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NEW STEROIDAL DERIVATES

WITH POTENTIAL ANTIOXIDANT AND/OR ANTITUMOURAL

ACTIVITIES

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INTRODUCTION

During my graduation thesis, focused on the oxidative chemistry of 17 β -estradiol (**17 β E**) in biomimetic conditions, I had the opportunity to understand what “to engage a topic of research” means; mine was addressed to define, on chemical bases, structural modifications of estrogens and other steroids derivatives in pathologic conditions associated to oxidative stress.

17 β E represents with estrone and estriol a particular group of steroid hormones (estrogen 18C, i.e. eighteen carbon atoms) with estrogenic properties in mammals and men.¹ In particular, **17 β E** is the major specific activity hormone and it plays a key role in the control of sexual behaviour and development as well as in a variety of processes related to reproduction.

These functions were discovered in 1922 owing to the studies of American biochemists Edward Doisy and Edgar Allen on mouse ovaric extracts. The same Doisy isolated 10 mg of **17 β E** from 4 ton of sow ovaric tissue in 1935. After the structural characterization of the estrogens, two Swiss scientists Karl Miescher e Georg Anner prepared estrone and **17 β E** *via* a chemical synthesis. This step had a great practical and social outcome because the synthesis of these hormones allowed the estrogen pharmaceutical use. The isolation from natural sources such as animal tissues would have required ovaries of two million sows to obtain 10 g of **17 β E**.

In 1941 Hans Selye demonstrated the anaesthetic and sedative function of progesterone and its modulation of intracellular mediators.² The presence of steroidal receptors on the cell membranes prompted a French researcher, Emile Baulieu, to look for steroids in nervous system. After some efforts steroidal hormones were found in animal brain.³ Baulieu introduced the term “neurosteroid” to designate steroids that are synthesized in the nervous system either *de novo* from cholesterol or by in situ metabolism of blood-borne precursors, and that accumulate in the nervous system to levels that are at least in part independent of steroidogenic gland secretion rates.⁴ In contrast to the circulating steroid hormones, which act at a distance from their gland of origin, on brain and neurons at relatively low concentrations (endocrine effect), neurosteroid hormones act in the nervous system in an auto/paracrine configuration.⁴

The main activity of these neurosteroids in the central and peripheral nervous system appear to be a neuroprotective and neurotrophic action.⁵ Indeed they also may induce some kind of cancer and this have been attributed, at least in part, to the inherent susceptibility of the phenolic A-ring to enzymatic or chemical oxidation.⁶ So the investigation of structural modifications suffered by **17 β E** in oxidative settings is central for the understanding of the molecular mechanism underlying these properties. In addition, beyond the specific relevance to the steroid sector, the oxidation of estrogen

compounds represents an attractive research issue because of its potential as convenient entry to complex functionalized scaffolds of academic and industrial interest.

In view of that, this work comes as an in-depth study on estrogen oxidative chemistry with a double target. The understanding of the molecular mechanisms of neuro- and cytoprotective activities as well as carcinogenic activities of **17 β E** on one side; the exploitation of oxidative transformations as access routes to new steroidal scaffolds of potential relevance to biomedical and pharmaceutical matters, on the other.

The results are collected in two chapters: the first contains studies on oxidative chemistry of **17 β E** in biomimetic conditions; the second describes procedures for the preparation of estrogenic derivatives that are halogenated, oxy-functionalized and/or desaturated at the steroidal backbone.

CHAPTER I

Oxidative chemistry of 17 β E related to biological activity

17 β E and estrogenic hormones originate from cholesterol by a series of enzyme catalyzed processes mainly located at the ovary, the kidney cortex, the placenta and for a few species, at the testicle.⁷ The biosynthesis of 17 β E, including enzymes involved in each step, is summarized schematically in figure 1.

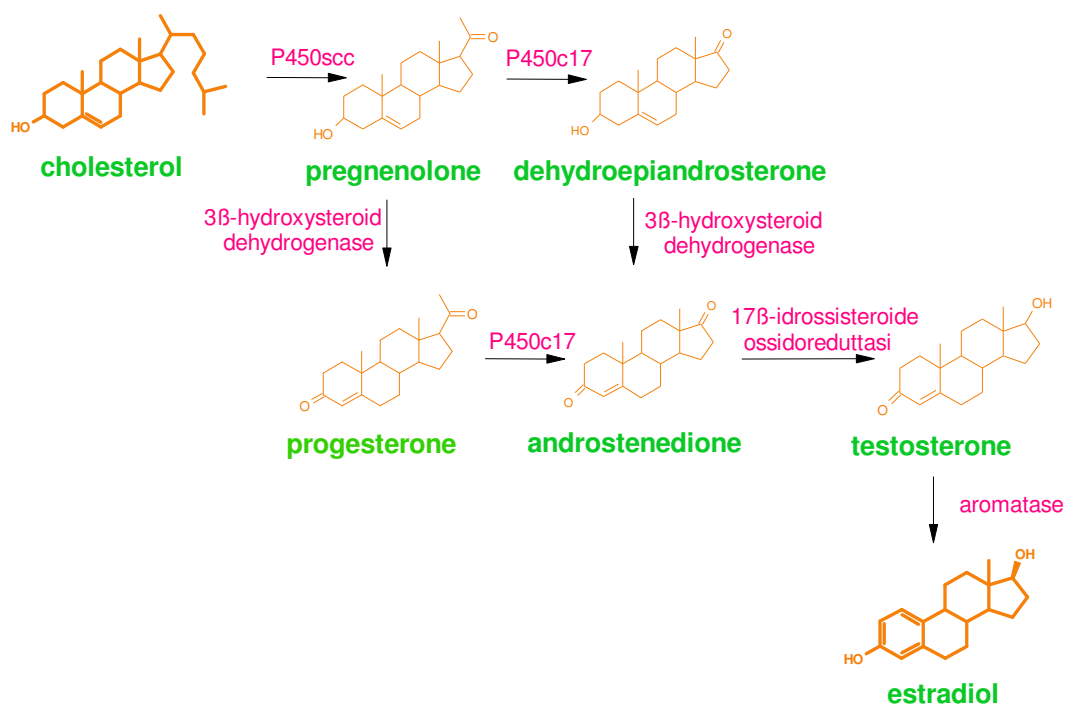


Fig. 1: biosynthetic pathways of 17 β -estradiol

The first step in this scheme of steroid biosynthesis corresponds to the formation of pregnenolone (PREG) from cholesterol. This reaction is catalyzed by an enzyme located at the inner mitochondrial membrane, the cytochrome P450_{scc}, which removes 6 carbons from the side-chain of the cholesterol molecule (scc for side-chain cleavage). The newly formed PREG then leaves the mitochondria for the endoplasmic reticulum, where it is converted to testosterone, the androgenic hormone direct precursor of 17 β E, by two alternative metabolic pathways which differ in the sequence of these two steps: a) oxidation of the alcoholic function at the C3 position of PREG and isomerization of the double bond in C5-C6 by the enzyme 3 β -hydroxysteroid dehydrogenase, b) oxidation at the C17 with removal of the two-carbon residue by the cytochrome P450_{c17} enzyme.⁵

Finally an aromatase does oxidize testosterone to the phenolic steroid E, with a simultaneous demethylation at carbon 10.

The action of estrogens on target cells (cells that synthesize nuclear proteins known as estrogen receptors) is a genomic one and carries on by regulating the transcription of hormone-sensitive genes. Once the steroids (i.e. estrogens) are formed, they easily diffuse, thanks to their lipophilicity, out of the cell membrane and into the blood where a plasma protein may transport them and release at the lipidic membrane of the target cells. Upon binding of estrogen to its protein receptor on the

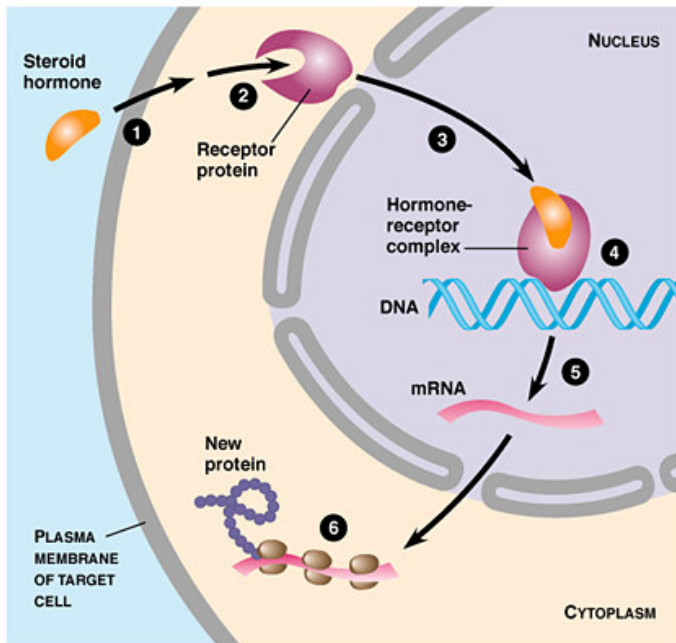


Fig. 2: the genomic mechanism of action estrogens

nuclear membrane, the receptor undergoes a conformational change leading to transformation into its active form. The receptors then dimerize and bind to the estrogen response element (ERE) of the targeted gene. The binding, therefore, induces a complex with RNA polymerase which activates the transcription of specific genes in mRNA molecules leading to the production of proteins by that cell to cause the physiological response.⁸ Through a genomic mechanism, estrogen hormones regulate menstrual cycle,

induction of luteolysis and other processes associated to reproduction such as embryo's nest in the uterus and prolactin biosynthesis.

Other functions, that are typical of steroidal hormones, are the control of muscle tissue growth, the morphological determination of bones and modulation of various inflammatory responses.¹

Recent studies have documented steroid hormones (mainly estrogenic hormones) influence on processes in central nervous system activity. Although these functions still await elucidation, current views attribute neuroprotective and neurotrophic properties of E and correlated metabolites.⁹⁻¹¹ Clinical studies demonstrate that estrogen replacement therapy in postmenopausal women may enhance cognitive function, decrease the risk of brain injury associated with ischemia and neurotrauma.¹²⁻¹⁵ Moreover E reduces neurodegeneration associated with Alzheimer's and Parkinson's diseases¹⁶⁻¹⁹ and influences memory and cognitive processes.²⁰ Similar results have been obtained from studies on animal models in which estrogen administration significantly attenuates the degree of striatal dopamine depletion by neurotoxins (MPTP, 6-hydroxydopamine

and methamphetamine).²¹⁻²⁵ A number of studies establish that estrogen replacement therapy decreases cardiopathic risk and thrombosis, showing a protective effect of estrogens on the cardiovascular system, and that **E** decreases low density cholesterol in blood.²⁶⁻²⁸

Following the observation of the various functions of **17βE** and other steroid hormones in central nervous system the search for steroidogenic pathways in the brain was undertaken.^{4, 5, 29} In fact the synthesis of **17βE** from pregnenolone has been evidenced in the astrocytes, star shaped glial cells that form cerebral connective tissue and that are present in gray and white matter of the brain.^{4, 30} In these tissues are also active metabolic transformations of **17βE** in hydroxylated derivatives known as catechol estrogens (CEs) catalized by enzymes (i.e. cytochrome P450 and estradiol 2-hydroxylase).³¹⁻³³

Currently, the term neurosteroid or neuroactive steroid is applied to those steroids that are synthesized in the nervous system or *de novo* from cholesterol or by in situ metabolism of blood-borne precursors.⁴ These steroids act, such as other psychoactive substances (benzodiazepine, barbiturates, convulsivants) as if they were allosteric modulators of the γ -aminobutyric acid receptor type A (GABA_A-R), NMDA, and sigma-1 receptor activities.^{34, 35} There are steroids which either positively or negatively modulate the GABA_A-R, for instance some are GABA_A agonists (3 α -hydroxy-5 α -pregan-20-one and androsterone), some are GABA_A antagonists (pregnenolone e DHEAS).⁴

Several studies have shown that estrogen receptors (ER) play a pivotal role in mediating neuroprotective actions of **17βE**. They are distributed widely throughout the central nervous system, act as intracellular transcription factors and are part of the nuclear receptor super-family.³⁶ There are two ER subtypes, ER α and ER β , and the deletion of ER α completely abolishes the protective actions of **17βE** in all central nervous system; whereas the ability of **17βE** to protect against brain injury is totally preserved in absence of ER β .^{37, 38} These results clearly establish that the ER α subtype is a critical mechanistic link in mediating the neuroprotective effects of **17βE**. Moreover, other potential means by which estrogen can function as a neuroprotectant are: its capacity to affect monoamine oxidase³⁹ (enzyme that rules the turn-over of catecholamine transmitters) and its influence on membrane morphology and fluidity.⁴⁰

In vitro experiments show inhibition of lipidic peroxidation,⁴¹ production of prostaglandine and trombossani⁴² and of lipopolysaccharidic inflammatory mediators.⁴³

Despite lots of in vitro and in vivo experiments, very little is known about the neuroprotection of **E** in oxidative stress conditions at molecular level.^{44, 45}

This property is independent of estrogen stereochemistry and a mechanistic hypothesis proposes **E** as lipophilic scavenger of oxygenated species, generated in oxidative stress conditions and related

to neurodegenerative processes.^{46, 47} This interpretation is based on evidence that the isomers 17 α and 17 β exert comparable protective effects,³⁹ so it appears reasonable to rule out receptorial effects, and on biochemical data that show the sensitivity of **E** to oxidation, generating hydroxylated products (catechol estrogens). Therefore **E** performs the neuroprotection by inactivating ROS (H₂O₂, \cdot OH, O₂⁻) and generating catechol estrogens. The ROS are produced in biological systems in physiological conditions, but in particular situations (i.e. presence of xenobiotics, cytotoxic drugs, ionizing ray) they are over-produced, as in oxidative stress conditions.

In view of that, a detailed elucidation of the structural modifications suffered by **E** in oxidative settings is central for the understanding of the nongenomic effects of estrogens. Despite the many prospects offered by oxidative manipulation of estrogens, current knowledge in the field is surprisingly limited. Indeed when I began the experimental activity here reported the only known oxidation products include, besides the catechol estrogens, a 10 β -hydroxyestra-1,4-dien-3-one derivative arising by peracid-induced photooxygenation or oxidation by Fenton reagent,⁴⁸ a series of benzylic oxidation species of estrone methyl ether,⁴⁹ and two dimers obtained by chemical and enzymatic oxidation of **E**, namely, the symmetric 2,2' and 4,4' dimers.⁵⁰ Other studies have appeared reporting formation of oligomer species by oxidation of **E**, but their characterization relied only on evaluation of chemical physical properties.⁵¹ More recently, a convenient synthetic access to O-linked dimers of **E** was reported⁵² in the frame of a study of the NADPH-dependent metabolism of **E** by human liver microsomes and cytochrome P450 enzymes. These latter studies and the vast body of literature on the oxidative coupling of phenols⁵³ suggest that oxidative conversion of **E** and related estrogens in vivo can lead to an array of oligomeric products, yet their nature and biological properties have remained so far poorly elucidated.

The study of oxidative chemistry of **E** also plays a pivotal role in the individuation of the molecular mechanism for carcinogenic proprieties associated to estrogen. In fact if estrogen replacement therapy has a variety of beneficial effects in vivo including protection against osteoporosis, coronary heart disease and stroke, Alzheimer's disease,⁵⁴ it is also true that disputed observations exist showing that, exposure to long-term high-dose estrogen replacement therapy increases the risk of developing breast or endometrial cancer in women.⁵⁵

Also for these activities the molecular mechanisms involved in the estrogens still remains both controversial and elusive. The role of estrogens in the induction of cancer has generally been related to stimulation of proliferation by receptor-mediated processes, that can lead an increased cell production. Although hormonal effects can mediate cell proliferation by receptor-mediated

processes, a genotoxic event seems to be needed to produce mutations, the permanent genetic changes at the origin of cancer.⁵⁶

A proposed mechanism might involve metabolism of estrogens to catechols, which are then oxidized to redox active/electrophilic *o*-quinones that could initiate the carcinogenic process by binding to cellular macromolecules.⁵⁵ For example, it is well established that the endogenous estrogens, estrone and **17βE**, are metabolized via two major pathways: 16α-hydroxylation (not shown) and formation of CE, the 2-hydroxy and 4-hydroxy derivatives (2OHE and 4OHE respectively) by P450 enzymes. Usually, these two CE are mainly inactivated by *O*-methylation catalyzed by catechol-*O*-methyltransferases. This conjugating pathway is protective because only the methylated and thus inactivated CE may be oxidized to semiquinones and quinones by peroxidases or P450 cytochromes.⁵⁷

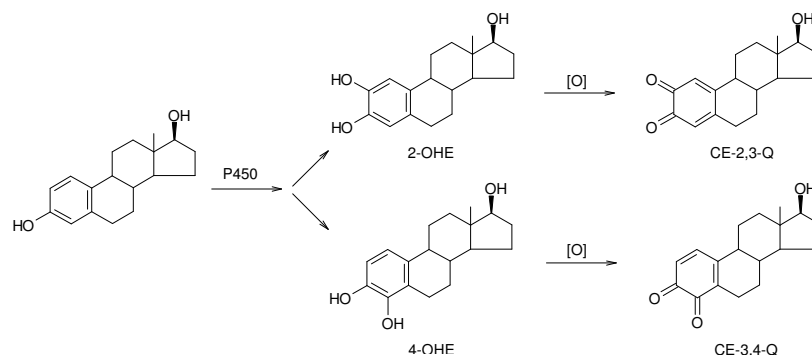


Fig. 3: metabolic oxidative pathways of 17β-estradiol

Catechol estrogen *o*-quinones (CEQs) are the reactive metabolites of catechol estrogens that are likely candidates for the ultimate estrogen carcinogenesis because of two main mechanisms. They can undergo redox cycling with the semiquinone radical, generating superoxide radicals ($O_2^{\cdot-}$) mediated through cytochrome P450/P450 reductase and/or other enzymatic/chemical redox couples.⁵⁸

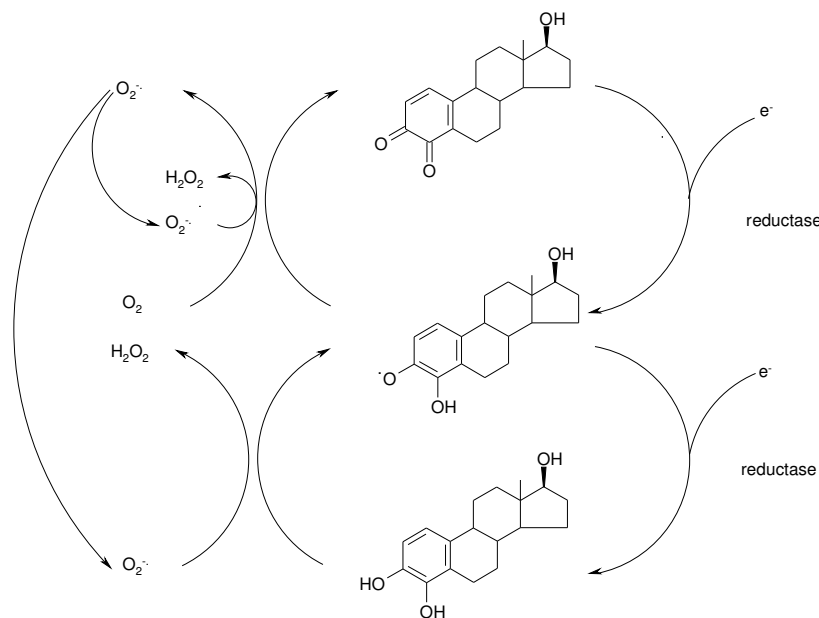


Fig. 4: catechol estrogens redox cycling

The reaction of $O_2^{\cdot-}$ with hydrogen peroxide, formed by enzymatic or spontaneous dismutation of $O_2^{\cdot-}$, in the presence of trace amounts of iron or other transition metals gives hydroxyl radicals. The hydroxyl radicals are powerful oxidizing agents that may be responsible for damage to essential functional groups of macromolecules. For example oxidation of cysteine residues in proteins leads to disulfide bond formation which can dramatically alter structure and function. Hydroxyl radicals can also catalyze oxidation of lipids generating lipid hydroperoxides, and elevated levels in lipid hydroperoxides may damage structural and functional integrity of membranes because of the increase of permeability and the membrane enzymes inactivity. Furthermore the degradation of lipid hydroperoxides generates aldehydes and hydrocarbons that are very cytotoxic products.

Finally, these radicals may cause oxidation of the phosphate-sugar backbone and/or the purine/pyrimidine residues of DNA. The resulting mutated bases could cause mispairing and mistake during the replication.

The second potential mechanism of estrogen carcinogenesis is based on the electrophilicity of *o*-quinones which can react with nucleophilic sites of DNA (nitrogenous bases) by Michael addition to form stable adducts that remain in DNA. Unless these alkylated bases are promptly repaired, miscoding may result during DNA replication, leading to mutations.

Recent model studies with CEQs and deoxynucleosides or bases showed that different types of adducts are obtained:⁵⁶ stable adducts that contain the deoxyribose moiety and that remain bound to DNA, depurinating adducts in which the glycosidic bond of dG or dA is destabilized, leading to loss from DNA and formation of apurinic sites.

CEQs derived from 4OHE and 2OHE (CE-3,4-Q and CE-2,3-Q respectively) react differently with nucleosides and DNA because of their distinctive chemical properties. CE-2,3-Q binds to the exocyclic amino groups of dA and dG to form adducts that retain the deoxyribose moiety whereas CE-3,4-Q bind exclusively to the N-7 of Gua and to the N-3 of Ade, resulting in destabilization of the glycosidic bond and subsequent depurination.⁵⁶

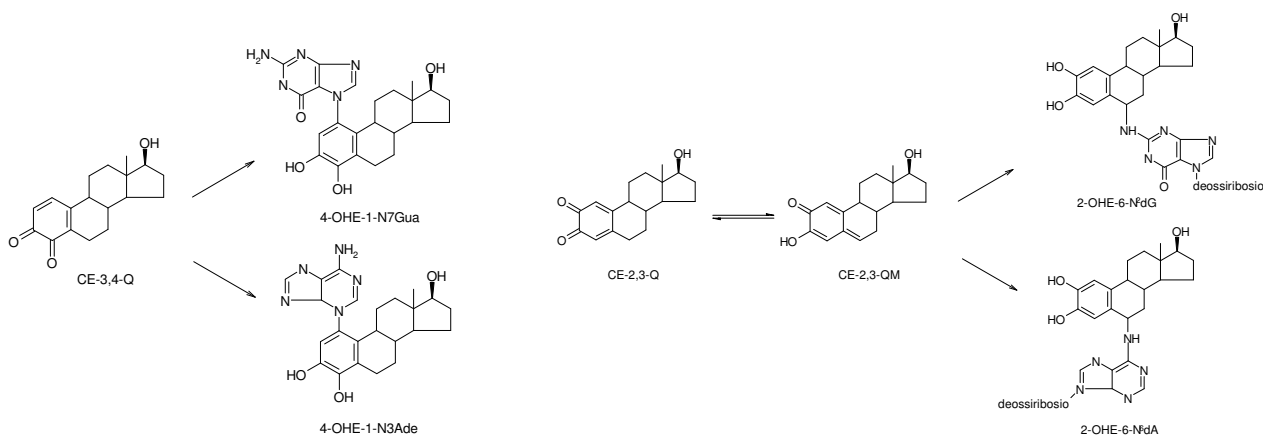


Fig. 5: CEQs adducts with dA and dG

The species shown above result to arise via a Michael addition of nucleophilic groups in dG e dA to CEQs. In the reaction of CE-3,4-Q with dA or dG, attack of the nitrogen nucleophile at C1 is the result of 1,4-addition with the respect to the C3 carbonyl. This is in contrast to sulfur nucleophiles, which attack at the C2 position, a 1,6-addition with respect to the C4 carbonyl. Molecular orbital calculations have shown that, in neutral species, C1 bears more positive charge than C2 and the orbital LUMO has an higher coefficient at C2 than C1. Thus, soft nucleophiles, such as thiols, will attack at C2, while harder nucleophiles, such as nitrogen and oxygen, will attack C1. The products of the reaction of CE-2,3-Q with dG or dA are the result of a 1,6-Michael addition to the quinone after initial tautomerization of quinone to the more electrophilic quinone methide. Several studies indicate that CE-2,3-Q is more stable than quinone methide isomer. However the products of 1,6-addition demonstrate that only the quinone methide isomer, presents in small quantities, reacts with these nucleotides.⁵⁷

These results are sustained by *in vivo* studies reporting, for instance, that the depurinating adducts, identical to those formed *in vitro*, have been identified in rat mammary gland after injection of CE-3,4-Q.⁵⁷

Several lines of evidence suggest that the 4-hydroxyestrogens are critical intermediates in the pathways leading to estrogen-induced cancer. For example 4OHE induces renal tumors in hamsters, whereas 2OHE does not.⁵⁹ Furthermore, an estrogen 4-hydroxylase activity has been identified not only in hamster kidney but also in other human and animal organs prone to estrogen-induced cancer, such as uterus, pituitary.⁵⁷

The biological consequences of bulky DNA adducts and/or apurinic sites are difficult to assess. It is possible that the loss of these adducts generates apurinic sites in DNA, which have high potential to produce mutations in critical genes (oncogenes and tumor suppressor genes). This series of events can initiate cancer in a variety of human tissues.⁵⁶ A similar relationship, for instance, exists between depurinating polycyclic aromatic hydrocarbons-DNA adducts and oncogenic mutations generated in cells culture.⁵⁷

The CE are inactivated by conjugation reactions such as glucuronidation and sulfation or by *O*-methylation. If these conjugating pathways are insufficient and/or ineffective, the competitive oxidation pathway of CE turns out their semiquinones and quinones; usually these reactive quinones are inactivated by reduction to CE catalyzed by quinone reductase enzyme or by conjugation with GSH catalyzed by glutathione-S-transferase enzyme. If these inactivating pathways are nonetheless insufficient and/or ineffective, the CEQs may show their cytotoxic proprieties.⁵⁶

The conjugation CEQ-GSH results to be a detoxification way in biological systems because GSH reacts very rapidly with *o*-quinones and successfully competes with other nucleophilic residues in vivo and in vitro, it decreases the number of electrophilic sites in the quinone and by increasing the hydrophilicity, facilitates excretion.⁶⁰

