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TITLE: Analogs of Estrogen Metabolites as Probes of Estrogen-Induced Tumorigenesis

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
A series of C-4 hydroxyalkylestradiol analogs were synthesized as metabolically stable analogs of 4-hydroxyestradiol (4-OHE2) in order to separate the receptor activation and redox cycling properties. The Stille cross-coupling and the carboxymethylation reactions were used for the synthesis of these analogs. The 4-hydroxyalkyl estrogens and catechol estrogens were compared in potentiometric and DNA-damaging studies. The non-redox cycling estrogen analogs were unable to induce DNA damage, whereas catechol estrogens produced DNA damage. A novel synthetic route was developed for synthesis of catechol estrogens from 2- and 4-substituted formyl estradiols. In addition, several estrogen analogs were synthesized as potential inhibitors of estrogen hydroxylases, the enzymes responsible for catechol estrogen biosynthesis. 2-Methoxymethylestradiol (2MME2) was identified as a novel inhibitor of tubulin polymerization in vitro. Finally, a novel synthetic route was developed for constructing benzopyrones, present in various natural products that interact with enzymes and receptors of therapeutic importance in breast and prostate cancer. Readily available salicylic acids and terminal alkynes were used as building blocks for the benzopyrone ring system. This synthetic approach utilizes readily available starting materials, mild and high yielding reactions with good functional group tolerance, and is ideal for developing combinatorial libraries of the benzopyrone ring system.
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1.0 Introduction

Chemical efforts in this project have concentrated on synthesis of stable catechol estrogen mimics and on similar nonsteroidal estrogens analogs. Synthetic work during this grant is described in this report and the attached manuscripts. This research concentrated on the following:

1) **4-Hydroxyalkyl and 4-aminoalkyl estrogens**: Synthesis of 4-hydroxyalkyl and 4-aminoalkyl estrogens were completed.

2) **Estrogen A-ring fused analogs**: 2,3- and 3,4-Methylenedioxy, 2,3- and 3,4-dihydropyranyl and dihydrofuranyl estradiols have been synthesized as potentially selective inhibitors of estrogen 2- and 4-hydroxylase, the enzyme isoforms responsible for forming catechol estrogens. Also,

3) **Catechol Estrogens**: Catechol estrogens are chemically unstable and easily decompose on air oxidation. The chemical instability makes catechol estrogens difficult to synthesize. Low yields and complex product mixtures mark the literature methods. We employ a Baeyer Villiger oxidation of formylestradiols to give protected catechols. The CE's are then produced under a reducing environment thereby circumventing oxidative complications.

4) **Alkoxyalkyl estrogens**: We have synthesized a series of 2-substituted alkoxyalkyl estradiols. These were evaluated as inhibitors of tubulin polymerization.

5) **Novel synthetic approaches for benzopyrone nucleus**: We are investigating various synthetic approaches for synthesis of benzopyrone combinatorial libraries.

In addition, biological evaluation of the 2-hydroxyalkyl, 4-hydroxyalkyl, and 2-methoxymethyl estradiol analogs were performed. These studies are also described in this report and the attached manuscripts.
2.0 Synthesis of 4-Hydroxyalkyl Estrogens

2.1 INTRODUCTION: A primary goal of this project was to study the role of catechol estrogens in initiation of breast tumors. As explained in the previous section catechol estrogens, oxidative metabolites of estrogens, are implicated as possible causative agents in estrogen-induced tumorigenesis. Catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates like quinones, semiquinones, and arene oxides. These highly reactive moieties may be cytotoxic via reaction with proteins and nucleic acids. Furthermore, the catechol estrogens have been shown to produce a variety of ROS, such as hydroxide, peroxide, and superoxide radicals, which can produce mutations leading to tumor initiation. Researchers in this field generally agree that of the two possible catechol estrogens, 2-hydroxyestradiol (2-OHE\textsubscript{2}) is not tumorigenic, whereas 4-hydroxyestradiol (4-OHE\textsubscript{2}) is the primary tumor initiator.\textsuperscript{1} Although the oxidative metabolism of 4-hydroxyestradiol is considered important for mediating the tumorigenic effects there were reports in the literature indicating that 4-OHE\textsubscript{2} is similar to estradiol in its ability to bind to and activate the classical estrogen receptor.\textsuperscript{2}

Interestingly, the interaction of 4-OHE\textsubscript{2} with ER appears to occur with a reduced dissociation rate compared with estradiol, suggesting that the association of 4-OHE\textsubscript{2} with ER may last longer than for the parent hormone. These results suggest that 4-OHE\textsubscript{2} has the potential to activate ER and induce gene transcription and set up the cascade of estrogen's mitogenic responses. Thus the tumorigenic potential of 4-OHE\textsubscript{2} may be a result of either the ER-mediated mitogenic events or its oxidative metabolism. It is important to understand the exact nature of involvement of 4-OHE\textsubscript{2} in the tumor initiation-progression events in order to develop better approaches for treatment and prevention of breast cancer. We were specifically interested in evaluating the relative importance of oxidative damage vs. the receptor-mediated pathways for the potential tumor induction by 4-OHE\textsubscript{2}. In order to differentiate between the two pathways we proposed the synthesis of 4-hydroxyalkyl estradiols 19, 20, and 21 as stable analogs of 4-OHE\textsubscript{2}.

![4-Hydroxyestradiol](image)

Figure 2.1 Biochemically Stable Analogs of 4-OHE\textsubscript{2}

These compounds lack the catechol moiety and so were not expected to undergo redox cycling and produce oxidative stress. On the other hand, like in 4-OHE\textsubscript{2}, the hydroxyl groups at positions 3- and 4- in these compounds can participate in hydrogen bonding.
interactions with receptor and/or enzyme binding sites. As a result, the 4-OHE$_2$ analogs were expected to have similar ER binding profile as 4-OHE$_2$. We envisaged to use these compounds as chemical probes to distinguish between receptor mediated events vs. redox cycling events in 4-OHE$_2$ induced tumorigenesis. Additionally, 4-OHE$_2$ is both chemically and biochemically unstable and its use in biological studies is difficult. The analogs with hydroxyalkyl chains should be considerably more stable than the catechol estrogens and therefore provide stable analogs to study the various biochemical effects of 4-OHE$_2$ as discussed in Section 1.5.

2.2 REGIOSPECIFIC FUNCTIONALIZATION OF 4-POSITION IN ESTRADIOLS: Previously, our lab had synthesized a series of 2-hydroxyalkyl estradiols 22, 23, 24 as chemically stable analogs of 2-hydroxyestradiol. These compounds had exhibited similar estrogen receptor affinity and pS2 gene induction to the catechol estrogen 2-hydroxyestradiol. The chemical synthesis of 2-hydroxyalkyl estrogens (22-24) relied on a key formylation reaction (Figure 2.2). Estradiol protected as its bis-MOM ether 25 underwent a selective ortho-lithiation at the 2-position to form 2-lithioestradiol derivative, which was reacted with freshly distilled DMF to produce a quaternary intermediate that provided the formyl derivative 26 upon acidification. The formyl derivative 26 was reduced with NaBH$_4$ to provide the hydroxymethyl analog 22 or homologated using well established protocols to provide 23 and 24. As this strategy had worked to provide the 2-hydroxyalkyl analogs in excellent yields, a similar strategy was adopted for the synthesis of 4-hydroxyalkyl analogs. Thus, a method was needed to regiospecifically prepare 4-lithioestradiol, which could then be converted to the 4-formyl derivative using conditions developed for the synthesis of 2-hydroxyalkylestradiols. It was expected that a suitably protected 4-haloestradiol upon treatment with an alkylolithium would undergo halogen-lithium exchange reaction and regiospecifically provide the 4-lithiated estradiol.

![Figure 2.2 Synthesis of 2-Formylestradiol](image)
The synthesis commenced by brominating estradiol with 1 equivalents of N-bromosuccinimide in ethanol, the required 4-bromoestradiol 27 precipitated from the reaction mixture and was obtained in 54% yield after recrystallization. Comparable results were obtained for bromination using N-bromoacetamide; however, when estradiol was treated in a similar fashion with N-iodoacetamide, mostly unreacted estradiol was recovered. Analysis of the filtrate from the bromination reaction by reverse phase HPLC revealed a mixture of 4-bromoestradiol (5-10%), 2-bromoestradiol 28 (~15%) and 2,4-dibromoestradiol 29 (~25%) along with unreacted estradiol. When bromination was attempted with 2 equivalents of NBS, 4-BrE2 failed to precipitate out of the reaction mixture. HPLC analysis of the reaction mixture revealed that the estradiol was completely consumed and 2,4-dibromoestradiol 29 was the major product. Several attempts to separate the components of this mixture using flash chromatography and preparative TLC were unsuccessful. In order to evaluate the feasibility of separation after capping the free hydroxyls, the mixture of 27, 28 and 29 was dissolved in pyridine and treated with excess acetic anhydride to convert the estrogens to their acetylated derivatives. However, several attempts to separate the acetylated bromoestrogens were unsuccessful.

Figure 2.3 Bromination of Estradiol
Based on the experience of using MOM protecting groups during the synthesis of the 2-hydroxyalkyl series, the hydroxyl groups of 4-bromoestradiol were masked as their MOM ethers. Thus, 4-bromoestradiol was protected as its bisMOM ether 30 with chloromethylmethyl ether and DIPEA in >90% yields. The reaction was successfully carried out on a 10g scale. The good yield is a significant improvement over a published procedure by Pert-Ridley to access 30, wherein poor yields and complex product mixtures were obtained on larger scales (>0.5g) and necessitated purification by HPLC.\(^5\)

A solution of 30 in THF at -78°C was treated with various organolithium reagents and stirred for three hours after which the reaction was quenched with D\(_2\)O or freshly distilled TMSCl. A clean formation of 32 indicated a successful halogen-lithium exchange reaction.

Halogen-Li exchange reaction was attempted with three organolithiums namely, n-butyllithium \((n-\text{BuLi})\), methylolithium, and sec-butyllithium. \(n-\text{BuLi}\) provided the cleanest reaction and quantitative formation of 4-dueteroestradiol 32. The position of the dueterium label was confirmed by NMR and mass-spectral analysis. No formation of 2-duetero estradiol was detected in the reaction with \(n-\text{BuLi}\). The time for the lithium exchange reaction was optimized by treating the solution of 30 with 1.2 equivalents of \(n-\text{BuLi}\) for various time periods (0.5hr, 0.75hr, 1.0hr, 2.0hr and 3.0hr), and was found to be
complete after one hour. Once the halogen-lithium exchange reaction was optimized to regiospecifically provide 4-lithioestradiol 31, efforts were concentrated on using this reaction to introduce various substituents at the C-4 position of estrogen.

2.3 ATTEMPTS TO FUNCTIONALIZE 4-LITHIOESTRADIOL WITH VARIOUS CARBON ELECTROPHILES: The reaction conditions developed for synthesis of 2-formylestradiol 26 from 2-lithioestradiol were employed for conversion of the 4-lithioestradiol derivative to the corresponding 4-formyl derivative. Thus 31 was treated with an excess of freshly distilled DMF and warmed to room temperature. The reaction mixture was acidified with aqueous HCl and the products were isolated after the usual workup and purification. However, employing these conditions provided only modest yields (34%) of the 4-formylestradiol derivative 33. Dehalogenated estradiol 25 was the major product isolated (65%) from the reaction. There is a possibility that the dehalo analog could result from a decomposition of the 4-formyl derivative. TLC analysis of the reaction mixture showed the formation of the dehalo analog prior to the hydrolysis reaction, which indicates that the 4-lithio derivative has considerably lower reactivity as compared to the

Figure 2.5 Preliminary Synthesis of 4-Hydroxyethylestradiol

corresponding 2-lithioderivative. Additionally the C-4 position is sterically hindered because of the allylic strain from position 6, and so the introduction of a reasonably
bulky N'-dimethylformyl group proved to be difficult. In a preliminary attempt to synthesize the hydroxyalkyl derivatives, the formyl derivative 33 was methylenated with CH₂=PPh₃ in a Wittig reaction to give the vinyl compound 34 in quantitative yield. Hydroboration of this vinyl derivative gave the 4-hydroxyethyl-bisMOM estradiol 35 in 35% yield, which upon refluxing with methanol and PPTS gave the desired hydroxyalkyl derivative 20. However the yields of this reaction sequence were very low (<7%), and so efforts on improving the 4-formylation reaction continued.

<table>
<thead>
<tr>
<th>No.</th>
<th>Electrophile</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MOMCl</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>Ethylene Oxide</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>DMF; HCl</td>
<td>33 (34%), 25</td>
</tr>
<tr>
<td>4</td>
<td>N-methyl formamide</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Allylbromide</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Allylbromide, TBAI</td>
<td>Complex Mixture</td>
</tr>
<tr>
<td>7</td>
<td>CO₂</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 2.1 Attempted Reaction of Various Electrophiles with 30

Several other electrophiles were investigated alongside DMF for their ability to react with 4-lithioestradiol and effect a C-4 alkyl functionalization (Table 2.1). Attempts to react the 4-lithio estradiol with ethylene oxide and methoxymethyl chloride were unsuccessful and only 25 was isolated from these reactions. Reaction of 30 with allyl-bromide predominantly gave the dehalo product 25 (60%) along with an unidentified product (~30%) that appeared to be hydroxylated at the 4-position based on mass-spectral analysis. Reaction with rigorously purified allylbromide eliminated the hydroxylated product but provided only the dehalogenated product. An attempt to increase the reactivity by in situ generation of allyl iodide using an iodide source like tetra-butyl ammonium iodide in conjunction with allyl bromide failed to provide the desired allylated product. It was found that transmetallation of the 30 with Cul, and subsequent treatment with allyl bromide gave the desired allylated product 36 as a mixture with the dehalo product 25. This mixture was chromatographically inseparable and so was subjected as such to a hydroboration reaction. The desired hydroxypropyl alcohol 37 could be obtained in ca 85% yield (32% overall). In contrast to the other electrophiles examined, CO₂ was the only electrophile that could be used successfully to functionalize...
the 4-position of estradiol in good yield. Thus, reaction of 4-lithioestradiol with \( \text{CO}_2 \) provided exclusively the 4-carboxy estradiol 38 derivative in 99% yield. No formation of the dehalogenated product 25 was noted in this reaction.

Figure 2.6 Reaction of 30 with Allylbromide and Carbon Dioxide

2.4 SYNTHESIS OF 4-SUBSTITUTED ESTRADIOLS BY THE STILLE COUPLING REACTION: Clearly, the attempts to functionalize the C-4 position of estradiol via the 4-lithioestradiol 31 failed to provide the desired 4-hydroxyalkyl estrogens in good yields. An alternative approach was considered, as 30 would be an ideal electrophilic partner for C-C bond forming reactions using \( \text{Pd}^0 \) chemistry. Specifically, 30 could be reacted with 4-vinyl- or 4-allyltin reagents in the presence of \( \text{Pd}^0 \) catalyst to provide the 4-vinyl- 34 and 4-allyl-estradiols 36, respectively.\(^6\) These alkenyl derivatives after hydroxylation and deprotection would provide 20 and 21, two of the desired hydroxyalkyl estradiols. Alternatively, 30 could undergo CO insertion reaction in presence of a hydride source and \( \text{Pd}^0 \) catalyst to yield the 4-formylestradiol 33, that could be reduced to the hydroxymethyl derivative 19.\(^7\)

8
Exploratory reactions were performed using 30 and tributylvinyltin to identify the optimum conditions for the Stille coupling reaction. A variety of catalysts, co-catalysts, solvent and reagent proportions were investigated as shown in **Table 2.2**. The successful conditions involved refluxing a solution of 30 with 2.1 equivalents of the stannane, 5 mol% of the Pd(PPh₃)₄ in DMF as the solvent. Preliminary observations indicated that high temperatures were required because oxidative addition of the palladium did not occur at useful rates below 120°C. It was also important to deoxygenate the reaction mixture after adding the Pd catalyst and prior to reflux in order to prevent oxygen from poisoning the catalysis cycle. Completion of the reaction was usually indicated by deposition of black Pd residue on the walls of the reaction flask.

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Catalyst</th>
<th>Solvent</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylacrylate</td>
<td>Pd(OAc)₂, P(O-tol)₃</td>
<td>Et₃N, 100°C</td>
<td>No reaction</td>
</tr>
<tr>
<td>Vinyltributyltin, 1equiv.</td>
<td>PhCH₂PdCl(PPh₃)₂ 10mol%</td>
<td>Toluene</td>
<td>No reaction</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>DPPE-PdCl, PPh₃ LiCl</td>
<td>Toluene</td>
<td>No reaction</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>Pd(PPh₃)₄, 5mol%</td>
<td>Toluene, 100°C</td>
<td>75% unreacted 3% 34</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>Pd(PPh₃)₄, 25mol%</td>
<td>Toluene, 100°C</td>
<td>15% 34</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>Pd(PPh₃)₄, 15mol%</td>
<td>Toluene, 100°C</td>
<td>15% 34</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>Pd(PPh₃)₄, 15mol%</td>
<td>Dioxane</td>
<td>Decomposition</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>Pd(PPh₃)₄, 15mol% CuI, 8 mol%</td>
<td>Dioxane</td>
<td>Decomposition</td>
</tr>
<tr>
<td>Vinyltributyltin</td>
<td>Pd(PPh₃)₄, 15mol% CuI, 8 mol%</td>
<td>Toluene</td>
<td>Decomposition</td>
</tr>
<tr>
<td>Vinyltributyltin, 2equiv.</td>
<td>Pd(PPh₃)₄, 15mol%</td>
<td>Toluene, 100°C</td>
<td>25% 34</td>
</tr>
<tr>
<td>Vinyltributyltin, 2equiv.</td>
<td>Pd(PPh₃)₄, 15mol%</td>
<td>DMF, 130°C</td>
<td>&gt;75% 34</td>
</tr>
<tr>
<td>Vinyltributyltin, 2equiv.</td>
<td>Pd(PPh₃)₄, 5mol%</td>
<td>DMF, 130°C</td>
<td>90% 34</td>
</tr>
</tbody>
</table>

**Table 2.2** Stille Reaction Conditions Explored for Functionalization of 30.
The \( \text{Pd(PPh}_3\text{)}_4 \), used for this reaction was prepared using the procedure described by Coulson \textit{et al.} and stored under argon. Employing these reaction conditions, the desired 4-vinyl bisMOM estradiol 34 was obtained in 90% yield by reacting 30 with tributyvinyltin (Figure 2.7). Using allyl tributyltin as the nucleophile the 4-allyl derivative 36 was obtained in 94% yield (Figure 2.9). The 4-butyl estradiol derivative was sometimes isolated as an impurity at 2-3%. In order to demonstrate the versatility of the Stille reaction, 30 was treated with phenyl tributyltin and the corresponding 4-phenylderivative 41 was obtained in 85% yield (Figure 2.7). Thus a bulky substituent could be introduced in the sterically hindered 4-position of estradiol using the Stille reaction. The Stille reaction conditions developed were later used for the synthesis of 4-methoxymethyl derivatives as discussed in Chapter 3.

A solution of \( \text{BH}_3 \)-etherate was added dropwise to a solution of the 36, and stirred for an hour at room temp. Oxidation of the reaction mixture with NaOH and 30% \( \text{H}_2\text{O}_2 \) gave the 4-hydroxypropyl derivative 37 in 82% yield (Figure 2.9). Refluxing of 37 with PPTS in MeOH provided 21 in 84% yield. When the vinyl derivative 34 was treated similarly, only 30% of the desired primary alcohol 35 was obtained. Refluxing 35 with PPTS in MeOH provided 20. Along with the primary alcohol, a 30% mixture of diastereomeric secondary alcohols 39 and 12% of diastereomeric mixture of partially deprotected secondary alcohols 40 were recovered. In order to improve the stereoselectivity, bulky hydroborating agents like catechol borane and 9-BBN were used for the hydroboration of 34. However, the yields of the primary alcohol did not improve significantly. This situation was very intriguing, especially given the facility with which the 2-vinylestradiol underwent hydroboration-oxidation to provide the corresponding primary alcohol in >80% yield. The influence of the MOM-protecting groups on the hydroboration of 34 was investigated. As it was found impossible to deprotect MOM groups from 34 without effecting polymerization of the vinyl compound, 27 was acetylated and benzylated in separate reactions to provide 41 and 42, which were subjected to the Stille coupling conditions. The Stille coupling with 41 failed to provide the corresponding vinyl estradiol, however Stille reaction of 42 with tributylvinyltin provided 43 in 84% yield. This vinyl derivative was subjected to hydroboration-oxidation, however only 34% of the hydroxyethyl estradiol 44 was isolated. Thus, changing the hydroxyl protecting groups to benzyl groups did not influence the outcome of the hydroboration reaction. The low yields in hydroboration are probably a combination of stereoelectronic and conformational effects in 34.
Figure 2.7 Synthesis of 4-Hydroxyethylestradiol and 4-Phenylestradiol
Another way to access the hydroxyethyl compound is oxidative cleavage of the terminal double bond in the 4-allylestradiol 36. In order to explore this option and improve upon the yields of hydroxyethyl derivatives, 36 was subjected to ozonolysis. Ozone gas was bubbled through a solution of 36 in dichloromethane at -78°C, the starting material was completely consumed in 10 minutes indicating ozonide formation. The ozonide was subsequently treated with NaBH$_4$, dimethylsulfide and triphenylphosphine in separate
reactions. In all the cases a complex inseparable mixture of products was formed and neither the desired alcohol nor the aldehyde were formed upon decomposition of the ozonide. Additionally, the attempt to oxidatively cleave the terminal double bond in 36 using KMnO₄ and NaIO₄ to provide the corresponding carboxyl derivative 45 was unsuccessful. It is interesting to note that reaction of 34 with KMnO₄ and NaIO₄ provided the corresponding carboxyl derivative 38 in quantitative yield.

2.5 Synthesis of 4-Hydroxymethyl Estradiol: Since the hydroxyethyl 20 and the hydroxypropyl 21 derivatives were synthesized, attention was turned to the synthesis of hydroxymethyl derivative 19. As an extension of the Pd-catalyzed C-C bond forming reactions used for synthesis of 20 and 21, 30 was used for a Pd-catalyzed CO insertion reaction. It was anticipated that 30 would form an aryl-Pd complex that would undergo CO insertion to form aryl-carbonyl-Pd complex 46. Then 46 would be converted into the aldehyde 33 in the presence of a suitable hydride source. The various catalyst, reagent, and temperature conditions attempted for the CO insertions are shown, in Table 2.3. However, none of the reaction conditions yielded any CO insertion product. Even reactions carried out in pressure tubes charged with CO failed to produce any CO insertion product.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Catalyst</th>
<th>Solvent</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO, Vinyltributyltin</td>
<td>Pd(PPh₃)₂Cl₂</td>
<td>Toluene-HMPA</td>
<td>20% 34</td>
</tr>
<tr>
<td>Pressure tube</td>
<td></td>
<td>60/100°C</td>
<td></td>
</tr>
<tr>
<td>CO, tetramethyltin</td>
<td>Pd(PPh₃)₂Cl₂</td>
<td>Toluene-HMPA</td>
<td>No reaction</td>
</tr>
<tr>
<td>Pressure tube</td>
<td></td>
<td>80/100°C</td>
<td></td>
</tr>
<tr>
<td>CO, Bu₃SnH</td>
<td>Pd(PPh₃)₂Cl₂</td>
<td>Toluene-HMPA</td>
<td>Mixture of 25 and starting</td>
</tr>
<tr>
<td>Pressure tube</td>
<td></td>
<td>80/100°C</td>
<td>material</td>
</tr>
<tr>
<td>CO, Slow-addition</td>
<td>Pd(PPh₃)₄</td>
<td>Toluene-HMPA</td>
<td>Mixture of 25 and starting</td>
</tr>
<tr>
<td>of Bu₃SnH</td>
<td></td>
<td>60-90°C</td>
<td>material</td>
</tr>
<tr>
<td>CO, Slow-addition</td>
<td>Pd(PPh₃)₂Cl₂</td>
<td>Toluene</td>
<td>Mixture of 25 and starting</td>
</tr>
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<td>of Bu₃SnH</td>
<td></td>
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<td>material</td>
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<td>CO, Slow-addition</td>
<td>PhCH₂Pd(PPh₃)₂Cl</td>
<td>Toluene-HMPA</td>
<td>Mixture of 25 and starting</td>
</tr>
<tr>
<td>of Bu₃SnH</td>
<td></td>
<td>90°C</td>
<td>material</td>
</tr>
<tr>
<td>CO, Slow-addition</td>
<td>Pd(PPh₃)₄</td>
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</tr>
<tr>
<td>of Bu₃SnH</td>
<td></td>
<td></td>
<td>material</td>
</tr>
</tbody>
</table>

Table 2.3 Conditions Explored for CO Insertion Reaction with 30
In general, aryl iodides are better substrates for CO insertion reaction than aryl bromides. Effort focused on synthesis of a 4-iodoestradiol derivative that could be used for the coupling reaction. Two exploratory CO insertion reactions attempted with MOM protected 4-iodoestradiol derivative proved unsuccessful, so we turned back to the lithiation reaction. As previously indicated, reaction of 4-lithioestradiol with CO₂ formed the corresponding carboxy derivative 38 in excellent yield. Thus, the reaction was repeated but instead of isolating the acid 38, it was treated with diazomethane to form the corresponding methyl ester 47 in 77% yield. Ester 47 was reduced with LiAlH₄ to the corresponding alcohol 48 in 90% yield; refluxing 48 with PPTS in methanol, provided 19 in < 15% yield. The overall yield of 19 was higher if the MOM groups were removed prior to the reduction with LiAlH₄. Refluxing 47 with PPTS in methanol formed 49 in 80% yield. 4-hydroxymethyl was produced from 49 in 64% yield by LiAlH₄ reduction.

