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Synthesis and cytotoxic evaluation of two novel anthraquinone derivatives

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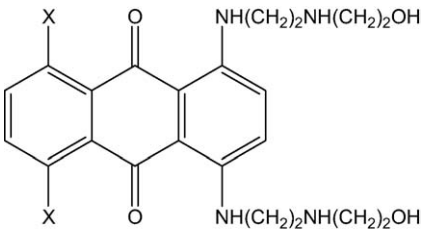
Abstract

The antitumor activity of dihydroxyanthracenediones such as mitoxantrone on a panel of cancer cell lines during the last 30 years, led investigators to synthesize thousands of anthracycline analogs and test their cytotoxicity to identify compounds superior to the parent drugs in terms of increased therapeutic effectiveness, reduced toxicity or both. To achieve this, new synthesized congeners either have different side arms or have extra rings on their skeletons. Following these studies, we proposed total synthesis of 2-amino-*N*-[4-(2-amino-3-hydroxy-propionylamino)-9,10-dioxo-9,10-dihydroanthracene-1-yl]-3-hydroxy-propionamide (V) and 6-amino-hexanoic acid [4-(5-amino-pentanoylamino)-9,10-dioxo-9,10-dihydro-anthracen-1-yl]-amide (VI). Acetylation of 1,4-diaminobenzene using acetyl chloride and reaction with phthalic anhydride under a Friedel–Crafts reaction and then cyclization gave 1, 4-diamino-anthraquinone. This compound was reacted with two amino acids (L-serine and 6-amino hexanoic acid) in their ester forms, using ethyl chloroformate as a coupling agent. Hydrolyzing esterified compounds gave their amino substituted derivatives. These compounds with diamine side arms are supposed to provide better intercalation with DNA. Synthesized novel ametantrone derivatives were tested against a panel of cancer cells (KB, Hela, MDA-MB-468 and K562), using MTT assay. The results showed that tested compounds inhibited the growth of cancer cells at micromolar concentrations. However, compound (VI) was more cytotoxic than compound (V) probably because of its longer side chains and better intercalation with DNA.

Keywords: Anthraquinone synthesis; Ametantrone derivatives; Cytotoxicity; Cancer cells

1. Introduction

Anthraquinones have been used as useful compounds in treating all kinds of cancers especially breast cancer tumors for a long time. The discovery of the antitumor activity of 1,4-bis[(aminoalkyl)amino]anthracene-9,10-diones such as ametantrone (a) and mitoxantrone (b) has led to numerous synthetic and pharmacological studies on the tumoricidal mechanisms of these anthraquinone congeners [1]. Mitoxantrone is used clinically either alone or in combination with other chemotherapeutic agents to treat a variety of human cancers, particularly lung carcinoma, leukemia, melanoma and lymphoma, Hodgkin's disease and breast cancer [2]. Mitoxantrone inhibits RNA and DNA synthesis and intercalates with DNA, although some researchers showed that non-intercalative mechanisms were involved in its cytotoxicity against L1210 leukemia cells [3]. In the case of breast cancer, Katsumata et al. [4] in their studies concluded that combination treatment with mitoxantrone hydrochloride, vincristine sulfate and prednisolone is an effective treatment for breast cancer.



Ametantrone (a, X= H)
Mitoxantrone (b, X= OH)

