Nitrosation, Nitration, and Autoxidation of the Selective Estrogen Receptor Modulator Raloxifene by Nitric Oxide, Peroxynitrite, and Reactive Nitrogen/Oxygen Species

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The regulation of estrogenic and antiestrogenic effects by selective estrogen receptor modulators (SERMs) provides the basis for use in long-term therapy in cancer chemoprevention and postmenopausal osteoporosis. However, the evidence for carcinogenic properties within this class requires study of potential pathways of toxicity. There is strong evidence for the elevation of cellular levels of NO in tissue treated with SERMs, including the benzothiophene derivative, raloxifene, in part via up-regulation of nitric oxide synthases. Therefore, the reactions of 17β-estradiol (E_2), raloxifene, and an isomer with NO, peroxynitrite, and reactive nitrogen/oxygen species (RNOS) generated from NO_2^-/H_2O_2 systems were examined. Peroxynitrite from bolus injection or slow release from higher concentrations of 3-morpholinosydnonimine (SIN-1) reacted with the benzothiophenes and E_2 to give aromatic ring nitration, whereas peroxynitrite, produced from the slow decomposition of lower concentrations of SIN-1, was relatively unreactive toward E_2 and yielded oxidation and nitrosation products with raloxifene and its isomer. The oxidation and nitrosation products formed were characterized as a dimer and quinone oxime derivative. Interestingly, the reaction of the benzothiophenes with NO in aerobic solution efficiently generated the same oxidation products. Stable quinone oximes are not unprecedented but have not been previously reported as products of RNOS-mediated metabolism. The reaction of glutathione (GSH) with the quinone oxime gave both GSH adducts from Michael addition and reduction to the corresponding *o*-aminophenol. The ready autoxidation of raloxifene, observed in the presence of NO, is the first such observation on the reactivity of SERMs and is potentially a general phenomenon of significance to SERM chemical toxicology.

Introduction

Tamoxifen is the therapy of choice in the treatment of all stages of hormone-dependent breast cancer and in breast cancer chemoprevention, despite the increased, demonstrated risk associated with endometrial cancer (*1*−*3*). Tamoxifen is regarded as a prototype selective estrogen receptor modulator (SERM). The goal of subsequent generations of SERMs has been to develop a potent, pure antiestrogen in mammary tissue, devoid of estrogenic uterine stimulation that manifests beneficial estrogenic activity toward bone, lipids, and the vasculature. The benzothiophene SERM, raloxifene (**1**), in clinical use in postmenopausal osteoporosis and in clinical trials as both a chemopreventive agent and a cardiovascular risk modifier, holds such promise (*4*−*6*). A further member of the benzothiophene SERM family, arzoxifene, has been shown to be significantly more potent than raloxifene in experimental breast cancer chemoprevention (*7*). The chronic, long-term drug treatment envisioned for SERM therapy in otherwise healthy individuals requires a full understanding of the potential cytotoxic and carcinogenic mechanisms associated with SERMs.

There is a sound body of research linking estrogen and SERM biological activity with NO signaling, in particular in mediating the cardiovascular effects produced by estrogens and SERMs. Increased cellular levels of NO have been shown to result from both activation and up-regulation of constitutive NOS[1] (eNOS and nNOS) (*8*−*11*). Evidence also exists for elevated NO levels resulting from induction of iNOS, from potentiation of iNOS activity, and from antiestrogenic inhibition of iNOS down-regulation (*12*−*15*). Both iNOS and eNOS are found at elevated levels in human breast tumors and

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[1] Abbreviations: ASI-3, 3-*p*-fluorophenylsydnonimine hydrochloride or 5-amino-3-(3-fluorophenyl)-1,2,3-oxadiazolium chloride; DHR, dihydrorhodamine 6G; DTPA, diethylenetriaminepentaacetic acid; ER, estrogen receptor; E_2, 17β-estradiol; GSH, glutathione; NONOate, diazeniumdiolate salt; NOS, nitric oxide synthase; HMBC, heteronuclear multiple bond correlation; HRP, horseradish peroxidase; HMQC, heteronuclear single quantum correlation; RNOS, reactive nitrogen/oxygen species; SIN-1, 3-morpholinosydnonimine hydrochloride or 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride; SIN-1C, 4-morpholinylimino acetonitrile; SPE/NO, spermine NONOate, $H_2N(CH_2)_3NH(CH_2)_4-N(NONO)-(CH_2)_3NH_3$.

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human breast apocrine metaplasia (*16*). In addition, a correlation has been reported between tumor grade progression and increased NOS activity in many cancers, including ovarian and uterine tumor tissue (*17*−*20*), although the interplay between NO and peroxynitrite and tumor promotion vs cytoprotection is complex (*21*−*23*).

NO may manifest both cytoprotective and cytotoxic properties, the latter argued to result largely through the strongly oxidizing cytotoxin, peroxynitrite, generated from the very rapid reaction of NO with superoxide ($O_2^{\bullet-}$) (*24*). Numerous biomolecules have been shown to undergo oxidation and nitration reactions with peroxynitrite, compatible with the chemistry of the peroxynitrite radical homolysis products, $HO^{\bullet}/CO_3^{\bullet-}$ and NO_2 (*25*−*27*). In particular, the formation of nitrotyrosine from the nitration of tyrosine residues of proteins has been suggested as a biomarker for inflammatory conditions associated with tissue injury mediated by peroxynitrite and other RNOS (*28*). Frequently, up-regulation of iNOS is proposed as an initiator of oxidative and nitrosative stress, since iNOS produces larger fluxes of NO than the constitutive isoforms and hence higher concentrations of peroxynitrite. Moreover, RNOS, in particular NO_2, may be derived from biological precursors other than peroxynitrite (*29*, *30*).

The carcinogenic effects of SERMs may result from hormonal effects (*31*); however, a number of pathways have been proposed for chemical carcinogenesis in which genotoxic events are initiated by SERMs or their metabolites through (i) binding to a cellular macromolecule resulting in covalent modification or other disruption of cellular function and (ii) redox cycling resulting in depletion of cellular reducing factors and loss of antioxidant capacity (*32*). SERMs are polyaromatic phenols, susceptible to oxidative metabolism, and capable of forming a number of reactive and electrophilic metabolites including orthoquinones and quinone methides that may covalently modify biomolecules including DNA (*33*). The potential reactivity of SERMs, in the presence of elevated NO concentrations and of peroxynitrite, includes the formation of oxidation products, which may be similar to those formed through other oxidative metabolic processes, but also the potential formation of nitration and nitrosation products.

The widespread observation of tyrosine nitration, by the action of peroxynitrite or other NO-derived nitrating systems, indicates that nitration of other phenol biomolecules, such as estrogens themselves, would be likely in biological systems. We hypothesized that the autoxidation of more readily oxidizable polyaromatic phenolics, such as the SERMs, might lead to reaction not only with peroxynitrite but also even with NO itself. The work presented herein, reporting on the formation of metabolites of estradiol and novel metabolites of raloxifene and an isomer, represents the first study directed at potential estrogen and antiestrogen metabolites resulting from reactions with peroxynitrite, RNOS, and NO.

Experimental Section

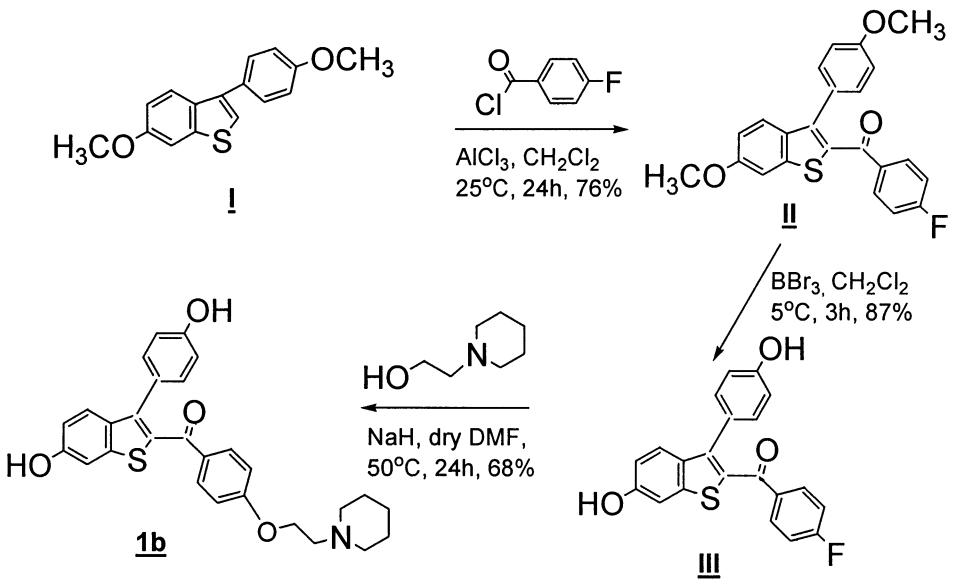
All chemicals unless otherwise stated were obtained from Sigma (St. Louis, MO), Aldrich Chemicals (Milwaukee, WI), or BDH (Toronto, Canada). Fluorescence measurements were made on a SpectraMaxGemini XS fluorescence reader, and UV−vis absorbance measurements were made on a Beckman DU 7400. HPLC method A: Shimadzu LC10A, with water-thermostated autosampler and with UV−vis detection at 280 nm employing gradient elution on a Jones Genesis C18 150 mm × 4.6 mm 5 μm column eluting with a gradient of 20−75% CH_3OH: (0.25% $HClO_4$, 0.25% CH_3CO_2H) at 0.7 mL/min over 40 min. HPLC method B: Waters Alliance 2690 HPLC with a PDA detector (996) and Peltier-thermostated autosampler (Waters, Milford, MA) employing gradient elution on a Supelco ODS-2 C18 250 mm × 4.6 mm, 5 μm column, protected by a Waters Delta-Pak RP-18 guard column. The absorption was monitored at 200−400 nm and measured at 280 nm for estradiol and 286 nm for **1**. The mobile phase consisted of 5% MeOH and 0.1% HCO_2H in water (A) and acetonitrile (B), run from 5% B to 65% B over 60 min at 1 mL/min, with an injection volume of 10 μL. HPLC method C: Agilent 1100 HPLC system, XTerraTM C18 2.1 mm× 100 mm 3.5 μm column, 40 min linear gradient of 5−85% MeCN/(aqueous 5% MeOH and 0.1% formic acid) at 0.25 mL/min, and detection at 285 and 296 nm. LC/MS/MS spectra were obtained using an Agilent LC/MSD Ion Trap SL mass spectrometer equipped with an Agilent 1100 HPLC system or a Micromass Quattro II triple-quadropole mass spectrometer equipped with an electrospray ionization detector and an HP 1050 HPLC system, as described previously (*33*). Higher resolution MS spectra were obtained on a Q-TOF mass spectrometer with a Waters 2690 HPLC System.

