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Acid-Promoted Reaction of the Stilbene Antioxidant Resveratrol with Nitrite Ions: Mild Phenolic Oxidation at the 4′-Hydroxystiryl Sector Triggering Nitration, Dimerization, and Aldehyde-Forming Routes

Lucia Panzella,[†] Maria De Lucia,[†] Carmine Amalfitano,[‡] Alessandro Pezzella,[†] Antonio Evidente,[‡] Alessandra Napolitano,[†] and Marco d'Ischia*[,†]

Department of Organic Chemistry and Biochemistry and Dipartimento di Scienze del Suolo, delle Piante e dell'Ambiente, University of Naples "Federico II", Naples, Italy

dischia@cds.unina.it

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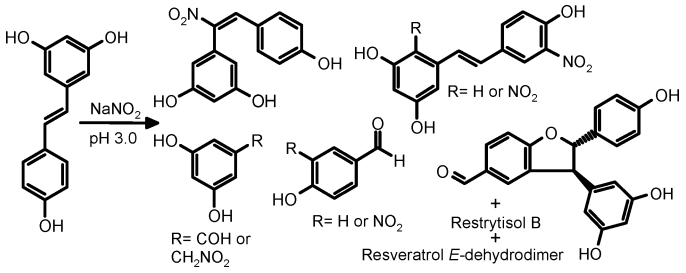

In 0.1 M phosphate buffer, pH 3.0, and at 37 °C, resveratrol ((*E*)-3,4′,5-trihydroxystilbene, **1a**), an antioxidant and cancer chemopreventive phytoalexin, reacted smoothly at 25 μM or 1 mM concentration with excess nitrite ions (NO_2^-) to give a complex pattern of products, including two novel regioisomeric α-nitro (**3a**) and 3′-nitro (**4**) derivatives along with some (*E*)-3,4′,5-trihydroxy-2,3′-dinitrostilbene (**5**), four oxidative breakdown products, 4-hydroxybenzaldehyde, 4-hydroxy-3-nitrobenzaldehyde, 3,5-dihydroxyphenylnitromethane, and 3,5-dihydroxybenzaldehyde, two dimers, the resveratrol (*E*)-dehydrodimer **6** and restrytisol B (**7**), and the partially cleaved dimer **2**. The same products were formed in the absence of oxygen. $^1H,^{15}N$ HMBC and LC/MS analysis of the crude mixture obtained by reaction of **1a** with $Na^{15}NO_2$ suggested the presence of 3,4′,5,β-tetrahydroxy-α-nitro-α,β-dihydrostilbene (**8**) as unstable intermediate which escaped isolation. Under similar conditions, the structurally related catecholic stilbene piceatannol ((*E*)-3,3′,4,5′-tetrahydroxystilbene, **1b**) gave, besides (*E*)-3,3′,4,5′-tetrahydroxy-β-nitrostilbene (**3b**), 3,4-dihydroxybenzaldehyde and small amounts of 3,5-dihydroxybenzaldehyde. Mechanistic experiments were consistent with the initial generation of the phenoxyl radical of **1a** at 4′-OH, which may undergo free radical coupling with NO_2 at the α- or 3′-position, to give eventually nitrated derivatives and/or oxidative double bond fission products, or self-coupling, to give dimers. The oxygen-independent, NO_2^- -mediated oxidative fission of the double bond under mild, physiologically relevant conditions is unprecedented in stilbene chemistry and is proposed to involve breakdown of hydroxynitro(so) intermediates of the type **8**.

Introduction

Resveratrol (5-[2-(4-hydroxyphenyl)vinyl]benzene-1,3-diol, **1a**) and its metabolite piceatannol (4-[2-(3,5-dihydroxyphenyl)-vinyl]benzene-1,2-diol, **1b**) are polyphenolic phytoalexins produced by various plants, including grapes, berries, and peanuts, in response to microbial attack.[1] Currently, they are under intensive study for their many potent biological activities, suggesting a potential role in the prevention of coronary heart disease and as cancer chemopreventive agents.[2] Besides acting

* Corresponding author. Phone: +39-081674132. Fax: +39-081674393.
[†] Department of Organic Chemistry and Biochemistry.
[‡] Dipartimento di Scienze del Suolo.

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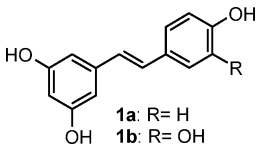
as inhibitors of specific enzymes such as cyclooxygenase,[3,4] **1a** has been shown to be an efficient scavenger of cytotoxic oxygen and nitrogen species.[5−9] Studies of structure−activity relationships indicate that the antioxidant activity of **1a** stems from the peculiar oxygenation pattern on the planar stilbenic skeleton, featuring as a crucial determinant of the radical scavenger activity the 4′-OH group, synergistically supported by the 3- and 5-OH groups on the resorcin moiety. The efficiency of the 4′-OH group as a hydrogen donor is enhanced by the trans double bond, which increases both its acidity[10] and the resonance stabilization energy of the phenoxyl radical derived from H-atom abstraction, as confirmed by semiempirical (PM3)[11] and DFT[12−14] analysis.



Because of the central relevance to the antioxidant activity of **1a** as well as to the process of biotransformation by the plant pathogens into a range of oligomer species,[15−17] the oxidation chemistry of **1a** has been the subject of considerable interest, and several aspects have been clarified.[18−20] Little is known, by contrast, on the reaction of **1a** with reactive nitrogen species derived from nitric oxide (NO),[21] the only relevant paper dealing with the peroxynitrite-induced conversion to oxidation products.[5] This represents a considerable gap in stilbene chemistry considering that NO-derived species are important mediators/contributory factors in the inflammatory response and in carcinogenesis.[22] The major physiologic metabolite of NO is nitrite (NO_2^-), which is present at high levels (30−210 μM) in saliva and is also found in polluted drinking waters, vegetables (e.g. spinaches), fertilizers, and preserved/pickled meats.[23,24] Within the stomach and other acidic compartments supporting nitrous acid (HNO_2) formation,[25] NO_2^- may cause nucleobase deamination and interstrand cross-link formation and production of mutagenic *N*-nitrosamines.[26] Determining the susceptibility of **1a** to react with acidic NO_2^- and the identity of the reaction products is therefore of particular interest to predict the possible transformations and fate of **1a** in the stomach in the presence of high NO_2^- levels. In this connection it is worth noting that **1b** has recently been shown to react efficiently with acidic nitrite via a regioselective nitration at the double bond sector.[27] Studies of the nitr(os)ation chemistry of **1a** are also expected to provide a convenient entry to novel stilbene derivatives of potential synthetic and pharmacological interest, e.g. in the field of cyclooxygenase inhibitors and antiestrogenic compounds.[3,4,28]

This study describes the isolation and structural characterization of the main products formed by acid-promoted reaction of **1a** with NO_2^- under mild conditions, with the view to filling a major gap in the chemistry of this bioactive stilbene and to gaining an improved background for further studies of the biological activity of this phytoalexin. Further interest of this study stems from the potential bioactivity of the oxidation/nitration products of **1a** against phytopathogenic fungi which is currently under scrutiny.

