Supporting Information

Inhibiting C-4 methyl sterol oxidase with novel diazaborines to target fungal plant pathogens

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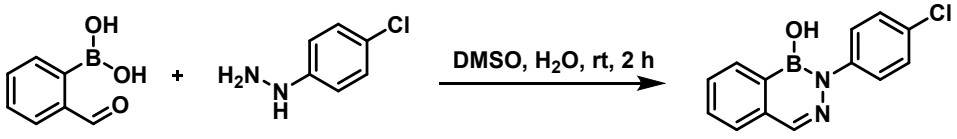
Supplementary File 1

Compound synthesis and characterization

Chemical shifts are expressed in ppm relative to an internal standard, tetramethylsilane (ppm = 0.00). The following abbreviations were utilized to describe peak patterns when appropriate: br = broad, s = singlet, d = doublet, and m = multiplet. Purity traces were acquired using an Agilent Technologies 1200 Series HPLC connected to a 6130 Mass Spectrometer. A Phenomenex Kinetex 2.6 µm C18 50 × 4.6 mm column was used at room temperature with a flow of 1 mL/min. The LC-MS method described in Supplementary Table 1 was used to analyze all compounds and detection performed at 214 nm and 254 nm. **Supplementary Table 1: LC/MS method for purity traces of compounds 3 – 8 with detection at 214 nm or 256 nm.**

Time (min)	H_2O + 0.1% Formic Acid (%)	Acetonitrile + 0.1% Formic Acid (%)
0	95	5
4	5	95
5	5	95
5.5	95	5

3 2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol (BN100286)



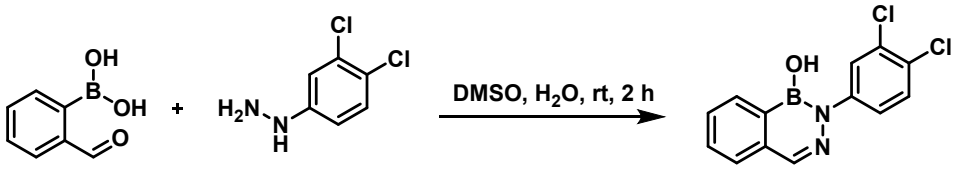
A mixture of (2-formylphenyl)boronic acid (1 g, 6.6 mmol) and (4-chlorophenyl)hydrazine (937 mg, 6.6 mmol) in DMSO/H_2O (2 mL/8 mL) was stirred at room temperature for 2 h. The reaction mixture was filtered, the filter cake was washed with hexane, dried in vacuo to give ***2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol*** (590 mg, yield 34%) as a white solid. ^{1}HNMR (400 MHz, DMSO-d_6): δ 9.13 (s, 1H), 8.41 (d, J = 7.5 Hz, 1H), 8.22 (s, 1H), 7.84-7.81 (m, 2H), 7.72-7.58 (m, 3H), 7.46 (d, J = 8.6 Hz, 2H) ppm. MS (ESI): mass calcd. For

$C_{13}H_{10}BClN_2O$ 256.06, m/z found 257.0 $[M+H]^+$. HPLC purity: 95.89% at 214 nm and 95.58% at 254 nm.

4 2-(2-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol (BN100311)

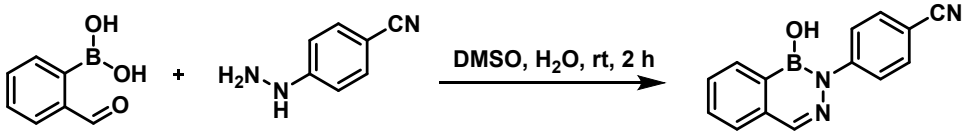
This title compound was prepared following the procedure employed in the synthesis of **2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol** as a white solid. Yield, 7%. **^{1}HNMR** (400 MHz, DMSO-d_6): δ 8.76 (s, 1H), 8.35 (d, J = 7.5 Hz, 1H), 8.15 (s, 1H), 7.81 (dt, J = 14.8, 7.4 Hz, 2H), 7.68 (t, J = 7.2 Hz, 1H), 7.59 (d, J = 7.3 Hz, 1H), 7.47-7.36 (m, 3H) ppm. MS (ESI): mass calcd. For $C_{13}H_{10}BClN_2O$ 256.06, m/z found 257.0 $[M+H]^+$. HPLC purity: 95.39% at 214 nm and >99.99% at 254 nm.

5 2-(3,4-dichlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol (BN100312)



This title compound was prepared following the procedure employed in the synthesis of **2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol** as a light yellow solid. Yield, 21%. **^{1}HNMR** (400 MHz, DMSO-d_6): δ 9.40 (s, 1H), 8.40 (d, J = 7.5 Hz, 1H), 8.25 (s, 1H), 7.91 (d, J = 2.0 Hz, 1H), 7.87-7.75 (m, 2H), 7.69-7.65 (m, 3H) ppm. MS (ESI): mass calcd. For $C_{13}H_9BC_{l2}N_2O$ 290.02, m/z found 291.0 $[M+H]^+$. HPLC purity: 94.60% at 214 nm and 96.87% at 254 nm.

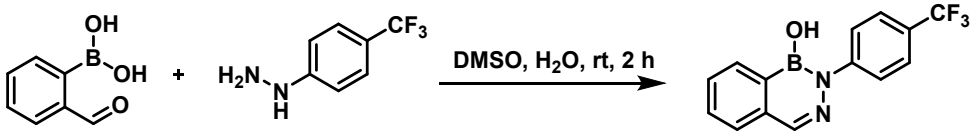
6 4-(1-hydroxybenzo[d][1,2,3]diazaborinin-2(1H)-yl)benzonitrile (BN100352)



This title compound was prepared following the procedure employed in the synthesis of **2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol** as a white solid. Yield, 11%. **^{1}HNMR** (400 MHz, DMSO-d_6): δ 9.51 (s, 1H), 8.43 (d, J = 7.6 Hz, 1H), 8.29 (s, 1H), 7.94-7.78 (m, 6H), 7.70 (t,

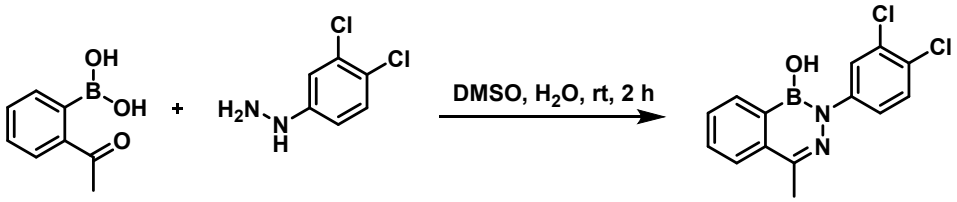
J = 7.3 Hz, 1H) ppm. MS (ESI): mass calcd. For $C_{14}H_{10}BN_3O$ 247.09, m/z found 248.0 $[M+H]^+$. HPLC purity: 97.86% at 214 nm and 95.27% at 254 nm.

7 2-(4-(trifluoromethyl)phenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol (BN100397)



This title compound was prepared following the procedure employed in the synthesis of **2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol** as a white solid. Yield, 79%. **^{1}HNMR** (400 MHz, DMSO-d_6): δ 9.37 (s, 1H), 8.44 (d, J = 7.5 Hz, 1H), 8.27 (s, 1H), 7.92-7.76 (m, 6H), 7.70 (dd, J = 10.6, 3.9 Hz, 1H) ppm. MS (ESI): mass calcd. For $C_{14}H_{10}BF_3N_2O$ 290.08, m/z found 291.0 $[M+H]^+$. HPLC purity: 95.95% at 214 nm and 95.39% at 254 nm.

