Quinones

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Synthesis and Intracellular Redox Cycling of Natural Quinones and Their Analogues and Identification of Indoleamine-2,3-dioxygenase (IDO) as Potential Target for Anticancer Activity**

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In memory of Adam David Przeslak

Abstract: *Natural quinones, often linked with cellular oxidation processes, exhibit pronounced biological activity. In particular, the structurally unique isothiazolonaphthoquinone aulosirazole, isolated from blue-green alga, possesses selective antitumor cytotoxicity, although its mechanism of action is unknown. The first synthesis of aulosirazole uses a route centered upon a late-stage regioselective Diels–Alder reaction. The structurally related natural product pronqodine A, an inhibitor of prostaglandin release, and analogues thereof, were also prepared for comparison. Biological evaluation of the compounds identified one potential target as the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO). The isothiazoloquinones are also efficient substrates for the human quinone reductase NQO1, and undergo intracellular NQO1-dependent redox cycling resulting in the generation of reactive oxygen species, and at lower doses have the potential to alter the ratio of intracellular oxidized to reduced pyridine nucleotides.*

For centuries, naturally occurring compounds have played a fundamental role in the discovery of new medicines. Despite the advent of a range of new drug discovery models, such as diversity-oriented synthesis, lead-oriented synthesis, and fragment-based drug discovery, natural products continue to provide inspiration for medicinal chemists.[1–4] Many naturally occurring substances contain heterocyclic rings, with the alkaloids being one of the most important groups. Often derived in nature from α-amino acids, they exhibit wide-ranging pharmacological properties. One interesting subgroup of alkaloids contains both nitrogen and sulfur atoms, and over the years, a number of such N,S-containing compounds have been discovered, attracting the attention of biological and synthetic chemists alike.[5–7] Whereas the simple N,S heterocycle thiazole is relatively common in nature, and, for example, plays a vital role in the function of thiamine (vitamin B_1), its isomer isothiazole, which contains a nitrogen–sulfur bond, is unknown in nature as a simple monocyclic ring structure. There are, however, some examples of ring-fused isothiazoles, such as the brassica-derived phytoalexins, brassilexin, and sinalexin (Figure 1).[8,9] Other examples of this extremely rare class of natural products, which has fewer than 10 members, include the *Streptomyces*-derived, neuroprotective isothiazolopyridine SF2738F,[10,11] and the quinones pronqodine A, an inhibitor of prostaglandin release isolated from a *Streptomyces* strain,[12] and aulosirazole, a tumor-selective cytotoxin (Figure 1).[13]

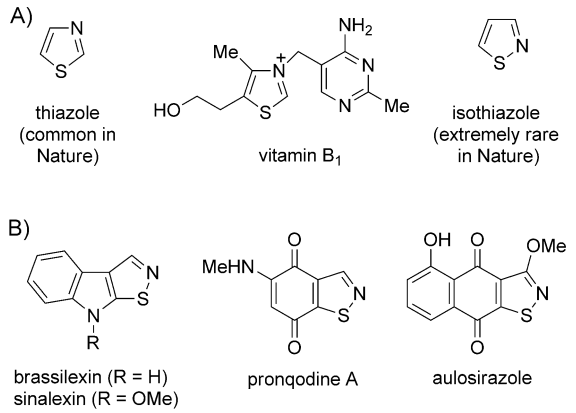

Figure 1. Isothiazole ring system. A) Comparison of thiazoles and isothiazoles; B) some naturally occurring benzisothiazoles, including the quinones pronqodine A and aulosirazole.

