SUPPORTING INFORMATION

Total synthesis and algaecidal activity of questiomycins against harmful bloom forming dinoflagellates

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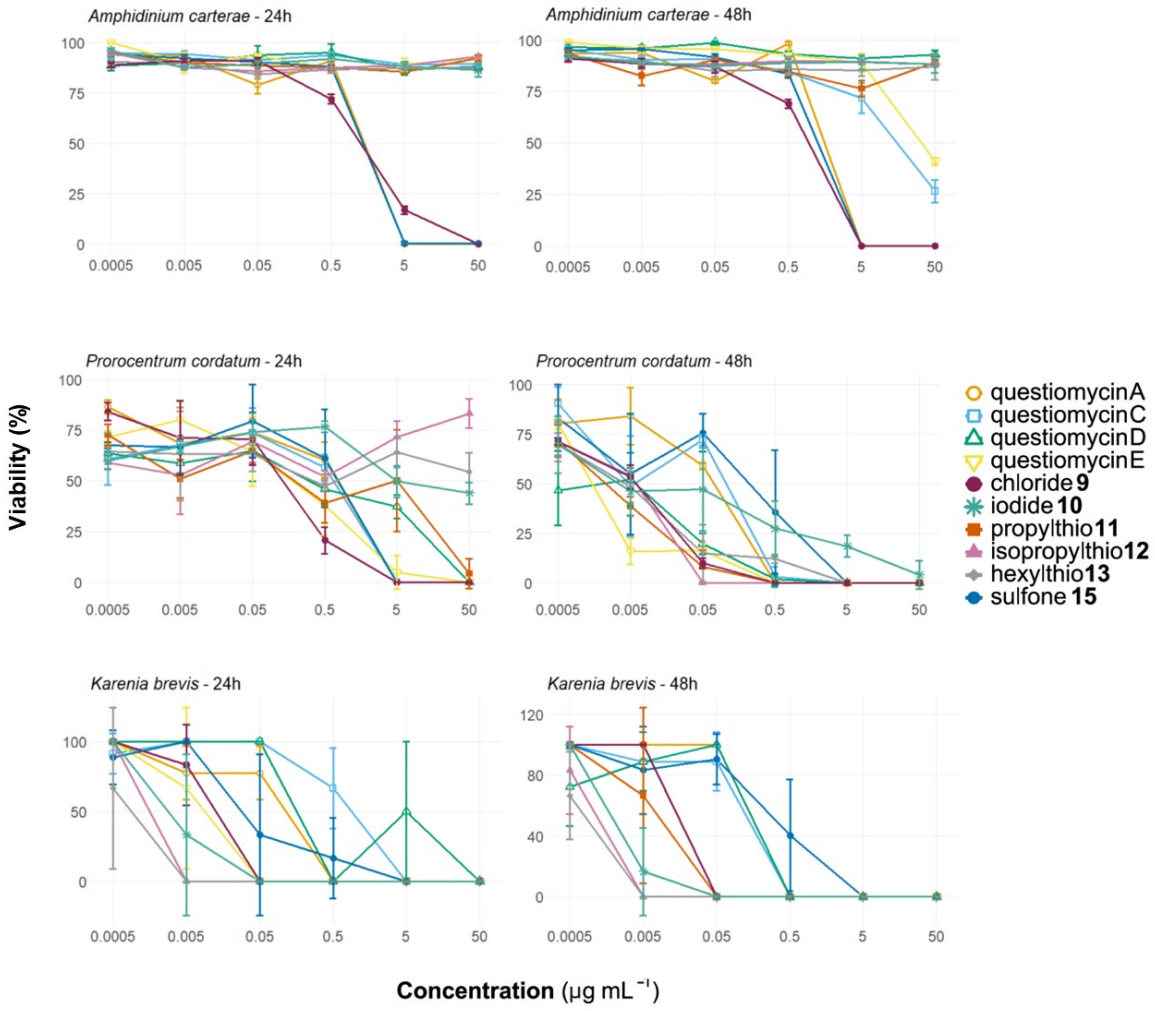


Figure S1. Cell viability in three algae treated with questiomycin A, C–E and analogues. Data are from 24 and 48 hours post treatment. Percent viability of cells in each treatment is based on Evans Blue staining. Points and error bars are the mean and standard deviation of triplicate measurements.

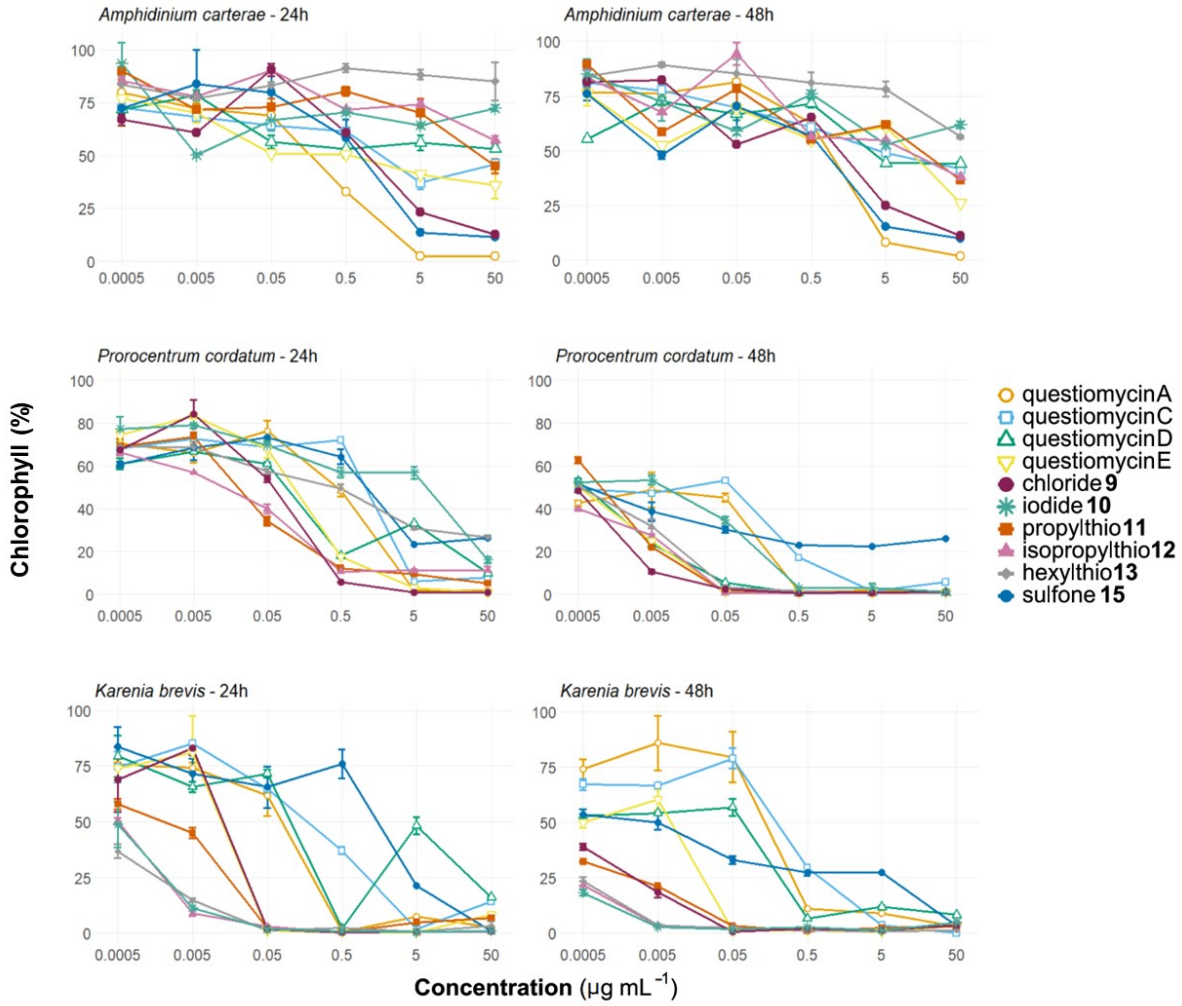


Figure S2. Chlorophyll percentage in three algae treated with questiomycins A, C–E and analogues. Data are from 24 and 48 hours post treatment. Percent chlorophyll fluorescence of each treatment relative to a positive control without questiomycin treatment. Points and error bars are the mean and standard deviation of triplicate measurements.

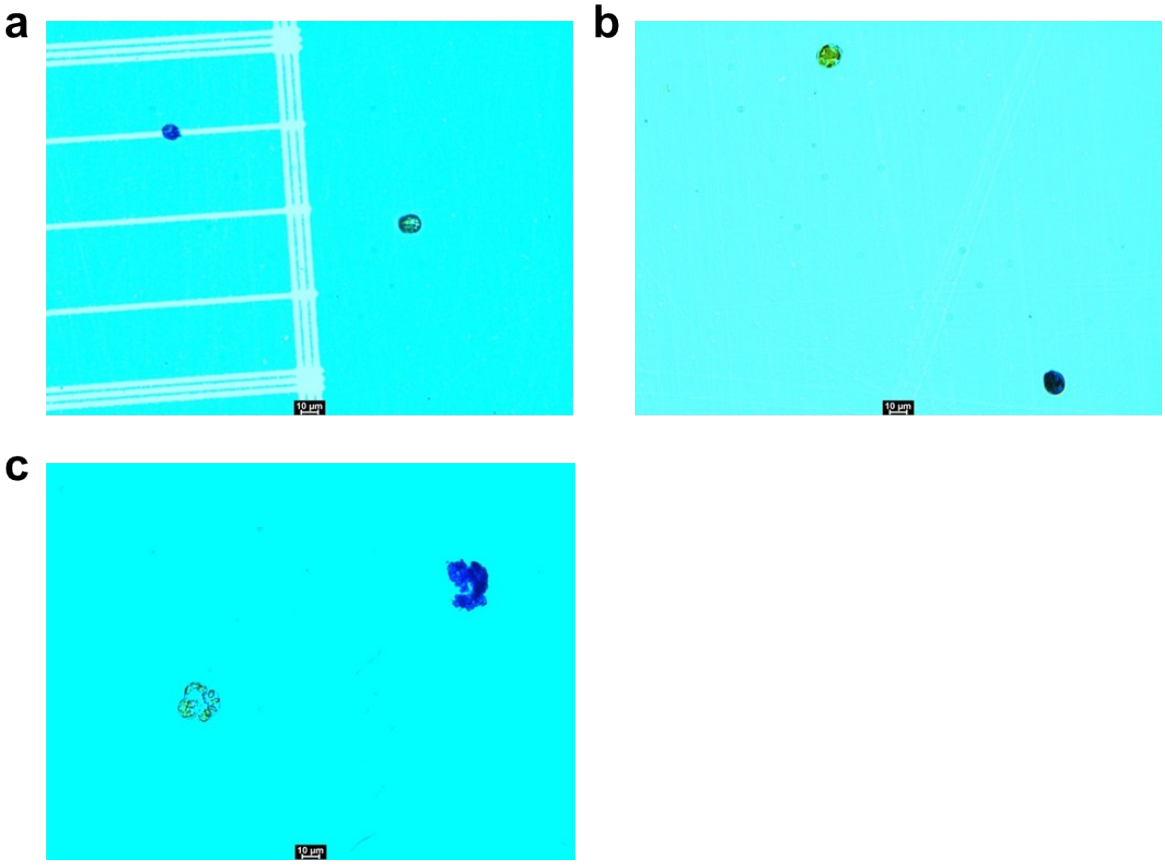


Figure S3. Live/dead light microscopy images of algal cells. Cells were treated with the mortal stain Evans Blue. Images were captured in fields of view that contained both live (green, unstained) and dead (blue, stained) cells for (a) *Amphidinium carterae,* (b) *Karenia brevis*, and (c) *Prorocentrum cordatum*. Scale bars at the bottom of each image are 10 microns.

Table S1. Lethal concentration (LC_{50}) and effective concentration (EC_{50}) of questiomycins after 48 h of exposure. Reported values are the mean and standard error of triplicate measurements and are in units of μM (cf. **Table 1**, reported in units of μg ml^{-1}).

	Amphidium carterae		***Karenia brevis***		***Prorocentrum cordatum***	
	LC_{50}	**EC_{50}**	**LC_{50}**	**EC_{50}**	**LC_{50}**	**EC_{50}**
questiomycin A	7.38±0.04	4.01±0.04	0.52±0.09	0.63±0.07	0.34±0.01	<0.002 [a]
questiomycin C	57±9	nd	1.8±0.0	0.70±0.02	0.3±0.1	<0.002 [a]
questiomycin D	nd	nd	6±5	0.26±0.02	<0.002 [a]	0.012±0.0003
questiomycin E	113±2	36±2	0.04±0.01	<0.002 [a]	0.007±0.0003	<0.002 [a]
chloride **9**	3.8±0.1	4.8±0.2	0.05±0.01	<0.002 [a]	0.024±0.004	<0.0002 [a]
iodide **10**	nd	nd	0.012±0.003	<0.002 [a]	0.104±0.098	0.021±0.003
propylthio **11**	nd	6.4±0.2	0.031±0.01	<0.002 [a]	0.007±0.002	0.010±0.001
isopropylthio **12**	nd	33±2	0.003±0.001	<0.002 [a]	0.021±0.002	<0.0002 [a]
hexylthio **13**	nd	nd	0.002±0.001	<0.002 [a]	0.015±0.003	0.015±0.002
sulfone **15**	4.33±0.08	2.52±0.02	0.6±0.4	0.014±0.005	1.4±0.7	nd

[a] Response <50% at lowest concentration tested.

nd, not determined (<2-fold-change in response across all tested concentrations).

Algal culture methods:

Algae strains *Amphidinium carterae* CCMP121 (originally isolated from the Caribbean Sea) and *Prorocentrum cordatum* CCMP1329 (originally isolated from Great South Bay, NY, USA) were obtained from the National Center for Marin e Algae and Microbiota (NCMA). *Karenia brevis* FWC1010 was isolated by Florida Fish and Wildlife Conservation Commission from Bayboro Harbor, Florida, USA in 2016. Algal cultures were maintained in a growth chamber at 22 °C under a 12:12 light:dark diurnal cycle at 40 µmol photons m^{-2} s^{-1}.

All algae were grown in a synthetic ocean water (SOW) salt solution according to NCMA guidelines and supplemented with nutrients as follows:

- *A. carterae* was grown with L1-Si recipe nutrients prepared using NCMA guidelines.
- *K. brevis* and *P. cordatum* were grown with L1-Si/20 recipe, with 1/20 concentrations of the L1 recipe for trace metals, vitamins, nitrogen and phosphorus sources and substitution of nitrate with ammonium.

Algal cultures were transferred to fresh medium every 2-3 weeks to maintain exponential phase. Doubling times were approximately 3-4 days for *A. carterae*, 5-7 days for *P. cordatum*, and 5-9 days for *K. brevis*. All experiments were carried out in late exponential phase.

Chlorophyll and viability measurements:

All questiomycins and synthetic derivatives were dissolved in DMSO to arrive at stock concentrations of 10 mg mL^{-1}. Subsequently, stocks were serially diluted in DMSO, and 5 µL of each dilution was added to 1 mL of culture to arrive at final treatment concentrations of 0.0005, 0.005, 0.05, 0.5, 5, and 50 µg mL^{-1}. Control cultures (with no questiomycins or derivatives) were treated with 5 µL of solvent only.

Chlorophyll *a* fluorescence was measured using a Turner Designs fluorometer (model: 7200-002, San Jose, CA, USA) with the Blue Module (excitation 441/82 nm and emission 660-710 nm) following standard fluorometric protocols. A blank measurement was taken using filtered culture medium with each questiomycin or derivative to account for background fluorescence. Chlorophyll % response in questiomycin treatment cultures was determined by

comparison to solvent-only control cultures. Each measurement was performed in triplicate, to generate a mean value with standard deviation.

