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# Highly selective and controllable synthesis of arylhydroxylamines by the reduction of nitroarenes with an electron-withdrawing group using a new nitroreductase *Ba*NTR1

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Supporting Information

Table of Contents:

## 1. General information

All nitroarenes were purchased from Aladdin Chemicals Co. Ltd. (Shanghai, China) with >97% purity. Other chemicals were of reagent grade or better. *E. coli* DH5α and *E. coli* BL21 (DE3) were used for recombinant vector amplification and recombinant protein expression respectively. Both strains were routinely grown in Luria–Bertani medium at 37°C unless otherwise stated. Kanamycin acid sulphate (50 $\mu g.mL^{-1}$) was used for the selection of recombinant strains. Plasmid pMD18-T for the cloning of PCR products was obtained from TaKaRa (Dalian, China) and plasmid pET-28a (+) for the heterogeneous expression was obtained from Novagen (Shanghai, China). Nitroreductase activity was determined in 1 mL final volume of 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM nitroarene and 0.1 mM NADPH. One unit of the enzyme activity (IU) was defined as the amount of enzyme required to oxidize 1 μmol NADPH per minute at 30°C and pH 7.0, and was measured by following the initial and linear decrease in absorbance at 340 nm.[S1] The conversion of substrates and selectivity of products were determined by HPLC (Shimazu, China) equipped with a reverse-phase C18 column (Inerstill ODS-4, *Φ* 4.6 mm × 250 mm × 5 μm). The samples were eluated by a mobile phase of methanol-water-citric acid: 60/40/1 (v/v/g) in flow rate of 0.8 mL/min and monitored at 254 nm with an UV detector. $^{1}H$ NMR and $^{13}C$ NMR were measured on a Bruker Avance 400 MHz and 500 MHz spectrometer with chemical shifts reported as ppm (using TMS as internal standard).

## 2. Screening, purification and characterization of the nitroreductases

### *2.1 Screening of nitroreductase capable of reducing nitroarenes to arylhydroxylamines*

The genome mining approach was employed to discover the enzyme capable of reducing nitroarenes to corresponding arylhydroxylamines. Two known nitroreductases from *Bacillus licheniformis* DSM 13 (yfkO and NfrA; SwissProt, Q65MG6 and Q65DM9, respectively) were used as probes. A series of putative nitroreductase genes were selected as the candidates from the NCBI database on the basis of the pBLAST searching with the two probes. Twenty four genes were cloned and expressed in *E. coli* BL21

(DE3) (**Table S1**). Among them, the enzyme *Ba*NTR1 from *Bacillus amyloliquefaciens* showed the highest activity towards reducing 4-cyanonitrobenzene to corresponding hydroxylamine (**Fig. S1**). Therefore, the *Bm*NTR1 was chosen as a potential biocatalyst for further studies.

**Table S1. List of genes cloned and expressed in this study.**

| Genbank accession No. | Gene | Source | aa | Primer |
|---|---|---|---|---|
| E1UNN3 | *Ba*NTR1 | *Bacillus amyloliquefaciens* | 258 | CGCGGATCCATGTCAGATA AAAAACACGA<br>CCGCTCGAGTTATACCCAC TTCACAACGT |
| E1UT19 | *Ba*NTR2 | | 221 | CCGGAATTCATGATATATAGAGAAGGAGG<br>CCGCTCGAGTTAATTTTTTTGAAAACCTT |
| D5DEH9 | *Bm*NTR1 | *Bacillus megaterium* | 223 | CGCGGATCCATGAACCATA CAGATAAAAA<br>CCGCTCGAGTTATTTTACC CACTGTACAA |
| D5DW24 | *Bm*NTR2 | | 249 | CCGGAATTCATGAACTCAG TTATTGAAAC<br>CCGCTCGAGTTATTTCTTGTTAAATCCTT |
| B4AGS2 | *Bp*NTR1 | *Bacillus pumilus* | 224 | CGCGGATCCATGCGAGACA AAGAGCAATT<br>CCGCTCGAGTTAACGAATC CAAACGGCTA |
| Q6FX82 | *Cg*NTR1 | *Candida glabrata* | 193 | CCGGAATTCATGAGCTTAC TGGATGATTT<br>CCGCTCGAGTTAGTTGAAG ATTTCGACTT |
| Q6FRQ1 | *Cg*NTR2 | | 205 | CCGGAATTCATGACCAAGC ATTCACCTAC<br>CCGCTCGAGTTACTCATAC ACTTTGACTA |
| Q6CUX4 | *Kl*NTR1 | *Kluyveromyces lactis* | 196 | CCGGAATTCATGTCTGCTG CTGCCACTAA<br>CCGCTCGAGCTAGTTCAAG ATTTTGACAG |
| DAA07458 | *By*NTR1 | Baker's yeast | 193 | CCGGAATTCATGTCTGCTG TTGCAACTTA<br>CCGCTCGAGTTAATTGAAG ATTTCAACAT |
| DAA07459 | *By*NTR2 | | 193 | CCGGAATTCATGTCCCCAA CTGGAAACTA<br>CCGCTCGAGTCAGTGATAA ACGTTGATTA |
| Q88GR1 | *Pp*NTR1 | *Pseudomonas putida* | 224 | CCGGAATTCATGATCGACT GGGCAATTGT<br>CCGCTCGAGTTATTCTGAG TGAAAGGTAG |
| A5W253 | *Pp*NTR2 | | 242 | CCGGAATTCATGCTTATTG ATCGCTTGCG<br>CCGCTCGAGTTTATTCTGAG TGAAAGGTCG |
| Q0K1B9 | *Cn*NTR1 | *Cupriavidus necator* | 227 | CCGGAATTCATGGAGGCAA TCCAGATGAC<br>CCGCTCGAGTCACCGGTCG ACGAACGAGA |
| CAJ93091 | *Cn*NTR2 | | 231 | CCGGAATTCATGGACGAAC TGAAGCGCAT<br>CCGCTCGAGCTAGACCGGC GCGCTGTCAT |
| CAJ95552 | *Cn*NTR3 | | 223 | CGCGGATCCATGAAAGTCA GCCAAGCCGT<br>CCCAAGCTTTCAGATGCCC ATGAACTCCG |
| CAJ92904 | *Cn*NTR4 | | 228 | CCGGAATTCATGAGCCGTA TCGCTATTCC<br>CCGCTCGAGCTATACCAAA TCCAACACAT |
| CAJ92283 | *Cn*NTR5 | | 195 | CCGGAATTCATGTCCCAGA TCGACAACGC<br>CCGCTCGAGTCAGACGATA CGGCAGGCTT |
| ABX32991 | *Da*NTR1 | *Delftia acidovorans* | 212 | CCGGAATTCATGGCCAGGCACCGCGCCAG<br>CCGCTCGAGTTTCAGCCGCCCGTGGCCACGG |
| ABX34950 | *Da*NTR2 | | 220 | CCGGAATTCATGAACGTTG AACAGGCCTT<br>CCGCTCGAGTTTCAGTCGAAG CCGCGCATCT |
| ABX36864 | *Da*NTR3 | | 209 | CCGGAATTCATGAGCACCA ACGACAGATC<br>CCGCTCGAGTTCAGCCTTCG TCGGCCGCGC |
| ABX37233 | *Da*NTR4 | | 241 | CCGGAATTCATGGACTCCG CATTTCCCAT<br>CCGCTCGAGTTCATTCCCCC AGCACTTTGA |
| ADB75839 | *Go*NTR1 | *Geodermatophilus obscurus* | 199 | CGCGGATCCATGGAGTTCG CTGACGTCGT<br>CCCAAGCTTTCACCAGCGT CCGCGGTGGA |

| | | | |
|---|---|---|---|
| ADB77573 | *Go*NTR2 | 222 | CGCGGATCCATGACCGGCA GCGAGTGGCC<br>CCCAAGCTTTCAGGAGTCG ACGCGGGCGG |
| ADB75707 | *Go*NTR3 | 335 | CGCGGATCCATGGACCAGC AGCAGTGGAC<br>CCCAAGCTTTCACCGCGCC GACTCGAACC |

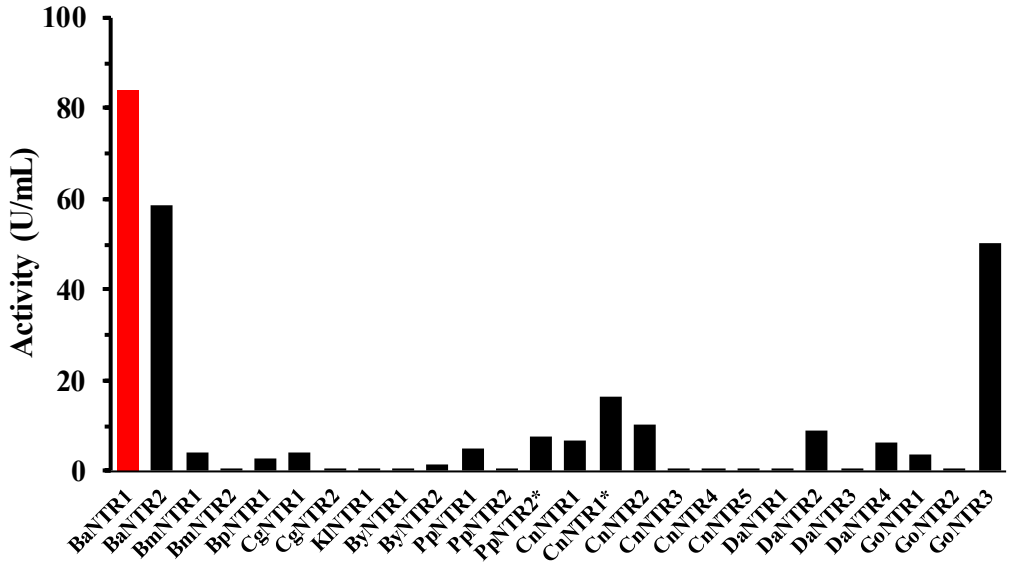



**Fig. S1**. The activities of various putative nitroreductases. The activity was measured under the standard conditions using 4-cyanonitrobenzene as the indicative substrate. The activity (U/mL) indicates that of culture broth; * Expressed in pET-43(a) vector.

*2.2 Purification of nitroreductase BaNTR1*

The recombinant nitroreductase *Ba*NTR1 with an *N*-terminal His-tag was rapidly purified to electrophoretic homogeneity by nickel affinity chromatography. The samples of cell-free extract and purified target protein were analyzed by SDS-PAGE (**Fig. S2**), which revealed that the purified target protein has a molecular size of about 25.7 kDa (**Fig. S2, lane 3**).