Raloxifene (**1a**) was synthesized by adaptation of literature procedures (*34*). The synthesis of 6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene proceeded in two steps starting from 3-mercaptoanisole and 4-bromoacetylanisole (*35*). Subsequent Friedel−Kraft acylation, followed by demethylation with BBr_3 and emplacement of the *N*-(2-hydroxyethyl)piperidine side chain, yielded raloxifene as the free base, which was purified by silica gel flash chromatography using 2:3 hexane/EtOAc followed by 1:1 MeOH/EtOAc (*34*). Isoraloxifene (**1b**) was synthesized from 4-methoxy-α[(3-methoxyphenyl)thio]acetophenone as shown in Scheme 1.

6-Methoxy-3-(4-methoxyphenyl)benzo[b]thiophene (I). Compound **I** was obtained by intramolecular dehydration of 4-methoxy-α[(3-methoxyphenyl)thio]acetophenone in the presence of concentrated H_3PO_4 (purity confirmed by ^{31}P NMR analysis) as follows: acid (17.75 g) was placed into a 150 mL round-bottom flask and heated with stirring to 85 °C. 4-Methoxy-α[(3-methoxyphenyl)thio]acetophenone (3 g, 0.0104 mol) was then added portionwise at such a rate that the temperature did not exceed 100 °C. After addition was complete, the reaction mixture was stirred, at 85 °C, for 2 h and then cooled to 70 °C and slowly poured into rapidly stirring ice water. The crude product separated as a light brown viscous liquid. The water was removed, and the organic phase was dissolved in acetone. The acetone solution was dried over $MgSO_4$ and concentrated under reduced pressure resulting in 2.75 g of light brown viscous oil. After the oil was purified on a silica gel column (eluted with 40% dichloromethane in hexanes), the desired product was obtained as a colorless viscous liquid with a yield of 1.53 g (54.4%). ^1H NMR (500 MHz, DMSO-d_6): δ 3.83 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 7.06 (1H, dd, $J_{H4-H5} = 8.9$ Hz, $J_{H5-H7} = 2.3$ Hz, H_5), 7.08 (2H, d, $J = 8.8$ Hz, $H_{3'}$), 7.5 (1H, s, H_2), 7.53 (2H, d, $J = 8.8$ Hz, $H_{2'}$), 7.63 (1H, d, $J = 2.3$ Hz, H_7), 7.74 (1H, d, $J = 8.9$ Hz, H_4). ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.18, 55.49, 114.5, 114.8, 120.9, 123.2, 129.8, 131.7, 136.6, 142, 157.2, 158.9.

Synthesis of (4-Fluorophenyl)[6-methoxy-3-(4-methoxyphenyl)benzo[b]thien-2-yl]methanone (II). 4-Fluorobenzoyl chloride (0.5165 g, 0.00325 mol) was added dropwise to a solution of $AlCl_3$ (0.596 g, 0.0039 mol) in 7 mL of dichloromethane, under an inert atmosphere. 6-Methoxy-3-(4-methoxyphenyl)benzo[b]thiophene (0.8802 g, 0.00325 mol), dissolved in 4 mL of dichloromethane, was added in two portions, and the resulting dark red reaction mixture was allowed to stir overnight at room temperature. It was then poured into ice water, neutralized with 5 M NaOH solution, and extracted three times with DCM. The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure to yield 0.9358 g (76%) of product after chromatography (SiO_2; 33% hexanes in CH_2Cl_2). ^1H NMR (300 MHz, $CDCl_3$): δ 3.77 (3H, s, OCH_3), 3.93 (3H, s,

Scheme 1



OCH_3), 6.76 (2H, d, J = 8.77 Hz, $H_{3'}$), 6.82 (2H, t, J = 8.7 Hz, J_F = 8.7, H_c), 7.03 (1H, dd, J_{H4-H5} = 9 Hz, J_{H5-H7} = 2.3 Hz, H_5), 7.17 (2H, d, J = 8.77 Hz, $H_{2'}$), 7.36 (1H, d, H_7), 7.59 (2H, dd, J = 8.7 Hz, J_F = 8.7, H_d), 7.64 (1H, d, J = 9 Hz, H_4).

Synthesis of (4-Fluorophenyl)[6-hydroxy-3-(4-hydroxyphenyl)benzo[b]thien-2-yl]methanone (III). A solution of (4-fluorophenyl)[6-methoxy-3-(4-methoxyphenyl)benzo[b]thien-2-yl]methanone (0.9358 g, 0.0023 mols) in 10 mL of dichloromethane was placed in a dry round-bottom flask, and an inert atmosphere was established and maintained during the reaction. The solution was cooled to 0−5 °C, using a 2-propanol/ice bath, and BBr_3 (1.7928 g, 0.0071 mol) was added through a septum. The dark red reaction mixture was stirred for an additional 30 min at lower temperature. The water bath was removed, and the solution was stirred for another 3 h at room temperature and then poured into ice water. A yellow solid precipitated, which was collected by filtration. Further precipitation from methanol/water gave 0.9 g of **III**. ^{1}H NMR (300 MHz, acetone-d_6): δ 6.71 (2H, d, J = 8.5 Hz, $H_{3'}$), 6.94 (2H, t, J = 8.7 Hz, J_F = 8.7, H_c), 7.04 (1H, dd, J_{H4-H5} = 9.0 Hz, J_{H5-H7} = 2.0 Hz, H_5), 7.1 (2H, d, J = 8.5 Hz, $H_{2'}$), 7.43 (1H, d, J = 2.0 Hz, H_7), 7.57−7.63 (overlapped) (3H, m, H_d, H_4), 8.68 (2H, broad, OH).

Synthesis of Isoraloxifene (1b). 1-Piperidylethanol (0.5385 g, 0.004168 mol) was added to a suspension of NaH (0.11 g, 0.0045 mol) in 2.5 mL of dry DMF under argon. Once the H_2 evolution ceased, a solution of (4-fluorophenyl)[6-hydroxy-3-(4-hydroxyphenyl)benzo[b]thien-2-yl]methanone (0.3796 g, 0.001042 mol) in 2.5 mL of dry DMF was added dropwise in one portion. The dark red reaction mixture was stirred overnight at 50 °C. The DMF was removed under reduced pressure, and the resulting concentrate was portioned in water/ethyl acetate mixture. The organic layer was separated, and the aqueous solution was extracted two times with EtOAc. The organic phase was dried over $MgSO_4$ and concentrated under vacuum. Purification by silica gel flash chromatography, using 2:3 hexane/EtOAc followed by 1:1 MeOH/EtOAc, gave 0.318 g (64.4%) of isoraloxifene as a free base. ^{1}H NMR (400 MHz, MeOH-d_4): δ 1.47 (2H, m, $N(CH_2CH_2)_2CH_2$), 1.62 (4H, m, $N(CH_2CH_2)_2CH_2$), 2.53 (4H, m, $N(CH_2CH_2)_2CH_2$), 2.74 (2H, t, OCH_2CH_2N), 4.1 (2H, t, OCH_2CH_2N), 6.61 (2H, d, J = 8.8, $H_{3'}$), 6.69 (2H, d, J = 8.8, H_c), 6.93 (1H,dd, J_{H4-H5} = 8.8, J_{H5-H7} = 2.0, H_5), 7.03 (2H, d, J = 8.8, $H_{2'}$), 7.26 (1H, d, J = 2 Hz, H_7), 7.48 (2H, d, J = 8.8, H_d), 7.58 (1H, d, J = 8.8, H_4), 9.57 (1H, broad, OH), 10.1 (1H, broad, OH). ^{13}C NMR (75 MHz, MeOH-d_4): δ 25.00, 26.45, 55.90, 58.67, 66.54, 108.14, 114.88, 116.28, 116.99, 127.14, 127.38, 131.80, 132.9, 133.18, 133.98, 135.14, 143.53, 144.17, 158.65, 159.23, 163.43, 192.66. Also see Table 1.

