A Modified Procedure for the Preparation of Mitoxantrone

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The anticancer drug mitoxantrone is commercially prepared in four synthetic steps starting from readily available chrysazin. A new and modified procedure for a similar process of mitoxantrone preparation has been successfully applied in small-scale preparation of mitoxantrone in our laboratories. This new process was designed to be practicable under Philippine setting. Success of the synthesis was established by full physico-chemical and spectral characterization of all intermediates and of the final mitoxantrone product. The cytotoxic activity of the synthesized mitoxantrone was tested against MCF-7 breast cancer cell lines and was found to be similar to commercially available mitoxantrone.

Key Words: anticancer compound, synthesis, anthraquinone, chemotherapy, chrysazin, cytotoxicity

INTRODUCTION

Mitoxantrone or 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthraquinone is a member of the anthraquinone class of antineoplastic agents, and is one of the most potent compounds of this class. Cheng & Ze-Cheng (1978) first described its efficacy as a cancer cure and with potent antiviral, antibacterial, immunomodulatory, and antitumor activities (Chang 1992). It is used in the treatment of leukemia, lymphoma, breast and ovarian cancer, acute nonlymphocytic leukemia, acute myeloid leukemia, and Hodgkin’s lymphoma. It has also gained approval of the US Food and Drug Administration (FDA) for the treatment of secondary-progressive multiple sclerosis (Immunex 1996).

Moreover, mitoxantrone is the first chemotherapy drug that FDA approved for the treatment and relief of pain in patients with advanced hormone refractory prostate cancer (Immunex 1996). Clinical efficacy is comparable to the popular anticancer drug Adriamycin (doxorubicin), but with less toxic side effects (Cheng & Zee-Cheng 1978).

Mitoxantrone has been commercially available since 1983. Since then, mitoxantrone was used in the treatment of several cancers; hence, the process for its production, yield obtained, and costs involved are constantly studied for optimization.

US Patent 4,197,249 (Murdock & Durr 1980) describes the preparation of mitoxantrone from leuco-tetrahydroxyanthraquinone as shown in the reaction scheme shown below.

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In the synthesis of mitoxantrone, there are four steps of chemical transformation involved. A key intermediate is the leuco-tetrahydroxyanthraquinone. According to Chang (1992), it is prepared in three steps. First, chrysazin is subjected to nitration in the presence of 20% oleum under ice cooling to afford 4,5-dinitrochrysazin. The reaction reportedly produces an undesired isomer (2,4-dinitrochrysazin) in less than 5% yield. The total yield for this step was claimed to be around 80%. The contaminating isomer was removed by recrystallization in DMF-benzene-ethanol mixture. The second step involves reduction of the nitro functional group using iron metal in sulfuric acid. The reaction was claimed to proceed in more than 90% yield to afford an essentially pure 4,5-diaminochrysazin compound. The third step involves conversion of 4,5-diaminochrysazin to leuco-tetrahydroxyanthraquinone-tetrahydroxyanthraquinone. This reaction was claimed to proceed in almost quantitative yield with no further purification of this intermediate, which was reported by the same workers (Chang & Cheng 1995) to be hygroscopic, oxygen- and light-sensitive; and thus, was used immediately in the fourth step of synthesis.

Murdock & Durr (1980) reported the preparation of mitoxantrone from the intermediate leuco-tetrahydroxyanthraquinone. The reaction involves condensation of the leuco-tetrahydroxyanthraquinone intermediate with an amino alcohol (2-(2-aminoethylamino)-ethanol) to form a Schiff base, which was oxidized to the final product mitoxantrone with the use of either dry or wet air.

The literature abounds in the number of citations published since the invention and synthesis of mitoxantrone. Different approaches have been tried to prepare mitoxantrone using a completely different set of starting materials (Krapcho et al. 1991).
In the method of mitoxantrone synthesis reported by Krapcho (Figure 3), the following reaction sequence is involved:

The above route involves more advanced reaction techniques such as the handling of flammable secondary butyl lithium in an orthometallation reaction, and the use of extremely reactive boron tribromide. The overall strategy relies on initial ring-forming reactions to build the anthraquinone framework. The amino alcohol side chain is installed at the very last stage by nucleophilic displacement of a fluoride leaving group.

Some workers, however, chose to adapt the original synthesis with a variation in any of the four steps (Schwabe et al. 1992; Pozdnyakovich et al. 1995). Still others disclosed new methods and processes in the purification of mitoxantrone itself (Niclas et al. 1983; Chang et al. 1990). Others reported the synthesis of mitoxantrone analogs (Zagotto et al. 1992; Zagotto et al. 1997; Johnson et al. 1997; Potter et al. 2002).