The reaction of CEQs with a variety of sulphur nucleophiles (RSH where R = Cys, NAcCys, GS) is very intriguing in estrogen carcinogenesis.⁶⁰ GSH adds at the 1- and 4- position in the case of *o*-quinones from 2OHE, giving two mono-GSH adducts and one GSH adduct, and only at the 2 position for the 4OHE *o*-quinone.⁵⁵ (fig.X)

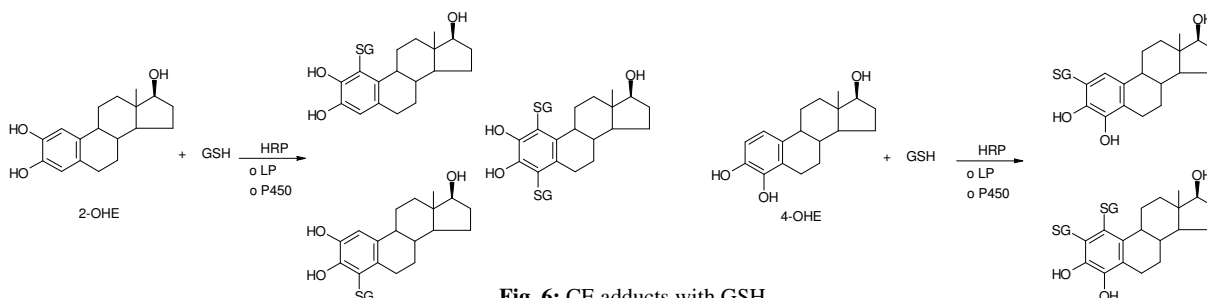


Fig. 6: CE adducts with GSH

The CEQ-GSH conjugates and DNA adducts are identified in vivo and vitro cultures and are potentially useful as biomarkers to indicate both DNA damage and potential susceptibility to estrogen-related cancer.⁶⁰

It has been shown that in vitro the *o*-quinones of both 2OHE and 4OHE isomerize nonenzymatically to highly electrophilic *p*-quinone methides. CE-2,3-Q forms two quinone methides: a quinone methide stabilized by two alchyl substituents on the metylene group in the C ring and a quinone methide with only one alchyl substituent in the B ring. In contrast, CE-3,4-Q

only isomerized to the potentially more stable C ring *p*-quinone methide as the B ring *o*-quinone methide was not detected.⁵⁵

The different structure of CE quinone methide with *o*-quinones results in a much more reactive electrophile and in a reduced capacity for redox chemistry. Consequently, reactions of quinone methides in biological systems are characterized by non-enzymatic Michael additions at the exocyclic methylene carbon, generating benzylic adducts of peptides, proteins and nucleic acids.⁵⁵

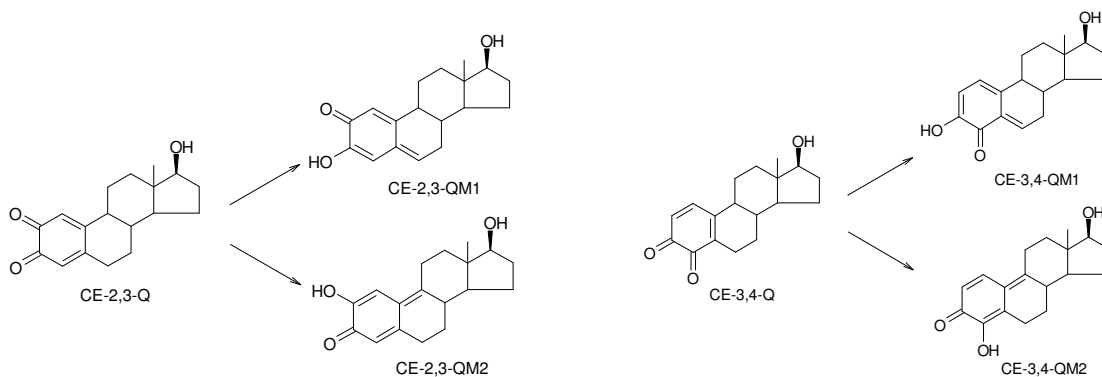


Fig. 7: isomerisation CE *o*-quinone to quinone methide

In vitro experiments suggest that in cells with low levels of GSH the formation of these potent electrophiles represents the major reaction pathway for estrogen *o*-quinones.⁵⁵

These results, therefore, are index of the complexity of the CEQs conversion pathway and little attention has been paid to the nature of the products formed by conversion of the CEs beyond the *o*-quinone stage. Yet, several interesting conversion pathways may be predicted based, for example, on recent studies of 2-hydroxy-8,9-dehydroestrone oxidation, leading to 2- and 4-hydroxyequilenin.^{54, 61}

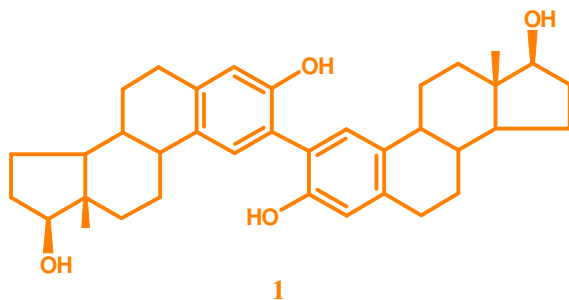
Oxidation of 17 β E by the peroxidase/H₂O₂ system⁶²

In preliminary experiments, the ability of various chemical and enzymatic oxidants to induce conversion of 17 β E to products was briefly investigated under different reaction conditions. With the chemical oxidants tested, i.e., persulfate, ferricyanide, and ceric ammonium nitrate, little or no substrate conversion was observed (HPLC and TLC evidence) in aqueous buffers or biphasic media in a broad range of pH values. With ferricyanide, slow substrate consumption was obtained only in 0.1 M NaOH, as previously reported.⁵⁰

By contrast, a substantial substrate consumption was observed with the peroxidase/H₂O₂ system, with formation of a number of products whose chromatographic and spectral properties were suggestive of oligomer species. Moreover the occurrence of peroxidase in mammalian tissues responsive to estrogen activity, such as uterus,⁶³ induced us to embark on the isolation and detailed characterization of the products formed by peroxidase/H₂O₂ oxidation of 17 β E.

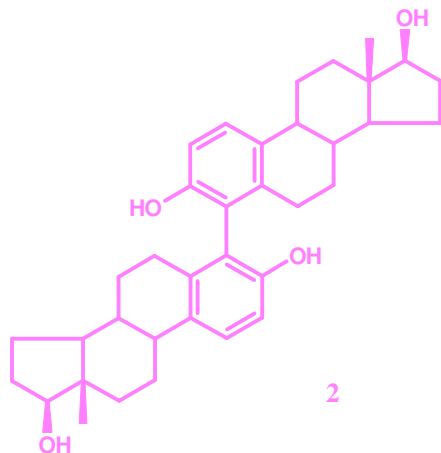
In a typical preparative scale reaction, 17 β E at 0.3 mM concentration was allowed to react with peroxidase (1U/mL) and hydrogen peroxide (2 mol equiv). After 60 min, with >98% substrate consumption, the mixture was extracted with ethyl acetate following careful acidification to pH 5.0. PLC fractionation afforded seven main chromatographic bands at R_f = 0.68, 0.55, 0.45, 0.43, 0.33, 0.22, and 0.10 (eluant A) designated A-G, in that order. Of these, only fraction D consisted of a single species pure enough for spectroscopic analysis, whereas remaining fractions required further fractionation. The most polar band G was made of chromatographically ill-defined products, and their identity was not investigated.

Spectral data (¹H and ¹³C NMR) of the product from band D were in agreement with a C₂-symmetric dimer (molecular ion peak at m/z 542). Homo- and heteronuclear correlation experiments allowed straightforward formulation of the product as **1**.



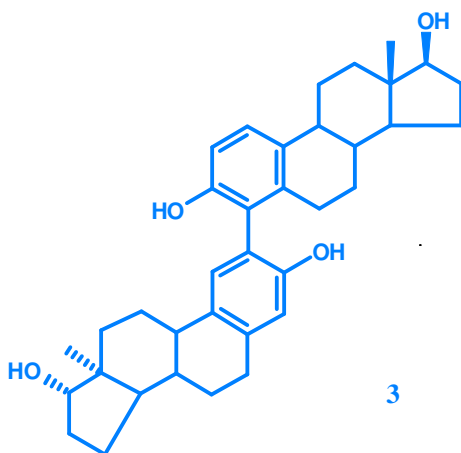
Chromatographic band A consisted of an intimate mixture of two closely related species which could be separated by preparative HPLC. The products showed nearly identical ¹H NMR spectra

featuring in the aromatic region two doublets ($J = 8.8$ Hz) at around δ 7.3 and 6.9, suggesting the C_2 -symmetric 4,4' dimer **2**.



On this basis, the two products were regarded as atropoisomers arising by a restricted rotation around the sterically crowded biphenyl 4,4' linkage. No appreciable interconversion of the rotational isomers was observed by heating to 110 °C at which temperature the products began to decompose significantly. This implies that the activation energy barrier is greater than 22.5 kcal mol⁻¹.

The constituents of chromatographic band C as purified by HPTLC displayed very similar ¹H NMR spectra showing in the aromatic region two singlets at about δ 7.0 and 6.8 and two doublets ($J = 8.4$ Hz) at about δ 7.3 and 6.9, suggesting two atropoisomers of a 2,4'-linked dimer (**3**).



This view was confirmed by dynamic ¹H NMR experiments (see figure 8). Line shape analysis at a temperature around coalescence allowed calculation of the mean lifetime of the atropoisomers, and a free energy of activation of 21.5 ± 0.5 kcal mol⁻¹ was determined by application of the Eyring equation:

$$k = \frac{RT}{hN} e^{\frac{-\Delta G^\ddagger}{RT}}$$

k = rate constant
 R = universal gas constant
 N = Avogadro's number
 h = Plank constant

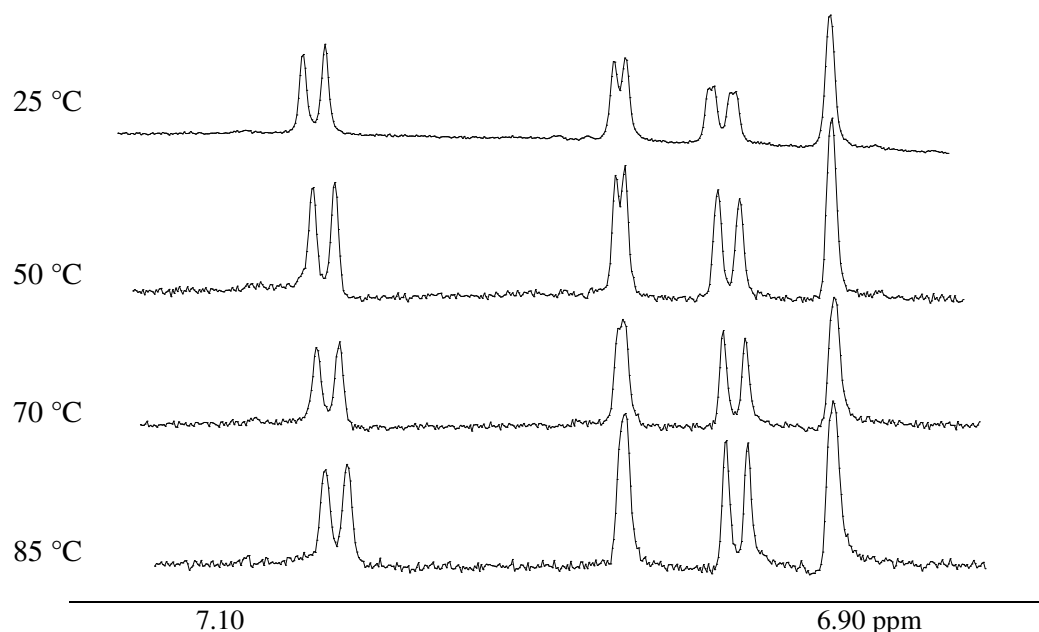
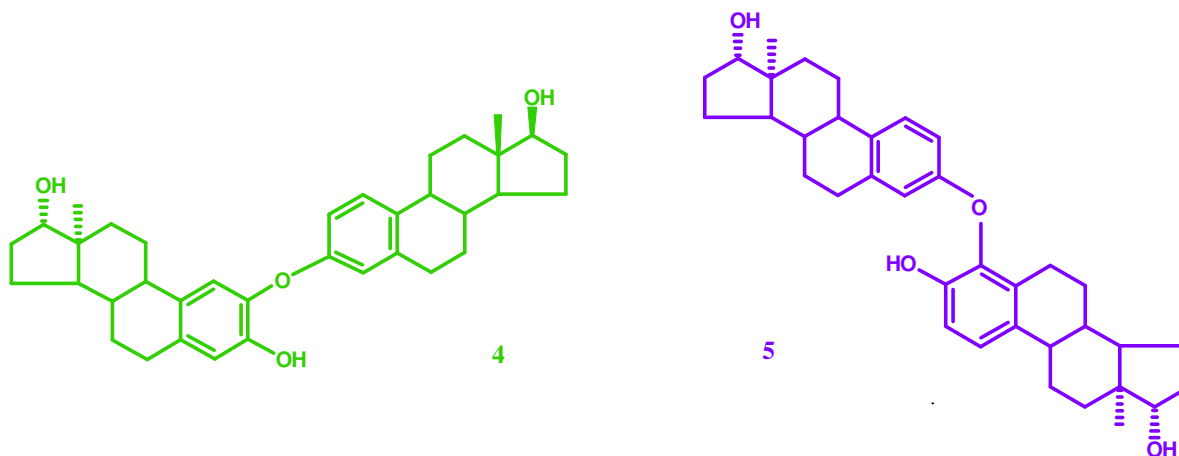


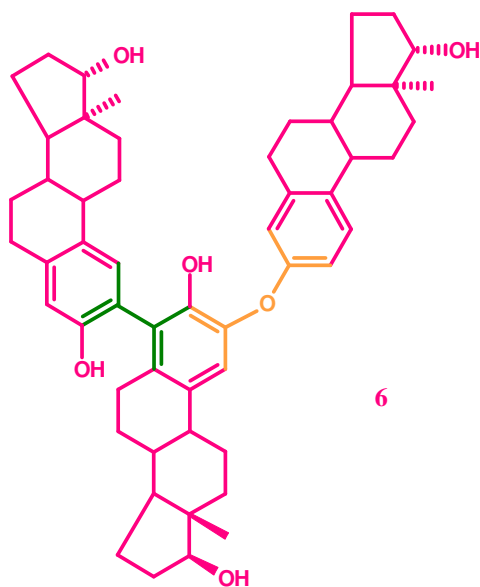
Fig. 8: ^1H NMR spectra of atropisomeric dimers **4a** and **4b** mix 1: 1 at several temperatures

The two components of chromatographic band B were separated by preparative HPLC. The mass and NMR data led to straightforward formulation of the compounds as the O-linked dimers **4** and **5**.⁵²



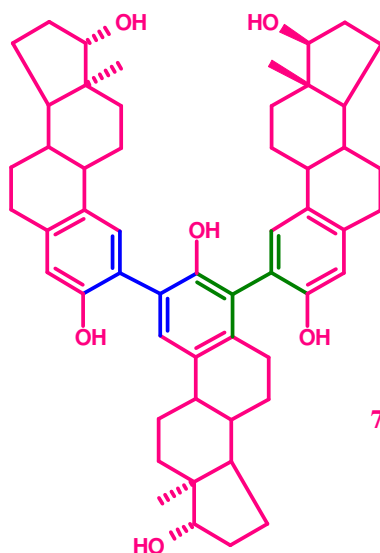
Extensive 2D proton-proton and proton-carbon correlation experiments allowed complete assignment of the aromatic resonances. All products from chromatographic bands E and F exhibited molecular ion peaks at m/z 812 in the EI-MS spectrum indicating trimeric structures. Products from band E as obtained in pure form by HPLC separation showed very close proton spectra and were regarded as atropisomers. Both displayed in the aromatic region an ABX spin system and three singlets around δ 6.7, 6.9, and 7.0, consistent with a trimer in which a central estradiol unit is linked

to the 2-position of an outer moiety and to the oxygen of the other unit. On the basis of the HMBC correlation data, it was possible to assign the signals at δ 7.04 and 6.75, for the faster HPLC eluted compound, to the H-1 and H-4 protons of the same estradiol unit. The shielding effect caused by the oxygen bridge, observed also in dimers **4** and **5**, and the presence of a weak but well discernible cross-peak between the proton resonance at δ 7.04 and a substituted C-4 carbon resonance at δ 124.3 allowed straightforward assignment of the former signal to the H-1 proton of the central unit leading eventually to assign the trimers structure **6**.

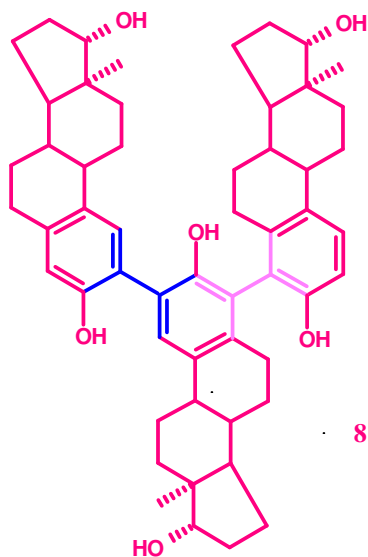


In these, the atropisomerism is apparently the result of the restricted rotation around the 2,4' linkage. NMR line shape analysis around the coalescence temperature allowed calculation of a free energy of activation of 20.9 ± 0.4 kcal mol⁻¹.

Of the four main HPLC-separable products in band F, those eluting at 24 and 80 min (eluant II) interchanged on heating, suggesting again an atropisomer relationship, whereas those eluting at 34 and 37 min (eluant II) were not affected by heating to 100 °C. The aromatic region of the ¹H NMR spectrum of the products eluted at 24 and 80 min displayed five singlets, a feature which was compatible with the trimeric structure **7**.



Complete assignment of the proton and carbon signals in the aromatic region was achieved on the basis of the data of the correlation experiments. In particular, in the case of the slower eluting isomer (**7b**), the singlets at δ 7.21 and δ 6.76 were assigned to H-1 and H-4 protons of the same unit, respectively, on the basis of the 3J and 2J long-range contacts exhibited with C-3 and C-2 at δ 151.1 and 123.0, respectively. A 3J contact between the latter carbon and the H-1' proton at δ 7.32 of another unit provided support to the 2,2' linkage between two of the trimer units. The free energy activation for the interconversion was calculated as 21.3 ± 0.5 kcal mol $^{-1}$. The ^1H and ^{13}C spectra of the other two constituents of band F were likewise very similar. The aromatic regions of the proton spectra displayed three singlets and two doublets ($J = 8.4$ Hz), a pattern of resonance that was compatible with either of the two trimeric structures in which the estradiol units were linked through the 2,4':2',4'' or the 2,2':4',4'' positions. The lack of appreciable interconversion on heating, observed also for the atropoisomers of the 4,4' dimer **2**, strongly argued in favor of structure **8**.



This assignment was confirmed by analysis of the proton-carbon correlation spectra showing contacts matching those of the 2,2' subunit of trimer **7**. Interestingly, when the oxidation of **17βE** was carried out with the substrate at 0.3 μM concentration, that is, at a concentration close to physiological values, substrate consumption was >95% at 1 h and the main reaction products were the dimers **1** and **3**, whereas the O-linked dimers **4** and **5** were formed only in very small amounts and dimer **2** and trimers **6-8** were below detection limits. For comparative purposes, the oxidation of **17βE** with manganese dioxide in chloroform was briefly investigated. Under these conditions, a smooth oxidation of **17βE** (>99% consumption after 20 h) was observed with formation of dimers **2** and **3** as main species (about 30% overall formation yields) but with no detectable **1**, **4**, and **5**. The different product patterns obtained at lower substrate concentration, or using manganese dioxide in chloroform, suggest that the generation and mode of coupling of phenoxyl radicals is under the influence of several factors. For example, tenuous steric factors may become significant under high dilution conditions, thus accounting for the lack of formation of the relatively hindered dimer **2**, whereas solvent effects may explain the prevalence of C-coupling products, i.e. dimers **2** and **3** in chloroform, also furnishing a suggestion for preparative purposes requiring regiochemically more restricted products patterns. The sterically hindered biphenyl linkage in **2**, **3**, and **6-8** represents a stereogenic element which adds to those already present in **17βE**. For all isolated products featuring such structural system, configuration at the biphenyl linkage (and thus absolute stereochemistry) was established by the exciton chirality method on the basis of the Cotton effect associated with the phenolic transition 1L_a , whose vector nearly overlaps that joining the C10-C3-O centers.⁶⁴ This transition is observed at around 220 nm in **17βE** in EtOH, but the formation of biphenyl linkages and the presence of other substituents, such as the O-linked unit in **6a,b**, cause shift to longer wavelengths. The relative directions of the 1L_a transition dipoles and of the biphenyl bonds allowed

assignment of positive screwness configuration (*P*) to those isomers exhibiting positive Cotton effect independently from the regiochemistry of the biphenyl linkage. Indeed, geometry optimization of the oligomer structures (MM+) showed that the dihedral angle between the planes of the aromatic rings of the biphenyl system has an absolute value ranging from 43° 22' to 45° 15' for 2,4' linkages and from 90° 02' to 94° 92' for 4,4' linkages. On the basis of the angles between 1L_a transition vectors and the dihedral intersection line, trigonometric calculations gave the range +37 to +77° for the angle between the dipole transition moments in the case of a positive dihedral angle. These values are significantly smaller than 110°, which is the theoretical zero point at which for polyphenyl systems featuring right-handed screwness^{64c} the sign of the exciton split of CD Cotton effect changes from positive to negative, allowing straightforward molecular configuration assignment. The choice of 1L_a transition arises also from the clearly defined monosigned Cotton effect at around 230 nm in all isolated products, whereas the Cotton effect at ca. 280 nm (transition 1L_b) was less defined for nearly all products, with the exception of those featuring 4,4'- biphenyl linkages. On this basis, the negative Cotton effect of the first HPLC eluted atropoisomer of **3** and **6** (i.e. **3a** and **6a**) indicates a negative helical orientation of phenol transition moments that means an *M* molecular chirality, while the first eluted isomer of **2** has the *P* configuration (Figure 9 A-C).

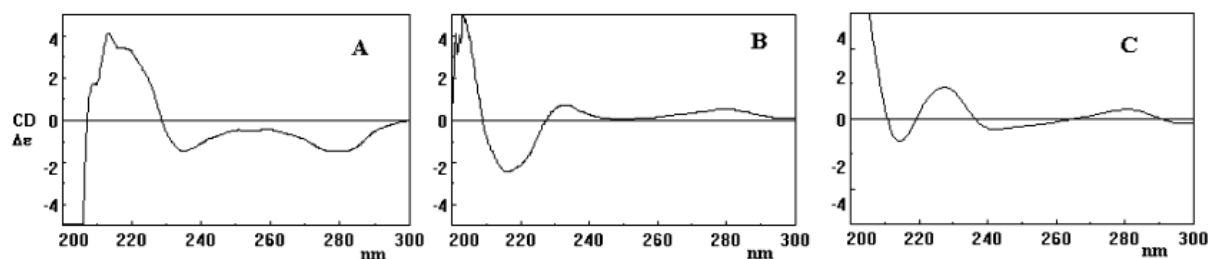


Fig. 9: CD spectra of compounds **2a** (A), **3a** (B), and **6a** (C).

In the case of **7** (i.e. 2,2':4',2''-triestradiol) and **8** (i.e. 2,2':4',4''-triestradiol) the first eluted isomers share the *M* configuration at the 4',2'' biphenyl linkage and at the 4',4'' linkage, respectively (Figure 10 A,B).

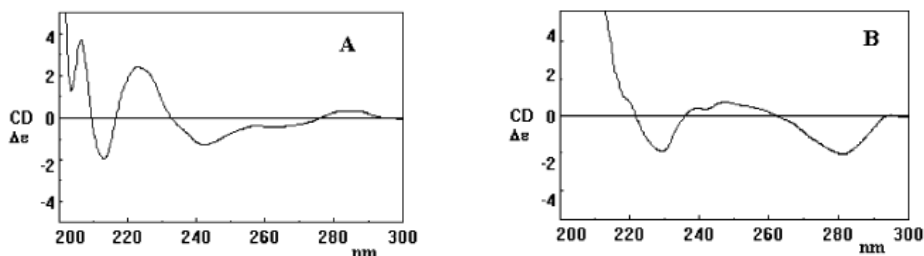


Fig. 10: CD spectra of compounds **7a** (A) and **8a** (B).

Mechanistically, formation of oligomer products **1-8** by peroxidase/H₂O₂ promoted oxidation of **17βE** can be interpreted as involving generation and coupling of phenoxyl radicals from **17βE**.

