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Benzofuran-, benzothiophene-, indazole- and benzisoxazole-quinones: Excellent substrates for NAD(P)H:quinone oxidoreductase 1

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ABSTRACT

A series of heterocyclic quinones based on benzofuran, benzothiophene, indazole and benzisoxazole has been synthesized, and evaluated for their ability to function as substrates for recombinant human NAD(P)H:quinone oxidoreductase (NQO1), a two-electron reductase upregulated in tumor cells. Overall, the quinones are excellent substrates for NQO1, approaching the reduction rates observed for menadione.

1. Introduction

Quinones are widespread in nature,[1] and participate in important biological redox processes. For example, the ubiquinones act as electron-transfer agents in the respiratory chain, and the heterocyclic pyrroloquinolinequinone (coenzyme PQQ) is a redox co-factor. Indeed heterocyclic derivatives form an important subset of quinones,[2] often possessing potent biological activity, for example as phosphatase inhibitors.[3] The best known heterocyclic quinone is the clinically used cancer therapeutic agent, the indolequinone natural product mitomycin C (MMC) **1**,[4–7] although other heterocyclic quinones such as streptonigrin **2** have also been widely studied.[8]

Given the biological importance of quinone reduction, our own studies have focused on the two-electron reduction of quinones by the enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, QR1, EC 1.6.99.2).[9–12] In particular, we have investigated a broad range of indolequinone based substrates and inhibitors,[13–17] the quinoline-5,8-dione system found in streptonigrin,[18] and both benzimidazole-4,7-diones **3** and benzothiazole-4,7-diones **4** (Fig. 1).[19] Others have also studied 5-undecyl-6-hydroxybenzothiazole-4,7-dione (UHDBT), an analogue of ubiquinone, as an inhibitor of electron transport by binding to cytochrome bc_1,[20] whilst the benzimidazole quinones have been widely investigated as analogues of MMC.[21–27]

Despite the aforementioned studies, we sought to extend the range of heterocyclic quinones studied as substrates for NQO1, focusing on benzofurans, benzothiophenes, and indazoles. Furoquinones are quite well described, and although most of their naturally occurring compounds are naphthofuranquinones,[1] relatively simple derivatives such as acamelin **5** are known.[28] Benzothiophene quinones are less common, although caldariellaquinone **6** appears to fulfil an important redox role in thermophilic and acidophlic archaea of the *Sulfolobus* genus that lack ubiquinones as electron-transfer agents.[29,30] On the other hand, indazolequinones are somewhat rarer, although compounds such as **7** have been prepared as MMC analogues,[31] whilst others have been investigated as substrates for carbonyl reductase.[32] Therefore in an attempt to widen the group of NQO1 substrates/inhibitors, and to probe further the active site of the enzyme, we have explored a new series of heterocyclic quinones based on benzofuran, benzothiophene, indazole and benzisoxazole.

2. Results and discussion

2.1. Chemistry

In order to make meaningful comparisons with the more widely studied indolequinones, we initially elected to investigate

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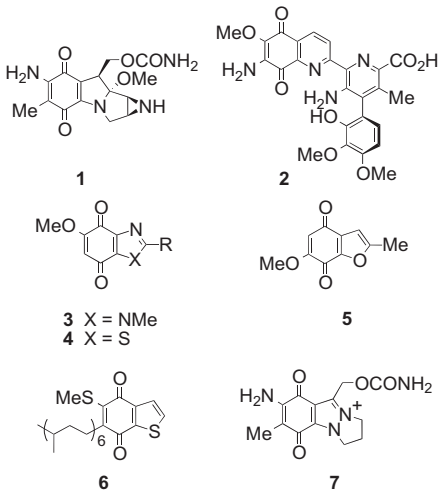
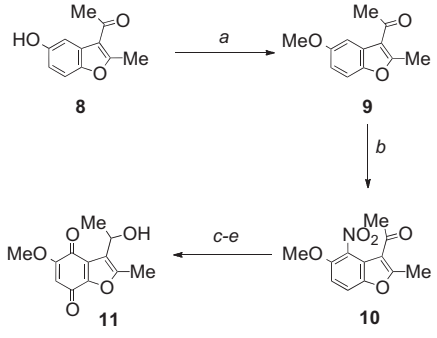


Figure 1. Some heterocyclic quinones.

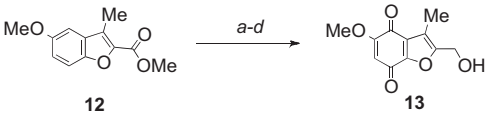
relatively simple 5-methoxy-heterocyclic quinones in the benzofuran and benzothiophene series. The synthesis of quinone **11** started with the known benzofuran **8**, readily prepared from benzoquinone in a Nenitzescu type reaction.[33] Methylation gave the known 5-methoxy derivative **9**, nitration of which gave a mixture of the desired 4-nitro compound **10** along with its 6-nitro isomer in excellent overall yield, but in a 1:2 ratio. Although nitrobenzofuran **10** could be isolated, it was more convenient to reduce the mixture of nitro compounds to the corresponding amines, reduce the ketone with sodium borohydride and then oxidize the aniline with Fremy's salt and purify the desired quinone **11** at the final stage (Scheme 1). The intermediate iminoquinone was not observed and was presumably readily hydrolyzed under the reaction conditions.

The isomeric benzofuranquinone **13**, with the alcohol group at the 2-position, was synthesized from the known benzofuran **12**, readily available from 4-methoxyphenol by carbene O–H insertion, and cyclization.[34] The sequence of nitration, reduction and oxidation to the quinone was carried out without purification of the intermediate compounds and delivered the pure benzofuranquinone **13** in 53% over the four steps (Scheme 2).

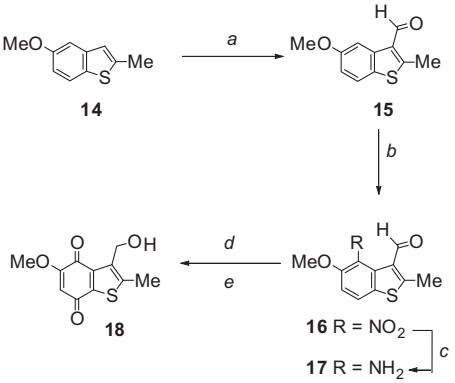
In the benzothiophene series, we started with the known 5-methoxy-2-methylbenzothiophene **14** (Scheme 3).[35] Formylation under Vilsmeier conditions gave the desired 3-aldehyde **15**, but in poor yield (20%), the major product being the unwanted 4-formyl compound. Nevertheless, subjecting aldehyde **15** to the usual sequence of nitration, reduction, and final oxidation to the



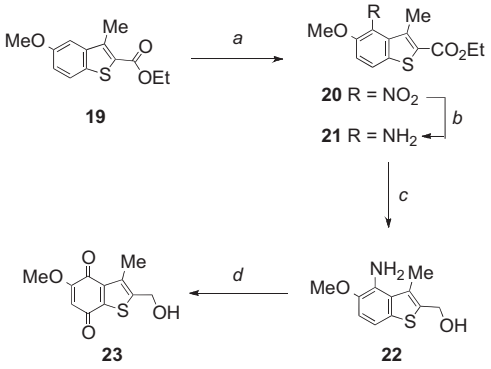
Scheme 1. Reagents and conditions: (a) KH, MeI, DMF, rt (42%); (b) fuming HNO_3, AcOH, rt (81% as 1:2 mixture of 4- and 6-nitro isomers); (c) Sn, HCl, EtOH, reflux; (d) $NaBH_4$, MeOH, rt; (e) Fremy's salt, NaH_2PO_4, aq acetone, rt (20% over three steps).



Scheme 2. Reagents and conditions: (a) HNO_3, AcOH, rt; (b) Sn, HCl, EtOH, rt; (c) $LiAlH_4$, THF, 0 °C; (d) Fremy's salt, NaH_2PO_4, aq acetone, rt (53% over four steps).



Scheme 3. Reagents and conditions: (a) $POCl_3$, DMF, CH_2Cl_2, 0–50 °C (20%, plus 40% of 4-formyl isomer); (b) HNO_3, AcOH, rt (61%); (c) Sn, HCl, EtOH, reflux (71%); (d) $NaBH_4$, MeOH, rt; (e) Fremy's salt, NaH_2PO_4, aq acetone, rt (41% over two steps).

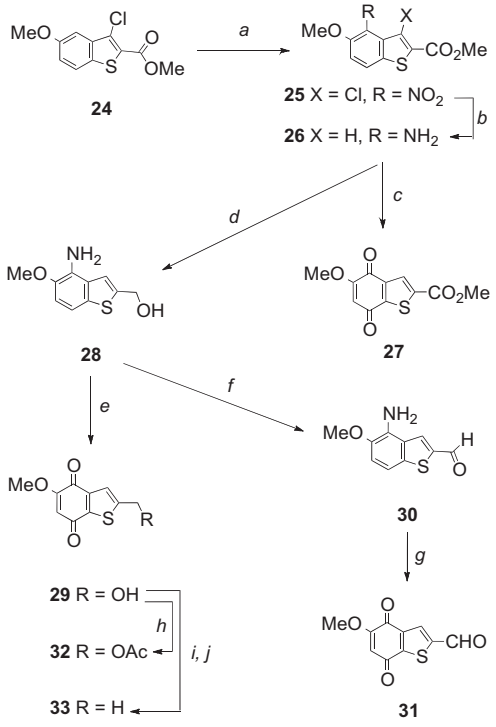


Scheme 4. Reagents and conditions: (a) HNO_3, AcOH, rt (74%); (b) Sn, HCl, EtOH, reflux (86%); (c) $LiAlH_4$, THF, rt (80%); (d) Fremy's salt, NaH_2PO_4, aq acetone, rt, then aq HCl (2 M), acetone. (67%).

