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A series of C-4 hydroxyalkylestradiol analogs were synthesized as metabolically stable analogs of 4-hydroxyestradiol (4-OHE_2) 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 ($2MME_2$) 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_2) is not tumorigenic, whereas 4-hydroxyestradiol (4-OHE_2) is the primary tumor initiator.[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_2 is similar to estradiol in its ability to bind to and activate the classical estrogen receptor.[2]

Interestingly, the interaction of 4-OHE_2 with ER appears to occur with a reduced dissociation rate compared with estradiol, suggesting that the association of 4-OHE_2 with ER may last longer than for the parent hormone. These results suggest that 4-OHE_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_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_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_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_2.

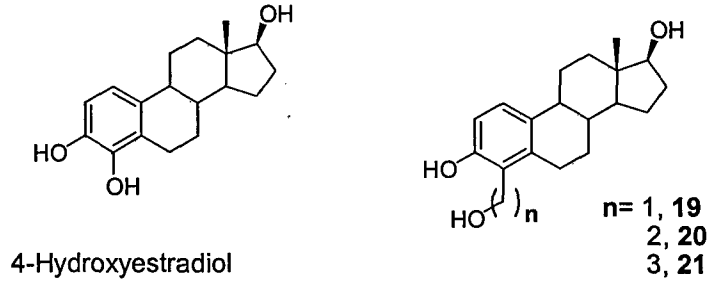


Figure 2.1 Biochemically Stable Analogs of 4-OHE_2

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_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.[3] These compounds had exhibited similar estrogen receptor affinity and pS2 gene induction to the catechol estrogen 2-hydroxyestradiol. The chemical synthesis of 2-hdyroxyalkyl estrogens (**22-24**) relied on a key formylation reaction (**Figure 2.2**).[4] 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 alkyllithium would undergo halogen-lithium exchange reaction and regiospecifically provide the 4-lithiated estradiol.

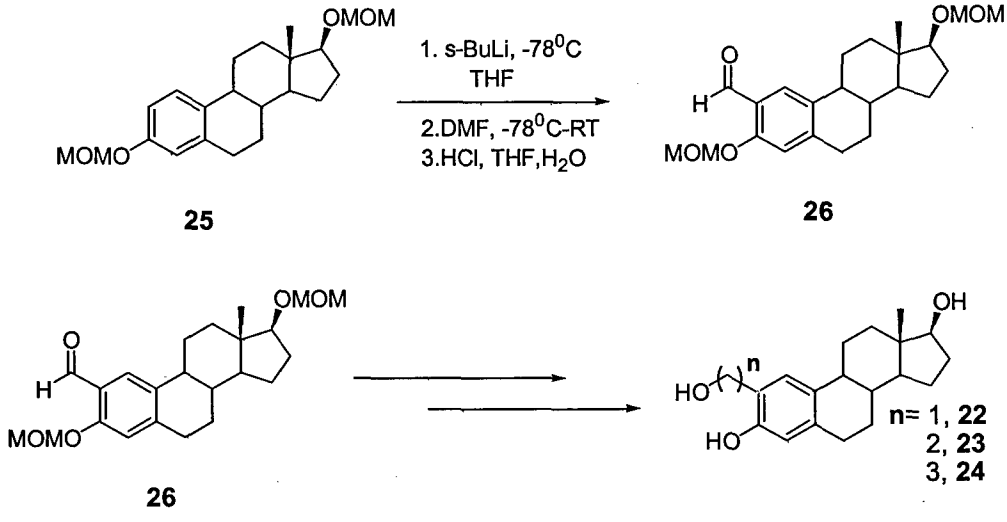


Figure 2.2 Synthesis of 2-Formylestradiol

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.

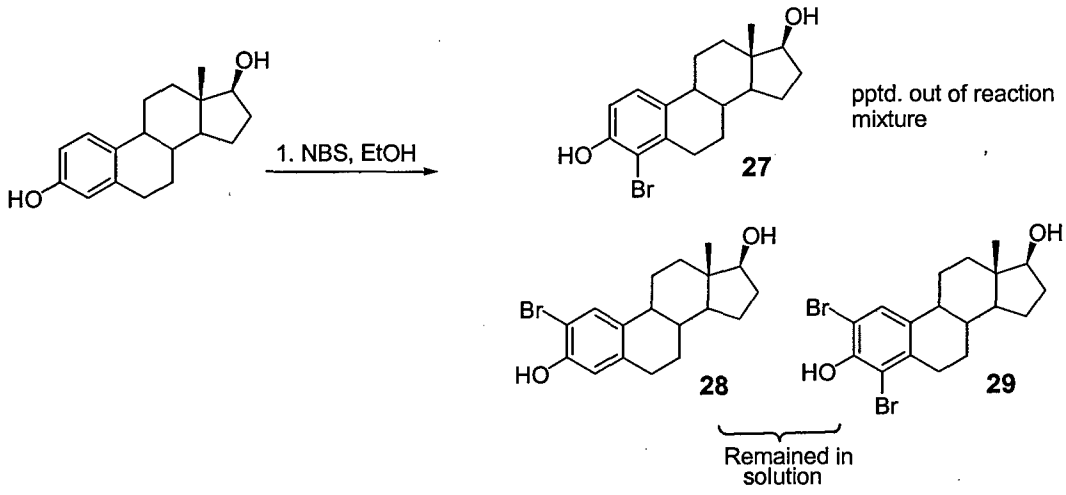


Figure 2.3 Bromination of Estradiol

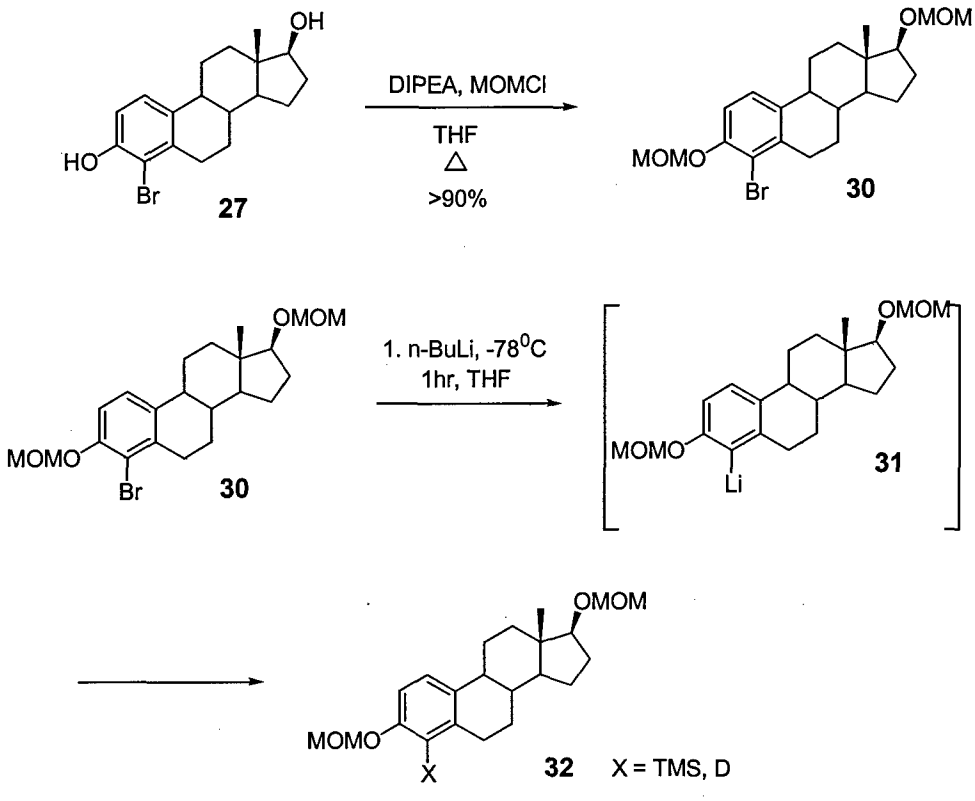


Figure 2.4 Halogen-Lithium Exchange Reaction of 4-Bromoestradiol

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_2O 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*-BuLi), methyllithium, and *sec*-butyllithium. *n*-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*-BuLi. The time for the lithium exchange reaction was optimized by treating the solution of **30** with 1.2 equivalents of *n*-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

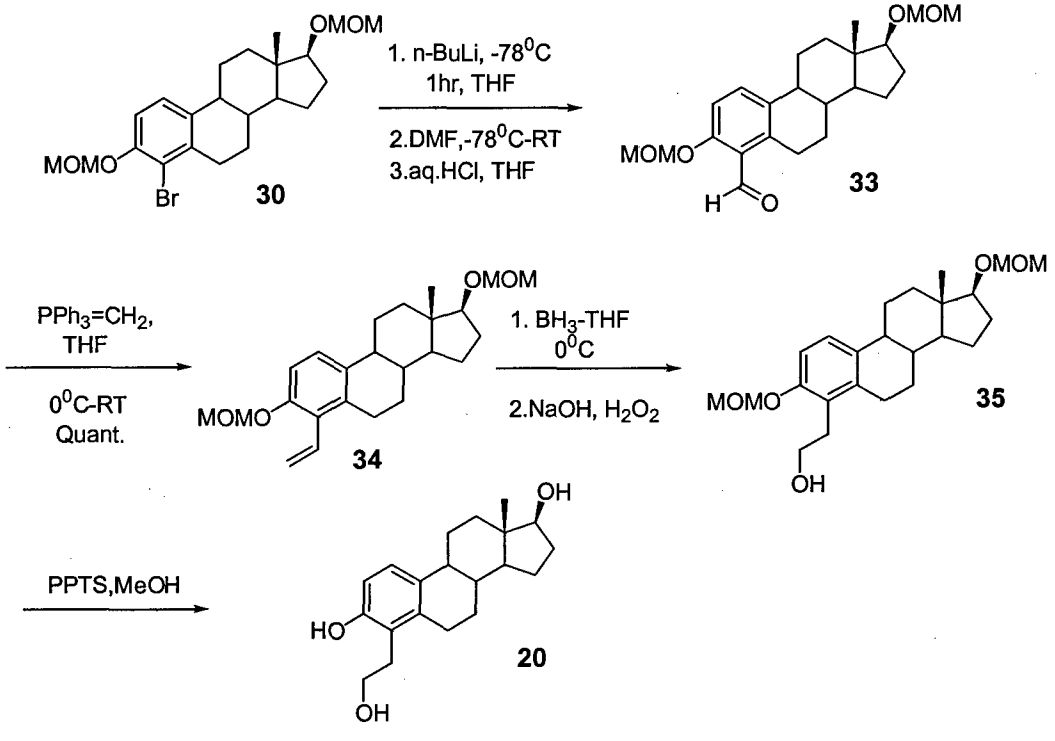


Figure 2.5 Preliminary Synthesis of 4-Hydroxyethylestradiol

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 CH_2=PPh_3 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.

No.	Electrophile	Result
1	MOMCl	**25**
2	Ethylene Oxide	**25**
3	DMF; HCl	**33 (34%), 25**
4	N-methyl formamide	**25**
5	Allylbromide	**25**
6	Allylbromide, TBAI	**Complex Mixture**
7	CO_2	**38**

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 CuI, 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, CO_2 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 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.

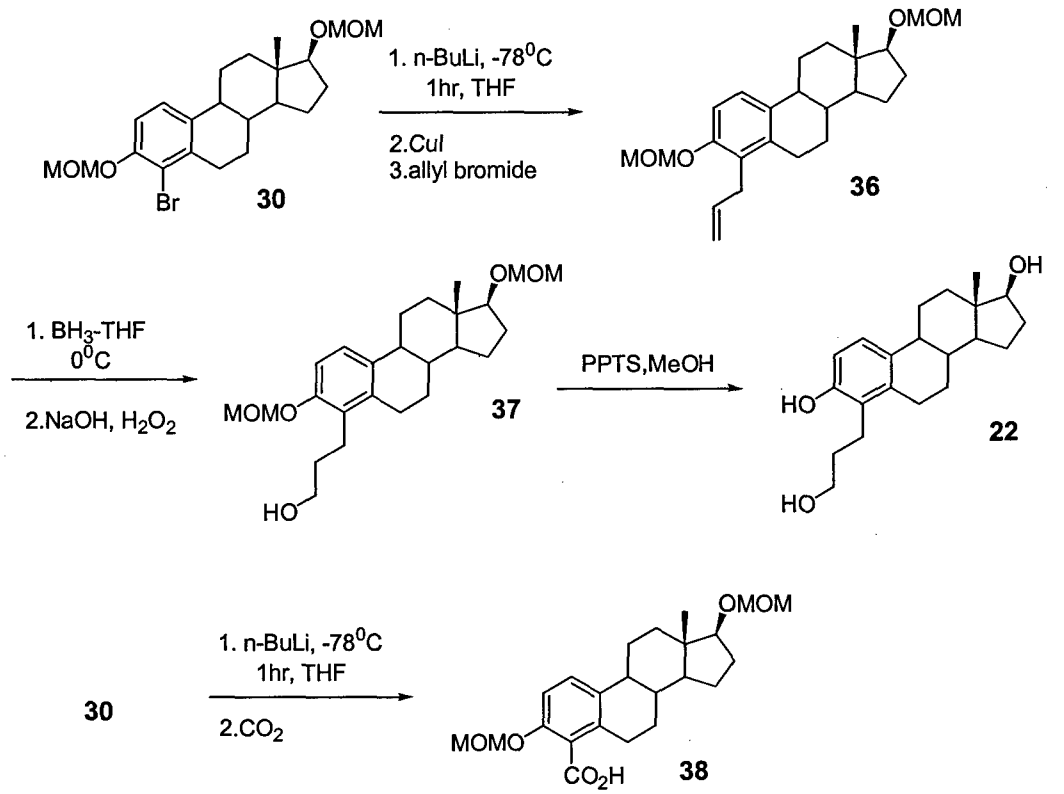
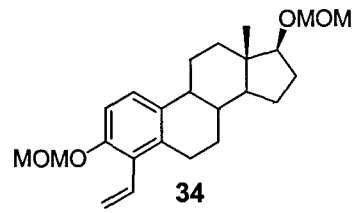


Figure 2.6 Reaction of **30** with Allylbromide and Carbon Dioxide

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 Pd^0 chemistry. Specifically, **30** could be reacted with 4-vinyl- or 4-allyltin reagents in the presence of 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 Pd^0 catalyst to yield the 4-formylestradiol **33**, that could be reduced to the hydroxymethyl derivative **19**.[7]



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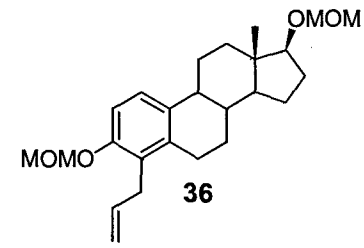


36

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 $Pd(PPh_3)_4$ 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.

Nucleophile	Catalyst	Solvent	Result
Methylacrylate	$Pd(OAc)_2$, $P(O\text{-}tol)_3$	Et_3N, 100°C	No reaction
Vinyltributyltin, 1equiv.	$PhCH_2PdCl(PPh_3)_2$ 10mol%	Toluene	No reaction
Vinyltributy tin 1equiv.	DPPE-PdCl, PPh_3 LiCl	Toluene	No reaction
Vinyltributyl tin 1equiv.	$Pd(PPh_3)_4$, 5mol%	Toluene, 100°C	75% unreacted 3% **34**
Vinyltributyl tin 1equiv.	$Pd(PPh_3)_4$, 25mol%	Toluene, 100°C	15% **34**
Vinyltributyl tin 1equiv.	$Pd(PPh_3)_4$, 15mol%	Toluene, 100°C	15% **34**
Vinyltributyl tin 1equiv.	$Pd(PPh_3)_4$, 15mol%	Dioxane	Decomposition
Vinyltributyl tin 1equiv.	$Pd(PPh_3)_4$, 15mol% CuI, 8 mol%	Dioxane	Decomposition
Vinyltributyl tin 1equiv.	$Pd(PPh_3)_4$, 15mol% CuI, 8 mol%	Toluene	Decomposition
Vinyltributyl tin, 2 equiv.	$Pd(PPh_3)_4$, 15mol%	Toluene, 100°C	25% **34**
Vinyltributyl tin 2 equiv.	$Pd(PPh_3)_4$, 15mol%	DMF, 130°C	>75% **34**
Vinyltributyl tin 2equiv.	$Pd(PPh_3)_4$, 5mol%	DMF, 130°C	90% **34**

Table 2.2 Stille Reaction Conditions Explored for Functionalization of **30**.

The $Pd(PPh_3)_4$, used for this reaction was prepared using the procedure described by Coulson *et al.* and stored under argon.[8] 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 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% H_2O_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.[3] 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**.

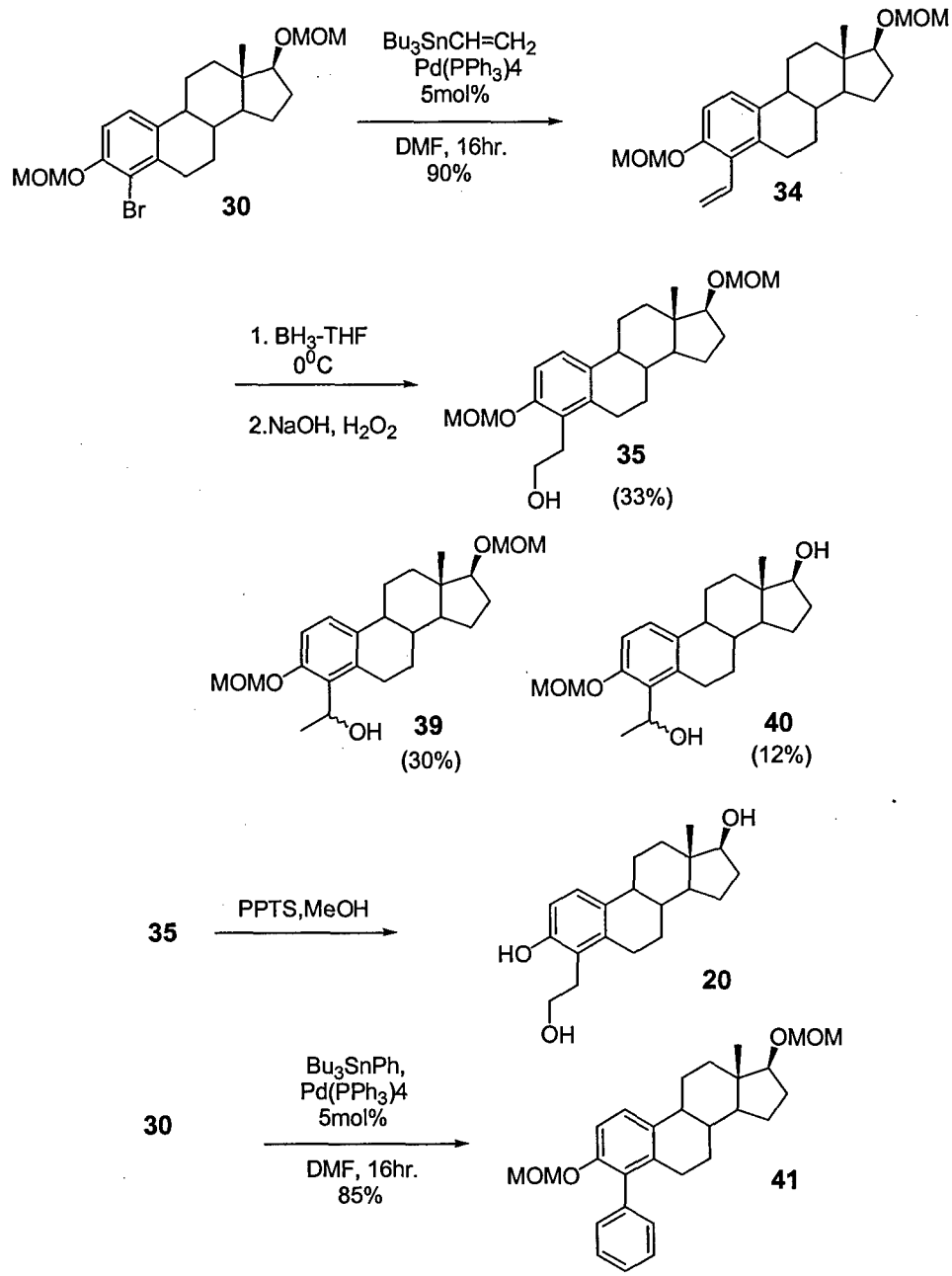


Figure 2.7 Synthesis of 4-Hydroxyethylestradiol and 4-Phenylestradiol

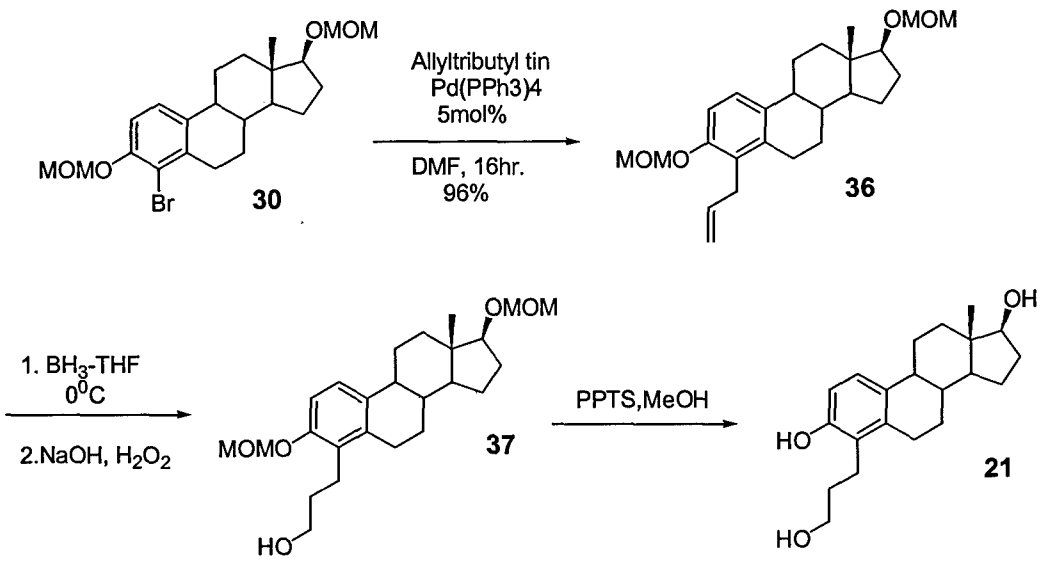


Figure 2.8 Alternative Precursors for Stille Reaction

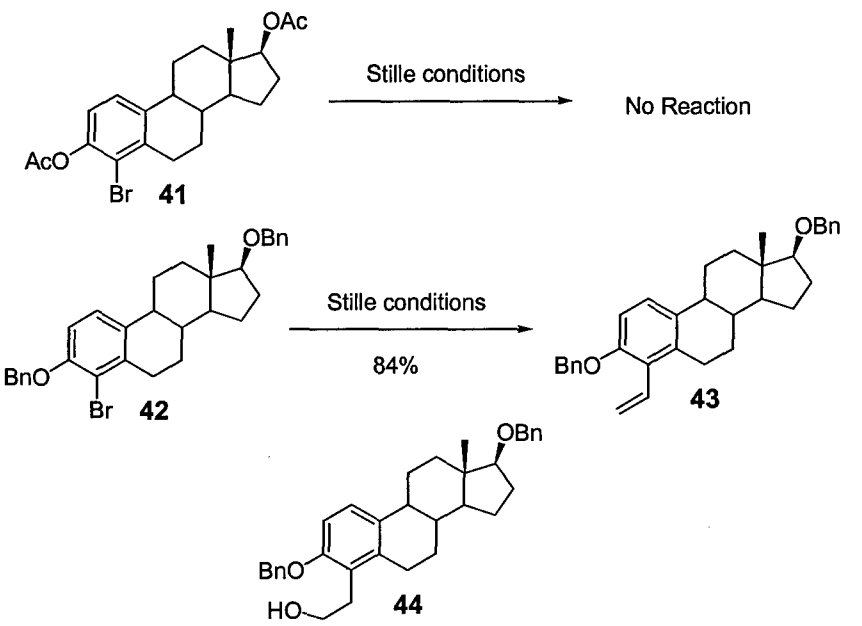
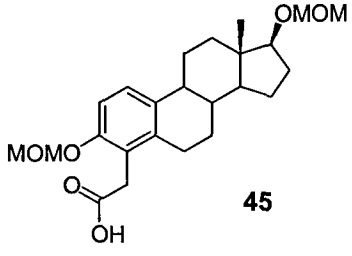


Figure 2.8 Synthesis of 4-Hydroxypropylestradiol

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 $KMnO_4$ and $NaIO_4$ to provide the corresponding carboxyl derivative **45** was unsuccessful. It is interesting to note that reaction of **34** with $KMnO_4$ and $NaIO_4$ provided the corresponding carboxyl derivative **38** in quantitive 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.[7] 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.



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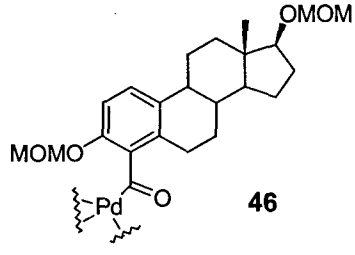


46

Reagents	Catalyst	Solvent	Result
CO, Vinyltributyltin Pressure tube	$Pd(PPh_3)_2Cl_2$	Toluene-HMPA 60/100°C	20% 34
CO, tetramethyltin Pressure tube	$Pd(PPh_3)_2Cl_2$	Toluene-HMPA 80/100°C	No reaction
CO, Bu_3SnH Pressure tube	$Pd(PPh_3)_2Cl_2$	Toluene-HMPA 80/100°C	Mixture of **25** and starting material
CO, Slow-addition of Bu_3SnH	$Pd(PPh_3)_4$	Toluene-HMPA 60-90°C	Mixture of **25** and starting material
CO, Slow-addition of Bu_3SnH	$Pd(PPh_3)_2Cl_2$	Toluene 60-90°C	Mixture of **25** and starting material
CO, Slow-addition of Bu_3SnH	$PhCh_2Pd(PPh_3)_2Cl$	Toluene-HMPA 90°C	Mixture of **25** and starting material
CO, Slow-addition of Bu_3SnH	$Pd(PPh_3)_4$	Toluene	Mixture of **25** and starting material

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.[9] 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 CO_2 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 $LiAlH_4$ 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 $LiAlH_4$. Refluxing **47** with PPTS in methanol formed **49** in 80% yield. 4-hydroxymethyl was produced from **49** in 64% yield by $LiAlH_4$ reduction.

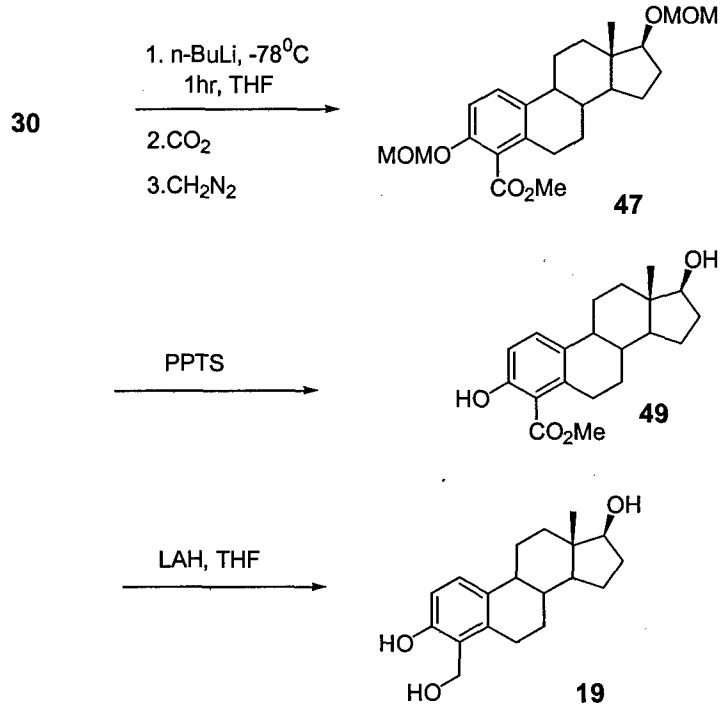


Figure 2.8 Synthesis of 4-Hydroxymethyl Estradiol

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.[10,11,12] 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 nitroestrones were converted to the corresponding nitroestradiols by $NaBH_4$ reduction. The nitroestradiols were further reduced to the corresponding aminoestradiols **53** and **54** with sodium hydrosulfite. We found that the nitroestradiols were reduced more efficiently by catalytic hydrogenation (55 psi, 5% Pd-C in methanol). The *ortho*-aminophenols 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.