8 2-(3,4-dichlorophenyl)-4-methylbenzo[d][1,2,3]diazaborinin-1(2H)-ol (BN100433)

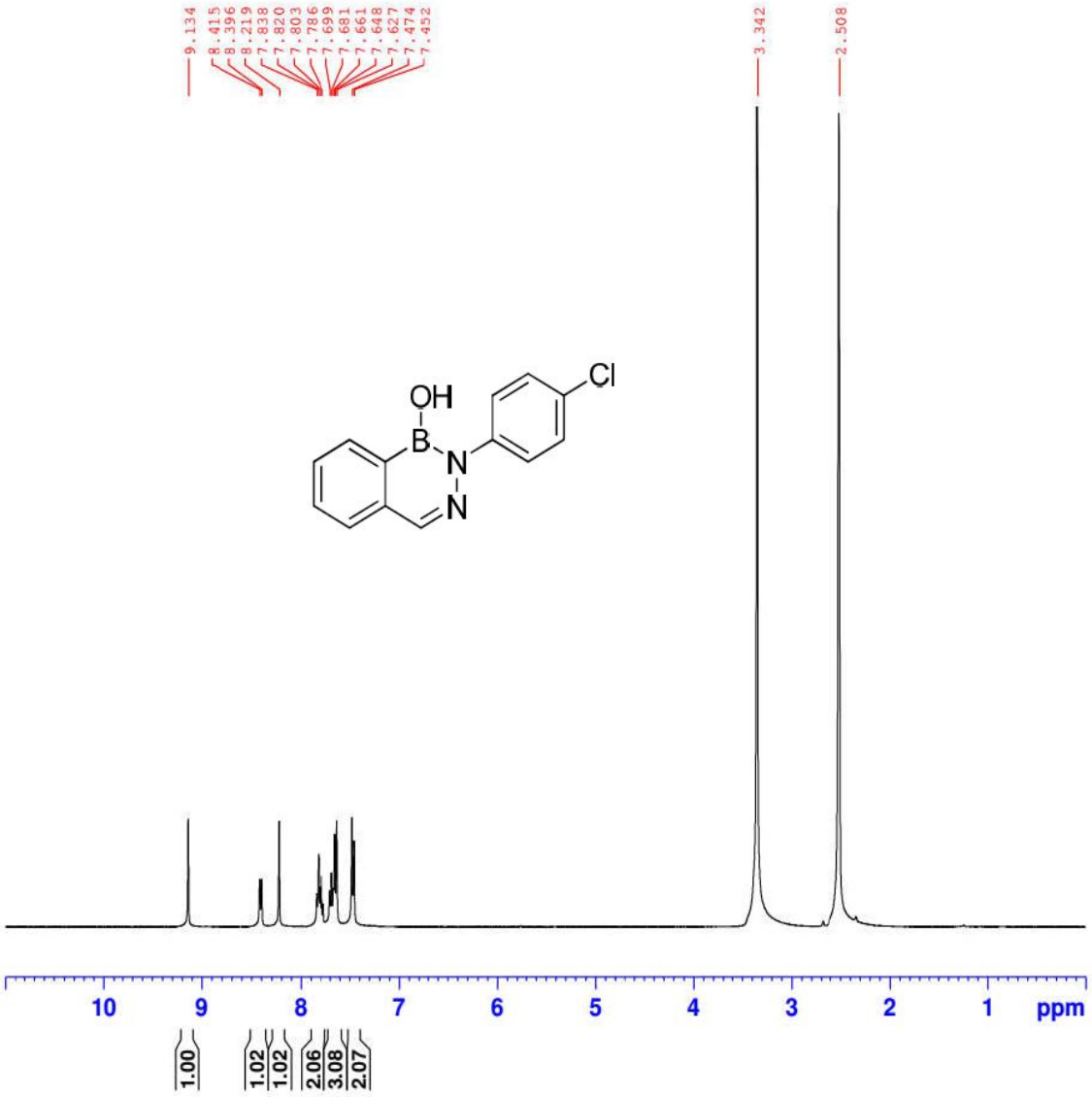


This title compound was prepared following the procedure employed in the synthesis of **2-(4-chlorophenyl)benzo[d][1,2,3]diazaborinin-1(2H)-ol** as a white solid. Yield, 71%. **^{1}HNMR** (400 MHz, DMSO-d_6): δ 9.25 (s, 1H), 8.42 (d, J = 7.4 Hz, 1H), 7.96-7.88 (m, 2H), 7.81 (t, J = 7.6 Hz, 1H), 7.75-7.61 (m, 3H), 2.56 (s, 3H) ppm. MS (ESI): mass calcd. For $C_{14}H_{11}BCl_2N_2O$ 304.03, m/z found 305.0 $[M+H]^+$. HPLC purity: 96.13% at 214 nm and 96.85% at 254 nm.

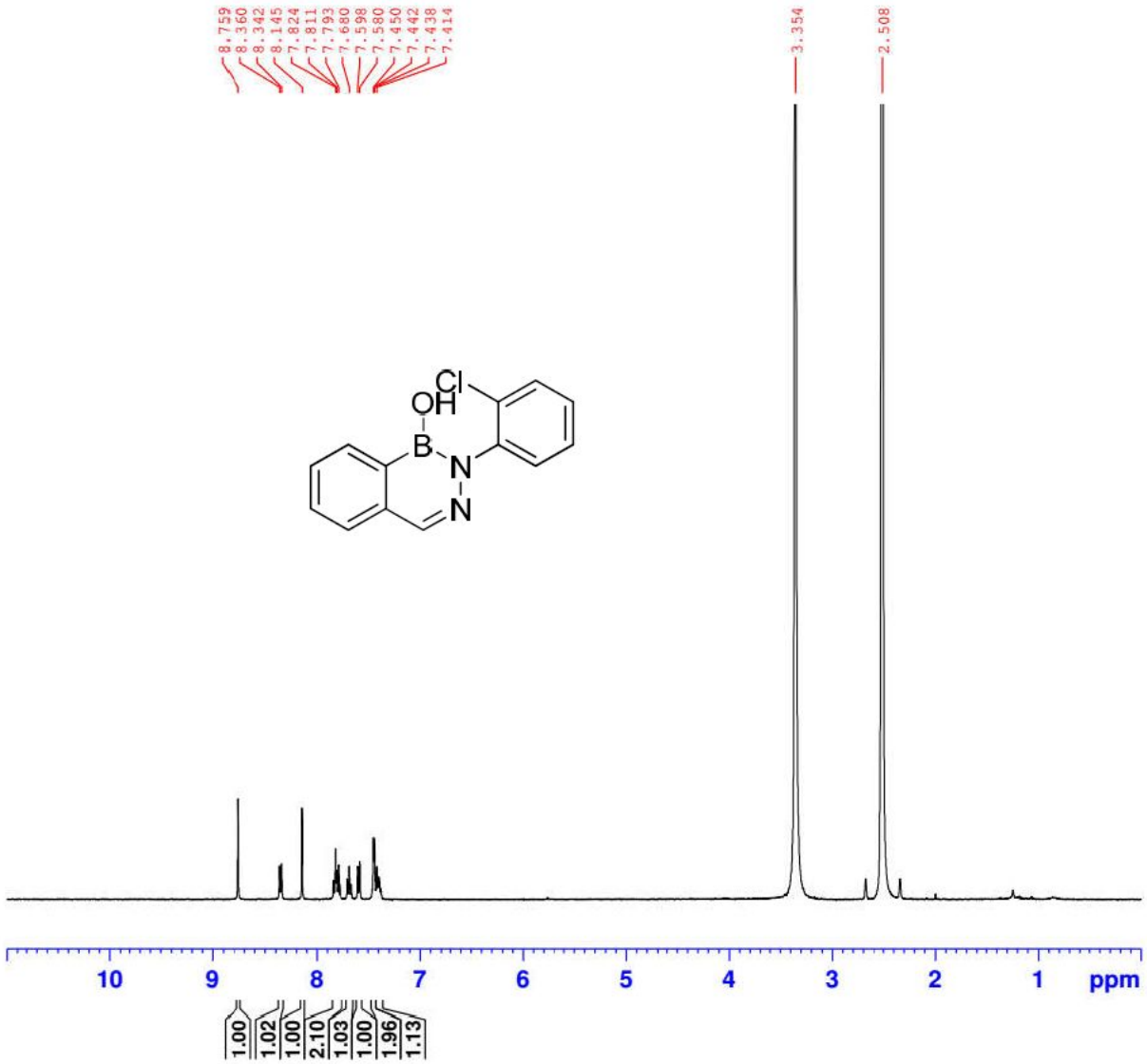
Supplementary File 2:

NMR spectra of compounds synthesized for this study

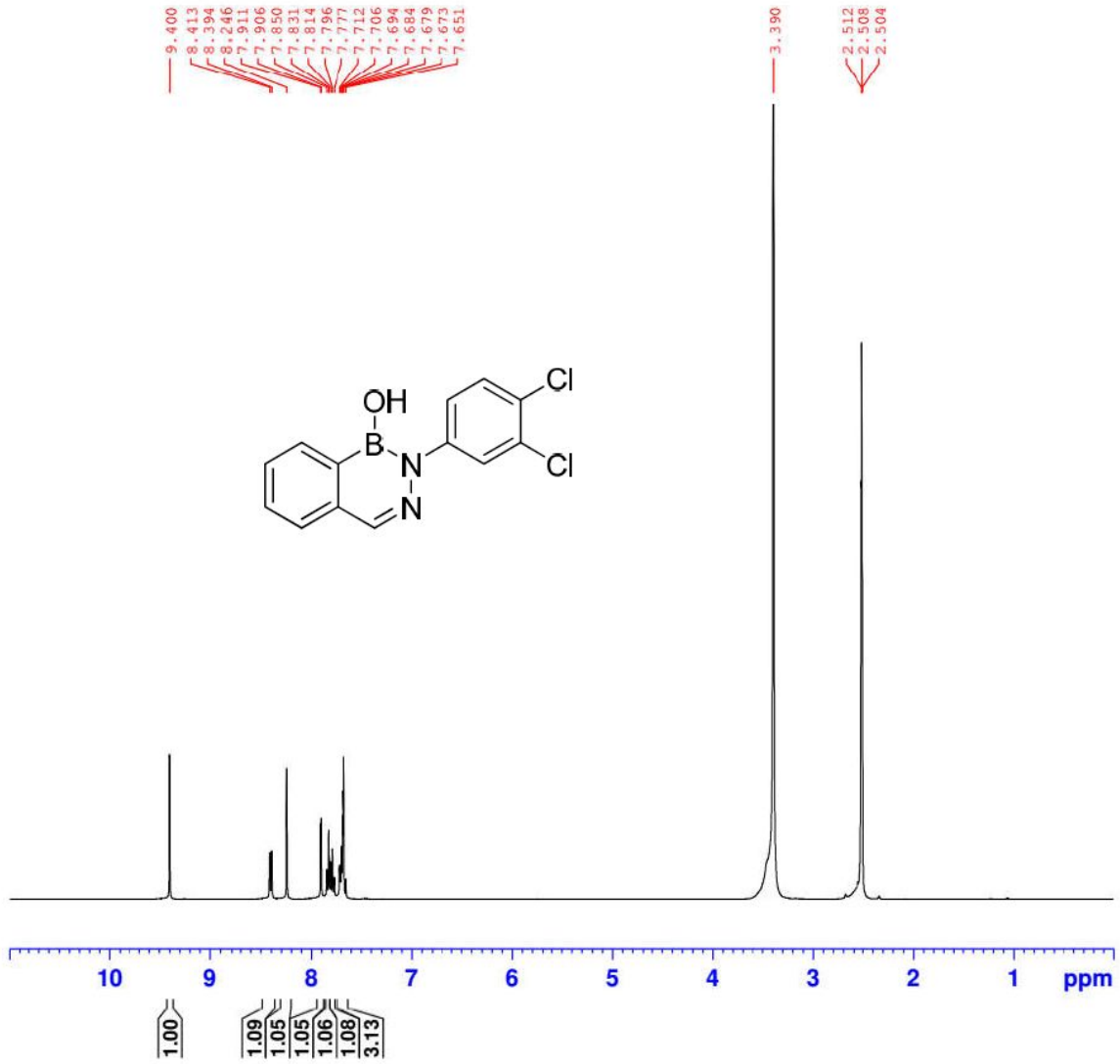
^{1}H NMR (400 MHz, DMSO-d_6) of compound **3**



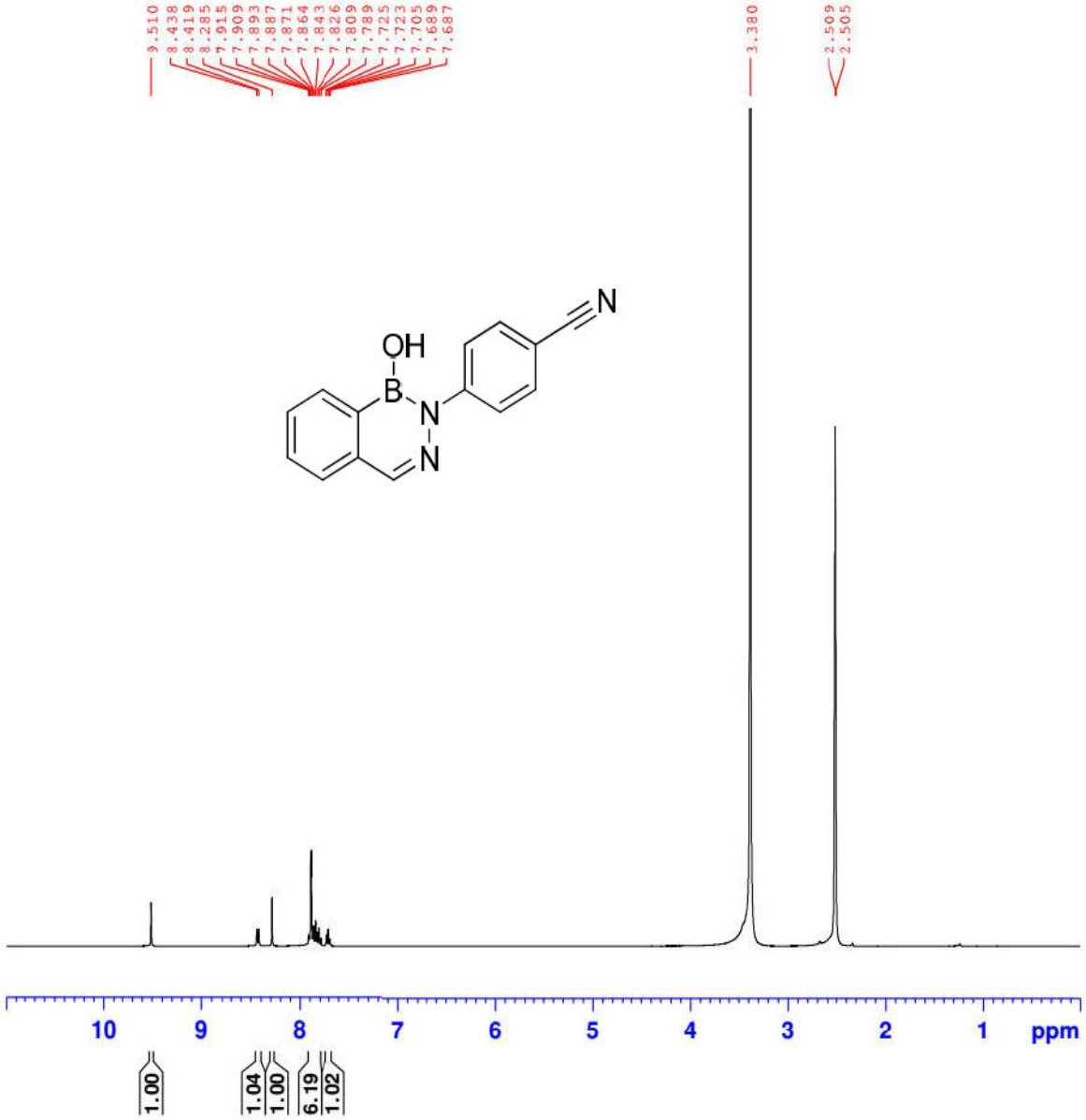
^{1}H NMR (400 MHz, DMSO-d_6) of compound **4**



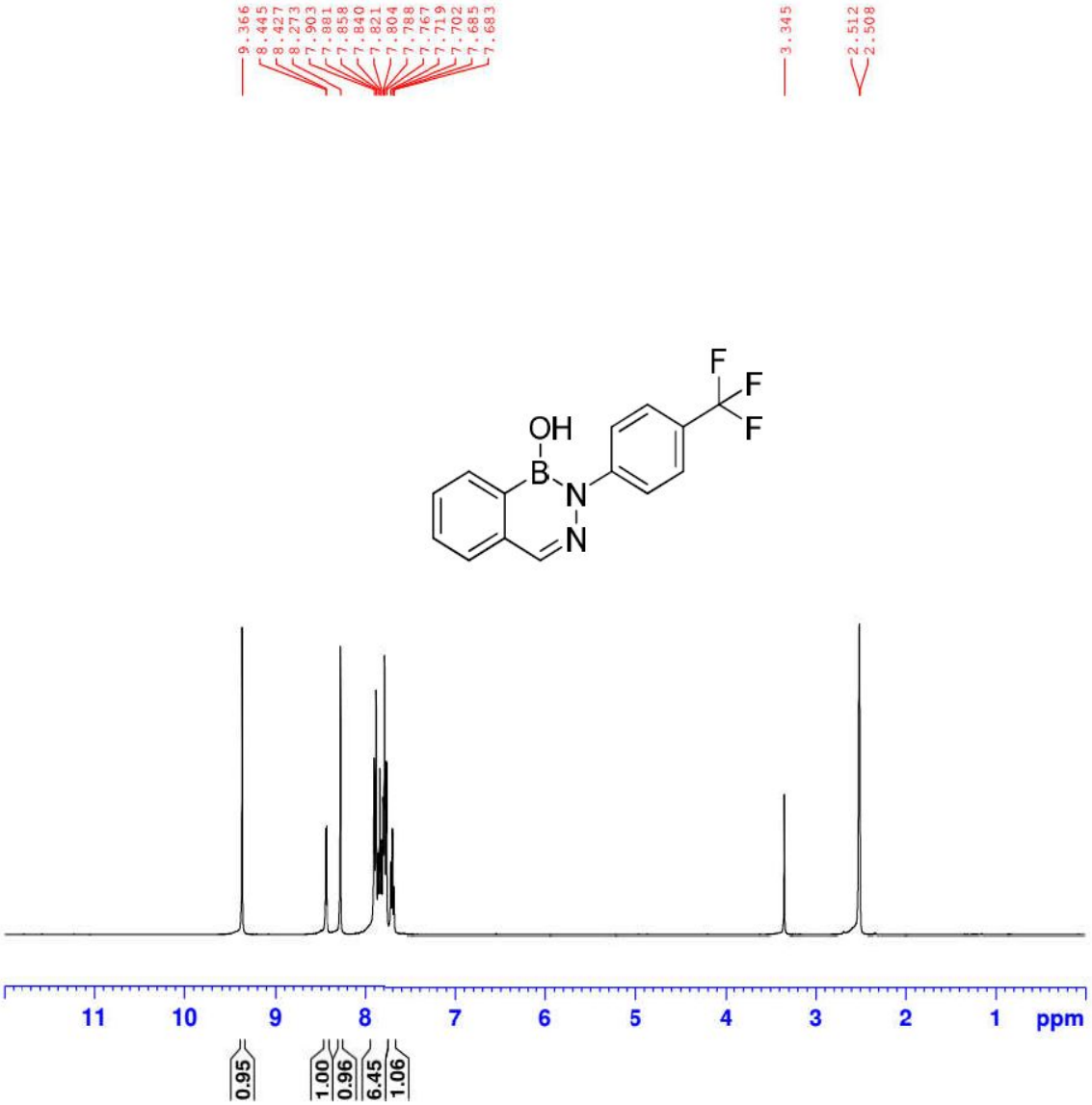
^{1}H NMR (400 MHz, DMSO-d_6) of compound **5**