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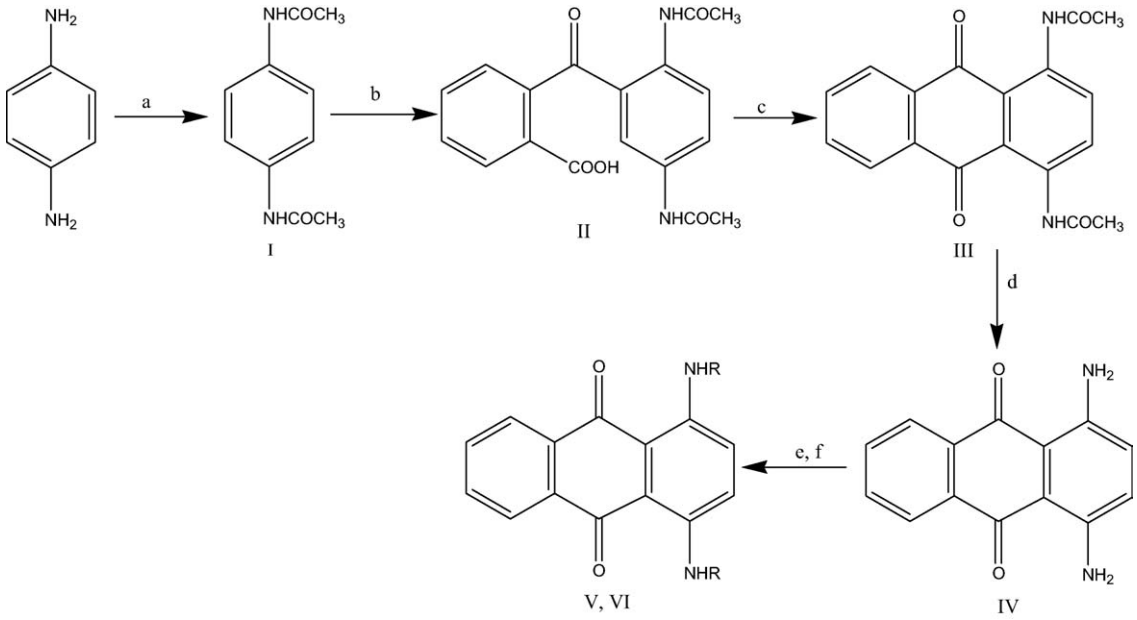

Scheme 1. Total synthesis of ametantrone derivatives; a) CH_3COCl, CH_3COOH, CH_3COONa, room temperature, 4 h; b) phthalic anhydride, H_3BO_3, H_2SO_4; c) H_3BO_3, H_2SO_4;d) NaOH (15%), reflux 3 h; e) $C_2H_5OCOCH(NH_2)CH_2OH$ to give "V" in which R=$CONH(NH_2)CH_2OH$; f) $C_2H_5OCO(CH_2)_5NH_2$ to give "VI" in which R=$CO(CH_2)_5NH_2$; for other reaction condition see methods and materials.

Mitoxantrone and its derivatives are loosely related to the anthracyclines such as doxorubicin and daunorubicin which bind strongly to DNA and present their antitumor activity by this mechanism. Unlike anthracyclines, which display a dose-dependent cardiotoxicity, dihydroxyanthraquinones (DHAQ) show a very low incidence of cardiac failure when used clinically [2]. Low incidence of cardiotoxicity of DHAQ derivatives led researchers to conduct more studies on the structure activity relationship (SAR) of these congeners. Introduction of an extra ring (D) into this system was designed to introduce a non-polar moiety into the molecule and thus provide a substituted benz(a)anthracene ring system. This compound displayed anticancer activity against both murine and human tumor cell lines [2]. All these new analogs have been synthesized and tested to identify compounds superior to the parent drugs in terms of increased therapeutic effectiveness, reduced toxicity or both [5]. Morreal et al. in their studies used phthalic anhydride and 6-methoxy-1,2,3,4-tetrahydronaphthalene in a reaction to synthesize 8,11-bis[[2-[(2-hydroxyethyl)amino] ethyl]amino]-1,2,3,4-tetrahydro-6-methoxy-7,12-bena[a] anthraquinone in five steps [2]. On the other hand, Krapcho et al. [1] synthesized and evaluated symmetrical and unsymmetrical substituted 1,4-bis[(aminoalkyl)amino]-anthracene-9,10-diones and 1,4-bis[(aminoalkyl)amino]-5,8-dihydroxyanthracene-9,10-diones starting from 1,4-difluoroanthracene-9,10-dione and 1,4-difluoro-5,8-dihydroxyanthracene-9,10-dione to substitute fluoride groups by diamines at room temperature in pyridine leading to the desired compounds. Biological evaluation of synthesized compounds by this group showed that some of congeners were active against cancer cells in a concentration of <24 ng/ml which is somehow similar to doxorubicin [1]. Following our previous studies [6], here we decided to synthesize some novel anthraquinone derivatives related to ametantrone with a different synthetic strategy. In this study, benzene-1,4-diamine and phthalic anhydride were used to synthesize the novel compounds as shown in Scheme 1.

