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Synthesis of carbazoloquinone derivatives and their antileukemic activity *via* modulating cellular reactive oxygen species

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ABSTRACT

Carbazoloquinone alkaloids are of great interest as privileged structures for anticancer drug molecules. The purpose of this study was to investigate the structure-activity relationships of carbazoloquinone derivatives as anticancer agents. A series of carbazoloquinones including murrayaquinone A, koeniginequinones A and B, and related analogues were therefore prepared. Palladium-catalyzed intramolecular cyclization reaction mechanism was well elucidated by DFT calculations. Treatment of the synthesized derivatives showed cytotoxicity on human leukemia HL-60 cells in a dose-dependent fashion. In addition, murrayaquinone A and β-brazanquinone elevated cellular levels of reactive oxygen species (ROS), thereby triggering apoptosis. Our findings emphasize the excellent potential of carbazoloquinone derivatives as ROS-inducing anticancer agents.

Keywords: Carbazoloquinone alkaloid; Murrayaquinone A; DFT calculation; Reactive oxygen species; Apoptosis

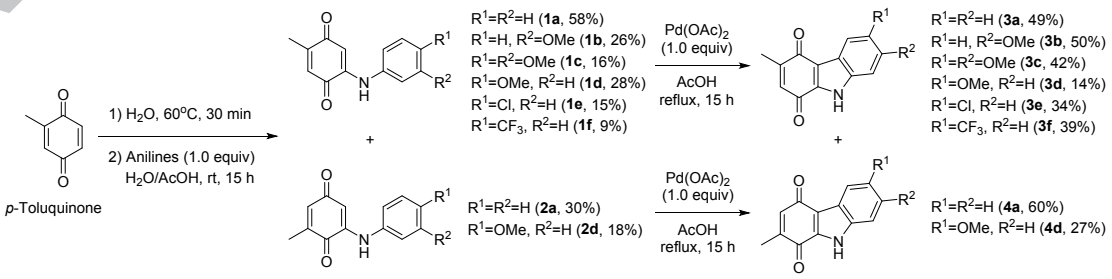
Quinone molecules are widespread in nature, and some of them have been used to treat cancer (e.g., doxorubicin, daunorubicin, and mitomycin C).[1] Anticancer quinones show remarkable potential and distinctive modes of actions, and because of that, the exploration of natural quinone constituents for cancer chemotherapy attracts a great deal of attention.[2–4] There is continued raising interest in the development of new chemotherapeutic agents containing the quinone motif.

Carbazoloquinones (carbazole-1,4-quinones) constitute a subclass of natural carbazole alkaloids occurring in the *Rutaceae* (citrus) family of plants.[5,6] The *Murraya* genus of flowering shrubs is the greatest source of these constituents. Their basic framework comprises fused 1,4-quinone and indole structures derived from anthranilic acid and prenyl pyrophosphate.[7] These quinones have been reported to possess a broad range of biological properties such as anticancer, anti-inflammatory, antibacterial, and anti-infective properties.[8–11] Murrayaquinone A (3-methyl-1*H*-carbazole-1,4(9*H*)-dione) was firstly isolated from the root bark of *Murraya euchrestifolia* Hayata.[12] This quinone displayed significant cytotoxicity against human skin melanoma SK-MEL-5 and human colon adenocarcinoma Colo-205 cells.[8] Additionally, murrayaquinone A was documented as a potential antibacterial agent because of its pronounced inhibition against both of *Escherichia coli* and *Staphylococcus aureus*.[9] Murrayaquinone A also inhibited against Trichomonas (*Trichomonas gallinae*) parasites.[11] Methoxylated analogues, koeniginequinones A and B were found primarily in the stem bark of curry tree, *Murraya koenigii* Spreng.[13]

Earlier observations prompted us to take up the carbazoloquinone unit as an active pharmacophore for further diversification to exploit its medicinal potential. In continuation of our work on naturally occurring quinones and related synthetic analogues,[14,15] the present study focuses on the structure-activity relationships of

carbazoloquinone derivatives as anticancer agents. Furthermore, the underlying mechanisms associated with those effects were studied.

