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Stilbazulenyl Nitrone (STAZN): A Nitronyl-Substituted Hydrocarbon with the Potency of Classical Phenolic Chain-Breaking Antioxidants

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Abstract: Stilbazulenyl nitrone (STAZN), **8**, a nitronyl-substituted hydrocarbon, is a novel second-generation azulenyl nitrone with significantly enhanced potency as a chain-breaking antioxidant vs conventional α-phenyl nitrones previously investigated as antioxidant therapeutics. A convenient ^{1}H NMR-based assay for assessing the potency of chain-breaking antioxidants has shown that STAZN is ca. 300 times more potent in inhibiting the free radical-mediated aerobic peroxidation of cumene than is PBN and the experimental stroke drug NXY-059. Such levels of antioxidant efficacy are unprecedented among archetypal α-phenyl nitrone spin traps. Furthermore, STAZN outperforms such classical phenolic antioxidants as BHT and probucol and rivals the antioxidant potency of Vitamin E in a polar medium comprised of 80% cumene and 20% methanol. The Volodarskii electron-transfer mechanism involving the intermediacy of the STAZN radical cation has been implicated in attempts to ascertain the basis for the increased potency of STAZN over the three α-phenyl nitrones PBN, S-PBN, and NXY-059.

Introduction

A wide body of evidence suggests that free radicals act as pathological agents in a range of human maladies, with much recent attention focusing on their role in senescence,[1] atherosclerosis,[2] and neurodegenerative conditions[3] such as stroke, Alzheimer's disease, ALS, and Parkinson's disease. Stemming from the seminal work of Novelli et al.,[4] nitrone spin traps have been widely tested as antioxidant therapeutics to counter free radical-mediated damage.[5] The vast majority of the nitrones studied in this context are α-phenyl nitrones such as PBN, **1**, and congeners.[6] Most notable in this regard is the 2,4-bissodium sulfonate derivative of PBN known as NXY-059, **2**, which has been slated by AstraZeneca to enter Phase IIb/III clinical trials for the treatment of acute stroke in early 2002.[7] Over the past five years, our research has involved the study of azulenyl nitrones, a novel class of nitrones first prepared in our laboratory.[8,9] In view of the fact that PBN ($E_{1/2}$ = 1.47 V vs SCE),[10] S-PBN (**3**, $E_{1/2}$ = 1.34 V vs SCE),[10] and in all likelihood NXY-059 (estimated $E_{1/2}$ = 1.20 V vs SCE) possess oxidation potentials far higher than important biological chain-breaking antioxidants[11] such as β-carotene ($E_{1/2}$ = 0.76 V vs SCE) and Vitamin E ($E_{1/2}$ = 0.24 V vs SCE), we prepared the azulenyl nitrones AZN (**4**, $E_{1/2}$ = 0.84 V vs SCE)[12] and w-AZN (**5**, $E_{1/2}$ = 0.63 V vs SCE), which have been shown to exhibit enhanced radical scavenging activity both in vitro and in vivo vs that of PBN[8,9] and S-PBN.[13]

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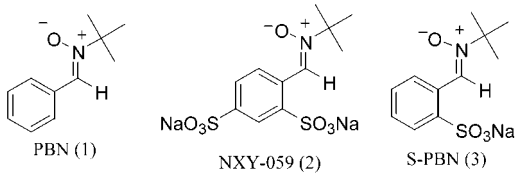
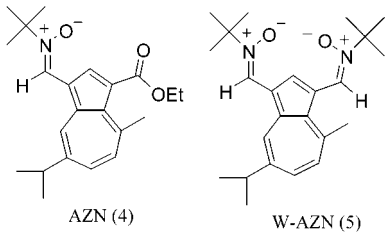
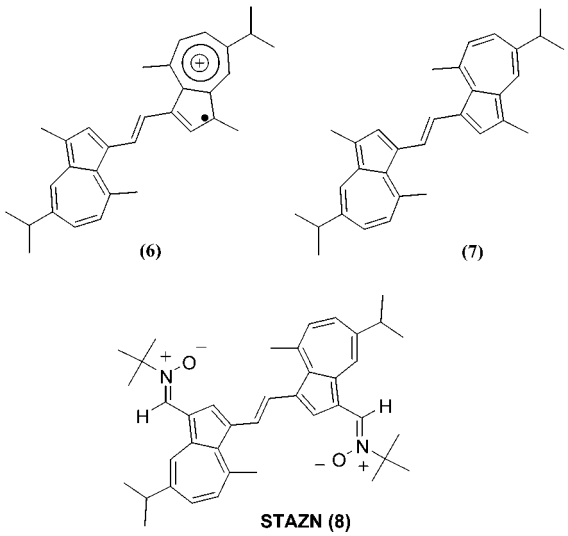