Products **2**−**4** were obtained by treatment of **1a** or **1b** (0.1 mM) with SIN-1 (1 mM) overnight (**2** and **4**) or peroxynitrite (0.5 mM) (**3**) for 1 h, respectively. Reaction mixtures were reduced and extracted with EtOAc, and the organic extracts were concentrated prior to flash chromatography on C18 Reverse Phase silica gel (Silicycle). Products **2**−**4** were isolated to 98, 90, and 98% purity, respectively, as assessed by NMR and HPLC analysis (the balance of sample **3** was identified as **1b**). The nitration products **5** and **7a**,**b** were isolated from a $NO_2^-/H_2O_2/FeCl_3$ nitration mixture, similar to that used for analytical work but without buffering and on a larger scale, such that the reaction mixture was at approximately pH 3. The acidic nitration conditions allowed for increased yields of nitroaromatics. Reactions were carried out at 37 °C for 4 h by incubating E_2 or raloxifene (0.2 mM) in 20 mM $FeCl_3$ containing 0.4 mM $NaNO_2$ and 0.4 mM H_2O_2 (final pH of 3), with subsequent addition of aliquots of H_2O_2 at 20 min intervals. The nitroestradiol isomers were purified by flash chromatography on silica gel and identified as 2-nitroestradiol (**7a**) and 4-nitroestradiol (**7b**) by ^{1}H NMR and LC/MS/MS (*36*). The synthesis of 7-nitroraloxifene (**5**) was achieved by incubation of raloxifene with $FeCl_3/NaNO_2/H_2O_2$ as described above and isolation by flash chromatography on silica gel. Both 7-nitroraloxifene (**5**) and 3′,7-dinitroraloxifene were separated and identified by NMR and LC/MS/MS analysis (Table 1). Synthetic adducts were used to identify reaction products by coinjection in reaction mixtures.

The sydnonimines SIN-1 and ASI-3 were synthesized by minor adaptation of literature procedures (*37*, *38*). Peroxynitrite was synthesized by two routes (*39*): first, from reaction of *i*-amyl nitrite with aqueous $NaOH/H_2O_2$ followed by exhaustive washing to remove excess organic nitrite; second, from the reaction of aqueous $NaNO_2$ with an acidic H_2O_2 solution with rapid alkaline quenching. In both cases, excess H_2O_2 was removed with MnO_2, and UV−vis spectroscopy was used to quantify peroxynitrite and check for contamination.

DHR was obtained from reduction of rhodamine 6G in a vigorously stirred mixture of 1:1 CH_2Cl_2/H_2O, by portionwise addition of $NaBH_4$ until no further bleaching of the colored solution was observed. After 1 h, the pale pink organic layer was separated, the water layers were washed with dichloromethane, and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The product was purified in 79% yield after silica gel flash chromatography using 9:1 EtOAc/CH_2Cl_2 and stored in the cold and dark. Excitation and emission wavelengths of 528 and 554 nm, respectively, allowed measurement of DHR (5 μM) oxidation, which was normalized to 0% corresponding to DHR and 100% corresponding to rhodamine 6G (5 μM) fluorescence.

Table 1. Physical Data for 1a,b−5

^{1}H Chemical shifts (ppm), multiplicity, coupling constant (Hz), integral, at 300 MHz and UV−vis absorbances (nm)

	H_4	H_5	H_7	$H_{3'}$	$H_{2'}$	H_c	H_d	UV−vis λ_{max}
1b[a]	7.52 d J = 8.8 1H	6.95 dd J = 8.8, 2 1H	7.35 d J = 2 1H	6.63 d J = 8.0 2H	7.04 d J = 8.0 2H	6.74 d J = 8.0 2H	7.48 d J = 8.0 2H	283
2[a]	7.33 d J = 9.6 1H	6.52 d J = 9.6 1H		6.62 d J = 8.2 2H	6.96 d J = 8.2 2H	6.79 d J = 8.4 2H	6.49 d J = 8.4 2H	291, 358, 468
1a[a]	7.42 d J = 8.8 1H	6.85 dd J = 8.8, 2 1H	7.26 d J = 2 1H	6.65 d J = 8.6 2H	7.18 d J = 8.6 2H	6.85 d J = 8.8 2H	7.71 d J = 8.8 2H	288
4[a]	7.36 d J = 9.0 1H	7.09 d J = 8.9 1H		6.63 d J = 8.4 2H	7.02 d J = 8.4 2H	6.81 d J = 8.7 2H	7.51 d J = 8 2H	292
1b [b]	7.52 d J = 8.8 1H	6.93 dd J = 8.8 1H	7.216 d J = 2.0 1H	6.61 d J = 8.4 2H	7.03 d J = 8.8 2H	6.69 d J = 8.8 2H	7.48 d J = 8.8 2H	
1a[b]	7.42 d J = 8.8 1H	6.88 dd J = 8.7, 2.2 1H	7.26 d J = 2.2 1H	6.63 d J = 8.7 2H	7.18 d J = 8.7 2H	6.85 d J = 8.9 2H	7.71 d J = 8.9 2H	
3[b]	7.53 d J = 9.3 1H	6.80 d J = 9.2 1H		6.61 d J = 8.4 2H	6.99 d J = 8.6 2H	6.72 d J = 8.8 2H	7.49 d J = 8.8 2H	299 356
5[b]	7.31 d J = 9.0 1H	6.73 J = 9.0 1H		6.66 d J = 8.5 2H	7.16 J = 8.5 2H	6.9 J = 8.7 2H	7.66 J = 8.7 2H	300

^{13}C chemical shifts (ppm) at 75 or 90 MHz

	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C(O)	$C_{3'}$	$C_{2'}$	C_c	C_d
1a[a]	140.5	139.5	123.1	114.8	155.8	107.1	132.5	129.9	192.8	115.5	129.5	114.4	131.6
1b[a]	144.2	143.2	126.6	117.0	158.2	108.0	132.5	133.2	189.9	116.0	132.3	114.6	132.6
2[a]	142.2	141.6	133.4	129.3	180.7	157.1	127.3	127.7	190.1	114.3	131.2	113.5	131.1
4[a]	141.0	140.3	123.5	116.1	152.6	116.0	132.1	130.5	192.8	116.0	130.1	115.1	132.3
5[a]	140.2	135.3	129.1	123.9	163.4	128.4	129.3	129.2	192.55	115.7	129.5	114.5	132

[a] In DMSO-d_6. [b] In MeOH-d_4.

Results

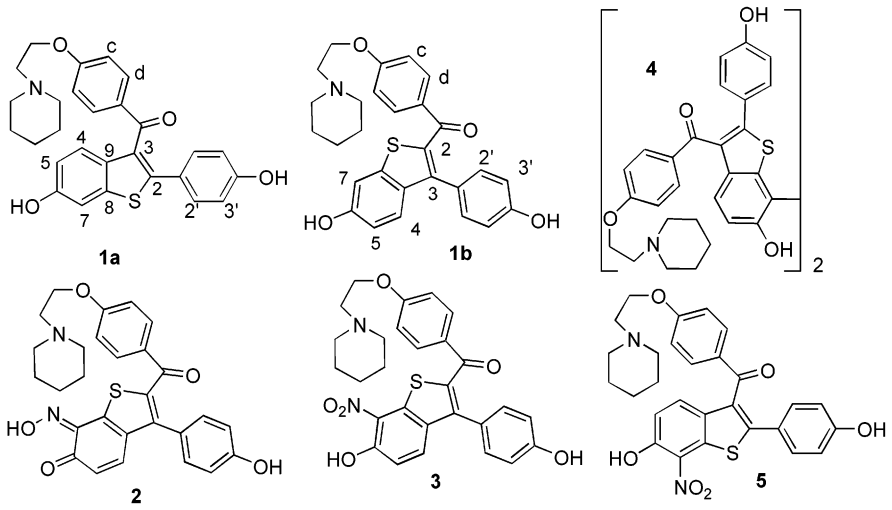
Sources of Peroxynitrite, NO, and NO_2. Sources of bolus peroxynitrite were prepared from two different synthetic procedures. Peroxynitrite preparations are very rarely pure; for example, the two standard syntheses used herein, organic and inorganic nitrite, respectively, are contaminants. Use of two separate sources minimizes data due to artifacts and contaminants; however, bolus peroxynitrite concentrations do not mimic a physiological situation but simply allow identification of possible chemical species resulting from reactions with peroxynitrite. To provide sustained release of lower concentrations of peroxynitrite, likely to be of more physiological relevance, two sydnonimines, SIN-1 and ASI-3, were used as sources. Sydnonimines undergo decomposition in neutral aqueous solution to release NO and, in the presence of oxygen, superoxide. The rapid reaction of NO with superoxide yields peroxynitrite. SIN-1 has been employed frequently as a reliable biomimetic source of peroxynitrite, although one must be aware that trapping or inhibition of superoxide formation may result in SIN-1 behaving as an NO source (*40*). SIN-1 itself undergoes decomposition by a stepwise mechanism, generating peroxynitrite and SIN-1C as final products, whereby peroxynitrite is produced in a continuous but nonlinear flux (*41*). The oxidation of DHR derivatives is particularly sensitive to peroxynitrite and HOCl; thus, it is a selective but not specific probe, providing in simple systems a reliable quantification of peroxynitrite release readily monitored by fluorescence and absorbance spectrophotometry (*42*).