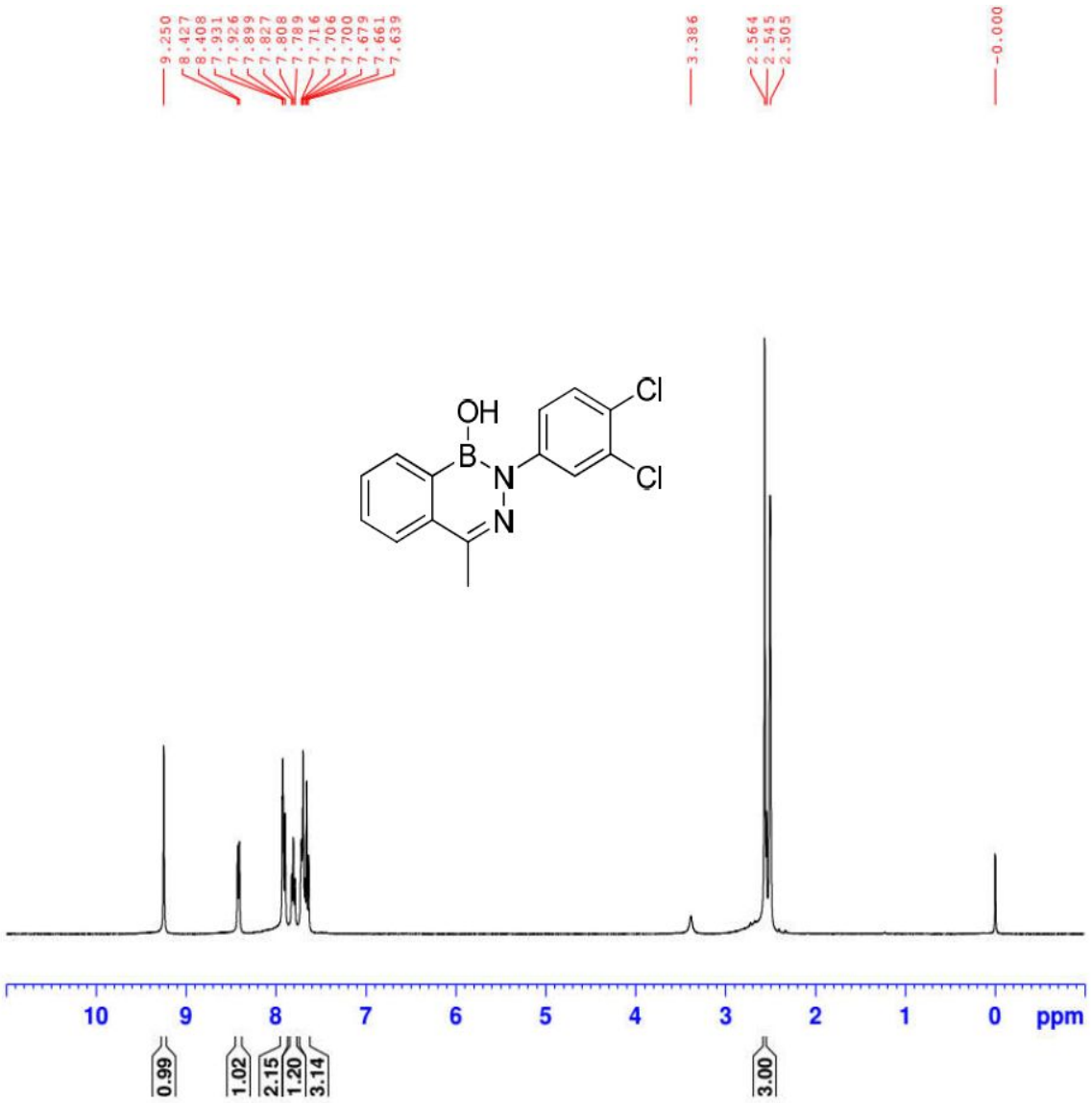
^{1}H NMR (400 MHz, DMSO-d_6) of compound **6**



^{1}H NMR (400 MHz, DMSO-d_6) of compound **7**



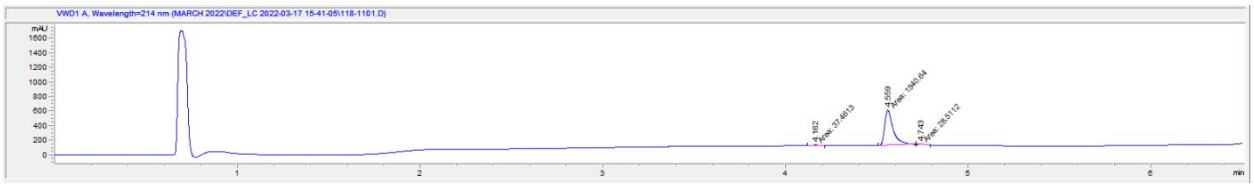
^{1}H NMR (400 MHz, DMSO-d_6) of compound **8**



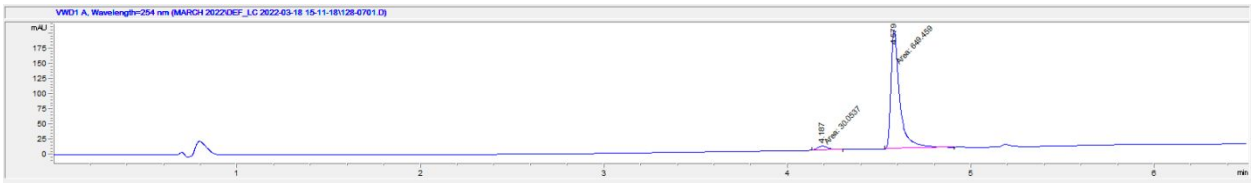
Supplementary File 3:

LC/MS spectra of compounds synthesized for this study

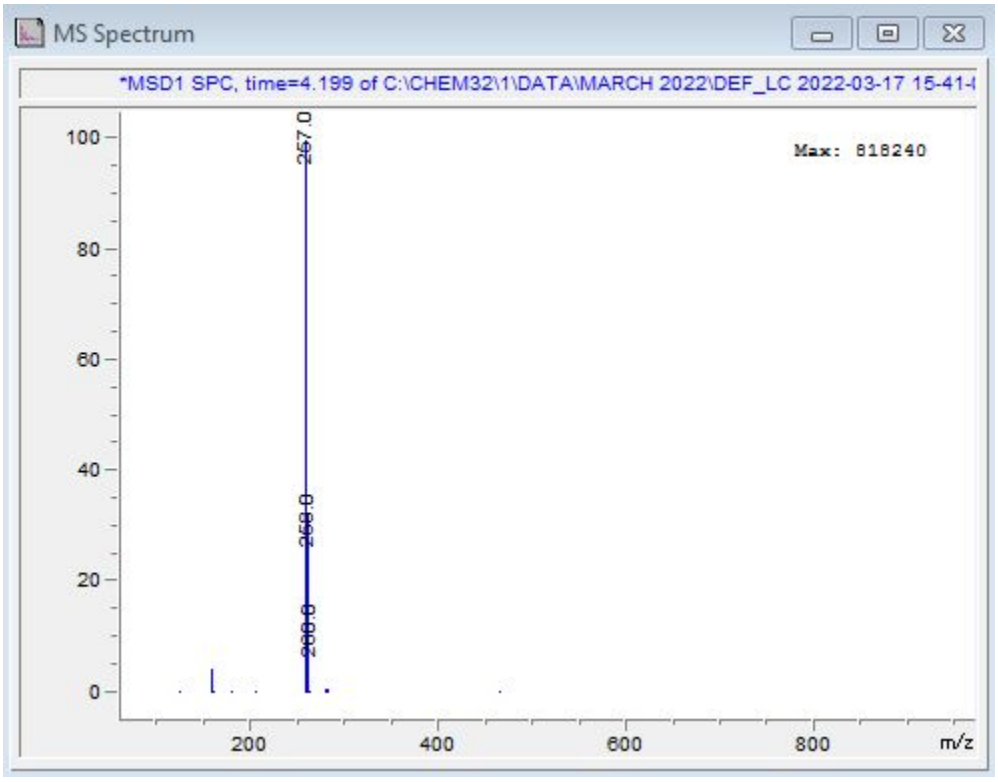
LC spectrum of compound **3** measured at 214 nm



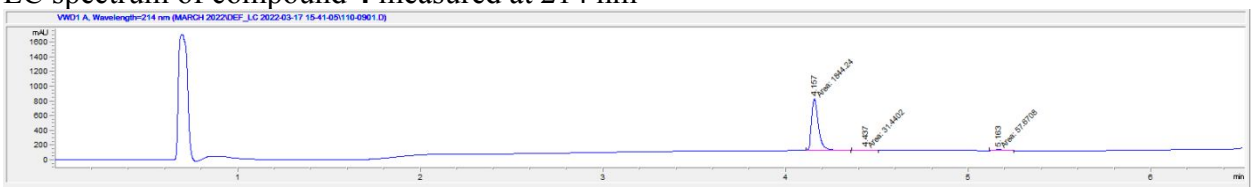
LC spectrum of compound **3** measured at 254 nm



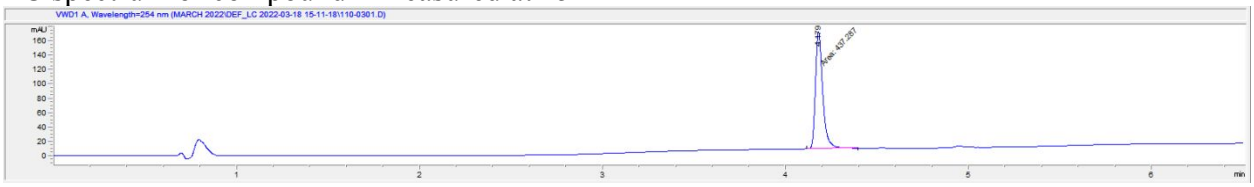
MS spectrum of compound **3**



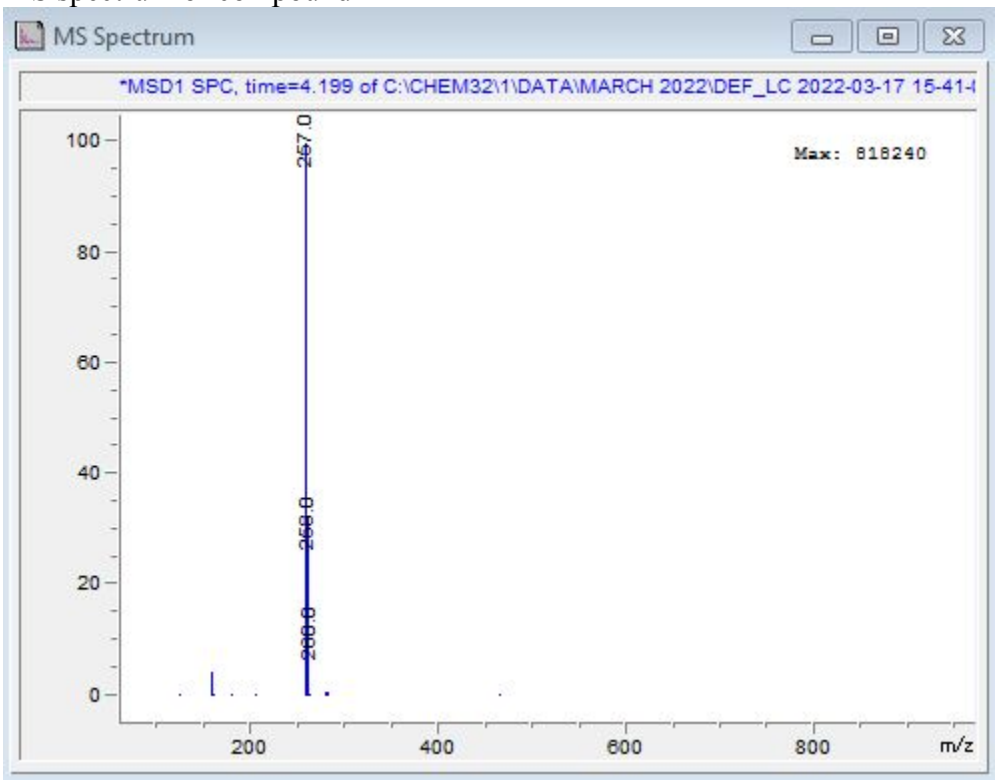
LC spectrum of compound **4** measured at 214 nm



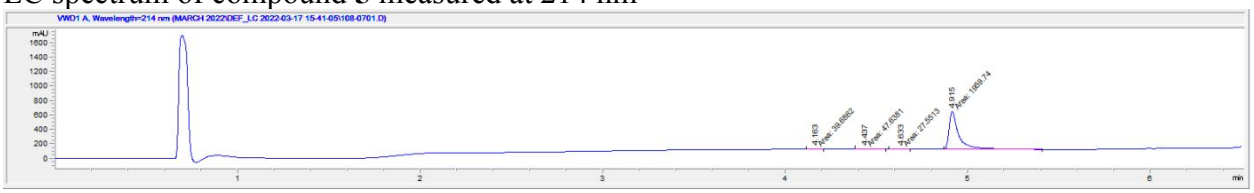
LC spectrum of compound **4** measured at 254 nm



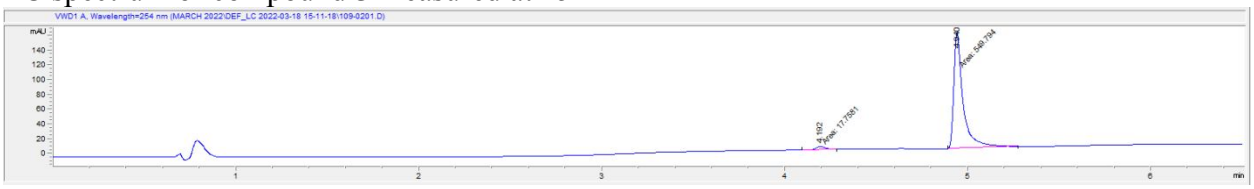
MS spectrum of compound **4**



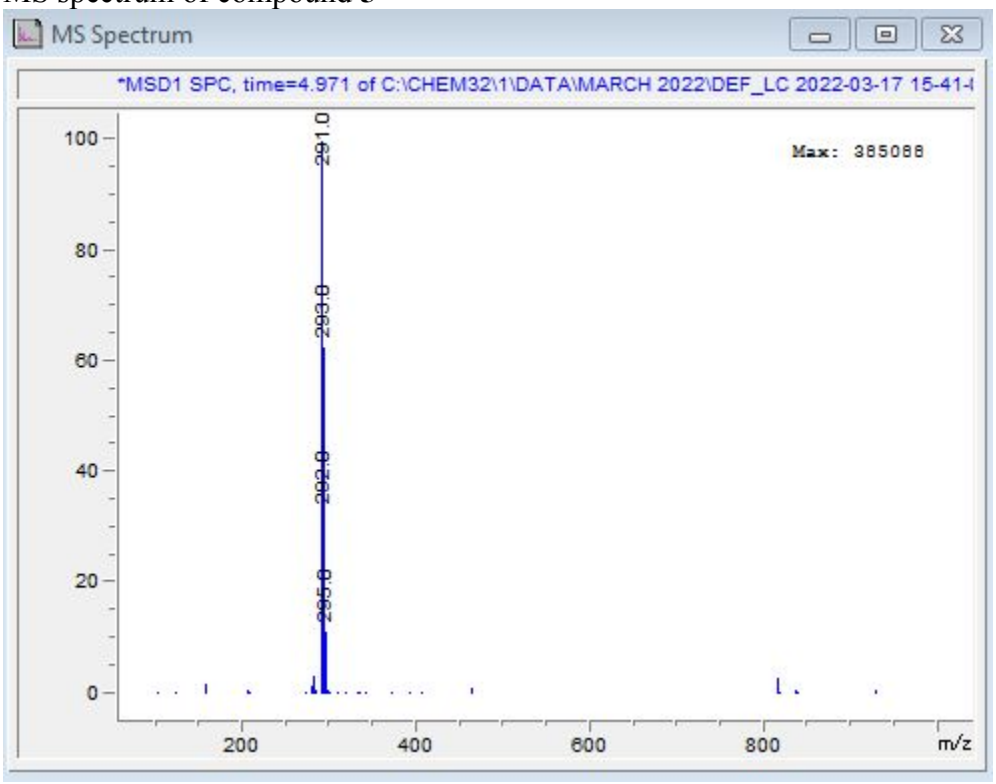
LC spectrum of compound **5** measured at 214 nm



