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Novel Rhein Analogues as Potential Anticancer Agents

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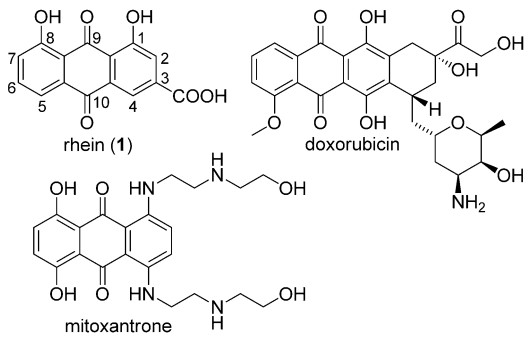
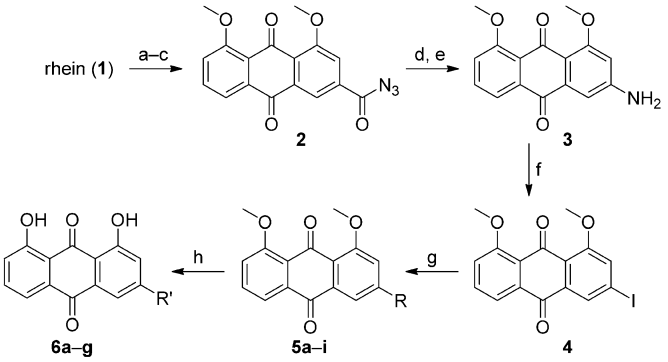
Two series of rhein analogues were synthesized with modification at the 3-position. Their cytotoxicities were evaluated using an MTT assay. Among all the compounds synthesized, one compound showed the best potency, with an IC_{50} value of 2.7 μM against the HeLa cell line and 0.6 μM against the MOLT4 cell line.

Introduction

Rhein is a natural product isolated from rhubarb (*Rheum palmatum*) that has an anthraquinone scaffold. Studies have shown that it has moderate anticancer activities against various tumor cell lines[1] and is very well tolerated by the human body when used as a laxative.[2] The anticancer properties of rhein are less well studied[2b] than those of other anthraquinone compounds extracted from rhubarb, such as emodin and aloe-emodin.[3] Various mechanisms of action have been proposed for rhein based on its cytotoxic effect through the induction of apoptosis,[1a,c,4] p53 up-regulation,[4] inhibition of ERK phosphorylation,[2a] disruption of mitochondrial function,[1c,5] and impairment of glucose uptake,[6] among others.[2b] Despite all these studies, the low-to-moderate activity of rhein (IC_{50}: 12–100 μM)[1] against tumors means that no further development is warranted until its potency can be improved.[2b] In contrast, several highly potent anthraquinone compounds such as mitoxantrone and doxorubicin have already been approved for clinical use. However, many such approved anthraquinone drugs have severe cardiac toxicity problems.[7] Furthermore, the complex structure of doxorubicin, along with its main source being from biosynthesis, makes it very costly.[8] Therefore, we are interested in the development of anthraquinone-based anticancer drugs by starting from the rhein scaffold, because it has shown some promise, bears structural similarity to other anthraquinone drugs, is well tolerated in humans, and is structurally fairly simple. Herein we report the optimization of rhein at the 3-position.



A commonly proposed mechanism for the cytotoxicity of anthraquinone compounds is their noncovalent binding to DNA duplexes, probably through intercalation.[3c,d,9] The binding event could distort the DNA conformation, leading to subsequent inhibition of DNA topoisomerase activities.[3d,9,10] Because the overall planar structure of anthraquinone is most likely responsible for its cytotoxicity, we purposely avoided modifications to this part. Thus, in this study two series of rhein analogues were designed through modification at the 3-position. The first series represents replacement of the 3-carboxyl group of rhein with an aromatic ring for increased diversity (Scheme 1). The second series features conversion of the



Scheme 1. Synthesis of rhein analogues. *Reagents and conditions:* a) NaH, MeI, DMF, ice bath, overnight; b) NaOH in H_2O/EtOH, 50 °C, 1 h; c) Et_3N, Ph_2PON_3, DMF, RT, 1 h; d) dioxane, reflux, 30 min; e) NaOH in H_2O, reflux, 4 h; f) isopentyl nitrite, CH_2I_2, THF, 50–60 °C, 2 days; g) $RB(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, DMF, 70–80 °C; h) PhSH, K_2CO_3, NMP, 140–160 °C.

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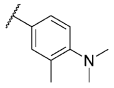
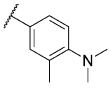
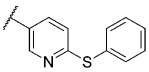
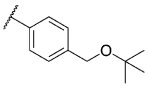
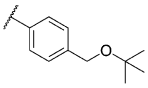
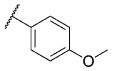
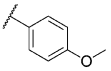
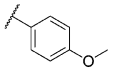
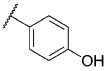
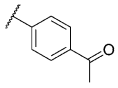
3-carboxyl group into an amide side chain, which is further derivatized with secondary amines, with the purpose of increasing diversity, water solubility, and potential ionic interactions with the DNA phosphate backbone.[3c,9,11]

Results and Discussion

Synthesis of the first series of analogues started with methylation of the phenolic hydroxy groups using iodomethane. This was followed by conversion of the 3-carboxyl group into an acyl azide (compound **2**). After Curtius rearrangement in dioxane and hydrolysis in a solution of sodium hydroxide, compound **3** was obtained with an amino group at the 3-position. An iodo group was introduced through a Sandmeyer-like reaction of **3** with isopentyl nitrite and diiodomethane in tetrahydrofuran. The aromatic groups were installed at the 3-position through Suzuki coupling of iodo compound **4** with the appropriate boronic acids. Afterward, the final products were obtained through deprotection of the hydroxy groups with thiophenol in the presence of potassium carbonate in *N*-methyl-2-pyrrolidone (Table 1). Compound **4** was also subjected to thiophenol-mediated demethylation, but the iodine atom was replaced by thiophenol in the reaction, probably through an addition/elimination reaction due to the strong nucleophilicity of the deprotonated thiophenol. A similar reaction also occurred with **5d**, with replacement of the fluorine atom on the pyridine ring by thiophenol. The demethylation of **5f** was particularly sensitive to experimental conditions. Heating at 150 °C for 20 min selectively removed the methyl groups from both protected anthraquinone hydroxy groups to yield **6f** as the product, leaving the methyl group on −R intact. Further demethylation of **6f** to **6g** was achieved by increasing the temperature to 170–180 °C and increasing the reaction time to 1 h. Meanwhile, deprotection of compounds **5g–i** was unsuccessful; the reaction did not generate the expected products probably because the thiophenol demethylation conditions were too harsh, leading to degradation.