The quinone chromophore that is present in the isothiazoles pronqodine A and aulosirazole adds further biological interest. Quinones are inextricably linked with oxidative processes in cells, and were probably present in very early unicellular organisms about 2 billion years ago. Ubiquinones (coenzymes Q) are present in virtually all aerobic organisms, from bacteria to higher plants and animals, in which they play a major role in electron transport in the respiratory chain. In view of the pronounced biological activity shown by these

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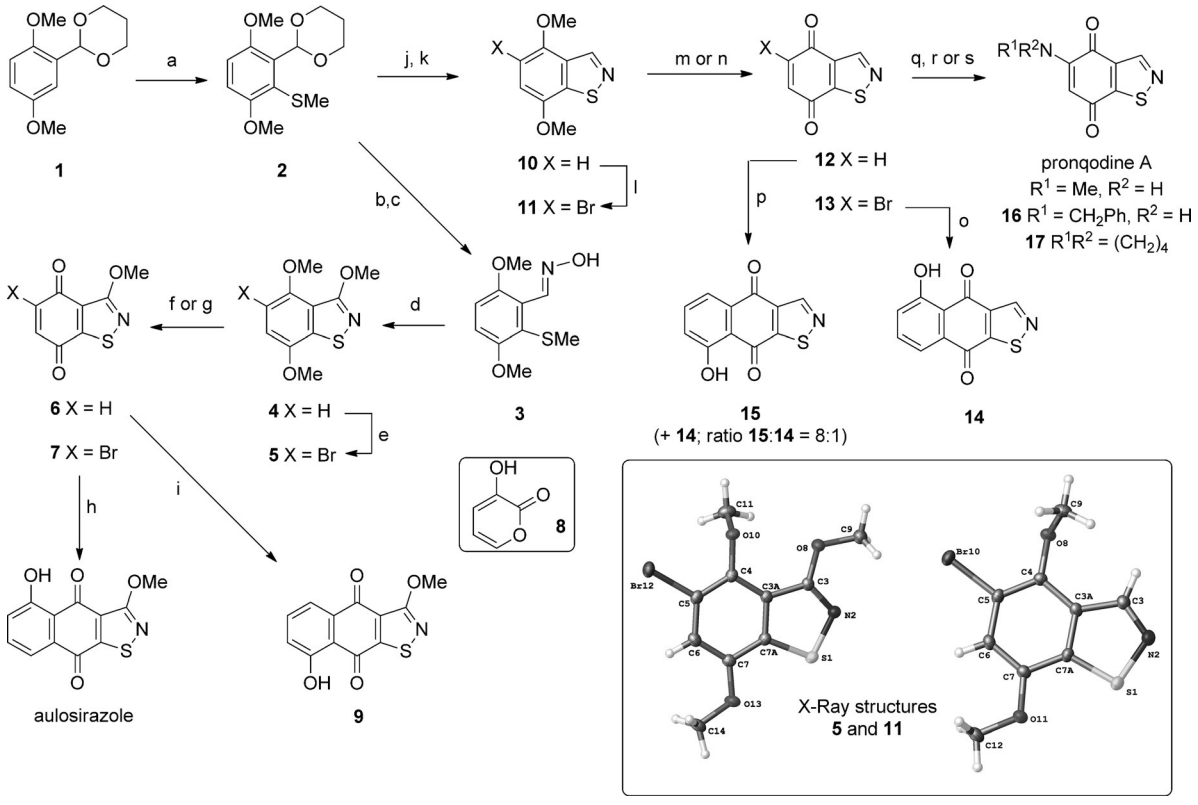
highly unusual isothiazolequinone structures, we now report the results of a combined chemistry–biology investigation, which resulted in the first chemical synthesis of the natural product aulosirazole (derived from blue-green algae), the identification of indoleamine-2,3-dioxygenase (IDO) as its potential biological target, its metabolism by the human quinone reductase NQO1 in comparison with pronqodine A, and the intracellular redox cycling of analogues.