![Chemical Structure](image)

**Figure 2.8 Synthesis of 4-Hydroxymethyl Estradiol**

### 2.6 Conclusions:
The three target 4-hydroxyalkyl estrogens were synthesized in good yields for use in biochemical studies. The synthesis of 4-hydroxyethyl and propyl estradiols was accomplished by oxidative hydroboration of 4-alkenyl estradiols, which were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenyl stannane. The key reaction for the synthesis of 4-hydroxymethyl estradiol involved a halogen-lithium exchange reaction to form 4-lithioestradiol. The lithioestradiol was trapped as the 4-carboxyestradiol, and this compound was esterified and reduced to the desired 4-hydroxymethyl estradiol. The Stille cross-coupling reaction
and the carboxymethylation reaction used to synthesize the target compounds represent two efficient, previously unexplored synthetic routes for the regiospecific functionalization of the C-4 position of estrogens. These synthetic strategies have enabled us to synthesize variously functionalized estrogen molecules with interesting biochemical properties. The biological evaluation of the 4-hydroxyalkyl estradiols will be discussed in Chapter 4.
3.0 Synthesis of Catechol Estrogens and Other Biochemically-related Estrogen Analogs

3.1 SYNTHESIS OF CATECHOL ESTROGENS: Estrogen metabolism by cytochrome P450 enzymes is discussed in detail in Section 1.4 (See Figure 1.11 for a general scheme of estrogen metabolism). A major emphasis of this research project was to study the biochemical roles of catechol estrogens. To this end we developed the synthesis of biochemically stable analogs of 2- and 4-hydroxy estradiols. In addition to these analogs, both the catechol estrogens, namely 2- and 4-hydroxyestradiol (2-OHE$_2$ and 4-OHE$_2$, respectively) were required for routine use in biological studies and also as intermediates for synthesis of other estrogen analogs. As a result we investigated a cost-effective chemical synthesis of 2- and 4-OHE$_2$, to provide ready access to large quantities of catechol estrogens required for biological studies. Catechol estrogens are chemically unstable and easily decompose by air oxidation. The chemical instability makes catechol estrogens difficult to synthesize and handle. Stubbenrauch and Knuppen have reported a synthesis of catechol estrogens that relied on an oxidation of ortho- aminophenols to quinones, which were then reduced to the catechols without isolation. These authors began the synthesis by nitration of estrone to give a mixture of readily separable 2- and 4-nitroestrones (50 39% and 51 42% respectively). The nitroestradiols were converted to the corresponding aminoestradiols with sodium hydrosulfite. We found that the nitroestradiols were reduced more efficiently by catalytic hydrogenation (55 psi, 5% Pd-C in methanol). The aminoestradiols were converted into the catechols by a two step reaction sequence carried out in a single flask. In the first step, the aminophenol was oxidized to the corresponding quinone by sodium metaperiodate. This reaction presumably proceeds via a quinonimine intermediate such as 54 (Figure 3.1), which undergoes hydrolysis in the acidic reaction media to form the ortho-quinone 55. The formation of the desired ortho-quinones was hampered by the coupling reactions of the quinonimine intermediates with the unreacted starting material. In order to avoid this complication, the oxidation was carried out in an inverse manner, where in the aminophenol was added to a solution of sodium metaperiodate and the reaction mixture was vigorously stirred while maintaining high dilution conditions. The quinone was extracted in chloroform and reduced with potassium iodide in acetic acid to the corresponding catechol. Using this procedure we expected to form 2- and 4-OHE$_2$ starting from 2- and 4-aminoestradiol, respectively. In our numerous attempts to employ this method, we were unable to reproduce the excellent results reported by the authors for the inverse oxidation of the aminophenols to the corresponding catechol. The procedure was moderately successful for the synthesis of 2-hydroxyestrone (2-OHE$_1$) in 35% yield; however 2-OHE$_1$ obtained herein was contaminated with several unidentified impurities and attempts at recrystallization or chromatography resulted in decomposition of the catechol. 4-OHE$_1$ was obtained in < 15 % yield for the inverse oxidation step. In order to minimize the oxidative complications and ensure the stability of the products upon synthesis, all the synthetic manipulations were attempted in a glove box charged with dry argon; however, this precaution also failed to provide the desired products in the purity and amounts needed.
As the inverse oxidation approach was unsuccessful, alternative approaches for synthesis of catechol estrogens were investigated. Figure 3.2 shows an alternate method for the synthesis of 2-OHE$_2$ that relies on a Dakin oxidation of the 2-acetyl estradiol 56 to form the corresponding catechol. The authors employed a Friedel-Craft's acylation to access...
the 2-acetyl estradiol 56 in moderate yields. Ketone 56 was treated with an alkaline solution of hydrogen peroxide in diglyme, resulting in the formation of 2-OHE₂. A similar strategy was employed for the synthesis of 2-OHE₁. There is no mention of synthesis of 4-acetyl estrogens and the corresponding use in the synthesis of 4-hydroxy estrogens using this method. Several attempts to carefully reproduce the reaction conditions reported in this paper were unsuccessful, and failed to yield the desired catechols. The authors note that the success of the reaction is dependent on a fine balance of reaction conditions, which we were unable to reproduce. Thus our quest for a more reliable and reproducible method for catechol estrogen remained unfulfilled.

Although the Dakin oxidations did not deliver the desired catechols, it was nevertheless an attractive approach and we realized that a mechanistically similar transformation, namely the Baeyer-Villiger oxidation, can be employed for converting the 2- and 4-formyl estrogens into the corresponding catechols. Literature survey revealed that the Baeyer-Villiger oxidation of aromatic aldehydes and ketone by peroxy acids is indeed a widely applicable method for the synthesis of phenols. Organic peroxy acids such as peroxyacetic acid, trifluoroperoxyacetic acid, 4-nitro- and 3,5-dinitroperoxybenzoic acids can carry out this oxidation. Most frequently, meta-chloroperoxybenzoic acid (MCPBA) is employed to effect the transformations. The mechanisms of Dakin and Baeyer-Villiger oxidations are shown in Figure 3.3.
3.1.1 SYNTHESIS OF 2-HYDROXYESTRADIOL: A suitably protected 2-formylestradiol derivative 26 was the starting point for synthesis of 2-OHE₂ using the Baeyer-Villiger chemistry. We had previously prepared 26, however we were unsure as to how the catechols would hold up to the deprotection of MOM groups. We employed benzyl protecting groups because the oxidatively labile catechol estrogens were expected to be stable in the reductive conditions employed for deprotection of benzyl groups. Thus 26 was treated with 6M HCl in THF to provide the free formyl derivative 57 in 98% yield. The formyl derivative was protected to give bis-benzyl estradiol 58 in 65% yield. As we were exploring the Baeyer-Villiger oxidations with 26 and 58, Cushman et al. reported the conversion of 59 into 2-hydroxyderivative 61 with MCPBA in 58% yield.¹⁶ We
found that when a solution of 58 in dichloromethane was treated with MCPBA and p-TSOH at room temperature for 3 hours under argon, a mixture of the formate ester 59 along with the free phenol 60 (approx. ratio 3:1) was isolated after workup and flash chromatography. Refluxing the mixture of 59 and 60 in methanol with 4-5 drops of conc. H₂SO₄ for 3 hours hydrolyzed the formyl ester and the phenol 60 was isolated in over 70% yield for the two steps. Phenol 60 upon catalytic hydrogenation (10% Pd-C, 55 psi hydrogen and THF-MeOH) provided the crude 2-hydroxyestradiol, which was purified using preparative RP-HPLC with 40% CH₃CN/ H₂O as the mobile phase. The purity of the isolated 2-hydroxyestradiol was confirmed by co-elution with a known standard of 2-hydroxyestradiol (Figure 3.5). The yield for the hydrogenation reaction was 75-80%, and the 2-OHE₂ was stored in the freezer without any appreciable decomposition for several months.

\[\text{Figure 3.4 Synthesis of 2-Hydroxyestradiol}\]
In the above synthetic scheme MOM groups are essential for ortho-lithiation and introduction of formyl group at the 2-position. The lithiation at the 2-position is enhanced by the coordinating and directing property of the MOM group as shown in Figure 3.6. Subsequent to formylation, the MOM groups were replaced by benzyl groups to facilitate the removal of protecting groups in a reducing environment. The manipulation of protecting groups undermines the synthetic efficiency of the protocol. We anticipated eliminating the protecting group manipulations by using just one protecting group, namely, the benzylxymethyl group (BOM). BOM group has an oxygen atom in a similar position as the MOM-group to direct lithiation at the 2-position, and additionally the BOM group can be removed by catalytic hydrogenation. Thus, the desirable properties of both MOM and benzyl groups are present in the BOM group. The corresponding BOM protected estradiol 61 was synthesized in moderate yields (45%) using DIPEA and BOMCl in THF. Unfortunately, several attempts at ortho-lithiation with 61 were unsuccessful as treatment with n-butyllithium deprotonated the benzylic hydrogen from the BOM group at the 3-position; thus this approach was abandoned. In subsequent halogenation experiments we have discovered that if bis-benzylestradiol 62 is treated with bromine in AcOH, bromination predominantly occurs at 2-position to yield 2-bromobisbenzyl estradiol 63. 63 can be converted into 2-formyl derivative 57 by treatment with n-BuLi and freshly distilled DMF in excellent yields. This alternative synthesis of 57 eliminates one step in the reaction sequence for 2-OHE₂ and increases the overall yield up to 00%.

Figure 3.7 Alternative Synthesis of 57
3.1.2 Synthesis of 4-Hydroxyestradiol: The key intermediate for 4-OHE\textsubscript{2} synthesis using the Baeyer-Villiger approach is the 4-formyl bisbenzyl estradiol derivative 65. As 2-bromobisbenzyl estradiol 64 could be successfully formylated, we decided to investigate a similar approach for the 4-bromo derivative. 4-Bromoestradiol was converted into 4-bisbenzyl protected estradiol 42. 42 when treated with \textit{n}-BuLi at -78°C followed by freshly distilled DMF and subsequent hydrolysis provided 64 in 44% yield. In an attempt to improve the yields of the formyl derivative other synthetic approaches were investigated. An attempt was made to generate a Grignard reagent at 4-position by treating 42 with Mg\textsuperscript{2+} and 1,2-dibromoethane.\textsuperscript{18} Although we were able to form the Grignard reagent the yields of the formyl derivative obtained by quenching the Grignard with DMF were unimpressive (5-10%). Several reaction conditions similar to those documented in Table 2.3 were attempted with an aim to achieve a CO insertion reaction into the 4-position. However, none of the reaction conditions provided any CO insertion product. Since aryl iodides are better substrates for the CO insertion reaction, the CO insertion reaction was investigated with a suitably protected 4-iodoestradiol derivatives. However the attempted CO insertions were not successful and so this avenue was not further explored. An approach to access the formyl derivative by ozonolysis of 43 also proved unsuccessful.

The formyl derivative 64 was subjected to Baeyer-Villiger oxidation with MCPBA and \textit{p}-TsOH (Figure 3.9). The reaction mixture upon stirring at room temperature turned dark brown in color, the formate ester 65 was isolated from this mixture in 44% yield. Acidic hydrolysis of 65 formed the hydroxy derivative 66 in only 35% yield. Phenol 66 was subjected to hydrogenolysis (5%Pd-C, 55 psi, THF-MeOH), the reaction mixture upon work up provided thick brown oil from which a pale brown precipitate crashed out. NMR analysis of the product revealed presence of 4-OHE\textsubscript{2} along with other unidentifiable impurities. Attempted recrystallization of the residue with acetone and methanol failed and the residue decomposed rapidly upon standing on the bench. This indicates that the 4-OHE\textsubscript{2} is very unstable as compared to 2-hydroxyestradiol, and considerable care has to be taken during its synthesis. It is interesting to note that the 4-formyl- and 4-hydroxy derivatives 64 and 66, respectively, are considerably less stable than the corresponding 2-substituted analogs 58 and 60.

![Figure 3.8 Synthesis of 4-Hydroxyestradiol](image-url)
3.2 SYNTHESIS OF POTENTIAL INHIBITORS OF ESTROGEN HYDROXYLASES: Estrogen hydroxylases are members of the CYP450 family of enzymes that are responsible for converting estrogens into hydroxylated metabolites. Most of the oxidative metabolism of estrogens takes place in the liver; however, some estrogen-metabolizing isoforms of CYP450 are selectively expressed in certain extrahepatic tissues. There are distinct isoforms responsible for effecting hydroxylations at 2- and the 4- position, that result in formation of the catechol estrogens. In humans, CYP 1A2 and 3A are mainly responsible for the hepatic 2-hydroxylation, whereas CYP 3A4 is believed to be responsible for extrahepatic 2-hydroxylation. In contrast to 2-hydroxylation which is the predominant pathway in hepatic tissue, 4-hydroxylation is a dominant metabolite formed in several extrahepatic tissues. CYP 1B1 is an important enzyme responsible for the 4-hydroxylation of estrogens in human breast and uterine tissues. As discussed in Section 1.5, catechol estrogens have important and unique biological actions in several extrahepatic tissues where they are produced. Access to selective inhibitors of the enzymes that are responsible for producing catechol estrogens, are valuable tools in controlling the tissue levels of catechol estrogens and studying their biochemical effects. Additionally, if catechol estrogens are conclusively implicated in estrogen tumor initiation process, compounds inhibiting the formation of catechol estrogens will have important therapeutic applications. With these long-term goals in mind, our lab has previously identified 2,3- and 3,4-methylenedioxy estradiols 67, 68, 2-aminomethyl estradiol 69, and 2-bromoestradiol as competitive inhibitors of estrogen hydroxylases. There is a renewed interest to evaluate these compounds as selective inhibitors of different isoforms of hydroxylases in conjunction with a new non-radioactive assay that is presently being developed in our research group.

**Figure 3.9** Synthesis of A-ring Fused Heterocyclic Estrogens
We have synthesized several additional compounds for evaluation as inhibitors of various isoforms of estrogen hydroxylases. Compounds 70 and 71 were synthesized as one-oxygen analogs of 67 and 68. The synthesis relied on an intramolecular cyclodehydration reaction under Mitsunobu conditions. DEAD was added dropwise to a solution of steroid 20 or 23 and PPh3 in THF and stirred at room temperature. A TLC of the reaction mixture revealed that the starting material was completely consumed within 10 minutes. Indeed, the desired cyclized products were isolated in quantitative yields upon standard workup and flash chromatography. When the hydroxypropyl analogs 21 and 24 were reacted under similar conditions, the corresponding dihydropyranyl analogs 71, 73 were also formed in excellent yields.

![Figure 3.10 Synthesis of 4-Aminoalkylestradiols](image)

In addition to hydroxyalkyl estrogens, aminoalkyl estrogens are active as inhibitors of estrogen hydroxylases. In order to extend the SAR data on this series of compounds and to complement the series of 2-aminoalkyl estrogens synthesized in our lab, we have completed the synthesis of the 4-aminoalkyl estrogens 77, 78 and 79. Treatment of the bisMOM-protected 4-hydroxyalkylestradiols 19, 20, and 21 with phthalimide under Mitsunobu conditions using PPh3 and DEAD yielded derivatives 74, 75, and 76 in 70-80% yields. Subsequent hydrazinolysis in refluxing ethanol formed the bisMOM-protected aminoestradiols, which upon treatment with methanolic HCl gave the desired aminoalkyl estradiols in good yields.

3.2 SYNTHESIS OF 2- AND 4-METHOXYMETHYL ESTRADIOLS: Recently, 2-methoxy estradiol (2MeOE2), an endogenous metabolite of estradiol, was shown to possess cytotoxic properties in cancer cell cultures. 2MeOE2 causes uneven chromosome distribution, faulty spindle formation, and inhibition of DNA synthesis and mitotic arrest.
in cell-culture systems. D'Amato et al. have shown that 2MeOE₂ inhibits in vitro tubulin polymerization by interacting at the colchicine binding site. Interaction of 2-MeOE₂ with tubulin results in a tubulin polymer with altered morphology, and stability. Sato et al. investigated the effects of 30 natural steroids in a Chinese hamster V79 cell line and identified 2MeOE₂ as the most potent microtubule disruptive agent amongst the steroids studied. Interestingly, Fotsis et al. have reported that 2-methoxyestradiol inhibits angiogenesis in vitro and suppresses tumor growth. They suggest that interactions of 2MeOE₂ with tubulin networks of growing vascular cells may be responsible for the angiogenic activity. 2-MeOE₂ can be potentially exploited as a lead structure to develop novel antitubulin and antiangiogenic agents. Indeed, Cushman et al. have looked at a series of 2-alkoxy, 2-alkyl and 2-thioalkyl estrogen derivatives and identified 2-ethoxyestradiol and 2-propenyl estradiol as being more potent inhibitors of tubulin polymerization than 2-methoxyestradiol. The 2- and 4-methoxyestradiols are methylated derivatives of the corresponding catechol estrogens. We thought it would be an interesting idea to methylate hydroxyalkyl estrogens, the analogs of catechol estrogens, and compare the biological activities of the resulting compounds with the methoxyestrogens. The preliminary target compounds that we decided to synthesize were 80 and 81. The synthesis of 81 was straightforward based on the chemistry previously developed in our laboratory. Estradiol, protected as its bis-MOM ether 25, underwent a selective ortholithiation at 2-position, and the resulting aryllithium was quenched with freshly distilled DMF to give the corresponding formyl derivative 26. Reduction of the aldehyde with NaBH₄ in MeOH formed the bis-MOM protected benzylic alcohol 82 in 87% yield. The alcohol was readily methylated with MeI to yield the methoxymethyl derivative 83 in 93% yield, which when refluxed with PPTS in MeOH provided 80 in 83% yield.

While the above approach provided 80 readily, it was not employed for synthesis of 81, primarily due to the inability to synthesize the 4-formyl and 4-hydroxymethyl bisMOM protected estradiol derivatives in high yields. Concurrently, we were investigating the potential of Stille coupling reactions for introducing C-4 alkyl substituents on estradiol. In order to explore the limits of the Stille coupling reaction and to develop one-step synthesis of 81, we decided to synthesize methoxymethyltributyltin 84 was synthesized as a coupling partner for 30. Tributyl tin chloride was reduced with LAH to yield tributyltin hydride (73%), which was purified by vacuum distillation. Treatment of the tributyl tin hydride with LDA, followed by a solution of MOMCl, formed the desired stannane 84, which was isolated in 63% yield after chromatography. Stille couplings between 30 and 84 were attempted in DMF using 2 equivalents of stannane and either Pd(PPh₃)₄ or Pd(PPh₃)₂Cl₂ as the palladium catalyst. The reaction temperatures were initially maintained around 80°C as stability of the stannane was a concern. However, considerable amount of unreacted 30 was isolated from these reactions, indicating that higher temperatures were required for the oxidative insertion of Pd into the aryl-halogen bond. When reaction temperatures were increased to 120°C, all of the starting material was consumed during the reaction. However, in all the reactions attempted, only 30-40% of the desired coupling product 85 was isolated. Dehalogenated estradiol 25 was isolated in almost equal amounts from these reactions along with 5-10% of the 4-butyl bisMOM
estradiol. The lower yields are a reflection of the lower tendency for the transfer of the methoxymethyl group as compared to allyl or vinyl groups from the corresponding tributylstannanes. A solution of hexamethylditin treated with methyl lithium was reacted with MOMCl and the resulting trimethylmethoxymethyltin was isolated after workup and chromatography. It was used in the coupling reaction; however, the yields of 85 did not improve. The protected methoxymethyl derivative 85 was refluxed with PPTS in MeOH to form 81 in 67% yield.

**Figure 3.11** Synthesis of 2- and 4-Methoxymethyleneestradiol
Figure 3.12 Synthesis of 2- and 4-Methoxyestradiols

The methoxymethyl estradiols 80 and 81 and several A-ring substituted estrogen analogs were evaluated in a tubulin polymerization assay. The results of these assays are presented in Chapter 4. Along with other estrogen analogs, we also needed substantial quantities of 2-and 4- methoxyestradiols for these assays and subsequent biochemical studies. 2-Methoxyestradiol was synthesized with modifications to the method described by Cushman et al., and 60 synthesized as an intermediate in the synthesis of 2-OHE2 was methylated with methyl iodide to form the corresponding methylated derivative 86. Debenzylation of 86 by catalytic hydrogenation provided 2-MeOE2 in 82% yield. Over one gram of 2-MeOE2 was synthesized using this method. 4-methoxy estradiol was synthesized from 4-bromoestradiol by a copper-promoted aromatic nucleophilic substitution reaction. A solution of 4-bromoestradiol in DMF was treated with a solution of NaOMe in presence of CuCl2 formed 4-MeOE2 88, which was obtained in 65% yield after chromatography and recrystallization.28
4.0 Biological Evaluation of Estrogen Analogs

4.1 ESTROGEN RECEPTOR BINDING AND GENE EXPRESSION STUDIES: The affinities of the synthetic estradiols were assessed in whole cell estrogen receptor (ER) binding assays using MCF-7 human mammary cancer cells. Previous experience with this assay has indicated that the whole-cell binding assay provides similar relative binding affinities (RBA) for the estrogen receptor as those obtained using isolated estrogen receptor preparations. Additionally, by using a cell-based assay the cellular uptake and stability of the compounds being tested can be evaluated. The EC\textsubscript{50} value for estradiol binding to the estrogen receptor in these whole cell assays was found to be 0.180 nM. The synthetic hydroxyestrogen analog with the highest ER affinity was 19, exhibiting an EC\textsubscript{50} value of 364 nM. Overall, the 4-substituted estradiol homologs exhibited significantly weaker affinity for ER as compared to estradiol with RBAs of 0.49, 0.29 and 0.05 (taking RBA of estradiol as 100) for compounds 19, 20 and 21 respectively. 4-OHE\textsubscript{2} was evaluated for ER binding in this assay and displayed an RBA of 0.36, which is similar to the RBA of the hydroxyalkyl analogs. The relative estrogenic activities of the catechol estrogen analogs were evaluated by examining the abilities of the synthetic compounds to induce ER-dependent gene expression in MCF-7 cells. The induction of transcription of the pS2 gene in human MCF-7 mammary carcinoma cells is a primary response to estrogen exposure. The induction of pS2 mRNA expression by estradiol, 4-OHE\textsubscript{2} and the analogs 19-21 was determined by RNA dot blot analysis. The EC\textsubscript{50} value for estradiol induction of pS2 mRNA was 0.03 nM. The estradiol homologs exhibited activity significantly weaker than that of estradiol for pS2 mRNA induction, with relative activities of 0.257, 0.02 and 0.001 for compounds 19, 20 and 21 respectively. In addition to these assays, the effect of 19 on the growth of hormone-dependent MCF-7 cells was investigated. The mitogenic activity was determined by measuring [\textsuperscript{3}H]thymidine incorporation at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 \textmu M. Alcohol 19 did not affect cellular DNA synthesis in this breast cancer cell line, whereas estradiol at a concentration of 1nM significantly increased MCF-7 cell growth. The ER-binding, pS2 gene expression, and MCF-7 growth assays were performed by the biochemists in our research group and the experimental details of these procedures can be found in the two publications describing this work.

A number of literature reports have shown that 4-OHE\textsubscript{2} is similar to estradiol in its ability to bind and activate the classical ER. Martucci and Fishman reported the binding affinities of catechol estrogens for rat uterine cytoplasmic ER. They found that 2-hydroxylation decreased ER affinity to a greater extent than 4-hydroxylation. The authors showed that 4-OHE\textsubscript{2} had a RBA of 45 and 2-OHE\textsubscript{2} had an RBA of 24 for ER binding; compared to estradiol RBA 100. Merriam and co-workers reported similar results for estrogen receptors in from rat brain, pituitary, and uterus. These findings were mirrored in studies by Kirchoff et al. working with ER from hypothalamus and pituitary cytosol. Dickson and co-workers reported that when present in a 50-fold molar excess, the 2- and 4-OHE\textsubscript{2} are capable of inhibiting [\textsuperscript{3}H]estradiol binding to partially purified cytoplasmic receptor sites. More recently van Aswegen et al. studied the binding of catechol estrogens, using estrogen receptors in cytosol prepared from human
breast cancers. They found that the relative affinity of 2-OHE$_2$ was identical to estradiol, and 4-OHE$_2$ had a RBA approximately 1.5 times higher than estradiol. Thus, our receptor binding data is not in agreement with the previous literature reports; however, it is important to realize that none of the published studies have looked at ER in intact MCF-7 cells. Many estrogen ligands are known to display tissue and species specific binding characteristics and so, the lower ER affinity for 4-OHE$_2$ seen in our assay may be because we are using ER in MCF-7 cells. In addition, all the binding data present in the literature came from assays performed on isolated estrogen receptor preparations as opposed to the whole cell method employed in our experiments. We did find that analogs 19 and 20 exhibited similar ER affinity and induction of pS2 gene transcription as 4-OHE$_2$, and so these compounds can be viewed as chemically stable analogs of 4-OHE$_2$ and used in the experiments probing the involvement of catechol estrogens in tumor formation. However, it remains important to compare 4-OHE$_2$ and the 4-hydroxyalkyl analogs in an ER assay system where 4-OHE$_2$ has previously displayed high RBA, as this would more conclusively prove that these analogs behave like 4-OHE$_2$ in receptor recognition.