2. Experimental

2.1. Reagent and instruments

All chemicals were obtained from Aldrich Chemical Company Ltd. (Dorset) except for doxorubicin which was obtained from (David Bull Labs.), and all solvents were purchased from Fisons (Loughborough, UK). Reagents were used as purchased and solvents were dried using type $4\text{-}A^0$ molecular sieves. Proof of chemical purity was established by spectral (infrared IR, elemental analysis and MS) and analytical (TLC and chemical analysis) techniques. Polyester coated by silica gel GF_{254} as TLC plates (Aldrich, Dorset) were pre-washed with methanol/dichloromethane (50:50) and activated in an oven at 110 °C for 1 h before use and visualized by UV light at 254 nm developed by 50% conc. H_2SO_4 in methanol or by iodine vapor. Melting points (m.p.) were determined on an electrothermal apparatus, IA9000 series, (Essex, UK) and are uncorrected. IR spectra were obtained in a 1% KBr disc using a Perkin Elmer 297 IR spectrophotometer. Mass spectra were recorded on an AEI (Kratos) MS902 updated with an MSS Console and data system, Acc V 8 kV, 70 EV, 300 μA^0 emission; fast atom bombardment (FAB) spectra were obtained using a Kratos MS50 spectrometer. Elemental analysis for C, H and N were obtained using a Carlo Erba 1106 Elemental Analyzer and weighed using a Mettler MT 5 microbalance.

2.2. Chemistry

2.2.1. Synthesis of N-(4-acetylamino-phenyl)-acetamide (I)

Acetyl chloride (7 ml, 98.1 mmol) was added to a solution of benzene-1,4-diamine (5 g, 46.3 mmol) and anhydrous sodium acetate (3 g, 36.6 mmol) in acetic acid (50 ml), dropwise and the resulting mixture was stirred for 4 h at room temperature. The reaction mixture was poured slowly into a mixture of ice/water (200 ml) and kept at 4 °C overnight. Then the precipitated product was filtered and recrystallized from methanol to give the title compound (6.6 g, 74% yield) as pink-brown flaky crystals; m.p. 303–304 °C; R_f 0.78 (chloroform/ethanol, 1:2). IR (KBr)/cm^{-1}, 3200 (NH), 1700 (C=O). Elemental analysis: ($C_{10}H_{12}N_2O_2$) C, H, N.

2.2.2. Synthesis of 2-(2,5-bis-acetylamino-benzoyl)-benzoic acid (II)

Compound I (2.0 g, 10.4 mmol) was added to a mixture of boric acid (H_3BO_3) (2 g, 32.3 mmol) and phthalic anhydride (2 g, 13.5 mmol) in H_2SO_4 (7 ml, 98% purity). The reaction mixture was stirred at 130–140 °C in an oil bath for almost 2 h. The reaction was stopped by adding cold water (0 °C, 20 ml) and precipitated was filtered and recrystallized from ethanol to give the title compound (1.44 g, 41% yield) as yellowish flaky crystals. M.p. 220 °C, IR (KBr)/cm^{-1}, 3400–2500 carboxylic groups (COOH), 3200 (NH), 1700 (C=O), *m/z*, 295 (M–COOH, 5%), 282 (M–NH–CO–CH_3, 20%), 218 (M^+-C_6H_5–COOH, 38%), 191 [M^+-C_6H_5 (CO)–COOH, 100%] 177 [M-C_6H_4 (C=O)–COOH–CH_3, 50%], 135 (M-C_6H_5–NHCOCH$_3$, 40%), 105 [C_6H_5 (C=O), 15%]. Elemental analysis: ($C_{18}H_{16}N_2O_5$) C, H, N.