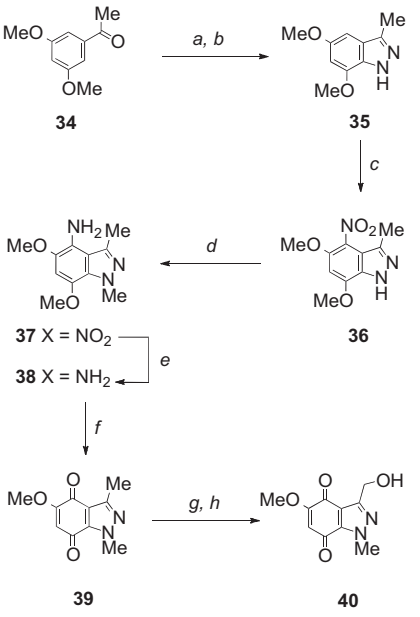
quinone, provided the benzothiophenequinone **18** in reasonable overall yield (Scheme 3).

By analogy with the conversion of benzofuran **12** into benzofuranquinone **13**, the known starting material,[36] ethyl 5-methoxy-3-methylbenzothiophene-2-carboxylate **19**, was converted into benzothiophenequinone **23** by the now familiar sequence of nitration, reduction and final oxidation to the quinone (Scheme 4).

A range of 3-unsubstituted benzothiophenequinones was also prepared as shown in Scheme 5. Reaction of 3-methoxycinnamic acid with thionyl chloride and methanol readily provided the known benzothiophene-2-carboxylate starting material **24** in 37% yield.[37] Nitration, followed by hydrogenation over Pd/C resulted in reduction of the nitro group with concomitant hydrogenolysis of the chlorine, and gave the 4-aminobenzothiophene **26**, which was either directly oxidized to quinone **27**, or converted into quinone **29** by initial reduction of the ester (Scheme 5). Benzothiophenequinones **31**–**33** were also prepared by standard transformations as shown in Scheme 5.

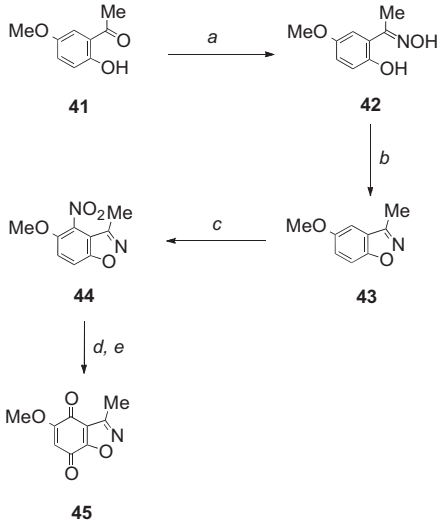


Scheme 5. Reagents and conditions: (a) HNO_3, AcOH, reflux (87%); (b) H_2, Pd/C, MeOH–THF (83%); (c) Fremy's salt, NaH_2PO_4, aq acetone, rt (75%); (d) $LiAlH_4$, THF, rt (78%); (e) Fremy's salt, NaH_2PO_4, aq acetone, rt (88%); (f) MnO_2, CH_2Cl_2, reflux (38%); (g) Fremy's salt, NaH_2PO_4, aq acetone, rt (79%); (h) Ac_2O, pyridine, rt (69%); (i) MsCl, Et_3N, CH_2Cl_2, rt; (j) $LiAlH_4$, THF, rt (5% over two steps).



Scheme 6. Reagents and conditions: (a) $Cl_3CCH_2O_2CN{=}NCO_2CH_2Cl_3$, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt; (b) Zn, AcOH, rt (35% over two steps); (c) HNO_3, AcOH, rt (81%); (d) MeI, KOH, DMSO, rt (49%); (e) Sn, HCl, EtOH, reflux (96%); (f) Fremy's salt, NaH_2PO_4, aq acetone, rt (78%); (g) NBS, AIBN, CCl_4; (h) $AgNO_3$, aq acetone (36% over two steps).

The synthesis of indazolequinones started from the dimethoxyindazole **35**, itself prepared by the literature method from 3,5-dimethoxyacetophenone **34** by electrophilic amination with bis(trichloroethyl) azodicarboxylate followed by zinc reduction and cyclization.[38] Nitration, methylation, reduction and oxidation



Scheme 7. Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, EtOH, pyridine, rt (98%); (b) DIAD, Ph_3P, THF, rt (100%); (c) HNO_3, AcOH, rt (91%); (d) Sn, HCl, EtOH, reflux; (e) Fremy's salt, NaH_2PO_4, aq acetone, rt (20% over two steps).

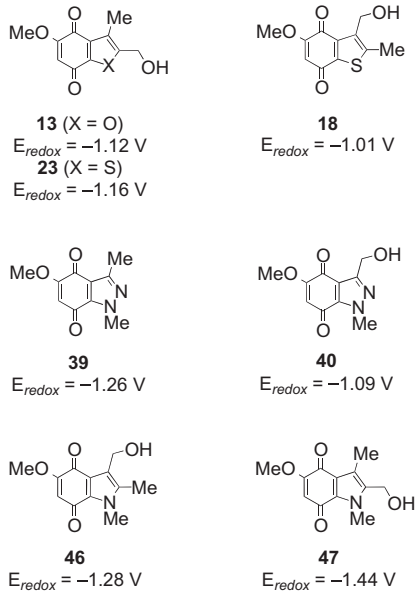


Figure 2. E_{redox} values (v. Fc) for benzofuran-, benzothiophene- and indazole-quinones **13**, **18**, **23**, **39** and **40** compared to related indolequinones **46** and **47**.[13,14]

delivered the required indazolequinone **39** (Scheme 6). The methyl group was further functionalized by radical bromination and conversion into the 3-hydroxymethylindazolequinone **40** (Scheme 6).

Finally an example of a benzisoxazolequinone was prepared. 2-Hydroxy-5-methoxyacetophenone **41** was converted into its oxime **42** that underwent facile intramolecular Mitsunobu reaction to provide the benzisoxazole **43** in quantitative yield. Thereafter, nitration, reduction and oxidation as before gave the required benzisoxazolequinone **45** (Scheme 7)

Reduction potentials were measured for benzofuran-, benzothiophene- and indazole-quinones **13**, **18**, **23**, **39** and **40** using cyclic voltammetry in DMF as solvent with tetra-*n*-butylammonium tetrafluoroborate as supporting electrolyte as previously described.[13] The E_{redox} values, with reference to ferrocene (Fc), are shown in Figure 2; values for the related indolequinones **46** and **47** are also shown. The data show that whilst the indazole quinone **39** has a similar redox potential to the indolequinones (E_{redox} v. Fc

Table 1
Metabolism of heterocyclic quinones by recombinant human NQO1

Entry	Ring	Compd	X	R^2	R^3	NQO1[a] (ave) (μmol/min/mg)	NQO1[a] (init) (μmol/min/mg)
1	A	**46**	NMe	Me	CH_2OH	1.25 ± 0.03[b]	nd[c]
2	A	**47**	NMe	CH_2OH	Me	2.49 ± 1.27[d]	nd[c]
3	A	**11**	O	Me	CH(OH)Me	87.9 ± 25.6	585 ± 32
4	A	**13**	O	CH_2OH	Me	80.8 ± 6.7	1025 ± 54
5	A	**18**	S	Me	CH_2OH	nd[c]	236 ± 35
6	A	**23**	S	CH_2OH	Me	nd[c]	468 ± 68
7	A	**27**	S	CO_2Me	H	53.9 ± 3.8	555 ± 71
8	A	**29**	S	CH_2OH	H	63.8 ± 11.7	927 ± 89
9	A	**31**	S	CHO	H	27.4 ± 5.7	897 ± 64
10	A	**32**	S	CH_2OAc	H	24.5 ± 7.9	900 ± 86
11	A	**33**	S	Me	H	5.51 ± 0.87	553 ± 66
12	B	**39**	NMe	—	Me	19.6 ± 5.3	55.3 ± 5.1
13	B	**40**	NMe	—	CH_2OH	38.1 ± 7.1	247 ± 14
14	B	**45**	O	—	Me	27.6 ± 3.2	290 ± 28

[a] Ave and init refer to average and initial rates of metabolism as measured by the HPLC and spectrophotometric assays, respectively; average rates are determined from irreversible NADH oxidation whereas initial rates are determined from cytochrome c reduction. For reference, initial rate for menadione reduction was 1225 ± 15 μmol/min/mg.
[b] Ref. 13.
[c] nd = not determined.
[d] Ref. 14.

−1.20 to −1.40 V), the other heterocyclic quinones are considerably easier to reduce. Consistent with this finding, quinone **39** had the lowest reduction rate by NQO1 (Table 1).

2.2. Enzyme studies

The new heterocyclic quinones were evaluated for their ability to act as substrates for NQO1. We used two assays for studying quinone metabolism by recombinant human NQO1 based on HPLC and spectrophotometry. The former HPLC system is capable of quantifying both NADH oxidation and quinone reduction, and gives average rates of reduction over a 30–40 min period.[39,13] Quinone reduction is reversible due to redox cycling of the hydroquinone, so results (Table 1) are reported as μmol NADH oxidized min^{-1} mg^{-1} NQO1. The alternative spectrophotometric method uses cytochrome *c* as the terminal electron acceptor and gives initial rates of reduction that are generally higher than the HPLC method.[18] Nevertheless the relative order of metabolism is essentially the same with the two methods, and with the exception of entries 1, 2, 5 and 6, both methods were used to enable reliable comparison between the new heterocyclic quinones. Interestingly, quinones **27** and **33** had similar initial reduction rates, but the average rate for **27** was 10-fold higher than for **33**. This suggests that the **27** hydroquinone redox cycles more efficiently than the **33** hydroquinone, most likely due to the electron-withdrawing methyl carboxylate group present on **27**.[40] In contrast to our previous studies on indolequinones,[13,39] electron-withdrawing groups did not appear to increase rates of reduction for the benzothiophene series.

The new quinones are all excellent substrates for rhNQO1. In the benzofuran and benzothiophene series, reduction rates were higher when the hydroxyalkyl substituent was at the C-2 position rather than C-3 (Table 1), possibly due to stabilizing hydrogen-bonding interactions with key amino acid residues in the NQO1 active site. As with the benzimidazole- and benzothiazole-quinones,[19] all of the new quinones were much better substrates for NQO1 than the widely studied indolequinones,[13,39,14] as seen by comparison with indolequinones **46** and **47** included in Table 1 for comparison. In fact, the reduction rates for benzofuran- and benzothiophene-quinones **13**, **29**, **31** and **32** approach the initial reduction rate observed for menadione (1225 ± 15 μmol/min/mg),[19] a simple naphthoquinone that has been used to measure activity of the enzyme, making these compounds some of the best NQO1 substrates seen to date.

