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A Study of the Reaction of Different Phenol Substrates with Nitric Oxide and Peroxynitrite

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Abstract: The reactivity of different phenol substrates with nitric oxide and peroxynitrite was investigated. In general, nitration is the major reaction with peroxynitrite, while reactions with aqueous solutions of nitric oxide led to mixtures of nitro and nitroso derivatives depending upon the phenol. Nitrosation occurs on phenol substrates bearing a free *para*- position with respect to the OH group with the exception of 1-naphthol, which afforded a 1:1 mixture of the 2- and the 4-nitroso derivatives. Chromans **7** and **8** showed the highest reactivity with peroxynitrite, which suggests that they can act as efficient scavengers of this toxic intermediate. In both cases the corresponding 5-nitro derivative was the only reaction product detected. Finally, the fact that chroman **8** reacts with nitric oxide to afford the *p*-quinone derivative **22a** in 90% yield suggests that this antioxidant could also be of potential use as specific nitric oxide tracer in biological tissues.

Keywords: Phenols. Antioxidants. Nitric oxide. Peroxynitrite.

Peroxynitrite ($ONOO^-$) and its conjugated acid, peroxynitrous acid (ONOOH), have become the subject of intense research in the biological sciences during the last decade.[1] This interest is based on the realization that peroxynitrite is produced in living matter by the reaction of nitric oxide (NO·) with superoxide ($O_2^{\cdot-}$). The high reactivity of this toxic species causes the oxidation of endogen compounds such as glutathione, cysteine, deoxyribose and tyrosine.[2] In this sense, 3-nitrotyrosine has been adopted as biological tracer for the presence of peroxynitrite in biological matrix molecules.[3,4] On the other hand, nitric oxide (NO) is a molecule of great biological importance because of its implication in processes related to vascular and immunologic systems and neurotransmission, although it is known that its overproduction exerts strong influence on pathologies like diabetes, stroke and neuronal degeneration.[5]

Thus, it is conceivable that radical species such as nitric oxide and highly reactive oxidation agents such as peroxynitrite may be generated in the same compartment, which could cause deleterious effects on the biological matrix.[6,7] Therefore, the availability of radical scavenger antioxidants exhibiting an additional protection in front of peroxynitrite could represent a valuable tool for preventive therapeutic strategies. Recently, the group of B. Ames reported that a component of natural tocopherols, that is γ-tocopherol, fulfills both requirements. Although not being as effective as α-tocopherol as a radical scavenger, this phenol can trap membrane-soluble electrophilic nitrogen oxides by forming adducts through its activated 5-position, which in turn is blocked in α-tocopherol.[8] Unfortunately, γ-tocopherol is difficult to make in large quantities, which justifies the search for structurally more simple compounds that could mimic its double protective effects.

In this context, we found that a simple tocopherol analogue, *i.e.*, 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2*H*)-benzopyran (**8**, Table 1), exhibited a potent inhibitory activity of the lipid peroxidation induced on rat liver microsomes. This activity was comparable or higher than that elicited by BHT or α-tocopherol under the conditions assayed.[9,10] Furthermore, the low toxicity effects shown by **8** and its ability to be embedded into liposomes, led to an exploration of the potential use of this antioxidant for reducing the undesired side effects

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produced by the administration of liposome encapsulated antitumoral drugs.[11] In this sense, compound **8**, which is currently under clinical trials in cancer therapy, was envisaged as an α-tocopherol surrogate. On the other hand, the fact that **8** contains two non-substituted and highly activated aromatic positions, led us to anticipate that this compound could be also considered as a γ-tocopherol analogue, in particular regarding its potential capacity to react with nitrating species such as peroxynitrite.

The fact that the 5-nitroderivative of γ-tocopherol was synthesized in good yields by Hoglen *et al.* using a nitrosating agent (sodium nitrite in acetic acid)[12] was somehow intriguing to us, since under these conditions the concomitant formation of the corresponding nitroso derivative could be also expected. At this point, the possibility of establishing some sort of distinction between the reactivity of peroxynitrite (nitrating agent) and of nitric oxide (nitrosating agent) depending upon the phenol substrate, was considered. This information could be useful for evaluating the potential protective effects that phenol antioxidants could offer to the adverse effects caused by the formation of peroxynitrite from nitric oxide and superoxide or by nitric oxide itself in cellular compartments. In addition, the finding of a specific biological tracer for the attack of nitric oxide with peroxynitrite might be of interest for biological studies on these intermediates. Accordingly, in the present contribution the reactivity of simple phenols bearing different substitution and activation at the aromatic ring, such as phenol itself (**1**), 2,6-dimethylphenol (**2**), 4-methoxyphenol (**3**) and 4-chlorophenol (**4**), with peroxynitrite and nitric oxide was investigated. In addition, 1-naphthol (**5**) was also selected as substrate because of its importance as a fine chemical, as well as tyrosine (**6**) due to its use as a peroxynitrite tracer in biological studies. Finally, the reactivity of two γ-tocopherol analogues was also examined: our antioxidant model **8** and phenol **7**, in which a methyl group replaces the phytyl side-chain present in γ-tocopherol.

Results and Discussion

The synthetic procedure for obtaining chroman **7** is shown in Scheme 1. A similar sequence, which has been described elsewhere, was used for the preparation of chroman **8**.[9] Concerning the nitrosophenols prepared as standards, *i.e.*, compounds **9**, **12**, **16** and **17** (Table 1), it should be mentioned that they exist as an equilibrium mixture between the nitroso derivative and the corresponding quinone monooxime.[13,14,15] Reactions of the different phenols with peroxynitrite and nitric oxide were monitored by HPLC using as internal standards: nitrobenzene for simple phenols and 1-naphthol, 4-chlorophenylalanine for tyrosine and the acetoxy derivatives from **7** and **8** for these γ-tocopherol analogues. The results obtained are summarized in Table 1.

