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17β-Estradiol nitration by peroxidase/H_2O_2/NO_2^-: a chemical assessment

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Abstract—Nitration of 17β-estradiol by H_2O_2 and nitrite in the presence of various peroxidases, viz. horseradish peroxidase, lactoperoxidase, and peroxidase-containing homogenates from bovine uteri, was systematically investigated to assess on a chemical basis its potential relevance to the mechanisms of impairment of estrogen functions under oxidative/nitrosative stress conditions. In the presence of excess nitrite 17β-estradiol reacted smoothly to give 2-nitroestradiol (**1**), 4-nitroestradiol (**2**), and 2,4-dinitroestradiol (**3**). With 10–300 μM estradiol, formation yields of **1**–**3** were 12–55%, but dropped to 1% or less at lower estrogen concentration, for example, 1 μM, or in plasma as the reaction medium. Time course analysis showed that **2** is the prevalent nitration product under conditions of slow generation of nitrating species, suggesting some regioselectivity for estradiol nitration at C-4, whereas **1** prevails with bolus addition of reagents, due to faster degradation of **2**. Competition experiments carried out with $^{15}NO_2^-$ showed that **2** is about twice more susceptible to nitration than **1** as determined by ^{15}N NMR analysis of the resulting **3**. The biological effects of **1** and **2** were preliminarily tested on in vitro bovine embryo cultures. When **1** and **2** were substituted to the standard 17β-estradiol in the oocyte maturation, a significant decrease in both cleavage and blastocyst efficiency was observed in the case of **1** but not **2**. Overall, these results suggest that estradiol nitration is a potential pathway of hormonal dysfunction and toxicity but would require elevated estrogen levels of questionable physiological relevance.

1. Introduction

Nitration reactions targeted to cellular constituents contribute to the broad range of geno- and cytotoxic mechanisms in oxidative stress-based disease states.[1] At loci of inflammation, macrophages, neutrophils, and other cells of the immune system become activated to secrete several mediators of the inflammatory cascade including abnormally high fluxes of nitric oxide (NO). Under oxidative stress conditions, NO is partly diverted from its physiological roles via interaction with reactive oxygen species (e.g., superoxide), leading to aberrant production of peroxynitrite, nitrogen dioxide, and other reactive nitrogen species purportedly responsible for nitration of biomolecules.[2–4] Peroxynitrite is commonly regarded as the main nitrating agent in vivo, but increasing evidence indicates that nitrite ions, the main physiologic metabolite of NO, may also be involved. Relatively high levels of NO_2^- are found in physiological fluids (e.g., 0.5–3.6 μM in plasma and up to 210 μM in saliva)[5] increasing dramatically under pathological conditions.[6] Following oxidation, for example, by peroxidases such as horseradish peroxidase, lactoperoxidase, myeloperoxidase, and eosinophil peroxidase, or by Fenton-like reagents nitrite may be converted to the nitrating agent NO_2.[3,7–9]

The most typical target of nitrating species is protein tyrosine, giving 3-nitrotyrosine.[1,3,7,8] Although tyrosine nitration may occur in healthy subjects with no apparent dysfunction in nitrated proteins,[10] it has been reported to alter the functional properties of a variety of enzymes[11] and has been implicated in the pathogenesis of a range of diseases.[12] 3-Nitrotyrosine per se appears to be involved in tubulin degradation[13,14] and in neurodegeneration.[15]

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Catecholamines are another class of phenolic substrates that are susceptible to nitration, for example, by the peroxidase/H_2O_2/NO_2^- system, leading to regioselective conversion to 6-nitrocatecholamines.[16,17]

The widespread observation of tyrosine nitration by NO-derived nitrating systems indicates that nitration of other phenolic biomolecules, such as estrogens, would be likely in biological systems. Estrogens are responsible for the development of most secondary sex characteristics in females, and appear also to play important roles in the nervous system,[18] whereby they are ranked among the neurosteroids. NO synthase (NOS) occur in female reproductive tract[19] and in the central nervous system.[20] Elevated levels of NOSs are found in human breast tumors[21] and a correlation has been reported between tumor grade progression and increased NOS activity in many cancers,[22] although the actual relationship remains to be assessed.

Recently, preliminary data on the nitration of 17 β-estradiol by different systems has been reported, as part of a study on the nitrosation and nitration of the selective estrogen receptor modulator raloxifene.[23] In this, horseradish peroxidase/H_2O_2/NO_2^- was shown to be the most effective nitrating system for estradiol. We report herein a systematic study of the nitration of estradiol promoted by horseradish and mammalian peroxidases with a view to assessing the chemical feasibility of the process in vitro and the underlying reaction pathways. The effects of nitroestradiols on the in vitro maturation of bovine oocytes were also explored.

2. Results and discussion

2.1. Estradiol nitration: isolation of reaction products

Preliminary experiments were aimed at isolating and characterizing the products formed by nitration of estradiol. To this aim, estradiol (300 μM) was exposed to the horseradish peroxidase (HRP)/H_2O_2/NO_2^- system,[3] using 600 μM H_2O_2 and a 10-fold excess nitrite, as well as to peroxynitrite, an established phenol nitrating agent in vitro and in vivo.[8] In both cases, HPLC analysis showed the generation of three main products (Fig. 1).

The products eluted under peaks A and C were identified as the known 2-nitroestradiol (**1**) and 4-nitroestradiol (**2**), respectively, by straightforward spectral analysis and comparison of the chromatographic behavior with those of synthetic samples.[24] The same products were recently described in preliminary experiments with the HRP/H_2O_2/NO_2^- system as well as in the reactions of estradiol (0.2 mM) with SIN-1 in 40% acetonitrile–phosphate buffer, pH 7.4, and with spermine-NONOate.[23] The third peak (marked as B) gave a product with a molecular ion peak at m/z 362, suggesting a dinitro derivative. The ^{1}H NMR spectrum displayed only a single proton in the aromatic region at δ 8.11 giving a cross peak in the ^{1}H, ^{13}C HMQC spectrum with a carbon signal at δ 123.45. 2D homo- and heterocorrelated NMR analysis eventually allowed