The results presented here complement our previous work on bioreductive activation of indolequinone antitumor agents by NQO1. Novel heterocyclic quinones have been synthesized, characterized and studied biologically as substrates for recombinant human NQO1. These data add to our understanding of the structural requirements for efficient metabolism by the quinone reductase enzyme.

3. Experimental section

3.1. General chemistry experimental details

Commercially available reagents were used throughout without purification unless otherwise stated. Light petroleum refers to the fraction with bp 40–60 °C and was distilled before use. Ether refers to diethyl ether. Reactions were routinely carried out under a nitrogen or argon atmosphere. Analytical thin layer chromatography was carried out on aluminum-backed plates coated with silica gel, and visualized under UV light at 254 and/or 360 nm. Flash chromatography was carried out on silica gel. Fully characterized compounds were chromatographically homogeneous.

Infrared spectra were recorded in the range 4000–600 cm^{-1} using FT spectrometers. NMR spectra were carried out at 300 and 400 MHz (^1H frequencies, corresponding ^{13}C frequencies are 75 and 100 MHz). Chemical shifts are quoted in ppm with TMS as internal standard. *J* values are recorded in Hz. In the ^{13}C spectra, signals corresponding to CH, CH_2 or CH_3 groups, as assigned from DEPT, are noted; all others are C. High and low resolution mass spectra were recorded on a Micromass GCT TDF High Resolution mass spectrometer, or at the EPSRC Mass Spectrometry Service (Swansea).

3.2. Synthesis of benzofuranquinones

3.2.1. 3-Acetyl-5-methoxy-2-methylbenzofuran 9

3-Acetyl-5-hydroxy-2-methylbenzofuran **8**[33] (1.62 g, 8.5 mmol) in DMF (30 mL) was added to a stirring suspension of potassium

hydride (0.61 g, 15.2 mmol) in DMF (50 mL) at 0 °C. The mixture was stirred at room temperature for 45 min. Iodomethane (1.80 g, 12.7 mmol) was added dropwise at 0 °C and the mixture allowed to warm to room temperature. After 2 h saturated ammonium chloride solution was added and the mixture extracted with ethyl acetate. The ethyl acetate layer was washed thoroughly with hydrochloric acid (1 M), dried ($MgSO_4$) and concentrated. The crude product was purified by chromatography eluting with dichloromethane to yield the *title compound* as a colorless solid (0.74 g, 42%), mp 69–70 °C (lit.,[41] mp 72 °C); δ_H (300 MHz; $CDCl_3$) 7.48 (1H, d, *J* 2.6, 4-H), 7.33 (1H, d, *J* 8.9, 7-H), 6.89 (1H, dd, *J* 8.9, *J* 2.6, 6-H), 3.88 (3H, s, OMe), 2.76 (3H, s, Me), 2.62 (3H, s, Me).

3.2.2. 3-Acetyl-5-methoxy-2-methyl-4-nitrobenzofuran 10

To a solution of 3-acetyl-5-methoxy-2-methylbenzofuran **9** (0.217 g, 1.1 mmol) in acetic acid (4 mL) cooled to 0 °C was added a mixture of fuming nitric acid (0.5 mL) and acetic acid (2 mL). The mixture was stirred at room temperature for 2 h and then poured on to an ice/water mixture and the precipitate obtained was filtered off and dried. NMR analysis of the mixture showed a 2:1 ratio of 6-nitro and 4-nitro products (0.21 g, 81%), used without further purification. Small quantities of each isomer were obtained by preparative TLC (dichloromethane elution) and were identified by ^1H NMR spectroscopy: 3-acetyl-5-methoxy-2-methyl-4-nitrobenzofuran **10**, δ_H (300 MHz; $CDCl_3$) 7.50 (1H, d, *J* 9.0, ArH), 7.03 (1H, d, *J* 9.0 ArH), 3.93 (3H, s, OMe), 2.74 (3H, s, Me), 2.49 (3H, s, Me); 3-acetyl-5-methoxy-2-methyl-6-nitrobenzofuran, δ_H (300 MHz; $CDCl_3$) 7.97 (1H, s, ArH), 7.76 (1H, s ArH), 4.02 (3H, s, OMe), 2.83 (3H, s, Me), 2.62 (3H, s, Me).

3.2.3. 3-(1-Hydroxyethyl)-5-methoxy-2-methylbenzofuran-4,7-dione 11

To a mixture of 3-acetyl-5-methoxy-2-methyl-4-nitrobenzofuran **10** and its 6-nitro isomer (1:2 ratio) (0.80 g, 3.4 mmol) in ethanol (100 mL) were added tin powder (4.00 g, 34.0 mmol) and hydrochloric acid (3 M; 40 mL). The mixture was heated under reflux for 30 min. Upon cooling the solution was decanted from the tin and neutralized with saturated aqueous sodium hydrogen carbonate. The mixture was extracted with ethyl acetate and the organic layer dried ($MgSO_4$) and concentrated, to yield a mixture of 4- and 6-amino derivatives (0.56 g) that was used directly. Sodium borohydride (0.10 g, 2.6 mmol) was added to a stirred solution of the above mixture (0.56 g, 2.6 mmol) in methanol (50 mL). After 20 min, acetone (10 mL) was added. The solvent was removed and the residue dissolved in ethyl acetate and washed with water. The organic layer was dried ($MgSO_4$) and concentrated. To a solution of the crude product in acetone (40 mL) was added a solution of potassium nitrosodisulfonate (1.00 g, 3.7 mmol) in sodium dihydrogen phosphate buffer (0.3 M; 40 mL). The mixture was stirred at room temperature for 1 h. The acetone was removed in vacuo, and the resulting residue was extracted with dichloromethane and washed with water. The organic layer was dried (Na_2SO_4) and concentrated. The crude material was purified by chromatography eluting with dichloromethane/ethyl acetate (19:1) and recrystallized (dichloromethane/ether) to yield the title compound (0.052 g, 20%, over three steps based on the 4-nitro starting material), as an orange crystalline solid, mp 157–158 °C; λ_{max} (MeOH)/nm 436 ($\log\varepsilon$ 3.18), 320 (3.66), 260 (3.98); λ_{max} (KBr)/cm^{-1} 3414, 3060, 2990, 2915, 1683, 1657, 1607, 1587; δ_H (300 MHz; $CDCl_3$) 5.76 (1H, s, 6-H), 4.81 (1H, m, C*H*Me), 3.85 (3H, s, OMe), 3.78 (1H, d, *J* 10.7 Hz, OH), 2.38 (3H, s, Me), 1.45 (3H, d, *J* 6.7 Hz, CH*Me*); δ_C (100 MHz; $CDCl_3$) 179.2 (C), 175.4 (C), 159.8 (C), 153.4 (C), 150.6 (C), 125.0 (C), 124.8 (C), 105.7 (CH), 62.4 (CH), 56.9 (Me), 24.4 (Me), 12.2 (Me).

3.2.4. 2-Hydroxymethyl-5-methoxy-3-methylbenzofuran-4,7-dione 13

To a solution of methyl 5-methoxy-3-methylbenzofuran-2-carboxylate **12**[34] (0.20 g, 0.91 mmol) in acetic acid (6 mL), cooled to −10 °C was added a mixture of concentrated nitric acid (1 mL) and acetic acid (4 mL). The mixture was stirred at room temperature for 1 h. The reaction mixture was poured in an ice/water mixture and the resulting precipitate filtered off and dried. The crude material was obtained as a 2.8:1 ratio of 4- and 6-nitro products and used directly in the next step. To a suspension of the above mixture in ethanol (30 mL) were added tin powder (0.48 g, 4.0 mmol) and hydrochloric acid (3 M; 7 mL). The mixture was stirred at room temperature for 2 h. The solution was decanted from the excess tin and neutralized with saturated aqueous sodium hydrogen carbonate. The suspension obtained was added to an equal volume of water. The mixture was extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and concentrated. The crude material was used directly in the next step without purification.

To a suspension of lithium aluminum hydride (0.091 g, 2.4 mmol) in THF (10 mL) at 0 °C was added a solution of the above mixture of benzofurans in THF (10 mL) and the reaction was stirred for 15 min. The reaction mixture was quenched by the addition of water (0.2 mL), sodium hydroxide (1 M; 0.2 mL) and silica gel (2 g). The granular precipitate was filtered off through a pad of Celite. The filtrate was dried ($MgSO_4$) and concentrated in vacuo to give the alcohol that was used directly in the next step without purification, or characterization. To a solution of the benzofuran-2-methanol in acetone (50 mL) was added a solution of potassium nitrosodisulfonate (0.64 g, 2.4 mmol) in sodium dihydrogen phosphate buffer (0.3 M; 50 mL). The mixture was stirred at room temperature for 1 h. The excess acetone was removed in vacuo. The resulting residue was extracted with dichloromethane and washed with water. The organic layer was dried (Na_2SO_4) and concentrated. The crude product was purified by chromatography eluting with ethyl acetate/dichloromethane (1:4) and recrystallized (ethyl acetate) to yield the *title compound* as an orange solid (0.108 g, 53%, over four steps); mp 205–206 °C; (found: C, 58.4; H, 4.5. $C_{11}H_{10}O_5 + 0.2H_2O$ requires C, 58.5; H, 4.6); (found: M^+, 222.0528. $C_{11}H_{10}O_5$ requires 222.0528); λ_{max} (KBr)/cm^{-1} 3452, 3057, 2939, 1691, 1660, 1610, 1587; δ_H (300 MHz; $CDCl_3$) 5.80 (1H, s, 6-H), 4.69 (2H, d, *J* 4.0 Hz, CH_2OH), 3.86 (3H, s, OMe), 2.31 (3H, s, Me), 1.98 (1H, br s, OH); δ_C (100 MHz; acetone) 177.8 (C), 175.3 (C), 160.3 (C), 156.6 (C), 149.2 (C), 125.4 (C), 116.7 (C), 105.7 (CH), 56.4 (Me), 54.0 (CH_2), 7.6 (Me); *m*/*z* (EI) 222 (M^+, 35%), 193 (13), 151 (23), 109 (25), 69 (100).

