Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 40 (2005) 1341–1345
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Short communication

The molecular mechanisms involved in the cytotoxicity of alkannin derivatives

Hai-Qiang Wu [a,b], Zhi-Shu Huang [b,*], Xian-Zhang Bu [b], Yu-Dong Shen [a,b], Zhu-Lin Zhang [b], Bing-Fen Xie [c], Zong-Chao Liu [c], Lian-Quan Gu [a,b,*], Albert S.C. Chan [d]

[a] *School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, China*
[b] *School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510080, China*
[c] *Cancer Center, Sun Yat-Sen University, Guangzhou 510060, China*
[d] *Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong*

Received 3 March 2005; revised and accepted 30 May 2005

Available online 29 June 2005

Abstract

In order to better understand the molecular aspects of the cytotoxic action mechanisms, the cytotoxicity of alkannin derivatives, **1**–**10**, on five human tumor cell lines were examined and their standard redox potentials in aprotic medium were tested by means of cyclic voltammetry. It was suggested that the oxidative potential is closely related to the cytotoxicity. The more negative the oxidative potential of the hydroquinones, the higher cytotoxicity of these derivatives. The results of the compounds **5**, **7**, **9** and **10** with bad leaving groups, have higher cytotoxic action is not agreed with the bioreductive alkylation mechanism of quinones. It indicates that the molecular mechanism involving cytotoxicity of alkannin derivatives may favor the mechanism of production of reactive oxygen.

Keywords: Alkannin derivatives; Cytotoxicity; Redox potential; Molecular mechanism

1. Introduction

As important naturally occurring pigments that are widely distributed in nature and play vital roles in the biochemistry of living cells and exert relevant biological activities, anthraquinones (AQ), naphthoquinones (NQ), and heteronaphthoquinones (HQ) exhibit cytostatic and antimicrobial activities. This may be due to their ability to act as potent inhibitors of electron transport [1], uncouplers of oxidative phosphorylation [2], intercalating agents in the DNA double helix [3], bioreductive alkylating agents of biomolecules [4], and producers of reactive oxygen radicals [5]. Their cellular toxicity has been ascribed to two basic mechanisms: one, the highly reactive "double-armed" or "single-armed" quinone methides, which are formed by losing an anionic leaving group originally bound β position (or other vinylogous positions) after bioreduction, adduct to cellular constituents, probably mainly thiol-containing proteins or other biomoleculars, resulting in their inactivation [6–10]. Moore and Scheuer [11], Ahn et al. [12] suggested that alkannin or shikonin and related derivatives could function in this manner (Fig. 1), and the redox cycling plays a prominent role on their cytotoxicity.

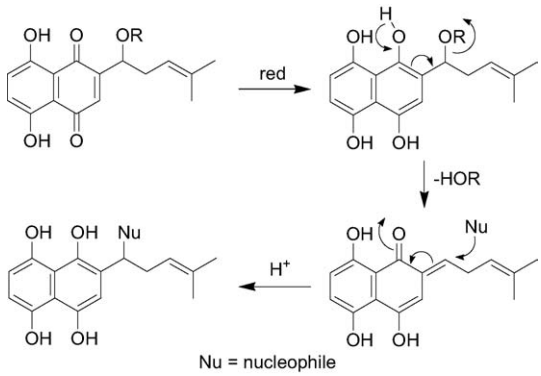


Fig. 1. A proposal bioreductive alkylation mechanism of shikonin (alkannin).

* Corresponding authors. Tel.: +86 20 8411 5536; fax: +86 20 8411 0272.
E-mail addresses: huangzhishu@hotmail.com (Z.-S. Huang), cedc42@zsu.edu.cn (L.-Q. Gu).