Aulosirazole was isolated from the blue-green alga *Aulosira fertilissima* over two decades ago, and its unusual isothiazolonaphthoquinone structure was determined by NMR spectroscopy and X-ray crystallography.[13] The compound was reported to exhibit tumor-selective cytotoxicity, although no mechanism of action was advanced. The biological activity and unique chemical structure of aulosirazole prompted us to undertake its chemical synthesis and to investigate its potential molecular therapeutic targets. In planning a synthesis of the tricyclic core of aulosirazole, we elected to use a late-stage Diels–Alder reaction of a suitable benzoquinone dienophile to build the phenolic ring of the natural product.[14–16] Thus, the synthesis of the benzoquinone dienophile started from the cyclic acetal **1** of 2,5-dimethoxybenzaldehyde, which underwent an *ortho* lithiation at C6,[17,18] followed by reaction with dimethyl disulfide to give the sulfide **2** in 50% yield. Subsequent hydrolysis of the acetal and reaction with hydroxylamine gave oxime **3** and set the stage for the formation of the isothiazolone ring initiated by chlorination.[19] The dropwise addition of sulfuryl chloride to oxime **3** in chlorobenzene at 80 °C was essential for this transformation to be successful and give an intermediate benzisothiazolone that was immediately methylated to give the desired 3-methoxybenz[*d*]isothiazole **4** (Scheme 1).

We anticipated, in accordance with pioneering work by Brassard,[20–23] that a halogen atom at C5 in the quinone would be necessary to confer high levels of regioselectivity in the subsequent Diels–Alder reaction with an electron-rich diene. Therefore, 3-methoxybenz[*d*]isothiazole **4** was brominated to give the corresponding 5-brominated compound **5**, the structure of which was confirmed by X-ray crystallography (Scheme 1). Both the dimethoxy-functionalized compounds **4** and **5** were readily converted into the corresponding benzoquinones **6** and **7** by oxidation with cerium(IV) ammonium nitrate (CAN). With the quinone dienophiles **6** and **7** in hand, our attention turned to a suitable diene for the Diels–Alder reaction. The use of α-pyrones as dienes in Diels–Alder reactions was pioneered by Corey,[24] and have since been



Scheme 1. Syntheses of aulosirazole, pronqodine A and various analogues. Reagents and conditions: a) *n*BuLi, Et_2O, hexanes, −25 °C, 18 h; dimethyl disulfide, THF, RT, 4.5 h, 50%; b) conc. HCl, THF, RT, 1.5 h, 82%; c) $NH_2OH{\cdot}HCl$, NaOH, EtOH, H_2O, RT, 20 h, 79%; d) sulfuryl chloride, PhCl, RT, 80 °C, 1 h; dimethyl sulfate, K_2CO_3, DMF, RT, 16 h, 18%; e) Br_2, AcOH, RT, 1.5 h, 49%; f) CAN, H_2O, MeCN, RT, 1 h, 59%; g) CAN, H_2O, MeCN, RT, 45 h, 61%; (h) pyrone **8**, NEt_3, $CHCl_3$, RT, 40 min, 13%; i) pyrone **8**, NEt_3, $CHCl_3$, RT, 15 min, 50%; j) conc. HCl, THF, RT, 1.5 h, 82%; k) $NH_2OH{\cdot}HCl$, formic acid, 100 °C, 1.5 h, 63%; l) Br_2, $CHCl_3$, RT, 3 h, 66%; m) CAN, H_2O, MeCN, RT, 1 h, 59%; n) CAN, H_2O, MeCN, RT, 1 h, 82%; o) pyrone **8**, NEt_3, CH_2Cl_2, RT, 14 h, 30%; p) pyrone **8**, NEt_3, $CHCl_3$, RT, 30 min, 30%; q) $MeNH_2$, $CeCl_3{\cdot}7H_2O$, EtOH, RT, 1 h, 42%; r) benzylamine, $CeCl_3{\cdot}7H_2O$, EtOH, RT, 30 min, 38%; s) pyrrolidine, $CeCl_3{\cdot}7H_2O$, EtOH, RT, 30 min, 60%. CAN = cerium(IV) ammonium nitrate.