The intermediate nitro compound methyl 5-methoxy-3-methyl-4-nitrobenzofuran-2-carboxylate, can be isolated and characterized. To a solution of methyl 5-methoxy-3-methylbenzofuran-2-carboxylate **12** (0.130 g, 0.6 mmol) in acetic acid (5 mL), cooled to −10 °C was added a mixture of concentrated nitric acid (0.5 mL) and acetic acid (3 mL). The mixture was stirred at room temperature for 1 h. The reaction mixture was poured in an ice/water mixture and the resulting precipitate filtered off and dried. The crude material was obtained as a 3:1 ratio of 4- and 6-nitro products (0.12 g, 80%). Purification by chromatography eluting with dichloromethane gave methyl 5-methoxy-3-methyl-4-nitrobenzofuran-2-carboxylate, mp 146–149 °C; λ_{max} (KBr)/cm^{-1} 3103, 2996, 2945, 1706, 1629, 1588; δ_H (300 MHz; $CDCl_3$) 7.64 (1H, d, *J* 9.1 Hz, ArH), 7.20 (1H, d, *J* 9.1 Hz, ArH), 3.99 (3H, s, OMe), 3.96 (3H, s, OMe), 2.47 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 160.0 (C), 148.8 (C), 147.6 (C), 143.3 (C), 133.6 (C), 123.0 (C), 121.5 (C), 115.1 (CH), 113.7 (CH), 57.7 (Me), 52.4 (Me), 8.9 (Me).

3.3. Synthesis of benzothiophenequinones

3.3.1. 5-Methoxy-2-methylbenzothiophene-3-carboxaldehyde 15

To a mixture of dry DMF (19.6 mL, 169.40 mmol) and dry dichloromethane (9.1 mL) was added dropwise phosphorus oxychloride (16.4 mL, 169.40 mmol) at 0 °C. The ice bath was removed. The reaction mixture was stirred for an additional 30 min and cooled in an ice bath. 5-Methoxy-2-methylbenzothiophene **14**[35] (3.02 g, 16.94 mmol) was added portionwise to the solution over 5 min at 0 °C. The reaction mixture was stirred and heated to 50 °C overnight, poured into an ice-cold aqueous sodium hydroxide (1 M). The mixture was extracted with dichloromethane, dried over $MgSO_4$, filtered, and concentrated. The residue obtained was purified by chromatography, eluting with dichloromethane, to yield the *title compound* (390 mg, 20%) as a yellow oil; (found: MH^+, 207.0477. $C_{11}H_{10}O_2S + H$ requires 207.0480); ν_{max} (film)/cm^{-1} 2957, 2930, 2834, 1735, 1670, 1597, 1559, 1455, 1413, 1351, 1270, 1247, 1224, 1151; δ_H (300 MHz; $CDCl_3$) 10.31 (1H, s, CHO), 8.14 (1H, d, *J* 2.5, 4-H), 7.60 (1H, d, *J* 9.0, 7-H), 7.00 (1H, dd, *J* 9.0, 2.5, 6-H), 3.88 (3H, s, OMe), 2.89 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 184.0 (CH), 159.6 (C), 159.3 (C), 138.9 (C), 130.5 (C), 129.3 (C), 122.6 (CH), 116.0 (CH), 106.4 (CH), 56.1 (Me), 14.8 (Me); *m*/*z* (CI) 207 (MH^+, 100%), 179 (10); and the *4-formyl derivative* (809 mg; 40%) as a beige solid; mp 119–120 °C; (found: MH^+, 207.0477. $C_{11}H_{10}SO_2 + H$ requires 207.0480); ν_{max} (KBr)/cm^{-1} 3421, 2910, 2846, 1661, 1566, 1454, 1430, 1314, 1243, 1183, 1131, 1071; δ_H (300 MHz; $CDCl_3$) 10.68 (1H, s, CHO), 8.09 (1H, br s, 3-H), 7.86 (1H, d, *J* 8.8, ArH), 6.96 (1H, d, *J* 8.8, ArH), 3.97 (3H, s, OMe), 2.63 (3H, s, Me); δ_C (100 MHz; $CDCl_3$) 190.7 (CH), 162.3 (C), 148.0 (C), 139.9 (C), 134.1 (C), 129.7 (CH), 122.7 (CH), 118.1 (C), 108.6 (CH), 55.8 (Me), 17.0 (Me); *m*/*z* (CI) 207 (MH^+, 100%), 179 (10).

3.3.2. 5-Methoxy-2-methyl-4-nitrobenzothiophene-3-carboxaldehyde 16

To a solution of 5-methoxy-2-methylbenzothiophene-3-carboxaldehyde **15** (388 mg, 1.88 mmol) in acetic acid (3.2 mL), cooled to 0–5 °C was added a mixture of nitric acid (638 µl, 9.42 mmol) in acetic acid (4.7 mL). The mixture was stirred at room temperature overnight. The reaction mixture was poured into ice/water, neutralized with a saturated aqueous sodium hydrogen carbonate, extracted with dichloromethane, dried over $MgSO_4$, filtered, and the filtrate evaporated under reduced pressure and azeotroped with toluene to remove the remaining acetic acid. The crude product obtained was purified by chromatography, eluting with dichloromethane, to yield the *title compound* (286 mg; 61%) as a yellow solid; mp 205–207 °C; (found: MH^+, 252.0333. $C_{11}H_9NO_4S + H$ requires 252.0331); ν_{max} (KBr)/cm^{-1} 2975, 2941, 2845, 2771, 1679, 1598, 1536, 1459, 1425, 1375, 1271, 1117, 1082; δ_H (300 MHz; $CDCl_3$) 10.05 (1H, s, CHO), 7.80 (1H, d, *J* 8.9, ArH), 7.66 (1H, d, *J* 8.9, ArH), 3.97 (3H, s, OMe), 2.90 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 183.7 (CHO), 160.4 (C), 150.4 (C), 131.0 (C), 129.4 (C), 128.8 (C), 125.1 (CH), 111.4 (CH), 57.7 (Me), 16.4 (Me), one C unobserved; *m*/*z* (CI) 252 (MH^+, 100%), 222 (65), 206 (30), 194 (10).

3.3.3. 4-Amino-5-methoxy-2-methylbenzothiophene-carboxaldehyde 17

To a suspension of 5-methoxy-2-methyl-4-nitrobenzothiophenecarboxaldehyde **16** (280 mg, 1.12 mmol) in ethanol (23 mL) was added tin powder (601 mg, 5.02 mmol) and hydrochloric acid (3 M; 8.1 mL). The mixture was stirred and heated under reflux for 1 h. Upon cooling, the reaction mixture was decanted from the excess of tin and neutralized with a saturated aqueous solution of $NaHCO_3$. The suspension obtained was filtered through Celite and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to yield the *title compound* (175 mg, 71%) as a yellow solid; mp 88–90 °C; (found: MH^+, 222.0587. $C_{11}H_{11}NO_2S + H$ requires 222.0589); ν_{max} (KBr)/cm^{-1} 3437, 2960, 2921, 2852, 1655, 1602, 1559, 1459, 1421, 1340, 1224, 1201; δ_H (300 MHz; $CDCl_3$) 10.08 (1H, s, CHO), 6.96 (1H, d, *J* 8.5, ArH), 6.91 (1H, d, *J* 8.5, ArH), 6.02 (2H, br s, NH_2), 3.89 (3H, s, OMe), 2.85 (3H, s, Me); δ_C (100 MHz; $CDCl_3$) 184.7 (CH), 160.8 (C), 144.2 (C), 134.3 (C), 132.1 (C), 130.9 (C), 123.1 (C), 110.5 (CH), 108.6 (CH), 56.5 (Me), 14.9 (Me); *m*/*z* (CI) 222 (MH^+, 100%), 206 (5).

3.3.4. 3-Hydroxymethyl-5-methoxy-2-methylbenzothiophene-4,7-dione 18

Sodium borohydride (44 mg, 2.31 mmol) was added in one portion to a solution of 4-amino-5-methoxy-2-methylbenzothiophene-3-carboxaldehyde **17** (170 mg, 0.77 mmol) in dry methanol (21 mL) cooled at 0 °C. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was evaporated off and the crude product obtained was purified by chromatography, eluting with ethyl acetate–dichloromethane 1:1, to yield the unstable *amino alcohol intermediate* as a yellow solid that was used in the next step with no further purification.

To a solution of the *amino alcohol intermediate* dissolved in acetone (50 mL) was added a solution of potassium nitrosodisulfonate (907 mg, 3.32 mmol) in sodium dihydrogen phosphate buffer (0.3 M; 40 mL). The reaction was stirred at room temperature for 1 h. The excess acetone was removed in vacuo. The resulting residue was extracted with dichloromethane and the combined organic layer evaporated off. The residue obtained was purified by chromatography, eluting with dichloromethane, to yield the *title compound* (75 mg, 41%) as a yellow crystalline solid; mp 196–198 °C; (found: MH^+, 239.0372. $C_{11}H_{10}O_4S + H$ requires 239.0378); λ_{max} (acetonitrile)/nm 200 ($\log\varepsilon$ 4.14), 232 (4.12), 288 (4.18), 348 (3.47), 416 (3.21); ν_{max} (KBr)/cm^{-1} 3425, 2925, 2852, 1663, 1636, 1601, 1447, 1344, 1324, 1255, 1220, 1120, 1086, 1070; δ_H (300 MHz; $CDCl_3$) 5.96 (1H, s, 6-H), 4.68 (2H, s, CH_2OH), 3.88 (3H, s, OMe), 2.53 (3H, s, Me); δ_C (100 MHz; $CDCl_3$) 179.5 (C), 178.1 (C), 160.1 (C), 144.4 (C), 142.9 (C), 138.4 (C), 137.0 (C), 108.1 (CH), 56.9 (Me), 56.5 (CH_2), 13.7 (Me); *m*/*z* (CI) 239 (MH^+, 85%), 223 (50), 221 (100).