For cell viability analysis, we tested Trypan Blue, Neutral Red, and Evans Blue stains. We established live (exponential cultures) and dead controls (culture treated with $CuSO_4$)[1] to examine the quality and effectiveness of stains to distinguish cell viability by microscopy. The mortal stain Evans Blue (EB) had best results in terms of its ability to stain the cells. A 0.1% working solution of EB was made in phosphate buffered saline (PBS). This solution was passed through 0.2µm filter to remove any undissolved particles. An aliquot of 50 µl of culture was stained with 17 µl EB at a final concentration of ~0.025% for viability assessment. Algae were stained for 15 min and subsequently heat shocked for 1 min 45 s at 37 °C seconds to stop motility. Heat shock was not required for *K. brevis*. Cells were visualized and counted on a Leica DM 2500 LED microscope within 5 mins of staining using a hemocytometer (Neubauer). The hemocytometer was cleaned thoroughly with 70% ethanol and dried before each use to ensure accurate readings. Using a pipette, 10 µL of the prepared algal suspension was loaded onto the edge of the coverslip, allowing capillary action to draw the suspension into the chamber without air bubbles or overflow. For each measurement, cells in at least four large grid squares (each comprising 16 smaller squares) were counted. Cells touching the top and left edges of the gridlines were included in the count, while those touching the bottom and right edges were excluded to avoid double-counting. All counts were performed in triplicates by counting three fields of view. The final % viability was expressed as mean ± standard deviation based on triplicate counts. Dark blue cells which took the EB stain were considered dead and green cells were considered live (**Fig. S3**). The viability percentage of cells was calculated using the following formula:

Viability (%) = (Number of viable cells/Total number of cells)×100

We estimated the lethal concentration for 50% mortality (LC_{50}) using the cell viability data and the effective concentration for 50% response (EC_{50}) using the chlorophyll data. We applied the linear interpolation method using the formula below.

$$\log_{10}(LC_{50} \text{ or } EC_{50}) = \log_{10}(C_1) + [(50 - R_1) / (R_2 - R_1)] \times [\log_{10}(C_2) - \log_{10}(C_1)]$$

where:

C_1, C_2 = concentrations before and after 50% response, respectively,

R_1, R_2 = responses (%) corresponding to C_1 and C_2, respectively.

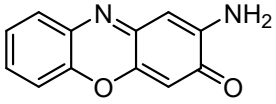
In treatments where the response was below 50% (i.e. >50% cell death or chlorophyll reduction) for the lowest tested concentration, we report LC_{50}/EC_{50} as less than the lowest tested concentration (<0.0005 μg mL^{-1}). For treatments where the response showed <2-fold change across the concentration curve, we did not determine LC_{50}/EC_{50}. All data visualizations were conducted in R software.

Synthetic chemistry

General Synthetic Methods

All reactions were performed under an inert atmosphere of N_2 unless otherwise specified. Analytical thin layer chromatography (t.l.c.) was conducted on aluminium-backed 2 mm thick silica gel 60 GF_{254}. Visualization was aided by UV light. Melting points were recorded on a digital melting point apparatus and are uncorrected. Infrared spectra were recorded using an FT-IR total attenuated reflectance spectrometer as neat compounds using a diamond-coated ZnSe accessory. Proton nuclear magnetic resonance spectra (^{1}H NMR, 500 MHz) and proton decoupled carbon nuclear magnetic resonance spectra (^{13}C NMR, 125 MHz) were obtained in deuterated chloroform and DMSO-d_6 with residual protonated solvent as internal standard. Abbreviations for multiplicity are s, singlet; d, doublet; t, triplet; q, quartet; p, pentet. Flash chromatography was performed on a Biotage system using silica gel 60. High resolution mass spectra (HRMS) were obtained by ionizing samples using electro-spray ionization (ESI) and a time-of-flight mass analyzer. Pyridine was dried over 4 Å molecular sieves. Hexanes refers to petroleum ether, boiling range 40-60 °C. Dichloromethane and THF were collected from a dry solvent apparatus (Glass Contour of SG Water, USA) as per the procedure of Pangborn *et al*.[2]

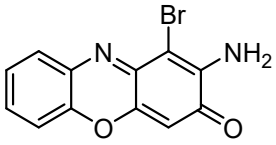
2-Amino-3*H*-phenoxazin-3-one (questiomycin A; 1)



Co(salen) (0.30 g, 0.92 mmol, 0.01 equiv.) was added to a solution of *ortho*-aminophenol (10.0 g, 91.6 mmol) in MeOH (500 mL) and H_2O (200 mL). The reaction mixture was stirred vigorously in air at rt for 3 d. A dark precipitate was obtained and collected by filtration, and washed extensively with cold water to afford **questiomycin A** (**1**) as a red solid (8.33 g, 83%). **mp:** 244–248 °C, (lit. 248–250 °C). **IR:** *v* 3411, 3305, 1582, 1571, 1471, 1172, 839, 758, 583 cm^{-1}. **^{1}H NMR (500 MHz, d_6-DMSO):** δ 7.70 (d, *J* = 7.7 Hz, 1 H), 7.51–7.45 (m, 2 H), 7.39 (t, *J* = 7.3 Hz, 1 H), 6.81 (br s, 2 H), 6.36 (s, 2 H) ppm. **^{13}C NMR (126 MHz, d_6-DMSO):** δ 180.2, 148.9, 148.3, 147.4, 141.9, 133.7, 128.8, 128.0, 125.3, 115.9, 103.4, 98.4 ppm. **HRMS**

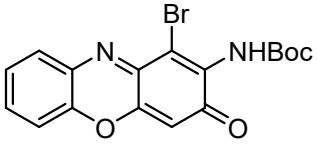
(ESI$^+$): *m/z* 213.0699 $[M+H]^+$. Calcd for $C_{12}H_9N_2O_2^+$ 213.0664. The spectral data were consistent with that reported.[3]

2-Amino-1-bromo-3*H*-phenoxazin-3-one (questiomycin E; 4)



N-Bromosuccinimide (2.26 g, 12.7 mmol) was added to compound **1** (1.50 g, 7.07 mmol) in $CHCl_3$ (400 mL) at 0 °C, and the mixture was stirred at rt for 16 h. On completion, the organic phase was washed with sat. aq. $NaHCO_3$ (100 mL) and brine (100 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified using flash column chromatography (CH_2Cl_2) to afford **questiomycin E** (**4**) as an orange solid (1.89 g, 92%). **mp:** 253.5–255 °C. **IR:** *v* 3313, 1572, 1365, 1304, 1181, 771 cm^{-1}. **^{1}H NMR (500 MHz, *d*$_6$-DMSO):** δ 7.81 (d, *J* = 7.6 Hz, 1 H), 7.56–7.52 (m, 2 H), 7.44 (m, 1 H), 6.99 (brs, 2 H), 6.40 (s, 1 H) ppm. **^{13}C NMR (125 MHz, *d*$_6$-DMSO):** δ 177.5, 149.6, 145.7, 144.0, 142.1, 133.2, 123.0, 128.5, 125.6, 115.9, 102.5, 94.8 ppm. **HRMS (ESI$^+$):** *m/z* 292.9740 $[M+H]^+$. Calcd for $C_{12}H_8BrN_2O_2^+$ 292.9749. The spectral data were consistent with that reported.[3]

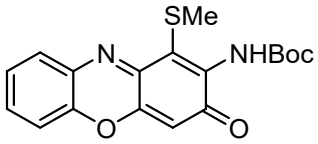
tert-*Butyl (1-bromo-3-oxo-3*H*-phenoxazin-2-yl)carbamate (6)*



Di-*tert*-butyl dicarbonate (2.47 g, 11.32 mmol) was added to a solution of compound **4** (2.06 g, 7.08 mmol) in pyridine (20 mL) under an atmosphere of N_2 at 0 °C, followed by the addition of DMAP (0.91 g, 7.43 mmol). The solution was then warmed to rt over 2 h and stirred for another 16 h. On completion, H_2O (100 mL) was added, and the aqueous phase was extracted with Et_2O (50 mL × 5). The combined organic solution was filtered through a short column of silica gel. The solvent was removed under reduced pressure to give compound **6** as an orange solid (2.3 g, 83%). **mp:** 175.5–176.5 °C. **IR:** *v* 2980, 1801, 1766, 1711, 1636, 1576, 1369, 1250, 1162, 1116, 850, 769 cm^{-1}. **^{1}H NMR (500 MHz, $CDCl_3$):** δ 7.94 (d, *J* = 7.9 Hz, 1 H), 7.63–7.60 (m, 1 H), 7.44–7.40 (m, 1 H), 7.37 (d, *J* = 8.2 Hz, 1 H), 6.39 (s, 1 H), 1.43 (s, 9 H) ppm. **^{13}C NMR (126 MHz, $CDCl_3$):** δ 178.4, 149.7, 149.0, 145.2, 143.8, 142.1, 133.9, 133.1,

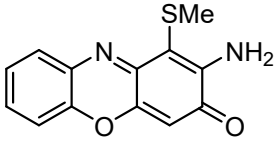
131.0, 130.6, 125.8, 116.0, 105.5, 83.7, 27.8 ppm. **HRMS (ESI$^+$):** *m/z* 391.0289 $[M+H]^+$. Calcd for $C_{17}H_{16}BrN_2O_4^+$ 391.0293.

tert-*Butyl (1-(methylthio)-3-oxo-3*H*-phenoxazin-2-yl)carbamate (7)*



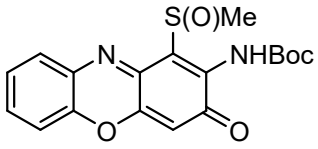
NaSMe (0.107 g, 1.53 mmol) was added to a solution of compound **6** (0.60 g, 1.53 mmol) in dry THF (20 mL) at 0 °C. The solution was warmed to rt over 1 h. On completion, sat. aq. NH_4Cl (10 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (20 mL × 3), the combined organic solution was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified using flash column chromatography (EtOAc in hexane = 10–50%) to afford compound **7** (0.34 g, 62%) as an orange solid. **mp:** 182.0–182.2 °C. **IR:** *v* 2978, 2929, 1761, 1629, 1369, 1284, 1153, 1113, 851, 768 cm^{-1}. **^{1}H NMR (500 MHz, $CDCl_3$):** δ 7.89 (dd, *J* = 8.0, 1.4 Hz, 1 H), 7.57 (m, 1 H), 7.39 (m, 1 H), 7.34 (dd, *J* = 8.3, 1.0 Hz, 1 H), 6.35 (s, 1 H), 2.62 (s, 3 H), 1.45 (s, 9 H) ppm. **^{13}C NMR (126 MHz, $CDCl_3$):** δ 179.2, 150.1, 150.0, 147.6, 143.8, 141.5, 140.5, 133.4, 132.8, 130.9, 125.7, 116.1, 106.1, 83.5, 28.0, 18.0 ppm. **HRMS (ESI$^+$):** *m/z* 359.1079 $[M+H]^+$. Calcd for $C_{18}H_{19}N_2O_4S^+$ 359.1066.

2-Amino-1-(methylthio)-3*H*-phenoxazin-3-one (questiomycin D; 3)



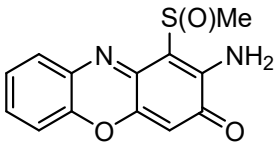
Trifluoroacetic acid (0.5 mL) was added to a solution of compound **7** (0.080 g, 0.22 mmol) in dry CH_2Cl_2 (5 mL) at 0 °C. The solution was warmed to rt over 16 h. On completion, the solvent was removed under reduced pressure. The residue was purified using flash column chromatography (EtOAc in hexane = 20–60%) to afford **questiomycin D** (**3**) as a dark red solid (0.049 g, 86%). **mp:** 249–251 °C. **IR:** *v* 3317, 2922, 1567, 1406, 1217, 1182, 853, 760 cm^{-1}. **^{1}H NMR (500 MHz, d_6-DMSO):** δ 7.82 (d, *J* = 7.8 Hz, 1 H), 7.61–7.47 (m, 2 H), 7.43 (m, 1 H), 6.97 (s, 1 H), 6.39 (s, 1 H), 2.31 (s, 3 H) ppm. **^{13}C NMR (126 MHz, d_6-DMSO):** δ 178.5, 150.2, 148.9, 146.5, 141.9, 133.4, 129.5, 128.6, 125.5, 115.8, 103.4, 102.1, 17.2 ppm. **HRMS (ESI$^+$):** *m/z* 259.0536 $[M+H]^+$. Calcd for $C_{13}H_{11}N_2O_2S^+$ 259.0541. The spectral data were consistent with that reported.[3]

tert-*Butyl-(1-(methylsulfinyl)-3-oxo-3*H*-phenoxazin-2-yl)carbamate (8)*



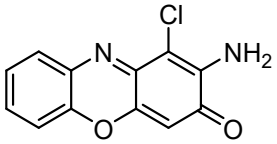
meta-Chloroperoxybenzoic acid (0.043 g, 0.25 mmol) was added to a solution of compound **7** (0.10 g, 0.28 mmol) in CH_2Cl_2 (5 mL) at 0 °C. The solution was stirred at rt for 3 h. On completion, the solvent was removed under reduced pressure. The residue was purified using flash column chromatography (EtOAc in hexane = 10–40%) to afford compound **8** as a bright red solid (0.09 g, 86%). **mp:** 169–170 °C. **IR:** *v* 2979, 2930, 1768, 1713, 1635, 1575, 1367, 1279, 1248, 1160, 1115, 847, 773 cm^{-1}. **^{1}H NMR (500 MHz, $CDCl_3$):** δ 7.85 (dd, *J* = 8.0, 1.5 Hz, 1 H), 7.61 (ddd, *J* = 8.4, 7.5, 1.6 Hz, 1 H), 7.41 (td, *J* = 8.0, 1.2 Hz, 1 H), 7.36 (dd, *J* = 8.3, 1.1 Hz, 1 H), 6.44 (s, 1 H), 3.25 (s, 3 H), 1.50, 1.48 (2 s, 9 H, rotamers) ppm. **^{13}C NMR (126 MHz, $CDCl_3$):** δ 179.3, 150.5, 150.4, 149.3, 145.8, 143.9, 141.4, 140.4, 134.2, 132.5, 131.0, 126.1, 116.5, 106.8, 39.1, 28.2, 28.1 ppm. **HRMS (ESI$^+$):** *m/z* 375.1007 $[M+H]^+$. Calcd for $C_{18}H_{19}N_2O_5S^+$ 375.1015.

2-Amino-1-(methylsulfinyl)-3*H*-phenoxazin-3-one (questiomycin C; 2)



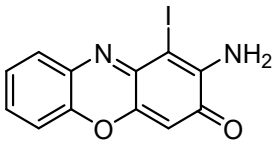
Trifluoroacetic acid (0.2 mL) was added to a solution of compound **8** (0.022 g, 0.059 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C. The solution was warmed to rt over 16 h. On completion, the volatile was removed under reduced pressure. The residue was purified using flash column chromatography (EtOAc: hexane = 20–60%) to afford **questiomycin C** (**2**) as a dark red solid (0.014 g, 88%). **mp:** 267–268 °C. **IR:** *v* 3374, 3234, 2917, 1572, 1407, 1312, 1185, 852, 766, 600 cm^{-1}. **^{1}H NMR (500 MHz, d_6-DMSO):** δ 8.45 (s, 1 H), 7.70 (d, *J* = 7.8 Hz, 1 H), 7.53 – 7.52 (m, 2 H), 7.42 (ddd, *J* = 8.4, 5.4, 3.3 Hz, 1 H), 7.32 (s, 1 H), 6.46 (s, 1 H), 2.99 (s, 3 H) ppm. **^{13}C NMR (126 MHz, d_6-DMSO):** δ 178.4, 148.9, 147.5, 144.6, 142.2, 132.6, 129.8, 128.1, 125.7, 116.1, 104.5, 103.7 [signal for Me obscured by solvent signal] ppm. **HRMS (ESI$^+$):** *m/z* 275.0487 $[M+H]^+$. Calcd for $C_{13}H_{11}N_2O_3S^+$ 275.0490. The spectral data were consistent with that reported.[3]

2-Amino-1-chloro-3*H*-phenoxazin-3-one (9)



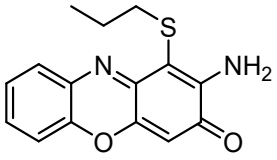
N-Chlorosuccinimide (0.164 g, 1.23 mmol.) was added to compound **1** (0.20 g, 0.94 mmol) in $CHCl_3$ (100 mL) at 0 °C. The solution was stirred at rt for another 16 h. On completion, the organic phase was washed with sat. aq. $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified using flash column chromatography (CH_2Cl_2) to afford compound **9** as a red solid (0.19 g, 82%). **mp:** 246–248 °C. **IR:** *v* 3342, 2971, 1739, 1593, 1435, 1366, 1229, 1217, 905, 758 cm^{-1}. **^{1}H NMR (500 MHz, *d*$_6$-DMSO):** δ 7.81 (d, *J* = 7.7 Hz, 1 H), 7.52–7.54 (m, 2 H), 7.44 (d, *J* = 3.5 Hz, 1 H), 7.06 (s, 2 H), 6.39 (s, 1 H) ppm. **^{13}C NMR (126 MHz, *d*$_6$-DMSO):** δ 177.8, 149.3, 143.9, 143.6, 142.2, 133.1, 129.9, 128.5, 125.6, 115.9, 103.1, 102.5 ppm. **HRMS (ESI$^+$):** *m/z* 247.0271 $[M+H]^+$. Calcd for $C_{12}H_8ClN_2O_2^+$ 247.0274.