For construction of the carbazoloquinone core, the palladium-catalyzed intramolecular cyclization of arylamino-*p*-toluquinones is a common approach.[16] Initially, naturally occurring carbazoloquinones were prepared as follows: the addition of anilines into *p*-toluquinone in acidic conditions furnished the required 2-anilino-5-methyl-1,4-benzoquinones **1a**–**1c** in 16–58% yields (Scheme 1). When aniline (R^1=R^2=H) was used, 2-anilino-6-methyl-1,4-benzoquinone **2a** was formed as a byproduct in 30% yield. Its methyl position on quinone was determined by HMQC and HMBC techniques. It is thought that this reaction proceeds through hydroquinone intermediates,[17] which are immediately converted into the quinones (aerial oxidation).[18,19] Tandon VK and Maurya HK reported aqueous conditions accelerate this type addition.[20] Refluxing **1a**–**1c** with an equimolar amount of palladium(II) acetate ($Pd(OAc)_2$) in acetic acid allowed access to murrayaquinone A (**3a**, 49% yield), koeniginequinones A (**3b**, 50% yield) and B (**3c**, 42% yield). Through the same route, other derivatives bearing 6-methoxy (-OMe), 6-chloro (-Cl), and 6-trifluoromethyl ($-CF_3$) substituents on their benzene ring were synthesized. Detailed synthetic conditions and spectroscopic data of compounds are given in the Supplementary data.

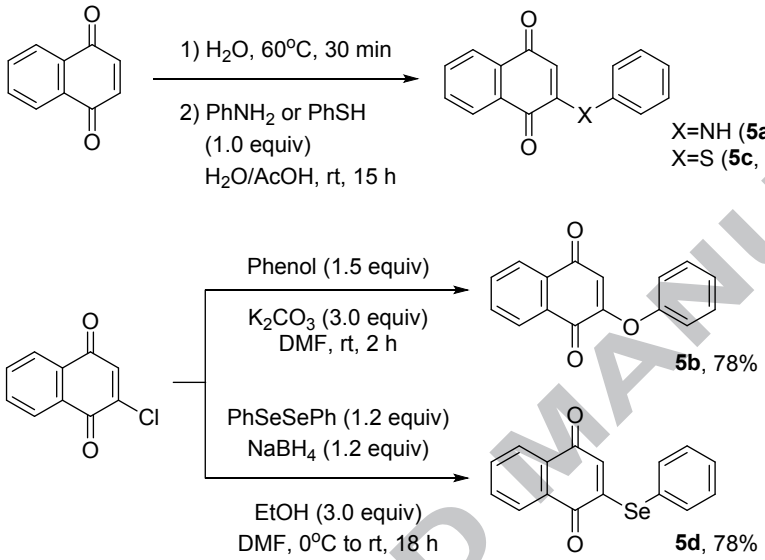


Scheme 1. Synthesis of carbazoloquinone derivatives.

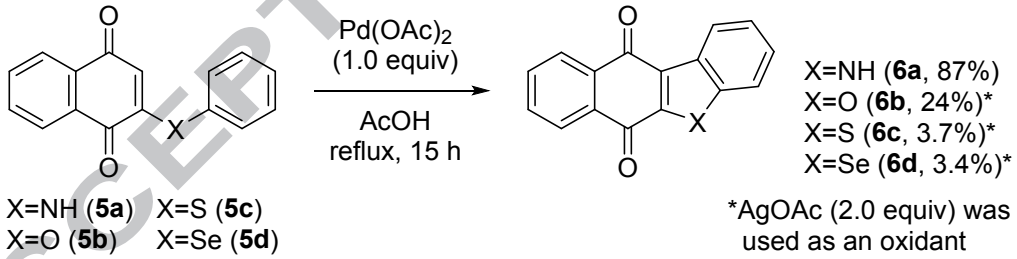
As a crucial step for the structure-activity relationships, we evaluated the synthetic carbazoloquinone derivatives (**3** and **4**) for *in vitro* cytotoxicity against human leukemia HL-60 cells using the CCK-8 assay method.[21] The cells were treated with increasing concentrations of each compound for 48 h, with DMSO as control. Even at a concentration of 10 μM, most derivatives exhibited high cytotoxicity with cell viability of less than 20% (% of control). Their IC_{50} values are as follows: **3a** (0.73 μM), **3b** (1.21 μM), **3c** (0.55 μM), **3d** (3.11 μM), **3e** (0.24 μM), **3f** (0.59 μM), **4a** (0.29 μM), and **4d** (5.06 μM), respectively. The cytotoxic effects of derivatives **3a**, **3c**, **3e**, **3f**, and **4a** are two orders of magnitude greater than that of 5-fluorouracil (5-FU: anticancer chemotherapy drug,[22] IC_{50}: 42.8 μM). There is not much difference between the compounds tested. In other words, a functional group on the benzene ring of carbazoloquinones has unexpectedly less impact on their cytotoxicity.