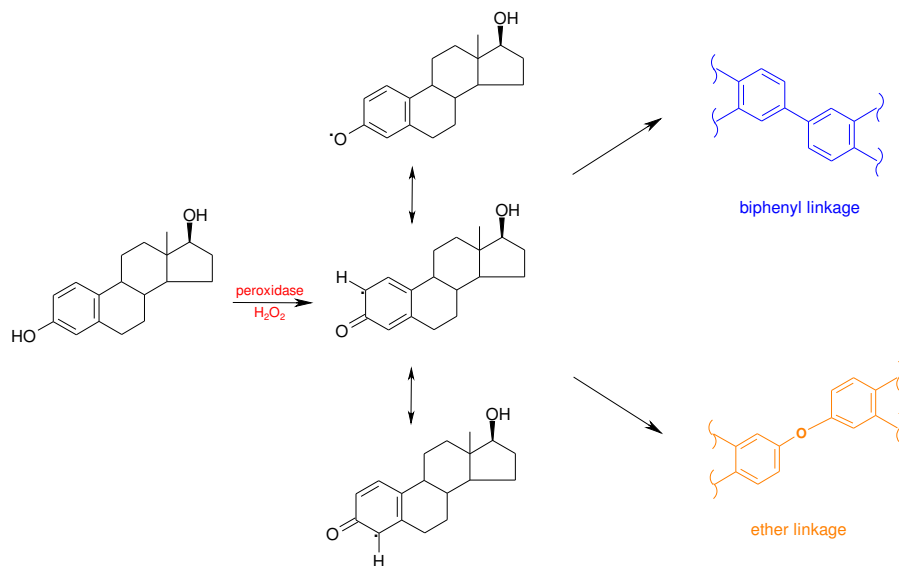


Fig. 11: generation and coupling of phenoxyl radicals from estradiol

In the presence of H₂O₂, ferric peroxidase (ground state) generates the ferryl π cation (compound I) via two electron oxidation. Compound I can then be reduced to compound II, the ferryl form of the enzyme, which has higher oxidative equivalents than the resting ferric form.^{63b}

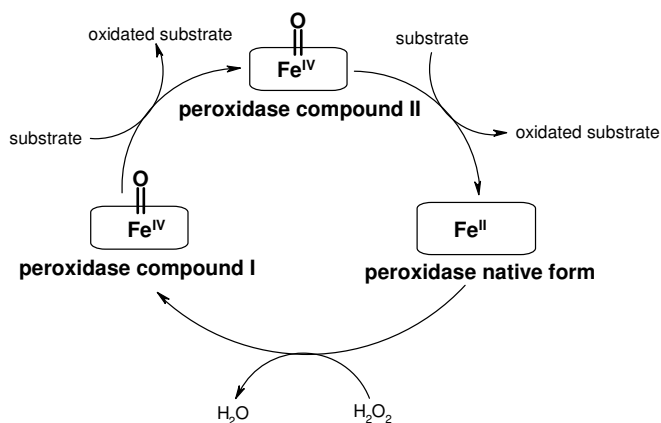


Fig. 12: schematic peroxidase action

Both compounds I and II can oxidize the phenolic moiety of **17βE** to give the phenoxyl radical. From inspection of the SOMO and Mulliken spin densities of the phenoxyl radical of **17βE** reported in a previous study,⁶⁵ no appreciable difference was anticipated in the reactivity of **17βE** through the 2 and 4 positions, in accord

with experimental evidence. Coupling through the oxygen center is clearly a reflection of the high spin density at this site, in conformity with the known patterns of oxidative coupling of phenols.

Concluding Remarks. Highlights of this study include (a) the first isolation and complete characterization of trimeric steroids linked through C-C and C-O-C bonds, (b) the first example, to the best of our knowledge, of atropoisomerism in steroidal systems, generated by steric hindrance to

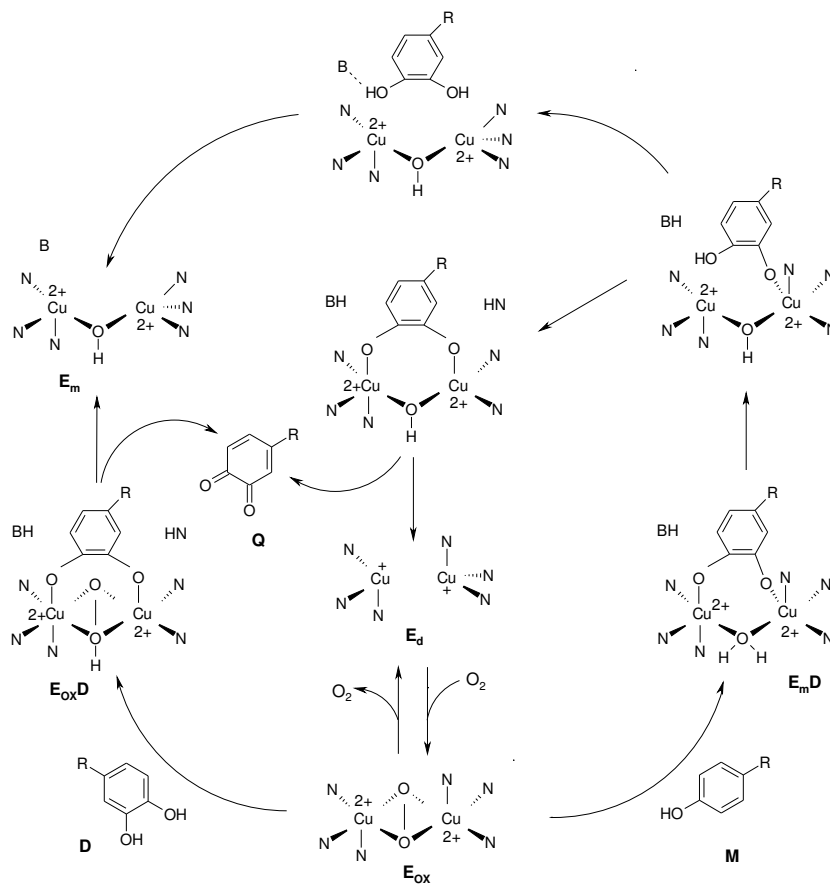
free rotation at 2,4'- and 4,4'-biphenyl linkages, and (c) the exploitation of peroxidase/H₂O₂ as an efficient and clean oxidizing system in estrogen chemistry.

From the biomedical point of view, the present results offer an improved background to elucidate the chemical nature and fate of the products derived from the antioxidant and radical scavenging reactions or from oxidative changes of the estrogens at sites of inflammation and active metabolic transformation. In the light of the suggested role of **17βE** as OH radical scavenger, generation of these oligomers may represent an alternative outcome of the radical scavenging action in addition to quinol formation.⁴⁸ Oligomers **5** and **6** resemble the photodegradation products of ethinyl estradiol,⁶⁶ and their formation by autoxidation and photodegradation of **17βE** containing drugs can be predicted. C₂-symmetric dimers bear considerable similarity to stereochemically related products⁶⁷ currently under scrutiny because of their antiestrogenic activity and may represent attractive prototypes/leads for the rational design of new bioactive steroids.

Finally, atropisomeric estradiol oligomers are analogous to *para*-polyaryls, which exhibit attractive structural features, such as helicity, and other connected unusual chemical-physical properties underlying a number of applications in material science.⁶⁸

Oxidation of 17βE by the tyrosinase/O₂ system⁶⁹

An oxidizing system that could model oxidative transformations of 17βE *in vivo*, thus enabling chemical studies of CEQs generation and fate, is tyrosinase.⁷⁰⁻⁷² Tyrosinase is the key enzyme of melanogenesis in epidermal melanocytes⁷² and possesses both monophenol monooxygenase activity (EC 1.14.18.1, tyrosine, 3,4-dihydroxyphenylalanine:oxygen oxidoreductase) and *o*-diphenoloxidase activity (EC 1.10.3.1, *o*-diphenol:oxygen oxidoreductase). Tyrosinase can act on monophenols (M) because of the mixture of met- (E_m) and oxy-tyrosinase (E_{ox}) which exists in the native form of the enzyme. The latter form is active on monophenols, while the former is not. However, the kinetics are complicated because monophenols can bind to both enzyme forms. This situation becomes even more complex since the products of the enzymatic reaction, the *o*-quinones, are unstable and continue evolving to generate *o*-diphenols (D) in the medium. A proposed kinetic mechanism to explain the enzyme's action is based on structural aspects developed by Solomon (Scheme z).⁷³ Briefly, E_{ox} would start the turnover by acting on M, which is hydroxylated to generate E_mD. At this point the enzyme may oxidise D to Q, generating E_d, or release D producing E_m, which would bind with M to produce the inactive form E_mM.



Scheme 1. Proposed mechanism tyrosinase's action

Tyrosinase-like proteins have recently been identified in estrogen-responsive tissues as well as in the nervous system.⁷⁴⁻⁷⁶ Such enzymes from nuclear extracts were found to possess both cresolase and catecholase activity, as well as estrogen binding properties, and may thus play a role in the conversion of **17βE** into its hydroxylated metabolites.

Mushroom tyrosinase was selected as a convenient oxidizing system for producing CEQs from **17βE** and for monitoring their fate under physiologically relevant conditions. At variance with the mammalian enzyme from melanoma cells,⁷⁷⁻⁷⁹ which is apparently unable to induce estradiol hydroxylation, mushroom tyrosinase displays efficient cresolase activity toward this estrogen and is highly effective in causing catechol estrogen oxidation to CEQs.

The reaction of **17βE** with the tyrosinase/O₂ system was examined with substrate concentration in the range 1nM-30μM in phosphate buffer at pH 7.4. For product determination, a work up procedure was developed, involving mild reductive treatment of the reaction mixture with sodium borohydride to halt the oxidation and convert quinonoid species to their reduced forms, followed by acidification to pH 5.0 and extraction with ethyl acetate. Substrate consumption and product formation were tracked by HPLC analysis of the ethyl acetate-extractable fraction.

Typical elution profiles of mixtures obtained by oxidation of **17βE** at 1 nM and 30μM concentration are reported in Figure 13a and 13b, respectively. A more complex reaction pathway is observed at higher substrate concentration. A preliminary ESI(-)-MS analysis of the HPLC eluates following ethyl acetate extraction indicated the presence of the catechol estrogens 2OHE and 4OHE along with a series of species with mass spectra suggestive of transformation products of 2OHE and 4OHE.

To test this hypothesis, in another series of experiments the tyrosinase-catalyzed oxidation of 2OHE and 4OHE was investigated under similar conditions.

Tyrosinase promoted oxidation of 2OHE and 4OHE. The HPLC elution profiles of the oxidation mixtures obtained by oxidation of 2OHE and 4OHE at 30 μM concentration are shown in Figure 1c and d. The data suggest that peaks I and III-VII in Figure 13b arise from 2OHE whereas peak II derives from 4OHE. Accordingly, the oxidation of these catechol estrogens was repeated on a preparative scale and the products eluted under the main peaks were isolated by preparative HPLC and were characterized by extensive 2D NMR and mass spectrometric analysis.

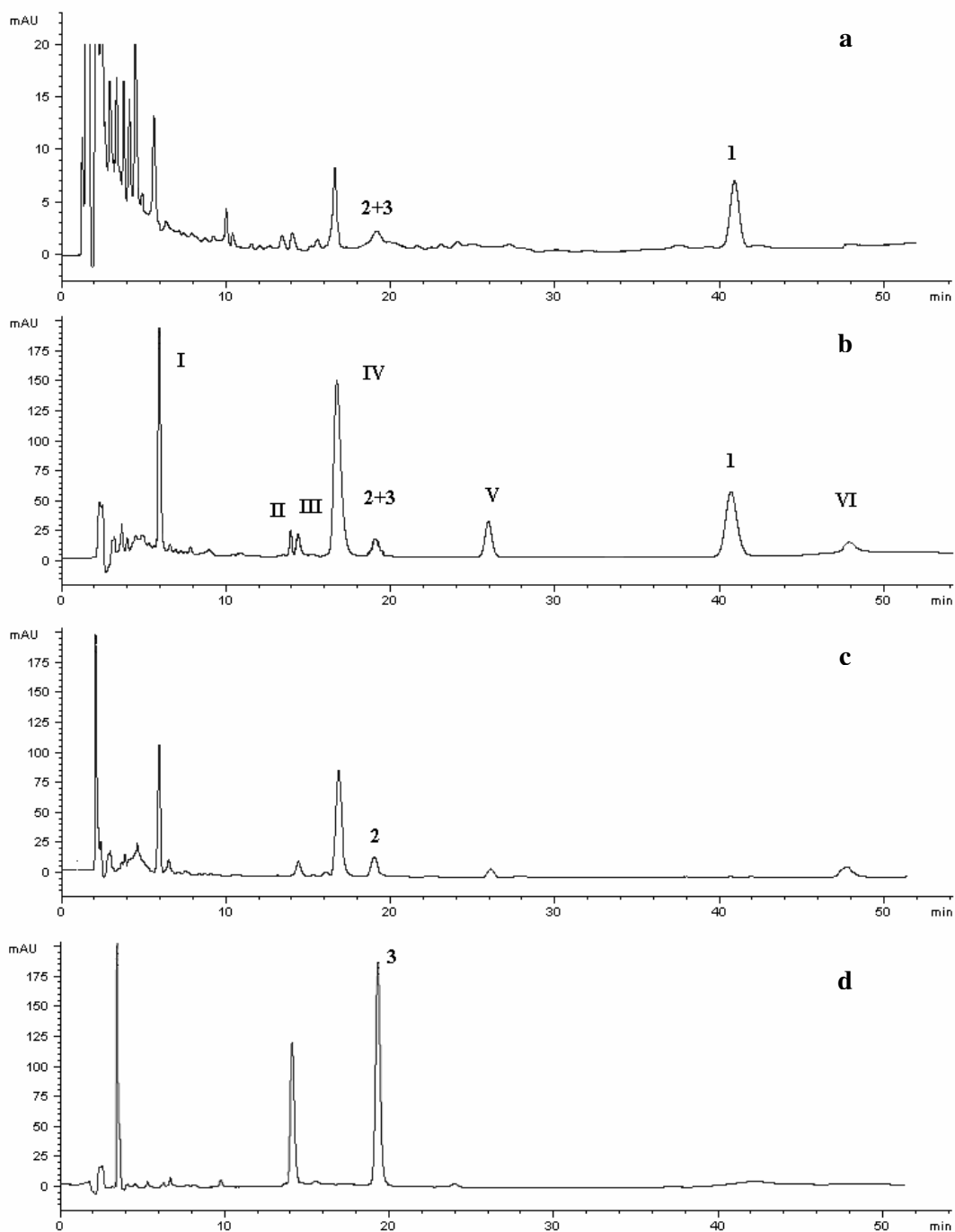
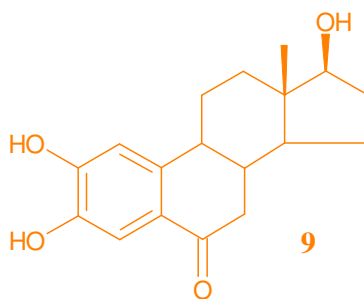


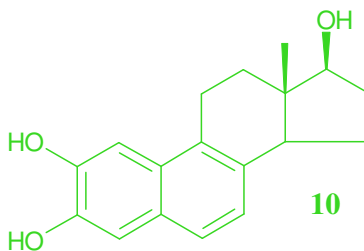
Fig. 13: Elutographic profiles of the tyrosinase mixture reaction of **17 β E** at 1 nM (trace a), 30 μ M (trace b), 2OHE (**2**) at 30 μ M (trace c) and 4OHE (**3**) at 30 μ M (trace d) after 3 h reaction time.

Preparative scale oxidation of 2OHE was carried out with 60 μM substrate and 5U/mL tyrosinase in vigorously stirred phosphate buffer at pH 7.4.

The ^1H and ^{13}C NMR spectra, as well as ^1H , ^{13}C HMBC and HMQC experiments, suggested that the product eluted under peak I was 6-oxo-2-hydroxyestradiol (**9**). This conclusion was supported by a carbonyl carbon signal at δ 200.3, the lack of the benzylic methylene protons resonating at δ 2.83, and the pseudomolecular ion peak at m/z 301 ($[\text{M}-\text{H}]$, ESI(-)). Product **9** was previously obtained by MnO_2 promoted oxidation of 2OHE in an organic medium,⁸⁰ but has never been described among the products formed by oxidation of **17 β E** or 2OHE under physiologically relevant conditions.

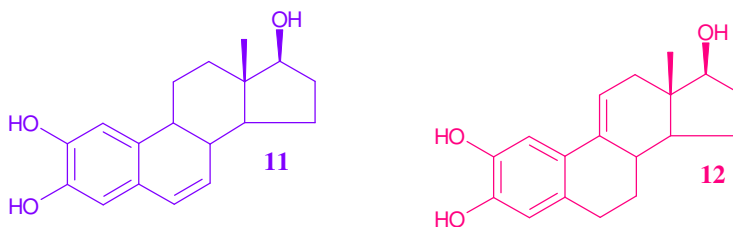


The mass spectrum of the product eluted under peak III indicated a molecular mass 4 units lower than that of 2OHE. The aromatic region of the proton spectrum, featuring two singlets and two doublets, was suggestive of a dihydroxynaphthalene moiety. This conclusion was corroborated by ^1H , ^{13}C HMBC experiments and comparison of the UV absorbance pattern with that of 2-hydroxyequilenin,⁸¹ allowing eventually formulation of the product as 6,7,8,9-dehydro-2-hydroxyestradiol (**10**). Compound **10** bears considerable resemblance to 2-hydroxyequilenin, which is produced by oxidative metabolism of 8,9-dehydroestrone via 2-hydroxy-8,9-dehydroestrone;⁶¹ however, to the best of our knowledge, it has never been obtained by oxidation of **17 β E** or 2OHE.



Peak IV proved to be due to an intimate mixture of two related products sharing the molecular mass of a dehydro derivative of 2OHE. Fractionation on silver nitrate-impregnated TLC plates allowed

eventually to isolate the products in pure form. These were subjected to extensive 2D homo- and heterocorrelation experiments and formulated as 6,7-dehydro-2-hydroxyestradiol (**11**) and 9,11-dehydro-2-hydroxyestradiol (**12**). In the case of **12**, significant NOE contacts could be observed between the H-1 and H-11 protons.

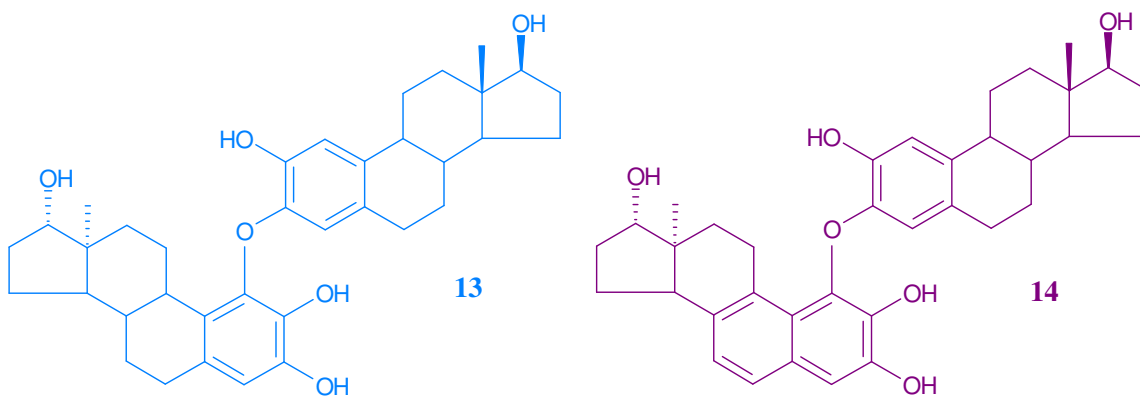


Products **11** and **12** were fairly stable in both acidic and alkaline media, without appreciable interconversion or degradation.

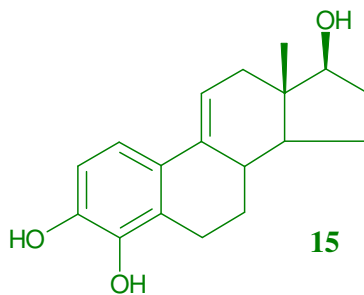
Mass spectra of products from peaks V and VI suggested dimeric structures, one of which (peak VI) was 4 mass units lower than expected for a dimer. Extensive spectral analysis, including 2D NMR, eventually allowed characterization of the products as the dimers **13** and **14**, respectively, in which the monomer units were linked through ether bridges.

For product **13**, substitution at the 1-position of one catechol ring was deduced from distinct long range couplings between the benzylic CH₂ carbons on the 6- and 6'-positions and two aromatic protons at δ 6.28 and 6.08, indicating that the adjacent 4- and 4'-positions were unsubstituted. The involvement of the 3-OH group of the other catechol ring in the ether bridge was inferred from chemical shift analysis and, in particular, from the upfield shift of one of the H-4 protons of ca. 0.5 ppm with respect to 2OHE (δ 6.58), suggesting an aryl ring on the adjacent OH group. Brief inspection of the geometry optimized structure of **13** (MM+) consistently indicated a spatial proximity of the H-4 proton to the aryl ring in a number of rotamers, whereby a shielding of at least 0.5 ppm can be expected.⁸² Furthermore, the less pronounced upfield shift of the other H-4 proton (δ 6.28) was in accord with the shielding effect of a *para* phenoxy group.

A survey of the literature indicated that a product related to **13**, *i.e.* a 2-hydroxyestrone dimer featuring an *o*-quinone moiety, has been described.⁸³ However, the close similarity of the aromatic proton spin systems and the facility of catechols to lose H₂ in the EI source to give [M-2]⁺ peaks⁸⁴ raises the possibility that the product described by the previous authors was in fact the analog of **13** in the estrone series.

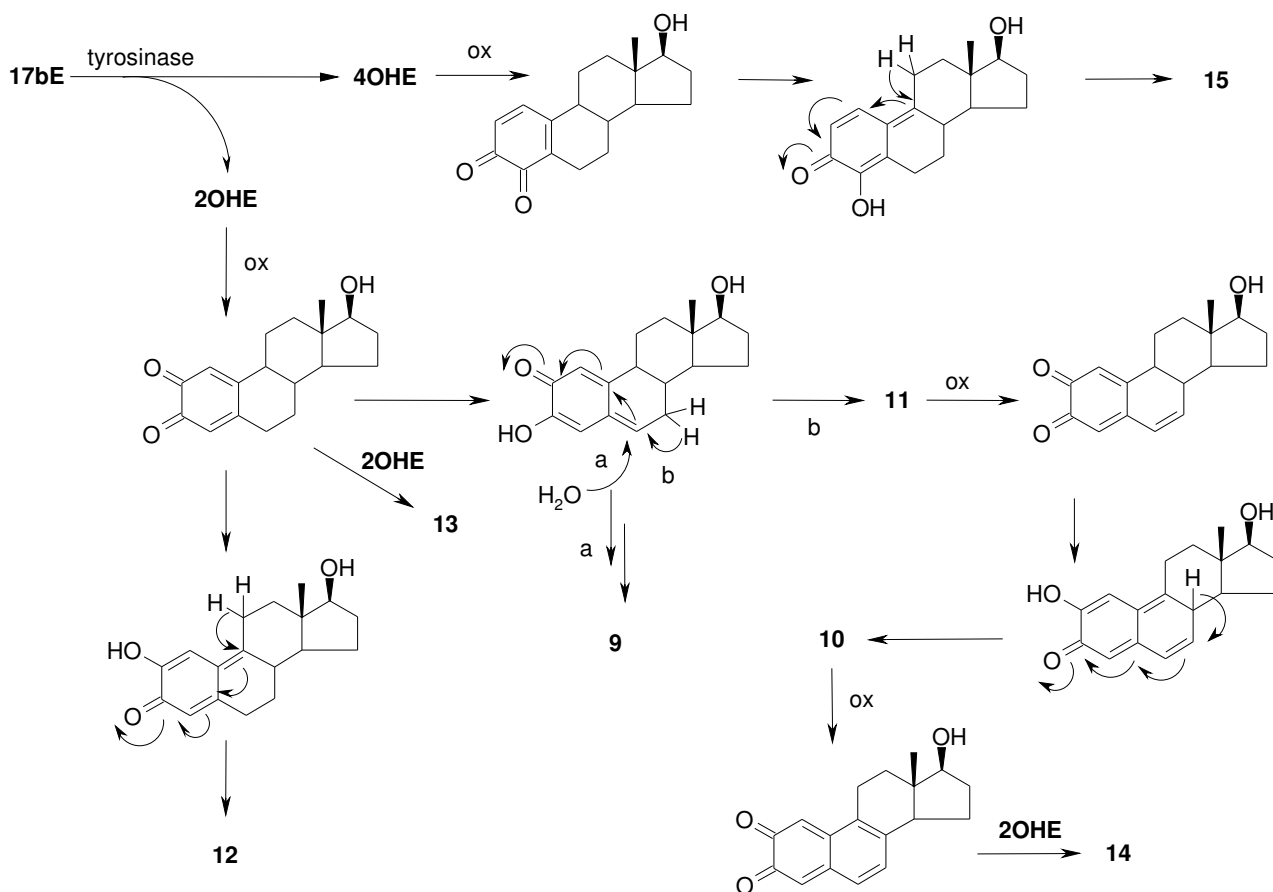


Oxidation of 4OHE under the reaction conditions adopted for 2OHE proceeded at a slightly lower rate with respect to 2OHE ($2.8 \times 10^{-10} \text{ Ms}^{-1}$ and $2.3 \times 10^{-9} \text{ Ms}^{-1}$, respectively), supporting previous observations on the higher oxidizability of 2-hydroxyestrogens compared to 4-hydroxyestrogens,⁸⁵ to give a single major product (peak II figure 13). This products was isolated and characterized as 9,11-dehydro-4-hydroxyestradiol (**15**) on the basis of extensive NMR analysis and mass spectral data.



At variance with previous observations on the oxidation of 2-hydroxyestrone in an organic solvent,⁸³ we were unable to identify C-C coupling products of 2OHE and 4OHE in the oxidation mixtures. With the above products available, separate analyses were run to determine changes in product distribution with estrogen concentrations. The results showed that with **17 β E** at 1-10 nM concentration, main reaction products included 6-oxo-2-hydroxyestradiol (**9**), 6,7-dehydro-2-hydroxyestradiol (**11**), 9,11-dehydro-2-hydroxyestradiol (**12**) and 9,11-dehydro-4-hydroxyestradiol (**15**), as apparent from Figure 1a, while 6,7,8,9-dehydro-2-hydroxyestradiol (**10**) and the dimeric products **13** and **14** were formed only at higher concentrations of **17 β E**, e.g. 1-30 μM .

Mechanistic remarks. Products **9-15** conceivably arise by conversion of catechol estrogens to the corresponding CEQs which may subsequently partition among different competing pathways, as schematically outlined in Scheme 2.



Scheme 2. Schematic outline of the proposed mechanism of formation of products **9-15**

Most of these pathways are proposed to involve transient quinone methide intermediates. These may undergo addition of water, as in the oxidative route to **9**, or may isomerize to give the unsaturated derivatives **10-12** and **15** after oxidation/aromatization steps. Isomerization to *p*-quinonemethides⁸⁶ is an established conversion route of *o*-quinones which usually does not require enzymatic assistance.^{87, 88} However, the possibility that this reaction was promoted by laccase, which is present as a contaminant in the commercial preparations of mushroom tyrosinase,⁸⁹ was considered. To this aim, the oxidation reactions were also carried out with carefully purified enzyme preparations according to reported procedures,⁸⁹ and were found to give product patterns that were superimposable to those obtained with the crude commercial enzyme. Moreover, addition

of a specific tyrosinase inhibitor (tropolone)⁹⁰ resulted in nearly complete suppression of substrate consumption and product formation.