shown to undergo a one-pot Diels–Alder reaction, decarboxylation, and oxidation to give peri-hydroxynaphthoquinones.[25–27] In accordance with a reported procedure, the required hydroxy-bearing α-pyrone **8** was prepared in one step from commercially available mucic acid.[28] To our delight, upon carrying out the Diels–Alder reaction with bromobenzoquinone **7**, aulosirazole was formed directly, albeit in poor yield (Scheme 1). The ^{1}H and ^{13}C NMR spectroscopic data of the synthetic material closely matched those reported for the natural product (Figure S1). As expected, the nonbrominated quinone dienophile **6** also underwent Diels–Alder reaction with the hydroxy-bearing α-pyrone **8**, and gave the regioisomeric hydroxynaphthoquinone **9** (Scheme 1), the NMR spectroscopic data of which significantly differed from the natural product (Figure S2).

For comparison purposes, the corresponding desmethoxy isothiazolonaphthoquinones **14** and **15** were also synthesized. Deprotection of acetal **2** was followed by reaction with hydroxylamine hydrochloride in formic acid, which proceeded smoothly to give benzisothiazole **10** directly in 63% yield. As before, bromination gave the 5-bromo-bearing compound **11**, the structure of which was confirmed by X-ray crystallography (Scheme 1), and oxidation of the dimethoxy compounds **10** and **11** gave the corresponding benzoquinones **12** and **13**. Diels–Alder reaction of bromoquinone **13** with the α-pyrone **8** gave desmethoxyaulosirazole **14**, while the use of benzoquinone **12** as dienophile resulted in an inseparable mixture of naphthoquinones **15** and **14** in the ratio 8:1 (Scheme 1).

Pronqodine A, a structurally related isothiazolobenzoquinone, was recently isolated from the soil bacterium *Streptomyces* sp. MK832-95F2, and shown to inhibit prostaglandin release from cancer cells.[12] Therefore, in view of its isothiazoloquinone core, which it shares with aulosirazole, we included pronqodine A and two analogues thereof in our biological evaluations. The natural product was readily prepared from isothiazolobenzoquinone **12** by a published method,[12] and the novel analogues **16** and **17** were obtained by reaction with benzylamine and pyrrolidine, respectively (Scheme 1).

Although the original report from 1994 stated that aulosirazole possessed tumor-selective cytotoxicity,[13] the mechanism of action has never been investigated. In planning the biological evaluation of aulosirazole and its analogues, we were struck by its similarity to a series of naphthoquinones and related compounds that are reported to be potent inhibitors of indoleamine-2,3-dioxygenase (IDO; Figure 2). In particular, IDO is an essential target for the antitumor activity of the naphthoquinone menadione.[29] IDO catalyzes the first step in tryptophan catabolism, the oxidative cleavage of the indole 2,3 bond to give *N*-formylkynurenine, and is thought to play a major role in suppressing the immune response.[30–36] Inhibitors of IDO have potential as anticancer medicines,[29] and the first compounds in this class are now entering the clinic.[37,38] Therefore, it seemed logical to screen aulosirazole and its analogues as inhibitors of IDO. We were also mindful of the biological activity ascribed to pronqodine A,[12] in which the quinone reductase NAD(P)H quinone oxidoreductase 1 (NQO1), formerly known as DT-diaphor-