2.2.3. Synthesis of N-(4-acetylamino-9,10-dioxo-9,10-dihydro-anthracene-1-yl)-acetamide (III)

Compound II (4 g, 11.7 mmol) and H_3BO_3 (3.71 g, 60 mmol) were dissolved in H_2SO_4 (98%, 10 ml) and stirred at a temperature of 130–140 °C for 20 min. The reaction was stopped by slowly pouring into a mixture of ice/water (200 ml). The precipitate was then filtered and crystallized from methanol to give pure title compound (2 g, 54% yield) as white crystals. M.p. 200 °C, IR (KBr)/cm^{-1}; 3200 (–NH), 1700, 1650 (C=O), *m/z*, 322 (M^+, 5%), 238 [MH_2^+-(–CO–CH_3)$_2$, 20%], 148 ($C_8H_4O_3$, 30%), 105 (C_7H_5O, 100%). Elemental analysis: ($C_{18}H_{14}N_2O_4$), C, H, N.

2.2.4. Synthesis of 1,4-diamino-anthraquinone (IV)

Compound III (1 g, 3.1 mmol) was mixed with sodium hydroxide (15%, 50 ml) and refluxed for 3 h. The product was extracted with a mixture of isopropyl alcohol/chloroform (2:1), (3 × 30 ml). Solvents were then evaporated in vacuum and the crude solid was heated in $CHCl_3$, filtered and pentane was added to the filtrate. On cooling, the title compound (0.32 g, 43% yield) was obtained as brown crystals. M.p. 268–270 °C. IR (KBr)/cm^{-1}: 3200–3400 (NH_2), 1650 (C=O), *m/z*: 238 (M^+, 5%), 224 (M–NH_2, 10%), 179 ($C_8H_7N_2O_3$, 36%), 162 ($C_8H_6N_2O_2$, 100%), 93 (C_6H_7N, 90%). Elemental analysis: ($C_8H_6N_2O_2$), C, H, N.

2.2.5. Synthesis of 2-amino-3-hydroxy-propionic acid ethyl ester (HCl)

L-Serine (1 g, 9.5 mmol) was dissolved in ethanol (10 ml) under dry HCl gas and stirred for 30 min. Extra ethanol was then removed in vacuo and the residual thick syrup triturated with diethyl ether to give title compound (1.25 g, 98% yield); m.p. 130 °C (130–131 °C) [7]. IR (KBr)/cm^{-1}: 3400–3200 (OH, NH_2), 1650 (C=O). Elemental analysis: ($C_5H_{11}NO_5$), C, H, N.

2.2.6. Synthesis of 2-amino-N-[4-(2-amino-3-hydroxy-propionylamino)-9,10-dioxo-9,10-dihydroanthracene-1-yl]-3-hydroxy-propionamide (V)

Compound IV (0.2 g, 0.84 mmol) was dissolved in cold (0 °C) and dry dimethyl formamide (DMF) (5 ml) and triethylamine (0.224 ml, 1.6 mmol) was added and stirred to mix at –10 °C under nitrogen. Ethylchloroformate (0.12 ml, 1.25 mmol) was added dropwise and stirred. Finally a solution of 2-amino-3-hydroxy-propionic acid ethyl ester (0.14 g, 1.05 mmol) in dry DMF (5 ml) was added to the reaction mixture dropwise and stirred at –10 °C for 30 min, followed by another 30 min stirring at room temperature. The reaction mixture was diluted with cold water and extracted with CH_2Cl_2 (3 × 50 ml), washed with 0.2 M HCl, brine and water, respectively and dried over $MgSO_4$. Removal of the solvent and recrystallization from benzene/water (90:10) gave the title compound (170 mg, 50% yield) as brown-orange crystals. M.p. 200 °C, IR (KBr)/cm^{-1} 3400–3200 (NH_2, OH), 1700 (C=O), *m/z*: 413 (M^+, 40%), 250 ($C_{15}H_8NO_3$, 95%), 164 ($C_8H_8N_2O_2$, 100%), 149 ($C_8H_{10}N_2O$, 45%). Elemental analysis: ($C_{20}H_{20}N_4O_6$), C, H, N.

