Small molecule induced control in duplex and triplex DNA-directed chemical reactions

Mikkel F. Jacobsen, Jens B. Ravnsbæk and Kurt V. Gothelf

The Danish National Research Foundation: Centre for DNA Nanotechnology (CDNA) at the Interdisciplinary Nanoscience Center (iNANO) and the Department of Chemistry, University of Aarhus, Langelandsgade 140, 8000 Aarhus C, Denmark.

General Experimental Methods

Standard Chemical Reactions:

Unless otherwise stated, all reactions were carried out under nitrogen or argon. Reactions were monitored by thin-layer chromatography (TLC) analysis on Merck® silica gel 60 F_{254} TLC plates. Spots were visualized by exposure to ultraviolet (UV) light (254 nm), or by staining with a 5% solution of phosphormolybdenic acid (PMA) in ethanol or basic aqueous potassium permanganate ($KMnO_4$) and then heating. Flash chromatography was carried out using Merck® silica gel 60 (230-400 mesh). *N*,*N*-Dimethylformamide (DMF) was dried over 3Å molecular sieves. Tetrahydrofuran (THF) was dried by distillation from Na/benzophenone. All other solvents were of HPLC grade quality and used as such. All reagents unless otherwise noted are commercially available and were purchased at the highest commercial quality and used without further purification. NMR spectra were recorded at 400 MHz (^{1}H NMR) and at 100 MHz (^{13}C NMR) on Varian® Gemini Systems, and calibrated to TMS or to the residual solvent peak. The following abbreviations are used for NMR data: s, singlet; d, doublet; q, quartet; qn, quintet; t, triplet; m, multiplet; br, broad; app, apparent. Coupling constants are rounded to nearest 0.5 Hz. All compounds synthesized were determined to be >95% pure by ^{1}H NMR. Melting points are uncorrected. Compound **5**,[1] **10**[2] and **12**[3] were prepared according to literature procedures.

DNA Reactions:

All oligonucleotides were obtained from DNA Technology A/S (Risskov, Denmark, www.dna-technology.dk). Determination of concentration of oligonucleotides by UV-VIS spectroscopy was performed using a Nanodrop® ND-1000 instrument (Nanodrop Technologies, USA). Reverse-phase High Performance Liquid Chromatography (RP-HPLC) was performed on an Agilent® 1200 Series HPLC system (Agilent Technologies, USA) with fraction collector and UV-detection at 260 nm, fitted with a Phenomenex C18 Clarity-Oligo RP column (3 μm, 50x4.6 mm) (Phenomenex, USA). MALDI-TOF mass spectrometry was performed on a Bruker Autoflex® LRF20 instrument (Bruker Daltonik GmbH, Germany) with FlexControl and FlexAnalysis software using an AnchorChip® target. Microcon centrifugal filters YM-3 (Millipore®, USA) were used for desalting reaction mixtures from click chemistry. Denaturing polyacrylamide gel electrophoresis was performed on a Mini Protean system equipped with a Powerpac Universal electricity-supply from Bio-rad® (Bio-rad, USA), and staining with aq. ethidium bromide solution. Gels were visualized using an Ingenius Analysis System (Syngene, UK) with GeneSnap and GeneTools software. Thermal denaturation experiments were performed on a Cary 100 Bio UV-VIS instrument (Varian Inc., USA) equipped with Peltier block temperature-controller and using Varian WinUV software. All experiments with DNA were carried out using water purified by a Milli-Q®-Ultrapure Water Purification System (Millipore). Bathophenanthrolinedisulfonic acid, disodium salt (**1**, BPDS ligand) was obtained from Fisher Scientific. Triplex DNA binders coralyne chloride (**3**) and naphthylquinoline (**4**) were obtained from Chemos and Aldrich, respectively. Compound **2**[4] was prepared according to a literature procedure.

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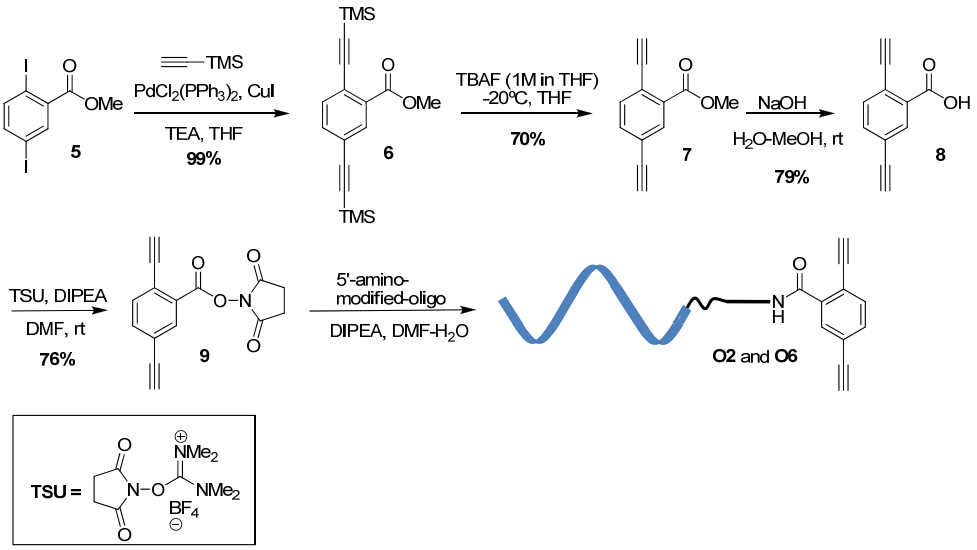
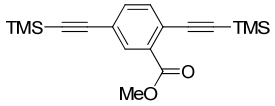
[1] D. Lesuisse, G. Lange, P. Deprez, D. Bénard, B. Schoot, G. Delettre, J.-P. Marquette, P. Broto, V. Jean-Baptiste, P. Bichet, E. Sarubbi, E. Mandine, *J. Med. Chem.* **2002**, *45*, 2379-2387.
[2] G. Eglinton, R. A. Raphael, R. G. Willis, J. A. Zabkiewicz, *J. Chem. Soc.* **1964**, 2597-2603.
[3] U. Henriksen, O. Buchardt, P. E. Nielsen, *Photochem. Photobiol., A* **1991**, *57*, 331-342.
[4] T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853-2855.