In a 1964 paper, Hunig et al. assigned the correct structure to the perchlorate salt of the remarkably stable hydrocarbon radical cation **6**, which was among other preparative methods, synthesized from azulene **7** by one-electron oxidation.[14] In that report, Hunig and co-workers emphasized the significance of their finding by stating that **6** appeared to be the first known stable radical cation of a hydrocarbon. It is worth noting that **6** (perchlorate salt) was found to have moderate stability in dichloromethane solution. Such stability for a radical cation is rare and, due to our interest in the one-electron oxidation reactions of nitrone antioxidants,[12] attracted our attention. With the expectation that azulenyl nitrones possessing certain structural features in both AZN and **7** would exhibit improved antioxidant behavior over other previously examined nitrones, we set out to expeditiously prepare members of this novel class of compounds. We herein report on the synthesis and extraordinary antioxidant properties of such a second-generation azulenyl nitrone to which we have assigned the acronym STAZN (stilbazulenyl nitrone, **8**). Furthermore, we describe a convenient ^{1}H NMR-based assay for assessing the potency of chain breaking antioxidants.



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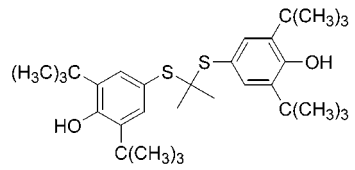
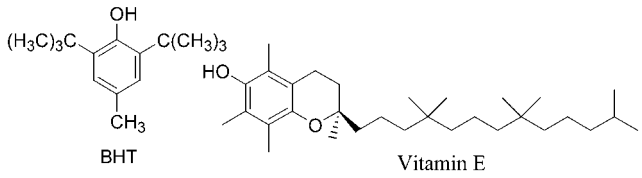
Results

Synthesis of STAZN (8). Our synthetic efforts have resulted in the efficient five-step preparation of STAZN from guaiazulene as illustrated in Scheme 1. Thus, the known red aldehyde **9** was prepared in two steps from guaiazulene according to the method of Kurokawa et al., which involves acylation of guaiazulene with trichloroacetic anhydride (76%) followed by oxidation with DDQ in the presence of water (84%).[15] A one-pot Haloform/Hunsdiecker process consisting of treatment of **9** with barium hydroxide followed by exposure to NBS afforded the bromoaldehyde **10** in 94% yield. Stille coupling of **10** with *E*-bis[(tri-*n*-butyl)stannyl]ethylene[16] in hot toluene provided bisaldehyde **11** (81%), which was converted to STAZN in 75% yield (36% overall from guaiazulene) by reaction with *N*-*tert*-butylhydroxylamine hydrochloride in pyridine at 100 °C.

Results of the Antioxidant Assay. Our assay is based on the well-established oxidation of cumene to cumene hydroperoxide by atmospheric oxygen at 37 °C in the presence of the free radical initiator AIBN.[17,18] The progress of the peroxidation reaction is conveniently followed and quantified by ^{1}H NMR spectroscopy by monitoring the appearance and increasing integration of the singlet arising from the methyl groups of cumene hydroperoxide.

The use of a long, low-power, selective pulse[19] allowed the growth of the cumene hydroperoxide methyl group singlet to be accurately measured in the presence of a huge excess of cumene. This was shown by using known concentrations of commercially available cumene hydroperoxide to calibrate the peak areas. The concentration of internal standard (bis-1,4-(trichloromethyl)benzene) was shown to be invariant under the reaction conditions. No cumene hydroperoxide was detected when the experiment was performed in the absence of AIBN or the absence of oxygen (by freeze−pump−thawing the reaction mixture).