Next, we examined the effects of substructures located directly on the quinone core on their efficacy. A methyl group was replaced by a benzene ring. Conversions of the pyrrole to other five-membered heterocycles such as furan, thiophene, and selenophene were carried out. Although the addition of aniline and benzenethiol into *p*-benzoquinone gave the desired products **5a** and **5c** in 32% and 64% yields, the reactions with phenol and benzeneselenol did not work at all under the same conditions. As an alternative, 2-chloro-*p*-benzoquinone was employed for the two cases. The nucleophilic substitution of phenol to 2-chloro-*p*-benzoquinone in the presence of K_2CO_3 in DMF worked well, giving **5b** in 78% yield. Since selenol (R-SeH) easily condenses into the diselenide (R-Se-Se-R),[23] phenylselenolate anion (Ph-Se^-) was generated *in situ* from diphenyl diselenide (Ph-Se-Se-Ph) by hydride reduction using $NaBH_4$. The anion successfully attacked to 2-chloro-*p*-benzoquinone, affording **5d** in 78% yield (Scheme 2). The $Pd(OAc)_2$-catalyzed intramolecular

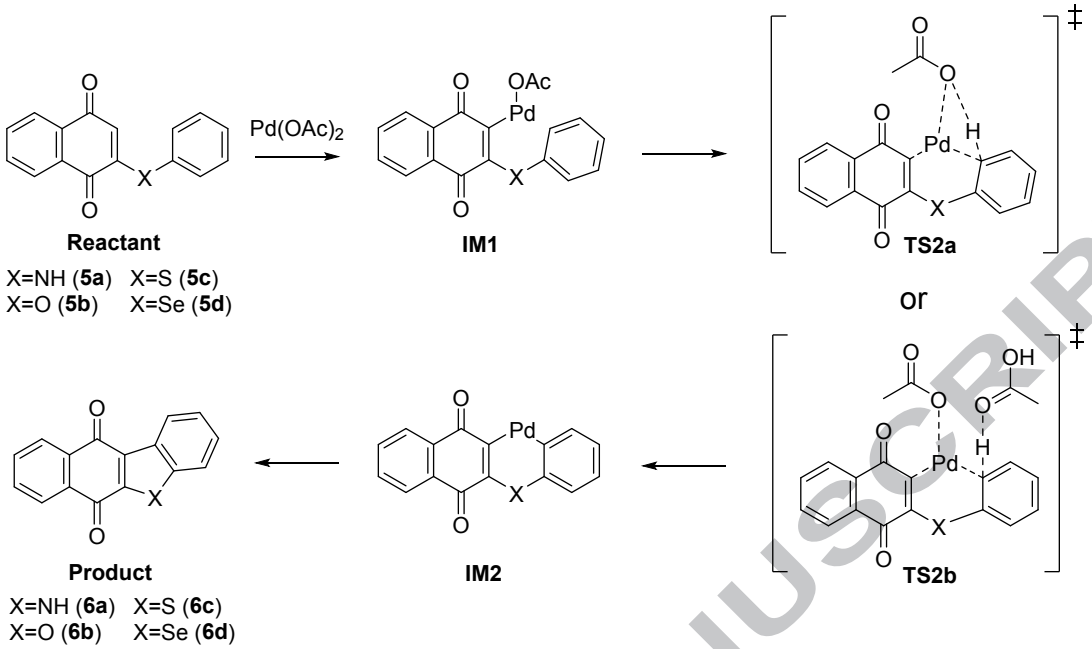
cyclization of **5a** succeeded in **6a** in 87% yield. Cyclized products **6b**–**6c** were obtained in yields of 24% for β-brazanquinone (benzo[*b*]naphtho[2,3-*d*]furan-6,11-dione) **6b**, 3.7% for **6c**, and 3.4% for **6d** when AgOAc was used as an oxidant.[24,25] It is worth mentioning that the yields were affected by the heteroatom (X atom) in five-membered rings (Scheme 3).