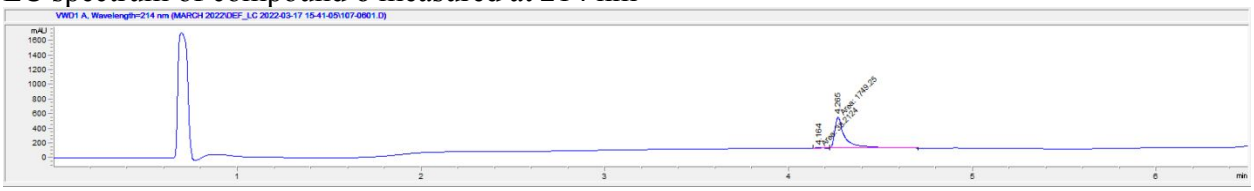
LC spectrum of compound **5** measured at 254 nm



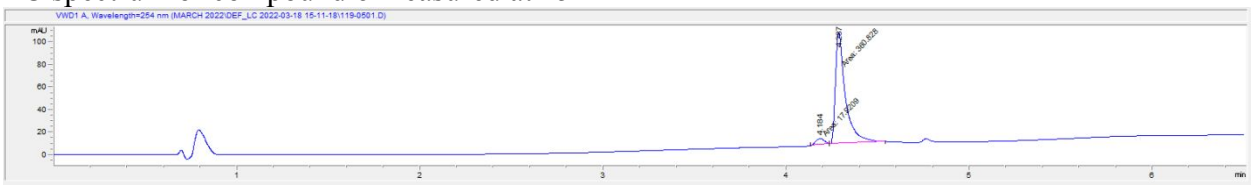
MS spectrum of compound **5**



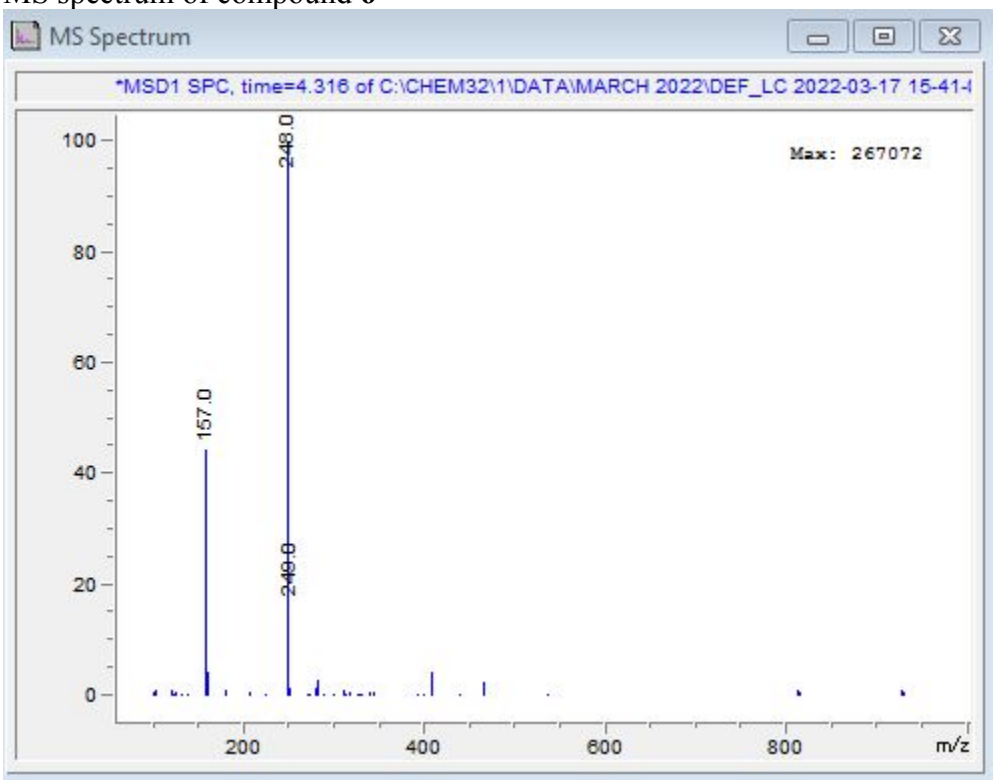
LC spectrum of compound **6** measured at 214 nm



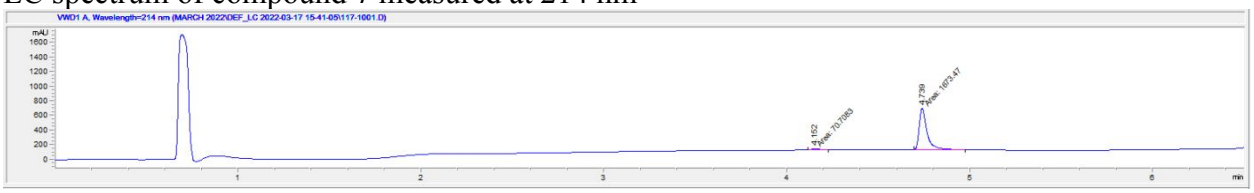
LC spectrum of compound **6** measured at 254 nm



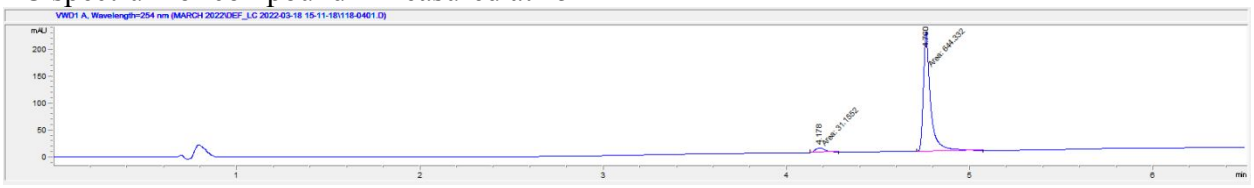
MS spectrum of compound **6**



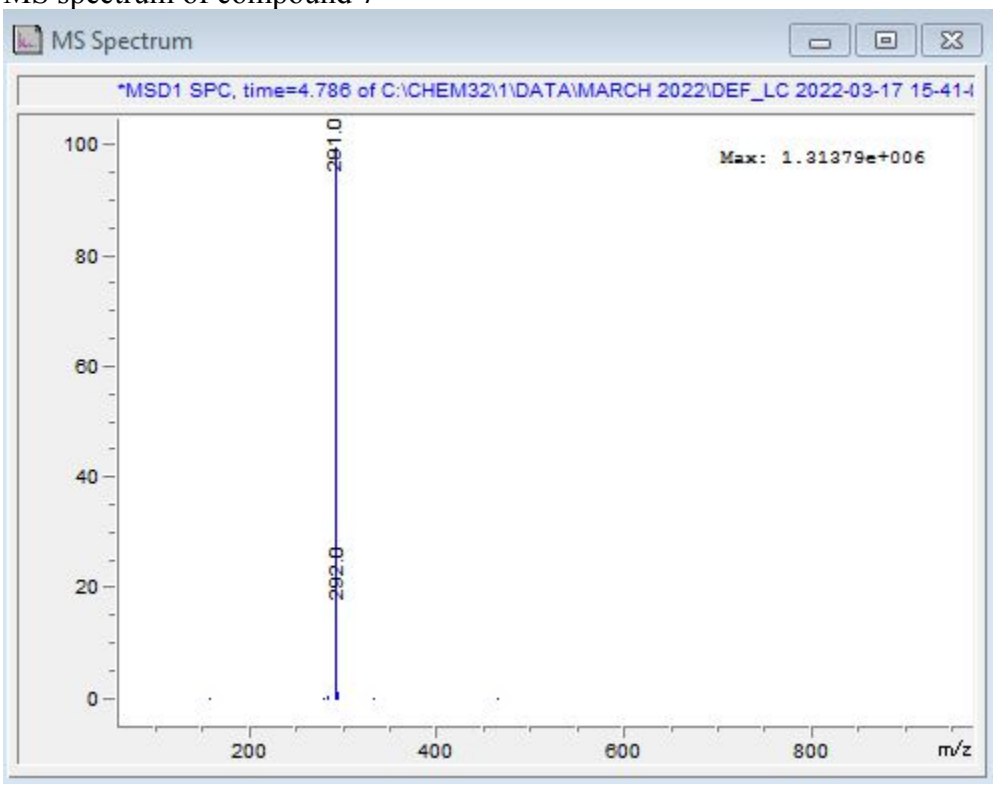
LC spectrum of compound **7** measured at 214 nm