Whereas the CE-2,3-Q may give rise to two isomeric quinone methide intermediates, which appear to contribute equally to product formation when generated in the nM range, the 3,4-quinone can give rise to only one quinone methide, and it is possible that this difference accounts for the simpler oxidation mixture of 4OHE. In Scheme 1, dimeric derivatives **13** and **14** are proposed to arise from different coupling reactions. This conclusion was supported by separate experiments showing that **13** is by no means converted to **14** when exposed to tyrosinase at pH 7.4, under the usual reaction conditions. However, redox cycling with intermediate oxidation products can not be ruled out. Indeed, dimer formation from catechol compounds may occur either by a one-electron transfer via a charge transfer complex^{83,91} or by addition of 2OHE to the *o*-quinone *via* the 3-OH group.

Structures **13** and **14** would reveal a similar pattern of reactivity of the *o*-quinones of 2OHE and **10**, favoring coupling at the hindered 1-position. In the case of 2OHE, this behavior was largely anticipated on the basis of the mode of dimerization of 2-hydroxyestrone.⁸³ Yet, it was less obvious for **10**, which would behave on oxidation like a highly reactive 2,3-naphthoquinone.^{92, 93}

Effect of nucleophiles on catechol estrogen oxidation. In further experiments, the effects of some nucleophilic compounds on the oxidation of the catechol estrogens at 10 μ M concentration were investigated to assess the possible influence of potential biological targets in tumor induction and other estrogen-induced toxic responses. Selected nucleophiles included adenine,⁹⁴ thymine,⁹⁵ cytosine,⁹⁵ 2-deoxyguanosine,⁸⁰ imidazole⁸³ and glutathione^{96, 97} for which the ability to trap CEQs was established. The effect of these compounds on the formation of **9-11** from 2OHE and of **15** from 4OHE was determined by comparing the HPLC traces of the organic extracts with those of control experiments carried out in the absence of additives.

The data in graphics 1-4 of figure 14 indicated that with both catechol estrogens, glutathione was by the far the most effective inhibitor of product formation, due likely to the ability of thiols to cause quinone reduction and/or trapping.⁹⁶

Appreciable changes in the relative product yields were observed with the other nucleophiles, suggesting that they affected the reaction course at different stages and with different modalities. Thus, free DNA bases were more effective at inhibiting product formation from 4OHE than from 2OHE. However, even in presence of a 4-fold excess of the nucleophiles, formation of products **9-11** and **15** remained significant with the substrates at 10 μ M concentration, and this would *a fortiori* be so at lower more physiological concentrations. In all cases, catechol estrogen consumption was

not affected by the additives, indicating that the inhibitory effects were indeed due to the trapping of oxidized intermediates.

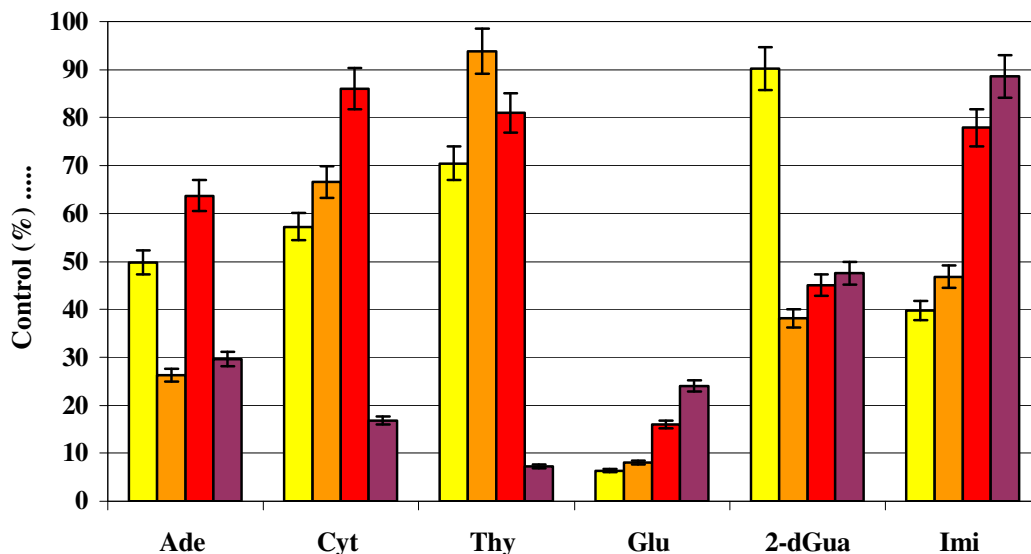


Figure 14. Effect of various nucleophiles on the formation of compounds **9-11** and **15** by tyrosinase catalyzed oxidation of 2OHE and 4OHE, respectively. Percent of control \pm SD is reported. Nucleophiles (adenine (Ade), cytosine (Cyt), thymine (Thy), glutathione (Glu), 2-deoxyguanosine (2-dGua), imidazole (Imi)) were at 4-fold molar excess with respect to substrates at 60 mM. Compound **9**: yellow bars; Compound **10**: orange bars; Compound **11**: red bars; Compound **15**: deep red bars.

Concluding remarks

The results of this study have yielded further insights into the conversion pathways of the catechol estrogens 2OHE and 4OHE beyond the *o*-quinone stage. A battery of products have been isolated and spectrally characterized, for use as standards for the identification of novel estrogen metabolites.

Although some of the products described here are not entirely unprecedented, as they have been previously reported as such or as their estrone analogs, to the best of our knowledge none of them has been obtained by tyrosinase-catalyzed oxidation of **17 β E**, nor by reaction of **17 β E** with other enzymes or chemical oxidants under biomimetic conditions. From the chemical viewpoint, the generation of product **10** by oxidation of 2OHE, and the formation of the novel dimer **14** by attack of 2OHE to the *o*-quinone of **10** appears to be of particular interest.

The use of commercially available mushroom tyrosinase, though of questionable relevance to mammalian systems, was necessary for preparative scale reactions. This enzyme may yet be taken

as a convenient model of the tyrosinase-like activity described in uterine and estrogen responsive tissues, since it displays an efficient monophenol monooxygenase (cresolase) activity. Furthermore, most of the previous studies on the tyrosinase-estradiol interaction were performed with the mushroom enzyme.^{54, 78, 98}

While products **9**, **11**, **12** and **15** were formed by oxidation of the estrogens at nanomolar concentrations, products **10**, **13** and **14** became detectable at substrate concentrations in the micromolar range. Thus, their formation may be implicated only under circumstances in which abnormally high concentrations of **17 β E** are present in settings of oxidative stress, *e.g.* during estrogen replacement therapy. In this connection, the finding that in the micromolar concentration range the 2,3-naphthoquinone from **10** can act as an efficient Michael acceptor toward the 3-OH hydroxyl function of 2OHE is biologically significant, as it may be taken to suggest that 2OHE can be converted to other reactive quinone intermediates in addition to CE-2,3-Q.

Clearly, more studies are required before the new products can be ranked among estradiol metabolites, and their toxicity or DNA-binding properties have to be tested in comparison with catechol estrogens and the CEQs. These latter are commonly regarded as the ultimate carcinogenic species produced by the oxidative metabolism of estrogens *via* the catechol estrogen pathway; however, the results of this study raise the possibility that at low physiological concentrations the actual mechanisms of estrogen-induced DNA damage and toxicity may be more complex than implied by commonly accepted views centered only on CEQ formation.

CHAPTER II

Oxidative Transformations of 17 β E

Beyond the specific relevance to the steroid sector, the oxidation of estrogen compounds represents an attractive research issue because of its potential as convenient entry to complex functionalized scaffolds of academic and industrial interest, e.g. in asymmetric synthesis⁹⁹ and supramolecular chemistry,¹⁰⁰ in the quest for innovative lead compounds in anticancer therapy,¹⁰¹ or for liquid crystal preparations,¹⁰² where **17 β E** and related compounds are commonly employed.

Halogenated, oxy-functionalized and/or desaturated on steroidal backbone estrogenic derivatives find interesting application in several fields so that simple and in good yield procedures for preparation of these compounds are useful to gram scale preparations.

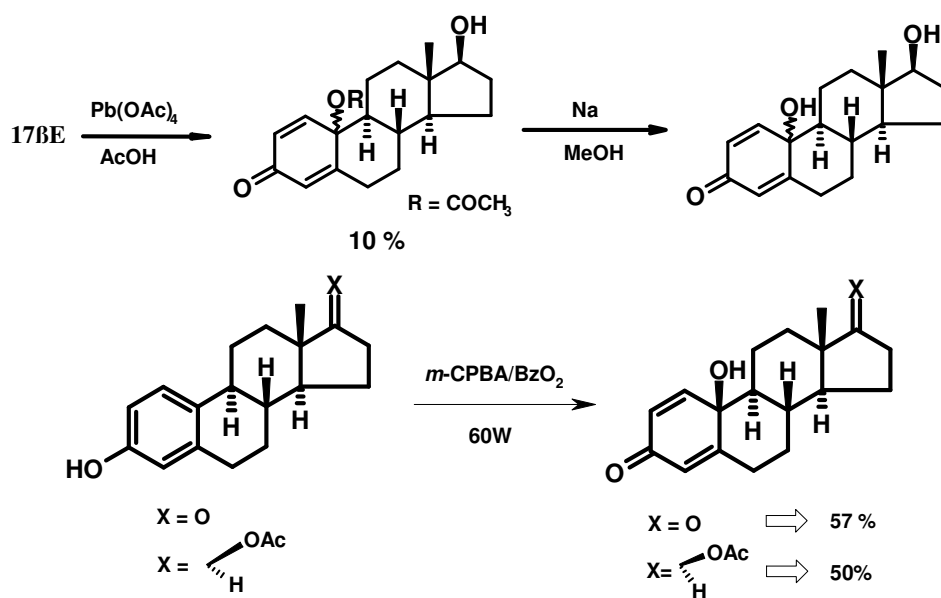
For example, in spite of considerable interest of catechol estrogens for toxicological studies and as starting materials for novel steroidal derivatives with antiestrogenic properties, their availability is limited by the lack of a facile and expeditious preparative procedure.¹⁰³ Classical approaches based on phenolic nitration involve at least three steps and, for the synthesis of 2OHE and 4OHE, reductive conversion of the 17-oxo group of an estrone derivative.^{103c} Moreover, protection/deprotection steps with chromatographic separations are often required. Use of potassium nitrosodisulfonate (FREMY's salt) for the one-step chemical conversion of estrone/estradiol to a mixture of the corresponding 2,3- and 3,4-quinones had been reported earlier by Gelbke et al.^{103e} The method involved a laborious work-up and ended up with very low overall yields of catechol estrogens.

Another class of estradiol-related compounds that have found application in estrogen replacement therapy, prevention, and treatment of osteoporosis,¹⁰⁴ in the detection and treatment of hormone dependent tumors,¹⁰⁵ or for the prevention and therapy of ophthalmic diseases¹⁰⁶ are 10 β -substituted-17 β -hydroxyestra-1,4-dien-3-ones. In addition, the identification of the 10-hydroxy derivative (*p*-quinol) in the redox cycling mechanisms underlying the putative antioxidant and cytoprotective properties of **17 β E**,¹⁰⁷ suggested its employment as prodrug of antioxidants,¹⁰⁸ while the 10 β -halo-derivatives have been shown to be valuable tools for probing interactions at estrogen receptors.¹⁰⁹

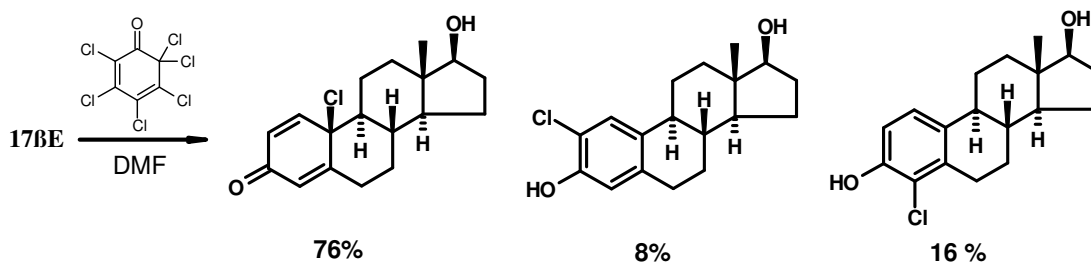
A number of synthetic approaches to 10 β -substituted-17 β -hydroxyestra-1,4-dien-3-ones have been reported,¹¹⁰⁻¹¹³ but they often require manipulation of estrone and **17 β E** derivatives, lengthy protection/deprotection steps or functional group modifications, resulting in complex mixtures of products. The best procedures for preparation of *p*-quinol derivatives are below reported: in the

first, oxidation of **17 β E** with lead tetraacetate results in a 10% yield of the corresponding *p*-quinol acetate that is hydrolyzed by sodium metal in absolute methanol.¹¹¹

The second procedure involves oxidation of 17 β -estradiol monoacetate with *m*-chloroperbenzoic acid and a catalytic amount of (BzO)₂ for 1.5 hours under irradiation with a 60 W tungsten lamp leading to the product in 52% yield, along with an epoxy-derivative.¹¹⁴ Other procedures are also available that afford the desired product, but in lower yields.



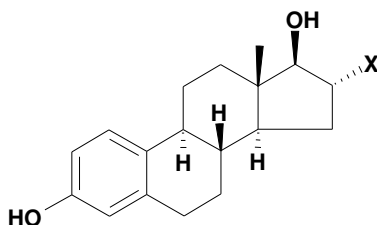
Access routes to 10 β -halo derivatives of **17 β E** have also been reported,¹¹⁵⁻¹¹⁷ though expensive organic chlorinating agents are employed and complex mixtures of products are usually obtained requiring separation and purification steps. For example, access to the 10 β -chloro derivative **2** is based on the reaction of **17 β E** with 2,3,4,5,6,6-hexachloro-2,4-cyclohexadienone in DMF which affords the desired compound in ca 75% yield together with 25% of other chlorinated derivatives.¹¹⁷



An example of estradiol-related compounds that have found application in asymmetric synthesis are steroidal BINOL-type ligands. Axially chiral nonracemic binaphthyls have achieved an important place in asymmetric metal-catalyzed synthesis since it was shown that their diol or bis-phosphine derivatives can introduce high degrees of enantioselectivity in several chemical transformations, e.g.

the Ti-alkoxide mediated addition of R_2Zn compounds to aldehydes, $LiAlH_4$ reductions, carbonyl ene reactions, aldol additions, cyanohydrin formation or hydrogenation reactions.¹¹⁸⁻¹²⁰ Therefore, the preparation of new chiral binaphthyl ligands is a subject of increasing interest and the development of a synthetic route making the BINOL-type ligands available in its diastereopure forms has achieved a significant place in asymmetric synthesis.

Also halogenation of **17 β E** and generally of aromatic compounds is a highly useful reaction for providing precursors to a number of organometallic species of potential relevance in the synthesis of natural products and pharmaceutically important compounds¹²¹. For instance antiestrogen therapy is the most widely used endocrine manipulation for the treatment of breast cancer, especially in postmenopausal women. Unfortunately, the compounds presently available possess mixed agonistic/antagonistic activity, thus potentially limiting their therapeutic efficacy. Following the observations that halogenation of **17 β E** can increase the affinity of its binding (expressed as RBA) to the estrogen receptor, a series of new steroidal antiestrogens (an example is below reported) have been synthesized.¹²²



Moreover aryl bromides and iodides are of importance in organic synthesis because of their utility to transition-metal-catalyzed cross coupling reactions to furnish complex functionality.¹²³ Then, the halogens on aromatic rings have been used as potential protecting or blocking groups in synthetic organic chemistry thanks to reductive elimination of halogens.^{124, 125} In fact the 2- and 4-haloestrogens are readily methoxylated to give the corresponding 2- and 4-methoxy derivatives regioselectively, that are used for treating neoplasm (such as lung neoplasm, breast neoplasm, melanoma, prostate neoplasm, pancreatic neoplasm, brain neoplasm).¹²⁶

Many established methods for the direct introduction of halogen atom into aromatic molecules have been reported.¹²⁷ Aryl iodides are usually more difficult to prepare than the other corresponding aryl halides due to the low electrophilic strength of iodine. Hence, synthetic methods involving a source of I^+ as the reactive species seem to be the most convenient procedures for the direct iodination of arenes. Generally, arenes can be iodinated by iodine in the presence of a Lewis acid, a hydrogen iodide trap or most commonly in the presence of an oxidizing agent.¹²⁸

All these examples of steroidal derivatives support their significance in applicative and academic fields and their preparation constitutes a synthetic intriguing challenge, as well as practical value.

Catechol estrogen preparation¹²⁹

As previously described, the catecholestrogens are important products of metabolic transformation of the estrogens arising by P450-mediated hydroxylation of **17 β E** and estrone, respectively, at the 2- and 4-positions of the phenolic A-ring.¹³⁰ Because of their implication in the mechanisms of estrogen-related carcinogenesis,¹³¹ as well as their interest in toxicological studies and as starting materials for novel steroidal derivatives with antiestrogenic properties, a synthesis on preparative scale is necessary.

In connection with our studies on the oxidation of estrogens, we have developed a simple and convenient one-pot procedure for the preparation of the catecholestrogens of **17 β E** and estrone, which involves use of the hypervalent iodine (V) reagent *o*-iodoxybenzoic acid (IBX)¹³² under carefully controlled reaction and work-up conditions. Despite its early description (1893),¹³³ IBX languished, essentially forgotten, until the 1980s when the seminal works of Dess and Martin initiated a renaissance in interest in the chemistry of hypervalent iodine(V) reagents.¹³⁴ Within the past decade, the use of IBX as a reagent has grown dramatically, a surge driven by an improved method for its synthesis,¹³² and by explorations into its chemistry that have unveiled its versatility in mediating a wide array of transformations with far-reaching synthetic applicability. In particular, investigations have revealed that IBX is a powerful single electron-transfer (SET) agent that readily accepts new heteroatom-based ligands and, thus, can 1) effect the oxidation of ketones, aldehydes, and silyl enol ethers to the corresponding α,β -unsaturated carbonyl compounds,¹³⁵⁻¹³⁷ 2) oxidize benzylic positions,¹³⁵ 3) facilitate the cyclization of *N*-aryl amides, (thio)carbamates, and ureas to afford various heterocycles and amino sugars,¹³⁸⁻¹⁴¹ and 4) convert monophenol to *o*-quinones.¹⁴² In light of this last potential, we have developed an IBX-mediated *o*-hydroxylation of estrogens.

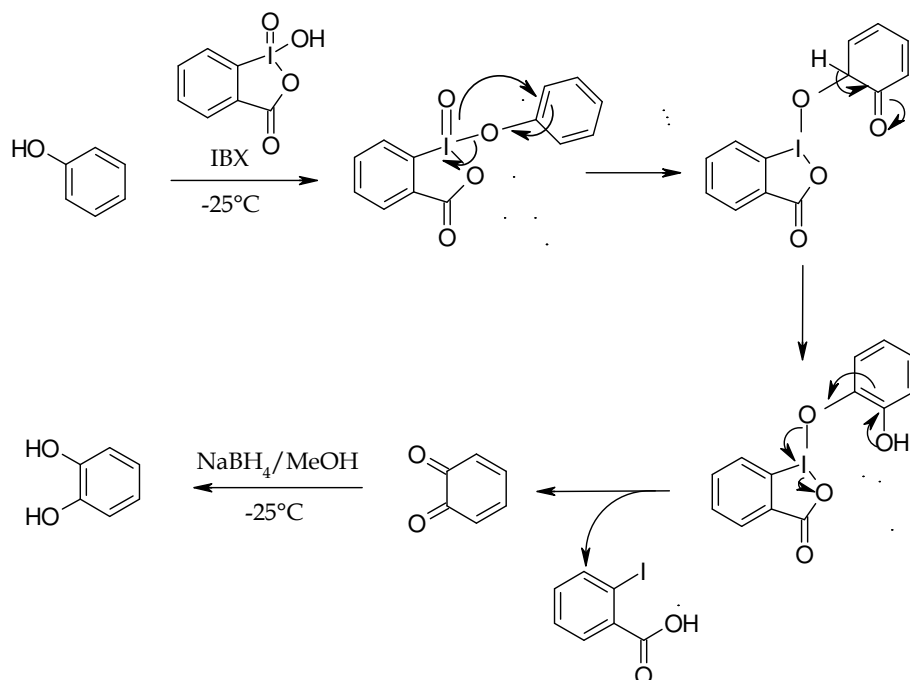
In a typical procedure, solid IBX was added to a solution of **17 β E** or estrone (**16**) in CHCl₃/MeOH mixture at -25 °C. After 24 h methanolic NaBH₄ was then added at -25 °C under vigorous stirring and NaBH₄ excess was removed by a mild acidification with acetic acid. The mixture was washed with saturated NaCl solution containing 10% sodium dithionite buffered at pH 7.0 with sodium phosphate. The two catechol estrogens were separated by preparative TLC.

This procedure differs from previous IBX-mediated oxidations¹⁴²⁻¹⁴⁴ in that the key steps are run in the cold, to prevent quinone conversion to intractable materials in the chloroform-containing medium,⁸⁵ and the critical reductive treatment is efficiently carried out with methanolic NaBH₄ under homogeneous phase conditions. The latter treatment allowed product recovery in good-to-high yields, without affecting the carbonyl at C-17 of estrone substrates **17** and **18**. Chemoselective reduction of the quinone moiety, sparing the carbonyl function of **17** and **18**, was made possible by the low temperature maintained during NaBH₄ treatment and the cold acid quenching of the

mixture; partial reduction occurred when the treatment was performed at room temperature and/or the cold acid quenching step was omitted prior to solvent evaporation.

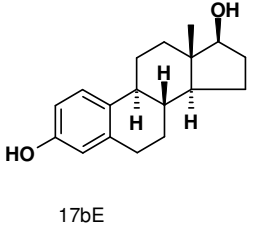
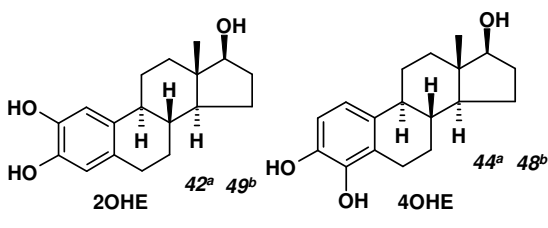
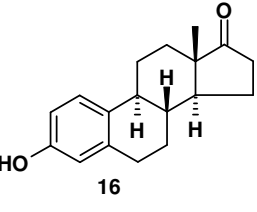
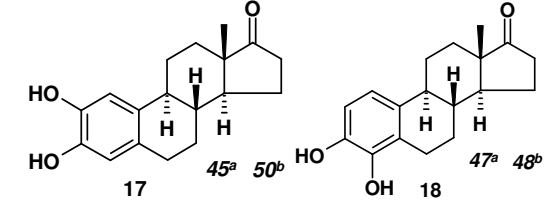
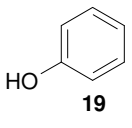
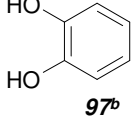
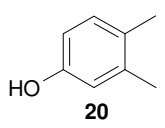
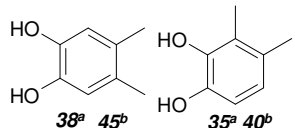
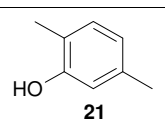
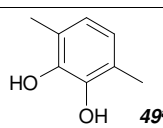
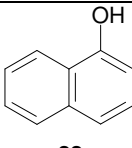
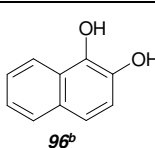
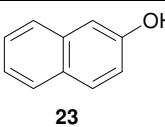
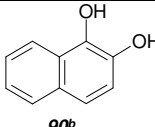
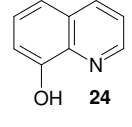
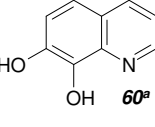
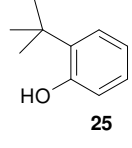
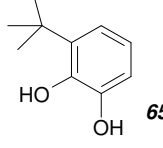
For comparative purposes, and to assess its potential and scope for phenolic oxidation, the procedure was extended to a number of representative substrates. These included the parent phenol (**19**), 3,4-dimethylphenol (**20**), 2,5-dimethylphenol (**21**), 1-naphthol (**22**), 2-naphthol (**23**), 8-hydroxyquinoline (**24**), and 2-tert-butylphenol (**25**). Reaction conditions, products and yields are provided in Table 1.

The reaction proceeded smoothly with complete substrate consumption in all cases and resulted in good to-high product yields. Data in Table 1 indicate that the reaction allows for the regioselective conversion of monophenols to *o*-diphenols (catechols), and that in few cases, that is, **17βE**, estrone, and **20**, two ortho regioisomers are produced in comparable yields. This is a reflection of the comparable steric hindrance and reactivity on the two positions ortho to the OH group. The IBX-induced conversion of phenol to catechol with a brief mechanistic description^{142,143} is illustrated in Scheme 3.