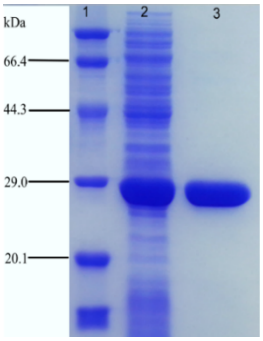
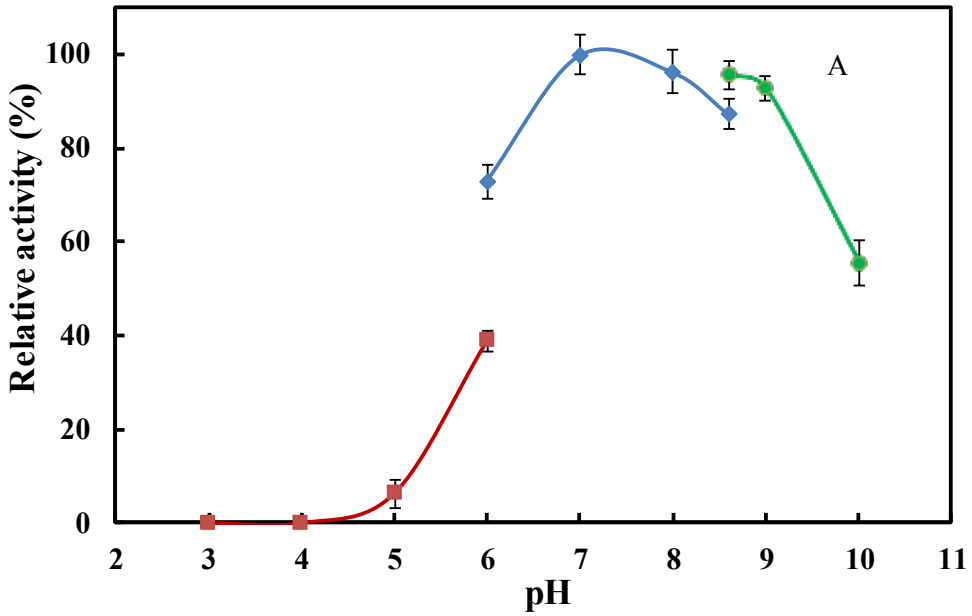



**Fig. S2.** The SDS–PAGE analysis of the purified protein *Ba*NTR1. *Lane 1,* protein markers; *Lane 2,* cell-free extract; *Lane 3,* purified protein.

*2.3 Characterization of the nitroreductase BaNTR1*

The effect of pH on the purified *Ba*NTR1 activity was examined in the range of 3.0–10.0. As shown in **Fig. S3A**, the optimal activity was observed at pH 7.0. The optimum temperature of nitroreductase *Ba*NTR1 was investigated by the measuring the activity of *Ba*NTR1 at different temperatures (**Fig. S3B**). The highest activity was observed at 35 °C.



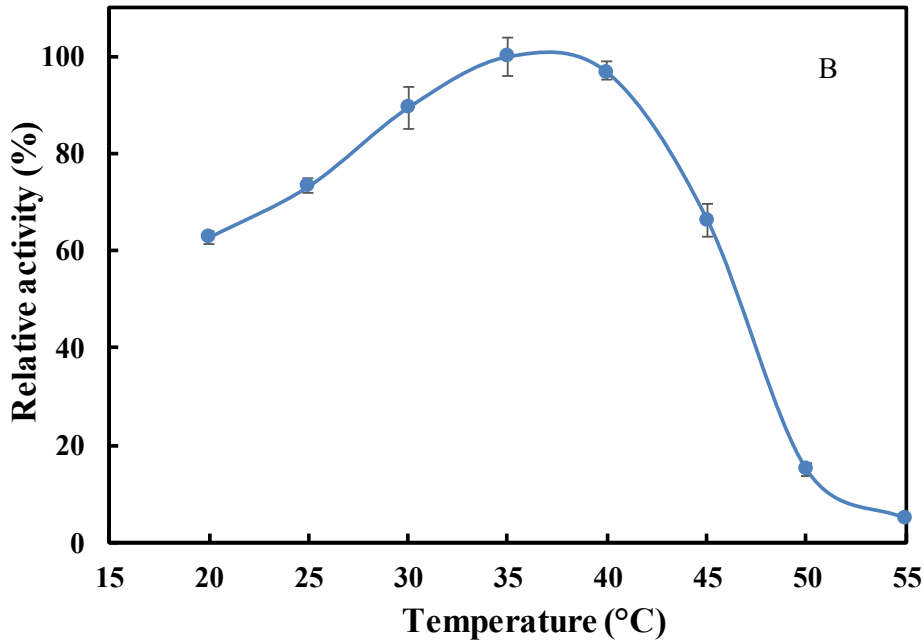



**Fig. S3.** Effects of pH and temperature on activity of the purified nitroreductase *Ba*NTR1. A: The activity was measured using the standard assay procedure in different buffers of 100 mM: *square*: citrate (pH 3.0–6.0), *diamond*: phosphate (pH 6.0–8.5) and *cycle:* Gly–NaOH (pH 8.5–10.0). B: The optimal temperature was measured in sodium phosphate buffer (100 mM, pH 7.0). Relative activity was expressed as a percentage of maximum activity under the experimental conditions.

The thermostability of the purified *Ba*NTR1 was examined at temperatures of 40°C, 50°C and 60°C (**Fig. S4**). The results showed that the nirtroreductase *Ba*NTR1 was relative stable at 40°C and 50°C with half-lives of 64 h and 20 h, respectively. However, higher temperature (60°C) resulted in a rapid loss of nitroreductase activity.

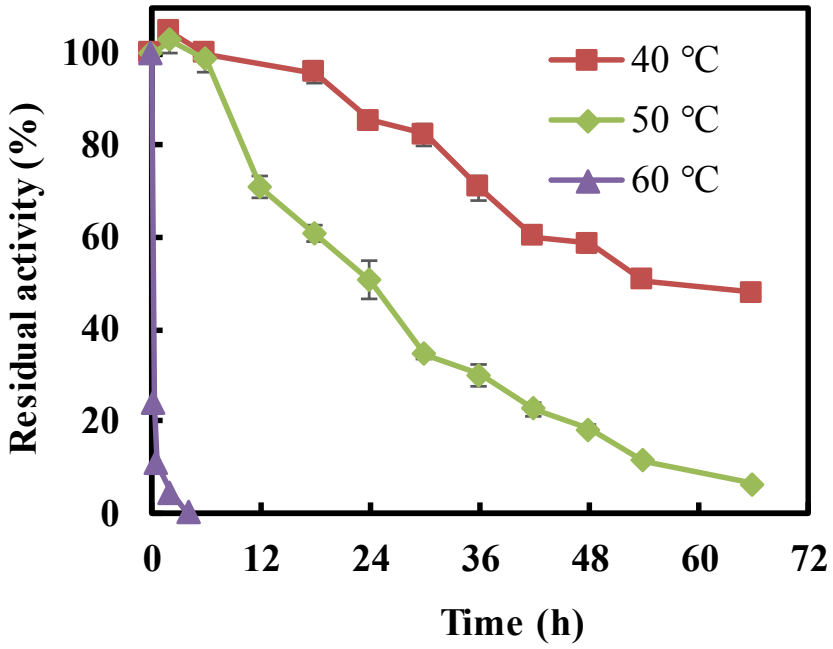



**Fig. S4.** Thermostability of the purified enzyme *Ba*NTR1 at different temperatures. Samples were pre-incubated in sodium phosphate buffer (100 mM, pH 7.0) at different time intervals to determine the residual activity using the standard assay protocol. The residual activity was expresssed as a percentage of the activity measured initially without any pre-incubation.

## 3. General procedures

To prepare the lyophilized enzyme powders, recombinant protein was expressed in *E. coli* BL21 cells. The cultures was grown at 37 °C in 1L LB medium containing 50 μg.mL$^{-1}$ kanamycin acid sulphate.

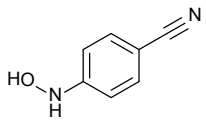
When $OD_{600}$ reached 1.8-2.0, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the cells were incubated at 16 °C for a further period of 24 h. Cells were harvested by centrifugation, washed twice with physiological saline and resuspended in 20 mM sodium phosphate buffer (pH 7.0) and the resultant cell suspension was disrupted by high pressure homogenizer, followed by centrifugation (10,000 × *g* for 20 min) to remove the cell debris. The supernatant was rapidly frozen at -80 °C and finally dried using a vacuum freeze drier.

A typical reaction was performed at 30 °C in a 150-mL reactor using an NADPH regeneration system consisting of crude enzyme powder nitroreductase *Ba*NTR1 and crude enzyme powder glucose dehydrogenase (*Bm*GDH). The reaction mixture of totally 100 mL was composed of 100 mM substrate (nitroarenes), 100 mg of nitroreductase, 0.3 mM $NADP^+$, 400 mM glucose and 100 mg of *Bm*GDH in 0.1 M sodium phosphate buffer (pH 7.0). The reaction mixture was stirred by magnetic agitation and the pH was automatically adjusted to 7.0 by titrating 1 M NaOH solution.

When the reaction was terminated, the reaction mixture was extracted three times with equal volume of ethyl acetate. The separated organic phase was combined and dried over anhydrous $MgSO_4$. The crude product was recovered by removal of solvent under reduced pressure and further purified by silica gel column chromatography using dichloromethane-acetone as an eluent. The product was characterized by $^1$H NMR and $^{13}$C NMR.

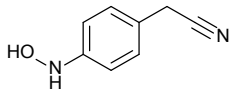
## 4. $^1$H NMR and $^{13}$C NMR spectra

**_N_-(4-Cyanophenyl)hydroxylamine**



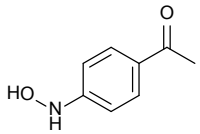

Yield: 1.09 g (82%), pale yellow solid. $^1$H NMR (400 MHz, $(CD_3)_2CO$) δ = 8.48 (s, 1H), 8.09 (s, 1H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H); $^{13}$C NMR (100 MHz, $(CD_3)_2CO$) δ 155.51, 133.01, 119.50, 112.64, 101.53.

**_N_-(4-(Cyanomethanyl)phenyl)hydroxylamine**



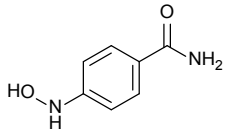
Yield: 0.72 g (49%), pale yellow solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.36 (brs, 2H), 7.14 (d, $J$ = 8.5 Hz, 2H), 6.85 (d, $J$ = 8.5 Hz, 2H), 3.86 (s, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 152.02, 128.75, 121.73, 120.19, 113.73, 22.12.

**_N_-(4-Acetylphenyl)hydroxylamine**



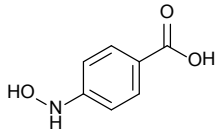
Yield: 0.97 g (65%), pale yellow solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.01 (s, 1H), 8.69 (s, 1H), 7.80 (d, $J$ = 8.6 Hz, 2H), 6.83 (d, $J$ = 8.6 Hz, 2H), 2.45 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 196.11, 156.38, 130.30, 128.15, 111.37, 26.56.