Scheme 1

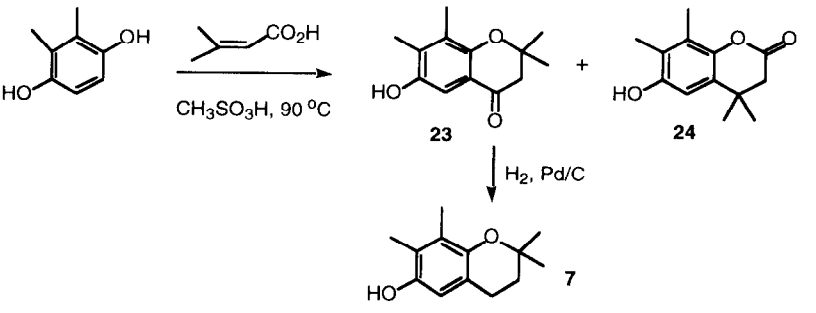
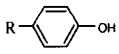
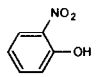
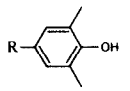
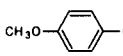
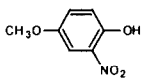
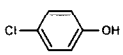
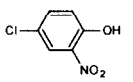
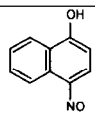
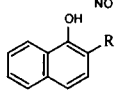
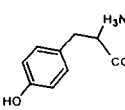
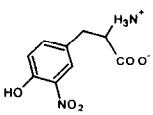
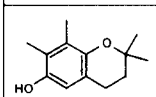
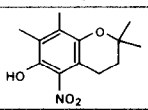
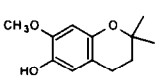
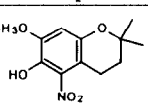
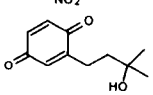


Table 1. Reactivity of different phenols with nitric oxide or peroxynitrite.

starting material	products		reactivity with NO[a]		reactivity with ONO_2^- [e]
			time (min)	yield[b] (%)	yield (%)
 1		R= NO, 9	15 30 60	29 59 76	n.d.[f]
		R= NO_2, 10	15 30 60	0.5 3.6 6	0.3
		11	15 30 60	2.8 6 9	0.6
 2		R= NO, 12	5 10	50 65	n.d.
		R= NO_2, 13	5 10	14 30	1.5
 3		14	2	80	0.4
 4		15	15 30 60 2 h 8 h	n.d. 3 10 12 46[c]	<0.1%
 5		16	5	48	1.2
		R= NO, 17	5	45	n.d.
		R= NO_2, 18	5	3	7
 6		19	5 15 30 60 120 8 h	3 9 16 21 30 45[d]	8
 7		20	5	68	15
 8		21	5	6	10
		22a	5	90	n.d.

[a] NO was used as 1 mM aqueous solutions prepared following the method described in the experimental section. [b] All the reactions were completed in 1 h, except for 4-chlorophenol and tyrosine. [c] The conversion of 4-chlorophenol was around 90% after 8 h (HPLC). [d] The conversion of tyrosine was around 60% after 8 h (HPLC). [e] The reaction time was 2 min in all cases. [f] n.d. Not detected.

Reactivity of phenol substrates 1-8 with nitric oxide. As shown, the reactivity of phenols with nitric oxide depended upon the nature, and most importantly, the position, of substituents with respect to the hydroxy phenol group. Actually, with the exception of 1-naphthol (**5**) which afforded a 45% yield of the *o*-nitroso derivative **17**, all other phenol substrates assayed containing free *ortho* positions with respect to the OH group gave the corresponding *o*-nitro derivative (cf. phenols **1**, **3**, **4**, **6** and **7**, since the case of chroman **8** will be discussed in more detail below), and no *o*-nitroso derivative was detected. In cases where the *para* position was free (cf. **1**, **2** and **5**), the *p*-nitroso derivative was the major compound produced. In the case of phenol (**1**) and 1-naphthol (**5**), which both have non substituted *ortho* and *para* positions, nitrosation was accompanied by a small amount of *ortho*-nitration (nitration at the *para* position was observed for **1** and not for **5**). Finally, the formation of the *para*-nitro derivative was only significant for phenol **2** (close to 1:2 molar ratio with respect to the corresponding nitroso derivative), a substrate lacking a free *ortho*-position. Concerning the reaction rates, they depended upon the aromatic ring activation: the more activated the phenol (*i.e.*, **2**, **3**, **5**, **7** and **8**), the higher the rate of nitration or nitrosation. The only exception was tyrosine. Reactions were completed in one hour for all phenols, except for 4-chlorophenol and tyrosine. For these substrates the yield of *o*-nitro derivative was around 50 % after 8 h.

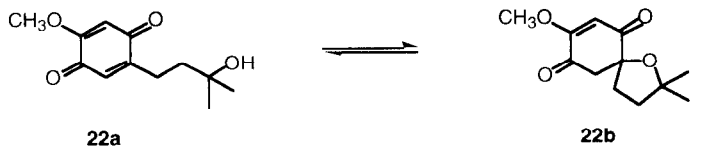
Reactivity of phenol substrates 1-8 with peroxynitrite. For the case of reactions with peroxynitrite and due to the instability of this reagent, assays were limited to 2 minutes, which limited the extent of the conversions attained. As shown in Table 1, formation of nitrophenols was observed in all cases assayed and the nitration took place at both *ortho* and *para* positions with respect to the hydroxyl group. As expected, no *ortho*-nitroso derivatives were detected with the exception of 1-naphthol (**5**) where a small amount of 4-nitroso-1-naphthol (**16**) was formed. On the other hand, the extent of the reaction with peroxynitrite was dependent on the substrate. Thus, for simple phenols (**1**-**4**), the yield of nitration was less than 2% and no clear correlation between the activation of the aromatic ring and the extent of the reaction could be deduced, although with the most deactivated substrate, that is 4-chlorophenol (**4**), only 0.1% of the corresponding nitro derivative **15** was detected. It is worth noting that within the low conversion yields obtained, those found for tyrosine (**6**), chroman **7** and chroman **8** were significantly higher (8-15%). In particular, the yields obtained for the latter two substrates suggest that these compounds can act as efficient peroxynitrite scavengers and thus offer additional protection to biological matrices[16], as occurs with γ-tocopherol[12] and compounds such as catechin polyphenols and ergothionine.[17,18]

A particular case: the reaction of chroman 8 with nitric oxide. As can be observed in Table 1, although chroman **8** exhibited a similar reactivity to compound **7** with peroxynitrite, its reaction with nitric oxide led to substantially different results; thus, in addition to the formation of 6% of the 5-nitro derivative **21**, a new major product, more polar according to the reverse-phase HPLC analysis, was detected. A detailed study of the HPLC profile under different elution conditions showed that this product was a mixture of two compounds in a 45:55 area ratio. When this mixture was separated by semi-preparative HPLC, followed by concentration through SEP-PACK cartridges, the initial mixture was again present in the HPLC profiles of the separated compounds. This result indicated that these two compounds were in equilibrium in aqueous medium. In fact, they could only be separated with ≥ 90% purity (^{1}H-NMR, HPLC) after a very rapid HPLC purification. It was also observed that the above interconversion did not occur in the solid state or in neutralized and dried solutions of chlorinated solvents.