Scheme 3. Proposed mechanism of *o*-hydroxylation/oxidation of phenol

Table 1. IBX-promoted o-hydroxylation of estrogens and other phenols

Substrate	Concn (mM)/IBX equiv	Solvent	Reaction time	Product(s)/yield(s)(%)
 17bE	20/2.5	CHCl ₃ /MeOH 3/2	24 h	 2OHE 42 ^a 49 ^b 4OHE 44 ^a 48 ^b
 16	20/2.5	CHCl ₃ /MeOH 3/2	24 h	 17 45 ^a 50 ^b 18 47 ^a 48 ^b
 19	90/2.0	CHCl ₃ /MeOH 4/1	20 min	 97 ^b
 20	100/1.5	CHCl ₃	12 h	 38 ^a 45 ^b 35 ^a 40 ^b
 21	100/1.5	CHCl ₃	12 h	 49 ^a
 22	70/1.5	CHCl ₃	12 h	 96 ^b
 23	70/1.5	CHCl ₃	12 h	 90 ^b
 24	60/2.0	CHCl ₃ /MeOH 4/1	1 h	 60 ^a
 25	60/2.0	CHCl ₃ /MeOH 4/1	1 h	 65 ^a

^a Determined on products isolated by TLC on silica (impurities below ¹HNMR detection limits)

^b Formation yield (determined by HPLC by comparing peak area with external calibration curves)

Use of cold methanolic NaBH₄ after oxidation of phenols **19–25** proved to be critical for efficient quinone reduction since, with CHCl₃ or a CHCl₃/MeOH mixture as the solvent, a simple reductive work-up with sodium dithionite at room temperature¹⁴³ was not entirely satisfactory leading in some cases to a poor recovery of the catechol products. In a previous paper¹⁴⁴ Quideau et al. Reported oxidation of 2,5-dimethylphenol (**21**) with Stabilized IBX (SIBX), a non-explosive alternative to IBX, in THF to give as main product a dimeric species arising from hydroxylation at the carbon bearing the methyl group. We did not notice the presence of that dimer in our mixture, so it is likely that the different regioselectivity observed in the present study reflects the change of solvent and/or temperature favoring attack of the oxygen to the unsubstituted position of the aromatic ring. The potential of IBX for the conversion of phenols to *o*-quinones and catechols was also underscored in a recent paper,¹⁴² in which a number of phenols substituted with electron-donating groups were shown to undergo regioselective oxidation. The procedure described herein not only stands comparison with the previous one, as judged from the reported oxidation of **23** in CDCl₃,¹⁴² but expands its scope to include the estrogens and several other phenolic compounds. Particularly worthy of note is the conversion in good yield of phenol itself, for which the IBX-mediated hydroxylation was reported to be unsuccessful.¹⁴²

10 β -substituted 17 β -hydroxyestra-1,4-dien-3-ones preparation¹⁴⁵

As part of our studies toward the preparation of functionalized steroidal scaffolds of potential investigative value and/or to be evaluated as pharmaceutical agents, we have developed operationally simple and convenient procedures for the one-pot conversion of **17 β E** to 10 β ,17 β -dihydroxyestra-1,4-dien-3-one (**26**) and 10 β -chloro-17 β -hydroxyestra-1,4-dien-3-one (**27**).



Several oxidants, such as potassium permanganate, lead tetraacetate, diacetoxyiodobenzene, sodium periodate, potassium persulfate were tested for their ability to bring about direct conversion of **17 β E** to the quinol **26**. Of these, potassium permanganate proved to be the most efficient in producing the desired quinol in good yield. The reaction was investigated under different experimental conditions, and an optimized procedure was eventually developed, using a acidic water/ethyl acetate 1:1 as solvent and 2 molar equivalents of the oxidant. By this method, complete substrate consumption was observed in less than one minute, and the desired quinol **26** was obtained in 75% isolated yield in pure form in the organic phase. Product identity was determined by spectral analysis and comparison with literature data; the stereochemistry of the C-10 centre was confirmed from the CD spectrum¹⁴⁶ showing a negative Cotton effect (figure 15) similar to that reported for 10 β -substituted compounds.^{117, 146-148}

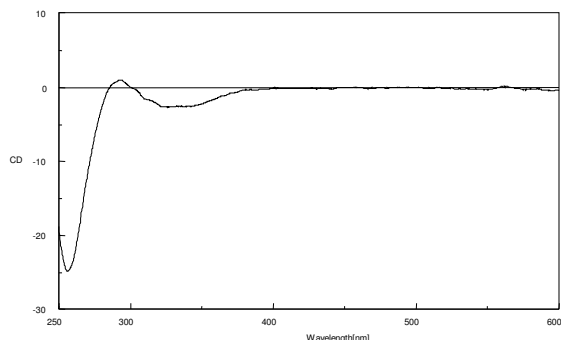


Fig.15: CD spectrum of compound **26**

To the best of our knowledge, this is the first method for preparing **26** by direct oxidation of **17 β E** without functional group protection and chromatographic separation.

10 β -Chloro-17 β -hydroxyestra-1,4-dien-3-one (**27**) was prepared in 44% isolated yield (48% formation yield as determined by HPLC) by treating **17 β E** with 2 molar equivalents of NaClO₂. After 30 min, substrate

consumption was complete, and compound **27** was obtained by extraction of the mixture with ethyl acetate, followed by chromatographic purification. Configuration at C-10 was determined by CD analysis.¹¹⁷ Under these reaction conditions no formation of **26** was observed.

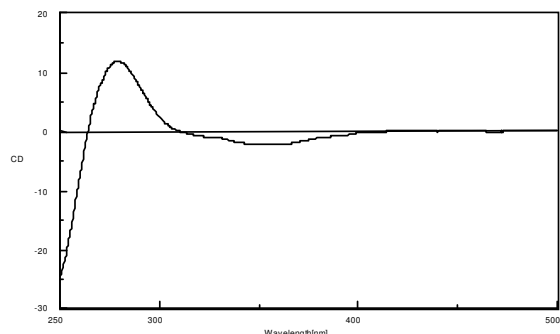
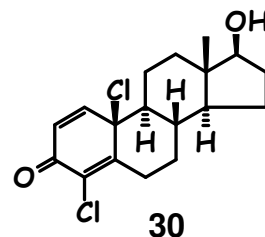
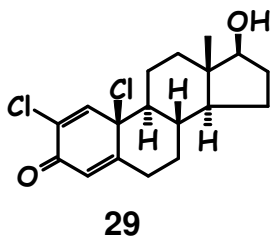
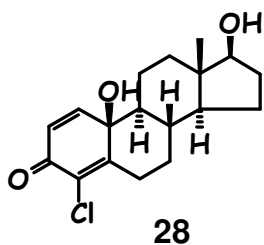


Fig. 16: CD spectrum of compound **27**

components which were purified by preparative TLC and subjected to complete spectral analysis. The most polar compound was characterized as the novel 4-chloro-10 β ,17 β -dihydroxyestra-1,4-dien-3-one (**28**). The CD spectrum supporting the assignment of configuration at C-10 is shown in figure 17. The other two products were identified as 2,10 β -dichloro-17 β -hydroxyestra-1,4-dien-3-one (**29**), and 4,10 β -dichloro-17 β -hydroxyestra-1,4-dien-3-one (**30**) previously obtained by chlorination of **17 β E** with *N*-chloro imide reagents.¹¹⁷



The procedures here disclosed for the preparation of the 10 β -substituted 17 β -hydroxyestra-1,4-dien-3-ones **26** and **27** are amenable to gram scale preparations and represent valuable alternatives to previous methodologies because of the lack of protection/deprotection steps, simple work-up, and use of cheap, non-toxic reagents.

For both products **26** and **27** complete resonances assignment including identification of β protons (Table 2) was obtained by 2D (COSY, HMQC, HMBC, and ROESY) NMR analysis. Coupling constants (Table 3) were obtained by DQF-COSY experiments.

Careful analysis of the reaction mixture of **17 β E** with NaClO₂ revealed the presence of minor

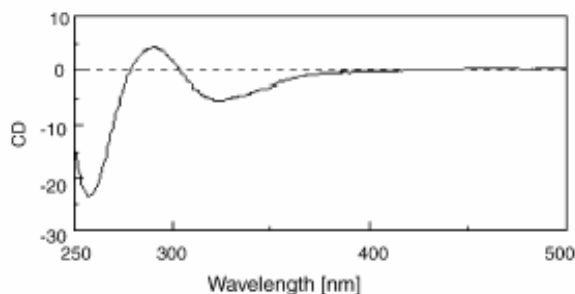


Fig. 17: CD spectrum of compound **3**

Table 2. Selected NMR data for compounds **26-27**^a

	26		27	
	δ_C	δ_H	δ_C	δ_H
C-1	151.1	6.98	147.9	7.13
C-2	131.3	6.34	126.6	6.18
C-3	186.0	-	185.0	-
C-4	126.1	6.17	123.8	6.06
C-5	165.2	-	161.1	-
C-6	32.3	2.45 (β), 2.33	32.3	2.85 (β), 2.41
C-7	33.4	1.95 (β), 1.06	32.3	1.96 (β), 1.03
C-8	35.1	1.97	35.8	1.96
C-9	55.6	1.20	53.4	1.34
C-10	76.3	-	67.7	-
C-11	22.7	1.83 (β), 1.68	22.9	1.89 (β), 1.80
C-12	36.3	1.99 (β), 1.05	35.9	1.91 (β), 1.12
C-13	43.2	-	43.0	-
C-14	49.9	0.92	49.4	1.02
C-15	23.6	1.33 (β), 1.62	23.5	1.38 (β), 1.61
C-16	30.4	1.45 (β), 2.04	30.4	1.50 (β), 2.09
C-17	81.6	3.65	81.4	3.65
C-18	11.0	0.83	11.0	0.85

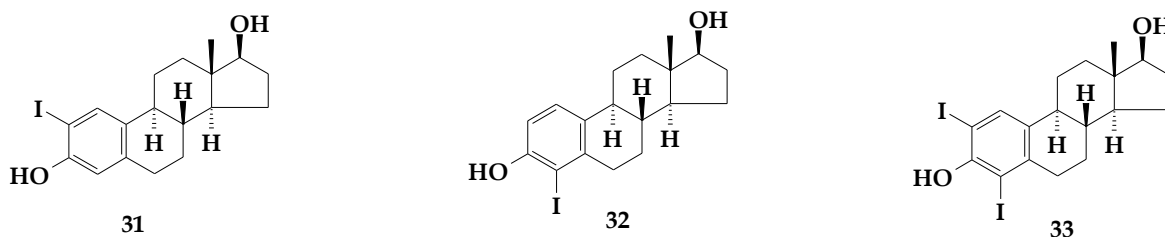
^a spectra were taken in CDCl₃.**Table 3.** *J*(H,H) absolute values (Hz)

Atoms	26	27
1,2	10.4	10.4

2,4	2.0	2.0
4,6 β	1.7	1.6
6 α ,6 β	13.2	13.2
6 α ,7 α	4.4	4.4
6 α ,7 β	2.6	2.4
6 β ,7 α	13.4	13.6
6 β ,7 β	4.4	4.8
7 α ,7 β	13.0	12.0
7 α ,8 β	11.2	11.2
7 β ,8 β	n.d.	n.d.
8 β ,9 α	12.9	12.0
8 β ,14 α	10.8	11.0
9, 11 α	4.0	4.8
9, 11 β	11.6	n.d.
11 α ,11 β	13.2	13.2
11 α ,12 α	4.5	4.5
11 α ,12 β	2.2	2.0
11 β ,12 α	12.0	12.0
11 β ,12 β	n.d.	4.4
12 α ,12 β	13.4	11.2
14 α ,15 α	7.4	6.8
14 α ,15 β	12.5	11.6
15 α ,15 β	11.8	11.6
15 α ,16 α	10.2	8.8
15 α ,16 β	3.4	3.6
15 β ,16 α	5.4	5.6
15 β ,16 β	11.4	12.0
16 α ,16 β	12.9	9.2
17 α ,16 α	8.6	7.6
17 α ,16 β	8.3	8.0

Halogenation of 17 β E

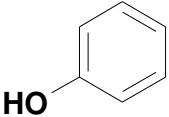
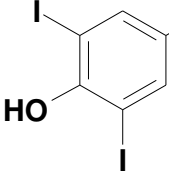
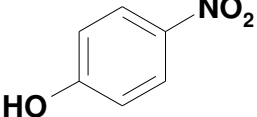
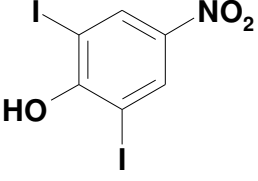
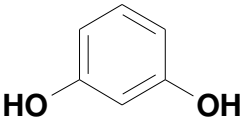
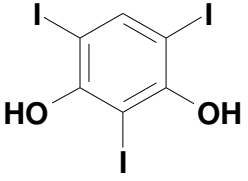
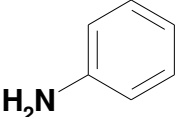
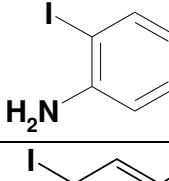
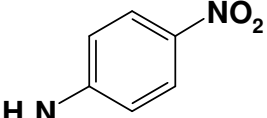
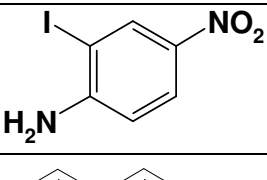
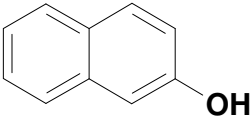
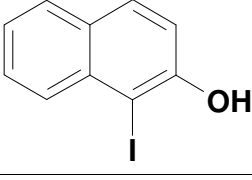
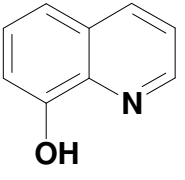
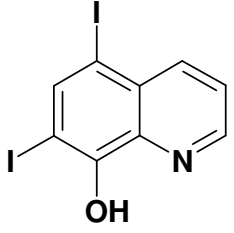
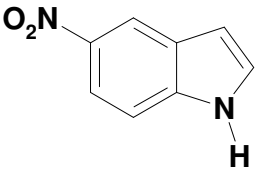
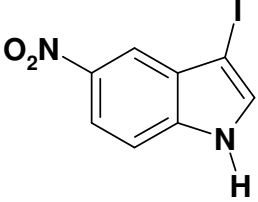
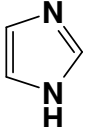
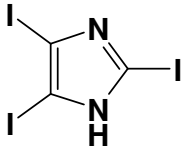
In connection with our ongoing program on NaClO₂ mediated oxidations of 17 β E, we envisioned a simple route, which might involve iodination of 17 β E to give 2-, 4-iodoestradiol and 2,4-diiodoestradiol (**31**, **32** and **33** respectively). The iodination involves use of NaI as the iodine source that is added to NaClO₂ reaction mixture. Although the mechanism for this iodination and for other methods reported in literature is uncertain, it is proposed that the oxidation of an iodide anion results in the in situ formation of an electrophilic iodonium species (I⁺), which is responsible for iodination of the aromatic nucleus.¹⁴⁹ Regulation of the equivalents of the iodinating reagents allows to select **31**, **32** and **33**. So iodination of 17 β E with NaClO₂/NaI at the equivalents ratio of 0.5/0.5 gives **31** with no total substrate consumption. Using NaClO₂/NaI 4/8, TLC analysis of the reaction confirms the formation of **32** and **33** in the ratio of 1/1 with complete conversion of substrate.



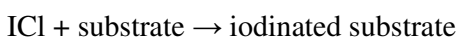
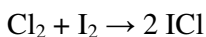
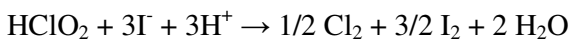
To establish the scope and potential of the methodology, a variety of representative aromatic compounds have been subjected to nuclear iodination and the results are shown in Table 4.

The peculiarity of procedure consists in total consumption of substrate and complete conversion to only iodinated product, as demonstrated by NMR and mass spectroscopy analysis. Moreover, the reagents reported for iodination of aromatic substrates include *N*-iodosuccinimide,¹⁵⁰ I₂-mercury salts,¹⁵¹ I₂-nitrogen dioxide,¹⁵² I₂-chromium oxide,¹⁵³ iodine monochloride,¹⁵⁴ NaOCl-NaI,¹⁵⁵ NH₄I-oxone[®],¹⁵⁶ and most of these reagents are complicated, costly or use-toxic heavy metal catalysts with potential environmental problems due to the generation of hazardous waste. So a quick, inexpensive, easy and environmentally benign method for iodination is looked-for and this procedure has such features. Also, by this methodology 3-iodo-5-nitroindole has been obtained with good yield and this is worthy of note because is the first preparation reported in the literature.

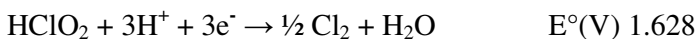
Table 4. Iodination of selected phenols by NaClO₂/NaI method

Substrate	Concn (mM) NaClO ₂ eq/NaI eq	Reaction time	Product	Yield (%)
	27 2/4	1h		80
	18 2/4	30min		95
	23 2/4	30min		73
	27 2/4	1.5h		98
	18 4/8	10min		95
	17 1/2	18h		87
	17 1/2	40min		98
	62 1/2	30min		75
	147 2/4	3h		45

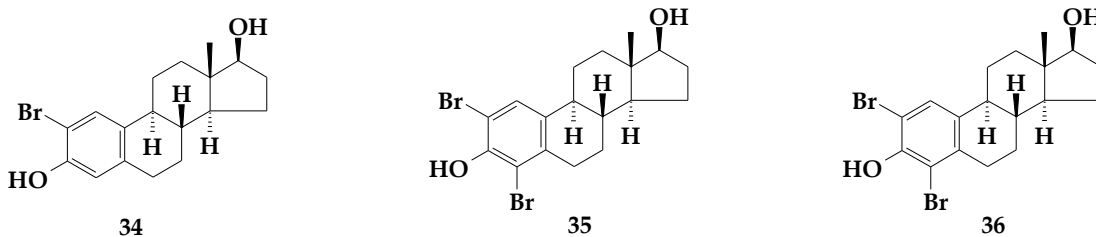
A possible mechanism of this iodination technique is based on capability of NaClO_2 to oxidize iodide anion in presence of acid to liberate I_2 . At the same time ClO_2^- is reduced to Cl_2 and, according to a reported hypothesis,¹²⁸ iodine monochloride, presumably formed from the liberated halogens, may act as the electrophile.



In view of standard reduction potential of $\text{ClO}_2^-/\text{Cl}_2$ and I^-/I_2 , the mechanism outlined above is likely from electrochemical point of view.



During iodination experiments it was added NaBr instead of NaI and bromination of estradiol ring A was noted. Reaction conditions are identical to iodination procedure and also in that case modulation of brominating reagents allows to select mono- and dibromination. In particular reaction of **17 β E** with $\text{NaClO}_2/\text{NaBr}$ in the equivalents ratio of 0.5/0.5 gives 2-bromoestradiol (**34**) and no total substrate consumption. Using $\text{NaClO}_2/\text{NaBr}$ 2/4, TLC analysis of the reaction confirms the formation of 2,4-dibromoestradiol (**35**) and 2,4-dibromoestrone (**36**) with complete conversion of substrate.

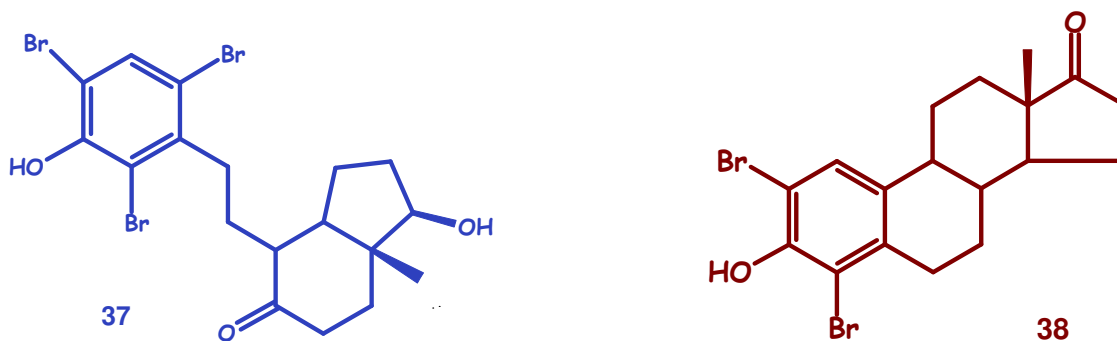


Another method of estradiol bromination has been performed by the use of NaBrO_3 combined with a reducing reagent such as $\text{Na}_2\text{S}_2\text{O}_4$. This coupling serves as an effective bromohydroxylation reagent of olefins,¹⁵⁷ alkynes, and allylic alcohols¹⁵⁸ and an oxidizing agent of primary alcohols,¹⁵⁹ diols, and ethers.¹⁶⁰ Moreover the $\text{NaBrO}_3/\text{Na}_2\text{S}_2\text{O}_4$ reagent in organic synthesis facilitates the R-bromination of alkylbenzenes under a two-phase system using ethyl acetate as solvent under ambient conditions.¹⁶¹ This procedure has never been applied to phenolic substrates, so we have used this reagent to obtain, possibly, brominated estradiol.

Owing to the difficulty of isolating any brominating species derived from $\text{NaBrO}_3/\text{Na}_2\text{S}_2\text{O}_4$ reagent in $\text{AcOEt}/\text{H}_2\text{O}$ and the complexity of the reaction medium employing the two-phase system, it seems rather hazardous to make an accurate assessment about the nature of the brominating species involved in this reaction. However, Kikuchi et al.¹⁶¹ have made several proposals, which seem to

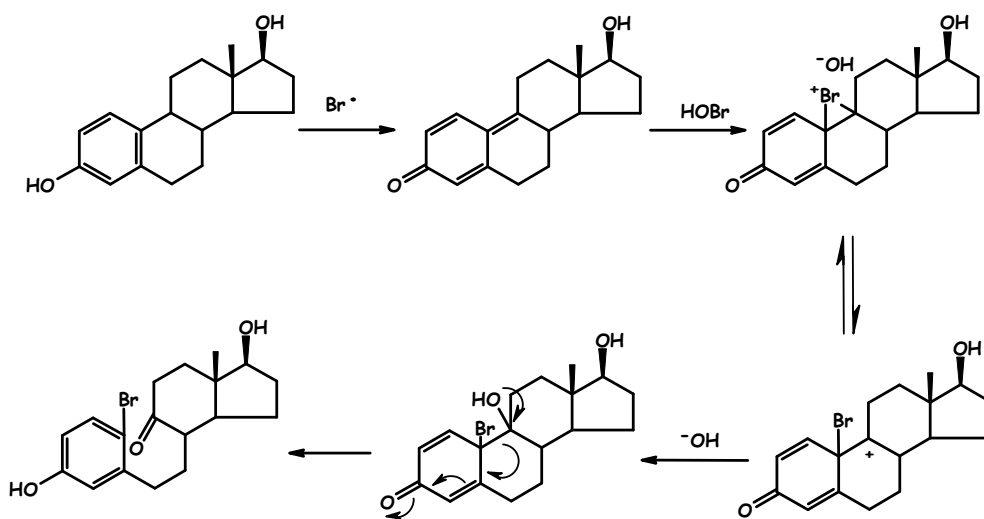
agree with their experimental results. The reaction is initiated by the generation of HOBr from NaBrO₃/ Na₂S₂O₄ in the aqueous phase, and then the resulting HOBr is thought to decompose gradually in aqueous solution with liberation of Br[•] or a Br[•] equivalent, which moves from the aqueous phase to the ethyl acetate phase, in which the substrate is dissolved. As a consequence, the bromination of **17βE** takes place.

To a two-phase system comprised of ethyl acetate involving **17βE** and aqueous NaBrO₃ (30 equiv) was added dropwise aqueous Na₂S₂O₄ (30 equiv) over a period of about 15 min under stirring and the mixture was allowed to react at room temperature for 4 h. The reaction produced 3-hydroxy-2,4,10-tribromo-9,10-secoestra-1(10),2,4-trien-9-one (**37**) along with 2,4-dibromoestrone (**38**).



The proposed mechanism of secoestra-derivate formation is outlined in scheme 4 and involves oxidation of **17βE** to quinone methide by Br[•], formation of bridged bromonium ion and B ring opening by hydroxide ion.

Besides the general pharmaceutical use of halogenated derivatives of estrogens, the halogenated secosteroidal compounds have been shown to be potential estrogen receptor-based imaging agents for human breast tumors.¹⁶²



Scheme 4. Schematic outline of the proposed mechanism of formation of product **37**

OXIDATION OF 17 β E IN SOLVENT FREE

Solvent free conditions have been employed in oxidation reactions of 17 β E to obtain steroidal derivatives by simple, eco-friendly and good-to-high yielding procedures. In fact solvent removal reduces environmental impact and inserts the processes in the green chemistry that is aimed to development of eco-compatible synthetic methodologies. Moreover solid phase procedure allows of simple work-up of reaction mixture and removes solubility problems of 17 β E in basic and acid conditions.

Optimized experimental procedure for preparation and work-up of reaction mixture is below reported:

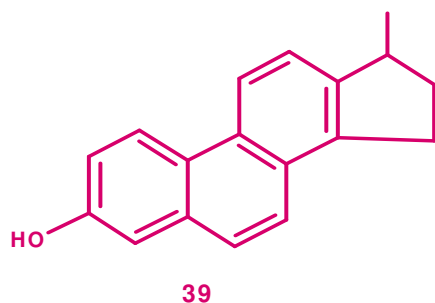
1. a mixture of substrate and oxidant is finely powdered in agate mortar and pestle;
2. the mixture is put in a test tube and kept at elevated temperature for variable time intervals.
3. when substrate consumption is complete (TLC evidence), the mixture is diluted with water, acidified and extracted with ethyl acetate;
4. if necessary, the crude mixture is subjected to chromatographic purification.

Among the tested chemical oxidants, FeCl₃ and FeNH₄(SO₄)₂ have provided interesting results.

In both of the oxidants, solid phase conditions have been optimized varying the temperature, the reaction time and the oxidant equivalents to improve substrate conversion and reaction yields.

Reaction with FeNH₄(SO₄)₂

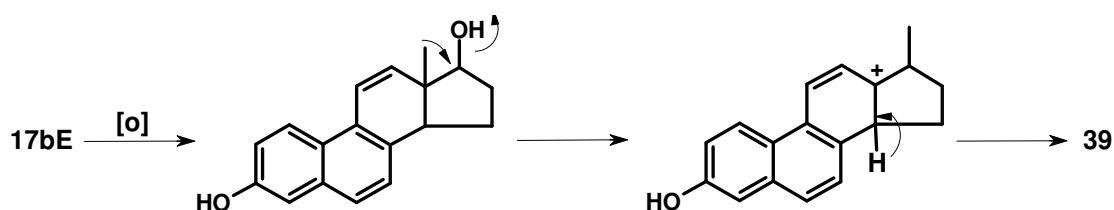
The oxidation of 17 β E is carried out with 2 equivalents of FeNH₄(SO₄)₂ for 6 h at 150 °C and gives a principal product identified as 3'-methyl-7-hydroxy-1,2-cyclopentenophenanthrene (**39**) on basis of spectral analysis. Pseudo-molecular ion peak at *m/z* 247 (ESI(-)MS) is an indication of molecular dehydration and dehydrogenation. ¹H NMR shows in the aromatic region seven signals (four doublets and an ABX system) and five, as well as methyl signal, in the aliphatic region. The scalar contact of methyl signal with H17 and the shift of this proton from 3.60 to 3.40 ppm provides support to the methyl migration together with the desaturation of rings B and D to afford a phenanthrene system.