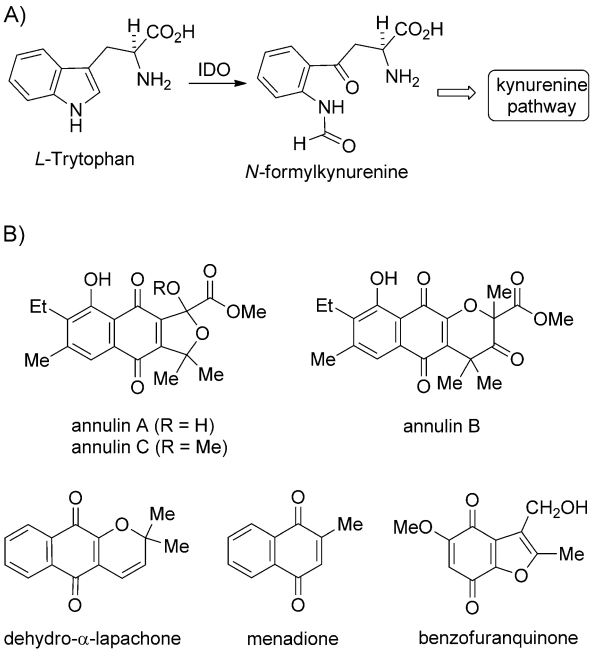


Figure 2. Aspects of indoleamine-2,3-dioxygenase (IDO). A) Oxidative cleavage of the 2,3 bond in L-tryptophan, the first and rate-determining step in the kynurenine pathway in mammalian cells; B) some quinone-based inhibitors of IDO.

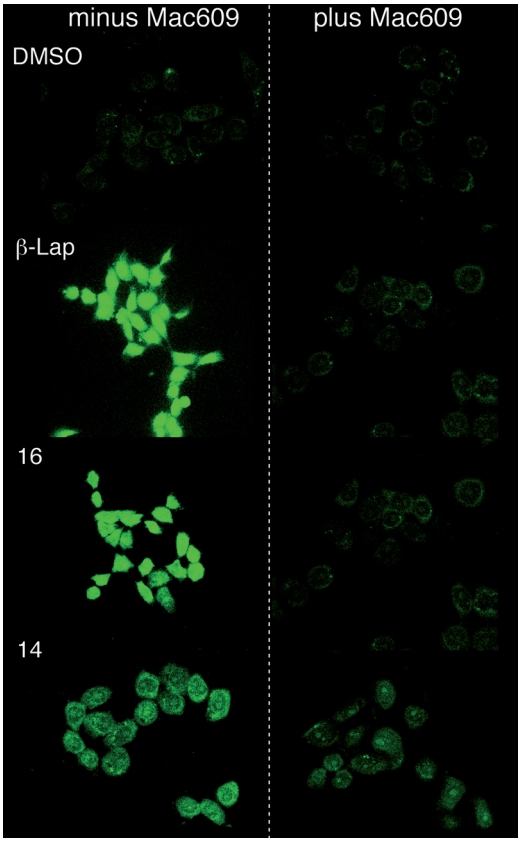
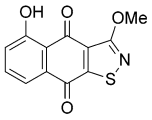
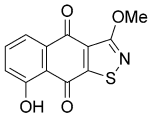
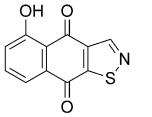
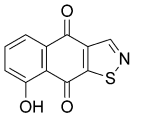
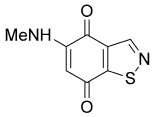
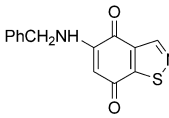
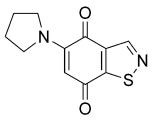
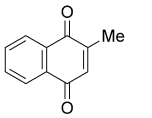
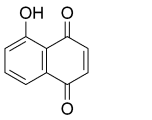


Figure 3. Compounds **14** and **16** undergo NQO1-dependent redox cycling in human breast cancer cells. MDA468/NQ16 cells were pretreated with the NQO1 inhibitor MAC609 (1 μM) for 30 min, then treated with DMSO or quinones (2 μM) for 1 h. The redox-sensitive dye CellROX green was added to cells 30 min after the quinones.

ase, plays a key role in the bioactivation by prior reduction of the quinone, and therefore the bioreduction of aulosirazole and analogues by NQO1 was also of interest.[39,40]