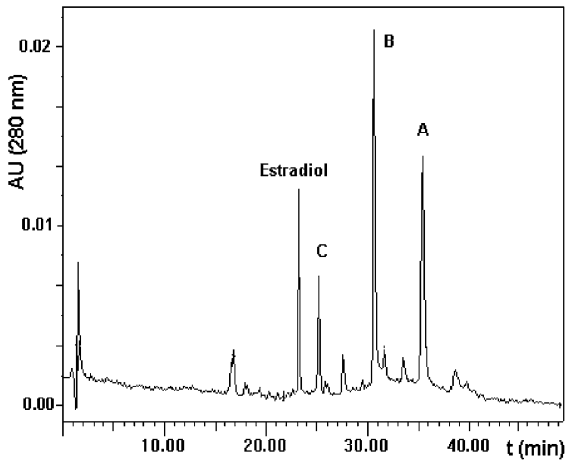
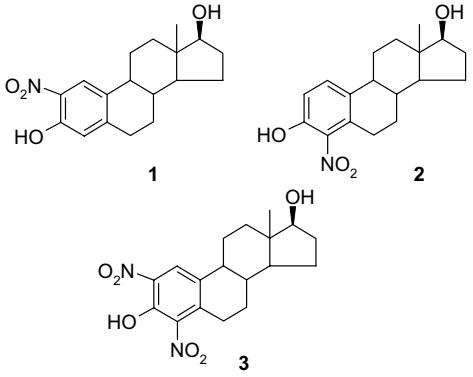


Figure 1. HPLC elution profile of the products formed by reaction of estradiol with HRP/H_2O_2/NO_2^- system. Estradiol (300 μM) in 0.1 M phosphate buffer pH 7.4 was treated with HRP (1 U/mL), NO_2^- (3.0 mM) and H_2O_2 (600 μM) portionwise over 40 min and the mixture analyzed at 50 min reaction time (eluant II).

identification of the product as 2,4-dinitroestradiol (**3**). The structural identity of the product was secured by comparison with a synthetic sample obtained by HNO_3 nitration of estradiol,[24,25] as well as by its formation by separate nitration of **1** or **2** by HRP/H_2O_2/NO_2^-.



2.2. Kinetic experiments

In a subsequent series of experiments, the kinetics of estradiol decay and nitroestradiol formation induced by various peroxidases were investigated with the substrate at 300 μM concentration and a 10-fold excess nitrite. Figure 2 compares the results obtained with a bolus addition of 600 μM H_2O_2 (graph A) and using 560 μM glucose/glucose oxidase as a physiologically relevant source of H_2O_2 (graph B) with HRP in the presence of NO_2^-. In both cases, the three main nitration products were clearly detectable.

In the reaction with the HRP/H_2O_2/NO_2^- system, estradiol consumption was about 85% at 1 h reaction time with an overall yield of nitration products of about 12%. Apparently, the low mass balance was accounted for by a complex mixture of products (possibly oxida-

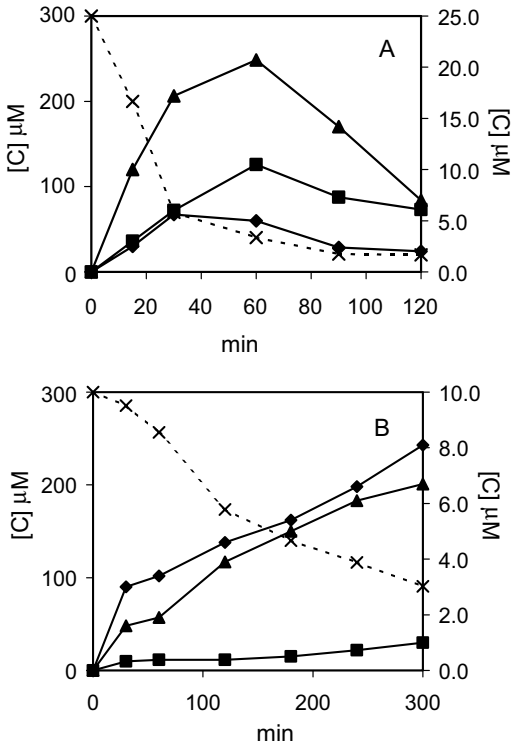


Figure 2. Time course of estradiol nitration and formation of **1**–**3** by the peroxidase/H_2O_2/NO_2^- system. Estradiol (300 μM) was incubated with HRP (1.0 U/mL), NO_2^- (3.0 mM) and H_2O_2 (600 μM) or glucose (560 μM), glucose oxidase (0.1 U/mL) as detailed in Section 4. Data are the means of three experiments, and SD <5%. Plot A: left axis (dashed line), consumption of estradiol (×), and right axis (solid line), formation of **1** (▲), **2** (◆), and **3** (■). Plot B: as in Plot A with generation of H_2O_2 by the glucose/glucose oxidase system.

tion products) all in very low amounts, which could not be identified. At 50% substrate consumption, **1** prevailed over **2** in the HRP/H_2O_2/NO_2^- reaction, whereas with the glucose/glucose oxidase system the products were formed in comparable amounts.

The reaction with the HRP/H_2O_2/NO_2^- system was also run with the substrate at 1.0 μM concentration and all other reagents maintained at the same molar ratios. At 2 h reaction time estradiol consumption was about 60% and **1** and **3** were formed in about 0.5% yield, whereas **2** was not detectable.

To assess the feasibility of estradiol nitration in a physiological fluid, the estrogen was reacted at 300 μM concentration with the HRP/H_2O_2/NO_2^- system using human plasma as the medium. At 2 h reaction time about 50% substrate consumption was observed and both **1** and **3** were formed at ca 0.3 and 0.4 μM concentration, respectively, whereas **2** was below detection limits.

The relative susceptibility of estradiol and tyrosine to nitration by HRP/H_2O_2/NO_2^- and peroxynitrite was compared under the reported conditions.[3] The results in Table 1 indicated that estradiol is at least as susceptible to nitration as tyrosine.

Table 1. Estradiol and tyrosine nitration by HRP/H_2O_2/NO_2^- and peroxynitrite

Substrate[a]	Product	Product yield (μM)[b]	
		Peroxynitrite	HRP/H_2O_2/NO_2^-
Tyrosine	3-Nitrotyrosine	6.1 ± 1.2	10.4 ± 1.5
Estradiol	**1**	<0.1	21.5 ± 2.4
	2	<0.3	6.3 ± 0.7
	3	3.1 ± 0.5	9.0 ± 0.8

[a] At 300 μM concentration.
[b] Determined at 60 min reaction time by HPLC analysis as detailed under Section 4. Data shown are the means of three experiments ± SD.

Figure 3 reports data obtained for estradiol nitration using lactoperoxidase (LPO)/hydrogen peroxide and tandem glucose/glucose oxidase/LPO as oxidizing systems under the above conditions.