This is the first reported isolation of this compound, being the sole related compound described in the literature some derivatives functionalized by alkyl groups on phenolic oxygen. For example, 3'-methyl-7-methoxy-1,2-cyclopentenophenanthrene was obtained heating methylated estradiol, 3-methoxy-estra-1,3,5(10)-trien-17 β -ol, with 78% (w/w) sulphuric acid for 20 minutes at 100 °C.¹⁶³ The reaction mixture required then separation and purification step. By the solvent free procedure, instead, complete substrate consumption is observed and the aromatised and dehydrated product is obtained in 67 % isolated yield in pure form.

The hypothesized mechanism, outlined in scheme 5, involves dehydrogenation without opening of D ring, dehydration, methyl migration with tertiary carbocation formation and proton extraction to obtain cyclopenta[*a*]phenanthrene. This hypothesis derives from isolation of a naphthalenic intermediate after 3 hours of reaction.

The stereochemistry of C17 is not well defined, the measurement of optical rotatory power $[\alpha]_{25^\circ C}^{589nm} = 0.016^\circ$ supports a racemic mixture proposal.

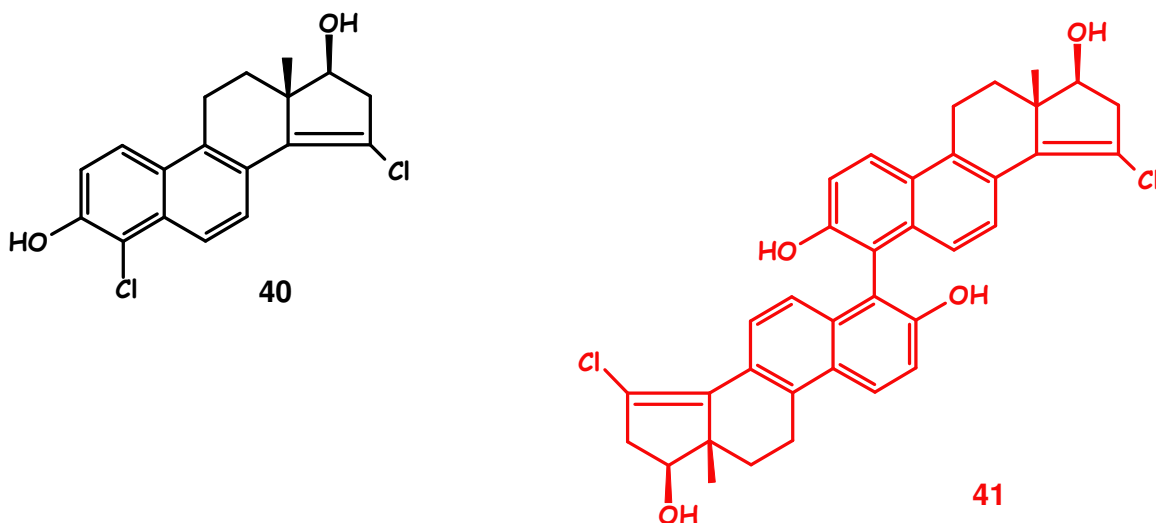


Scheme 5. Schematic outline of the proposed mechanism of formation of products **39**

The cyclopenta[*a*]phenanthrenic derivatives of **17 β E** are considered potential anticholesteremic and hypolipidemic agents, in fact in vivo experiments have shown a significant plasma cholesterol lowering in hypercholesteremic rats treated with these compounds.¹⁶⁴

Reaction with FeCl₃

The oxidation of **17βE** is carried out with 8 equivalents of FeCl₃ for 6 h at 60 °C and gives two main products, not known in the literature, obtained through PLC fractionation. The mass and NMR data led to straightforward formulation of desaturated and chlorinated species, in particular a monomer (4,15-dichloroestra-1,3,5(10)-trien-3,17β-diol) (**40**) and a C₂-symmetric 4,4'-di[15-chloroestra-1,3,5(10)-trien-3,17β-diol] (**41**)



In fact, the monomer exhibits pseudo-molecular ion peak at m/z 333 (ESI(-)MS) and two peaks at 335 [M+2], 337 [M+4] in a ratio 100: 65: 12, indicating two chlorine atoms presence. The dimer shows pseudo-molecular ion peak at m/z 597 (ESI(-)MS) with an isotopic pattern like monomer. Moreover the two products show nearly identical ¹H NMR spectra featuring in the aromatic region four doublets and in the aliphatic region six multiplets as well as methyl and H17 signals.

The dimer is a very interesting compound because of its bissteroidal BINOL-type structure. BINOL and BINAP, which are employed in numerous catalytic and stoichiometric asymmetric reactions, and have attracted great attention over the last several years as ligands with C₂-symmetry. Nevertheless there are at least two drawbacks for these compounds. The enantiomers of BINOL and/or BINAP have to be separated by 1) transforming the racemic compounds into diastereoisomeric derivatives; 2) separation of these derivatives; and 3) retransformation into BINOL or BINAP.¹⁶⁵ Therefore the development of a synthetic approach making the BINOL-type ligands available in their diastereopure forms is a subject of increasing interest.

It is possible to hypothesize diastereoselectivity of this reaction leading to the chlorinated dimer on basis of collected data and on previous knowledge of rotational isomers of estradiol dimers,⁶² but a crystallographic analysis could resolve any doubts.

The dimer exhibits two asymmetric centers (C13 and C17) in every unit and an axially chiral center at the biphenyl linkage, so the two possible rotational isomers are diastereoisomers. Hence their physical and chemical properties have to be different. Indeed HPLC, TLC, NMR analysis of chromatographic band do not evidence the presence of two species. Furthermore no appreciable interconversion of the rotamers is observed by heating to 180°C at which temperature the product begins to decompose significantly as previously shown for C_2 -symmetric 4,4' dimer [cfr. page 15].

CONCLUSIONS

In this work estrogen oxidative chemistry has been studied addressing two main issues: the elucidation of the molecular mechanisms of neuro- and cytoprotection as well as carcinogenic activities that **17 β E** exerts in several biological compartments; the exploitation of oxidative transformations as access routes to new steroidal scaffolds of potential biomedical and pharmaceutical relevance.

So the work may be ideally divided in two sections: experiments carried out under biomimetic conditions to examine **17 β E** biological activity related to its redox properties; and experiments using oxidative transformation as preparative tools for new steroidal derivatives or of compounds well known in literature and already used.

If it is true the redox behaviour of **17 β E** may be considered the key of its cytoprotective activity^{46, 47} it is also that the same reactivity is involved in estrogenic induced carcinogenesis.^{55, 56, 57} According to literature cytoprotection is essentially ascribed to an antioxidant activity showed by **17 β E** under oxidative stress condition^{46,47} while formation of highly reactive oxidation derivatives of **17 β E** (i.e. 2- and 4-hydroxyestradiol) plays a pivotal role in the molecular mechanism of estrogen carcinogenesis associated to an alteration of cellular redox homeostasis in the tissues with high levels of estrogens (ovary, uterus...). In fact **17 β E** is metabolised in vivo to catechols, which are then oxidized to redox active/electrophilic *o*-quinones that could initiate the carcinogenic process by binding to DNA and depurination processes.⁵⁷

On the other hand new steroidal derivatives based on **17 β E** modification appears to be promising and sometimes effective scaffold for a wide range of application from pharmaceutical to high tech fields. In this prospective oxidative chemistry may be an innovative access tool to unprecedented estradiol functionalized derivatives often difficult if not impossible to get by traditional synthetic approach.

In this double frame the first issue was addressed carrying out biomimetic oxidation in aqueous phosphate buffers using as oxidants the enzyme systems peroxidase/H₂O₂ and tyrosinase/O₂, both evidencing the susceptibility of **17 β E** to oxidation in such conditions.

In fact a substantial substrate consumption was observed with the peroxidase/H₂O₂ system, with formation of a number of products whose chromatographic and spectral properties were suggestive of oligomer species. In particular five dimers and three trimers have been isolated and in the case of the trimers and a dimer is the first isolation reported in the literature. The coupling involves the only A ring through the formation of biphenyl and ether-biphenyl linkage. In all C-coupling products

involving C4, the sterically hindered biphenyl linkage represents a stereogenic element, which adds to those already present in **17βE**. For all isolated products featuring such structural system, configuration at the biphenyl linkage (and thus absolute stereochemistry) was established by the exciton chirality method on the basis of the Cotton effect, and interconversion energy was also determined by dynamic NMR. Overall this study confirms **17βE** antioxidant properties and provides coupling products of **17βE** that lay the foundation for the future studies aimed to develop novel estrogen derivatives based on oligomeric scaffolds.

17βE oxidative transformations induced by tyrosinase/O₂ have also been investigated on the base of putative molecular mechanism underling carcinogenesis induction. Tyrosinase is the key enzyme of melanogenesis in epidermal melanocytes⁷² and possesses both monophenol monooxygenase activity (EC 1.14.18.1, tyrosine, 3,4-dihydroxyphenylalanine:oxygen oxidoreductase) and *o*-diphenoloxidase activity (EC 1.10.3.1, *o*-diphenol:oxygen oxidoreductase). Tyrosinase-like proteins have recently been identified in estrogen-responsive tissues as well as in the nervous system.⁷⁴⁻⁷⁶ Such enzymes from nuclear extracts were found to possess both cresolase and catecholase activity, as well as estrogen binding properties, and may thus play a role in the conversion of **17βE** into its hydroxylated metabolites.

Mushroom tyrosinase was selected as a convenient oxidizing system for producing CEQs from **17βE** and for monitoring their fate under physiologically relevant conditions. At variance with the mammalian enzyme from melanoma cells,⁷⁷⁻⁷⁹ which is apparently unable to induce estradiol hydroxylation, mushroom tyrosinase displays efficient cresolase activity toward this estrogen and is highly effective in causing catechol estrogen oxidation to CEQs.

The reaction of **17βE** with the tyrosinase/O₂ system was examined with substrate concentration in the range 1nM-30μM in phosphate buffer at pH 7.4. HPLC analysis of the ethyl acetate-extractable fraction the oxidation mixture evidenced a complex reaction pathway including the presence of the catechol estrogens 2OHE and 4OHE along with a series of species with mass spectra suggestive of transformation products of 2OHE and 4OHE. The main peaks were isolated by preparative HPLC and were characterized by extensive 2D NMR and mass spectrometric analysis. The same products, with more high yields, have been obtained by the tyrosinase-catalyzed oxidation of 2OHE and 4OHE prepared by a simple and convenient one-pot procedure developed ad hoc and involving phenol IBX-mediated *o*-hydroxylation.¹²⁹ Hence these observations have made easier products isolation on a preparative scale and have evidenced tyrosinase capability to hydroxylate **17βE** and to oxidize CE, generating species that result from *o*-quinones. Some products, and in particular the

naphtalenic one, have been difficultly isolated because they easily react with CE to give dimeric species.

To test the potential significance of this **17 β E** oxidative pathway, the effects of some nucleophilic compounds on the oxidation of the catechol estrogens were investigated. Selected nucleophiles included adenine,⁹⁴ thymine,⁹⁵ cytosine,⁹⁵ 2-deoxyguanosine,⁸⁰ imidazole⁸³ and glutathione^{96, 97} for which the ability to trap CEQs was established. The data indicated that with both catechol estrogens, glutathione was by far the most effective inhibitor of product formation, due likely to the ability of thiols to cause quinone reduction and/or trapping.⁹⁶

Appreciable changes in the relative product yields were observed with the other nucleophiles, suggesting that they affected the reaction course at different stages and with different modalities. Thus, free DNA bases were more effective at inhibiting product formation from 4OHE than from 2OHE. However, even in presence of a 4-fold excess of the nucleophiles, formation of products **9-11** and **15** remained significant with the substrates at 10 μ M concentration, and this would *a fortiori* be so at lower more physiological concentrations. In all cases, catechol estrogen consumption was not affected by the additives, indicating that the inhibitory effects were indeed due to the trapping of oxidized intermediates. The results yield further insights into the conversion pathways of the CEs beyond the *o*-quinone stage and the isolated products could potentially be able to interact with biological macromolecules. So cytotoxicity mechanism of estrogens could be more complex than commonly accepted one and based on adducts of estrogen *o*-quinones with DNA or proteins.

In connection with the second issues, use of oxidative transformations as access routes to new steroidal scaffolds, a number of different oxidants and/or halogenating agents as well as reaction medium were exploited including aqueous, organic, biphasic and solvent free conditions.

Indeed halogenated, oxy-functionalized and/or desaturated on steroidal backbone estrogenic derivatives find interesting application in several fields and simple and in good yield preparation procedures are useful to gram scale preparations.

Valuable results are obtained through preparation of 10 β -substituted-17 β -hydroxyestra-1,4-dien-3-ones, class of estradiol-related compounds that have found application in estrogen replacement therapy, prevention, and treatment of osteoporosis,¹⁰⁴ in the detection and treatment of hormone dependent tumors,¹⁰⁵ or for the prevention and therapy of ophthalmic diseases.¹⁰⁶ 10 β -hydroxy- and 10 β -chloroderivate were prepared. The former, also defined p-quinol, was produced by KMnO₄ (2eq) using an acidic water/ethyl acetate 1:1 as solvent. By this method, complete substrate consumption was observed in less than one minute, and the desired quinol was obtained in 75% isolated yield in pure form in the organic phase. The stereochemistry of the C-10 centre was

confirmed from the CD spectrum¹⁴⁶ showing a negative Cotton effect similar to that reported for 10 β -substituted compounds.^{117, 146-148} This one-pot procedure represents an alternative to previous methodologies because of the lack of protection/deprotection steps, simple work-up, and use of cheap, non-toxic reagents. In addition, the identification of the *p*-quinol in the redox cycling mechanisms underlying the putative antioxidant and cytoprotective properties of **17 β E**,¹⁰⁷ suggested its employment as prodrug of antioxidants,¹⁰⁸ while the 10 β -halo-derivatives have been shown to be valuable tools for probing interactions at estrogen receptors.¹⁰⁹ 10 β -Chloro-17 β -hydroxyestra-1,4-dien-3-one was prepared by treating **17 β E** with 2 molar equivalents of NaClO₂ in acidic aqueous methanol. Also in this case, the procedure represents an alternative to previous methodologies involving expensive organic chlorinating agents and separation and purification of complex reaction mixture.

Introducing NaI in NaClO₂ reaction mixture, was obtained a practical and versatile estradiol iodination method. In particular, varying NaClO₂ and NaI equivalents was possible to chemoselect iodination position on estradiol A ring. To establish the scope and potential of the methodology, a variety of representative aromatic compounds have been subjected to nuclear iodination and it was resulted rapid and chemoselective. Halogenations of **17 β E** and generally of aromatic compounds are highly useful reactions for providing the precursors to a number of organometallic species applicable in the synthesis of natural products and pharmaceutically important compounds.¹²¹ Aryl iodides are usually more difficult to prepare than the other corresponding aryl halides due to the low electrophilic strength of iodine. Generally, arenes can be iodinated by iodine or iodide in the presence of complex, expensive, toxic organic oxidizing agents. This method, indeed, requires cheap, non-toxic reagent and eco-friendly reaction conditions.

Substituting anion with Br⁻ was obtained a simple and cheap bromination method of estradiol phenolic ring and also in this case varying NaClO₂ and NaBr equivalents is possible to chemoselect brominated product.

Solvent free conditions have been employed in oxidation reactions of **17 β E** to obtain steroidal derivatives by simple, eco-friendly and good-to-high yielding procedures. In fact solvent removal reduces environment impact and inserts the processes in the green chemistry that is based on development of eco-compatible synthetic methodologies. Among the tested chemical oxidants, FeCl₃ and FeNH₄(SO₄)₂ have provided interesting results.

The oxidation of **17 β E** by FeCl₃ (8 eq for 6 h at 60 °C) gives two principal desaturated and chlorinated products, not known in literature. The mass and NMR data are suggestive of a monomer dichlorinated in C4 and C15 and a C₂-symmetric 4,4' dimer totally conjugated. The dimer is a very interesting compound because of its bissteroidal BINOL-type structure which are employed in

numerous catalytic and stoichiometric asymmetric reactions as chiral ligands and auxiliaries with C_2 -symmetry. The reaction peculiarity is the interesting yield (25%) and the elevated stereoselectivity that avoids laborious steps of derivatization and retransformation associated with separation of stereoisomers mixture.

In the case of $FeNH_4(SO_4)_2$, the oxidation of **17 β E** is carried out with 2 equivalents of oxidant for 6 h at 150 °C and gives a principal product characterized by methyl migration in position 17, OH leaving and B and C ring aromatisation. This solvent free procedure allows to obtain one-pot aromatisation of two condensed rings and represents an alternative to the literature methodologies requiring harsh oxidation conditions as well as purification steps.

In summary, main results afforded by the study here reported are may be collected under this tree point:

1. isolation of new estradiol-related species;
2. thorough examination of potential metabolic pathways of estrogens oxidation *in vivo*;
3. development of synthetic strategies for steroidal derivatives and aromatic compounds;

highlighting both the relevance of oxidative chemistry of **17 β E** to biological processes and the potential of oxidative transformations in the search and synthesis of new derivative of applicative interest.

Experimental Section

General Methods. 17β -Estradiol (**17 β E**), manganese (IV) dioxide activated 5 μm (85%), hydrogen peroxide (30% w/w solution in water), glutathione, 2-deoxyguanosine, cytosine, thymine, adenine, imidazole, 2-iodobenzoic acid, oxone (2 $\text{KHSO}_5\text{-KHSO}_4\text{-K}_2\text{SO}_4$), sodium borohydride, sodium dithionite, potassium permanganate, sodium chlorite, sodium iodide, sodium bromide, phenol, 3,4-dimethylphenol, 2,5-dimethylphenol, 1-naphthol, 2-naphthol, 8-hydroxyquinoline, 2-*tert*-butylphenol were from Aldrich Chemie. Horseradish peroxidase (donor: H_2O_2 oxidoreductase; EC 1.11.1.7) type II and mushroom tyrosinase (EC 1.14.18.1) and tropolone were from Sigma. *O*-Iodoxybenzoic acid (IBX) was freshly prepared from 2-iodobenzoic acid according to a reported procedure.¹³²

Melting points were obtained with a Gallenkamp apparatus. Elemental analyses were performed with a Perkin-Elmer CHN analyzer mod. 2400. Ultraviolet spectra were performed using a diode array Hewlett Packard spectrophotometer model 8453E. CD spectra were taken on Spectropolarimeter Jasco J-715 at 25 °C using solutions of the products in ethanol exhibiting absorbance values in the range 0.1–0.2 at 220 nm. ^1H (^{13}C) NMR spectra were recorded at 400.1 (100.6) MHz using a Bruker DRX-400MHz instrument fitted with a 5mm ^1H broadband gradient probe with inverse geometry. ^1H , ^1H Correlation spectroscopy (COSY), ^1H , ^{13}C heteronuclear multiple quantum coherence (HMQC), ^1H , ^{13}C heteronuclear multiple bond correlation (HMBC), rotating Overhauser effect spectroscopy (ROESY) and double-quantum-filtered correlation spectroscopy (DQF-COSY) experiments were run at 400.1MHz using standard pulse programs from the Bruker library. Electrospray ionization mass spectrometry (ESIMS) spectra were recorded in negative or positive ion mode with a Waters ZQ quadrupole mass spectrometer on samples dissolved in methanol. High resolution electrospray ionization (HRESI) mass spectra were obtained with a Finnegan MAT 90 instrument. Analytical and preparative thin layer chromatography (TLC) analyses were performed on F254 0.25 and 0.5mm silica gel plates. Silver nitrate-impregnated silica gel plates were prepared as described.¹⁶⁶ Sephacryl S 200 HR from Amersham Bioscience was used for tyrosinase purification. HPLC was carried out on an Agilent mod. 1100 apparatus equipped with a UV detector set at 280nm using octadecylsilane coated columns, 250 \times 4.6 mm, or 22 \times 250, 5 μm particle size, (Sphereclone, Phenomenex) for analytical or preparative runs, respectively at a flow rate of 1.0 mL/min or 15 mL/min.

TLC eluants:

40:60 cyclohexane-ethyl acetate (eluant A)

60:40 cyclohexane-ethyl acetate (eluant B)

95:5 ethyl acetate-cyclohexane containing 0.5% acetic acid (eluant C)

50:50 benzene-ethyl acetate containing 0.01% acetic acid (eluant D)

98:2 chloroform-methyl alcohol (eluant E)

HPLC eluants:

70:30 H₂O-acetonitrile (eluant I)

60:40 H₂O-acetonitrile (eluant II)

90:10 H₂O-acetonitrile (solvent A), acetonitrile (solvent B), 0-5 min 30% B, 5-30 min 30-55% B, 30-40 min 55% B (eluant III).

67:30:3 H₂O-acetonitrile-acetic acid (eluant IV)

50:50 1% acetic acid-acetonitrile (eluant V)

PEROXIDASE CATALYZED REACTION

Oxidation of 17βE by the Peroxidase/H₂O₂ System: General Procedure. To a solution of **17βE** (5 mg, 1.9×10^{-5} mol) in methanol (5 mL) were added 0.1 M phosphate buffer, pH 7.4 (60 mL), and peroxidase (1 U/mL) sequentially. The mixture was then treated with hydrogen peroxide in aliquots ($8 \times 2.5 \times 10^{-6}$ mol) every 10 min while being kept under stirring at room temperature. At different time intervals the reaction was carefully acidified at pH 5.0 and extracted three times with ethyl acetate (3 x 60 mL). The combined organic layers were dried over sodium sulfate and analyzed by HPLC (eluant III) and TLC (eluant A). In other experiments the reaction was carried out as above with the substrate at 3×10^{-7} M concentration using peroxidase (0.02 U/mg) and hydrogen peroxide (1 mol equiv)

Oxidation of 1 by MnO₂. A solution of **17βE** (10 mg, 3.7×10^{-5} mol) in chloroform (10 mL) was treated with MnO₂ (64 mg, 8×10^{-4} mol) and kept overnight at room temperature. The solid was removed by centrifugation, and the mixture was taken to dryness, taken up in methanol, and analyzed by HPLC (eluant III) and TLC (eluant A).

Isolation of Compounds 1-8. For preparative purposes, reaction of **17βE** with peroxidase/H₂O₂ was run as described above using 500 mg (1.84×10^{-3} mol) of the starting material at 3.0×10^{-4} M concentration. After workup of the reaction mixture, the residue obtained (480 mg) was fractionated by PLC (eluant A) to give seven fractions. Fraction A (15 mg, $R_f = 0.68$ eluant A) was further purified by preparative HPLC (eluant II) to give pure **2a** (5 mg, $t_r = 8$ min, eluant II, 1% yield) and **2b** (5 mg, $t_r = 17$ min, eluant II, 1% yield). Fraction B (25 mg, $R_f = 0.55$ eluant A) was fractionated by PLC (eluant I) to give pure **4** (8 mg, $t_r = 27$ min, eluant II, 1.6% yield) and **5** (8 mg, $t_r = 29$ min, eluant II, 1.6% yield). Fraction C (15 mg, $R_f = 0.45$ eluant A) was purified by HPTLC (eluant E) to afford **3a** (5 mg, $t_r = 9$ min, eluant II, 1% yield) and **3b** (5 mg, $t_r = 10$ min, eluant II, 1% yield). Fraction D (20 mg, $R_f = 0.43$, eluant A) consisted of pure **1** ($t_r = 14$ min, eluant II, 4% yield). Fraction E (22 mg, $R_f = 0.33$, eluant A) was purified by preparative HPLC (eluant II) to afford **6a** (3 mg, $t_r = 31$ min, eluant II, 0.6% yield) and **6b** (3 mg, $t_r = 32$ min, eluant II, 0.6% yield). Fraction F (14 mg, $R_f = 0.22$, eluant A) was fractionated by preparative HPLC (eluant II) to give four bands corresponding to pure **7a** (3 mg, $t_r = 24$ min, eluant II, 0.6% yield), **7b** (3 mg, $t_r = 80$ min, eluant II, 0.6% yield), **8a** (3 mg, $t_r = 34$ min, eluant II, 0.6% yield), and **8b** (3 mg, $t_r = 37$ min, eluant II, 0.6% yield).

yield). Fraction G (35 mg, $R_f = 0.10$ eluant A) was found to consist of a complex pattern of species and was not further purified.

2,2'-Bis[estra-1,3,5(10)-trien-3,17 β -diol] (1). UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CD₃OD), δ (ppm): 0.75 (s, 3H x 2), 1.0-1.8 (m, 8H x 2), 1.9-2.1 (m, 4H x 2), 2.15 (m, 1H x 2), 2.25 (m, 1H x 2), 2.85 (m, 1H x 2), 3.64 (m, 1H x 2), 6.31 (s, 1H x 2), 7.14 (s, 1H x 2). ¹³C NMR (CD₃OD), δ (ppm): 12.6 (2 x CH₃), 24.1 (2 x CH₂), 29.3 (2 x CH₂), 31.5 (2 x CH₂), 31.8 (4 x CH₂), 38.8 (2 x CH₂), 41.1 (2 x CH), 45.1 (2 x C), 46.1 (2 x CH), 52.1 (2 x CH), 83.3 (2 x CH), 118.1 (2 x CH), 127.0 (2 x C), 130.3 (2 x CH), 134.7 (2 x C), 139.1 (2 x C), 153.0 (2 x C). EI/MS (m/z): 542, [M]⁺. HREIMS (m/z): calcd mass for C₃₆H₄₆O₄, 542.3396; found, 542.3401.