The analysis of the spectral data led us to postulate the *para*-quinone structure **22a** for the yellow, less

polar compound and the spiro derivative **22b** for the colorless component of this equilibrium mixture (Scheme 2). The HPLC-MS and HRMS determination showed a molecular peak at *m/z* 224 for both compounds.

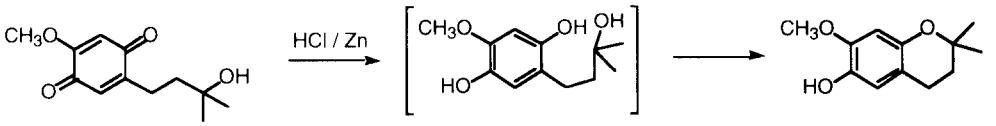
Scheme 2



22a **22b**

The ^{13}C-NMR data showed the presence of two carbonyl groups for each structure: **22a** presented the absorptions between 180-190 ppm, while those assigned to **22b** appeared at 190-200 ppm. This hypothesis was supported by the IR spectra, where bands at 1676, 1650 cm^{-1} and 1720, 1683 cm^{-1} were observed for **22a** and **22b**, respectively. These data suggested that α,β-unsaturated carbonyl groups were present in both compounds. In addition, the IR spectrum of **22a** showed a broad band centered at 3450 cm^{-1} and a sharp one at 3600 cm^{-1}. The ^{1}H-NMR data showed the aromatic hydrogen atoms of both compounds appearing at higher field than those of chroman **8**, thus suggesting a more olefinic character for them. It was also observed that the methylene groups at C-3 and C-4 in chroman **8** became non-equivalent in these structures. Moreover, it was clear that for the case of **22b** one olefin hydrogen atom had been incorporated into a new methylene group, which indicated that the equilibrium between **22a** and **22b** involved the saturation of one carbon-carbon double bond. This hypothesis was supported by the corresponding DEPT experiments. An independent confirmation of the structure of **22a** was obtained from its treatment with hydrochloric acid and zinc. The HPLC monitoring of the reaction course showed the conversion of the quinone into a more polar compound, to which the hydroquinone structure **25** was assigned. This intermediate was quantitatively converted into the expected chroman **8** during the work-up (Scheme 3). The identification of **22a** and **22b** permitted to evaluate in 90% their formation yield from the reaction of chroman **8** with nitric oxide. This is the highest yield among all reaction products found in the present study.

Scheme 3



22a **25** **8**

p-Quinone structures such as **22a** are well known in the family of natural tocopherols.[19] They had been found in the reaction of α-tocopherol with peroxynitrite[8] and of γ-tocopherol with some agents such $NO_2^+BF_4^-$.[12] Actually, the quinone derived from α-tocopherol is the major oxidative metabolite of this compound *in vivo*. However, to the best of our knowledge no reports on the existence of an equilibrium between structures such as **22a** and **22b** has been reported for natural tocopherols. In this context, we suggest that an intramolecular Michael and retro-Michael reaction account for the establishment of the above equilibrium. On the other hand,

the formation of **22a** and **22b** was detected in the reaction of chroman **8** with nitric oxide but not with peroxynitrite. This specificity and the high conversion yields of the reaction suggest that the formation of the mixture of **22** from chroman **8** could be considered as an attractive candidate for differentiating the presence of nitric oxide with peroxynitrite in biological tissues. Another possibility could be the use of phenol substrates that selectively afford nitroso derivatives in their reaction with nitric oxide, as it is the case of 4-nitrosophenol (**9**) from phenol, 2,6-dimethyl-4-nitrosophenol (**12**) from 2,6-dimethylphenol, and 2-nitroso-1-naphthol (**17**) from 1-naphthol (cf. Table 1). However, the use of these compounds would present several drawbacks: nitroso derivatives are generally considered as deleterious compounds, the formation yields obtained at short reaction times are considerably lower that for chroman **8** derived products and, as has been mentioned above, they undergo equilibration with the corresponding quinone monooxime derivatives,[13,14,15] which might complicate their analysis and detection.

In summary, the results of the present study show that the reactivity of different phenol substrates with nitric oxide and peroxynitrite does not permit the establishment of clear-cut differences. In general, nitration is the major reaction with peroxynitrite, while nitrosation with nitric oxide only seems to occur on phenol substrates bearing a free *para*-position with respect to the OH group. From the compounds studied, chromans **7** and **8** showed the highest reactivity with peroxynitrite, which suggests that they can act as efficient scavengers of this toxic intermediate. In both cases, the corresponding 5-nitro derivative is the only reaction product detected. Finally, the fact that chroman **8** reacts with nitric oxide to afford the *p*-quinone derivative **22a** in 90% yield suggests that this chroman could be of potential use as specific nitric oxide tracer in biological tissues. Research along this line is in progress in our laboratory.

Experimental Section

Melting points were determined with a Koffler apparatus and were not corrected. The NMR spectra (^{1}H-NMR, 300 MHz; ^{13}C-NMR, 75 MHz) were recorded with a Varian Unity 300 spectrometer. Unless otherwise indicated, they were performed in $CDCl_3$ solutions and chemical shifts are given in ppm downfield from tetramethylsilane for ^{1}H and deuterochloroform for ^{13}C. The IR spectra were recorded by using a Bomem model MB120 apparatus and absorptions are given in cm^{-1}. The GC-MS-EI spectra (70 eV) were obtained using a Fisons model MD 800 mass spectrometer coupled to a Fisons GC 8000 apparatus, which was equipped with a 25 m HP-5 capillary column. The HPLC-MS-APCI spectra were obtained with a HP 1090 Liquid Chromatograph and a HP 1100 Mass Spectrometer operating in positive mode. High-resolution mass spectra (HRMS) were performed on a VG Autospec-Q apparatus (Mass Spectrometry Service, IIQAB). Elemental analyses were carried out with Carlo Erba 1108 instrument (Microanalysis Service, IIQAB). The HPLC monitoring at the analytical scale was carried out using a HP-1100 modular system while an Applied Biosystems 783 Programmable Absorbance Detector and Waters Millipore 510 pumps were used for the preparative purifications.