3.3.5. Ethyl 5-methoxy-3-methyl-4-nitrobenzothiophene-2-carboxylate 20

To a solution of ethyl 5-methoxy-3-methylbenzothiophene-2-carboxylate **19**[36] (480 mg, 1.92 mmol) in acetic acid (3.2 mL), cooled to 0–5 °C was added a mixture of nitric acid (1.3 mL, 19.20 mmol) in acetic acid (4.8 mL). The mixture was stirred at room temperature overnight. The reaction mixture was dropped in ice/water, extracted with dichloromethane, dried over $MgSO_4$, filtered, evaporated and azeotroped with toluene to remove the acetic acid. The crude product obtained was purified by chromatography, eluting with dichloromethane, to yield the *title compound* (417 mg, 74%) as a light yellow solid; mp 158–160 °C; (found: MH^+, 296.0589. $C_{13}H_{13}NO_5S + H$ requires 296.0592); ν_{max} (KBr)/cm^{-1} 3422, 2988, 2939, 2850, 1711, 1608, 1527, 1446, 1370, 1297, 1247, 1193, 1174, 1151, 1051; δ_H (300 MHz; $CDCl_3$) 7.85 (1H, d, *J* 8.9, ArH), 7.26 (1H, d, *J* 8.9, ArH), 4.40 (2H, q, *J* 7.1, OCH_2Me), 3.98 (3H, s, OMe), 2.64 (3H, s, Me), 1.41 (3H, t, *J* 7.1, CH_2Me); δ_C (100 MHz; $CDCl_3$) 162.6 (C), 148.6 (C), 137.1 (C), 136.7 (C), 133.8 (C), 131.5 (C), 130.6 (C), 125.1 (CH), 113.3 (CH), 61.7 (CH_2), 57.3 (Me), 14.2 (Me), 12.1 (Me); *m*/*z* (CI) 296 (MH^+, 100%), 279 (8), 266 (15).

3.3.6. Ethyl 4-amino-5-methoxy-3-methylbenzothiophene-2-carboxylate 21

To a suspension of ethyl 5-methoxy-3-methyl-4-nitrobenzothiophene-2-carboxylate **20** (370 mg, 1.25 mmol) in ethanol (27 mL)

was added tin powder (676 mg, 5.64 mmol) and hydrochloric acid (3 M; 9 mL). The mixture was stirred and heated under reflux for 1 h. Upon cooling, the reaction mixture was decanted from the excess of tin and neutralized with a saturated aqueous solution of $NaHCO_3$. The suspension obtained was filtered through Celite and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure to yield the *title compound* (285 mg, 86%) as a yellow solid; mp 120–123 °C; (found: MH^+, 266.0844. $C_{13}H_{15}NO_3S + H$ requires 266.0851); ν_{max} (KBr)/cm^{-1} 3460, 3368, 2968, 2925, 1705, 1621, 1536, 1471, 1440, 1332, 1259, 1209, 1178, 1144, 1094, 1055, 1009; δ_H (300 MHz; $CDCl_3$) 7.11 (1H, d, *J* 8.6, ArH), 7.03 (1H, d, *J* 8.6, ArH), 4.35 (2H, q, *J* 7.0, O*CH*$_2$Me), 3.89 (3H, s, OMe), 3.06 (3H, s, Me), 1.40 (3H, t, *J* 7.0, CH_2*Me*); NH_2 not observed; δ_C (100 MHz; $CDCl_3$) 165.7 (C), 145.5 (C), 143.3 (C), 136.7 (C), 136.5 (C), 130.5 (C), 127.3 (C), 115.3 (CH), 113.4 (CH), 63.1 (CH_2), 59.0 (Me), 18.1 (Me), 16.4 (Me); *m*/*z* (CI) 266 (MH^+, 100%), 265 (40), 220 (10).

3.3.7. 4-Amino-5-methoxy-3-methylbenzothiophene-2-methanol 22

To a suspension of lithium aluminum hydride (162 mg, 4.23 mmol) in dry THF (6 mL) at 0 °C was added a solution of ethyl 4-amino-5-methoxy-3-methylbenzothiophene-2-carboxylate **21** (280 mg, 1.06 mmol) in dry THF (3 mL). The mixture was allowed to warm up to room temperature and stirred for 2 h. The mixture was cooled to 0 °C and quenched by the addition of water (0.5 mL), aqueous sodium hydroxide (1 M; 0.5 mL) and silica gel. The granular precipitate was filtered off through a pad of Celite. The filtrate was dried over $MgSO_4$, filtered and concentrated in vacuo to yield the *title compound* (188 mg, 80%) as an orange solid; mp 114–115 °C; (found: MH^+, 224.0753. $C_{11}H_{11}NO_2S + H$ requires 224.0745); ν_{max} (KBr)/cm^{-1} 3436, 3352, 3273, 2923, 2851, 1599, 1462, 1265, 1203, 1157, 1136, 1044, 1019; δ_H (300 MHz; $CDCl_3$) 7.12 (1H, d, *J* 8.6, ArH), 6.90 (1H, d, *J* 8.6, ArH), 4.75 (2H, s, C*H*$_2$OH), 3.88 (3H, s, OMe), 2.54 (3H, s, Me); OH, NH_2 not observed; δ_C (100 MHz; $CDCl_3$) 142.3 (C), 134.8 (C), 131.7 (C), 131.1 (C), 127.5 (C), 126.8 (C), 110.6 (CH), 109.1 (CH), 56.9 (CH_2), 55.5 (Me), 13.4 (Me); *m*/*z* (CI) 224 (MH^+, 60%), 223 (M^+, 80), 206 (100), 194 (15).

3.3.8. 2-Hydroxymethyl-5-methoxy-3-methylbenzothiophene-4,7-dione 23

To a solution of 4-amino-5-methoxy-3-methylbenzo-thiophene-2-methanol **22** (180 mg, 0.81 mmol) in acetone (48 mL) was added a solution of potassium nitrosodisulfonate (889 mg, 3.26 mmol) in sodium dihydrogen phosphate buffer (0.3 M; 39 mL). The reaction was stirred at room temperature for 1 h. The excess acetone was removed in vacuo. The resulting residue was extracted with dichloromethane and the combined organic layer concentrated. The residue obtained was stirred at room temperature in a 1:1 mixture of hydrochloric acid (2 M)-acetone for 1 h. The acetone was removed in vacuo. The resulting residue was extracted with dichloromethane. The organic layer was washed with water, dried over $MgSO_4$, filtered and evaporated under reduced pressure to yield the *title compound* (130 mg, 67%) as an orange crystalline solid; mp 215–216 °C; (found: MH^+, 239.0379. $C_{11}H_{10}O_4S + H$ requires 239.0378); λ_{max} (acetonitrile)/nm 200 (log ε 3.86), 232 (3.88), 284 (3.90), 352 (3.24), 412 (3.09); ν_{max} (KBr)/cm^{-1} 3385, 3054, 2920, 2843, 1677, 1638, 1600, 1538, 1461, 1442, 1342, 1327, 1253, 1227, 1134; δ_H (300 MHz; $CDCl_3$) 5.96 (1H, s, 6-H), 4.87 (2H, s, C*H*$_2$OH), 3.87 (3H, s, OMe), 2.47 (3H, s, Me), 1.71 (1H, br s, OH); δ_C (100 MHz; $CDCl_3$) 180.0 (C), 176.9 (C), 160.4 (C), 146.6 (C), 143.2 (C), 136.5 (C), 135.7 (C), 107.7 (CH), 58.2 (CH_2), 56.7 (Me), 13.2 (Me); *m*/*z* (CI) 239 (MH^+, 100%), 221 (30).

3.3.9. Methyl 3-chloro-5-methoxy-4-nitrobenzothiophene-2-carboxylate 25

Methyl 3-chloro-5-methoxybenzothiophene-2-carboxylate **24**[37] (1.00 g, 3.90 mmol) was dissolved in acetic acid (20 mL). Fuming nitric acid (0.25 mL) was added carefully, and the mixture was stirred at room temperature before being heated under reflux overnight. After cooling, the mixture was poured into water (125 mL), and the precipitate collected, washed with water and dried to give the *title compound* (1.03 g, 87%) used without further purification. A sample was recrystallized from methanol to give yellow crystals, mp 170–173 °C; (found: M^+, 300.9821. $C_{11}H_8{}^{35}ClNO_5S$ requires 300.9812); λ_{max} (KBr)/cm^{-1} 1721, 1607, 1534, 1514, 1438, 1374, 1293, 1241; δ_H (300 MHz; $CDCl_3$) 7.86 (1H, d, *J* 9.0, ArH), 7.33 (1H, d, *J* 9.0, ArH), 3.99 (3H, s, OMe), 3.97 (3H, s, OMe); δ_C (100 MHz; $CDCl_3$) 160.8 (C), 149.4 (C), 132.1 (C), 130.1 (C), 127.4 (C), 125.4 (CH), 122.8 (C), 114.6 (CH), 57.4 (Me), 52.9 (Me); 1 C unobserved; *m*/*z* (EI) 303/301 (MH^+, 100%).

3.3.10. Methyl 4-amino-5-methoxybenzothiophene-2-carboxylate 26

The 3-chloro-4-nitrobenzothiophene **25** (1.00 g, 3.3 mmol) and sodium acetate (0.60 g, 7.3 mmol) were dissolved in methanol (200 mL) and THF (55 mL). Palladium-on-carbon (5%; 0.5 g) was added, and the mixture was shaken under a hydrogen atmosphere for 5 h. The mixture was filtered through Celite, the filtrate evaporated, and the residue purified by chromatography eluting with light petroleum / ethyl acetate (4:1) to give the *title compound* (0.69 g, 88%) as a pale yellow solid, mp 149–151 °C; (found: C, 55.6; H, 4.7; N, 5.7. $C_{11}H_{11}NO_3S$ requires C, 55.7; H, 4.7; N, 5.9%); λ_{max} (KBr)/cm^{-1} 3445, 3359, 1702, 1625, 1528, 1470, 1206; δ_H (300 MHz; $CDCl_3$) 8.01 (1H, d, *J* 0.8, 3-H), 7.20 (1H, dd, *J* 8.6, 0.8, ArH), 7.09 (1H, d, *J* 8.6, ArH), 4.27 (2H, br s, NH_2), 3.93 (3H, s, OMe), 3.91 (3H, s, OMe); δ_C (100 MHz; $CDCl_3$) 163.3 (C), 142.9 (C), 136.0 (C), 132.4 (C), 131.8 (C), 127.8 (C), 126.5 (CH), 113.7 (CH), 111.4 (CH), 56.8 (Me), 52.3 (Me); *m*/*z* (EI) 237 (M^+, 70%), 222 (100), 194 (35), 83 (68).