To anticipate the objection that the antioxidant performance of STAZN, with two nitrone groups, cannot fairly be compared to other mononitrones, the concentration of the mononitrones studied was double or more that of STAZN. At concentrations needed to see antioxidant behavior from these mononitrones in the 80% cumene/20% benzene-d_6 solvent system (10 μM or

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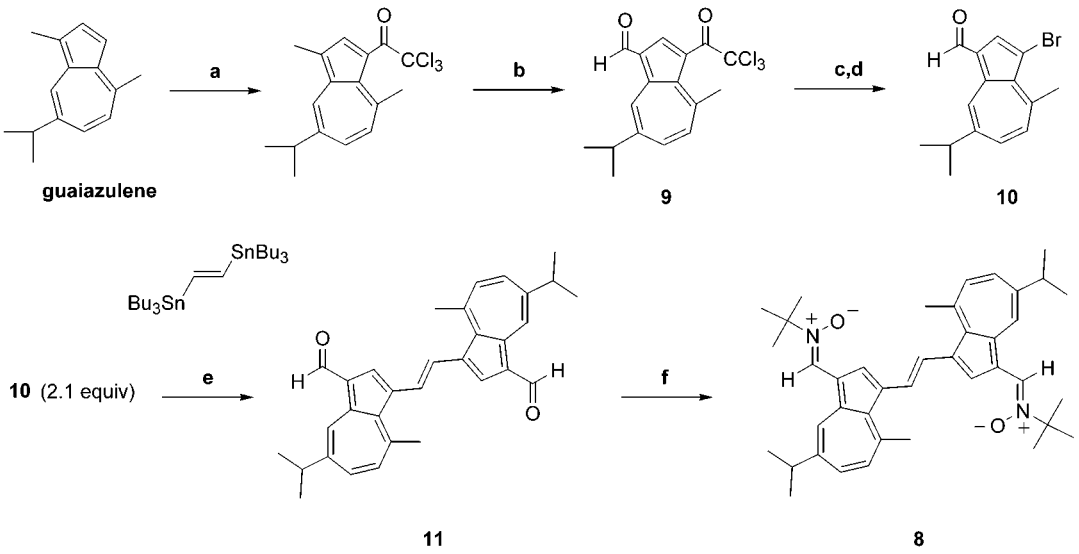
Scheme 1 [a]



[a] Conditions: (a) 4 equiv of TCAA, CH_2Cl_2; (b) 3 equiv of DDQ, acetone/water (9:1 v/v); (c) $Ba(OH)_2$, acetone/water (9:1 v/v), 50 °C; (d) 1.5 equiv of NBS, acetone/hexane (e) $Pd(PPh_3)_4$ (5 mol %), toluene 105 °C; (f) 4 equiv of *N-tert*-butyl hydroxylamine HCl, 4 equiv of $MgSO_4$, pyridine, 100 °C.

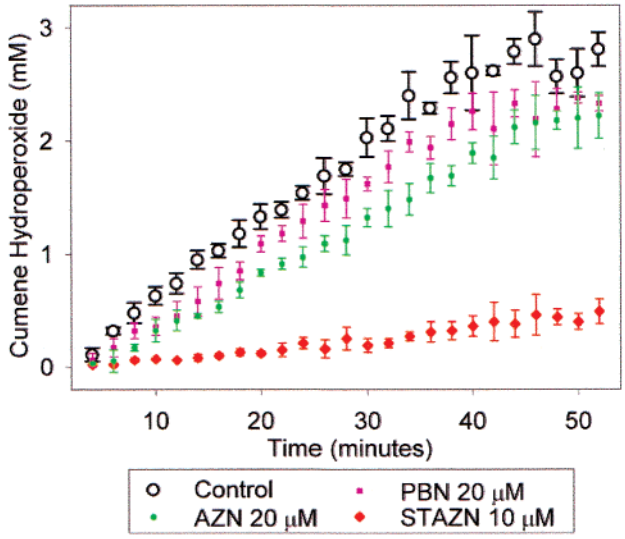


Figure 1. Plot of cumene hydroperoxide vs time for 80% cumene 20% benzene-d_6 PBN (*N-tert*-butylphenyl nitrone); AZN (azulenyl nitrone).