Results and Discussion

Acid-Promoted Reaction of 1a with NO_2^-. Product Characterization. In a first series of experiments **1a** (1×10^{-3} M) was reacted with NO_2^- (5 molar equiv) in 0.1 M phosphate buffer, pH 3.0. Reverse phase HPLC analysis of the reaction mixture after 3 h indicated complete substrate consumption and the presence of a complex pattern of products (Figure 1, plot A), two of which (t_R 29.2, product V, and t_R 37.9 min, product VII) displayed intense UV absorption at 320 nm.

The complexity of the reaction mixture was confirmed by TLC analysis of the ethyl acetate extractable fraction which showed seven bands at R_f 0.36, 0.40, 0.48, 0.55, 0.69, 0.78, and 0.84, two of which (R_f 0.36 and 0.55) exhibited a marked bathochromic shift on exposure to alkali. At lower concentrations of both **1a** (2.5×10^{-5} M) and NO_2^- (8 molar equiv added with stirring over 2 h at 15 min intervals of time), that is, under conditions aimed to model interactions that may occur in the gastric compartment during digestion following continuous elevated nitrite intake, the product pattern was slightly different (Figure 1, plot B). In particular, formation of VII and the product

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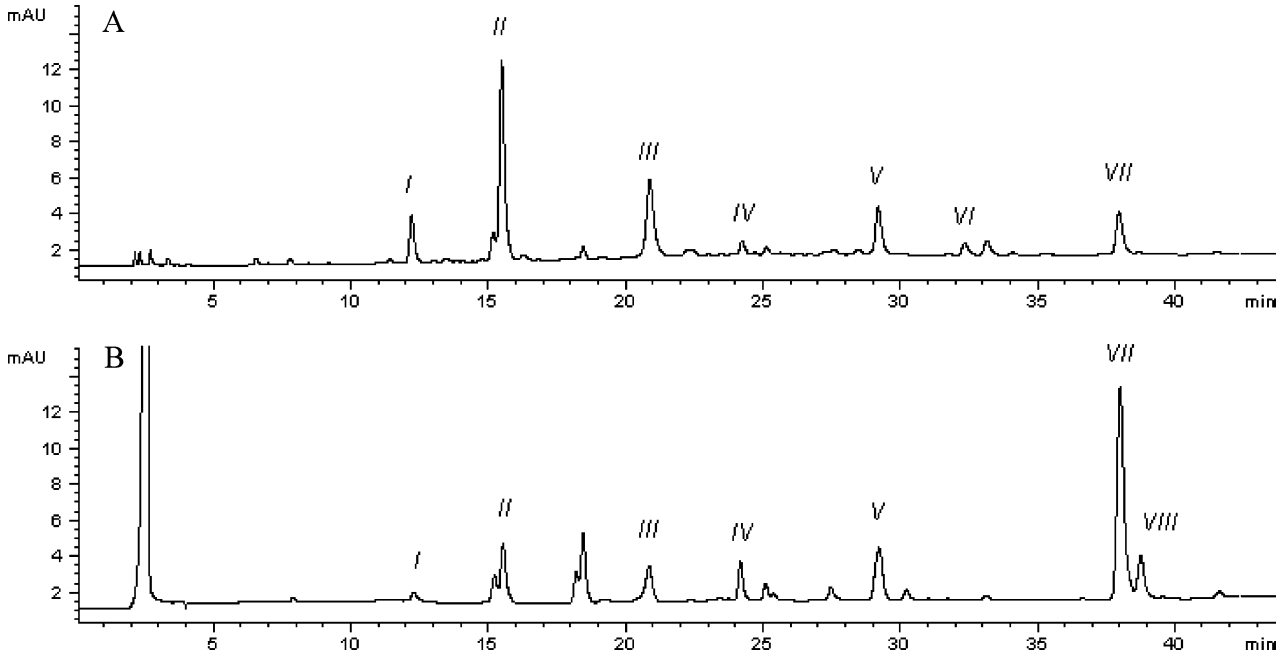
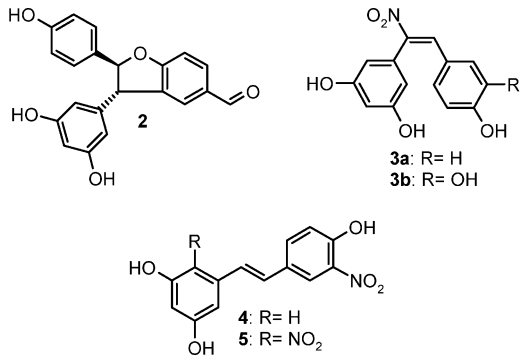


FIGURE 1. HPLC elution profile of the reaction mixture of **1a** with NO_2^- in 0.1 M phosphate buffer, pH 3.0, at 37 °C, at 3 h reaction time at different concentrations: plot A, 1.0×10^{-3} M **1a**, 5.0×10^{-3} M NO_2^-; plot B, 2.5×10^{-5} M **1a**, 2.0×10^{-4} M NO_2^-. Elution conditions: eluant A, detection at 280 nm.

at t_R 24.2 min (IV) was enhanced, while an abatement of those at t_R 12.2 (I), 15.5 (II), 20.9 (III), and 32.3 (VI) min was observed; moreover, novel species, for example that eluted at t_R 38.7 min (VIII), were present. Under such conditions (2.5×10^{-5} M **1a**, 1 mM NO_2^-), a pseudo-first-order rate constant of $(9.9 \pm 0.5) \times 10^{-3}$ s^{-1} for **1a** decay was determined.

For products isolation, the reaction of **1a** (1×10^{-3} M) with NO_2^- (5 molar equiv) was run on a preparative scale and the ethyl acetate extractable fraction was subjected to careful TLC fractionation.

The compound at R_f 0.40 (VI) was identified as the dimer **2**, previously isolated from *Smilax bracteata* rhizomes,[29] while products at R_f 0.69 and 0.84, corresponding to II and III in the elutogram in that order, were identified as 4-hydroxybenzaldehyde (4%) and 4-hydroxy-3-nitrobenzaldehyde (1%), respectively, by comparison with authentic samples.



The product at R_f 0.36 (V) gave a pseudomolecular ion peak $[M - H]^-$ in the ESI-/MS spectrum at *m*/*z* 272, suggesting a nitrated derivative of **1a**. The ^1H NMR spectrum featured the expected resonances for unchanged resorcin and 4-substituted phenol moieties but lacked the pair of doublets for the trans protons on the stilbene double bond. These were replaced by a 1H singlet appearing downfield at δ 8.14, suggesting a strong deshielding effect caused by a spatially close nitro group. A distinct cross-peak in the ^1H, ^{13}C HMBC spectrum between the resorcin proton resonances at δ 6.36 and a deshielded carbon signal at δ 147.7 supported nitration on the α-position of the stilbene system. On this basis, the product was formulated as (*E*)-3,4′,5-trihydroxy-α-nitrostilbene (**3a**). The isolated yield of **3a** was 4%.