The diazeniumdiolate, SPE/NO ($t_{1/2}$ approximately 45 min at pH 7.4), provides a continuous flux of NO, which has frequently been used as a slow release source of NO (*43*, *44*). The use of pure NO gas, unless a special low-flow apparatus is employed, is contraindicated because even after rigorous sparging with NaOH, NO_2 can be produced from the rapid termolecular reaction of NO with O_2. The point has been made by ourselves and others that care should be taken in the use of NO donors to assay the effects of NO itself, because of biological activity or chemical reactivity attributable to the intact NO donor or to reactive intermediates on the pathway to NO (*43*, *45*). However, all available evidence supports the tenet that simple diazeniumdiolates act as reliable sources of NO, cleanly yielding amine and NO.

In addition to peroxynitrite, systems containing NO_2^- and H_2O_2 are capable of tyrosine nitration via NO_2 release in the presence of transition metals, Fe-heme, or peroxidase enzyme catalysts (*29*, *30*, *46*, *47*). These systems have been argued to mimic physiological situations in which NO_2^- acts as a source of RNOS under situations of disrupted metal ion homeostasis or through catalysis by myeloperoxidase. It is noted that elevated NO_2^-/NO_3^- levels will result from elevated NO release and oxidative metabolism.

Reactions with Bolus Peroxynitrite. The reactions of **1a**,**b** with bolus peroxynitrite, at pH 7.4, 22 °C, were monitored by HPLC with UV detection for bolus additions of peroxynitrite (0.1−0.5 mM), revealing a number of products. Major products for reactions of both **1a**,**b** were the mononitrated adducts, **3** and **5**, which were isolated

Scheme 2



by chromatography and characterized spectroscopically (Table 1). These compounds proved to be the products of nitration at the 7-position of the benzothiophene ring, as demonstrated by NMR and MS analysis (Table 1 and Scheme 2). Reaction of **1a**,**b** with peroxynitrite (0.1−0.5 mM) at 22 °C in 40% MeCN/phosphate buffer (100 mM, pH 7.4) gave 10−40% yields of the relevant nitration products. The relevance of bolus injections of peroxynitrite to concentrations of peroxynitrite likely to be encountered in vivo is problematic, but this experiment does demonstrate the possibility of formation of nitroaromatics from raloxifene and the particular susceptibility of the 7-position to nitration.

Reactions with SIN-1 as a Peroxynitrite Source. Reactions in aerobic solutions of SIN-1 (0.1 mM) provide a better model for potential reactions of peroxynitrite formed at physiologically relevant rates. Oxidation of DHR is selective for peroxynitrite, and as expected, DHR oxidation by SIN-1 was concentration-dependent, but this oxidation was also observed to be inhibited by added **1b** in a concentration-dependent manner (Figure 1). HPLC monitoring of the reaction of SIN-1 with **1b** (0.1 mM), at pH 7.4, 22 °C, showed the formation of one major oxidation product (**2**, RT = 26.5 min), the rate of formation of which corresponded qualitatively to the rate of formation of SIN-1C and peroxynitrite from SIN-1

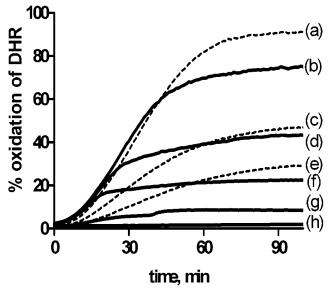


Figure 1. Inhibition of peroxynitrite oxidation of DHR by **1b**. Time course of DHR (5 μM) oxidation by peroxynitrite generated from SIN-1. SIN-1 only: dashed lines (a) 10, (c) 4, and (e) 2 μM. SIN-1 (10 μM) with addition of **1b**: (b) 20, (d) 50, (f) 100, and (g) 200 μM. Control in the absence of SIN-1 with **1b** (10 μM) (h). Stock solutions were prepared in MeCN (**1b**), cold buffer (SIN-1), and DMF (DHR) to give <4% organic solvent in phosphate buffer (100 mM, pH 7.4, 200 μL), at 37 °C, with reaction initiated by SIN-1. Data obtained in triplicate were normalized to 100% fluorescence of rhodamine and 0% fluorescence of DHR (both 5 μM) and averaged.

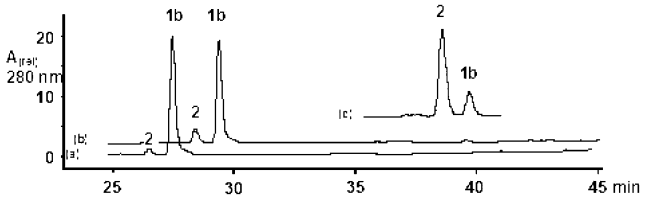


Figure 2. HPLC analysis of reaction of **1b** with peroxynitrite produced by decomposition of SIN-1. HPLC chromatograms for incubations of **1b** (0.1 mM) with SIN-1 (0.1 mM) at 1 (a) and 6 h (b). INSET portion of chromatogram showing **1b** and **2** at 11 h, from reaction of **1b** with SIN-1 (1 mM) (c). All reactions were run in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) at 22 °C, using HPLC method A for analysis. Chromatograms are stacked and offset for ease of viewing; A_{rel} is relative absorbance at 280 nm.

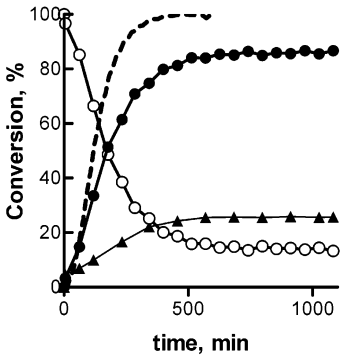


Figure 3. Time course of peroxynitrite oxidation of **1b**. Formation of **2** (●, ▲) and decay of **1b** (○) on reaction with SIN-1 (1 mM, ●, ○; and 0.1 mM, ▲) as compared with formation of SIN-1C from decomposition of SIN-1 (0.1 mM dashed line), all in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) at 22 °C, as measured by integration of peaks in HPLC chromatograms at 280 nm, using method A for analysis.

(Figures 2 and 3). Reaction of **1b** with SIN-1 (0.1 or 1.0 mM) gave remarkably clean conversion to **2** (Figure 2), allowing purification and isolation. Candidate structures for compound **2** would include products of dimerization and oxidation at N and S (peroxynitrite oxidation of methionine is documented (*48*)). However, the identity of **2** was confirmed as the quinone oxime by a combination of MS and NMR analysis. A MW for **2** of 502 was confirmed by observation of parent ions and the expected daughter ions in LC/MS/MS spectra using both positive ($[M + H]^+ = 503$) and negative ($[M - H]^+ = 501$) ion

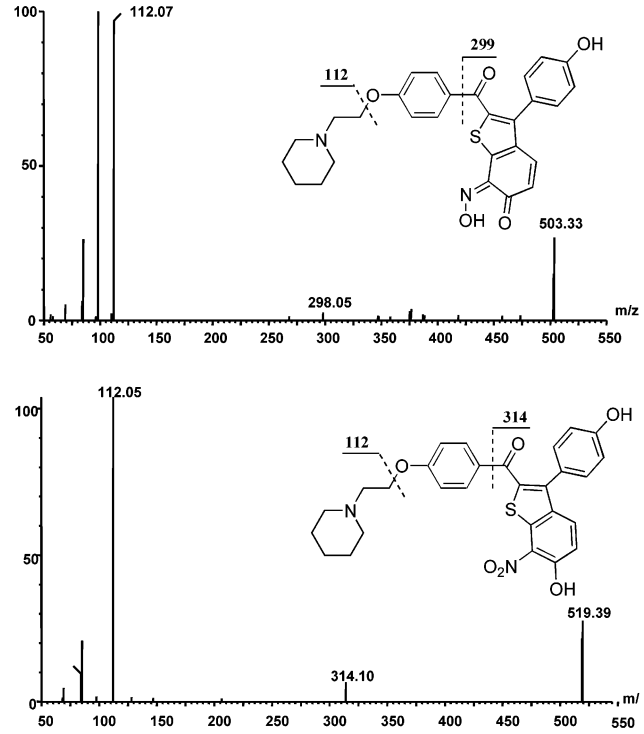


Figure 4. LC/MS spectra for **2** and **3** obtained in positive detection mode, showing parent and daughter ions.

