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Synthetic atpenin analogs: Potent mitochondrial inhibitors of mammalian and fungal succinate-ubiquinone oxidoreductase

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ABSTRACT

Atpenins and harzianopyridone represent a unique class of penta-substituted pyridine-based natural products that are potent inhibitors of complex II (succinate-ubiquinone oxidoreductase) in the mitochondrial respiratory chain. These compounds block electron transfer in oxidative phosphorylation by inhibiting oxidation of succinate to fumarate and the coupled reduction of ubiquinone to ubiquinol. From our investigations of complex II inhibitors as potential agricultural fungicides, we report here on the synthesis and complex II inhibition for a series of synthetic atpenin analogs against both mammalian and fungal forms of the enzyme. Synthetic atpenin **2e** provided optimum mammalian and fungal inhibition with slightly higher potency than natural occurring atpenin A5.

Succinate-ubiquinone oxidoreductase (SQR, complex II) plays a critical role in ATP production by enabling electron transfer as an integral component of mitochondrial respiration and the Krebs cycle. SQR specifically catalyzes the oxidation of succinate to fumarate with concomitant reduction of ubiquinone to ubiquinol (Fig. 1). The enzyme is a four subunit membrane-bound dehydrogenase that comprises a FAD-containing flavoprotein, a subunit containing multiple iron-sulfur clusters and two smaller hydrophobic cytochrome b containing subunits that anchor the catalytic portion to the mitochondrial membrane.[1,2]

Harzianopyridone (initially isolated from *Trichoderma harzianum* and later obtained from the soil fungus *Trichoderma* sp.), atpenin B and atpenin A5 (both isolated from Penicillium sp.) are representative of the atpenin class of penta-substituted pyridine-based natural products that were reported to be potent inhibitors of SQR (Fig. 2).[3] Harzianopyridone is generally represented in the literature as the 4-hydroxy-2-pyridone tautomer whereas atpenins B and A5 are usually shown as the 2,4-dihydroxypyridine tautomer, assignments in both cases inferred from X-ray crystallographic structure analyses.[4,5]

Atpenins bind to the quinone-binding site (Q-site) of complex II but in a slightly different fashion than that of the natural substrate ubiquinone as evidenced by an X-ray crystal study where atpenin A5 was co-crystallized with procaryotic SQR.[6]

The agricultural fungicides carboxin and boscalid are examples of the carboxamide agrochemical class that also inhibit complex II. Like atpenin A5, carboxin reportedly blocks binding of ubiquinone to the active site.[7,8] The carboxamide class of chemistry remains of high interest to the agrochemical research community where ongoing efforts have given rise to new complex II-inhibiting broad-

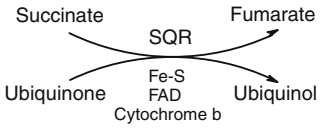

Figure 1. SQR activity in mitochondrial respiration.

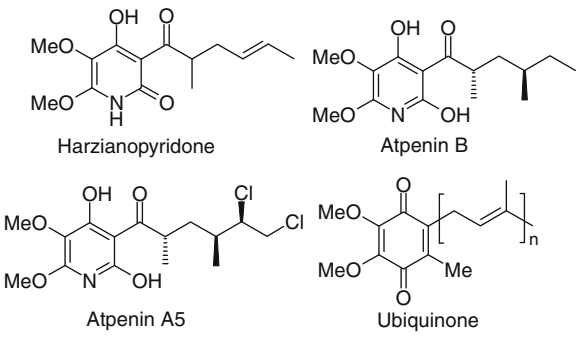

Figure 2. Structures of harzianopyridone, atpenins, and the natural substrate ubiquinone.

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spectrum antifungal product candidates such as penthiopyrad and bixafen.[9,10]

Activity against filamentous fungi, that is, *Trichophyton* sp., has been reported for atpenins with some in vivo activity reported for harzianopyridone against agriculturally relevant pathogens.[11,12] Although we found atpenin A5 to be an extremely potent inhibitor of mammalian and fungal SQR (vide infra), only modest levels of in vivo activity were observed in a fungal growth assay against several pathogens of agricultural significance. However, our interest in complex II as a biological target prompted us to explore a series of synthetic atpenin analogs with the goal of improving in vivo efficacy, preferably with higher selectivity for the fungal enzyme form. Here, we report on the synthesis and inhibitory activity against mammalian and fungal SQR (with observed fungicidal activity) for a series of synthetic analogs, focusing on structures of formulae **1** and **2** (see Fig. 3).