Two cancer cell lines, HeLa and MOLT4, were chosen for cytotoxicity evaluations, representing adherent tumor cells and suspension tumor cells, respectively. MTT assays were performed for quantitative evaluation of in vitro cytotoxicity.[12] Test results of this series of analogues showed that the most potent compound, **4a**, has an IC_{50} value at the single-digit micromolar level against both HeLa and MOLT4 cell lines (Figure 1). Relative to rhein, the potency increased by at least 30-fold in both cancer cell types with introduction of a phenylthio group at the 3-position. However, the rest of the compounds did not show such improved activity, particularly in the MOLT4 cell line (Table 2). Analogues **6b** and **6c** even showed decreased cytotoxicity against MOLT4 relative to rhein. In HeLa cells, **6a**, **6c**, and **6g** showed a moderate improvement in potency. Interestingly, the difference between **6f** and **6g**, with demethylation of the phenol hydroxy group at the 3-position, leads to a ~10-fold increase in potency against HeLa cells. Previous studies have reported that hydroxyanthraquinones with a 3-hydroxy group have greater anticancer potency than those with a methoxy group at the 3-position.[1e] Here we observed similar phenomena with the side chain phenol hydroxy group. In general, for this series of analogues, direct attachment of an aromatic ring to the anthraquinone at the 3-position does not result in increased potency. This might be due to the planar structure of anthraquinone being compromised by steric strain around the biaryl C–C bond, leading to decreased DNA intercalation.[10] Spacing the aromatic ring away from anthraquinone by one sulfur atom appears to greatly alleviate this situation.

Table 1. Demethylation reactions in the synthesis of series 1 rhein analogues.

Methylated	R	Demethylated	R′	Yield [%]
4		**4a**		54
5a		**6a**		41
5b		**6b**		47
5c		**6c**		71
5d		**6d**		47
5e		**6e**		78
5f		**6f**		61
6f		**6g**		28
5g		–	NA[a]	NA
5h		–	NA	NA
5i		–	NA	NA

[a] NA: not applicable.

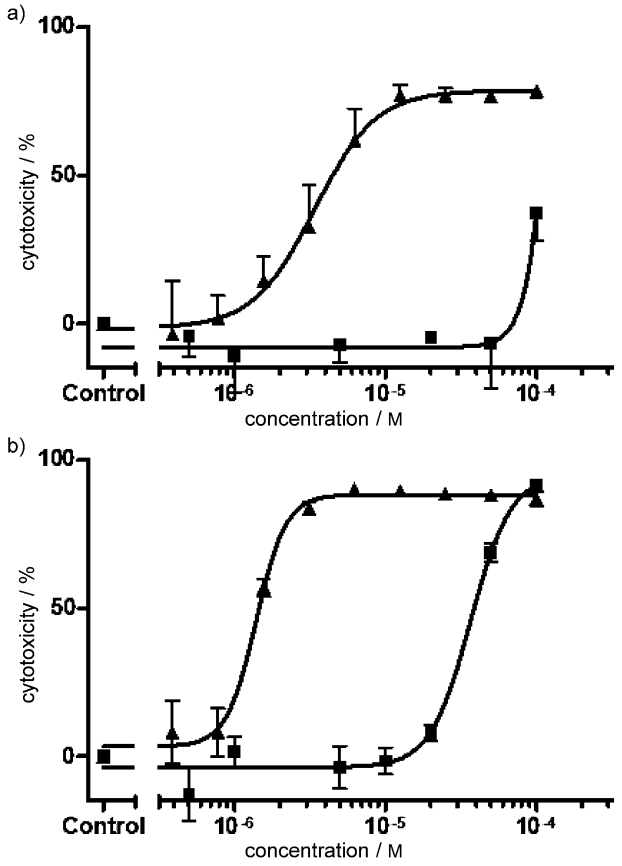


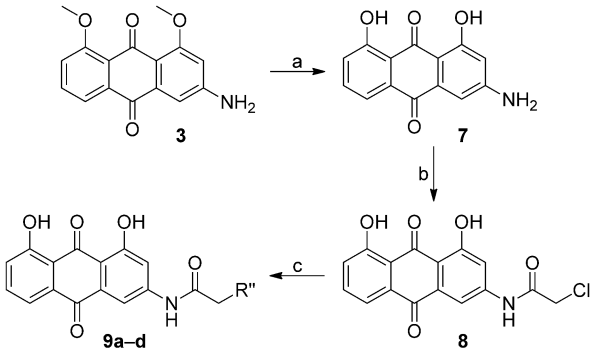
Figure 1. MTT assay dose–response curves of a) HeLa and b) MOLT4 cells in the presence of rhein [■: a) $IC_{50} = 3.7 \times 10^{-4}$ M, b) $IC_{50} = 3.7 \times 10^{-5}$ M] and compound **4a** [▲: a) $IC_{50} = 3.4 \times 10^{-6}$ M, b) $IC_{50} = 1.4 \times 10^{-6}$ M].

Table 2. Cytotoxicity evaluation of series 1 rhein analogues by MTT assay.

Compd	IC_{50} [μM] HeLa	MOLT4
Rhein	>100	37
4a	3.4	1.4
6a	16	14[b]
6b	>100[a,b]	>100[a,b]
6c	68	>100
6d	>100	35
6e	>100	22
6f	>100[a,b]	29[b]
6g	9.9	21[b]

[a] The actual IC_{50} value should be >100 μM, as fewer than 50% of cells were killed even at the highest concentration. [b] $R^2 < 0.8$ for nonlinear regression in sigmoidal model fitting; see Supporting Information for further details.

The second series of compounds was designed with a focus on modifying the 3-amine of rhein analogues. As illustrated in Scheme 2, compound **7** was obtained after demethylation of **3** using the same method as for the first series of analogues. The 3-amino group of **7** was acylated with chloroacetyl chloride to yield **8**. Four secondary amines were then treated with amide **8** to give compounds **9a–d** (Figure 2). The cytotoxicities of this



Scheme 2. Synthesis of series 2 rhein analogues. *Reagents and conditions:* a) PhSH, K_2CO_3, NMP, 150–160 °C, 1 h; b) chloroacetyl chloride, dioxane, RT, 2 h; c) secondary amine, dioxane, 80–90 °C.