The object of this study was to develop a new preparation process for mitoxantrone. The present process could be conducted under Philippine conditions and with the available research capability. Furthermore, the process was designed to be flexible to scale up for bench scale (multigram scale) production of mitoxantrone in...
a cost-effective method. The new route was intended to achieve the same overall transformations with the use of completely different set of reagents in some steps of the chemical synthesis.

**MATERIALS AND METHODS**

All solvents (JT Baker) used were of analytical grade. Distilled deionized water was used for quenching the reaction. The progress of reaction was monitored via TLC using commercially available Silica Gel 60F254 TLC plates (Merck), with I2 visualization. Purification was achieved by recrystallization. Evaporation was conducted in an Eyela Rotary Vacuum Evaporator N-N series with an Eyela Water Bath SB-650.

Products were characterized by using a BIO-RAD FTS 40-A FT-IR Spectrophotometer for IR Data; LCQ Finnigan MAT LC-MS, Finnigan MAT 95 Strap (EI), and Finnigan LCQ (ESI) for MS Data; a Shimadzu Differential Thermogravimetric Analyzer (TGA) DTG-60H for melting point determination; Shimadzu UV-1601PC, UV-Mini 1240 for UV-VIS absorption spectroscopy; and JEOL Lambda 400 (400 MHz) for NMR data.

The overall scheme for a new process of mitoxantrone preparation is described below (Figure 4):

![Image of chemical reactions]

**Figure 4.** The new process for preparation of mitoxantrone

The preparation of 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthraquinone or mitoxantrone comprises the steps of:

(a) reacting chrysazin (or 1,8-dihydroxyanthraquinone) with concentrated nitric acid and oleum (20% fuming sulfuric acid) to produce a mixture of 4,5-dinitrochrysazin or 1,8-dihydroxy-4,5-dinitroanthraquinone (II) and 2,4-dinitrochrysazin or 1,8-dihydroxy-2,4-dinitroanthraquinone;

(b) isolating (II) from the mixture obtained in step (a) by heating (~60°C) the mixture in dilute sodium sulfate solution followed by recrystallization;

(c) reacting (II) obtained in step (b) with stannous dichloride by refluxing the reaction mixture in absolute ethanol to obtain the compound of the formula (III); that is, 4,5-diaminochrysazin or 1,8-diamino-4,5-dihydroxyanthraquinone;

(d) reacting (III) obtained in step (c) with sodium dithionite in boiling aqueous sodium hydroxide solution;

(e) coupling leuco-1,4,5,8-tetrahydroxyanthraquinone, simply referred to as leuco-tetrahydroxyanthraquinone, of the formula (IV) obtained in step (d) with 2-(2-aminoethyl)amino)ethanol in pyridine to afford a Schiff base in a known manner; and, converting said Schiff base obtained in step (e) into 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthraquinone or mitoxantrone (I) by bubbling oxygen gas in a solution of the Schiff base in absolute ethanol at 55-60°C.

**Synthesis of 4,5-dinitrochrysazin (II):** Chrysazin (20 g, 83.3 mmol) was added to a stirred solution of boric acid
Cytotoxicity towards MCF-7 cells was assessed in MTT-microtiter plate tetrazolium cytotoxicity assay adapted from Mossman (1983). The experiment was performed using the standard MTT assay. This assay was originally described by Mossman (1983) and has since been modified by others.

**MTT Assay:** Cytotoxicity towards MCF-7 cells was assessed in MTT-microtiter plate tetrazolium cytotoxicity assay. This assay was originally described by Mossman (1983) and has since been modified by others.

**IC₅₀ Determination:** The IC₅₀ values of mitoxantrone (Sigma) and the synthesized mitoxantrone were determined using the MTT Assay mentioned above. In this experiment, a stock mixture of 10 mg/mL for both compounds was used. A 10-fold dilution was made from each stock solution. The final working concentrations ranged from 0.05 µg/mL to 5.0 µg/mL for each compound. Fractional survival values from the different doses were used to compute for the IC₅₀. The IC₅₀s were determined using ICPIN, a TOXstat computer software.

**RESULTS & DISCUSSION**

Synthesis of 4,5-dinitrochrysazin: The technical-grade oleum used in the synthesis of 4,5-dinitrochrysazin was from Stepan industry, a local detergent manufacturing plant. They supplied practical-grade oleum that was...
analyzed to be around 20% SO$_3$, together with an unknown colored organic material (Table 1).