0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.05.004

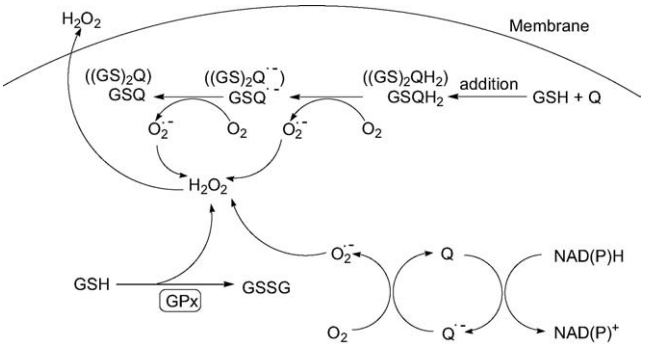


Fig. 2. Mechanism involving production of reactive oxygen. GS–QH_2 or $(GS)_2QH_2$ = mono- or diglutathione conjugated hydroquinone; GPx = glutathione peroxidase.

Many researchers put forward another mechanism involving production of reactive oxygen (Fig. 2) [13–17]. The intracellular reactive oxygen (such as superoxide anion and H_2O_2) production by quinones is thought to be a consequence of O_2 reduction by semiquinone and hydroquinone intermediates. The semiquinones are formed via one-electron reduction of the corresponding quinones catalyzed by, e.g. NADPH-cytochrome P_{450} reductase, and the hydroquinones maybe formed via one-electron continued reduction of semiquinones or two-electron reduction of quinones by DT-diaphorases. This process, called redox cycling, generates $O_2\bullet^-$ and H_2O_2. Quinones can also be converted to hydroquinone form via a nucleophilic addition of glutathione (GSH) leading to GSH–quinone adducts, and then the oxidation of the GSH–quinone adducts might couple with reduction of oxygen to generate reactive oxygen. The production of reactive oxygen may thereby cause oxidative stress which lead to apoptosis.

β,β-Dimethylacrylalkannin **1** was isolated from the root of *Arnebia euchroma*, a well-known Chinese herb used as drugs for many years. In the previous report [18], we have studied the oxidation-reductive alkylation and oxidation-conjugated addition of **1** with a variety of nucleophiles, and most of the reaction products, which are formed by autooxidation of nucleophile–quinone adducts, showed higher cytotoxicity than the original material. In order to better understand the molecular aspects of the mechanisms of cytotoxicity and the SAR of these compounds, in this work, the cytotoxicity of 10 alkannin derivatives, which included in the natural product **1** and semi-synthetic compounds **2**–**10** derived from **1** (Fig. 3), were examined on five human tumor cell lines. And a molecular mechanism involved in cytotoxicity of these compounds is presented, based on the analysis of the relationship of their redox potentials and cytotoxicity.

2. Results and discussion

2.1. Cytotoxic activity of naphthazarin derivatives

The concentration of alkannin derivatives for 50% inhibition (IC_{50}) on the human nasopharyngeal carcinoma cell line (CNE_2), human lung adenocarcinoma cell line (GLC-82), human breast carcinoma cell line (MD453), human cervices cell line (HeLa) and human nonnasality epithelium carcinoma cell line (KB) were determined using MTT method and the results were summarized in Table 1. These results clearly showed that **5**, **7**, **9** and **10** are the most cytotoxic compounds for all five cell lines compared with **1**, and the cytotoxicity of the same one compound may be significant different for dif-