3.3.11. Methyl 5-methoxy-4,7-dioxobenzothiophene-2-carboxylate 27

A mixture of 4-aminobenzothiophene **26** (50 mg, 0.21 mmol) and potassium nitrosodisulfonate (230 mg, 0.84 mmol) in acetone (7.5 mL) and sodium dihydrogen phosphate buffer (0.3 M; 7.5 mL) was stirred at room temperature overnight. The acetone was evaporated, water (70 mL)was added, and the mixture was extracted with dichloromethane (3 × 25 mL). The combined extracts were dried (Na_2SO_4), evaporated and the residue purified by chromatography eluting with dichloromethane to give the *title compound* (40 mg, 75%) as a dark yellow solid, mp 234 °C; (found: C, 52.8; H, 3.3. $C_{11}H_8O_5S$ requires C, 52.4; H, 3.2%); λ_{max} (KBr) 3088, 2960, 1720, 1689, 1633, 1597, 1534, 1439, 1244, 1268, 1085; δ_H (300 MHz; $CDCl_3$) 8.15 (1H, s, 3-H), 6.08 (1H, s, 6-H), 3.94 (3H, s, OMe), 3.90 (3H, s, OMe); δ_C (75 MHz; $CDCl_3$) 179.6 (C), 175.1 (C), 161.4 (C), 160.8 (C), 148.0 (C), 139.3 (C), 138.5 (C), 130.7 (CH), 109.0 (CH), 57.0 (Me), 53.1 (Me); *m*/*z* (EI) 252 (M^+, 100%), 237 (34).

3.3.12. 4-Amino-5-methoxybenzothiophene-2-methanol 28

A solution of the benzothiophene **26** (2.00 g, 8.43 mmol) in dry THF (28 mL) was added dropwise to lithium aluminum hydride (1.07 g, 28.1 mmol) in THF (55 mL). The mixture was stirred at room temperature for 16 h, and then water was carefully added. The mixture was filtered through Celite, and extracted with ethyl acetate (3 × 30 mL). The combined extracts were washed with water (30 mL), dried ($MgSO_4$), and evaporated to a solid that was recrystallized from methanol to give the *title compound* (1.38 g, 78%), mp 130–132 °C; (found: C, 57.2; H, 5.3; N, 6.6. $C_{10}H_{11}NO_2$ requires C, 57.4; H, 5.3; N, 6.7%); λ_{max} (KBr)/cm^{-1} 3401, 3310, 3016,

1602, 1480, 1463; δ_H (300 MHz; $CDCl_3$) 7.19 (1H, d, *J* 8.6, ArH), 7.09 (1H, s, 3-H), 6.95 (1H, d, *J* 8.6, ArH), 4.87 (2H, s, CH_2), 3.90 (3H, s, OMe); OH, NH_2 not observed; δ_C (75 MHz; $CDCl_3$) 144.0 (C), 143.3 (C), 133.7 (C), 130.4 (C), 128.6 (C), 117.1 (CH), 111.9 (CH), 110.7 (CH), 61.0 (CH_2), 56.8 (Me); *m*/*z* (EI) 209 (M^+, 62%), 194 (100), 166 (25).

3.3.13. 2-Hydroxymethyl-5-methoxybenzothiophene-4,7-dione 29

A mixture of the 4-aminobenzothiophene **28** (0.32 g, 1.5 mmol) and potassium nitrosodisulfonate (1.61 g, 6.0 mmol) in acetone (47 mL) and sodium dihydrogen phosphate buffer (0.3 M; 47 mL) was stirred at room temperature for 1 h. Work-up as described above gave the *title compound* (0.29 g, 88%) as a dark orange solid, mp 213 °C (from methanol); (found: C, 53.3; H, 3.3. $C_{10}H_8O_4S$ requires C, 53.6; H. 3.60%); λ_{max} (KBr)/cm^{-1} 3413, 3062, 2986, 2947, 1680, 1628, 1598, 1572, 1326, 1246, 1140, 1084, 1043, 864, 792; δ_H (300 MHz; $CDCl_3$) 7.43 (1H, s, 3-H), 5.99 (1H, s, 6-H), 4.91 (2H, d, *J* 6.0, C*H*$_2$OH), 3.88 (3H, s, OMe), 2.05 (1H, t, *J* 6.0, CH_2O*H*); δ_C (100 MHz; $CDCl_3$) 180.2 (C), 175.9 (C), 160.6 (C), 156.3 (C), 142.3 (C), 139.2 (C), 121.5 (CH), 108.7 (CH), 58.8 (CH_2), 57.3 (Me); *m*/*z* (EI) 224 (M^+, 100%) 209 (28), 194 (35), 125 (26).

3.3.14. 4-Amino-5-methoxybenzothiophene-2-carboxaldehyde 30

A solution of the benzothiophene-2-methanol **28** (0.90 g, 4.30 mmol) in dichloromethane (100 mL) was stirred with manganese(IV) oxide (3.78 g, 43 mmol) under reflux for 24 h. The solution was filtered through Celite, washed through with dichloromethane, and the combined filtrate and washings concentrated under vacuum. The residue was purified by chromatography eluting with light petroleum/ethyl acetate (4:1) to give the *title compound* (0.29 g, 33%) as a pale yellow solid, mp 128–130 °C; (found: C, 57.9; H, 4.3; N, 6.5. $C_{10}H_9NO_2S$ requires C, 57.9; H, 4.4; N, 6.8%); λ_{max} (KBr)/cm^{-1} 3474, 3373, 2826, 1667, 1520, 1483; δ_H (300 MHz; $CDCl_3$) 10.00 (1H, s, CHO), 7.97 (1H, s, H-3), 7.19 (1H, d, *J* 8.6, ArH), 7.09 (1H, d, *J* 8.6, ArH), 4.42 (2H, br s, NH_2), 3.90 (3H, s, OMe); δ_C (75 MHz; $CDCl_3$) 184.4 (CH), 143.0 (C), 142.2 (C), 136.2 (C), 133.2 (C), 130.8 (CH), 127.7 (C), 114.6 (CH), 111.9 (CH), 56.7 (Me); *m*/*z* (EI) 207 (M^+, 70%), 192 (100), 164 (40), 77 (35).

3.3.15. 2-Formyl-5-methoxybenzothiophene-4,7-dione 31

A mixture of the 4-aminobenzothiophene **30** (50 mg, 0.24 mmol) and potassium nitrosodisulfonate (260 mg, 0.96 mmol) in acetone (7.5 mL) and sodium dihydrogen phosphate buffer (0.3 M; 7.5 mL) was stirred at room temperature overnight. Work-up as described above gave the *title compound* (42 mg, 79%) as a dark orange solid, mp 218 °C; (found: C, 53.9; H, 2.8. $C_{10}H_6O_4S$ requires C, 54.0; H, 2.7%); λ_{max} (KBr)/cm^{-1} 3070, 1688, 1649, 1600, 1522, 1325, 1249, 1148, 1079, 867; δ_H (300 MHz; $CDCl_3$) 10.03 (1H, s, CHO), 8.16 (1H, s, 3-H), 6.12 (1H, s, 6-H), 3.92 (3H, s, OMe); δ_C (75 MHz; $CDCl_3$) 183.0 (CH), 179.4 (C), 174.9 (C), 161.0 (C), 149.6 (C), 147.6 (C), 138.7 (C), 132.6 (CH) 109.2 (CH), 57.1 (Me); *m*/*z* (EI) 222 (M^+, 100%), 207 (32), 192 (37).

3.3.16. 2-Acetoxymethyl-5-methoxybenzothiophene-4,7-dione 32

Acetic anhydride (2.7 mL) was added to a solution of the alcohol **29** (75 mg, 0.33 mmol) in pyridine (16 mL), and the mixture was stirred overnight at room temperature. The mixture was diluted with water (27 mL), and extracted with dichloromethane (3 × 50 mL). The combined extracts were washed with water (50 mL) and brine (50 mL), dried ($MgSO_4$) and evaporated. The residue was purified by chromatography eluting with dichloromethane/ethyl acetate (19:1) to give the *title compound* as a yellow solid (58 mg, 66%), mp 153–155 °C; (found: C, 54.0; H, 3.7. $C_{12}H_{10}O_5S$ requires C, 54.1; H, 3.8%); λ_{max} (KBr)/cm^{-1} 3091, 2983, 2963, 1725, 1677, 1642, 1602, 1534, 1467, 1438, 1333, 1232, 1141, 1085, 1030, 962, 854, 798; δ_H (300 MHz; $CDCl_3$) 7.49 (1H, s, 3-H), 5.99 (1H, s, 6-H), 5.26 (2H, s, CH_2), 3.88 (3H, s, OMe), 2.13 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 179.7 (C), 175.6 (C), 170.3 (C), 160.3 (C), 146.0 (C), 144.6 (C), 138.7 (C), 125.5 (CH), 108.5 (CH), 60.2 (CH_2), 56.8 (Me), 20.7 (Me); *m*/*z* (EI) 266 (M^+, 32%), 251 (8), 224 (100), 207 (36), 178 (20).

3.3.17. 5-Methoxy-2-methylbenzothiophene-4,7-dione 33

(a) A stirred solution of the alcohol **29** (200 mg, 0.89 mmol) in dichloromethane (11 mL) at −10 °C was treated with triethylamine (140 mg, 0.19 mL, 1.34 mmol). After 10 min, methanesulfonyl chloride (110 mg, 0.08 mL, 0.98 mmol) was added, and the mixture allowed to stir at room temperature overnight. Water (11 mL) and dichloromethane (30 mL) were added, the organic layer separated, washed with water (50 mL), brine (50 mL), dried ($MgSO_4$) and evaporated to give the *mesylate* as a yellow solid, used without any purification.

