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Synthesis of new *p*-quinone methide containing morpholine fragment: access to (diarylmethyl)phosphonamidates with antitumor activity

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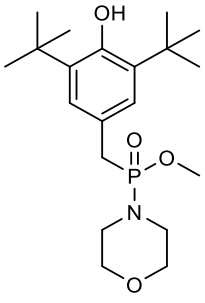
General

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on an Avance-500, Avance-400 high-resolution pulse NMR spectrometer (Bruker). The instrument was tuned to frequencies of 500.13 (^{1}H), 202.46 (^{31}P), and 125.76 MHz (^{13}C) of 399.93 (^{1}H), 161.90 (^{31}P), and 100.57 MHz (^{13}C) to provide resonance conditions. The ^{1}H and ^{13}C chemical shifts were measured relative to residual signals of the solvent (DMSO-d_6, acetone- d_6, $CDCl_3$), and the chemical shift of phosphorus was measured relative to the signal of the standard (H_3PO_4,). Matrix-activated laser desorption/ionization (MALDI) mass spectra were detected on a MALDITOF/TOF time-of-flight mass spectrometer (Bruker Daltonics). IR spectra were recorded on a Vector 22 FT-IR spectrometer (Bruker) in a range of 400—4000 cm^{-1}. Crystalline samples were studied in KBr pellets. Elemental analyses were carried out on a Carlo-Erba EA 1108 instrument.

O-Methyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)phosphonochloridate **1** was synthesized by the procedure[S1] from *O,O*-dimethyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)phosphonate.

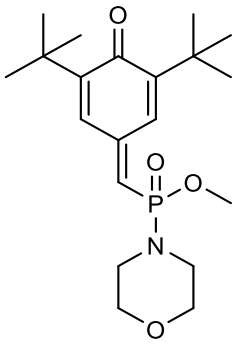
Synthetic procedures and compound characterization data



Methyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)(morpholino)phosphinate 2.

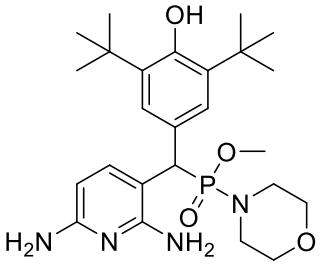
To a solution of *O*-methyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)phosphonochloridate **1** (0.2 g, 6.02 mmol) in dioxane (5 ml) morpholine (1.05 ml, 12.05 mmol) was added. The mixture was kept at room temperature until precipitation of salt which was filtered off. Dioxane was removed, an orange product **2** was obtained and dried in a vacuum (1 h, 20°C, 1 Torr). Yield 91%, m.p. 113-117ºC. IR (KBr), $см^{-1}$: 773 (P–C); 951 (P–N); 1032, 1049 (P–O–C_{alk}); 1115 (C–O–C); 1229 (P=O); 1598 (C=C_{arom}); 3628 (OH). ^{1}H NMR (DMSO-d_6, 500.13 MHz), δ, ppm: 1.38 (s, 18H, $C(CH_3)_3$), 2.87 – 2.91 (m, 4H, $NC\underline{H}_2CH_2O$), 3.02 (d, 2H, CHP, $^{2}J_{PH}$ = 24.4), 3.33 – 3.38 (m, 4H, $NCH_2C\underline{H}_2O$), 3.52 (d, OCH_3, $^{3}J_{PH}$ = 11.0),

6.80 (s, 1H, OH), 7.03 (d, 2H, C<u>H</u>CC(CH$_3$)$_3$, $^4J_{HP}$ = 2.2). ^{13}C{^{1}H} NMR (DMSO-*d*$_6$, 125.76 MHz), *δ*, ppm: 30.91 (C(<u>C</u>H$_3$)$_3$), 32.57 (d, CHP, $^1J_{PC}$ = 126.8), 34.95 (<u>C</u>(CH$_3$)$_3$), 44.15 (NCH$_2$<u>C</u>H$_2$O), 50.31 (d, OCH$_3$, $^2J_{PC}$ = 7.1), 66.81 (d, N<u>C</u>H$_2$CH$_2$O, $^2J_{PC}$ = 5.1), 123.85 (d, <u>C</u>HCC(CH$_3$)$_3$, $^2J_{PC}$ = 7.7), 126.47 (d, <u>C</u>HCC(CH$_3$)$_3$, $^3J_{PC}$ = 6.5), 139.61 (<u>C</u$_{arom}$C(CH$_3$)$_3$), 152.85 (COH). ^{31}P NMR (DMSO-*d*$_6$, 202.46 MHz), *δ*, ppm: 31.1. Anal. Calcd for Calc. for $C_{20}H_{34}NO_4P$ (%): C, 62.95; H, 8.67; N, 3.66; P, 8.08. $C_{20}H_{34}NO_4P$. Found (%): C, 62.64; H, 8.84; N, 3.65; P, 7.89. HRMS (MALDI-TOF) m/z for $C_{20}H_{34}NO_4P$: calc. 383.4 [M], found 383.3 $[M]^+$; 406.3 $[M+Na]^+$.

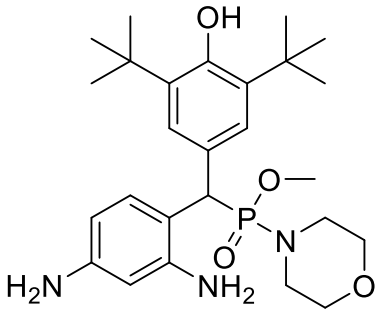


Methyl [(3,5-di-*tert*-butyl-4-oxocyclohexa-2,5-dien-1-ylidene)methyl]-(morpholino)phosphinate 3. To a solution of compound **2** (2 g, 5.0 mmol) in benzene (50 ml) was added a solution of $K_3[Fe(CN)]_6$ (6 g, 18 mmol) in a solution KOH (2N, 40 ml). The reaction mixture was stirred at room temperature for 5 h. The colored benzene solution was separated and washed until neutral. Benzene was removed to leave an orange-brown product **3** which was dried in a vacuum (2 h, 20°C, 1 mm Torr). Yield 95%, m.p. 96-98ºC. IR (KBr), см$^{-1}$: 757 (P–C); 987 (P–N); 1025, 1053 (P–O–C$_{alk}$); 1120 (C–O–C); 1126 (P=O); 1600 (C=C$_{arom}$); 1710 (C=O). ^{1}H NMR (DMSO-*d*$_6$, 500.13 MHz), *δ*, ppm: 1.24 (s, 18H, C(CH$_3$)$_3$), 3.07 (m, 4H, NC<u>H</u>$_2$CH$_2$O), 3.56 (t, 4H, NCH$_2$C<u>H</u>$_2$O, $^3J_{HH}$ = 4.6), 3.63 (d, OCH$_3$, $^3J_{PH}$ = 11.4), 6.67 (d, 1H, CHP, $^2J_{PH}$ = 14.8), 7.10 (s, 1H, C<u>H</u>CC(CH$_3$)$_3$), 8.00 (s, 1H, C<u>H</u>CC(CH$_3$)$_3$). ^{13}C{^{1}H} NMR (DMSO-*d*$_6$, 125.76 MHz), *δ*, ppm: 29.70 (C(<u>C</u>H$_3$)$_3$), 35.33, 35.56 (<u>C</u>(CH$_3$)$_3$), 43.86 (NCH$_2$<u>C</u>H$_2$O), 50.82 (d, OCH$_3$, $^2J_{PC}$ = 5.9), 66.86 (d, N<u>C</u>H$_2$CH$_2$O, $^2J_{PC}$ = 5.1), 129.04, 135.02 (d, <u>C</u>HCC(CH$_3$)$_3$, $^3J_{PC}$ = 7.5), 130.86 (d, CHP, $^1J_{PC}$ = 219.8), 143.32 (d, <u>C</u>HCC(CH$_3$)$_3$, $^2J_{PC}$ = 5.5), 149.42, 149.56 (<u>C</u$_{arom}$C(CH$_3$)$_3$), 186.37 (C=O). ^{31}P NMR (DMSO-*d*$_6$, 202.46 MHz), *δ*, ppm: 17.7. Anal. Calcd for Calc. for $C_{20}H_{32}NO_4P$ (%): C, 62.98; H, 8.46; N, 3.67; P, 8.12. $C_{20}H_{32}NO_4P$. Found (%):C, 62.28; H, 8.14; N, 3.60; P, 8.02. HRMS (MALDI-TOF) m/z for $C_{20}H_{32}NO_4P$: calc. 381.2 [M], found 382.3 $[M]^+$.