**_N_-(4-Aminocarbonylphenyl)hydroxylamine**



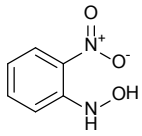
Yield: 0.87 g (58%), pale yellow solid. $^{1}$H NMR (500 MHz, DMSO-$d_6$) δ 8.67 (s, 1H), 8.51 (s, 1H), 7.72 (d, $J$ = 8.5 Hz, 2H ), 7.69 (brs, 1H), 7.02 (brs, 1H), 6.81 (d, $J$ = 8.5 Hz, 2H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 167.89, 154.52, 128.46, 124.48, 111.33.

**_N_-(4-Hydroxycarbonylphenyl)hydroxylamine**



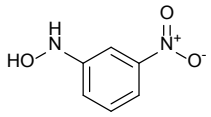
Yield: 0.78 g (51%), pale yellow solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 12.32 (brs, 1H), 8.88 (s, 1H), 8.60 (s, 1H), 7.75 (d, $J$ = 8.7 Hz, 2H), 6.82 (d, $J$ = 8.7 Hz, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 167.82, 156.22, 131.03, 120.82, 111.67.

**_N_-(2-Nitrophenyl)hydroxylamine**



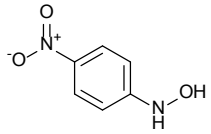
Yield: 0.54 g (35%), reddish brown solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.96 (s, 1H), 9.38 (s, 1H), 8.06 (d, $J$ = 8.5 Hz, 1H), 7.62 (t, $J$ = 7.7 Hz, 1H ), 7.47 (d, $J$ = 8.5 Hz, 1H), 6.83 (t, $J$ = 7.7 Hz, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 147.69, 136.69, 130.95, 125.84, 117.62, 115.24.

**_N_-(3-Nitrophenyl)hydroxylamine**



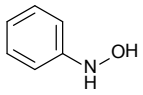
Yield: 0.65 g (42%), reddish brown solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 8.87 (s, 1H), 8.73 (d, $J$ = 1.8 Hz, 1H), 7.62 (s, 1H), 7.57 (d, $J$ = 8.0 Hz, 1H), 7.43 (t, $J$ = 8.1 Hz, 1H), 7.19 (d, $J$ = 8.0 Hz, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 153.77, 148.90, 130.23, 119.33, 113.87, 106.68.

**_N_-(4-Nitrophenyl)hydroxylamine**



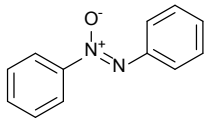
Yield: 0.89 g (57%), brown solid. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.66 (s, 1H), 9.08 (s, 1H), 8.06 (d, $J$ = 9.2 Hz, 2H), 6.81 (d, $J$ = 9.2 Hz, 2H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 157.26, 138.16, 126.21, 110.47.

**_N_-Phenylhydroxylamine**



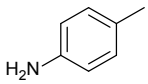
Yield: 0. 65 g (60%), pale yellow solid. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.30 (t, *J* = 7.9 Hz, 2H), 7.02 (m, 3H), 6.68 (brs, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 149.28, 129.07, 122.79, 115.16.

**1, 2-Diphenyldiazene oxide**



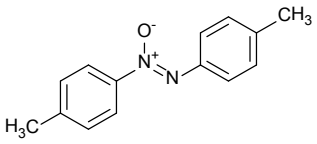
$^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, *J* = 7.5 Hz, 2H), 8.19 (d, *J* = 7.5 Hz, 2H), 7.63 – 7.43 (m, 5H), 7.38 (t, *J* = 7.4 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 148.38, 144.04, 131.64, 129.67, 128.85, 128.76, 125.59, 122.39.

**4-Aminotoluene**



1H NMR (400 MHz, CDCl3) δ 7.00 (d, *J* = 8.0 Hz, 2H), 6.64 (d, *J* = 8.3 Hz, 2H), 3.41 (s, 2H), 2.28 (s, 3H).

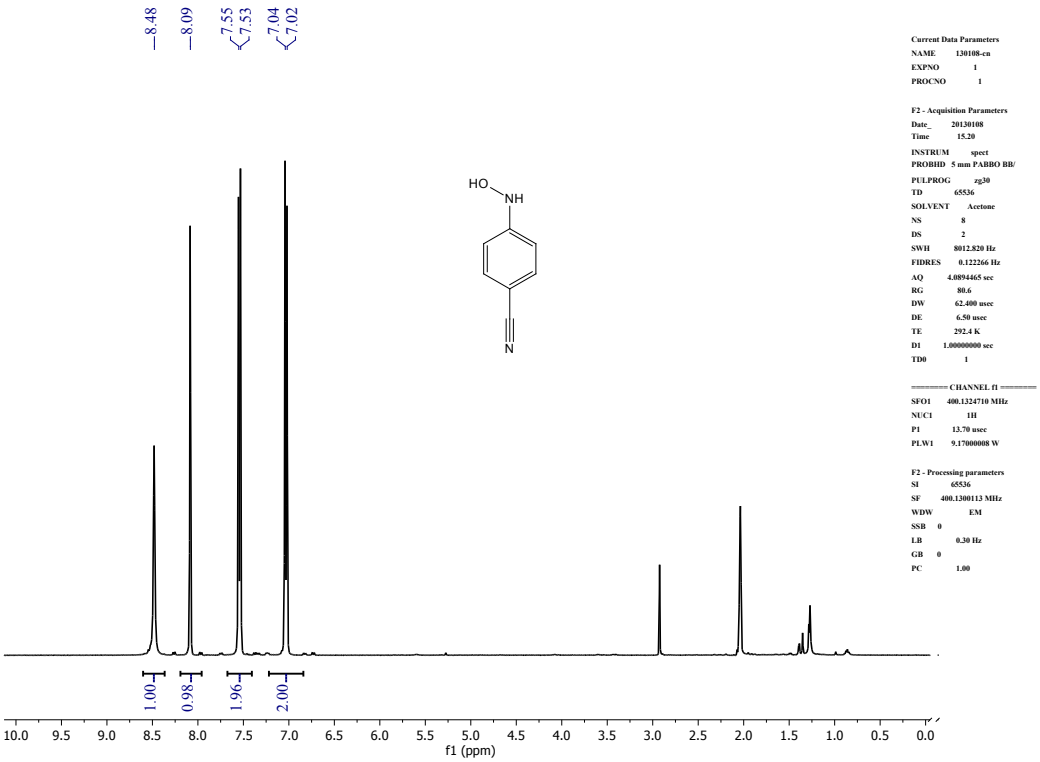
**1, 2-Bis(4-methylphenyl)diazene oxide**



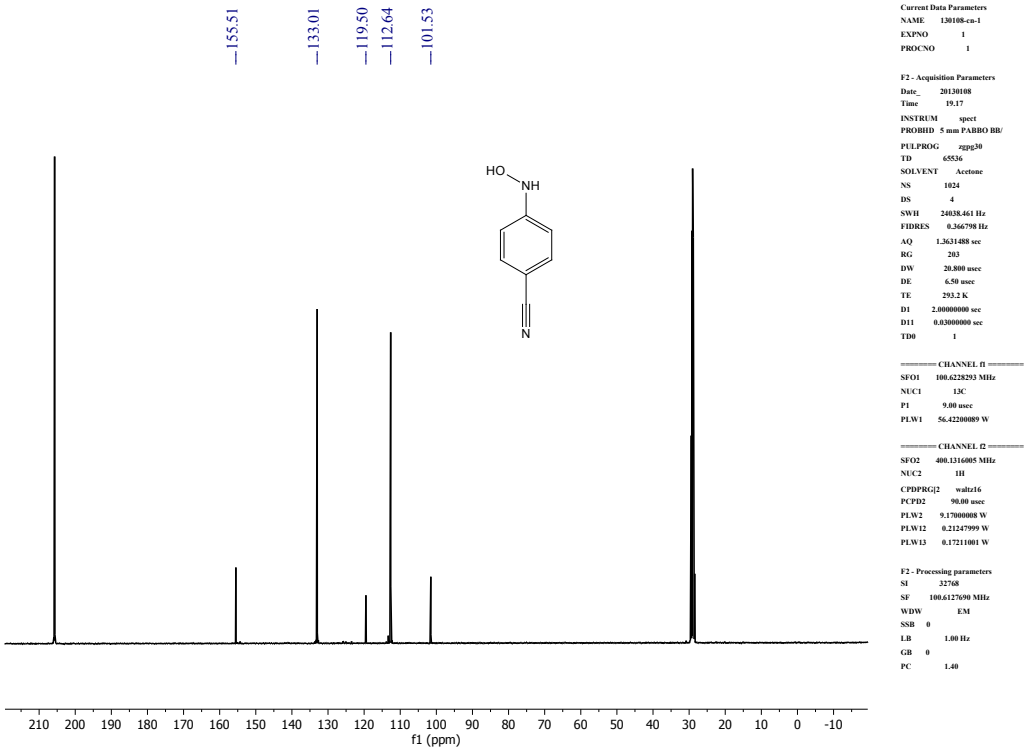
$^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, *J* = 8.5 Hz, 2H), 8.13 (d, *J* = 8.5 Hz, 2H), 7.29 (d, *J* = 8.6 Hz, 4H), 2.44 (s, 3H), 2.42 (s, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 141.92, 141.84, 140.01, 129.69, 129.29, 125.65, 122.71, 122.13, 21.55, 21.29.

## 5. References

S1. H. Y. Kim and H. G. Song, *Appl Microbiol Biotechnol*, 2005, **68**, 766-773.