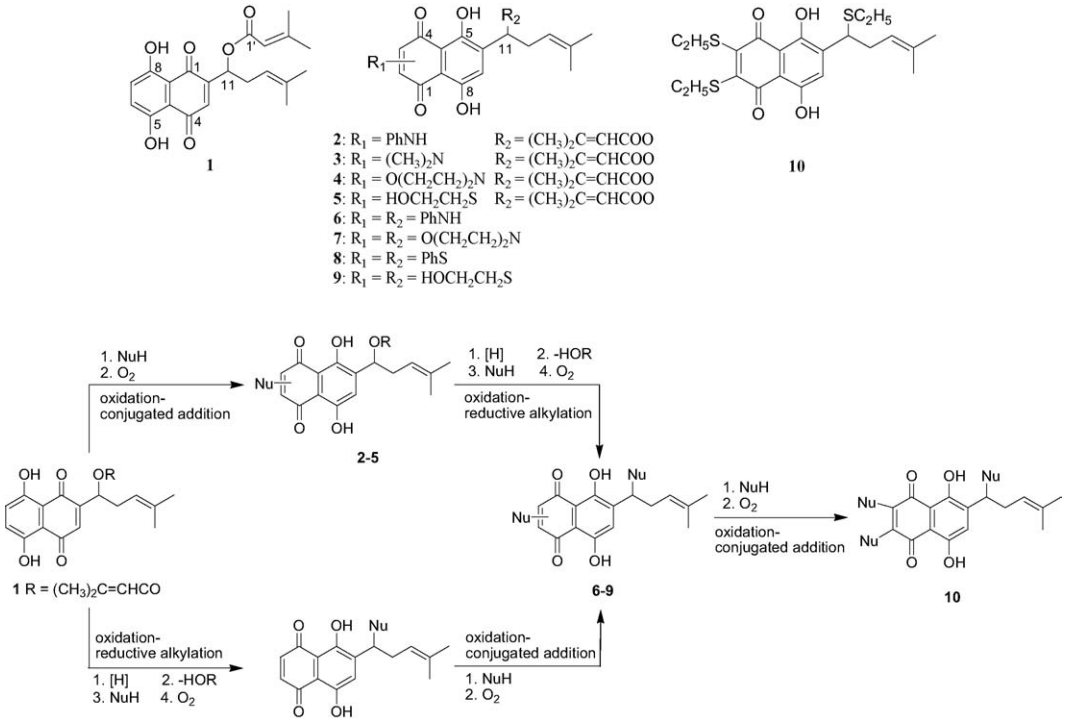


Fig. 3. Structures of alkannin derivatives **1**–**10** and semi-synthesis of compounds **2**–**10** [18].

Table 1
IC_{50} values on the human cancer cell lines and *E*p values of **1–10**

Compounds	The human cancer cell lines and IC_{50} (μM)					*E*p (V)			
	CNE_2	GLC-82	MD453	HeLa	KB	–*E*pc1	–*E*pc2	–*E*pa2	–*E*pa1
1	–	–	–	–	–	0.357	1.181	1.353	0.656
2	–	–	–	–	–	0.606	1.225	1.412	0.560
3	–	13.1	–	–	–	0.598	1.212	1.367	0.521
4	17.9	5.4	6.41	13.1	6.5	0.554	1.173	1.393	0.586
5	8.2	2.2	2.6	5.9	10.4	0.645	1.103	**1.625**	0.754
6	–	–	23.6	–	18.4	0.632	1.173	1.496	0.613
7	4.3	2.2	1.3	3.8	3.3	0.454	1.079	**1.645**	0.618
8	2.4	2.4	16.0	17.4	3.3	0.638	1.181	1.612	0.534
9	5.0	1.7	0.71	2.7	4.2	0.632	1.173	**1.634**	0.625
10	8.8	5.4	3.0	10.0	7.6	0.883	1.593	**1.652**	1.038
HCPT*	1.0	0.02	1.3	1.2	0.08	–	–	–	—

–: No significant cytotoxicity and the IC_{50} value is higher than 25 μM; *: positive control.

ferent cell lines, such as **4** and **8**. At the same time, no obvious cytotoxicity was found in **1**, **2**, **3**, and **6**.

2.2. Cyclic voltammetry (CV) of *1–10*

CV scans were carried out in DMF for **1–10** and the CV voltammograms were shown in Fig. 4. The redox potential (*E*pc1, *E*pc2; *E*pa2, *E*pa1) of these substances are list in Table 1. Generally, *E*pc1, *E*pc2 could represent the ability of substance (quinone) be reduced to semiquinone and hydroquinone, respectively, while the *E*pa2, *E*pa1 represent the reductive ability of hydroquinone and semiquinone, respectively, under electrochemistry condition.