2-Amino-1-iodo-3*H*-phenoxazin-3-one (10)



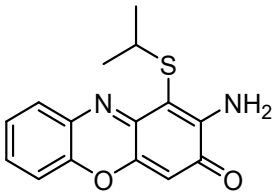
N-Iodosuccinimide (0.276 g, 1.23 mmol) was added to compound **1** (0.20 g, 0.94 mmol) in $CHCl_3$ (100 mL) at 0 °C. The solution was stirred at rt for another 16 h. On completion, the organic phase was washed with sat. aq. $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified using flash column chromatography (CH_2Cl_2) to afford compound **10** as an orange solid (0.29 g, 90%). **mp:** 240–241.5 °C. **IR:** *v* 3015, 2971, 1739, 1435, 1366, 1229, 1217, 900 cm^{-1}. **^{1}H NMR (500 MHz, *d*$_6$-DMSO):** δ 7.79 (d, *J* = 7.8 Hz, 1 H), 7.56–7.51 (m, 2 H), 7.44 (m, 1 H), 6.82 (br s, 2 H), 6.38 (s, 1 H) ppm. **^{13}C NMR (125 MHz, *d*$_6$-DMSO):** δ 177.2, 149.98, 149.9, 145.8, 142.6, 133.9, 130.3, 128.8, 126.0, 116.1, 103.0, 74.3 ppm. **HRMS (ESI$^+$):** *m/z* 338.9619 $[M+H]^+$. Calcd for $C_{12}H_8IN_2O_2^+$ 338.9630.

2-Amino-1-(propylthio)-3H-phenoxazin-3-one (11)



Sodium propane-1-thiolate (0.075 g, 0.767 mmol) was added to a solution of compound **7** (0.20 g, 0.511 mmol) and 1-propanethiol (0.093 mL, 1.02 mmol) in dry THF (5 mL) at 0 °C. The solution was warmed to rt over 16 h. On completion, sat. aq. NH_4Cl (10 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was dissolved in dry CH_2Cl_2 (5 mL), cooled to 0 °C, and then trifluoroacetic acid (0.5 mL) was added. The mixture was warmed to rt over 16 h. On completion, sat. aq. $NaHCO_3$ (5 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a red residue that was purified using flash column chromatography (EtOAc in hexane = 10–30%) to afford compound **11** as a dark red solid (0.073 g, 50%). **mp:** 291.5–293.5 °C. **IR:** *v* 3323, 3015, 2971, 1739, 1568, 1366, 1229, 1217, 853, 761 cm^{-1}. **^{1}H NMR (500 MHz, $CDCl_3$):** δ 7.92 (d, *J* = 7.7 Hz, 1 H), 7.45–7.48 (m, 1 H), 7.42–7.37 (m, 2 H), 6.41 (s, 1 H), 5.99 (brs, 2 H), 2.88 (t, *J* = 7.2 Hz, 2 H), 1.63–1.55 (m, 2 H), 0.99 (t, *J* = 7.2 Hz, 3 H) ppm. **^{13}C NMR (126 MHz, $CDCl_3$):** δ 178.8, 150.7, 149.5, 147.1, 142.7, 134.0, 129.9, 129.7, 125.4, 115.9, 104.2, 102.7, 37.0, 23.4, 13.7 ppm. **HRMS (ESI$^+$):** *m/z* 287.0851 $[M+H]^+$. Calcd for $C_{15}H_{15}N_2O_2S^+$ 287.0854.

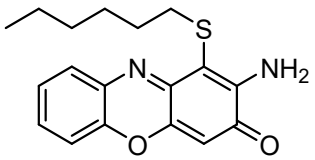
2-Amino-1-(isopropylthio)-3H-phenoxazin-3-one (12)



Sodium propane-2-thiolate (0.075 g, 0.767 mmol) was added to a solution of compound **7** (0.20 g, 0.511 mmol) and 2-propanethiol (0.095 mL, 1.02 mmol) in dry THF (5 mL) at 0 °C. The solution was warmed to rt over 16 h. On completion, sat. aq. NH_4Cl (10 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (10 mL × 3), the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was retaken in dry CH_2Cl_2 (5 mL) and cooled to 0 °C, followed by the addition of trifluoroacetic acid (0.5 mL). The mixture was then warmed to rt over 16 h. On completion, sat. aq. $NaHCO_3$ (5 mL) was added,

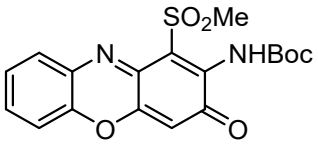
and the aqueous phase was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a dark red residue that was then purified using flash column chromatography (EtOAc in hexane = 10–30%) to afford compound **12** as a dark red solid (0.091 g, 61%). **mp:** 197–199 °C. **IR:** *v* 3328, 3016, 2971, 1739, 1568, 1366, 1229, 1217, 900, 761 cm^{-1}. **^{1}H NMR (500 MHz, $CDCl_3$):** δ 7.91 (d, *J* = 7.8 Hz, 1 H), 7.47–7.43 (m, 1 H), 7.40–7.34 (m, 2 H), 6.42 (s, 1 H), 6.00 (br s, 2 H), 3.61 (septet, *J* = 6.5 Hz, 1 H), 1.28 (d, *J* = 6.5 Hz, 6 H) ppm. **^{13}C NMR (126 MHz, $CDCl_3$):** δ 178.9, 150.7, 149.9, 147.3, 142.6, 134.0, 129.9, 129.7, 125.4, 115.9, 104.3, 102.6, 38.9, 23.5 ppm. **HRMS (ESI^+):** *m/z* 287.0847 $[M+H]^+$. Calcd for $C_{15}H_{15}N_2O_2S^+$ 287.0854.

2-Amino-1-(hexylthio)-3*H*-phenoxazin-3-one (13)



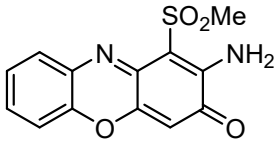
Sodium hexane-1-thiolate (0.108 g, 0.767 mmol) was added to a solution of compound **7** (0.20 g, 0.511 mmol) and 1-hexanethiol (0.144 mL, 1.02 mmol) in dry THF (5 mL) at 0 °C. The solution was warmed to rt over 16 h. On completion, sat. aq. NH_4Cl (10 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (10 mL × 3), the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was retaken in dry CH_2Cl_2 (5 mL) and cooled to 0 °C, followed by the addition of trifluoroacetic acid (0.5 mL). The mixture was then warmed to rt over 16 h. On completion, sat. aq. $NaHCO_3$ (5 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a dark red residue that was then purified using flash column chromatography (EtOAc in hexane = 10–30%) to afford compound **13** as a dark red solid (0.072 g, 43%). **mp:** 246.5–248 °C. **IR:** *v* 3320, 2971, 2924, 1739, 1568, 1452, 1366, 1228, 1217, 922, 853, 761 cm^{-1}. **^{1}H NMR (600 MHz, $CDCl_3$):** δ 7.96 (d, *J* = 7.7 Hz, 1 H), 7.46 (t, *J* = 7.7 Hz, 1 H), 7.42–7.35 (m, 2 H), 6.42 (s, 1 H), 6.01 (brs, 2 H), 2.90 (t, *J* = 7.4 Hz, 2 H), 1.58–1.52 (m, 2 H), 1.42–1.35 (m, 2 H), 1.31–1.18 (m, 4H), 0.85 (t, *J* = 6.9 Hz, 3 H) ppm. **^{13}C NMR (151 MHz, $CDCl_3$):** δ 178.7, 150.8, 149.6, 147.0, 142.7, 133.8, 130.0, 129.6, 125.5, 115.9, 104.3, 102.7, 35.0, 31.6, 30.0, 28.7, 22.7, 14.2 ppm. **HRMS (ESI^+):** *m/z* 329.1320 $[M+H]^+$. Calcd for $C_{18}H_{21}N_2O_2S^+$ 329.1324.

tert-*Butyl (1-(methylsulfonyl)-3-oxo-3*H*-phenoxazin-2-yl)carbamate (14)*



meta-Chloroperoxybenzoic acid (0.106 g, 0.62 mmol) was added to a solution of compound **7** (0.10 g, 0.28 mmol) in CH_2Cl_2 (5 mL) at 0 °C. The solution was stirred at rt for 5 h. On completion, the solvent was removed under reduced pressure. The residue was purified using flash column chromatography (EtOAc in hexane = 20–50%) to afford compound **14** as a bright red solid (0.096 g, 88%). **mp:** 159–161.5 °C. **IR:** *v* 2979, 2927, 1806, 1771, 1640, 1571, 1332, 1287, 1252, 1151, 1117, 851, 775 cm^{-1}. **^{1}H NMR (500 MHz, $CDCl_3$):** δ 7.91 (d, *J* = 8.0 Hz, 1 H), 7.64 (m, 1 H), 7.44 (t, *J* = 7.7 Hz, 1 H), 7.38 (d, *J* = 8.3 Hz, 1 H), 6.51 (s, 1 H), 3.47 (s, 3 H), 1.46 (s, 9 H) ppm. **^{13}C NMR (126 MHz, $CDCl_3$):** δ 179.0, 149.6, 149.3, 144.9, 143.4, 141.8, 135.7, 134.6, 132.4, 131.1, 126.3, 116.3, 107.2, 84.3, 44.9, 28.0 ppm. **HRMS (ESI^+):** *m/z* 391.0958 $[M+H]^+$, Calcd for $C_{18}H_{19}N_2O_6S^+$ 391.0964.

2-Amino-1-(methylsulfonyl)-3*H*-phenoxazin-3-one (15)



Trifluoroacetic acid (0.2 mL) was added to a solution of compound **14** (0.055 g, 0.14 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C. The solution was warmed to rt over 16 h. On completion, the solvent was removed under reduced pressure. The residue was purified using flash column chromatography (EtOAc in hexane = 20–60%) to afford compound **15** as a brown solid (0.037 g, 90%). **mp:** 241.0–241.1 °C. **IR:** *v* 3408, 3308, 2925, 1593, 1578, 1307, 1114, 784, 764 cm^{-1}. **^{1}H NMR (500 MHz, d_6-DMSO):** δ 8.32 (d, *J* = 2.4 Hz, 1 H), 7.87–7.73 (m, 2 H), 7.63–7.52 (m, 2 H), 7.47 (m, 1 H), 6.57 (s, 1 H), 3.54 (s, 3 H) ppm. **^{13}C NMR (126 MHz, d_6-DMSO):** δ 177.4, 149.6, 146.8, 144.3, 141.9, 132.3, 130.4, 128.6, 125.9, 116.0, 104.2, 104.1, 45.9 ppm. **HRMS (ESI^+):** *m/z* 291.0440 $[M+H]^+$, Calcd for $C_{13}H_{11}N_2O_4S^+$ 291.0434.

Supplementary references

1 J. Hu, D. E. Berthold, Y. Wang, X. Xiao and H. D. Laughinghouse, *Harmful Algae*, 2022, **120**, 102347.

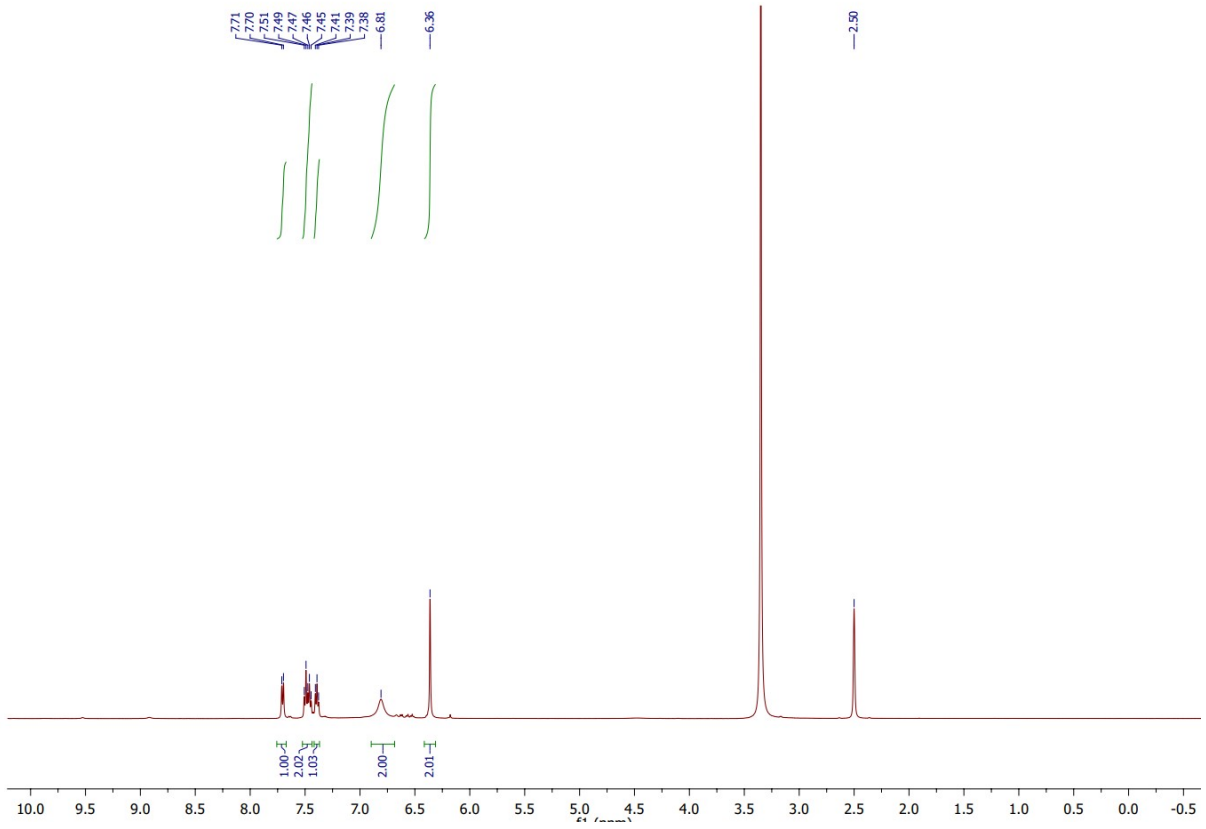
2 A. B. Pangborn, M. A. Giardello, R. H. Grubbs, R. K. Rosen and F. J. Timmers, *Organometallics*, 1996, **15**, 1518-1520.