Nitrobenzene and 4-chlorophenylalanine (internal standards for HPLC), tyrosine and 3-nitrotyrosine were from Aldrich. The 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) used for titration of NO aqueous solutions, was also from Aldrich.

Starting phenols and internal standards. The preparation of chroman **8** has been described

elsewhere.[9] Compound **7** was prepared using a two step synthetic procedure shown in scheme 1. Thus, a mixture of 2,3-dimethyl-1,4-hydroquinone (1.38 g, 10 mmol), 3-methylbut-2-enoic acid (1.0 g, 10 mmol) and methanesulfonic acid (25 ml) was stirred vigorously for 1 h at 90 °C (GC monitoring). The brown orange mixture was poured onto 120 g of ice, extracted with methyl *tert*-butyl ether (3 x 60 ml), washed with water, brine and dried over $MgSO_4$. The residue obtained from solvent evaporation was purified by flash chromatography on silicagel eluting with 20:1 CH_2Cl_2:EtOAc to give a pale yellow solid (1.3 g, 60% yield), and a white solid (0.52 g, 24% yield), which were identified as the benzopyranones **23** and **24**, respectively:

3,4-Dihydro-6-hydroxy-2,2,7,8-tetramethyl-1(2H)-benzopyran-4-one (23)[20]: m.p: 189-191 °C (lit: 190-191.5 °C); ^{1}H-NMR: 1.44 (s, 6 H, CH_3), 2.16 (s, 3 H, CH_3), 2.22 (s, 3 H, CH_3), 2.67 (s, 2 H, H-3), 5.08 (s, 1 H, OH), 7.16 (s, 1 H, H-5); ^{13}C-NMR: 11.91, 12.97 (CH_3), 26.73 (CH_3), 48.76 (C-3), 78.49 (C-2), 107.2 (C-5), 117.4, 127.3, 134.1 (C-4a, C-7, C-8), 147.9, 152.5 (C-6, C-8a), 193.7 (C-4). **3,4-Dihydro-6-hydroxy-4,4,7,8-tetramethyl-1(2H)-benzopyran-2-one (24)**[20]: m.p: 146-148 °C (lit: 145-147 °C); ^{1}H-NMR: 1.29 (s, 6 H, CH_3), 2.17 (s, 3 H, CH_3), 2.23 (s, 3 H, CH_3), 2.57 (s, 2 H, H-3), 5.16 (s, 1 H, OH), 6.63 (s, 1 H, H-5); ^{13}C-NMR: 11.80, 12.31 (CH_3), 27.58 (CH_3), 33.12 (C-4), 43.68 (C-3), 107.8 (C-5), 122.6, 126.3, 129.5 (C-8, C-7, C-4a), 142.7, 150.2 (C-6, C-8a), 169.1 (C-2).

3,4-Dihydro-6-hydroxy-2,2,7,8-tetramethyl-1(2H)-benzopyran (7). A solution containing the chromanone **23** (0.30 g, 1.4 mmol), EtOH (30 ml) and few drops of $HClO_4$ was subjected to catalytic hydrogenation (70 atm and 20 °C), in the presence of 0.30 g of 10% Pd/C. After 3 hours 0.15 g of catalyst were added and the reaction was prolonged for 3 more hours under the above conditions. The crude reaction mixture was filtered over Celite®, evaporated and purified by flash chromatography on silicagel using 30:1 hexane:EtOAc as eluent mixture to render a crystalline solid identified as chroman **7**[21] (0.25 g, 90% yield): m.p: 75-77 °C (lit: 75.5-76.5 °C); ^{1}H-NMR: 1.29 (s, 6 H, CH_3), 1.75 (t, 2 H, J = 6.7 Hz, H-3), 2.11 (s, 3 H, CH_3), 2.13 (s, 3 H, CH_3), 2.69 (t, 2 H, J = 6.7 Hz, H-4), 4.22 (s, 1 H, OH), 6.39 (s, 1 H, H-5); ^{13}C-NMR: 11.85, 11.89 (CH_3, CH_3), 22.63 (C-4), 26.93 (CH_3), 32.95 (C-3), 73.39 (C-2), 112.17 (C-5), 118.0, 121.6, 125.7 (C-4a, C-8, C-7), 145.8, 146.2 (C-8a, C-6).

The rest of the starting phenols were commercially available from Aldrich.

The acetoxy derivatives from **7** (**7a**) and **8** (**8a**) used as internal standards for HPLC, were prepared as follows: acetic anhydride (100 µl, 1.06 mmol) was added to a solution of **7** or **8** (0.24 mmol) in pyridine (100 µl) and the mixture was stirred for 30 min at room temperature (GC monitoring). Then,the excess of reagents was removed by vacuum distillation to leave a white solid residue which was identified as the acetoxy derivative **7a** or **8a**, respectively. **3,4-Dihydro-6-acetoxy-2,2,7,8-tetramethyl-1(2H)-benzopyran (7a)**: m.p: 71-73 °C (lit[22]: 70.5-71.5); IR (KBr): 2974, 2937, 1749, 1479, 1425, 1371, 1209; ^{1}H-NMR: 1.31 (s, 6 H, CH_3), 1.75 (t, 2 H, J = 6.7 Hz, H-3), 2.02 (s, 3 H, CH_3), 2.10 (s, 3 H, CH_3), 2.29 (s, 3 H, CH_3CO), 2.72 (t, 2 H, J = 6.7 Hz, H-4), 6.58 (s, 1 H, H-5); ^{13}C- NMR: 11.94, 12.66 (CH_3), 20.80, 22.57 (CH_3CO, C-4), 27.10 (CH_3), 32.66 (C-3), 73.92 (C-2), 118.8 (C-5), 118.2,125.8, 127.0 (C-4a, C-8, C-7), 141.7, 149.7 (C-8a, C-6), 170.0 (CO); MS: 248 (M^+, 43), 207 (23), 206 (100), 191 (14), 151 (69), 150 (85). **3,4-Dihydro-6-acetoxy-7-methoxy-2,2-dimethyl-1(2H)-benzopyran (8a)**: m.p: 61-63 °C; IR (KBr): 2974, 2933, 1760, 1627, 1512, 1448, 1213, 1188; ^{1}H-NMR: 1.32 (s, 6 H, CH_3), 1.76 (t, 2 H, J = 6.7 Hz, H-3), 2.28 (s, 3 H, CH_3CO), 2.67 (t, 2 H, J = 6.6 Hz, H-4), 3.75 (s, 3 H, CH_3O), 6.40 (s, 1 H, H-5), 6.71 (s, 1 H, H-8); ^{13}C-NMR: 20.61, 21.69 (CH_3CO, C-4), 26.82 (CH_3), 32.67 (C-3), 55.82 (CH_3O), 74.38 (C-2), 101.6, 112.2 (C-8, C-4a), 122.6 (C-5), 132.7 (C-6), 150.1, 152.2 (C-7, C-8a), 169.6 (CO); MS: 250 (M^+, 14), 208 (80),