$^{1}$H NMR spectra of *N*-(4-cyanophenyl)hydroxylamine

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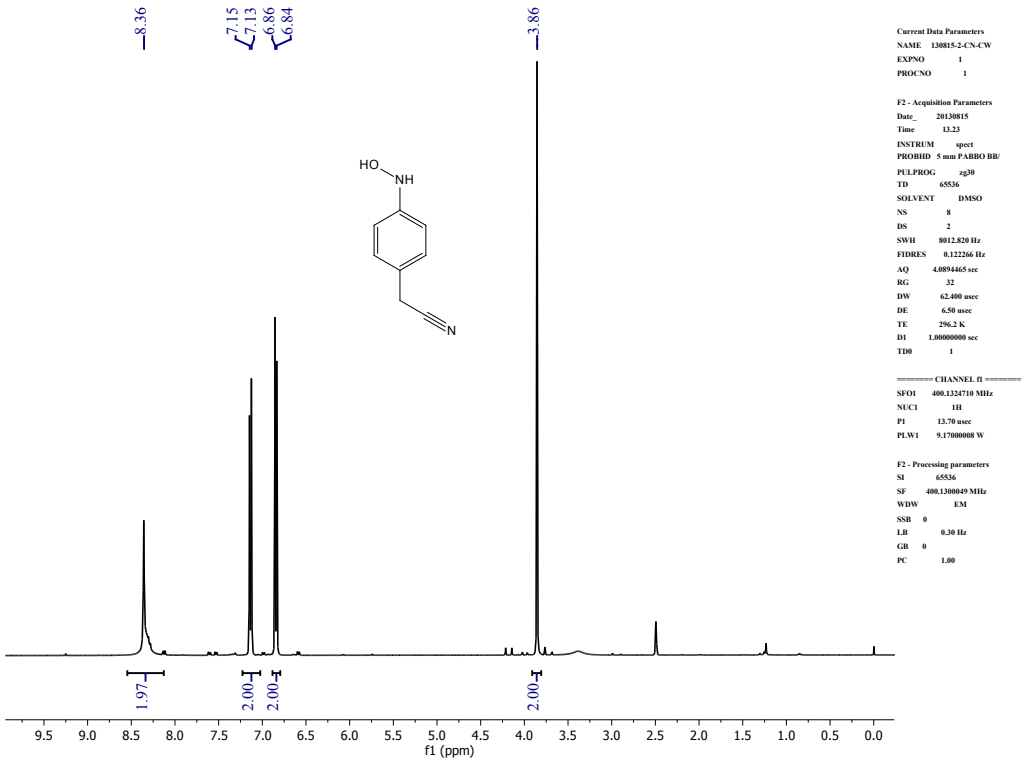
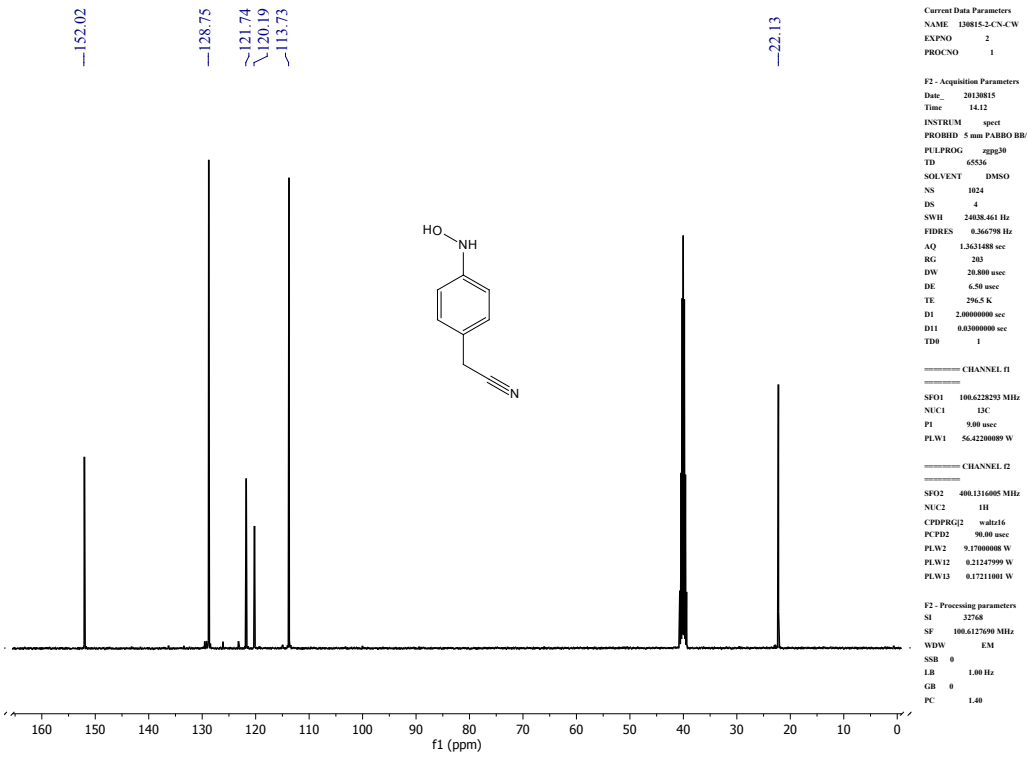
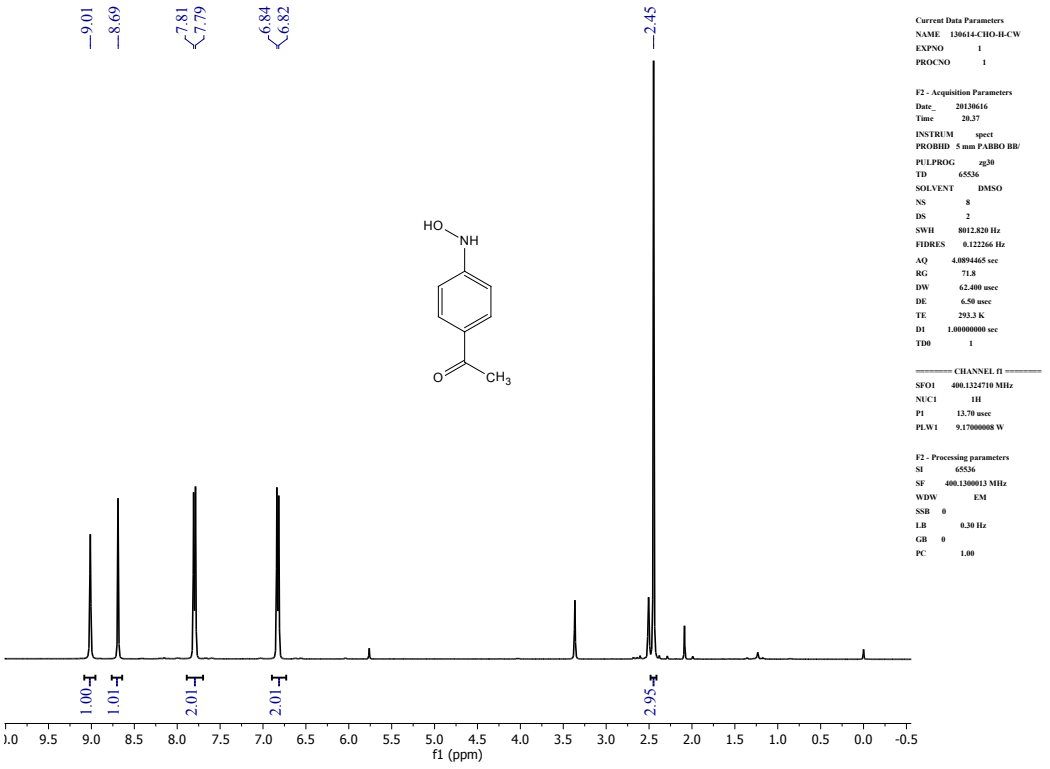