4.2 MEASUREMENT OF OXIDATION/REDUCTION POTENTIALS: The redox cycling behavior of catechol estrogens and the homologs was studied using cyclic voltammetry (CV). The oxidation potential was used to determine the likelihood that a compound would be oxidized in the surrounding matrix. The degree of reversibility, expressed as $\Delta E$, was used to indicate the ability of an oxidized product to be reduced back to its initial state. Compounds, which do not exhibit a reduction peak, and compounds with a large $\Delta E$ value, for example, would not be considered reversible and would not be able to participate in redox cycling. The CV studies were performed in an aqueous medium at physiological pH in an attempt to simulate biological environment. The potentiometric measurements indicate that both 2-OHE$_2$ and 4-OHE$_2$ were quasi-reversible with $\Delta E$'s of 55 mV and 60 mV respectively, and with nearly equal half wave potentials ($E_{1/2}$) of 263 ± 10 mV and 265 ± 10 mV vs the NHE, respectively (Table 4.1). This indicates that the catechol estrogens are indeed capable of redox cycling in a physiologic matrix. Differences in oxidation potentials or $\Delta E$ could possibly explain differences in toxicity; however, both 2-OHE$_2$ and 4-OHE$_2$ exhibited nearly equal electrochemical properties under physiological conditions, indicating enzymatic influences are responsible for differential toxicity of these two compounds. The hydroxyalkyl analogs 19 and 22 each exhibited one anodic peak at 595 ± 10 mV and 597 ± 10 mV Vs the NHE respectively (Table 4.1) which was not reduced at the electrode surface. Although the end products were not studied, the CV profile is consistent with a mechanism involving loss of 2 e' with generation of the phenoxonium intermediate followed by hydroxylation primarily in the one position (Figure 4.4b). In order to show that the phenolic group was oxidized, a CV of 2,3-dihydropyranyl-estradiol 73 was obtained with no oxidation peak within the water window (data not shown). The two methoxy metabolites 87 and 88 were nearly identical in their potentiometric behavior. Each compound exhibited one anodic peak ($E_{p,a2}$) that decreased in current with each scan and a second anodic peak ($E_{p,a1}$) followed by one cathodic peak ($E_{p,c1}$) that both increased in current with each scan. This CV was consistent with oxidation of the phenolic moiety followed by irreversible
demethylation and reversible reduction of the resultant quinone (Figure 4.4 c). The Ep,a1 and Ep,c1 peaks were nearly identical to the oxidation and reduction peaks of 22 (Table 4.1). The slight shift in E½ may be due to generation of MeOH at the electrode surface. Therefore, the very rate limiting process of spontaneous demethylation would be necessary before any redox could occur. The half wave potential of the validation standard 4-methyl-catechol agrees within 30 mV of the literature value obtained under similar conditions.

Figure 4.1. CV of 2-Hydroxyestradiol

Figure 4.2. CV of 2-Hydroxymethyl Estradiol 22
Figure 4.3 CV of 2-Methoxy Estradiol

Figure 4.4 (a) Reversible Oxidation/Reduction of 2-OHE₂ (b) Irreversible Oxidation of 2-Hydroxymethyl Estradiol (c) Oxidative Demethylation of 2-Methoxy Estradiol
Peak potentials are expressed in mV

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^a Std. Err. ± 10 mV, ^b Potentials corrected vs. NHE at pH 7.4

Table 4.1 Peak Potentials for Catechol estrogens and Analogs Vs Ag/AgCl at pH 7.4

4.3 8-Oxo-dG Fomation by CE's and CE Analogs with and without Cu(II). The hydroxyalkyl estradiol analogs are being used to understand receptor-mediated and redox-mediated events in estrogen induced tumorigenesis. As discussed in Chapter 1 the catechol estrogens can undergo redox cycling and produce DNA damage. In the preliminary in vitro studies by Mobley et. al, calf thymus DNA was exposed to varying amounts of catechol estrogens and their non-redox cycling counterparts. The DNA was analyzed at specific time intervals using reverse phase HPLC coupled with an electrochemical detector (ECD), and elevated levels of 8-oxo-dG in the DNA samples were measured as a marker of oxidative DNA damage. Calf thymus DNA was exposed to 100 μM of catechol estrogen or analog and ascorbic acid, with and without 100 μM Cu(II)SO₄ for 3 hours (Figure 4.5). Ascorbic acid is known to increase 8-oxo-dG in vitro and was therefore used as a positive control. Cu(II)SO₄ was included in the experiments due to the noted redox coupling with hydroquinones. There was a slight increase in 8-oxo-dG formation over the non-incubated DNA (7.3± 0.9) for the various negative controls which included DNA incubated alone (11.7± 1.0), DNA + 100 μM Cu(II)SO₄ (14.3± 2.0), and the addition of DMSO which had no effect on the system. Increases in 8-oxo-dG over controls were significant for 2-OHE₂ (21.0± 0.6), 4-OHE₂ (15.5± 1.7) and ascorbic acid (31.3± 3.0). The addition of Cu(II) significantly increased 8-oxo-dG levels in the 2-OHE₂ (1189± 119.3), 4-OHE₂ (1256.4± 74.9) and ascorbic acid (1016.3± 89.0) samples over those samples containing no Cu(II). The hydroxyalkyl and the methoxy analogs did not increase 8-oxo-dG levels even with the addition of Cu(II). Although 2-OHE₂ induced significantly more 8-oxo-dG than did 4-OHE₂ when incubated alone, both CE’s generated nearly equal DNA damage when Cu(II) was added. Cu(II) increased the DNA damaging potential of both catechol estrogens by nearly 50 fold, illustrating the great significance of copper on CE toxicity. These results are in good agreement with what would be predicted from the potentiometric data. Numerous studies have shown that catechol estrogens are capable of inducing DNA damage at high
concentrations. However, the minimal concentrations needed to induce such damage often goes unreported. In this study calf thymus DNA was exposed to increasing concentrations of 2-OHE$_2$ (0.1 μM to 100 μM) with and without the addition of 10 μM Cu(II)SO$_4$ for 3 hours (Figure 4.6). The induction of 8-oxo-dG by 2-OHE$_2$ was compared to non-incubated DNA, DNA incubated alone, or DNA incubated in the presence of Cu(II)SO$_4$. High concentrations of 2-OHE$_2$ induced 8-oxo-dG formation over incubated controls (carrier, 11.0±0.2; carrier + Cu(II)SO$_4$, 13.4±0.6) at no less than 100 μM alone (13.2±0.6) and no less than 10 μM in the presence of Cu(II)SO$_4$ (45.7±1.1). The addition of lower concentrations of 2-OHE$_2$ decreased 8-oxo-dG formation to those levels found in the non-incubated control (7.0±0.2) at 1.0 μM (7.6±0.2) and 10 μM (7.6±0.2) when added alone and at 0.1 μM (9.8±0.4) and 1.0 μM (10.6±0.1) with the addition of Cu(II)SO$_4$. These results are reinforced by reports that 2-OHE$_2$ exhibits antioxidant activity in lipid peroxidation studies. Our data indicates that 2-OHE$_2$ is primarily oxidized through a 2 e⁻ transfer mechanism as may be expected from the CV experiments measured under aqueous conditions at pH 7.4. The addition of Fenton catalysts such as copper and the reaction of residual superoxide through a Haber-Weiss mechanism would produce DNA damaging hydroxyl radicals. This would explain why high concentrations of catechol estrogens and the addition of copper are necessary before any oxidative DNA damage occurs.

Figure 4.5. Calf thymus DNA was exposed to 100 μM of CE or CE analog with and without the addition of 100 μM Cu(II)SO$_4$ for 3 hours in PBS (pH 7.4) at 37°C. The induction of 8-oxo-dG was compared to that of the following controls: non-incubated DNA* (C$_1$), DNA incubated alone (C$_2$), and DNA incubated in the presence of Cu(II)SO$_4$ (C$_3$). Data points are means of N=3 ± SD for all samples.
Figure 4.6. Calf thymus DNA was exposed to increasing concentrations of 2-HE₂ (0.1 μM to 100 μM) with and without the addition of 10 μM Cu(II)SO₄ for 3 hours in PBS (pH 7.4) at 37°C. The induction of 8-oxo-dG by 2-HE₂ was compared to that of the following controls; non-incubated DNA* (C₁), DNA incubated alone (C₂), and DNA incubated in the presence of Cu(II)SO₄ (C₃). Data points are means of N=3 ± SD for all samples.
4.4 Inhibition of Tubulin Polymerization: D'Amato et al. have developed specific assay conditions for studying interactions of 2MeOE2 with tubulin. Using these conditions a number of estrogenic analogs were screened in a simple in vitro tubulin polymerization assay. Tubulin solutions were preincubated with the compound to be screened for 15 minutes at 37°C, these mixtures were transferred to cuvettes and chilled on ice. GTP was added to the reaction mixtures and polymerization was initiated by warming to room temperature. The polymerization was followed spectroscopically at 350 nm and the extent of polymerization at the twenty minute time point was compared with the controls to estimate the % inhibition of polymerization. Colchicine and 2MeOE2 were used as positive controls. All the compounds were evaluated at a uniform concentration of 3μM in the assay. Colchicine inhibited polymerization by 64% at 3μM whereas at the same concentration 2MeOE2 and 80 inhibited the polymerization by around 34% and 44% respectively. The extent of inhibition was considerably less when these compounds were evaluated at 1μM concentrations. 4-methoxymethyl estradiol 81 and 4-MeOME2 were inactive in this assay (inhibition <5%). The hydroxyalkyl estradiols exhibited moderate activity (inhibition 15-20%). The results of screening of various estrogens are shown in Table 4.2. This preliminary screening identified 80 as being slightly more effective than 2-MeOE2 in preventing tubulin polymerization. It is important to realize that this is a preliminary screening assay and more experiments to investigate the kinetics of inhibition, determination of IC50 and competitive binding experiments with [3H] colchicine need to be performed to investigate in detail the nature of interaction of 80 with tubulin polymer.

<table>
<thead>
<tr>
<th>Compound (3μM)</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>64%</td>
</tr>
<tr>
<td>2-MeOE2</td>
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<tr>
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<tr>
<td>2-MeOME2</td>
<td>44%</td>
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<tr>
<td>4-MeOME2</td>
<td>No inhibition</td>
</tr>
<tr>
<td>2-Hydroxymethyl E2</td>
<td>15%</td>
</tr>
<tr>
<td>2-Hydroxyethyl E2</td>
<td>24%</td>
</tr>
<tr>
<td>2-Hydroxypropyl E2</td>
<td>12%</td>
</tr>
<tr>
<td>2,3-(2H)-benzofuranyl E2</td>
<td>15%</td>
</tr>
</tbody>
</table>

Table 4.2 Screening of Estrogenic Compounds as Tubulin Polymerization Inhibitors
Cushman et al. have evaluated a series of 2-substituted estrogens as tubulin polymerization inhibitors and have observed that the size of the substituent at the 2-position plays a critical role in determining its interaction with tubulin.\(^{16}\) They found that 2-ethoxy and 2-propenylestradiol were more potent and 2-aminoethyl estradiol was equipotent to 2-MeOE\(_2\) in the tubulin polymerization assay. Based on these observations they concluded that the optimal substituent in the 2-position for inhibition of tubulin polymerization appears to be one with three atoms from the second row of periodic table and which could increase the electron density around the aromatic ring. It is interesting to note that compound 80 identified as a potential inhibitor meets the above criteria. Several research laboratories investigating the mechanisms of cytotoxicity of 2-MeOE\(_2\), have recently pointed out that the cytotoxic effects of 2-MeOE\(_2\) are probably not due to its effects on depolymerization of tubulin. They suggest that 2-MeOE\(_2\) may exert its effects by disruption of tubulin dynamics, or interfering with the dynamics of the mitotic spindle or activating specific signaling pathways leading to apoptosis. It has also been recently noted that treatment with 2-MeOE\(_2\) increases the insoluble polymerized fraction of tubulin similar to Taxol, in contrast to the microtubule depolymerizing drugs such as Cochine and Vinca alkaloids. It would be very interesting to compare the activity of 2-MeOE\(_2\) and analogs like 80 in the various biochemical studies attempting to delineate the mechanisms of apoptic induction by 2-MeOE\(_2\).
5.0 Nonsteroidal Analogs – Benzopyrone Libraries

We found that 2-methoxymethyl estradiol is a more potent inhibitor of tubulin polymerization than 2-methoxy estradiol. Recently, Cushman et al. have shown that 2-ethoxy, 6-oxo or 6-oximino estradiols are potent inhibitors of tubulin polymerization. This report came at about the same time that we were looking into the possibility of transferring the SAR from the estradiol series and making nonsteroidal antitubulin agents. Several molecules with benzopyrone ring system have shown to be ligands for the estrogen receptor. Additionally, there are literature reports of some benzopyrones being active as tubulin polymerization inhibitors. Interestingly if we compare the benzopyrones with Cushman’s 6-oxo estradiols, we can readily see that the 6-oxo group in these inhibitors corresponds with the 3-keto group of the benzopyrones. The benzopyrone ring system provides an ideal template for nonsteroidal inhibitors of tubulin polymerization. We reasoned that the benzopyrone ring system could be exploited to develop novel, potent antitubulin agents. Additional literature survey indicated that the benzopyrone ring system is present in a number of natural products including flavonoids. They form a biologically interesting group of molecules interacting with a number of enzymes and receptors of pharmacological significance. Some of these compounds have shown activity as tyrosine kinase inhibitors, PKC inhibitors, antiinflammatory, antiangiogenic agents, and antiestrogenic agents. These represent important molecular targets for developing new therapies for controlling and treating breast cancer. So we decided to exploit the benzopyrone nucleus on a broader perspective using the modern combinatorial chemistry techniques. The details of the development of this project are given in the following section.

5.1 Synthetic Plan for Benzopyrone Combinatorial Library: The usefulness of Pd-catalyzed carbonylative cyclization in obtaining carbonyl-containing heterocycles such as benzopyrones and quinolones was independently demonstrated by S. Torii and V. Kalinin. Kalinin reacted o-iodophenol 166, and terminal alkynes (2 equivalents) in diethylamine at 120°C under CO (20 atm) atmosphere in the presence of PdCl₂(dppf), to form 2-substituted benzopyrones 168 in yields of 50-81%. S. Torii prepared 2-substituted 1,4-dihydro-4-oxo-quinolines 169 by reacting 2-iodoaniline 167 and either aryl or aliphatic terminal alkynes (2 equivalents) in high pressure CO atmosphere (20 atm CO, 120°C PdCl₂(PPh₃)₂). The yields with aliphatic acetylenes ranged from 60 to 80%, whereas the aryl acetylenes generally provided the cyclized product in > 85% yield. Under modified conditions (1.2 equivalent of alkyne, 60°C under 1 atm of CO in DMF with DBU as the base and Pd(OAc)₂(dppf)₂ as the catalyst), the heteroannulations resulted in mixtures of 6- and 5-membered rings 168 and 170, respectively. Similarly, the five-membered indoxyl derivatives and alkynyl ketone were isolated in a ratio of 5:1 upon heteroanuulation of o-iodoaniline with phenylacetylene under milder conditions (1 mmol alkyne, 1 atm CO, 80°C, 5 mol% Pd(PPh₃)₄, anisole).
The first step in the heteroannulation reaction is an oxidative insertion of Pd (0) into the aryl iodide to form aryl-Pd (II) complex 171; CO inserts into the aryl-Pd complex to form an acyl-Pd complex 172. The terminal alkyne attacks the acyl-Pd complex to form 173, which collapses with extrusion of Pd (0) and generates the alkynyl ketone 174. The mechanism of cyclization of the alkynyl ketone 174 and the factors governing the formation of 5- vs. 6- membered rings are not clearly understood. The outcome of the cyclization and the product distribution is influenced by the nature of the Pd catalyst, the substituents on the alkynyl ketone and temperature and solvent used for the reaction.

**Figure 5.11** Heteroannulation Reactions of o-Iodophenols and o-Iodoanilines
Excellent substituent tolerance and generally mild reaction conditions make the Pd (0) catalyzed C-C bond formation a very powerful tool in the arsenal of a combinatorial chemist. These reactions have been used extensively to construct or diversify combinatorial libraries in both solution and solid phase. However, the heteroannulation reactions discussed above could not be directly employed for combinatorial applications as they suffered from two main drawbacks: (i) forcing conditions such as high temperatures and high pressure CO environment and (ii) lack of control on the outcome of cyclization products. An acyl-Pd complex, such as 172 which is the precursor for formation of alkynyl ketones, can also be generated by oxidative insertion of Pd(0) and into acid chlorides. We reasoned that using salicyloyl chloride instead of o-iodophenol to form an acyl-Pd species would obviate the forcing temperature and CO-pressure conditions required for formation of alkynyl ketones. Also, masking the phenolic hydroxyl with a suitable protecting group would prevent the oxypalladation reactions of alkynyl ketones leading to mixtures of 5-and 6-membered ring systems. The desired benzopyrones could be constructed under separate and controlled conditions that preclude formation of the five membered rings. Thus the proposed synthetic plan to adapt the heteroannulation reactions for use in combinatorial chemistry is depicted in Figure 5.13.
This synthetic approach has several features that make it attractive for combinatorial chemistry applications. Salicylic acids and terminal alkynes are the proposed building blocks for the benzopyrone nucleus. There are over 50 different salicylic acids and over 30 different terminal alkynes commercially available that could be used as diversity inputs. More importantly though, if needed, additional salicylic acids and terminal alkynes can be readily synthesized from simple starting materials using well established chemistry. A key requirement of combinatorial chemistry, i.e. the ready availability of building blocks, is satisfied by this route. The reaction conditions are mild and should be display wide substituent tolerance as is the case with Pd (0) catalyzed reactions. Pd-chemistry is extensively employed in SPOS; the above synthetic route should be easily adaptable for the solid phase. This approach looked promising and so feasibility studies were undertaken to study the reaction conditions in detail.

5.2 PRELIMINARY STUDIES TO INVESTIGATE COUPLING REACTIONS BETWEEN SALICYLOYL CHLORIDE AND TERMINAL ALKYNES: The coupling reactions between salicyloyl chlorides and terminal alkynes were studied using three acid chlorides 177, 178 and 179. Acid chloride 177 was used to study the effect of the free o-hydroxy group on the coupling reaction. Also, it was possible that we could identify reaction conditions that could provide the alkynyl ketones in good yields, without having to protect the phenolic hydroxyl. The silyl protecting group was chosen to mask the phenolic hydroxyl, because the deprotection of silyl groups could be easily achieved without affecting the integrity of the alkynone. The benzyl group was the other choice for a protecting group, although the deprotection of simple benzyl group in the presence of the alkynone could
prove difficult. We were ultimately interested in using photochemically cleavable forms of benzyl protecting groups. This strategy would be especially valuable for solid phase synthesis as the photochemically cleavable benzyl group could be incorporated into the linker and conditions could be developed for cyclative release of the benzopyrones. There are several methods for synthesis of acid chlorides; however, the most commonly employed method involves the conversion of carboxylic acids into acid chlorides by treatment with thionyl or oxalyl chloride in presence of catalytic amount of DMF. Accordingly, salicylic acid could be converted into salicyloyl chloride 177 by treatment with thionyl chloride, the salicyloyl chloride obtained was washed thoroughly with toluene and used for coupling reactions without any further purification. Salicylic acid was readily converted into the bisbenzylated form 180 either by reacting with benzylbromide and KOH in DMF or alternatively by a Mitsunobu reaction with 2 equivalents of benzyl alcohol. The Mitsunobu route was preferred for higher yields and shorter reaction times. The benzyl ester was hydrolyzed by refluxing with NaOH in EtOH to provide 181 in 84% yield after recrystallization from Hexane/EtOAc. 181 was cleanly converted into the corresponding acid chloride 178 using oxalyl chloride. Salicylic acid was converted into the bisTBS protected salicylic acid 182 by reacting with TBSCI and imidazole in DMF. Several attempts to hydrolyze the silyl ester (Table 5.1) to form 183 yielded only salicylic acid. It is possible that 183 formed by hydrolysis of silyl ester undergoes a silyl transfer from the ortho-OTBS group to form 184. This silyl
ester is then easily hydrolyzed to salicylic acid. Salicylic acid was protected with TBDPSCI with imidazole in DMF to form 185. The silyl ester in 185 proved resistant to hydrolysis under milder conditions, while under more forcing conditions only the unprotected salicylic acid was isolated along with unreacted starting material.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Hydrolysis Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AcOH:H₂O:THF (1:1:1) 30°C</td>
</tr>
<tr>
<td>2</td>
<td>AcOH:H₂O:THF (1:1:1) 0°C</td>
</tr>
<tr>
<td>3</td>
<td>K₂CO₃, MeOH, THF 30°C</td>
</tr>
<tr>
<td>4</td>
<td>K₂CO₃, MeOH, THF 0°C</td>
</tr>
<tr>
<td>5</td>
<td>K₂CO₃, MeOH</td>
</tr>
<tr>
<td>6</td>
<td>MeOH, 40°C</td>
</tr>
<tr>
<td>7</td>
<td>LiOH, MeOH:H₂O:THF (1:1:4)</td>
</tr>
<tr>
<td>8</td>
<td>LiOH, CH₂Cl₂, MeOH</td>
</tr>
</tbody>
</table>

**Table 5.1 Conditions for Hydrolysis of Silyester in 183 and 185**

Wissner et al. reported the reaction of tert-butyldimethylsilyl esters with oxalyl chloride in presence of catalytic amounts of DMF as an effective way to generate acid chlorides under neutral conditions. The proposed mechanism for this conversion is shown in **Figure 5.15**. The side products of this reaction namely TBSCI, CO and CO₂ are all volatile and easily removed from the reaction mixture. Oxalyl chloride (1.2mmol) was added to a cold solution of 182 (1 mmol) in dichloromethane with 3 drops of DMF; the resulting solution was stirred for 18 hours with warming to room temperature. When the reaction was quenched with ethanol, ethyl ester 186 was isolated in 88% yield indicating the clean formation of acid chloride.
The acid chlorination protocol was attempted on protected salicylic acid derivatives 187 and 188, and the corresponding ethyl esters 189 and 190 were isolated in > 85% yield. The reaction of 4-hydroxy and 5-bromo salicylic acids with TBSCl and imidazole in DMF failed to provide the bis-silylated derivatives 187 and 188 in good yields. The compounds could be synthesized in quantitative yields by carrying out the silylation with
TBSCI in dichlormethane using Et$_3$N as the base. This silylation protocol provided the bissilylated derivatives in quantitative yields in all the examples attempted.

5.3 COUPLING REACTIONS OF SALICYLOYL CHLORIDES AND TERMINAL ALKYNES: Acyl halides are reactive compounds and react with nucleophiles even without a catalyst. However, when treated with Pd (0) catalyst, they are further activated for nucleophilic attack by formation of an acyl-Pd species. There are a few reports of reactions of alkynyl nucleophiles with acyl halides to form alkynyl ketones. The choice of reaction conditions for such acylations is crucial, because the alkynyl ketones formed in the reaction have comparable reactivity to the acyl halides and form side products such as tertiary carbinol and Michael adducts. In general, Zn, Sn and Cu acetylides are used for these reactions and are presumably too unreactive for further reaction with the product ynone, and give little tertiary alcohol formation. One of the commonly used methods is to react Zn-acetylides with acid chlorides, and this reaction is found to be much faster and better yielding when catalyzed by Pd(PPh$_3$)$_4$ or Pd(PPh$_3$)$_2$Cl$_2$. Alkynes are treated with butyllithium to generate lithio-alkynes, which when treated with anhydrous zinc chloride provide the alkynyl zinc reagents. (1-Alkynyl)-tributyl stannanes are also used for

\[ R'-\equiv-M + R'Cl \rightarrow R'-\equiv-Zn/Cu/SnR_3 \]

Alkynylketone

**Coupling of Acid chlorides with alkynyl metals**

\[ R'\equiv-H \rightarrow n-BuLi \quad [R'\equiv-Li] \quad \text{ZnCl}_2\text{or CuI} \quad \text{or R}_3\text{SnCl} \rightarrow R'-\equiv-Zn/Cu/SnR_3 \]

**Sonogashira Cu-Pd catalyzed coupling of terminal alkyne and acid chloride**

\[ R'-\equiv-M + R'\equiv-Cl \rightarrow \text{Pd(PPh}_3)_2\text{Cl}_2 \quad \text{CuI} \rightarrow R'-\equiv-R' \]

*Figure 5.16 Reactions of Acid Chlorides and Terminal Alkynes* generating alkynyl ketones from acid chlorides under Pd catalysis. The alkynyl stannanes are prepared by reaction of alkynyl lithium with tributyltin chloride. A serious limitation for use in combinatorial chemistry is that a wide range of alkynyl tin reagents are not readily available for use as building blocks. Condenstion of copper(I) salts of alkynes with acylhalides also provides a useful synthesis of alkynyl ketones. However, the preparation of Cu-acetylides is relatively troublesome, especially on a large scale. All these procedures are more involved than the Sonogashira copper-palladium catalyzed
coupling of terminal alkynes with acid chlorides. In this method terminal alkynes and acyl halides are coupled in Et$_3$N in presence of CuI and Pd(PPh$_3$)$_2$Cl$_2$ as catalysts to provide alkynyl ketones in good yields. The advantage of this method is that it is a one

\[
\text{O} \quad \text{Cl} + \quad \text{O} \quad \text{Cl} \quad \text{Pd$_2$(dba)$_3$} \quad \text{Trioctylamine} \\
\text{177} \quad \text{194} \quad \text{Anisole} \quad \text{191}
\]


**Figure 5.17 Attempted Coupling of Salicyloyl Chloride with Phenylacetylene**

step condensation reaction and employs mild reaction conditions. For reasons of operational simplicity, this approach was most attractive for application in combinatorial chemistry. There is one literature example of a salicyloyl chloride coupling with phenyl acetylene. Chiusoli et al. observed that reaction of salicyloyl chloride with phenylacetylene in trioctylamine at 50°C for six hours with Pd$_2$(dba)$_3$ as the catalyst, provided the alkynyl ketone in 56% yield. Interestingly, aurone and flavone were also isolated in yields of 19% and 14%, respectively. These authors report that an attempt to effect the coupling using Sonogashira method was unsuccessful. The reaction outcome and product distribution is influenced by the free phenolic hydroxyl in the ortho position. We attempted the coupling of 177 with phenyl acetylene using various conditions shown in Figure 5.17. The alkynyl ketone was isolated in <15% yield.
using Pd$_2$(dba)$_3$ as catalyst in Et$_3$N. An attempted Stille reaction with allyltributyltin also failed to give any coupling product.