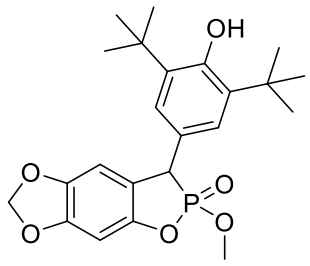
General procedure for the synthesis of compounds 4, 5. To a solution of compound **3** (0.2 g, 0.5 mmol) in dioxane (5 ml) were added 2,6-diaminopyridine or 1,3-diaminobenzene (0.5 mmol, 0.06 g). The mixture was kept at room temperature for 24 h, and the solvent was removed. The product was purified by silica gel column chromatography in acetone for **4** and in dichloromethane-methanol (9.5:0.5) for **5**. Fractions containing product (monitored by TLC, mass spectrometry MALDI-TOF) were combined, the solvent was evaporated, and the residue was dried in a vacuum (3 h, 20°C, 1 mm Torr).



Methyl [(3,5-di-*tert*-butyl-4-hydroxyphenyl)(2,6-diaminopyridin-3-yl)methyl](morpholino)phosphinate 4. Yield 60%, m.p. 134–136 °C. IR (KBr), см$^{-1}$: 703 (P–C), 971 (P-N), 1035 (P–O–C_{alk}), 1113 (C–O–C), 1226 (P=O), 1616 (C=C_{arom}), 3367, 3455 (NH_2); 3627 (OH). ^{1}H NMR (acetone-d_6, 500.13 MHz), δ, ppm: 1.42, 1.44, 1.45, 1.46 (all s, 18H, C(CH_3)$_3$), 2.80, 2.83, 3.04, 3.37 (all m, 8H, CH_{2morph}), 3.47, 3.56 (all d, 3H, OCH_3, $^3J_{HH}$ = 10.8), 4.27, 4.30, 4.37 (all d, 1H, CHP, $^2J_{PH}$ = 24.7), 4.85, 4.94 (all s, 2H, NH_2), 5.11, 5.31 (all s, 2H, NH_2), 5.87, 5.91, 6.26 (all d, 1H, CH_{Py}, $^3J_{HH}$ = 8.1), 6.02 (s, 1H, OH), 7.25, 7.41, 7.51 (all s, 2H, C<u>H</u>CC(CH_3)$_3$), 7.63, 7.76, 8.19 (all d, 1H, CH_{Py}, $^3J_{HH}$ = 8.3). ^{13}C{^{1}H} NMR (acetone-d_6, 125.76 MHz), δ, ppm: 29.91, 29.96 (C(<u>C</u>H_3)$_3$), 34.32, 34.39 (<u>C</u>(CH_3)$_3$), 43.27 (d, CHP, $^1J_{PC}$ = 141.3), 44.18 (N<u>C</u>$H_2$$CH_2$O), 44.27 (N<u>C</u>$H_2$$CH_2$O), 49.60 (d, OCH_3, $^2J_{PC}$ = 7.5), 66.50 (NCH_2<u>C</u>H_2O), 66.63 (NCH_2<u>C</u>H_2O), 97.02, 97.28 (CH_{Py}), 105.95 (C_{Py}), 126.18, 126.545 (d, <u>C</u>HCC(CH_3)$_3$, $^3J_{PC}$ = 7.8), 128.50 (<u>C</u>CHP), 136.83, 137.42 (<u>C</u>C(CH_3)$_3$), 139.66, 140.01 (CH_{Py}), 152.92 (COH), 156.09 (d, CNH_2, $^3J_{PC}$ = 9.2), 157.23 (CNH_2). ^{31}P NMR (acetone-d_6, 202.46 MHz), δ, ppm: 30.5, 30.9, 31.0, 31.3. Anal. Calcd for Calc. for $C_{25}H_{39}N_4O_4P$ (%): C 62.43; H 8.18; N 11.76; P 6.49. Found (%): C 62.21; H 8.11; N 11.42; P 6.31. HRMS (MALDI-TOF) m/z for $C_{25}H_{39}N_4O_4P$: calc. 490.3 [M], found 491.3 $[M+H]^+$, 514.3 $[M+Na]^+$, 530.4 $[M+K]^+$.



Methyl [(3,5-di-*tert*-butyl-4-hydroxyphenyl)(2,4-diaminophenyl)methyl](morpholino)phosphinate 5. Yield 58%, m.p. 118–120 °C. ^{1}H NMR ($CDCl_3$, 399.93 MHz), δ, ppm: 1.41, 1.42 (all s, 18H, C(CH_3)$_3$), 2.78, 2.99, 3.33, 3.45 (all m, 8H, CH_{2morph}), 3.59, 3.62 (all d, 3H, OCH_3, $^3J_{HH}$ = 10.8), 4.25, 4.33 (all d, 1H, CHP, $^2J_{PH}$ = 24.7), 5.12, 5.16, 5.20 (all s, 4H, NH_2), 5.96, 6.05 (all d, 1H, CH_{arom}, $^3J_{HH}$ = 1.6), 6.04 (s, 1H, OH), 6.13, 6.17 (all dd, 1H, CH_{arom}, $^3J_{HH}$ = 8.1, 2.1), 7.34, 7.35 (all d, 2H, C<u>H</u>CC(CH_3)$_3$, $^3J_{HH}$ = 1.6), 7.25, 7.39 (all dd, 1H, CH_{Py}, $^3J_{HH}$ = 8.3, 1.7). ^{13}C{^{1}H} NMR ($CDCl_3$, 101.57 MHz), δ, ppm: 30.87, 30.92 (C(<u>C</u>H_3)$_3$), 34.89, 34.92 (<u>C</u>(CH_3)$_3$), 44.86, 44.90 (N<u>C</u>$H_2$$CH_2$O), 45.37 (d, CHP, $^1J_{PC}$ = 128.3), 51.10, 51.33 (d, OCH_3, $^2J_{PC}$ = 10.1), 57.51, 57.55 (N<u>C</u>$H_2$$CH_2$O), 104.44, 104.59 ($CH_{arom}$), 107.03, 107.72 ($C_{arom}$), 126.98, 127.17 (d, <u>C</u>HCC(CH_3)$_3$, $^3J_{PC}$ = 8.0), 127.51 (<u>C</u>CHP), 136.83, 131.86 (d, CH_{arom}, $^3J_{PC}$ = 6.1), 136.58 (<u>C</u>C(CH_3)$_3$), 145.88 (d, CNH_2, $^3J_{PC}$ = 9.1), 146.49 (CNH_2), 153.54 (COH). ^{31}P NMR ($CDCl_3$, 161.57 MHz), δ, ppm: 31.68, 31.54. Anal. Calcd for Calc. for $C_{25}H_{39}N_4O_4P$ (%): C 63.78; H 8.24; N 8.58; P 6.33. Found (%): C 63.41; H 8.18, N 8.27; P 6.15. HRMS (ESI) m/z for $C_{26}H_{40}N_3O_4P$: calc. 489.59 [M], found 490.22 $[M+H]^+$.



3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-2-methoxy-3*H*-[1,3]dioxolo[4',5':4,5]benzo[1,2-*d*][1,2]oxaphosphole 2-oxide 6. To a solution of of compound **3** (0.397 g, 1 mmol) in CH_2Cl_2 (5 ml) was added sesamol (0.138g, 1 mmol) and two drops of CF_3SO_3H. The mixture was stirred at room temperature for 4 h. The precipitate was filtered off, washed with diethyl ether and dried in a vacuum (2 h, 20°C, 1 mm Torr). Yield 60%. m.p. 240–241 °C. IR (KBr), $см^{-1}$: 767 (P–C); 927 (C–O); 1035 (P–O–C_{alk}); 1233 (P=O); 1621 (C=C_{arom}); 3627 (OH). ^{1}H NMR (DMSO-d_6, 399.93 MHz), δ, ppm: 1.34 (s, 18H, $C(CH_3)_3$), 3.79 (d, 3H, OCH_3, $^3J_{PH}$ = 11.2), 4.62 (d, 1H, CHP, $^2J_{PH}$ = 18.9), 6.03 (d, 2H, CH_2, $^1J_{OH}$ = 19.5), 6.73 (s, 1H, CH_{ses}), 6.88 (d, 1H, C<u>H</u>CC$(CH_3)_3$, $^4J_{PH}$ = 2.3), 6.94 (s, 1H, CH_{ses}). ^{13}C NMR (DMSO-d_6, 100.57MHz), δ, ppm: 30.74 (C(<u>C</u>$H_3)_3$), 34.96 (<u>C</u>$(CH_3)_3$), 42.64 (d, CHP, $^1J_{PC}$ = 148.3), 53.73 (d, OCH_3, $^2J_{PC}$ = 7.1), 96.57 (d, CH_{ses}, $^3J_{PC}$ = 11.1), 101.98 (CH_2), 107.47 (d, CH_{ses}, $^3J_{PC}$ = 17.1), 120.05 (d, C_{ses}, $^2J_{PC}$ = 6.8), 125.26 (d, <u>C</u>HCC$(CH_3)_3$, $^3J_{PC}$ = 6.8), 126.68 (d, <u>C</u>CHP, $^2J_{PC}$ = 6.1), 139.78 (<u>C</u>C$(CH_3)_3$), 143.90 (C_{ses}), 146.71 (C_{ses}), 148.01 (COH_{ses}), 153.57 (COH). ^{31}P NMR (DMSO-d_6, 100.57 MHz), δ, ppm: 46.65. Anal. Calcd for Calc. for $C_{23}H_{29}O_6P$ (%): C 63.74; H 6.75; P 7.14. Found (%): C 63.88; H 6.76; P 7.16. HRMS (MALDI-TOF) m/z for $C_{23}H_{29}O_6P$: calc. 432.2 [M], found 433.1 $[M+H]^+$, 472.1 $[M+K]^+$.