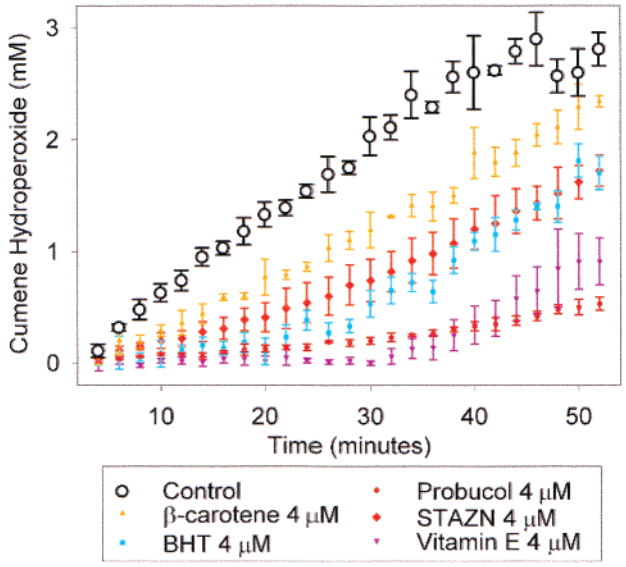


Figure 2. Plot of cumene hydroperoxide vs time for 80% cumene 20% benzene d_6 BHT (butylated hydroxytoluene).

greater), some of the conventional antioxidants (e.g. BHT, probucol, and Vitamin E) abolished all oxidation and STAZN nearly did so. Therefore, the performance of STAZN was compared to that of the conventional antioxidants at concentrations less than 10 μM (in both of the investigated solvent systems).

Figures1−4 reveal that STAZN does indeed possess remarkable potency as a nitrone-based chain-breaking antioxidant. Inspection of Figure 1 shows that STAZN markedly outperforms PBN and AZN—even at half the concentration of these two mononitrones. According to the data in Figure 1, the antioxidant potency of these three nitrones in decreasing order is STAZN, AZN, and last PBN. The modest outperformance of PBN by AZN is in line with previous experiments.[8] It is clear that STAZN displays a far superior antioxidant profile when compared to either PBN or AZN.

From the experiments performed in 80:20 cumene/benzene-d_6 displayed In Figure 2, it is evident that while STAZN is outperformed by probucol and Vitamin E, it (STAZN) surpasses the antioxidant efficacy of β-carotene and rivals that of BHT. To assess the antioxidant potency of STAZN vs the polar nitrones S-PBN and NXY-059, the assay was conducted with methanol-d_3 as cosolvent instead of benzene-d_6. The results, shown in Figure 3, clearly indicate that STAZN is substantially more effective in suppressing the free radical-driven peroxidation of cumene than is PBN, S-PBN, NXY-059, or AZN. Indeed, even at 100 times the concentration of STAZN, NXY-059 is still significantly outperformed by STAZN. We find that a 3 μM solution of STAZN rivals the performance of a 1000 μM solution of NXY-059, thus demonstrating that STAZN is more than 300 times as potent as is NXY-059 in this assay (data not shown). That STAZN is between 2 and 3 orders of magnitude more efficacious in this regard than NXY-059 is an important validation of our expectations and bodes well for the potential utilization of this new class of azulenyl nitrones as antioxidant therapeutics.