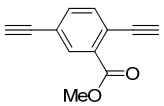
Synthesis of Dialkyne-modified Oligonucleotides O2 and O6:



Scheme 1. Synthesis of dialkyne-modified oligonucleotides **O2** and **O6**.



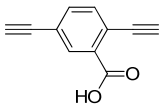
Methyl 2,5-bis((trimethylsilyl)ethynyl)benzoate (6).[5] To solution of **5**[1] (582 mg, 1.50 mmol), $PdCl_2(PPh_3)_2$ (105 mg, 0.15 mmol) and CuI (29 mg, 0.15 mmol) in THF (10 mL) was added triethylamine (2.1 mL, 15 mmol) followed by trimethylsilylacetylene (1.27 mL, 9.00 mmol) at rt. The reaction mixture was stirred for 2 h at rt, and water (10 mL) was added. The aqueous mixture was extracted several times with CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was subjected to column chromatography (hexane-CH_2Cl_2, 5:1 then 3:2) to afford the product **6** (492 mg, 99%) as yellowish oil: R_f = 0.4 (hexane-CH_2Cl_2, 3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.50 (s, 2H), 3.91 (s, 3H), 0.27 (s, 9H), 0.25 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.2, 134.5, 134.5, 134.0, 132.7, 123.3, 123.1, 103.5, 103.0, 102.0, 97.6, 52.3, 0.1, 0.0; HRMS (ES) *m/z*: $[M+Na]^+$ calcd for $C_{18}H_{24}NaO_2Si_2$, 351.1213; found, 351.1214.



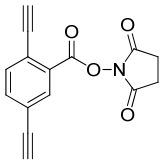
Methyl 2,5-diethynylbenzoate (7). To a solution of **6** (164 mg, 0.50 mmol) in THF (10 mL) was added quickly TBAF (1.1 mL, 1.0 M in THF) at -20°C. The mixture was stirred for 10-20 s and quenched with sat. aq. NH_4Cl (10 mL). The aqueous mixture was extracted several times with CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was subjected to column chromatography (hexane-CH_2Cl_2, 1:1) to afford the product **7** (65 mg, 70%) as a reddish solid: mp 49-50°C; R_f = 0.2 (hexane-CH_2Cl_2, 3:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.56-7.57 (m, 2H), 3.93

[5] F. Balavoine, D. Madec, C. Mieskowski, *Tetrahedron Lett.* **1999**, *40*, 8351-8354.

(s, 3H), 3.49 (s, 1H), 3.21 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.7, 135.1, 135.0, 134.1, 132.8, 123.0, 122.7, 84.3, 82.1, 81.7, 80.1, 52.5; HRMS (ES) *m/z*: $[M+Na]^+$ calcd for $C_{12}H_8NaO_2$, 207.0422; found, 207.0429.



2,5-Diethynylbenzoic acid (8). To a solution of **7** (100 mg, 0.54 mmol) in MeOH (10 mL) was added NaOH (2.7 mL, 1.0 M in H_2O) at rt. The mixture was stirred for 2 h at rt. Aq. HCl (700 μL, 1.0 M) was added, and the mixture was extracted several times with CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$ and evaporated to dryness *in* vacuo to afford pure **8** (73 mg, 79%) as a yellow solid: mp 115-116°C; R_f = 0.4 (EtOAc); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.84 (s, 1H), 7.55-7.60 (m, 2H), 4.48 (s, 1H), 4.42 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 163.6, 132.9, 132.8, 131.9, 130.6, 120.8, 120.6, 82.1, 80.0, 79.5, 78.0; HRMS (ES) *m/z*: $[M+Na]^+$ calcd for $C_{11}H_6NaO_2$, 193.0265; found, 193.0268.

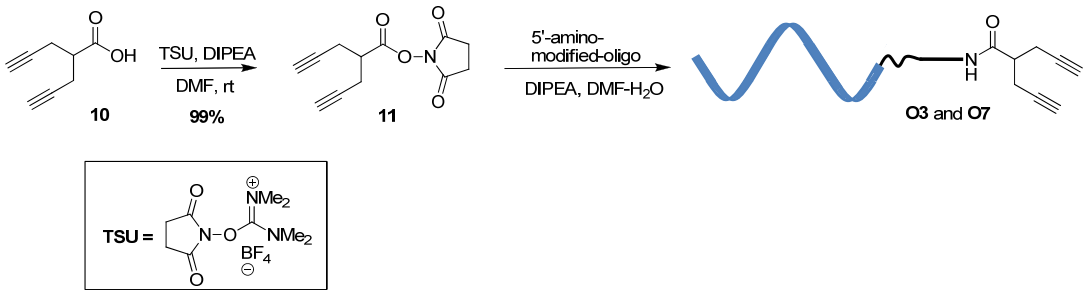


2,5-Dioxopyrrolidin-1-yl 2,5-diethynylbenzoate (9). To a solution of **8** (17 mg, 0.10 mmol) in DMF (0.50 mL) was added DIPEA (35 μL, 0.20 mmol) followed by *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSU) (33 mg, 0.11 mmol). The reaction mixture was stirred for 1.5 h at rt, and evaporated to dryness *in vacuo*. The residue was subjected to column chromatography (hexane-EtOAc, 1:1) to yield **9** (20 mg, 76%) as yellow crystals: mp >150°C (decomp.); R_f = 0.2 (hexane-EtOAc, 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, *J* = 1.5 Hz, 1H), 7.69 (dd, *J* = 1.5, 8.0 Hz, 1H), 7.65 (d, *J* = 8.0 Hz, 1H), 3.55 (s, 1H), 3.26 (s, 1H), 2.91 (s, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.1, 160.1, 136.8, 135.3, 134.8, 127.4, 124.4, 123.2, 86.2, 81.5, 81.1, 80.5, 25.8; HRMS (ES) *m/z*: $[M+Na]^+$ calcd for $C_{15}H_9NaO_4$, 290.0429; found, 290.0422.