3 S. Umetsu, M. Kanda, I. Imai, R. Sakai and M. J. Fujita, *Molecules*, 2019, **24**, 4522.

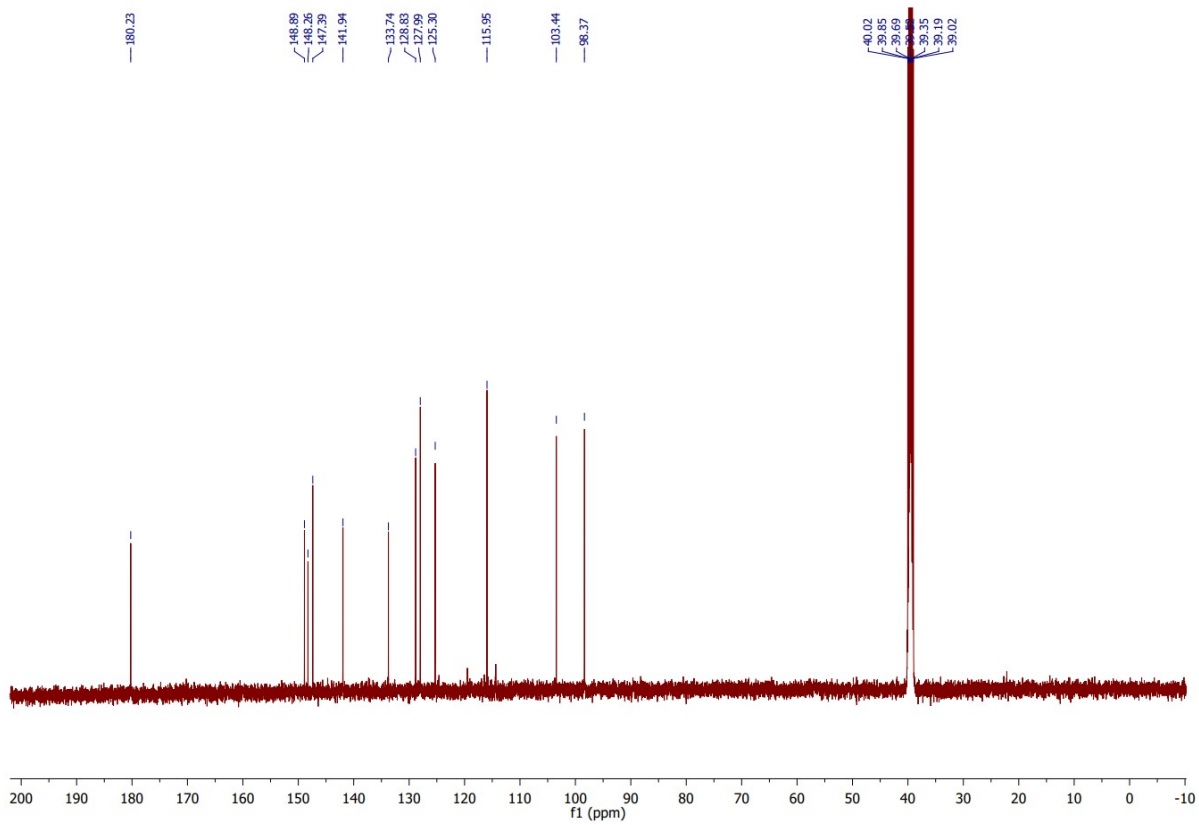
NMR spectra

2-Amino-3*H*-phenoxazin-3-one (questiomycin A; 1)

^{1}H NMR spectrum

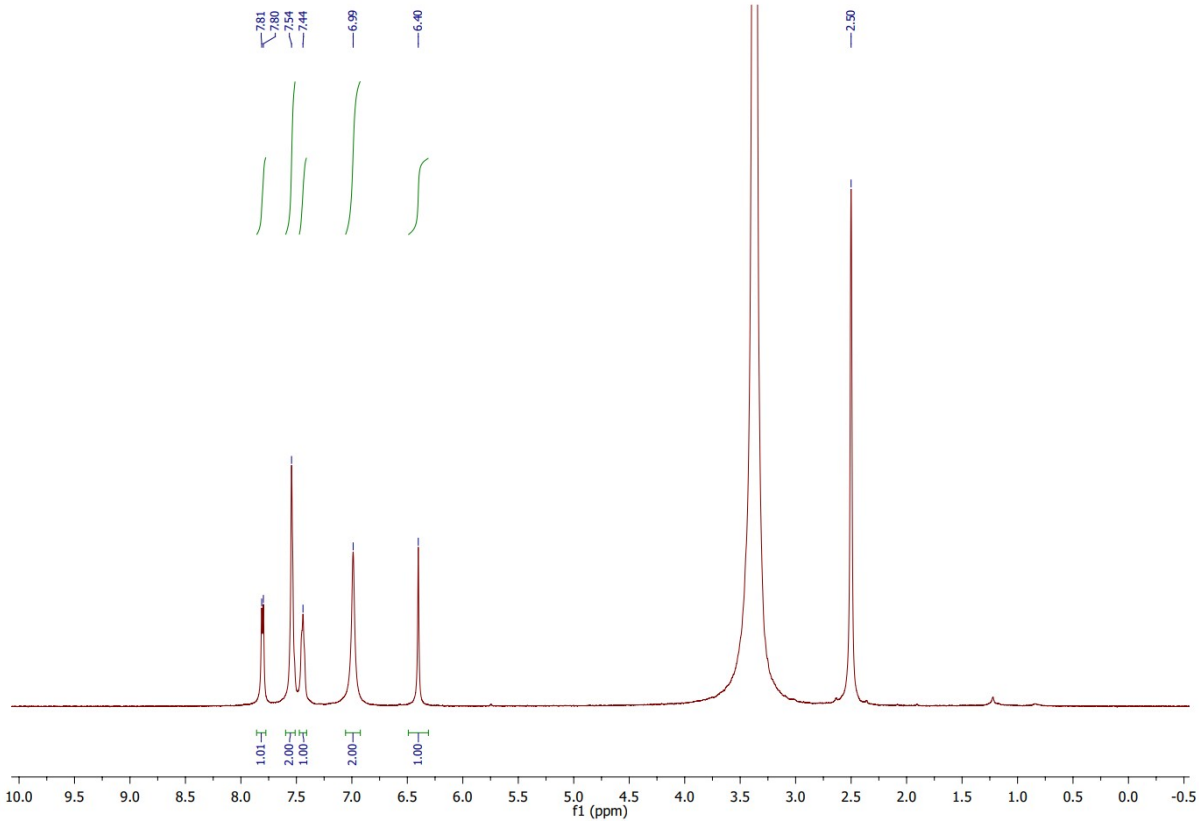


^{13}C NMR spectrum

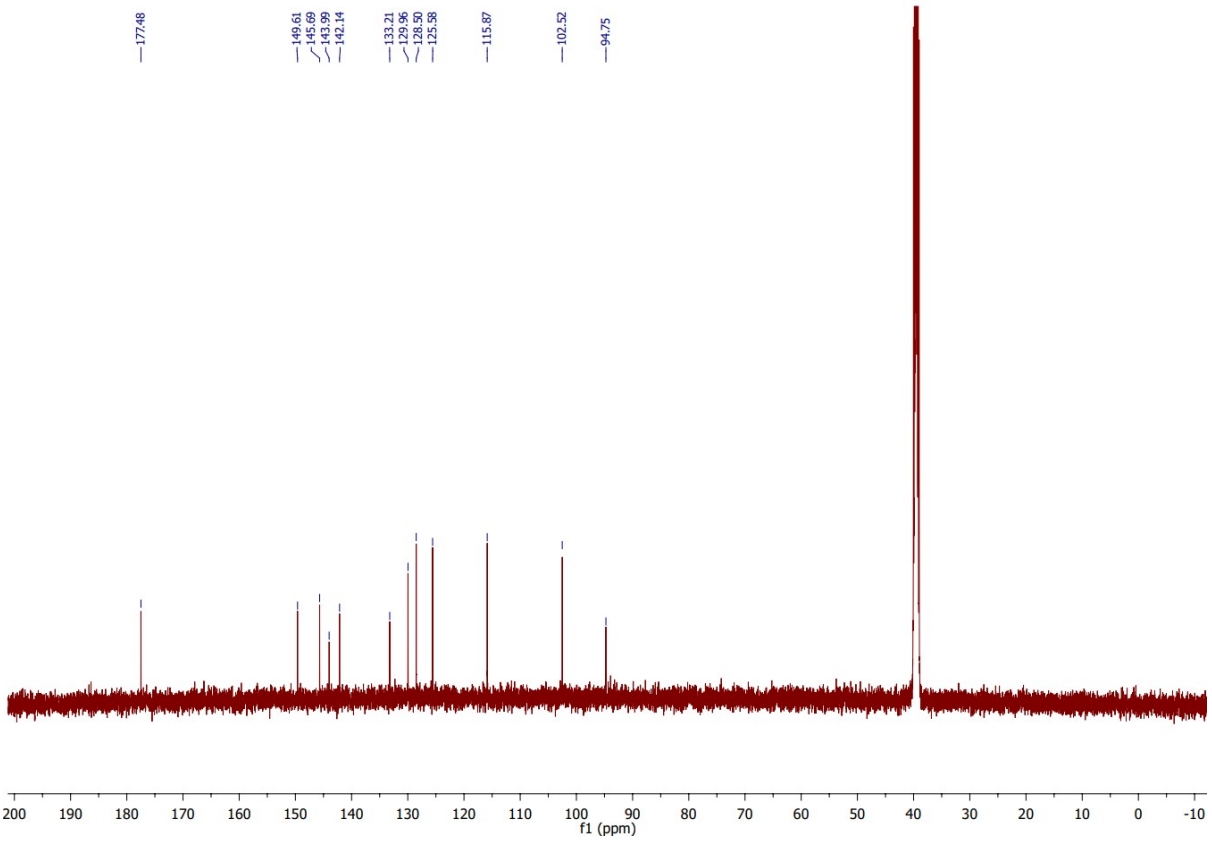


2-Amino-1-bromo-3*H*-phenoxazin-3-one (questiomycin E; 4)

^{1}H NMR spectrum

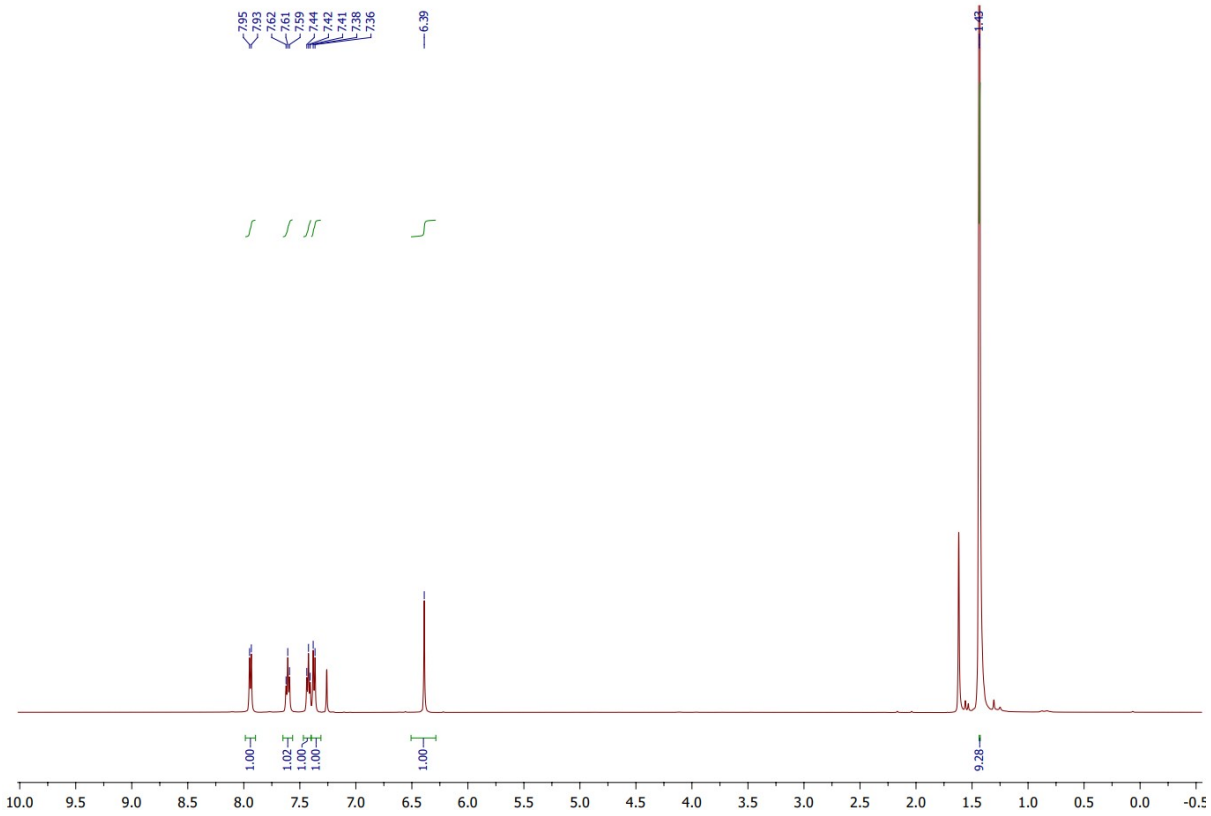


^{13}C NMR spectrum

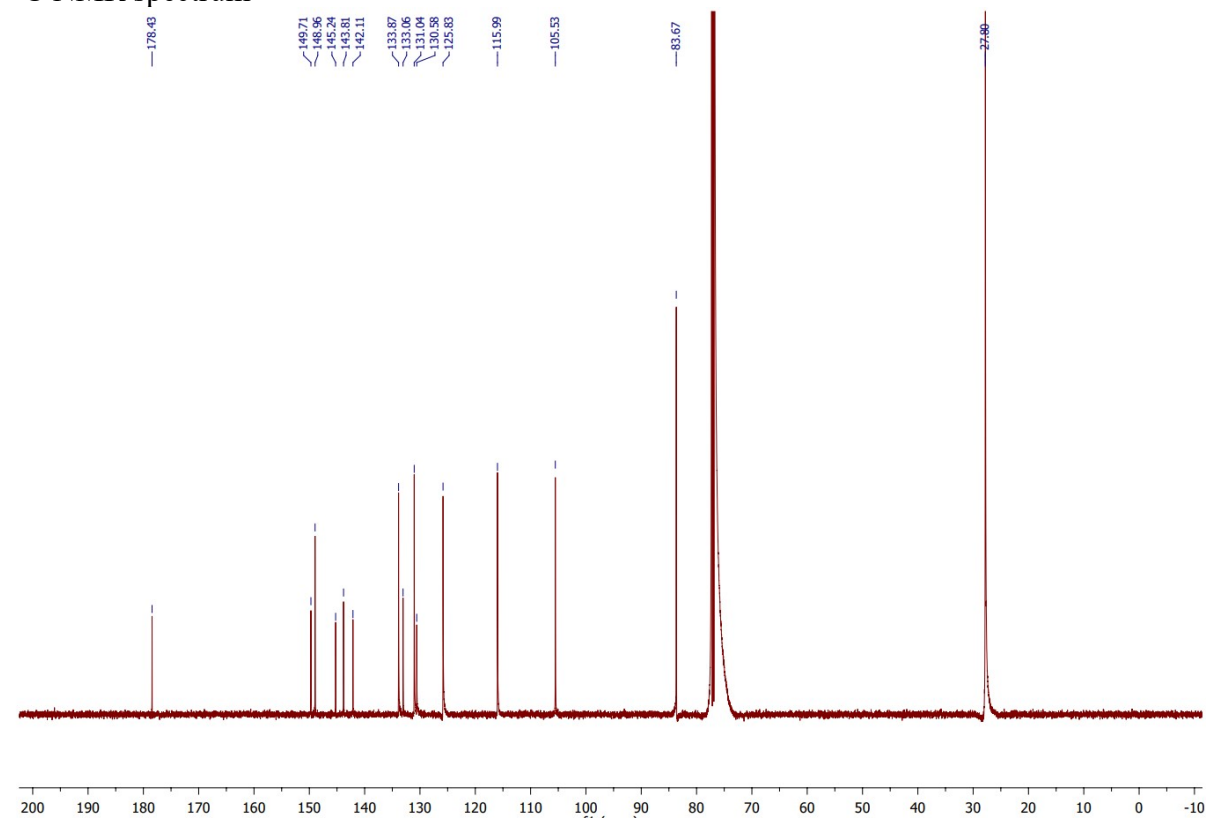

177.48
149.61
145.69
143.99
142.14
133.21
129.96
128.50
125.58
115.87
102.52
94.75
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