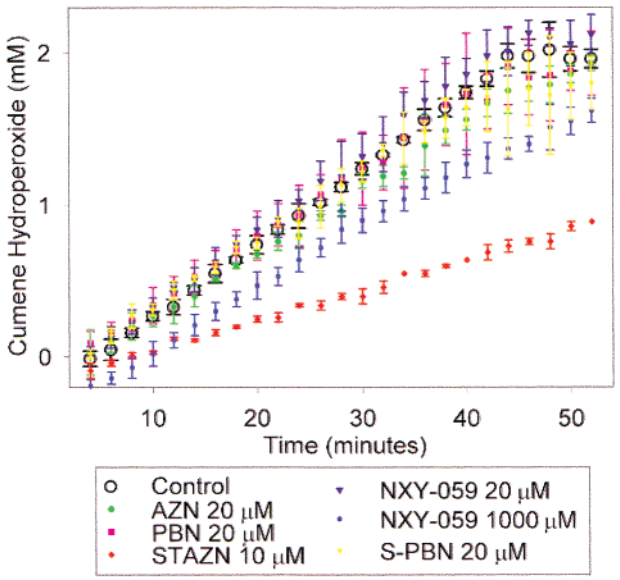


Figure 3. Plot of cumene hydroperoxide vs time for 80% cumene 20% methanol d_3, S-PBN (*N*-*tert*-butyl(2-sulfophenyl)nitrone, sodium salt); NXY-059 = (N-*tert*-butyl(2,4-disulfophenyl)nitrone, disodium salt).

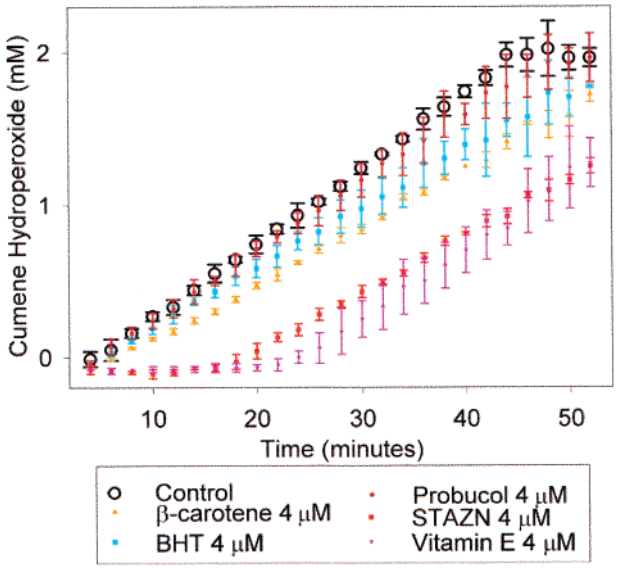
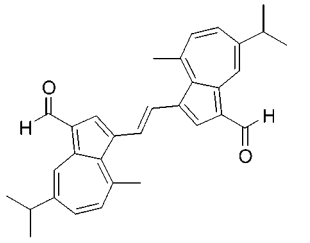


Figure 4. Plot of cumene hydroperoxide vs time for 80% cumene 20% methanol d_3.

Interestingly, as shown in Figure 4, a dramatic solvent effect is observed (vide infra) when the antioxidant potency of STAZN is compared to that of the conventional antioxidants that were employed in the experiments that generated the data in Figure 2. Thus, in the more polar medium containing methanol in place of benzene, STAZN exhibits superior antioxidant behavior vs that observed for β-carotene, BHT, and probucol. Furthermore, while STAZN was outperformed by Vitamin E in the less polar medium, it rivals the efficacy of Vitamin E in the more polar solution. The ability of STAZN—a nitrone bearing nothing more than a hydrocarbon core—to closely mirror the antioxidant potency of Vitamin E is especially noteworthy.

Peroxyl Radical-Induced Formation of Aldehyde 12 from STAZN. Experiments were performed to determine the fate of STAZN under the reaction conditions by isolating products from the reaction of the nitrone with free radicals. The exposure of STAZN to 10 equiv of AIBN in air at 37 °C yields aldehyde **12** as the major product of the reaction of STAZN with peroxyl radicals in the nonpolar solvent system (80% cumene/20% benzene-d_6). Several unidentified trace products were also observed.