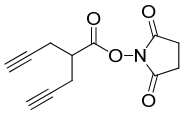
Reaction of NHS-Ester 9 with Amino-modified Oligonucleotides

5'-amino modified oligonucleotides (20 nmol) were reacted with NHS-ester **9** (40 μL, 400 nmol, 20 mM solution in DMF) in 330 μL of H_2O:DIPEA:DMF (1:1:3) overnight. The reaction mixture was diluted with water (400 μL) and extracted three times with 500 μL portions of CH_2Cl_2. The aq. residue was evaporated to dryness, and the obtained pellets were redissolved in H_2O. The crude DNA conjugate-solutions were subjected to purification by preparative RP-HPLC eluting with TEAA (0.1 M)-MeCN solution. The collected fractions were freeze-dried and the obtained pellets of **O2** and **O6** were redissolved in H_2O and quantified by UV-VIS spectroscopy. Their purity was ascertained *via* analytical RP-HPLC and they were characterized by MALDI-TOF MS (see page S7).

Synthesis of Dialkyne-modified Oligonucleotides O3 and O7:



Scheme 2. Synthesis of dialkyne-modified oligonucleotides **O3** and **O7**.

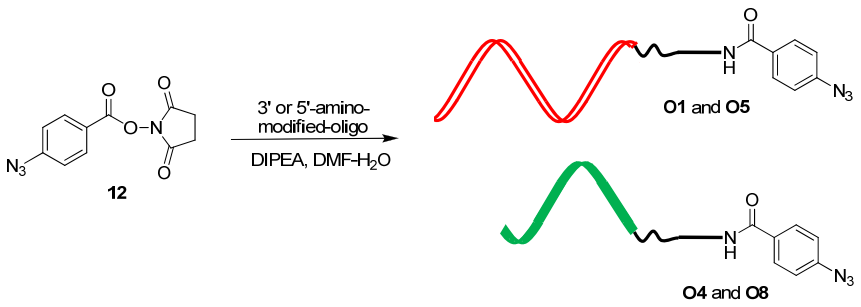


2,5-Dioxopyrrolidin-1-yl 2-(prop-2-ynyl)pent-4-ynoate (11). To a solution of **10**[2] (55 mg, 0.40 mmol) in DMF (0.5 mL) was added DIPEA (139 μL, 0.80 mmol) followed by *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSU) (133 mg, 0.44 mmol). The reaction mixture was stirred for 1 h at rt, and evaporated to dryness *in vacuo*. The residue was subjected to column chromatography (CH_2Cl_2 then CH_2Cl_2-EtOAc, 4:1) to yield **11** (93 mg, 99%) as a white solid: mp 127-128°C; R_f = 0.6 (CH_2Cl_2-EtOAc, 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.11 (qn, J = 6.5 Hz, 1H), 2.83 (s, 4H), 2.76-2.79 (m, 4H), 2.11 (t, J = 2.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.8, 168.0, 79.0, 71.7, 41.0, 25.7, 20.0; HRMS (ES) *m/z*: $[M+Na]^+$ calcd for $C_{15}H_9NNaO_4$, 256.0586; found, 256.0594.

Reaction of NHS-Ester 11 with Amino-modified Oligonucleotides

The dialkyne-DNA conjugates **O3** and **O7** were synthesized as described above for **O2** and **O6**, except NHS-ester **11** was employed instead. Their purity was ascertained *via* analytical RP-HPLC and they were characterized by MALDI-TOF MS (see page S7).

Synthesis of Azide-modified Oligonucleotides O1, O4, O5 and O8:



Scheme 3. Synthesis of azide-modified oligonucleotides **O1**, **O4**, **O5** and **O8**.

Reaction of NHS-Ester 12 with Amino-modified Oligonucleotides

The azide-DNA conjugates **O1**, **O4**, **O5** and **O8** were synthesized as described above for **O2** and **O6**, except NHS-ester **12**[3] was employed instead. Their purity was ascertained *via* analytical RP-HPLC and they were characterized by MALDI-TOF MS (see page S7).

Triplex-Programmed Click Chemistry Experiments:

Experimental Procedure

Preformed duplexes were made by mixing equimolar amounts of **O1**, **O5** or **O9** (250 pmol) and **O2**, **O3**, **O6** or **O7** (250 pmol) with phosphate buffer (1.3 μL, 1.0 M, pH 4.3), aq. NaCl (5 μL, 2.0 M) at rt, and diluting with water to an approx. volume of 110 uL. The duplex solutions was heated to 90°C for 5 min and allowed to cool slowly to rt. Oligonucleotide **O4** or **O8** (250 pmol) and triplex DNA binder **3** or **4** (2.5 μL, 600 μM solution in H_2O) was added subsequently to a total volume of approx. 115 μL. The mixtures were incubated overnight at 5°C (fridge). The mixtures were allowed to warm slowly to rt (1 h). Ligand (**1** or **2**), $CuSO_4$ and sodium ascorbate were added to final concentrations of 1.4 mM, 200 μM and 2 mM, respectively. The reaction mixtures (final conc.: 2 μM in each oligonucleotide, 10 mM phosphate buffer, 80 mM NaCl, pH 5.0) were allowed to react for 2 h at rt, and desalted on Microcon YM-3 centrifugal filters using three cycles. MALDI-TOF and denaturing PAGE analysis was subsequently performed on the retentates.

Denaturing PAGE Analysis of Reactions

Each retentate was diluted to a final volume of 50 μL (5 μM conc. of triplex assuming 100% conversion). The retentates (6 μL) were mixed with bromophenol blue (BPB) loading solution (6 μL, 0.05% BPB in formamide) and loaded onto 15% polyacrylamide gels (8 M urea) and subjected to electrophoresis (150V, 1.5 h) using tris-HCl buffer (50 mM, pH 8.4). The gels were stained with aq. ethidium bromide, followed by UV-visualization of the bands by illumination at 254 nm.

MALDI-TOF MS Analysis of Reactions

Retentates were co-crystallized using 3-hydroxypicolinic acid (Fluka)-diammonium citrate (Merck) solution on the target, and analyzed in both linear and reflector mode.

Isolation of Intramolecular Triplexes

For the isolation of triplexes **T1**-**T4**, each corresponding reaction was scaled up four times. The retentates from desalting on Microcon YM-3 centrifugal filters were subjected to purification by preparative RP-HPLC, as detailed above for **O2** and **O6**.