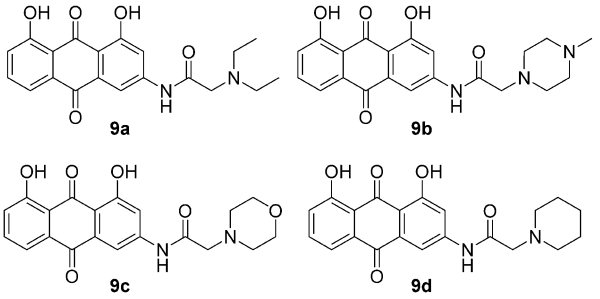


Figure 2. Series 2 rhein analogues **9a–d**.

series of analogues were evaluated by using the same method as for the first series. Generally speaking, compounds in this second series have much greater potency than the first series. Among all analogues, compound **8** showed the best activity (Table 3). The IC_{50} value of **8** against HeLa approaches that of the positive control, doxorubicin (Figure 3), and **8** is the only compound among all the analogues with a sub-micromolar IC_{50} value against the MOLT4 cell line. The potent activity of **8** might be attributed to its unique structural feature of combining an alkylating agent with an intercalation moiety. Such a proposition has been made in earlier studies.[3a] At this point, it is important to note that α-chloroacetamide alone is not ex-

Table 3. Cytotoxicity evaluation of series 2 rhein analogues by MTT assay.

Compd	IC_{50} [μM] HeLa	MOLT4
rhein	>100	37
7	17	10
8	2.7	0.6
9a	6.1	3.0
9b	5.8	3.1
9c	13[a]	25
9d	33	4.1
doxorubicin	0.98	0.04

[a] $R^2 < 0.8$ for nonlinear regression in sigmoidal model fitting; see Supporting Information for further details.

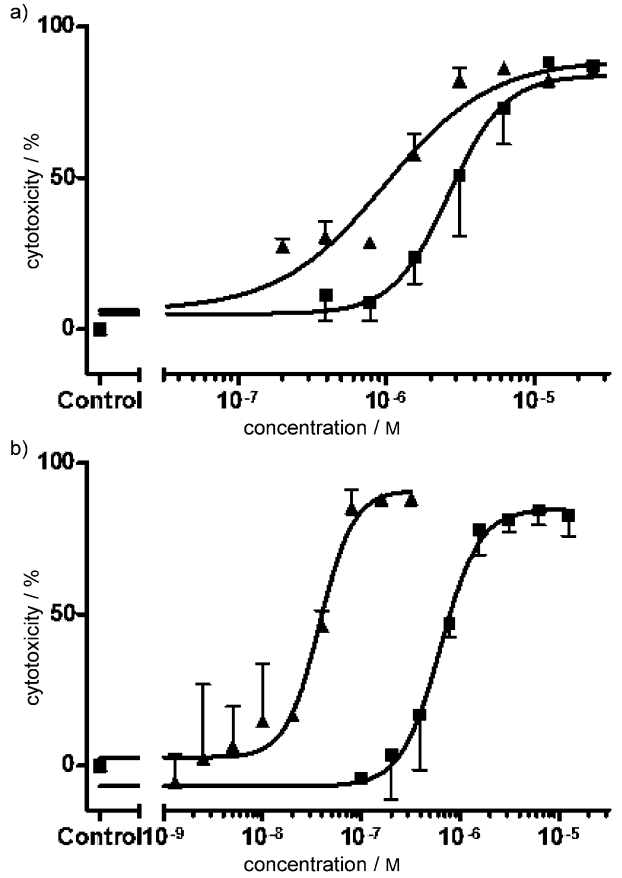


Figure 3. MTT assay dose–response curves of a) HeLa and b) MOLT4 cells in the presence of compound **8** [■: a) $IC_{50}=2.7\times10^{-6}$ M, b) $IC_{50}=6.3\times10^{-7}$ M] and doxorubicin [▲: a) $IC_{50}=9.8\times10^{-7}$ M, a) $IC_{50}=3.8\times10^{-8}$ M].

pected to be able to account for the observed cytotoxicity, as published studies have shown that it is only mildly cytotoxic, with an IC_{50} value of ~0.1 mM (against CHO cells).[13] Besides **8**, compound **7** also showed higher potency than most compounds in the first series except **4a**. These phenomena suggest that the amine/amide modification has an improved chance of success than aryl derivatization at the 3-position. Previous reports by other research groups suggest that the ring structure side chains are not critical for the high cytotoxicity of these anthraquinone compounds,[14] and the dicationic side chain gives better activity than the monocationic ones.[3c] In the second series, we found that **9b** shows better activity than **9c** and **9d**, but similar activity to that of **9a**. Compound **9b** shares similar structural features with mitoxantrone, but it has a cyclic instead of a linear diaminoalkyl side chain. There have been previous studies on the anticancer properties of aminoanthraquinone, but the alkylamino side chains were functionalized at the 1, 2, 4, 5, and 8-position(s).[3c,9,11,14,15] Herein we demonstrate that 3-position amine/amide modification can also significantly potentiate the anticancer activity of anthraquinone.

Conclusions

In summary, two series of rhein analogues were synthesized through modification at the 3-position. Their cytotoxicities were determined by MTT assays against the HeLa and MOLT4 cell lines. Among all 14 compounds generated, compounds **4a** and **8** stood out for their significantly higher cytotoxicity than the others. Especially important is compound **8**, which has an IC_{50} value similar to that of doxorubicin against HeLa cells and is active at sub-micromolar concentrations against MOLT4.

Experimental Section

Chemistry

General: Rhein was purchased from Nanjing ZeLang Medical Technology Co. (P.R. China) and used directly without further purification. Other starting materials and solvents were purchased from Aldrich or Acros. For all reactions, analytical grade solvents were used. Anhydrous solvents were used for all moisture-sensitive reactions. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 NMR spectrometer in deuterated solvent with TMS ($\delta=0.00$ ppm) or residual solvent as the internal reference. Deuterium solvents were purchased from Cambridge Isotope Laboratories, Inc. Mass spectra were recorded on a Waters Micromass LC-Q-TOF micro-spectrometer or an ABI4800 MALDI-TOF-TOF mass spectrometer at the Georgia State University Mass Spectrometry Facilities.