tert-*Butyl (1-bromo-3-oxo-3*H*-phenoxazin-2-yl)carbamate (6)*

^{1}H NMR spectrum

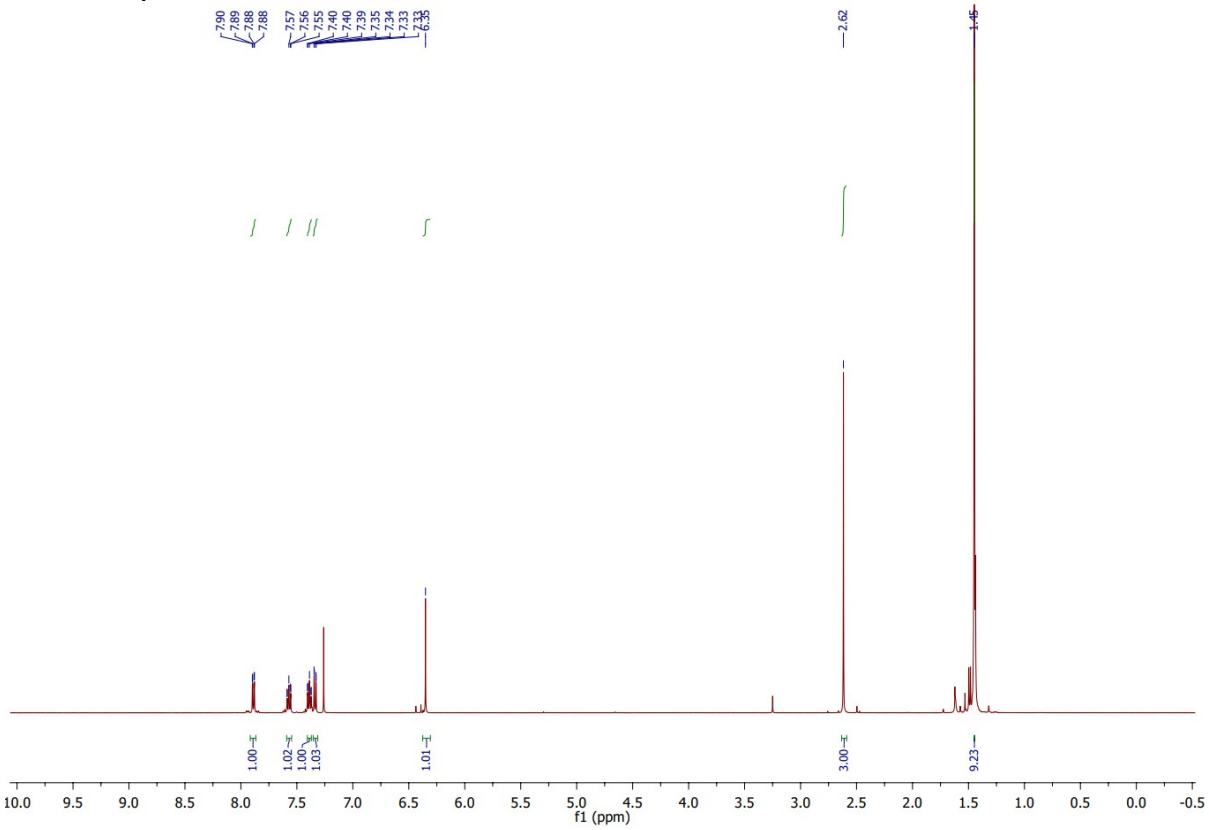


^{13}C NMR spectrum

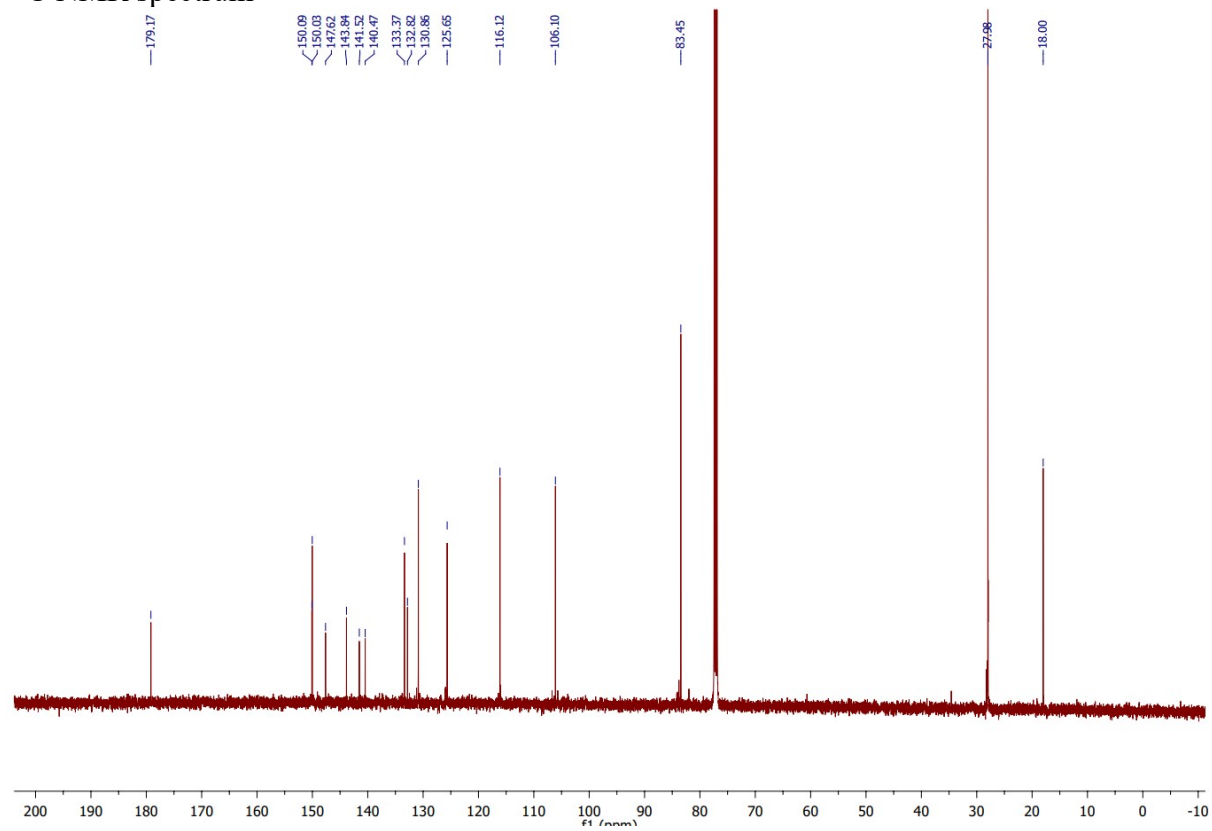


tert-*Butyl (1-(methylthio)-3-oxo-3*H-*phenoxazin-2-yl)carbamate (7)*

^{1}H NMR spectrum

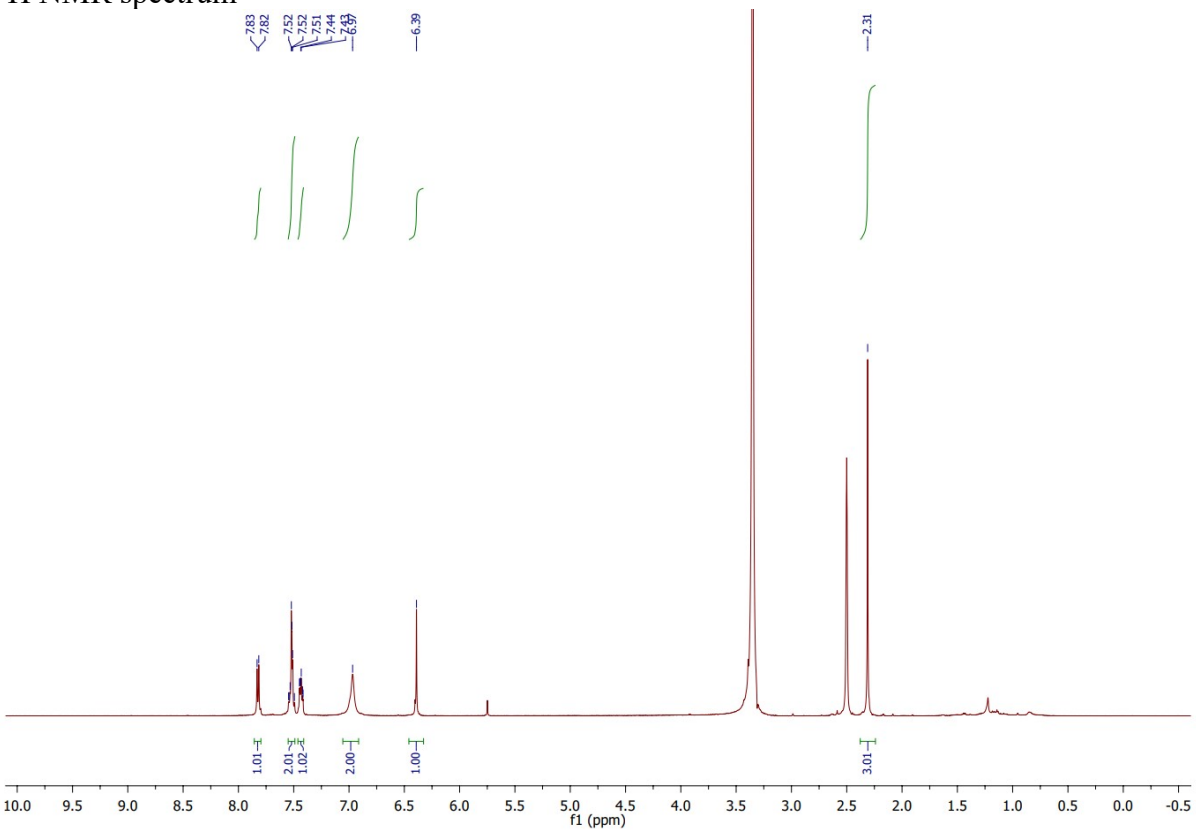


^{13}C NMR spectrum

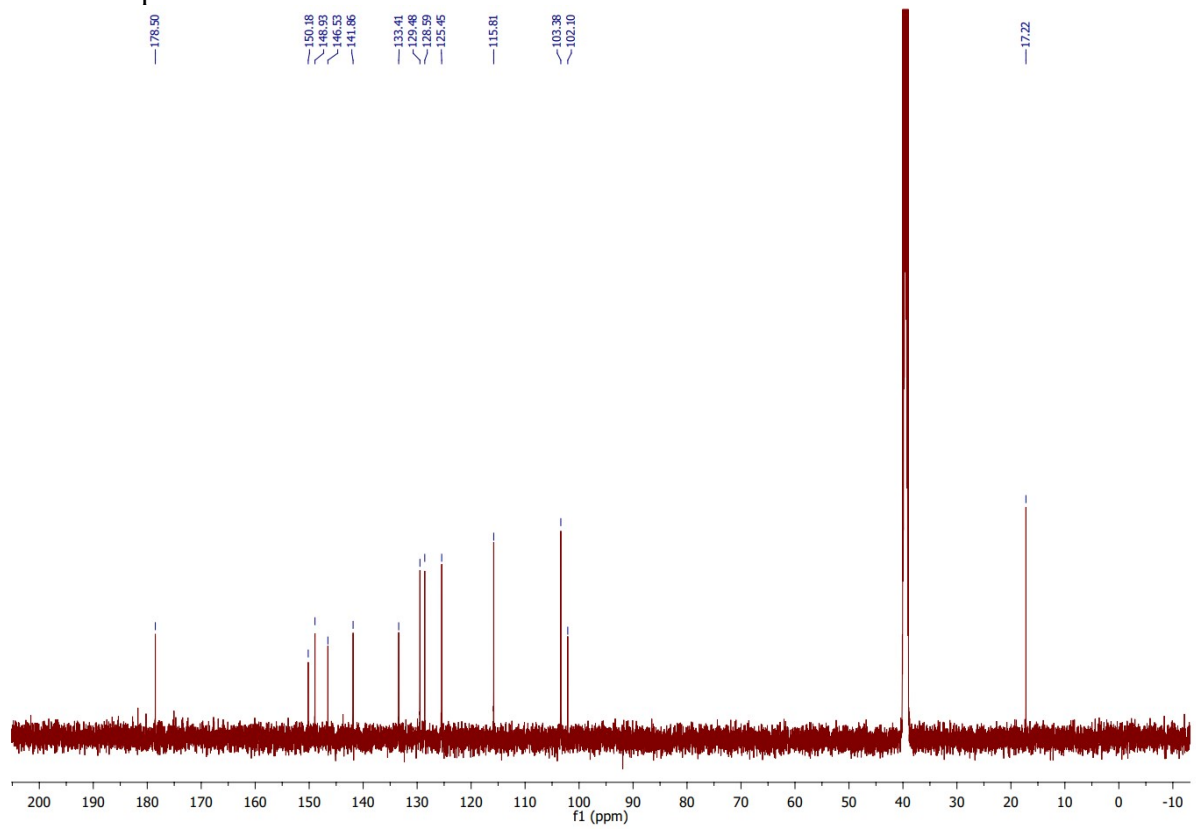


2-Amino-1-(methylthio)-3*H*-phenoxazin-3-one (questiomycin D; 3)