Thermal Denaturation Experiments:

Oligonucleotide samples were prepared as 2 μM solutions (in each strand) in PBS-solution (10 mM phosphate buffer, 80 mM NaCl, pH 5.0) and cooled overnight at 5°C. The cooled samples were subjected to

two cycles in the range 5-90°C starting with heating and using a temperature gradient of 0.5°C/min, and observing at 260 nm. To avoid condensation below rt, the cuvette chamber was flushed with Ar gas. T_m values were determined as the maximum of the first derivative of the melting curve on the first heating ramp due to considerable hysteresis for intermolecular triplexes. Each T_m value is the average of three independent experiments with an error of ±0.5°C.

MALDI-TOF MS data for Oligonucleotides O1-O9, T1-T4, Duplex and Hoogsteen Products

Oligonucleotide	**Calc. exact mass**	**Found**
O1	5718	5719
O2	5926	5928
O3	5893	5892
O4	3881	3881
O5	5982	5983
O6	6190	6190
O7	6157	6153
O8	4145	4143
O9	5394	5393

Triplex	**Calc. exact mass**	**Found**
T1	16284	16275
T2	16317	16361
T3	15492	15491
T4	15525	15524

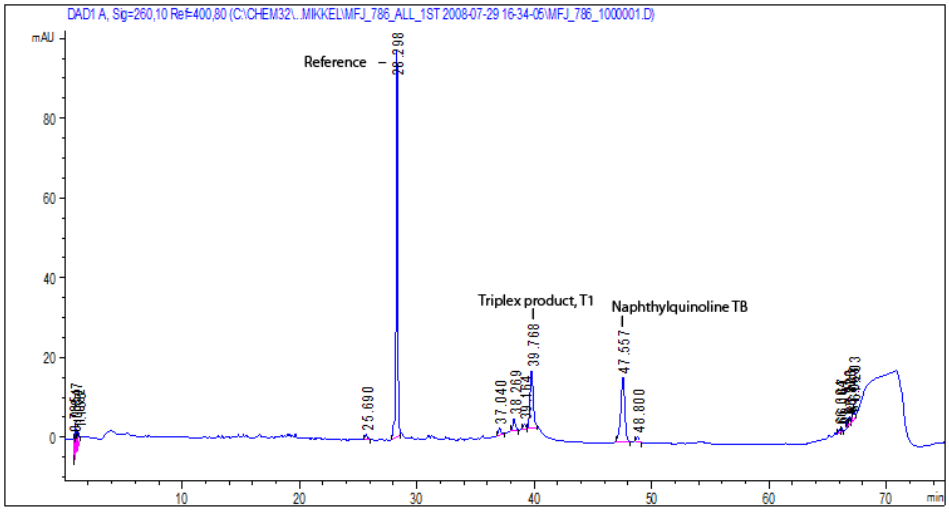
Product	**Calc. exact mass**	**Found**
Duplex (O5+O6)	12172	12157
Duplex (O5+O7)	12139	12126
Duplex (O1+O2)	11644	11690
Duplex (O1+O3)	11611	11642

Product	**Calc. exact mass**	**Found**
Hoogsteen (O6+O8)	10335	10337
Hoogsteen (O7+O8)	10302	10328
Hoogsteen (O2+O4)	9807	9812
Hoogsteen (O3+O4)	9774	9789

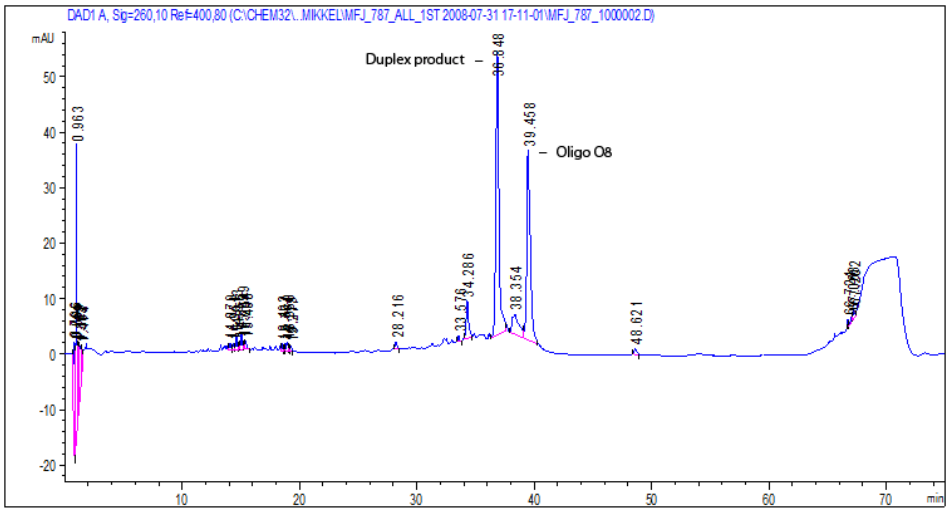
The deviations of observed masses of click chemistry products vs calculated masses compared to **O1-O9** are due to the broad peaks obtained (see below), and the fact that only linear mode of operation gave acceptable signal-to-noise ratios, even when using concentrated samples (> 50 μM) and rigorous cleaning of the target.