Biological methods

Cells and Materials

For the experiments, we used tumor cell cultures M-HeLa clone 11 (epithelioid carcinoma of the cervix, subline HeLa., clone M-HeLa), MCF7 - human breast adenocarcinoma (pleural fluid); from the collection of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg); human liver cells (Chang liver) from the collection and the Research Institute of Virology of the Russian Academy of Medical Sciences (Moscow).

Cytotoxicity assay

The cytotoxic effect on cells was determined using the colorimetric method of cell proliferation - the MTT test.[S2,S3] NADP-H-dependent cellular oxidoreductase enzymes can, under certain conditions, reflect the number of viable cells. These enzymes are able to reduce the tetrazolium dye (MTT) - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble blue-violet formazan, which crystallizes inside the cell. The amount of formazan formed is proportional to the number of cells with active metabolism. Cells were seeded on a 96-well Nunc plate at a concentration of 5×10^3 cells per well in a volume of 100 μl of medium and cultured in a CO_2 incubator at 37°C until a monolayer was formed. Then the nutrient medium was removed and 100 μl of solutions of the test drug in the given dilutions were added to the wells, which

were prepared directly in the nutrient medium with the addition of 5% DMSO to improve solubility. After 48 h of incubation of the cells with the tested compounds, the nutrient medium was removed from the plates and 100 μl of the nutrient medium without serum with MTT at a concentration of 0.5 mg ml^{-1} was added and incubated at 37°C for 4 h. Formazan crystals were added 100 μl of DMSO to each well. Optical density was recorded at 540 nm on an Invitro logic microplate reader (Russia). The experiments for all compounds were repeated three times.

References

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S2 A. V. Smolobochkin, A. S. Gazizov, L. J. Yakhshilikova, D. D. Bekrenev, A. R. Burilov, M. A. Pudovik, A. P. Lyubina, S. K. Amerhanova and A. D. Voloshina, *Chem. Biodiversity*. 2022, **19**, e202100970.

S3 L. K. Kibardina, A. V. Trifonov, A. B. Dobrynin, M. A. Pudovik, A. R. Burilov, A. D. Voloshina, A. G. Strelnik and A. S. Gazizov, *Mendeleev Commun*., 2021, **31**, 664.