Quéguiner et al. reported the first syntheses of racemic atpenin B and harzianopyridone by a unique metalation route involving an innovative 'clockwise' functionalization of a pyridine nucleus.[13,14] By a modification of that method, we made a series of compounds of formulae **1** and **2** from commercially available 2,3-dimethoxypyridine (**3**).

Scheme 1 outlines the syntheses of key pyridine precursors to both **1** and **2**. Lithiation of **3** with 1.5 equiv of *n*-butyl lithium (more than 1 equiv of base was used, presumably to overcome chelation by the methoxy groups of **3** as reported by Quéguiner et al.) followed by low temperature addition of trimethylborate and subsequent oxidation in peracetic acid afforded 2,3-dimethoxy-4-pyridone that was protected as the *N*,*N*-diisopropyl carbamate **4** (the precursor to compounds of formula **1**) via acylation on oxygen with *N*,*N*-diisopropyl chloroformate in the presence of silver carbonate.[15] Electrophilic bromination of **4** (vs introduction via lithiation by the method of Quéguiner et al.) provided a good yield of 3-bromopyridine **5** that was allowed to undergo a 'halogen dance' in the presence of LDA and a small amount of bromine as a concentrated solution in THF to afford 2-bromopyridine **6**. Metal–halogen exchange on **6** and trapping with trimethylborate followed by oxidation in peracetic provided 2-pyridone **7**. Alkylation on oxygen with SEMCl and sodium hydride afforded the SEM protected pyridyl carbamate **8**, which served as a precursor to atpenin analogs of formula **2**.

Preparation of keto 4-pyridones of formula **1** from pyridyl carbamate **4** is shown in Scheme 2. Lithiation of **4** with an excess of butyl lithium followed by quenching with a series of aldehydes gave alcohol adducts that underwent PCC oxidation to afford the ketopyridyl carbamates **9**. Cleavage of the carbamate protecting group on **9** by heating in methanolic KOH afforded compounds of formula **1** (R values listed in Table 1) following isolation upon acidic workup.

Structurally closer to natural occurring atpenins, analogs of formula **2** were made from **8** as outlined in Scheme 3. Lithiation of **8**

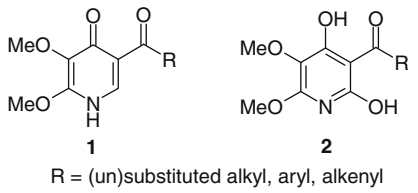
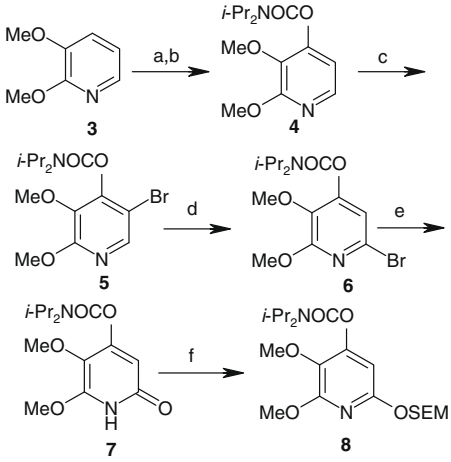
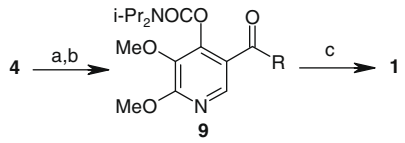


Figure 3. Synthetic atpenin targets.



Scheme 1. Reagents and conditions: (a) (i) 2.5 M *n*-BuLi (1.5 equiv), THF, −70 °C→0 °C, 1 h; (ii) trimethylborate (2.5 equiv), −70 °C to −50 °C; (iii) 32 wt % $MeCO_3H$ in acetic acid (2 equiv), −50 °C→10 °C, 86%; (b) *N*,*N*-diisopropyl chloroformate (1.5 equiv), Ag_2CO_3 (1.5 equiv) toluene, 110 °C, 3 h, 72%; (c) Br_2 (2.5 equiv), CCl_4, 0 °C→25 °C, 24 h, 82%; (d) LDA (3 equiv), Br_2 (cat.), THF, −70 °C to −40 °C, 1 h, 75%; (e) (i) 2.5 M *n*-BuLi (1.5 equiv), THF, −70 °C; (ii) trimethylborate (2.5 equiv), −70 °C; (iii) 32 wt % $MeCO_3H$ in acetic acid (2 equiv), −70 °C→10 °C, 66%; (f) SEMCl (1.2 equiv), NaH (1.3 equiv), THF, 0 °C→25 °C, 1 h, 65%.