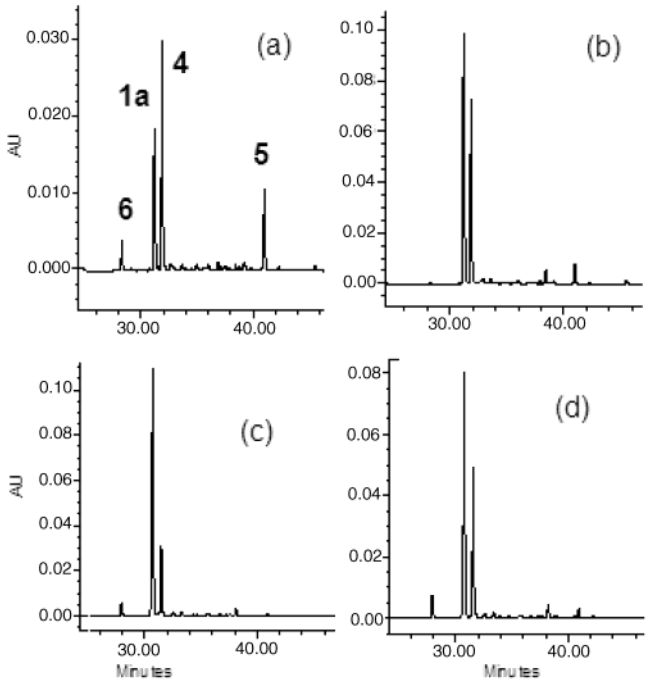


Figure 5. HPLC analysis of reactions of **1a** with (a, b) peroxynitrite produced by decomposition of SIN-1 and (c, d) NO produced from SPE/NO. HPLC chromatograms for incubations of **1a** (0.1 mM) with (a) SIN-1 (1.0 mM) at 120 min, (b) SIN-1 (0.1 mM) at 90 min, (c) SPE/NO (0.1 mM) at 60 min, and (d) SPE/NO (0.1 mM) at 180 min. All reactions run in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) at 22 (SIN-1) or 37 °C (SPE/NO), using HPLC method B for analysis. Absorbance units (AU) at 286 nm are arbitrary.

detection (Figure 4) and by Q-TOF ($[M + H]^+ = 503.16$), corresponding to a product with addition of N and O (**1b** + O + N − H). ^{1}H NMR analysis of **2**, with assignments supported by two-dimensional NMR experiments (including COSY, HMBC, and HMQC), showed substitution only

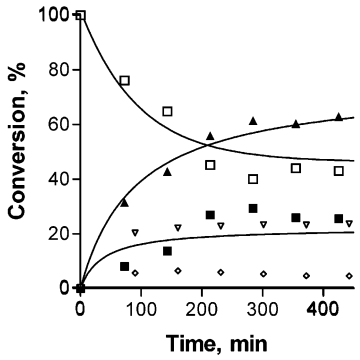


Figure 6. Formation of dimer **4** (▲), nitroraloxifene **5** (■), and decay of raloxifene **1a** (□) on reaction of **1a** (0.1 mM) with SIN-1 (1 mM) or formation of **4** (▽) and **5** (◊) on reaction with SIN-1 (0.1 mM). All reactions in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) at 22 °C, as measured by integration of peaks in HPLC chromatograms at 286 nm, using method B. Peak areas were corrected using measured ϵ_{286} for each species. A 100% conversion to dimer would represent 50 mol % dimer. Curves are for ease of visualization and do not represent verified kinetic models.

at the 7-position of the benzothiophene ring (Table 1). The ^{13}C NMR spectrum showed significant downfield shifts at C_{3-7} of the benzothiophene relative to **1b**, with the value for C_6 definitive for and C_7 compatible with quinoid carbonyl carbons (Table 1). Comparison with the ^{13}C NMR spectrum obtained for the comparator quinone oxime of 2-naphthol (1,2-naphthalenedione-1-oxime) confirmed the identity of **2**. HPLC analysis of the incubation of **1b** with the more labile sydnonimine, ASI-3, which generates peroxynitrite and therefore NO_2 more rapidly than SIN-1, showed the formation of both the nitrosation product (**2**) and the nitration product (**3**) (data not shown).

The reaction of **1a** with SIN-1 at pH 7.4, 22 °C, was qualitatively similar to that of **1b**, showing formation of a major product (**4**) with similar retention time to that of the substrate (Figures 5 and 6). However, product **4** was identified as the dimer of **1b** rather than the quinone oxime by a combination of MS and NMR analysis. A MW for **4** of 944 was confirmed by observation of parent ions and the expected daughter ions in LC/MS/MS spectra using both positive ($[M + 2H]^{2+} = 473$; $[M + H]^+ = 945$; $[M + H - (N\text{-Et-piperidine})]^+ = 834$) and negative ($[M - H]^- = 943$) ion detection and by Q-TOF ($[M + 2H]^{2+} = 473.17$). Furthermore, ^{1}H NMR and ^{13}C NMR analysis of **4**, with assignments supported by two-dimensional NMR experiments (including COSY, HMBC, and HMQC), showed substitution only at the 7-position of the benzothiophene ring (Table 1) and a spectrum similar to that of **1a** with no evidence for significant shifts such as for quinoid carbonyl carbons (Table 1). Dimer **4** was also produced on reaction of **1a** with bolus peroxynitrite. 7-Nitroraloxifene (**5**) was identified as a product of reaction, being favored at higher fluxes of peroxynitrite, as expected from results with bolus peroxynitrite by coinjection with an authentic synthetic sample and by LC/MS analysis of the reaction mixtures (Figure 5). The product at RT = 28.2 min was identified as the quinone oxime of raloxifene (**6**) by LC/MS analysis ($[M + H]^+ = 503$) (Figure 5).

Decomposition of SIN-1 in atmospherically equilibrated solution yields fluxes of NO and $O_2^{\bullet-}$ via peroxynitrite, NO_2, and $CO_3^{\bullet-}$. Reactions of **1a**,**b** in these reaction

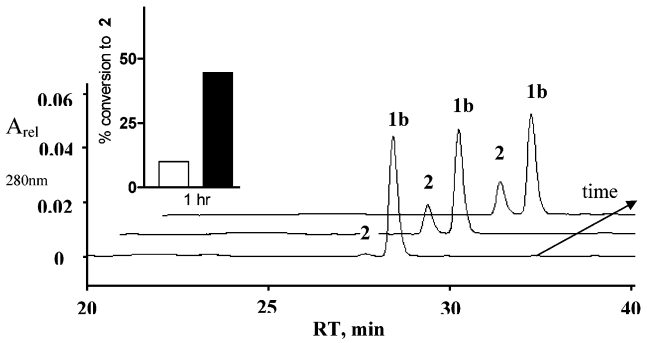


Figure 7. HPLC analysis of reaction of **1b** in the presence of NO. HPLC chromatograms showing incubations of **1b** (0.1 mM) with SPE/NO (0.1 mM: lower, 5 min; middle, 2 h; upper, 4 h). Inset: Oxidation of **1b** under aerobic (solid bars) and anaerobic (open bars) conditions, showing % conversion to product **2** at 1 h ([**2**]/{[**1b**] + [**2**]} expressed as %), after incubation of **1b** (0.1 mM) with SPE/NO (0.2 mM), as quantified by integration of signals in HPLC. All reactions in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) at 22 °C, using HPLC method A. Chromatograms are stacked and offset for ease of viewing.

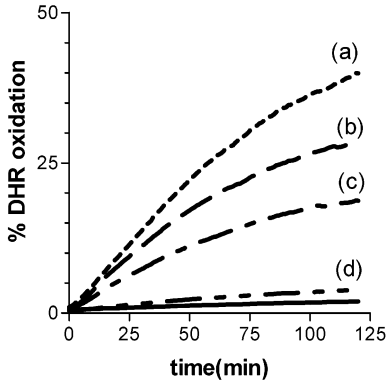


Figure 8. Oxidation of DHR in the presence of **1b** and NO. Time course of DHR (5 μM) oxidation in aerobic solutions containing SPE/NO (50 μM) and **1b**: (a) 10, (b) 100, (c) 200, and (d) 500 μM. The solid line is a control reaction in the absence of **1b**. Reactions were carried out in aerobic phosphate buffer (100 mM, pH 7.4, 200 μL) at 22 °C. Data obtained in triplicate were normalized to 100% fluorescence of rhodamine and 0% fluorescence of DHR (both 5 μM) and averaged.

mixtures at neutral pH yields nitrosation products (**2** and **6**), nitration products (**3** and **5**), and dimerization products (**4**). The product distribution and reaction kinetics clearly depend on substrate and reaction conditions. The object of this initial study was to delineate the reactivity of SERMs toward RNOS and the products that might result. A mixed medium of water/acetonitrile at pH 7.4, containing O_2 and CO_2 in atmospheric equilibrium, was chosen to avoid precipitation of potential products. Product distribution can be expected to be modified by changes in solvent, pH, [CO_2], and [O_2]. The preference for nitrosation over dimerization observed for **1b** relative to **1a** might be expected to result from steric hindrance to dimerization.