The band eluting at R_f 0.48 proved positive to the Griess reagent for nitroso compounds or nitrite-releasing species.[30] On NMR analysis it was shown to consist of an intimate mixture of two products at a 2:1 ratio. The major product was identified as 3,5-dihydroxybenzaldehyde (compound eluting under peak I in the elutogram of Figure 1) by comparison of the spectral features with those of an authentic sample. The signals of the ^1H NMR spectrum pertaining to the minor component included those typical of a resorcin moiety and a 2H singlet at δ 5.48 for a methylene group apparently linked to a nitro group (^{13}C NMR: δ 81.8). Accordingly, the compound was assigned the structure of (3,5-dihydroxyphenyl)nitromethane. LC/ESI+/MS analysis of the R_f 0.48 band showed, in addition to a peak due to 3,5-dihydroxybenzaldehyde (t_R 15.1 min), a peak at t_R 13.9 min displaying a pseudomolecular ion peak $[M + Na]^+$ at *m*/*z* 192.

The product at R_f 0.55 (VII) was evidently an isomer of **3a**, as inferred from the pseudomolecular ion peak $[M - H]^-$ in the ESI-/MS spectrum at *m*/*z* 272. Inspection of the ^1H NMR spectrum revealed the characteristic signals for the trans double bond (2H singlet at δ 7.14 in $(CD_3)_2CO$, appearing as a couple of doublets ($J = 16.4$ Hz) at δ 6.96 and 7.01 in CD_3OD) and the resorcin ring but indicated a substituted phenol ring, as denoted by an ABX spin system (doublet at δ 7.20, double

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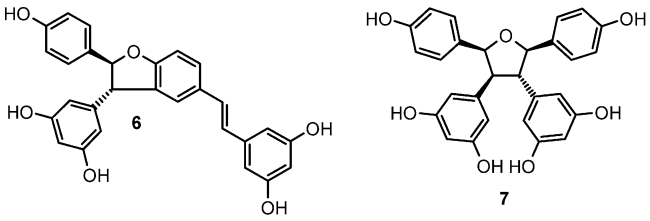
TABLE 1. NMR Spectral Data (ppm) for 3a, 4, and 5 ($(CD_3)_2CO$)

	3a		4		5	
	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C
1		133.9		139.8		136.8
2	6.36 (d, 2.0)	109.3	6.61 (d, 2.0)	106.0		130.5
3		160.1		159.6		156.0
4	6.53 (t, 2.0)	104.6	6.33 (t, 2.0)	103.4	6.53 (d, 2.0)	103.0
5		160.1		159.6		162.0
6	6.36 (d, 2.0)	109.3	6.61 (d, 2.0)	106.0	6.79 (d, 2.0)	107.5
α		147.7	7.14 (s)	130.5	7.42 (d, 16.0)	125.5
β	8.14 (s)	134.8	7.14 (s)	126.4	7.15 (d, 16.0)	130.0
1′		123.3		131.3		129.8
2′	7.21 (d, 8.8)	134.3	8.23 (d, 2.4)	123.3	8.27 (d, 2.0)	123.5
3′	6.79 (d, 8.8)	116.4		135.3		135.0
4′		160.9		154.5		154.0
5′	6.79 (d, 8.8)	116.4	7.20 (d, 8.8)	121.0	7.25 (d, 8.0)	120.5
6′	7.21 (d, 8.8)	134.3	7.97 (dd, 8.8, 2.4)	135.5	8.00 (dd, 8.0, 2.0)	134.8

doublet at δ 7.97, and deshielded doublet at δ 8.23). These data, along with 2D NMR analysis, allowed formulation of the product as (*E*)-3,4′,5-trihydroxy-3′-nitrostilbene (**4**).[31] The product was isolated in 1% yield. Notably, **4** was also obtained in 45% formation yield by reaction of **1a** (3.5×10^{-2} M) with NO_2^- (0.35 M) in acetonitrile containing 2.5% acetic acid. In this conditions no detectable formation of **3a** was observed.

The compound at R_f 0.78 was characterized as a dinitro compound (ESI+/MS: pseudomolecular ion peaks $[M + H]^+$ and $[M + Na]^+$ at *m*/*z* 319 and 341, respectively). The ^{1}H NMR spectrum showed the signals for a trans double bond, a substituted phenol ring (doublet at δ 7.25, double doublet at δ 8.00 and deshielded doublet at δ 8.27), and two doublets (J = 2.0 Hz) at δ 6.53 and 6.79. On this basis the product was formulated as (*E*)-3,4′,5-trihydroxy-2,3′-dinitrostilbene (**5**) (1% yield). NMR data assignments for **3a**, **4**, and **5** are reported in Table 1.

With the attempt to isolate the products IV and VIII, the reaction of **1a** (2.5×10^{-5} M) and NO_2^- (8 molar equiv) was run on preparative scale. TLC fractionation of the ethyl acetate extracts allowed isolation of four main bands at R_f 0.09, 0.36, 0.55, and 0.69. The product at R_f 0.09, corresponding to VIII, was identified as the resveratrol (*E*)-dehydrodimer **6** (4% yield) by comparison with literature data,[17,18] whereas the species at R_f 0.36, 0.55, and 0.69 were identified as **3a** (3%), **4** (5%), and 4-hydroxybenzaldehyde (4%), in that order.



Because of the difficulties to isolate product IV by the above procedure, an alternative approach was pursued, involving column chromatography of the ethyl acetate extract of the reaction mixture on Sephadex LH-20 followed by preparative HPLC. This methodology allowed isolation of the product that could be identified as restrytisol B[15] (**7**) (1%) by NMR analysis and comparison with literature data. The stereochemical features of **7** were deduced from the ^{1}H NMR spectrum: in particular, the splitting patterns of the aliphatic protons at δ 3.40 (t, J = 9.2 Hz), 3.96 (t, J = 9.2 Hz), 5.00 (d, J = 9.6 Hz), and 5.50 (d, J = 8.6 Hz) were in agreement with those reported for a cis−trans−trans configuration[15] as illustrated in structure **7**.

At 3×10^{-6} M concentration, **1a** reacted with NO_2^- (0.2×10^{-3} M, added in eight portions at 15 min intervals) to give mainly the two nitration products **3a** and **4** and 3,5-dihydroxybenzaldehyde and 4-hydroxybenxaldehyde, with little or no detectable dimers formation (HPLC evidence).

Close inspection of the aqueous phase after extraction and workup revealed the presence in all cases of chromatographically ill-defined materials, presumably oligomers and polymers, which could not be identified. Whether other dimers, e.g. restrytisols A and C,[15] are produced in the mixture remains uncertain, although, if present, they would be only minor constituents. In no case, however, could *trans*-ϵ-viniferin[32,33] be detected (HPLC evidence in mixtures spiked with an authentic sample).