4,4'-Bis[estra-1,3,5(10)-trien-3,17 β -diol] (2a). UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CDCl₃), δ (ppm): 0.80 (s, 3H x 2), 1.1-1.7 (m, 8H x 2), 1.78 (m, 1H x 2), 1.95 (m, 1H x 2), 2.11 (m, 1H x 2), 2.15-2.30 (m, 2H x 2), 2.30-2.40 (m, 2H x 2), 3.73 (t, J) 8.2 Hz, 1H x 2), 6.87 (d, J) 8.8 Hz, 1H x 2), 7.30 (d, J) 8.8 Hz, 1H x 2). ¹³C NMR (CDCl₃), δ (ppm): 11.9 (2 x CH₃), 23.9 (2 x CH₂), 27.1 (2 x CH₂), 27.8 (2 x CH₂), 28.1 (2 x CH₂), 31.4 (2 x CH₂), 37.5 (2 x CH₂), 39.1 (2 x CH), 44.0 (2 x C), 45.0 (2 x CH), 50.9 (2 x CH), 82.6 (2 x CH), 113.6 (2 x CH), 119.9 (2 x C), 128.0 (2 x CH), 134.4 (2 x C), 137.8 (2 x C), 152.0 (2 x C). EI/MS (m/z): 542, [M]⁺. HREIMS (m/z): calcd mass for C₃₆H₄₆O₄, 542.3396; found, 542.3393.

2b. UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CDCl₃), δ (ppm): 0.81 (s, 3H x 2), 1.1-1.7 (m, 8H x 2), 1.79 (m, 1H x 2), 1.99 (s, 1H x 2), 2.12 (m, 1H x 2), 2.15-2.30 (m, 2H x 2), 2.30-2.40 (m, 2H x 2), 3.74 (t, $J = 8.2$ Hz, 1H x 2), 6.86 (d, $J = 8.8$ Hz, 1H x 2), 7.32 (d, $J = 8.8$ Hz, 1H x 2). EI/MS (m/z): 542, [M]⁺. HREIMS (m/z): calcd mass for C₃₆H₄₆O₄, 542.3396; found, 542.3397.

2,4'-Bis[estra-1,3,5(10)-trien-3,17 β -diol] (3a). UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CDCl₃), δ (ppm): 0.79 (s, 6H), 1.1-1.8 (m, 16H), 1.9-2.0 (m, 4H), 2.10 (m, 2H), 2.25 (m, 2H), 2.32 (m, 2H), 2.50 (m, 2H), 2.85 (m, 2H), 3.73 (m, 2H), 6.77 (s, 1H), 6.85 (d, $J = 8.4$ Hz, 1H), 7.00 (s, 1H), 7.29 (d, $J = 8.4$ Hz, 1H). ¹³C NMR (CDCl₃), δ (ppm): 11.9 (CH₃), 23.9 (CH₂), 27.3 (CH₂), 27.8 (CH₂), 28.0 (CH₂), 28.3 (CH₂), 30.4 (CH₂), 31.4 (CH₂), 31.7 (CH₂), 37.50 (CH₂), 37.52 (CH₂), 39.0 (CH), 39.5 (CH), 44.0 (C), 44.8 (CH), 45.0 (CH), 50.8 (CH), 83.3 (CH), 113.6 (CH), 116.7 (CH), 117.4 (C), 120.5 (C), 127.9 (CH), 128.3 (CH), 133.3 (C), 134.5 (C), 136.9 (C), 140.1 (C), 152.3 (C). EI/MS (m/z): 542, [M]⁺. HREIMS (m/z): calcd mass for C₃₆H₄₆O₄, 542.3396; found, 542.3403.

3b. UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CDCl₃), δ (ppm): 0.78 (s, 6H), 1.1-1.8 (m, 16H), 1.9-2.1 (m, 4H), 2.1-2.3 (m, 4H), 2.3-2.5 (m, 4H), 2.92 (m, 2H), 3.72 (m, 2H), 6.78 (s, 1H), 6.87 (d, $J = 8.4$ Hz, 1H), 6.99 (s, 1H), 7.29 (d, $J = 8.4$ Hz, 1H). EI/MS (m/z): 542, [M]⁺. HREIMS: calcd mass for C₃₆H₄₆O₄, 542.3396; found, 542.3401.

2-[[17 β]-17-Hydroxy-19-norpregna-1,3,5(10)-trien-3-yl]-oxy]estra-1,3,5(10)-trien-3,17 β -diol (4). UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CDCl₃), δ (ppm): 0.77 (s, 6H), 1.1-1.6 (m, 16H), 1.6-1.8 (m, 2H), 1.90 (m, 2H), 1.95 (m, 1H), 2.0-2.2 (m, 5H), 2.32 (m, 1H), 2.83 (m, 3H), 3.71 (m, 2H), 6.70 (d, $J = 2.4$ Hz, 1H), 6.74 (s, 1H), 6.76 (dd, $J = 8.4, 2.4$ Hz, 1H), 6.87 (s, 1H), 7.22 (d, $J = 8.4$ Hz, 1H). ¹³C NMR (CDCl₃), δ (ppm): 11.8 (CH₃), 23.9 (CH₂), 27.0 (CH₂), 27.1 (CH₂), 27.9 (CH₂), 28.0 (CH₂), 29.9 (CH₂), 30.4 (CH₂), 31.4 (CH₂), 37.4 (CH₂), 37.5 (CH₂), 39.40 (CH), 39.45 (CH), 44.0 (C), 44.8 (CH), 50.8 (CH), 82.6 (CH), 114.8 (CH), 116.7 (CH), 117.6 (CH), 117.7 (CH), 127.4 (CH), 133.7 (C), 134.2 (C), 135.8 (C), 139.3 (C), 141.5 (C), 146.2 (C), 156.0 (C). EI/MS (m/z): 542, [M]⁺. HREIMS (m/z): calcd mass for C₃₆H₄₆O₄, 542.3396; found, 542.3399.

4-[[17 β]-17-Hydroxy-19-norpregna-1,3,5(10)-trien-3-yl]-oxy]estra-1,3,5(10)-trien-3,17 β -diol (5). UV [λ_{\max} (CH₃OH)]: 288 nm. ¹H NMR (CDCl₃), δ (ppm): 0.78 (s, 6H), 1.1-1.8 (m, 16H), 1.8-1.9 (m, 2H), 1.9-2.0 (m, 2H), 2.0-2.2 (m, 4H), 2.33 (m, 2H), 2.40 (m, 1H), 2.73 (m, 1H), 2.81 (m,

2H), 3.73 (m, 2H), 6.60 (d, $J = 2.4$ Hz, 1H), 6.62 (dd, $J = 8.4, 2.4$ Hz, 1H), 6.86 (d, $J = 8.4$, 1H), 7.10 (d, $J = 8.4$ Hz, 1H), 7.17 (d, $J = 8.4$ Hz, 1H). ^{13}C NMR (CDCl_3), δ (ppm): 11.8 (CH_3), 23.9 (CH_2), 24.6 (CH_2), 27.0 (CH_2), 28.0 (CH_2), 28.2 (CH_2), 30.5 (CH_2), 31.4 (CH_2), 37.4 (CH_2), 39.0 (CH), 39.5 (CH), 44.0 (C), 44.8 (CH), 50.8 (CH), 82.7 (CH), 112.8 (CH), 113.8 (CH), 115.6 (CH), 123.6 (CH), 127.5 (CH), 131.5 (C), 134.4 (C), 135.5 (C), 139.4 (C), 139.7 (C), 147.4 (C), 155.7 (C). EI/MS (m/z): 542, $[\text{M}]^+$. HREIMS (m/z): calcd mass for $\text{C}_{36}\text{H}_{46}\text{O}_4$, 542.3396; found, 542.3402.

2-[[17 β]-17-Hydroxy-19-norpregna-1,3,5(10)-trien-3-yl]-oxy]-4,2'-bis[estra-1,3,5(10)-trien-3,17 β -diol] (6a). UV [λ_{max} (CH_3OH): 288 nm. ^1H NMR (CDCl_3), δ (ppm): 0.78 (s, 3H), 0.79 (s, 3H) 0.80 (s, 3H), 1.1-1.7 (m, 21H), 1.70-1.85 (m, 3H), 1.85-2.00 (m, 5H), 2.1-2.2 (m, 5H), 2.2-2.3 (m, 3H), 2.35 (m, 2H), 2.45 (m, 1H), 2.55 (m, 1H), 2.85 (m, 4H), 3.74 (m, 3H), 6.75 (s, 1H), 6.76 (d, $J = 2.4$ Hz, 1H), 6.80 (dd, $J = 8.4, 2.4$ Hz, 1H), 6.98 (s, 1H), 7.04 (s, 1H), 7.23 (d, $J = 8.4$, 1H). ^{13}C NMR (CDCl_3), δ (ppm): 11.9 (CH_3), 23.9 (CH_2), 27.1 (CH_2), 27.3 (CH_2), 27.9 (CH_2), 30.5 (CH_2), 31.4 (CH_2), 31.7 (CH_2), 37.5 (CH), 38.9 (C), 39.5 (C), 44.0 (CH), 44.8 (CH), 45.1 (CH), 50.9 (CH), 82.7 (CH), 115.4 (CH), 116.4 (CH), 117.8 (CH), 118.4 (CH), 119.8 (C), 124.3 (C), 127.4 (CH), 128.3 (CH), 132.3 (C), 133.6 (C), 133.9 (C), 134.3 (C), 136.0 (C), 139.3 (C), 142.2 (C), 144.6 (C), 151.6 (C), 155.9 (C). EI/MS (m/z): 812, $[\text{M}]^+$. HREIMS (m/z): calcd mass for $\text{C}_{54}\text{H}_{68}\text{O}_6$, 812.5016; found, 812.5035.

6b. UV [λ_{max} (CH_3OH): 288 nm. ^1H NMR (CDCl_3), δ (ppm): 0.77 (s, 3H), 0.78 (s, 3H) 0.79 (s, 3H), 1.1-1.7 (m, 24 H), 1.7-2.0 (m, 5H), 2.0-2.3 (m, 8H), 2.40 (m, 2H), 2.50 (m, 2H), 2.87 (m, 4H), 3.72 (m, 3H), 6.75 (d, $J = 2.4$ Hz, 1H), 6.77 (s, 1H), 6.81 (dd, $J = 8.4, 2.4$ Hz, 1H), 6.98 (s, 1H), 7.03 (s, 1H), 7.24 (d, $J = 8.4$, 1H). EI/MS (m/z): 812, $[\text{M}]^+$. HREIMS (m/z): calcd mass for $\text{C}_{54}\text{H}_{68}\text{O}_6$, 812.5016; found, 812.5026.

2,2'4'2''-Tris[estra-1,3,5(10)-trien-3,17 β -diol] (7a). UV [λ_{max} (CH_3OH): 288 nm. ^1H NMR (CDCl_3), δ (ppm): 0.78 (s, 3H), 0.80 (s, 6H), 1.1-1.9 (m, 24H), 1.9-2.0 (m, 6H), 2.1-2.4 (m, 10H), 2.42 (m, 2H), 2.90 (m, 3H), 3.73 (m, 3H), 6.76 (s, 1H), 6.79 (s, 1H), 7.04 (s, 1H), 7.20 (s, 1H), 7.32 (s, 1H). EI/MS (m/z): 812, $[\text{M}]^+$. HREIMS (m/z): calcd mass for $\text{C}_{54}\text{H}_{68}\text{O}_6$, 812.5016; found, 812.5005.

7b. UV [λ_{max} (CH_3OH): 288 nm. ^1H NMR (CDCl_3), δ (ppm): 0.77 (s, 3H), 0.78 (s, 6H), 1.1-1.9 (m, 24H), 1.9-2.0 (m, 5H), 2.0-2.2 (m, 5H), 2.2-2.5 (m, 8H), 2.90 (m, 3H), 3.73 (m, 3H), 6.76 (s, 1H), 6.78 (s, 1H), 7.04 (s, 1H), 7.21 (s, 1H), 7.32 (s, 1H). ^{13}C NMR (CDCl_3), δ (ppm): 11.8 (CH_3), 23.9 (CH_2), 24.0 (CH_2), 24.5 (CH_2), 27.2 (CH_2), 27.9 (CH_2), 29.3 (CH_2), 30.1 (CH_2), 30.4 (CH_2), 30.5 (CH_2), 31.4 (CH_2), 37.5 (CH_2), 39.1 (CH), 39.4 (CH), 39.6 (CH), 44.0 (C), 44.8 (CH), 45.1 (CH), 50.9 (CH), 82.7 (CH), 116.9 (CH), 118.2 (CH), 121.9 (C), 122.5 (C), 123.0 (C), 128.6 (CH), 128.9 (CH), 130.0 (CH), 133.3 (C), 133.9 (C), 137.4 (C), 138.3 (C), 140.0 (C), 150.9 (C), 151.1 (C). EI/MS (m/z): $[\text{M}]^+$. HREIMS (m/z): calcd mass for $\text{C}_{54}\text{H}_{68}\text{O}_6$, 812.5016; found, 812.5027.

2,2'4'4''-Tris[estra-1,3,5(10)-trien-3,17 β -diol] (8a). UV [λ_{max} (CH_3OH): 288 nm. ^1H NMR (CDCl_3), δ (ppm): 0.79 (s, 3H), 0.80 (s, 3H), 0.81 (s, 3H), 1.2-1.8 (m, 24H), 1.8-2.0 (m, 6H), 2.0-2.2 (m, 4H), 2.2-2.4 (m, 8H), 2.89 (m, 3H), 3.74 (m, 3H), 6.76 (s, 1H), 6.88 (d, $J = 8.4$ Hz, 1H), 7.22 (s, 1H), 7.32 (d, $J = 8.4$, 1H), 7.34 (s, 1H). EI/MS (m/z): $[\text{M}]^+$. HREIMS (m/z): calcd mass for $\text{C}_{54}\text{H}_{68}\text{O}_6$, 812.5016; found, 812.5003.

8b. UV [λ_{max} (CH_3OH): 288 nm. ^1H NMR (CDCl_3), δ (ppm): 0.79 (s, 3H), 0.80 (s, 6H), 1.1-1.8 (m, 24H), 1.8-2.0 (m, 5H), 2.1-2.2 (m, 5H), 2.2-2.5 (m, 8H), 2.8-2.9 (m, 3H), 3.73 (m, 3H), 6.76 (s, 1H), 6.89 (d, $J = 8.4$ Hz, 1H), 7.22 (s, 1H), 7.33 (d, $J = 8.4$, 1H), 7.36 (s, 1H). ^{13}C NMR (CDCl_3), δ (ppm): 11.8 (CH_3), 11.9 (CH_3), 23.8 (CH_2), 24.0 (CH_2), 24.5 (CH_2), 26.5 (CH_2), 28.1 (CH_2), 29.7

(CH₂), 30.5 (CH₂), 31.1 (CH₂), 31.4 (CH₂), 37.5 (CH₂), 38.6 (CH), 39.5 (CH), 42.1 (C), 44.0 (CH), 44.9 (CH), 48.8 (CH), 50.8 (CH), 82.7 (CH), 118.3 (CH), 118.6 (CH), 118.9 (C), 122.6 (C), 122.7 (C), 128.2 (CH), 130.1 (CH), 130.2 (CH), 132.6 (C), 134.3 (C), 136.3 (C), 136.5 (C), 138.2 (C), 148.0 (C), 151.5 (C), 152.1 (C). EI/MS (*m/z*): 812, [M]⁺. HREIMS (*m/z*): calcd mass for C₅₄H₆₈O₆, 812.5016; found, 812.5024.

TYROSINASE CATALYZED REACTIONS

Oxidation of 17βE by the Tyrosinase/O₂ system: General Procedure. To a solution of 17βE (5 mg, 1.9 × 10⁻⁵ mol) predissolved in methanol (1 mL), 0.1 M phosphate buffer (600 mL) (pH 7.4) was added followed by tyrosinase in two aliquots at 3 h intervals up to a 10 U/mL final concentration. After 18 h, the reaction mixture was treated with NaBH₄ (1 mg), carefully acidified to pH 5.0, and extracted with ethyl acetate (3 × 150 mL). The combined organic layers were dried over sodium sulfate and analyzed by HPLC (eluant IV). In other experiments, the reaction was carried out as above with the substrate at concentrations varying in the range of 1 nM to 30 μM and tyrosinase at 1 U/mL final concentration when the substrate was 1 nM to 1 μM. When required, tropolone (1 nM) was added to the incubation mixture with the substrate at 1 μM concentration.

Oxidation of 2OHE and 4OHE by Tyrosinase. To a solution of 2OHE or 4OHE (5 mg, 1.7 × 10⁻⁵ mol) in methanol (1 mL), 0.1 M phosphate buffer (300 mL) (pH 7.4) was added followed by tyrosinase in two aliquots at 3 h intervals up to a 5 U/mL final concentration. The resulting pale yellow solution was taken under stirring for 18 h and then treated with NaBH₄, carefully acidified to pH 5.0, and extracted three times with ethyl acetate (3 × 60 mL). The combined organic layers were dried over sodium sulfate and analyzed by HPLC (eluant IV). In other experiments, the reaction was carried out as above with the substrate at concentrations varying in the range of 1-60 μM.

Isolation of Compounds 9-15. For preparative purposes, the oxidation of 2OHE with tyrosinase was run as described above using 100 mg (3.5 × 10⁻⁴ mol) of the starting material at 60 μM concentration. After work up of the reaction mixture as above, the residue obtained (90 mg) was fractionated by HPLC (eluant IV) to give five fractions extracted with AcOEt. Fraction A (8 mg, *t_R* 7 min) consisted of pure **9** (8% yield), while fraction B (2 mg, *t_R* 26 min) contained compound **10** (2% yield). Fraction C (7 mg, *t_R* 31 min) was found to be a mixture of compounds **11** and **12** based on NMR analysis and was further purified by fractionation on silver nitrate-impregnated TLC plates (eluant C) to afford compounds **12** (*R_f* 0.45, 3 mg, 3% yield) and **11** (*R_f* 0.50, 2 mg, 2% yield). Fractions D (5 mg, *t_R* 56 min) and E (5 mg, *t_R* 82 min) contained compounds **13** (5% yield) and **14** (5% yield). The reaction of 4OHE was run under the same conditions using 100 mg of the starting material at 60 μM concentration. After work up as above, preparative HPLC fractionation (eluant IV) followed by AcOEt extraction of the eluates afforded one main fraction (6 mg, *t_R* 35 min) consisting of compound **15** (6% yield).

2-Hydroxy-6-oxo-estra-1,3,5(10)-trien-3,17β-diol (9). UV λ_{max} (CH₃OH): 235, 280 and 321 nm. ESI(-)/MS: *m/z* 301 [M - H]. ESI(-)HRMS calculated mass for C₁₈H₂₁O₄ [M - H], 301.1440; found, *m/z* 301.1432. ¹H NMR and ¹³C NMR (CD₃OD) spectra were consistent with those reported (28).

2-Hydroxy-estra-1,3,5,6,8-pentaene-3,17β-diol (10). UV λ_{max} (CH₃OH): 241 and 288 nm. ¹H NMR (CD₃OD) δ (ppm): 0.65 (s, 3H), 3.01-3.11 (m, 2H), 3.89 (m, 1H), 6.93 (d, *J* = 8.4 Hz, 1H, H-7), 7.04 (s, 1H, H-4), 7.22 (s, 1H, H-1), 7.36 (d, *J* = 8.4 Hz, 1H, H-6). ¹³C NMR (CD₃OD) δ (ppm): 11.5 (CH₃), 24.3 (CH₂), 26.3 (CH₂), 36.5 (CH₂), 44.3 (C), 48.1 (CH), 81.0 (CH), 113.3 (CH), 118.5 (CH), 127.4 (CH), 130.0 (C), 131.2 (C), 132.3 (C), 137.2 (C), 148.3 (C), 149.0 (C). ESI(-)/MS: *m/z* 283 [M - H]. ESI(-)HRMS calculated mass for C₁₈H₁₉O₃ [M - H], 283.1334; found, *m/z* 283.1379.

2-Hydroxy-estra-1,3,5(10),9-tetraen-3,17 β -diol (11). UV λ_{\max} (CH₃OH): 230, 270, 290, 316 nm. ¹H NMR (CD₃OD) δ (ppm): 0.77 (s, 3H), 2.55-2.75 (m, 2H), 3.75 (t, J = 8.8 Hz, 1H), 5.98 (m, 1H, H-11), 6.44 (s, 1H, H-4), 6.99 (s, 1H, H-1). ¹³C NMR (CD₃OD) δ (ppm): 12.0 (CH₃), 25.4 (CH₂), 30.4 (CH₂), 30.7 (CH₂), 31.1 (CH₂), 40.8 (CH₂), 43.2 (C), 51.2 (CH), 83.1 (CH), 111.8 (CH), 116.7 (CH), 118.0 (CH), 128.5 (C), 129.8 (C), 137.5 (C), 145.1 (C), 146.2 (C). ESI(-)/MS: m/z 285 [M - H]. ESI(-)HRMS calculated mass for C₁₈H₂₁O₃ [M - H], 285.1491; found, m/z 285.1517.

2-Hydroxy-estra-1,3,5,6-tetraen-3,17 β -diol (12). UV λ_{\max} (CH₃OH): 222, 283, 316 nm. ¹H NMR (CD₃OD) δ (ppm): 0.76 (s, 3H), 3.67 (t, J = 8.4 Hz, 1H, H-17), 5.75 (dd, J = 9.6, 2.0 Hz, 1H, H-7), 6.29 (dd, J = 9.6, 2.8 Hz, 1H, H-6), 6.50 (s, 1H, H-4), 6.71 (s, 1H, H-1). ¹³C NMR (CD₃OD) δ (ppm): 11.9 (CH₃), 24.4 (CH₂), 26.0 (CH₂), 31.3 (CH₂), 40.9 (CH), 44.1 (CH), 45.3 (C), 52.0 (CH), 82.8 (CH), 112.9 (CH), 115.1 (CH), 128.5 (C), 129.2 (CH), 130.3 (CH), 133.2 (C), 144.4 (C), 145.7 (C). ESI(-)MS: m/z 285 [M - H]. ESI(-)HRMS calculated mass for C₁₈H₂₁O₃ [M - H], 285.1491; found, m/z 285.1479.

2-Hydroxy-1-[(17 β)-2,17-dihydroxy-19-norpregna-1,3,5(10)-trien-3-yl]oxy]estra-1,3,5(10)trien-3,17 β -diol. (13). UV λ_{\max} (CH₃OH): 290 nm. ¹H NMR (CD₃OD) δ (ppm): 0.64 (s, 3H), 0.69 (s, 3H), 2.55 (m, 1H), 2.80 (m, 1H), 3.57 (m, 1H), 3.67 (m, 1H), 6.08 (s, 1H, H-4 \square), 6.29 (s, 1H, H-4), 6.80 (s, 1H, H-1 \square). ¹³C NMR (CD₃OD) δ (ppm): 12.2 (CH₃), 12.3 (CH₃), 27.1 (CH₂), 28.7 (CH₂), 20.3 (CH₂), 31.5 (CH₂), 38.3 (CH₂), 38.6 (CH₂), 38.9 (CH₂), 39.0 (CH₂), 41.8 (CH), 44.5 (C), 44.9 (C), 46.2 (CH), 47.4 (CH), 51.1 (CH), 51.2 (CH), 83.0 (CH), 118.2 (CH), 123.4 (C), 127.1 (C), 132.3 (C), 135.5 (C), 142.6 (C), 145.2 (C), 145.1 (C). ESI(-)/MS: m/z 573 [M - H]. ESI(-)HRMS calculated mass for C₃₆H₄₅O₆ [M - H], 573.3216; found, m/z 573.3203.

2-Hydroxy-1-[(17 β)-2,17-dihydroxy-19-norpregna-1,3,5(10)-trien-3-yl]oxy]estra-1,3,5,6,8-pentaen-3,17 β -diol. (14). UV λ_{\max} (CH₃OH): 238 and 289 nm. ¹H NMR (CD₃OD) δ (ppm): 0.65 (s, 3H), 0.77 (s, 3H), 3.08-3.11 (m, 2H), 3.66 (m, 1H), 3.70 (m, 1H), 6.14 (s, 1H, H-4 \square), 6.62 (d, J = 8.8 Hz, 1H, H-7), 6.81 (s, 1H, H-4), 7.17 (d, J = 8.8 Hz, 1H, H-6), 7.19 (s, 1H, H-1 \square). ¹³C NMR (CD₃OD) δ (ppm): 10.5 (CH₃), 11.0 (CH₃), 24.1 (CH₂), 28.3 (CH₂), 30.4 (CH₂), 31.0 (CH₂), 32.4 (CH₂), 36.3 (CH₂), 38.3 (CH₂), 44.2 (C), 45.1 (C), 48.4 (CH), 52.7 (CH), 105.0 (CH), 113.1 (CH), 116.2 (CH), 122.5 (CH), 125.7 (CH), 127.2 (C), 130.2 (C), 131.1 (C), 132.2 (C), 134.2 (C), 135.3 (C), 144.3 (C), 145.0 (C), 146.2 (C), 147.5 (C). ESI/MS: m/z 569 [M - H]. ESI(-)HRMS calculated mass for C₃₆H₄₁O₆ [M - H], 569.2903; found, m/z 569.2935.

4-Hydroxy-9,11-dehydroestra-1,3,5(10)-trien-3,17 β -diol (15). UV λ_{\max} (CH₃OH): 225 and 273 nm. ¹H NMR (CD₃OD) δ (ppm): 0.78 (s, 3H), 1.1-1.5 (m, 5H), 1.94-2.10 (m, 2H), 2.19 (m, 1H), 2.50 (m, 2H), 2.97 (m, 2H), 3.74 (t, J = 8.5 Hz, 1H), 6.05 (m, 1H, H-10), 6.58 (d, J = 9.0 Hz, 1H, H-2), 6.97 (d, J = 9.0 Hz, 1H, H-1). ¹³C NMR (CD₃OD) δ (ppm): 12.0 (CH₃), 24.9 (CH₂), 25.4 (CH₂), 31.2 (CH₂), 31.3 (CH₂), 40.4 (CH), 40.8 (CH₂), 43.2 (C), 50.0 (CH), 83.1 (CH), 114.4 (CH), 116.8 (CH), 118.1 (CH), 125.7 (C), 129.0 (C), 137.8 (C), 144.2 (C), 146.4 (C). ESI/MS: m/z 285 [M - H]. HREIMS calculated mass for C₁₈H₂₂O₃ [M - H], 285.1491; found, m/z 285.1525.