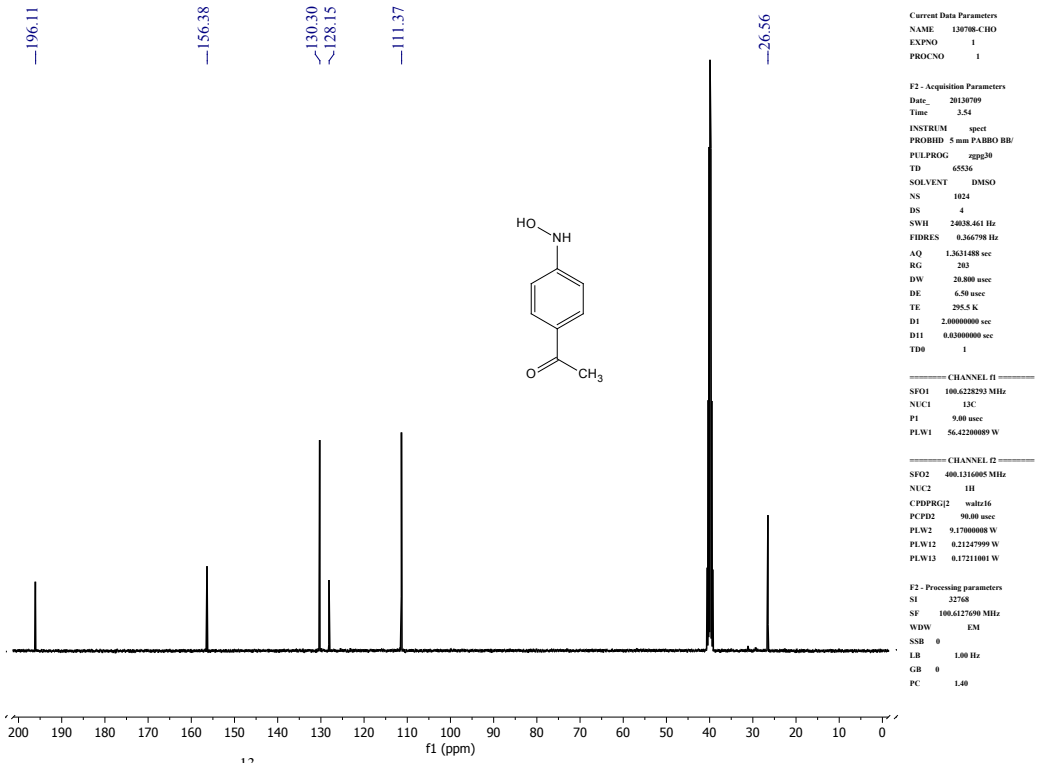

$^{1}$H NMR spectra of *N*-(4-(cyanomethanyl)phenyl)hydroxylamine



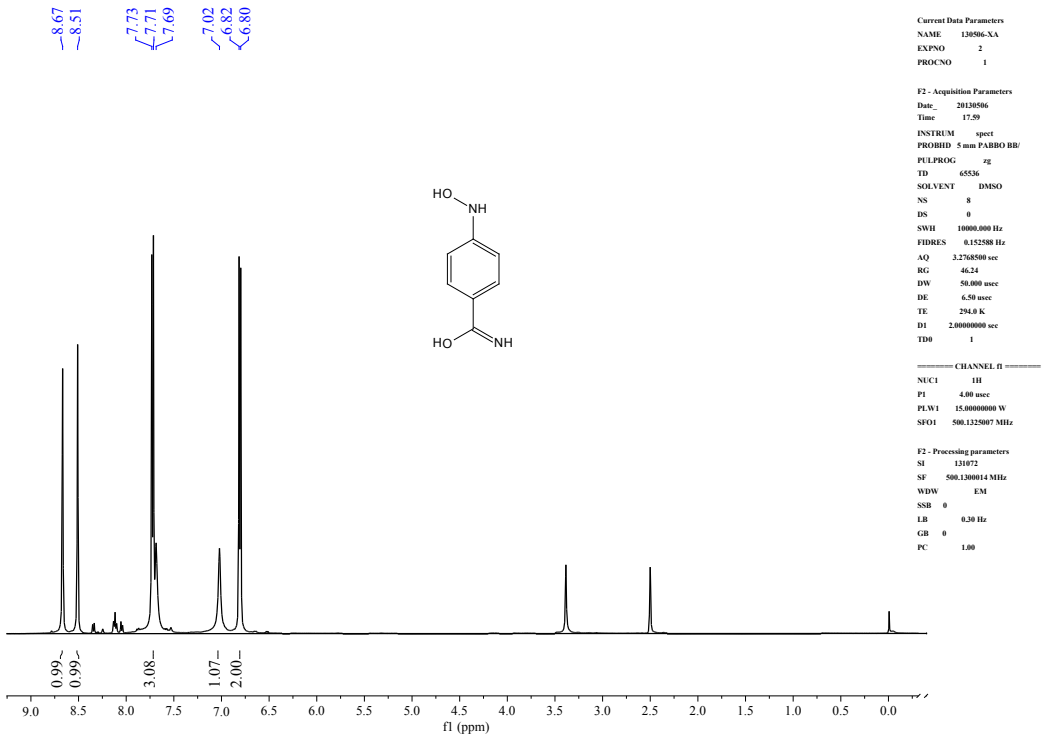

$^{13}$C NMR spectra of *N*-(4-(cyanomethanyl)phenyl)hydroxylamine



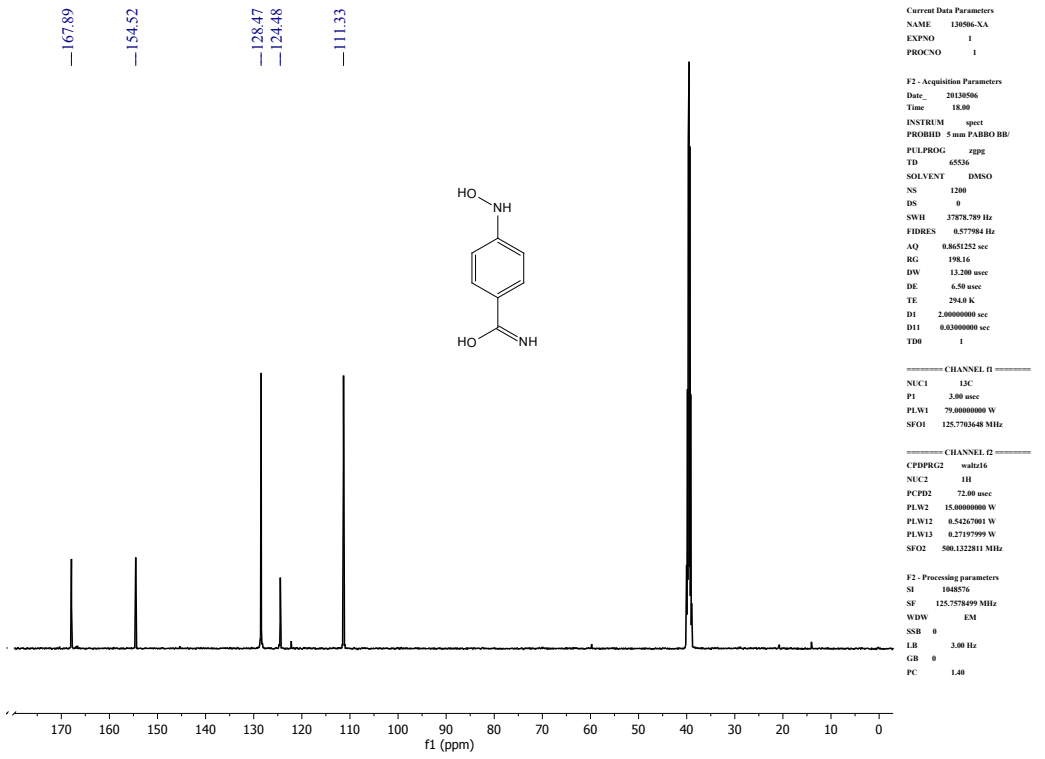

$^{1}$H NMR spectra of *N*-(4-acetylphenyl)hydroxylamine



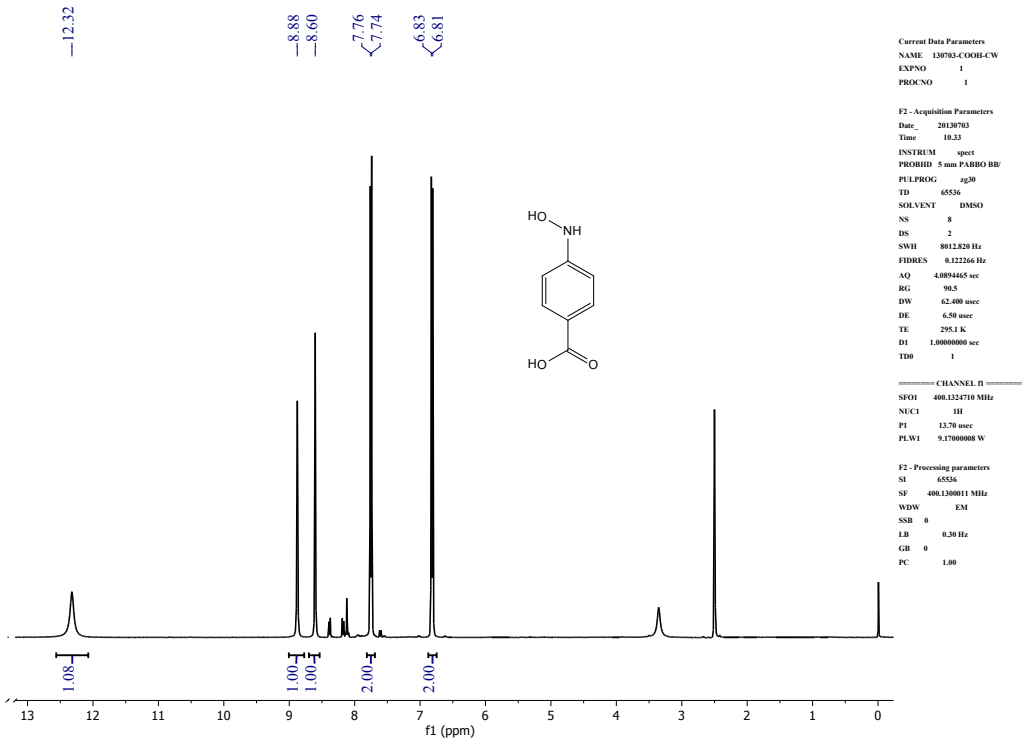

$^{13}$C NMR spectra of *N*-(4-acetylphenyl)hydroxylamine



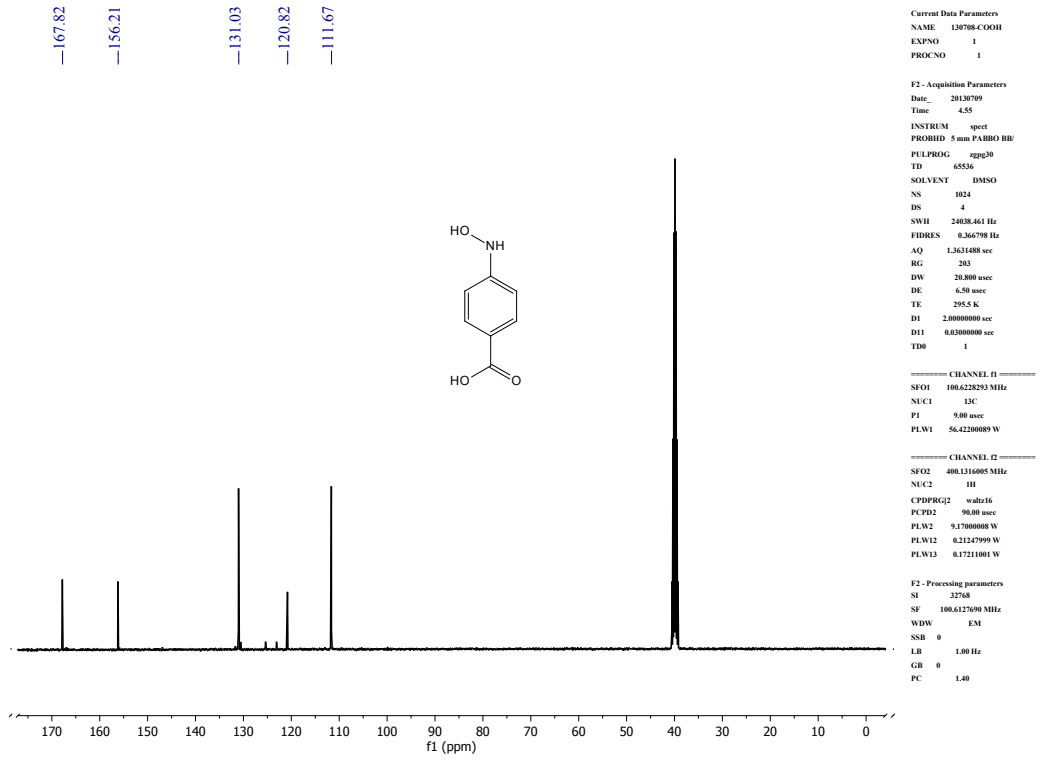

$^{1}$H NMR spectra of *N*-(4-aminocarbonylphenyl)hydroxylamine