^{1}H NMR spectrum

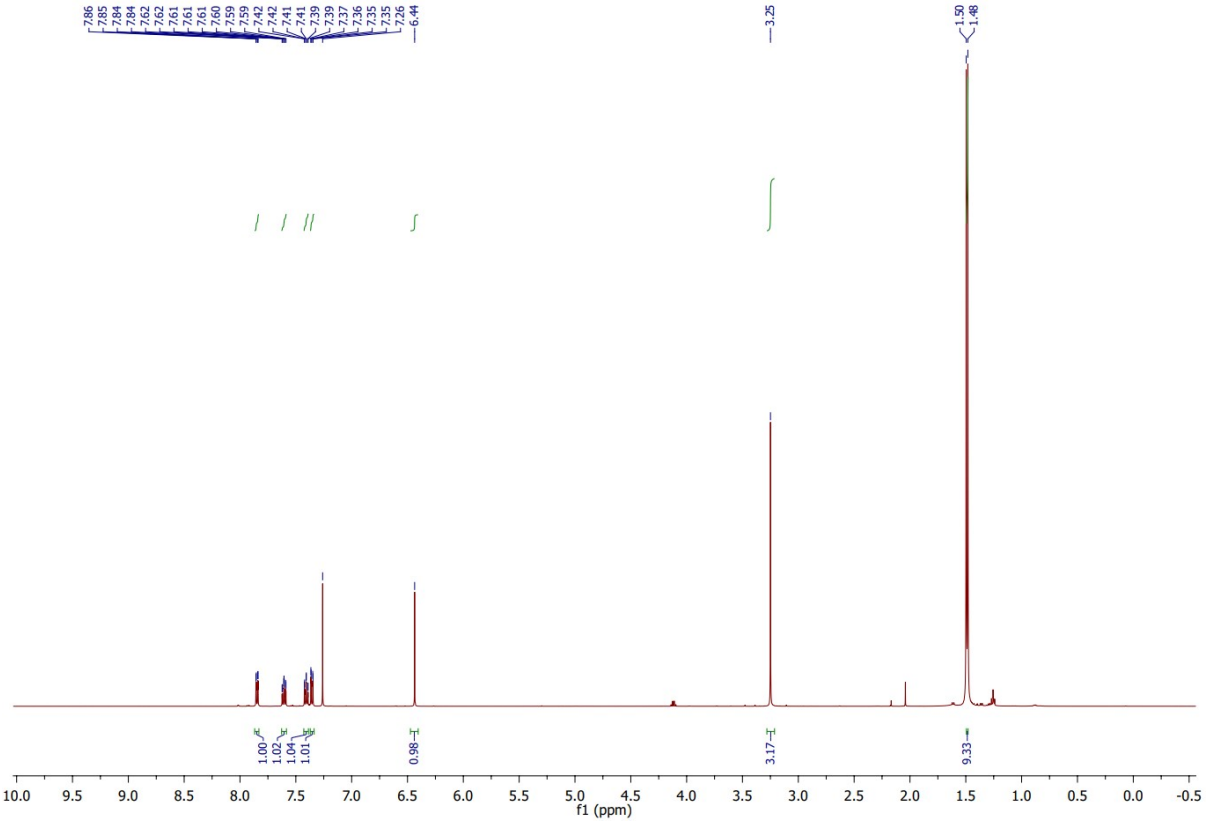


^{13}C NMR spectrum

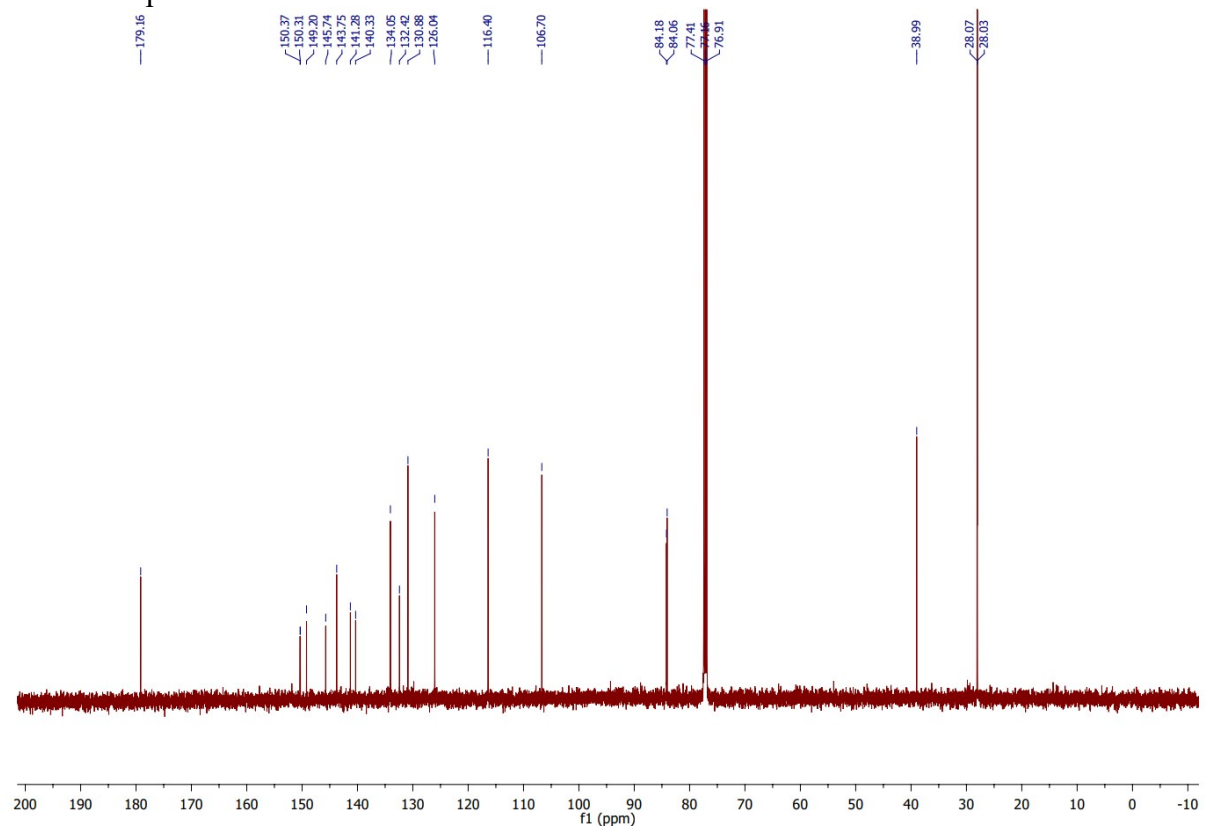


tert-*Butyl (1-(methylsulfinyl)-3-oxo-3*H*-phenoxazin-2-yl)carbamate (8)*

^{1}H NMR spectrum

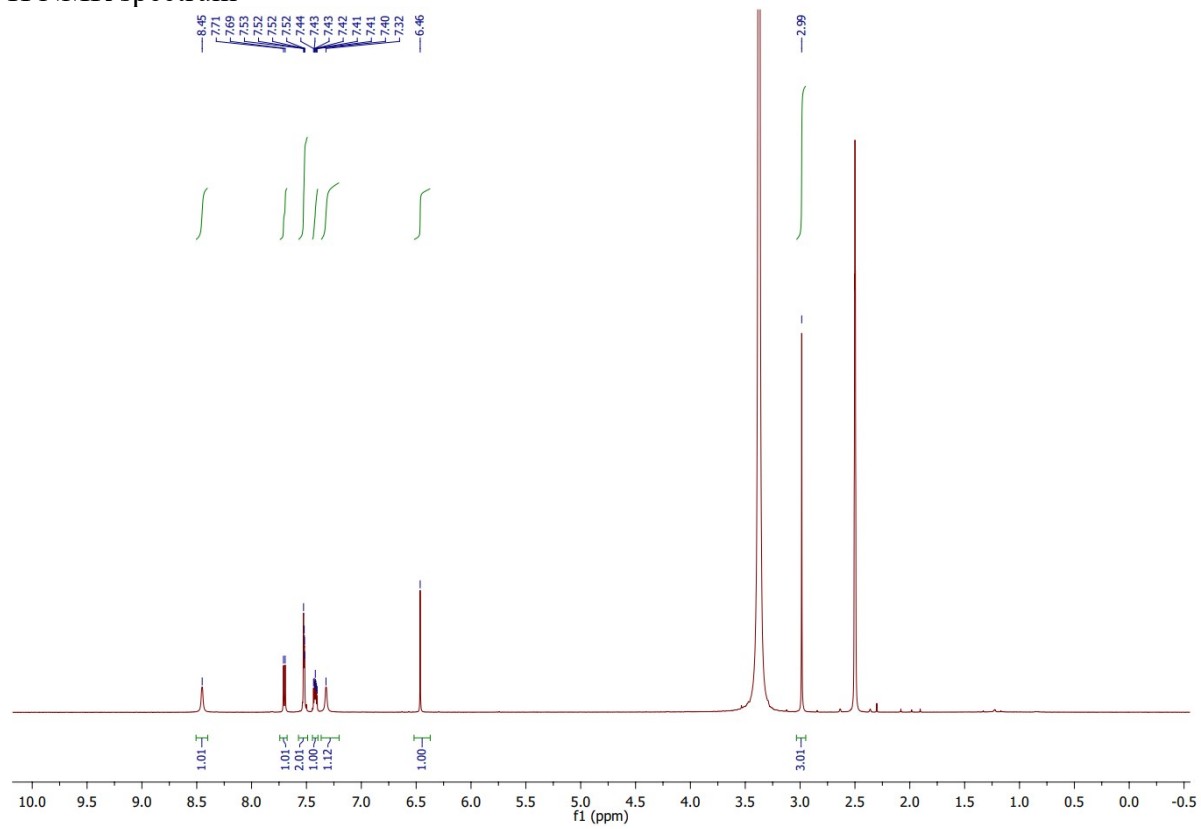


^{13}C NMR spectrum

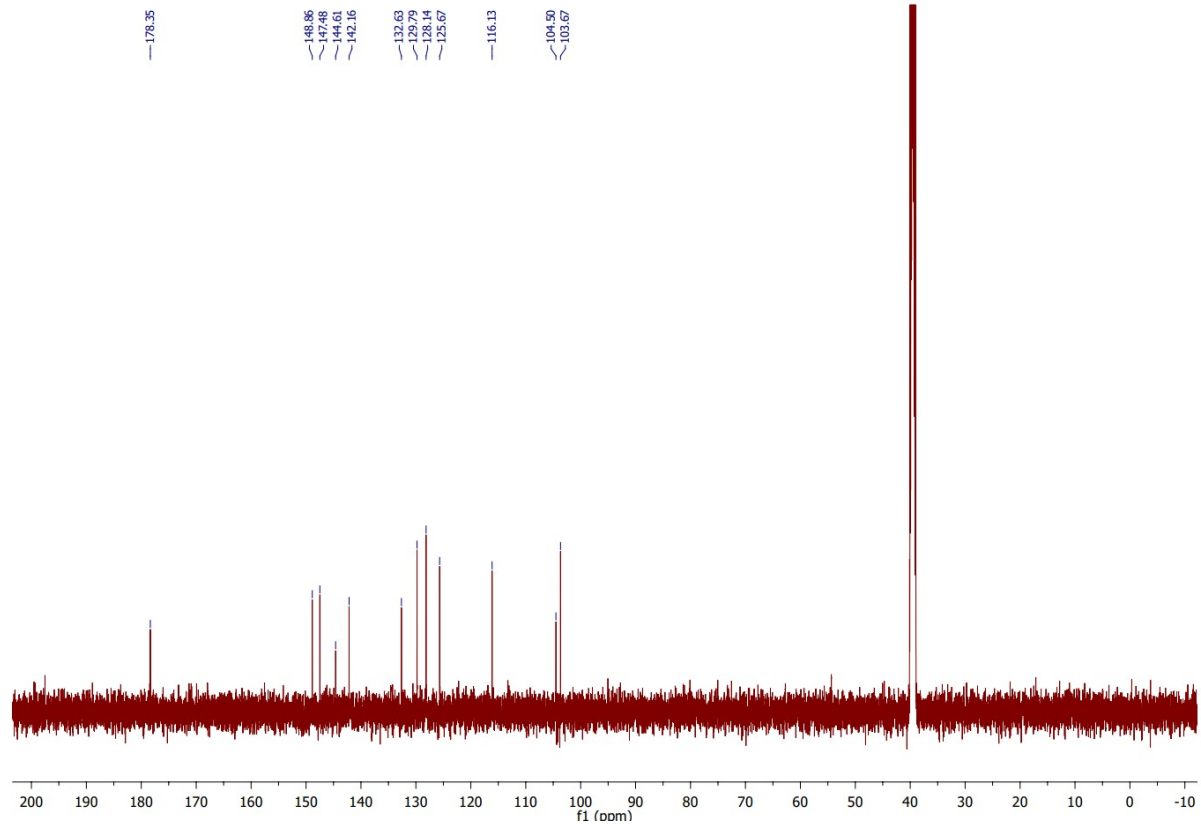


2-Amino-1-(methylsulfinyl)-3H-phenoxazin-3-one (questiomycin C; 2)

^{1}H NMR spectrum

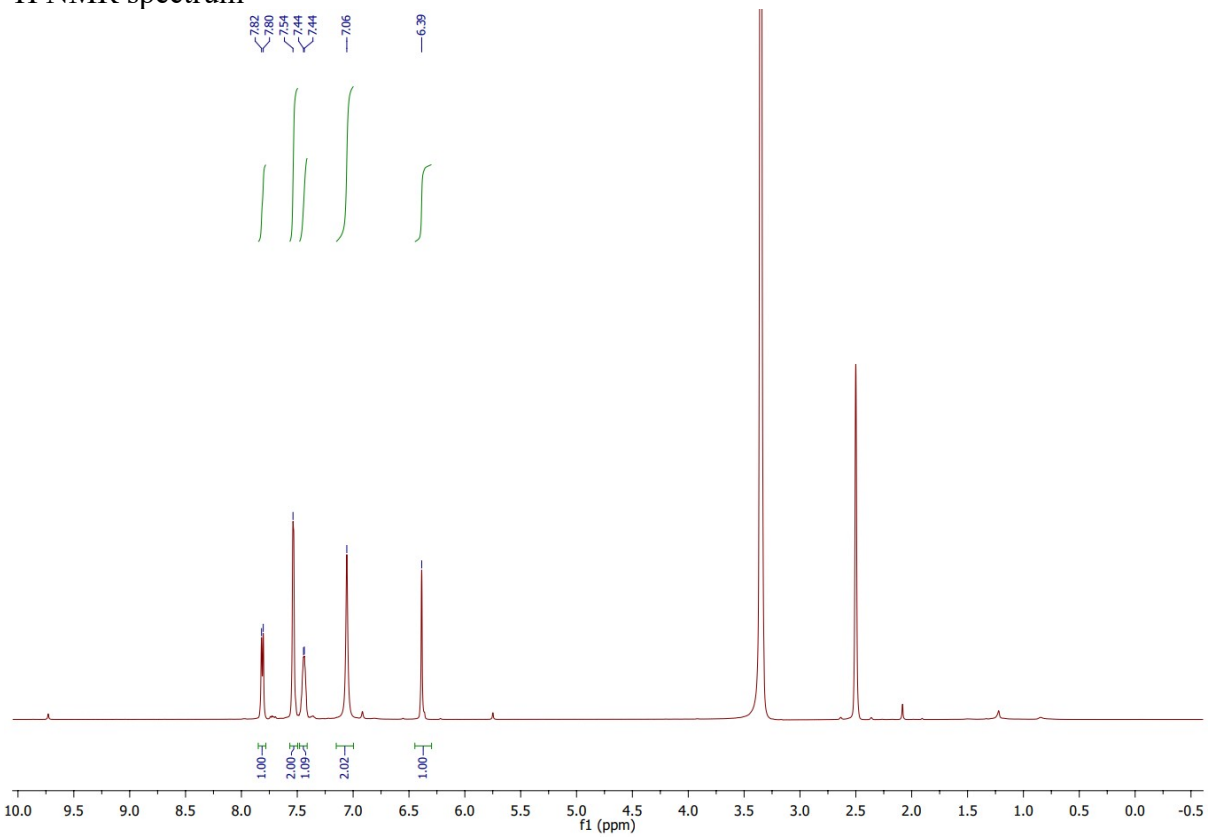


^{13}C NMR spectrum

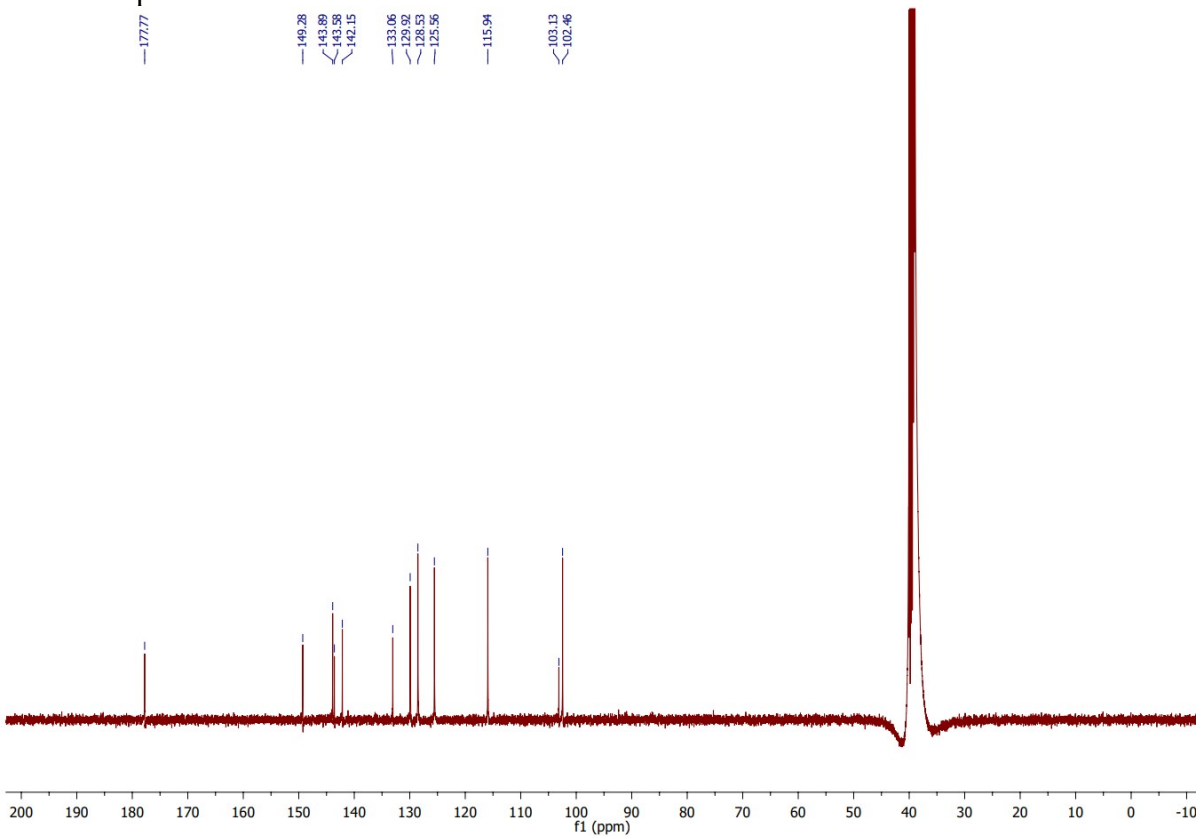


2-Amino-1-chloro-3*H*-phenoxazin-3-one (9)