2.3. Discussion

2.3.1. Bioreduction alkylation is not responsible for cytotoxicity of the studied compounds

In the bioreduction alkylation pathway of cell killing by benzoquinone and NQ, there are two key elements that could influence the degree of tumor-inhibitory activity. One is the leaving ability of the leaving group at C-2 or C-2 and C-3 positions of 1,4-quinone ring, respectively. Substances with the best leaving group, give the highest values of *T*/*C* (the ratio of survival time of treated to control animals) [6–10,19–25]. Another is the ability of substance being reduced to intermediate of semiquinone or hydroquinone. Although the role of redox potentials in the biological activity is still inconclusive, they were measured in several cases and it is reasonable that the easer the substance be reduced, the more stable the intermediate formed.

Unexpectedly, the studied substance in this work show no definite relation between *E*pc1 (or *E*pc2) and cytotoxicity on tested five human tumor cell lines, substance with both low or high *E*pc1 (or *E*pc2) could exhibit higher inhibitory activities or lower activities (Table 1). In addition to, comparing the structure and activity of **2**, **4**, **5** with that of **6**, **7**, **9**, respectively, the R_2 ($(CH_3)_2C$=CHCOO–) of the former should be better leaving group than in **6**, **7**, **9**, however, the experiment results ($IC_{50}^{6} < IC_{50}^{2}$, $IC_{50}^{7} < IC_{50}^{4}$, $IC_{50}^{9} < IC_{50}^{5}$) indicate that better leaving groups did not lead to a better cytotoxicity.

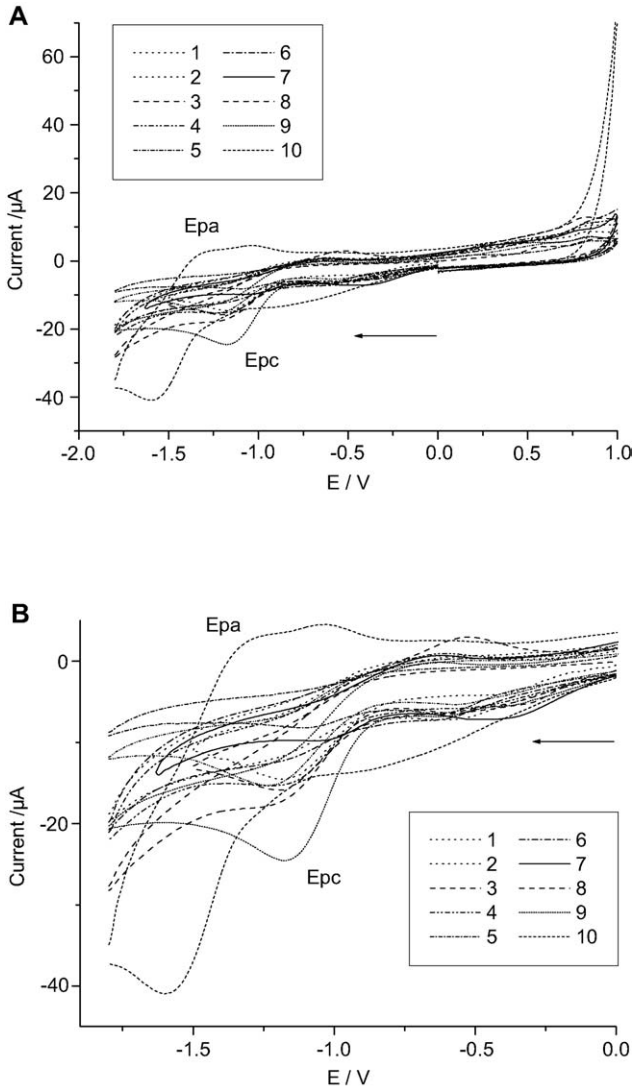


Fig. 4. Cyclic voltammograms of **1–10**.
In DMF + 0.1 M TBAP, Pt, $HglHgCl_2|Cl^-$ (0.1 M), c**1** = 5 mM, $v = 0.100$ V s^{-1}.