RP-HPLC analysis of click reactions:

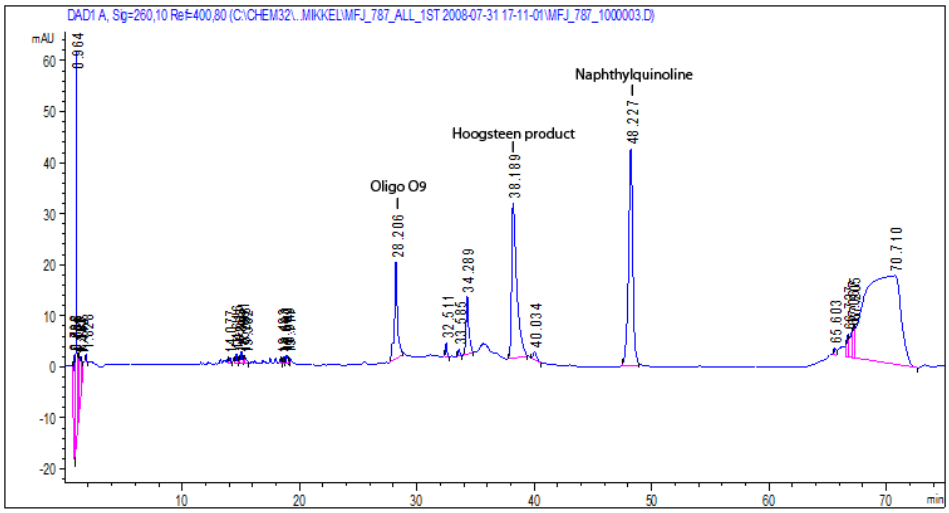
- Triplex reaction (Lane 1):

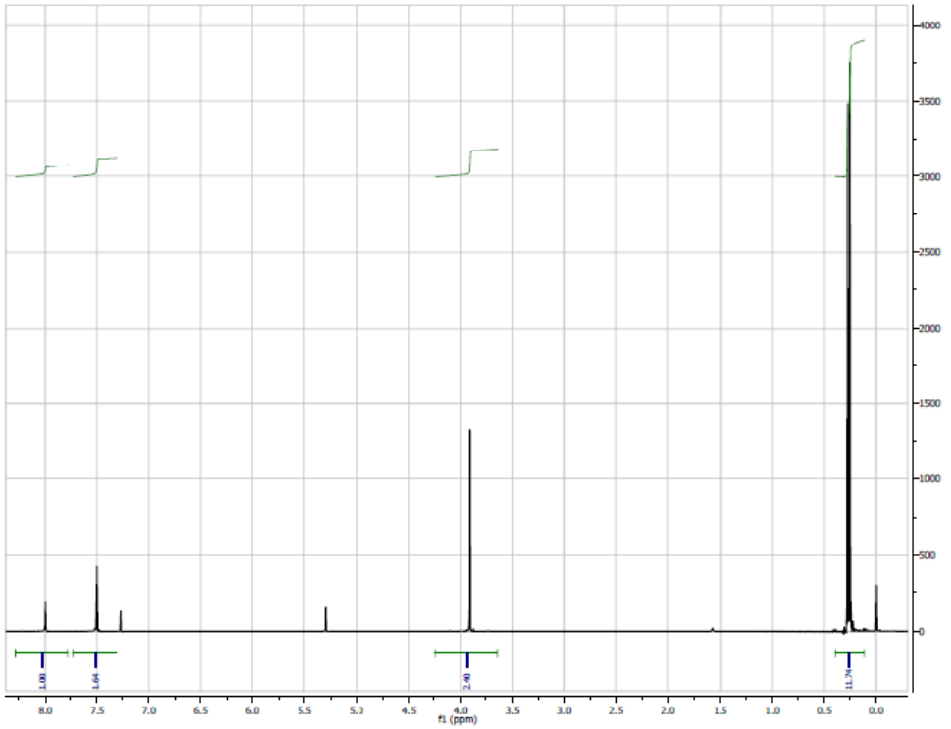


- Duplex reaction (Lane 3):

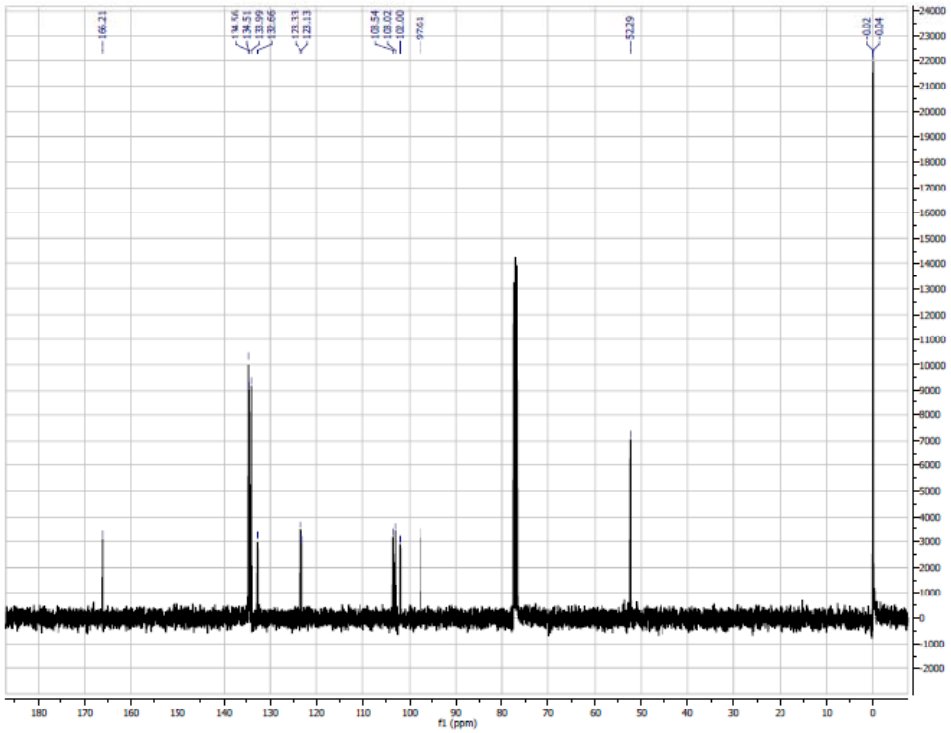


- Hoogsteen reaction (Lane 6):