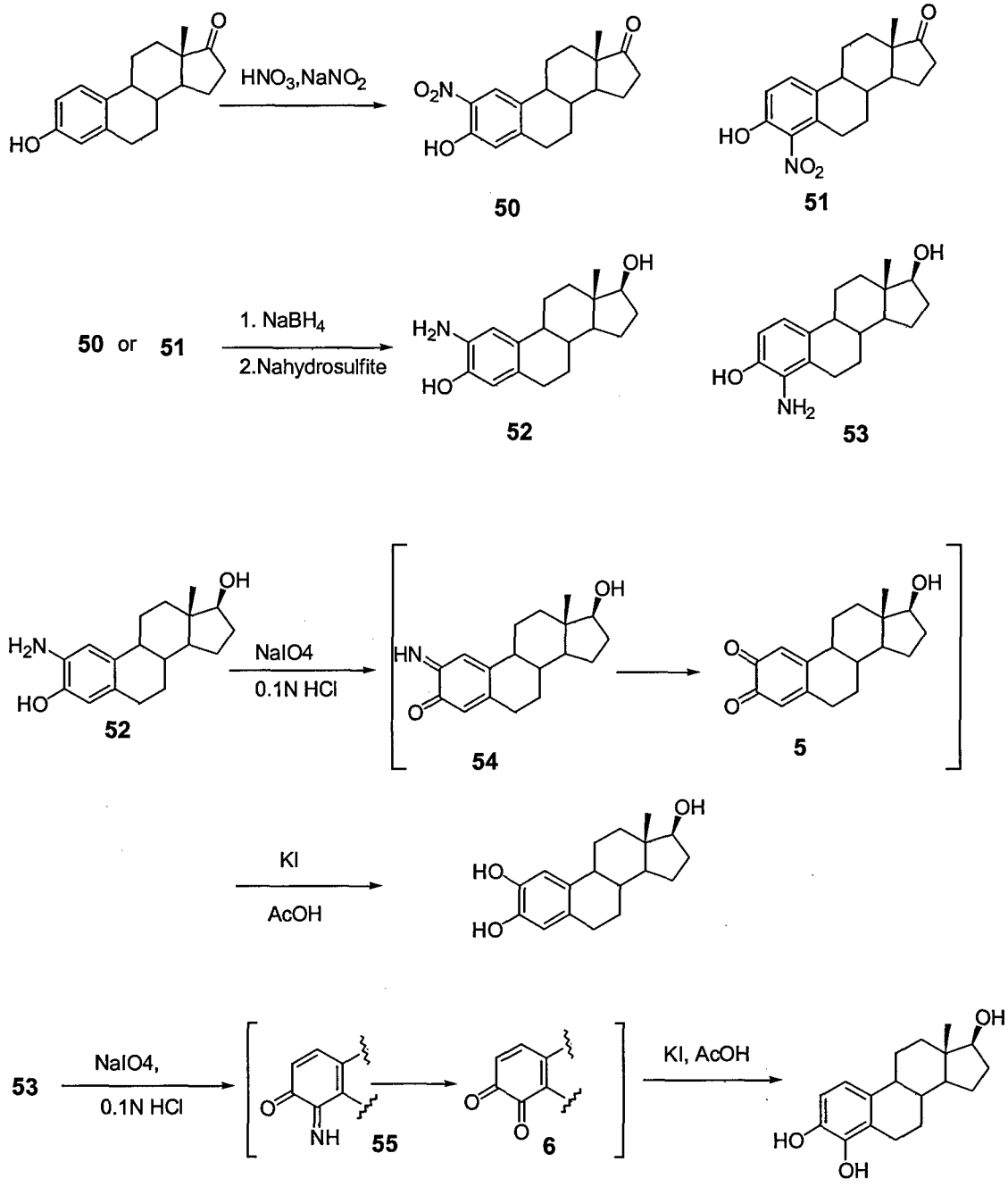


Figure 3.1 Stubbenrauch and Knuppen's Catechol Estrogen Synthesis

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.[13] The authors employed a Friedel-Craft's acylation to access

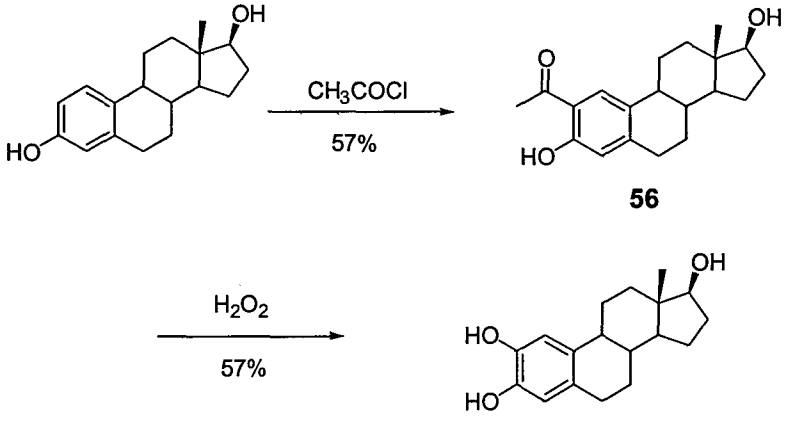


Figure 3.2 Dakin Oxidation of Acetyl Estrogens

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-OHE_2. A similar strategy was employed for the synthesis of 2-OHE_1. 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.[14] Organic peroxy acids such as peroxyaceticacid, trifluoroperoxyaceticacid, 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**.[15]

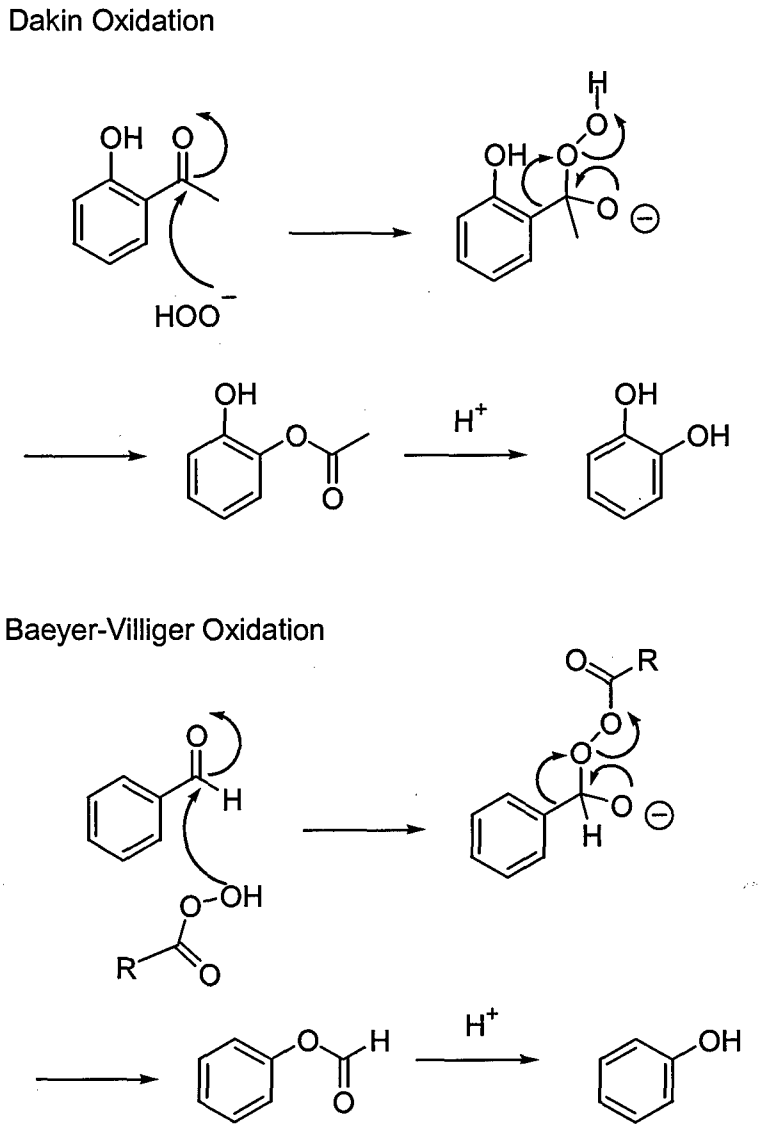


Figure 3.3 Comparitive mechanisms of Dakin and Baeyer-Villiger Oxidation

3.1.1 SYNTHESIS OF 2-HYDROXYESTRADIOL: A suitably protected 2-formylestradiol derivative **26** was the starting point for synthesis of 2-OHE_2 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. H_2SO_4 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% CH_3CN/ H_2O 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-OHE_2 was stored in the freezer without any appreciable decomposition for several months.

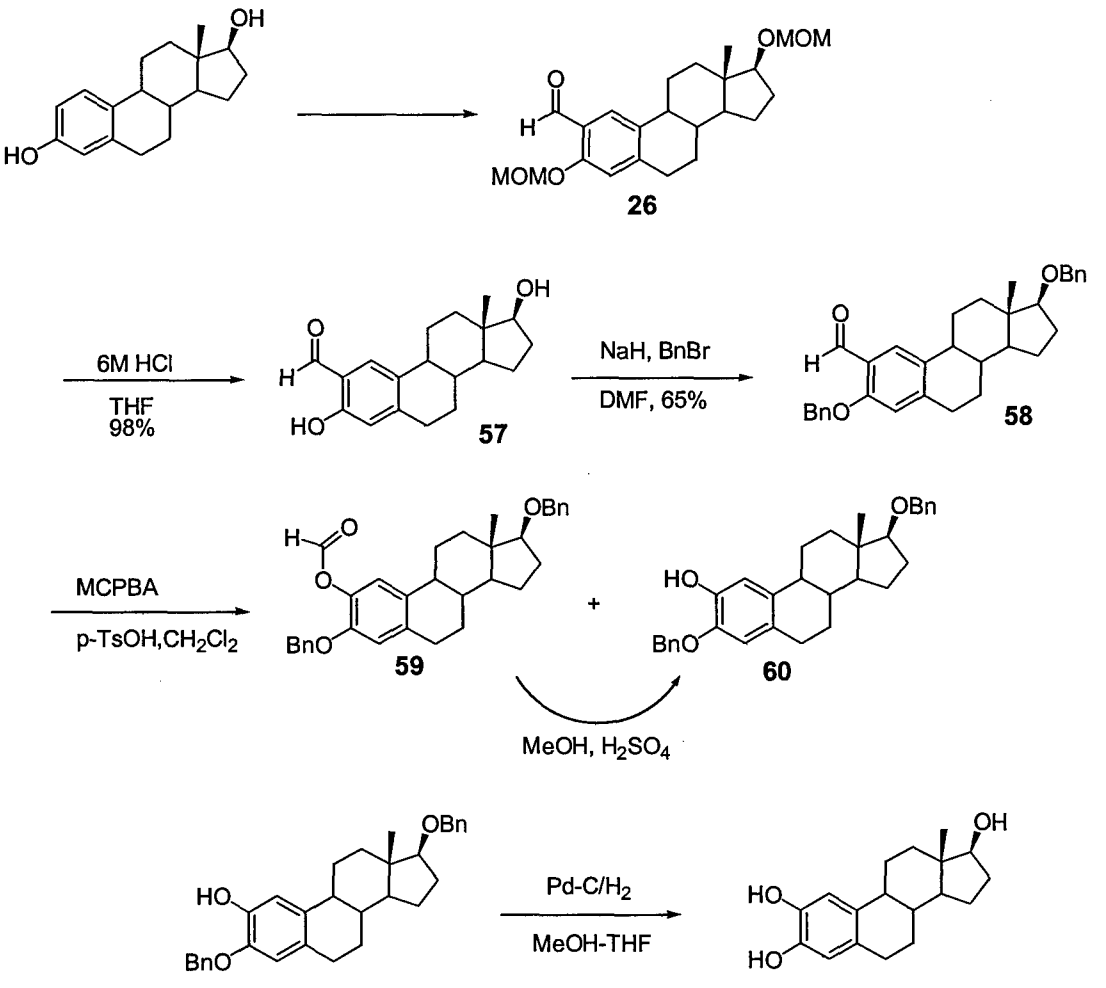


Figure 3.4 Synthesis of 2-Hydroxyestradiol

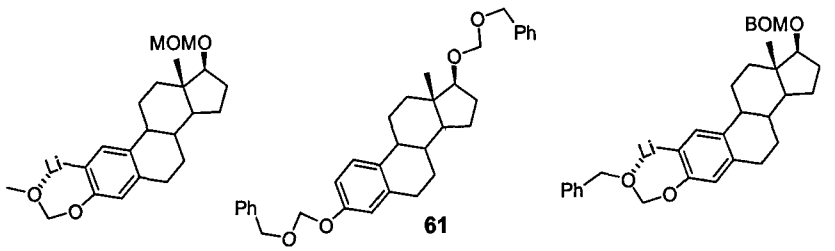


Figure 3.6 Suggested Replacement of MOM group with BOM Protecting group

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-postion 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 benzyloxymethyl group (BOM).[17] 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-postion 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-OHE_2 and increases the overall yield up to 00%.

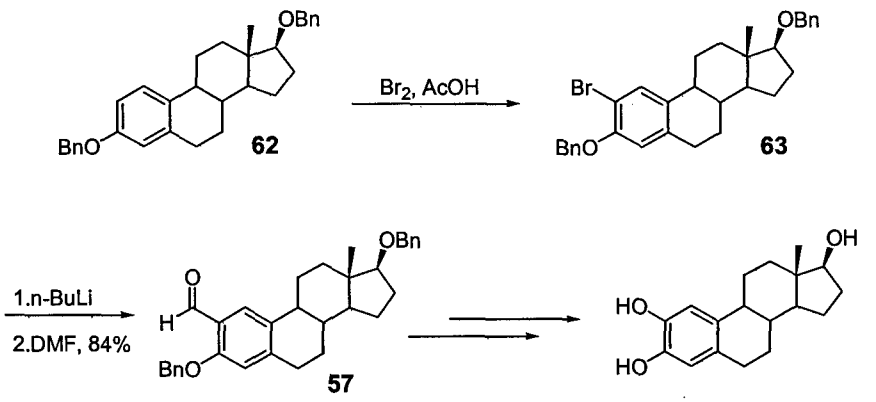


Figure 3.7 Alternative Synthesis of **57**

3.1.2 SYNTHESIS OF 4-HYDROXYESTRADIOL: The key intermediate for 4-OHE_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 *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^{+2} and 1,2-dibromoethane.[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 *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_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_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**.

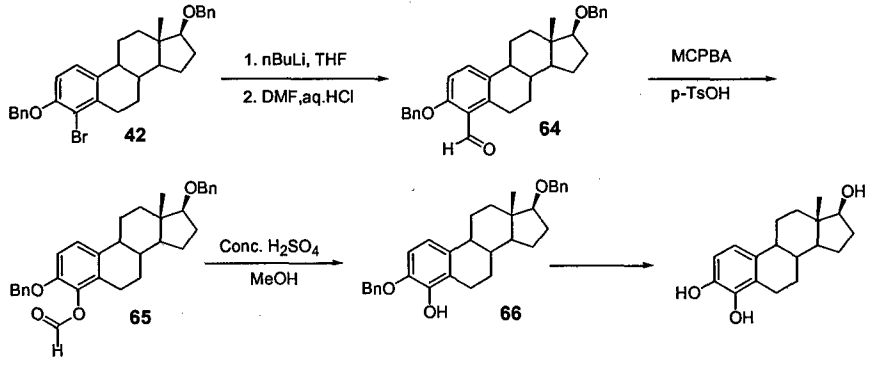


Figure 3.8 Synthesis of 4-Hydroxyestradiol

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.[19] 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.[2,19,20] 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.[19,20] 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.[21,22] 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.

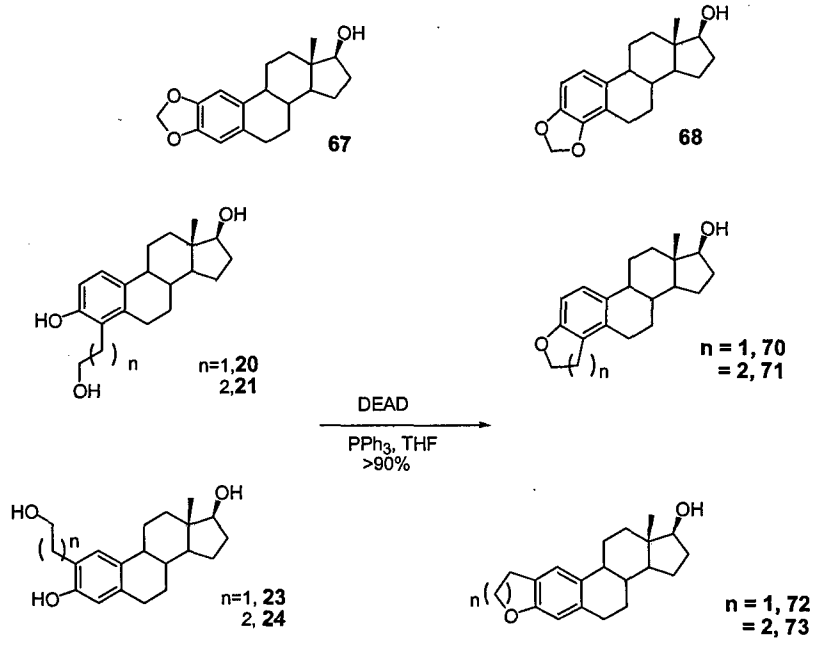


Figure 3.9 Synthesis of A-ring Fused Heterocyclic Estrogens

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 PPh_3 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.

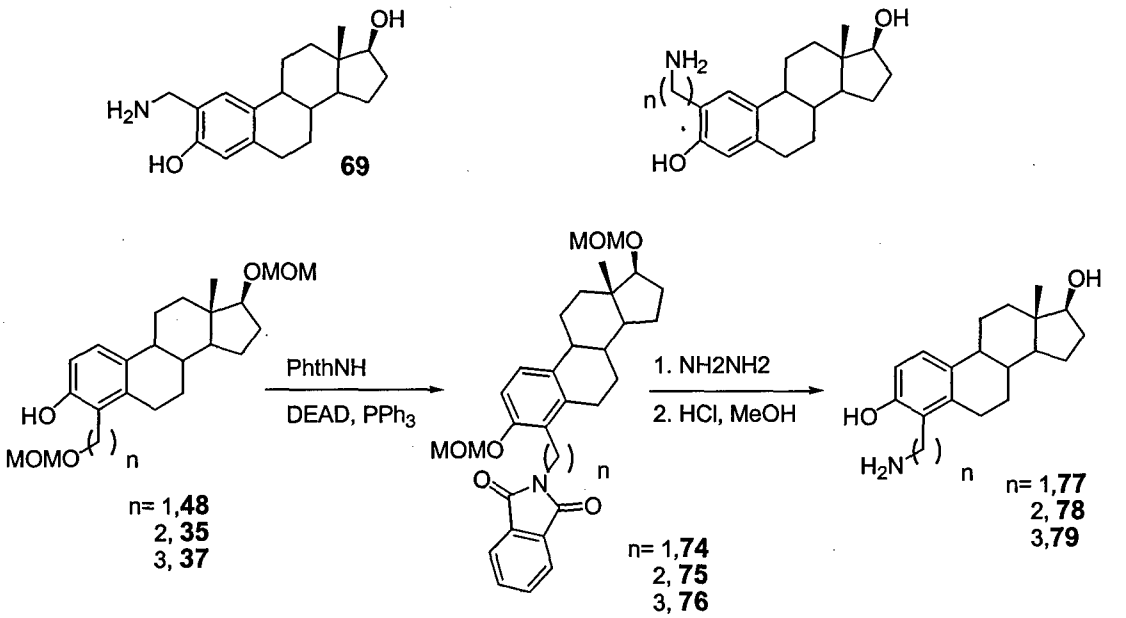


Figure 3.10 Synthesis of 4-Aminoalkylestradiols

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 PPh_3 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 ($2MeOE_2$), an endogenous metabolite of estradiol, was shown to possess cytotoxic properties in cancer cell cultures.[23] $2MeOE_2$ 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 $2MeOE_2$ inhibits *in vitro* tubulin polymerization by interacting at the colchicine binding site.[24] Interaction of $2\text{-}MeOE_2$ 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 $2MeOE_2$ as the most potent microtubule disruptive agent amongst the steroids studied.[25] Interestingly, Fotsis *et al.* have reported that 2-methoxyestradiol inhibits angiogenesis *in vitro* and suppresses tumor growth.[26] They suggest that interactions of $2MeOE_2$ with tubulin networks of growing vascular cells may be responsible for the angiogenic activity. $2\text{-}MeOE_2$ 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.[16] 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 $NaBH_4$ 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.[27] Stille couplings between **30** and **84** were attempted in DMF using 2 equivalents of stannane and either $Pd(PPh_3)_4$ or $Pd(PPh_3)_2Cl_2$ 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.

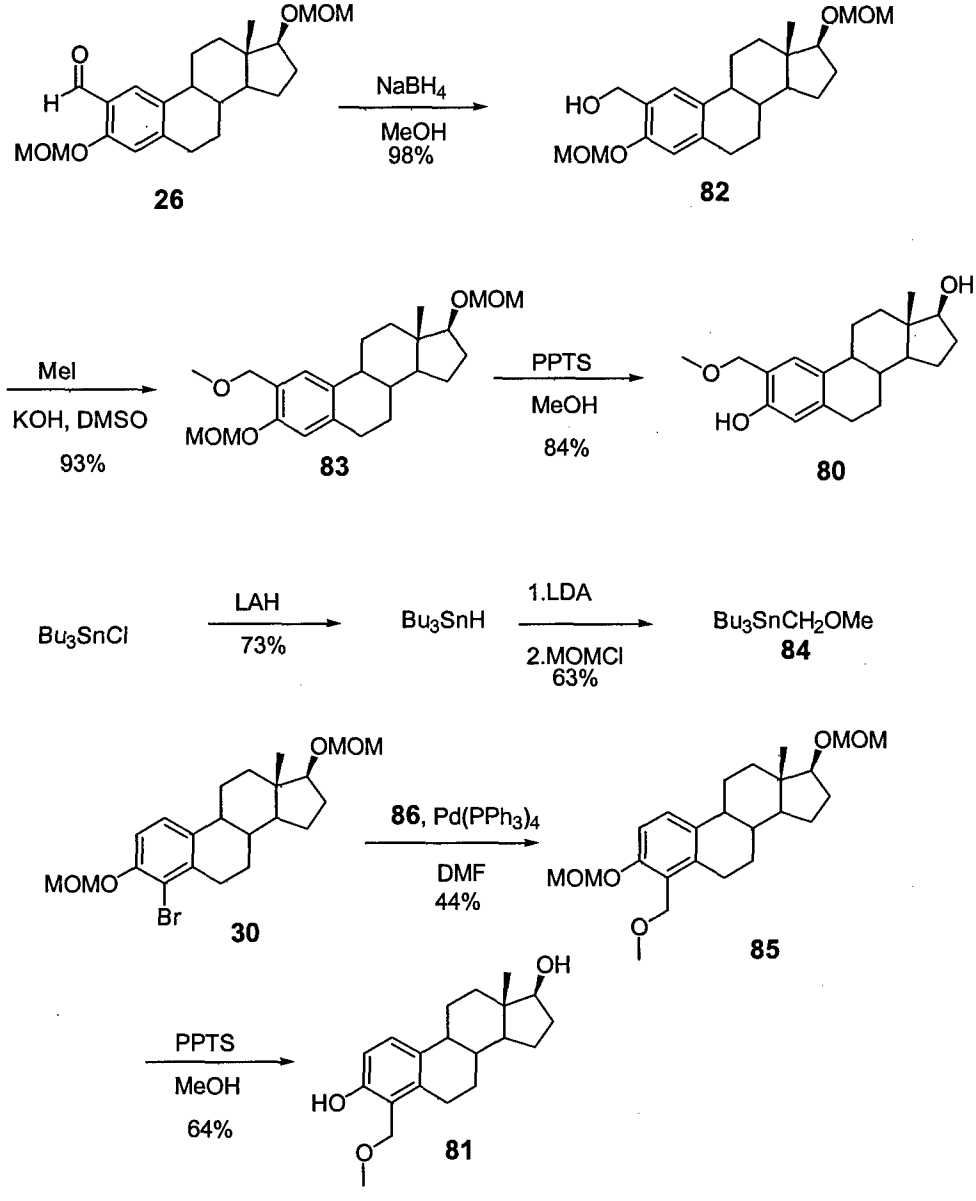


Figure 3.11 Synthesis of 2- and 4-Methoxymethylestradiol

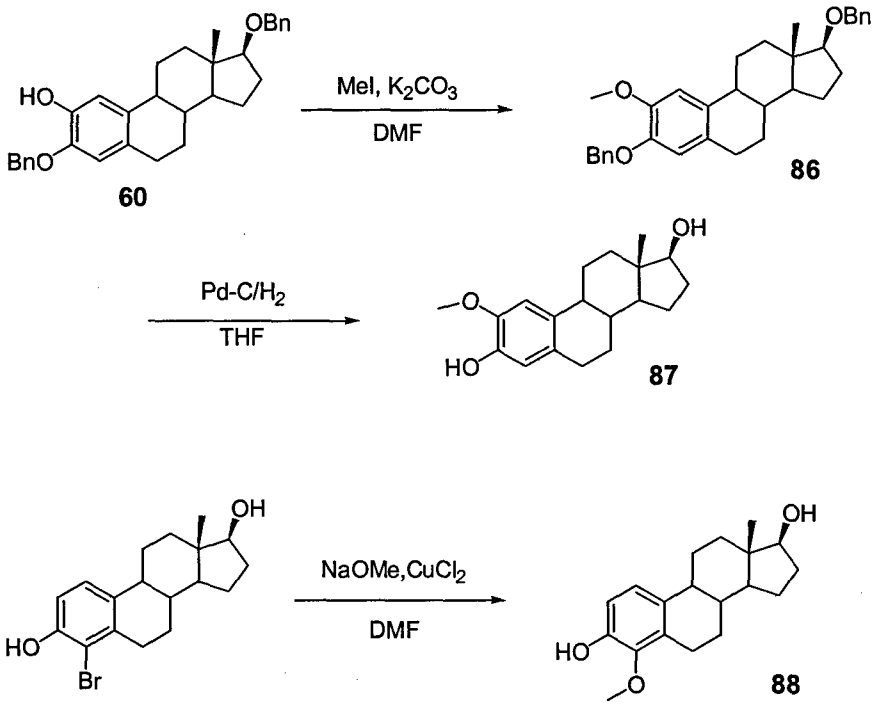


Figure 3.12 Synthesis of 2- and 4-Methoxyestradiols

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-OHE_2 was methylated with methyl iodide to form the corresponding methylated derivative **86**. Debenzylation of **86** by catalytic hydrogenation provided 2-$MeOE_2$ in 82% yield. Over one gram of 2-$MeOE_2$ 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 $CuCl_2$ 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.[3] Additionally, by using a cell-based assay the cellular uptake and stability of the compounds being tested can be evaluated. The EC_{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_{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_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.[29] The induction of pS2 mRNA expression by estradiol, 4-OHE_2 and the analogs **19-21** was determined by RNA dot blot analysis. The EC_{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 [^{3}H]thymidine incorporation at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 μM. 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.[3,29]

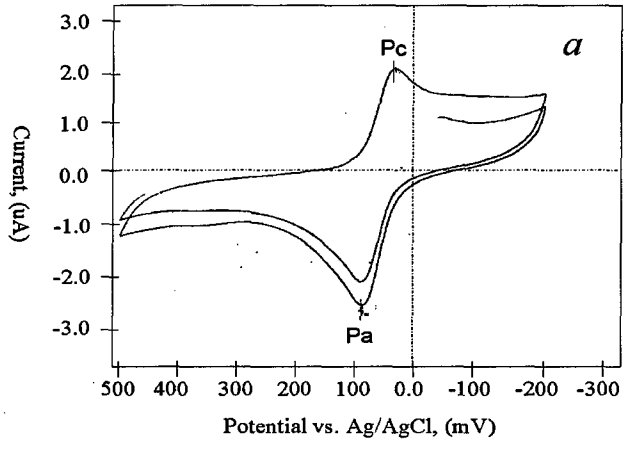
A number of literature reports have shown that 4-OHE_2 is similar to estradiol in its ability to bind and activate the classical ER.[2] Martucci and Fishman reported the binding affinities of catechol estrogens for rat uterine cytoplasmic ER.[30] They found that 2-hydroxylation decreased ER affinity to a greater extent than 4-hydroxylation. The authors showed that 4-OHE_2 had a RBA of 45 and 2-OHE_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.[31] 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_2 are capable of inhibiting [^{3}H]estradiol binding to partially purified cytoplasmic receptor sites.[32] 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.[33] 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 voltammetery (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 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. 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 ΔE'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 ΔE 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 4.4 b**).[34] 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 (Ep,a2) that decreased in current with each scan and a second anodic peak (Ep,a1) followed by one cathodic peak (Ep,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*).[34] 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