The coupling experiments using 177 emphasized the need for protecting the phenolic hydroxyl during the coupling reaction. We examined the coupling reactions between phenyl acetylene and acid chlorides 178 and 179, using the Sonogashira coupling method. Acid chloride (1mmol) in Et$_3$N was treated with 1 equivalent of phenyl acetylene, 1 mg of Cul and 1 mg of Pd(PPh$_3$)$_2$Cl$_2$. The reaction was stirred for 15 hours under argon, and products isolated after workup and flash chromatography. The reaction with 179 provided the alkynyl ketone 203 in >80% yield. Reaction of 178 provided a mixture of products that were inseparable on a flash column. NMR study of this mixture revealed the presence of the desired ketone along with debenzylated, decarbonylated products and unidentified impurities. The alkynyl ketone was not formed when the coupling reaction of 178 and 179 with phenylacetylene was attempted with only the copper salts (Cul or CuCl) in absence of Pd catalyst. In addition to Pd(PPh$_3$)$_2$Cl$_2$, the coupling of 178 and phenylacetylene was attempted with Pd(PPh$_3$)$_4$ and PhCH$_2$Pd(PPh$_3$)$_2$Cl$_2$ as the catalysts. However, complex and unidentifiable product mixtures were obtained upon work up and chromatography in both these cases. In order to explore the functional group tolerance on the alkyne component, 179 was coupled with alkynes 196, 198, and 199 using the conditions used for coupling phenylacetylene. These couplings failed to provide the expected alkynyl ketones. None of the products isolated from these reaction mixtures could be clearly identified. Several different reagent-ratios, solvent and catalyst combinations were attempted using 179 and 199 as the coupling partners. It was noted that by using 3 equivalents of 199 for 1 equivalent of 179 with Pd(PPh$_3$)$_2$Cl$_2$/Cul in Et$_3$N the alkynyl ketone 208 was isolated in 45% yield along with homocoupled alkyne 210. Literature survey revealed that homocoupling of alkynes by Pd/Cu catalyst system is catalyzed in presence of molecular oxygen. Argon gas was bubbled to deoxygenate the reaction mixture and minimize the alkyne homo-coupling product. When this precaution was taken, the alkynyl ketone 208 was isolated in 84% yield. Additional experimentation with aliphatic and aromatic alkynes revealed that using 7 equivalents of aliphatic or 4 equivalents of aromatic alkynes in conjunction with Pd/Cu catalysts and Et$_3$N as the solvent proved to be the optimal reaction conditions. The results of the coupling reactions of 179 with different terminal alkynes are shown in Figure 5.18.
The alkyne couplings with 179 provided the desired alkynyl ketones in good yields. The reaction is tolerant of a variety of functional groups on the alkyne, as can be seen from the successful couplings with alkynes 194-200. The terminal alkynes 201 and 202 failed to provide the coupling products. The free double bond in 202 probably interacts with the Pd catalyst and participates in the coupling reactions. The reaction mixture instantly turned dark black when the alkyne 201 was added, and a precipitate was formed within a few minutes. The alkyne being conjugated to an electron-withdrawing group probably

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**Figure 5.18 Coupling of Terminal Alkynes with 179**

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*Isolated yields after workup and flash chromatography*
leads to Michael addition products of the organic base on the propargyl ester. Functional
groups in the carbonyl oxidation state can be used for coupling if protected as acetals, as
is the case in the alkyne 197.

The coupling reactions of substituted salicyloyl chlorides 211-215 were examined in
order to investigate the functional group compatibility of the salicylic acid component. 4-
methoxysalicylic acid was prepared in 54% yield by methylation of 2,4-dihydroxy
benzoic acid using dimethylsulfate. 5-phenylsalicylic acid was prepared by an aqueous
Suzuki coupling of 5-bromosalicylic acid and phenyl boronic acid in the presence of
Pd(OAc)$_2$ with Na$_2$CO$_3$ as the base. All the free salicylic acids were converted into
their bis-(t-butyl-dimethylsilyl) forms with TBSCl and Et$_3$N in CH$_2$Cl$_2$, in quantitative
yields. The results from these couplings are shown in Figures 5.19-5.22.

![Chemical structure](image)

**Coupling of 211 with terminal alkynes**

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* Isolated yields after workup and flash chromatography

**Figure 5.19 Sonogashira Couplings of 211**
Figure 5.20 Sonogashira Couplings of 212

Coupling of 212 with terminal alkynes

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<tr>
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<td>( = )</td>
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<td>( = )</td>
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* Isolated yields after workup and flash chromatography
Coupling of 213 with terminal alkynes

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<td>196</td>
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<td>198</td>
<td>239 (78%)</td>
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* Isolated yields after workup and flash chromatography

Figure 5.21 Coupling of 213 and 215 with Terminal Alkynes

Coupling of 214 with terminal alkynes

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<tbody>
<tr>
<td>194</td>
<td>240 (83%)</td>
</tr>
<tr>
<td>195</td>
<td>241 (40%)#</td>
</tr>
<tr>
<td>196</td>
<td>242 (82%)</td>
</tr>
<tr>
<td>198</td>
<td>243 (64%)</td>
</tr>
</tbody>
</table>

* Isolated yields after workup and flash chromatography
The presence of halogens on the aromatic ring in salicylic acid presented some problems for the coupling reaction. Generally, aryl chlorides do not react with alkynes under the Sonogashira coupling conditions; however, coupling of 214 with toluyl alkyne 195 resulted in a mixture of products 241 and 241a. The chloro group being para to the acid chloride is activated for coupling with a fairly electron-rich alkyne like 195. When Cl is present in the 5-position (meta to the acid chloride) as in 215, it does not participate in the coupling reaction. When 5-bromosalicyloyl chloride derivative 216 was coupled with
The product isolated was a mixture of 216 and 216a. Lowering the temperature of the reaction to 0°C did not prevent the bromo group from coupling to the alkyne.

The reaction tables in Figure 5.18-5.22 demonstrate that the one-pot acid chlorination of silylesters of salicylic acids and subsequent coupling with terminal alkynes provides the alkylnyl ketones in good yields. The reaction sequence shows good functional group tolerance on both the salicylic acid and alkyne components. The alkylnyl ketones were found to be relatively unstable upon standing at room temperature and started decomposing with desilylation reactions. The stability of the alkylnyl ketones is greater if they are stored in the freezer after chromatography. The reactions are very clean, use a very small amount of Pd catalyst and copper iodide (1 mg/ mmol of the alkyne). The Pd catalyst used is easily prepared from PdCl₂, is relatively cheap and is stable for long term storage.

In addition to using terminal alkynes, the coupling of acid chlorides with alkenyl stannanes was investigated. Pd-catalyzed coupling of 179 with allyltributyl tin was investigated. Several catalyst and solvent combinations were examined as shown in Figure 5.23. None of the standard Stille coupling conditions reported for coupling of acid chlorides with alkenyl stannanes worked in this case. The coupled products 248 and 249 could be obtained in good yield only when CuI was used as a cocatalyst according to conditions described by Ye et al. (Figure 5.24). The conditions identified for coupling of stannanes should provide an alternate route to introduce diversity during combinatorial library design.

Figure 5.23 Stille coupling with 178
5.4 Cyclizations of alkynones to the benzopyrone ring system: As the one-pot acid chlorination/ Sonogashira coupling provided the o-(t-butyldimethylsilyloxy)phenyl alkynyl ketones in good yields, the cyclization reactions to form the benzopyrone ring system were investigated. The nucleophilic attack of the unmasked phenolic hydroxyl on the alkynone could proceed by two routes, a 5-exo-dig pathway to provide 5 membered aurones or the 6-endo-dig mode to provide the benzopyrones. Both these cyclization modes are "favored" according to Baldwin's cyclization rules. The original cyclization rules postulated an acute approach angle of about 60° in dig systems and stated that the endo-dig closures are generally preferred, rather than the exo-dig ones, for the formation of five and six membered rings. However, subsequent experimentation suggested that in the case of electronically unbiased acetylenes, exo-dig cyclizations are actually favored.

Miranda et al. studied the cyclizations of o-hydroxyaryl phenyl ethynyl ketones 250. They found that suitable variation of reaction conditions provides a certain degree of control over the direction of cyclization (6-endo-dig vs. 5-exo-dig). These authors showed that when K₂CO₃ in refluxing acetone was used for cyclization, the 6-endo-dig cyclization was the preferred pathway leading to formation of benzopyrones. However, when NaOEt in ethanol or K₂CO₃ in ethanol was used to effect cyclization, the 5-exo-dig cyclization mode was preferred and the aurones were the only products formed. These observations were explained on the basis of the stability of the vinyl carbanion intermediates 251 and 252. The authors reasoned that the vinyl carbanion 252 is the more stable, thermodynamic product whereas the vinyl carbanion 253 is the kinetically favored, less stable species. 253 is preferentially formed in presence of a protic solvent.

![Figure 5.24 Cyclization of o-Hydroxyphenyl Alkynyl Ketones](image)
and picks up a proton in producing the aurone; however, in absence of a protic solvent 253 is unstable and undergoes a β-elimination to the phenolate ion 251. Thus, under aprotic conditions the thermodynamically stable vinyl carbanion 252 is the dominant species that stays around to form the benzopyrones. The authors noted that the flavones are readily deprotonated by LDA in THF at -78°C, giving a 3-lithio derivative that is stable at that temperature. Aurones, on the other hand, react sluggishly with LDA and suffer a ring opening to form the phenolate ion, demonstrating the low stability of vinyl carbanions like 253. More recently Saito et al. studied the cyclization of o-hydroxy phenyl alkynyl ketones as a part of synthetic studies aimed toward antitumor antibiotic

\[ \text{Figure 5.25. Cyclization Experiments by Saito.} \]

Kapurimycin A3 and its analogs, and came up with the same conclusions as Miranda et al. These researchers generated a phenoxide ion under aprotic conditions in situ by desilylation of o-silyloxyphenyl ethynyl ketones with KF and 18-crown-6 in DMF. Under these conditions, the cyclization proceeded to give exclusively the benzopyrones in excellent yields. However when protic solvents were present during cyclization, analogous to findings of Miranda et al., the 5-exo-dig pathway was preferred and the aurones were formed predominantly. The authors explain these observations using similar reasoning as Miranda et al. by invoking the stability of the intermediate vinyl carbanions. As Saito et al. used alkynyl ketones similar to the ones we had synthesized, it was decided to explore the cyclizations of the alkynyl ketones using the method described by these authors. The results of cyclization using KF/18-crown-6 in anhydrous DMF are shown in Table 5.2. In all these cases, only the six membered benzopyrones were formed, and no aurones were isolated.
<table>
<thead>
<tr>
<th>No.</th>
<th>Alkynone</th>
<th>Benzopyrone</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>203</td>
<td>262</td>
<td>85%</td>
</tr>
<tr>
<td>2</td>
<td>204</td>
<td>263</td>
<td>60%</td>
</tr>
<tr>
<td>3</td>
<td>205</td>
<td>264</td>
<td>56%</td>
</tr>
<tr>
<td>4</td>
<td>206</td>
<td>265</td>
<td>75%</td>
</tr>
<tr>
<td>5</td>
<td>207</td>
<td>-</td>
<td>Decomposition</td>
</tr>
<tr>
<td>6</td>
<td>208</td>
<td>267</td>
<td>75%</td>
</tr>
<tr>
<td>7</td>
<td>222</td>
<td>269</td>
<td>88%</td>
</tr>
<tr>
<td>8</td>
<td>223</td>
<td>270</td>
<td>64%</td>
</tr>
<tr>
<td>9</td>
<td>233</td>
<td>276</td>
<td>72%</td>
</tr>
<tr>
<td>10</td>
<td>235</td>
<td>-</td>
<td>Decomposition</td>
</tr>
</tbody>
</table>

Table 5.2 Cyclization of Alkynones with KF/18-C6

The KF/18-Crown-6/DMF method worked fairly well for the cyclization of the alkynyl ketones. The yields were acceptable in most cases attempted, although the alkynones 207 and 235 could not be cyclized with this method. In addition to this method, we were interested in identifying alternative cyclization protocols that would not only be higher yielding but also provide an opportunity to introduce functional groups at the 3-position. We reasoned that, if the alkynones were first converted to enaminoketones such as 258 and then subjected to TBS deprotection, the system would be prone to undergo Michael addition followed by elimination of secondary amine to exclusively yield the benzopyrones. This pathway would effectively eliminate the 5-exo-dig cyclization option. In addition, the enaminoketone functionality would make it possible to introduce functional groups at the 3-position.67
Figure 5.26 Proposed Cyclization via Enaminoketone Intermediates

Diisopropylamine reacted sluggishly with an ethanolic solution of 203, so the reaction mixture was refluxed for 24 hours and after workup the enaminoketone 259 was isolated in quantitative yield. However, when a solution of 203 was refluxed with dimethyl and diethyl amine, the benzopyrone 262 was isolated in 75% yield, instead of the enaminoketones. TLC analysis revealed that the starting material was consumed within two hours and subsequent NMR analysis of the reaction mixture after 10 hours of reflux revealed a mixture of enaminoketone and benzopyrone.

Figure 5.27 Formation of Enaminoketones from Alkynones

Pure samples of enaminoketones 260 were prepared by stirring an alcoholic solution of the alkynone with excess secondary amine (5-10 equivalents) for up to two hours. These
enaminoketones, when heated to 45°C in methanol, formed the benzopyrones. Five equivalents of diethylamine were added to a solution of alkynone 261 in ethanol. After starting material had disappeared within 45 minutes, excess diethylamine was removed under vacuum and the reaction mixture was filtered through a short pad of silica gel. NMR analysis of the residue revealed a single isomer of the enaminone (Figure 5.28 A). This residue was suspended in ethanol and refluxed. After 10 hours a small sample of the reaction mixture was concentrated, and NMR analysis of this concentrate revealed a mixture of enaminone and benzopyrone (Figure 5.28 B). The enaminone was refluxed for a total of 18 hours, and the isolated product was the benzopyrone 264 (Figure 5.28 C). This cyclization method provides a simple way to convert the α-hydroxy phenylalkynyl ketones into 6-membered benzopyrones without forming the competing aurones. In general solutions of alkynones in methanol or ethanol were treated with 5-10 equivalents of secondary amine. When TLC indicated that the starting material had disappeared, the excess secondary amine was removed from the reaction mixture under vacuum and the residue was resuspended in methanol and stirred at 45°C for about 15 hours. The reaction mixture was concentrated and benzopyrones were isolated in excellent yields after flash chromatography. Results of cyclization of a series of alkynones using diethlymine are shown in Table 5.3. Pilot experiments with alkynone 203 showed that different secondary amines, such as dimethylamine (2M solution in THF), N-benzylethylamine and pyrrolidine, gave identical cyclization results.
<table>
<thead>
<tr>
<th>No.</th>
<th>Alkynone</th>
<th>Benzopyrone</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>203</td>
<td>262</td>
<td>92%</td>
</tr>
<tr>
<td>2</td>
<td>204</td>
<td>263</td>
<td>95%</td>
</tr>
<tr>
<td>3</td>
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<td>96%</td>
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<td>206</td>
<td>265</td>
<td>78%</td>
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<td>5</td>
<td>207</td>
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<td>6</td>
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<tr>
<td>7</td>
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<td>8</td>
<td>223</td>
<td>270</td>
<td>88%</td>
</tr>
<tr>
<td>9</td>
<td>224</td>
<td>271</td>
<td>74%</td>
</tr>
<tr>
<td>10</td>
<td>225</td>
<td>272</td>
<td>89%</td>
</tr>
<tr>
<td>11</td>
<td>226</td>
<td>273</td>
<td>80%</td>
</tr>
<tr>
<td>12</td>
<td>231</td>
<td>274</td>
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</tr>
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<td>13</td>
<td>233</td>
<td>276</td>
<td>92%</td>
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<tr>
<td>14</td>
<td>236</td>
<td>277</td>
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<td>15</td>
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<td>278</td>
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<td>16</td>
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<td>279</td>
<td>83%</td>
</tr>
<tr>
<td>17</td>
<td>247</td>
<td>280</td>
<td>76%</td>
</tr>
</tbody>
</table>

**Table 5.4** DEA Cyclizations of Alkynones
6.0 References


protected phenols using directed-Ortho-metalation reaction, followed by quenching with CO₂.


7.0 Appendix

7.1 Manuscripts (copies attached):


7.2 Degree Awarded (copy of title page and tables of contents attached):

Abhijit S. Bhat, Ph.D. Dissertation, Medicinal Chemistry  
The Ohio State University, Summer Quarter 1999

7.3 Abstracts and Presentations:


Synthesis and Biological Evaluation of 4-(Hydroxyalkyl)estradiols and Related Compounds

Carl J. Lovely, Abhijit S. Bhat, Holly D. Coughenour, Nancy E. Gilbert, and Robert W. Brueggemeier

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and the OSU Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210
Synthesis and Biological Evaluation of 4-(Hydroxyalkyl)estraediols and Related Compounds

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Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and the OSU Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210

Received March 13, 1997

A series of synthetic estrogens containing hydroxyalkyl side chains at the C-4 position of the A ring were designed as metabolically stable analogs of 4-hydroxyestradiol, a catechol estrogen. These synthetic steroids would facilitate investigations on the potential biological role of catechol estrogens and also enable further examination of the structural and electronic constraints on the A ring in the interaction of estrogens with the estrogen receptor. Catechol estrogens are implicated as possible causative agents in estrogen-induced tumorigenesis. 4-Hydroxyestradiol has weaker affinity for the estrogen receptor and exhibits lower estrogenic activity in vivo; on the other hand, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates. This report describes the synthesis and initial biochemical evaluation of 4-(hydroxyalkyl)estrogens and 4-(aminoalkyl)estraediols. The 4-(hydroxyalkyl)estrogens were prepared by oxidative hydroboration of 4-alkenylerstradiols. The alkenyleratestradiols were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkenylstannane. The (4-aminoalkyl)estrogens were prepared from the hydroxyalkyl derivatives with phthalimide under Mitsunobu conditions. The substituted estrogens were evaluated for estrogen receptor binding activity in MCF-7 human mammary carcinoma cells, and 4-(hydroxymethyl)estradiol 1 exhibited the highest affinity with an apparent EC₅₀ value of 364 nM. The relative activities for mRNA induction of the pS2 gene in MCF-7 cell cultures by the 4-(hydroxyalkyl)estrogens closely parallel the relative binding affinities. 4-(Hydroxymethyl)estradiol 1 did not stimulate the growth of MCF-7 cells at concentrations up to 1 μM. Thus, 4-(hydroxymethyl)estradiol 1 exhibited similar estrogen receptor affinity as the catechol estrogen, 4-hydroxyestradiol, and may prove useful in the examination of the biological effects of 4-hydroxyestrogens.

Introduction

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle, reproduction, and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Currently, one out of nine American women will develop breast cancer in her lifetime. Approximately 60% of all breast cancer patients have hormone-dependent breast cancer, with these cancers characterized as containing estrogen receptors and requiring estrogen for tumor growth. The possible biochemical roles of estrogens in the development of breast cancer remain to be fully elucidated.

Epidemiological studies have shown that women with breast cancer have higher estrogen levels than healthy control women and that estrogen levels are higher in populations characterized by high breast cancer rates. An estimated 60–70% of human breast cancers are associated with sex hormone exposure. The fact that an early menarche and a late menopause are important risk factors for breast cancer suggests a role of the female sex hormones in the etiology of the disease. Also, studies in experimental animals have shown estrogens to induce tumors in hormone-responsive tissues like mammary tissue, uterus, cervix, and pituitary. Although estrogens have been implicated as carcinogens, the exact biochemical mechanisms by which estrogens may be tumorigenic remain to be established.

Catechol estrogens, oxidative metabolites of estrogens, have been suggested as possible causative agents in estrogen-induced tumorigenesis. Estrogens are converted to 2-hydroxy and 4-hydroxy derivatives by cytochrome P-450 hydroxylases. Both 2-hydroxyestradiol and 4-hydroxyestradiol have weaker affinity for the estrogen receptor than estradiol and exhibit significantly lower estrogenic activity in vivo. However, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates like quinones, semiquinones, and arene oxides. These highly reactive moieties may be cytotoxic via reaction with proteins and nucleic acids. Furthermore, the catechol estrogens have been shown to produce a variety of reactive oxygen species (ROS), such as the hydroxide, peroxide, and superoxide radicals. These ROS have shown cytotoxic and genotoxic effects in several independent studies. Contrasting reports exist in the literature in regard to the tumorigenic potential of 2-hydroxyestradiols vs 4-hydroxyestradiols. Liehr et al. recently reported that microsomes prepared from human mammary adenocarcinoma and fibroadenoma have predominantly 4-hydroxylation activity, suggesting a mechanistic role of 4-hydroxyestradiol in tumor formation. An earlier report demonstrated that 4-hydroxyestradiol formation is predominant in tissues susceptible to estrogen-induced tumorigenesis like Syrian hamster kidney and...
rat pituitary, whereas 2-hydroxyestradiol formation is predominant in rodent livers where tumors are not produced under similar conditions. In contrast, Li and Trush found that 2-hydroxyestradiol produced oxidative damage and strand breaks of double-stranded DNA in the presence of micromolar concentrations of Cu(II), whereas 4-hydroxyestradiol failed to produce any DNA damage.

In order to investigate the role of estrogen metabolites in tumor initiation and progression, we have designed, prepared, and reported on a series of 2-hydroxyalkyl derivatives. The receptor binding and gene expression potential of these synthetic analogs closely parallels that of 2-hydroxyestradiol. Additionally, these compounds are not able to undergo oxidative metabolism at the 2-position. As a continuing part of this study, we have now prepared the corresponding 4-(hydroxyalkyl)estradiols 1-3. These compounds were designed to provide 4-hydroxy-substituted estrogens that are not able to undergo further oxidative metabolism. On the other hand, compounds 1-3 do contain hydroxyl groups at the 3- and 4-positions that are available for hydrogen bonding during protein interactions with receptors and/or enzymes. The 4-(aminoalkyl)estrogens, compounds 4-6, were also synthesized from the hydroxyalkyl derivatives to further elucidate electronic factors at the C-4 position that influence biological activity. Therefore, these analogs may prove useful as chemical probes for differentiating receptor-mediated vs redox-mediated events in estrogen-induced tumorigenesis. The synthesis and initial biochemical evaluation of these 4-hydroxyestradiol metabolite analogs are reported in this paper.

**Results and Discussion**

**Chemistry.** In our earlier work, the 2-(hydroxyalkyl)estradiols were prepared via homologation of a protected 2-formylestradiol 7. Pert and Ridley have previously demonstrated that the analogous 4-formylestradiol 8 could be prepared from 10 by lithium-halogen exchange and subsequent reaction of the organolithium with DMF. Unlike the preparation of 7, wherein yields in excess of 80% were routinely realized, only modest yields of 8 could be obtained. As this synthetic intermediate would be required in large quantities, the homologation of 8 was not considered to be the optimal route available for the preparation of 1-3. Alternatively, the bisMOM-protected 4-bromoestradiol 10 was envisioned to be a suitable partner for a Stille cross-coupling reaction. Introduction of an appropriate unsaturated group, vinyl or allyl, would afford the hydroxyethyl and hydroxypropyl derivatives, respectively, after hydroboration and oxidation.

The synthesis commenced by brominating estradiol with N-bromosuccinimide in ethanol (Scheme 1), from which the required 4-bromoestradiol 9 precipitated and was obtained in 54% yield after recrystallization. The bromoestradiol was protected in 75% yield as its bisMOM ether 10 with chloromethyl methyl ether, diisopropylethylamine in THF at reflux. Using vinyltributyltin as the alkenyl donor, exploratory experiments were performed to determine the optimal reaction conditions required for the cross-coupling reaction. Thus, reaction of 10 with tetrakis(triphenylphosphine)-palladium(0) in DMF afforded the desired 4-vinyl-bisMOM-estradiol 11 in 90% yield after heating at reflux overnight. Under similar reaction conditions, 10 was treated with allyltributyltin, affording 4-allyl-bisMOM-estradiol 12 in 94% yield. Using well-established chemistry, the unsaturated estradiols 11 and 12 were converted into alcohols 2 and 3. Thus, hydroboration of 11 with BH₃·THF, followed by oxidative workup of the alkylborane with basic hydrogen peroxide, gave the desired alcohol 13. The allylestradiol 12 was transformed into 14 in a similar fashion in 75% yield. Subsequent treatment of alcohols 13 or 14 with pyridinium p-toluenesulfonate (PPTS) gave the targeted triols in 70% and 61% yields, respectively.

An attempt was made to prepare the 4-formylestradiol 8 by way of a Stille-like reductive carbonylation as a prelude to preparing alcohol 1. Treatment of 10 with carbon monoxide, tributyltin hydride, and tetrakis(triphenylphosphine)palladium(0) in DMF at reflux failed to yield 8. A control reaction in which 8, prepared by the Pert and Ridley method, was heated for several hours in refluxing DMF demonstrated that it was thermally labile. Indeed, a sample of 8 deteriorated simply on standing at room temperature for a few days.