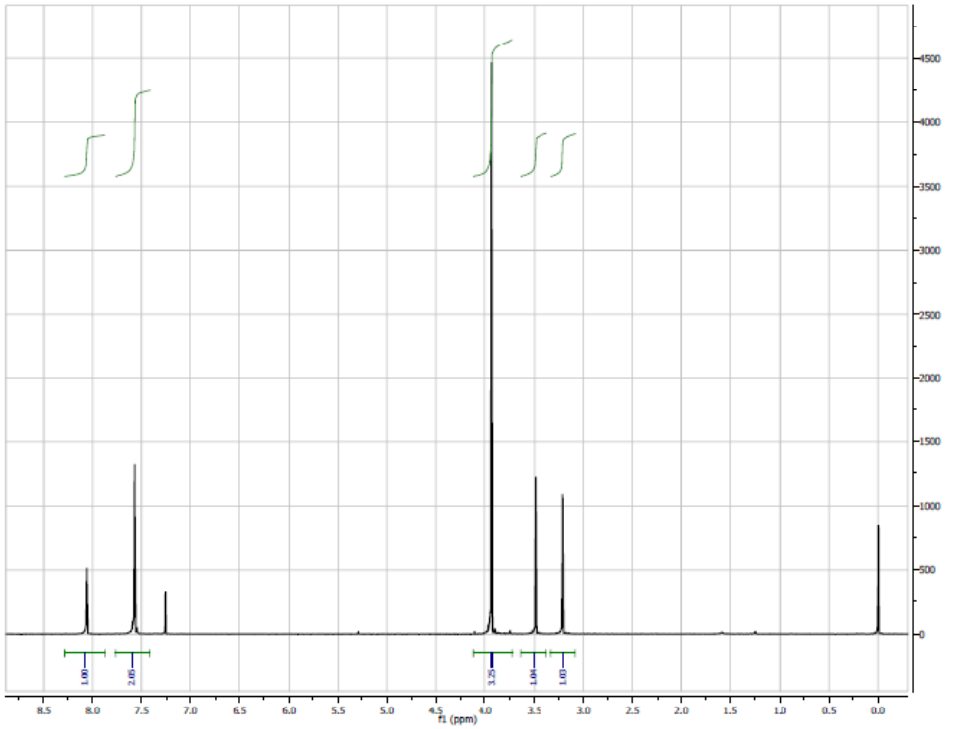




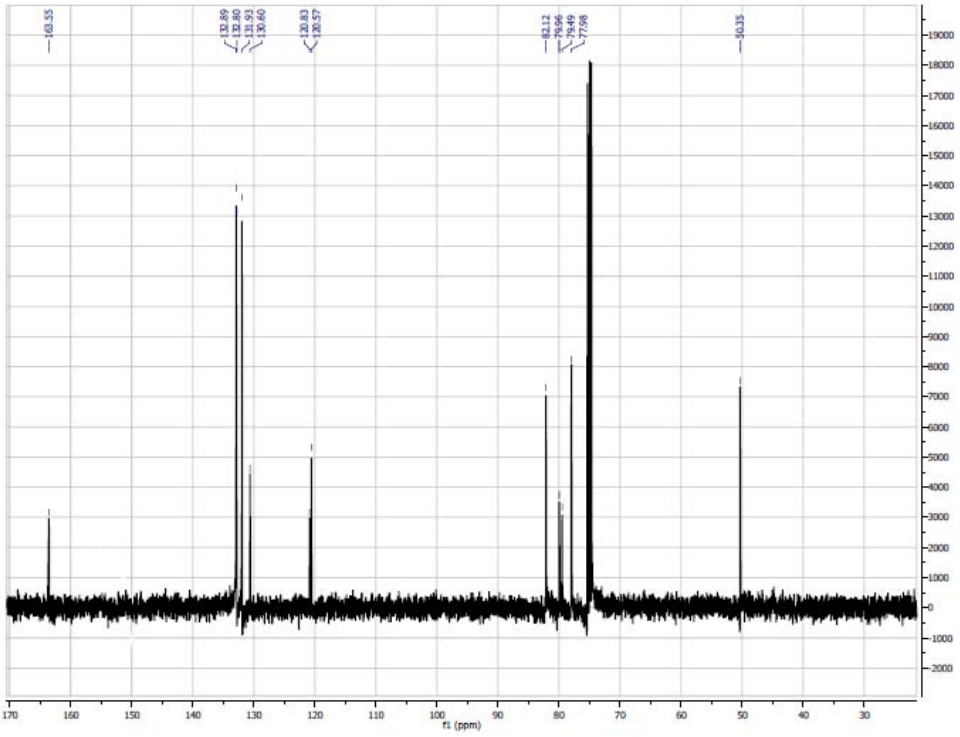
^{1}H NMR Spectra (400 MHz) of **6** in $CDCl_3$



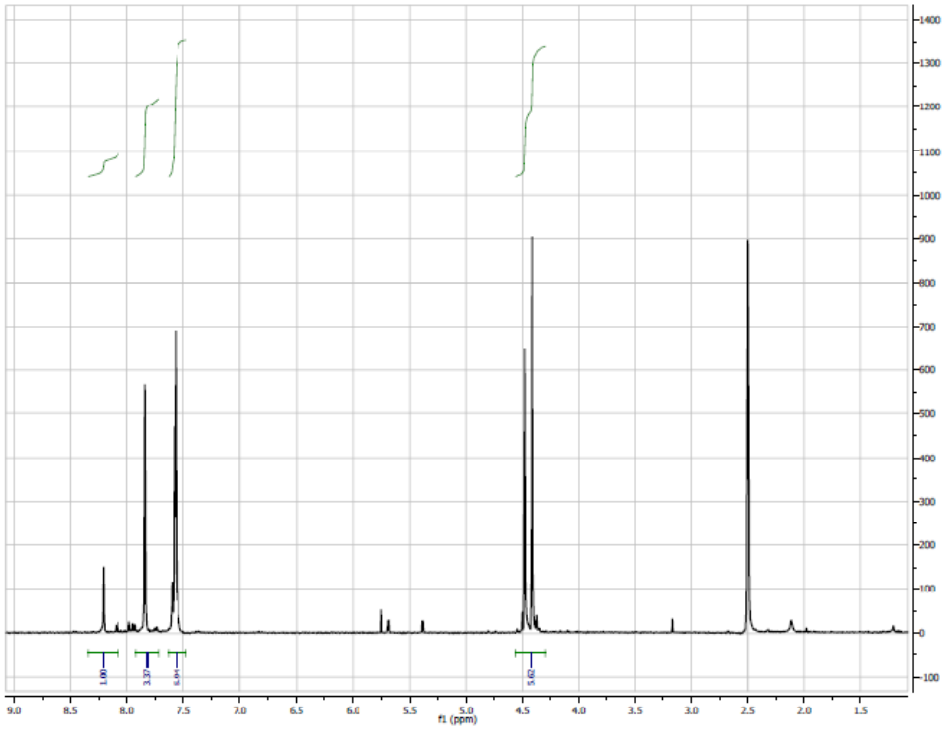
^{13}C NMR Spectra (100 MHz) of **6** in $CDCl_3$