153 (100). Elemental analysis for $C_{14}H_{18}O_4$. Calculated: C, 67.18; H, 7.25. Found: C, 67.00; H, 7.26.

Synthesis of the nitro and nitroso standards. General procedure: the different nitro and nitroso phenols were synthesized following the method for nitrous acid induced nitration of phenols with minor modifications.[12,23] Briefly, glacial acetic (2 ml) was added to a solution of the phenol (0.54 mmol) in EtOH (50 ml), followed by addition of 2% $NaNO_2$ solution (30 ml). Reactions were monitored by GC and after completion, they were neutralized with 20% KOH solution and diluted with 100 ml of water. The product was extracted into methyl *tert*-butyl ether, washed with water, brine and dried over $MgSO_4$. The residue obtained from solvent evaporation was purified by preparative TLC on silicagel using the appropriate hexane/EtOAc eluent mixtures (see below).

Phenol derivatives. 4-Nitrosophenol (9): this compound was purified by preparative TLC using a 3:1 hexane:EtOAc mixture as eluent and it was isolated as a brown solid in 65% yield: m.p. 130-132 °C (lit[24]: 133 °C); ^{1}H-NMR ($CDCl_3$/DMSO): 6.54, 6.50, 7.22, 7.24, 7.77, 7.79, 8.92 (br, 1 H, OH); MS: 123 (M^+, 100), 107 (77). **4-Nitrophenol (10)**: this compound was purified by preparative TLC using a 3:1 hexane:EtOAc 3:1 mixture as eluent (10% yield). **2-Nitrophenol (11)**: this compound was purified by preparative TLC using a 3:1 hexane:EtOAc mixture as eluent (3% yield).

2,6-Dimethylphenol derivatives. 2,6-Dimethyl-4-nitrosophenol (12): this compound was purified by preparative TLC using a 10:1 hexane:EtOAC mixture as eluent and it was isolated as a yellow solid in 20% yield: m.p. 145-147 °C (lit[24]: 169-170 °C); ^{1}H-NMR: 2.04, 2.07 (s, 6 H, CH_3), 6.97, 7.58 (s, 2 H); MS: 151 (M^+, 100), 134 (30). **2,6-Dimethyl-4-nitrophenol (13)**: this compound was purified by preparative TLC using a 10:1 hexane:EtOAC mixture as eluent and it was isolated as a yellow solid in 46% yield: m.p. 170-172 °C (lit[24]: 169-170 °C); ^{1}H-NMR: 2.32 (s, 6 H, CH_3), 7.93 (s, 2 H); MS: 167 (M^+, 66), 151 (3), 137 (38), 91 (83), 77 (100).

4-Methoxy-2-nitrophenol (14): this compound was purified by preparative TLC using a 30:1 hexane:EtOAc mixture as eluent and it was isolated as an orange solid in 78% yield: m.p. 79-80 °C (lit[25]: 80 °C); ^{1}H-NMR: 3.83 (s, 3 H, CH_3O), 7.09 (d, J = 9 Hz, 1 H, H-6), 7.23 (dd, J = 9, 3 Hz, 1 H, H-5), 7.51 (d, J = 3 Hz, 1 H, H-3), 10.35 (s, 1 H, OH), 8.95 (s, 1 H, OH); MS: 169 (M^+, 100), 154 (6), 152 (5), 139 (7), 124 (10).

4-Chloro-2-nitrophenol (15): this compound was purified by preparative TLC using a 5:1 hexane:EtOAC mixture as eluent and it was isolated as an orange solid in 16% yield: m.p. 85-87 °C (lit[25]: 85-87 °C); ^{1}H-NMR: 7.18 (d, J= 9 Hz, 1 H), 7.57 (dd, J = 9, 2.5 Hz, 1 H), 8.15 (d, J = 2.5 Hz, 1 H), 10.52 (s, 1 H, OH); MS: 173 (M^+, 100), 175(M^+ + 2, 30), 156 (10), 158 (3), 143 (23), 145 (7), 127 (22), 129 (7), 115 (39), 117 (11), 99 (49), 101 (16).

1-Naphthol derivatives. 4-Nitrosonaphthol (16): this compound was purified by preparative TLC using a 3:1 hexane:EtOAc mixture as eluent and it was isolated as a pale brown solid in 42% yield: m.p. 194-195 °C (lit[26]: 197); ^{1}H-NMR: 6.67 (d, J= 10.5 Hz, 1 H, H-2), 7.57, 7.65, (ddd, J= 7.5 , 7.5, 1.5 Hz, 1 H, H-6, H-7), 8.17 (dd, J= 7.5 , 1.5 Hz, 1 H, H-5), 8.17 (dd, J= 7.5 , 1.5 Hz, 1 H, H-8), 8.02 (d, J= 10.5 Hz, 1 H, H-3); MS: 173 (M^+, 77), 156 (13), 143 (13), 130 (18), 128 (18), 115 (100). **2-Nitrosonaphthol (17)**: this compound was purified by preparative TLC using a 3:1 hexane:EtOAc mixture as eluent and it was isolated as a pale brown solid in 17% yield: m.p. 142-145 °C (lit[26]: 142-146 °C); ^{1}H-NMR ($CDCl_3$/DMSO): 6.9 (d, J= 9.6 Hz, 1 H), 6.96 (d, J= 9.6 Hz, 5/9 H), 7.20 (d, J= 9.6 Hz, 4/9 H), 7.27 (d, J= 7.6, 4/9 H), 7.41 (t, J= 7.6, 4/9 H), 7.47 (d, J= 7.6, 5/9 H), 7.49 (dt, J= 7.6, 1.2 Hz, 5/9 H), 7.62 (t, J= 7.6 Hz, 4/9 H), 7.74 (dt, J= 7.6,