1,8-Dimethoxy-3-methylcarboxylate-anthraquinone: Rhein (10 g, 35 mmol) was suspended in dry DMF (300 mL) in a round-bottom flask, and the mixture was cooled in an ice bath under N_2. The suspension was treated with NaH (9 g, 225 mmol; 60% dispersed in mineral oil) and the color of the mixture turned from yellow to deep red. The reaction flask outlet was connected to a bubbler sealed with mineral oil. As the bubbling reached a steady and slow speed, MeI (18 mL, 290 mmol) was added through a syringe in one shot. The mixture was kept in the ice bath, with the reaction temperature slowly increasing to RT as the ice melting away. After stirring overnight, the reaction mixture was diluted with H_2O (1 L), and the aqueous suspension was repeatedly extracted with CH_2Cl_2 (1×vol) until TLC indicated no significant amount of product in the extraction. The combined organic extracts were concentrated to ~250 mL. MeOH (4×volume) was added to dilute the solution, and the mixture was cooled at 4 °C in a refrigerator. The desired product precipitated from solution as a yellow solid (8.9 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.46$ (d, 1H, $J=1.6$ Hz), 7.94 (d, 1H, $J=1.2$ Hz), 7.87–7.85 (dd, 1H, $J=0.8$, 7.6 Hz), 7.67 (t, 1H, $J=8.0$ Hz), 7.32 (d, 1H, $J=8.4$ Hz), 4.07 (s, 3H), 4.02 (s, 3H), 4.00 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=183.3$, 182.4, 165.6, 159.7, 135.0, 134.9, 134.8, 134.4, 126.9, 124.0, 120.0, 119.2, 118.4, 118.3, 56.9, 56.7, 52.9 ppm; HRMS: m/z $[M+H]^+$ calcd for $C_{18}H_{15}O_6$: 327.0869, found: 327.0857.

1,8-Dimethoxy-3-carboxy-anthraquinone: 1,8-Dimethoxy-3-methylcarboxylate-anthraquinone (8.9 g, 27 mmol) was suspended in EtOH (45 mL) and treated with a solution of NaOH in EtOH/H_2O (0.46 M, 90 mL, 1:1 *v/v*). The mixture was stirred at 50 °C for 1 h, and the reaction color turned from yellow to deep red. The suspension was cooled in an ice bath and acidified with HCl (1 M). The solution color changed to light yellow and precipitation was observed. The precipitate was isolated by filtration to give the desired product as a yellow solid (8.9 g, 100%). ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta=8.17$ (d, 1H, $J=1.6$ Hz), 7.89 (d, 1H, $J=1.2$ Hz),

7.76–7.69 (m, 2H), 7.54 (d, 1H, $J=8.0$ Hz), 3.99 (s, 3H), 3.93 ppm (s, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta=182.5$, 180.6, 165.6, 158.8, 158.7, 135.4, 134.3, 134.2, 133.9, 126.1, 123.4, 119.1, 118.4, 118.2, 118.1, 56.4, 56.3 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{17}H_{12}O_6$: 311.0556, found: 311.0547.

1,8-Dimethoxy-anthraquinone-3-carboxyl azide (2): 1,8-Dimethoxy-3-carboxy-anthraquinone (8.9 g, 28 mmol) was dissolved in a mixture of DMF (93 mL) and Et_3N (4.1 mL, 57 mmol). The deep red solution was cooled in an ice bath and treated dropwise with diphenylphosphoryl azide (6.2 mL, 29 mmol) while stirring. The solution was warmed to RT and stirred for 1 h. The mixture was diluted with H_2O (1.2 L), and the suspension was filtered to give the desired product as a light yellow solid (9.0 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.43$ (d, 1H, $J=1.6$ Hz), 7.90 (d, 1H, $J=1.2$ Hz), 7.86–7.84 (dd, 1H, $J=0.8$, 7.6 Hz), 7.67 (t, 1H, $J=8.0$ Hz), 7.33 (d, 1H, $J=8.0$ Hz), 4.07 (s, 3H), 4.02 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=183.1$, 182.3, 171.4, 159.9, 159.8, 135.3, 135.1, 134.7, 134.6, 127.8, 124.0, 120.0, 119.3, 118.5, 117.6, 57.1, 56.8 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{17}H_{12}N_3O_5$: 338.0777, found: 338.0769.

1,8-Dimethoxy-3-aminoanthraquinone (3): Acyl azide **2** (9.0 g, 27 mmol) was refluxed in dry dioxane (200 mL) for 30 min under N_2. TLC monitoring showed the disappearance of **2**. The mixture was concentrated in vacuo and diluted with aq NaOH (1 M, 400 mL). The suspension was refluxed for 4 h and then cooled to RT. The precipitate was removed by filtration, rinsed with acetone, and discarded. The filtrate was extracted repeatedly with CH_2Cl_2 (1×vol) until TLC indicated no significant amount of product in the extraction, and the organic layers were combined and concentrated in vacuo to give the desired product as a deep red solid (4.0 g, 53%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.78$ (d, 1H, $J=7.6$ Hz), 7.55 (t, 1H, $J=8.0$ Hz), 7.25 (m, 1H), 7.03 (d, 1H, $J=2.0$ Hz), 6.44 (d, 1H, $J=2.0$ Hz), 3.96 (s, 3H), 3.91 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=184.8$, 181.8, 162.3, 159.8, 151.9, 136.6, 135.0, 133.3, 124.5, 119.1, 118.6, 115.7, 104.7, 102.7, 56.8, 56.5 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{16}H_{14}NO_4$: 284.0923, found: 284.0914.

1,8-Dimethoxy-3-iodo-anthraquinone (4): Aminoanthraquinone **3** (2.0 g, 7.1 mmol), isopentyl nitrite (6.0 mL, 45 mmol) and diiodomethane (15 mL, 186 mmol) were mixed and stirred in dry THF (200 mL) at 50–60 °C for 2 days under N_2. The mixture was concentrated in vacuo, and the residue was purified by column chromatography (EtOAc/hexane, 1:1) to give the desired product as a yellow solid (1.4 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.14$ (d, 1H, $J=1.6$ Hz), 7.80–7.78 (dd, 1H, $J=0.8$, 8.0 Hz), 7.64 (t, 1H, $J=8.0$ Hz), 7.60 (d, 1H, $J=1.2$ Hz), 7.30 (d, 1H, $J=8.0$ Hz), 4.00 (s, 3H), 3.99 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=182.9$, 182.3, 159.7, 159.6, 135.0, 134.3, 128.2, 127.1, 123.8, 123.3, 119.2, 118.5, 100.7, 57.0, 56.7 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{16}H_{12}O_4I$: 394.9780, found: 394.9785.