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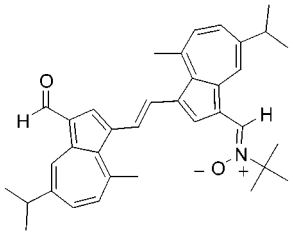

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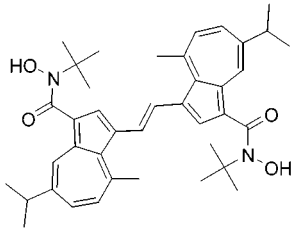
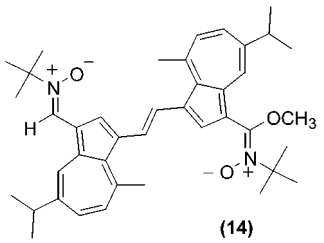


(13)

Peroxyl Radical- and Aminium Salt-Induced Formation of Hydroxamic Acid 13 from STAZN. In AIBN-initiated reactions of STAZN with peroxyl radicals in the more polar medium (80% cumene/20% methanol-d_3), a product was isolated and identified as bis-hydroxamic acid **13**. A small amount of aldehyde **12** in addition to a number of unidentified trace products were also formed. From the reaction of STAZN with $TBPA^{\bullet+}$ (tris(4-bromophenyl)aminium hexachloroantiminate) in cumene with subsequent exposure to methanol at 37 °C a product was isolated by chromatography that was identical to **13** as determined by TLC, HRMS, and ^1H and ^{13}C NMR. A low-resolution mass peak at $MH^+ = 607$ was also observed, which we attribute to the monohydroxamic acid derivative of STAZN.

Isolation of the Intermediary Methoxynitrone 14. The unstable alkoxynitrone **14** could be isolated from reaction mixtures in which STAZN was aerobically exposed to AIBN in 80% cumene/20% methanol at 37 °C or by reacting STAZN with $TBPA^{\bullet+}$ in cumene followed by addition of methanol at 37 °C.



(14)

Double and Quadruple Spin Adducts. To show that STAZN does indeed trap radicals, an experiment was performed to sequester carbon-centered radicals in an anaerobic reaction. Treatment of STAZN with an excess of AIBN in benzene under argon at 60 °C for 4 h gave rise to the double spin adduct **15** and quadruple spin adduct **16**. These unstable compounds were

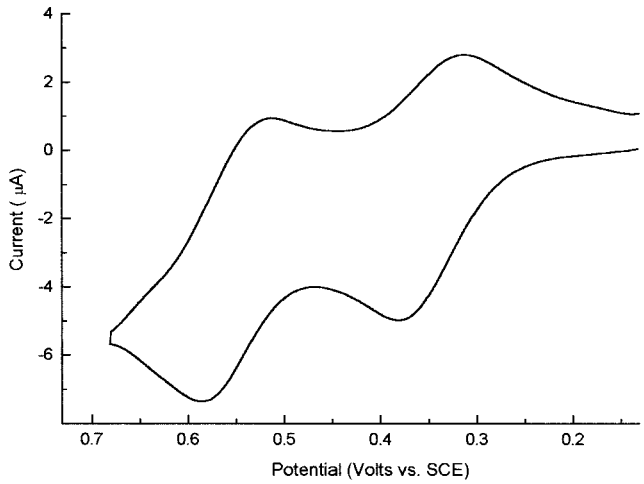
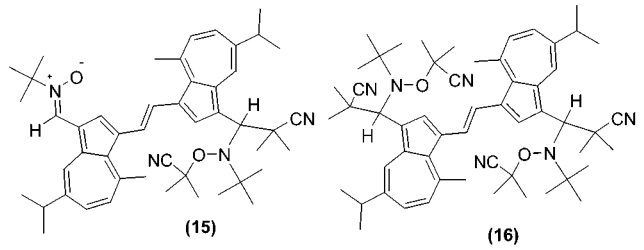


Figure 5. Cyclic voltammogram of a 1 mM solution of STAZN in CH_2Cl_2 at a scan rate of 100 mV s^{-1}.

formed by the addition of two and four cyanoisopropyl radicals respectively and were identified by HRMS.



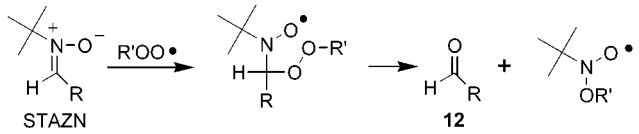
Mechanistic Studies Employing ^{18}O-Labeled Methanol. To shed light on the mechanism of formation of bis-hydroxamic acid **13** ($MH^+ = 623$), experiments were conducted with ^{18}O-methanol in place of ^{16}O-methanol. Mass spectral analysis clearly shows a new mass peak increased by four mass units ($MH^+ = 627$) that is consistent with incorporation of two ^{18}O atoms.