Acid-Promoted Reaction of 1b with NO_2^-. Product Characterization. At 2×10^{-5} M concentration, **1b** reacted with NO_2^- (4 molar equiv added in four portions at 30 min intervals of time) in 0.1 M phosphate buffer, pH 3.0, at 37 °C to give the nitro derivative **3b** as the major product.[27] Close inspection of the mixture revealed small amounts of two additional species, of which one was isolated and identified as 3,4-dihydroxybenzaldehyde (19%) while the other was identified as 3,5-dihydroxybenzaldehyde by comparison of chromatographic properties with an authentic sample.

Effects of Oxygen, Oxidant, and pH on Product Stability and Distribution from 1a. No significant change in product distribution was observed when the reaction of 1×10^{-3} M **1a** with NO_2^- was run under an oxygen-depleted atmosphere, care being taken to avoid contact with air prior to workup. Furthermore, the reaction of **1a** with NO_2^- was also run under an $^{18}O_2$ atmosphere and products were analyzed for incorporation of the label. LC/ESI+/MS analysis of the reaction mixture confirmed the expected lack of incorporation of ^{18}O within the main reaction products, including notably the aldehyde derivatives. Taken together, these observations ruled out any significant involvement of O_2 in the nitration, dimerization, and aldehyde-forming pathways.

To gain some insights into the mechanism of dimerization and oxidative cleavage, the behavior of **1a** with one-electron oxidants at acidic pH was investigated. Oxidation of 0.25×10^{-3} M **1a** with 0.25×10^{-3} M CAN in 0.1 M phosphate buffer, pH 3.0, resulted in the formation of **6** and **7** as the main products as well as of 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde in comparable amounts but in much lower yields than in the reaction with NO_2^-. Interestingly, oxidation of **1a** with $K_3Fe(CN)_6$ at pH 7.0 led to the formation of both aldehyde products and the dimer **6** but no detectable **7**, suggesting that the latter reflects a specific acid-mediated oxidation pathway of **1a**.

To establish possible relationships between reaction products, additional experiments were directed to investigate the fate of isolated aldehydes, nitrated derivatives, and dimers on exposure to NO_2^- under the usual reaction conditions. Careful monitoring of the reaction course by HPLC or TLC at various intervals of time showed that 4-hydroxybenzaldehyde, 3,5-dihydroxyben-

(31) For the sake of simplicity, throughout this paper, the same numbering system as for **1a** was adopted for **4**, which assigns numbers 1−6 to the resorcin moiety and numbers 1′−6′ to the phenol moiety.

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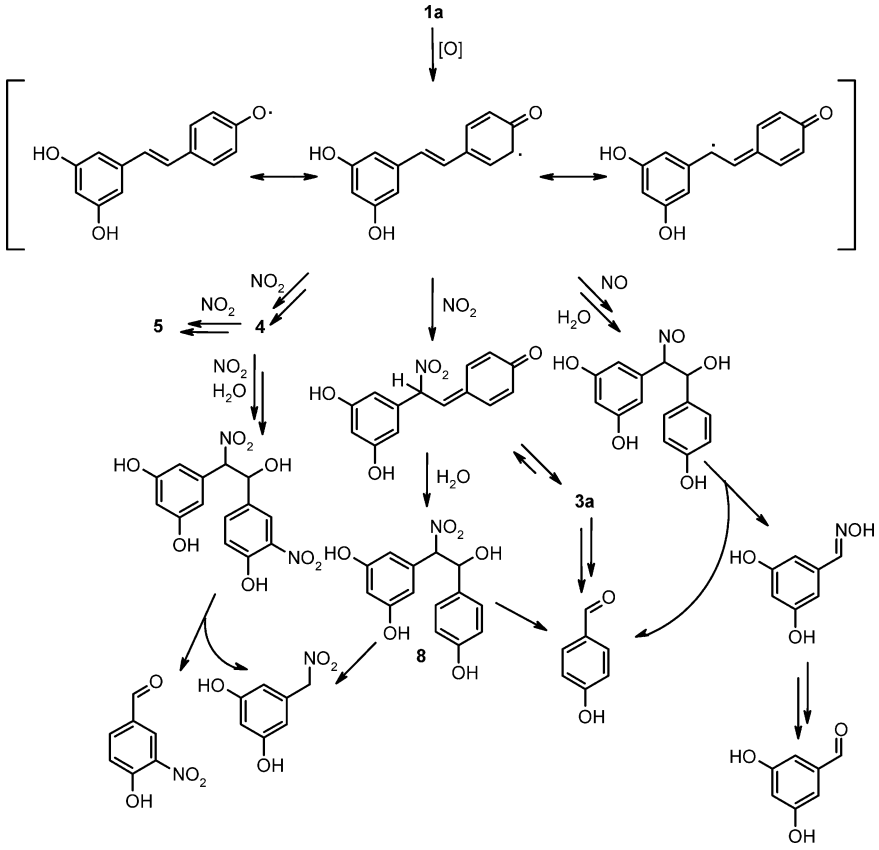
SCHEME 1



zaldehyde, and (3,5-dihydroxyphenyl)nitromethane in the R_f 0.48 band remained unchanged in 0.1 M phosphate buffer, pH 3.0, at 37 °C, with or without added NO_2^-, after 24 h. Conversely, under the typical reaction conditions, **4** smoothly decayed to give 4-hydroxy-3-nitrobenzaldehyde and (3,5-dihydroxyphenyl)-nitromethane, while **3a** gave rise to 4-hydroxybenzaldehyde as the main product. Under the same conditions **6** gave the cleaved dimer **2**.

Mechanistic Issues. The products obtained by exposure of **1a** to acidic NO_2^- suggest competing reaction channels that lead to nitration, dimerization, and cleavage of the stilbene double bond. When NO_2^- is exposed to acids, nitrous acid (HNO_2, pK_a = 3.25)[34] is formed which decomposes according to the following equilibria:

$$NO_2^- + H^+ \rightleftarrows HNO_2$$

$$HNO_2 + H^+ \rightleftarrows NO^+ + H_2O$$

$$NO^+ + NO_2^- \rightleftarrows N_2O_3$$

$$N_2O_3 \rightleftarrows NO + NO_2$$

To distinguish between several possible mechanistic pathways, the *O*,*O*,*O*-trimethyl derivative of **1a**, prepared by a reported procedure,[11] was allowed to react with acidic NO_2^- under the same conditions used for **1a**, and HPLC analysis did not reveal appreciable conversion to products. This observation would suggest that reaction of **1a** with acidic NO_2^- proceeds via an initial oxidative step (probably via H-atom transfer,[10,35] though electron transfer has also been proposed[12]) leading to the delocalized 4′-phenoxyl radical as a common intermediate from which the nitration, dimerization, and aldehyde-forming paths depart. Oxidation of **1a** (E^{ox}_p = +1.14 V vs saturated calomel electrode in CH_3CN)[19] may be brought about by HNO_2 (the reduction potential for the equation $HNO_2 + H^+ + e^- = NO + H_2O$ is +0.996 V at pH = 0)[36] or by the NO_2 produced by decomposition of HNO_2 (the reduction potential for $NO_2 + e^- = NO_2^-$ is 0.99 V).[37]

Formation of nitration products would involve coupling of the phenoxyl radical with NO_2 at the 3′- and α-positions[38,39] (Scheme 1). According to this scheme, double bond nitration to give **3a** follows from a nitro quinone methide intermediate and is in line with the reactivity of the 4′-phenoxyl radical at the α-position predicted by computational studies at the semiempirical[11] and the DFT[12,13] levels. This mechanism is akin to that proposed for nitration of **1b** leading to **3b**[27] and reflects again the dominant role of the 4-OH group in directing the reactivity of phenolic stilbenes toward the double bond. However, at variance with **1a**, **1b** does not undergo significant ring nitration.