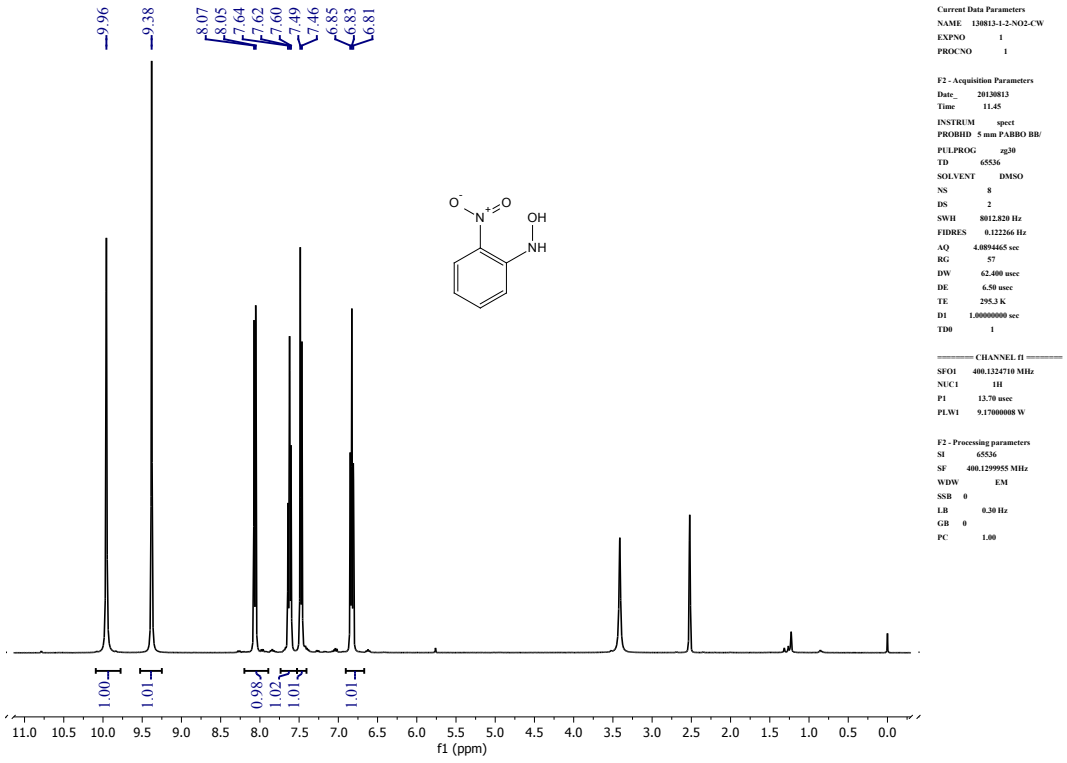

$^{13}$C NMR spectra of *N*-(4-aminocarbonylphenyl)hydroxylamine



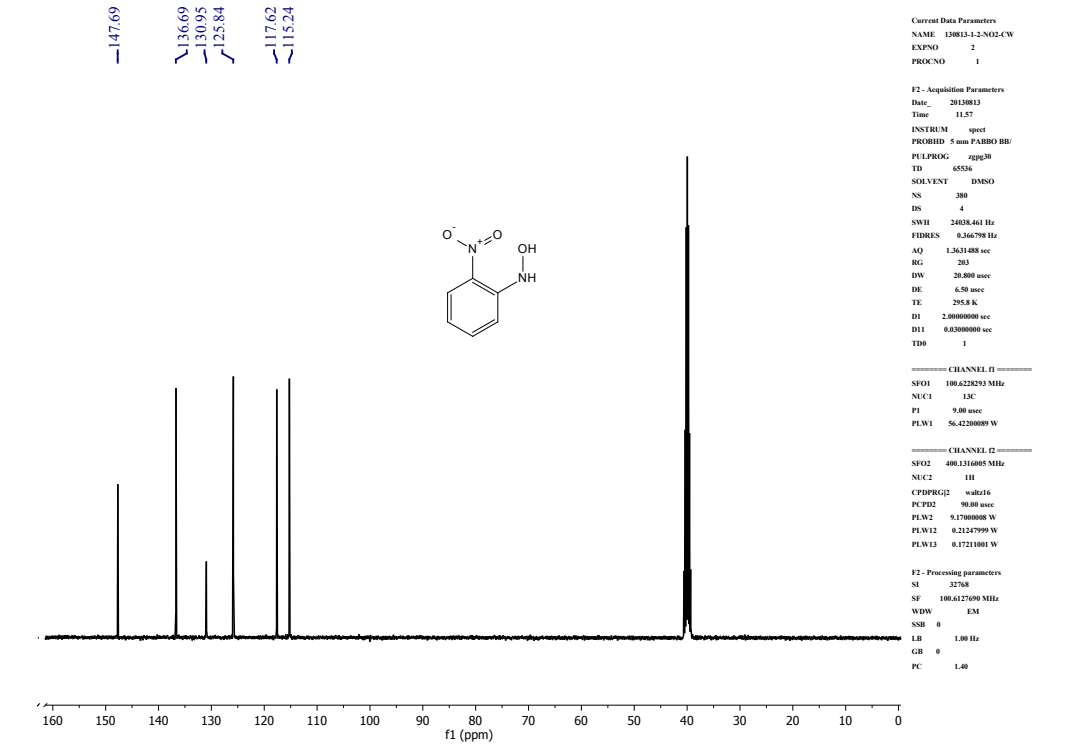

$^{1}$H NMR spectra of *N*-(4-hydroxycarbonylphenyl)hydroxylamine



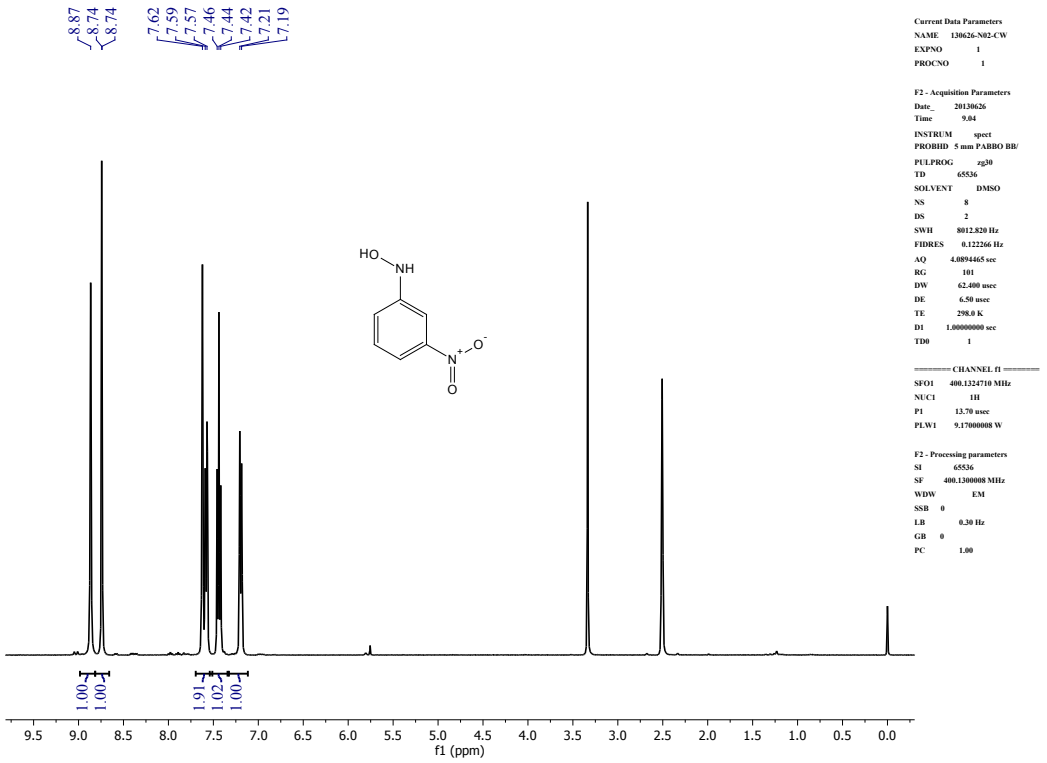

$^{13}$C NMR spectra of *N*-(4-hydroxycarbonylphenyl)hydroxylamine



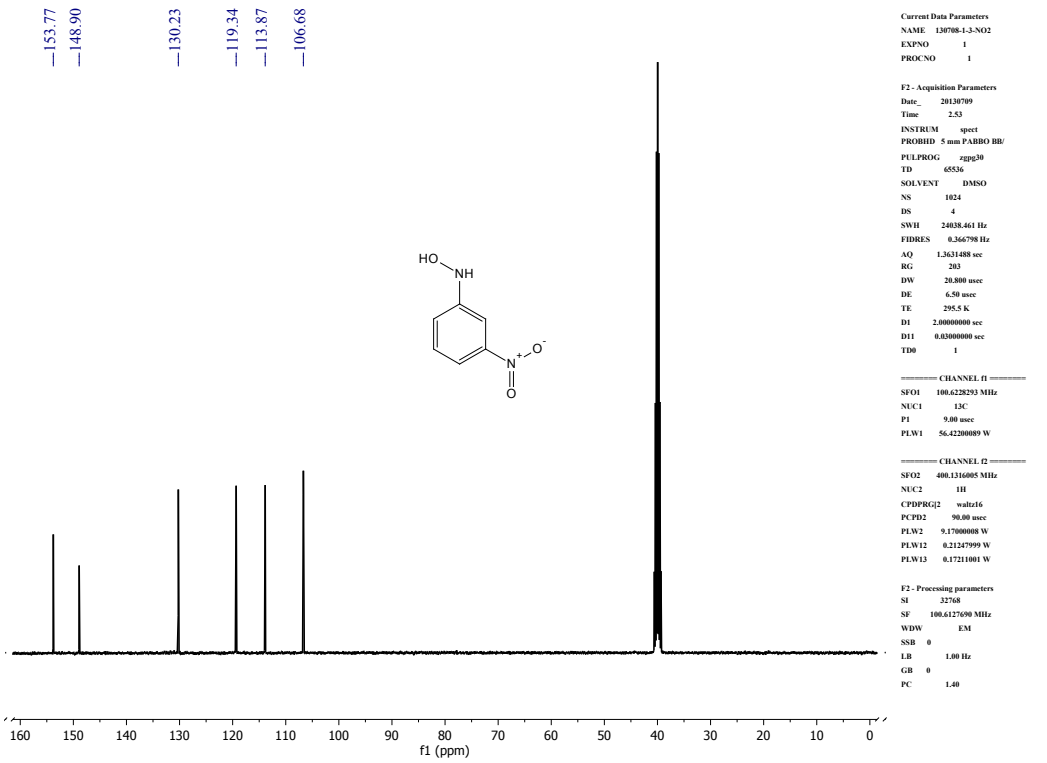

$^{1}$H NMR spectra of *N*-(2-nitrophenyl)hydroxylamine



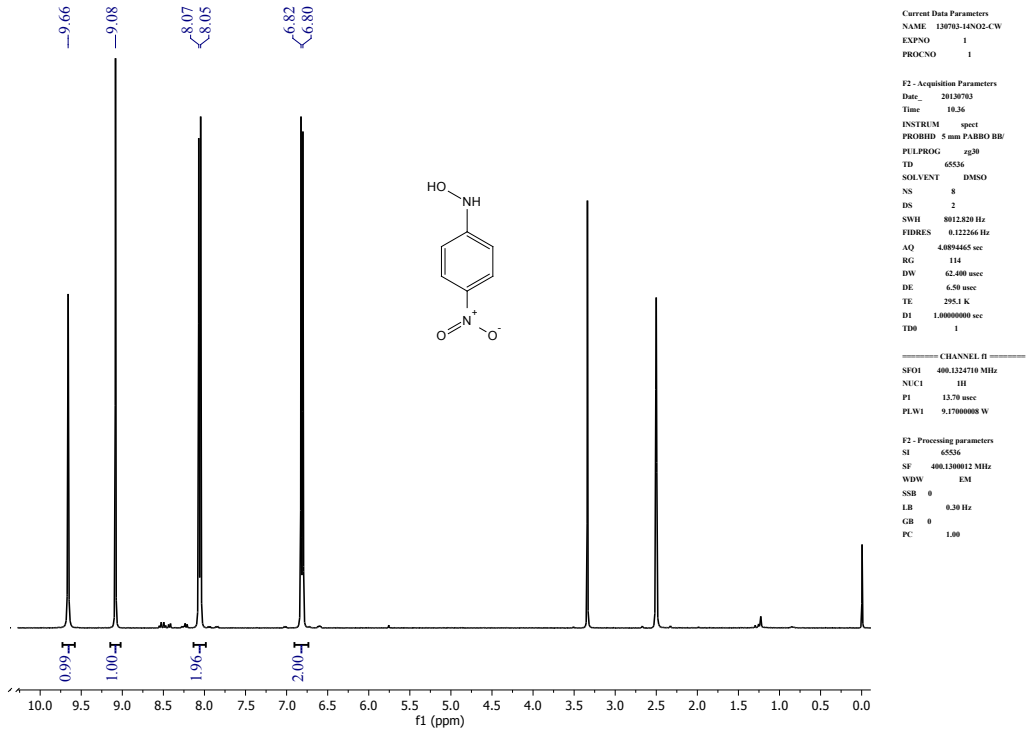