2.2.7. Synthesis of 6-amino-hexanoic acid ethyl ester

6-Aminohexanoic acid (1 g, 7.6 mmol) was dissolved in absolute ethanol (10 ml) under dry HCl gas and stirred for 30 min. Extra ethanol was then removed to provide colorless oil. Boiling point 83 °C (81–82 °C) [8]. IR (cm^{-1}): 3200 (NH_2), 1650 (C=O). Elemental analysis: ($C_8H_{17}NO_2$), C, H, N.

2.2.8. Synthesis of 6-amino-hexanoic acid [4-(5-amino-pentanoylamino)-9,10-dioxo-9,10-dihydro-anthracene-1-yl]-amide (VI)

Title compound was prepared in the same procedure mentioned for compound (V), using a solution of compound IV (0.2 g, 0.84 mmol) and 6-amino-hexanoic acid ethyl ester (0.14 g, 0.88 mmol) in DMF to provide 190 mg (49% yield) as brown solid. M.p. 320 °C, IR (KBr)/cm^{-1} 3380 (NH_2), 1700, 1650 (C=O), *m/z*: 464 (M^+, 5%), 378 [M^+-$(CH_2)_5$–NH_2, 28%], 362 (M^+-$C_5H_{14}N_2$, 50%), 208 (9,10-anthracenedione, 30%), 148 ($C_4H_4O_3$, 28%), 132 ($C_4H_4O_2$, 58%),108 ($C_6H_8N_2$, 100%). Elemental analysis: ($C_{26}H_{32}N_4O_4$), C, H, N.

2.3. Biological studies

2.3.1. Cell lines

Hela (human cervix carcinoma), KB (human caucasian/epidermal carcinoma), MDA-MB-468 (human breast adenocarcinoma) and K562 (human leukemia) cell lines were purchased from Pasture Institute of Iran in Tehran. They were grown in RPMI-1640 [each 500 ml of RPMI-1640 was supplemented with 10% of fetal calf serum, 5 ml of penicillin/streptomycin (50 IU ml^{-1} and 500 μg ml^{-1}, respectively), 5 ml of sodium pyruvate (1 mM), $NaHCO_3$ (1 g) and 5 ml of L-glutamine (2 mM)]. Completed media was sterilized by 0.22 μm microbiological filters after preparation and kept at 4 °C before using.

2.3.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT)-based cytotoxicity assay

The cytotoxic effects of synthesized compounds against previously mentioned human tumor cell lines were determined by a rapid colorimetric assay, using MTT bromide and compared with untreated controls [9]. This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells, into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolution in dimethyl sulfoxide (DMSO) [10]. Briefly, 200 μl of cells (5×10^4 cells per ml of media) were seeded in 96-well microplates and incubated for 24 h (37 °C, 5% CO_2 air humidified). Then 20 μl of final concentration of each compound was added and incubated for another 72 h in the same condition. Doxorubicin was used as a positive control. The first column of each microplate was used as negative control (containing no compound or doxorubicin). To evaluate cell survival, 20 μl of MTT solution (5 mg/ml in phosphate buffer solution) was added to each well and incubated for 3 h. Then gently 150 μl of old medium containing MTT was replaced by DMSO and pipetted to dissolve any formed formazan crystals. Absorbance was then determined at 540 nm by ELISA plate reader. Each concentration was assayed in eight wells and repeated 4–6 times. The drug concentration which inhibited 50% of tumor growth (IC_{50}) was determined. Percent of cell survival in negative control was assumed 100%.