As in the case of the HRP-catalyzed reaction, **2** was the prevailing product under conditions of slow generation of nitrating species (Fig. 3B), whereas the situation was reversed with bolus addition of reagents (Fig. 3A).

Figure 4 shows formation of **1** and **2** from 10 μM estradiol with LPO (1.0 U/mL) and H_2O_2 (20 μM), as a function of nitrite concentration. The data, determined in the early stages of the reaction when nitroestradiol consumption was not significant, indicated the preferential formation of **2** over the whole range of nitrite concentrations. The profile of estradiol consumption

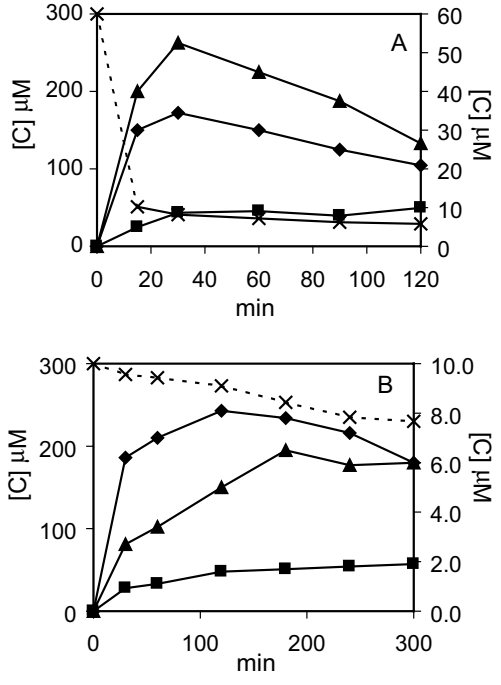


Figure 3. Time course of estradiol nitration and formation of **1**–**3** by the LPO/H_2O_2/NO_2^- system. Estradiol nitration was carried out as described in the legend to Figure 2. Data are the means of three experiments, and SD <5%. Plot A: left axis (dashed line), consumption of estradiol (×), and right axis (solid line), formation of **1** (▲), **2** (◆) and **3** (■). Plot B: as in Plot A with generation of H_2O_2 by the glucose/glucose oxidase system.

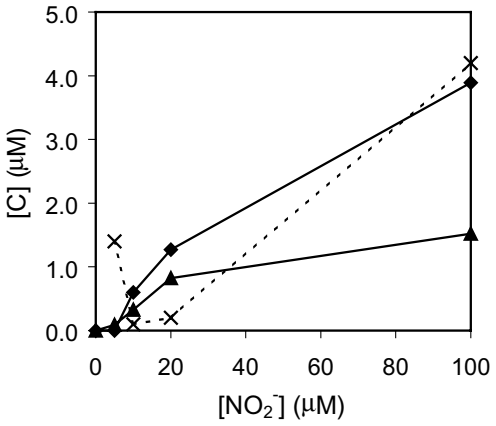


Figure 4. Formation of **1** and **2** and estradiol consumption as a function of nitrite concentration. Estradiol (10 μM) was incubated with LPO (1U/mL) and H_2O_2 (20 μM) and the mixture was analyzed at 4 h reaction time as detailed in Section 4. Data are the means of three experiments, and SD <10%. Solid line: **1** (▲), **2** (◆); dashed line: estradiol (×).

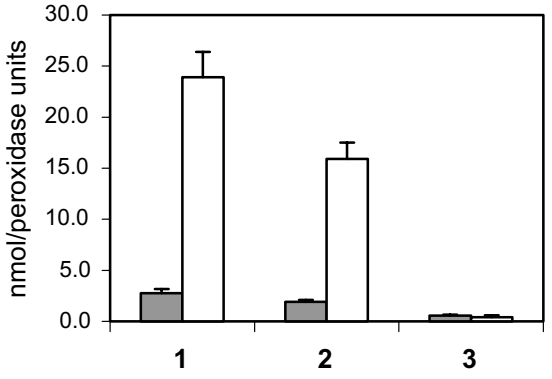


Figure 5. Yields of nitroestradiols obtained from estradiol by action of different peroxidase-containing fractions from bovine uterus. Estradiol (300 μM) was incubated with SF (gray bars) or MF (open bars) fractions in the presence of 32 mM $CaCl_2$ and in the presence of hydrogen peroxide (600 μM) and nitrite (3.0 mM) in 0.05 M phosphate buffer (pH 7.2). Shown are the mean + SD values for three separate experiments.

and mass balance data suggest that estradiol oxidation is somewhat inhibited at the higher nitrite concentrations, whereas at low nitrite concentrations other reaction pathways are operative in addition to nitration. In the presence of 10-fold excess nitrite, an about 60% consumption of estradiol is associated with formation of nitroestradiols **1** and **2** in around 80% overall yield based on reacted material. On the other hand, at lower nitrite to substrate ratios, for example, in the presence of 20 μM nitrite, estradiol is completely consumed with formation of nitroestradiols in less than 20% yield.

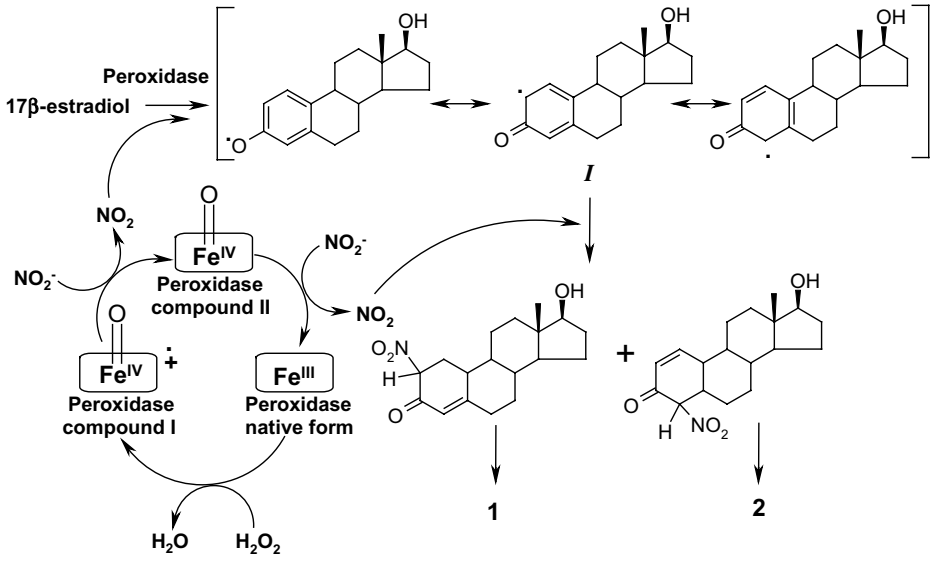
The data in Figures 2–4 were suggestive of a different susceptibility to decomposition of **1** and **2**. A competition experiment was therefore performed, in which an equimolar mixture of **1** and **2** (10 μM each) was reacted with the LPO/H_2O_2/NO_2^- system. The results showed that **2** was far more reactive to oxidation than **1**. At 15 min reaction time, the consumption of **1** and **2** was about 40% and 80%, respectively.