(b) The above product was dissolved in THF (11 mL) and added to lithium aluminum hydride (330 mg, 8.63 mmol) in THF (9 mL). The mixture was stirred overnight at room temperature, before the careful addition of water. The mixture was filtered through Celite, and the filtrate extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with saturated aqueous sodium hydrogen carbonate (10 mL), water (10 mL), dried ($MgSO_4$) and evaporated. The residue was purified by chromatography to give the *title compound* (9 mg, 5%) as a yellow solid, mp 217–220 °C; (found: M^+, 208.0194. $C_{10}H_8O_3S$ requires 208.0194); λ_{max} (KBr)/cm^{-1} 3066, 2923, 1680, 1640, 1596, 1535, 1468, 1329, 1246, 1083, 968, 858, 792; δ_H (300 MHz; $CDCl_3$) 7.22 (1H, s, 3-H), 5.94 (1H, s, 6-H), 3.86 (3H, s, OMe), 2.56 (3H, s, Me); δ_C (75 MHz; $CDC1_3$) 179.9 (C), 175.9 (C), 160.0 (C), 148.5 (C), 142.4 (C), 139.4 (C), 124.1 (CH), 108.3 (CH), 56.7 (Me), 15.9 (Me); *m*/*z* (EI) 208 (M^+, 64%), 193 (21), 178 (30), 149 (47), 71 (44), 69 (100).

3.4. Synthesis of indazolequinones

3.4.1. 5,7-Dimethoxy-3-methylindazole 35

To a solution of 3,5-dimethoxyacetophenone **34** (1.58 g, 8.81 mmol) in dry dichloromethane (44 mL) were added bis(trichloroethyl) azodicarboxylate (3.35 g, 8.81 mmol) and BF_3·EtO (539 μl, 4.40 mmol). The mixture was stirred overnight at room temperature, quenched with aqueous ammonium acetate solution (25%; 70 mL) and extracted with ethyl acetate (4 × 70 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product obtained was purified by flash chromatography. A mixture of hydrazine intermediate and starting material (20:1) was obtained. To a solution of the above product in glacial acetic acid (49 mL) was added zinc dust (4.94 g, 75.55 mmol). The mixture was stirred at room temperature for 1 h and water (50 mL) followed by aqueous sodium hydroxide (1 M) were added until pH = 10. The mixture was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography, eluting with ethyl acetate–light petroleum 1:1 to yield the *title compound* (590 mg; 35%) as a beige solid; mp 154–156 °C (lit.,[38] mp 155–156 °C); δ_H (300 MHz; $CDCl_3$) 11.72 (1H, br s, NH), 6.64 (1H, d, *J* 2.3, ArH), 6.44 (1H, t, *J* 2.3, ArH), 3.95 (3H, s, OMe), 3.80 (3H, s, OMe), 2.45 (3H, s, Me).

3.4.2. 5,7-Dimethoxy-3-methyl-4-nitroindazole 36

To a solution of 5,7-dimethoxy-3-methylindazole **35** (200 mg, 1.04 mmol) in acetic acid (8 mL), cooled to 0–5 °C, was added a mixture of nitric acid (69 μl, 1.04 mmol) in acetic acid (1 mL). The mixture was stirred at room temperature for 1 h. The reaction

was quenched by addition of brine, extracted with ethyl acetate, dried over $MgSO_4$, filtered, evaporated and azeotroped with toluene to remove the acetic acid. The crude product obtained was purified by flash chromatography, eluting with ethyl acetate/light petroleum (1:1), to yield the *title compound* (201 mg, 81%) as a bright yellow crystalline solid, recrystallized from dichloromethane–pentane; mp 219–220 °C; (found: C, 50.7; H, 4.5; N, 17.6. $C_{10}H_{11}N_3O_4$ requires C, 50.6; H, 4.7; N, 17.7%); (found: MH^+, 238.0829. $C_{10}H_{11}N_3O_4$ + H requires 238.0828); ν_{max} (KBr)/cm^{-1} 3398, 3149, 2917, 1597, 1517, 1308, 1223, 1115, 1059; δ_H (300 MHz; $CDCl_3$) 13.43 (1H, s, NH), 6.53 (1H, s, ArH), 4.05 (3H, s, OMe), 4.00 (3H, s, OMe), 2.50 (3H, s, Me); δ_C (100 MHz; d_6-DMSO) 148.9 (C), 148.2 (C), 138.6 (C), 129.0 (C), 124.3 (C), 114.7 (C), 94.8 (CH), 58.3 (Me), 56.6 (Me), 13.2 (Me); *m*/*z* (CI) 238 (MH^+, 95%), 221 (55), 207 (100), 192 (80).

3.4.3. 5,7-Dimethoxy-1,3-dimethyl-4-nitroindazole 37

To a stirred solution of 5,7-dimethoxy-3-methyl-4-nitroindazole **36** (116 mg, 0.60 mmol) in DMSO (2 mL) was added potassium hydroxide (135 mg, 2.42 mmol). The mixture was stirred at room temperature for 30 min and iodomethane (150 μl, 2.42 mmol) was added dropwise to the solution. The reaction mixture was then stirred at room temperature for a further 4 h. The crude mixture was diluted with ethyl acetate, washed thoroughly with hydrochloric acid (2 M), dried over $MgSO_4$, filtered and evaporated. The crude product obtained was purified by flash chromatography, eluting with ethyl acetate/light petroleum (1:1), to yield the *title compound* (71 mg, 49%) as a bright yellow crystalline solid, recrystallized from dichloromethane–pentane; mp 168–169 °C; (found: C, 52.8; H, 5.1; N, 16.9. $C_{11}H_{13}N_3O_4$ requires C, 52.6; H, 5.2; N, 16.7%); (found: M^+, 251.0910. $C_{11}H_{13}N_3O_4$ requires 251.0906); ν_{max} (KBr)/cm^{-1} 3435, 2929, 2841, 1577, 1522, 1324, 1280, 1220, 1050; δ_H (300 MHz; $CDCl_3$) 6.45 (1H, s, ArH), 4.19 (3H, s, NMe), 4.02 (3H, s, OMe), 3.97 (3H, s, OMe), 2.40 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 149.0 (C), 148.6 (C), 138.9 (C), 128.9 (C), 126.3 (C), 117.1 (C), 94.6 (CH), 58.9 (Me), 56.4 (Me), 39.0 (Me), 13.4 (Me); *m*/*z* (EI) 251 (M^+, 100%), 234 (28), 221 (29), 206 (72), 175 (40). No nOe enhancement observed after pre-irradiation of the Me group at 2.39 ppm. 4.9% nOe enhancement observed on the aromatic proton at 6.45 ppm after pre-irradiation at 4.02 ppm (5-OMe). 2.3% nOe enhancement observed at 3.97 ppm (7-OMe) after pre-irradiation of NMe at 4.19 ppm. 3.5% nOe enhancements observed on the aromatic proton at 6.45 ppm and 1.3% nOe enhancement observed at 4.02 ppm (5-OMe) after pre-irradiation at 3.97 ppm (7-OMe).

3.4.4. 4-Amino-5,7-dimethoxy-1,3-dimethylindazole 38

To a suspension of 5,7-dimethoxy-1,3-dimethyl-4-nitroindazole **37** (55.3 mg, 0.22 mmol) in ethanol (3.7 mL) were added tin powder (120 mg, 0.99 mmol) and hydrochloric acid (3 M; 1.5 mL). The mixture was heated under reflux for 1 h. Upon cooling, the solution was decanted from the excess tin and neutralized with a saturated aqueous solution of sodium hydrogen carbonate. The precipitate obtained was extracted with ethyl acetate. The organic layer was filtered through a pad of Celite, dried over $MgSO_4$, filtered and evaporated under reduced pressure to yield the *title compound* (47 mg; 96%) as a colorless solid; mp 79–80 °C; (found: M^+, 221.1163. $C_{11}H_{15}N_3O_2$ requires 221.1164); ν_{max} (KBr)/cm^{-1} 3434, 3343, 2927, 2836, 1590, 1527, 1453, 1351, 1271, 1197, 1111; δ_H (300 MHz; $CDCl_3$) 6.48 (1H, s, 6-H), 4.11 (3H, s, NMe), 3.84 (3H, s, OMe), 3.82 (3H, s, OMe), 2.66 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 139.9 (C), 138.4 (C), 138.3 (C), 130.5 (C), 123.2 (C), 116.3 (C), 99.3 (CH), 59.2 (Me), 56.4 (Me), 38.3 (Me), 14.5 (Me); *m*/*z* (EI) 221 (M^+, 27%), 206 (100), 191 (14), 163 (8).

3.4.5. 5-Methoxy-1,3-dimethylindazole-4,7-dione 39

To a solution of 4-amino-5,7-dimethoxy-1,3-dimethylindazole **38** (0.250 g, 1.15 mmol) in acetone (70 mL) was added a solution of potassium nitrosodisulfonate (1.250 g, 4.59 mmol) in sodium dihydrogen phosphate buffer (0.3 M, 58 mL). The reaction was stirred at room temperature for 1 h. The excess acetone was removed in vacuo. The resulting residue was extracted with dichloromethane. The organic layer was washed with water, dried over $MgSO_4$, filtered, evaporated under reduced pressure to yield the *title compound* (208 mg, 78%) as a bright yellow crystalline solid, recrystallized from dichloromethane–pentane; mp 185–186 °C; (found: C, 58.2; H, 4.8; N, 13.6. $C_{10}H_{10}N_2O_3$ requires C, 58.2; H, 4.9; N, 13.6%); (found: M^+, 206.0683. $C_{10}H_{10}N_2O_3$ requires 206.0691); λ_{max} (acetonitrile)/nm 272 (log ε 3.91), 320 (3.84); ν_{max} (KBr)/cm^{-1} 1680, 1657, 1590, 1529, 1510, 1340, 1216, 1018; δ_H (300 MHz; $CDCl_3$) 5.79 (1H, s, 6-H), 4.16 (3H, s, NMe), 3.85 (3H, s, OMe), 2.49 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 177.8 (C), 176.1 (C), 162.0 (C), 149.0 (C), 137.4 (C), 117.1 (C), 107.3 (CH), 57.0 (Me), 38.4 (Me), 12.9 (Me); *m*/*z* (EI) 206 (M^+, 100%), 191 (85), 177 (62), 123 (30).