Copies of NMR spectra

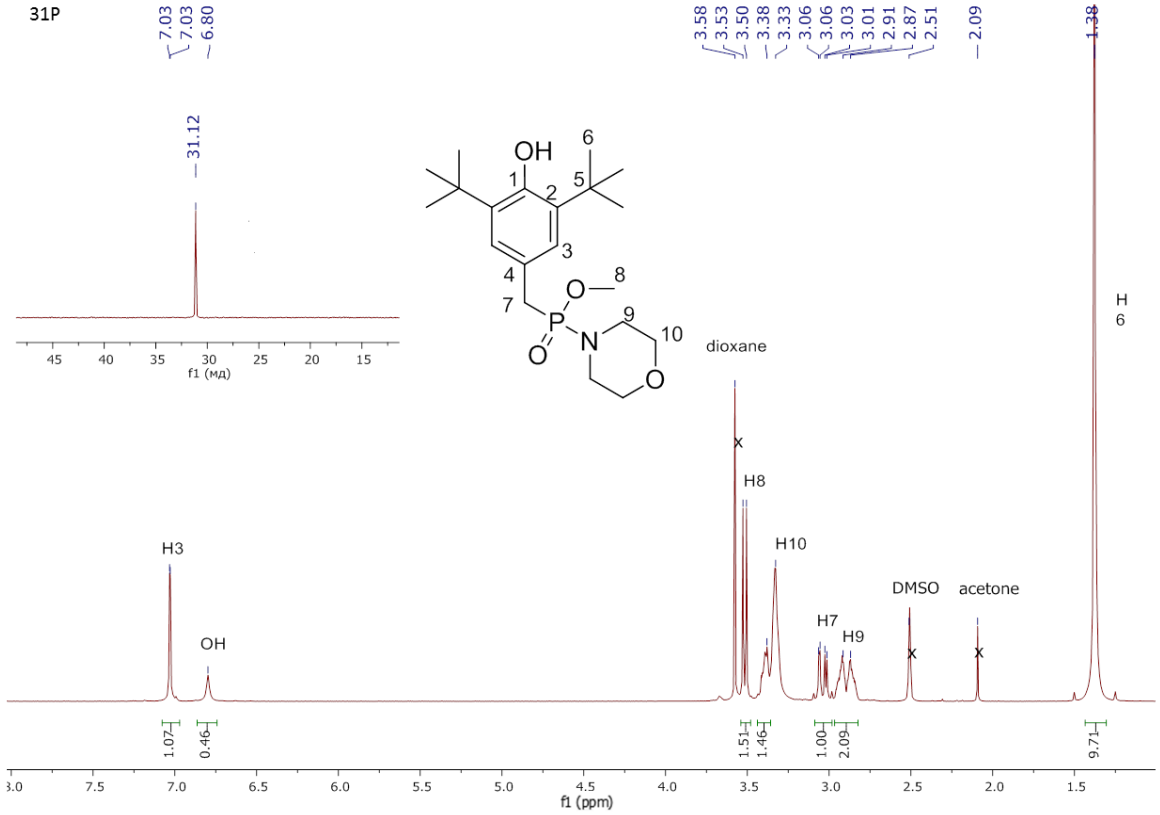

Figure S1. ^{1}H, ^{31}P NMR (DMSO-d$_6$, 500.13 MHz, 25 °C) of compound **2**

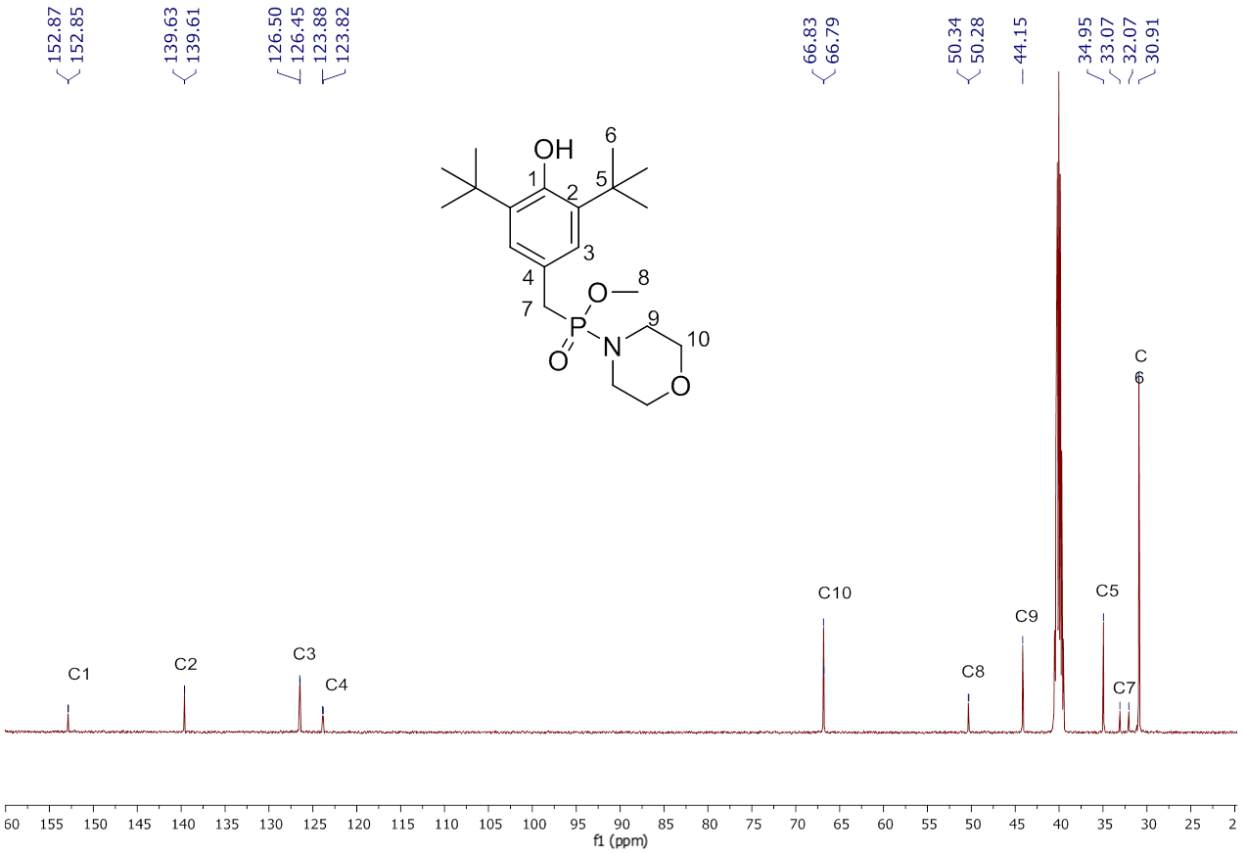

Figure S2. ^{13}C{1H} NMR (DMSO-d$_6$, 125.76 MHz, 25 °C) of compound **2**

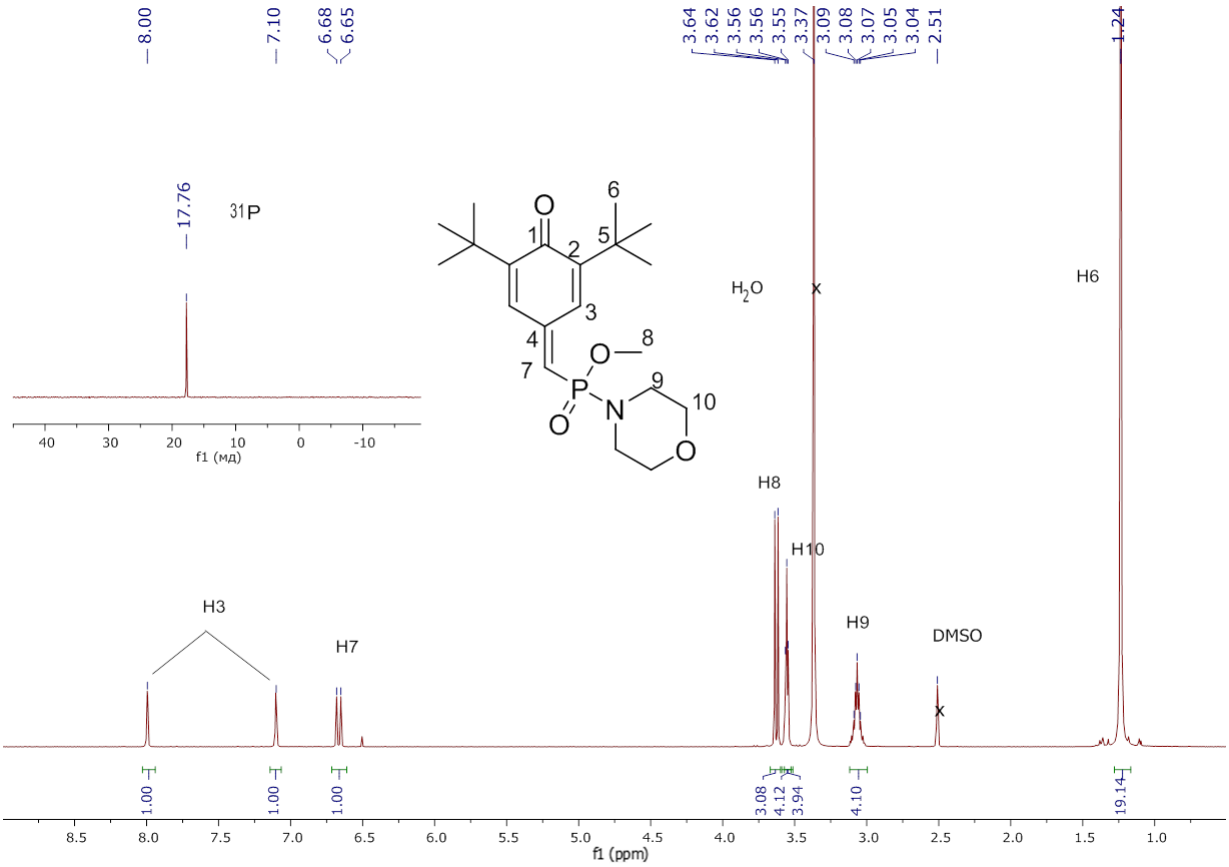