General procedure for the synthesis of 5a–i by Suzuki coupling of 4 with RB(OH)$_2$: A mixture of **4** (0.13 mmol), $RB(OH)_2$ (0.23 mmol), $Pd(PPh_3)_4$ (0.013 mmol) and K_2CO_3 (0.38 mmol) in dry DMF (5 mL) was heated at 70–80 °C for 1→16 h under N_2. The mixture was then diluted with CH_2Cl_2 (40 mL) and washed with H_2O (2 or 3×50 mL). The organic layer was concentrated in vacuo, and the residue was purified by column chromatography (EtOAc/hexane or EtOAc/CH_2Cl_2). After removal of the solvent, the solid was further purified by dissolving in a minimal amount of CH_2Cl_2 and then precipitated through hexane addition.

1,8-Dimethoxy-3-(3′,5′-dimethyl-isoxazol-4′-yl)anthraquinone (5a): Yield: 50 mg, 92%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.84$–7.82 (dd, 1H, $J=0.8$, 7.6 Hz), 7.73 (d, 1H, $J=1.6$ Hz), 7.65 (t, 1H, $J=8.0$ Hz), 7.34–7.31 (dd, 1H, $J=0.8$, 8.4 Hz), 7.14 (d, 1H, $J=1.6$ Hz), 4.02 (s, 3H), 4.01 (s, 3H), 2.48 (s, 3H), 2.33 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=183.9$, 182.5, 166.5, 160.0, 159.7, 158.3, 136.7, 135.3, 134.8, 134.2, 124.0, 123.2, 119.3, 119.2, 118.5, 118.2, 115.8, 56.8, 56.7, 12.0, 11.1 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{21}H_{18}NO_5$: 364.1185, found: 364.1169.

1,8-Dimethoxy-3-(thiophen-2′-yl)anthraquinone (5b): Yield: 45 mg, 87%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.08$ (d, 1H, $J=2.0$ Hz), 7.87–7.85 (dd, 1H, $J=0.8$, 7.6 Hz), 7.65 (t, 1H, $J=8.0$ Hz), 7.55–7.54 (dd, 1H, $J=1.2$, 3.6 Hz), 7.46 (d, 1H, $J=1.2$ Hz), 7.43–7.42 (dd, 1H, $J=0.8$, 5.2 Hz), 7.32 (d, 1H, $J=8.0$ Hz), 7.17–7.15 (dd, 1H, $J=4.8$, 3.6 Hz), 4.08 (s, 3H), 4.02 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=184.1$, 182.4, 160.4, 159.8, 142.5, 139.9, 135.5, 134.9, 134.0, 128.7, 127.3, 125.6, 124.2, 122.8, 119.2, 118.5, 116.3, 114.6, 56.8, 56.7 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{15}O_4S$: 351.0691, found: 351.0682.

1,8-Dimethoxy-3-(4′-dimethylamino-3′-methylphenyl)anthraquinone (5c): Yield: 63 mg, 100%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.05$ (d, 1H, $J=1.6$ Hz), 7.86–7.84 (dd, 1H, $J=1.2$, 7.6 Hz), 7.62 (t, 1H, $J=8.0$ Hz), 7.51–7.48 (m, 2H), 7.45 (d, 1H, $J=1.6$ Hz), 7.31–7.29 (dd, 1H, $J=0.8$, 8.4 Hz), 7.10 (d, 1H, $J=8.0$ Hz), 7.17–7.15 (dd, 1H, $J=4.8$, 3.6 Hz), 4.07 (s, 3H), 4.01 (s, 3H), 2.77 (s, 6H), 2.41 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=184.4$, 182.8, 160.3, 159.7, 153.8, 146.9, 135.1, 135.0, 133.9, 132.8, 132.4, 130.2, 125.4, 124.3, 122.2, 119.2, 118.8, 118.3, 117.3, 115.9, 56.8, 56.7, 44.1, 19.1 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{25}H_{24}O_4N$: 402.1705, found: 402.1704.

1,8-Dimethoxy-3-(6′-fluoropyridin-3′-yl)anthraquinone (5d): Yield: 46 mg, 99%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.53$ (d, 1H, $J=1.6$ Hz), 8.10 (td, 1H, $J=2.0$, 8.0 Hz), 8.01 (d, 1H, $J=1.6$ Hz), 7.86 (d, 1H, $J=8.0$ Hz), 7.67 (t, 1H, $J=8.0$ Hz), 7.42 (s, 1H), 7.33 (d, 1H, $J=8.4$ Hz), 7.01–7.07 (dd, 1H, $J=2.8$, 8.8 Hz), 4.09 (s, 3H), 4.03 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=184.0$, 182.5, 160.5, 159.8, 146.5, 146.3, 142.4, 140.2, 140.1, 135.6, 134.9, 134.3, 133.3, 124.1, 123.6, 119.3, 118.6, 117.6, 116.2, 110.3, 109.9, 57.0, 56.8 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{21}H_{15}NO_4F$: 364.0985, found: 364.0977.

1,8-Dimethoxy-3-(4′-*tert*-butoxymethyl-phenyl)anthraquinone (5e): Yield: 30 mg, 55%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.06$ (s, 1H), 7.85 (s,1H, $J=5.2$ Hz), 7.65 (s, 3H), 7.47 (s, 3H), 7.30 (m, 1H), 4.52 (s, 2H), 4.07 (s, 3H), 4.01 (s, 3H), 1.32 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=184.3$, 182.8, 160.2, 159.7, 146.8, 141.1, 138.2, 135.2, 135.0, 134.0, 128.2, 127.3, 124.2, 122.7, 119.2, 118.3, 117.6, 116.4, 73.8, 63.9, 56.8, 56.7, 27.9 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{27}H_{27}O_5$: 431.1858, found: 431.1863.

