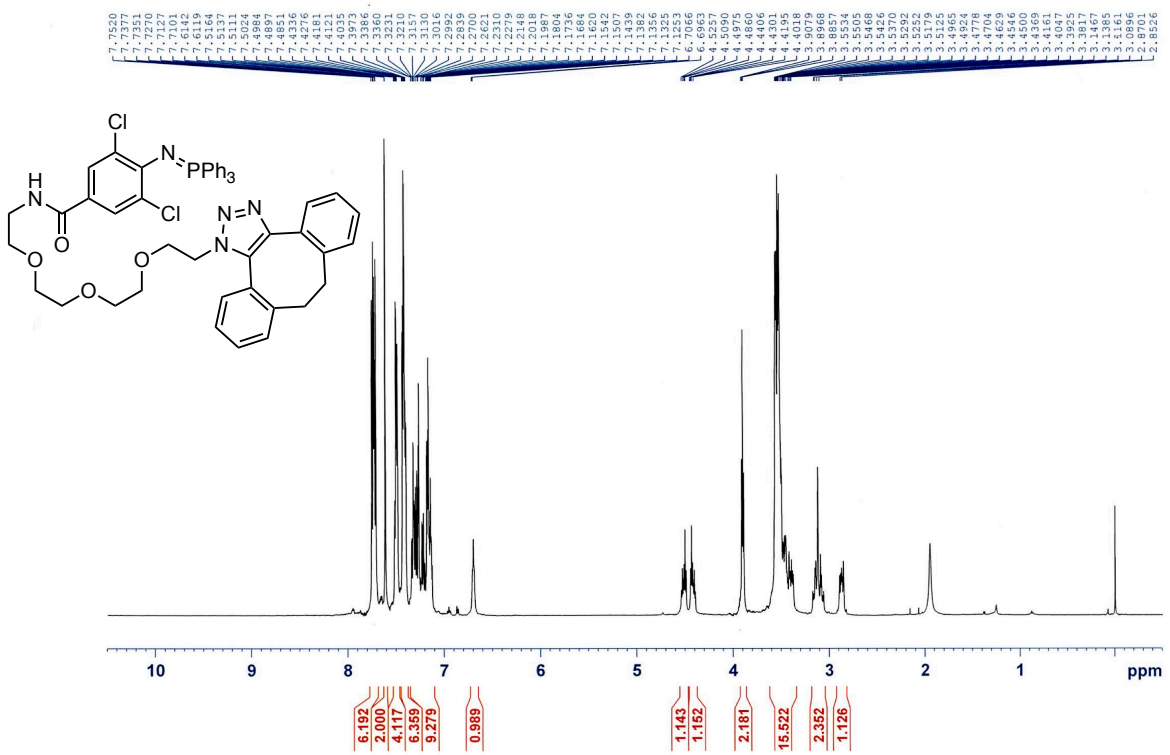
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of
4-amino-*N*-(11-azido-3,6,9-trioxaundecyl)-3,5-dichlorobenzamide ($CDCl_3$)