In view of the instability of 8, alternate routes for the preparation of other related derivatives were developed. Attempts have been made by Pert and Ridley to introduce an ester group by trapping the organolithium, generated from 10 and n-BuLi with alkyl chlorofor-mates; these reactions were unsuccessful. Treatment of 10 with organolithium (vide supra) and carbon dioxide, followed by acidification and subsequent esterification with diazomethane, yielded the methyl ester 15 in 75% yield (Scheme 2). The MOM protecting groups were removed using PPTS in methanol at reflux in 88% yield. Subsequent reduction of the ester 16 with lithium aluminum hydride gave the benzyl alcohol 1 in 51% yield.

The preparation of the 4-substituted amines was accomplished using chemistry similar to that employed for the 2-substituted analogs previously reported. Pert and Ridley have previously demonstrated that the analogous 4-formylestradiol 8 could be prepared from 10 by lithium-halogen exchange and subsequent reaction of the organolithium with DMF. Unlike the preparation of 7, wherein yields in excess of 80% were routinely realized, only modest yields of 8 could be obtained. As this synthetic intermediate would be required in large quantities, the homologation of 8 was not considered to be the optimal route available for the preparation of 1-3. Alternatively, the bisMOM-protected 4-bromoestradiol 10 was envisioned to be a suitable partner for a Stille cross-coupling reaction. Introduction of an appropriate unsaturated group, vinyl or allyl, would afford the hydroxyethyl and hydroxypropyl derivatives, respectively, after hydroboration and oxidation.

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The preparation of the 4-substituted amines was accomplished using chemistry similar to that employed for the 2-substituted analogs previously reported.
**Scheme 1**

- **Reagents and conditions:**
  1. N-bromosuccinimide, EtOH, 54%;
  2. MOMCl, i-Pr₂NEt, THF, Δ, 75%;
  3. Pd(PPh₃)₄, CH₂=CHSnBu₃, DMF, Δ, 90%;
  4. MOMCl, i-Pr₂NEt, THF, Δ, 94%;
  5. Pd(PPh₃)₄, CH₂=CHSnBu₃, DMF, A, 90%;
  6. Pd(PPh₃)₄, CH₃CH=CHSnBu₃, DMF, A, 94%;
  7. BHS-THF, THF, 0 °C, NaOH, H₂O₂, A, 11-13 39%, 12-14, 82%;
  8. PPTS, MeOH, A, 13-2 80%, 14-3 61%.

---

**Scheme 2**

- **Reagents and conditions:**
  1. n-BuLi, THF, -78 °C, CO₂, -78 °C - rt;
  2. CH₂N₂, Et₂O, 0 °C, 76%;
  3. PPTS, MeOH, Δ, 83%;
  4. LiAlH₄, THF, 0 °C - rt, 51%.

---

**Biology**

The affinities of the synthetic 4-hydroxyestradiol analogs for the estrogen receptor were assessed in whole cell estrogen receptor binding assays using MCF-7 human mammary cancer cells. The whole cell binding assay provides similar relative binding affinities (RBAs) for the estrogen receptor as those obtained using isolated estrogen receptor preparations. In addition, the cellular uptake and stability of analogs in the whole cell assay can be assessed. The EC₅₀ value for estradiol binding to the estrogen receptor in these whole cell assays was found to be 0.180 nM (Table 1). The synthetic hydroxyestradiol analog with the highest estrogen receptor affinity was 4-(hydroxymethyl)estradiol 1, exhibiting an EC₅₀ value of 364 nM. Overall, the 4-substituted estradiol homologs exhibited significantly weaker affinity for the estrogen receptor than estradiol.

---

**Scheme 3**

- **Reagents and conditions:**
  1. PPh₃, DEAD, THF; H₂N₂H₂, EtOH, Δ; HCl, MeOH.
with relative binding affinities (RBA; estradiol = 100) ranging from 0.49 for compound 1 to 0.05 for compound 3 (Table 1, Figure 1).

The relative estrogenic activities of the 4-hydroxyestradiol analogs were evaluated by examining the abilities of the synthetic compounds to induce estrogen-dependent gene expression in human breast cancer cells. In human MCF-7 mammary carcinoma cells, the induction of transcription of the pS2 gene is a primary response to estrogen. The induction of pS2 mRNA expression by estradiol, 4-hydroxyestradiol, and 4-(hydroxyalkyl)estradiol analogs 1–3 was determined by RNA dot blot analysis. The EC50 value for estradiol induction of pS2 mRNA was found to be 0.030 nM. The estradiol homologs exhibited activity significantly weaker than that of estradiol for pS2 mRNA induction, with relative activities (estradiol = 100) ranging from 0.257 for compound 1 to 0.001 for compound 3 (Table 2, Figure 2).

The effects of 4-(hydroxyalkyl)estradiol 1 on the growth of hormone-dependent MCF-7 breast cancer cells was investigated since the compound exhibited the highest estrogen receptor affinity of the 4-hydroxyalkyl analogs synthesized. This mitogenic activity was determined by measuring [3H]thymidine incorporation at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 μM. 4-(Hydroxyalkyl)estradiol did not affect cellular DNA synthesis in this breast cancer cell line, whereas estradiol at a concentration of 1 nM significantly increased MCF-7 cell growth (Figure 3).

**Conclusions**

The Stille cross-coupling and the carboxymethylation reaction reported here represent two efficient, previously unexplored synthetic routes for the functionalization of the 4-position of estradiol. The synthesis of the 4-(hydroxyalkyl)estrogens was accomplished by oxida-
tive hydroboration of 4-alkenylestradiols, which were obtained via a Stille cross-coupling between a MOM-protected 4-bromoestradiol and an alkynylstannane. The 4-(aminoalkyl)estrogens were prepared from the hydroxyalkyl derivatives with phosphite under Mitsunobu conditions.

The substituted estradiols were evaluated for estrogen receptor binding activity using whole cell receptor binding assays in MCF-7 human mammary cell cultures. The 4-(hydroxyalkyl)estradiols had significantly lower affinity for the estrogen receptor when compared with the endogenous ligand, estradiol. 4-(Hydroxymethyl)estradiol (1) exhibited the highest affinity of the synthetic compounds, with an apparent EC50 value of 364 nM, and it exhibited an affinity similar to that of the endogenous metabolite, 4-hydroxyestradiol, in the whole cell assays. On the other hand, the 4-(aminoalkyl)estradiols (4–6) exhibited either extremely weak or no affinity for the estrogen receptor.

 Estradiol acts through the nuclear estrogen receptor to induce the transcription of a variety of hormone-responsive genes in target tissues, and induction of pS2 gene transcription is a primary response to estrogen observed in human MCF-7 mammary carcinoma cells. The 4-(hydroxyalkyl)estradiols had significantly decreased efficacy for the induction of pS2 mRNA levels in MCF-7 cells when compared with the endogenous ligand, estradiol. Again, 4-(hydroxymethyl)estradiol (1) was the most potent among the synthetic compounds, with an apparent EC50 value of 11.7 nM. This synthetic compound was more effective than the endogenous metabolite, 4-hydroxyestradiol, which exhibited an apparent EC50 value of 65.4 nM.

Thus, the 4-(hydroxyalkyl)estradiols 1–3 exhibited both significantly weaker estrogen receptor affinities and abilities to induce pS2 gene expression in MCF-7 cell cultures. These results are consistent with the established structure–activity relationships of estrogens and the limitations of A ring substitutions on the estrogen molecule in producing estrogen receptor-mediated responses. On the other hand, 4-(hydroxymethyl)estradiol (1) exhibited similar estrogen receptor affinity and similar induction of pS2 gene transcription as the catechol estrogen, 4-hydroxyestradiol. This catechol estradiol has been implicated as a possible causative agent in estrogen-induced tumorigenesis; however, in vitro and in vivo investigations with 4-hydroxyestradiol are difficult due to its chemical and biochemical instability. Thus, 4-(hydroxymethyl)estradiol (1) may be viewed as a chemically stable catechol estrogen homolog and may therefore prove useful in examination of the role of catechol estrogens in normal physiology and in pathological states, such as estrogen-induced tumorigenesis.

**Experimental Methods**

**Synthesis: General Information.** Estradiol was purchased from Steroids (Wilton, NH). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee) and were used as received unless otherwise indicated. Anhydrous solvents were dried by standard procedures. Amines were stirred over CaH2, distilled, and then stored over KOH pellets. Silica gel TLC plates (60 F254) were purchased from Analtech Inc. (Newark, NE) and visualized with a UV lamp and/or 5% ethanolic phosphomolybdic acid followed by charring. All intermediates were purified by flash column chromatography on silica gel (Merck Kieselgel 60) using the indicated mixtures of hexanes and ethyl acetate. Melting points were determined in open capillaries on a Thomas-Hoope capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Laser Precision Analytical RFX-40 FTIR spectrometer in the phase indicated. 1H NMR and 13C NMR were recorded on an IBM AF250 spectrometer at 250 and 67.5 MHz, respectively. In CDCl3 solutions unless otherwise indicated using the residual protosolvent signal as internal reference. Mass spectra were obtained at The Ohio State University Chemical Instrumentation Center on either a VG 70-2505, a Nicolet FTMS-200 or a Finnigan MAT-900 mass spectrometer. Elemental analyses were performed on Oneida Research Services, Inc. (Whitesboro, NY).

4-Bromoestr-1,3,5(10)-trien-3,17β-diol, 3,17β-Bis(methoxymethoxy) Ether (12): A solution of 1 (440 mg, 0.66 mmol) in DMF (15 mL) was deoxygenated by bubbling argon through it for 15 min. The solution was heated at reflux overnight, cooled to room temperature and diluted with ether (50 mL), washed with 5% NH4OH (15 mL), water (4 x 20 mL), and brine (3 x 20 mL), dried (MgSO4), and concentrated. The residue was purified by column chromatography (SiO2, hexane/ethyl acetate) to yield 94 mg (44%) of the desired compound as a colorless solid: mp 97-98°C (lit. mp 105°C).

4-Bromoestr-1,3,5(10)-trien-3,17β-diol, 3,17β-Bis(methoxymethoxy) Ether (11): A solution of 11 (1.50 g, 0.30 mmol) and allyl tri-n-butylstannane (2.0 mmol) in toluene (10 mL) was heated at reflux overnight. The mixture was allowed to cool, and then concentrated. The residue was purified by column chromatography (SiO2, hexane/ethyl acetate, 10:1) to give 1.28 g (94%) of the desired compound as a colorless oil, which solidified on standing to a colorless waxy solid: mp 95-100°C. The crude product was purified by flash column chromatography (SiO2, hexane/ethyl acetate, 9:1) to afford a pale yellow solid, which was reccrystallized from hexane to give 4.78 g (72%) of the desired compound as a colorless solid: mp 88-89°C (lit. mp 97-98°C).

The substituted estradiols were evaluated for estrogen receptor binding activity using whole cell receptor binding assays in MCF-7 human mammary cell cultures. These results are consistent with the established structure–activity relationships of estrogens and the limitations of A ring substitutions on the estrogen molecule in producing estrogen receptor-mediated responses. On the other hand, 4-(hydroxymethyl)estradiol (1) exhibited similar estrogen receptor affinity and similar induction of pS2 gene transcription as the catechol estrogen, 4-hydroxyestradiol. This catechol estradiol has been implicated as a possible causative agent in estrogen-induced tumorigenesis; however, in vitro and in vivo investigations with 4-hydroxyestradiol are difficult due to its chemical and biochemical instability. Thus, 4-(hydroxymethyl)estradiol (1) may be viewed as a chemically stable catechol estrogen homolog and may therefore prove useful in examination of the role of catechol estrogens in normal physiology and in pathological states, such as estrogen-induced tumorigenesis.

**4-(Hydroxyalkyl)estriadiols and Related Compounds**

(1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.97-5.82 (1H, m), 5.17 (2H, s), 4.97 (1H, s), 4.92 (1H, dd, J = 1.5, 7.1 Hz), 4.91 (1H, d, J = 7.0 Hz), 3.61 (1H, J = 10.2 Hz), 3.45 (2H, s), 3.42 (2H, J = 6.6 Hz), 3.37 (3H, s), 3.29 (3H, s). 

**Carbonyl Bis(methoxymethoxy)ether (13).** A solution of the alcohol 14 (173 mg, 0.41 mmol) and pyridine (5 mL) was heated at reflux for 24 h. After cooling, the reaction mixture was diluted with ethyl acetate (50 mL), dried with MgSO4, and concentrated. The residue was recrystallized from methanol/water to give 23 mg (61%) of the desired alcohol as a colorless solid: mp 240-242 ºC; IR (KBr, cm-1) 3329, 3245, 2971, 2916, 2864, 1591, 1491, 1471, 1446, 1425, 1379, 1362, 1280, 1089, 1059, 1034, 1003, 814; % H NMR (DMSO) 10.28 (1H, s), 6.90 (1H, d, J = 8.5 Hz), 6.56 (1H, d, J = 8.5 Hz), 4.47 (1H, d, J = 8.4 Hz), 4.41 (1H, t, J = 5.2 Hz), 3.84-3.36 (2H, m), 2.90-2.45 (1H, J = 6.6 Hz), 1.84-1.50 (3H, m), 1.39-1.04 (4H, m), 0.95-0.54 (3H, m). 

**4-(Hydroxyethyl)estra-1,3,5(10)-triene-3,17ß-diol 2.** A solution of the alcohol 14 (173 mg, 0.41 mmol) and pyridine (5 mL) was heated at reflux for 24 h. After cooling, the reaction mixture was diluted with ethyl acetate (50 mL), dried with MgSO4, and concentrated. The residue was recrystallized from methanol/acetone to give 23 mg (61%) of the desired alcohol as a colorless solid: mp 240-242 ºC; IR (KBr, cm-1) 3329, 3245, 2971, 2916, 2864, 1591, 1491, 1471, 1446, 1425, 1379, 1362, 1280, 1089, 1059, 1034, 1003, 814; % H NMR (DMSO) 10.28 (1H, s), 6.90 (1H, d, J = 8.5 Hz), 6.56 (1H, d, J = 8.5 Hz), 4.47 (1H, d, J = 8.4 Hz), 4.41 (1H, t, J = 5.2 Hz), 3.84-3.36 (2H, m), 2.90-2.45 (1H, J = 6.6 Hz), 1.84-1.50 (3H, m), 1.39-1.04 (4H, m), 0.95-0.54 (3H, m).
4-(Hydroxyimino)-3,17-bis(methoxymethoxy)estra-1,3,5(10)-triene (17). L-IAH (400 mg, 9.19 mmol) was added portionwise to a solution of 18 (0.5 g, 1.20 mmol) in THF (30 mL). L-IAH was not isolated to wash the reaction mixture left at room temperature over 1 h and then stirred for 4 h. Water (0.34 mL, 15% NaOH (0.34 mL), and water (1.00 mL) were added successively, and then the resulting granule precipitate was removed by filtration through a pad of Celite and MgSO4 (1:1). After concentration of the filtrate it was chromatographed to give 0.43 g (90%) of the desired alcohol as a colorless oil, which slowly crystallized: mp 80–81 °C; IR (KBr, cm⁻¹) 3479, 2964–2914, 1657, 1583, 1461, 1411, 1405, 1385, 1252, 1245, 1222, 1193, 1079, 1065, 1054, 1041, 933, 805, 743, 726, 699, 673, 601, 579, 491, 470, 467, 456, 443, 430, 376, 357, 330, 320, 309, 283, 275, 257, 252, 248, 243, 226, 217, 214, 196, 192, 187, 165, 163, 157, 147, 139, 133, 129, 114, 109, 104, 102, 99, 96, 92, 94, 90, 89, 77, 72, 70, 66, 62, 58, 54, 50, 44, 40, 33, 31, 26, 24, 23, 20, 17, 12, 11, 7, 6, 5, 4, 3, 2, 1. NMR (CDCl3) δ 8.43 (1H, s), 6.47 (1H, d, J = 7.8 Hz, 3.35–3.50 (1H, m), 2.98–2.80 (1H, m), 2.78–2.63 (1H, m), 2.30–2.05 (1H, m), 2.03–1.86 (1H, m), 1.85–1.63 (3H, m), 0.67 (3H, s); 13C NMR 153.1, 136.3, 130.7, 124.7, 124.4, 112.7, 79.9, 54.7, 49.5, 43.7, 42.6, 37.9, 36.5, 29.8, 26.8, 26.2, 25.4, 22.6, 11.0; MS m/z (M⁺) = 302.1875, obsd 302.1883. Anal. (C21H22O2) C, H, N.

4-(Aminomethyl)estra-1,3,5(10)-triene-3,17-diol (4): 90%; mp > 270 °C; IR (KBr, cm⁻¹) 3444–2868, 1620, 1591, 1509, 1491, 1473, 1450, 1379, 1352, 1233, 1284, 1261, 1219, 1201, 1188, 1080, 1057, 1007, 945, 814; 1H NMR (DMSO-d6) 8.42 (4H, brs), 7.15 (1H, d, J = 8.6 Hz), 6.75 (1H, d, J = 8.6 Hz), 4.48 (1H, brs), 3.89 (1H, s), 3.52 (1H, t, J = 8.2 Hz), 2.94–2.70 (2H, m), 2.30–2.22 (1H, m), 1.95–1.70 (3H, m), 1.65–1.49 (6H, s), 13C NMR 152.9, 153.4, 151.0, 125.5, 116.8, 111.5, 78.8, 42.6, 41.5, 36.7, 35.7, 32.6, 25.2, 25.1, 21.6, 10.0; MS m/z (M⁺ – H) calcd 301.2037, obsd 301.2042. Anal. (C21H22N2O2) C, H, N.

4-(Aminomethyl)-1,3,5(10)-triene-3,17-diol (5): 87%; mp > 270 °C; IR (KBr, cm⁻¹) 3355, 3299, 3059, 2865, 1589, 1471, 1447, 1383, 1362, 1281, 1270, 1142, 1086, 1066, 1020, 943, 809; 1H NMR (DMSO-d6) 8.5 (4H, brs), 6.92 (1H, d, J = 8.12 Hz), 6.54 (1H, d, J = 8.03 Hz), 4.49 (1H, brs), 3.52 (1H, t, J = 8.3 Hz), 2.80–2.55 (4H, m), 0.65 (3H, s); 13C NMR 153.7, 134.9, 131.2, 131.3, 113.0, 79.5, 49.7, 44.9, 42.4, 38.1, 36.2, 29.9, 27.0, 26.3, 26.1, 22.9, 11.3; MS m/z (M⁺ – H) calcd 315.2189, obsd 315.2201.

4-(Aminomethyl)-1,3,5(10)-triene-3,17-diol (6): 86%; mp > 270 °C; IR (KBr, cm⁻¹) 3362, 3276, 3056, 2922, 2925, 2863, 1635, 1589, 1488, 1443, 1280, 1208, 1133, 1058, 813; 1H NMR (DMSO-d6) 9.2 (1H, brs), 7.91 (3H, s), 6.94 (1H, d, J = 8.49 Hz), 6.62 (1H, d, J = 8.38 Hz), 4.50 (1H, d, J = 4.6 Hz), 3.40–3.34 (4H, m), 2.78–2.73 (4H, m), 0.63 (3H, s); 13C NMR 152.6, 143.4, 130.8, 124.4, 133.2, 121.2, 79.8, 49.5, 43.7, 42.8, 40.5, 37.8, 36.4, 29.8, 26.9, 26.3, 26.1, 25.6, 22.0, 21.0, 11.0; MS m/z (M⁺ – H) calcd 329.2385, obsd 329.2354.

Biological Evaluations. General Information. 2,4,6,7-Tetrahydro-3H-Estradiol (98.4 µg/mmol, [3H]-E2) was purchased from Dupont/NEC (Boston, MA) and was used as received. MCF-7 human breast adenocarcinoma cells were obtained from ATCC, and cells were incubated in a humidified CO₂ incubator (Forma model 3052) with 5% CO₂ atmosphere. A modified Eagle's minimum essential medium (MEM) supplemented with essential amino acids (1.5×), vitamins (1.5×), nonessential amino acids (2×), and L-glutamine (1×) was obtained from Gibco BRL (Long Island, NY) and was used for maintaining the cells. The sterilized liquid medium was prepared by the OSU Comprehensive Cancer Center by dissolving the powder in water containing sodium chloride (0.9%/w/v), pyruvic acid (0.1%/w/v), and sodium bicarbonate (1.5% w/v) and the pH adjusted to 6.8. Fetal calf serum was obtained from Gibco BRL. Steroids were removed from heat-inactivated fetal calf serum by two treatments with dextran-coated charcoal at 57 °C. Tissue culture flasks and supplies were obtained from Corning Glass Works (Corning, NY). Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formamide 963 (Dupont/NEC) as the counting solution. Probes for RNA dot blot analysis (pS2-ATCC 57137; 36B4-ATCC 65917) were obtained as purified plasmids from the American Type Culture Collection and amplified by PCR for use in hybridization. Primers used were synthesized by OLGOS, ETC (Wilsonville, OR) and were as follows:
Membranes were baked at 80 °C for 1 h and then incubated for at least 3 h in a prehybridization solution containing 5 x SSPE, 5 x Denhardt's Reagent, 2% SDS, 100 μg/mL salmon sperm DNA, and 0.5 and 36B4. DNA was prepared as described above and used to make 32P-radio labeled probes using random primers in the RadPrime Kit (Gibco BRL). Probes with specific activity ranging from 5.0 x 10^6 to 2.0 x 10^7 cpm/ng were used. Membranes, probes separately for pS2 or 36B4, were incubated for 48 h or 24 h, respectively, in hybridization solution containing 5 x SSPE, 5 x Denhardt's reagent, 1% SDS, 100 μg/mL salmon sperm DNA, 10% PEG, and 50% formamide. The membranes were washed in 0.1 x SSPE, 60° C, 0.1 x SSPE, 60° C, and 0.1 x SSPE, 60°, 65° C. Phosphor screens were exposed for at least 1 h and scanned on the PhosphorImager SI (Molecular Dynamics). Quantification of the signal was performed using ImageQuaNT software (Molecular Dynamics). The apparent EC50 value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal induction of pS2 mRNA and was calculated by a nonlinear regression analysis (GraphPad Prism, Version 2.0, GraphPad Software Inc., San Diego, CA).

Cell Growth Assay. Human mammary carcinoma cell lines were maintained in 75-cm² plastic flasks at 37 °C in a modified Eagle's MEM (10 mL) containing 10% fetal calf serum and gentamycin (20 μg/mL). For cell growth determinations, the mammary carcinoma cells were divided into 9.4 cm² wells at approximately 100 000 cells/well in modified MEM (2 mL) containing 10% steroid-free fetal calf serum and gentamycin (20 μg/mL). After 2 days of growth, the media was changed to serum-free, modified MEM and experiments initiated. To determine dose-dependent effects, varying concentrations of 4-(hydroxymethyl)estradiol (3 nM to 10 μM in 5 μL of 95% ethanol) were added and incubated for 4 days. Effects on cell division were measured by the addition of [3H]thymidine (1 μCi/well), followed by incubation for 2 h, cell lysis, and determination of [3H]thymidine incorporation into DNA. Each experiment was carried out in quadruplicate, and test compounds were evaluated at least three different times. Statistical differences between control and treated groups were determined using the Student's t test.

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Measurement of Oxidative DNA Damage by Catechol Estrogens and Analogues in Vitro

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Measurement of Oxidative DNA Damage by Catechol Estrogens and Analogues in Vitro

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The growth-promoting effects of estrogens in hormone-dependent tumor tissues involve receptor-mediated pathways that are well-recognized; however, the role of estrogens in tumor initiation remains controversial. Estrogen metabolites, primarily the catechol estrogens (CE's), have been implicated in tumor initiation via a redox cycling mechanism. We have developed metabolically stable CE analogues for the study of receptor versus redox cycling effects on DNA damage. Comparisons between hydroxy estriadiols (HE_E2), methoxy estriadiols (ME_E2), and hydroxymethyl estriadiols (HME_E2) in potentiometric and DNA damaging studies were made. DNA damage was assessed in calf thymus DNA using 8-oxo-2'-deoxyguanosine (8-oxo-dG) as a genotoxic marker for oxidative stress. Increases in the number of 8-oxo-dG/10^6 G were significant for each 2-HE_E2 and 4-HE_E2. Cu(II)SO_4, a transition metal known to catalyze the redox cycling of o-quinones, substantially increased the amount of DNA damage caused by both CE's. However, DNA damage was only observed at concentrations of 10 μM or higher, much greater than what is found under physiologic conditions. Furthermore, the presence of endogenous antioxidants such as glutathione, SOD, and catalase drastically reduced the amount of DNA damage induced by high concentrations of 2-HE_E2. There was no DNA damage observed for the non-redox cycling HME_E2's, making these compounds useful probes in the study of receptor-mediated carcinogenesis. Thus, both 2-HE_E2 and 4-HE_E2 are capable of producing oxidative DNA damage at micromolar concentrations in vitro. However, since the amount of CE's has not been shown to surpass nanomolar levels in vivo, it is unlikely that free radical production via redox cycling of CE's is a causative factor in human tumorigenesis.

Introduction

The possible biochemical role(s) of estrogens and related compounds in the development of estrogen-dependent breast cancer remains to be elucidated. Estrogens produce normal physiological effects by binding to specific nuclear receptor proteins. The steroid—receptor complex then interacts with sequence specific estrogen response elements (ERE) in target cell chromatin to induce gene expression and promote growth of target cells such as breast epithelial cells and estrogen-dependent mammary carcinoma cells.