In another series of experiments, estradiol nitration was investigated in crude tissue homogenates from bovine uteri. Two cellular fractions were obtained, which contained peroxidase activity: a soluble fraction (SF) and a microsomal fraction (MF) (see Section 4). The results in Figure 5 indicated that both fractions contain significant levels of peroxidase capable of mediating conversion of estradiol to nitroestradiols **1**–**3** in the presence of H_2O_2 and nitrite. For comparative purposes, in these experiments substrate and reagent concentrations were identical with those adopted in the case of the HRP and LPO-induced reactions.

Omission of nitrite in the incubation mixture resulted in substrate consumption without appreciable formation of nitroestradiols. In the absence of Ca^{2+} the peroxidase containing fractions proved unable to mediate nitroestradiol formation, supporting the role of peroxidase in the nitrite-dependent nitration. Omission of H_2O_2 resulted in no detectable nitroestradiol formation. Under the reaction conditions investigated nitroestradiol yields obtained with the MF fraction were significantly higher than those of the SF fraction. Product **1** invariably prevailed over the isomer **2** while dinitroestradiol **3** was formed to a lower extent.

2.3. Mechanistic issues

Ample experimental evidence indicates that the active forms of HRP as well as of mammalian peroxidase lactoperoxidase and myeloperoxidase are competent in bringing about oxidation of NO_2^- to NO_2.[3,26] The ability of peroxidase including HRP and uterine peroxidase to effect oxidation of estradiol is also documented[27] and it can therefore be expected that estradiol may compete with nitrite for oxidation by peroxidase compound **I** and **II** under the reaction conditions examined. Such a competition may account for the observed decrease in estradiol consumption with increasing nitrite/estradiol molar ratios (Fig. 4). Nitroestradiols **1** and **2** would then result from coupling of the semiquinone radical ***I*** arising from one electron oxidation of estradiol, with NO_2 (Scheme 1). Such a mechanism is similar to that proposed for the haem-peroxidase generation of 3-nitrotyrosine by cooperative oxidation of nitrite and tyrosine.[1] Given the susceptibility of monophenolic compounds to undergo radical nitration by NO_2,[28] generation of ***I*** from estradiol brought about by NO_2 followed by radical coupling should be also considered a feasible route to nitroestradiols **1**–**3**. In the case of tyrosine nitration, the higher rate of reaction of tyrosine with peroxidase compound II at pH 7 ($1.6\times10^4\ M^{-1}\ s^{-1}$)[29] with respect to that of nitrite ($5.5\times10^2\ M^{-1}\ s^{-1}$)[30] suggests that the enzyme can bring about oxidation of both substrates. The lack of kinetic data for the reaction of estradiol with peroxidase does not allow to extend this conclusion to the case of estradiol nitration.



Scheme 1. Proposed mechanism of formation of nitroestradiols **1** and **2** by the peroxidase/H_2O_2/NO_2^- system.

Data in Figures 2–4 indicate that **2** prevails under conditions of slow generation of the oxidizing species, for example, with the tandem glucose/glucose oxidase–peroxidase system, at relatively low substrate concentration or in the early stages of the reaction, whereas **1** is the main product with bolus addition of reagents or whenever the oxidizing/nitrating species are in large excess or are generated at rapid rate. These lines of evidence indicate that **2** is the prevalent nitration product but is oxidized to a greater extent than **1** under forcing reaction conditions.

Calculation of Mulliken atomic spin densities for the geometry optimized structure of estradiol phenoxyl radical in vacuo and with the polarizable continuum model,[31] to keep into account possible solvent effects, gave values of 0.340 and 0.300 at C-2 and of 0.237 and 0.296 at C-4, respectively, which were not compatible with the apparent regioselectivity at C-4 for estradiol nitration. Likewise, inspection of SOMO isosurfaces[31] did not reveal appreciable differences between the 2- and 4-positions. Interestingly, however, a comparison of the energy minimized conformations[32] for each of the α and β stereoisomers of the 2- and 4-nitroketone arising by free radical coupling of estradiol phenoxyl radical with NO_2 (see Scheme 1) revealed that the stereoisomers of the 4-nitroketone were more stable than those of the 2-nitroketone. In particular, the most stable conformer of the 4-nitro series, viz. the 4β-nitro conformer having the O–N–C–H dihedral angle of 0.3°, displayed an energy 1.03 kcal/mol lower than that of the most stable conformer of the 2-nitro series, viz. the 2α-nitro conformer having the O–N–C–H dihedral angle of −26° (Fig. 6). Whether this has any bearing on the regiochemical outcome of estradiol nitration is difficult to assess at the present level of analysis.

The relative susceptibility of **1** and **2** to undergo further nitration to give **3** was then investigated. Under identical conditions, that is with substrate at 300 μM concentration, in the presence of nitrite (10 M equiv), HRP (2.0 U/mL) and H_2O_2 (2 M equiv), and at 30 min reaction time, **2** was consumed to a higher extent than **1** (30% vs 20%) leading to **3** in 16% yield versus 10% yield in the case of **1**. Furthermore, a competition experiment was performed in which an equimolar mixture of the nitroestradiols was oxidized by peroxidase/H_2O_2 in the presence of an excess of $^{15}NO_2^-$. The reaction was halted at 50 min, and the resulting **3** was isolated and subjected to NMR analysis (Scheme 2). The ^1H, ^{15}N HMBC spectrum (Fig. 7) indicated two distinct ^{15}N signals at δ 369.5 and 369.8, the former due to labeled nitro group at C-2 (well discernible ^3J correlation with the aromatic proton resonance at δ 8.11 (H-1)) and the latter to the labeled nitro group at C-4. The assignment was confirmed by separate preparation and analysis of the mono ^{15} N-labeled derivatives of **3**. The integrated areas of the nitrogen signals, under conditions ensuring complete relaxation of the nuclei, were in the approximate ratio of 2:1, denoting a prevalent location of the