Figure S3. ^{1}H, ^{31}P NMR (DMSO-d_6, 500.13 MHz, 25 °C) of compound **3**

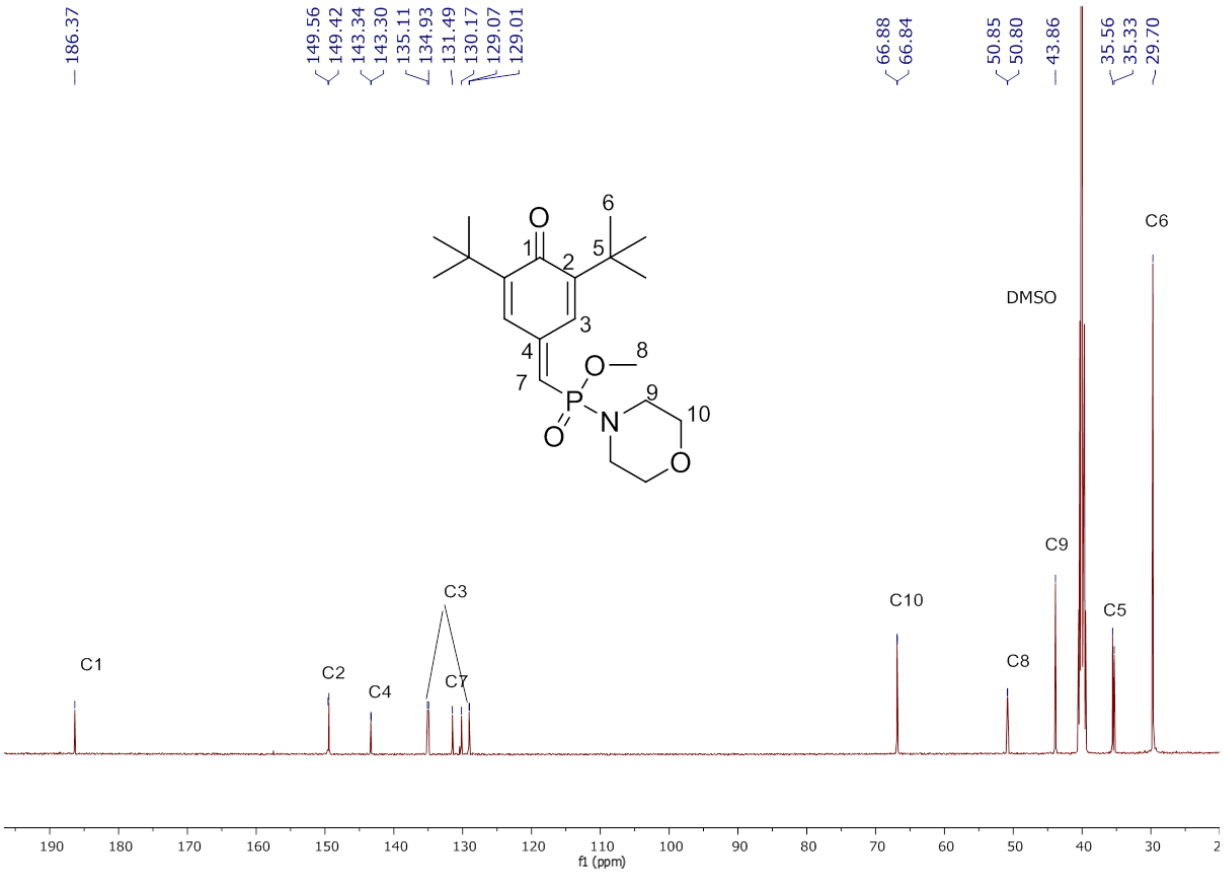

Figure S4. $^{13}C\{1H\}$ NMR (DMSO-d_6, 125.76 MHz, 25 °C) of compound **3**

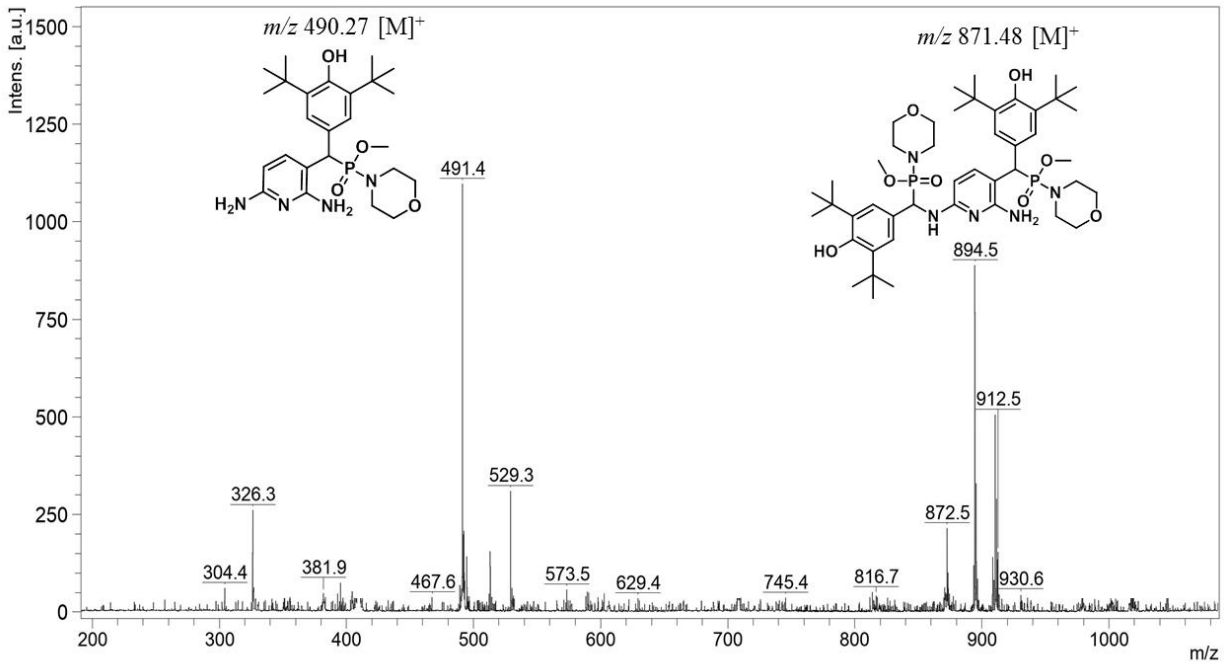

Figure S5. Mass spectrometry (MALDI/TOF) of the reaction mixture of the interaction of compound **3** with 2,6-diaminopyridine (24 h)