5/9 Hz, 1.2 H), 8.24 (d, J= 7.6 Hz, 4/9 H), 8.36 (dd, J= 7.6, 1.2 Hz, 5/9 H); MS: 173 (M^+, 84), 156 (100), 143 (22), 130 (43), 128 (56), 115 (66). **2-Nitronaphthol (18)**: this compound was purified by preparative TLC using a 3:1 hexane:EtOAc mixture as eluent and it was isolated as a pale brown solid in 8% yield: m.p. 125-127 °C (lit[27]: 127-128 °C); ^{1}H-NMR: 7.32 (d, J= 9 Hz, 1 H), 7.61 (tt, J= 7.5 , 1.5 Hz, 1 H), 7.71 (tt, J= 7.5 , 1.5 Hz, 1 H), 7.8 (d, J= 9 Hz, 1 H), 7.99 (d, J= 9 Hz, 1 H), 8.50 (d, J= 9 Hz, 1 H); MS: 189 (M^+, 100), 172 (11), 159 (13), 131 (13).

3,4-Dihydro-6-hydroxy-5-nitro-2,2,7,8-tetramethyl-1(2H)-benzopyran (20). Purification of this compound by preparative TLC on silicagel using a 30:1 hexane:EtOAc mixture as eluent gave an orange solid in 62% yield: m.p: 79-81 °C; IR (KBr): 3428, 2978, 2929, 1595, 1540, 1455, 1262, 1175; ^{1}H-NMR: 1.32 (s, 6 H, CH_3), 1.74 (t, 2 H, J = 6.7 Hz, H-3), 2.19 (s, 2 H, CH_3) 2.23 (s, 2 H, CH_3), 3.03 (t, 2 H, J = 6.7 Hz, H-4), 10.7 (s, H, OH); ^{13}C-NMR: 12.04, 13.29 (CH_3, CH_3), 22.01 (C-4), 26.48 (CH_3), 32.47 (C-3), 73.38 (C-2), 113.2, 125.3, 132.0, 137.0 (C4a, C-5, C-7, C-8), 145.3, 148.4 (C-6, C-8a); MS: 251 (M^+, 82), 219 (62), 218 (100), 202 (45), 178 (68), 150 (67). Elemental analysis for $C_{13}H_{17}O_4$. Calculated: C, 62.13; H, 6.82; N, 5.57. Found: C, 62.09; H, 6.81; N 5.49.

3,4-Dihydro-2,2-dimethyl-6-hydroxy-7-methoxy-5-nitro-1(2H)-benzopyran (21). This nitro derivative was purified by preparative TLC on silicagel using a 3:1 hexane:EtOAc mixture as an eluent and it was isolated as a bright yellow solid in 50% yield. NOE experiments confirmed that the nitration took place only at C-5. **21**: m.p. 115-117 °C; IR (CCl_4): 3554, 2979, 2937, 1732, 1544, 1444, 1272; ^{1}H-NMR: 1.33 (s, 6 H, CH_3), 1.77 (t, *J* = 6.7 Hz, 2 H, H-3), 2.88 (t, *J* = 6.7 Hz, 2 H, H-4), 3.88 (s, 3 H, CH_3O), 6.61 (s, 1 H, H-8), 8.95 (s, 1 H, OH); ^{13}C- NMR: 20.32 (C-4), 26.29 (CH_3), 32.21 (C-3), 56.38 (CH_3O), 74.12 (C-2), 106.8, 106.9 (C-4a, C-8), 135.8,,138.7 (C-5, C-6), 146.7, 147.8 (C-7, C-8a); MS: 253 (93), 235 (16), 220 (100), 203 (47), 204 (40), 198 (62), 180 (52). Elemental analysis for $C_{12}H_{15}NO_5$. Calculated: C, 56.91; H, 5.97; N, 5.53. Found: C, 56.58; H, 6.12; N, 5.13.[16]

Synthesis of 22a and 22b. Compound **22** was obtained in a 17% yield from the reaction of chroman **8** with nitrous acid as described above, but after extraction with methyl *tert*-butyl ether to remove the 5-nitro derivative, a second extraction with EtOAc was performed to take the mixtrure of **22**. This mixture was purified by semi-preparative HPLC (Tracer Kromasil 100, C18, 5 μ, 25 x 0.46 cm column) using a 30:70 MeOH:water mixture as eluent at a rate flow of 4 ml/min. Under these conditions **22b** and **22a** eluted at 42.2 and 53.5 min, respectively. The collected eluates were diluted with water to reach 5:95 MeOH:water mixtures and were passed through a SEP-PACK (Millipore Waters C-18, Part. num. 20515, 360 mg/cartridge) column. Thus, starting from 15 mg of the mixture, 4 mg of a 10:90 **22a:22b** and 8 mg of a 95:5 **22a:22b** samples were finally isolated. **2-Methoxy-5(3'-hydroxy-3'-methyl)butyl-1,4-benzoquinone (22a)**: IR (CCl_4): 3600, 3450, 3024, 2975, 1676, 1650, 1604, 1151, 1139; ^{1}H-NMR: 1.28 (s, 6 H, CH_3), 1.65 (m, 2 H), 2.55 (m, 2 H), 3.82 (s, 3 H, CH_3O), 5.93 (s, 1 H), 6.53 (s, 1 H); ^{13}C- NMR: 24.17 (CH_2), 29.27 (2 x CH_3), 41.76 (CH_2), 56.23 (CH_3O-), 70.59 (C-OH), 107.75 (CH), 130.37 (CH), 150.83 (CH), 158.58 (CH), 182.5 (CO), 187.4 (CO); MS: 209 (10), 191 (6), 181 (6), 177 (8), 168 (38), 166 (21), 151 (21), 137 (29), 69 (41), 59 (100). HRMS for $C_{12}H_{16}O_4$. Calculated: 224.1048. Found: 224.1043. **1-Oxaspiro[4.5]-2,2-dimethyldec-8-ene-7,10-dione (22b)**: IR (CCl_4): 2974, 1720, 1683, 1604, 1174, 1139; ^{1}H-NMR: 1.28 (s, 3 H, CH_3), 1.32 (s, 3 H, CH_3), 1.88 (3 H), 2.47 (m, 1 H), 2.92 (d, J = 16 Hz, 1 H), 3.07 (d, J = 16 Hz, 1 H), 3.81 (s, 3 H, CH_3O), 6.00 (s, 1 H); ^{13}C- NMR: 28.09 (CH_3), 29.18 (CH_3), 34.37 (CH_2), 37.98 (CH_2), 50.89 (CH_2), 56.37 (CH_3O), 84.31 ((CH_3)$_2$C-O), 85.62, 111.4 (CH), 162.15 (C-OCH_3), 190.8 (CO), 196.9 (CO); MS: 224