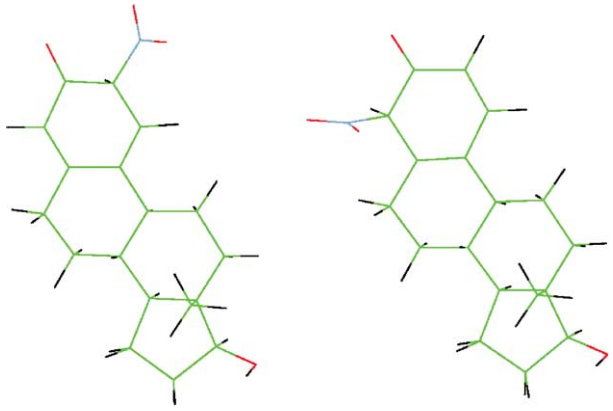
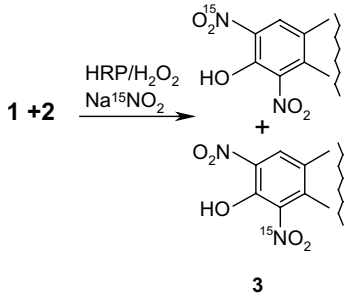


Figure 6. Energy minimized structures of nitroketones formed by coupling of estradiol phenoxy radical with NO_2 at C-2 and C-4 position.[32]



Scheme 2. Formation of ^{15}N labeled **3** by reaction of **1** and **2** with peroxidase/H_2O_2 in the presence of $^{15}NO_2^-$.

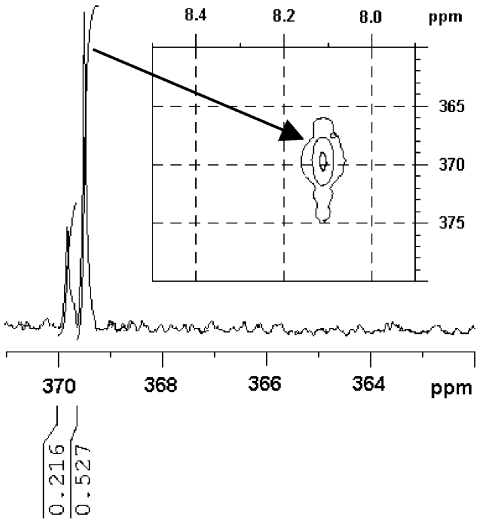


Figure 7. Selected region of the ^{15}N NMR spectrum of **3** obtained by reaction of equimolar **1** and **2** with HRP/H_2O_2 in the presence of $^{15}NO_2^-$. The integrated areas of ^{15}N resonances are reported below the scale. Inset: shown is the correlation peak between the proton resonance at 8.11 ppm and the ^{15}N resonance at 369.49 ppm in the ^1H, ^{15}N HMBC spectrum.

label on the 2-position due to the faster formation of the phenoxyl radical from **2**.

A useful interpretative basis of the higher reactivity exhibited by **2** with respect to **1** is provided by the structural properties of the compounds.[33] The crystal structure of **1** indicated that the nitro-oxygens are coplanar to the aromatic ring, thus ensuring efficient electron conjugation. In contrast, in **2** the nitro group is significantly twisted out of the plane of the aromatic ring and is thus nonconjugating. This entails a decreased influence of the nitro group on the electronic properties of the phenol ring making the reactivity of **2** somewhat closer to that of the parent estrogen.

2.4. Effects of nitroestradiols on in vitro oocyte maturation

In a final set of experiments, the effect of **1** and **2** on bovine oocyte maturation in vitro was investigated, to determine the possible influence of nitroestradiols on

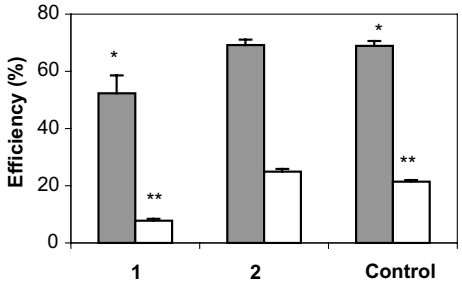


Figure 8. Effect of nitroestradiols **1** and **2** in comparison with estradiol (control) on the efficiency of cleavage and blastocyst production during the in vitro maturation of cattle oocytes. Shown are the means of three replicates + SD. Gray bars: cleavage; open bars: blastocysts. Concentration of estradiols was 1 μg/mL. COC used for the experiments with **1** or **2** or 17β-estradiol were 76, 94, and 85 in the order. * $P<0.05$; ** $P<0.01$.

embryonic development. 17β-Estradiol is a component naturally present in the pre-ovulatory follicle and is involved in the oocyte meiotic competence.[34] As shown in Figure 8, substitution of 17β-estradiol with **1** (1 μg/mL) resulted in significant inhibition of embryo development, as evaluated by cleavage and blastocyst production.

Addition of 17β-estradiol (1 μg/mL) to incubation mixtures containing 1 μg/mL of **1** partially counteracted the inhibitory effects of **1** (data not shown). In all cases nitroestradiols **1** and **2** were recovered apparently unchanged from the incubation media.

3. Conclusions and biological relevance

Aim of the present study was to address whether aromatic nitration may be a potential conversion route of estradiol under oxidative and nitrosative stress conditions. Based on these results and previous[23] lines of evidence, peroxidase/H_2O_2/nitrite may be a candidate system to induce estradiol nitration. In female reproductive apparatus, peroxidase is present in the uterus,[35] and it is relevant that the uterine peroxidase has been found to be competent to bring about estradiol nitration in crude homogenates.[27] The observed formation of a dinitroderivative, along with two nitroderivatives, denoted a *chemical* susceptibility to nitration at least comparable to that of tyrosine, the nitratable biological target *par excellence*.

This notwithstanding, the balance of available data fails to meet expectations of substantial estradiol nitration in vivo, mainly because the reaction becomes significant at substrate concentrations in the μM range, that is far from physiological levels. Moreover, tyrosine, both free and protein bound, may be a more abundant target for nitrating species in vivo. However, the possibility that some nitration may occur under acute oxidative/nitrosative stress conditions accompanying inflammation and with abnormally high estrogen levels, for example, after administration for therapeutic purposes, should not be overlooked.