**Table 1. Assay for %SO$_3$ of available oleum from Stepan industry**

<table>
<thead>
<tr>
<th>Trial</th>
<th>%SO$_3$</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.85</td>
<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>20.98</td>
<td>0.19</td>
</tr>
</tbody>
</table>

The nitration proceeded as expected (Figure 5). The yield and composition of the resulting mixture, however, were consistently different from those reported in the accounts first published by Cheng & Zee-Cheng (1978), and later by Chang & Cheng (1995).

![Nitration of chrysazin](image)

To purify the desired 4,5-dinitrochrysazin (II), repeated recrystallizations from DMF-benzene-ethanol were tried but this could not afford the desired (II) as pure compound. To remove the undesired 2,4-dinitrochrysazin, the crude mixture was treated with a boiling solution of dilute sodium sulfite (Galinowski et al. 1983). After washing with water, the by-product of sodium sulfite treatment (a sulfaminic acid of 2,4-dinitrochrysazin) was successfully removed. A final purification was achieved by recrystallization of the resulting product with DMF-benzene-ethanol mixture. The data obtained for (II) are summarized in Table 2.

**Table 2. Physical and spectroscopic data for intermediates and final mitoxantrone product**

<table>
<thead>
<tr>
<th>Physical/ spectroscopic data</th>
<th>Synthesized 4,5-dinitrochrysazin (II)</th>
<th>Synthesized 4,5-diaminochrysazin (III)</th>
<th>Synthesized leuco-tetrahydroxyanthraquinone (IV)</th>
<th>Synthesized mitoxantrone (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed Melting point, °C</td>
<td>&gt; 300</td>
<td>&gt; 300</td>
<td>244</td>
<td>174</td>
</tr>
<tr>
<td>Literature (Chang &amp; Cheng 1995)</td>
<td>(CDCl$_3$) δ 12.07 (2H, s), 7.90 (2H, d, J = 9.0 Hz), 7.41 (2H, d, J = 9.0 Hz)</td>
<td>(acetone-$d_6$) δ 12.84 (2H, s), 7.33 (2H, d, J = 9.3 Hz), 7.18 (2H, d, J = 9.3 Hz)</td>
<td>(CDCl$_3$) δ 15.83 (2H, s), 9.81 (2H, s), 7.17 (2H, s), 3.05 (4H, s)</td>
<td>(DMSO-$d_6$) δ 13.30 (2H, s), 9.80 (2H, s), 7.15 (2H, s), 3.03 (2H, s)</td>
</tr>
<tr>
<td>1H NMR, ppm</td>
<td>(CDCl$_3$) δ 12.07 (2H, s), 7.90 (2H, d, J = 9.0 Hz), 7.41 (2H, d, J = 9.0 Hz)</td>
<td>(acetone-$d_6$) δ 12.87 (2H, s), 7.65 (2H, exch), 7.37 (2H, d, J = 9.3 Hz), 7.17 (2H, d, J = 9.3 Hz)</td>
<td>(CDCl$_3$) δ 15.81 (2H, s)</td>
<td>(CDCl$_3$) δ 13.30 (2H, s), 10.43 (2H, s), 6.99 (2H, s), 6.81 (2H, s), 3.73 (4H, m), 3.41 (4H, m), 2.99 (4H, m), 2.89 (4H, m)</td>
</tr>
<tr>
<td>Literature (Chang 1992)</td>
<td>(CDCl$_3$) δ 12.02 (2H, s), 7.86 (2H, d, J=9.2 Hz), 7.47 (2H, d, H=9.2 Hz)</td>
<td>(acetone-$d_6$) δ 12.87 (2H, s), 7.65 (2H, exch), 7.37 (2H, d, J=9.3 Hz), 7.17 (2H, d, J=9.3 Hz)</td>
<td>(CDCl$_3$) δ 15.81 (2H, s)</td>
<td>(CDCl$_3$) δ 13.30 (2H, s), 10.43 (2H, s), 6.99 (2H, s), 6.81 (2H, s), 3.73 (4H, m), 3.41 (4H, m), 2.99 (4H, m), 2.89 (4H, m)</td>
</tr>
<tr>
<td>$^{13}$C NMR</td>
<td>(DMSO-$d_6$) δ 182.7, 154.3, 146.6, 124.8, 123.6, 114.9, 106.9, 60.3, 51.2, 48.1, 42.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

see table continuation on the next page
Synthesis of 4,5-diaminochrysazin: In the second step of mitoxantrone synthesis as outlined above, 4,5-dinitrochrysazin (II) was subjected to reduction with iron metal in sulfuric acid to afford 4,5-diaminochrysazin (III). In a modification of this method, stannous dichloride was used as the reducing agent as shown below (Figure 6).