Cyclic Voltammetry of STAZN. As expected, the oxidation potential of STAZN is exceptionally low vs conventional nitrones previously studied as antioxidant therapeutics. As can be seen from Figure 5, the first oxidation is reversible, with an $E_{1/2}$ of 0.33 V vs SCE and a peak-to-peak separation of 64 mV. The second oxidation is quasireversible and occurs at 0.54 V vs SCE with a peak-to-peak separation of 73 mV.

Discussion

While there is no clear consensus as of yet, several mechanisms have been put forth to account for the observed biological activity of nitrones.[5a,20] Much of the work in this area is predicated on the assumption that the spin trapping of harmful endogenous free radicals by nitrones would render nitrones effective antioxidants via their known propensity to generate stable nitroxide spin adducts devoid of the capacity to engage in chain propagation. Recent work, however, has cast doubt on

Scheme 2



the ability of archetypal nitrones such as PBN to function as classical chain-breaking antioxidants.[21] In this regard, Ames and co-workers have now attributed the in vivo antioxidant action of PBN to *N-tert*-butylhydroxylamine, a product of hydrolysis of PBN with roughly 20 times the potency of PBN in delaying the senescence of human diploid fibroblasts.[22] As can be seen from the data in Figures 2 and 4 (NMR assay), we have for the first time established that a nitronyl-substituted hydrocarbon (STAZN) can indeed perform as well as or better than classical phenolic chain-breaking antioxidants in a chemical system and might also do so in biological systems.

As previously mentioned, aldehyde **12** is produced in reactions of STAZN with peroxyl radicals in 80% cumene/20% benzene-d_6. Formation of aldehydes by rearrangement of peroxyl radical adducts of nitrones as shown in Scheme 2 has been previously described.[8,23] Aldehyde **12** does not form in the absence of AIBN. In the preparation of STAZN (by reaction of **11** with *N-tert*-butylhydroxylamine hydrochloride) a small amount of **12** is produced, which was found to be identical to the aldehyde **12** produced by the aforementioned reaction of STAZN with peroxyl radicals.

Scheme 3 shows our proposed mechanism for the peroxyl radical-mediated conversion of STAZN to bis-hydroxamic acid **13**. Thus, transfer of an electron from the electron-rich STAZN to a peroxyl radical generates the STAZN radical cation, which then suffers nucleophilic attack by methanol to provide an intermediary nitroxide via inverted spin trapping.[24] Oxidation of the nitroxide gives rise to a nitrosonium species that, upon loss of a proton, produces methoxynitrone **14**. Although unstable (as is the case for such alkoxynitrones),[25] **14** was isolated from the reaction mixture. An even more facile one-electron oxidation[26] converts **14** to its corresponding radical cation, which is then cleaved by methanol to generate an acyl nitroxide. The hydroxamic acid moiety is then formed upon reaction of the acyl nitroxide with methanol.[27] Iteration of the entire process with the second nitrone substituent leads to bis-hydroxamic acid **13**.

The literature contains ample precedent for the mechanism delineated in Scheme 3. Thus, extensive studies by Volodarskii's group have established that oxidation of nitrones in methanol can give rise to hydroxamic acids in a process termed "oxidative alkoxylation" that proceeds via the intermediacy of alkoxynitrones.[28] Furthermore, the accepted Volodarskii mechanism for