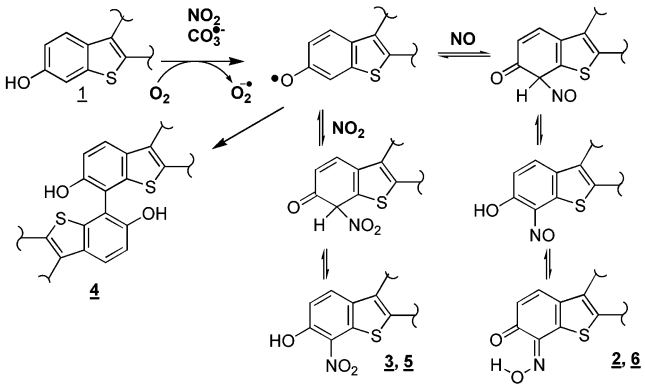
Reactions with NO. HPLC analysis of the reactions of **1a** or **1b** (0.1 mM) in the presence of the slow NO donor SPE/NO (0.1 mM) showed the ready formation of similar products to those observed in reactions in the presence of SIN-1, which is consistent with formation of peroxynitrite (or its dissociation product NO_2) in the reaction mixture (Figures 5 and 7). Consideration of the reduction potentials of NO/NO^- (−0.8 V), $O_2/O_2^{\bullet-}$ (−0.16 V), and 1,2-naphthaquinone (−0.56 V) supports peroxynitrite formation from initial generation of superoxide and oxidation to the phenoxyl radical **1**$^\bullet$ as the most chemically reasonable mechanism (eqs 1 and 2), in preference to direct oxidation of raloxifene by NO (*49*, *50*).

$$\mathbf{1} + O_2 \rightarrow \mathbf{1}^\bullet + H^+ + O_2^{\bullet-} \quad (1)$$

$$NO + O_2^{\bullet-} \rightarrow ONOO^- \quad (2)$$

The requirement for oxygen in the reaction of **1** with NO was confirmed by carrying out the reaction of **1b** in solutions deoxygenated by bubbling with N_2 for 10 min (Figure 7). That the reaction proceeds via formation of peroxynitrite was supported by the observation that the mixture of **1b** and SPE/NO gave concentration-dependent oxidation of DHR, whereas the individual components had no reactivity toward DHR (Figure 8). The low level of DHR oxidation observed in aerobic solutions of SPE/NO, relative to the efficient oxidation observed for SPE/NO + **1b**, argues against the importance of the termolecular reaction of NO with O_2 (which will generate

Scheme 3



NO_2 as oxidant). HPLC analysis of the formation of **2** from **1b** and SPE/NO showed no influence of the metal ion chelator, DTPA, but reaction was inhibited by added GSH, which is compatible with either reduction (repair) of the initial phenoxyl radical **1**$^\bullet$ or trapping of the radicals formed from peroxynitrite. The balance of the evidence supports an autoxidation mechanism, as described in Scheme 3 and eqs 1−8 (eq 5 describes oxidation of phenols and of phenolates).

$$ONOO^- + H^+ \rightarrow ONOOH \rightarrow NO_2 + HO^\bullet \quad (3)$$

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow NO_2 + CO_3^{\bullet-} \quad (4)$$

$$ArOH + ox^\bullet \rightarrow ArO^\bullet + H^+ + ox^- \quad (ox^\bullet = HO^\bullet, NO_2, \text{or } CO_3^{\bullet-}) \quad (5)$$

$$ArO^\bullet + NO_2 \rightarrow Ar(OH)NO_2 \quad (6)$$

$$ArO^\bullet + NO \rightarrow Ar(OH)NO \quad (7)$$

$$ArO^\bullet + ArO^\bullet \rightarrow [Ar(OH)]_2 \quad (8)$$

Reaction of Nitrosation Products with GSH. The reactivity of *o*-quinone oximes toward biomolecules such as GSH has not been explored; however, one might anticipate both redox chemistry and Michael addition reactions in simile with the reactivity of *o*-quinones.

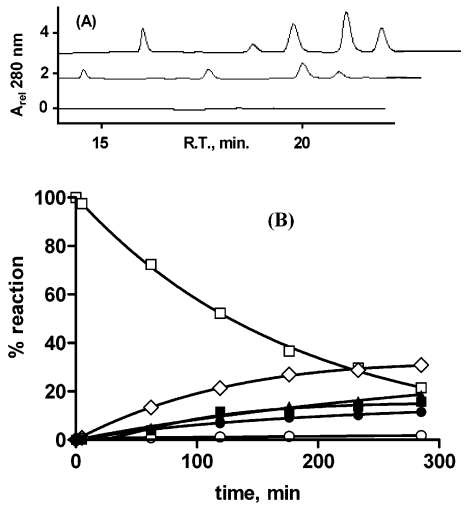
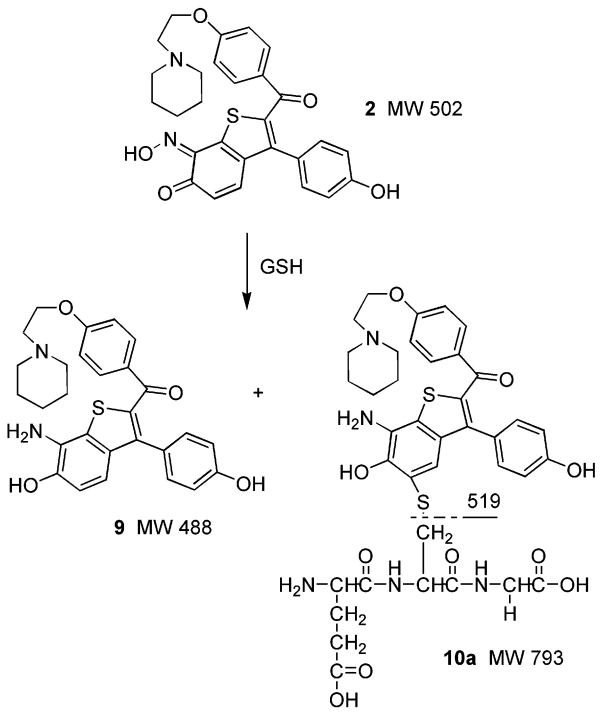


Figure 9. HPLC analysis of reaction of **2** with GSH. (**A**) HPLC chromatograms of incubations of **2** (0.16 mM) with GSH at 1 h (2 mM, lower; 20 mM, middle; 50 mM, upper) in phosphate buffer (100 mM, pH 7.4) in a water bath at 37 °C (280 nm). Chromatograms are stacked and offset for ease of viewing (RT is retention time; A_{rel} is relative absorbance at 280 nm). HPLC method A was used. (**B**) Plot of time course for reaction of **2** (0.16 mM) with GSH (20 mM), as measured by integration of peaks in HPLC chromatograms: **2**, □; products, ■, ▲, ○, ◊, ●.

Scheme 4



Using HPLC analysis, **1b** was observed to be inert toward GSH, but product **2** reacted with GSH to form an array of more hydrophilic products, which were shown to form in a time-dependent manner (Figure 9). The quinone oxime **2** possesses a number of electrophilic sites susceptible to addition of GSH; therefore, multiple adducts might be expected. Reaction was observed to be slow with low concentrations of GSH (2 mM), and even at 20 mM GSH, the $t_{1/2}$ for reaction of **2** was approximately 2 h (Figure 9). Oxidation of GSH by **2** will be compete with Michael addition of GSH, and in the presence of O_2, redox cycling of the reduction product of **2** has the potential to regenerate **2**, leading to loss of GSH, the effects of which would be accentuated at lower initial GSH concentrations. LC/MS/MS analysis of the reaction mixture of **2** (0.1 mM) with GSH (50 mM) allowed identification of two GSH adducts (**10a**,**b**), which would be expected to be the 4- and 5-adducts; however, these were not simple adducts of **2** but adducts of the reduction product, **9**, the *o*-aminophenol derivative (Scheme 4 and Figure 10). Competition between (i) reduction of **2** by GSH and (ii) addition of GSH to the electrophilic quinone oxime followed by subsequent reduction is compatible with the reaction products observed and the chemistry of quinone oximes (*51*).

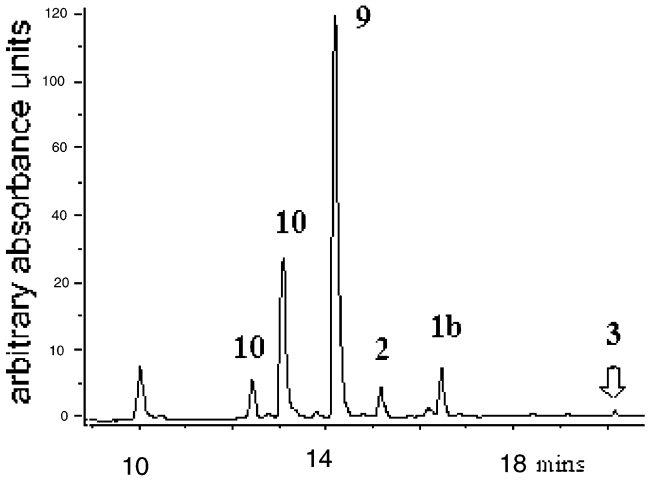


Figure 10. HPLC chromatogram from LC/MS/MS analysis of reaction of **2** (0.08 mM) with GSH (50 mM). Reaction in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) at 37 °C for 1 h, using HPLC method C for analysis. Absorbance units at 286 nm are arbitrary.