Oxygen-independent formation of aldehydes by oxidative fission of the double bond under mild conditions of physiologi-

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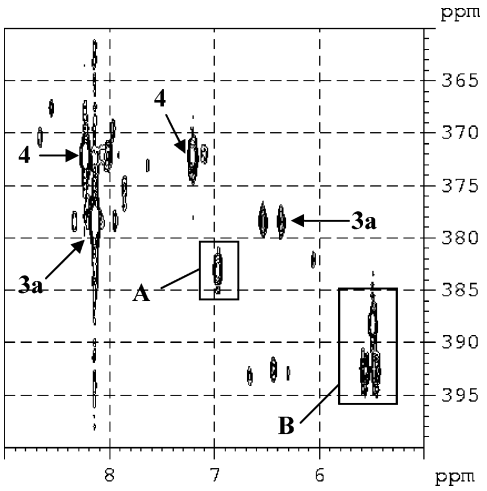

FIGURE 2. $^{1}H,^{15}N$ HMBC spectrum of the ethyl acetate extractable fraction of the reaction mixture of **1a** with $[^{15}N]NaNO_2$ at pH 3.0. Arrows indicate cross-peaks of identified products (see text).

cal relevance has apparently escaped the attention of previous workers in the chemistry on **1a** and related natural stilbene antioxidants. To the best of our knowledge, formation of 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde (as *O*-methyl derivatives) was reported only by harsh ozonolytic splitting of the double bond of the *O*,*O*,*O*-trimethyl derivative of **1a**.[40] It is also noteworthy that aldehyde formation is enhanced in the NO_2^--induced reaction compared to the CAN oxidation at pH 3.0, suggesting a specific NO_2^--dependent mechanism. The identification of (3,5-dihydroxyphenyl)nitromethane among the products argues strongly for an oxidative fission pathway involving nucleophilic attack of water to the nitro quinone methide intermediate in Scheme 1 to give 3,4′,5,*β*-tetrahydroxy-α-nitro-α,*β*-dihydrostilbene (**8**), which would undergo fragmentation to give 4-hydroxybenzaldehyde and (3,5-dihydroxyphenyl)nitromethane. Unfortunately, all attempts to isolate the postulated hydroxynitro derivative **8** proved unsuccessful, due to the apparent instability of this species during chromatographic separation and workup. In an attempt to demonstrate its formation, the reaction was carried out with 2.5×10^{-5} M **1a** and ^{15}N-labeled NO_2^- under the usual reaction conditions. Direct analysis of the crude ethyl acetate extractable fraction by $^{1}H,^{15}N$ HMBC revealed, as expected, two significant series of cross-peaks, correlating the proton resonances of **4** at δ 8.23 (appearing as a triplet ($J = 2.4$ Hz) because of the further splitting by coupling with ^{15}N) and δ 7.20 with a nitrogen signal at δ 373 and the proton signals of **3a** at δ 8.14 (doublet, $J =$ 4.0 Hz) and δ 6.36 with a nitrogen resonance at δ 378 (Figure 2). Another cross-peak correlating a proton signal at δ 6.95 with a nitrogen signal at δ 383 (Figure 2, region A) was considered to be indicative of the presence of the *Z* isomer of **3a**.[41]

In addition, two intense ^{15}N resonances were detectable at δ 388 and 392 (Figure 2, region B), denoting nitro groups linked to sp^3 carbons.[41,42] These resonances (cross-peaks with proton signals in the range δ 5.4−5.6) may be attributed to the nitrogens of (3,5-dihydroxyphenyl)nitromethane and **8** (mixture of diastereoisomers). Consistent with this interpretation, LC/ESI+/MS analysis of two separate mixtures obtained from reaction of **1a** with ^{15}N-labeled and unlabeled NO_2^- indicated in both cases a species eluted at t_R 14.1 min giving a pseudomolecular ion peak $[M + Na]^+$ at *m*/*z* 315 and 314, respectively, confirming the presence of **8** (Figure 3). Label incorporation was observed also in the case of **3a** (t_R 31.8 min) and **4** (t_R 46.5 min), showing pseudomolecular ion peaks $[M + H]^+$ and $[M + Na]^+$ at *m*/*z* 275 and 297, respectively.

Cleavage of a nitrohydroxy derivative akin to **8** from **4** could account for the formation of 4-hydroxy-3-nitrobenzaldehyde.

Formation of 3,5-dihydroxybenzaldehyde is however incompatible with reaction pathways involving **8**, since it does not seem to arise from (3,5-dihydroxyphenyl)nitromethane. A plausible route would be through hydrolysis of an oxime intermediate[43] produced by cleavage of a nitrosohydroxy species akin to **8**, arising by coupling of NO with the 4′-phenoxyl radical of **1a**. To test the proposed route, 3,5-dihydroxybenzaldoxime was prepared by reaction of 3,5-dihydroxybenzaldehyde with NH_2OH in 1.2 M sodium acetate at 80 °C[44] and exposed to NO_2^- under the usual reaction conditions: HPLC analysis of the reaction mixture showed a ca. 50% consumption of the oxime after 2 h, with concomitant formation of 3,5-dihydroxybenzaldehyde. 3,5-Dihydroxybenzaldoxime was also detected in trace amounts by careful HPLC analysis (t_R 11.4 min, eluant A) in the reaction mixture of **1a** (1×10^{-3} M) with NO_2^- (5×10^{-3} M).

Mechanisms similar to those described above can be envisaged for cleavage of **1b**.

Dimerization of **1a** is well documented[18,19,45] and occurs via coupling of the resulting phenoxyl radical. Formation of **7** in small amounts by NO_2^--induced oxidation of **1a** is however noteworthy, since this dimer was previously described only by enzymatic oxidation produced by a fungal grapevine pathogen[15] but was never obtained by chemical oxidation under mild conditions. Present data indicate that one-electron oxidants, like CAN, can induce formation of **7** and that the heterocyclic oxygen derives from H_2O rather than O_2, because of the apparent formation of this dimer under O_2-depleted atmosphere. A possible mechanism is depicted in Scheme 2.

This mechanism is akin to that proposed for the biogenesis of tricuspitadol A, a diastereoisomer of **7** isolated from *Parthenocissus tricuspidata*,[46] and for the formation of related tetrahydrofuran derivatives by chemical oxidation of caffeic acid under acidic conditions.[47] Formation of **7** in acidic but not in neutral medium is in line with previous observations and indicates that H_2O can act as a nucleophile toward the quinone methide only when acidic catalysis is provided.