1,8-Dimethoxy-3-(4′-methoxyphenyl)anthraquinone (5f): Yield: 56 mg, 98%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.03$ (d, 1H, $J=1.6$ Hz), 7.86–7.84 (dd, 1H, $J=1.2$, 7.6 Hz), 7.63 (m, 3H, $J=8.4$, 7.6 Hz), 7.43 (d, 1H, $J=2.0$ Hz), 7.30 (d, 1H, $J=8.4$ Hz), 7.01 (d, 2H, $J=8.8$ Hz), 4.07 (s, 3H), 4.01 (s, 3H), 3.87 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=184.4$, 182.8, 160.5, 160.3, 159.7, 146.5, 135.2, 135.1, 134.0, 131.7, 128.6, 124.2, 122.3, 119.2, 118.4, 117.2, 115.8, 114.7, 56.8, 56.7, 55.6 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{23}H_{19}O_5$: 375.1232, found: 375.1247.

1,8-Dimethoxy-3-(4′-acetylphenyl)anthraquinone (5g): Yield: 50 mg, 81%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.07$–8.05 (m, 3H, $J=1.6$, 8.0 Hz), 7.85–7.83 (dd, 1H, $J=0.8$, 7.6 Hz), 7.76 (d, 2H, $J=$

8.4 Hz), 7.65 (t, 1H, J=8.4, 7.6 Hz), 7.48 (d, 1H, J=1.6 Hz), 7.32 (d, 1H, J=8.0 Hz), 4.09 (s, 3H), 4.02 (s, 3H), 2.66 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=197.7, 184.0, 182.6, 160.3, 159.8, 145.4, 143.7, 137.2, 135.3, 134.9, 134.2, 129.2, 127.6, 124.1, 123.4, 119.2, 118.4, 117.9, 116.5, 56.9, 56.7, 26.9 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{24}H_{19}O_5$: 387.1232, found: 387.1241.

1,8-Dimethoxy-3-(furan-2′-yl)anthraquinone (5h): Yield: 34 mg, 78%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.06 (s, 1H), 7.83 (d, 1H, J=7.6 Hz), 7.61–7.52 (m, 3H), 7.27 (t, 1H, J=8.4, 4.0 Hz), 6.88 (d, 1H, J=3.2 Hz), 6.52 (s, 1H), 4.05 (s, 3H), 3.99 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=184.0, 182.0, 160.7, 160.0, 152.6, 143.8, 136.0, 135.7, 135.2, 133.8, 124.8, 123.0, 119.4, 118.9, 114.7, 112.8, 112.5, 108.8, 56.9 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{15}O_5$: 335.0919, found: 335.0935.

1,8-Dimethoxy-3-(pyridin-3′-yl)anthraquinone (5i): Yield: 35 mg, 100%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.93 (s, 1H), 8.68 (s, 1H), 8.03 (d, 1H, J=1.6 Hz), 7.99 (d, 1H, J=8.0 Hz), 7.84 (d, 1H, J=7.6 Hz), 7.64 (t, 1H, J=8.0 Hz), 7.46 (d, 1H, J=1.2 Hz), 7.43 (m, 1H), 7.32 (d, 1H, J=8.4 Hz), 4.09 (s, 3H), 4.02 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=183.9, 182.5, 160.3, 159.7, 150.0, 148.3, 143.4, 135.4, 135.0, 134.8, 134.7, 134.2, 123.9, 123.3, 119.1, 118.4, 117.6, 116.2, 56.8, 56.7 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{21}H_{16}NO_4$: 346.1079, found: 346.1084.

General procedure for the synthesis of 4a, 6a-f by thiophenol demethylation: A mixture of 1,8-dimethoxy-anthraquinone (0.07 mmol), PhSH (0.40 mmol) and K_2CO_3 (0.40 mmol) in dry *N*-methyl-2-pyrrolidone (NMP) (4 mL) was heated at 140–160 °C for 20–60 min under N_2. The mixture was then diluted with H_2O (150 mL), and the aqueous suspension was extracted with EtOAc (3×100 mL). The combined organic layers were and concentrated in vacuo. The residue was purified by column chromatography (EtOAc/hexane).

1,8-Dihydroxy-3-(phenylthio)anthraquinone (4a): Yield: 10 mg, 54%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.10 (s, 1H), 12.09 (s, 1H), 7.80–7.77 (dd, 1H, J=1.2, 7.6 Hz), 7.65 (t, 1H, J=8.0 Hz), 7.60–7.57 (m, 3H), 7.50 (d, 2H, J=1.6 Hz), 7.48 (d, 1H, J=1.6 Hz), 7.30–7.27 (dd, 1H, J=1.2, 7.6 Hz), 6.80 ppm (d, 1H, J=1.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.0, 181.8, 163.1, 162.6, 152.9, 137.1, 135.5, 133.6, 133.5, 130.4, 129.4, 125.0, 120.2, 119.6, 118.1, 116.1, 113.2 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{20}H_{11}O_4S$: 347.0378, found: 347.0377.

1,8-Dihydroxy-3-(3′,5′-dimethyl-isoxazol-4′-yl)anthraquinone (6a): Yield: 10 mg, 41%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.12 (s, 1H), 12.06 (s, 1H), 7.85 (d, 1H, J=7.2 Hz), 7.75 (d, 1H, J=1.6 Hz), 7.72 (t, 1H, J=8.0 Hz), 7.33 (d, 1H, J=7.6 Hz), 7.20 (d, 1H, J=1.6 Hz), 2.53 (s, 3H), 2.38 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.7, 181.7, 167.1, 163.0, 162.8, 140.4, 137.6, 134.3, 133.7, 125.1, 123.8, 120.4, 120.3, 116.0, 115.3, 115.0, 12.3, 11.3 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{19}H_{12}O_5N$: 334.0715, found: 334.0709.

1,8-Dihydroxy-3-(thiophen-2′-yl)anthraquinone (6b): Yield: 10 mg, 47%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.05 (s, 2H), 8.07 (s, 1H), 7.86 (d, 1H, J=6.8 Hz), 7.66 (t, 1H, J=7.6 Hz), 7.58 (s, 1H), 7.46 (m, 2H), 7.29 (d, 1H, J=8.0 Hz), 7.15 ppm (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.4, 181.9, 163.6, 163.0, 143.4, 142.1, 137.2, 134.7, 134.1, 128.9, 128.5, 126.7, 125.0, 120.3, 119.8, 117.7, 116.4, 115.0 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{18}H_9O_4S$: 321.0222, found: 321.0236.