Comparison with Reactions of Estradiol and Reactions with NO_2^-/H_2O_2 Systems. Preliminary experiments were conducted to compare the reactivity of **1** with the endogenous ER ligand, E_2, for which the 2- and 4-nitroestradiol derivatives were synthesized for product identification. Incubation of E_2 (0.2 mM) with SIN-1 (0.4 mM) at 22 °C in 40% MeCN/phosphate buffer (pH 7.4, 100 mM) for 4 h gave a low yield of nitroestradiols: approximately 0.9% **7a** (RT = 53 min) and 0.5% **7b** (RT = 43 min) based upon integration of HPLC signals at 286 nm corrected for relative extinction coefficients. Incubation of E_2 (0.2 mM) with SPE/NO (0.4 mM) in MeCN/buffer at 37 °C for 4 h gave similarly low yields of the nitroestradiols as measured by HPLC analysis (approximately 1.7% **7a** and 1.2% **7b**). However, when incubated using the same reaction conditions employed for **1** (substrate, 0.1 mM; SPE/NO or SIN-1, 0.1 mM), no reaction was detectable for E_2 + SPE/NO and a negligible reaction was observed for E_2 + SIN-1 (≤0.5% **7a** at 4 h). The reactions of **1a** (0.1 mM) and E_2 (0.2 mM) were examined by HPLC analysis in phosphate buffer (100 mM, pH 7.4) solutions containing $NaNO_2$ (0.1 or 0.2 mM), H_2O_2 (0.2 or 0.4 mM), and either (i) $FeCl_3$ (10 or 20 mM), (ii) hemin (1 or 2 mM), or (iii) HRP (0.2 mg/mL), to qualitatively define differences in reactivity and reaction products as compared to the SIN-1 and SPE/NO incubations. Low yields of **7a**,**b** were generally observed from reaction of E_2 in pH 7.4 buffered solutions (e.g., $E_2/NO_2^-/H_2O_2/FeCl_3$ at 0.2/0.4/0.4/20 mM and 37 °C gave approximately 1.8% **7a** and 1.3% **7b** at 4 h), with NO_2^-/H_2O_2/HRP proving the most reactive system (approximately 7.2% **7a** at 4 h). Raloxifene, **1a**, was relatively

unreactive in the hemin system, but dimer was observed from reaction in the $FeCl_3$ and HRP systems. After incubation of **1a** (0.1 mM) for 7 h at 37 °C with $NaNO_2$ (0.1 mM), H_2O_2 (0.1 mM), and HRP in buffer, approximately 25% conversion to dimer was observed by HPLC analysis. No evidence for the nitrosation of **1** and only small amounts of nitration product were observed in the NO_2^-/H_2O_2 systems, although in unbuffered solutions substantial nitration could be observed.

Discussion

RNOS-Mediated Metabolism. Physiological levels of NO are thought to be in the nanomolar range, except in exceptional cases, such as where iNOS is up-regulated, when micromolar levels may reached (*52*). Inorganic nitrite, NO_2^-, the primary metabolite of NO, on the other hand, is present physiologically at levels from micromolar to millimolar (*53*, *54*). Because the tyrosine residues of a large number of proteins have been observed to be nitrated in vivo, the biological nitration of phenol biomolecules including tyrosine is a real physiological phenomenon (*46*, *55*, *56*). That tissue and plasma nitrotyrosine levels may provide readily measurable early biomarkers for disease states emphasizes the need to understand the pathways of RNOS-mediated metabolism. Despite the current interest in nitrotyrosine, two aspects of RNOS-mediated metabolism have received less attention. First, there are important phenolic biomolecules and xenobiotics other than tyrosine that should also be susceptible to RNOS-mediated metabolism, including the estrogens and antiestrogens. Second, chemical studies on the nitration of simple phenols in aqueous solution by peroxynitrite demonstrate a range of products in addition to the nitroaromatic nitration products.

Of several chemical mechanisms leading to phenol nitration, the dominant pathway in vivo for formation of nitrotyrosine has not been defined (*46*, *47*). Possible nitration agents at neutral pH include (i) peroxynitrite, (ii) NO_2, and (iii) NO_2^-/H_2O_2 in the presence of peroxidase or transition metal catalysts, including Fe-heme. Furthermore, the reaction of tyrosyl radical with NO is capable of generating nitrotyrosine (*57*). In addition to tyrosine, there are reports of nitration of other important biomolecules, including tryptophan and catecholamine neurotransmitters such as norepinephrine and dopamine (*58*, *59*). 6-Nitronorepinephrine has been isolated and characterized from pig brain synaptosomes (*60*) and has been synthesized by the reaction of NO gas with norepinephrine in neutral aqueous solution, although some disagreement exists as to the mechanism of nitration (*58*, *61*). In these catecholamine reactions and in the reactions of other phenolic compounds with peroxynitrite, a variety of oxidation products have been reported, including dimerization, hydroxylation, quinone, and quinoid-derived products (*27*, *40*, *62*−*64*). It has been reported that a major product of serotonin oxidation by peroxynitrite is the *o*-quinone, 4,5-hydroxytryptaminedione, a potentially neurotoxic metabolite (*65*). In addition to oxidation products and nitrophenols, the reactions of simple phenols with bolus peroxynitrite have also been reported to yield nitrosophenol products (*62*, *63*).

An accepted mechanism for peroxynitrite-mediated nitration of a phenol such as tyrosine, at neutral pH, involves rapid oxidation by $HO^•$, $CO_3^{•-}$, or NO_2, followed by radical combination with NO_2 (*66*) (eqs 3−6). However, the initial phenoxyl radical ($ArO^•$) may be "repaired" by antioxidants, such as GSH, or trapped by other radicals, such as NO, in fast radical combination reactions ($k_2 = 10^9$ M^{-1} s^{-1}) (eqs 6−8). The nitrosophenols formed by addition of NO to a phenoxyl radical and subsequent reorganization have sometimes been assumed to be too unstable to be of relevance; however, nitrosotyrosine has been shown to be of importance in several systems, in which the $1e^-$ oxidation product, iminoxyl radical, has been directly observed (*57*, *67*−*69*). Subsequent $1e^-$ oxidation of the iminoxyl radical to an oxoammonium ion provides an alternative mechanism for nitrotyrosine formation.

Nitrosophenols exist in equilibrium with their quinone oxime tautomers. Several solution and solid phase studies are not entirely in accord, but for simple substituted phenols, both tautomers are likely to be observed in solvent-dependent equilibria (*70*−*73*). MO calculations support a substantial shift of the equilibrium toward the oxime tautomer with increasing aromatic conjugation, compatible with the experimental observation of 1-nitroso-2-naphthol existing entirely as the oxime tautomer in a variety of solvents (*72*, *73*). 1-Nitroso-2-naphthol itself was part of a study by Fischer and Mason on metabolic activation of the human carcinogen naphthalenamine: the nitrosonaphthol is stable to air oxidation and is oxidized chemically and enzymically to a relatively long-lived 2-oxo iminoxyl radical (*51*, *57*, *74*). If *o*-nitrosophenols and their quinone oxime tautomers are indeed observed to be products of RNOS-mediated metabolism of phenolic compounds, they can be expected to be biologically active (i) through redox cycling via the stable iminoxy radical, (ii) via strong metal ion chelation, and (iii) by *o*-quinone-like covalent modification of biomolecules. Furthermore, oxidative and reductive metabolism may yield nitrophenols and aminophenols, respectively.

Oxidation and Nitration. The RNOS-mediated metabolism of the SERM raloxifene was examined in a number of model reaction systems. Raloxifene (**1a**) was chosen because it is in clinical use for postmenopausal osteoporosis and in clinical trials for treatment of breast cancer and modification of cardiovascular risk and other postmenopausal indications (*75*, *76*). Two related compounds were studied for comparison, an isomer of raloxifene, herein coined isoraloxifene (**1b**), and E_2. Isoraloxifene is expected to manifest similar chemical/biological properties to raloxifene but without ER binding, whereas E_2 is an endogenous ER ligand. The systems used to generate RNOS and to model RNOS-mediated metabolism were (i) bolus peroxynitrite, (ii) sydnonimine peroxynitrite generators, (iii) a diazeniumdiolate NO generator, and (iv) NO_2^-/H_2O_2/(HRP, hemin, or $FeCl_3$).

The use of bolus peroxynitrite (usually 0.1−10 mM) to model physiological phenomena is problematic but, given the large number of such studies, is essential for comparison purposes. Sydnonimines, in aerobic solution, decompose to yield NO and superoxide, which because of the short half-life of peroxynitrite, constitutes a continuous source of low, more physiologically relevant concentrations of peroxynitrite. Unsurprisingly, product mixtures from reaction with bolus peroxynitrite and with sydnonimines are not always identical. The reactions of **1a**,**b** with bolus peroxynitrite or with SIN-1 gave as major products compounds with similar retention times to the parent on reverse phase HPLC. In the case of **1a**, this

product was isolated and characterized as a derivative of raloxifene modified at C-7, with NMR and MS spectroscopy compatible with this compound being the raloxifene dimer, **4**. The same product was observed on incubation of raloxifene with NO_2^-/H_2O_2 at neutral pH in the presence of HRP and $FeCl_3$. In the absence of buffer, reaction of raloxifene with $NO_2^-/H_2O_2/FeCl_3$ generated a number of products, including mono- and dinitroraloxifene derivatives, from which it was possible to isolate and characterize 7-nitroraloxifene, **5**. This nitration product was identified as a product in the incubation of **1a** with higher concentrations of bolus peroxynitrite and SIN-1 at neutral pH.