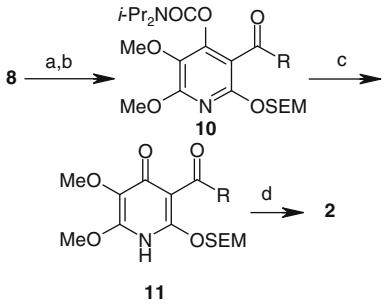


Scheme 2. Reagents and conditions: (a) (i) 2.5 M *n*-BuLi (3.0 equiv), THF, −70 °C, 1 h; (ii) RCHO (3.5 equiv), THF, −70 °C, 1 h, 80–90%; (b) PCC (3.0 equiv), CH_2Cl_2, molecular sieves, 25 °C, 2 h, 75–85%; (c) (i) 5 N KOH in MeOH, reflux, 2–3 h; (ii)10% aq acetic acid quench, 25 °C, 35–60%.

Table 1
Inhibitory activity of synthetic atpenins against mammalian and fungal complex II

Entry	R	Bovine IC_{50} (μM)	*Septoria n.* IC_{50} (μM)
1a	2-Butyl	>30	>30
1b	2-Chlorophenyl	>30	>30
1c	2-Phenoxyphenyl	>30	>30
1d	3-Chlorophenyl	6.56	>30
1e	3-Phenoxyphenyl	0.454	2.98
1f	4-Chlorophenyl	3.40	26.7
1g	4-Methoxyphenyl	>30	>30
1h	4-Phenoxyphenyl	0.492	4.08
1i	4-Biphenyl	0.795	2.75
1j	2-Naphthyl	12.8	20.7
2a	2-Butyl	0.096	2.58
2b	2-Hexyl	0.020	0.122
2c	Cyclohexyl	0.127	1.78
2d	3-Heptyl	0.125	0.234
2e	$CH(Me)(CH_2)_8Me$	0.003	0.004
2f	$CH(Me)(CH_2)_2$–$CH{=}C(Me)_2$	0.005	0.051
2g	4-Chlorophenyl	0.326	>30
2h	3-Phenoxyphenyl	0.112	2.33
2i	CH(Me)Ph	0.008	0.299
2j	$CH(Me)CH_2$-(4-*t*-BuPh)	0.008	0.024
2k	$CH(Ph)_2$	0.630	>30
14	4-Chlorophenyl	1.27	1.94
15	4-Chlorophenyl	>30	>30
16	4-Chlorophenyl	13.8	13.95
17	4-Chlorophenyl	>30	>30
18	4-Chlorophenyl	>30	>30
—	Atpenin A5	0.004	0.011
—	Boscalid	1.79	0.245

IC_{50} values represent the mean of triplicate determinations with a standard error of less than 5%.



Scheme 3. Reagents and conditions: (a) (i) 2.5 M *n*-BuLi (3.0 equiv), THF, −70 °C, 1 h; (ii) RCHO (4.0 equiv), THF, −70 °C, 1 h, 50–65%; (b) PCC (3.0 equiv), CH_2Cl_2, molecular sieves, 25 °C, 50–80%; (c) (i) 5 N KOH in MeOH, reflux, 2–3 h (ii) 10% aq acetic acid quench, 35–55%; (d) concd HCl, MeOH, reflux, 1–2 h, 70–80%.

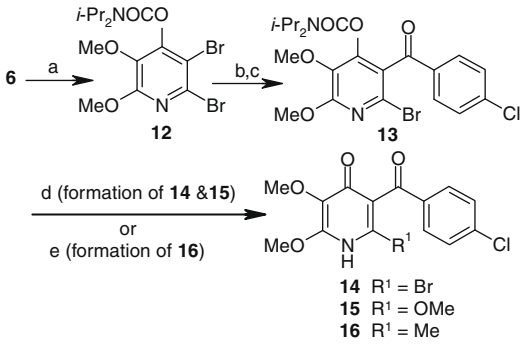
with an excess of butyl lithium and quenching with aldehydes gave alcohols that were oxidized with PCC to the ketopyridyl carbamates **10**. Cleavage of the carbamate protecting group by heating in methanolic KOH gave pyridones of formula **11** after acidic workup, generally in moderate yields. Heating **11** in acidic methanol resulted in removal of the SEM group with isolation of fully unprotected synthetic atpenins **2** (R values listed in Table 1).[16]

In Scheme 4, the preparation of several 4-chlorobenzoylpyridones (**14**–**16**) is outlined where, in place of the atpenin hydroxy group adjacent to the pyridine ring nitrogen, bromine, methoxy and methyl groups were inserted.