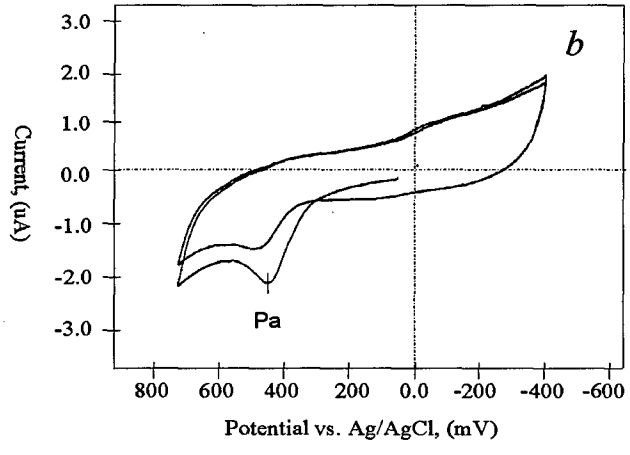


Figure 4.2. CV of 2-Hydroxymethyl Estradiol **22**

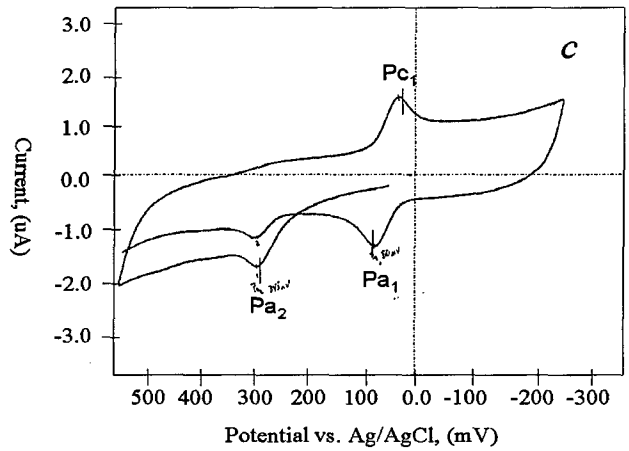


Figure 4.3 CV of 2-Methoxy Estradiol **87**

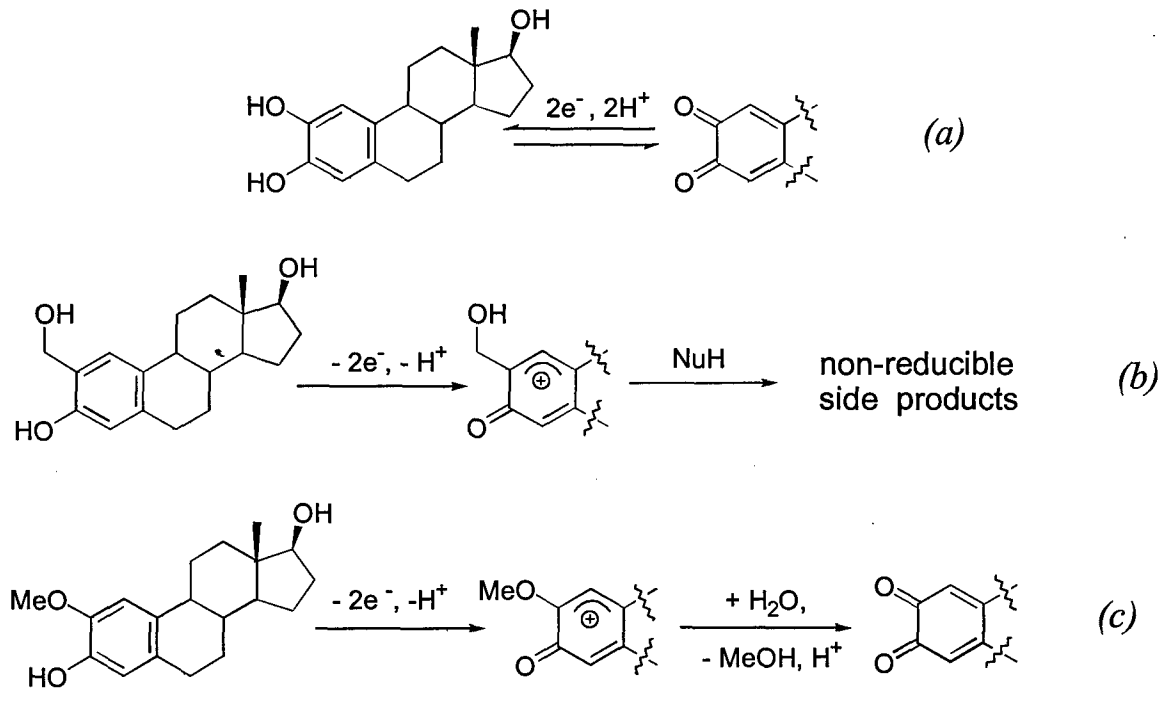


Figure 4.4 *(a)* Reversible Oxidation/Reduction of 2-OHE_2 *(b)* Irreversible Oxidation of 2-Hydroxymethyl Estradiol *(c)* Oxidative Demethylation of 2-Methoxy Estradiol

	Peak potentials are expressed in mV					
compound	Ep, a1	Ep, c1	Ep, a2	ΔE	E½[a]	E½[b]
2-OH-E_2	90	35	-	55	73	263
2-MeO-E_2	80	15	280	65	48	238
2-HMe-E_2	407	-	-	-	-	-
4-OH-E_2	95	35	-	60	75	265
4-MeO-E_2	80	25	345	55	53	243
4-HMe-E_2	405	-	-	-	-	-
4-Me-Catechol	140	50	-	90	95	285

[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.[35] Calf thymus DNA was exposed to 100 μM of catechol estrogen or analog and ascorbic acid, with and without 100 μM Cu(II)SO_4 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)SO_4 was included in the experiments due to the noted redox coupling with hydroquinones.[36] 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)SO_4 (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-OHE_2 (21.0± 0.6), 4-OHE_2 (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-OHE_2 (1189± 119.3), 4-OHE_2 (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-OHE_2 induced significantly more 8-oxo-dG than did 4-OHE_2 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.

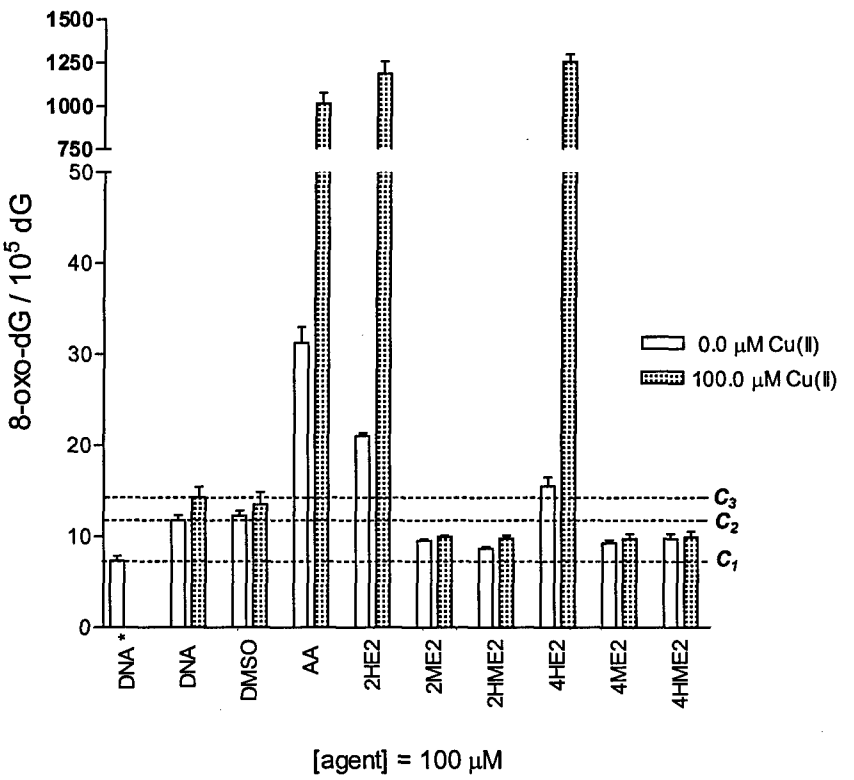


Figure 4.5. Calf thymus DNA was exposed to 100 μM of CE or CE analog with and without the addition of 100 μM Cu(II)SO_4 for 3 hours in PBS (pH 7.4) at 37°C. The induction of 8-oxo-dG was compared to that of the following controls; non-incubated DNA* (C_1), DNA incubated alone (C_2), and DNA incubated in the presence of Cu(II)SO_4 (C_3). Data points are means of N=3 ± SD for all samples.

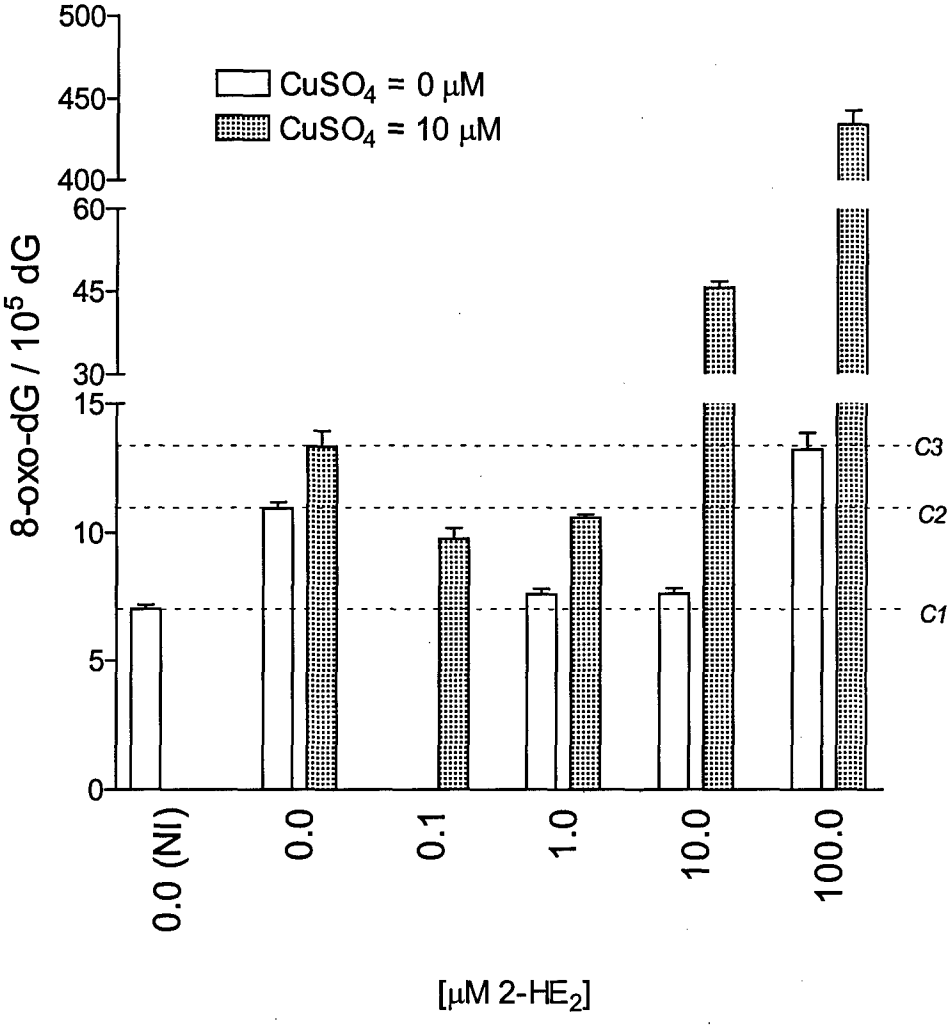


Figure4.6. Calf thymus DNA was exposed to increasing concentrations of 2-HE_2 (0.1 µM to 100 µM) with and without the addition of 10 µM Cu(II)SO_4 for 3 hours in PBS (pH 7.4) at 37°C. The induction of 8-oxo-dG by 2-HE_2 was compared to that of the following controls; non-incubated DNA* (C_1), DNA incubated 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.

4.4 INHIBITION OF TUBULIN POLYMERIZATION: D'Amato *et al.* have developed specific assay conditions for studying interactions of 2MeOE2 with tubulin.[24] 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 spectroscopicaly 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 $2MeOE_2$ 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 $2MeOE_2$ 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 $4\text{-}MeOE_2$ were inactive in this assay (inhibition <5%). The hydroxyalkyl estradiols exhibited moderate activity (inhibition15-20%). The results of screening of various estrogens are shown in **Table 4.2**. This preliminary screening identified **80** as being slightly more effective than $2\text{-}MeOE_2$ 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 IC_{50} and competitive binding experiments with [^{3}H] colchicine need to be performed to investigate in detail the nature of interaction of **80** with tubulin polymer.

Compound (3μM)	%Inhibition
Colchicine	**64%**
$2\text{-}MeOE_2$	**34%**
$4\text{-}MeOE_2$	**No inhibition**
$2\text{-}MeOME_2$	**44%**
$4\text{-}MeOME_2$	**No inhibition**
2-Hydoxymethyl E_2	**15%**
2-Hydoxyethyl E_2	**24%**
2-Hydoxypropyl E_2	**12%**
2,3-(2H)-benzofuranyl E_2	**15%**

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 Cochicine 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 – Benzypyrone 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.[37] 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.[38] Additionally, there are literature reports of some benzopyrones being active as tubulin polymerization inhibitors.[39] 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.[40] Some of these compounds have shown activity as tyrosine kinase inhibitors, PKC inhibitors, antiinflammatory, antiangiogenic agents, and antiestrogenic agents.[41] 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 modem 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 (20atm) atmosphere in the presence of $PdCl_2$(dppf), to form 2-substituted benzopyrones **168** in yields of 50-81%.[42] 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 $PdCl_2(PPh_3)_2$).[43] 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 1atm 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.[44] Similarly, the five-membered indoxyl derivatives and alkynyl ketone were isolated in a ratio of 5:1 upon heteroannulation of *o*-iodoaniline with phenylacetylene under milder conditions (1 mmol alkyne,1 atm CO, 80°C, 5 mol% $Pd(PPh_3)_4$, anisole).[45]

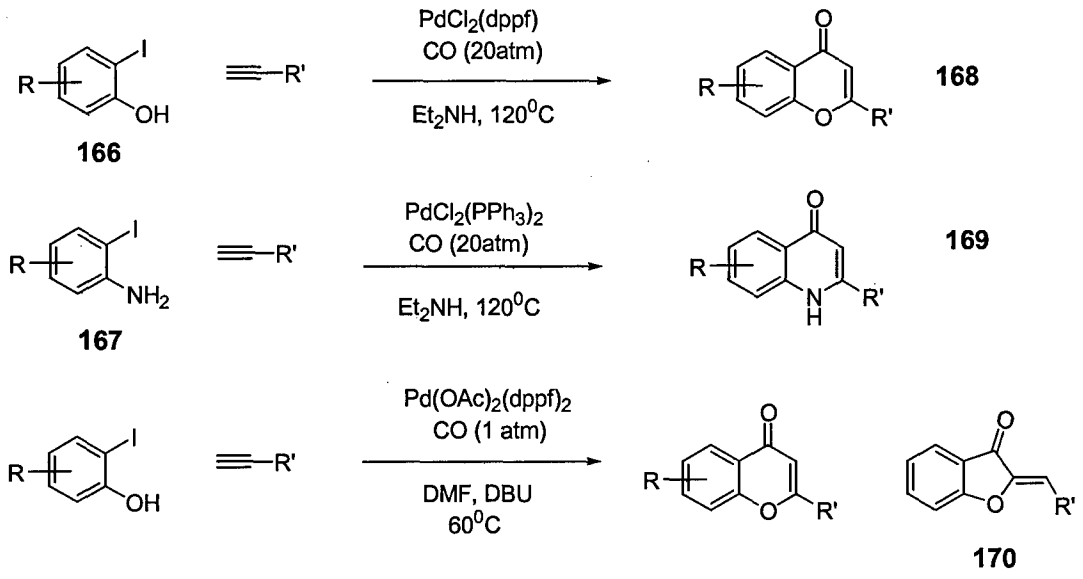


Figure 5.11 Heteroannulation Reactions of *o*-Iodophenols and *o*-Iodoanilines

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.

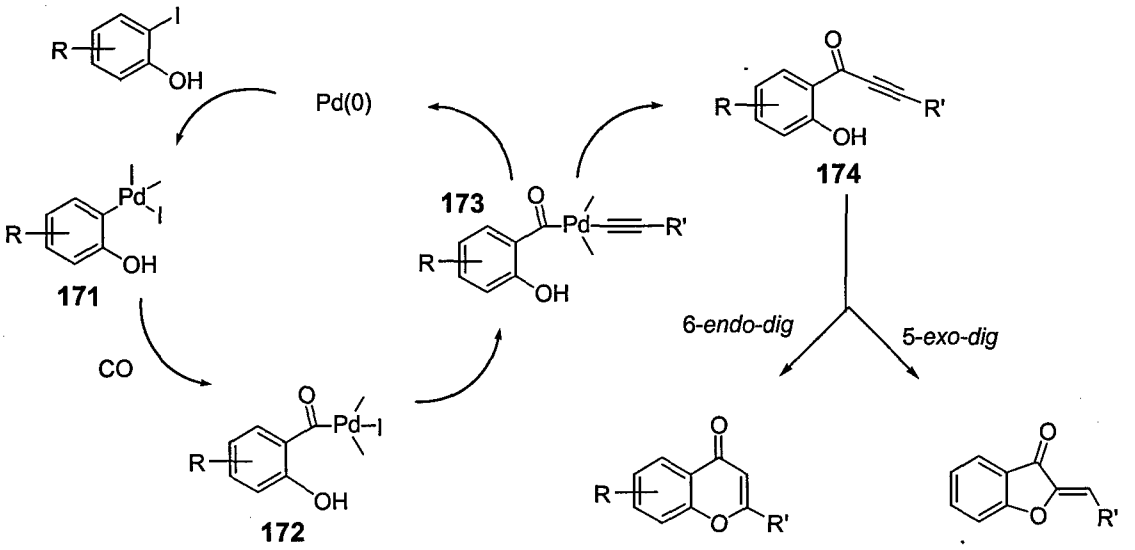
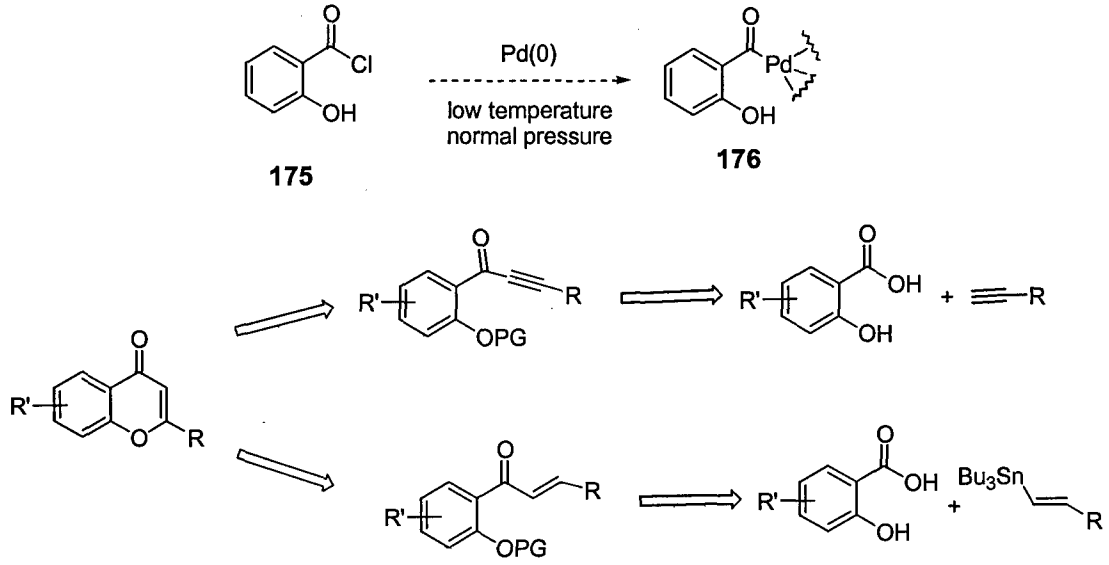


Figure 5.12 Mechanism of Heteroannulation Reaction

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.[46,47] 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.[48] 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**.

Figure 5.13 Synthetic Plan for Benzopyrone Combinatorial Library



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.[49] More importantly though, if needed, additional salicylic acids and terminal alkynes can be readily synthesized from simple starting materials using well established chemistry.[50] 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

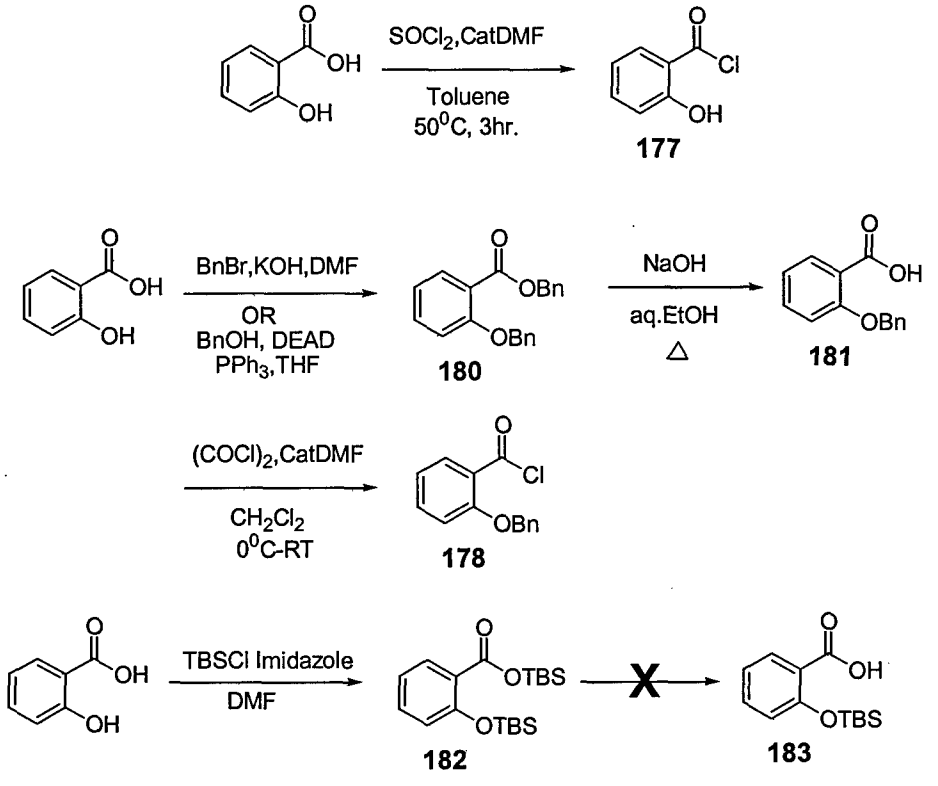


Figure 5.14 Acid Chlorination of Salicylic Acids

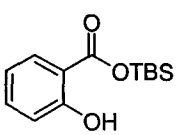
prove difficult. We were ultimately interested in using photochemically cleavable forms of benzyl protecting groups.[51] This strategy would be especially valuable for solid phase synthesis as the photochemically cleavable benzyl group could be incorporated into the linker and conditions could be developed 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 TBSCl 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 TBDPSCl 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.

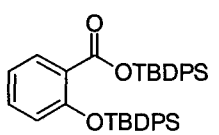
Entry	Hydrolysis Condition
1	AcOH:H_2O:THF (1:1:1) 30°C
2	AcOH:H_2O:THF (1:1:1) 0°C
3	K_2CO_3, MeOH, THF 30°C
4	K_2CO_3, MeOH, THF 0°C
5	K_2CO_3, MeOH
6	MeOH, 40°C
7	LiOH, MeOH:H_2O:THF (1:1:4)
8	LiOH, CH_2Cl_2, MeOH

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

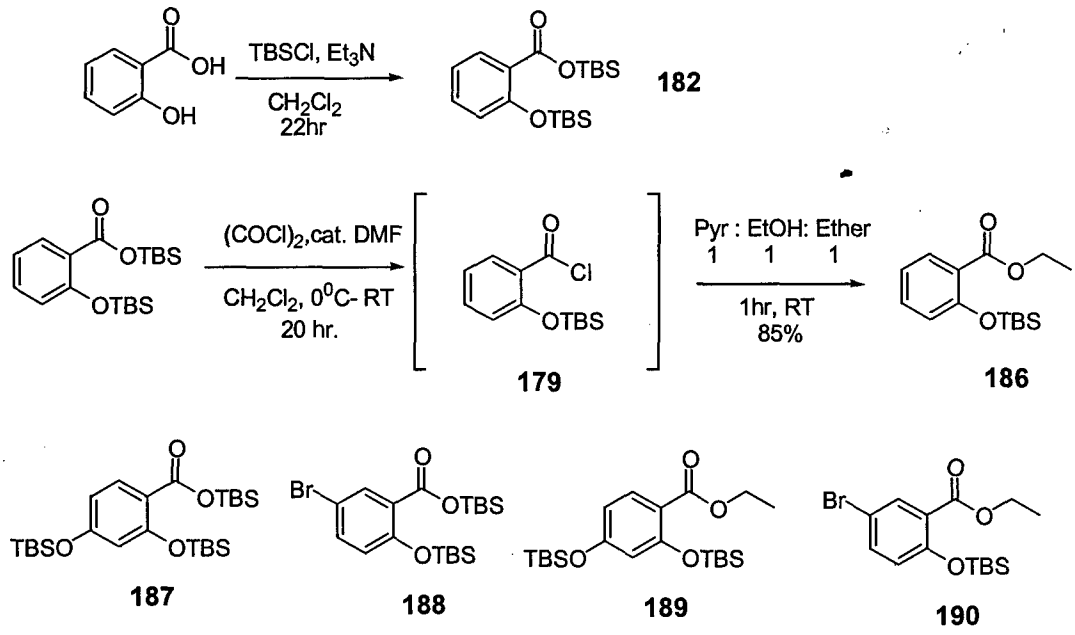
Wissner *et al.* reported the reaction of *tert*-butyldimethylsilyl esters with oxalyl chloride in presence of catalytic amounts of DMF as an effective way to generate acid chlorides under neutral conditions.[52] The proposed mechanism for this conversion is shown in **Figure 5.15**. The side products of this reaction namely TBSCl, CO and CO_2 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.



184



185



Mechanism of conversion of silyl ester to acid chloride

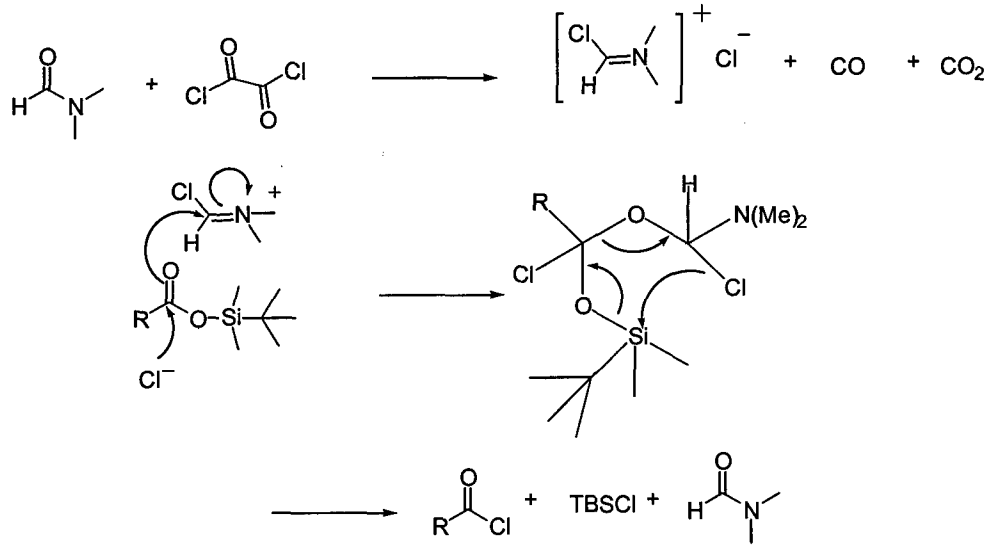


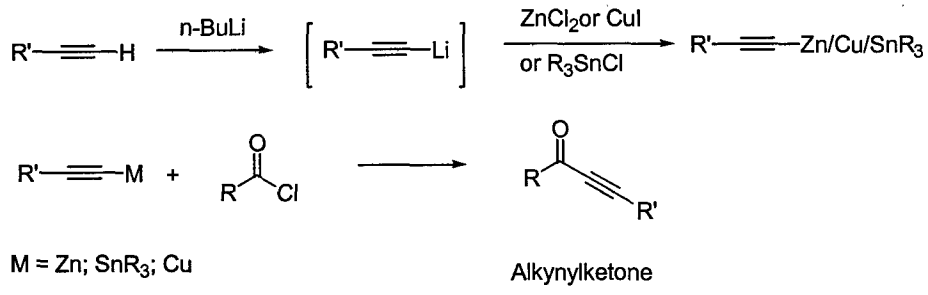
Figure 5.15 Conversion of Silyl-ester into Acid Chlorides

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

TBSCl in dichlormethane using Et_3N as the base.[53] 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 $Pd(PPh_3)_2Cl_2$.[54] 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

Coupling of Acid chlorides with alkynyl metals



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

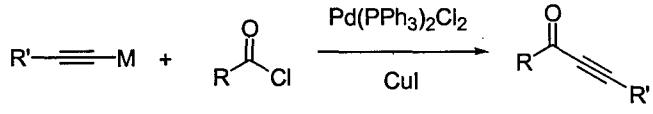
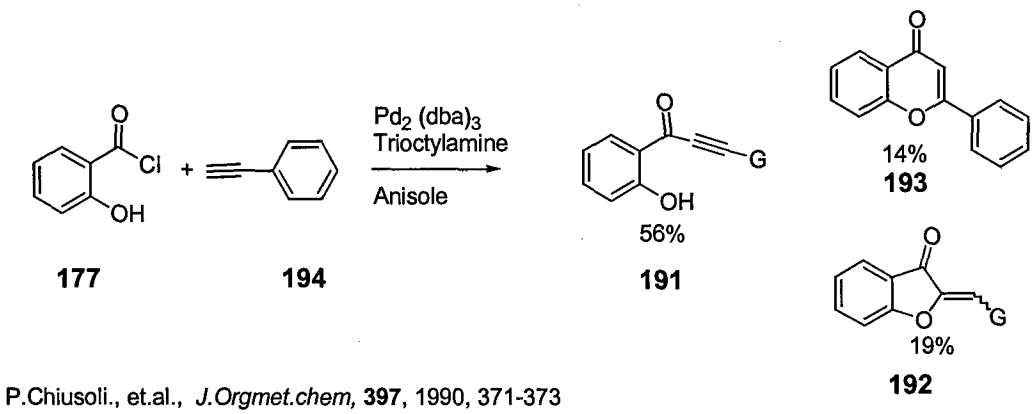


Figure 5.16 Reactions of Acid Chlorides and Terminal Alkynes

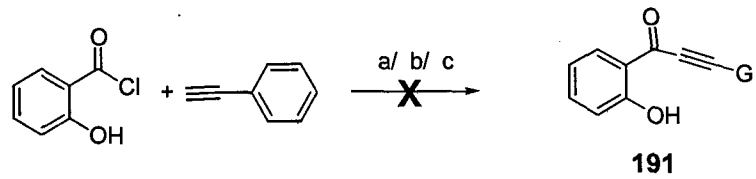
generating alkynyl ketones from acid chlorides under Pd catalysis.[55] The alkynyl stannanes are prepared by reaction of alkynyl lithium with tributyltin chloride. A serious limitation for use in combinatorial chemistry is that a wide range of alkynyl tin reagents are not readily available for use as 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.[56] All these procedures are more involved than the Sonogashira copper-palladium catalyzed

coupling of terminal alkynes with acid chlorides.[57] In this method terminal alkynes and acyl halides are coupled in Et_3N in presence of CuI and $Pd(PPh_3)_2Cl_2$ as catalysts to provide alkynyl ketones in good yields. The advantage of this method is that it is a one



177 194 191 193 192

P.Chiusoli., et.al., *J.Orgmet.chem,* **397**, 1990, 371-373



177 191

a: $Pd(PPh_3)_2Cl_2/CuI/Et_3N$
b: $Pd(PPh_3)_2Cl_2/Et_3N$/Toluene/50-60^{0}C
c: $Pd(PPh_3)_4/Et_3N$/Toluene/50-60^{0}C

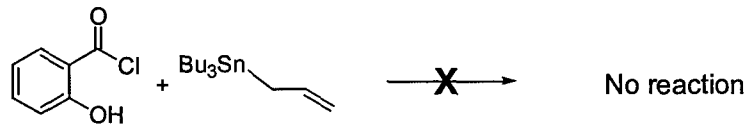
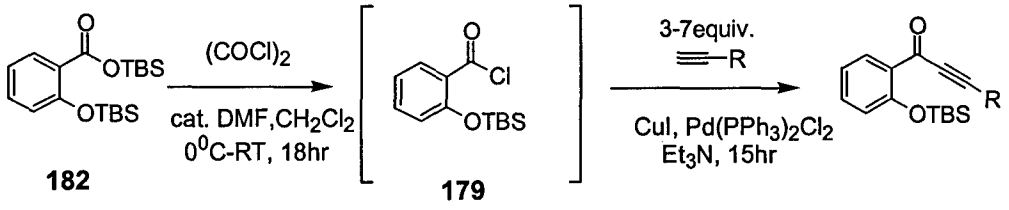


Figure 5.17 Attempted Coupling of Salicyloyl Chloride with Phenylacetylene

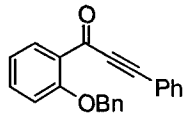
step condensation reaction and employs mild reaction conditions. For reasons of operational simplicity, this approach was most attractive for application in combinatorial chemistry. There is one literature example of a salicyloyl chloride coupling with phenyl acetylene **194**. Chiusoli *et al.* observed 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 **191** in 56% yield.[58] Interestingly, aurone **192** and flavone **193** 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 **191** was isolated in <15% yield

using $Pd_2(dba)_3$ as catalyst in Et_3N. An attempted Stille reaction with allyltributyltin also failed to give any coupling product.