Incubation of **1b** with SIN-1 (0.1 mM) at neutral pH in simile with **1a** gave one major product (**2**) of similar retention time to the parent by reverse phase HPLC. Compound **2** was isolated from reaction of isoraloxifene with SIN-1 and characterized by NMR and MS spectroscopy. Although derivatization at C-7 of the benzothiophene ring was indicated by two-dimensional ^1H and ^{13}C NMR experiments, the character of compound **2** is substantially different from that of the comparable raloxifene product **4**. In particular, the ^{13}C NMR spectrum showed significant downfield shifts at C_{3-7} of the benzothiophene relative to **1a**,**b** and **3**−**5**. A variety of MS techniques were applied giving in each case data consistent with a MW for **2** of 502.16. As discussed above, the only structure compatible with the spectroscopic data is that of a quinone oxime. In contrast to reaction with SIN-1, the reaction of **1b** with bolus peroxynitrite at pH 7.4 generated a number of products, the major of which, from HPLC monitoring, was isolated and characterized as 7-nitroisoraloxifene (**3**).

The mono- and dinitration products of E_2 have been reported previously, although not as products of RNOS-mediated metabolism in neutral aqueous solution (*36*, *77*−*79*). The objective in the study of E_2 was to compare the reactivity of this endogenous ER ligand and simple phenol against that of the polyaromatic phenolics, **1a**,**b**. The vast majority of SERMs reported to date have been polyaromatic phenolic compounds that would be expected to be highly susceptible to enzymic and chemical oxidation yielding both *o*-quinone and quinone methide reactive metabolites and catechols susceptible to further oxidation and redox cycling (*80*). The reactivity of E_2 toward RNOS would be anticipated to be similar to that of tyrosine, and low yields of the nitroestradiols **7a**,**b** were observed on reaction with higher concentrations of SIN-1, bolus peroxynitrite, and NO_2^-/H_2O_2/catalyst systems. E_2 was poorly reactive toward the low concentrations of SIN-1 used to study reaction with **1a**,**b** and was unreactive toward NO produced from lower concentrations of SPE/NO.

Autoxidation and Nitrosation. Low fluxes of NO were generated in aerobic solution at pH 7.4, using the NO donor, SPE/NO. Under these conditions, no reaction was observed for E_2, whereas the reactivity of **1a**,**b** was observed to be equivalent to that seen in the presence of SIN-1, readily generating raloxifene dimer (**4**), raloxifene quinone oxime (**6**), and isoraloxifene quinone oxime (**2**). Thus, in the presence of O_2 and NO, the benzothiophene SERM, raloxifene, and its isomer undergo reactions that are indicative of phenoxyl radical formation and yield the same products as seen from reaction with lower concentrations of peroxynitrite produced from SIN-1 (Scheme 3). This reactivity of **1a** with NO is in contrast to the reactivity with RNOS produced from NO_2^-/H_2O_2/catalyst systems, where quinone oxime is not observed. Preliminary experiments with other SERMs suggest that these reactions are not peculiar to the benzothiophene SERMs.

The data obtained for reaction of **1a**,**b** with low fluxes of NO in aerobic solution are compatible with an autoxidation mechanism, whereby strong oxidants are generated from degradation of peroxynitrite, which itself is formed from the initial autoxidation of **1** in the presence of NO (eqs 1−5; Scheme 3). In support of this mechanism, (i) oxygen was observed to be required for reaction, (ii) an aerobic solution of **1** and SPE/NO was observed to give oxidation of DHR in a concentration-dependent manner, and (iii) the metal ion chelator, DTPA, did not inhibit reaction. Conversely, added GSH quenched the reaction, which is compatible with either reduction of the initial phenoxyl radical or trapping of the oxidant radicals generated by peroxynitrite. Of interest are reports in the literature that catechols, including hydroxyestrogens (*81*−*83*), may generate peroxynitrite in the presence of NO, with evidence for consequent effects including DNA strand cleavage (*81*−*84*).

The reaction products observed in this work from the reactions of estrogens and antiestrogens with NO, peroxynitrite, and NO_2^-/H_2O_2 systems are compatible with reactions via the intermediate phenoxyl radicals ($ArO^\bullet$ in eqs 5−8). Oxidation of estradiol itself requires a strong oxidant such as $CO_3^{\bullet-}$, $HO^\bullet$, or even NO_2, which can be generated from bolus peroxynitrite or NO_2^-/H_2O_2/catalyst systems in atmospherically equilibrated solution. The polyaromatic phenolic benzothiophenes, raloxifene and isoraloxifene, however, may undergo autoxidation via peroxynitrite generation in the presence of NO. Because the rate constants for phenoxyl radical addition to NO, NO_2, and dimerization are all reported to be in the range of 10^8-10^9 M^{-1} s^{-1}, the major products observed will be largely determined by the concentrations of radicals in each system. Thus, at the relatively high concentrations of NO_2 generated from both bolus peroxynitrite, higher concentrations of SIN-1, or NO_2^-/H_2O_2/catalyst, nitration is observed. In reaction mixtures with NO or lower concentrations of SIN-1, the in situ reaction of NO with superoxide results in much lower concentrations of NO_2 that do not compete with NO or even the phenoxyl radical itself for trapping of the phenoxyl radical (Scheme 3). Dimerization is not expected to be of biological relevance itself but demonstrates the possibility of covalent modification of biomolecules via radical−radical reactions of the phenoxyl radical, **1**$^\bullet$. The regiospecificity for reaction of the benzothiophenes **1a**,**b** is compatible with the calculated relative spin density of the 6-oxobenzothiophene radical (B3LYP/6-31G**: C4, −0.06; C5, 0.11; C6, −0.04; C7, 0.20) rather than any direct role for the thiophene S.

NO, Estrogens, Antiestrogens, and RNOS-Mediated Metabolism. There is ample evidence that SERMs, including raloxifene, elevate cellular levels of NO; for example, in the brain cortex of raloxifene-treated rats, NO_2^-/NO_3^- levels were almost doubled relative to controls (*8*−*15*, *85*, *86*). Direct and indirect activation and up-regulation of constitutive and inducible NOS elevate NO levels, and important aspects of the biological activity of SERMs are mediated via NO signaling (*8*−*15*, *85*, *86*). The biological activity of SERMs is tissue selective and may be cytoprotective, cytotoxic, chemopreventive, or even carcinogenic; thus, there is considerable interest in

elucidating possible cyto- and genotoxic pathways that may result from both hormonal and chemical mechanisms. The latter include formation of reactive metabolites and consequent modification of cellular macromolecules and depletion of cellular antioxidants, as has been proposed for the prototypical SERM, tamoxifen (*33*, *87*).

The elevation of cellular NO levels, especially under conditions of oxidative stress, has the potential to generate oxidation, nitration, and nitrosation products from polyaromatic phenols, such as the SERMs and their metabolites, but such studies have not previously been reported. The data presented herein demonstrate that estrogens and SERMs are reactive toward RNOS-mediated metabolism. In the presence of a low flux of NO, raloxifene and an isomer, but not E_2, undergo oxidation and nitrosation probably via autoxidation and formation of peroxynitrite. The ready formation of nitrosation and dimerization products resulting from initial $1e^-$ oxidation in aerobic solution containing NO was observed. Of special interest was the formation of the nitrosation products, **2** and **6**. Although RNOS-derived nitration products, in particular nitrotyrosine, have been extensively researched, nitrosation products have received little attention (*67*, *69*). The quinone oxime, **2**, was isolated and was observed to function as an electrophilic and redox active metabolite toward GSH, generating GSH adducts and aminophenol products (Scheme 4).

We hypothesized that since SERMs elevate cellular levels of NO and on chemical grounds might be susceptible to RNOS-mediated metabolism, NO/SERM interactions may be of biological significance. The physiological relevance of reactions and reaction products observed in model systems may be questioned; for example, if present at physiological concentrations, the hydrophilic antioxidants GSH and ascorbate would inhibit almost all RNOS-mediated reactions in any aqueous model system. Nevertheless, the products of RNOS-mediated metabolism are clearly observed in vivo (*28*, *46*, *60*). Polyaromatic phenols, such as the SERMs, are good substrates for RNOS-mediated metabolism, including nitration, oxidation, and nitrosation. Nitration may simply perturb ER binding (*77*); however, the quinone oxime nitrosation products are reactive toward covalent modification of biomolecules and redox cycling. Further study of the chemical mechanisms and biological impact of the oxidation and nitrosation of SERMs and other conjugated phenols is warranted. Recently, Chen et al. reported that raloxifene was oxidized by cytochrome P450 3A4 to a metabolite that caused irreversible inhibition of this enzyme (*58*). Although the clinical significance of this work remains to be determined, it was suggested that SERMs with a decreased propensity for oxidative bioactivation might be desirable. The autoxidation of raloxifene in NO-containing solutions and RNOS-mediated nitrosation and nitration provide further examples of oxidative bioactivation pathways available to SERMs.

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