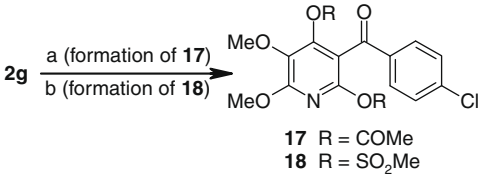
Bromination of **6** provided 2,3-dibromopyridine **12** which was selectively lithiated at the 3-position via metal halogen exchange. Trapping with 4-chlorobenzaldehyde gave an alcohol intermediate that was immediately oxidized with PCC to afford benzoylpyridine **13**. Removal of the carbamate protecting group by heating in alcoholic KOH followed by an acidic quench generated a mixture of **14** and **15** that were separated by chromatography. Treatment of **13** with $AlMe_3$ resulted in displacement of the remaining bromine at the 2-position with methyl where **16** was isolated upon de-protection in alcoholic KOH and acidic workup.

Synthesis of two bis-protected 3-benzoyl 2,4-dihydroxypyridines is shown in Scheme 5. Heating the benzoyl-substituted dihydroxypyridine **2g** (compound **2** where R is 4-chlorophenyl) in acetic anhydride gave the bis-acetylated adduct **17** and reaction with methane sulfonyl chloride in the presence of Hunig's base gave the bis-sulfonylated **18**.

Prepared compounds were evaluated for complex II activity in both a mammalian (bovine heart) and fungal (*Septoria nodorum*,



Scheme 4. Reagents and conditions: (a) Br_2, (5 equiv) CCl_4, 0 °C→25 °C, 3 days, 35%; (b) (i) 2.5 M *n*-BuLi (1.1 equiv), THF, −70 °C; (ii) 4-chlorobenzaldehyde (2.0 equiv), THF, −70 °C, 1 h; (iii) (c) PCC (2 equiv), CH_2Cl_2, molecular sieves, 25 °C, 80%; (d) (i) 5 N KOH in MeOH, reflux; (ii) 20% aq acetic acid quench, 25 °C, 35%; (e) (i) 2 M $AlMe_3$ in hexane (2 equiv) $Pd(PPh_3)_4$ (5 mol %) DME, 70 °C, 80%); (ii) 5 N KOH in MeOH, reflux; (iii) 20% aq acetic acid quench, 65%.



Scheme 5. Reagents and conditions: (a) acetic anhydride neat, 25 °C, 25% (b) methanesulfonyl chloride (3.5 equiv) 25 °C, *i*-Pr_2EtN (5 equiv), THF, 25 °C, 30%.

wheat glume blotch) sub-mitochondrial membrane assay. SQR inhibition was measured in plate-based tests where succinic acid served as the electron donor and the dye 2,6-dichlorophenol-indophenol (DCIP) was used as the final electron acceptor. Electron transfer from the succinate substrate to ubiquinone (with concomitant formation of ubiquinol) was measured spectrophotometrically by ubiquinol-dependent reduction of DCIP.[17] Table 1 summarizes mammalian and fungal inhibitory data as micromolar IC_{50} values.

Acyl pyridones of formula **1** where R = 2-butyl (**1a**), 2-chlorophenyl (**1b**), 2-phenoxyphenyl (**1c**), and 4-methoxyphenyl (**1g**) did not provide significant mammalian or fungal SQR inhibition below 30 μM. Analogs where R = 3-chlorophenyl (**1d**), 4-chlorophenyl (**1f**), and 2-naphthyl (**1j**) did afford mammalian IC_{50} values in the 3–13 μM range but with fungal IC_{50} values greater than 20 μM. Pyridones where R = 3-phenoxyphenyl (**1e**), 4-phenoxyphenyl (**1h**), and 4-biphenyl (**1i**) had higher potency with mammalian IC_{50} values slightly below 1 μM and fungal IC_{50} values in the 2-5 μM range.