In addition to the chemical elucidation of reaction pathways, the results of the present study have provided preliminary evidence for a potential inhibitory effect of **1** and, to a lesser extent, **2** on oocyte cleavage and embryonic development. Differential biological effects of isomeric nitroestradiols have already been noted and are confirmed in the present study. For example, they display different receptor binding affinities:[33] **1** bound to estrogen receptors with 1/1000th the affinity of estradiol and was inefficient in gene stimulation, whereas **2** displayed a relative binding affinity 40-fold greater than that of the 2-nitro derivative. Thus nitroestradiols, especially **1**, appear to be valuable candidate substances to modulate estradiol actions. Product **1** moreover is an efficient inhibitor of steroid alcohol sulfotransferase, an enzyme catalyzing metabolically important sulfation of steroids.[36] The toxicity data presented here point to nitroestradiols as potentially useful compounds for investigative purposes and could stimulate their search as novel endogenous modulators of receptor functions. A better understanding of their biological properties may open up new avenues for the therapy of malignant diseases, infertility, and CNS disorders.

4. Experimental

4.1. Materials

17β-Estradiol, tyrosine, 3-nitrotyrosine, D-glucose, potassium thiocyanate, ferrous sulfate heptahydrate, nonstabilized hydrogen peroxide (35% solution in water), [^{15}N]$NaNO_2$ (>99% isotopic enrichment) were from Aldrich Chemie. Horseradish peroxidase (HRP) (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) type II (167 pyrogallol U/mg, RZ $E_{430}/E_{275} = 2.0$), lactoperoxidase (LPO) from bovine milk (80 pyrogallol U/mg RZ $E_{412}/E_{280} = 0.76$), glucose oxidase from *Aspergillus niger* (β-D-glucose: oxygen 1-oxidoreductase EC 1.1.3.4) type II (20,000 U/mg) were obtained from Sigma and used without further purification. Peroxynitrite was prepared from sodium nitrite and hydrogen peroxide in acid using a quenched flow reactor as described,[37] and its concentration was determined spectrophotometrically ($\varepsilon = 1.67\,mM^{-1}\,cm^{-1}$ at 302 nm). Plasma samples from freshly drawn heparinized blood was deep frozen at −70 °C until use.

4.2. Animals

The uterus of 30 cycling 14–18 months old heifers were collected at abattoir at different times within a three month period (Sept–Dec) and promptly stored into a thermal bag at 4 °C. At the arrival to the laboratory, within 3 h from collection, the organs were layered on crushed ice and washed with saline solution at 4 °C. The uterine bodies and horns were opened along their length to remove endometrium slices. Pools of endometrium slices were stored at −80 °C. Ovaries from slaughtered cows were collected from the abattoir and transported in a thermal bag at 27–30 °C to the laboratory within 3–4 h of collection. The laboratory temperature was 30 °C.

4.3. Methods

UV and IR spectra were performed using a diode array and spectrophotometer. ^{1}H, ^{13}C spectra were recorded at 400.1 and 100.6 MHz, respectively. ^{1}H, ^{1}H COSY, ^{1}H, ^{13}C HMQC, ^{1}H, ^{13}C HMBC, and ^{1}H, ^{15}N HMBC experiments were run at 400.1 MHz using standard pulse programs from the Bruker library. For ^{15}N NMR experiments delay values up to 10 s were used. ^{15}N chemical shifts are relative to NH_3 (liquid, 298 K, 0.0 ppm). For EIMS spectra samples were ionized with a 70 eV beam, and the source was taken at 230 °C. Main fragmentation peaks are reported with their relative intensities (percent values are in brackets). Analytical and preparative TLC analyses were performed on F_{254} 0.25 and 0.5 mm silica gel plates or high performance TLC (HPTLC) using cyclohexane/ethyl acetate 60:40, eluant A. Column chromatography was performed using 60–230 mesh silica gel. Analytical and preparative HPLC was carried out with an instrument equipped with a UV detector set at 254 or 280 nm. Octadecylsilane coated columns, 4.6×250 mm (5 μM particle size) or 22×250 mm (10 μM particle size) were used for analytical or preparative runs at a flow rate of 1.3 or 15 mL/min, respectively. Elution conditions: 1% acetic acid/acetonitrile 40:60 (eluant I); 1% acetic acid/acetonitrile 90:10 (solvent A), acetonitrile (solvent B), from 20% to 55% solvent B gradient, 30 min, 55% solvent B, 10 min (eluant II); 0.05 M phosphate buffer, pH 3.0/methanol 90:10 (eluant III); 0.5% acetic acid/acetonitrile 60:40 (solvent A), acetonitrile (solvent B); 0% solvent B, 20 min, from 0% to 25% solvent B gradient, 10 min, 25% solvent B, 20 min (eluant IV).

Production of H_2O_2 by glucose oxidase was quantitated by oxidation of Fe (II) and formation of Fe (III)-thiocyanate complex.[3] Nitrite was determined spectrophotometrically at 538 nm using the Griess reagent (1% sulfanylamide, 0.1% naphthylethylenediamine in 2.5% phosphoric acid).[38]

4.4. Synthesis of nitroestradiols 1–3

The reaction was carried out by procedures previously reported[24,25] with modifications. In brief, to a solution of **1** (200 mg, 0.74 mmol) in glacial acetic acid (20 mL) taken at 70 °C in a water bath concentrated nitric acid (40 μL) was added under stirring. The mixture was allowed to stand for 18 h at rt, diluted with H_2O and extracted with ethyl acetate (3×10 mL). The organic layers dried over sodium sulfate were taken to dryness and the residue fractionated by silica gel chromatography (1.0×50 cm column) using cyclohexane/ethyl acetate (70:30 to 40:60 gradient mixtures) as the eluant to afford pure 2-nitroestradiol (**1**) (70 mg, 30% yield), 4-nitroestradiol (**2**) (59 mg, 25% yield). When nitration reaction was carried out as above but using higher amounts of HNO_3 (160 μL) work up and column chromatography of the reaction mixture afforded as the main product 2,4-dinitroestradiol (**3**) (30 mg, 12% yield).