Scheme 2. Addition of aniline, phenol, benzenethiol, and benzeneselenol.



Scheme 3. $Pd(OAc)_2$-catalyzed intramolecular cyclization of **5**.



Scheme 4. Proposed mechanism for $Pd(OAc)_2$-catalyzed intramolecular cyclization of **5**.

To reveal the reaction mechanism of $Pd(OAc)_2$-catalyzed intramolecular cyclization of **5**, we performed DFT calculations (see, SI for computational detail). The proposed mechanism and relative Gibbs free energy profile for the reaction are shown in Scheme 4 and Fig. 1, respectively. This reaction is stepwise reaction and has three transition states corresponding to coordination of Pd(OAc) (**TS1**), ring closure process to form six-membered ring (**TS2**), and elimination of Pd atom (**TS3**). According to the previous study,[16] we calculated two kinds of reaction pathways for **TS2** (**TS2a** and **TS2b**). In **TS2b**, AcOH participates to abstract H atom from phenyl ring, whereas AcOH does not participate in **TS2a**. Our DFT calculation implies that the ring closure reaction proceed *without* AcOH, because the relative Gibbs free energy of **TS2a** is 5.4 ~ 8.8 kcal/mol lower than that of **TS2b**. Since the relative Gibbs free energy of **TS1** and **TS2a** are higher than **TS3**, the rate-determining step is coordination of Pd(OAc) or ring closure reaction to form six-membered ring.

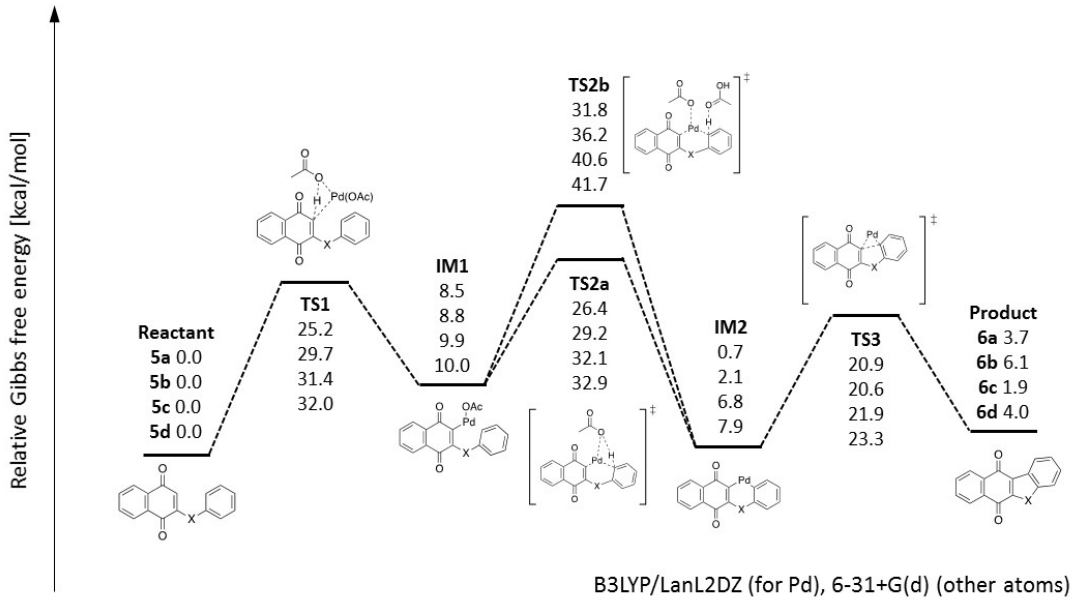


Fig. 1. Relative Gibbs free energy profile of $Pd(OAc)_2$-catalyzed intramolecular cyclization of **5**.