On the other hand, the role of estrogens in tumor initiation remains controversial. Possible tumorigenic effects of catechol estrogen formation and subsequent metabolism via quinones and/or semiquinones have been suggested in the literature (1). The cytochrome P450 isoforms responsible for CE formation can be induced in MCF-7 cells by the environmental toxins 2,3,7,8-tetrachlorodibenzop-dioxin (2) and are often constitutively induced in human breast tumor tissues (3, 4). Cytotoxic levels of CE's have been reported to transform cells in the BALB/c 3T3 assay (5) and are believed to be responsible for estradiol-induced renal tumor formation in the Syrian hamster model (6). It has been proposed that the CE genotoxicity mechanism involves the generation of free radicals by way of a redox cycling mechanism (Figure 1). Various compounds containing an o- or p-dihydroquinone moiety have been reported to redox cycle through processes catalyzed by oxidoreductases, peroxidases, and metals such as copper (7–9). For example, lactoperoxidase (LP), which is a breast tissue specific enzyme, has been reported to potentiate superoxide generation by hydroxyquinones (10–12). Copper has also been reported to potentiate CE-mediated strand breaks in vitro (9), and EPR measurements have confirmed the formation of semiquinones and reactive oxygen species (ROS) in the cytosolic extracts from MCF-7 cells after exposure to CE's (13). There are numerous reports of CE-induced DNA damage, which include DNA strand breaks (14), 8-oxo-2'-deoxyguanosine (8-oxo-dG) induction (6, 15, 16), and CE–DNA adduct formation (17–19).

A major problem is that the methodology for the determination of catechol estrogen formation and subsequent metabolism in various in vitro assay conditions utilizes high steroid concentrations that are unlikely to occur in vivo. Estradiol levels in serum in adult women
Figure 1. Potential genotoxicity mechanisms for catechol estrogens.

Material and Methods. 8-Oxo-dG was prepared as described below. The 2- and 4-HEs and MEs were purchased from Steraloids Inc. (Wilton, NH). Calf thymus DNA, 4-methylcatechol, 2′-deoxyguanosine, NH4OAc, ascorbic acid, GSH, mannitol, H2O2, alkaline phosphatase, and LP were all purchased from Sigma Ltd. (St. Louis, MO). Nucleoside P1 was purchased from Boehringer Mannheim (Indianapolis, IN). Caution: 2-HE and 4-HE are considered hazardous and should therefore be handled in an appropriate manner.

8-Oxo-2′-deoxyguanosine Synthesis and Purification. 8-Oxo-dG was prepared with modifications to the procedure outlined by Kasai and Nishimura (28). Thus, to 10 mL of a 0.4 mM solution of 8-dG dissolved in a 0.5 M Na2PO4 buffer at pH 7 were added 10 equiv of a 3% solution of H2O2 and 0.5 equiv of ascorbic acid. The reaction mixture was stirred vigorously at 37 °C as a steady stream of O2 was bubbled into the mixture for the duration of the reaction. After 2 h, 10 equiv of a 3% solution of H2O2 and 0.5 equiv of ascorbic acid were added, and the reaction mixture was stirred for an additional 2 h. The reaction mixture was concentrated to 15 mL at 40 °C (higher temperatures can cause degradation, and complete evaporation can lead to insoluble phosphate complexes). Purification was carried out with a preparative C-18 column at a flow rate of 4 mL/min, with 3% MeOH in H2O, with UV detection at 254 nm; 8-oxo-2′-dG elutes directly after 2′-dGuo. Yields varied from 8 to 12% after purification. Identification was confirmed by 1H NMR (DMSO-d6, 250 MHz) and coelution with a standard by HPLC.

Potentiometric Measurements. Potentiometric measurements were performed on a BAS CV-1B cyclic voltammeter. The sample cell included the platinum auxiliary, glassy carbon working, and Ag+/AgCl reference electrodes. The sweep rate was optimized at 100 mV/s, with a sensitivity of 2 μA/V and a filter rate of 0.1 s. Sample solutions were made with the addition of 10 mM stocks in DMSO into an N2-purged cell containing phosphate-buffered saline (PBS) (pH 7.4, Ca and Mg free) to give 100 μM working solutions. It was necessary to polish the glassy carbon electrode after each scan. The system was validated with 4-methylcatechol.
In Vitro DNA Damage Experiments. Freshly prepared 1 
µg/µL solutions of calf thymus DNA (200 µL) in PBS (Ca²⁺ and 
Mg²⁺ free at pH 7.4) were delivered to uncapped 12 mm × 75 
mm borosilicate glass culture tubes. The compounds of interest 
were added, and incubation was carried out in a shaking water 
bath at 37 °C for 3 h. The steroid stock solutions were made up 
in DMSO, while all other compounds were diluted in distilled 
water. Stock solutions were added to the calf thymus DNA such 
that a 1:100 dilution of the stock solution would give the 
appropriate working concentration. DNA was precipitated with 
the addition of 50 µL of 7 M NH₄OAc and 500 µL of 95% EtOH 
at -20 °C.

Enzymatic DNA Digestion. The precipitated DNA was 
transferred to a 0.5 mL heat-resistant microfuge tube and 
centrifuged at 12000g for 30 s. The pellet was washed with 70% 
EtOH followed by 90% EtOH, allowed to air-dry, inverted for 
10 min, and diluted in 200 µL of a 10 mM Tris solution at pH 
7. The DNA was denatured at 95 °C for 10 min and cooled on 
ice. This was followed by the addition of 10 units of nuclease 
P1 in 20 mM NaOAc buffer at pH 3.4 (final pH of 4.8) containing 
0.1 mM ZnCl₂ while the mixture was being heated to 65 °C for 
15 min. Incubation times longer than 15 min can cause an 
increase in 8-oxo-dG levels. The solution was cooled to 37 °C, 
and 20 units of alkaline phosphatase type VII-S was added 
in 200 mM Tris at pH 8.5 (final pH of 7.8), followed by digestion 
for 45 min. The final pH was adjusted to 6 with 0.05 M HCl 
and the mixture injected onto the HPLC/ECD system.

HPLC/ECD System. Separation of the hydrolyzed DNA was 
achieved on a Beckman HPLC model 126 system containing 
an ESA pulse dampener just prior to the sample injector and a 
YMC basic 5 µm, B-02-3, 15 cm reverse phase column. The 
optimum isocratic system contained 5% MeOH in 0.1 M NaPO₄/ 
0.1 M NH₄Ac buffer at pH 6 with a flow rate of 0.8 mL/min. 
Detection was carried out using a Beckman model 167 UV 
detector at 280 nm and an ESA Coulochem Detector II with 
the guard cell removed, the conditioning cell at 100 mV, and 
the sample cell at 350 mV. Data acquisition was carried out on 
a Metrabit analog to digital converter. The detection limit for 
8-oxo-dG was 50 fmol on the column, and the R² was 0.999 for a 
standard curve covering the experimental range.

2-HE₂ Oxidation by Lactoperoxidase. Spectroscopic 
control studies of the LP-catalyzed oxidations were carried out on a 
Pharmacia Ultraspec III UV/VIS spectrophotometer. All reactions 
were carried out in a 3 mL quartz cuvette containing 100 
µM CE or CE analogue and 2 units of LP in 2 mL of PBS at 25 
°C, and initiated with the addition of 10 µM H₂O₂. H₂O₂ (10 
µM) was added every 2 min, and scans were taken every 4 min.

Results and Discussion

Measurements of Oxidation and Reduction Potentials. The term "redox cycling" refers to a reversible process of oxidation and reduction. Under highly reverse-

able and otherwise optimal conditions, a very small amount of redox active material has the potential to generate a slow but endless supply of ROS. Mechanisms by which chemicals exhibit carcinogenic activity often include redox cycling with generation of ROS and subsequent covalent binding with modification to proteins and DNA. Potentiometry can be used to determine which damaging mechanism, if any, is most likely. Cyclic voltammetry was used to examine the toxic potential of the CE's and CE analogues. The oxidation potential was used to determine the likelihood that a compound would be oxidized in the surrounding matrix. The degree of reversibility, expressed as ΔE, was used to indicate the ability of an oxidized product to be reduced back to its initial state. Compounds which do not exhibit a reduction peak and compounds with a large ΔE value, for example, would not be considered reversible and would not be able to participate in redox cycling.

With regard to the CE's, both 2-HE₂ (Figure 2a) and 
4-HE₂ were quasi-reversible (Scheme 1a), exhibiting ΔE's 
of 55 and 60 mV, respectively, and nearly equal half-wave 
potentials (E½) of 263 ± 10 and 265 ± 10 mV versus the 
NHE, respectively (Table 1). This would indicate that 
the CE's are indeed capable of redox cycling in a physi-
ologic matrix. Differences in oxidation potentials or ΔE 
values could possibly explain differences in toxicity; 
however, both 2-HE₂ and 4-HE₂ exhibited nearly equal 
electrochemical properties under physiological conditions. 
This would imply that different rates of oxidation in vivo 
would likely be due to enzymatic rather than chemical 
influences.

The 2-HME₂ (Figure 2b) and 4-HME₂ each exhibited 
one anodic peak at 597 ± 10 and 595 ± 10 mV versus the 
normal hydrogen electrode (NHE), respectively (Table 
1), and neither compound was reduced at the electrode 
surface. Although the end products were not studied, 
electrochemical investigations of polyalkylated phenols
DNA Damage by Catechol Estrogens and Analogues


Scheme 1. Oxidation/Reduction Schemes for the Estrogen Analogues

(a) Reversible oxidation and reduction of 2-HE2, (b) irreversible oxidation of 2-HME2, and (c) oxidative demethylation of 2-ME2.

Table 1. Peak Potentials for CE's and CE Analogues versus Ag/AgCl at pH 7.4

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_{p,al}$ (mV)</th>
<th>$E_{p,al}$ (mV)</th>
<th>$E_{p,a2}$ (mV)</th>
<th>$E_{p,cl}$ (mV)</th>
<th>$E_{cl}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-HE2</td>
<td>90</td>
<td>35</td>
<td>-</td>
<td>55</td>
<td>73</td>
</tr>
<tr>
<td>2-ME2</td>
<td>80</td>
<td>15</td>
<td>280</td>
<td>65</td>
<td>48</td>
</tr>
<tr>
<td>2-HME2</td>
<td>407</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-HE2</td>
<td>95</td>
<td>35</td>
<td>280</td>
<td>65</td>
<td>48</td>
</tr>
<tr>
<td>4-ME2</td>
<td>80</td>
<td>25</td>
<td>345</td>
<td>55</td>
<td>53</td>
</tr>
<tr>
<td>4-HME2</td>
<td>405</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-methylcatechol</td>
<td>140</td>
<td>50</td>
<td>-</td>
<td>90</td>
<td>95</td>
</tr>
</tbody>
</table>

* Standard error ± 10 mV. * Potentials corrected vs the NHE at pH 7.4.

have been thoroughly reviewed (29). A reasonable mechanism under neutral conditions would include the loss of two electrons with generation of the phenoxonium intermediate followed by hydroxylation primarily in the 1-position (Scheme 1b). This type of end product would lead to an oxidation-reduction profile consistent with that observed for the HME2's. The HME2's were only oxidized at high oxidation potentials with an end product that is incapable of participating in reOXD CYCLING, thus making this type of "capped" CE analogue excellent for use in receptor studies that may involve estrogen metabolites without inducing damage by reOXD CYCLING.

With regard to methoxyestrogens, 2-ME2 is found at a much higher concentration in the serum than the CE's, and the potentiometric properties of the ME2 have not been reported. Important questions are how these metabolites behave under oxidative conditions and if they participate in redox cycling. The redox properties of the 2- and 4-methoxyestradiol metabolites were nearly identical (Figure 2c). Each compound exhibited one anodic peak ($E_{p,a2}$) that decreased in current with each scan and a second anodic peak ($E_{p,al}$) that both increased in current with each scan. This potentiometric profile is consistent with oxidation of the phenolic moiety followed by irreversible demethylation and reversible reduction of the resultant quinone (Scheme 1c) (29). The $E_{p,al}$ and $E_{p,al}$ peaks were nearly identical to the oxidation and reduction peaks of 2-HE2 (Table 1). The slight shift in $E'_{1/2}$ may be due to generation of MeOH at the electrode surface. Therefore, the very rate limiting process of spontaneous demethylation at a relatively high oxidation potential would be necessary before reOXD CYCLING could occur. The half-wave potential of the validation standard 4-methylecatechol is within 30 mV of the literature value obtained under similar conditions (30).

8-Oxo-dG Formation by CE's and CE Analogues with and without Cu(II). Calf thymus DNA was exposed to 100 μM CE or CE analogue with and without the addition of 100 μM Cu(II)SO4 for 3 h in PBS (pH 7.4) at 37 °C. The induction of 8-oxo-dG was compared to that of the following controls: nonincubated DNA* (C1), DNA incubated alone (C2), and DNA incubated in the presence of Cu(II)SO4 (C3). Data points are means ± SD (n = 3) for all samples.
Although tors as indicated. (Figure 4) with the prior addition of specific ROS inhibition of ROS was measured as a percent decrease in the level of 8-oxo-dG formation above those levels found in cells and in vivo. It is important to determine the type to incubated controls (carrier, 11 ± 0.2; carrier and Cu-

Figure 4. Determination of ROS production by 2-hydroxy estradiol. 2-HE$_2$ (100 µM) was incubated in the presence of Cu(II) (100 µM) for 3 h in PBS (pH 7.4) at 37 °C in the presence of a copper chelator or ROS scavenging agent as shown. Concentrations were as follows: 1.0 mM GSH, 1.0 mM sodium azide, 200 µM BCS, 1.0 mM MgCl$_2$, 200 units of SOD, 1.0 mM mannitol, and 200 units of catalase. Data points are means ± SD (n = 3) for all samples.

The values for the number of 8-oxo-dG/10$^5$ dG in controls include nonincubated DNA (7.3 ± 0.9), DNA incubated alone (11.7 ± 1), and DNA administered with 100 µM Cu(II)SO$_4$ (14.3 ± 2). There was no increase in the amount of DNA damage by the carrier solvent DMSO.

Inhibitors of 8-oxo-dG formation were significantly for 2-HE$_2$ (21 ± 0.6), 4-HE$_2$ (15.5 ± 1.7), and ascorbic acid (31.3 ± 3). The addition of Cu(II) significantly increased 8-oxo-dG levels in the 2-HE$_2$ (1190 ± 119), 4-HE$_2$ (1280 ± 74.9), and ascorbic acid (1020 ± 89) samples. The HMDE's and ME's did not increase 8-oxo-dG levels even with the addition of Cu(II), and actually appear to behave in a protective fashion, causing less DNA damage than that found in the incubated controls. Although 2-HE$_2$ induced more ROS damage than did 4-HE$_2$ when incubated alone, both CE's generated nearly equal amounts of DNA damage when Cu(II) was added. Cu(II) increased the DNA-damaging potential of both CE's by nearly 50-fold, illustrating the great significance of copper in CE toxicity.

Identification of ROS Formed by CE and Cu(II). Specific ROS inhibitors were chosen to help elucidate the damaging mechanisms that may be especially significant in cells and in vivo. It is important to determine the type of ROS generated, as this can help predict other types of damage that may occur in proteins, polyunsaturated fatty acids (PUFAs), carbohydrates, and DNA (33, 34). Inhibition of ROS was measured as a percent decrease in the amount of 8-oxo-dG formed versus a positive control containing 2-HE$_2$ and Cu(II)SO$_4$. Calf thymus DNA was exposed to 100 µM 2-HE$_2$ and 100 µM Cu(II)SO$_4$ for 3 h (Figure 4) with the prior addition of specific ROS inhibitors as indicated.

Sodium azide, acting primarily as an O$_2^-$ scavenger (1250 ± 58.7, 6% decrease), and mannitol as an "OH scavenger (1170 ± 44.6, 1% decrease) were far less effective at lowering the level of 8-oxo-dG formation. However, catalase, an H$_2$O$_2$ scavenger (26.7 ± 5.0, 98% decrease), and SOD, an O$_2^-$ scavenger (6.8 ± 0.8, 99% decrease), lowered the 8-oxo-dG level to nearly that of background. The high efficacies of catalase and SOD indicate that peroxide and superoxide are the primary reactive oxygen species formed in this system. The less efficacious singlet oxygen and hydroxyl radical scavengers indicate that these reactive oxygen species may be formed secondarily through Heiber-Wiese and Fenton chemistry at a very close proximity to the DNA.

There were similar decreases in the level of 8-oxo-dG formation for the copper chelator BCS (168.0 ± 76.4, 86% decrease), for the copper chelator/reducing agent glutathione (GSH) (458.7 ± 61.2, 61% decrease), and in the anaerobic system (289 ± 15.6, 76% decrease). GSH is noted for its radical scavenging abilities; however, GSH is also known to chelate metal ions such as copper (8). In the BCS copper ion complex, the formal potential is raised such that Cu(II)-BCS is easily reduced, forming the Cu(I)-BCS complex which is difficult to oxidize and rendering the copper ineffective in activating O$_2$. The high efficacy of the copper chelators along with the free radical scavenging activity of GSH points to a mechanismic role for the Cu(I)/Cu(II) couple as a one-electron transfer agent. This one-electron transfer may result in the formation of the CE semiquinone and superoxide radical anion. The significant decrease in the level of 8-oxo-dG formation in the anaerobic system reiterates the necessity for O$_2$ in this system.

Magnesium and calcium (data not shown) were also added to the buffer separately and were found to have no effect on 8-oxo-dG production. Various biological buffers have been reported to decrease the level of 8-oxo-dG formation in the H$_2$O$_2$-Cu(II)-DNA system which is believed to be due to complexation with and deactivation of Cu(II) (12). We have found that the presence of magnesium or calcium often alleviates this effect, possibly through competitive interactions with anionic sites in the buffer and on the calf thymus DNA which is purchased as the sodium salt.

Concentration Requirements for Oxidatively Induced DNA Damage. Numerous studies have reported that CE's are capable of inducing DNA damage. However, the "minimal" concentrations needed to induce such damage often go unreported. In this study, calf thymus DNA was exposed to increasing concentrations of 2-HE$_2$ (0.1-100 µM) with and without the addition of 10 µM Cu(II)SO$_4$ for 3 h (Figure 5). The level of induction of 8-oxo-dG by 2-HE$_2$ was compared to that by nonincubated DNA, DNA incubated alone, or DNA incubated in the presence of Cu(II)SO$_4$. High concentrations of 2-HE$_2$ induced 8-oxo-dG formation at greater levels compared to incubated controls (carrier, 11 ± 0.2; carrier and Cu(II)SO$_4$, 13.4 ± 0.6) at 100 µM alone (13.2 ± 0.6) and at 10 µM in the presence of Cu(II)SO$_4$ (45.7 ± 1.1). The addition of lower concentrations of 2-HE$_2$ did not increase the level of 8-oxo-dG formation above those levels found in the nonincubated control (7 ± 0.2) at 1.0 µM (7.6 ± 0.2) and 10 µM (7.6 ± 0.2) when added alone, and at 0.1 µM (9.8 ± 0.4) and 1.0 µM (10.6 ± 0.1) with the addition of Cu(II)SO$_4$. These results are reinforced by reports that 2-HE$_2$ decreases the level of lipid peroxidation at lower concentrations (35). The DNA damage and potentiometric studies both indicate that under physiological conditions, in the absence of Fenton catalysts, 2-HE$_2$ is primarily
DNA Damage by Catechol Estrogens and Analogues

Figure 5. Effect of estrogen concentration on the formation of 8-oxo-dG. Calf thymus DNA was exposed to increasing concentrations of 2-HE2 (0.1 to 100 μM) with and without the addition of 10 μM Cu(II)SO4 for 3 h in PBS (pH 7.4) at 37 °C. The induction of 8-oxo-dG by 2-HE2 was compared to that of the following controls: nonincubated DNA (C1), DNA incubated alone (C2), and DNA incubated in the presence of Cu(II)SO4 (C3). Data points are means ± SD (n = 3) for all samples.

Table 2. Lactoperoxidase Effects on CE-Induced 8-Oxo-dG Formation

<table>
<thead>
<tr>
<th>experiment</th>
<th>8-oxo-dG/10⁶ dG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT DNA only</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>100 μM H2O2</td>
<td>10.7 ± 0.3</td>
</tr>
<tr>
<td>100 μM H2O2 and 100 μM 2-HE2</td>
<td>50.0 ± 2.0</td>
</tr>
<tr>
<td>2 units of LP, 100 μM H2O2, and 100 μM 2-HE2</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td>2 units of LP, 100 μM H2O2, and 100 μM 4-HE2</td>
<td>6.2 ± 0.9</td>
</tr>
</tbody>
</table>

* Data points are means ± SD (n = 3) for all samples.

Lactoperoxidase Influence on the DNA-Damaging Potential of CE’s. The LP-catalyzed one-electron oxidation of estradiol and o-quinones has been reported (11, 12). For this reason, we have taken a close look at 8-oxo-dG formation by CE in the presence of LP. Calf thymus DNA was exposed to 100 μM 2-HE2 or 4-HE2, 2 units of lactoperoxidase (LP), and 100 μM H2O2 for 3 h. Controls were performed with the addition of either H2O2 alone or H2O2 with 2-HE2. There was a significant increase in the level of 8-oxo-dG formation in the 2-HE2/hperoxide control (50 ± 2), with levels decreasing to that of background for both CE’s (6.5 ± 0.4 with 2-HE2 and 6.2 ± 0.9 with 4-HE2) when LP was added. The results in Table 2 indicate that LP may behave in a protective fashion. A one-electron oxidation pathway of LP would produce the CE semiquinone and resultant ROS with expected increases in the level of 8-oxo-dG, which is not observed. It is likely that the peroxide-driven CE oxidation by LP proceeds through a two-electron transfer mechanism and not through a one-electron transfer mechanism as may be expected. This is in partial agreement with spectroscopic studies, which illustrate accelerated oxidation of 2-HE2 by LP (Figure 6). The spectroscopic studies also indicate that 2-ME2 and 2-HME2 are virtually inert toward the effects of LP (data not shown). Although LP has been shown to decrease the amount of CE-induced oxidative DNA damage in vitro, the potentially toxic effect of the resultant quinone should not be ignored.

Figure 6. Lactoperoxidase oxidation of 2-hydroxy estradiol. Overlapping absorption spectra of 2-HE2 (100 μM) as it became increasingly oxidized by LP (2 units) and H2O2. H2O2 (10 μM) was added every 2 min, while scans were taken every 4 min.

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<table>
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<td>100 μM H2O2 and 100 μM 2-HE2</td>
<td>50.0 ± 2.0</td>
</tr>
<tr>
<td>2 units of LP, 100 μM H2O2, and 100 μM 2-HE2</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td>2 units of LP, 100 μM H2O2, and 100 μM 4-HE2</td>
<td>6.2 ± 0.9</td>
</tr>
</tbody>
</table>

* Data points are means ± SD (n = 3) for all samples.

Conclusion

Potentiometric studies were carried out with both 2-HE2 and 4-HE2, with each demonstrating quasi-reversibility at pH 7.4. The similarity in the degree of reversibility, expressed as ΔE, and the similarity in formal potentials between these two compounds were somewhat surprising. There has been some debate over differences in the nonenzymatic oxidation of 2-HE2 and 4-HE2. For example, 2-HE2 has been reported to form significant DNA strand breaks in vitro while 4-HE2 failed to generate any strand breaks under the same conditions (9). In addition, 8-oxo-dG was reportedly induced in calf thymus DNA exposed to 4-HE2 incubated with a microsomal extract from Syrian hamster liver but not with 2-HE2 (16). In agreement with the potentiometric data, we have shown that 8-oxo-dG can be induced by both CE’s, and that although 2-HE2 was slightly more damaging than 4-HE2 alone, the amount of DNA damage was nearly equal when Cu(II) was present. Our studies indicate that there is a significant influence by copper on the DNAdamaging potential of both 2-HE2 and 4-HE2 in vitro, which is possibly more significant than previously believed. However, a minimum of 10 μM 2-HE2 was necessary even in the presence of copper to induce 8-oxo-dG levels that were greater than controls. Furthermore, at concentrations of <10 μM, 2-HE2 behaves as an antioxidant, reducing the level of 8-oxo-dG below control levels. The effect of LP on the HE2’s was shown to be protective with respect to 8-oxo-dG formation, acting through a two-electron oxidation pathway, resulting in less ROS generation than that of the controls containing either 2-HE2 or peroxide alone.

Potentiometric studies were also carried out with the HME2’s and ME2’s. These compounds are incapable of redox cycling and therefore would not be expected to cause formation of significant amounts of 8-oxo-dG. The lack of 8-oxo-dG formation by these analogues is consis-
tent with the potentiometric data. Spectroscopic studies indicate that the ME's and HME's were inert toward the LP-catalyzed oxidation by peroxide.

Thus, both the 2- and 4-HEQ's are capable of causing significant DNA damage at high micromolar concentrations while in the presence of Cu(II) in vitro. Although copper concentrations have been reported to reach into the micromolar range under some circumstances (31, 32), the concentrations of CE's have not been shown to surpass sub-nanomolar levels in serum (5). Furthermore, serum estradiol concentrations equal to or less than approximately 2.5 ng/mL are sufficient to induce tumorigenesis in vivo in the ACI rat mammary tumor model and the Syrian hamster kidney tumor model (36, 37), suggesting that concentrations of CE metabolites are below nanomolar levels. In addition, considerations for many cellular defenses such as conjugating enzymes, DNA binding proteins, and DNA repair have not been addressed in this paper. However, SOD, catalase, and antioxidants were shown to be effective in reactions with deoxyribonucleosides.

Therefore, both the 2- and 4-HEQ's are capable of causing oxidative DNA damage in reactions with deoxyribonucleosides. Moreover, the presence of 100 pM GSH is required for the in vitro oxidation of GSH via redox cycling of CE's is a causative factor in E. L. Oakley, G. G., Devanaboyina, U., Robertson, L. W., and Gupta, R. C. (1996) Oxidative DNA damage induced by activation of polychlorinated biphenyls (PCBs): Implications for PCB-induced oxidative stress in breast cancer. Chem. Res. Toxicol. 9, 1285–1292.


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DNA Damage by Catechol Estrogens and Analogues

(32) National Academy of Sciences (1977) Copper/Medical and Biological Effects of Environmental Pollutants, Washington, DC.