^{1}H NMR spectrum

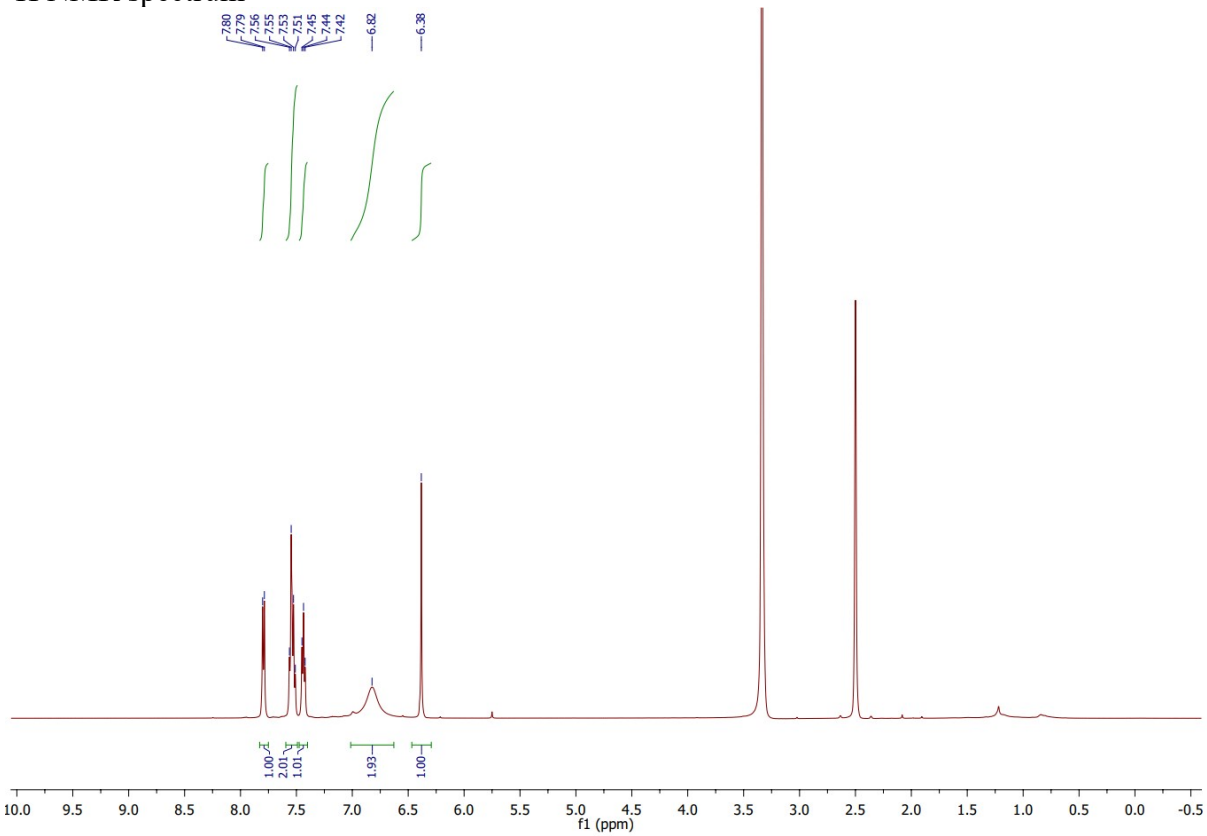


^{13}C NMR spectrum

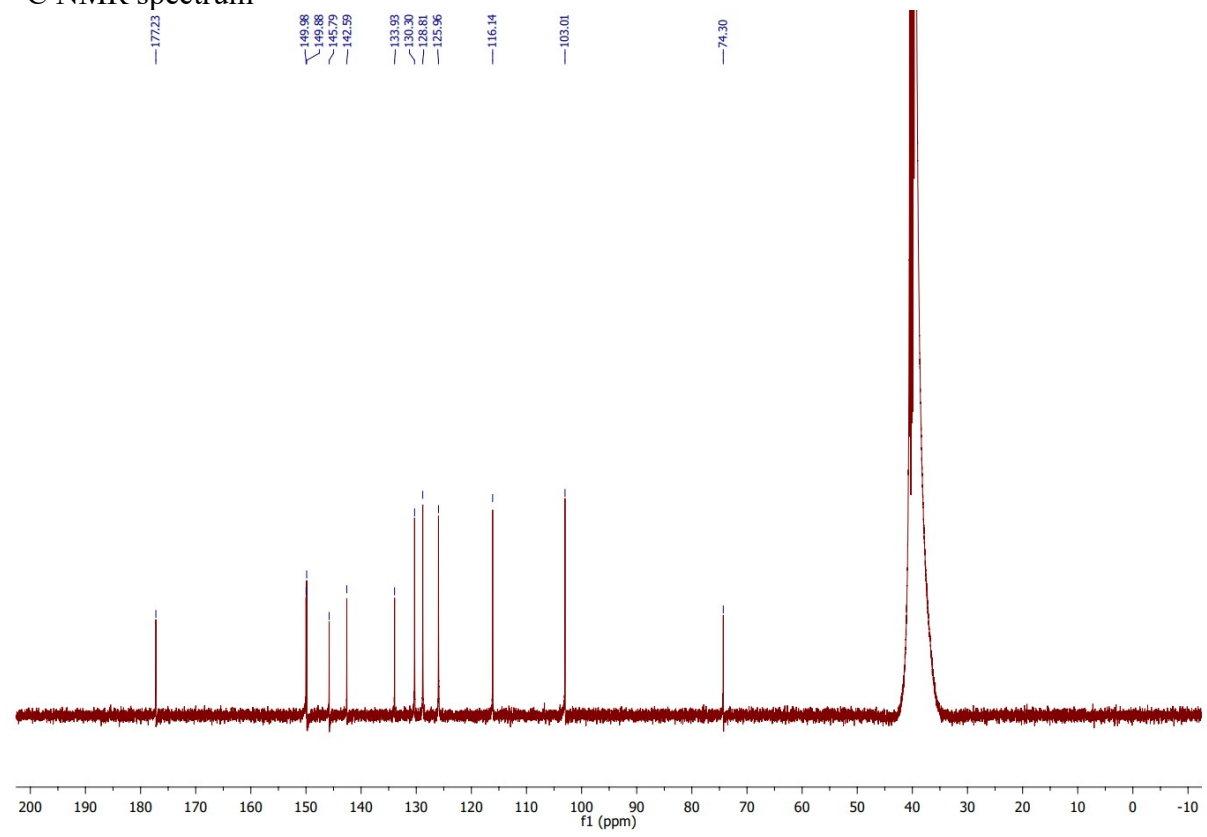


2-Amino-1-iodo-3*H*-phenoxazin-3-one (10)

^{1}H NMR spectrum

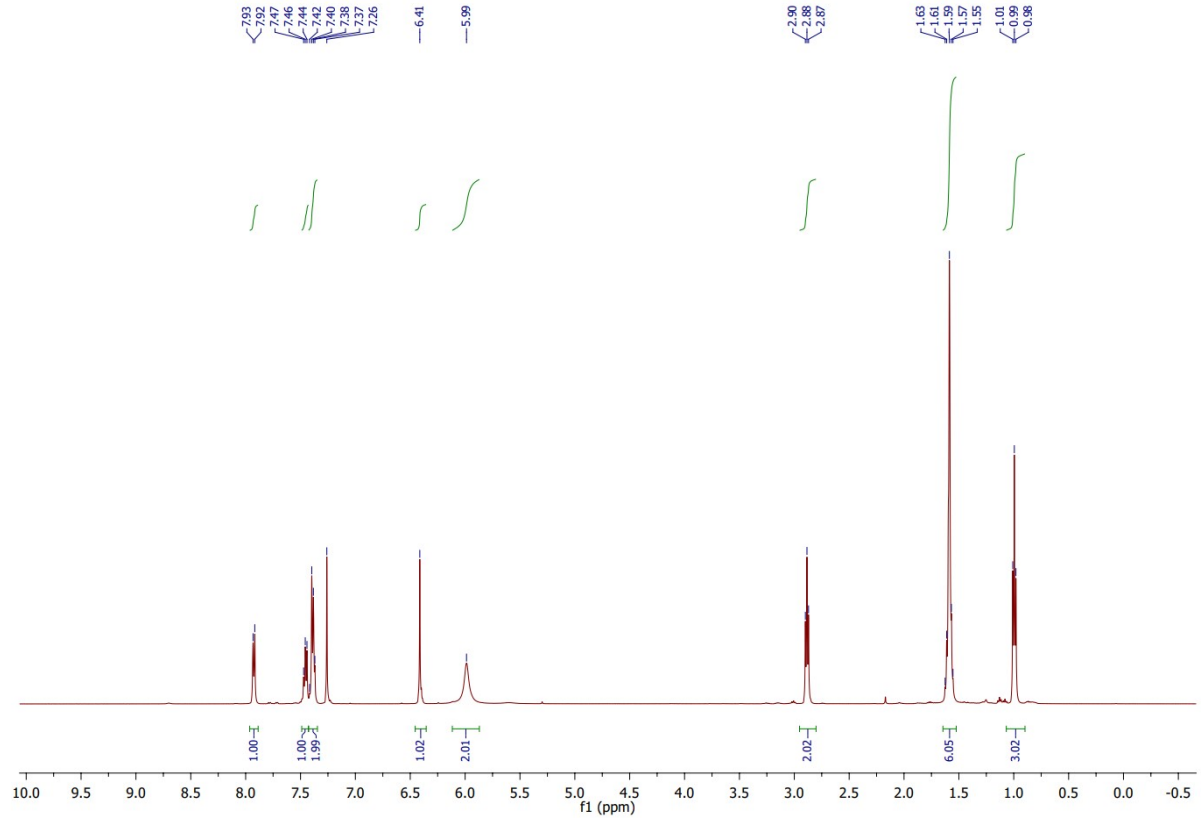


^{13}C NMR spectrum

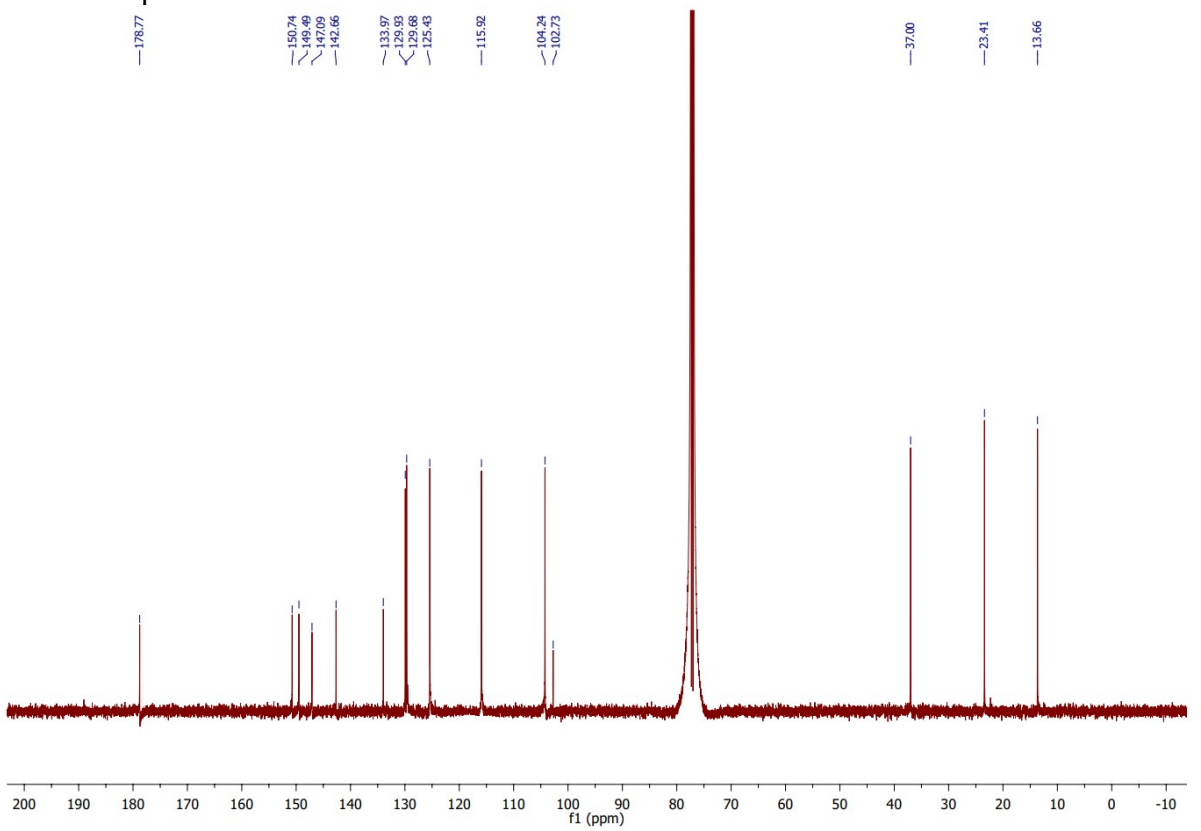


2-Amino-1-(propylthio)-3H-phenoxazin-3-one (11)

^{1}H NMR spectrum

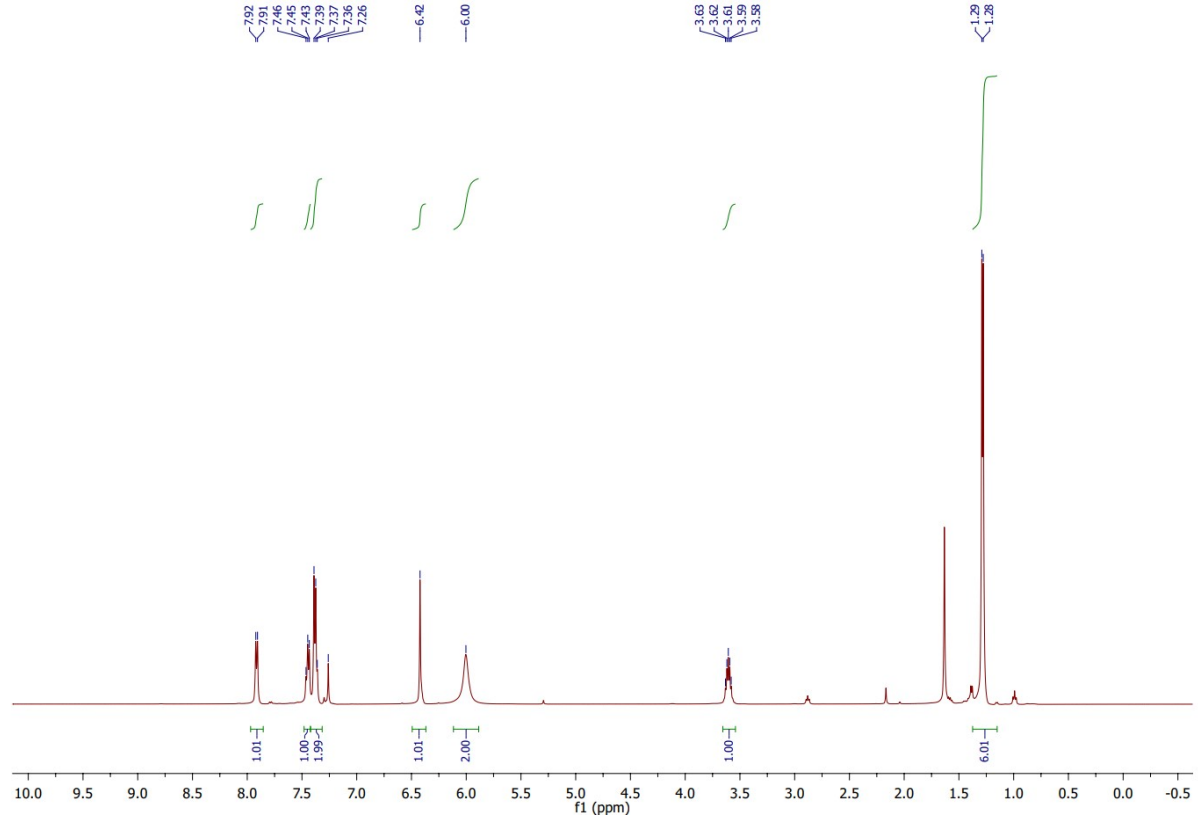


^{13}C NMR spectrum

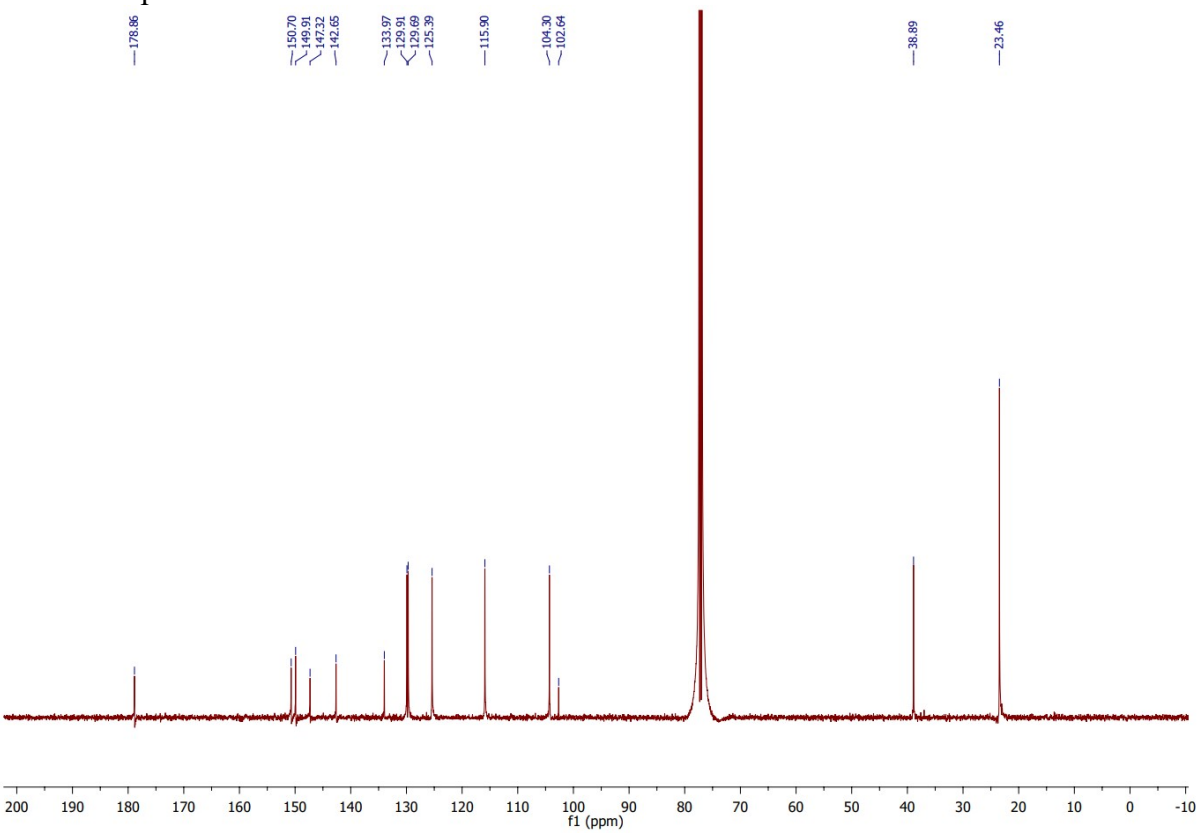


2-Amino-1-(isopropylthio)-3H-phenoxazin-3-one (12)