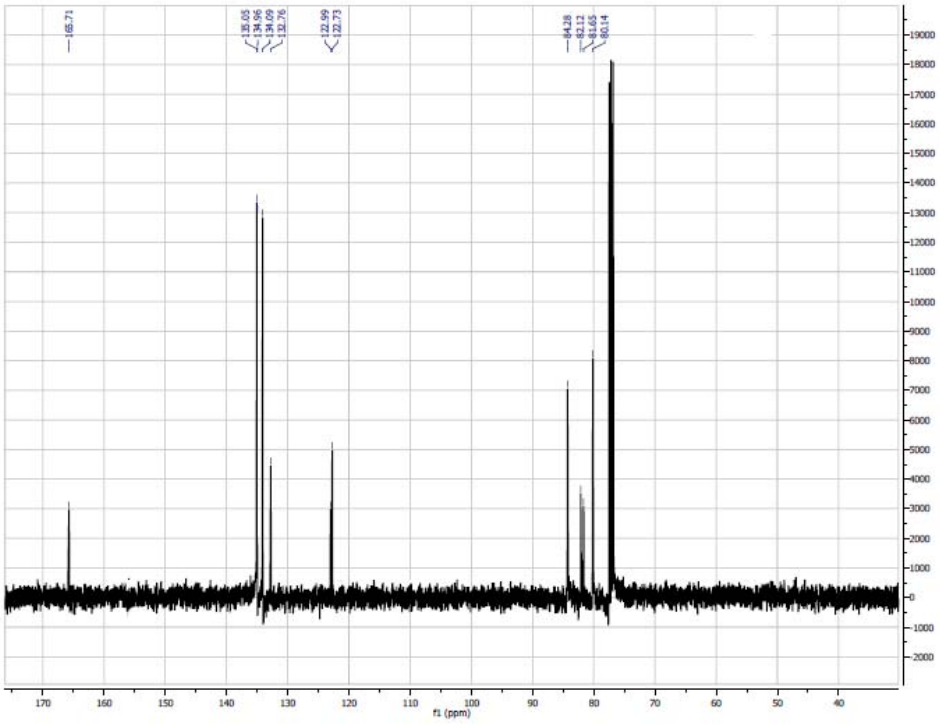
^{1}H NMR Spectra (400 MHz) of **7** in $CDCl_3$



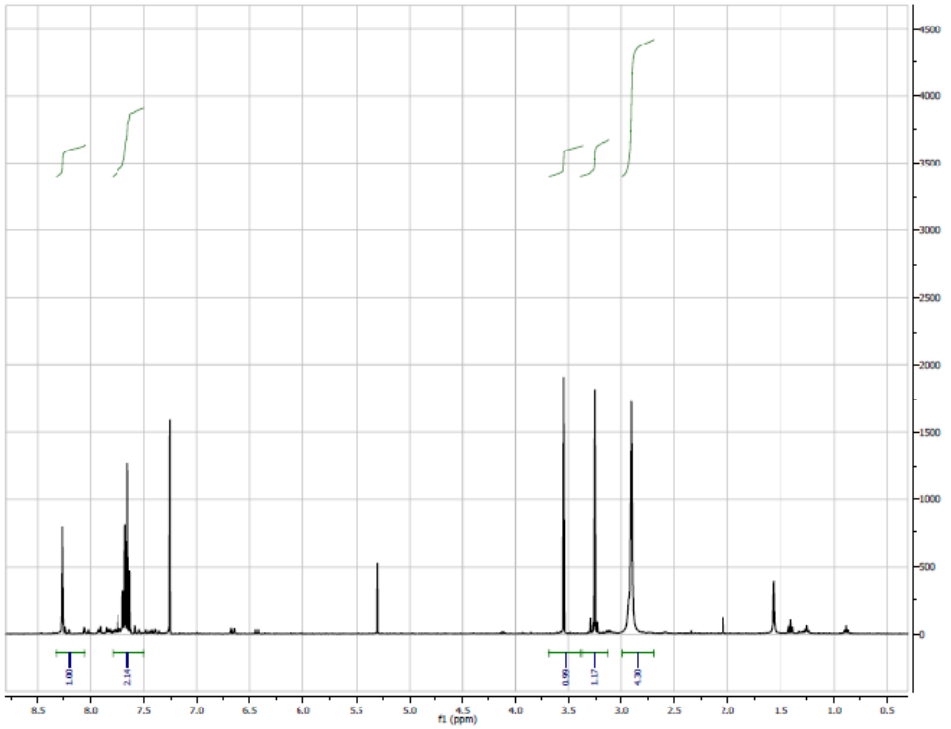
^{13}C NMR Spectra (100 MHz) of **7** in $CDCl_3$



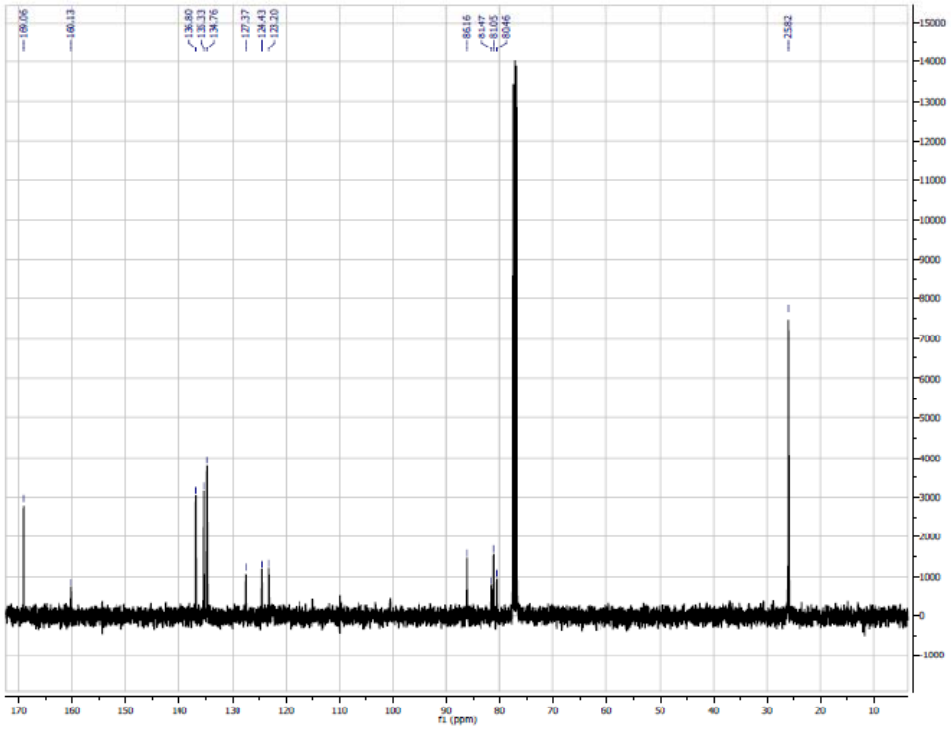
^{1}H NMR Spectra (400 MHz) of **8** in DMSO-d_6