These results show that the leaving group, redox potentials and activities seems irrespective and can not agree with the bioreductive alkylating mechanism on cell killing. It is sug-

gested that the bioreduction alkylation (Fig. 1) is not responsible for the cell killing by these compounds.

2.3.2. The studied substances behave under reactive oxygen production mechanism

Interestingly, in this work, it is found that the oxidative potential of hydroquinones (*E*pa2, Table 1) is related the cytotoxicity of these compounds. For **5**, **7**, **9** and **10** whose cytotoxicities are higher, their *E*pa2 are more negative than others (*E*pa2 = –1.625, –1.645, –1.634, and –1.652 V, respectively). The more negative the *E*pa2, the higher the cytotoxicity. This result indicates our derivatives behave under the mechanism of production of reactive oxygen on the studied cell lines.

From the structure (substitutes) of view, substitutes with better electro-donating ability could enhance the electron density of the whole molecule, leading to more reductive ability of hydroquinone, it should be more active on the cell killing. It is consists with our data that the substance (**5**, **7**, **9**, **10**) with best activity exhibits better electro-donating. These compounds, which alkyl amine or alkyl thio was attached to the conjugated π-system or C-11 position, can cause a more negative *E*pa2 of relative hydroquinone form (Scheme 1).

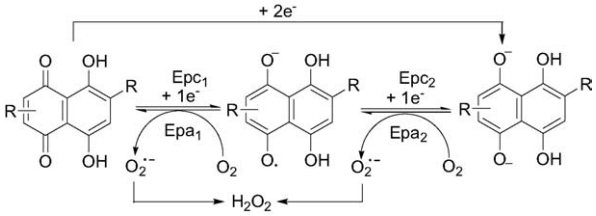
It is noticeable that the *E*pa1 of **1**–**10** are not so accordant with their cytotoxicity, suggesting the highest reductive state of these substance, hydroquinone plays a more important role in the cell-killing pathway than the semiquinone does.

3. Conclusion

As a continuation of our studies using natural and synthetic products as cancer chemopreventive agents, the cytotoxicity of 10 alkannin derivatives (**1**–**10**) on five human tumor cell lines were examined here and their standard redox potentials in aprotic medium were also determined by means of CV, in order to better understand the molecular mechanisms involved in cytotoxic action of compounds.

These results indicated that the reactive oxygen production mechanism should be responsible for the cell cytotoxic action of these compounds. Since our studied compounds are the products of oxidation-reductive alkylation and oxidation-conjugated addition of **1** with a variety of nucleophiles, the production of reactive oxygen by GSH–quinone adducts pathway seems to be impossible (Fig. 2).

The possible molecular mechanism cytotoxicity of alkannin derivatives involved in maybe: first, they undergo two-electron reduction or two steps of one-electron reduction to yield the corresponding hydroquinone, then the hydroquinone participates in redox cycling to generate reactive oxygen species, superoxide anion and H_2O_2, and these reactive oxygen species are poisonous for cells. The stronger the electron-pushing effect of substitute, the more unstable of the hydroquinone, the more negative of *E*pa of them and the higher cytotoxiciry of compounds.



Scheme 1.

4. Experimental

4.1. Materials

β,β-Dimethylacrylalkannin **1** is isolated from the dry roots of *A. euchroma*. 2- or 3-phenylamino-6-(11-β,β-dimethylacryl)isohexenylnaphthazarin **2**, 2- or 3-dimethylamino-6-(11-β, β-dimethylacryl)isohexenylnaphthazarin **3**, 2- or 3-morpholino-6-(11-β, β-dimethylacryl)iso- hexenylnaphthazarin **4**, 2- or 3-(2-hydroxyethanethio)-6-(11-β,β-dimethylacryl) isohexenyl-naphthazarin **5**, 2, 11- or 3, 11-bis(phenylamino)-6-isohexenylnaphthazarin **6**, 2, 11- or 3, 11-bis(morpholino)-6-isohexenylnaphthazarin **7**, 2, 11-, or 3, 11-bis(phenylthio)-6-isohexenyl- naphthazarin **8**, 2, 11- or 3, 11-bis- (2-hydroxyethanethio)-6-isohexenylnaphthazarin **9** and 2, 3, 11-triethanethio-6-isohexenylnaphthazarin **10** were synthesized using compound **1** as starting material by ourselves [18]. Above derivatives all have two enantiomers (*R* and *S*, at C-11) but all the enantiomers did not separated since there is not significant difference in terms of therapeutical activities between the two enantiomers [26].