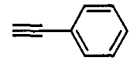
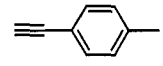
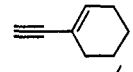
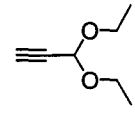
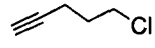
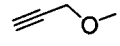
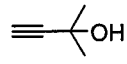
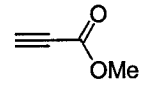
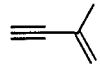
The coupling experiments using **177** emphasized the need for protecting the phenolic hydroxyl during the coupling reaction. We examined the coupling reactions between phenyl acetylene and acid chlorides **178** and **179**, using the Sonogashira coupling method. Acid chloride (1mmol) in Et_3N was treated with 1 equivalent of phenyl acetylene, 1 mg of CuI and 1 mg of $Pd(PPh_3)_2Cl_2$. 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 (CuI or CuCl) in absence of Pd catalyst. In addition to $Pd(PPh_3)_2Cl_2$, the coupling of **178** and phenylacetylene was attempted with $Pd(PPh_3)_4$ and $PhCH_2Pd(PPh_3)_2Cl_2$ 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 $Pd(PPh_3)_2Cl_2$/ CuI in Et_3N 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.[59] 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_3N 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**.

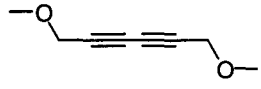


Coupling of 179 with terminal alkynes



194

	—R	Product **ketone** (Yield)*
194		**203** (92%)
195		**204** (95%)
196		**205** (96%)
197		**206** (78%)
198		**207** (85%)
199		**208** (84%)
200		**209** (74%)
201		**NR**
202		**NR**



210

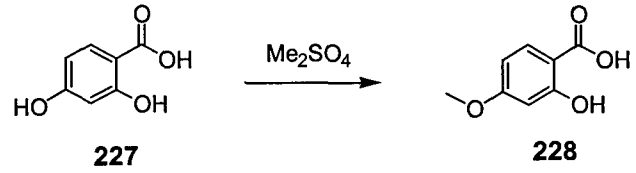
* Isolated yields after workup and flash chromatography

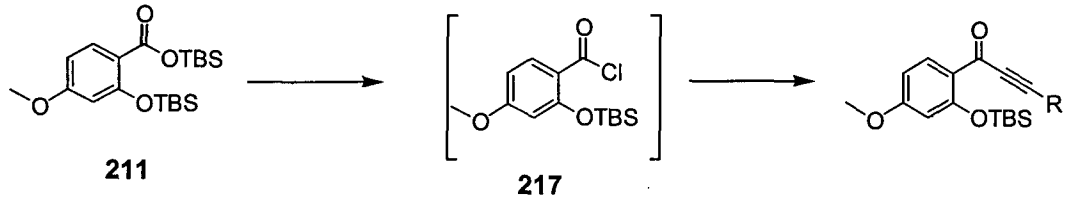
Figure 5.18 Coupling of Terminal Alkynes with **179**

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

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.[60] 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_2CO_3 as the base.[61] All the free salicylic acids were converted into their bis-(*t*-butyl-dimethylsilyl) forms with TBSCl and Et_3N in CH_2Cl_2, in quantitative yields. The results from these couplings are shown in Figures **5.19-5.22**.





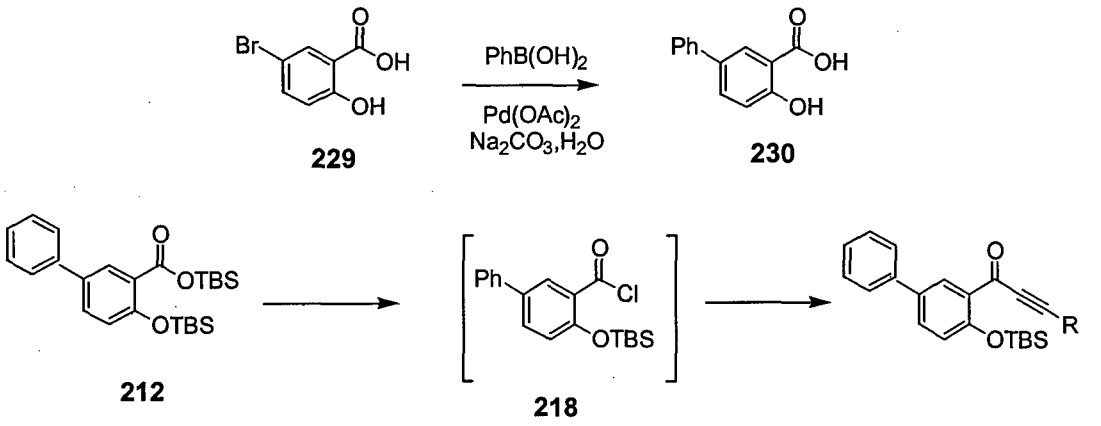
Coupling of **211** with terminal alkynes

	≡—R	Product **ketone** (Yield)*
194		**222** (92%)
195		**223** (79%)
196		**224** (74%)
197		**225** (84%)
198	Cl	**226** (42%)

* Isolated yields after workup and flash chromatography

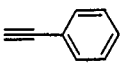
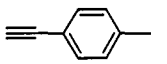
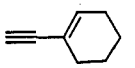
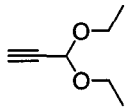
Figure 5.19 Sonogashira Couplings of **211**

Figure 5.20 Sonogashira Couplings of **212**

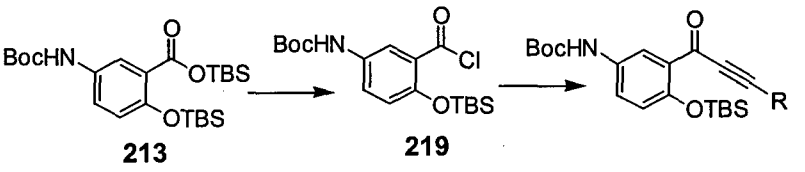


229 **230** **212** **218**

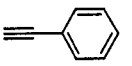
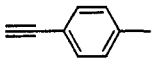
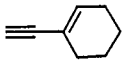
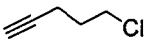
Coupling of **212** with terminal alkynes

	—R	Product **ketone** (Yield)*
194		**231** (90%)
195		**232** (84%)
196		**233** (73%)
197		**234** (81%)
198		**235** (78%)

* Isolated yields after workup and flash chromatography

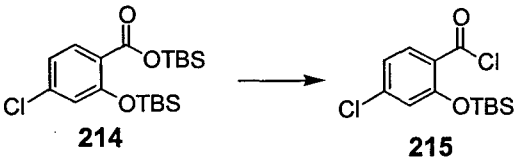


Coupling of **213** with terminal alkynes

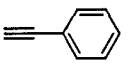
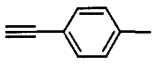
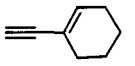
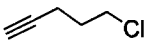
	≡—R	Product **ketone** (Yield)*
194		**236** (90%)
195		**237** (84%)
196		**238** (73%)
198		**239** (78%)

* Isolated yields after workup and flash chromatography

Figure 5.21 Coupling of **213** and **215** with Terminal Alkynes

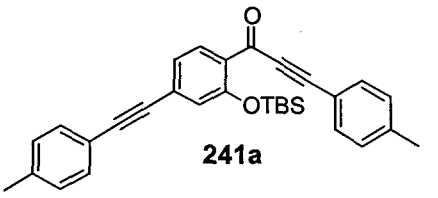


Coupling of **214** with terminal alkynes

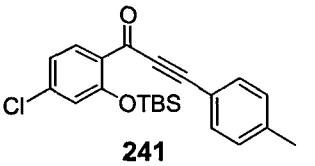
	≡—R	Product **ketone** (Yield)*
194		240 (83%)
195		241 (40%)#
196		242 (82%)
198		243 (64%)

* Isolated yields after workup and flash chromatography

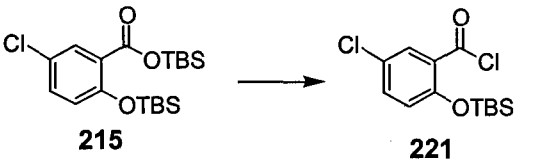
#:Mixture



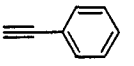
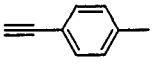
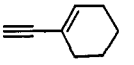
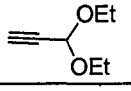
241a



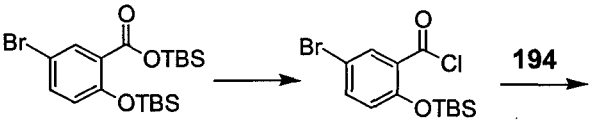
241



Coupling of **213** with terminal alkynes

	≡—R	Product **ketone** (Yield)*
194		**244** (84%)
195		**245** (77%)
196		**246** (74%)
197		**247** (70%)

* Isolated yields after workup and flash chromatography



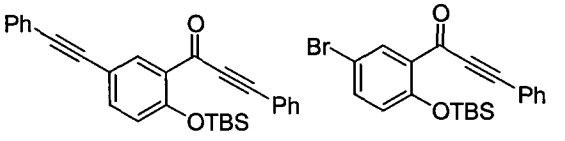


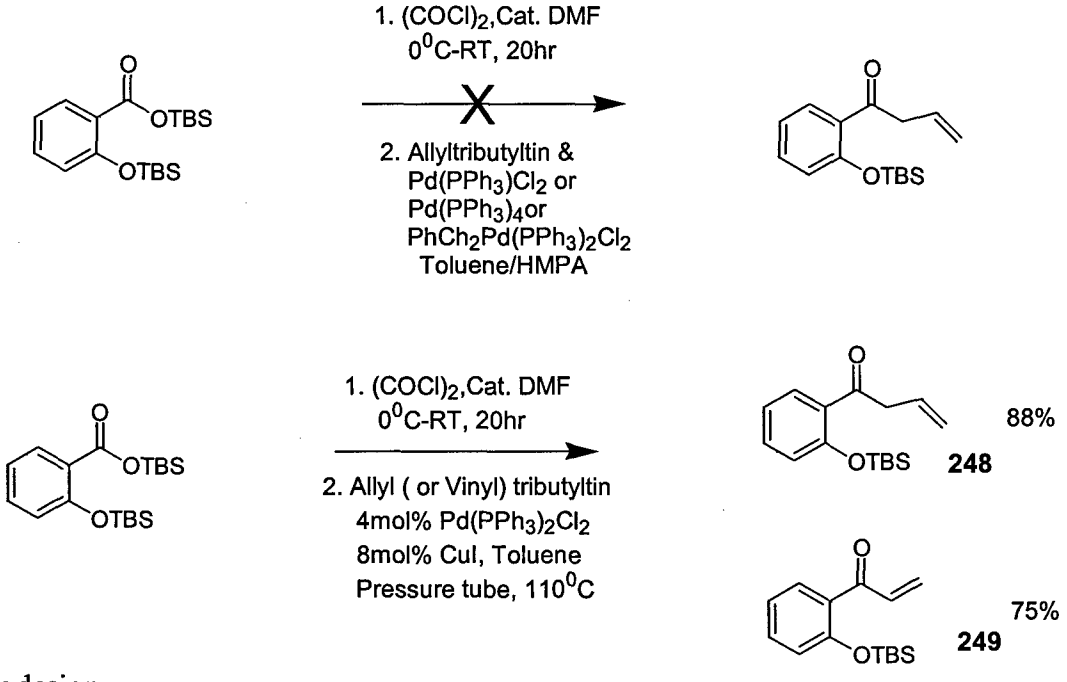
Figure 5.22 Sonogashira Coupling of **213**.

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

194, 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 alkynyl ketones in good yields. The reaction sequence shows good functional group tolerance on both the salicylic acid and alkyne components. The alkynyl ketones were found to be relatively unstable upon standing at room temperature and started decomposing with desilylation reactions. The stability of the alkynyl 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 $PdCl_2$, 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**).[62] 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.[63] 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.[64] Miranda *et al.* studied the cyclizations of *o*-hydroxyaryl phenyl ethynyl ketones **250**.[65] 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 K_2CO_3 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 K_2CO_3 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

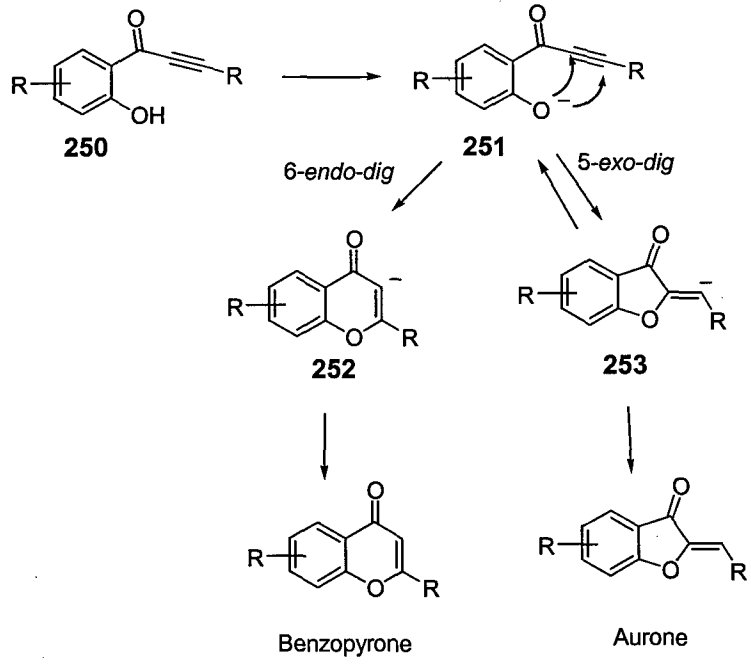


Figure 5.24 Cyclization of *o*-Hydroxyphenyl Alkynyl Ketones

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

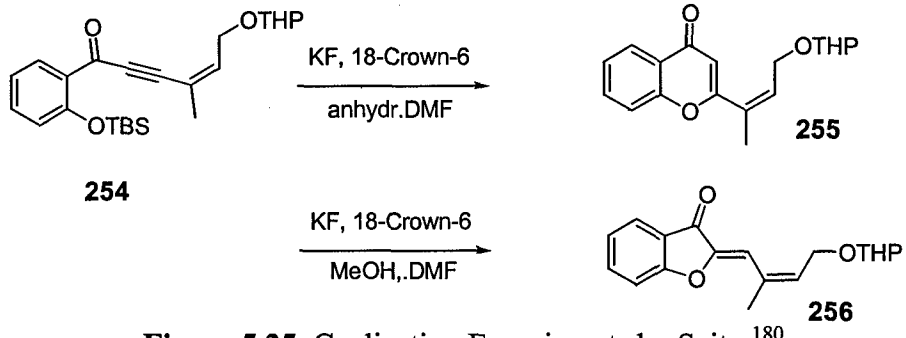


Figure 5.25. Cyclization Experiments by Saito.[180]

Kapurimycin A_3 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.[66] 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.

No.	Alkynone	Benzopyrone	Yield
1	**203**	**262**	85%
2	**204**	**263**	60%
3	**205**	**264**	56%
4	**206**	**265**	75%
5	**207**	-	Decomposition
6	**208**	**267**	75%
7	**222**	**269**	88%
8	**223**	**270**	64%
9	**233**	**276**	72%
10	**235**	-	Decomposition

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]

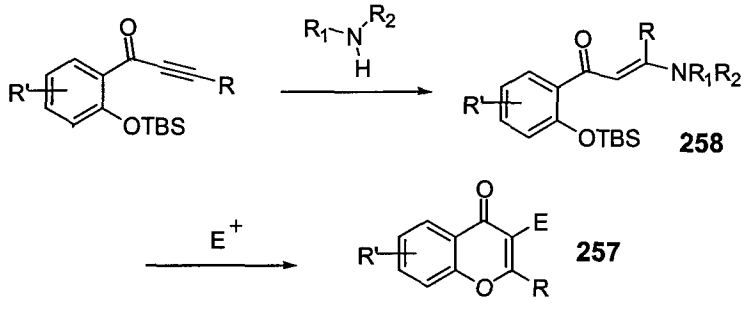


Figure 5.26 Proposed Cyclization via Enaminoketone Intermediates

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.

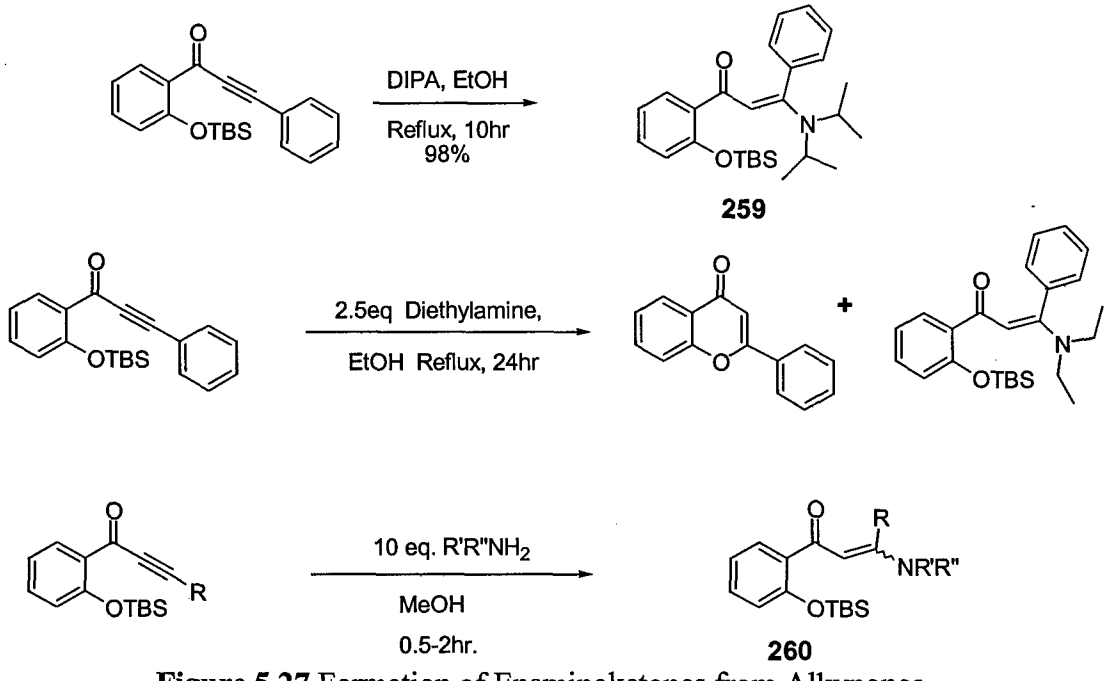


Figure 5.27 Formation of Enaminoketones from Alkynones

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 *o*-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 dimethyamine (2M solution in THF), N-benzylethylamine and pyrrolidine, gave identical cyclization results.

No.	Alkynone	Benzopyrone	Yield
1	**203**	**262**	92%
2	**204**	**263**	95%
3	**205**	**264**	96%
4	**206**	**265**	78%
5	**207**	**266**	85%
6	**208**	**267**	84%
7	**222**	**269**	90%
8	**223**	**270**	88%
9	**224**	**271**	74%
10	**225**	**272**	89%
11	**226**	**273**	80%
12	**231**	**274**	94%
13	**233**	**276**	92%
14	**236**	**277**	79%
15	**242**	**278**	85%
16	**244**	**279**	83%
17	**247**	**280**	76%

Table 5.4 DEA Cyclizations of Alkynones

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7.0 Appendix

7.1 **Manuscripts** (copies attached):

1. C.J. Lovely, **A.S. Bhat**, H.D. Coughenour, N.E. Gilbert, and R.W. Brueggemeier, Synthesis and biological evaluation of 4-(hydroxy-alkyl)estradiols and related compounds. *J. Med. Chem.*, **40**, 3756-3764 (1997).

2. J.A. Mobley, **A.S. Bhat**, and R.W. Brueggemeier, Measurement of oxidative DNA damage by catechol estrogen analogs *in vitro*. *Chem. Res. Toxicol.*, **12**, 270-277 (1999).

3. A.S. Bhat, J.L. Whetstone, and R.W. Brueggemeier, Novel synthetic routes suitable for constructing benzopyrone combinatorial libraries. *Tetrahedron Letters*, **40**, 2469-2472 (1999).

4. R.W. Brueggemeier, **A.S. Bhat**, C.J. Lovely, H.D. Coughenour, S. Joomprabutra, S.H. Weitzel, D.D. Vandre, F. Yusuf, and W.E. Burak, Jr. Comparison of endocrine and antitumor activity of 2-methoxyestrogens. *Cancer Res.*, submitted (1999).

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**:

1. **A.S. Bhat**, C.J. Lovely, N.E. Gilbert, H.D. Coughenour, J.A. Mobley, and R.W. Brueggemeier, Estrogen analogs as mechanistic probes in hormonal carcinogenesis. 25th National Medicinal Chemistry Symposium, Ann Arbor, MI, June 21-25, 1996, Abst. 23.

2. J. Mobley, **A.S. Bhat**, H. Coughenour, C. Lovely, M. Liberto, N. Gilbert, and R.W. Brueggemeier, Differential oxidative DNA damage by catechol estrogen analogs. 1997 Gordon Research Conference on Hormonal Carcinogenesis, Tilton School, Tilton, NH, July 27 - August 1, 1997.

3. **A.S. Bhat**, J. Mobley, H. Coughenour, C. Lovely, M. Liberto, N. Gilbert, and R.W. Brueggemeier, Non-redox cycling analogs of estrogen metabolites as chemical probes to evaluate the tumorigenic potential of catechol estrogens. Era of Hope, 1997 DoD Breast Cancer Research Program Meeting, Washington, DC, November 1-4, 1997.

4. **A.S. Bhat**, J.L. Windholtz, and R.W. Brueggemeier, New synthetic approaches for benzopyrone combinatorial libraries. 39th Annual Buffalo Medicinal Chemistry Symposium, SUNY at Buffalo, Buffalo, NY, May 17-20, 1998.

5. **A.S. Bhat**, J.L. Windholtz, and R.W. Brueggemeier, New synthetic approaches for benzopyrone combinatorial libraries. 26th National Medicinal Chemistry Symposium, Virginia Commonwealth University, Richmond, VA, June 14-18, 1998, Abst. E2.

6. J.A. Mobley, **A.S. Bhat**, and R.W. Brueggemeier, Examination of oxidative DNA damage by catechol estrogen analogs *in vitro*. Xth International Congress on Hormonal Steroids, Quebec City, Canada, June 17-21, 1998.

7. **A.S. Bhat**, J.L. Windholtz, and R.W. Brueggemeier, Synthesis of benzopyrone combinatorial libraries. 214th American Chemical Society National Meeting, Boston, MA, August 23 - 27, 1998, Abstract No. MEDI 0156.

8. W.E. Burak, Jr., L. DePalatis, F. Yusuf, **A.S. Bhat**, R.W. Brueggemeier, 2-Methoxymethyl estradiol inhibits the growth of MDA-MB-435 breast cancer xenografts. Association for Academic Surgery, 32nd Annual Meeting, Seattle, WA, Nov. 19-22, 1998.

9. **A.S. Bhat**, J.L. Windholtz, and R.W. Brueggemeier, Novel approaches for the synthesis of diverse benzopyrone libraries. 3rd Lake Tahoe Symposium on Molecular Diversity, Lake Tahoe, CA, Jan. 24-29, 1999.

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

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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)estradiols. The 4-(hydroxyalkyl)estrogens were prepared by oxidative hydroboration of 4-alkenylestradiols. The alkenylestradiols 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 estradiols 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 EC_{50} 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.[1] 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.[2] 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.[3] Also, studies in experimental animals have shown estrogens to induce tumors in hormone-responsive tissues like mammary tissue, uterus, cervix, and pituitary.[4] 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.[5] Both 2-hydroxyestradiol and 4-hydroxyestradiol have weaker affinity for the estrogen receptor than estradiol and exhibit significantly lower estrogenic activity *in vivo*.[5] However, the catechol estrogens are prone to further oxidative metabolism and can form reactive intermediates like quinones, semiquinones, and arene oxides.[6,7] These highly reactive moieties may be cytotoxic via reaction with proteins and nucleic acids.[8,9] Furthermore, the catechol estrogens have been shown to produce a variety of reactive oxygen species (ROS), such as the hydroxide, peroxide, and superoxide radicals.[10–12] These ROS have shown cytotoxic and genotoxic effects in several independent studies.[10,13,14]

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-hydroxylase activity, suggesting a mechanistic role of 4-hydroxyestradiol in tumor formation.[15] An earlier report demonstrated that 4-hydroxyestradiol formation is predominant in tissues susceptible to estrogen-induced tumorigenesis like Syrian hamster kidney and

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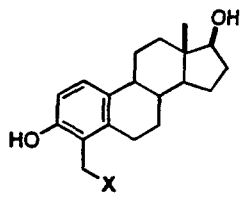
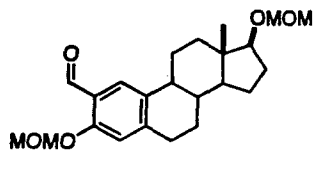
rat pituitary, whereas 2-hydroxyestradiol formation is predominant in rodent livers where tumors are not produced under similar conditions.[16-18] In contrast, Li and Trush found that 2-hydroxyestradiol produced oxidative damage and strand breaks of double-stranded DNA in the presence of micromolar concentrations of Cu(II), whereas 4-hydroxyestradiol failed to produce any DNA damage.[13,19]

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.[20] 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.