![Figure 6. Reduction of dinitrochrysazin](image)

It was observed that when iron was used as reducing agent, composition of the reaction mixture was made up of unreacted starting material, partially reduced 4,5-dinitrochrysazin (II) and 4,5-diaminochrysazin (III). However, no separation scheme exists in the literature for the isolation of the product from the contaminating substances. It was believed that the solution to this problem could be the pre-activation of iron metal to elemental iron with the use of hydrogen gas. The equipment for this kind of hydrogenation is not available in the Philippines. To circumvent this problem, a different reducing agent was employed. Stannous dichloride is a stable solid that does not require special precautions for handling. The reaction setup allows the use of a magnetic stirrer. The use of iron, on the other hand, necessitates the use of a mechanical stirrer due to the paramagnetic properties of iron. The tin by-products were removed by sequential acid-water-base-water washings. The conversion of (II) to (III) was complete and quantitative. The data obtained for 4,5-diaminochrysazin (III) are summarized in Table 2.

Synthesis of leuco-tetrahydroxyanthraquinone: The third step was adapted from the literature (Potter et al. 2002) as shown in the scheme below (Figure 7). Since the desired leuco-tetrahydroxyanthraquinone product (IV) is sensitive to light and oxygen, the reaction and its work-up was conducted with a careful exclusion of air. Table 2 shows the data for the leuco-tetrahydroxyanthraquinone product obtained.

![Figure 7. Preparation of leuco-tetrahydroxyanthraquinone (IV) from 4,5-diaminochrysazin](image)
Synthesis of Mitoxantrone: Again, the procedure for the fourth step was adapted from published material (Figure 8). The only modification used was the use of oxygen gas as oxidant and absolute ethanol as solvent in the last step. Table 2 shows the data for the synthesized mitoxantrone.

![Figure 8. Synthesis of mitoxantrone](image)

Biological activity of synthesized mitoxantrone: MCF-7 cells were cultured in the presence of commercial mitoxantrone hydrochloride or synthesized mitoxantrone and a 10-fold dilution was performed. Cell survival was determined using the MTT Assay (Table 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC50 values mg/mL in breast cancer cell line MCF-7 (average of two trials)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mitoxantrone hydrochloride</td>
<td>0.329</td>
<td>0.004</td>
</tr>
<tr>
<td>synthesized mitoxantrone</td>
<td>0.411</td>
<td>0.107</td>
</tr>
</tbody>
</table>

To summarize, several innovations were introduced in this process. First, chrysazin was subjected to nitration reaction employing an excess of nitrating agent to afford a mixture of 4,5-dinitrochrysazin (II) and 2,4-dinitrochrysazin. The two isomers, consistently produced at a ratio of 1:1 to 3:2, were separated by reacting the crude mixture with dilute sodium bisulfite (Galinowski et al. 1983), followed by recrystallization of the resulting solid with DMF-benzene-ethanol mixture to afford the pure 4,5-dinitrochrysazin (II). The use of dilute sodium sulfite solution to purify the desired dinitro intermediate has not been reported in the literature for mitoxantrone synthesis.

Furthermore, 4,5-dinitrochrysazin (II) was subjected to stannous dichloride reduction in absolute ethanol to afford the desired 4,5-diaminochrysazin (III). Moreover, stannous dichloride has not been used as reducing agent in previously reported mitoxantrone syntheses.

The third step in the synthetic route was similar to those reported in the literature (Pozdnyakovich et al. 1995). Thus, 4,5-diaminochrysazin (III) was reacted with sodium dithionite to yield leuco-tetrahydroxyanthraquinone (IV).

The leuco-tetrahydroxyanthraquinone compound (IV) was reacted with 2-(2-aminoethylamino)-ethanol in pyridine (Chang 1992), which was removed after the formation of Schiff base. The mixture was diluted with absolute ethanol and oxygen gas was bubbled through it. In the literature, dry air is used as the oxidant instead of pure oxygen gas. The reaction time in this study decreased five-fold when pure oxygen gas as oxidant and absolute ethanol as solvent were used instead of dry air.

CONCLUSION

This modified method for the synthesis of mitoxantrone can be adopted for local production of the drug under Philippine conditions. The overall chemical process is shown below which is according to US Pat No 4,197,249 (steps 3-4) and Cheng (steps 1-2). The procedure accomplished the same overall
transformations described by Chang (1992) with the use of a completely different set of reagents in some steps of the process. Mitoxantrone prepared using the methods described in this paper had a similar level of cytotoxicity to the breast cancer cell line MCF7 with the commercial mitoxantrone hydrochloride (SIGMA) standard. Physical, spectroscopic, and cytotoxic activity properties were obtained to confirm the identity of the synthesized mitoxantrone. The procedure is also amenable to further optimization and scale up (multigram scale) for production of mitoxantrone at a lower cost.

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