2.3.3. Statistical analysis

All results are expressed as the mean ± S.D. of at least three experiments. *P* values for significance were determined using the two-tailed Students *t*-test. Significance was assumed at 5% level.

3. Results and discussion

The total synthesis of anthraquinones with proposed side arms was performed successfully. In this study m.p., IR and elemental analysis were used to verify the structures of the synthesized compounds. All synthetic data are shown in material and methods section. The biological effect of final synthesized compounds against a panel of cancer cell lines was evaluated via the MTT cytotoxicity assay and the results are summarized in Table 1. As it shown in this table, these compounds are cytotoxic in micromolar concentrations, though they are almost as effective as doxorubicin, as a positive control in this study.

Table 1
In vitro cytotoxic activity of compounds V and VI in comparison to doxorubicin

Compounds	IC_{50} (μM)			
	KB	Hela	MDA-MB-468	K562
Doxorubicin	14.1 ± 1.5	24.7 ± 1.1	4.2 ± 1.1	3.8 ± 0.7
Compound V	22.3 ± 2.1	25.2 ± 1.8	15.6 ± 1.6	12.5 ± 2.3
Compound VI	16.2 ± 1.9	20.3 ± 1.9	10.7 ± 1.1	7.8 ± 0.9

To perform the first step of these reactions, acetic anhydride and acetic acid were used for acetylation of benzene-1,4-diamine. Analysis of the obtained product revealed that the product contain three acetyl groups and is not *N*-(4-acetylamino-phenyl)-acetamide. Therefore, these reagents were replaced by acetyl chloride and the reaction was successfully completed.

The synthesis of the anthraquinone backbone was carried out by Friedel–Crafts intramolecular cyclization reaction with the aid of H_3BO_3 and conc. H_2SO_4. $AlCl_3$, as a usual reagent for Friedel–Crafts reaction, reacts in benzene as solvent which was not a good solvent for dissolving *N*-(4-acetylamino-phenyl)-acetamide. Triflic acid was used by other researchers for this purpose [11]. Polyphosphoric acid (PPA) is usually used for cyclization, but in this study the longer time needed for completion of reaction with PPA causes polymerization and low yield. Therefore, H_3BO_3, in the presence of conc. H_2SO_4 [2] was used to catalyze this reaction which gave higher product yield and reduced the reaction time to 20 min.

Certain amino acids (L-serine and 6-aminohexanoic acid) were used as specific side arms in the synthesis of anthraquinone derivatives. These side arms, which are weakly related to mitoxantrone and ametantrone side chains, not only provide the proper length (4–12 atoms) to produce hydrogenic or covalent bonds with DNA base pairs, but possess nucleophilic groups such as OH or NH_2 to intercalate more easily with DNA three dimensional structures. Although some researchers have applied simultaneous derivatization of functional groups by an aqueous-phase chloroformate-mediated reaction for determination of amino acids [12,13]. Amino acids solubility was increased by esterification prior to coupling reaction.

The final step in the reaction of these compounds is an amidation process. To increase the yield, the reaction was performed in dry solvents under N_2 gas and ethyl chloroformate was used as coupling agent. Carbodiimides such as [1-ethy1-3 (3-dimethylaminopropyl) carbodiimide hydrochloride] (EDC) or alkyl chloroformates (e.g. ethyl chloroformate) activate the COOH in the carboxylic acid moiety

and forms the intermediate *O*-acylisourea, which can chemically bind to exposed amino groups, such as those in compound IV. In this procedure, the product obtained is essentially pure and in quantitative yield [14]. In the similar way, Antonini et al. [15] used ethyl chloroformate to synthesize pyrimido-acridine derivatives as potential anticancer agents.

Synthesized compounds V and VI both have a similar structure to ametantrone but compound VI, which has longer side chains, is more cytotoxic than compound V, suggesting that this structure is more suitable for DNA intercalating. Although the efficacies of these compounds are almost the same as doxorubicin, more research should be carried out to evaluate their cardiotoxicity in comparison to doxorubicin.

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