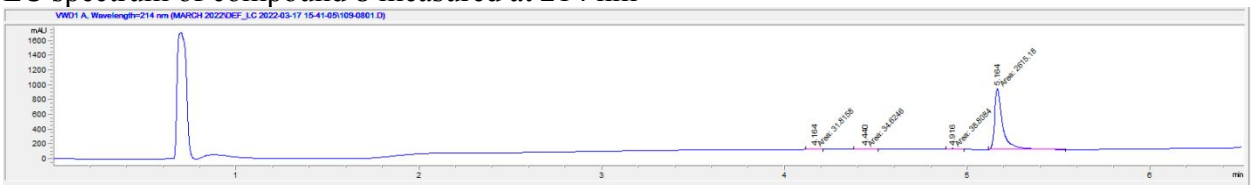
LC spectrum of compound **7** measured at 254 nm



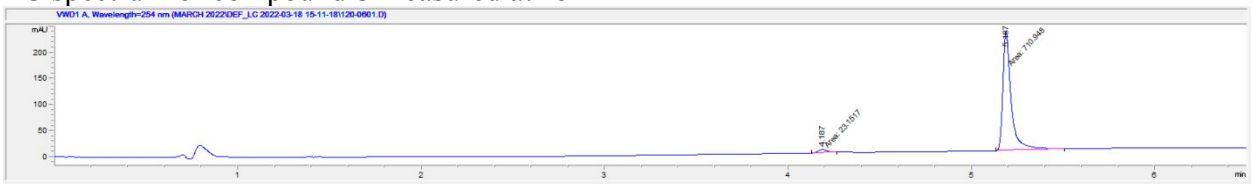
MS spectrum of compound **7**



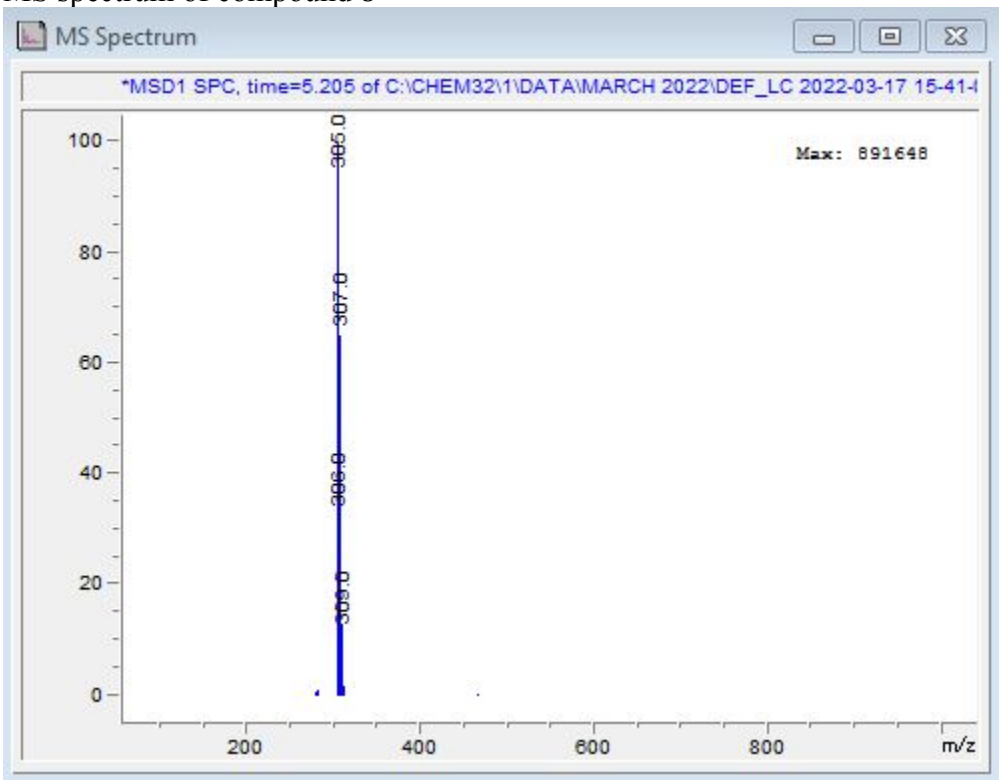
LC spectrum of compound **8** measured at 214 nm



LC spectrum of compound **8** measured at 254 nm

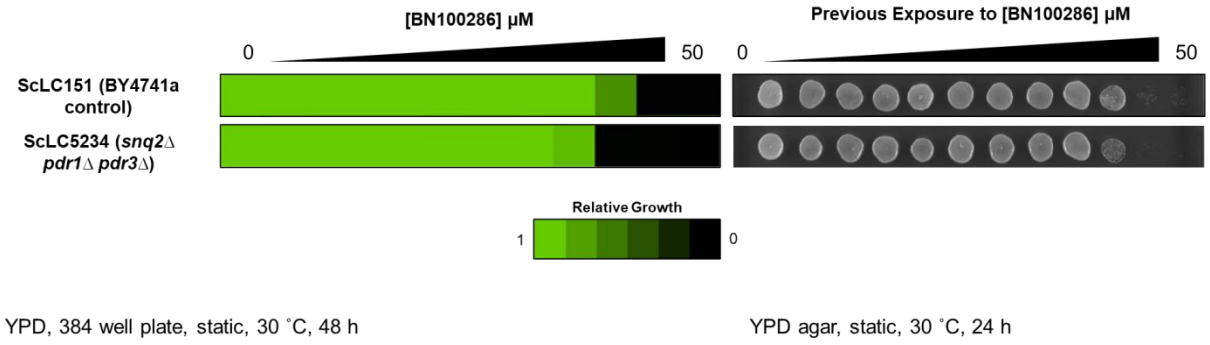


MS spectrum of compound **8**



Supplementary File 4

BN100286 (Compound 3) MIC assays and spotting assays for fungicidal activity against wild-type and drug-sensitized (*snq2*Δ *pdr1*Δ *pdr3*Δ) *S. cerevisiae*



Supplementary File 5

Detailed biological and biochemical methods

Culture conditions for fungi

Plant pathogens were maintained on potato dextrose agar (PDA) or V8 agar and spores were isolated from the cultures after 1-2 weeks of incubation at room temperature (20-22 °C) with 12 hours fluorescent light (Philips, F40LW). The final concentrations of all inocula were 1×10^5 CFU/ml. All yeast strains were archived in 25% glycerol and stored at -80 ℃. Yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30 ℃. For propagation of plasmids with hygromycin B marker, yeast strains were grown in YPD plus 600 µg/ml hygromycin B.

Antifungal MIC testing

Assays were performed in flat-bottom, 96-well microtiter plates (Greiner Bio-One™, Greiner Bio-One North America, Inc., Monroe, NC). Compounds were diluted into sterile half-strength broth media for *in vitro* testing, such that the DMSO final concentration was not greater than 1% (v/v). MICs were determined in triplicate in a final volume of 0.2 ml/well with antifungal concentrations of 0.2 – 25 µg/ml (2-fold serial dilution) from 25 µg/ml; control studies with 0 µg/ml of compounds were performed in parallel for each plate. Plates were sealed with clear polyester film (VWR), incubated at 22 °C and progress of fungal growth measured at 72 hours.

Yeast strain construction

Strains used in this study and details regarding their construction are provided in Supporting Information File 4. To prepare yeast cultures for transformation, cells were grown as 10 ml cultures until the optical density (OD_{600}) was between 0.3 – 0.6. Cells were pelleted and resuspended in 60 µl of 100 mM lithium acetate, followed by 15-minute incubation at 30 ℃. Cells were mixed with

10 µl of linear DNA with 10 µl of boiled salmon sperm DNA (10 mg/ml) and 300 µl of PEG mixture (40% polyethylene glycol, 100 mM lithium acetate) and incubated at 30 ℃ for 30 minutes followed by 42 ℃ for 20 minutes. Cells were recovered in 10 ml YPD at 30 ℃ for 4 hours before plating on YPD agar plus 600 µg/ml hygromycin B. Plates were incubated at 30 ℃ for 48 hours.