1: X=OH
2: X=CH_2OH
3: X=CH_2CH_2OH
4: X=NH_2
5: X=CH_2NH_2
6: X=$CH_2CH_2NH_2$



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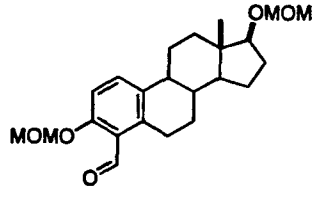

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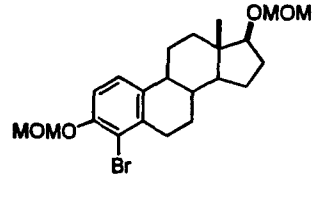


10

Results and Discussion

Chemistry. In our earlier work, the 2-(hydroxyalkyl)estradiols were prepared via homologation of a protected 2-formylestradiol **7**.[20,21] 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.[22] 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.[23] Introduction of an appropriate unsaturated group, vinyl or allyl, would afford the hydroxyethyl and hydroxypropyl derivatives, respectively, after hydroboration and oxidation.

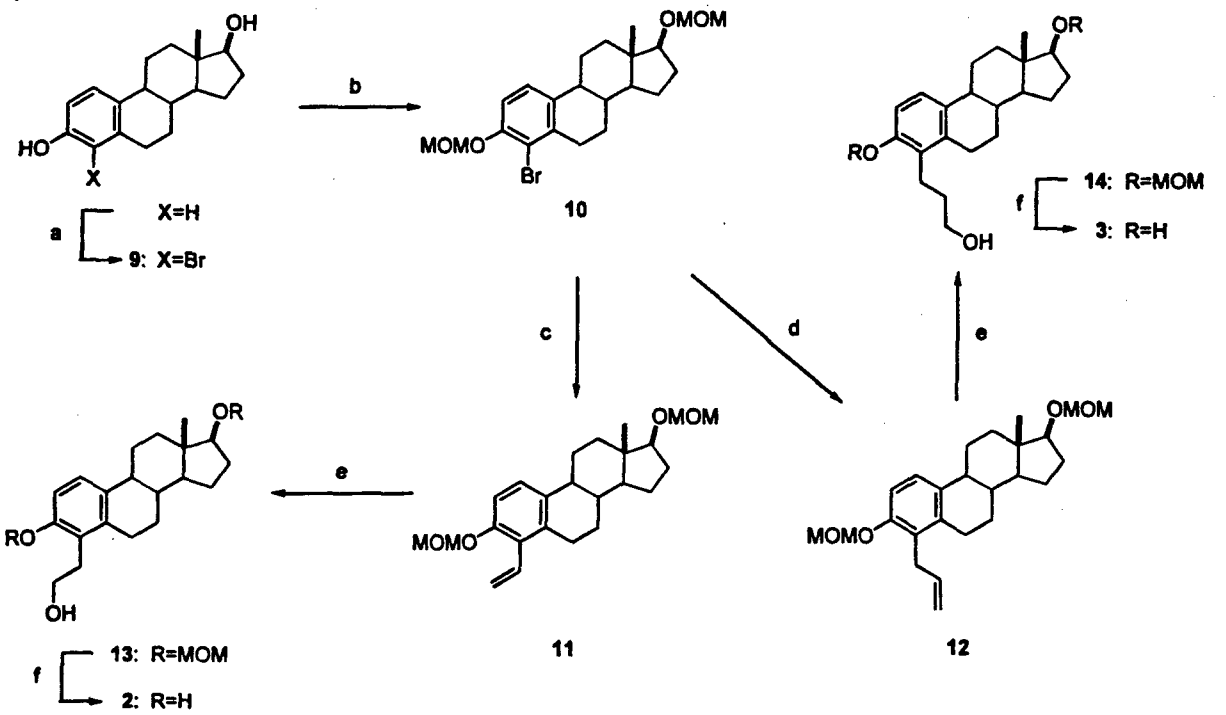
The synthesis commenced by brominating estradiol with *N*-bromosuccinimide in ethanol (Scheme 1), from which the required 4-bromoestradiol **9** precipitated and was obtained in 54% yield after recrystallization. The bromoestradiol was protected in 75% yield as its bisMOM ether **10** with chloromethyl methyl ether, diisopropylethylamine in THF at reflux.[20] 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) (0.06 molar equiv) and vinyltributyltin (2.1 molar equiv) in dry deoxygenated 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 BH_3·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.[22] 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 chloroformates; 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 76% 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.

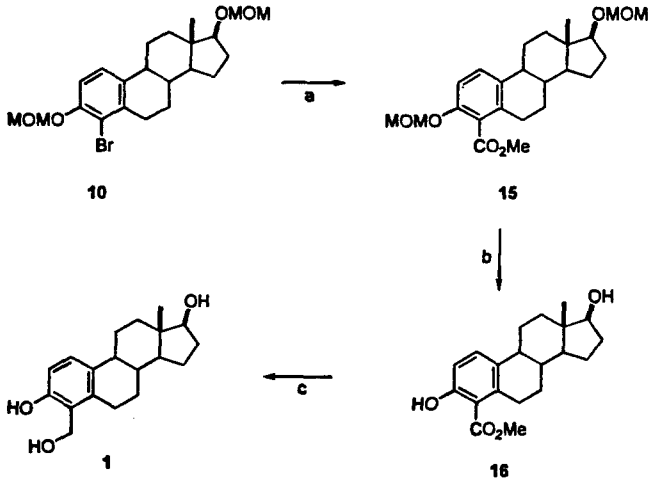
The preparation of the 4-substituted amines was accomplished using chemistry similar to that employed for the 2-substituted analogs previously reported.[20,21]

Scheme 1[a]



[a] Reagents and conditions: (a) *N*-bromosuccinimide, EtOH, 54%; (b) MOMCl, *i*-Pr_2NEt, THF, Δ, 75%; (c) $Pd(PPh_3)_4$, CH_2=$CHSnBu_3$, DMF, Δ, 90%; (d) $Pd(PPh_3)_4$, CH_3CH=$CHSnBu_3$, DMF, Δ, 94%; (e) (i) BH_3·THF, THF, 0 °C, (ii) NaOH, H_2O_2, Δ, **11** → **13** 39%, **12** → **14**, 82%; (f) PPTS, MeOH, Δ, **13** → **2** 80%, **14** → **3** 61%.

Scheme 2[a]



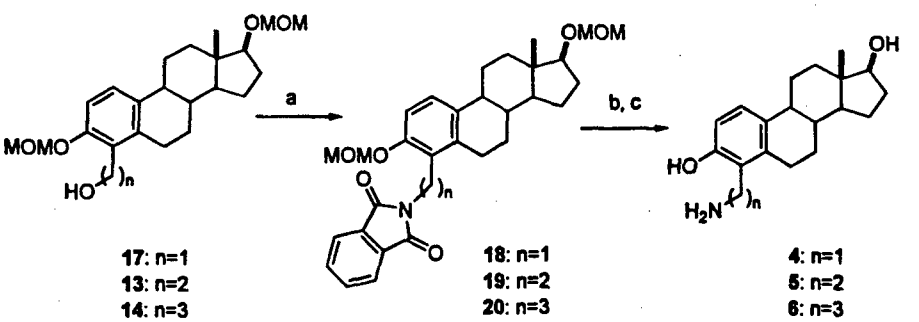
[a] Reagents and conditions: (a) (i) *n*-BuLi, THF, −78 °C, (ii) CO_2, −78 °C → rt; (iii) CH_2N_2, Et_2O, 0 °C, 76%; (b) PPTS, MeOH, Δ, 83%; (c) $LiAlH_4$, THF, 0 °C → rt, 51%.

Treatment of the bisMOM-protected 4-(hydroxylalkyl)-estradiols (**13, 14, 17**) with phthalimide under Mitsunobu conditions using triphenylphosphine (PPh_3) and diethyl azodicarboxylate (DEAD) yielded derivatives **18**–**20** in 70–80% yield (Scheme 3). Subsequent hydrazinolysis in refluxing ethanol gave the bisMOM-protected aminoestradiols which, upon treatment with methanolic HCl, gave the desired 4-(aminoalkyl)estradiols **4**–**6** in good yields.

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.[20] The whole cell binding assay provides similar relative binding affinities (RBAs) for the estrogen receptor as those obtained using isolated estrogen receptor preparations.[20] 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 0.180 nM (Table 1). The synthetic hydroxyestrogen analog with the highest estrogen receptor affinity was 4-(hydroxymethyl)estradiol **1**, exhibiting an EC_{50} value of 364 nM. Overall, the 4-substituted estradiol homologs exhibited significantly weaker affinity for the estrogen receptor than estradiol,

Scheme 3[a]



[a] Reagents and conditions: (a) PhthNH, DEAD, Ph_3P, THF; (b) NH_2NH_2, EtOH, Δ; (c) HCl, MeOH.

Table 1. Estrogen Receptor Affinity of 4-Substituted Estradiol Analogs

steroid	compd	EC_{50} (M)	log EC_{50} ± SD	RBA
estradiol		1.80×10^{-10}	−9.744 ± 0.102	100.00
4-hydroxyestradiol		5.06×10^{-7}	−6.295 ± 0.092	0.36
4-(hydroxymethyl)estradiol	1	3.64×10^{-7}	−6.438 ± 0.141	0.49
4-(hydroxyethyl)estradiol	2	6.20×10^{-7}	−6.207 ± 0.290	0.29
4-(hydroxypropyl)estradiol	3	3.32×10^{-6}	−5.479 ± 0.116	0.05
4-(aminomethyl)estradiol	4	NB[a]		
4-(aminoethyl)estradiol	5	2.50×10^{-6}	−5.600 ± 0.159	0.07
4-(aminopropyl)estradiol	6	NB		

[a] NB = no measurable binding of steroid at 10^{-5} M concentration.

Table 2. Induction of pS2 Gene Expression by 4-Substituted Estradiol Analogs

steroid	compd	EC_{50} (M)	log EC_{50} ± SD	% relative activity
estradiol		3.01×10^{-11}	−10.520 ± 0.217	100.00
4-hydroxyestradiol		6.54×10^{-8}	−7.184 ± 0.158	0.046
4-(hydroxymethyl)estradiol	1	1.17×10^{-8}	−7.933 ± 0.288	0.257
4-(hydroxyethyl)estradiol	2	1.48×10^{-7}	−6.829 ± 0.094	0.020
4-(hydroxypropyl)estradiol	3	2.95×10^{-6}	−5.530 ± 0.217	0.001

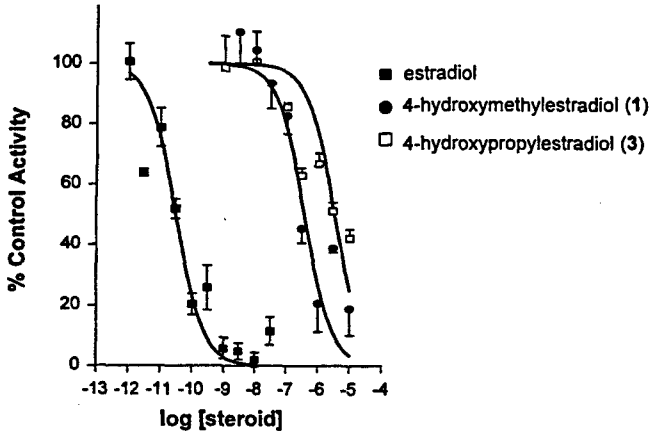


Figure 1. Estrogen receptor competitive binding assays for estradiol (■), 4-hydroxy-methylestradiol (●), and 4-hydroxypropylestradiol (□).

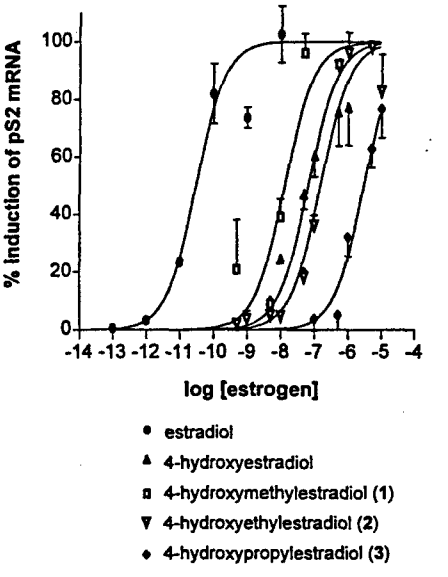


Figure 2. Induction of pS2 gene expression by estradiol (●), 4-hydroxyestradiol (▲), 4-(hydroxymethyl)estradiol (□), 4-(hydroxyethyl)estradiol (▽), and 4-(hydroxypropyl)estradiol (◆).

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.[24] The induction of pS2 mRNA expression by estradiol, 4-hydroxyestradiol, and 4-(hydroxyalkyl)estrogen analogs **1**−**3** was determined by RNA dot blot analysis. The EC_{50} 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-(hydroxymethyl)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-hydroxylalkyl analogs synthesized. This mitogenic activity was determined by measuring [^{3}H]thymidine incorporation[25] at day 4 at synthetic estrogen concentrations ranging from 2.5 to 10 μM. 4-(Hydroxymethyl)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).

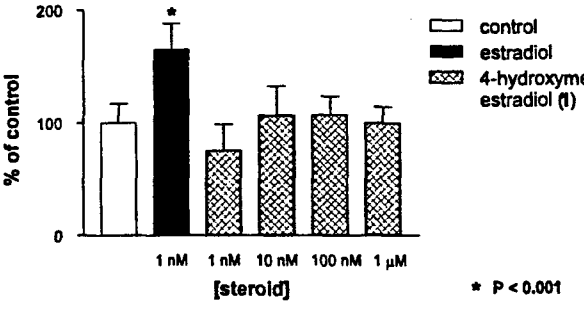


Figure 3. Comparison of mitogenic activities of estradiol (black bar), 4-(hydroxymethyl)estradiol (gray bar), and vehicle control (white bar) in MCF-7 human mammary carcinoma cell cultures.

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-

tivè hydroboration of 4-alkenylestradiols, which 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 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 EC_{50} 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.[22] 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 EC_{50} value of 11.7 nM. This synthetic compound was more effective than the endogenous metabolite, 4-hydroxyestradiol, which exhibited an apparent EC_{50} 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 Steraloids (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 CaH_2, distilled, and then stored over KOH pellets. Silica gel TLC plates (60 F_{254}) 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-Hoover 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 ^{13}C NMR were recorded on an IBM AF/250 spectrometer at 250 and 67.5 MHz, respectively, in $CDCl_3$ solutions unless otherwise indicated using the residual protiosolvent 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 by Oneida Research Services, Inc. (Whitesboro, NY).

4-Bromoestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (10). MOMCl (5.7 mL, 75 mmol) was added dropwise to a cold (0 °C) solution of 4-bromoestradiol (5.26 g, 15.0 mmol) and diisopropylethylamine (21.3 mL, 89.3 mmol) in THF (125 mL). On completion of the addition, the reaction mixture was allowed to warm up to room temperature, stirred for 1 h at the same temperature, and then heated at reflux overnight. The mixture was allowed to cool, and then saturated NH_4Cl solution (100 mL) was added. The mixture was extracted with EtOAc (4 × 100 mL), and the combined organic solutions were washed with saturated aqueous brine (100 mL), dried ($MgSO_4$), and concentrated. The crude product was purified by flash column chromatography (SiO_2, hexane/ethyl acetate, 9:1) to afford a pale yellow solid, which was recrystallized 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); IR (KBr, cm^{-1}) 2925–2785, 1597, 1578, 1473, 1452, 1444, 1402, 1385, 1306, 1261, 1234, 1224, 1205, 1176, 1155, 1122, 1107, 1088, 980, 914, 897, 856; ^1H NMR 7.20 (1H, d, J = 8.6 Hz), 6.95 (1H, d, J = 8.6 Hz), 5.22 (2H, s), 4.69 (2H, AB q, J = 6.6 Hz, $\Delta\nu$ = 3.5 Hz), 3.61 (1H, t, J = 8.4 Hz), 3.51 (3H, s), 3.37 (3H,s), 2.98 (1H, dd, J = 5.4, 17.9 Hz), 2.76–2.65 (1H, m), 2.31–2.11 (2H, m), 0.79 (3H, s); ^{13}C NMR 151.8, 137.8, 136.5, 124.8, 116.4, 113.5, 96.1, 95.4, 86.7, 56.2, 55.1, 50.1, 44.3, 43.0, 37.9, 37.3, 31.3, 28.2, 27.4, 26.6, 23.1, 11.7; MS m/z (M^+) calcd 440.1341, obsd 440.1388.

4-Ethenylestra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (11). A solution of **11** (440 mg, 1.0 mmol), vinyltributyltin (0.62 g, 2.0 mmol), and $Pd(PPh_3)_4$ (67 mg, 0.06 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% NH_4OH (15 mL), water (4 × 20 mL), and brine (3 × 20 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (SiO_2, 4:1 hexane/ethyl acetate) to yield 344 mg (90%) of the title compound as a colorless oil, which solidified on standing to a colorless waxy solid: mp 55 °C; IR (KBr, cm^{-1}) 2930, 2888, 2847, 2023, 1698, 1586, 1476, 1444, 1158, 1112, 1055, 1045, 927; ^1H NMR 7.16 (1H, d, J = 8.7 Hz), 6.94 (1H, d, J = 8.9 Hz), 6.40 (1H, dd, J = 11.7, 17.9 Hz), 5.55 (1H, dd, J = 2.3, 17.9 Hz), 5.52 (1H, dd, J = 2.3, 11.7 Hz), 5.12 (2H, s), 4.64 (2H, s), 3.60 (1H, t, J = 8.3 Hz), 3.45 (3H, s), 3.36 (3H, s), 2.91–2.65 (2H, m), 0.74 (3H.s); ^{13}C NMR 152.9, 136.2, 134.4, 131.3, 127.1, 125.0, 119.7, 112.7, 96.1, 95.0, 86.7, 56.0, 55.1, 10.3, 44.5, 43.0, 38.0, 37.5, 28.23, 28.20, 27.4, 26.6, 23.1, 11.7; MS m/z (M^+) calcd 386.2457, obsd 386.2443. Anal. ($C_{24}H_{34}O_4$) C, H.

4-(2′-Propenyl)estra-1,3,5(10)-triene-3,17β-diol 3,17β-Bis(methoxymethoxy) Ether (12). A solution of **11** (1.50 g, 3.41 mmol), $Pd(PPh_3)_4$ (250 mg, 0.22 mmol) and allyl tri-*n*-butyl stannane (2.28 g, 6.90 mmol) in DMF (50 mL) was deoxygenated by bubbling argon through it for 15 min, and then the solution was heated at reflux overnight. After cooling, the solution was decanted off from the palladium, and the residual precipitated palladium was washed with ethyl acetate. The reaction solution was diluted with ethyl acetate, washed with water (3 × 50 mL), and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 10:1) to give 1.28 g (94%) of the desired allyl compound **14** as a colorless oil: IR (neat, cm^{-1}) 2931, 2850, 2825, 1637, 1481, 1446, 1254, 1225, 1205, 1190, 1151, 1134, 1105, 1082, 1055, 1028, 1007, 918; ^1H NMR 7.15

(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), 4.65 (2H, AB q, J = 6.7, $\Delta\nu$ = 2.9 Hz), 3.61 (1H, t, J = 8.3 Hz), 3.45 (3H, s), 3.42 (2H, t, J = 6.6 Hz), 3.37 (3H, s), 2.91–2.66 (2H, m), 0.79 (3H, s); ^{13}C NMR 153.1, 136.5, 136.3, 134.3, 126.7, 124.1, 114.4, 111.7, 96.1, 94.8, 86.7, 55.9, 55.1, 50.3, 44.4, 43.0, 38.0, 37.5, 30.2, 28.2, 27.4, 26.7, 26.6, 23.1, 11.7; MS m/z (M^+) obsd 400.2613, calcd 400.2618. Anal. ($C_{25}H_{36}O_4$) C, H.

4-(2′-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol 3,-17β-Bis(methoxymethoxy) Ether (13). A solution of 1 M BH_3·THF (3.00 mL, 3.00 mmol) was added dropwise to a solution of **12** (286 mg, 0.75 mmol) in THF (6 mL) at 0 °C. On completion of the addition, the cooling bath was removed and the mixture stirred for 1 h; 1 M NaOH (3 mL) was added cautiously, and after the addition of 30% H_2O_2 (3 mL), the mixture was heated at reflux for 1 h. The mixture was allowed to cool, then ethyl acetate (75 mL) was added, and the organic solution was separated from the aqueous layer. The organics were washed with water (25 mL) and brine (25 mL), dried ($MgSO_4$), and concentrated. The residue was purified by MPLC (SiO_2, hexane/ethyl acetate, 2:1) to give 37 mg (12%) of a diastereomeric mixture of partially deprotected secondary alcohols, 92 mg (30%) of a mixture of two diastereomeric secondary alcohols, and 119 mg (39%) of the desired primary alcohol as a colorless oil, which crystallized on standing: mp 81–82 °C; IR (KBr, cm^{-1}) 3496, 3311, 2931, 2870, 2844, 2821, 1595, 1581, 1481, 1404, 1385, 1309, 1255, 1227, 1205, 1190, 1149, 1111, 1096, 1068, 1053, 1009, 912, 814; ^{1}H NMR 7.12 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.18 (2H, s), 4.65 (2H, AB q, J = 6.6, $\Delta\nu$ = 3.3 Hz), 3.78 (2H, t, J = 7.0 Hz), 3.61 (1H, t, J = 8.4 Hz), 3.46 (3H, s), 3.36 (3H, s), 2.97 (2H, t, J = 6.7 Hz), 2.96–2.88 (1H, m), 2.82–2.70 (1H, m), 0.79 (3H, s); ^{13}C NMR 153.6, 136.7, 134.6, 125.4, 124.4, 111.5, 96.1, 94.7, 86.7, 62.2, 56.0, 55.1, 50.3, 44.4, 43.0, 37.9, 37.4, 29.5, 28.2, 27.4, 27.1, 26.6, 23.1, 11.7; MS m/z (M^+) calcd 404.2563, obsd 404.2574. Anal. ($C_{24}H_{36}O_5$) C, H.

4-(3′-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol 3,-17β-Bis(methoxymethoxy) Ether (14). A solution of 1 M BH_3·THF (12.4 mL, 12.4 mmol) was added dropwise to a solution of **14** (1.23 g, 3.08 mmol) in THF (25 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 1 h. NaOH (1 M, 25 mL) was added cautiously, then 30% H_2O_2 (25mL) was added, and the resulting mixture was heated at reflux for 1 h. The aqueous reaction mixture was extracted with ethyl acetate (3 × 100 mL), and the organic layer was washed with water (100 mL), brine (100 mL) and dried ($MgSO_4$), and concentrated. Column chromatography (SiO_2, hexane/ethyl acetate, 2:1) of the residue gave 1.06 g (82%) of the desired alcohol as a colorless oil: IR (neat, cm^{-1}) 3442, 2927, 1479, 1254, 1205, 1151, 1105, 1053, 1024, 920; ^{1}H NMR 7.13 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.18 (2H, s), 4.65 (2H, AB q, J = 7.0, $\Delta\upsilon$ = 0 Hz), 3.67–3.37 (3H, m), 3.48 (3H, s), 3.37 (3H, m), 0.79 (3H, s), ^{13}C NMR 153.3, 136.2, 134.7, 128.6, 123.8, 111.5, 96.1, 95.0, 86.7, 62.4, 86.1, 55.1, 50.3, 44.4, 43.0, 38.0, 37.4, 32.0, 28.2, 27.4, 26.5, 23.1, 21.8, 11.7; MS m/z (M^+) calcd 418.2719, obsd 418.2712. Anal. ($C_{25}H_{38}O_5$) C, H.

4-(2′-Hydroxyethyl)estra-1,3,5(10)-triene-3,17β-diol (2). A solution of **13** (80 mg, 0.20 mmol) and pyridinium p-toluenesufonate (0.50 g, 2.00 mmol) in methanol (5 mL) was heated at reflux for 24 h. After the mixture as cooled to room temperature, ethyl acetate (50 mL) was added, and then the solution was washed with water (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was recrystallized from methanol and water to afford 50 mg (80%) of the alcohol: mp 229–230 °C; IR (KBr, cm^{-1}) 3338, 2966–2860, 1591, 1481, 1469, 1444, 1425, 1377, 1358, 1340, 1277, 1200, 1180, 1134. 1072, 1057, 1039, 1011, 818, 810; ^{1}H NMR (DMSO) 8.89 (1H, s), 6.92 (1H, d, J = 8.48 Hz), 6.56 (1H, d, J = 8.4 Hz), 4.62 (1H, br), 4.47 (1H, d, J = 4.8 Hz), 3.54–3.41 (1H, m), 2.84–2.59 (4H, m), 0.63 (3H, s); ^{13}C NMR 151.9, 135.5, 130.7, 123.2, 122.7, 112.1, 79.9, 59.9, 49.5, 43.7, 42.5, 37.8, 36.5, 29.8, 29.4, 27.0, 26.1, 22.6, 11.0; (M^+) calcd 316.2038, obsd 316.2032. Anal. ($C_{20}H_{28}O_3$·0.5H_2O) C, H.

4-(3′-Hydroxypropyl)estra-1,3,5(10)-triene-3,17β-diol (3). A solution of the alcohol **14** (173 mg, 0.41 mmol) and pyridinium p-toluenesulfonate (0.50 g, 2.0 mmol) in methanol (5 mL) was heated at reflux for 24 h. After cooling, the reaction mixture was diluted with ethyl acetate (50 mL), and then it was washed with water (3 × 25 mL) and brine (25 mL), dried, and concentrated. The residue was recrystallized from methanol/water to give 61 mg (61%) of the desired alcohol as a colorless solid: mp 240–242 °C; IR (KBr, cm^{-1}) 3392, 3249, 2971, 2916, 2864, 1591, 1491, 1471, 1446, 1425, 1379, 1362, 1280, 1080, 1059, 1034, 1003, 814, 808; ^{1}H NMR (DMSO) 10.28 (1H, s), 6.90 (1H, d, J = 8.5 Hz), 6.56 (1H, d, 8.5 Hz), 4.47 (1H, d, J = 4.8 Hz), 4.41 (1H, t, J = 5.2 Hz), 3.54–3.36 (2H, m), 2.80–2.45 (5H, m), 2.22–2.12 (1H, m), 2.09–2.00 (1H, m), 1.84–1.80 (3H, m), 1.59–1.43 (3H, m), 1.39–1.04 (7H, m), 0.54 (3H, s); ^{13}C NMR 152.6, 134.9, 130.8, 125.9, 122.8, 112.1, 79.9, 60.9, 49.6, 43.8, 42.6, 37.9, 36.5, 31.8, 27.0, 26.2, 25.9, 22.6, 21.8, 11.1; MS m/z (M^+) calcd 330.2195, obsd 330.2192. Anal. ($C_{21}H_{30}O_3$·0.25H_2O) C, H.

4-Carboxy-3,17β-Bis(methoxymethoxy)estra-1,3,5(10)-triene-3,17β-diol Methyl Ester (15). n-BuLi (3.5 mL, 5.65 mmol) was added dropwise to a solution of **10** (1.15 g, 2.61 mmol) in THF (50 mL) at −78 °C. After 1 h of stirring at this temperature, several pieces of dry ice were added, and then the cooling bath was removed. After being warmed to room temperature, the reaction mixture was diluted with ether (50 mL) and then extracted with 5% KOH (5 × 50 mL). The combined basic extracts were acidified to pH 5 with concentrated HCl and then extracted with ether (6 × 50 mL). The combined ethereal extracts were washed with brine (50 mL), dried ($MgSO_4$), and concentrated to give the crude acid. The acid was suspended in ether (20 mL), and an ethereal solution of diazomethane was added to it at 0 °C. After 30 min, sufficient acetic acid was added to the reaction mixture to discharge the yellow coloration. The reaction mixture was washed with $NaHCO_3$ (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), concentrated, and purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to give 0.82 g (76%) of the desired product as a colorless oil, which crystallized after a few days of standing at room temperature: mp 66–68 °C; IR (KBr, cm^{-1}) 2924, 2862, 1728, 1585, 1481, 1440, 1384, 1273, 1257, 1248, 1155, 1126, 1103, 1061, 1049, 1031, 793; ^{1}H NMR 7.25 (1H, d, J = 8.7 Hz), 6.93 (1H, d, J = 8.7 Hz), 5.13 (2H, s), 4.64 (2H, AB q, J = 7.0, $\Delta\upsilon$ = 0 Hz), 3.88 (3H, s), 3.59 (1H, t, J = 8.4 Hz), 3.44 (3H, s), 3.36 (3H, s), 2.79–2.73 (2H, m), 2.29–1.99 (4H, m), 1.95–1.83 (1H, m), 0.79 (3H, s); ^{13}C NMR 168.9, 151.6, 134.7, 134.6, 127.4, 124.8, 112.6, 96.2, 95.0, 86.7, 56.0, 55.1, 51.9, 50.1, 44.1, 43.0, 38.1, 37.3, 28.2, 26.8, 26.6, 26.4, 23.1, 11.7; MS m/z (M^+) calcd 418.2355, found 418.2337. Anal. ($C_{24}H_{34}O_6$) C, H.