(7), 206 (7), 191 (26), 176 (9), 153 (9), 127 (100). HRMS for $C_{12}H_{16}O_4$. Calculated: 224.1048. Found: 224.1052.

A small amount of Zn powder was added to a solution of 2 mg of **22a** in 0.25 ml of diluted 2N HCl. The yellow solution become colorless in one minute. After five minutes, the crude mixture was filtered, extracted with diethyl ether, dried over $MgSO_4$ and evaporated. The HPLC analysis of the residue showed the presence of compound **8** (Scheme 3).

HPLC Conditions for the quantification of nitro and nitroso phenol derivatives. The HPLC analyses were carried out using a Merck LiChrocart analytical column (5μm LiChrospher 100 RP-18, 12.5 x 0.4 cm). Reactions were monitored at λ= 286 nm for most phenols, and at λ= 230, 274 nm for tyrosine derivatives, using a rate flow of 1 ml/min in all cases. The elution conditions are indicated below for each substrate. Mobile phases consisted of different MeOH/water mixtures for most phenols, but a MeOH/Et_3N-HCOOH 0.05 M buffer mixture (pH= 3) was employed for the tyrosine derivatives. Nitrobenzene was used as internal standard for most phenol samples, 4-chlorophenylalanine was used for tyrosine samples, and the respective 6-acetoxy derivatives of chromans **7** and **8** were used for these substrates. 2 mM Methanol solutions of phenol derivatives, nitrobenzene, 4-chlorophenylalanine and the acetoxy derivatives were prepared and the respective calibration curves were calculated within the 0-10 nmol range ($r > 0.999$ for all cases).

Phenol derivatives. The following program of gradients was used: from minute 0 to minute 6, 20% MeOH, from minute 6 to 10 change to 40% MeOH and 40% MeOH was maintained up to minute 25. Under these conditions phenol, 4-nitrosophenol, 4-nitrophenol, 2-nitrophenol and nitrobenzene eluted at 8.7, 4.4, 12.3, 15.5 and 16.9 min, respectively.

2,6-Dimethylphenol derivatives. A 50:50 MeOH:water mixture was used. Under these conditions 2,6-dimethylphenol, 4-nitroso-2,6-dimethylphenol, 4-nitro-2,6-dimethylphenol and nitrobenzene eluted at 6.7, 3.9, 7.8 and 5.4 min, respectively.

4-Methoxyphenol derivatives. A 40:60 MeOH:water mixture was used. Under these conditions 4-methoxyphenol, 4-methoxy-2-nitrophenol and nitrobenzene eluted at 3.1, 12.1 and 9.3 min, respectively.

4-Chlorophenol derivatives. A 50:50 MeOH:water mixture was used. Under these conditions 4-chlorophenol, 4-chloro-2-nitrophenol and nitrobenzene eluted at 3, 8.7 and 4.8 min, respectively.

1-Naphthol derivatives. The following program of gradients was used: from minute 0 to minute 13, 40% MeOH in H_2O, from minute 13 to minute 15 change to 50% MeOH and 50% MeOH was maintained up to minute 17; from minute 17 to minute 22 change to 75% MeOH and 75% MeOH was maintained up to minute 27. Under these conditions 1-naphthol, 4-nitrosonaphthol, 2-nitrosonaphthol, 4-nitronaphthol and nitrobenzene eluted at 18.1, 14.1, 15.1, 26, and 10.4 min, respectively.

Tyrosine derivatives. The following program of gradients was used: from minute 0 to minute 3, 0% MeOH, from minute 3 to minute 8 change to 15% MeOH and 15% MeOH was maintained up to minute 15. Under these conditions tyrosine, 3-nitrotyrosine and 4-chlorophenylalanine eluted at 3.41, 9.68 and 13.07 min, receptively

Chroman 7 derivatives. A 70:30 MeOH: water mixture was used. Under these conditions chroman **7**, compound **20** and the acetoxy **7a** derivative eluted at 4.8, 14.3 and 8.6 min, respectively.

Chroman 8 derivatives. The following program of gradients was used: from minute 0 to minute 5, 45% MeOH in H_2O, from minute 5 to minute 7 change to 55% MeOH and 55% MeOH was maintained up to

minute 12; from minute 12 to minute 14 change to 45% MeOH and 45 % MeOH was maintained up to minute 25. Under these conditions chroman **8**, compounds **21** and **22** and the acetoxy derivative **8a** eluted at 9.95, 14.7, 3.8, 21.5, respectively. It should be noted that under these conditions compounds **22a** and **22b** were not resolved.

Preparation of aqueous NO solutions. Nitric oxide solutions were prepared as described previously.[28,29] Briefly, a 25 ml flask was filled with 10 ml of phosphate buffer solution (pH= 7.4). The oxygen was removed by vacuum degassing followed by bubbling with argon through a rubber seal. NO gas (15% in N_2, Abelló-Linde, S.A.) was purified by passage through 5 ml of a 1 M NaOH solution and then through the stirred buffer solution maintained at 0 °C. By this procedure approx. 1 mM NO solutions were obtained. The concentration of the NO solutions was measured by adding 0.250 ml aliquots to 10 ml of a 5 mM ABTS solution and determining the absorbance changes at 660 nm (e=12000 M^{-1} cm^{-1}).[28]

Reactions of phenols with NO aqueous solution. Reactions were carried out in 2 ml of a 1:4 methanol: 0.05 M potassium phosphate buffer (pH= 7.4) containing the corresponding phenol substrate (1 mM). Then, 2 ml of a 1 mM NO aqueous solution were added at 25 °C under vigorous stirring. At the specified time, 0.5 ml aliquots were added to 0.125 ml of a 2 mM methanol solution of the appropriate standard and the resulting samples were analyzed by HPLC. The results are shown in Table 1.