The relative energies of **TS1** and **TS2a** are the lowest in **5a**, whereas the highest values are found in **5d**. Our DFT calculation implies that the reaction of **5a** occurs more easily than **5b**–**5d**. This calculation result is in good agreement with the experimental yields of **6a**–**6d** (Scheme 3). To interpret the difference in reactivity among **5a**–**5d**, we focus on the atomic charge (natural charge) of carbon atom in quinone ring in **Reactant** and in phenyl ring in **IM1** (Fig. 2). These carbon atoms are negatively-charged and their negative charges become smaller in the order of **5a** > **5b** > **5c** > **5d**. The electron distribution around the carbon atom is, thus, clearly affected by X atom. Since positively-charged Pd atom attacks the carbon atom to proceed the reaction, it can be speculated that the negative charge on the carbon atom determines the reactivity of compound.

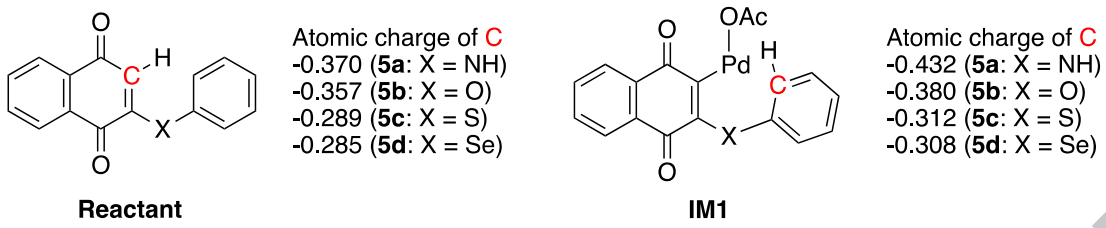


Fig. 2. Calculated atomic charge of C (red-colored) atom in **Reactant** and **IM1**.

To gain insights into the structure-activity relationships, the synthetic derivatives (**6**) were assessed their *in vitro* cytotoxicity on HL-60 cells. The furan derivative β-brazanquinone **6b** exhibited strong cytotoxicity with a low IC_{50} value of 0.41 μM. On the other hand, the IC_{50} values of pyrrole, thiophene, and selenophene derivatives **6a**, **6c**, and **6d** were over 50 μM. Comparing **6a** with murrayaquinone A (**3a**), the directly attached benzene ring on the quinone moiety decreased the potential. Very interestingly, the heteroatom in five-membered rings was most influential on cytotoxicity of these compounds.

Reactive oxygen species (ROS) including hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) are constantly generated in organisms.[26] Cells are particularly sensitive to ROS-resulted redox imbalance, thereby causing damages of biological macromolecules and genotoxicity.[27] Therefore, in this study, it was hypothesized that the potent carbazoloquinone derivatives exert inhibition of HL-60 cell proliferation *via* affecting cellular redox environment. Intracellular and extracellular ROS levels were measured following 3 h-treatment with murrayaquinone A (**3a**), **6a**, and β-brazanquinone (**6b**) by the nitroblue tetrazolium (NBT) reduction method.[28,29] Results are summarized in Fig. 3. When exposed to 1.0 μM of compounds **3a** and **6b**, intracellular ROS levels were increased approximately 2.3–2.4-fold compared to untreated controls, but the

increase in **6a**-treated cells was minimal. Comparatively high extracellular ROS levels were detected in **3a**- and **6b**-treated cell culture medium.