On the other hand, 3-acyl-2,4-dihydroxypyridines of formula **2** in most cases provided much higher levels of SQR inhibition with the mammalian enzyme form consistently showing greater sensitivity than the fungal form. Substituents where R = 2-butyl (**2a**) and 2-hexyl (**2b**) gave mammalian IC_{50} values in the 0.02–0.1 μM range but with fungal IC_{50} values 6–27-fold higher. Analogs where R = cyclohexyl (**2c**), 3-heptyl (**2d**), 4-chlorophenyl (**2g**), 3-phenoxyphenyl (**2h**), and $CH(Ph)_2$ (**2k**) were less active with both mammalian and fungal IC_{50} values above 0.1 μM.

Substituents where R = $CH(Me)(CH_2)_8Me$ (**2e**), $CH(Me)(CH_2)_2CH{=}C(Me)_2$ (**2f**), CH(Me)Ph (**2i**), and $CH(Me)CH_2$(4-*t*-butylphenyl) (**2j**) provided the highest levels of inhibition with mammalian IC_{50} values below 0.01 μM. Although the fungal IC_{50} value for **2i** was close to 0.3 μM, **2e**, **2f**, and **2j** all had fungal IC_{50} values close to or below 0.05 μM. Optimum activity was attained with **2e** which had mammalian and fungal IC_{50} values of 0.003 and 0.004 μM, respectively. Compound **2e** was found to be slightly more active than atpenin A5 which showed mammalian and fungal IC_{50} values of 0.004 and 0.011 μM, respectively.

Surprisingly, 2-bromopyrimidinone **14** showed mammalian and fungal inhibition in the IC_{50} range of 1–2 μM whereas 2-methoxypyrimidinone **15** had IC_{50} values above 30 μM. In between the activity of **14** and **15**, 2-methyl pyrimidinone **16** had mammalian and fungal IC_{50} values in the 13–14 μM range.

Prior to obtaining data revealing **2g** to be a poor inhibitor of fungal SQR, a mammalian IC_{50} value of 0.326 μM obtained early in this program prompted investigation of the bis-acetylated and bis-sulfonylated dihydroxy pyrimidines **17** and **18** as pro-forms where expression of in vivo activity might be improved.[18] As expected, IC_{50} values for both compounds were greater than 30 μM.

Although some of these synthetic atpenin analogs showed better fungal inhibition of SQR than the commercial complex II inhibiting fungicide boscalid (fungal IC_{50} = 0.245 μM), surprisingly, substantial levels of in vivo activity against *S. nodorum* were not observed for any of these atpenin analogs in both an antifungal growth assay or whole plant assay at 200 ppm. Limited in vivo activity on other agricultural pathogens was detected as well.

In summary, a series of synthetic atpenin analogs, were prepared via 'clockwise' functionalization of commercially available 2,3-dimethoxypyridine (**3**) via a modification of the synthetic method of Quéguiner.[13,14] Pyridones of formula **1** provided modest levels of inhibition relative to atpenin A5 with some analogs showing low micromolar affinity. On the other hand, dihydroxypyridines of formula **2** gave much higher levels of mammalian and fungal SQR inhibition with some compounds demonstrating low nanomolar binding. The long-chain 3-alkanoyl (13 carbons) substituted dihydroxypyridine **2e** provided optimum SQR inhibition and was slightly more active than atpenin A5 on both mammalian and fungal SQR. Evidently, the presence of stereochemical centers on the 3-acyl atpenin side chain (as in the case of atpenin A5) is not critical for high SQR potency. Unfortunately, similar to atpenin A5, no synthetic analogs showed higher affinity for fungal versus mammalian SQR or substantial levels of in vivo antifungal activity. The strong likelihood that poor in vivo activity resulted from an issue around fungal penetration (membrane/cell wall permeability), translocation within the pathogen, metabolism or efflux precluded our continued interest in atpenin analogs as potential agricultural fungicides.