CATECHOL ESTROGENS PREPARATION

Preparation of the Catechol Estrogens: General Procedure. Solid IBX (2.5 equiv) was added to a solution of **17 β E** or **2** (200 mg) in CHCl₃/MeOH 3: 2 v/v (40 mL) at -25 °C. A yellow-to-orange color developed and the mixture was stirred for 24 h. Methanolic NaBH₄ (15mg in 1 mL) was then added at -25 °C under vigorous stirring until the color disappeared (usually within 5min). After

mild acidification with acetic acid (200–500 μL) to remove excess NaBH_4 , the mixture was washed five times with equal volumes of a saturated NaCl solution containing 10% sodium dithionite buffered at pH 7.4 with sodium phosphate. Evaporation of the organic layer eventually furnished the desired products 3/4 or 5/6, which could be separated by preparative TLC (eluant D) on silica.

2-Hydroxy-estra-1,3,5(10)-trien-3,17 β -diol (2OHE). Pale yellow powder. UV (MeOH): λ_{max} 281 nm. ESI/MS m/z : 287 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_{18}\text{H}_{23}\text{O}_3$ ($\text{M}-\text{H}^+$) 287.1647, found 287.1649. ^1H NMR (CDCl_3), δ (ppm) selected signals: 0.78 (s, 3H, CH_3), 3.73 (t, $J = 8.8$ Hz, 1H, CHOH), 6.58 (s, 1H), 6.81 (s, 1H).

4-Hydroxy-estra-1,3,5(10)-trien-3,17 β -diol (4OHE). Pale yellow powder. UV (MeOH): λ_{max} 280 nm. ESI/MS m/z : 287 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_{18}\text{H}_{23}\text{O}_3$ ($\text{M}-\text{H}^+$) 287.1647, found 287.1648. ^1H NMR (CDCl_3), δ (ppm) selected signals: 0.77 (s, 3H, CH_3), 3.73 (t, $J = 8.8$ Hz, 1H, CHOH), 6.69 (d, $J = 8.4$ Hz, 1H), 6.76 (d, $J = 8.4$ Hz, 1H).

2-Hydroxyestrone (17). Pale yellow powder. UV (MeOH): λ_{max} 282 nm. ESI/MS m/z : 285 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_{18}\text{H}_{21}\text{O}_3$ ($\text{M}-\text{H}^+$) 285.1491, found 285.1491. ^1H NMR (CDCl_3), δ (ppm) selected signals: 0.78 (s, 3H, CH_3), 6.61 (s, 1H), 6.82 (s, 1H).

4-Hydroxyestrone (18). Pale yellow powder. UV (MeOH): λ_{max} 282 nm. ESI/MS m/z : 285 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_{18}\text{H}_{21}\text{O}_3$ ($\text{M}-\text{H}^+$) 285.1491, found 285.1490, found 288.1725. ^1H NMR (CDCl_3), δ (ppm) selected signals: 0.77 (s, 3H, CH_3), 6.67 (d, $J = 8.4$ Hz, 1H), 6.74 (d, $J = 8.4$ Hz, 1H).

Catechol. Brownish powder. UV (MeOH): λ_{max} 280 nm. ESI/MS m/z : 109 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_6\text{H}_5\text{O}_2$ ($\text{M}-\text{H}^+$) 109.0289, found 109.0291.

4,5-Dimethylcatechol. Pale brown powder. UV (MeOH): λ_{max} 280 nm. ESI/MS m/z : 137 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_8\text{H}_9\text{O}_2$ ($\text{M}-\text{H}^+$) 137.0603, found 137.0604. ^1H NMR (CDCl_3), δ (ppm) selected signals: 2.20 (s, 6H, CH_3), 5.51 (s, 2H).

3,4-Dimethylcatechol. Pale brown powder. UV (MeOH): λ_{max} 280 nm. ESI/MS m/z : 137 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_8\text{H}_9\text{O}_2$ ($\text{M}-\text{H}^+$) 137.0603, found 137.0605. ^1H NMR (CDCl_3), δ (ppm) selected signals: 2.21 (s, 3H, CH_3), 2.22 (s, 3H, CH_3), 5.53 (d, $J = 8.4$ Hz, 1H), 6.64 (d, $J = 8.4$ Hz, 1H).

3,6-Dimethylcatechol. Pale brown powder. UV (MeOH): λ_{max} 281 nm. ESI/MS m/z : 137 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_8\text{H}_9\text{O}_2$ ($\text{M}-\text{H}^+$) 137.0603, found 137.0604. ^1H NMR (CDCl_3), δ (ppm) selected signals: 2.22 (s, 6H, CH_3), 6.61 (s, 2H).

1,2-Dihydroxynaphthalene. Pale blue powder ESI/MS m/z : 159 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_{10}\text{H}_7\text{O}_2$ ($\text{M}-\text{H}^+$) 159.0446, found 159.0449.

7,8-Dihydroxyquinoline. Pale red powder ESI/MS m/z : 160 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_9\text{H}_6\text{O}_2\text{N}$ ($\text{M}-\text{H}^+$) 160.0398, found 160.0403. ^1H NMR (CDCl_3), δ (ppm): 7.22 (d, $J = 8.8$ Hz, 1H), 7.29 (dd, $J = 8.4, 4.4$, Hz, 1H), 7.31 (d, $J = 8.8$ Hz, 1H), 8.16 (dd, $J = 8.4, J = 1.6$, Hz, 1H), 7.29 (dd, $J = 4.4, J = 1.6$, Hz, 1H).

3-tert-Butylcatechol. Pale brown powder. UV (MeOH): λ_{max} 281 nm. ESI/MS m/z : 165 $[\text{M}-\text{H}^+]$; ESI-HRMS calculated for $\text{C}_{10}\text{H}_{13}\text{O}_2$ ($\text{M}-\text{H}^+$) 165.0915, found 165.0917. ^1H NMR (CD_3OD), δ

(ppm): 1.40 (s, 9H, CH₃), 6.55 (dd, J = 8.0, J = 7.5, Hz, 1H), 6.64 (dd, J = 8.0, J = 1.5, Hz, 1H), 6.70 (dd, J = 7.5, J = 1.5, Hz, 1H).

10 β -SUBSTITUTED 17 β -HYDROXYESTRA-1,4-DIEN-3-ONES PREPARATION

Preparation of 10 β , 17 β -dihydroxyestra-1,4-dien-3-one (1): General Procedure. A solution of **17 β E** (100 mg, 0.37 mmol) in ethyl acetate (16 mL) was added under vigorous stirring to a solution of potassium permanganate (116 mg, 0.74 mmol) in 0.05M aqueous HCl (16 mL). After 30 s, when substrate consumption was complete (TLC evidence), the mixture was extracted with ethyl acetate (3 \times 75 mL), the organic layers were collected, and taken to dryness to afford pure **26** (80 mg, 75% yield, R_f = 0.30) as crystals from ethyl acetate.

26: m.p. 217–219 °C; UV (MeOH): λ_{\max} 243 nm; ¹H and ¹³C NMR (see Table 1); ESI(–)MS m/z : 287([M–H][–]). Anal. calcd. for C₁₈H₂₄O₃: C, 74.97; H, 8.39. Found: C, 75.00; H, 8.42.

Preparation of 10 β -Chloro-17 β -hydroxyestra-1,4-dien-3-one (27): General Procedure. **17 β E** (100 mg, 0.37 mmol) in methanol (7 mL) was added under vigorous stirring to 0.01M aqueous HCl (20 mL) containing NaClO₂ (66 mg, 0.74 mmol). After 30 min reaction time, when substrate consumption was complete (HPLC evidence, eluant V), the mixture was extracted with ethyl acetate (3 \times 75 mL), the organic layers were collected, taken to dryness, and the residue fractionated on silica plates (eluant A) to afford pure **27** (50 mg, 44% yield, R_f = 0.49, R_T = 12.7 min) as colourless crystals from ethyl acetate, **28** (2 mg, 2% yield, R_f = 0.17, R_T = 5.5 min), **29** (7 mg, 6% yield, R_f = 0.55, R_T = 19.3 min), **30** (12 mg, 9% yield, R_f = 0.52, R_T = 21.2 min).

27: m.p. 158–160 °C rif. 117; UV (CH₃OH): λ_{\max} 241, 280 (sh) nm; ¹H and ¹³C NMR (see Table 3); ESI(+)MS m/z : 307 ([M+H]⁺, 100), 309 ([M+2+H]⁺, 35). Anal. calcd. for C₁₈H₂₃O₂Cl: C, 70.46; H, 7.56. Found C, 70.89; H, 7.45.

4-chloro-10 β , 17 β -dihydroxyestra-1,4-dien-3-one (28): UV (CH₃OH): λ_{\max} 249, 289 nm; ¹H NMR (CDCl₃), δ (ppm) 0.85 (3H, s), 1.03 (1H, m), 1.07 (2H, m), 1.09 (1H, m), 1.34 (1H, m), 1.45 (1H, m), 1.50 (1H, m), 1.64 (1H, m), 1.65 (1H, m), 1.86 (1H, m), 2.00 (2H, m), 2.09 (1H, m), 2.60 (1H, m), 3.17 (1H, m), 3.64 (1H, m), 6.31 (1H, d, J = 10.4 Hz), 7.12 (1H, d, J = 10.4 Hz); ¹³C NMR (CDCl₃), δ (ppm) 11.0 (CH₃), 22.8 (CH₂), 23.6 (CH₂), 28.7 (CH₂), 30.4 (CH₂), 31.9 (CH₂), 35.0 (CH), 36.1 (CH₂), 43.0 (C), 49.7 (CH), 55.3 (CH), 72.8 (C), 81.5 (CH), 126.9 (CH), 127.0 (C), 150.6 (CH), 160.6 (C), 178.7 (C); ESI(–)MS m/z : 321 ([M–H][–], 100), 323 ([M+2–H][–], 35).

2,10 β -dichloro-17 β -hydroxyestra-1,4-dien-3-one (29): UV (CH₃OH): λ_{\max} 253, 288 (sh) nm; ¹H NMR rif. 117; ESI(+)MS m/z : 341 ([M +H]⁺, 100), 343 ([M+2+H]⁺, 63), 345 ([M+4+H]⁺, 12).

4,10 β -dichloro-17 β -hydroxyestra-1,4-dien-3-one (30): UV (CH₃OH): λ_{\max} 247, 289 nm; ¹H NMR rif. 117; ESI(+)MS m/z : 341 ([M +H]⁺, 100), 343 ([M+2+H]⁺, 63), 345 ([M+4+H]⁺, 15).

HALOGENATION

Iodination of estradiol: General Procedure. A solution of **17 β E** (50 mg, 0.2 mmol) in 10 ml MeOH was added to a well-stirred solution of NaClO₂ (66 mg, 0.8 mmol) and NaI (220 mg, 1.6 mmol) in H₂O (10 ml). This mixture was treated with 0.5 mL HCl conc (6 mmol) and allowed to

stir at room temperature. The reaction was monitored by TLC. After completion of reaction (30 min), the mixture was diluted with water (20 mL) and extracted with ethyl acetate (3x30 mL). The combined organic layers were washed with an aqueous solution of sodium tiosulphate, and sodium chloride to remove excess iodine, and dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by PLC (eluant B) to give products **33** ($R_f=0.75$, 36 mg, 38 % yield) and **32** ($R_f=0.70$, 25 mg, 35% yield).

2,4-diiodo-17 β -estradiol (33): ESI(-)MS m/z : 523 [M-H]⁻; ¹H NMR (CDCl₃), δ (ppm): 0.72 (s, 3H), 3.68 (t, 1H), 7.57 (s, 1H).

4-iodo-17 β -estradiol (32): ESI(-)MS m/z : 397 [M-H]⁻; ¹H NMR (CD₃OD), δ (ppm): 0.78 (s, 3H), 3.55 (t, 1H), 6.60 (d, $J=8.8$ Hz, 1H), 7.08 (d, $J=8.8$ Hz, 1H).

2,4,6-triiodophenol: ESI(-)MS m/z : 471 [M-H]⁻; ¹H NMR (CDCl₃), δ (ppm): 7.95 (s, 2H).

2,6-diiodo-4-nitrophenol: ESI(-)MS m/z : 389 [M-H]⁻; ¹H NMR (acetone-*d*₆), δ (ppm): 8.62 (s, 2H).

2,4,6-triiodoresorcine: ESI(-)MS m/z : 486 [M-H]⁻; ¹H NMR (CDCl₃), δ (ppm): 7.93 (s, 1H).

2,4-diiodoaniline: ESI(+)MS m/z : 346 [M+H]⁺; ¹H NMR (CD₃OD), δ (ppm): 6.57 (d, $J=8.2$ Hz, 1H), 7.33 (dd, $J=8.2, 2.0$ Hz, 1H), 7.80 (d, $J=2.0$ Hz, 1H).

2-iodo-4-nitroaniline: ESI(+)MS m/z : 265 [M+H]⁺, 287 [M+Na]⁺; ¹H NMR (CDCl₃), δ (ppm): 6.70 (d, $J=8.7$ Hz, 1H), 8.04 (dd, $J=8.7, 2.4$ Hz, 1H), 8.54 (d, $J=2.4$ Hz, 1H).

1-iodo-2-naphthol: ESI(+)MS m/z : 269 [M+H]⁺; ¹H NMR (acetone-*d*₆), δ (ppm): 7.29 (d, $J=9.0$, 1H), 7.37 (t, $J=7.2$ Hz, 1H), 7.56 (t, $J=7.5$ Hz, 1H), 7.81 (d, $J=8.1$ Hz, 1H), 7.82 (d, $J=9.0$, 1H), 8.06 (d, $J=8.1$, 1H), 9.52 (s, 1H).

8-hydroxy-5,7-diiodoquinoline: ESI(+)MS m/z : 398 [M+H]⁺; ¹H NMR (CD₃OD), δ (ppm): 7.53 (dd, $J=8.4, 8.7$ Hz, 1H), 8.25 (dd, $J=8.4, 1.5$ Hz, 1H), 8.32 (s, 1H), 8.80 (dd, $J=8.7, 1.5$ Hz, 1H).

3-iodo-5-nitroindole: IR ν_{\max} 3460, 1755, 1622, 1525, 1472, 1341 cm⁻¹; ESI(+)MS m/z : 311 [M+Na]⁺; ¹H NMR (CD₃OD), δ (ppm): 7.51 (d, $J=9.0$ Hz, 1H), 7.56 (d, $J=2.2$ Hz, 1H), 8.05 (dd, $J=9.0, 2.2$ Hz, 1H), 8.24 (d, $J=2.1$ Hz, 1H); ¹³C NMR (CD₃OD), δ (ppm): 58.9 (C), 113.7 (CH), 118.8 (CH), 119.2 (CH), 131.4 (C), 134.9 (CH), 141.4 (C), 143.9 (C).

2,4,5-triiodoimidazole: ESI(-)MS m/z : 444 [M-H]⁻.

Iodination of estradiol: General Procedure. A solution of NaClO₂ (7 mg, 0.1 mmol) and NaI (14 mg, 0.1 mmol) in 5 mL MeOH was added **17 β E** (50 mg, 0.2 mmol) under stirring. This mixture was treated with 0.05 mL HCl conc (0.6 mmol) and allowed to stir at room temperature. The reaction was monitored by TLC. After 10 min, the mixture was diluted with water (20 mL) and extracted with ethyl acetate (3x25 mL). The combined organic layers were dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by PLC (eluant B) to give products **31** ($R_f=0.63$, 30 mg, 40 % yield) and **17 β E** ($R_f=0.50$, 10 mg).

2,-iodo-17 β -estradiol (31): ESI(-)MS m/z : 397 [M-H]⁻; ¹H NMR (CD₃OD), δ (ppm): 0.76 (s, 3H), 3.63 (t, 1H), 6.53 (s, 1H), 7.49 (s, 1H).

Bromination of estradiol: General Procedure. A solution of **17 β E** (50 mg, 0.2 mmol) in 10 ml MeOH was added to a well-stirred solution of NaClO₂ (33 mg, 0.4 mmol) and NaBr (76 mg, 0.8 mmol) in H₂O (10 ml). This mixture was treated with 0.5 ml HCl conc (6 mmol) and allowed to stir at room temperature. The reaction was monitored by TLC. After completion of reaction (30 min), the mixture was diluted with water (20 mL) and extracted with ethyl acetate (3x30 mL). The combined organic layers were dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by PLC (eluant B) to give products **36** (R_f = 0.80, 5 mg, 6 % yield) and **35** (R_f = 0.61, 28 mg, 35 % yield).

2,4-dibromo-17 β -estradiol (35): ESI(-)MS m/z : 429 ([M-H]⁻, 100), 431 ([M+2-H]⁺, 190), 433 ([M+4-H]⁺, 93); ¹H NMR (CD₃OD), δ (ppm): 0.72 (s, 3H), 3.63 (t, 1H), 7.34 (s, 1H).

2,4-dibromoestrone (36): ESI(-)MS m/z : 427 ([M-H]⁻, 100), 429 ([M+2-H]⁻, 192), 431 ([M+4-H]⁻, 95); ¹H NMR (CD₃OD), δ (ppm): 0.90 (s, 3H), 7.41 (s, 1H).

Bromination of estradiol: General Procedure. A solution of NaClO₂ (7 mg, 0.1 mmol) and NaBr (9.5 mg, 0.1 mmol) in 5 mL MeOH was added **17 β E** (50 mg, 0.2 mmol) under stirring. This mixture was treated with 0.1 ml HCl conc (1 mmol) and allowed to stir at room temperature. The reaction was monitored by TLC. After 10 min, the mixture was diluted with water (20 mL) and extracted with ethyl acetate (3x25 mL). The combined organic layers were dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by PLC to give products **34** (R_f = 0.65, 22 mg, 35 % yield) and **17 β E** (R_f = 0.55, 25 mg).

2-bromo-17 β -estradiol (34): ESI(-)MS m/z : 350 ([M-H]⁻, 100), 352 ([M+2-H]⁻, 98); ¹H NMR (CD₃OD), δ (ppm): 0.76 (s, 3H), 3.64 (t, 1H), 6.58 (s, 1H), 7.28 (s, 1H).

Bromination of estradiol by NaBrO₃/NaS₂O₄: General Procedure. To a solution of NaBrO₃ (832 mg, 6 mmol) in water (20 mL) was added **17 β E** (50 mg, 0.2 mmol) in ethyl acetate (25 mL), followed by a solution of NaS₂O₄ (96 mg, 6 mmol) in water (40 mL) over a period of about 15 min, and the mixture was stirred at room temperature for 30 min. The orange mixture was poured into 40 mL of ethyl acetate. After separation of the phases, the aqueous layer was extracted twice with ethyl acetate, and the combined organic layer was dried over MgSO₄. The solvents were then removed in vacuo, and the residue was purified by PLC (eluant A) to give compounds **37** (R_f = 0.59, 15 mg, 15 % yield) and **38** (R_f = 0.82, 5 mg, 6% yield).

3-hydroxy-2,4,10-tribromo-9,10-secoestra-1(10),2,4-trien-9-one (37): UV (CH₃OH): λ_{max} 218, 232, 296 nm; ¹H NMR (CDCl₃), δ (ppm) 1.08 (s, 3H), 1.46-1.61 (m, 4H), 1.79 (m, 1H), 2.00 (m, 2H), 2.17 (m, 1H), 2.40 (m, 1H), 2.42 (m, 1H), 2.55 (m, 2H), 2.85 (m, 1H), 3.13 (m, 1H), 3.74 (t, 1H), 7.67 (s, 1H); ¹³C NMR (CDCl₃), δ (ppm) 11.5 (CH₃), 25.0 (CH₂), 25.8 (CH₂), 31.9 (CH₂), 36.1 (CH₂), 36.1 (CH₂), 38.6 (CH₂), 44.2 (C), 50.6 (CH), 51.3 (CH), 81.1 (CH), 108.0 (C), 113.9 (C), 115.6 (C), 135.4 (CH), 142.4 (C), 149.9 (C), 212.6 (C); ESI(-)MS m/z : 523 ([M-H]⁻, 100), 525 ([M+2-H]⁻, 265), 527 ([M+4-H]⁻, 223), 529 ([M+6-H]⁻, 92).

2,4-dibromoestrone (38): UV (CH₃OH): λ_{max} 225, 290 nm; ¹H NMR (CDCl₃), 0.90 (s, 3H), 1.46-1.52 (m, 4H), 1.63 (m, 1H), 1.96 (m, 1H), 2.04-2.18 (m, 3H), 2.26 (m, 1H), 2.33 (m, 1H), 2.46-2.60 (m, 2H), 2.67 (m, 1H), 2.94 (m, 1H), 7.40 (s, 1H); ¹³C NMR (CDCl₃), δ (ppm) 13.7 (CH₃), 21.5 (CH₂), 26.1 (CH₂), 26.4 (CH₂), 30.9 (CH₂), 31.4 (CH₂), 35.8 (CH₂), 37.3 (CH), 43.9 (CH), 47.8 (C), 50.2 (CH), 106.5 (C), 113.2 (C), 128.5 (CH), 134.7 (C), 136.4 (C), 147.2 (C), 220.0 (C); ESI(-)MS m/z : 425 ([M-H]⁻, 100), 427 ([M+2-H]⁻, 146), 429 ([M+4-H]⁻, 93)

SOLVENT FREE REACTIONS

Oxidation of 17 β E by FeNH₄(SO₄)₂: General Procedure. A mixture of 17 β E (50 mg, 0.2 mmol) and FeNH₄(SO₄)₂ (177 mg, 0.4 mmol) was finely powdered by agate mortar and pestle. The mixture was then put in a test tube and kept at 150 °C for 6 h. The solid was diluted with water and (60 mL) and extracted with ethyl acetate (3x40 mL). The combined organic layers were dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product gave the pure product **39** (30 mg, 67 % yield).

3'-methyl-7-hydroxy-1,2-cyclopentenophenanthrene (39): UV: λ_{\max} (CH₃OH) 259 nm; ¹H-NMR (CDCl₃) δ (ppm) : 1.38 (d, 3H), 1.80 (m, 1H), 2.52 (m, 1H), 3.19 (m, 1H), 3.33 (m, 1H), 3.40 (m, 1H), 7.19 (dd, *J* = 8.8, 2.8 Hz, 1H), 7.23 (d, *J* = 2.8 Hz, 1H), 7.49 (d, *J* = 8.4 Hz, 1H), 7.61(d, *J* = 8.8 Hz, 1H), 7.74 (d, *J* = 8.8 Hz, 1H), 8.45 (d, *J* = 8.4 Hz, 1H), 8.56 (d, *J* = 8.8 Hz, 1H); ¹³C-NMR (CDCl₃) δ (ppm) : 20.5 (CH₃), 29.9 (CH₂), 34.3 (CH₂), 40.1 (CH), 111.8 (CH), 116.6 (CH), 120.8 (CH), 122.3 (CH), 124.3 (CH), 124.7 (CH), 125.4 (C), 126.0 (CH), 127.6 (C), 129.1 (C), 133.0 (C), 140.3 (C), 145.7 (C), 153.7 (C); ESI(-)MS: *m/z* 247 [M-H]⁻.

Oxidation of 17 β E by FeCl₃: General Procedure. A mixture of 17 β E (50mg, 0.2 mmol) and FeCl₃ (400 mg, 1.6 mmol) was finely powdered by agate mortar and pestle. The mixture was then put in a test tube and kept at 60 °C for 6 h. The solid was diluted with water and (60 mL) and extracted with ethyl acetate (3x40 mL). The combined organic layers were dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by PLC (eluant A) to afford product **40** (*R_f*= 0.47, 3 mg, 5 % yield) and **41** (*R_f*= 0.19, 26 mg, 25% yield).

4,15-dichloroestra-1,3,5(10)-trien-3,17 β -diol (40): UV: λ_{\max} (CH₃OH) 209, 234, 264, 290, 302, 315, 337, 356 nm; ¹H-NMR (CD₃OD) δ (ppm): 1.00 (s, 3H), 1.70 (m, 1H), 2.27 (m, 1H), 2.75 (m, 1H), 2.93 (m, 1H), 3.22 (m, 1H), 3.40 (m, 1H), 4.11 (t, 1H), 7.21(d, *J* = 9.2 Hz, 1H), 7.98 (d, *J* = 9.2 Hz, H) 8.00 (d, *J* = 9.2 Hz, H), 8.57(d, *J* = 9.2 Hz, 1H); ¹³C-NMR (CD₃OD) δ (ppm): 16.3 (CH₃), 25.9 (CH₂), 36.5 (CH₂), 47.2 (CH₂), 49.9 (C), 79.8 (CH), 116.1 (C), 120.0 (CH), 122.4 (C), 122.7 (CH), 126.1 (CH), 127.3 (C), 128.0 (CH), 129.7 (C), 133.6 (C), 134.5 (C), 138.3 (C), 153.3 (C); ESI(-)MS *m/z* 333 ([M-H]⁻, 100), 335 ([M+2-H]⁻, 65), 337 ([M+4-H]⁻, 12) [α]_D²⁵ = -64.8° (*c* 0.42×10⁻⁵, MeOH)

4,4'-di[15-chloroestra-1,3,5(10)-trien-3,17 β -diol] (41): UV: λ_{\max} (CH₃OH) 257, 273, 303, 315 nm; ¹H-NMR (CD₃OD) δ (ppm): 1.00 (s, 6H), 1.72 (m, 2H), 2.25 (m, 2H), 2.70 (m, 2H), 2.87 (m, 2H), 3.23 (m, 2H), 3.46 (m, 2H), 4.11 (t, 2H), 6.90 (d, *J* = 9.6 Hz, 2H), 7.31 (d, *J* = 9.2 Hz, 2H), 8.13 (d, *J* = 9.2 Hz, 2H), 8.22 (d, *J* = 9.6 Hz, 2H); ¹³C-NMR (CD₃OD) δ (ppm): 15.9 (2 CH₃), 25.5 (2 CH₂), 36.3 (2 CH₂), 46.8 (2 CH₂), 49.0 (2 C), 79.5 (2 CH), 117.6 (2 C), 119.6 (2 CH), 121.9 (2 C), 124.5 (2 CH), 126.2 (2 C), 126.5 (2 CH), 127.2 (2 CH), 129.1 (2 C), 134.3 (2 C), 135.9 (2 C), 138.3 (2 C), 155.1 (2 C); ESI(-)MS *m/z* 597 ([M-H]⁻, 100), 599 ([M+2-H]⁻, 70), 601 ([M+4-H]⁻, 20); [α]_D²⁵ = -188.37°(*c* 0.43×10⁻⁵, MeOH).

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