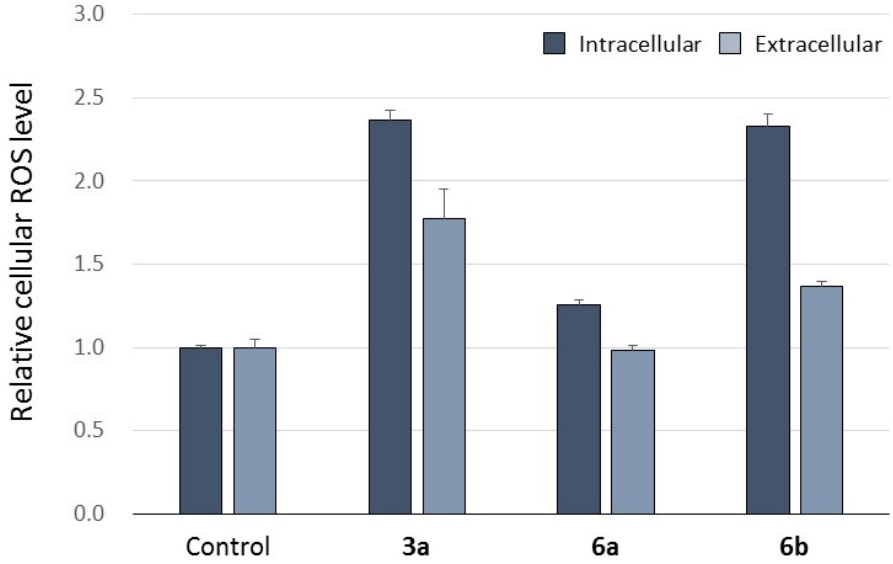


Fig. 3. Intracellular and extracellular ROS levels in compounds **3a**-, **6a**-, and **6b**-treated HL-60 cells. The cells were treated with 1.0 μM of **3a**, **6a**, and **6b** for 3 h. NBT reduction assay was performed to determine cellular ROS levels and the results were quantified relative to untreated controls (mean ± S.D., n = 3).

The nuclear morphology of HL-60 cells stained with Hoechst 33342 was observed. The cells in the rapid phase of growth were exposed to the derivatives **3a** and **6b** at a concentration of 1.0 μM for 24 h. The microscopic images are shown in Fig. 4. In the vehicle control group, the cells had uniform round- or oval-shaped nuclei with evenly distributed chromatin, while treatment with **3a** and **6b** resulted in alterations in chromatin features including crenation, condensation, and fragmentation, which are cues for the appearance of apoptosis.

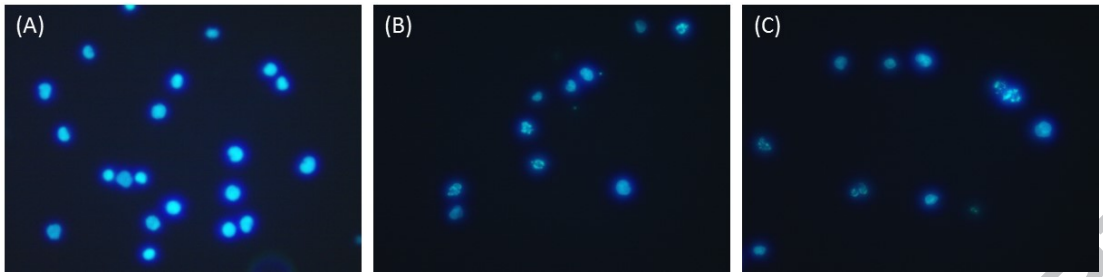


Fig. 4. Morphological changes of HL-60 cells induced by compounds **3a** and **6b** at a concentration of 1.0 μM. The cells were treated with **3a** and **6b** for 24 h, and were stained with Hoechst 33342. (A) Control; (B) Treatment with **3a**; (C) Treatment with **6b**.

In general, quinone molecules undergo redox cycling involved continuous generation of ROS through successive reduction and oxidation reactions.[30,31] There are several evidences showing that ROS act as critical mediators in cell apoptosis in the biological context.[32,33] A potent naturally occurring quinone β-lapachone isolated from *Tabebuia avellanedae* (*Bignoniaceae* family), displayed promising cytotoxic activity due to its ability to promote ROS-mediated apoptosis[34,35] and to disrupt redox homeostasis.[15] Aziridinylquinone RH-1 (2,5-diaziridinyl-3-hydroxymethyl-6-methyl-cyclohexa-2,5-diene-1,4-dione) induced apoptosis by the formation of ROS arising from its redox cycling.[36] It is believed that a drastic increase in cellular ROS accumulation and redox imbalance caused by **3a** and **6b** profoundly inhibited cell proliferation and induced apoptotic cell death. Mechanically, their quinone moieties can be reductively metabolized to the corresponding hydroquinones, which easily transfer electrons and protons to molecular oxygen under backformation of the original quinones.[37–39] It is presumed that ROS generated by their redox cycling contribute largely to cytotoxic actions of these carbazoloquinone derivatives.

In conclusion, the present study reports on the synthesis of carbazoloquinone derivatives and their inhibition on HL-60 cells *via* induction of ROS-mediated apoptosis. The promising antileukemic properties of murrayaquinone A (**3a**) and β-brazanquinone (**6b**) would be helpful in the synthesis of a library of quinone-based molecules for extensive anticancer studies, which would be used to develop more appropriate drug candidates. Further research continues in our laboratory and will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://XXXXXXXX.

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