1,8-Dihydroxy-3-(4′-dimethylamino-3′-methylphenyl)anthraquinone (6c): Yield: 20 mg, 71%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.13 (s, 1H), 12.05 (s, 1H), 8.03 (s, 1H), 7.81 (d, 1H, J=7.2 Hz), 7.64 (t, 1H, J=8.4 Hz), 7.53–7.49 (m, 2H), 7.42 (s, 1H), 7.26 (d, 1H, J=8.4 Hz), 7.06 (d, 1H, J=8.0 Hz), 2.80 (s, 6H), 2.40 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.3, 182.1, 163.2, 162.6, 154.5, 150.0, 137.0, 133.9, 133.8, 132.2, 131.4, 130.3, 125.5, 124.7, 120.7, 120.2, 118.7, 118.6, 116.2, 114.2, 44.0, 19.2 ppm; HRMS (ESI−): m/z $[M+H]^+$ calcd for $C_{23}H_{20}O_4N$: 374.1392, found: 374.1397.

1,8-Dihydroxy-3-(6′-phenylthio-pyridin-3′-yl)anthraquinone (6d): Yield: 11 mg, 47%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.01 (s, 1H), 11.98 (s, 1H), 8.76 (s, 1H), 8.00 (d, 1H, J=1.6 Hz), 7.83 (dd, 1H, J=1.2, 7.6 Hz), 7.74 (dd, 1H, J=2.4, 8.4 Hz), 7.68–7.62 (m, 3H), 7.45–7.43 (m, 4H), 7.29 (dd, 1H, J=1.2, 8.4 Hz), 7.03 ppm (d, 1H, J=8.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.8, 181.7, 163.6, 163.4, 163.0, 148.2, 146.7, 137.5, 135.4, 135.1, 134.8, 134.0, 130.9, 130.5, 130.0, 129.7, 125.1, 121.5, 121.4, 120.4, 118.4, 116.3, 115.5 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{25}H_{16}NO_4S$: 426.0800, found: 426.0800.

1,8-Dihydroxy-3-(4′-*tert*-butoxymethyl-phenyl)anthraquinone (6e): Yield: 22 mg, 78%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.08 (s, 1H), 12.04 (s, 1H), 8.04 (d, 1H, J=1.6 Hz), 7.81 (dd, 1H, J=1.2, 7.6 Hz), 7.67–7.63 (m, 3H), 7.48 (s, 1H), 7.45 (m, 2H), 7.27 (dd, 1H, J=1.2, 7.6 Hz), 4.51 (s, 2H), 1.33 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.6, 181.9, 163.1, 162.7, 150.0, 141.9, 137.2, 137.1, 134.0, 133.8, 128.2, 127.4, 124.8, 121.7, 120.3, 119.0, 116.1, 114.7, 73.9, 63.9, 27.9 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{25}H_{23}O_5$: 403.1545, found: 403.1543.

1,8-Dihydroxy-3-(4′-methoxyphenyl)anthraquinone (6f): Yield: 18 mg, 61%. ^{1}H NMR (400 MHz, $CDCl_3$): δ=12.06 (s, 1H), 12.00 (s, 1H), 8.05 (d, 1H, J=1.6 Hz), 7.83 (d, 1H, J=7.2 Hz), 7.64 (m, 3H, J=8.8, 7.2 Hz), 7.44 (d, 1H, J=1.6 Hz), 7.27 (d, 1H, J=8.4 Hz), 7.00 (d, 2H, J=8.8 Hz), 3.87 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=192.7, 182.1, 163.5, 162.9, 161.4, 150.0, 137.1, 134.4, 134.2, 131.0, 128.8, 124.8, 121.0, 120.2, 118.7, 116.5, 115.0, 114.6, 55.7 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{21}H_{13}O_5$: 345.0763, found: 345.0767.

1,8-Dihydroxy-3-(4′-hydroxyphenyl)anthraquinone (6g): A mixture of **6f** (15 mg, 0.043 mmol), PhSH (13 μL, 0.13 mmol) and K_2CO_3 (36 mg, 0.26 mmol) in dry NMP (1 mL) was heated at 170–180 °C for 1 h under N_2. The mixture was then diluted with H_2O (20 mL) and acidified with HCl (5 M). The aqueous suspension was extracted with EtOAc (1×vol), and concentrated in vacuo. The residue was purified by column chromatography (EtOAc/hexane, 1:4). Yield: 4 mg, 28%. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=11.97 (s, 2H), 9.99 (s, 1H), 7.90 (d, 1H, J=1.6 Hz), 7.80 (t, 1H, J=8.0 Hz), 7.73–7.70 (m, 3H), 7.56 (d, 1H, J=1.6 Hz), 7.38 (dd, 1H, J=1.2, 8.0 Hz), 6.92 ppm (d, 2H, J=8.4 Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=191.2, 181.4, 162.1, 161.3, 159.2, 148.6, 137.3, 133.7, 133.3, 128.6, 127.8, 124.5, 119.6, 119.4, 116.7, 116.1, 114.0 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{20}H_{11}O_5$: 331.0606, found: 331.0614.

1,8-Dihydroxy-3-aminoanthraquinone (7): A mixture of **3** (1.7 g, 6.0 mmol), PhSH (3.3 mL, 30 mmol) and K_2CO_3 (4.1 g, 30 mmol) in dry NMP (100 mL) was heated at 150–160 °C for 1 h under N_2. The mixture was then diluted with CH_2Cl_2 (400 mL) and washed with brine (4×500 mL). The organic layer was concentrated in vacuo. The residue was diluted with H_2O (250 mL) and hexane (250 mL), and a precipitate formed at the organic/aqueous interface. The precipitate was collected by filtration to give the desired product as a deep red solid (1.5 g, 98%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=12.42 (s, 1H), 12.23 (s, 1H), 7.70–7.63 (m, 2H), 7.30 (d, 1H, J=8.0 Hz), 7.16 (s, 2H), 7.04 (s, 1H), 6.25 ppm (s, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=187.5, 181.9, 164.9, 161.0, 157.5, 135.9,

134.7, 133.1, 124.3, 119.0, 115.9, 108.6, 105.4, 102.0 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{14}H_8O_4N$: 254.0453, found: 254.0441.