First we evaluated aulosirazole and its analogues as IDO inhibitors. Human IDO was obtained and purified as previously described.[41] Compounds were evaluated for their ability to inhibit the IDO-catalyzed oxidative degradation of L-tryptophan to *N*-formylkynurenine, subsequently converted into kynurenine by the cleavage of the *N*-formyl group with trichloroacetic acid, assayed by reaction with Ehrlich's reagent to produce a product with strong absorbance at 480 nm.[42] The data in Table 1 show the percentage of IDO catalytic activity remaining at four different inhibitor concentrations and the resultant IC_{50} values. These data demonstrate that analogues of both pronqodine A and aulosirazole are potent IDO inhibitors, with IC_{50} values one order of magnitude below what was observed for menadione, and identify IDO as a potential biological target for these compounds.

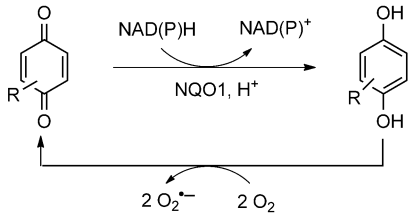
Table 1: Inhibition of rhIDO by aulosirazole and analogues.

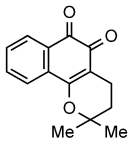
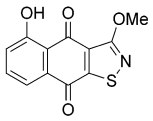
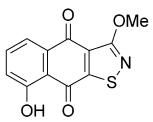
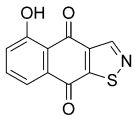
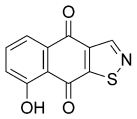
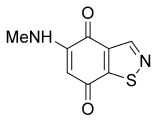
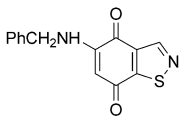
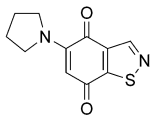
Compound	Structure	IC_{50} [nM]	Percent activity remaining of IDO at[a]			
			0.1 μM	1 μM	10 μM	100 μM
aulosirazole		80.3 ± 4.3	31.7 ± 0.4	7.2 ± 1.0	1.2 ± 0.4	2.1 ± 0.7[b]
9		71.3 ± 1.4	29.9 ± 0.6	2.2 ± 0.2	0	0
14		78.9 ± 1.3	36.0 ± 0.3	9.4 ± 0.2	2.5 ± 0.1	2.3 ± 0.1
15		80.8 ± 0.7	38.1 ± 0.4	10.5 ± 0.1	0.5 ± 0.2	0
pronqodine A		131.5 ± 28.5	51.1 ± 1.5	15.4 ± 0.1	3.0 ± 0.1	5.9 ± 0.1[b]
16		83.3 ± 2.9	40.0 ± 1.7	6.3 ± 0.2	0	0
17		541.5 ± 16.2	73.4 ± 1.4	25.7 ± 0.3	5.5 ± 0.1	2.6 ± 0.2
menadione		887.4 ± 15.8	86.0 ± 1.6	44.9 ± 0.6	10.8 ± 0.1	0
juglone		16413.5 ± 1197.6	94.2 ± 0.7	84.9 ± 0.3	53.5 ± 0.7	4.5 ± 0.1

[a] Results are presented as the mean ± standard deviation of three determinations. IDO inhibition was assayed as described in the methods section of the Supporting Information. [b] Because many of these quinones are highly colored, the apparent decrease in IDO inhibition observed with some compounds at 100 μM was due to interference from co-absorption (480 nm) between the kynurenine product and quinone.