Whole genome sequencing

Overnight cultures of resistant mutants were used to isolate genomic DNA for whole genome sequencing. DNA was prepared using the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Sequencing libraries were prepared using the Nextera XT Kit (Illumina) according to the manufacturer's instructions. Libraries were sequenced on the Illumina NextSeq platform using paired reads (300 bp). The sequence reads were de-multiplexed and trimmed to remove bases with Phred scores < Q30. Reads were aligned to S288C reference genome using Bowtie2[1], and the alignment was visualized using Integrative Genomics Viewer.[2] MuTect was used to identify unique mutations in resistant mutants.[3]

Culture conditions for sterol extraction

Two replicate cultures (biological replicates) of the *S. cerevisiae* parent and *tetO-ERG25* strain were grown overnight in 5 ml YPD and incubated at 30 °C. *S. cerevisiae* parent cultures were diluted to the final OD_{600} of 0.1 in 10 ml YPD and *tetO-ERG25* cultures were diluted to the final OD_{600} of 0.1 in 10 ml YPD or 10 ml YPD + doxycycline (DOX, 0.5 µg/ml) and incubated overnight at 30 °C. Overnight cultures were pelleted at 3,000 rpm for 5 minutes and washed once with 10 ml synthetic complete without ammonium sulfate + 2% glucose (SC) medium once. For the *S. cerevisiae* parent, cultures were diluted to the final OD_{600} of 1 in 50 ml SC medium + DMSO (equal volume), PF1163A (40 µM), or Compound **3** (50 µM) and incubated for 6 h at 30 °C. For the *tetO-ERG25* samples, cultures were diluted to the final OD_{600} of 1 in 50 ml SC medium –/+

DOX (10 μg/ml) and incubated for 6 h at 30 °C. Cells were pelleted at 3,000 rpm for 5 minutes at 4 °C and washed once with ice-cold phosphate buffered saline. Pellets were weighed and flash frozen with liquid nitrogen and stored at -80 °C.

Sterol extraction for derivatization

Total intracellular sterols of yeast were extracted as reported previously with slight modification.[4] Cell pellets were mixed with 3 ml extraction solution (25% KOH w/v, 64% methanol v/v) adding 30 μl of 2 mg/ml cholesterol (dissolved in chloroform) as an internal standard in borosilicate glass tubes and incubated at 80 °C water bath for 1 hour and vortexed every 20 mins. After incubation, the samples were cooled to room temperature and 1 ml water and 3 ml chloroform were added to each sample for extraction. The tubes were vortexed for 10 seconds 3 times then centrifuged at 2,500 rpm for 5 mins. Using Pasteur pipettes, the bottom organic phases were transferred to another borosilicate glass tubes and dried by Genevac (EZ-2 Series SP Scientific).

Derivatization for GC-MS

Samples were dissolved in 600 μl chloroform and 200 μl then transferred into 1 ml conical thick-walled glass reaction vials for derivatization, as previously reported.[5] The bottom of the tubes were suspended in a 40 ℃ water bath and lipid extracts were evaporated under a stream of N_2 gas at an airflow rate of 1 ml/min. The extract was dissolved in 20 μl pyridine and 30 μl MSTFA by flicking bottom of the vial then was incubated in a dry heating block at 37 ℃ for 30 min to derivatize. After derivatization, 20 μl was transferred into GC auto-sampler vials and 1 μl extract was injected into the GC-MS for analysis.

Sterol quantification

Samples were injected at 200 °C into a 30 m 122-5532 Agilent DB-5MS polysiloxane column (0.25 mm id) coated with 50% methyl and 50% phenyl groups coupled to an intermediate polarity

fused silica guard column 5 m in length with a 0.53 mm diameter. Helium was the carrier gas at a flow rate of 1 ml/min. The oven temperature was isocratic at 50 °C for 2.5 min and increased to 70 °C at a rate of 7.5 °C/min, then the temperature was programmed to 310 °C at a rate of 5 °C/min and held for 1 min. The oven was then cooled down to 50 °C before injection of the next sample. The MS detector was turned on at 6 min and the mass spectra was recorded at 0.9 scans/s with a scanning range of 50 to 700 m/z. The data were collected in SIM mode.[5] Peak areas of sterols were normalized to that of internal standards and the wet weights of each sample. Medians between the two technical of the two biological replicates of each sample were plotted as bar graph with individual data points using R and ggplot2.[6,7]

Supplementary File 6

Reagents and strains used in this study

Strain ID	Description	Genotype	Source
	B. cinerea		
	C. sublineolum		
	P. pachyrhizi		
ScLC5234	*S. cerevisiae* parent (*snq2Δ/snq2Δ pdr3Δ/pdr3Δ pdr1Δ/pdr1Δ*)	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+*	Ref 8
ScLC7140	Compound **3**-selected R3	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+; ERG25/ERG25^{C590A}*	This study
ScLC7141	Compound **3**-selected R9	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+; ERG25/ERG25^{C590A}*	This study
ScLC7423	*ERG25^{WT}/ ERG25^{WT}*	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+; ERG25/ERG25^{WT}::HYGB*	This study
ScLC7419	*ERG25^{WT}/ ERG25^{C590A}*	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+; ERG25/ERG25^{C590A}::HYGB*	This study
ADE13/ ade13Δ	*ADE13/ ade13Δ*	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+; ADE13/ade13Δ::KANMX*	Ref 8

ERG25 /erg25Δ	*ERG25/ erg25Δ*	*snq2Δ::KILeu2/snq2Δ::KILeu2; pdr3Δ::KIura3/pdr3Δ::KIura3; pdr1Δ::NATMX/pdr1Δ::NATMX; can1Δ::STE2pr-Sp_his5/can1Δ::STE2pr-Sp_his5; lyp1Δ/lyp1Δ; his3Δ/his3Δ; leu2Δ/leu2^0; ura3Δ/ura3Δ; met15Δ/met15Δ; LYS2+; ERG25/erg25Δ::KANMX*	Ref 8
tetO-ERG25	*tetO-ERG25*	*pERG25::kanR-tet07-TATA; URA3::CMV-tTA; MATa; his3-1; leu2-0; met15-0*	Ref 9

ScLC7140 and ScLC7141

See Experimental section for selection of resistant mutants.

ScLC7423: *ERG25^{WT}/ERG25^{WT}*

pLC1480 containing the HygB cassette flanked by upstream and downstream *ERG25^{C590A}* homology was linearized using NotI. This was used to transform ScLC5234 and plated on YPD + HygB. Transformant was checked for upstream (oLC9502/oLC274) and downstream (oLC9503/oLC6292) integration and WT allele was verified by Sanger sequencing.

ScLC7419: *ERG25^{WT}/ERG25^{C590A}*

pLC1480 containing the HygB cassette flanked by upstream and downstream *ERG25^{C590A}* homology was linearized using NotI. This was used to transform ScLC5234 and plated on YPD + HygB. Transformant was checked for upstream (oLC9502/oLC274) and downstream (oLC9503/oLC6292) integration and C590A was verified by Sanger sequencing.

Plasmids used in this study.

Plasmid ID	Description	Source
pLC3	pAG32, Yeast dominant selectable marker cassette, hygromycin B resistance marker, ampicillin resistance.	Ref 10
pLC1480	pLC3-*ERG25^{C590A}*. Hygromycin B resistance marker, ampicillin resistance.	This study

pLC1480: pLC3-*ERG25*^{C590A}

ERG25 open reading frame (ORF) homology and 3' homology were amplified from ScLC7140 gDNA using oLC9232/oLC9233 and oLC9234/oLC9235, respectively. pLC3 and *ERG25* ORF homology amplicon were digested with BamHI/AscI then ligated together. This plasmid and the *ERG25* 3' homology amplicon were digested with SacI/SpeI then ligated together. Completed plasmid was sequence verified.

Oligonucleotides used in this study.

Oligo ID	Description	Sequence (5' to 3')
oLC9502	ScERG25m54F	AGTAGCATAGAGGACTAAGG
oLC274	pJK863down-F	CTGTCAAGGAGGGTATTCTGG
oLC9503	ScERG25m1906R	TCCGTATTGTATCACGAAGG
oLC6292	pLC Tef-ter 189 F	TGGTCGCTATACTGCTGTCG
oLC9232	ScERG25p1F-BamHI	GCATGGATCCATGTCTGCCGTTTTCAACAA
oLC9233	ScERG25p1325R-AscI	GCATGGCGCGCCCGGTCCATGGATTAGATTAT
oLC9234	ScERG25p1345F-SacI	GCATGAGCTCTATCCACCCCACTCTTCGTA
oLC9235	ScERG25p1883R-SpeI	GCATACTAGTGCGCTCGCAATTTTCTAAGT

Supplementary File 7

Supplementary References

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