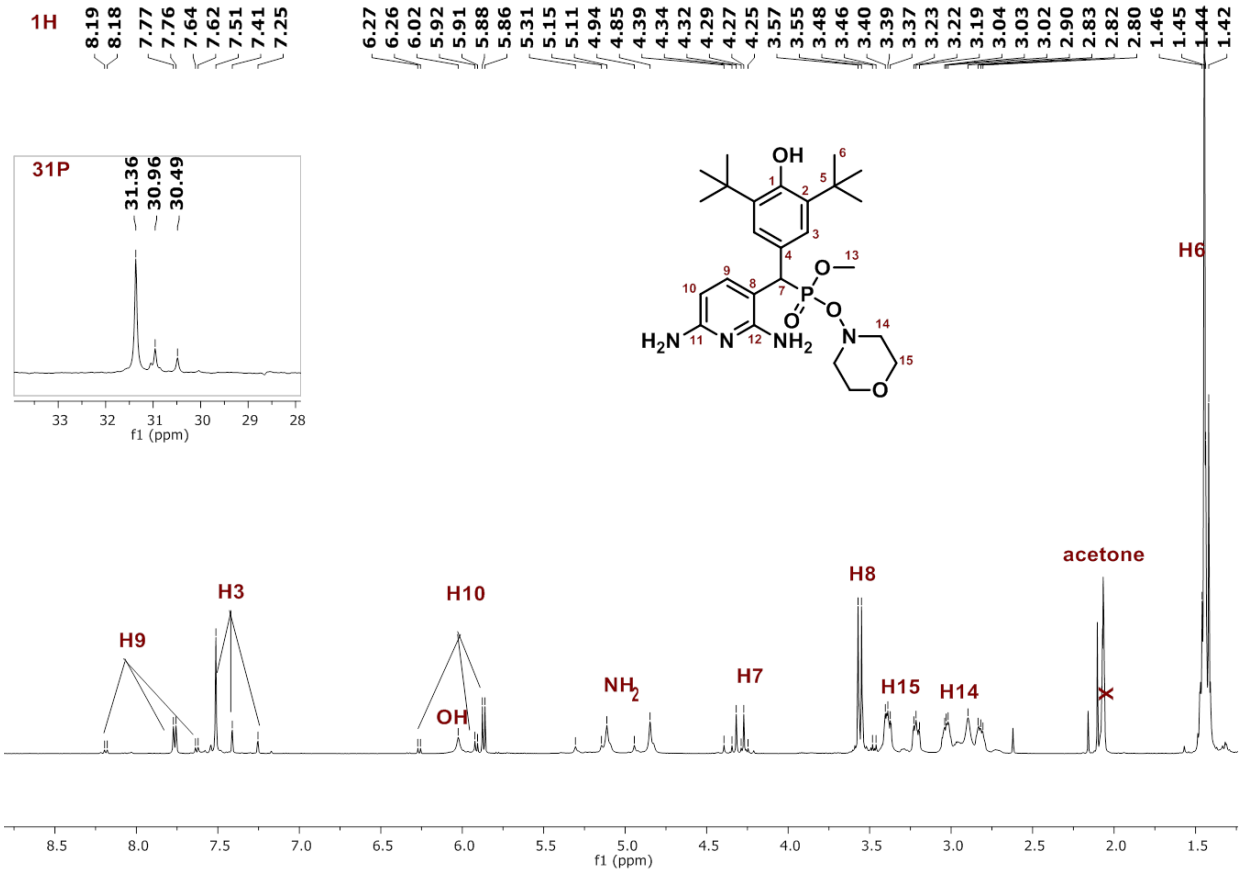

Figure S6. ^{1}H, ^{31}P NMR (acetone-d_6, 500.13 MHz, 25 °C) of compound **4**

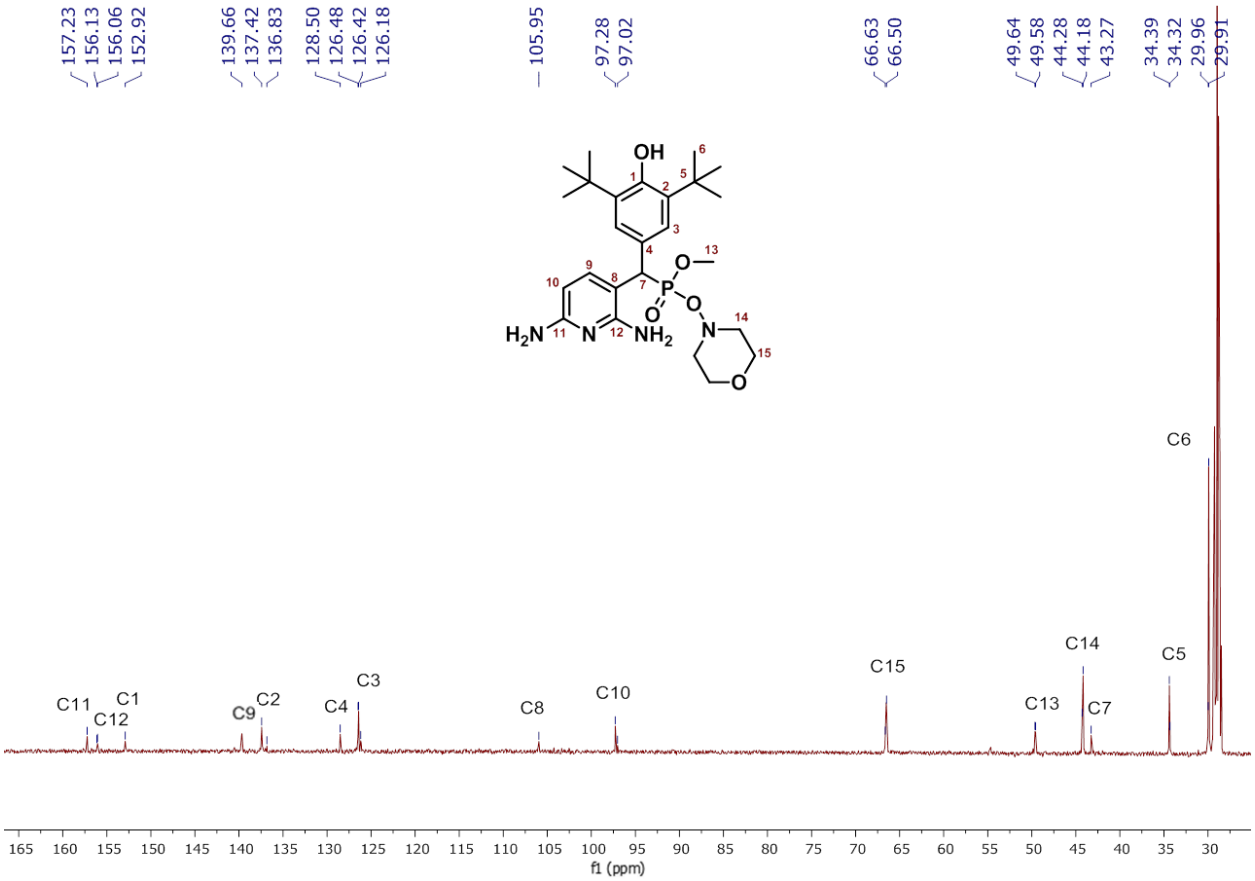

Figure S7. $^{13}C\{1H\}$ NMR (acetone-d_6, 125.76 MHz, 25 °C) of compound **4**

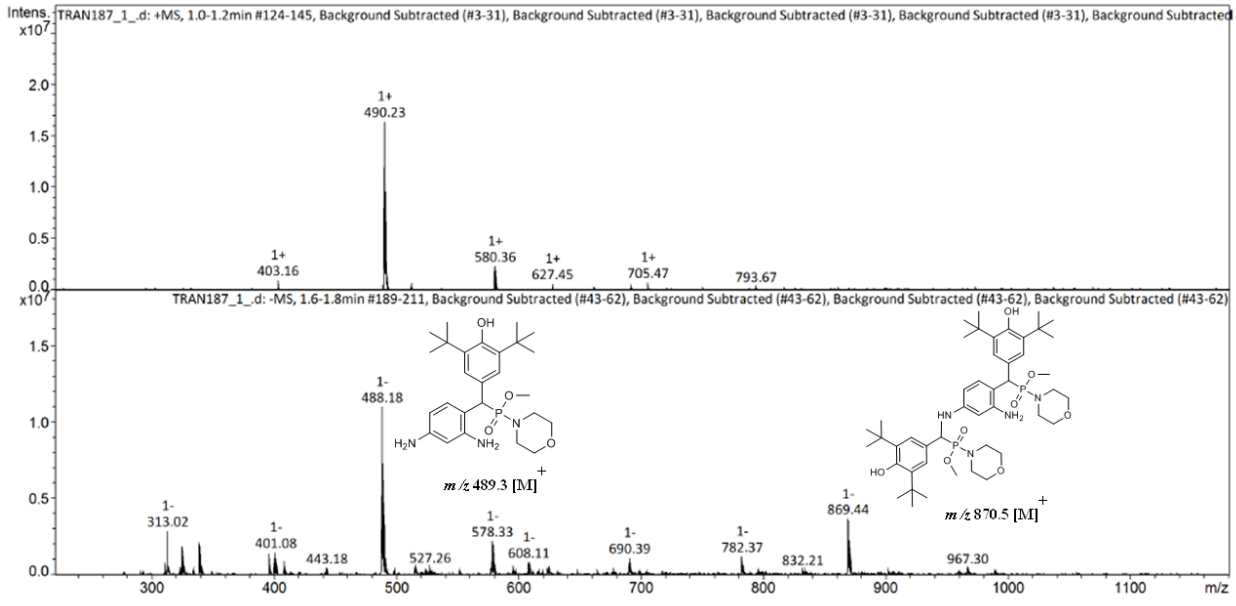

Figure S8. Mass spectrometry (MALDI/TOF) of the reaction mixture (24 h) of the interaction of compound **3** with 1,3-diaminobenzene