Cleaved dimer **2** may derive at least in part by NO_2^--induced oxidation of **6**. The likely mechanism would involve free radical addition of NO_2 to the double bond followed by recombination of the *β*-nitroalkyl radical with another molecule of NO_2 to give a nitronitrito adduct,[41,48,49] which would suffer subsequent

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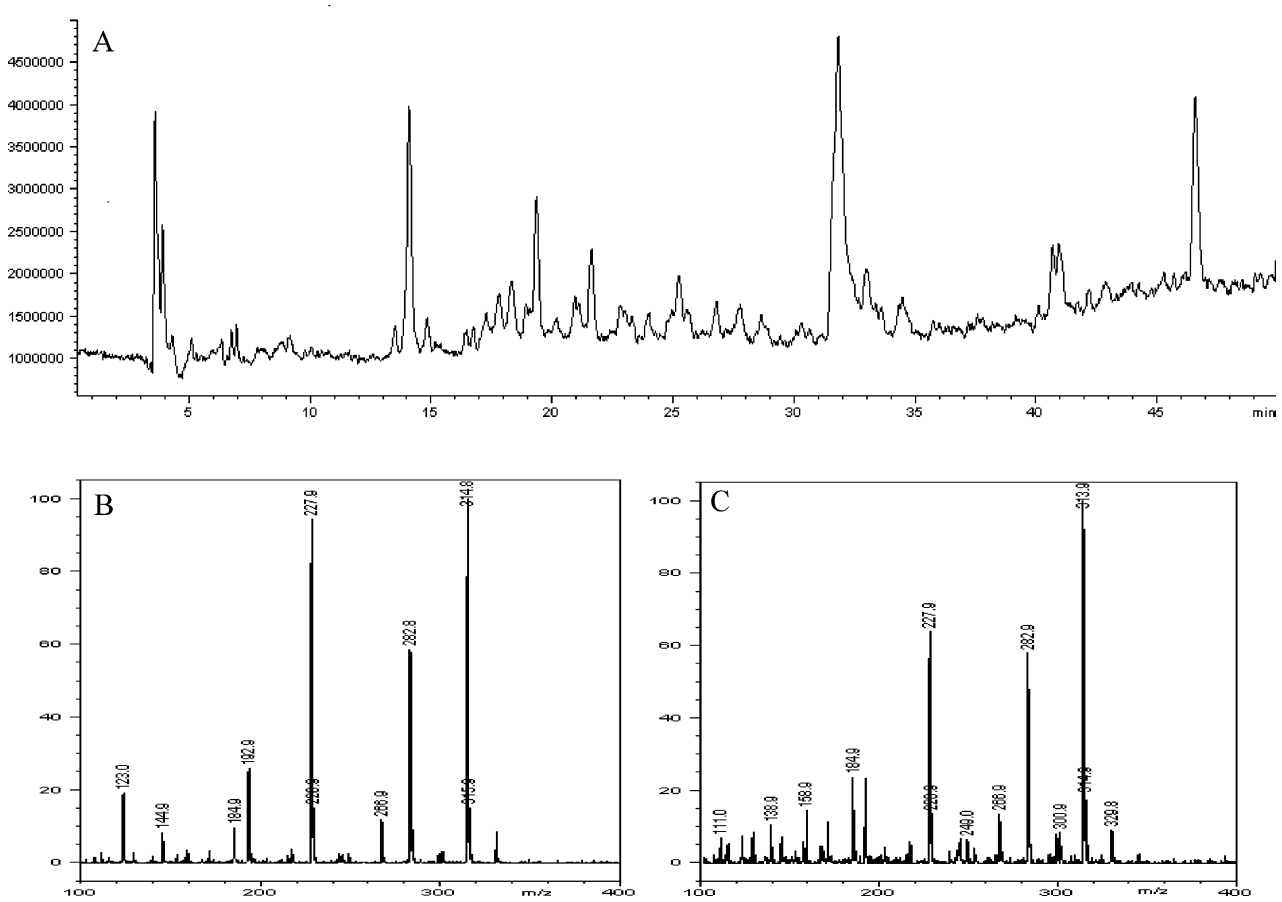

FIGURE 3. (A) LC/ESI+/MS elution profile of the ethyl acetate extractable fraction of the reaction mixture of **1a** with ^{15}N-labeled NO_2^- at pH 3.0. (B) ESI+/MS spectrum of the species eluting at t_R 14.1 min in the ethyl acetate extractable fraction of the reaction mixture of **1a** with ^{15}N-labeled NO_2^-at pH 3.0. (C) ESI+/MS spectrum of the species eluting at t_R 14.1 min in the ethyl acetate extractable fraction of the reaction mixture of **1a** with unlabeled NO_2^- at pH 3.0.

cleavage.[50] This mechanism, which does not require a phenolic oxidation step, would become operative under forcing conditions such as those leading to the formation of **2**.

The reaction pathways illustrated in Schemes 1 and 2 entail that product distribution mirrors the relative concentrations of reacting free radical species in the medium. The incomplete mass balance, due to the presence of other ill-defined species that escaped isolation and characterization, prevents a more detailed mechanistic analysis, so it is possible that other reaction pathways of **1a** are operative. However, the above schemes establish the central role of the 4′-OH group in directing the main reaction pathways of **1a** with acidic NO_2^- toward the double bond, which is a most significant outcome of this study.

In conclusion, the chemistry described in this paper discloses unusual reactions of phenolic stilbene antioxidants with NO_2^- in acidic medium, highlighting the hitherto overlooked susceptibility of **1a**,**b** to oxidative cleavage under mild conditions of physiological relevance, and the formation of **7** under nonenzymatic conditions. Nitration of **1a** is also of chemical interest as it may provide an entry to novel functionalized stilbene derivatives via proper manipulation of **3a** and **4**.

Experimental Section

Preparation of 3,5-Dihydroxybenzaldoxime. 3,5-Dihydroxybenzaldoxime was prepared by a general procedure reported in the literature.[44] Briefly, to 3,5-dihydroxybenzaldehyde (50 mg, 0.36 mmol) dissolved in water (4.2 mL) was added a solution of NH_2-OH·HCl (14 mg, 0.20 mmol) and $CH_3COONa \times 3\ H_2O$ (27 mg, 0.20 mmol) in water (4.8 mL), and the mixture was taken under stirring at 80 °C. After 2 h, the mixture was cooled and extracted with ethyl acetate (3 × 3 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in ethyl acetate and fractionated by preparative TLC to give 3,5-dihydroxybenzaldoxime (R_f 0.43, 30 mg, 54% yield).

3,5-Dihydroxybenzaldoxime: ^{1}H NMR δ 6.38 (1H, t, J = 2.0 Hz), 6.63 (2H, d, J = 2.0 Hz), 7.96 (1H, s); ESI+/MS:*m*/*z* 154 ($[M + H]^+$). 3,5-Dihydroxybenzaldoxime was reacted with $NaNO_2$ under the same conditions as for **1a**, and the reaction mixture was periodically analyzed by HPLC (gradient elution: water, solvent A; acetonitrile, solvent B; from 2 to 30% B, 0−25 min; from 30 to 60% B, 25−70 min; 60% B, 70−75 min, eluant A).