$^{13}$C NMR spectra of *N*-(2-nitrophenyl)hydroxylamine



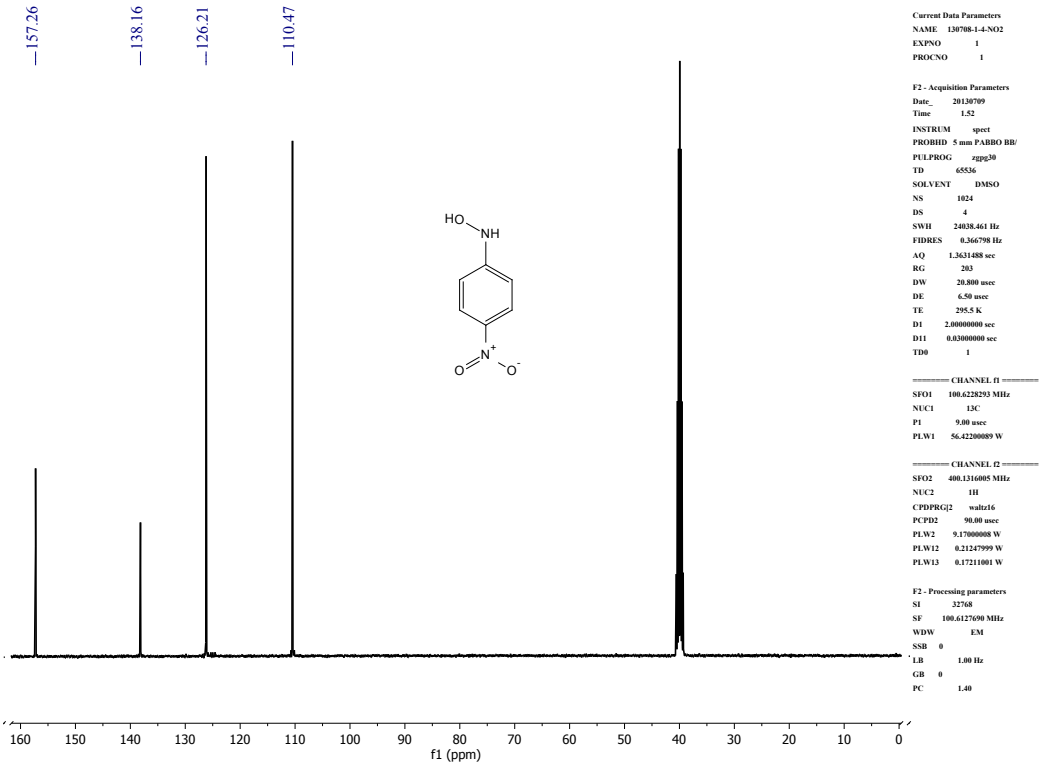

$^{1}$H NMR spectra of *N*-(3-nitrophenyl)hydroxylamine



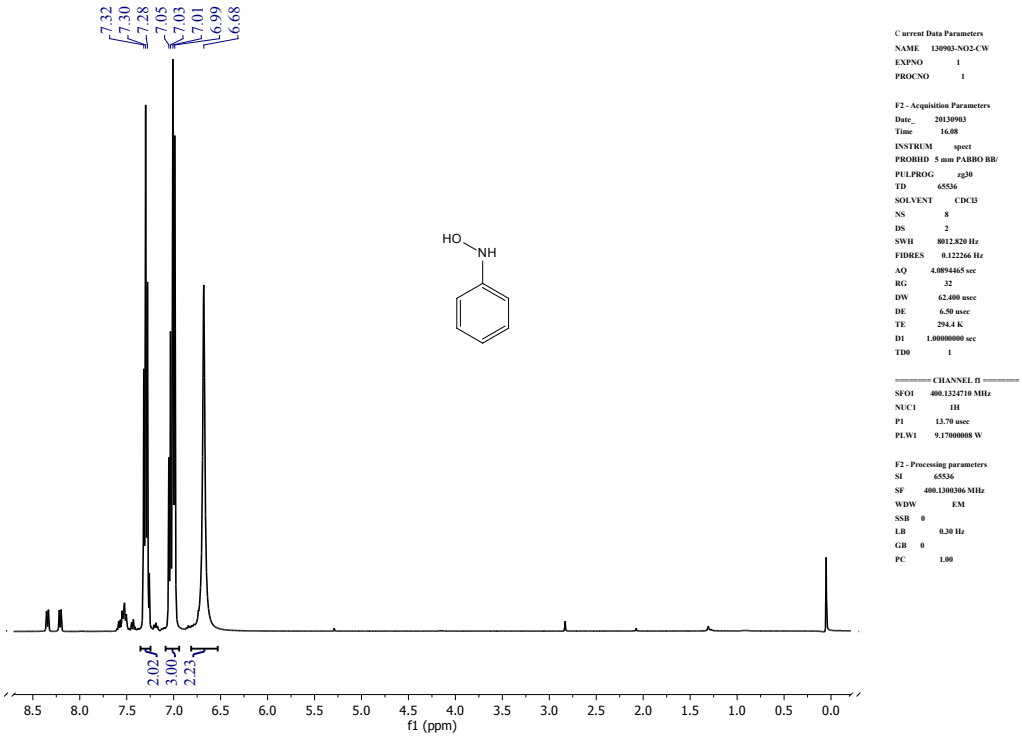

$^{13}$C NMR spectra of *N*-(3-nitrophenyl)hydroxylamine



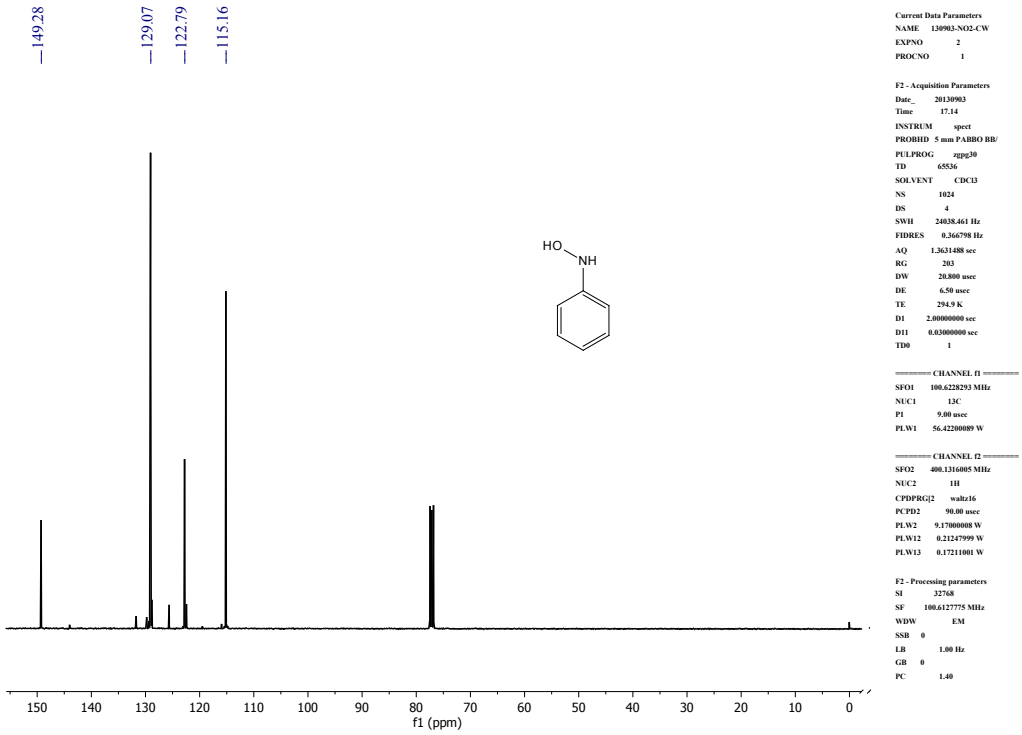

$^{1}$H NMR spectra of *N*-(4-nitrophenyl)hydroxylamine



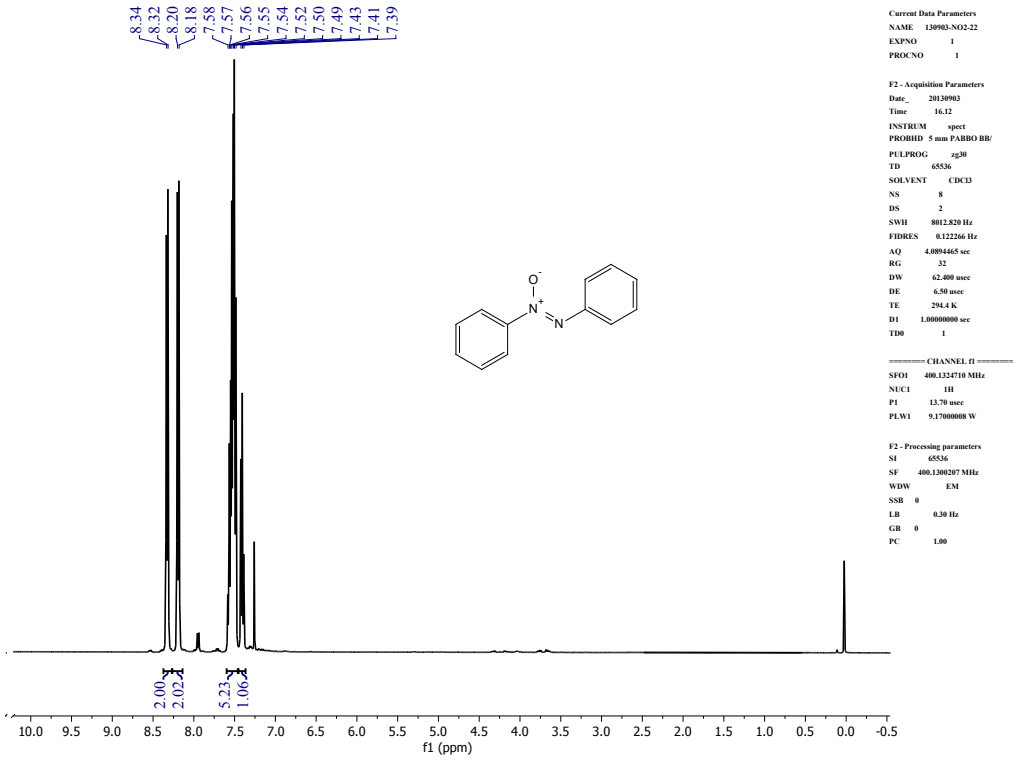

$^{13}$C NMR spectra of *N*-(4-nitrophenyl)hydroxylamine



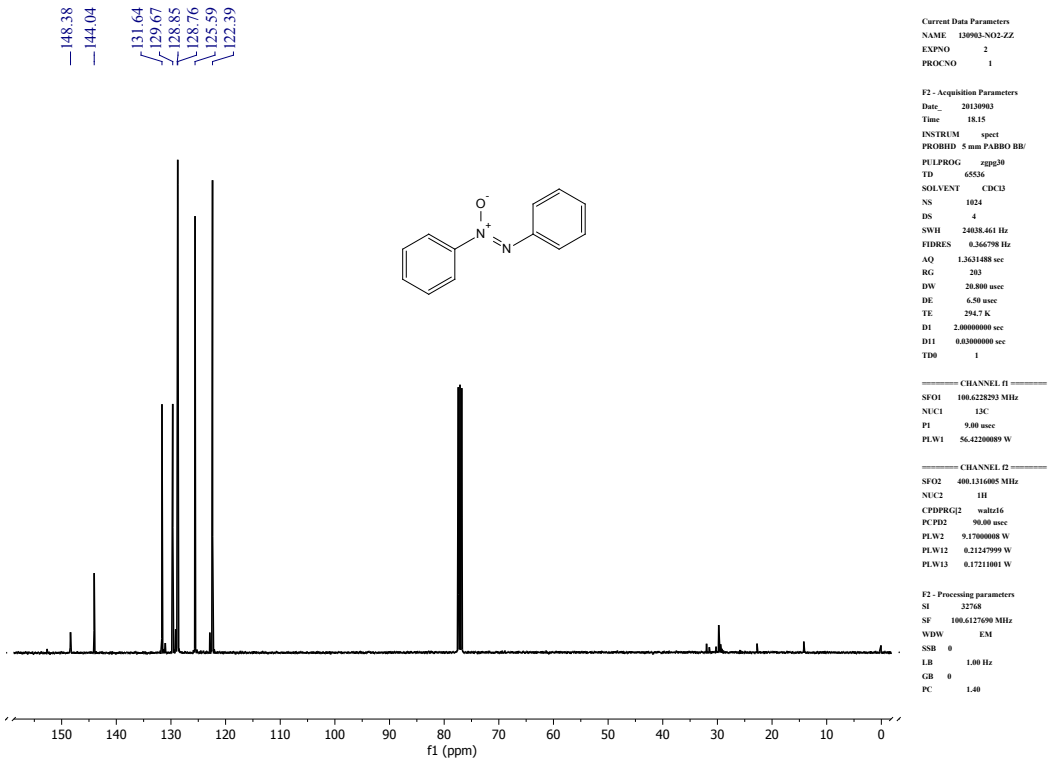