1,8-Dihydroxy-3-(2′-chloro-acetamido)anthraquinone (8): Chloroacetyl chloride (0.7 mL, 8.8 mmol) was injected slowly into a solution of **7** (1.5 g, 5.9 mmol) in dry dioxane (60 mL). The mixture was stirred at RT for 2 h. The reaction mixture was then diluted with H_2O (300 mL), and the suspension was extracted with CH_2Cl_2 (2× 200 mL). The combined organic extracts were and concentrated in vacuo. Purification by column chromatography (CH_2Cl_2/EtOAc, 2:1) gave the desired product as an orange solid (0.51 g, 26%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 12.07$ (s, 1H), 12.01 (s, 1H), 8.43 (s, 1H), 7.88 (s, 1H), 7.82 (d, 1H, $J = 7.2$ Hz), 7.68–7.63 (m, 2H), 7.29 (d, 1H, $J = 8.4$ Hz), 4.21 ppm (d, 1H, $J = 2.4$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 191.9, 181.5, 164.8, 164.4, 163.0, 144.8, 137.2, 135.2, 133.9, 125.2, 120.4, 116.3, 113.4, 113.2, 111.5, 43.1 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{16}H_9O_5NCl$: 330.0169, found: 330.0175.

General procedure for the synthesis of 9a–d: A mixture of **8** (0.3 mmol) and secondary amine (1.5 mmol) in dry dioxane (9 mL) was heated at 80–90 °C for 2 h. The reaction mixture was concentrated in vacuo, and the residue was dissolved in a minimal amount of CH_2Cl_2. The solution was then diluted with hexane (2× volume) and stored at −20 °C. Precipitation was observed, and filtration gave the desired product as a yellow solid.

1,8-Dihydroxy-3-(2′-diethylaminoacetamido)anthraquinone (9a): Yield: 42 mg, 36%. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 12.16$ (s, 2H), 9.90 (s, 1H), 8.03 (d, 1H, $J = 2.0$ Hz), 7.82 (d, 1H, $J = 7.6$ Hz), 7.66 (t, 1H, $J = 7.6$, 8.4 Hz), 7.57 (d, 1H, $J = 2.0$ Hz), 7.29 (d, 1H, $J = 8.4$ Hz), 3.21 (s, 2H), 2.69 (q, 4H, $J = 7.2$ Hz), 1.12 ppm (t, 6H, $J = 7.2$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 191.4$, 181.9, 171.2, 164.9, 162.7, 145.6, 136.9, 134.8, 133.7, 125.1, 120.2, 116.1, 112.5, 112.1, 111.2, 58.2, 49.2, 12.6 ppm; HRMS (ESI−): m/z $[M-H]^-$ calcd for $C_{20}H_{19}O_5N_2$: 367.1294, found: 367.1296.

1,8-Dihydroxy-3-(2′-(4″-methylpiperazin-1″-yl)acetamido)anthraquinone (9b): Yield: 54 mg, 45%. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.97$ (s, 2H), 10.51 (s, 1H), 7.97 (d, 1H, $J = 2.0$ Hz), 7.80–7.76 (m, 2H), 7.72–7.70 (dd, 1H, $J = 1.2$, 7.6 Hz), 7.37–7.35 (dd, 1H, $J = 1.2$, 8.4 Hz), 3.38 (s, 2H), 3.22 (s, 4H), 2.90 (s, 4H), 2.75 ppm (s, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 190.0$, 180.9, 168.9, 162.7, 161.1, 146.2, 136.7, 134.0, 133.1, 124.1, 119.1, 115.6, 111.3, 110.9, 59.8, 52.3, 48.8, 42.0 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{21}H_{22}O_5N_3$: 396.1559, found: 396.1556.

1,8-Dihydroxy-3-(2′-morpholino-acetamido)anthraquinone (9c): Yield: 100 mg, 82%. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.80$–11.40 (br s, 2H), 10.47 (s, 1H), 7.90 (d, 1H, $J = 2.0$ Hz), 7.78–7.74 (m, 2H), 7.66 (d, 1H, $J = 7.2$ Hz), 7.33 (d, 1H, $J = 8.4$ Hz), 3.66 (t, 4H, $J = 4.4$ Hz), 3.22 (s, 2H), 2.54 ppm (t, 4H, $J = 4.4$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 190.2$, 181.1, 169.6, 163.0, 161.3, 146.5, 137.0, 134.1, 133.2, 124.4, 119.4, 115.7, 111.4, 111.1, 66.0, 62.0, 53.1 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{20}H_{19}O_6N_2$: 383.1243, found: 383.1228.

1,8-Dihydroxy-3-(2′-piperidinyl-acetamido)anthraquinone (9d): Yield: 55 mg, 47%. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 12.50$–11.50 (br s, 2H), 9.65 (s, 1H), 7.92 (d, 1H, $J = 2.0$ Hz), 7.81–7.78 (dd, 2H, $J = 1.2$, 7.6 Hz), 7.64–7.60 (m, 2H), 7.27–7.25 (d, 1H, $J = 8.4$ Hz), 3.11 (s, 2H), 2.58 (t, 4H, $J = 5.2$ Hz), 1.69 (m, 4H), 1.53 ppm (t, 2H, $J = 5.6$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 191.6$, 181.8, 169.9, 165.0, 162.9, 145.9, 136.9, 135.1, 134.0, 125.0, 120.2, 116.3, 112.7, 112.3, 111.3, 63.1, 55.3, 26.5, 23.8 ppm; HRMS (ESI+): m/z $[M+H]^+$ calcd for $C_{21}H_{21}O_5N_2$: 381.1450, found: 381.1442.

Biology

MTT assay: Both HeLa and MOLT4 cell lines were purchased from the ATCC. MOLT4 cells were cultured in RPMI-1640 medium, while HeLa cells were cultured in MEM medium. Both media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For the cytotoxicity assays, cells were seeded into 96-well plate (2.5×10^4 in 100 μL per well for HeLa; 1.0×10^5 in 200 μL per well for MOLT4). Test compounds were dissolved or suspended in DMSO to make 10–20 mM stock solutions and diluted with culture medium to various concentrations (final DMSO concentration < 0.5%). Addition of test compound was performed immediately after seeding (MOLT4) or after adherent cells reached 40–50% confluence (HeLa). After incubation for 64–72 h at 37 °C in a humidified atmosphere with 5% CO_2, MTT (10 μL, 5 mg mL^{-1} in phosphate-buffered saline) was added to each well, and the plate was incubated for another 4 h. The culture medium was then aspirated and DMSO (100 μL) was added to each well. The 96-well plate was read using a microarray reader for optical density at 490 nm. All tests were performed in triplicate and the optical absorption readout was normalized to percentage of maximum cytotoxicity. Data were processed using GraphPad Prism 4, and the dose–response curves were fitted to a sigmoidal (or "logarithmic") model to calculate IC_{50} values. For compounds with IC_{50} values < 100 μM, the MTT assay was repeated.

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