The reductive metabolism of aulosirazole and pronqodine A and their analogues by recombinant human NQO1 (rhNQO1) was also studied, using a UV spectrophotometric assay designed to quantify the rate of NADH oxidation as previously described.[43] For NQO1 the rate of NADH oxidation is dependent upon the rate of quinone reduction and therefore can be used for comparison of reduction velocities. Reactions were run in the absence and presence of rhNQO1, and β-lapachone was included as reference, as it is known to undergo very efficient NQO1-dependent redox cycling.[44] The results are presented in Table 2 and show that both aulosirazole and pronqodine A and their analogues are good substrates for NQO1 and are rapidly reduced to unstable hydroquinones that spontaneously undergo autoxidation back to the parent quinone. These data confirm the earlier studies with pronqodine A, which showed that NQO1 could reduce pronqodine A to an unstable hydroquinone.[12] To determine whether aulosirazole and pronqodine A and their analogues could efficiently enter cells and undergo NQO1-dependent redox cycling, we examined the ability of these quinones to generate intracellular levels of reactive oxygen species in a human breast cancer cell line engineered to overexpress human NQO1 (MDA468/NQ16).[45] In these studies, MDA468/NQ16 cells were treated with β-lapachone (positive control) and representative isothiazoloquinones **14** and **16** in the presence and absence of the potent NQO1 mechanism-based inhibitor 6-methoxy-1,2-dimethyl-3-(4-pyridyloxymethyl)indole-4,7-dione (MAC609).[46] The generation of reactive oxygen species was monitored using the intracellular redox probe CellROX green in combination with confocal microscopy

Table 2: NQO1-mediated redox cycling. Rate of reduction of aulosirazole and analogues by rhNQO1.



Compound	Structure	Rate of NADH oxidation[a] minus rhNQO1 [nmol min^{-1}]	Rate of NADH oxidation[a] plus rhNQO1 [nmol min^{-1}]
β-lapachone		0.24 ± 0.05	48.68 ± 1.94
aulosirazole		0.66 ± 0.08	18.00 ± 0.41
9		0.66 ± 0.08	19.72 ± 0.44
14		0.50 ± 0.09	24.18 ± 0.62
15		0.62 ± 0.02	21.09 ± 0.67
pronqodine A		0.21 ± 0.11	28.85 ± 1.67
16		0.39 ± 0.12	52.49 ± 0.95
17		0.16 ± 0.06	9.66 ± 0.20

[a] Results are presented as the mean ± standard deviation of three determinations. Rates of NADH oxidation were determined as described in the methods section of the Supporting Information.

(Figure 3). Results from these experiments clearly demonstrate that compounds **14** and **16** readily enter cells and undergo NQO1-dependent redox cycling, which results in the generation of reactive oxygen species.

In these studies, aulosirazole and pronqodine A and their analogues have been shown to be potent IDO inhibitors and undergo NQO1-dependent bioactivation to unstable hydroquinones, which spontaneously undergo autoxidation resulting in the formation of reactive oxygen species and enzymatic oxidation of reduced pyridine nucleotides. The inhibition of IDO and the intracellular generation of reactive oxygen species make these quinones attractive molecules for the development as antitumor agents. Recent studies, however, have demonstrated that redox-cycling quinones may also have other therapeutic uses. Pronqodine A has been shown to regulate prostaglandin releases from human synovial cells,[12] and experiments in endothelial cells have shown that treatment with nontoxic concentrations of β-lapachone induced an NQO1-dependent increase in the NAD^+/NADH ratio as a consequence of β-lapachone redox cycling, which resulted in modulation of enzymatic pathways that control hypertension.[47–49]

The synthesis of aulosirazole, a structurally unique naturally occurring isothiazolonaphthoquinone, has been achieved using a route based upon a late-stage regioselective Diels–Alder reaction of an isothiazolobenzoquinone dienophile. Aulosirazole and pronqodine A, a related isothiazolobenzoquinone natural product, and their analogues are potent inhibitors of human IDO, however, their propensity to undergo NQO1-mediated reduction to unstable hydroquinones with the generation of reactive oxygen species may limit their use at high doses. At lower doses, these agents may be useful reagents to alter the ratio of intracellular oxidized to reduced pyridine nucleotides.

Keywords: Diels–Alder reaction · oxidoreductases · quinones · redox chemistry · sulfur/nitrogen heterocycles

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