4-Carboxy-3,17β-estra-1,3,5(10)-triene-3,17β-diol Methyl Ester (16). A solution of the ester **15** (0.44 g, 1.10 mmol) and pyridinium p-toluenesulfonate (2.77 g, 11.0 mmol) in MeOH (10 mL) was heated to reflux for 36 h. After cooling and addition of EtOAc (100 mL), the organic solution was washed with water (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was filtered through a short pad of silica gel (ethyl acetate/hexane, 1:2) to give 0.30 g (83%) of **16** as a colorless oil, which crystallized on standing: mp 135–136 °C (MeOH/H_2O); IR (KBr, cm^{-1}) 3435, 2920, 2866, 1718, 1591, 1446, 1427, 1383, 1361, 1344, 1288, 1267, 1230, 1217, 1190, 1171, 1136, 1122, 1059, 1039, 1011, 960; ^{1}H NMR 10.72 (1H, s), 7.38 (1H, d, J = 8.8 Hz), 6.80 (1H, d, J = 8.8 Hz), 3.93 (3H, s), 3.72 (1H, t, J = 8.4 Hz), 3.10–3.04 (2H, m), 2.29–2.06 (3H, m), 1.97–1.84 (2H, m), 0.77 (3H, s); ^{13}C NMR 172.0, 160.0, 139.3, 132.4, 132.1, 115.2, 112.7, 81.8, 51.9, 501, 44.7, 43.3, 37.9, 36.9, 30.7, 29.8, 27.3, 26.9, 23.0, 11.1; MS m/z (M^+) calcd 330.1831, found 330.1835. Anal. ($C_{20}H_{26}O_4$·0.5H_2O) C, H.

4-Hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (1). $LiAlH_4$ (70 mg, 1.89 mmol) was added portionwise to a solution of the ester **16** (51 mg, 0.15 mmol) in THF (5 mL) at 0 °C. On completion of the addition, the mixture was allowed to warm to room temperature and then stirred for 4 h. When the reaction was complete, water (0.07 mL), 15% NaOH (0.07 mL), and water (0.21 mL) were added. Once a granular precipitate had formed, it was removed by suction filtration through Celite, washed with MeOH, and concentrated to yield 23 mg

(51%) of the desired alcohol as a colorless solid: mp > 270 °C; IR (KBr, cm^{-1}) 3375, 3240, 2960, 2931, 2920, 2866, 2850, 1591, 1479, 1448, 1429, 1383, 1352, 1286, 1252, 1078, 1065, 1009, 820 ^1H NMR 9.01 (1H, s), 6.99 (1H, d, J = 8.5 Hz), 6.58 (1H, d, J = 8.5 Hz), 4.53 (1H, m), 4.48 (2H, AB q, J = 4.8, $\Delta\nu$ = 7.7 Hz), 3.55–3.50 (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); ^{13}C 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^+) calcd 302.1875, obsd 302.1883. Anal. ($C_{19}H_{26}O_3 \cdot 0.25H_2O$) C, H.

4-(Hydroxymethyl)-3,17β-bis(methoxymethoxy)estra-1,3,5(10)-triene (17). $LiAlH_4$ (340 mg, 9.19 mmol) was added portionwise to a solution of **15** (0.50 g, 1.20 mmol) in THF (30 mL) at 0 °C. The reaction mixture was allowed to warm to 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 grannular precipitate was removed by filtration through a pad of Celite and $MgSO_4$ (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, 2964–2814, 1597, 1583, 1481, 1441, 1400, 1385, 1255, 1242, 1228, 1153, 1107, 1092, 1063, 1045, 1031, 1005, 995, 955, 903; ^1H NMR 7.23 (1H, d, J = 8.7 Hz), 6.94 (1H, d, J = 8.7 Hz), 5.19 (2H, AB q, J = 6.7, $\Delta\nu$ = 3.4 Hz), 4.74 (2H, m), 4.64 (2H, AB q, J = 6.6, $\Delta\nu$ = 3.4 Hz), 3.61 (1H, t, J = 8.3 Hz), 3.48 (3H, s), 3.36 (3H, s), 3.10–2.92 (1H, m), 2.90–2.81 (1H, m), 2.33–1.91 (6H, m), 1.76–1.11 (9H, m), 0.79 (3H, m); ^{13}C NMR 153.9, 136.8, 135.1, 128.0, 126.2, 112.6, 96.1, 95.5, 86.7, 56.7, 56.3, 55.1, 50.2, 44.4, 43.0, 37.9, 37.4, 28.2, 27.2, 26.6, 26.5, 23.1, 11.7; MS m/z (M^+) calcd 390.2397, obsd 390.2391. Anal. ($C_{23}H_{34}O_5$) C, H.

Phthalimides 23–24: General Procedure. DEAD (0.42 mL, 2.40 mmol) was added dropwise to a solution of the alcohol **13**, **14**, or **17** (0.80 mmol), phthalimide (0.35 g, 2.40 mmol), and triphenylphosphine (0.63 g, 2.40 mmol) in THF (10 mL) at room temperature and then stirred overnight. The solvent was removed *in vacuo*, and then the residue was dissolved in EtOAc (100 mL), washed with 5% aqueous KOH (4 × 50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by chromatography (SiO_2, hexane/ethyl acetate, 4:1) to afford the substituted phthalimides **18–20** as colorless or pale yellow oils, which solidified on standing.

4-(Phthalimidoylmethyl)-3,17β-bis(methoxymethoxy)-estra-1,3,5(10)-triene (18): 79%; mp 104–106 °C; IR (KBr, cm^{-1}) 2951, 2925, 2868, 1711, 1479, 1396, 1385, 1348, 1255, 1151, 1113, 1092, 1055, 1028, 1009, 993, 962, 949, 918, 723; ^1H NMR 7.79–7.74 (2H, m), 7.69–7.64 (2H, m), 7.20 (1H, d, J = 8.7 Hz), 6.90 (1H, d, J = 8.7 Hz), 5.12 (2H, s), 4.90 (2H, s), 4.64 (2H, s), 3.60 (1H, t, J = 8.3 Hz), 3.36 (3H, s), 3.31 (3H, s), 3.20–3.13 (1H, m), 2.97–2.86 (1H, m), 2.29–1.94 (3H, m), 0.78 (3H, s); ^{13}C NMR 167.9, 137.6, 134.2, 133.7, 132.3, 126.0, 123.0, 122.0, 111.4, 96.1, 94.6, 86.7, 55.8, 55.1, 50.3, 44.4, 43.0, 37.8, 37.4, 34.3, 28.2, 27.4, 26.9, 26.5, 23.1, 11.7; MS m/z (M^+) calcd 519.2621, obsd 519.2634. Anal. ($C_{32}H_{39}NO_6$) C, H, N.

4-(Phthalimidoylethyl)-3,17β-bis(methoxymethoxy)-estra-1,3,5(10)-triene (19): 87%; mp 117–120 °C; IR (KBr, cm^{-1}) 2930, 2880, 1772, 1716, 1505, 1430, 1393, 1360, 1152, 1119, 1109, 1070, 1052, 998, 900, 720; ^1H NMR 7.83–7.67 (4H, m), 7.15 (1H, d, J = 8.68 Hz), 6.90 (1H, d, J = 8.67 Hz), 5.16 (2H, s), 4.64 (2H, AB q, J = 6.8, $\Delta\nu$ = 2.9 Hz), 3.81 (1H, t, J = 8.2 Hz), 3.48 (3H, s), 3.35 (3H, s), 3.03–2.75 (4H, m), 0.78 (3H, s); ^{13}C NMR 168.1, 153.6, 136.6, 134.4, 133.7, 132.3, 124.9, 124.6, 123.0, 111.2, 96.0, 94.5, 86.6, 56.0, 50.1, 44.3, 42.9, 37.8, 37.3, 36.8, 28.1, 27.3, 26.7, 26.5, 25.3, 23.0, 11.7; MS m/z (M^+) calcd 533.2767, obsd 533.2772 Anal. ($C_{31}H_{37}NO_6$) C, H, N.

4-(Phthalimidoylpropyl)-3,17β-bis(methoxymethoxy)-estra-1,3,5(10)-triene (25): 93%; mp 134–135 °C; IR (KBr, cm^{-1}) 2955, 2935, 2894, 2786, 1777, 1726, 1485, 1478, 1445, 1394, 1363, 1153, 1122, 1086, 1055, 1040, 922, 720; ^1H NMR 7.85–7.80 (2H, m), 7.73–7.67 (2H, m), 7.13 (1H, d, J = 8.7 Hz), 6.87 (1H, d, J = 8.7 Hz), 5.09 (2H, s), 4.64 (2H, AB q, J = 6.9, $\Delta\nu$ = 1.7 Hz), 3.78 (2H, t, J = 7.2 Hz), 3.59 (1H, t, J = 8.3 Hz), 3.39 (3H, s), 3.36 (3H, s), 2.88–2.64 (4H, m), 0.78 (3H, s); ^{13}C NMR 168.3, 153.0, 135.7, 134.1, 133.7, 132.2, 128.2, 123.7, 123.0, 111.2, 96.0, 94.4, 86.7, 55.8, 55.0, 50.2, 44.3, 42.9, 38.4, 37.8, 37.4, 28.1, 27.4, 26.6, 26.5, 23.3, 23.0, 11.6; MS m/z (M^+) calcd 547.1693, obsd 547.2908. Anal. ($C_{33}H_{41}NO_6$) C, H, N.

Amines 4–6: General Procedure: A solution of the phthalimides **18–20** (0.46 mmol) and hydrazine (1 mL) in ethanol (10 mL) was heated at reflux for 1 h. After cooling, ethyl acetate (50 mL) was added, and then the mixture was washed with 5% KOH solution (3 × 25 mL) and brine (25 mL), dried ($MgSO_4$), and concentrated. The residue was dissolved in methanol (5 mL) and cooled to 0 °C, and then HCl was bubbled through it for 15 min. The cooling bath was removed, and then stirring was continued for 3 h. The reaction mixture was concentrated and then redissolved in methanol, and the resulting green solution was decolorized with charcoal. After filtration and concentration, the residue was taken up in the minimum amount of methanol, and the product was precipitated out by the addition of ether, affording the amine salts as colorless or yellow solids.

4-(Aminomethyl)estra-1,3,5(10)-triene-3,17β-diol (4): 90%; mp > 270 °C; IR (KBr, cm^{-1}) 3444–2868, 1620, 1591, 1509, 1491, 1473, 1450, 1379, 1352, 1323, 1284, 1261, 1219, 1201, 1188, 1080, 1057, 1007, 945, 814; ^1H NMR (DMSO) 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), 0.64 (3H, s); ^{13}C NMR 152.9, 135.4, 130.1, 125.5, 116.8, 111.5, 78.8, 48.3, 42.6, 41.5, 36.7, 35.4, 32.9, 28.8, 25.6, 25.2, 24.8, 21.6, 10.0; MS m/z (M^+ − HCl) calcd 301.2037, obsd 301.2042. Anal. ($C_{19}H_{28}NO_2Cl \cdot H_2O$) C, H, N.

4-(Aminoethyl)estra-1,3,5(10)-triene 3,17β-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) 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 = 8.01 Hz), 2.80–2.55 (4H, m), 0.64 (3H, s); ^{13}C NMR 153.7, 134.9, 131.2, 123.1, 113.0, 79.8, 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^+ − HCl) calcd 315.2198, obsd 315.2201.

4-(Aminopropyl)estra-1,3,5(10)-triene-3,17β-diol (6): 86%; mp >270 °C; IR (KBr, cm^{-1}) 3362, 3276, 3056, 3022, 2962, 2925, 2863, 1635, 1589, 1489, 1443, 1280, 1208, 1133, 1058, 813; ^1H NMR (DMSO) 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), 3.51–3.44 (3H, m), 2.78–2.73 (4H, m), 0.63 (3H, s); ^{13}C NMR 152.6, 134.9, 130.8, 124.4, 123.2, 112.2, 79.8, 49.5, 43.7, 42.5, 40.5, 37.8, 36.4, 29.8, 26.9, 26.3, 26.1, 25.8, 22.6, 22.0, 11.0; MS m/z (M^+ − HCl) calcd 329.2355, obsd 329.2354.

Biological Evaluations. General Information. [2,4,6,7-^3H]Estradiol (98.4 Ci/mmol, ^3H-E_2) was purchased from Dupont/NEN (Boston, MA) and was used as received. MCF-7 human breast adenocarcinoma cells were obtained from ATCC, and cells were incubated in a humidified CO_2 incubator (Forma model 3052) with 5% CO_2 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 (0.487 g/L), pyruvic acid (0.11 g/L), and sodium bicarbonate (1.5 g/L) 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 Formula 963 (Dupont/NEN) 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 OLIGOS, ETC (Wilsonville, OR) and were as follows:

For pS2:	sense	5'-ATC CCT GAC TCG GGG TCG CCT TTG-3'
	antisense	5'-CAA TCT GTG TTG TGA GCC GAG GCA CAG-3'
For 36B4:	sense	5'-AAA CTG CTG CCT CAT ATC CG-3'
	antisense	5'-TTT CAG CAA GTG GGA AGG TG-3'

Probes were labeled by random priming with Klenow fragment. Analysis of the RNA dot blots were performed on a Molecular Dynamics PhosphoImager SI.

Whole Cell Estrogen Receptor Studies.[20] MCF-7 cells were maintained in a similar fashion as described above. 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 cm^2 wells on a six-well plate at (1.5−2) × 10^5 cells/well in modified MEM (2−3 mL) containing 10% steroid free fetal calf serum and gentamycin (20 mg/mL). After 12−24 h at 37 °C, the culture media was removed and replaced and with serum-free modified MEM media (888 μL) containing insulin (5.0 mg/L), transferrin (5.0 mg/L), glutamine (2 mM), and albumin (2.0 mg/mL). After 48 h, the media was removed, fresh serum-free-modified MEM media added, and the synthetic estrogens **1**−**6** at various concentrations (3 × 10^{-5} to 1 × 10^{-5} M, 100 μL) were added and incubated for 10 min at 37 °C. To determine total binding, [^{3}H]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 and then 95% ethanol (1 mL) was added, followed by standing for 30 min at room temperature. An aliquot (500 μL) of the ethanol solution was added to Formula 963 and 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 [^{3}H]estradiol was calculated by subtracting the nonspecific binding data from total binding data. The apparent EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal displacement of specific [^{3}H]estradiol binding and was calculated by a nonlinear regression analysis (GraphPad Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

pS2 Induction. MCF-7 cells were maintained in a similar fashion as described above. Cells were plated at a concentration of 5.5 × 10^5 cells/25 cm^2 flask. After 2 days of growth, the cells were rinsed with Ca^{2+}, Mg^{2+} free PBS and placed on defined media for 48 h. Defined media contained DMEM/F12 media (Gibco BRL) supplemented with human albumin (2.0 mg/ml), transferrin (5.0 mg/L), bovine insulin (5.0 mg/L), and *l*-glutamine (2 mM). After addition of fresh defined media, the cells were dosed with compound (10^{-10}−10^{-5} M), 10 nM 17β-estradiol (Sigma, St. Louis, MO), or carrier (95% ethanol). Each compound was tested in triplicate. After 24 h, total cellular RNA was isolated by an adaptation of the method of Chomczynski and Sacchi.[20] The cells were lysed with a 4 M guanidine isothiocyanate solution, and the lysate was acidified with 3 M sodium acetate, pH 5.2 (1:10 vol). After addition of 3 M NaOAc, pH 5.2 (1:10 vol), RNA was extracted twice using water-saturated phenol:chloroform:isoamyl alcohol (60:24:1) at pH 4.0. A final extraction using an equal volume of chloroform:isoamyl alcohol (25:1) was performed. RNA from the resulting aqueous layer was precipitated with an equal volume of 2-propanol at −20 °C for 1 h. The RNA was pelleted at 15000*g* for 30 min at 4 °C. The resulting pellet was washed twice with 70% ethanol and once with 95% ethanol. Dried pellets were resuspended in 30 uL of Dnase-, Rnase-free molecular biology grade water (Sigma Chemical Co.). Quantification of RNA in each sample was performed using the absorbance at 260 nm.

Dot Blot Analysis. A denaturing solution containing 50% formamide, 7% formaldehyde, and 1× SSPE was added to 15 μg of RNA from each sample. The RNA was denatured at 68 °C for 15 min. Two volumes of 10× SSPE was added to each sample. The samples were loaded onto a 0.45 μm, positively charged, nylon membrane (Schleicher and Schuell, Keene, NH) using gentle suction through a 96-well dot blot manifold (BioRad, Hercules, CA). Membranes probed for pS2 gene expression were loaded with 10 μg RNA, the remaining 5 μg was loaded onto a membrane probed for the control gene, 36B4. Membranes were baked at 80 °C for 1 h and then incubated for at least 3 h in a prehydridization solution containing 5× SSPE, 5× Denhardts Reagent, 2% SDS, 100 μg/mL salmon sperm DNA, and 50% formamide. pS2 and 36B4 cDNA was prepared as described above and used to make ^{32}P-radiolabeled probes using random primers in the RadPrime Kit (Gibco BRL). Probes with specific activity ranging from 5.0 × 10^5 to 2.0 × 10^6 cpm/ng were used. Membranes, probed separately for pS2 or 36B4, were incubated for 48 h or 24 h, respectively, in hybridization solution containing 5× SSPE, 5× Denhardts reagent, 1% SDS, 100 μg/mL salmon sperm DNA, 10% PEG, and 50% formamide. The membranes were washed in 0.5× SSPE, 60′, at 55 °C; 0.1× SSPE, 60′, 60 °C; and 0.1× 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 EC_{50} 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 Prizm, Version 2.0, GraphPad Software Inc., San Diego, CA).

Cell Growth Assay. Human mammary carcinoma cell lines were maintained in 75-cm^2 plastic flasks at 37 °C in a modified Eagle's MEM (10 mL) containing 10% fetal calf serum and gentamycin (20 mg/L). For cell growth determinations, the mammary carcinoma cells were divided into 9.4 cm^2 wells at approximately 100 000 cells/well in modified MEM (2 mL) containing 10% steroid-free fetal calf serum and gentamycin (20 mg/l). After 2 days, media was changed to serum-free-modified MEM and experiments initiated. To determine dose-dependent effects, varying concentrations of 4-(hydroxymethyl)-estradiol **1** (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 [^{3}H]thymidine (1 μCi/well), followed by incubation for 2 h, cell lysis, and determination of [^{3}H]thymidine incorporation into DNA. Each experiment was carried out in quadriplicates, and test compounds were evaluated in experiments performed 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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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 estradiols (HE_2's), methoxy estradiols (ME_2's), and hydroxymethyl estradiols (HME_2) 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/10^5 dG were significant for each 2-HE_2 and 4-HE_2. 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_2. There was no DNA damage observed for the non-redox cycling HME_2's, making these compounds useful probes in the study of receptor-mediated carcinogenesis. Thus, both 2-HE_2 and 4-HE_2 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[1] formation can be induced in MCF-7 cells by the environmental toxin 2,3,7,8-tetrachlorodibenzo-*p*-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 *o*-hydroquinones (*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

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[1] Abbreviations: BCS, bathocuproinedisulfonic acid; CE, catechol estrogen; CV, cyclic voltammetry; 2′-dG, 2′-deoxyguanosine; $E^1/_2$, half-wave potential; Ep,a, anodic peak potential; Ep,c, cathodic peak potential; ECD, electrochemical detector; E_1, estrone; E_2, estradiol; ER, estrogen receptor; GSH, glutathione; HE_2, hydroxy estradiol; HME_2, hydroxymethyl estradiol; LP, lactoperoxidase; ME_2, methoxy estradiol; NHE, normal hydogen electrode; 8-oxo-dG, 8-oxo-2′-deoxyguanosine; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase.

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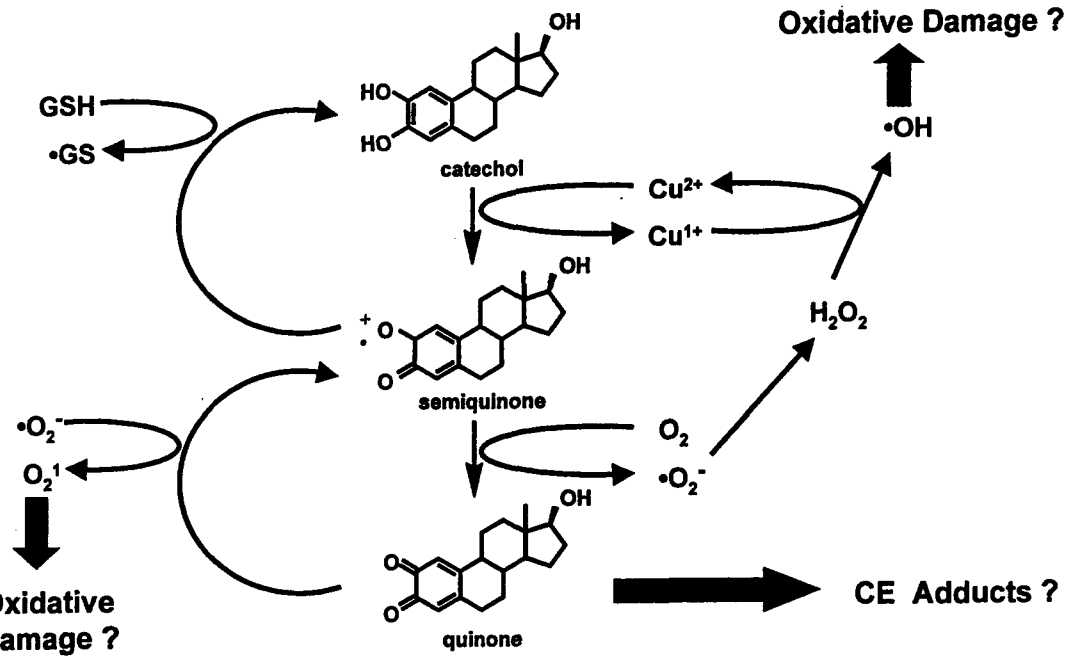


Figure 1. Potential genotoxicity mechanisms for catechol estrogens.

range from 40 to 350 pg/mL, approximately 0.1−1.0 nM. In many of the studies on catechol estrogen tumorigenesis, micromolar concentrations of CE or a nonphysiological matrix (e.g., organic solvents) were used. Therefore, further studies which show that a correlation exists between minimal CE concentrations and the amount of cellular damage are necessary. Other mechanisms which do not include redox cycling, such as a receptor-mediated pathway, should also be explored. We are interested in pursuing this possibility, while further studying the redox cycling aspect of estrogen-induced carcinogenesis using CE analogues synthesized in our lab.

The use of 8-oxo-dG as a DNA damage marker for oxidative stress is well-documented (*20*). 8-Oxo-dG is mutagenic, causing G−T transversion (*21*); this type of mutation has been found on the p53 gene in hepatic cells exposed to chronic inflammation (*22*). If handled properly, DNA can be digested without appreciable formation of background 8-oxo-dG or scission of the 8-oxo-dG ribosyl linkage. With the use of an HPLC system coupled to an electrochemical detector (ECD), 8-oxo-dG can be detected in the low-femtomole range, making it a good marker for ROS-generated DNA damage (*23*, *24*). However, the process of DNA isolation, cellular DNA repair, and the induction of antioxidant defenses make the measurement of 8-oxo-dG levels in vivo and in cell culture very difficult and often misleading (*25*). At present, there is very little information regarding the formation of 8-oxo-dG by the CE's.

We previously reported the synthesis and biological evaluation of various 2- and 4-hydroxyalkyl estradiols as metabolically stable CE analogues. In this study, we have compared hydroxy estradiols (HE_2's), methoxy estradiols (ME_2's), and hydroxymethyl estradiols (HME_2) in potentiometric and DNA damaging studies in a physiologically relevant buffer at pH 7.4. Potentiometric measurements were used to determine chemical reversibilities and half-wave potentials. The DNA-damaging potential of the CE's was quantified in calf thymus DNA using the genotoxic marker 8-oxo-dG. Varied concentrations of 2-HE_2 were used to determine the lowest concentration at which DNA damage is induced. Influences of copper and LP on CE-induced DNA damage were also studied, as well as the mechanism of ROS generation.

Experimental Procedures

Materials and Methods. 8-Oxo-dG was prepared as described below. The 2- and 4-HE_2's and ME_2's were purchased from Steraloids Inc. (Wilton, NH). The 2- and 4- HME_2's were prepared as previously described (*26*, *27*). Calf thymus DNA, 4-methylcatechol, 2′-deoxyguanosine, NH_4OAc, ascorbic acid, GSH, mannitol, H_2O_2, alkaline phosphatase, and LP were all purchased from Sigma Ltd. (St. Louis, MO). Nuclease P1 was purchased from Boehringer Mannheim (Indianapolis, IN). **Caution:** *2-HE_2 and 4-HE_2 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 100 mL of a 0.4 mM solution of 2-dG dissolved in a 0.5 M H_2NaPO_4 buffer at pH 7 were added 10 equiv of a 3% solution of H_2O_2 and 0.5 equiv of ascorbic acid. The reaction mixture was stirred vigorously at 37 °C as a steady stream of O_2 was bubbled into the mixture for the duration of the reaction. After 2 h, 10 equiv of a 3% solution of H_2O_2 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 H_2O, 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-d_6, 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 N_2-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 (Ca^{2+} and Mg^{2+} 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 NH_4OAc 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 12000*g* 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 $ZnCl_2$ 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 $NaPO_4$/0.1 M NH_4Ac 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 260 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 R^2 was 0.999 for a standard curve covering the experimental range.

2-HE_2 Oxidation by Lactoperoxidase. Spectroscopic 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 H_2O_2. H_2O_2 (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 reversible 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.

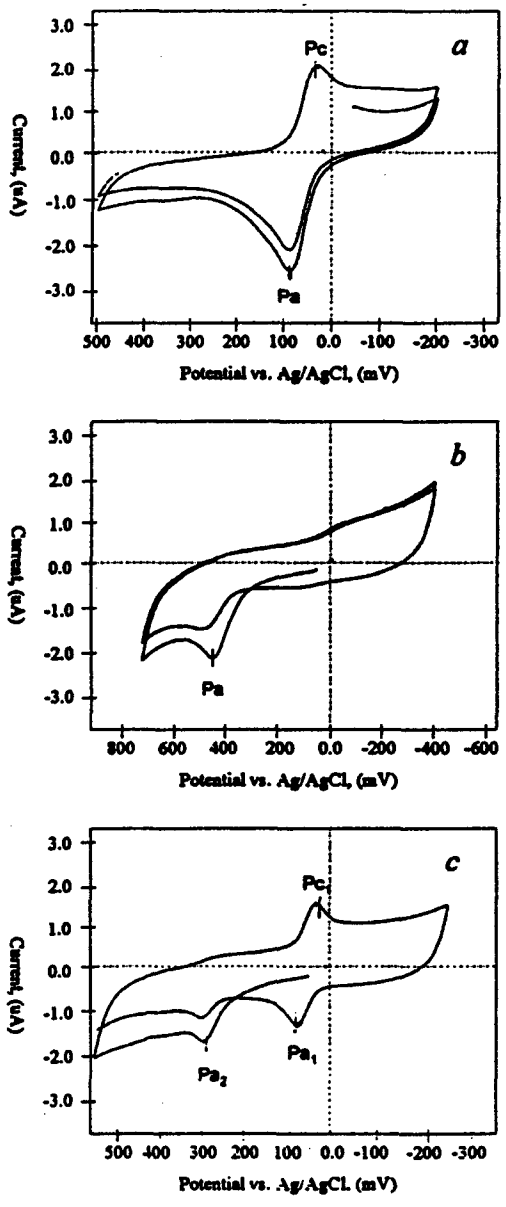
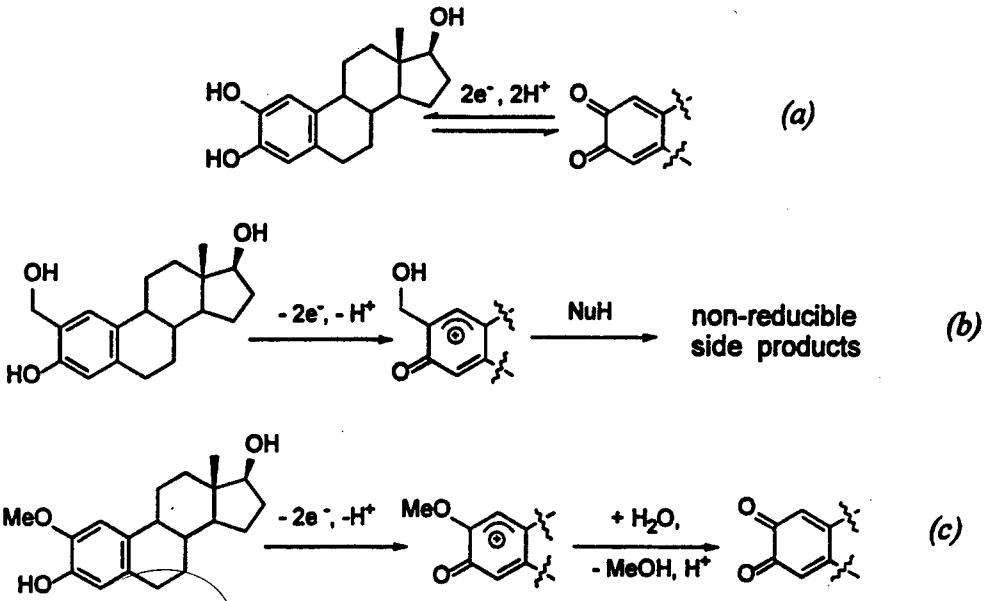


Figure 2. Cyclic voltammograms of estrogen analogues. Cyclic voltammograms of (a) 2-HE_2, (b) 2-HME_2, and (c) 2-ME_2 were obtained to determine redox peak potentials. The abbreviation Pa is the anodic peak potential (Ep,a), and Pc is the cathodic peak potential (Ep,c).