Reaction of 1a with $NaNO_2$. General Procedure. To a solution of **1a** (10 mg, 44 μmol) in methanol (0.5 mL) was added 0.1 M phosphate buffer (pH 3.0) (44 mL) followed by $NaNO_2$ (15 mg, 0.22 mmol), and the mixture was taken under vigorous stirring at room temperature. After 3 h, at complete consumption of the substrate (HPLC analysis, eluant A), the mixture was extracted with ethyl acetate (3 × 30 mL) and the combined organic layers were dried over Na_2SO_4 and taken to dryness. The residue was dissolved in methanol and analyzed by HPLC (eluant A), TLC, and LC/MS. In other experiments, the reaction of **1a** was run (i) as above with

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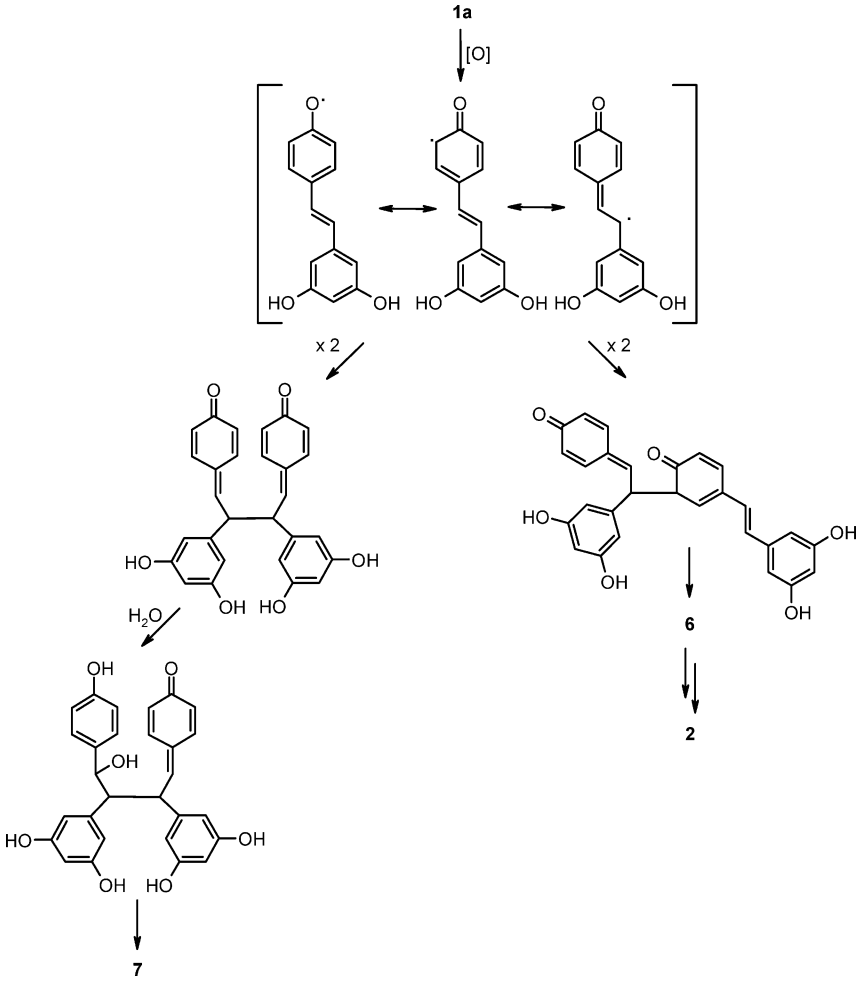
SCHEME 2



1a at 3×10^{-6} or 25×10^{-6} M concentration, with 0.2×10^{-3} M $NaNO_2$ added in eight portions at 15 min intervals, and at 37 °C, (ii) under an argon atmosphere, and (iii) under an $^{18}O_2$ atmosphere. When required, $Na^{15}NO_2$ was used in the reaction of 25×10^{-6} M **1a** and the mixture was worked up as above and directly analyzed by NMR and LC/MS. For kinetic experiments **1a** (2.5×10^{-5} M) was reacted with 1×10^{-3} M $NaNO_2$ added in one portion. In control experiments, the reaction was carried out under the conditions of the general procedure without added $NaNO_2$. Reaction of **1a** (3.5×10^{-2} M) with $NaNO_2$ (0.35 M) was also run in acetonitrile containing 2.5% acetic acid; the reaction course was followed by HPLC (eluant A). Reaction of 3,4′,5-trimethoxystilbene (2.5×10^{-4} M) with $NaNO_2$ (1×10^{-3} M) was carried out at pH 3.0, and the reaction course was followed by HPLC (gradient elution: water, solvent A; acetonitrile, solvent B; from 20 to 80% B, 0−45 min; 80% B, 45−55 min).

Reaction of 1a with CAN. To a solution of **1a** (10 mg, 44 μmol) in methanol (0.5 mL) was added 0.1 M phosphate buffer (pH 3.0) (175 mL) followed by CAN (24 mg, 44 μmol), and the mixture was taken under vigorous stirring. After 1 h, the mixture was extracted with ethyl acetate (3×50 mL) and the combined organic layers were dried over Na_2SO_4 and taken to dryness. The residue was analyzed by HPLC (eluant A).

Reaction of 1a with $K_3Fe(CN)_6$. To a solution of **1a** (10 mg, 44 μmol) in methanol (0.5 mL) was added 0.1 M phosphate buffer (pH 7.0) (175 mL) followed by $K_3Fe(CN)_6$ (14 mg, 44 μmol), and the mixture was taken under stirring. After 1 h, the mixture was acidified with 0.5 M HCl to pH 3 and extracted with ethyl acetate (3×50 mL), and the combined organic layers were dried over Na_2SO_4 and taken to dryness. The residue was analyzed by HPLC (eluant A).

Reaction of 1b with $NaNO_2$. Isolation of (*E*)-3,3′,4,5′-Tetrahydroxy-β-nitrostilbene (3b) and 3,4-Dihydroxybenzaldehyde. The reaction of **1b** with $NaNO_2$ was run as previously described,[27] and the mixture was analyzed by HPLC (gradient elution: 3% TFA, solvent A; acetonitrile, solvent B; from 2 to 30% B, 0−15 min; from 30 to 60% B, 15−45 min; 60% B, 45−55 min, eluant B). For preparative purposes the reaction was carried out using 45 mg of the starting material. After workup of the reaction mixture, the residue (65 mg) was fractionated by preparative HPLC (3% TFA−acetonitrile, 70:30 v/v) to give **3b**[27] (t_R 18.0 min eluant B, 36 mg, 68% yield) and 3,4-dihydroxybenzaldehyde (t_R 11.0 min eluant B, 5 mg, 19% yield).