$^{1}$H NMR spectra of *N*-phenylhydroxylamine



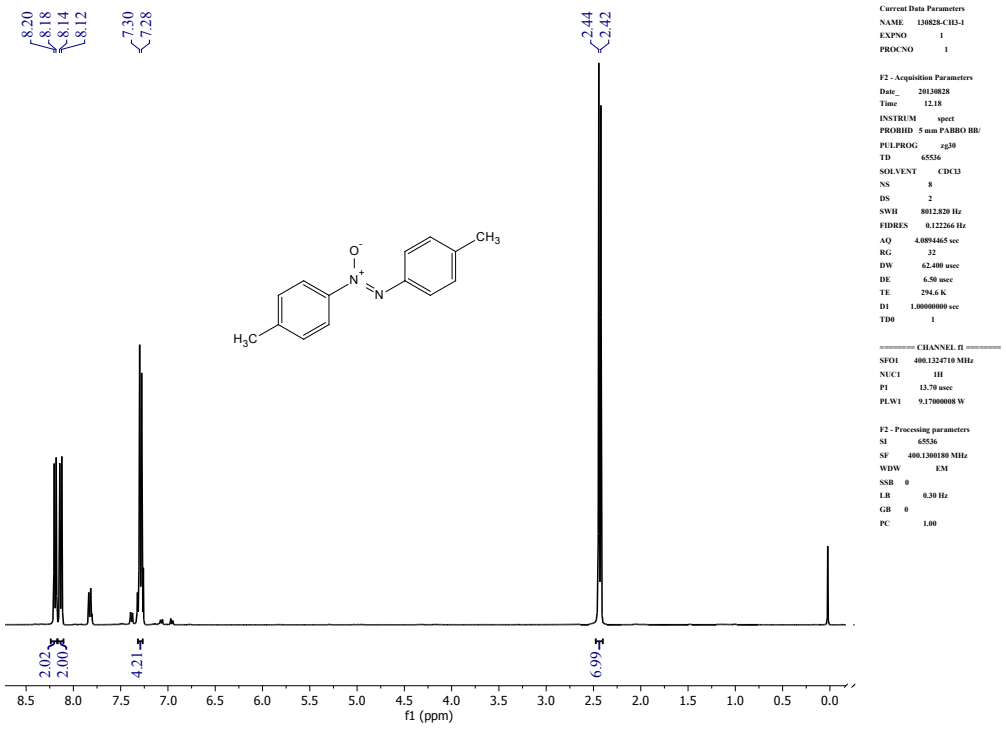

$^{13}$C NMR spectra of *N*-phenylhydroxylamine



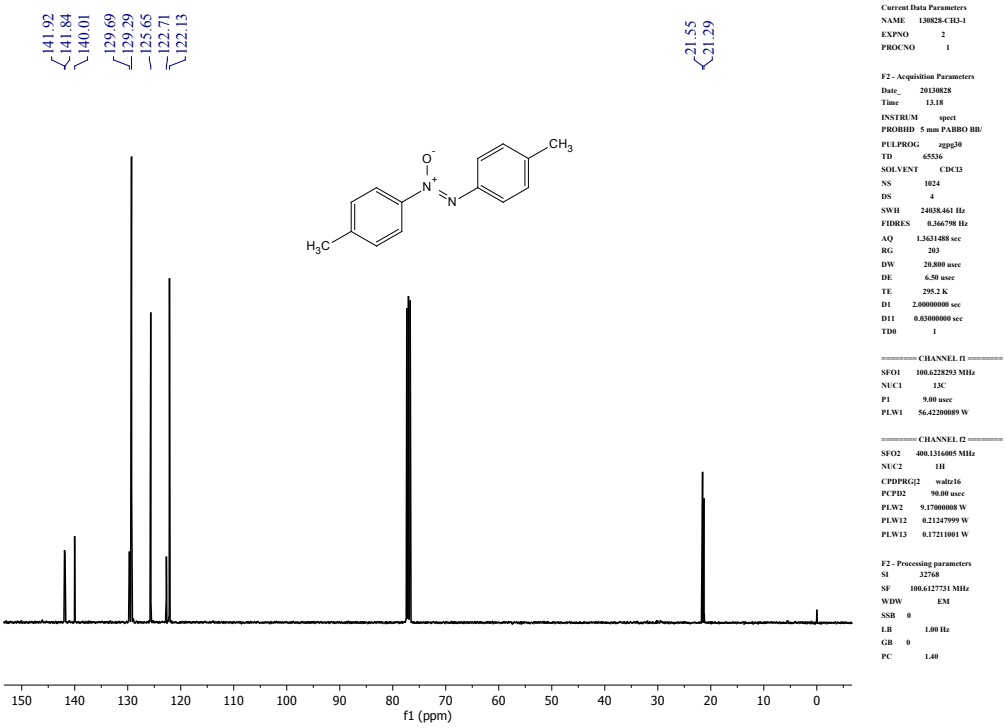

$^{1}$H NMR spectra of 1,2-diphenyldiazene oxide



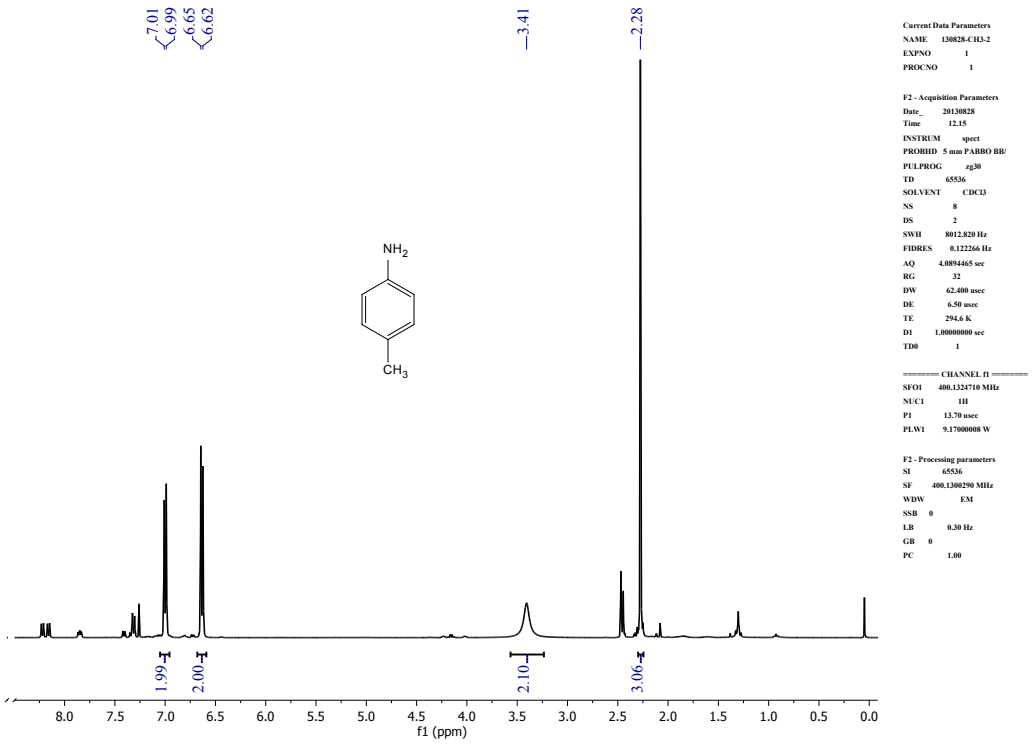

$^{13}$C NMR spectra of 1,2-diphenyldiazene oxide




$^{1}$H NMR spectra of 1, 2-bis(4-methylphenyl)diazene oxide




$^{13}$C NMR spectra of 1, 2-bis(4-methylphenyl)diazene oxide




$^{1}$H NMR spectra of 4-aminotoluene