4.2. Cytotoxic activity assay

The five cell lines used for the tests were human nasopharyngeal carcinoma cell line CNE_2, human lung adenocarcinoma cell line GLC-82, human breast carcinoma cell line MD453, human cervices carcinoma cell line HeLa and human nonnasality epithelium carcinoma cell line KB, which were kindly provided by E. Gong-Kan Feng (Cancer Center, Sun Yat-Sen University, Guangzhou, China). They were, respectively, incubated at 37°C in an incubator with 5% of CO_2. The media used was RPMI-1640 with 10% newborn calf serum (product of New Zealand). All other regents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification.

The cancer cells in exponential phase were placed in a 96-well plate, containing $1–2 \times 10^4$ per well. After incubating for 4 h the sample solutions were added to each well, respectively, making final concentrations of 0.1–25 μg ml^{-1}. The cells were continually incubated for 68 h. The groups treated by RPMI-1640 were used as control groups. To each well was added 10 μl (5 mg ml^{-1}) of MTT solution, and the incubation was continued for 4 h (total time 72 h). The formazane crystals thus formed were dissolved by 100 μl DMSO. After 15 min, the absorbance was detected in the microplate reader Bio-red 550 model at 570 nm wavelength. The IC_{50}

values were determined using a Litchfield and Wilcoxon I: confidence limits of ED_{50} pharmacological calculation system.

4.3. Electrochemical methods

DMF (Merck, Cuasol Grade) was treated with anhydrous cupric sulfate, filtered and distilled at reduced pressure through a glass Vigreux column (12 cm). Tetra-*n*-butylammonium perchlorate (TBAP) was prepared from the corresponding bromide (Aldrich) and perchloric acid (Aldrich). The resulting salt was washed with cold water until neutral pH, then methanol, recrystallized from ethyl acetate and thoroughly dried before use under high vacuum, at 75°C, for 2 days. Test solutions of the compounds ($c = 0.05$ mM) were prepared just before electrochemical experiments and the dissolved oxygen eliminated by bubbling the solution with dry Argon gas for 15 min. During the experiments the cell was kept in a dark Faraday cage to minimize photoreactions and external electric fields all the times. Other reagents were all commercial and used without further purification (AR).

CV was performed using a PAR model 273A/PAR EG&G potentiostat–galvanostat equipped with an HP 7090A measuring plotter system. A 586 IBM PC controlled the whole system and accompanying software package was used. A line of platinum wire was used as working electrode (area 0.6 mm^2), together with a platinum slice auxiliary electrode and an saturated Hg|$HgCl_2$ reference electrode carrying an anhydrous brine-bridge in one electrochemical cell. All the electrolysis was performed at room temperature and the scan rate was in the range 0.010–0.200 V s^{-1}.

8 (0.061 mg, 0.125 mmol), was dissolved in 25 ml of anhydrous DMF supplemented with 0.1 M TBAP as a supporting electrolyte. CV scans were carried out at 0.010–0.200 V s^{-1} after purging the reaction mixture with dry Argon gas for 15 min. The half-wave potential ($E_{1/2}$) was calculated as the midpoint of the anode and cathode potentials.

CV scans for other compounds were also carried out under the same conditions described above. The concentrations were fixed at 5 mM.

Acknowledgements

We are indebted to the National Nature Science Foundation of China (20272085, 30271601), the Guangdong Provincial Science Foundation (021770, 031594), and the Hong Kong Polytechnic University Area of Strategic Development Fund for financial support of this study.