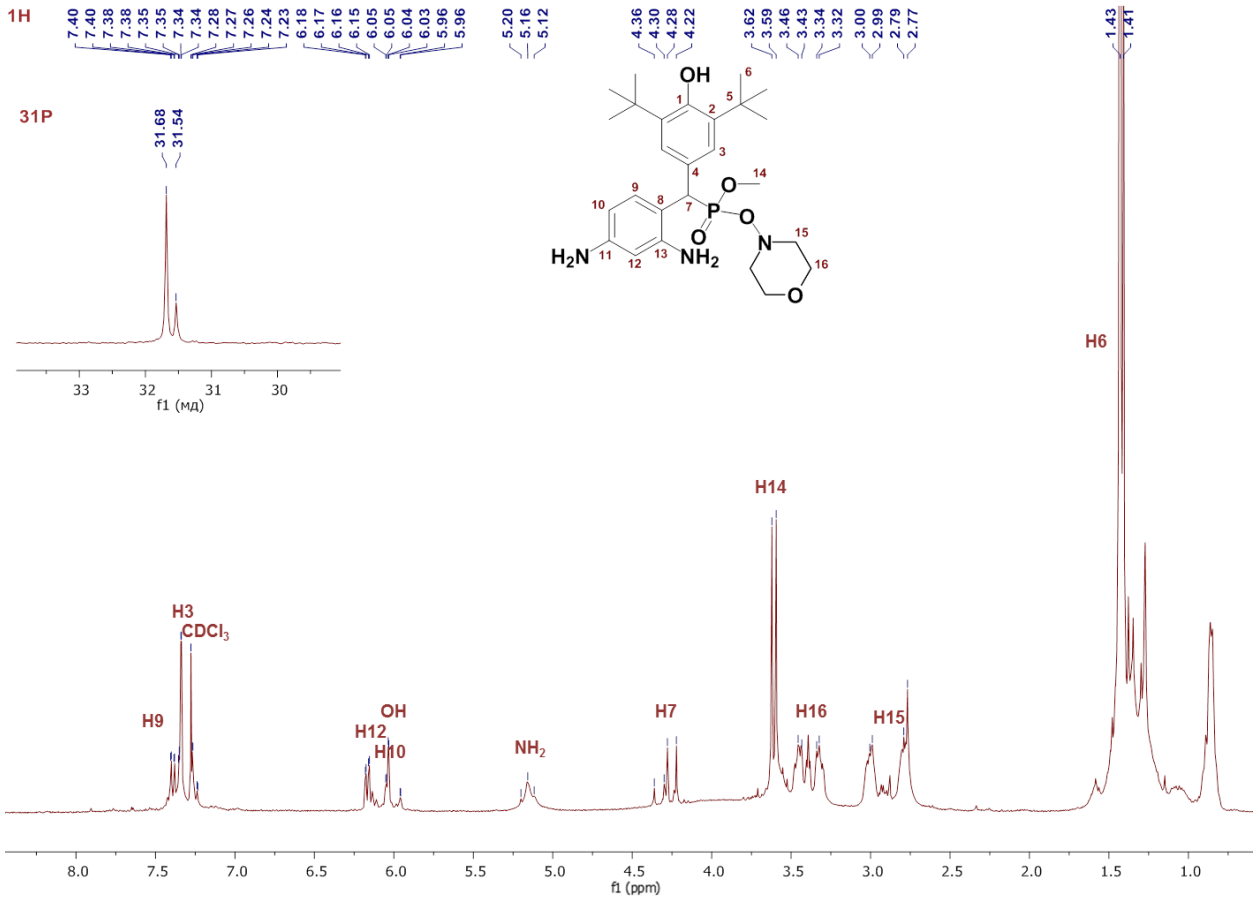

Figure S9. ^{1}H, ^{31}P NMR ($CDCl_3$, 399.93 MHz, 25 °C) of compound **5**

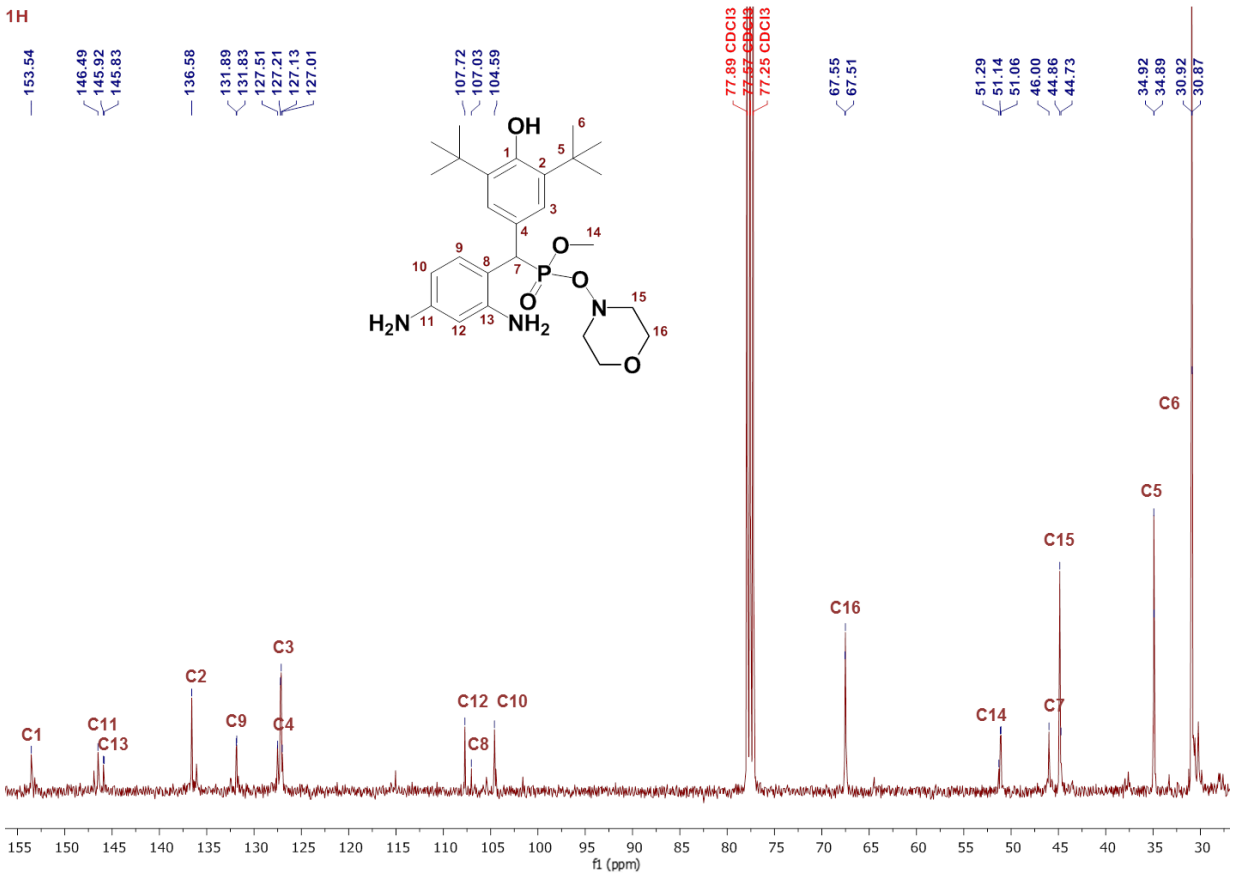

Figure S10. $^{13}C\{1H\}$ NMR ($CDCl_3$, 101.90 MHz, 25 °C) of compound **5**

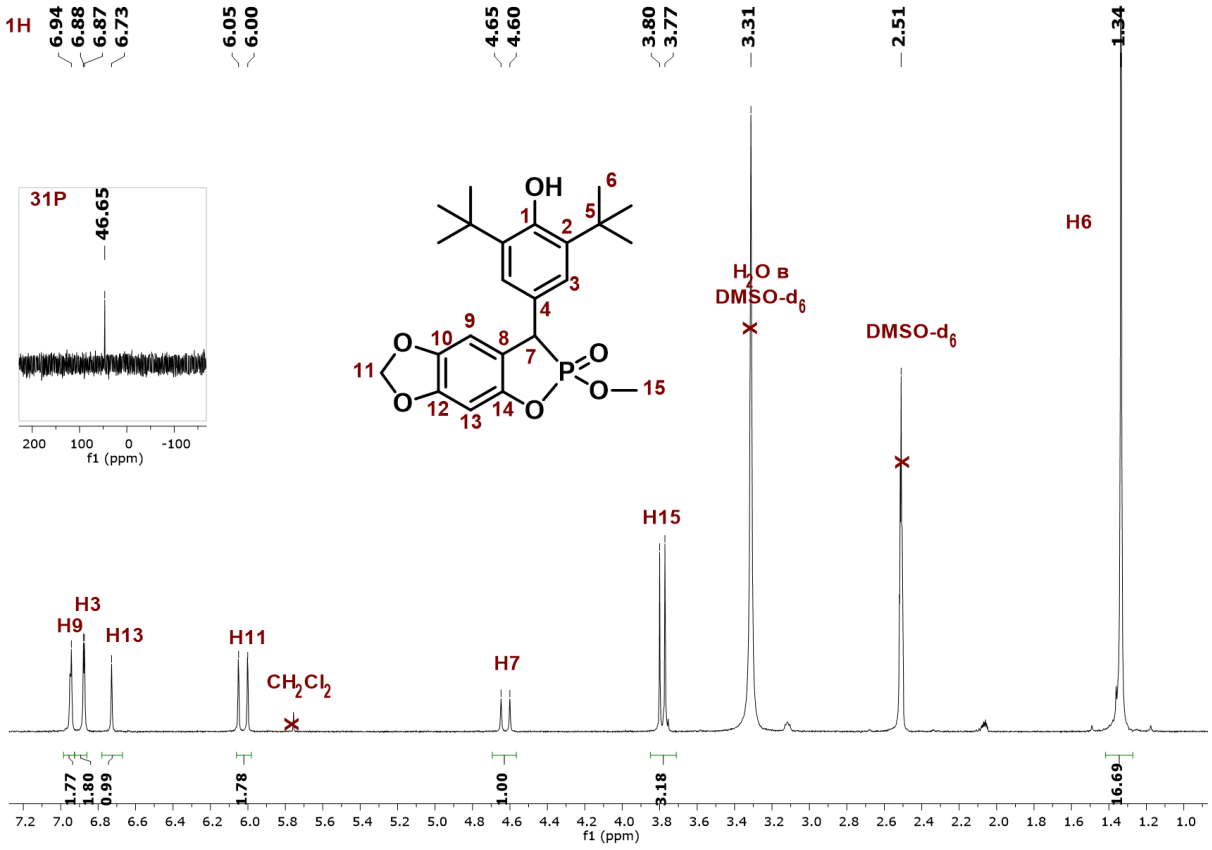

Figure S11. ^{1}H NMR (DMSO-d$_6$, 399.93 MHz, 25 °C) of compound **6**