Isolation of *rac*-(2*R*,3*R*)-3-(3,5-Dihydroxyphenyl)-2-(4-hydroxyphenyl)-2,3-dihydrobenzofuran-5-carbaldehyde (2), (*E*)-3,4′,5-Trihydroxy-α-nitrostilbene (3a), (*E*)-3,4′,5-Trihydroxy-3′-nitrostilbene (4),[31] (*E*)-3,4′,5-Trihydroxy-2,3′-dinitrostilbene (5), 3,5-Dihydroxybenzaldehyde, (3,5-Dihydroxyphenyl)nitromethane, 4-Hydroxybenzaldehyde, and 4-Hydroxy-3-nitrobenzaldehyde. For preparative purposes, the reaction of **1a** with $NaNO_2$ was carried out as in the general procedure using 400 mg of starting material. After workup of the reaction mixture, the residue (380 mg) was fractionated by preparative TLC to give **3a** (R_f 0.36, 18 mg, 4% yield, >95% purity), **2**[29] (R_f 0.40, 10 mg, 2% yield, >90%purity), **4** (R_f 0.55, 5 mg, 1% yield, >98% purity), 4-hydroxybenzaldehyde (R_f 0.69, 8 mg, 4% yield), **5** (R_f 0.78, 4 mg, 1% yield, >90% purity), and 4-hydroxy-3-nitrobenzaldehyde (R_f 0.84, 4 mg, 1% yield). The

fraction (5 mg) eluting at R_f 0.48 was found to consist of 3,5-dihydroxybenzaldehyde and (3,5-dihydroxyphenyl)nitromethane.

3a, **4**, 3,5-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde, or the R_f 0.48 band was exposed to $NaNO_2$ under the standard reaction conditions, and the products formed were analyzed by HPLC (eluant A) and TLC.

3a. UV λ_{max}: CH_3OH, 276, 356 nm; CH_3OH/0.1 M $NaHCO_3$, pH 8, 302, 451 nm. ^1H and ^{13}C NMR: see Table 1. HR ESI-/MS: found *m*/*z* 272.0563 ($[M - H]^-$), calcd for $C_{14}H_{10}NO_5$ *m*/*z* 272.0559.

4. UV λ_{max}: CH_3OH 303, 323, 396 nm; CH_3OH/0.1 M $NaHCO_3$, pH 8, 331, 464 nm. ^1H and ^{13}C NMR: see Table 1. ^1H NMR (CD_3OD): δ 6.20 (1H, t, $J = 2.0$ Hz), 6.49 (2H, d, $J = 2.0$ Hz), 6.96 (1H, d, $J = 16.4$ Hz), 7.01 (1H, d, $J = 16.4$ Hz), 7.13 (1H, d, $J = 8.8$ Hz), 7.83 (1H, dd, $J = 8.8$, 2.0 Hz), 8.14 (d, 1H, $J = 2.0$ Hz). HR ESI-/MS: found *m*/*z* 272.0555 ($[M - H]^-$), calcd for $C_{14}H_{10}NO_5$ *m*/*z* 272.0559.

5. UV λ_{max}: CH_3OH 300, 394 nm; CH_3OH/0.1M $NaHCO_3$, pH 8, 317, 399 nm. ^1H and ^{13}C NMR: see Table 1. HR ESI+/MS: found *m*/*z* 319.0561 ($[M + H]^+$), calcd for $C_{14}H_{11}N_2O_7$ *m*/*z* 319.0566; found *m*/*z* 341.0380 ($[M + Na]^+$), calcd for $C_{14}H_{10}N_2O_7Na$ *m*/*z* 341.0386.

***R*$_f$ 0.48 Band.** ^1H NMR resonances for (3,5-dihydroxyphenyl)nitromethane: δ 5.48 (2H, s), 6.42 (1H, t, $J = 2.0$ Hz), 6.50 (2H, d, $J = 2.0$ Hz). ^{13}C NMR resonances for (3,5-dihydroxyphenyl)nitromethane: δ 81.8 (CH_2), 103.9 (CH), 110.6 (2 × CH), 135.0 (C), 160.8 (2 × C). LC/ESI+/MS: t_R 13.9 min, *m*/*z* 192 ($[M + Na]^+$). HR ESI+MS: found 192.0279 ($[M + Na]^+$), calcd for $C_7H_7NO_4Na$ *m*/*z* 192.0273.

Isolation of *rac*-5-{(2*R*, 3*R*)-2-(4-Hydroxyphenyl)-5-[(1*E*)-2-(3,5-dihydroxyphenyl)vinyl]-2,3-dihydrobenzofuran-3-yl}benzene-1,3-diol (6). For preparative purposes the reaction of **1a** (2.5×10^{-5} M) with $NaNO_2$ (8 molar equiv) was carried out using 50 mg of starting material. After workup of the reaction mixture, the residue (45 mg) was fractionated by preparative TLC to give **6**[17,18] (R_f 0.09, 2 mg, 4% yield, purity >95%), **3a** (2 mg, 3% yield), **4** (3 mg, 5% yield), and 4-hydroxybenzaldehyde (1 mg, 4% yield). **6** was exposed to $NaNO_2$ under the standard reaction conditions, and the products formed were analyzed by HPLC (eluant A) and TLC.

Isolation of *rac*-5,5′-[(2*R*,3*R*,4*R*,5*S*)-2,5-Bis(4-hydroxyphenyl)-tetrahydrofuran-3,4-diyl]bis(benzene-1,3-diol) (7). The reaction of **1a** (2.5×10^{-5} M) with $NaNO_2$ (8 molar equiv) was carried out using 50 mg of the starting material. After workup of the reaction mixture, the residue (45 mg) was fractionated on a Sephadex LH-20 column (50 cm × 2 cm) using 95% ethanol as the eluant. Fractions were collected on the basis of HPLC analysis (eluant A) and further purified by preparative HPLC (water−acetonitrile, 70:30 v/v) to give **7**[15] (t_R 24.2 min eluant A, 1 mg, 1% yield, >95% purity).

Isolation of *trans*-ϵ-Viniferin. Wood from grapewine plants infected with fungi associated with esca (*Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea*) was lyophilized and milled under nitrogen to obtain a fine powder. After treatment with petroleum ether 40−60 to remove lipids, the powder was extracted twice (3 h and overnight) with methanol (1:15 w/v) in the dark with stirring. The extracts were filtered and taken to dryness. The residue was dissolved in methanol and purified by silica gel column chromatography using chloroform−methanol, 80:20 v/v, as the eluant. The fraction containing *trans*-ϵ-viniferin (HPLC analysis: water−acetonitrile gradient from 80:20 to 33:67 in 90 min, t_R 56.9 min) was further fractionated by preparative TLC (chloroform−methanol, 80:20 v/v) to give the pure compound[33] (R_f 0.51).

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Supporting Information Available: General experimental methods, ^1H NMR spectra of compound **2**, **5**, **6**, and **7**, ^1H NMR, ^{13}C NMR (and/or ^{13}C DEPT), ^1H,^{13}C HMQC, and ^1H,^{13}C HMBC spectra of compound **3a** and **4**, and ^1H NMR, ^{13}C NMR, ^1H,^1H COSY, and ^1H,^{13}C HMBC spectra of a reaction mixture of **1a** obtained with $^{15}NO_2^-$. This material is available free of charge via the Internet at http://pubs.acs.org.

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