4.5. 2-Nitroestradiol (1)[33]

(R_f 0.45, eluant A; t_R 39 min, eluant I); δ_H (acetone-d_6) 0.74 (3H, s), 1.11–1.52 (7H, m), 1.65 (1H, m), 1.85–1.96 (3H, m), 2.12 (1H, m), 2.29 (1H, m), 2.86 (2H, m), 3.64 (1H, m), 6.83 (1H, s), 7.90 (1H, s), 10.21 (1H, s); δ_C (acetone-d_6) 11.69 (CH_3), 23.88 (CH_2), 27.08 (CH_2), 27.50 (CH_2), 31.12 (CH_2), 31.78 (CH_2), 37.56 (CH_2), 39.33 (CH), 44.11 (C), 44.42 (CH), 51.07 (CH), 81.84 (CH), 119.86 (CH), 122.22 (CH), 133.06 (C), 134.85 (C), 150.11 (C), 153.37 (C). EIMS m/z 317 (M^+, 97), 287 (15), 271 (10), 258 (100). HREIMS calcd mass for $C_{18}H_{23}NO_4$ 317.1627 Da, found m/z 317.1618 Da.

4.6. 4-Nitroestradiol (2)[33]

(R_f 0.22, eluant A; t_R 28 min, eluant I); δ_H (acetone-d_6) 0.81 (3H, s), 1.20–1.52 (7H, m), 1.70 (1H, m), 1.80–2.20 (3H, m), 2.23 (1H, m), 2.32 (1H, m), 2.74 (2H, m), 3.67 (1H, m), 6.94 (1H, d, $J = 8.8$ Hz), 7.35 (1H, d, $J = 8.8$ Hz), 9.30 (1H, br s); δ_C (acetone-d_6) 11.69 (CH_3), 23.73 (CH_2), 24.94 (CH_2), 27.01 (CH_2), 27.23 (CH_2), 30.98 (CH_2), 37.60 (CH_2), 39.06 (CH), 44.01 (C), 44.74 (CH), 50.67 (CH), 81.79 (CH), 115.32 (CH), 128.89 (CH), 129.44 (C), 133.41 (C), 144.22 (C), 147.42 (C). EIMS m/z 317 (M^+, 100), 300 (73), 287 (20), 258 (79). HREIMS calcd mass for $C_{18}H_{23}NO_4$ 317.1627 Da, found m/z 317.1599 Da.

4.7. 2,4-Dinitroestradiol (3)[25]

(R_f 0.28 eluant A; t_R 36 min eluant I); δ_H (acetone-d_6) 0.80 (3H, s), 1.21–1.45 (7H, m), 1.70 (1H, m), 1.93–2.04 (3H, m), 2.25 (1H, m), 2.36 (1H, m), 2.78 (2H, m), 3.70 (1H, m), 8.11 (1H, s); δ_C (acetone-d_6) 11.36 (CH_3), 23.51 (CH_2), 25.19 (CH_2), 26.37 (CH_2), 26.83 (CH_2), 30.86 (CH_2), 37.19 (CH_2), 38.52 (CH), 43.81 (C), 44.02 (CH), 50.44 (CH), 81.47 (CH), 123.45 (CH), 133.70 (C), 133.81 (C), 138.72 (C), 145.82 (C), 148.81 (C). EIMS m/z 362 (M^+, 6), 332 (3), 317 (62), 258 (100). HREIMS calcd mass for $C_{18}H_{22}N_2O_6$ 362.1478 Da, found m/z 362.1455 Da.

4.8. Reaction of estradiol with HRP/H_2O_2/$NaNO_2$: isolation of 1–3

A solution of estradiol (100 mg, 0.37 mmol) in methanol (20 mL) was added to 0.1 M phosphate buffer pH 7.4 (1.2 L). The mixture was treated under vigorous stirring at room temperature with sodium nitrite (255 mg, 3.7 mmol) and HRP (1 U/mL). Hydrogen peroxide (10%) was added in 8 aliquots (0.09 mmol each) over 40 min. The reaction course was followed by HPLC analysis (eluant I or II). After 50 min the reaction was halted by addition of $Na_2S_2O_5$ (70 mg, 0.37 mmol) and the mixture was extracted with ethyl acetate (3×500 mL). The combined organic layers were dried over sodium sulfate and taken to dryness. The residue was fractionated by PLC (eluant A) to afford three main bands, which were found to consist of pure **1** (12 mg, 10% yield), **2** (5 mg, 4% yield) and **3** (10 mg, 8% yield). In separate experiments **1** or **2** were exposed to the HRP/H_2O_2/NO_2^- system under the above conditions and the reaction course was monitored by HPLC analysis (eluant I or II).

4.9. Oxidation of 1 and 2 in the presence of nitrite ions

A solution of the appropriate nitroestradiol (5 mg, 0.016 mmol) in methanol (1 mL) was added to 0.1 M phosphate buffer, pH 7.4 (52 mL) and treated with nitrite ions (11 mg, 0.16 mmol) and HRP (2.0 U/mL) under vigorous stirring. Hydrogen peroxide was added in 4 aliquots (0.008 mmol each) over 10 min. Aliquots of the reaction mixture were periodically withdrawn, extracted with ethyl acetate and analyzed by HPLC using eluant I. In either case 2,4-dinitroestradiol (**3**) was the only detectable reaction product. Identification and quantitation of reaction products was carried out by comparing retention times and integrated peak areas with external calibration curves for authentic samples. At 30 min reaction time consumption of **2** (t_R 6 min, eluant I) or **1** (t_R 17 min, eluant I) was 30% and 20%, respectively, and formation yields of **3** (t_R 12 min, eluant I) were 16% and 10%, respectively.

4.10. ^{15}N labeling experiments

An equimolar mixture of **1** and **2** (50 mg each, 0.15 mmol) dissolved in methanol (10 mL) was added to 0.1 M phosphate buffer, pH 7.4 (500 mL) and the solution was treated with [^{15}N] $NaNO_2$ (1.5 mmol) and peroxidase (1.0 U/mL). Hydrogen peroxide was added portionwise up to 0.30 mmol final concentration within 40 min. The reaction course was followed by HPLC (eluant II). At 50 min reaction time, when HPLC analysis revealed formation of **3** but with residual starting materials, the mixture was extracted with ethyl acetate (3×200 mL) and the combined organic layers were dried over sodium sulfate and taken to dryness. The residue was fractionated by PLC (eluant A) to afford labeled **3** (14 mg) δ_N (acetone-d_6) 369.49, 369.82; EIMS: m/z 363 (M^+, 30), 317 (M^+–$^{15}NO_2$, 46), 258 (M^+–$^{15}NO_2$–C_3H_7O, 100). In other experiments **1** or **2** were treated separately with [^{15}N] $NaNO_2$ and labeled **3** was isolated by fractionation of the reaction mixture as above.