References

[1] J.L. Vennerstrom, J.W. Eaton, J. Med. Chem. 31 (1988) 1269–1277.
[2] J.L. Howland, Biochim. Biophys. Acta 77 (8) (1963) 419–429.
[3] W.D. Wilson, R.L. Jones, in: Advances in Pharmacology and Chemotherapy, vol. 18, Academic Press, New York, 1981, pp. 177.
[4] H.W. Moore, J.O. Karlsson, in: Recent Advances in Phytochemistry, vol. 20, Plenum, New York, 1986, pp. 263.
[5] T.J. Monks, P. Hanzlik, G.M. Cohen, D. Ross, D.G. Graham, Toxicol. Appl. Pharmacol. 112 (1992) 2–16.
[6] T.-S. Lin, I. Antonini, L.A. Cosby, A.C. Sartorelli, J. Med. Chem. 27 (1984) 813–815.
[7] A.J. Lin, A.C. Sartorelli, J. Med. Chem. 19 (1976) 1336–1338.
[8] D.T. Witiak, P.L. Kamat, D.L. Allison, S.M. Liebowitz, R. Glaser, J.E. Holliday, M.L. Moeschberger, J.P. Schaller, J. Med. Chem. 26 (1983) 1679–1686.
[9] B.A. Teicher, A.C. Sartorelli, J. Med. Chem. 23 (1980) 955–960.
[10] A.J. Lin, A.C. Sartorelli, J. Org. Chem. 38 (1973) 813–815.
[11] R.E. Moore, P.J. Scheuer, J. Org. Chem. 31 (1966) 3272–3283.
[12] B.-Z. Ahn, G.-Y. Seng, K.-U. Baik, D.-E. Sok, Korean J. Med. Chem. 6 (1996) 98–100.
[13] K. Ollinger, J. Llopis, E. Cadenas, Arch. Biochem. Biophys. 275 (1989) 514–530.
[14] G.D. Buffinton, K. Ollinger, A. Brunmark, R. Cadenas, Biochem. J. 257 (1989) 561–571.
[15] P.R. Rich, D.S. Bendall, Biochim. Biophys. Acta 592 (1980) 506–518.
[16] D. Meisel, Chem. Phys. Lett. 34 (1975) 263–266.
[17] P.J. Wardman, Phys. Chem. Ref. Data 18 (1989) 1637–1755.
[18] Z.-S. Huang, H.-Q. Wu, Z.-F. Duan, B.-F. Xie, Z.-C. Liu, G.-K. Feng, L.-Q. Gu, A.S.C. Chan, Y.-M. Li, Eur. J. Med. Chem. 39 (2004) 755–764.
[19] A.J. Lin, R.S. Pardini, L.A. Cosby, B.J. Lillis, C.W. Shansky, A.C. Sartorelli, J. Med. Chem. 16 (11) (1973) 1268–1271.
[20] G. Prakash, E.M. Hodnett, J. Med. Chem. 21 (4) (1978) 369–374.
[21] F.C. Abreu, A.O. Lopes, M.A. Pereira, C.A. Imone, M.O.F. Goulart, Tetra. Lett. 43 (2002) 8153–8157.
[22] K.E. O'Shea, M.A. Fox, J. Am. Chem. Soc. 113 (2) (1991) 611–615.
[23] I. Antonini, T.S. Lin, L.A. Cosby, Y.R. Dai, A.C. Sartorelli, J. Med. Chem. 25 (6) (1982) 730–735.
[24] T.S. Lin, I. Antonini, L.A. Cosby, A.C. Sartorelli, J. Med. Chem. 27 (6) (1984) 813–815.
[25] I. Wilson, P. Wardman, T.S. Lin, A.C. Sartorelli, J. Med. Chem. 29 (8) (1986) 1381–1384.
[26] S. Tanaka, M. Tajima, M. Tsukada, M. Tabata, J. Nat. Prod. 49 (1986) 466–469.