Acknowledgment

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15. Quéguiner et al. reported that use of 2.2 equiv of *n*-butyl lithium gave optimum results, presumably due to chelation of an equivalent of base by the methoxy groups and ring nitrogen. However, we found that satisfactory product yields were obtained with 1.5 equiv of *n*-butyl lithium.
16. All new compounds gave satisfactory spectral data consistent with their structures. As a representative example of the general method for making keto dihydroxypyridine analogs of formula **2**, synthesis of 3-(4-chlorobenzoyl)-2,4-dihydroxy-5,6-dimethoxypyridine (**2g**) as outlined in Scheme 3 (steps a–d) is as follows: (step a) To a solution of 1 g (2.3 mmol) of 2,3-dimethoxy-6-(2-trimethylsilylethoxymethoxy)-4-pyridyl *N*,*N*-diisopropylcarbamate (**8**)[13] stirring in 15 mL of THF at −70 °C, *n*-butyllithium (2.5 M, 2.8 mL) was added dropwise. After stirring 1 h at −70 °C, 4-chlorobenzaldehyde (1.15 g, 8.2 mmol) in 5 mL of THF was added dropwise. The reaction stirred 2 h at −70 °C, quenched with 8 mL of a 1:1:1 mixture of ethanol, water, and glacial acetic acid, followed by extracting with dichloromethane. The separated organic layer was dried over magnesium sulfate and evaporated in vacuo to give orange oil. Purification by MPLC on silica gel (10–30% ethyl acetate in hexanes) yielded 0.92 g (70%) of the 3-(4-chlorophenyl)-methanol adduct of **8**, isolated as a clear oil. (step b) To a solution of this oil (0.75 g, 1.3 mmol) stirring in 10 mL of dichloromethane over 0.5 g of ground molecular sieves at room temperature, pyridium chlorochromate (0.91 g, 4.2 mmol) was added in portions over 1 min. The reaction was stirred at ambient temperature for 2 h, diluted with 20 mL of diethyl ether and filtered through a medium glass frit filter. Evaporating in vacuo gave a brown oil that was purified by MPLC on silica gel (10–20% ethyl acetate in hexanes) to afford 0.61 g (82%) of the benzoylpyridine **10** where R = 4-chlorophenyl as a clear oil. ^{1}H NMR (300 MHz), $CDCl_3$): δ 7.39 (s, 2H), 7.37 (s, 2H), 5.45 (s, 2H), 4.00 (s, 3H), 3.81 (s, 3H), 3.90–3.69 (m, 2H), 3.90–3.69 (m, 2H), 3.51–3.43 (m, 2H), 1.10 (dd J = 5.1, 6.2 Hz, 12H), 0.90–0.74 (m, 2H). (steps c and d) A 0.54 g (0.95 mmol) sample of **10** was dissolved in 12 mL of 5 N potassium hydroxide in methanol and heated at reflux for 1 h. After quenching with 10 mL of 10% aqueous acetic acid followed by neutralization with saturated sodium bicarbonate, the reaction mixture was extracted with ethyl acetate. The separated organic layer was dried over magnesium sulfate and concentrated in vacuo to afford 0.31 g of crude pyridone **11** where R = 4-chlorophenyl, isolated as a yellow oil (NMR confirmed removal of the SEM protecting group). The entire sample of **11** was heated in 10 mL of a 1:10 mixture of concentrated HCl and methanol at reflux for 1 h. The reaction mixture was concentrated in vacuo, treated with saturated aqueous sodium bicarbonate (20 mL) and extracted with dichloromethane. The separated organic layer was dried over magnesium sulfate and evaporated in vacuo to give a yellow solid. Purification by MPLC on silica gel (10–20% ethyl acetate in hexanes) afforded 0.17 g of the dihydroxypyridine **2g** (57%) as a yellow-tinted solid; ^{1}H NMR (300 MHz), $CDCl_3$): δ 7.51 (d, J = 8.6 Hz, 2H, ArH), 7.38 (d, J = 8.6 Hz, 2H, ArH), 3.83 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, acetone-d): δ 53.608, 59.957, 99.675, 124.357, 127.946, 130.063, 137.013, 139.005, 159.344, 159.674, 161.395, 196.513; IR (PTFE): 1647,1592 cm^{-1}; HRMS (APESI, M+) $C_{14}H_{12}NO_5Cl$: m/z calcd 309.704, m/z found 310.479 (M+); mp 210–211 °C.
17. The complex II assay was run in a 96-well microplate format using submitochondrial membranes prepared from bovine heart or *Septoria nodorum*. The assay included 20 mM succinate as the electron donor and decyl ubiquinone as the electron acceptor. The reaction was initiated by addition of succinate and allowed to proceed for 10 min. Reduction of the dye 2,6-dichlorophenol-indophenol (DCIP) was monitored by absorbance at λ = 600 nm.
18. Investigating pro-forms of a much more active fungal SQR inhibitor such as **2e** where there might be a higher possibility of attaining significant in vivo activity remains to be explored.