With regard to the CE's, both 2-HE_2 (Figure 2a) and 4-HE_2 were quasi-reversible (Scheme 1a), exhibiting ΔE's of 55 and 60 mV, respectively, and nearly equal half-wave potentials ($E^1/_2$) 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 physiologic matrix. Differences in oxidation potentials or ΔE values could possibly explain differences in toxicity; however, both 2-HE_2 and 4-HE_2 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-HME_2 (Figure 2b) and 4-HME_2 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

Scheme 1. Oxidation/Reduction Schemes for the Estrogen Analogues[a]



[a] (a) Reversible oxidation and reduction of 2-HE_2, (b) irreversible oxidation of 2-HME_2, and (c) oxidative demethylation of 2-ME_2.

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

compound	*E*p,a1 (mV)	*E*p,c1 (mV)	*E*p,a2 (mV)	Δ*E* (mV)	$E^1/_2{}^a$ (mV)	$E^1/_2{}^b$ (mV)
2-HE_2	90	35	–	55	73	263
2-ME_2	80	15	280	65	48	238
2-HME_2	407	–	–	–	–	–
4-HE_2	95	35	–	60	75	265
4-ME_2	80	25	345	55	53	243
4-HME_2	405	–	–	–	–	–
4-methylcatechol	140	50	–	90	95	285

[a] Standard error ± 10 mV. [b] 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 HME_2's. The HME_2's were only oxidized at high oxidation potentials with an end product that is incapable of participating in redox cycling, thus making this type of "capped" CE analogue excellent for use in receptor studies that may involve estrogen metabolites without inducing damage by redox cycling.

With regard to methoxyestrogens, 2-ME_2 is found at a much higher concentration in the serum than the CE's, and the potentiometric properties of the ME_2's 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,a1) followed by one cathodic peak (*E*p,c1) 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,a1 and *E*p,c1 peaks were nearly identical to the oxidation and reduction peaks of 2-HE_2 (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 redox cycling could occur. The half-wave potential of the validation standard 4-methylcatechol is within 30 mV of the literature value obtained under similar conditions (*30*).

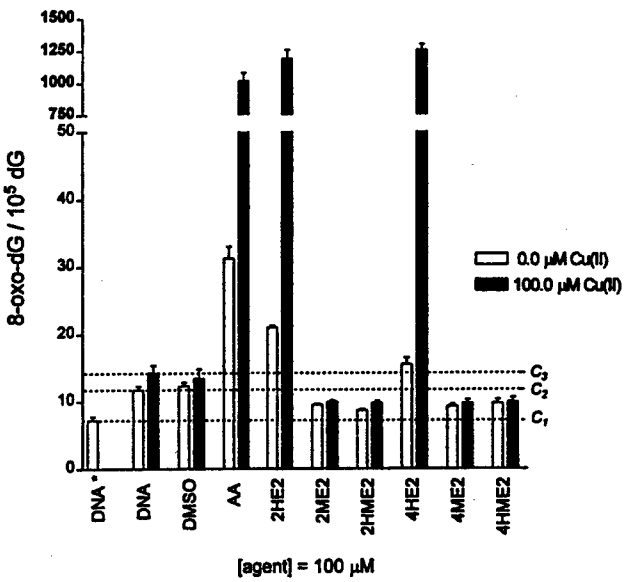


Figure 3. Formation of 8-oxo-dG by estrogen analogues. Calf thymus DNA was exposed to 100 μM CE or CE analogue with and without the addition of 100 μM Cu(II)SO_4 for 3 h in PBS (pH 7.4) at 37 °C. The induction of 8-oxo-dG was compared to that of the following controls: nonincubated DNA* (C_1), DNA incubated alone (C_2), and DNA incubated in the presence of Cu(II)SO_4 (C_3). Data points are means ± SD ($n = 3$) for all samples.

8-Oxo-dG Formation by CE's and CE Analogues with and without Cu(II). Calf thymus DNA was exposed to 100 μM CE, CE analogue, or ascorbic acid, with and without 100 μM Cu(II)SO_4 for 3 h (Figure 3). Ascorbic acid is known to increase 8-oxo-dG levels at high concentrations in vitro and was therefore used as a positive control (*30*). Cu(II)SO_4 was included in the experiments due to the noted redox coupling with hydroquinones, the low micromolar concentrations found in

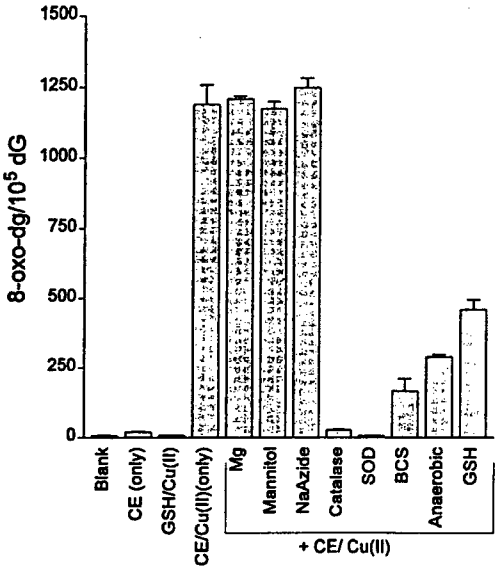


Figure 4. Determination of ROS production by 2-hydroxy estradiol. 2-HE_2 (100 μM) was incubated in the presence of Cu(II) (100 μM) for 3 h in PBS (pH 7.4) at 37 °C in the presence of a copper chelator or ROS quenching agent as shown. Concentrations were as follows: 1.0 mM GSH, 1.0 mM sodium azide, 200 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.

normal serum, and the reported association with histone proteins and dGuo in DNA (*8*, *23*, *31*, *32*).

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. Increases in the number of 8-oxo-dG/10^5 dG were significant 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 (1260 ± 74.9), and ascorbic acid (1020 ± 89) samples. The HME_2's and ME_2'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 significantly more 8-oxo-dG formation 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 (PUFA's), 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 a $^1O_2$ scavenger (1250 ± 58.7, 5% decrease), and mannitol as an $^{\bullet}OH$ scavenger (1170 ± 44.6, 1% decrease) were far less effective at lowering the level of 8-oxo-dG formation. However, catalase, an H_2O_2 scavenger (26.7 ± 5.0, 98% decrease), and SOD, an $O_2^{\bullet-}$ 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 (166.0 ± 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 mechanistic 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_2Q–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, and in the absence of Fenton catalysts, 2-HE_2 is primarily

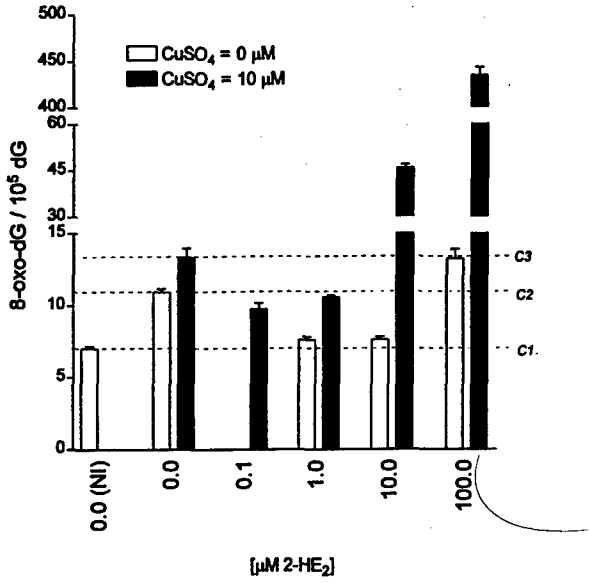


Figure 5. Effect of estrogen concentration on the formation of 8-oxo-dG. Calf thymus DNA was exposed to increasing concentrations of 2-HE_2 (0.1 to 100 μM) with and without the addition of 10 μM Cu(II)SO_4 for 3 h in PBS (pH 7.4) at 37 °C. The induction of 8-oxo-dG by 2-HE_2 was compared to that of the following controls: nonincubated 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 ± SD (n = 3) for all samples.

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

experiment	8-oxo-dG/10^5 dG
CT DNA only	7.3 ± 0.9
100 μM H_2O_2	17.0 ± 0.3
100 μM H_2O_2 and 100 μM 2-HE_2	50.0 ± 2.0
2 units of LP, 100 μM H_2O_2, and 100 μM 2-HE_2	6.5 ± 0.4
2 units of LP, 100 μM H_2O_2, and 100 μM 4-HE_2	6.2 ± 0.9

[a] Data points are means ± SD (n = 3) for all samples.

oxidized through a two-electron transfer mechanism with the formation of peroxide and water. Furthermore, Fenton catalysts such as copper appear to be necessary in forming DNA-damaging hydroxyl radicals at high CE concentrations.

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-HE_2 or 4-HE_2, 2 units of lactoperoxidase (LP), and 100 μM H_2O_2 for 3 h.

Controls were performed with the addition of either H_2O_2 alone or H_2O_2 with 2-HE_2. There was a significant increase in the level of 8-oxo-dG formation in the 2-HE_2/peroxide control (50 ± 2), with levels decreasing to that of background for both CE's (6.5 ± 0.4 with 2-HE_2 and 6.2 ± 0.9 with 4-HE_2) 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-HE_2 by LP (Figure 6). The

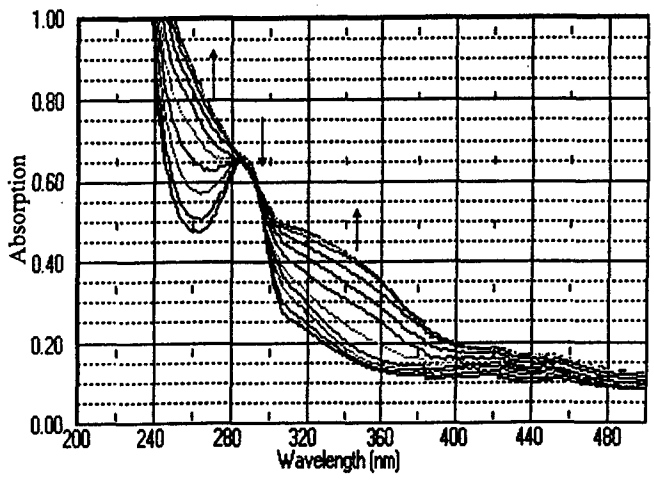


Figure 6. Lactoperoxidase oxidation of 2-hydroxy estradiol. Overlapping absorption spectra of 2-HE_2 (100 μM) as it became increasingly oxidized by LP (2 units) and H_2O_2. H_2O_2 (10 μM) was added every 2 min, while scans were taken every 4 min.

spectroscopic studies also indicate that 2-ME_2 and 2-HME_2 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.

Conclusion

Potentiometric studies were carried out with both 2-HE_2 and 4-HE_2, 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-HE_2 and 4-HE_2. For example, 2-HE_2 has been reported to form significant DNA strand breaks in vitro while 4-HE_2 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-HE_2 incubated with a microsomal extract from Syrian hamster liver but not with 2-HE_2 (*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-HE_2 was slightly more damaging than 4-HE_2 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 DNA-damaging potential of both 2-HE_2 and 4-HE_2 in vitro, which is possibly more significant than previously believed. However, a minimum of 10 μM 2-HE_2 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-HE_2 behaves as an antioxidant, reducing the level of 8-oxo-dG below control levels. The effect of LP on the HE_2'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-HE_2 or peroxide alone.

Potentiometric studies were also carried out with the HME_2's and ME_2'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_2's and HME_2's were inert toward the LP-catalyzed oxidation by peroxide.

Thus, both the 2- and 4-HE_2'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 GSH all suppressed 8-oxo-dG formation significantly in the presence of 100 μM 2-HE_2 and 100 μM Cu(II)SO_4. Taken as a whole, it is unlikely that free radical production via redox cycling of CE's is a causative factor in human tumorigenesis. The HME_2's were shown to be stable analogues of the HE_2's and should prove to be useful probes in the study of receptor-mediated carcinogenesis. Extension of this study to an ER positive breast cancer cell model is underway.

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TETRAHEDRON LETTERS

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.

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

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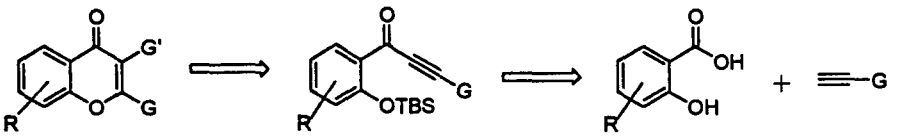
including flavonoids that interact with various enzymes and receptor systems of pharmacological significance.[2]

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



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 TBSCl and Et_3N in CH_2Cl_2 to generate the bisTBS protected salicylic acids ($\mathbf{A_1}$-$\mathbf{A_5}$) 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_3N was reacted with a variety of terminal alkynes ($\mathbf{B_1}$-$\mathbf{B_7}$) in the presence of catalytic amount of $Pd(PPh_3)_2Cl_2$ 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 ($\mathbf{A_2}$-$\mathbf{A_5}$) were coupled with phenyl acetylene ($\mathbf{B_1}$) 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 ($\mathbf{A_5}$) is successfully carried through the acid chlorination step, emphasizing the mild nature of reaction conditions. The one-pot acid chlorination-

[2] Molecules with benzopyrone ring system have shown to be active as tyrosine and protein kinase C inhibitors, antifungal and antiviral agents as well as antitubulin and antihypertensive agents. This nucleus can be exploited to develop novel antiinflammatory agents as well as selective modulators of estrogen receptors α/β, and adenosine receptor antagonists. Synthesis and screening of benzopyrone libraries would allow us to harvest the biological potential of these molecules.

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

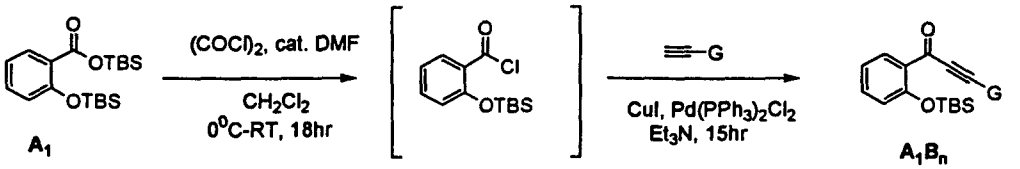


Table 1. Coupling of A_1 with terminal alkynes

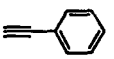
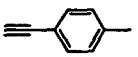
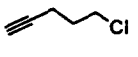
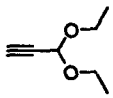
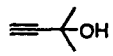
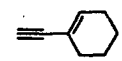
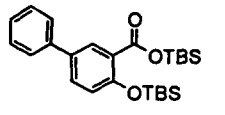
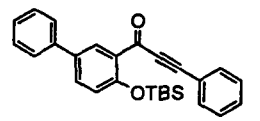
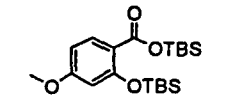
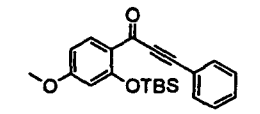
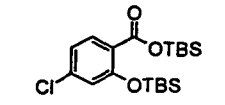
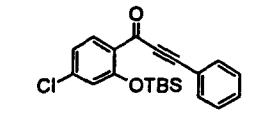
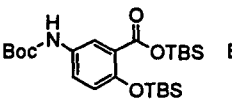
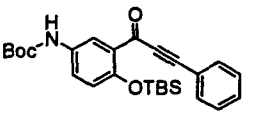
≡—G	Product A_1B_n (Yield)*
B_1 	A_1B_1 (92%)
B_2 	A_1B_2 (95%)
B_3 	A_1B_3 (85%)
B_4 	A_1B_4 (84%)
B_5 	A_1B_5 (78%)
B_6 	A_1B_6 (74%)
B_7 	A_1B_7 (96%)

Table 2. Coupling of B_1 with salicylic acids A_2-A_5

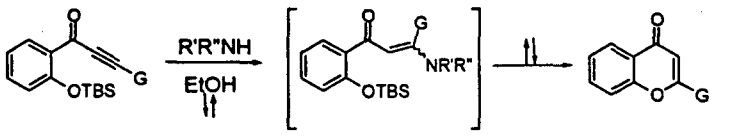
Salicylic acid	Product	A_nB_1 (Yield)*
A_2 		A_2B_1 (90%)
A_3 		A_3B_1 (92%)
A_4 		A_4B_1 (83%)
A_5 		A_5B_1 (78%)

* 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 alkynones 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



Alkynone
A_1B_n

Flavone

Alkynone	% Yield of Flavone*
A_1B_1	96%
A_1B_2	87%
A_1B_3	73%
A_1B_4	68%
A_1B_5	75%
A_1B_6	54%
A_1B_7	82%

*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 CH_2Cl_2 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, Et_3N(3mL) was added to the residue and argon was bubbled through the solution for five minutes. 5mmol of alkyne, 5mg $Pd(PPh_3)_2Cl_2$ and 5mg of CuI 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 diethylether, organics were washed with water, brine, dried (Na_2SO4) and concentrated, the residue was purified by flash chromatography (SiO_2; 18% EtOAc in Hexanes). All the coupling products were characterized by ^1H, ^{13}C NMR, IR and HRMS.

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COMPARISON OF ENDOCRINE AND ANTITUMOR ACTIVITY OF 2-METHOXY ESTROGENS

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Keywords: methoxyestrogens, antiproliferative effects, tubulin alterations

ABSTRACT

An estradiol metabolite, 2-methoxyestradiol (2-MeOE$_2$), 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-MeOE$_2$. 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-MeOMeE$_2$), 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-MeOMeE$_2$ inhibited tubulin polymerization *in vitro* at concentrations of 1 and 3 μM and was more effective than 2-MeOE$_2$. Both 2-MeOE$_2$ and 2-MeOMeE$_2$ were equally effective in suppressing growth and inducing cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cells. The cytotoxic effects of 2-MeOMeE$_2$ 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-MeOMeE$_2$ or vehicle control for 45 days. Treatment with 2-MeOMeE$_2$ 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-MeOMeE$_2$ 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-MeOE$_2$; Figure 1), one of the endogenous metabolites of estradiol (E$_2$), 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 IC$_{50}$'s ranging from 0.134 μM for 2-methoxy-estradiol to 15.7 μM for 2-hydroxyestradiol. Further investigations on possible mechanisms for this antiangiogenic effect have focused on the interactions of 2-MeOE$_2$ with tubulin (3,4), with 2-MeOE$_2$ inhibiting nucleation and propagation of tubulin assembly and being a competitive inhibitor of colchicine binding (K_i of 22 μM). Similar antitumor and antiangiogencic activities were observed in studies comparing 2-methoxyestradiol and taxol (5). Other proposed mechanisms for 2-MeOE$_2$ 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 ß-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-hydroxy-alkylestradiols 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-Hydroxymethylestradiol 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% CO_2 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 (2.7g, 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 CH_2Cl_2 (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 $MgSO_4$, and concentrated. The residue was purified by chromatography (SiO_2, 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; 13.75g, 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 (SiO_2, 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 ($CDCl_3$) 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 ($CDCl_3$) 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 $C_{20}H_{28}O_3$: 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 cm^2 wells on a six well plate at 1.5-2x10^5 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, [^{3}H]-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 [^{3}H]-estradiol was calculated by subtracting the nonspecific binding data from total binding data. The EC_{50} value for each synthetic estrogen analog represents the concentration of analog to produce a half-maximal displacement of specific [^{3}H]-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-MeOMeE_2 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-MeOMeE_2, 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 EC_{50} 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^{0}Cfor 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-$MeOE_2$ and 2-$MeOMeE_2$ 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 $MgCl_2$, 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 (0.5×10^4 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 3×10^6 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-MeOMeE$_2$) 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-MeOMeE$_2$) 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-MeOMeE$_2$, exhibiting an EC_{50} value of 42.4 nM, while 2-MeOE$_2$ exhibited an EC_{50} 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-MeOMeE$_2$ and 0.078 for 2-MeOE$_2$.

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-MeOMeE$_2$, and 2-MeOE$_2$ was determined by RNA dot blot analysis. The EC_{50} value for estradiol induction of pS2 mRNA was found to be 0.030 nM, while the EC_{50} values for 2-MeOMeE$_2$ and 2-MeOE$_2$ 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-MeOMeE$_2$ and 0.003 for 2-MeOE$_2$.

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-MeOE$_2$ with tubulin (3). Tubulin solutions were pre-incubated with the compound to be screened for 15 minutes at 37^{0}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 μM. 2-MeOMeE$_2$ produced a 30% reduction in MCF-7 cells and a 50% reduction in MDA-MB-231 cells at 1.0 μM.

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 μM 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 μM 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-MeOMeE$_2$-treated cells. Similar mitotic microtubule-staining patterns and chromosome misalignment were observed in 2-MeOE$_2$-treated cells, but only at much lower concentrations (0.87 μM) of 2-MeOE$_2$ (data not shown). The effects of 2-MeOE$_2$ on spindle morphology became progressively more pronounced as the concentration was increased from 0.87 to 8.7 μM. At concentrations of 2-MeOE$_2$ 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-MeOMeE$_2$ concentrations below 8.7 μM, and concentrations of 2-MeOMeE$_2$ 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-MeOE$_2$ (Figure 7E). Most microtubules were depolymerized after 24 hours of exposure to 15.8 μM 2-MeOE$_2$. 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-MeOMeE$_2$ (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-MeOMeE$_2$ 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 $2MeOE_2$ for 24 hours (Figure 7E'), suggesting that few cells escaped the mitotic arrest induced by this concentration of $2MeOE_2$.

To further examine the effects of $2\text{-}MeOE_2$ and $2\text{-}MeOMeE_2$, 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 $2\text{-}MeOE_2$ 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 $2\text{-}MeOMeE_2$ treatment spanning a similar concentration range of drug (Figure 8B). Since cells exposed to $2\text{-}MeOMeE_2$ retained mitotic spindles (see above), it appears that there was a relatively rapid transition from M to G_1. $2\text{-}MeOE_2$ treatment restricted M-to-G_1 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 $2\text{-}MeOMeE_2$ used in this study appeared to induce a delay in mitotic progression, but $2\text{-}MeOE_2$ induced a more stringent blockage of cells in mitosis.

Exposure of cells to either drug also stimulated an apoptotic response. $2\text{-}MeOE_2$ generated a greater amount of apoptosis than $2\text{-}MeOMeE_2$ as measured by TUNEL assay (Figure 8). The proportion of $2\text{-}MeOE_2$-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-MeOE$_2$, 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-MeOMeE$_2$. 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-MeOMeE$_2$ 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-MeOMeE$_2$ showed an approximate 50% reduction in mean tumor volume at treatment day 45 when compared to control animals (Figure 10). The 2-MeOMeE$_2$ treatment had no effect on final animal weight at the end of the study.

DISCUSSION

2-Methoxymethyl estradiol (2-MeOMeE$_2$) 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-methoxy-estrogens 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-MeOMeE$_2$, exhibiting an EC_{50} value of 42.4 nM, while 2-MeOE$_2$ exhibited an EC_{50} 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-MeOMeE$_2$ and 0.078 for 2-MeOE$_2$. 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 EC_{50} values for 2-MeOMeE$_2$ and 2-MeOE$_2$ 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-MeOMeE$_2$ and 0.003 for 2-MeOE$_2$.

The *in vitro* tubulin polymerization assay identified 2-MeOMeE$_2$ as being more effective than 2-MeOE$_2$ 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-MeOMeE$_2$-treated cells. The *in vivo* effects of 2-MeOMeE$_2$ in comparison to 2-MeOE$_2$ on microtubule morphology are in contrast to the *in vitro* results showing a greater effect of 2-MeOMeE$_2$ on microtubule polymerization. These results suggest that the greater effect of 2-MeOE$_2$ on microtubule morphology may be due to differential permeability of the two compounds. This would allow for a higher intracellular concentration of 2-MeOE$_2$ being achieved when both compounds are applied to the cultures at the same concentration.

The *in vivo* antitumor activity of 2-MeOMeE$_2$ 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-MeOMeE$_2$ 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-MeOMeE$_2$ and 2-MeOE$_2$ are equally effective in suppressing tumor growth in human breast tumor-bearing athymic mice.

In summary, 2-MeOMeE$_2$ is an estrogen analog with minimal estrogenic properties, inhibits tubulin polymerization at micromolar concentrations, and demonstrates antiproliferative effects *in vitro*. Micromolar concentrations of 2-MeOMeE$_2$ induce a delay in mitotic progression of treated cells, with 2-MeOE$_2$ inducing a more stringent blockage of cells in mitosis. The *in vivo* antitumor activity of 2-MeOMeE$_2$ 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 *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.

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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

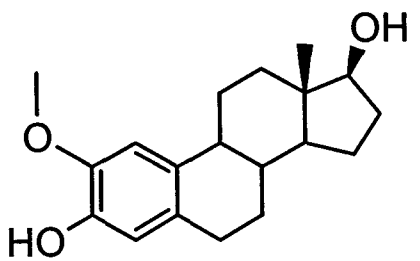
microtubules (B), resulting in a loss of chromosomal alignment (B'). 2-MeOMeE2 treatment had only a slight effect on the morphology of the spindle (C), however, chromosome misalignment was present (C'). A characteristic microtubule array (D) and single nucleus (D') are present in untreated interphase 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.

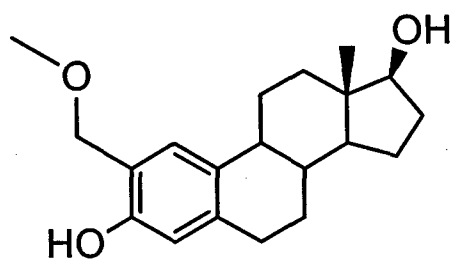
Figure 10. **Effect of 2-MeOMeE2 on Tumor Progression in Athymic Mice MDA-MB-231 Xenografts.** Athymic nude mice containing MDA-MB-435 (ER-) human breast cancer cell xenografts were treated i.p. with 2-MeOMeE2 at 50 mg/kg/day (○) or vehicle only (●). Data was analyzed using ANOVA (one way for repeated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

Figure 1.



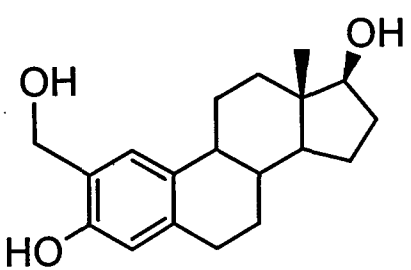
2-Methoxyestradiol

2-MeOE_2



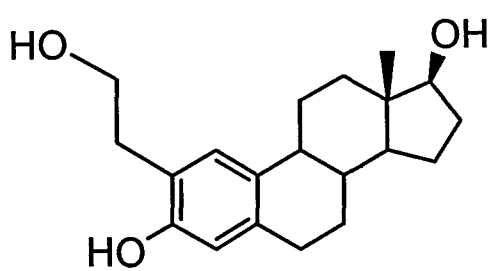
2-Methoxymethyl Estradiol

2-MeOMeE_2



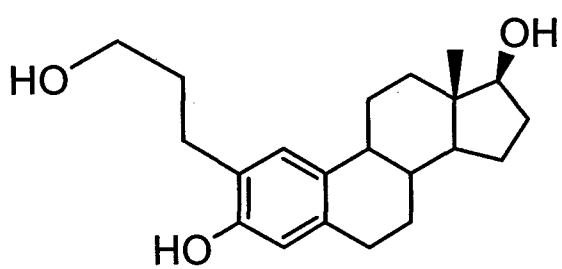
2-Hydroxymethyl Estradiol

2-HOMeE_2



2-Hydroxyethyl Estradiol

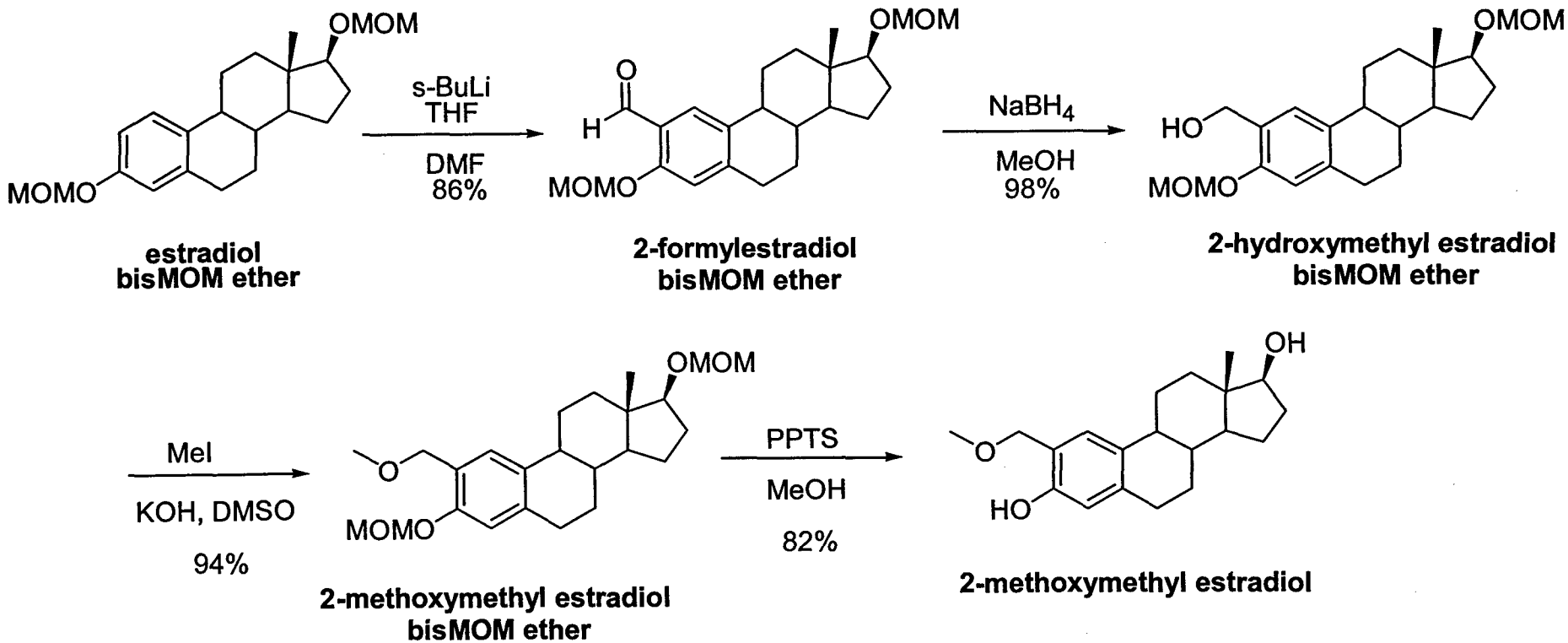
2-HOEtE_2



2-Hydroxypropyl Estradiol

2-HOPrE_2

Figure 2.



estradiol bisMOM ether

2-formylestradiol bisMOM ether

2-hydroxymethyl estradiol bisMOM ether

2-methoxymethyl estradiol bisMOM ether

2-methoxymethyl estradiol

Figure 3.