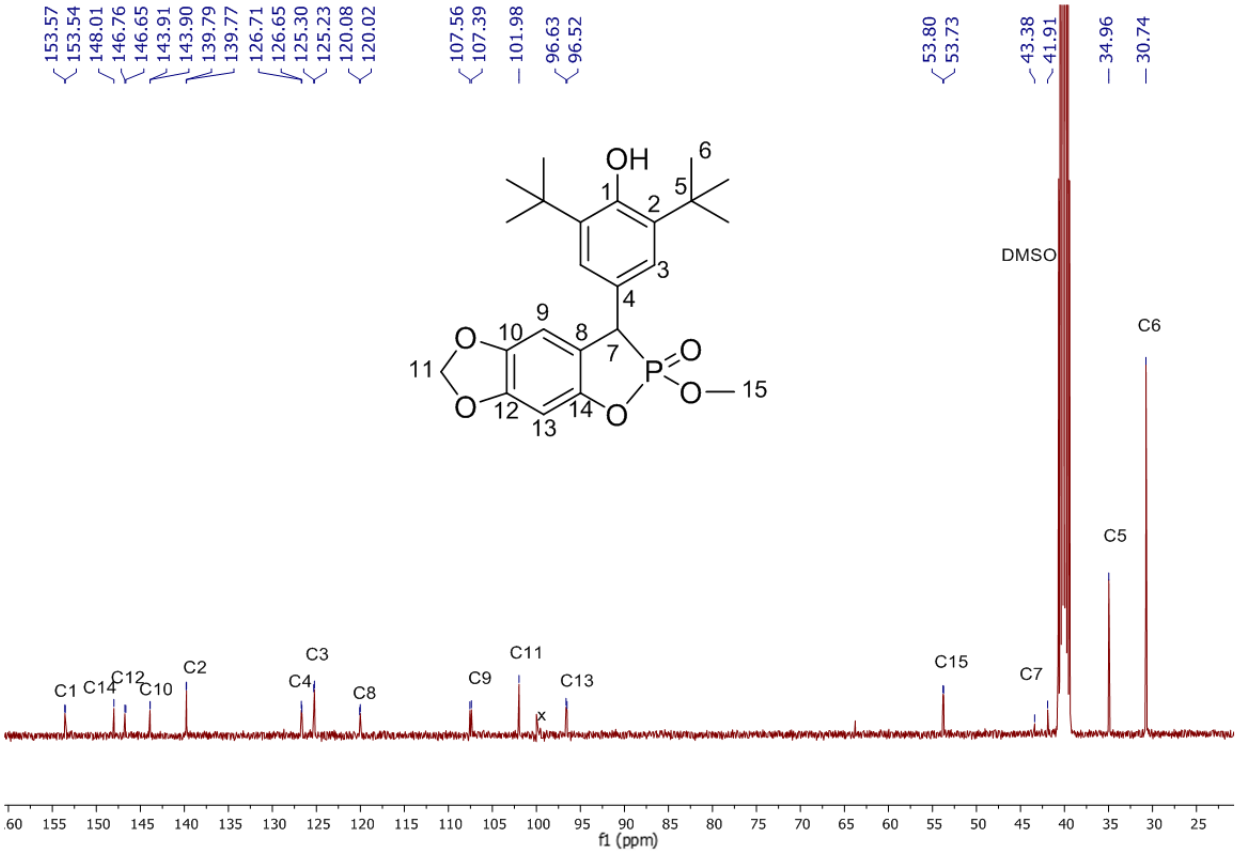

Figure S12. ^{13}C{1H} NMR (DMSO-d$_6$, 101.57 MHz, 25 °C) of compound **6**

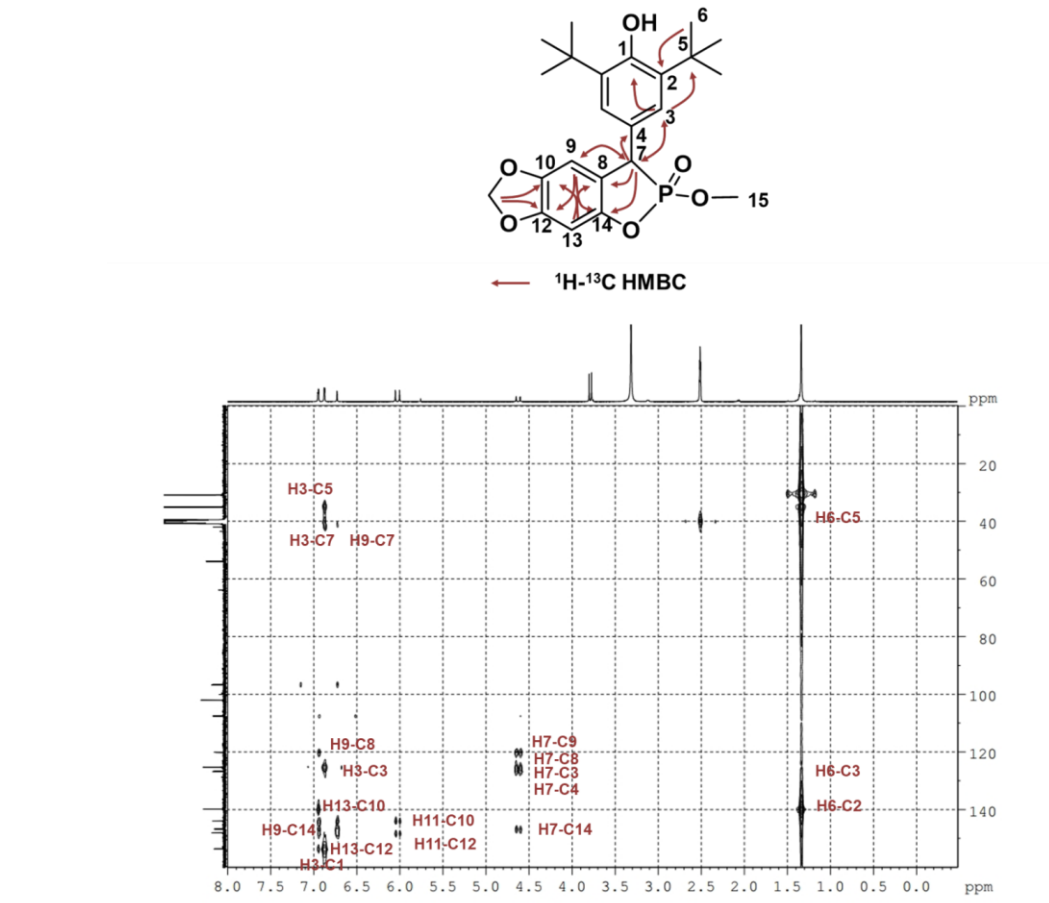

Figure S13. ^{1}H-^{13}C HMBC spectrum of compound **6**

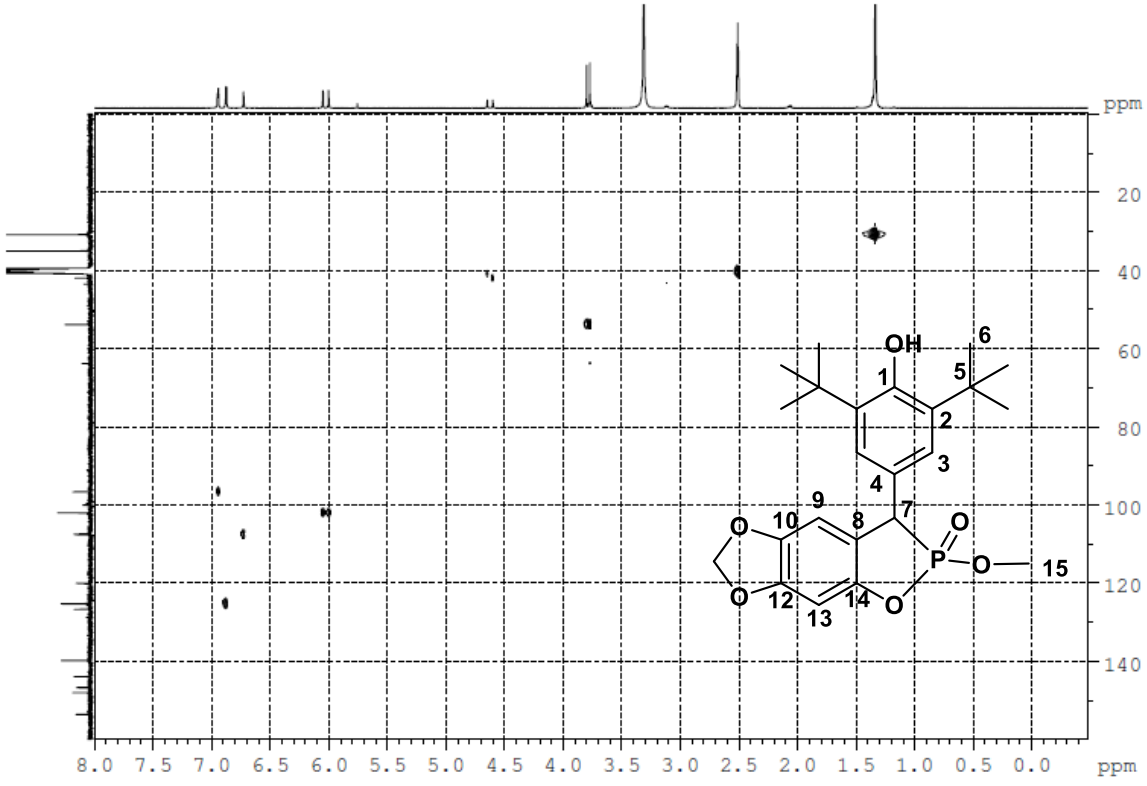

Figure S14. ^{1}H-^{13}C HSQC spectrum of compound **6**