^{1}H NMR spectrum

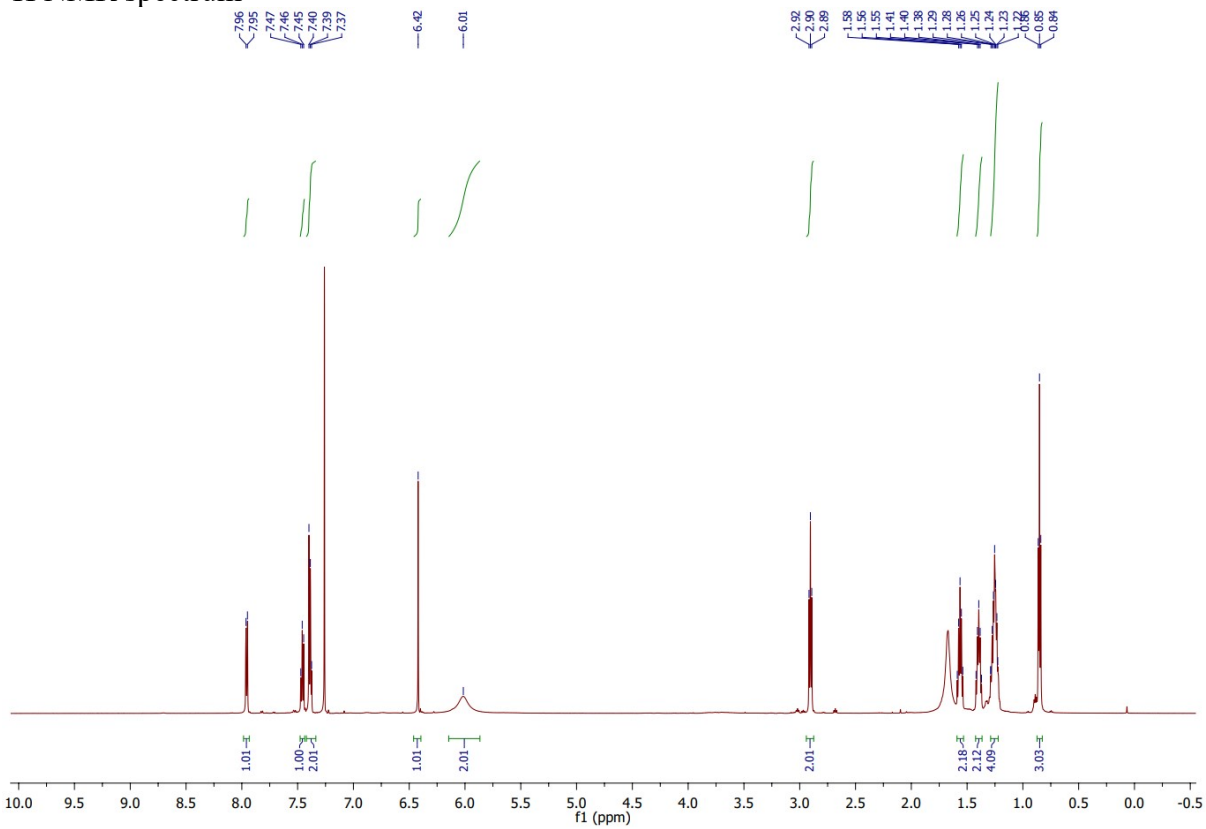


^{13}C NMR spectrum

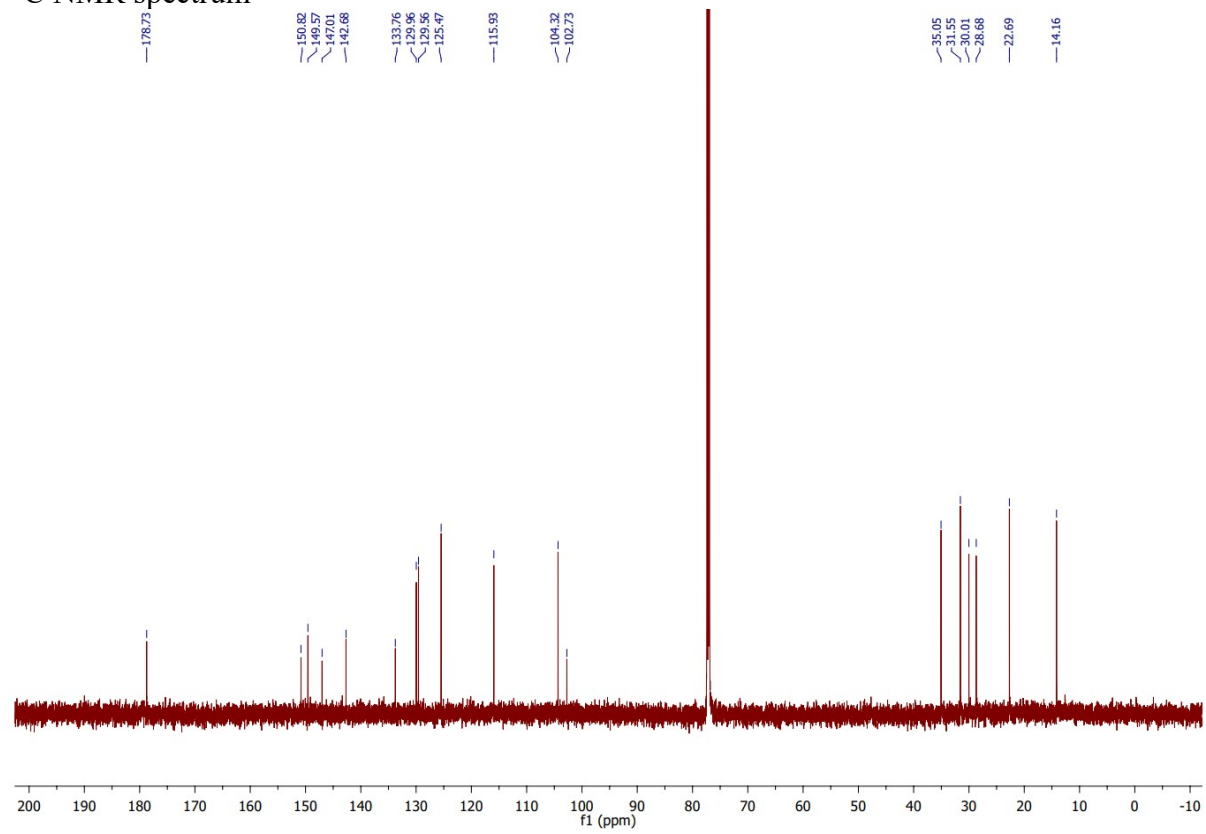


2-Amino-1-(hexylthio)-3*H*-phenoxazin-3-one (13)

^{1}H NMR spectrum

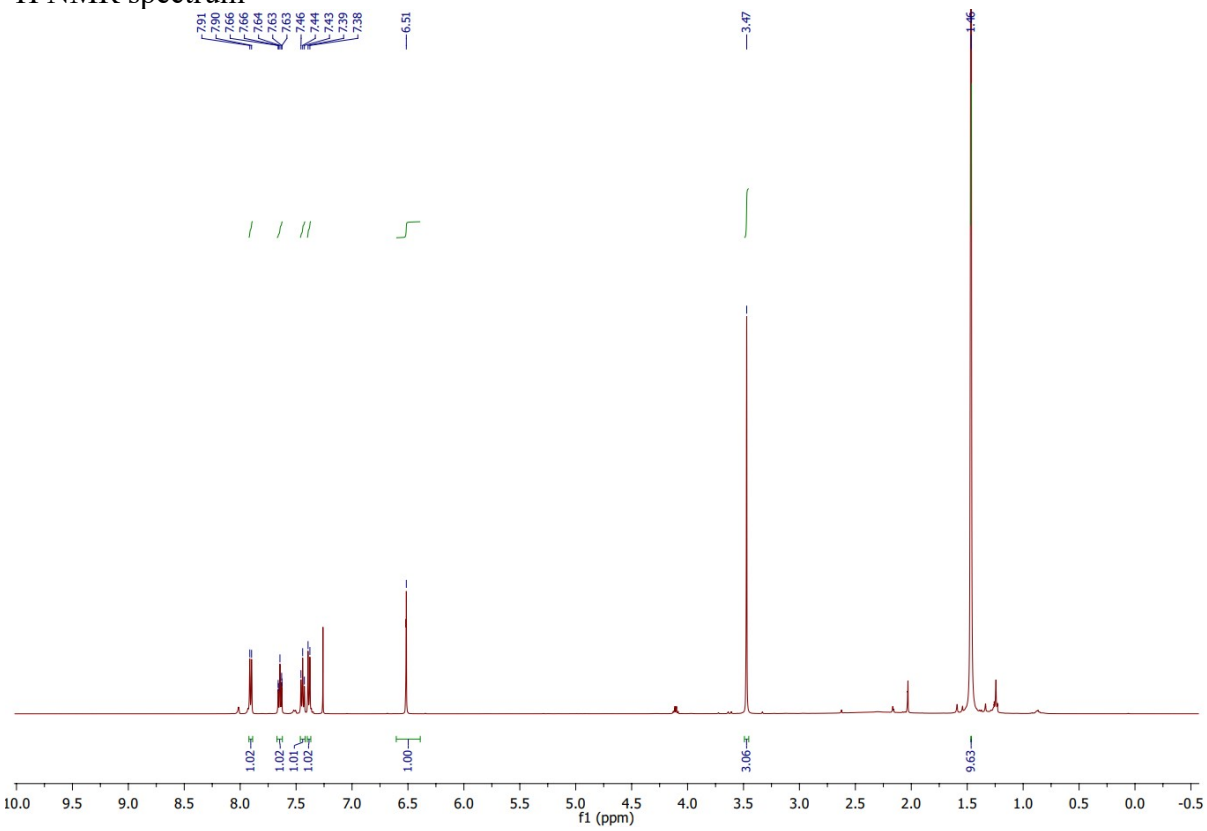


^{13}C NMR spectrum

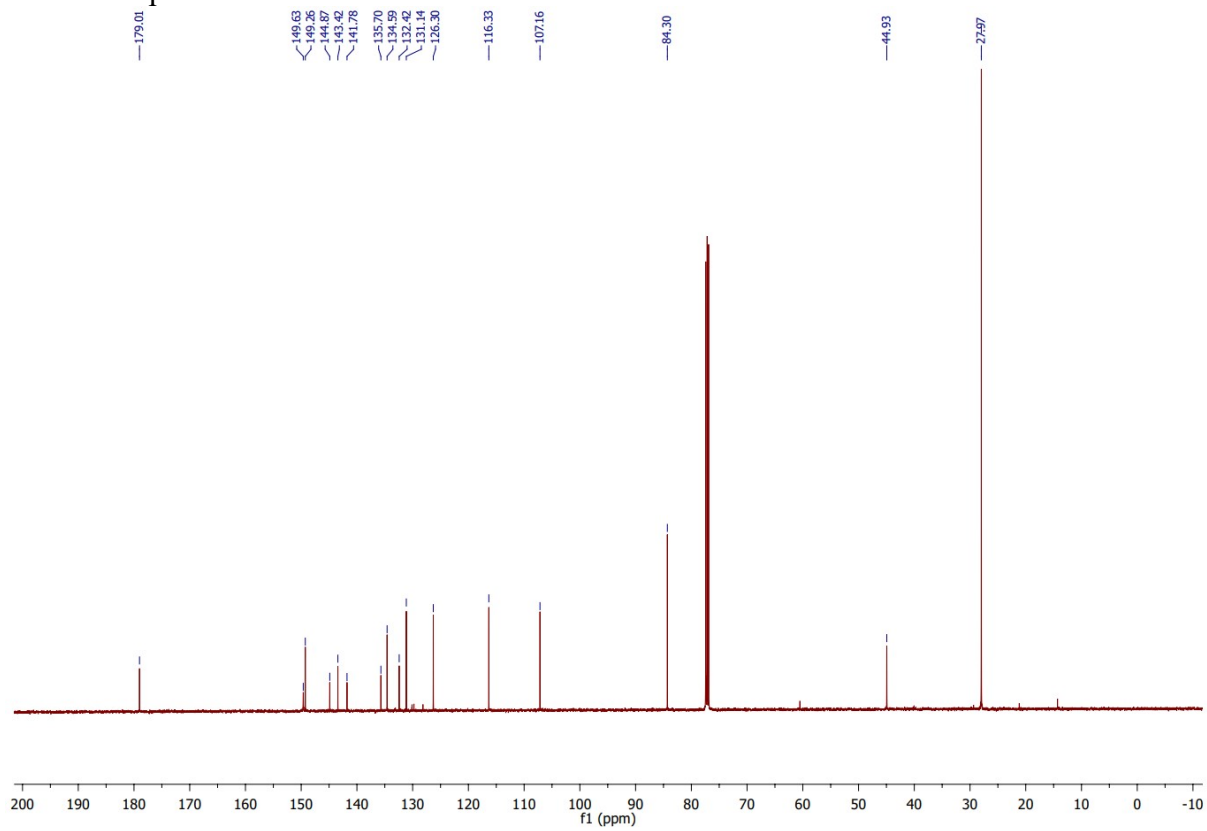


tert-*Butyl (1-(methylsulfonyl)-3-oxo-3*H-*phenoxazin-2-yl)carbamate (14)*

^{1}H NMR spectrum

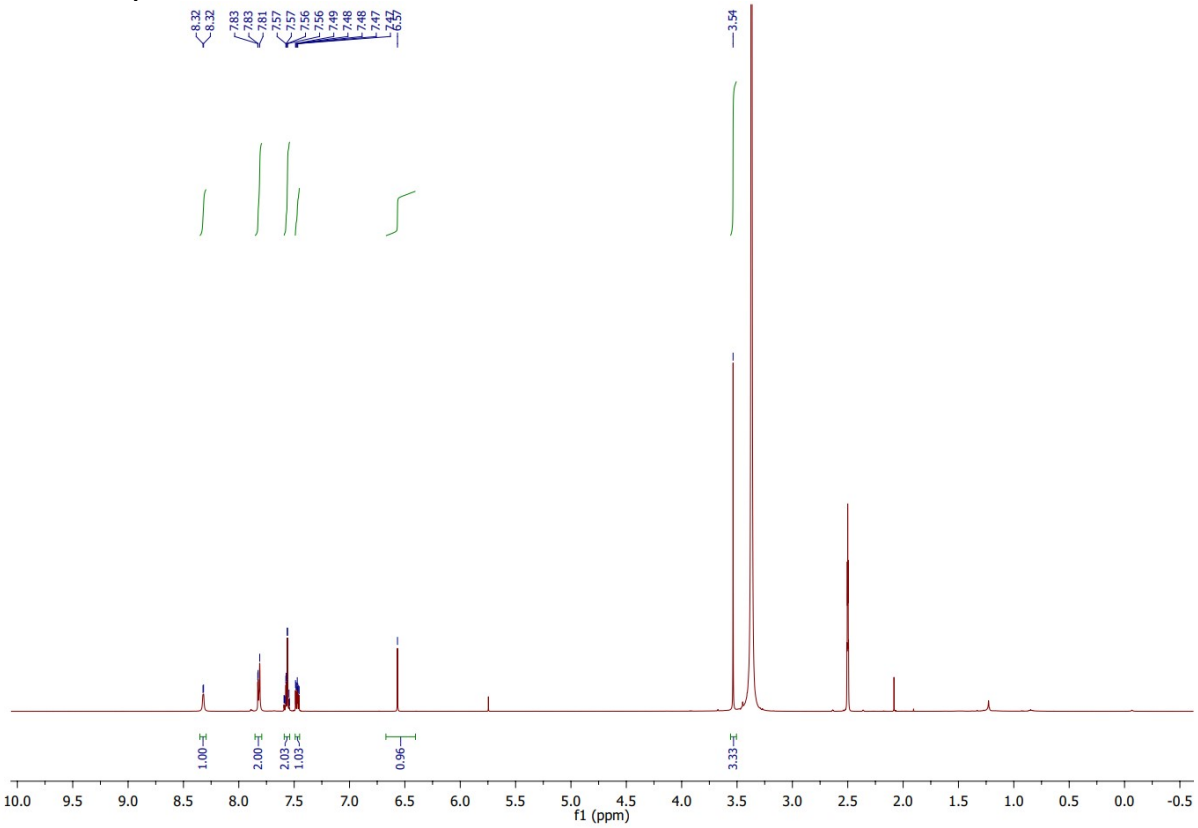


^{13}C NMR spectrum



2-Amino-1-(methylsulfonyl)-3*H*-phenoxazin-3-one (15)

^{1}H NMR spectrum



^{13}C NMR spectrum