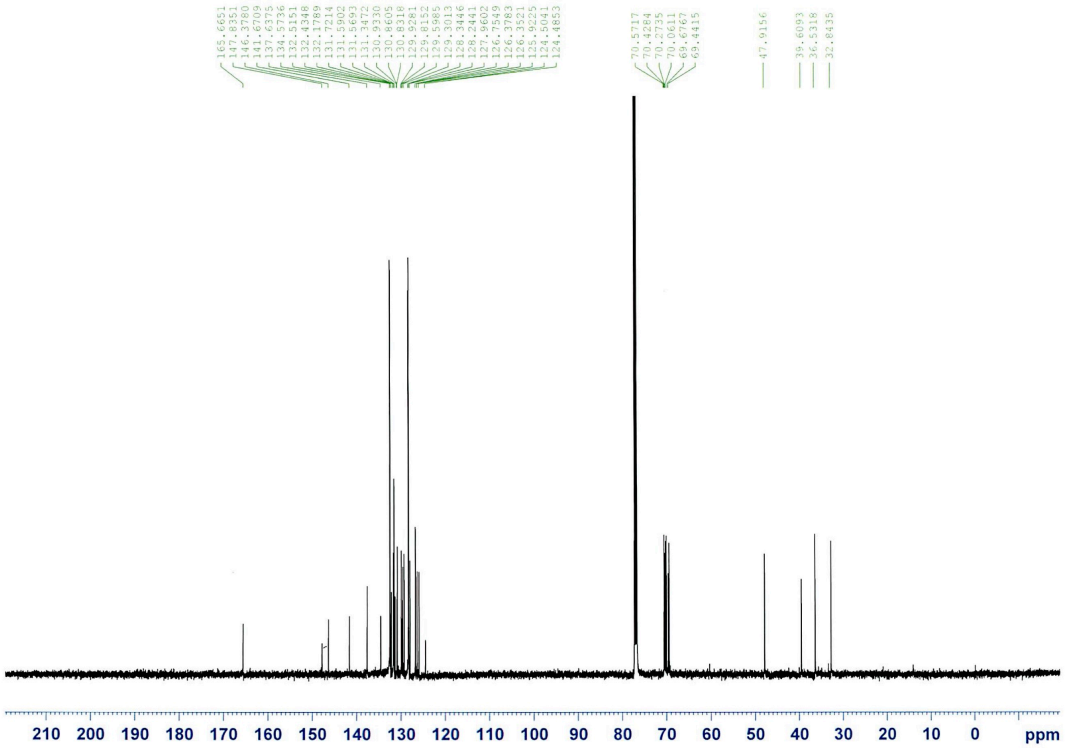


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **12** ($CDCl_3$)

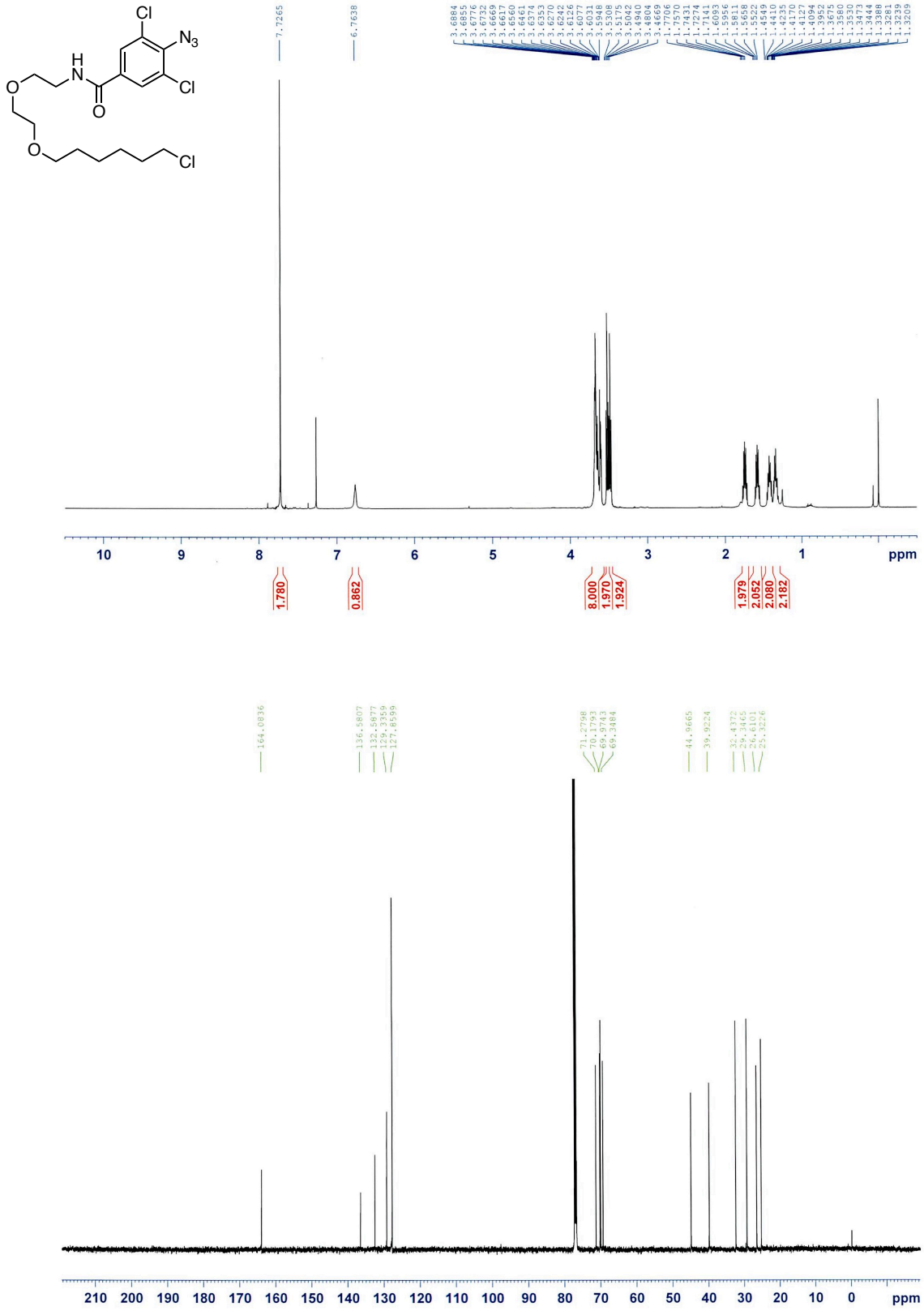


^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **13** ($CDCl_3$)