3.4.6. 3-Hydroxymethyl-5-methoxy-1-methylindazole-4,7-dione 40

A solution of 5-methoxy-1,3-dimethylindazole-4,7-dione **39** (51 mg, 0.25 mmol), AIBN (12 mg, 0.07 mmol) and *N*-bromosuccinimide (86 mg, 0.49 mmol) in CCl_4 (3 mL) was purged five times with vacuum followed with nitrogen. The reaction mixture was heated at reflux under a nitrogen atmosphere overnight. The mixture was concentrated under reduced pressure and purified by flash chromatography, eluting with dichloromethane, to yield the *bromide intermediate*, which was used in the next step with no further purification. The *bromide intermediate* dissolved in acetone (9 mL) and water (4 mL) was added to a suspension of silver nitrate (92 mg, 0.54 mmol) in an aqueous solution of acetone (50%; 13 mL). The reaction mixture was heated under reflux overnight and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue obtained was purified by flash chromatography, eluting with light petroleum–ethyl acetate 1:2, to yield the *title compound* (29 mg; 36%) as a yellow solid; mp 177–179 °C; (found: MH^+, 223.0721. $C_{10}H_{10}N_2O_4$ + H requires 223.0719); λ_{max} (acetonitrile)/nm 276 (log ε 4.23), 308 (3.74); ν_{max} (KBr)/cm^{-1} 3368, 2928, 1689, 1653, 1593, 1525, 1505, 1240, 1204, 1015; δ_H (300 MHz; $CDCl_3$) 5.87 (1H, s, 6-H), 4.86 (2H, s, CH_2OH), 4.17 (3H, s, NMe), 3.90 (3H, s, OMe); δ_C (75 MHz; $CDCl_3$) 177.5 (C), 177.0 (C), 161.8 (C), 152.6 (C), 137.8 (C), 117.4 (C), 107.7 (CH), 58.2 (CH_2), 57.2 (Me), 38.7 (Me); *m*/*z* (ES) 223 (MH^+, 95%), 164 (35), 149 (30), 90 (100).

3.5. Synthesis of benzisoxazolequinones

3.5.1. (*E*)-2-Hydroxy-5-methoxyacetophenone oxime 42

To a solution of 2-hydroxy-5-methoxyacetophenone **41** (2.50 g, 15.1 mmol) and hydroxylamine hydrochloride (2.10 g, 30.1 mmol) in ethanol (50 mL) was added dropwise dry pyridine (2.68 mL, 30.1 mmol). The reaction mixture was stirred at room temperature overnight and evaporated under reduced pressure. The crude product obtained was purified by flash chromatography, eluting with ethyl acetate–light petroleum 1:2, to yield the *title compound* (1.68 g, 98%) as a colorless solid; mp 112–113 °C (lit.,[42] mp 121 °C); (found: MH^+, 182.0813. $C_9H_{11}NO_3$ + H requires 182.0817); ν_{max} (KBr)/cm^{-1} 3348, 2964, 2920, 2831, 1641, 1497, 1404, 1368, 1284, 1232, 1208, 1176, 1052, 1011; δ_H (300 MHz; $CDCl_3$) 10.70 (1H, br s, OH), 7.53 (1H, br s, OH), 7.17 (1H, d, *J* 3.0, 6-H), 7.11 (1H, dd, *J* 9.0, 3.0, 4-H), 6.93 (1H, d, *J* 9.0, 3-H), 3.80 (3H, s, OMe), 2.62 (3H, s, Me); δ_C (100 MHz; $CDCl_3$) 159.3 (C),

152.3 (C), 151.6 (C), 118.7 (C), 117.7 (CH), 116.5 (CH), 113.1 (CH), 56.0 (Me), 10.9 (Me); *m*/*z* (CI) 182 (MH^+, 63%), 166 (100), 151 (15), 125 (5).

3.5.2. 5-Methoxy-3-methylbenzisoxazole 43

Diisopropyl azodicarboxylate (8.5 mL, 43.5 mmol) was added dropwise to a stirred solution of the oxime **42** (5.24 g, 29.0 mmol) and triphenylphosphine (11.35 g, 43.5 mmol) in dry THF (192 mL) under nitrogen. The reaction mixture was stirred at room temperature overnight, and evaporated under reduced pressure. The crude product was purified by chromatography, eluting with ethyl acetate/light petroleum (1:3), to yield the *title compound* (4.72 g, 100%) as a yellow solid; mp 28–30 °C (lit.,[43] mp not given); (found: MH^+, 164.0708. $C_9H_9NO_2$ + H requires 164.0712); ν_{max} (KBr)/cm^{-1} 2972, 2935, 1730, 1528, 1484, 1458, 1439, 1252, 1219, 1127, 1079, 1028; δ_H (300 MHz; $CDCl_3$) 7.44 (1H, d, *J* 9.0, 7-H), 7.16 (1H, dd, *J* 9.0, 2.3, 6-H), 6.96 (1H, d, *J* 2.3, 4-H), 3.87 (3H, s, OMe), 2.56 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 158.4 (C), 156.2 (C), 154.9 (C), 122.5 (C), 120.2 (CH), 110.5 (CH), 101.3 (CH), 55.9 (Me), 10.1 (Me); *m*/*z* (CI) 164 (MH^+, 100%), 148 (5).

3.5.3. 5-Methoxy-3-methyl-4-nitrobenzisoxazole 44

To a solution of nitric acid/sulfuric acid 9:1 (22 mL), cooled in a salt and ice bath, was added 5-methoxy-3-methylbenzisoxazole **43** (2.22 g, 13.6 mmol) portionwise. The mixture was stirred at room temperature overnight. The mixture was poured into ice/water, basified with a saturated aqueous sodium hydrogen carbonate, extracted with dichloromethane, dried over $MgSO_4$, and evaporated under reduced pressure to yield the *title compound* (2.58 g, 91%) as a yellow solid; mp 103–105 °C; (found: MH^+, 209.0551. $C_9H_8N_2O_4$ + H requires 209.0562); ν_{max} (KBr)/cm^{-1} 3092, 2954, 2850, 1525, 1513, 1475, 1371, 1325, 1267, 1060; δ_H (300 MHz; $CDCl_3$) 7.69 (1H, d, *J* 9.2, ArH), 7.36 (1H, d, *J* 9.2, ArH), 4.00 (3H, s, OMe), 2.50 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 158.2 (C), 153.0 (C), 149.1 (C), 131.7 (C), 116.9 (CH), 115.3 (C), 113.6 (CH), 58.1 (Me), 10.9 (Me); *m*/*z* (CI) 209 (MH^+, 100%), 192 (5).

3.5.4. 5-Methoxy-3-methylbenzisoxazole-4,7-dione 45

To a suspension of 5-methoxy-3-methyl-4-nitrobenzisoxazole **44** (2.5 g, 12.0 mmol) in ethanol (200 mL) was added tin powder (3.6 g, 120.2 mmol) and hydrochloric acid (3 M; 83 mL). The mixture was stirred and heated under reflux for 1 h. Upon cooling, the reaction mixture was decanted from the excess of tin and neutralized with a saturated sodium hydrogen carbonate. The suspension obtained was added to an equal volume of water. The precipitate and aqueous layer were stirred overnight with dichloromethane, filtered through Celite and the layers separated. The organic layer was dried over Na_2SO_4 and concentrated to yield the 4-amino compound, used in the next step with no further purification.

To a solution of the 4-amino compound in acetone (490 mL) was added a solution of potassium nitrosodisulfonate (8.9 g, 33.2 mmol) in sodium dihydrogen phosphate buffer (0.3 M; 400 mL). The reaction was stirred at room temperature for 1 h, and concentrated under reduced pressure. The residue was extracted with dichloromethane. The organic layer was washed with water, dried over $MgSO_4$, filtered, evaporated under reduced pressure to yield the *title compound* (250 mg, 20%) as a dark orange crystalline solid; mp 126–129 °C; (found: MH^+, 194.0451. $C_9H_7NO_4$ + H requires 194.0453); λ_{max} (acetonitrile)/nm 220 (log ε 3.92), 256 (3.89), 380 (3.30); ν_{max} (KBr)/cm^{-1} 3437, 3060, 2917, 1698, 1667, 1578, 1486, 1467, 1440, 1355, 1336, 1251, 1205, 1186, 1032, 1013; δ_H (300 MHz; $CDCl_3$) 5.98 (1H, s, 6-H), 3.93 (3H, s, OMe), 2.59 (3H, s, Me); δ_C (75 MHz; $CDCl_3$) 176.0 (C), 174.5 (C), 165.2 (C), 161.8 (C), 158.1 (C), 117.7 (C), 107.1 (CH), 57.9 (Me), 11.0 (Me); *m*/*z* (CI) 194 (MH^+, 100%), 166 (5).

3.6. Biology

3.6.1. HPLC analysis

Reduction of the quinones was followed by HPLC using an Alltech C18 (5 μm, 250 mm × 4.6 mm) column with a Waters HPLC system (2487 Dual λ Absorbance detector, two 515 HPLC pumps, 717plus Autosampler, Millennium32 Chromatography Manager). The solvent program used a linear gradient of 5–80% B over 10 min, 80% B for 5 min, then 80% B to 5% B over 5 min (solution A, 10 mM potassium phosphate buffer, pH 6.0; solution B, methanol). Reactions were run in 25 mM Tris–HCl (pH 7.4) containing 200 μM NADH (Sigma), 50 μM quinone, and recombinant human NQO1 (gift from David Ross, University of Colorado, Denver, CO). NADH oxidation was quantified at 340 nm following 30–40 min incubations at 22 °C.

3.6.2. Spectrophotometric method

Quinone reduction by recombinant human NQO1 was also quantified using a modification of an assay that uses cytochrome *c* as the terminal electron acceptor.[44] Reaction mixtures contained 1 mM NADH (Sigma), 25 μM quinone, 70 μM cytochrome *c* (Sigma) and 0.1–3.0 μg/mL rhNQO1 in 25 mM Tris–HCl (pH 7.4) with 0.07% BSA and 0.1% Tween-20. Reactions were run at least in triplicate at 22 °C in a Beckman DU 7500 spectrophotometer at 550 nm (molar absorptivity 21.1 mM^{-1} cm^{-1} for cytochrome *c*). Initial reduction rates (μmol cytochrome *c* reduced/min/mg NQO1) were calculated from the linear portion (0–30 s) of the reaction curves.

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