4.11. Kinetic experiments

To solutions of estradiol (300 μM) in 0.1 M phosphate buffer pH 7.4 in a water bath thermostated at 37 °C, sodium nitrite (3.0 mM) and HRP or LPO (1.0 U/mL final concentration) were added under vigorous stirring. Hydrogen peroxide was added portionwise over 10 min up to 600 μM final concentration. Aliquots of the reaction mixture were periodically withdrawn, treated with a solution of $Na_2S_2O_5$ in water (600 μM final concentration) to terminate oxidation, extracted with ethyl acetate, and analyzed by HPLC using eluant I or II. Similar

experiments were performed in which: (i) the substrate was at 1 μM; (ii) H_2O_2 was generated in situ using D-glucose (560 μM) and glucose oxidase (0.1 U/mL, final concentration); (iii) the substrate was at 10 μM with nitrite varying in the range 5–100 μM. In other experiments peroxynitrite was added to estradiol (300 μM) in 0.1 M phosphate buffer pH 7.4 portionwise at 5 min intervals up to 300 μM concentration. Aliquots of the reaction mixture were analyzed by HPLC (eluant I) following work up as above. When estradiol nitration was carried out in plasma as the medium, a solution of estradiol in methanol (20 μL) was added to plasma (2 mL) up to 300 μM concentration, followed by sodium nitrite (3.0 mM) and HRP (1.0 U/mL) and the mixture was taken at 37 °C. Hydrogen peroxide was added portionwise over 10 min up to 1.20 mM final concentration. At 2 h reaction time the mixture was worked up as above. In all experiments, identification and quantitation of reaction products were carried out by comparing retention times and integrated peak areas with external calibration curves for authentic samples. All experiments were run at least in triplicate.

4.12. Uterine peroxidase

Crude uterine peroxidases were extracted as previously reported.[39] Briefly, endometrium from uterine horns was finely minced, frozen on dry ice–acetone, reduced to a fine powder, and homogenized in ice cold 0.01 M Tris–HCl pH 7.2 (20% w/v), using a glass/glass homogenizer. Homogenates were centrifuged (Beckman, Model L8-70M) at 40,000×*g* for 30 min, at 4 °C. The supernatants represented the soluble fraction (SF). Pellets were homogenized again in ice cold 0.01 M Tris–HCl pH 7.2 (2 mL) containing 0.5 M $CaCl_2$ and centrifuged at 100,000×*g* for 30 min at 4 °C; the supernatants collected at this stage represented the calcium-extractable microsomial fraction (MF).

Peroxidase activity of either SF and MF fractions was determined by the *o*-phenylenediamine assay as follows. The incubation mixture contained 154 mM sodium citrate buffer, pH 5 (650 μL), 75 mM *o*-phenylenediamine (250 μL), 100 mM H_2O_2 (50 μL) and a suitable amount of the enzyme. SF and MF were adjusted to 0.25 M $CaCl_2$ before assay. The reaction course was followed spectrophotometrically by recording the absorbance at 496 nm at 37 °C. One unit was defined as the amount of enzyme required to produce one unit increase of the absorbance under the assay conditions. SF fraction, diluted 1:3 (v/v) with 0.05 M sodium phosphate buffer (pH 7.2), was incubated at 37 °C with estradiol (300 μM) in the presence of 32 mM $CaCl_2$. The reaction was started by addition of H_2O_2 (600 μM) and sodium nitrite (3.0 mM). Incubation with MF fraction diluted 1:3 (v/v) with 0.05 M sodium phosphate (pH 7.2) was carried out under the same conditions. Other experiments were carried out as above without addition of $CaCl_2$ or nitrite. In all cases, after 2 h, reaction mixtures were extracted three times with ethyl acetate, and the combined organic layers, dried over anhydrous sodium sulfate, were evaporated to dryness. The residue was taken up in methanol for HPLC analysis (eluant IV). Identification and quantitation of nitroestradiols were carried out as described above.

4.13. Tyrosine nitration

A solution of tyrosine (300 μM) in 0.1 M phosphate buffer pH 7.4 was treated sequentially with HRP (1 U/mL), sodium nitrite (3.0 mM) and hydrogen peroxide (600 μM) as described above for estradiol or with peroxynitrite portionwise up to 300 μM concentration. Aliquots of the reaction mixture were periodically withdrawn, treated with $Na_2S_2O_5$ (300 μM final concentration) in the case of the reaction with $HRP/H_2O_2/NO_2^-$ system and analyzed by HPLC (eluant III) for identification and quantitation of 3-nitrotyrosine.

4.14. Effect of 1 and 2 on in vitro maturation of cattle oocytes

In vitro production of cattle embryos was performed by the procedure previously described.[40] Immature oocytes were collected from 2 to 8 mm follicles by an 18-G needle under controlled pressure (50–70 mm Hg). Cumulus-oocyte complexes (COC) were isolated from the follicular fluid and washed three times with tissue culture medium (TCM199) supplemented with 0.05% polyvinylalcohol and 10 mM HEPES. The COC were transferred into the maturation medium within 4-well plates (Nunclon, Nunc, DK) (30 μL/COC) and left in an incubator at 39.0 °C in 5% CO_2 humidified air for 24 h.

Maturation medium was TCM199 supplemented with 10% fetal calf serum and 10 IU/mL luteinizing hormone and 0.1 IU/mL follicle stimulating hormone. This medium was further supplemented with either 17β-estradiol (1 μg/mL) or **1** (1 μg/mL) or **2** (1 μg/mL).

After maturation, COCs were freed from the cumulus cells by vortexing for 3 min and parthenogenetically activated by 5 min exposure to 7.5 μM Ca-ionophore, A23187, in Fert-TALP medium. The oocytes were then transferred in culture dishes (Nunclon, Nunc, DK) containing Fert-TALP medium supplemented with 2.5 mM 6-dimethylaminopurine and incubated for 3.5 h. Finally, the oocytes were transferred in synthetic oviductal fluid supplemented with essential and nonessential amino acids and bovine serum albumin (SOFaaBSA), covered with embryo-tested oil and cultured under controlled gaseous environment (5% CO_2, 7% O_2, 88% N_2) for 8 days post-activation for blastocyst development. Culture plates were changed every 2 days. The recovery of **1** or **2** in the incubation media was determined by ethyl acetate extraction followed by HPLC analysis (eluant I).

The developmental potential of cattle oocytes exposed either to **1** or **2** or control (17β-estradiol), measured as cleavage and blastocyst rates, was compared by ANOVA after arcsine transformation.

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