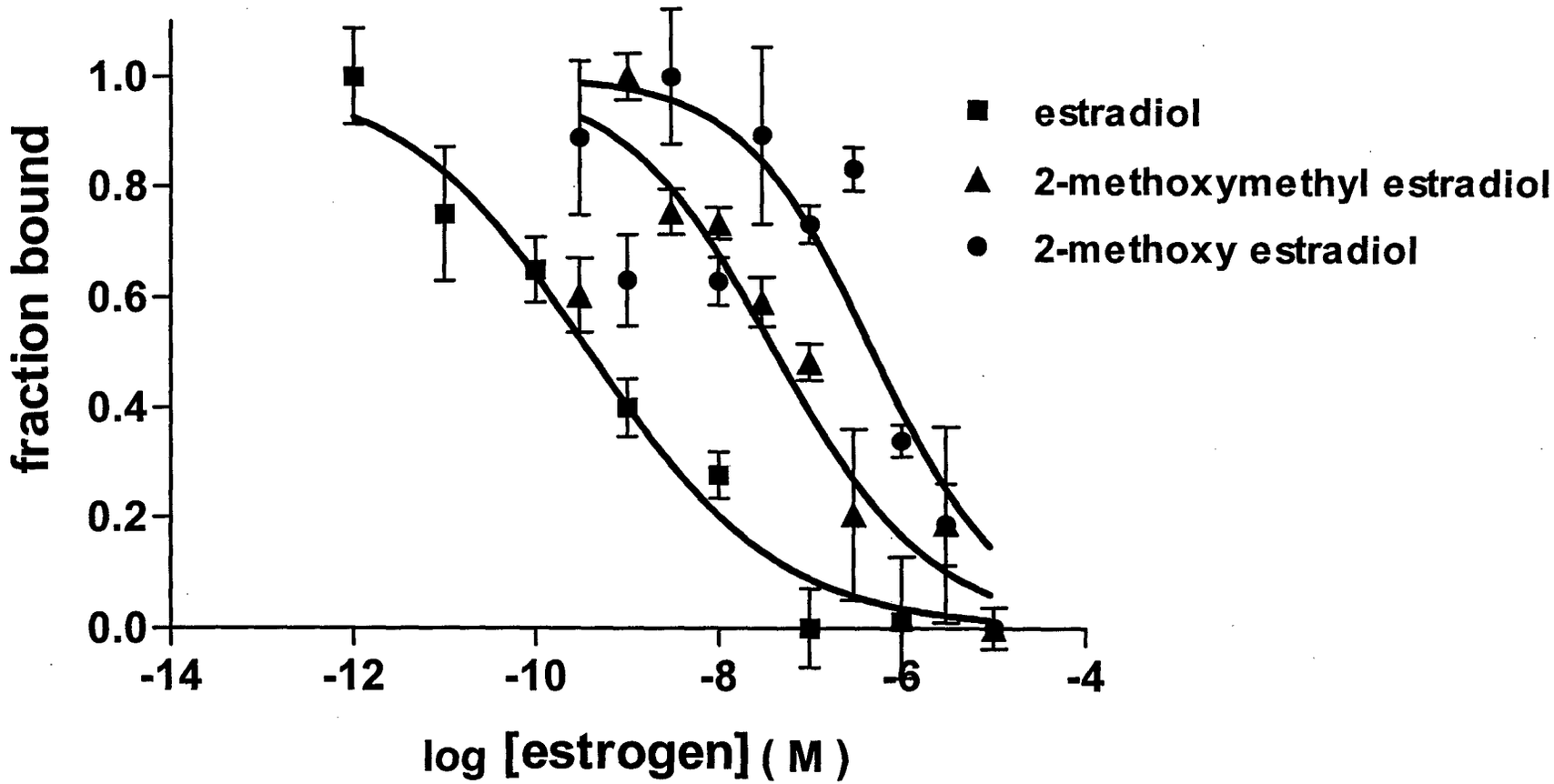

fraction bound
1.0
0.8
0.6
0.4
0.2
0.0
-14
-12
-10
-8
-6
-4
log [estrogen] (M)
estradiol
2-methoxymethyl estradiol
2-methoxy estradiol

Figure 4.

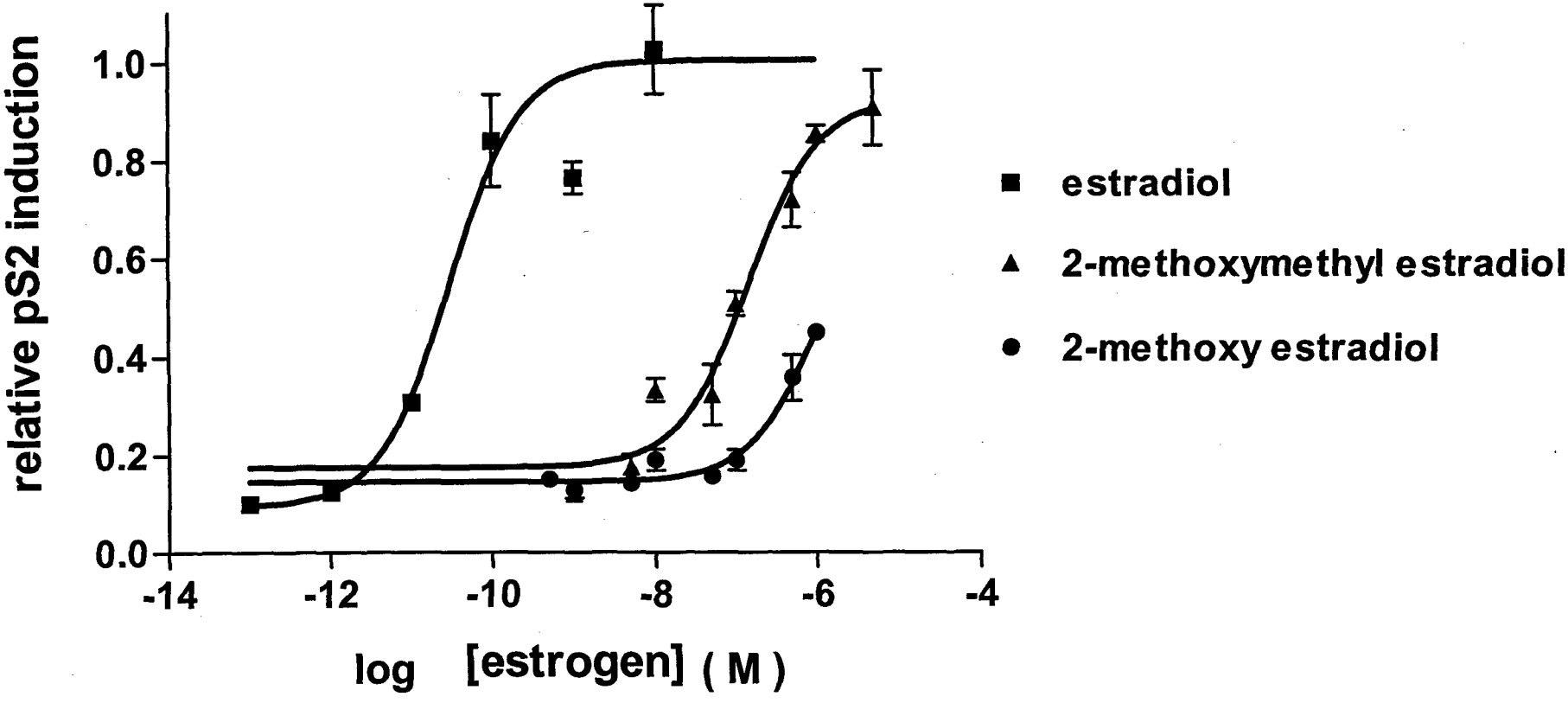
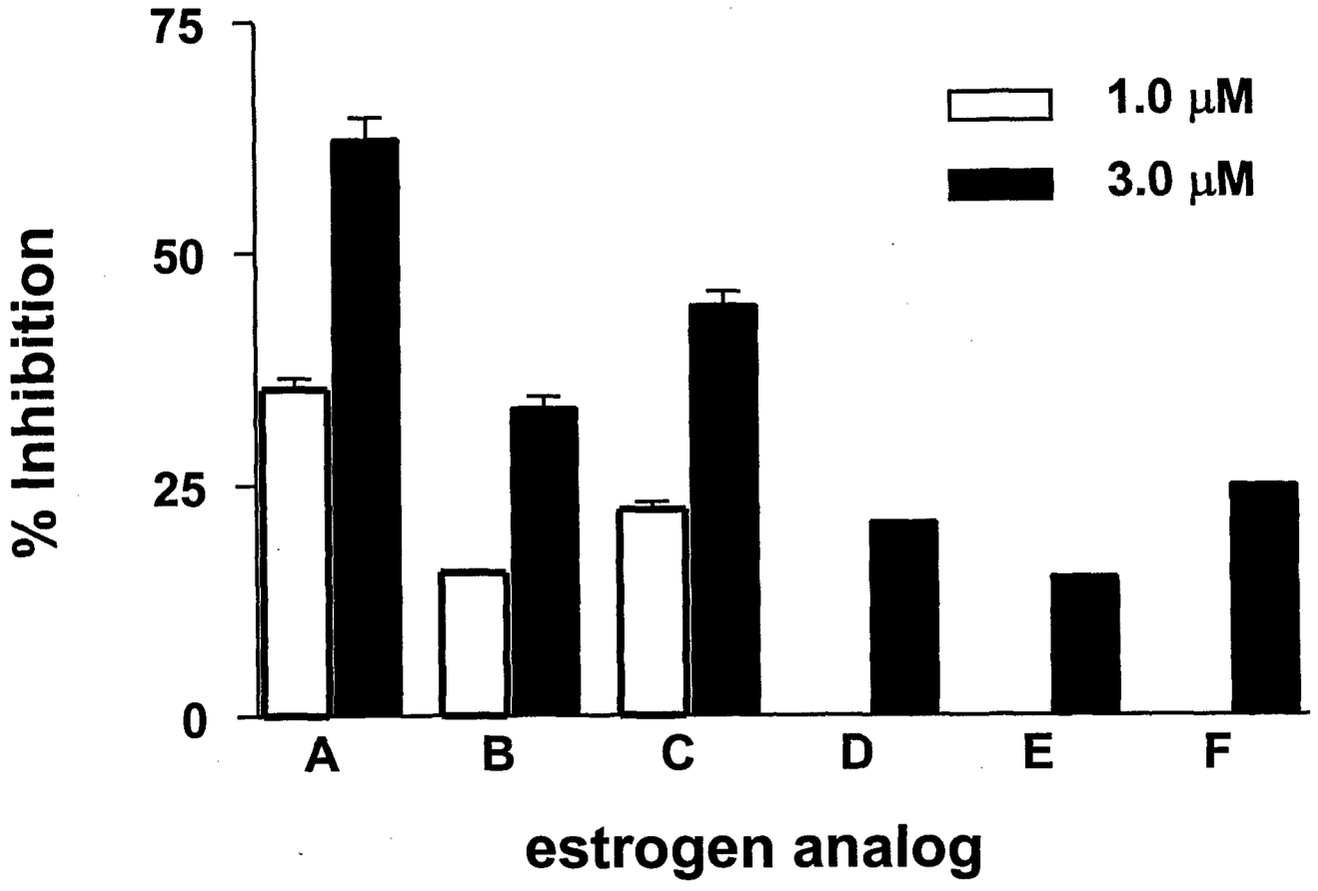

relative pS2 induction
1.0
0.8
0.6
0.4
0.2
0.0
-14
-12
-10
-8
-6
-4
log [estrogen] (M)
estradiol
2-methoxymethyl estradiol
2-methoxy estradiol

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.

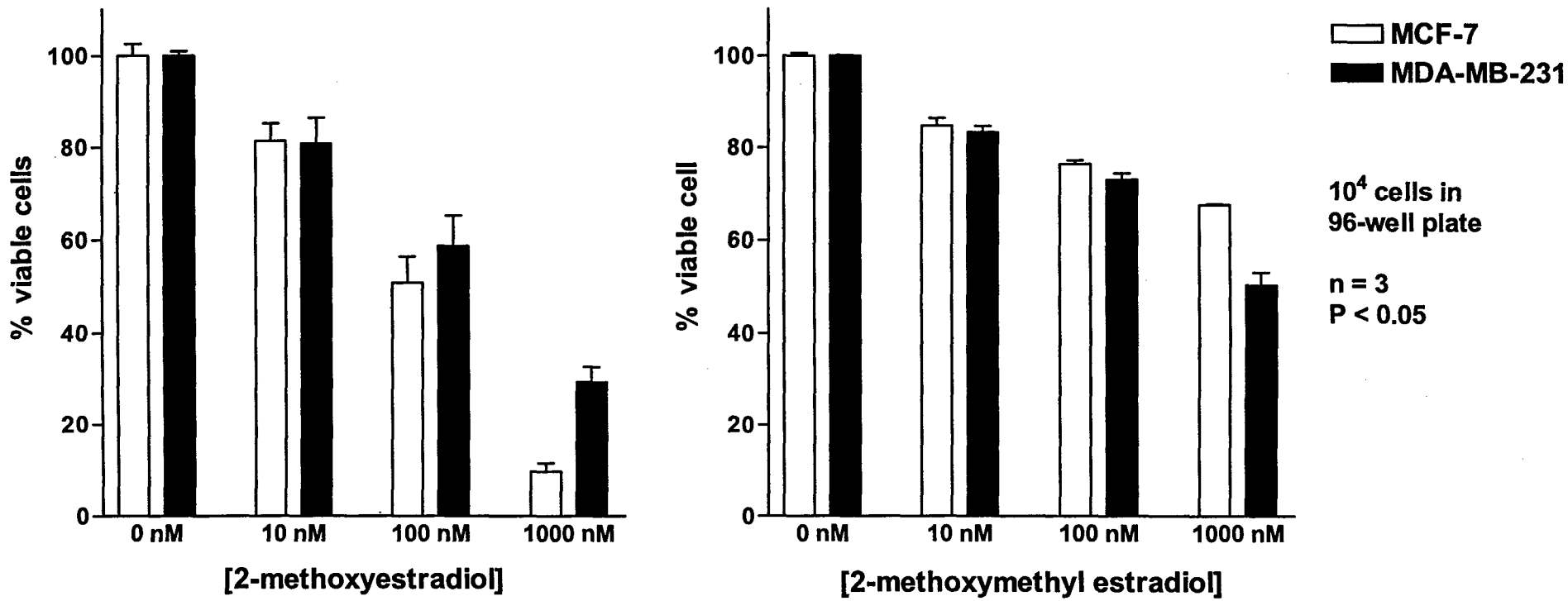

% viable cells
100
80
60
40
20
0
0 nM
10 nM
100 nM
1000 nM
[2-methoxyestradiol]
% viable cell
100
80
60
40
20
0
0 nM
10 nM
100 nM
1000 nM
[2-methoxymethyl estradiol]
MCF-7
MDA-MB-231
10^4 cells in
96-well plate
n = 3
P < 0.05

Figure 7.

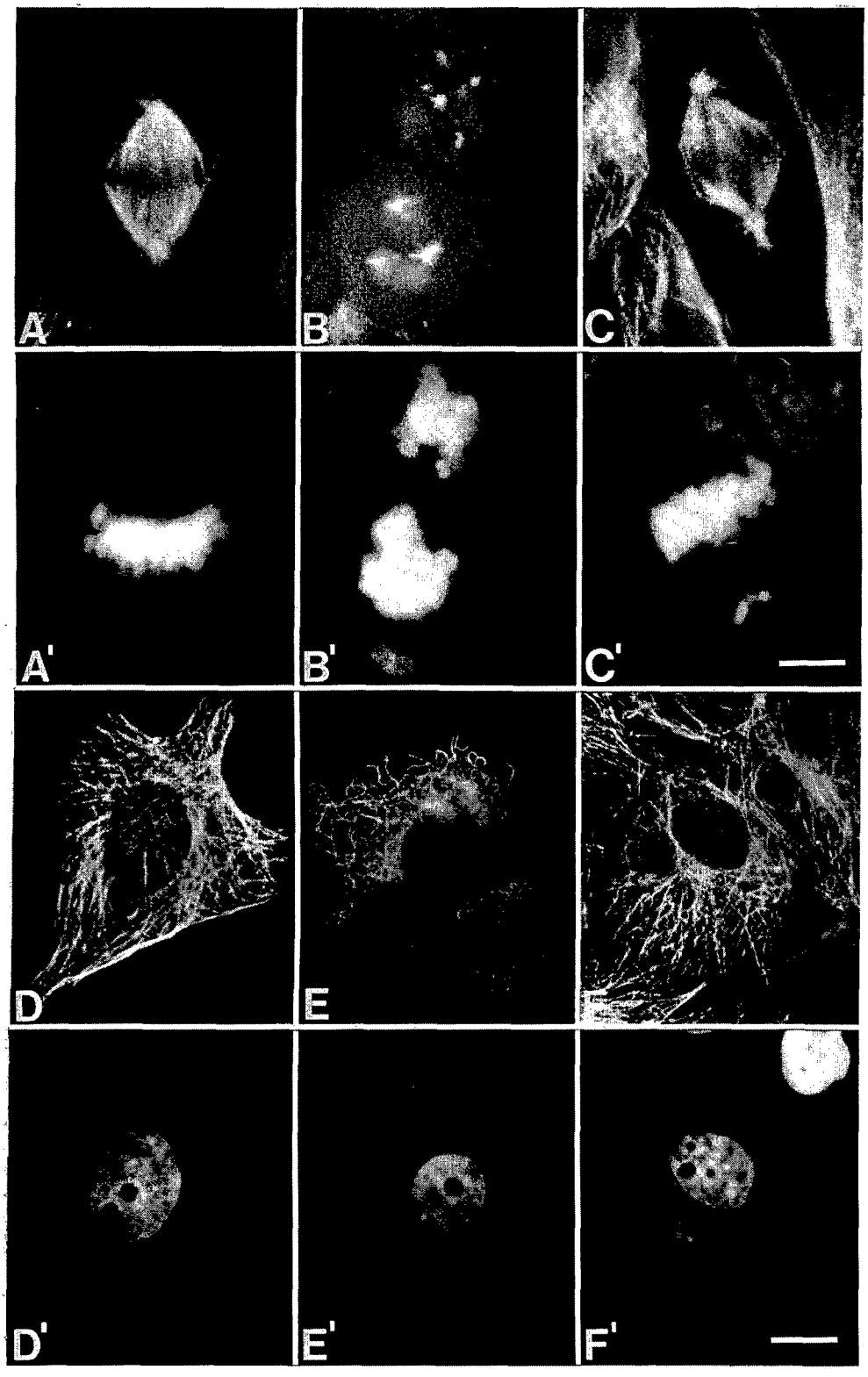


Figure 8.

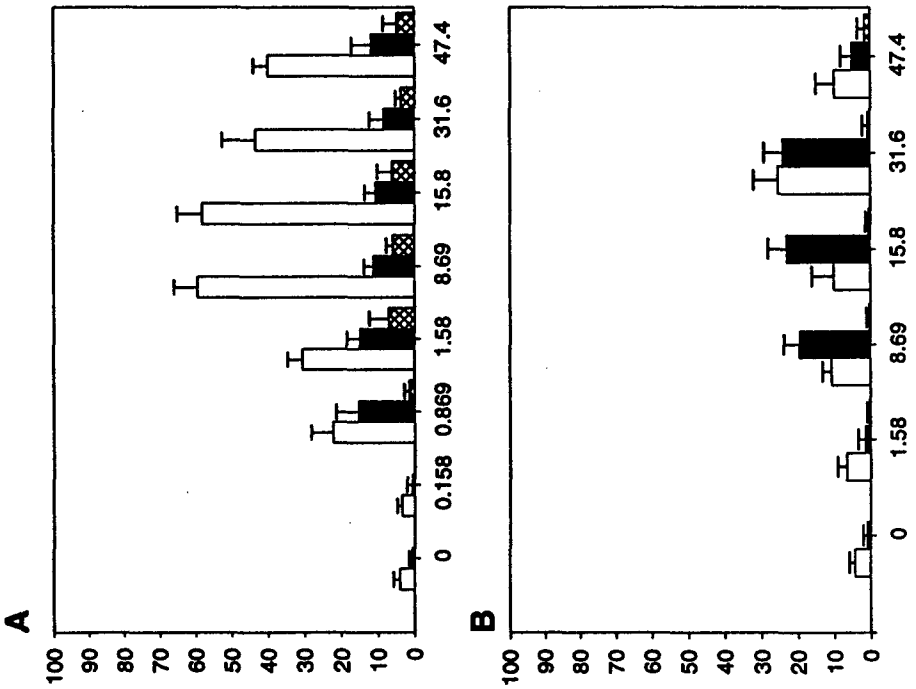


Figure 9.

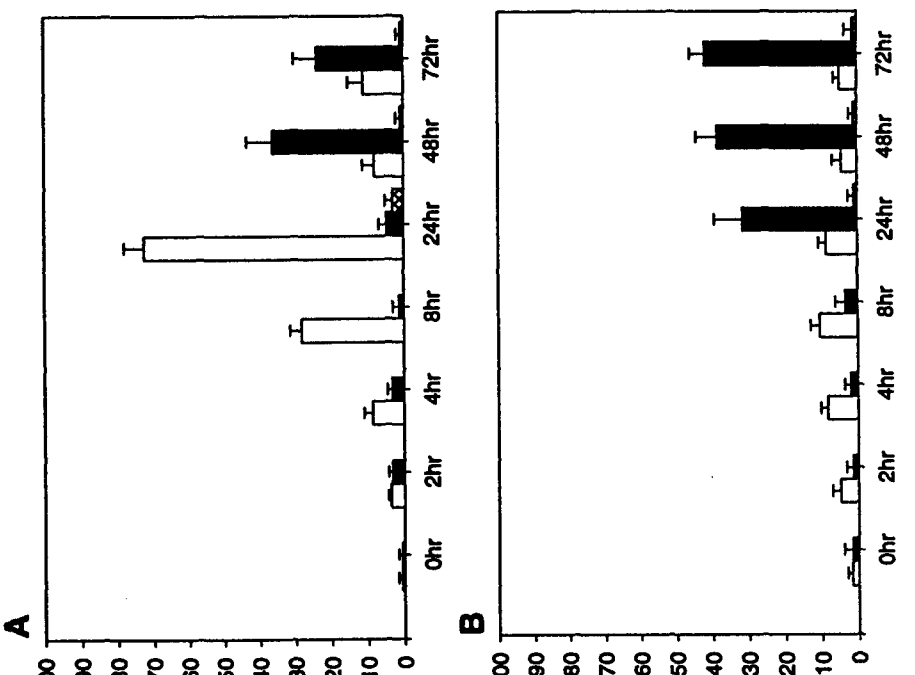
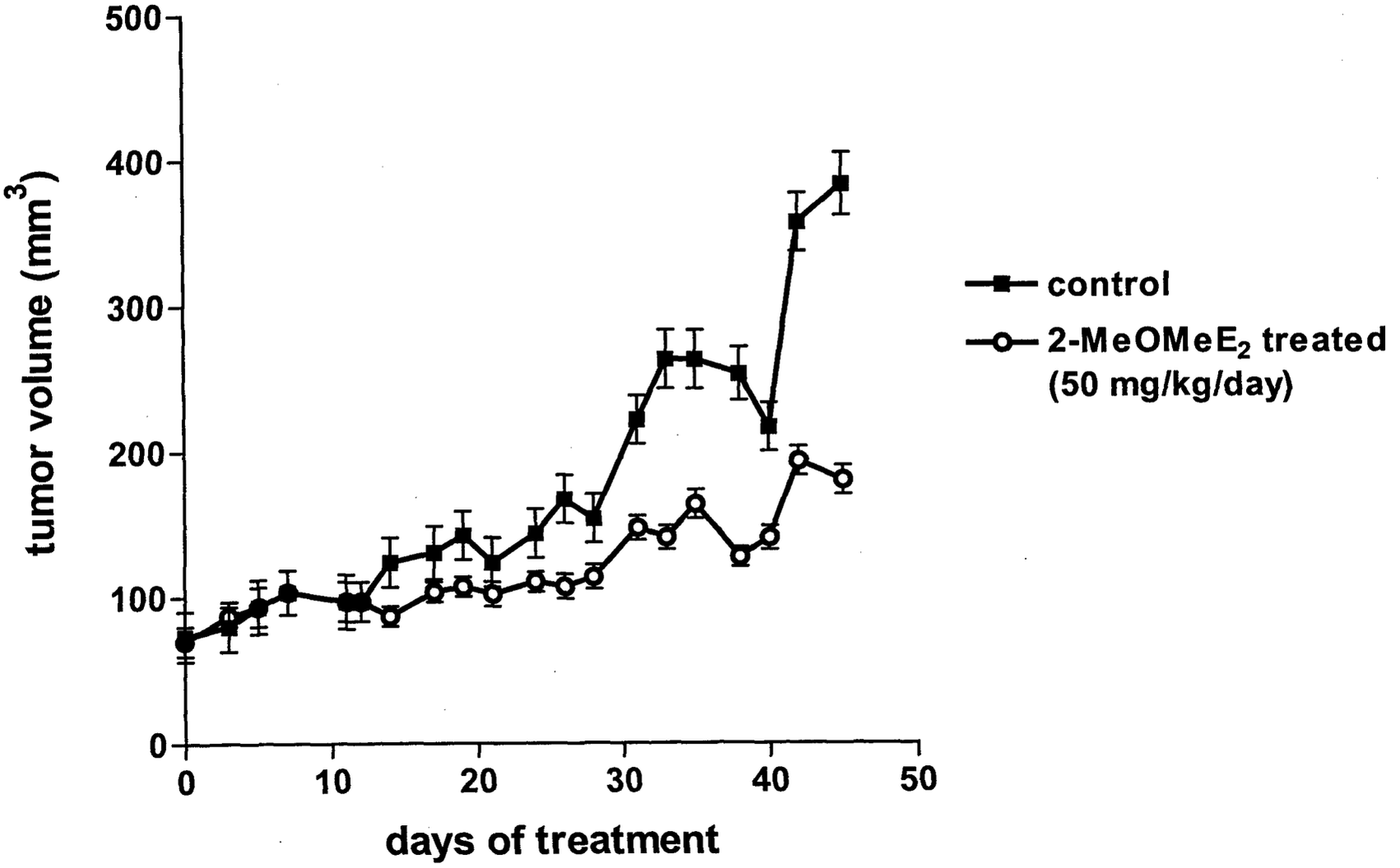


Figure 10.


500
400
300
200
100
0
tumor volume (mm3)
0
10
20
30
40
50
days of treatment
control
2-MeOMeE2 treated
(50 mg/kg/day)

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

Abhijit S. Bhat, B.S.(Pharm.Sci.)

* * * * *

The Ohio State University

1999

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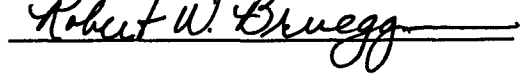

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ABSTRACT

4-Hydroxy estradiol (4-OHE_2) is an oxidative metabolite of estrogens that is implicated as a possible causative agent in estrogen-induced tumorigenesis. The tumorigenic potential of 4-OHE_2 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-OHE_2. 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-OHE_2 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, where as 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 ($2MME_2$) 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 an 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*-butylsilyloxy)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 alkynones 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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2. Novel Synthetic Approaches for Construction of Benzopyrone Combinatorial Libraries. Bhat, A. S.; Whetstone, J.L.; Brueggemeier, R. W. *Tet. Lett.* **1999**, *40*, 2469-72.
3. Measurement of Oxidative DNA Damage by Catechol Estrogens and Analogs, *in vitro* Mobley, J. A.; Bhat, A. S.; Brueggemeier, R. W. *Chem. Res. Toxicol.* **1999**, *12*, 270-77.

FIELD OF STUDY

Major Field: Pharmacy, Medicinal Chemistry

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