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Novel Synthetic Routes Suitable for Constructing Benzopyrone Combinatorial Libraries

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Abstract

A series of O-(t-butylsilyloxy)benzoyl chlorides generated from the corresponding silyl esters were coupled with a range of terminal alkynes to afford the corresponding alkynyl ketones. The alkynyl ketones were converted to enaminoketones and then cyclized to yield the desired benzopyrone ring system. This synthetic protocol utilizes readily available starting materials, mild and high yielding reactions with good functional group tolerance, and is ideal for developing combinatorial libraries centered around the benzopyrone ring system. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Benzopyrones; Flavonoids; Combinatorial chemistry; Sonogashira coupling

Synthesis and biological screening of a heterocyclic, small molecule library forms the backbone of most combinatorial chemistry programs today [1,2,3]. Molecular scaffolds that have been shown to interact with different receptor systems whose natural ligands bear no resemblance with each other are termed as “privileged structures” [4]. There is substantial interest in synthesizing libraries of privileged structures, with the hope that screening of such libraries would yield ligands for a diverse collection of pharmacological targets. The driving force behind synthesis and screening of privileged structure libraries is the underlying promise of reducing the synthetic effort required to generate lead structures for a range of biological targets. Benzodiazepines are examples of privileged structures that have been explored using combinatorial methods [1,2,3].

The benzopyrone ring system represents a privileged structure that is yet to be fully exploited by combinatorial chemistry [5,6]. This ring system is present in a number of natural products.
including flavonoids that interact with various enzymes and receptor systems of pharmacological significance.

The benzopyrone ring system presents a fairly rigid molecular framework, resistant to hydrophobic collapse, with multiple sites to introduce potential diversity elements. The prevalent literature methods for constructing benzopyrones are not ideally suited for making libraries as these methods suffer from harsh reaction conditions, poor substituent tolerance and low yields [7].

**Figure 1**

![Figure 1](image)

Heteroannulation reactions of o-iodophenols and terminal alkynes in presence of CO are known to produce mixtures of 6-membered benzopyrones and 5-membered aurones [8,9]. In our synthetic planning we proposed to use salicyloyl chlorides as the coupling partner of terminal alkynes in order to obviate high CO pressure conditions required for heteroannulations (Figure 1). Also, the phenolic hydroxyl is masked as a TBS ether in order to prevent the oxypalladation reactions leading to mixtures of 5- and 6- membered ring systems. The desired benzopyrone would be then constructed by 6-endo-dig cyclization of the alkynone under controlled conditions that preclude the formation of aurones.

Salicylic acids were treated with 2.2 equiv of TBSCI and Et$_3$N in CH$_2$Cl$_2$ to generate the bisTBS protected salicylic acids (A$_1$-A$_5$) in quantitative yield [10]. The bisTBS salicylic acids were reacted with 1.2 equiv of oxalyl chloride in presence of catalytic amounts of DMF to provide the corresponding acid chlorides [11]. The acid chlorides were used for the Sonogashira couplings without any further purification (Figure 2). The acid chloride in Et$_3$N was reacted with a variety of terminal alkynes (B$_1$-B$_7$) in the presence of catalytic amount of Pd(PPh$_3$)$_2$Cl$_2$ and CuI [12]. It was important to use 3-5 mole excess of terminal alkynes and deoxygenate the reaction mixture in order to reduce the amount of alkyne homocoupled byproducts (Glaser Coupling). Salicylic acids (A$_2$-A$_5$) were coupled with phenyl acetylene (B$_1$) to evaluate the effect of substitutions on the salicylic acid component over the coupling reactions. All of the coupling reactions gave desired alkynones in excellent yields (Table 1, 2). The acid sensitive NH-Boc function (A$_2$) is successfully carried through the acid chlorination step, emphasizing the mild nature of reaction conditions. The one-pot acid chlorination-
Sonogashira coupling, key for introducing diversity, displays a wide substituent tolerance in both the coupling partners and provides the desired alkynones in excellent yields.

Figure 2

![Diagram](image-url)

**Table 1. Coupling of A₁ with terminal alkynes**

<table>
<thead>
<tr>
<th>Product A₁B₁ (Yield)*</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁B₁ (92%)</td>
<td>=</td>
</tr>
<tr>
<td>A₁B₂ (95%)</td>
<td>O</td>
</tr>
<tr>
<td>A₁B₃ (95%)</td>
<td>H</td>
</tr>
<tr>
<td>A₁B₄ (94%)</td>
<td>H</td>
</tr>
<tr>
<td>A₁B₅ (78%)</td>
<td>O</td>
</tr>
<tr>
<td>A₁B₆ (74%)</td>
<td>H</td>
</tr>
<tr>
<td>A₁B₇ (96%)</td>
<td>O</td>
</tr>
</tbody>
</table>

**Table 2. Coupling of B₁ with salicylic acids A₂-A₅**

<table>
<thead>
<tr>
<th>Salicylic acid</th>
<th>Product A₂B₁ (Yield)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₂</td>
<td>A₂B₁ (90%)</td>
</tr>
<tr>
<td>A₃</td>
<td>A₂B₂ (92%)</td>
</tr>
<tr>
<td>A₄</td>
<td>A₂B₃ (83%)</td>
</tr>
<tr>
<td>A₅</td>
<td>A₂B₄ (78%)</td>
</tr>
</tbody>
</table>

*isolated yields after workup and flash chromatography

Upon removal of TBS group, the free phenolic hydroxyl can effect 6-endo-dig or 5-exo-dig cyclization to yield either benzopyrones or aurones respectively [13]. We reasoned that, if the alkynones were first converted to enaminoketones and then subjected to TBS deprotection, the system would be prone to undergo Michael addition followed by elimination of secondary amine to exclusively yield the desired benzopyrones. To our surprise, we discovered that conversion of the alkynones to enaminoketones and subsequent cyclization could be effected in a single step. Thus, ethanolic solutions of alkynones refluxed with 10 equiv of diethylamine for 24 hours underwent cyclization to give the benzopyrones via enaminoketone intermediates. TLC revealed that the starting material was consumed within two hours and subsequent NMR analysis of the reaction mixture after 10 hours of reflux revealed a mixture of enaminoketone and benzopyrone. Pure samples of the enaminoketones were prepared by stirring an alcoholic solution of the alkynone with 10 equiv of the secondary amine for two hours. These
enaminoketones when refluxed with excess diethylamine formed benzopyrones. The results of these cyclizations are shown in Figure 3. Similar results were obtained by refluxing the alkyrones with dimethylamine (2M solution in THF) and N-benzyl-ethylamine. This strategy effectively eliminates the 5-exo-dig cyclization option.

Figure 3. Cyclizations with Diethylamine

<table>
<thead>
<tr>
<th>Alkyrone</th>
<th>% Yield of Flavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁B₁</td>
<td>96%</td>
</tr>
<tr>
<td>A₁B₂</td>
<td>87%</td>
</tr>
<tr>
<td>A₁B₃</td>
<td>73%</td>
</tr>
<tr>
<td>A₁B₄</td>
<td>68%</td>
</tr>
<tr>
<td>A₁B₅</td>
<td>75%</td>
</tr>
<tr>
<td>A₁B₆</td>
<td>54%</td>
</tr>
<tr>
<td>A₁B₇</td>
<td>82%</td>
</tr>
</tbody>
</table>

Isolated yield after workup and flash chromatography

In summary, we have disclosed a novel way to construct the benzopyrone nucleus. This method utilizes readily available starting materials, mild reaction conditions and displays a wide substituent tolerance and therefore should prove useful in constructing libraries of benzopyrones not readily accessible by conventional synthetic protocols.

**Experimental:** Oxalyl chloride (1.1mmol) was added dropwise to a cold (0°C) solution of bisTBS salicylic acid (1mmol) in CH₂Cl₂ containing 3 drops of DMF. The resulting solution was stirred at 0°C for two hours and stirred at room temperature for 16 hours. Solvent was evaporated, Et₃N (3ml) was added to the residue and argon was bubbled through the solution for five minutes. 5mmol of alkyne, 5mg Pd(PPh₃)₂Cl₂ and 5mg of Cul were added and the reaction mixture was deoxygenated by bubbling argon gas for 10min and stirred at room temperature for 12 h. MeOH (5ml) was added to the reaction mixture and solvents evaporated, the residue was taken up in diethyl ether, organics were washed with water, brine, dried (Na₂SO₄) and concentrated, the residue was purified by flash chromatography (SiO₂; 18% EtOAc in Hexanes). All the coupling products were characterized by ¹H, ¹³C NMR, IR and HRMS.

**References**

COMPARISON OF ENDOCRINE AND ANTITUMOR ACTIVITY OF 2-METHOXY ESTROGENS


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ABSTRACT

An estradiol metabolite, 2-methoxyestradiol (2-MeOE2), has shown antiproliferative effects in both hormone-dependent and hormone-independent breast cancer cells. Previously, a series of 2-hydroxyalkyl estradiol analogs had been synthesized in our laboratories as potential probes for comparison of estrogen receptor (ER)-mediated vs. non-ER-mediated effects in breast cancer cells. A methoxy derivative of 2-hydroxymethyl estradiol was prepared for biological evaluation and comparison with 2-MeOE2. Estrogenic activity of the synthetic analogs was evaluated in two ways, one by examining affinity of the analogs for the estrogen receptor in MCF-7 cells and the other by examining the ability of the analogs to induce estrogen-responsive gene expression. The analog, 2-methoxymethyl estradiol (2-MeOMeE2), demonstrated weak affinity for the estrogen receptor (1.7% of estradiol) and weak ability to stimulate estrogen-induced expression of the pS2 gene (0.02% of estradiol). Antitumor activity was evaluated both in vitro and in vivo. The steroidal nucleus seems to be an attractive target for developing novel tubulin polymerization inhibitors. Additionally, such steroidal compounds may have low toxicity compared to the natural products known to interact with tubulin. Interestingly, 2-MeOMeE2 inhibited tubulin polymerization in vitro at concentrations of 1 and 3 μM and was more effective than 2-MeOE2. Both 2-MeOE2 and 2-MeOMeE2 were equally effective in suppressing growth and inducing cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cells. The cytotoxic effects of 2-MeOMeE2 are associated with alterations in tubulin dynamics, with the frequent appearance of misaligned chromosomes, a significant mitotic delay, and the formation of multinucleated cells. Assessment of in vivo antitumor activity was performed in athymic mice containing human breast tumor xenografts. Nude mice bearing MDA-MB-435 tumor xenografts were treated i.p. with 50 mg/kg/day of 2-MeOMeE2 or vehicle control for 45 days. Treatment with 2-MeOMeE2 resulted in an approximate 50% reduction in mean tumor volume at treatment day 45 when compared to control animals and had no effect on animal weight. Thus, 2-MeOMeE2 is an estrogen analog with minimal
estrogenic properties that demonstrates antiproliferative effects both in vitro and in the human xenograft animal model of human breast cancer.

INTRODUCTION

An estimated 186,000 new cases of breast cancer will be diagnosed, and 46,000 women in the U.S. will die from breast cancer in 1999 (1). The development of new drugs that facilitate better management and control of breast cancer is warranted, particularly with the increased incidence of breast cancer in the past two decades and the development of resistance to current chemotherapeutic agents. Recently, 2-methoxyestradiol (2-MeOE2; Figure 1), one of the endogenous metabolites of estradiol (E2), was shown to demonstrate in vitro inhibition of angiogenesis and suppress tumor growth (2). The antiangiogenic activity was found in fractionated human urine, with the most potent fractions containing catechol metabolites and nonpolar estrogen metabolites. Also in 1994, D'Amato et al. reported that 2-methoxyestradiol, a normal mammalian metabolite inhibits in vitro tubulin polymerization (3). These studies suggested that abnormal microtubule assembly may be responsible for the antiangiogenic activity. Examination of synthetic standards of estrogens for their ability to block bFGF-induced proliferation of bovine brain capillary endothelial cells identified several catechol estrogens and methoxyestrogens, with IC50's ranging from 0.134 μM for 2-methoxyestradiol to 15.7 μM for 2-hydroxyestradiol. Further investigations on possible mechanisms for this antiangiogenic effect have focused on the interactions of 2-MeOE2 with tubulin (3,4), with 2-MeOE2 inhibiting nucleation and propagation of tubulin assembly and being a competitive inhibitor of colchicine binding (Ki of 22 μM). Similar antitumor and antiangiogenic activities were observed in studies comparing 2-methoxyestradiol and taxol (5). Other proposed mechanisms for 2-MeOE2 antitumor activity include metaphase arrest, interference with mitotic spindle dynamics, and increased phosphorylation of Bcl-2 (6-8).
Various steroidal and nonsteroidal estrogens have demonstrated alteration and/or inhibition of microtubule polymerization and microtubule function in both the Chinese and Syrian hamster embryo cells in culture (9-11). *In vitro* experiments have demonstrated that certain estrogen metabolites bind covalently to the C-terminal region of the β-subunit of tubulin (9), suggesting that the interactions of estrogens and/or estrogen metabolites with tubulin and microtubule assembly may play an important epigenetic role. Important questions of whether this effect on tubulin and microtubules is due to the estrogen itself, the formation of a hydroxylated metabolite, or further metabolism of the 2-hydroxyestrogen via oxidative or peroxidative pathways (12-15) remains unanswered. Furthermore, demethylation of 2-methoxyestrogens is an additional pathway of estrogen metabolism (16) and may complicate the *in vivo* investigations of 2-methoxyestradiol as a potential anticancer agent.

In related studies on estrogen drug design and development, our laboratory has examined synthetic estrogens possessing hydroxyalkyl side chains at position C-2 of the A-ring (17,18). These compounds were designed in order to further elucidate the structural and electronic requirements of the estrogen receptor to A-ring modifications. Also, the steroidal agents were envisaged as being stable analogs of the estradiol metabolite, 2-hydroxyestradiol. These analogs contain the oxygen atoms at positions 2 and 3 with unshared pairs of electrons available for hydrogen bonding in protein interactions (receptor, enzyme); furthermore, these analogs are not susceptible to quinone/semiquinone formation and subsequent redox cycling, permitting analysis of the role of redox cycling (19). The homologous series of 2-hydroxyalkylestradiols has been prepared by chain extension of 2-formylestradiol, which in turn was prepared via ortholithiation of estradiol. The substituted estradiols were assayed for abilities to bind to the estrogen receptor in MCF-7 cells and to induce estrogen-responsive gene expression. The estradiol homologs exhibited significantly weaker affinity than estradiol for the MCF-7 cell estrogen receptor, with relative binding affinities (RBA; estradiol = 100) ranging from 1.11 for 2-hydroxymethylestradiol to 0.073 for 2-hydroxypropylestradiol. The
relative activities for mRNA induction of the pS2 gene by the estradiol homologs closely parallel the relative binding affinities for estrogen receptor in MCF-7 cells. 2-Hydroxy-methylestradiol exhibited similar estrogen receptor affinity and pS2 gene induction to the catechol estrogen, 2-hydroxyestradiol.

A methoxy derivative of 2-hydroxymethyl estradiol, 2-methoxymethyl estradiol (2-MeOMeE2; Figure 1), was synthesized and evaluated in this current study. Investigations of the estrogenic activity of 2-MeOMeE2 and 2-MeOE2 were performed in hormone-dependent MCF-7 human mammary cell cultures. Effects of these methoxyestrogen analogs and other hydroxyalkyl estradiols on tubulin polymerization were examined in vitro. The anti-neoplastic properties of this novel steroidal compound were evaluated in MCF-7 cells, in hormone-independent MDA-MB-231 cells, and in nude mice bearing the hormone-independent MDA-MB-435 tumor xenografts. These investigations include evaluation of antiproliferative activity, effects on cytoskeletal structure, and ability to reduce tumor volumes in vivo.

**EXPERIMENTAL PROCEDURES**

Materials and Methods: The 2-HOE2 and 2-MeOE2 were purchased from Steraloids. The 2-hydroxyalkyl estradiols were prepared as previously described (17,18). Biochemicals were purchased from Sigma Ltd. Cell cultures were maintained using a supplemented DMEM media (Gibco), without phenol red and containing 1.5x essential amino acids, 1.5x vitamin and 2x nonessential amino acids. The sterilized liquid media was prepared by the OSU Comprehensive Cancer Center by dissolving the powder into water containing sodium chloride (8.3mM), pyruvic acid (1.25mM) and sodium bicarbonate (17.5mM). Cells were maintained at 37°C, 5% CO2 and 85-95% humidity (Forma model 3052) using Corning culture flasks and plates. Cells were grown to 80% confluence and split as needed for experiments using a trypsin (0.5%) EDTA mixture (Gibco). MCF-7 cells, MDA-MB-231, and MDA-MB-435 were obtained from
The American Type Culture Collection (ATCC) and were stored in liquid nitrogen (-196°C) until needed.

Synthesis of 2-Methoxymethyl Estradiol (2-MeOMeE2):

2-(Methoxymethyl)estra-1,3,5(10)-triene-3,17β-diol. 2-Hydroxymethylestradiol bismethoxymethyl (bisMOM) ether was prepared using methodologies analogous to those previously described (17). Powdered KOH (1.49 g, 26.7 mmol) was added to DMSO (6.0 mL) and stirred for 5 minutes. A solution of 2-hydroxymethyl estradiol bisMOM ether (2.7 g, 6.67 mmol) in DMSO (7.0 mL) was added to the KOH-DMSO solution, followed by addition of methyl iodide (1.72 g, 13.35 mmol). The resulting solution was stirred at room temperature for 14 hours. The reaction mixture was poured in water (50 mL) and extracted with CH2Cl2 (3 x 40 mL). The combined organic layers were washed with a saturated solution of sodium thiosulfate (15 mL), water (3 x 40 mL), brine (40 mL), dried with MgSO4, and concentrated. The residue was purified by chromatography (SiO2, hexane/ethylacetate 4:1) to yield 2.64 g (94%) of 2-methoxymethyl estradiol bisMOM ether as a colorless oil. A solution of 2-methoxymethyl estradiol bisMOM ether (2.5 g, 6.19 mmol) and pyridinium p-toluenesulfonate (PPTS; 13.75 g, 61.9 mmol) in MeOH (70 mL) was heated at reflux for 24 hours. After cooling to room temperature, ethyl acetate (100 mL) was added and the solution washed with water (4 x 50 mL), brine (2 x 50 mL), dried (MgSO4) and concentrated. The residue was purified by chromatography (SiO2, hexane/ethyl acetate, 1/1) to afford 1.6 g (82%) of 2-methoxymethyl estradiol (2-MeOMeE2): mp 169°C; IR (KBr, cm⁻¹) 3332, 2918, 2864, 1723, 1621, 1513, 1427, 1261, 1072, 1008, 787; 1H NMR (CDCl3) 7.18 (1H, s), 6.9 (1H, s), 4.6 (2H, d, J = 3 Hz), 3.71 (1H, t, J = 8.5 Hz), 3.4 (3H, s), 2.88-2.75 (2H, m), 2.3-1.1 (14H,
m, 0.75 (3H, s); 13CNMR (CDCl₃) 153.9, 138.2, 125.09, 119, 116.3, 81.9, 77.1, 74.3, 58.06, 50.2, 43.9, 43.3, 38.9, 36.8, 30.7, 29.3, 27.2, 26.4, 23.1, 11.05; HRMS calcd. 316.2031, found 316.2039. Anal. Calcd for C₂₀H₂₈O₃: C, 75.91; H, 8.92. Found: C, 75.68; H, 8.78.

Whole Cell Estrogen Receptor Studies: MCF-7 cells from 90-100% confluent cultures were harvested by treatment with 0.01% trypsin solution, and the washed cell pellet was divided into 9.4 cm² wells on a six well plate at 1.5-2x10⁵ cells/well in modified MEM (2-3 mL) containing 10% steroid free fetal calf serum and gentamycin (20 mg/mL). The media was removed and then serum free MEM media (888 μL) containing insulin (5.0 mg/L), transferrin (5.0 mg/L), glutamine (2 mM) and albumin (2.0 mg/mL). The synthetic estrogen at various concentrations were added and incubated for 10 min at 37°C. To determine total binding, [³H]-estradiol (3.0 nM, 1.0 μCi) was added and the plates were then incubated for 1 h at 37°C. The cells were washed twice with PBS at 4°C then 95% ethanol (1 mL) was added, followed by standing for 30 min. at room temperature and then counted on a liquid scintillation counter. The blank samples with no cells and nonspecific binding samples, containing 6 μM unlabeled estradiol, were performed in a comparable manner. Specific binding of [³H]-estradiol was calculated by subtracting the nonspecific binding data from total binding data. The EC₅₀ value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal displacement of specific [³H]-estradiol binding and was calculated by a nonlinear regression analysis method (GraphPad Prizm, Version 2.0, San Diego, CA).

pS2 Gene Expression: As a measure of 2-MeOME₂ estrogenicity, the estrogen-regulated gene pS2 was measured in MCF-7 cells following steroid treatment. Twelve hours prior to RNA isolation, cells were treated with 2-MeOME₂, estradiol, or carrier (95% ethanol). Aliquots of RNA equal to 10 μg were loaded onto a 1.5% agarose:(0.66M) formaldehyde gel and electrophoresed for 2 h followed by transfer to nylon membranes. Membranes were hybridized
simultaneously with pS2 and 36B4 probes (10^6 cpm/mL each; specific activity of approximately 1 x 10^9 cpm/µg each) for 12-20 hours. The extent of pS2 induction normalized to the 36B4 signal in each lane and corrected for baseline pS2 expression on an Ambis scanning proportional counter. The EC50 value for each synthetic estrogen homolog represents the concentration of homolog to produce a half-maximal induction of pS2 and was calculated by a nonlinear regression analysis method (GraphPad Prizm, Version 2.0, San Diego, CA).

Tubulin Polymerization Assay: Tubulin solution (240 µL in MES buffer, pH 6.5, 0.5mM Mg^2+, conc. 1mg/mL) was incubated with 10µL of drug solution in DMSO at 37°C for 15 minutes. The samples were chilled on ice and GTP was added (2.5 µL of 100mM solution). Reaction mixtures were transferred onto cuvettes chilled on ice, base line was established and polymerization was followed at 350 nm for 45 minutes, with data points being sampled every 90 seconds. The per cent inhibition of assembly (blank incubation with no drug) after 20 minutes of incubation was used to compare potency of different drug solutions.

Effects on Cytoskeletal Structures by Immunofluorescence and TUNEL Analysis: LLC-PK cells (ATCC, Rockville, MD) were grown in M199 medium (Life Technologies, Gaithersburg, MD) plus 3% fetal bovine serum. Cells were plated on coverslips and allowed to grow for at least 16 hours before drug treatment. 2-MeOE2 and 2-MeOMeE2 were dissolved in DMSO and added to the culture medium at the indicated concentrations for the indicated times.

Cells were fixed with 4% paraformaldehyde in PHEM buffer (10 mM PIPES, 25 mM HEPES, 25 mM EGTA, 2 mM MgCl2, pH 6.9) and lysed with 0.5% Triton X-100 in phosphate-buffered saline with 0.002% sodium azide (PBSa). Following rinsing in PBSa, samples were blocked in 4% normal donkey serum and stained with mouse monoclonal anti-α and anti-β tubulin antibodies (Amersham, Arlington Heights, IL). Samples were washed and
incubated with Cy-3-conjugated Donkey-anti-Mouse antibodies (Jackson ImmunoResearch, West Grove, PA) then stained with 0.2 µg/ml 4′-6-diamidino-2-phenylindole (DAPI) during the final rinse steps. Apoptosis was measured by Apoptosis Detection System, Fluorescein kit (Promega, Madison, WI). Coverslips were mounted onto slides with Mowiol mounting media and examined by epifluorescence on a Zeiss Axioskop. Photomicrographs were recorded on Kodak T-Max 400 film. Cell counts were scored by observing tubulin, DAPI, and TUNEL staining. Six counts of approximately 70 cells each were used to determine the percent of mitotic, multinucleated, and apoptotic cells per timepoint. Data was graphed and statistically analyzed by SigmaPlot (Jandel).

Breast Cancer Cell Cytoxicity Assays: Cytotoxicity in the breast cancer cell lines, MCF-7 (ER+) and MDA-MB-231 (ER-), was determined using the MTS assay. For the MTS bioassay, breast cancer cells were plated into 96-well plate (0.5 x 10⁴ cells/well), and after 24 hours the culture medium was removed and cells washed with PBS. Cells were then treated with estrogen analogs (10 nM to 1 µM) in define media (100 µL/well) at 37°C for 24 hrs. After 24 hours, 20 µl of combined solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) was added into each well, and the culture plate incubated for 2 hours at 37°C. Absorbance at 490 nm was measured (reference wavelength is 700 nm) using a SPECTRAMax plate reader.

Breast Tumor Xenograft Growth Study: Congenitally athymic nude mice were inoculated (flank) with 3 x 10⁶ MDA-MB-435 (ER-) human breast cancer cells in 0.1 ml RPMI 1640 with 10% FBS. Once tumors were measurable, mice were randomized (n=8, each group) to treatment with 2-MeOMeE₂ (50 mg/kg/day, i.p.) or vehicle only. Animal weights and tumor
volumes (t.v.) were measured 2-3 times weekly for 6 weeks (t.v. = smallest diameter$^2$ x largest diameter x 0.52). Data was analyzed using ANOVA (one way for repeated measures).