Preparation of peroxynitrite solutions. Solutions of this reagent were prepared in a two-phase system using isoamyl nitrite and hydrogen peroxide.[30] Unreacted hydrogen peroxide was removed by passing the peroxynitrite solution through a column fitted with MnO_2,[30] which was pre-washed with water and 1M NaOH. Concentration of peroxynitrite solutions were determined spectrophotometrically at 302 nm (ε= 1670) in 1M NaOH.[31] The approximately 1M peroxynitrite solutions obtained were used immediately after their preparation.

Reactions of phenols with peroxynitrite. Reactions were carried out in 2 ml of a 1:4 methanol: 0.05 M potassium phosphate buffer (pH= 7.4) containing the corresponding phenol substrate (1 mM). Then, a solution of peroxynitrite in NaOH was added at 25 °C with vigorous stirring. The concentration of peroxynitrite in the final solution was 1 mM. By using this buffer, pH changes after the peroxynitrite addition were kept to a minimum. After 2 min, reactions were treated as follows. For simple phenol derivatives samples were mixed with 0.1 ml of a 2 mM nitrobenzene solution, extracted into hexane, evaporated, redissolved in 0.20 ml of methanol and analysed by HPLC. For 1-naphthol, tyrosine, chroman **7** and chroman **8** derivatives, samples were mixed with the internal standard (4-chlorophenylalanine and the corresponding 6-acetoxy derivatives, respectively), and analysed by HPLC. The results obtained are shown in Table 1.

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Notes and References

1. Beckman, J.S.; Beckman, T.W.; Chen, J; Marshall, P.A. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1620-

1624.
2. Koppenol, W.H.; Moreno, J.J.; Pryor; W.A.; Ischiropoulos, H.; Beckman, J.S. *Chem. Res. Toxicol.* **1992**, *5*, 834-842.
3. Halliwell, B. *FEBS Lett.* **1997**, *411*, 157-160.
4. Van der Vliet, A.; O'Neill, C.A.; Halliwell, B.; Cross, C.E.; Kaur, H. *FEBS Lett.* **1994**, *339*, 89-92.
5. Wink, D.A.; Mitchell, J.B. *Free Rad. Biol. Med.* **1998**, *25*, 434-456.
6. Hensley, K.; Tabatabaie, T.; Stewart, C.A.; Pye, Q.; Floyd, R.A. *Chem. Res. Toxicol.* **1997**, *10*, 527-532.
7. Fukuto, J.M.; Ignarro, L.J. *Acc. Chem. Res.* **1997**, *30*, 149-152.
8. Christen, S.; Woodall, A.A.; Shigenaga, M.K.; Southwell-Keely, P.T.; Duncan, M.W.; Ames, B.N. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 3217-3222.
9. Casas, J.; Gorchs, G.; Sánchez-Baeza, F.; Teixidor, P.; Messeguer, A. *J. Agric. Food Chem.* **1992**, *40*, 585-590
10. Irurre, Jr, J.; Casas, J.; Ramos, I.; Messeguer, A. *Bioorg. Med. Chem.* **1993**, *1*, 219-225.
11. Lipotec, S.A., European Patent EP 55239, **1994**.
12. Hoglen, N.C.; Waller, S.C.; Sipes, I.G.; Liebler, D.C. *Chem. Res. Toxicol.* **1997**, *10*, 401-407.
13. Pilichowski, J.F.; Boule, P.; Billard, J.P. *Can. J. Chem.* **1995**, *73*, 2143-2147.
14. Geraldes, C.F.G.C.; Silva, M.I.F. *Opt. Pur. Apl.* **1988**, *21*, 71-78.
15. Uffmann, H. *Tetrahedron Lett.* **1966**, *38*, 4631-4637.
16. Montoliu, C.; Llansola, M.; Yenes, S.; Messeguer, A.; Felipo, V. *Biochem. Pharmacol.* **1999**, *58*, 255-261.
17. Pannala, A.; Rice-Evans, C.A.; Halliwell, B.; Singh, S. *Biochem. Biophys. Res. Commun.* **1997**, *232*, 164-168.
18. Aruoma, O.I.; Whiteman, M.; England, T.G.; Halliwell, B. *Biochem. Biophys. Res. Commun.* **1997**, *231*, 389-391.
19. Kamal-Eldin, A.; Appelqvist, L-A. *Lipids* **1996**, *31*, 671-701.
20. King, M.M.; Cohen, L.A. *J. Am. Chem. Soc.* **1983**, *105*, 2752-2760.
21. Lars, J.; Nilsson, G.; Sievertsson, H.; Selander, H. *Acta Chem. Scand.* **1968**, *22*, 3160-3170.
22. Smith, L.I.; Tess, R.W.H. *J. Amer. Chem. Soc.* **1944**, *66*, 1523-1525.
23. Marcinkiewicz, S. *Acta Pol. Pharm.* **1967**, *24*, 375-378.
24. Vaughan, W.R.; Finch, G.K. *J. Org. Chem.* **1956**, *21*, 1201-1210.
25. Halfpenny, E.; Robinson, P.L. *J. Chem. Soc.* **1952**, 939-946.
26. Benson, W.R.; Gajan, R.J. *J. Chem. Soc.* **1966**, *31*, 2498-2502.
27. Edwards, Jr.,W.R.; Tate, C.W. *Anal. Chem.* **1951**, *23*, 826-830.
28. Nims, R.W.; Cook, J.C.; Krishna, M.C.; Christodoulou, D.; Poore, M.B.; Miles, A.M.; Grisham, M.B.; Wink, D.A. *Methods Enzymol.* **1996**, *268*, 93-105.
29. Nitric Oxide Protocols. Edited by Michael A. Titheradge. University of Sussex, Brighton, UK. Humana Press Totowa, New Jersey, 1998.
30. Uppu, R.M.; Pryor, A. *Anal. Biochem.* **1996**, *236*, 242-249.
31. Hughes, M.N.; Nicklin, H.G. *J. Chem. Soc. (A).* **1968**, 450-452.