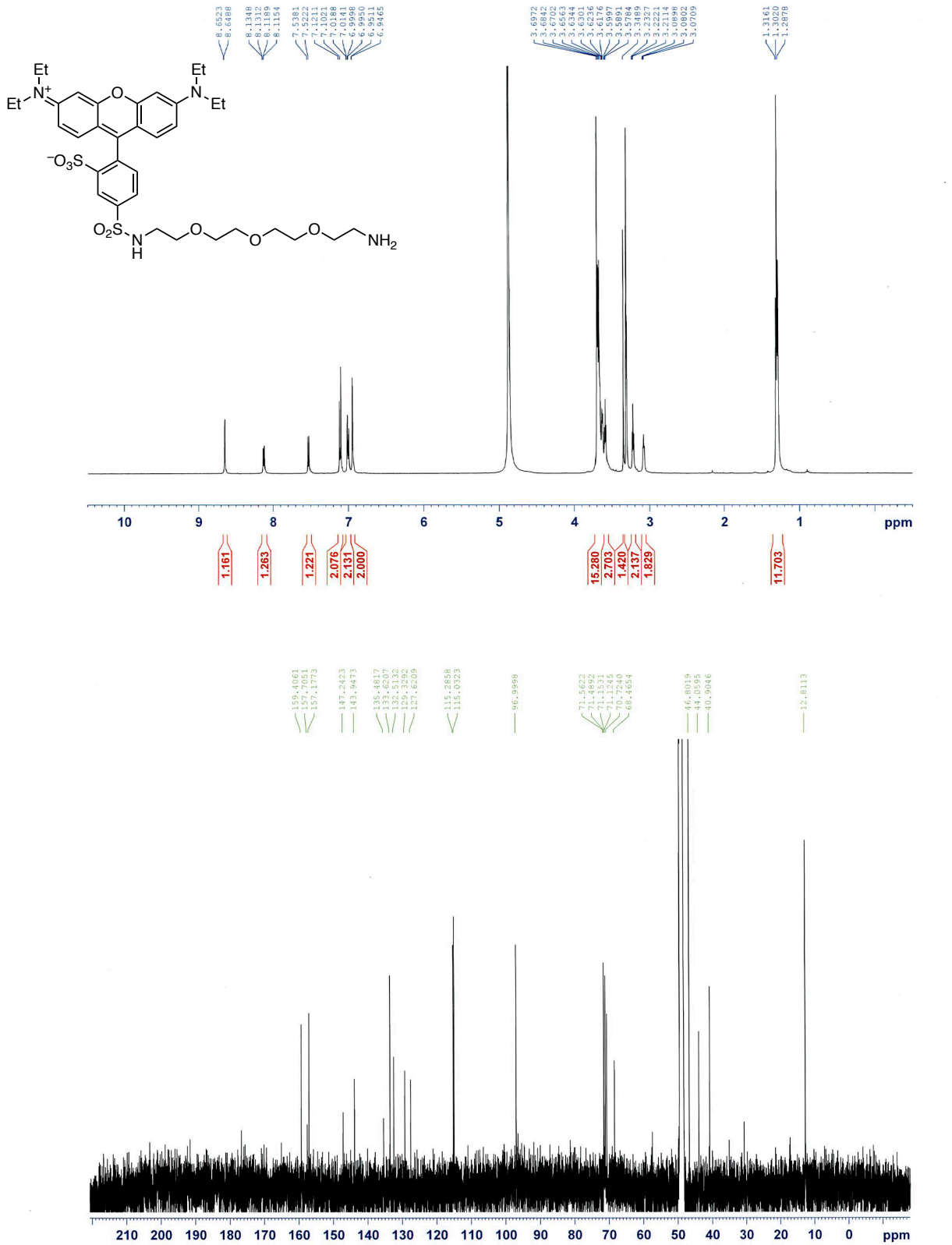




^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **14** ($CDCl_3$)



^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of *N*-(11-amino-3,6,9-trioxaundecyl)-4-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-3-sulfonatobenzenesulfonamide (CD_3OD)



^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra of **15** ($CDCl_3$)