RESULTS

Chemistry and Biochemistry of Methoxyestrogens:

The synthesis of 2-methoxymethyl estradiol (2-MeOMeE2) was accomplished in four steps from estradiol bis-methoxymethyl (bisMOM) ether using methodologies analogous to those previously described (Figure 2; 17,18). An ortholithiation of estradiol bisMOM ether, followed by reaction with dimethylformamide (DMF), provided 2-formylestradiol bisMOM ether in excellent yields (17,18). Reduction of the 2-formylestradiol bisMOM ether with lithium aluminum hydride provided 2-hydroxymethyl estradiol bisMOM ether. Methylation of 2-hydroxymethyl estradiol bisMOM ether was accomplished by reaction with methyl iodide and potassium hydroxide. Deprotection using pyridinium p-toluenesulfonate (PPTS) yielded 2-methoxymethyl estradiol (2-MeOMeE2) in an 82% overall yields.

The affinities of the 2-methoxyestrogen analogs for the estrogen receptor were assessed in whole cell estrogen receptor binding assays using MCF-7 human mammary cancer cells (18). The whole cell binding assay provides similar relative binding affinities (RBAs) for the estrogen receptor as those obtained using isolated estrogen receptor preparations (18). In addition, the cellular uptake and stability of analogs in the whole cell assay can be assessed. The EC$^{50}$ value for estradiol binding to the estrogen receptor in these whole cell assays was
found to be 0.387 nM (Figure 3). The 2-methoxyestrogen analog with the highest estrogen receptor affinity was 2-MeOMeE₂, exhibiting an EC₅₀ value of 42.4 nM, while 2-MeOE₂ exhibited an EC₅₀ value of 493 nM. Overall, the 2-methoxyestrogen analogs exhibited significantly weaker affinity for the estrogen receptor than estradiol, with relative binding affinities (RBA; estradiol = 100) of 0.91 for 2-MeOMeE₂ and 0.078 for 2-MeOE₂.

The relative estrogenic activities of the 2-methoxy estrogen analogs were evaluated by examining the abilities of the synthetic compounds to induce estrogen-dependent gene expression in human breast cancer cells. In human MCF-7 mammary carcinoma cells, the induction of transcription of the pS2 gene is a primary response to estrogen (20). The induction of pS2 mRNA expression by estradiol, 2-MeOMeE₂, and 2-MeOE₂ was determined by RNA dot blot analysis. The EC₅₀ value for estradiol induction of pS2 mRNA was found to be 0.030 nM, while the EC₅₀ values for 2-MeOMeE₂ and 2-MeOE₂ were 147 nM and 1.07 μM, respectively. Thus, the methoxyestradiol analogs exhibited significantly weaker activity than estradiol for pS2 mRNA induction, with relative activities (estradiol = 100) being 0.02 for 2-MeOMeE₂ and 0.003 for 2-MeOE₂.

Inhibition of Tubulin Polymerization:

A number of estrogenic analogs were screened in a simple in vitro tubulin polymerization assay using specific assay conditions developed by D'Amato et al. for studying interactions of 2-MeOE₂ with tubulin (3). Tubulin solutions were pre-incubated with the compound to be screened for 15 minutes at 37°C, and these mixtures were transferred to cuvettes and chilled on ice. GTP was added to the reaction mixtures and polymerization was
initiated by warming to room temperature. The polymerization was followed spectroscopically at 350 nm, and the extent of polymerization at 20 minutes was compared with the controls to estimate the % inhibition of polymerization. Colchicine and 2-MeOE₂ were used as positive controls. All the compounds were evaluated at a uniform concentration of 3µM in the assay (Figure 5). Colchicine inhibited polymerization by 64% at 3 µM whereas at the same concentration 2-MeOE₂ and 2-MeOMeE₂ inhibited the polymerization by around 34% and 44%, respectively. The extent of inhibition was considerably less when these compounds were evaluated at 1 µM concentrations. The hydroxyalkyl estradiols exhibited moderate activity (inhibition 15-20%). This assay identified 2-MeOMeE₂ as being more effective at concentrations of 1 and 3 µM than 2-MeOE₂ in preventing tubulin polymerization.

**Cytotoxic Activity in Breast Cancer Cell Cultures:**

The cytotoxic effects of 2-methoxy estrogens on hormone-dependent MCF-7 breast cancer cells and hormone-independent MDA-MB-231 breast cancer cells were investigated. Cytotoxic activity was determined using the MTS assay (21). This assay is a colorimetric method for determining the number of viable cell based upon the principle that the MTS reagent is bioreduced by dehydrogenase enzymes found in metabolically active cells into a formazan that is soluble in tissue culture medium. The quantity of formazan, which is directly proportional to the number of viable cells in the culture, is quantified by measuring absorbance at 490 nm. The breast cancer cells were incubated for 24 hours with 2-methoxyestradiol or 2-methoxymethylestradiol at concentrations ranging from 10 nM to 1.0 µM. At 24 hours, the MTS reagent was added, incubated for 2 hours, and formazan dye assayed at 490 nm. Both methoxy estrogens exhibited dose-dependent cytotoxicity on both MCF-7 cells and MDA-MB-
231 cells (Figure 6). 2-MeOE\(_2\) was more effective, producing a 90% reduction in MCF-7 cells and a 70% reduction in MDA-MB-231 cells at 1.0 \(\mu\)M. 2-MeOMeE\(_2\) produced a 30% reduction in MCF-7 cells and a 50% reduction in MDA-MB-231 cells at 1.0 \(\mu\)M.

*Effects of Methoxyestrogens on Cytoskeletal Structures:*

The *in vivo* effects of 2-MeOE\(_2\) and 2-MeOMeE\(_2\) on tubulin depolymerization were characterized in LLC-PK cells. Cells were treated with drug or vehicle for 24 hours and processed for immunofluorescence microscopy. Untreated cells displayed typical microtubule morphology in both mitotic and interphase cells (Figure 7A and 7D). Chromosomes were aligned at the metaphase plate of mitotic spindles (Figure 7A'), and interphase cells were mononucleated (Figure 7D').

Both drugs affected the spindle morphology of mitotic cells, however, 2-MeOE\(_2\)-treated cells exhibited more pronounced phenotypic alterations than 2-MeOMeE\(_2\)-treated cells. Not only was chromosomal alignment disrupted in 2-MeOE\(_2\)-treated cells, but depolymerization of mitotic microtubules was also evident (Figures 7B and 7B'). Several foci of short microtubules were present in cells treated with 15.8 \(\mu\)M 2-MeOE\(_2\), and the chromosomes appeared to be clustered around these microtubule foci. In comparison, the effects of similar concentrations of 2-MeOMeE\(_2\) were less severe (Figure 7C). For example, cells exposed to 15.8 \(\mu\)M 2-MeOMeE\(_2\) for 24 hours showed only slight alterations in spindle microtubule morphology (Figure 7C). Despite this limited effect on spindle microtubules, misaligned chromosomes were present near the spindle poles in the 2-MeOMeE\(_2\)-treated cells (Figure 7C'). These
results suggested that chromosome attachment or movement was partially disrupted in the 2-MeOMeE2-treated cells. Similar mitotic microtubule-staining patterns and chromosome misalignment were observed in 2-MeOE2-treated cells, but only at much lower concentrations (0.87 μM) of 2-MeOE2 (data not shown). The effects of 2-MeOE2 on spindle morphology became progressively more pronounced as the concentration was increased from 0.87 to 8.7 μM. At concentrations of 2-MeOE2 above 8.7 μM, morphological effects similar to those presented in Figure 7B were observed (data not shown). An effect on mitotic spindle morphology was not observed at 2-MeOMeE2 concentrations below 8.7 μM, and concentrations of 2-MeOMeE2 as great as 47 μM did not severely disrupt mitotic microtubule organization (data not shown).

Interphase microtubule arrays were also disrupted following treatment with 2-MeOE2 (Figure 7E). Most microtubules were depolymerized after 24 hours of exposure to 15.8 μM 2-MeOE2. The microtubules still present resembled the “curly” microtubules typical of the more stable subset of detyrosinated microtubules (22). The tyrosination state of these residual microtubules was not determined, however. A typical microtubule array was present in interphase cells exposed to 15.8 μM 2-MeOMeE2 (Figure 7F), however, many of the interphase cells in the treated population contained multiple nuclei (Figure 7F'). These results suggested that cells treated with 2-MeOMeE2 may have undergone a transient mitotic arrest, and then either completed mitosis with misaligned chromosomes or escaped the mitotic arrest and reverted to an interphase state. Due to the misalignment of the chromosomes in these cells (Figure 7C'), either of these events would give rise to interphase cells with multiple nuclei. In contrast, few multinucleated interphase cells were present in the population exposed to
15.8 μM 2MeOE₂ for 24 hours (Figure 7E'), suggesting that few cells escaped the mitotic arrest induced by this concentration of 2MeOE₂.

To further examine the effects of 2-MeOE₂ and 2-MeOMeE₂, cells were exposed to different concentrations of each drug for 24 hours and the mitotic, multinucleated, and apoptotic index was determined. Over a wide range of 2-MeOE₂ concentrations (0.869 - 47.4 μM), an approximately four- to twelve-fold increase in the percentage of mitotic cells in comparison to untreated populations was observed (Figure 8A). In contrast, only minimal increases in the mitotic index were observed after 2-MeOMeE₂ treatment spanning a similar concentration range of drug (Figure 8B). Since cells exposed to 2-MeOMeE₂ retained mitotic spindles (see above), it appears that there was a relatively rapid transition from M to G₁. 2-MeOE₂ treatment restricted M-to-G₁ progression much more effectively because of the near-complete loss of microtubules in mitotic spindles in these treated cells. While both drugs were capable of affecting progression through mitosis, the concentrations of 2-MeOMeE₂ used in this study appeared to induce a delay in mitotic progression, but 2-MeOE₂ induced a more stringent blockage of cells in mitosis.

Exposure of cells to either drug also stimulated an apoptotic response. 2-MeOE₂ generated a greater amount of apoptosis than 2-MeOMeE₂ as measured by TUNEL assay (Figure 8). The proportion of 2-MeOE₂-treated cells that became apoptotic was similar to the proportion that became multinucleated. The amount of apoptotic death may also be underrepresented in these studies due to detachment of dead and dying cells from the coverslip during treatment since only those cells attached to the coverslip were examined.
In a similar fashion, the effects of each estradiol derivative were also followed over time. After exposure to 2-MeOE₂, an accumulation of mitotic cells was apparent after 4 hours and peaked at 75% of the population by 24 hours (Figure 9A). Approximately 40% of the population was multinucleated at 48 hours. The decrease in the percentage of multinucleated cells at 72 hours probably reflects the loss of cells from the coverslip due to apoptosis. Significant accumulation of mitotic cells did not occur in populations treated with 15.8 μM 2-MeOMeE₂. Instead, multinucleated cells accumulated in the population within 24 hours. The multinucleated population remained constant over the remaining course of the experiment, perhaps due to a balance between their generation by ensuing mitosis and their elimination by apoptosis.

**Activity of 2-Methoxymethylestradiol in Tumor-bearing Mice:**

Assessment of *in vivo* antitumor activity was performed in athymic mice containing human breast tumor xenografts. Nude mice bearing MDA-MB-435 tumor xenografts were treated i.p. with 50 mg/kg/day of 2-MeOMeE₂ or vehicle control for 45 days. Differences in tumor volumes between treated animals and control animals were first observed two weeks into the study. Tumor-bearing mice treated with 2-MeOMeE₂ showed an approximate 50% reduction in mean tumor volume at treatment day 45 when compared to control animals (Figure 10). The 2-MeOMeE₂ treatment had no effect on final animal weight at the end of the study.
DISCUSSION

2-Methoxymethyl estradiol (2-MeOMeE2) was prepared in excellent overall yields via a four-step synthesis from estradiol bis-methoxymethyl ether. Both 2-methoxyestradiol and 2-methoxymethyl estradiol were evaluated for estrogen receptor binding activity using whole cell receptor binding assays in MCF-7 human mammary cell cultures. The 2-methoxyestrogens had significantly lower affinity for the estrogen receptor when compared with the endogenous ligand, estradiol. The 2-methoxyestrogen analog with the highest estrogen receptor affinity was 2-MeOMeE2, exhibiting an EC50 value of 42.4 nM, while 2-MeOE2 exhibited an EC50 value of 493 nM. Overall, the 2-methoxyestrogen analogs exhibited significantly weaker affinity for the estrogen receptor than estradiol, with relative binding affinities (RBA; estradiol = 100) of 0.91 for 2-MeOMeE2 and 0.078 for 2-MeOE2. The ability of the 2-methoxyestrogens to induce ER-mediated gene expression was evaluated by measuring pS2 gene transcription. The 2-methoxyestrogens had significantly decreased efficacy for the induction of pS2 mRNA levels in MCF-7 cells when compared with the endogenous ligand, estradiol. The EC50 values for 2-MeOMeE2 and 2-MeOE2 were 147 nM and 1.07 μM, respectively. Thus, the methoxyestradiol analogs exhibited significantly weaker activity than estradiol for pS2 mRNA induction, with relative activities (estradiol = 100) being 0.02 for 2-MeOMeE2 and 0.003 for 2-MeOE2.

The in vitro tubulin polymerization assay identified 2-MeOMeE2 as being more effective than 2-MeOE2 in inhibiting tubulin polymerization at concentrations of 1 and 3 μM. Cushman et al. evaluated a series of 2-substituted estrogens as tubulin polymerization inhibitors, and observed that the size of the substituent at the 2-position plays a critical role in
determining its interaction with tubulin (23). They found that 2-ethoxyestradiol and 2-proenylestradiol were more potent and 2-aminoethylestradiol was equipotent to 2-MeOE2 in the tubulin polymerization assay. Based on these observations they conclude that the optimal substituent in the 2-position for inhibition of tubulin polymerization appears to be one with three atoms from second row of periodic table and which could increase the electron density around the aromatic ring. Our results identified 2-MeOMeE2 as an effective inhibitor, and this compound meets the above criteria and is consistent with the conclusion.

Immunofluorescence staining of tubulin indicated that 2-MeOE2 had a greater effect than 2-MeOMeE2 on microtubule morphology. At concentrations of 8.7 μM, 2-MeOE2 inhibited bipolar spindle formation, blocked cells in mitosis, and disrupted interphase microtubules, whereas in 2-MeOMeE2-treated cells bipolar spindles were formed and there was no apparent effect on interphase microtubule arrays. However, normal spindle function was disrupted in 2-MeOMeE2-treated cells as indicated by the frequent appearance of misaligned chromosomes, a significant mitotic delay, and the formation of multinucleated cells. Ten-fold lower concentrations of 2-MeOE2 (0.87 μM) showed effects on microtubule morphology similar to those observed with 2-MeOMeE2. Recently, it has been suggested that the cytotoxic effects of 2-MeOE2 are probably not due to its depolymerization of microtubules, rather it may exert its effects by disruption of tubulin dynamics (6,8). Similar effects on spindle morphology have also been observed using taxol at low concentrations (24). At these low concentrations, taxol has been shown to block dynamics at the plus ends of microtubules while having no effect on minus end dynamics (25). Thus, the cytotoxic effects of 2-MeOMeE2 also appear to be associated with alterations in tubulin dynamics, since no depolymerization of microtubules was
observed *in vivo*. A disruption of mitotic spindle dynamics is likely responsible for the failure of some chromosomes to align properly at the metaphase plate and contributes to both the observed mitotic delay and subsequent multinucleation seen in 2-MeOMeE2-treated cells. The *in vivo* effects of 2-MeOMeE2 in comparison to 2-MeOE2 on microtubule morphology are in contrast to the *in vitro* results showing a greater effect of 2-MeOMeE2 on microtubule polymerization. These results suggest that the greater effect of 2-MeOE2 on microtubule morphology may be due to differential permeability of the two compounds. This would allow for a higher intracellular concentration of 2-MeOE2 being achieved when both compounds are applied to the cultures at the same concentration.

The *in vivo* antitumor activity of 2-MeOMeE2 was evaluated in athymic mice containing human breast MDA-MB-435 tumor xenografts. Reductions in tumor volumes in animals treated with 50 mg/kg/day were first observed two weeks into the study. Tumor-bearing mice treated with 2-MeOMeE2 showed an approximate 50% reduction in mean tumor volume at treatment day 45 when compared to control animals. These results are similar to the *in vivo* antitumor activity of 2-methoxyestradiol previously reported (2,5). Thus, both 2-MeOMeE2 and 2-MeOE2 are equally effective in suppressing tumor growth in human breast tumor-bearing athymic mice.

In summary, 2-MeOMeE2 is an estrogen analog with minimal estrogenic properties, inhibits tubulin polymerization at micromolar concentrations, and demonstrates antiproliferative effects *in vitro*. Micromolar concentrations of 2-MeOMeE2 induce a delay in mitotic progression of treated cells, with 2-MeOE2 inducing a more stringent blockage of cells in mitosis. The *in vivo* antitumor activity of 2-MeOMeE2 in the human xenograft animal
model of human breast cancer was observed at 50 mg/kg/day. This study supports further evaluation of 2-MeOMeE₂ pharmacodynamics, such as pharmacokinetics and metabolism, in \textit{in vivo} breast cancer models. Also, 2-MeOMeE₂ provides an additional analog for probing structure-activity relationships and for investigating the mechanism(s) of anticancer activity of 2-methoxyestrogen analogs.

**ACKNOWLEDGEMENTS**

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Figure Legends:

Figure 1. Chemical Structures of Methoxyestradiols and Hydroxyestrogens.

Figure 2. Synthesis of 2-Methoxymethylestradiol.

Figure 3. Estrogen Receptor Affinity of Estradiol and Methoxyestradiols in MCF-7 cells. Whole cell competitive binding assays for estrogen receptor were performed with estradiol ( ), 2-MeOMeE2 ( ▲ ), and 2-MeOE2 ( ○ ). Error bars represent standard deviation.

Figure 4. Ability of Estradiol and Methoxyestradiols to Induce pS2 Gene Expression in MCF-7 cells. The induction of pS2 mRNA was determined by northern blot analysis following 12-hour treatment of MCF-7 cells with estradiol ( ), 2-MeOMeE2 ( ▲ ), and 2-MeOE2 ( ○ ). Error bars represent standard deviation.

Figure 5. Inhibition of Tubulin Polymerization by Estradiol, Methoxyestradiols and Hydroxyestradiols. The per cent inhibition of tubulin assembly after 20 minutes of incubation was used to compare potencies of different drugs at 1.0 μM ( □□□□ ) and 3.0 μM ( □□□□ ). (A) colchicine [positive control], (B) 2-MeOE2, (C) 2-MeOMeE2, (D) 2-HOMeE2, (E) 2-HOEtE2, and (F) 2-HOPrE2. Error bars represent standard deviation.

Figure 6. Cytotoxic Activities of 2-MeOE2 and 2-MeOMeE2 on Human Breast Cancer Cell Cultures. Cytotoxicity in the breast cancer cell lines, MCF-7 cells ( □□□□ ) and MDA-MB-231 cells ( □□□□ ), was determined using the MTS assay. Error bars represent standard deviation.

Figure 7. Immunofluorescence Staining of Tubulin and DNA Patterns in LLC-PK Cells. A typical metaphase microtubule spindle (A) and chromosomal alignment by DAPI staining (A') is shown in an untreated mitotic cell. Panels (B) through (F) show staining of cells following 24-hour treatment with 15.8 μM 2-MeOE2 or 2-MeOMeE2. 2-MeOE2 exposure induced depolymerization of most mitotic...
microtubules (B), resulting in a loss of chromosomal alignment (B'). 2-MeOMeE₂ treatment had only a slight effect on the morphology of the spindle (C), however, chromosome misalignment was present (C'). A characteristic microtubule array (D) and single nucleus (D') are present in untreated interphase cells. 2-MeOE₂ induced depolymerization or a majority of the interphase microtubules (E), and cells contained a single DAPI-staining nucleus (E'). 2-MeOMeE₂ did not affect interphase microtubules (F), but many interphase cells in the population were multinucleated (F'). The bar in panel C' and F' equals 6 and 17 μm, respectively.

Figure 8. **Effect of 2-MeOE₂ and 2-MeOMeE₂ on LLC-PK cells.** The percentage of mitotic, multinucleated, and apoptotic LLC-PK cells were determined following a 24 hour treatment with increasing micromolar concentrations of 2-MeOE₂ and 2-MeOMeE₂. White bars indicate the percent of mitotic cells in the population remaining attached to coverslips after the 24 hour treatment with either 2-MeOE₂ (A) or 2-MeOMeE₂ (B). Black and hatched bars depict the percent of multinucleated and apoptotic cells, respectively. Error bars represent standard deviation.

Figure 9. **Time Course of 2-MeOE₂ and 2-MeOMeE₂ Effects on LLC-PK cells.** Cells were treated with 15.8 μM 2-MeOE₂ (A) or 15.8 μM 2-MeOMeE₂ (B) and the percentage of mitotic, multinucleated, and apoptotic cells were determined at various times. White bars indicate the percent of mitotic cells in the population remaining attached to coverslips after the indicated times. Black and hatched bars depict the percent of multinucleated and apoptotic cells, respectively. Error bars represent standard deviation.

Figure 10. **Effect of 2-MeOMeE₂ on Tumor Progression in Athymic Mice MDA-MB-231 Xenografts.** Athymic nude mice containing MDA-MB-435 (ER-) human breast cancer cell xenografts were treated i.p. with 2-MeOMeE₂ at 50 mg/kg/day (○) or vehicle only (●). Data was analyzed using ANOVA (one way for repeated measures) and error bars represent standard deviation.
Figure 1.

2-Methoxyestradiol
2-MeOEtE₂

2-Methoxymethyl Estradiol
2-MeOMeE₂

2-Hydroxyethyl Estradiol
2-HOEtE₂

2-Hydroxymethyl Estradiol
2-HOME₂
Figure 2.

 Estradiol bisMOM ether → 2-formylestradiol bisMOM ether

 Estradiol bisMOM ether → 2-hydroxymethyl estradiol bisMOM ether

 Estradiol bisMOM ether → 2-methoxymethyl estradiol bisMOM ether

 Estradiol bisMOM ether → 2-methoxymethyl estradiol
Figure 3.

- Estradiol
- 2-methoxymethyl estradiol
- 2-methoxy estradiol

fraction bound

log [estrogen] (M)
Figure 4.
Figure 5.

A = colchicine
B = 2-methoxy estradiol
C = 2-methoxymethyl estradiol
D = 2-hydroxymethyl estradiol
E = 2-hydroxyethyl estradiol
F = 2-hydroxypropyl estradiol
Figure 6.

10^4 cells in 96-well plate

n = 3
P < 0.05
Figure 7.
Figure 8.
Figure 10.

![Graph showing tumor volume (mm$^3$) over days of treatment for control and 2-MeOMeE$_2$ treated groups. The graph indicates a significant increase in tumor volume for the treated group compared to the control. The y-axis represents tumor volume in mm$^3$, and the x-axis represents days of treatment. The legend identifies the control group with solid squares and the 2-MeOMeE$_2$ treated group with open circles. The treated group shows a sharp increase in tumor volume at day 40, indicating a potential effect of the treatment.](image-url)
A: SYNTHESIS AND BIOCHEMICAL EVALUATION OF ESTROGEN ANALOGS
B: SYNTHETIC STRATEGIES FOR CONSTRUCTING BENZOPYRONE COMBINATORIAL LIBRARIES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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* * * * *

The Ohio State University

1999

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ABSTRACT

4-Hydroxy estradiol (4-OHE₂) is an oxidative metabolite of estrogens that is implicated as a possible causative agent in estrogen-induced tumorigenesis. The tumorigenic potential of 4-OHE₂ may be a result of its ability to bind ER and initiate mitogenic events or its oxidative metabolism and redox-cycling properties resulting in DNA lesions. In order to separate the receptor activation and redox cycling properties, a series of C-4 hydroxyalkylestradiol analogs were synthesized as metabolically stable analogs of 4-OHE₂. These compounds lack the catechol moiety and were not expected to undergo redox cycling and produce oxidative stress. On the other hand, the hydroxyl groups at position 3- and 4- in these compounds were expected to mimic the binding interactions of 4-OHE₂ with receptors and enzymes. The Stille cross-coupling and the carboxymethylation reactions used for the synthesis of these analogs represent two efficient and previously unexplored synthetic routes for the functionalization of the 4-position of estradiol. The 4-hydroxyalkyl estrogens and catechol estrogens were compared in potentiometric and DNA-damaging studies. These studies revealed that the non-redox cycling estrogen analogs are unable to induce DNA damage, whereas catechol estrogens produce DNA damage. A novel synthetic route was developed for synthesis of
catechol estrogens based on Baeyer-Villiger oxidation of 2- and 4-substituted formyl estradiols. In addition several estrogen analogs were synthesized as potential inhibitors of estrogen-hydroxylases, the enzymes responsible for metabolizing estrogens into catechol estrogens. 2-Methoxymethylestradiol (2MME₂) was identified as a novel inhibitor of tubulin polymerization in vitro.

The benzopyrone ring system is present in various natural products that interact with enzymes and receptors of therapeutic importance in breast and prostate cancer. In another project, a novel synthetic route was developed for constructing benzopyrone libraries. Readily available salicylic acids and terminal alkynes were used as building blocks for the benzopyrone ring system. A series of o-(O-t-buty1silyloxy)benzoyl chlorides generated from salicylic acids were coupled with a range of terminal alkynes to afford alkynyl ketones. The alkynyl ketones were converted to enaminoketones and cyclized to yield a benzopyrone ring system. Piperazinyl resin was used to effect a resin-capture of the alkylnones to yield support bound enaminoketones, which underwent an on-resin cyclization to provide the benzopyrone ring system. This synthetic approach utilizes readily available starting materials, mild and high yielding reactions with good functional group tolerance, and is ideal for developing combinatorial libraries centered around the benzopyrone ring system.
Dedicated

To

Suresh, Archana & Virunya
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PUBLICATIONS

Research Publications


FIELD OF STUDY

Major Field: Pharmacy, Medicinal Chemistry
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<td>CV of 2-Hydroxyethyl estradiol</td>
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<td>4.3</td>
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