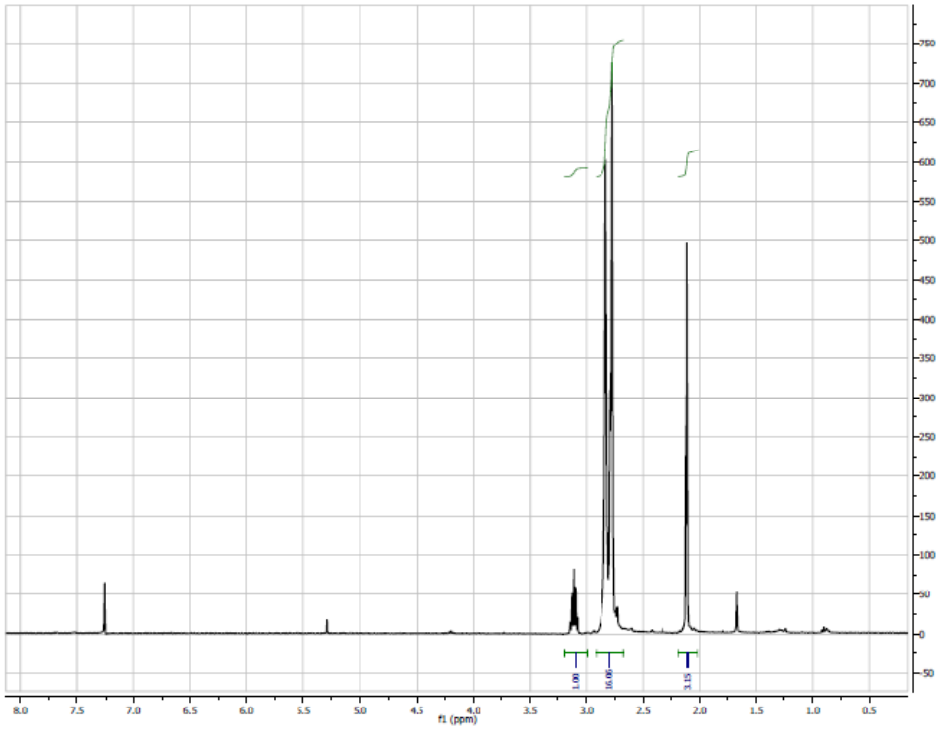
^{13}C NMR Spectra (100 MHz) of **8** in $CDCl_3$



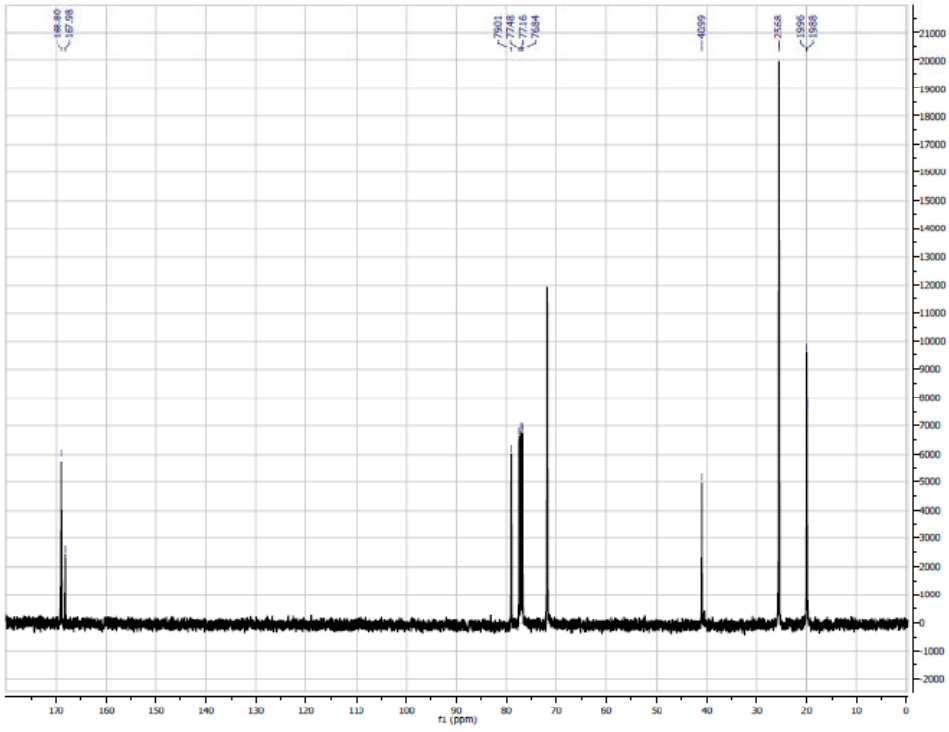
^{1}H NMR Spectra (400 MHz) of **9** in $CDCl_3$



^{13}C NMR Spectra (100 MHz) of **9** in $CDCl_3$



^{1}H NMR Spectra (400 MHz) of **11** in $CDCl_3$



^{13}C NMR Spectra (100 MHz) of **11** in $CDCl_3$