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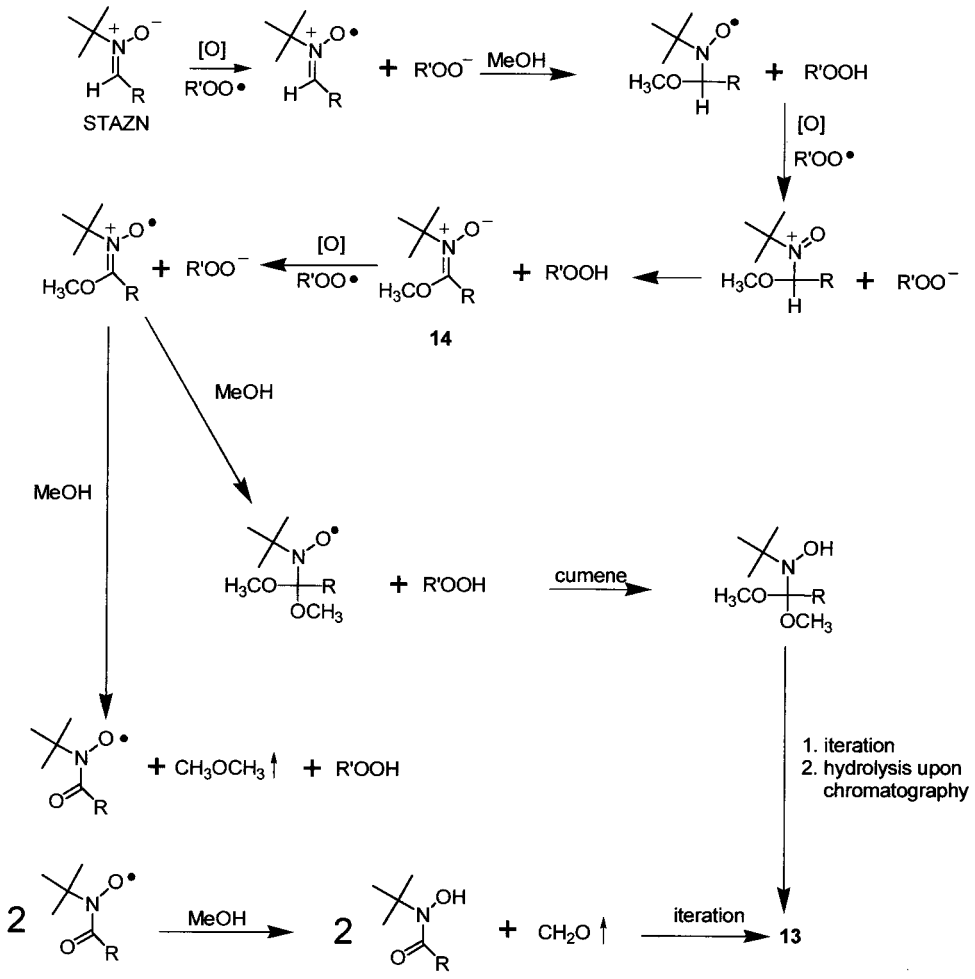
Scheme 3



the conversion of nitrones with low oxidation potentials (such as STAZN and alkoxynitrones such as **14**[26]) to hydroxamic acids is via the corresponding radical cations of such species. [28] The mechanism in Scheme 3 from the point of the intermediary methoxynitroxide onward is identical with that of Volodarskii's, with the exception that, in the present case, the oxidizing agent is a peroxyl radical. That the highly oxidizable STAZN is converted to methoxynitrone **14** via its (STAZN's) radical cation finds support in electrochemical studies by Volodarskii et al. which show that the oxidative alkoxylation pathway to alkoxynitrones does indeed occur starting from the nitrone radical cations themselves.[29] Moreover, bis-hydroxamic acid **13** is formed from STAZN by exposure to $TBPA^{\bullet+}$ followed by the addition of methanol. One-electron oxidation of nitrones to their radical cations by $TBPA^{\bullet+}$ is well documented.[24c] The results of experiments employing ^{18}O-labeled methanol are also in line with the Volodarskii mechanism of Scheme 3 in that the expected incorporation of two ^{18}O atoms is indeed observed by mass spectroscopic analysis. That the observed incorporation of the label is only ca. 30% can be rationalized by the alternative pathway for the formation of bis-hydroxamic acid **13** shown in Scheme 3. Thus, addition of methanol to the intermediary methoxynitrone radical cation gives rise to a dimethoxynitroxide that upon hydrogen atom abstraction from cumene produces thecorresponding hydroxamic acid dimethylacetal. Iteration followed by hydrolysis with unlabeled water during chromatography produces bis-hydroxamic acid **13** devoid of the isotopic label.

It is important to note that the mechanism in Scheme 3 also accounts for termination of the radical chain by the conversion of cumene peroxyl radical to cumene hydroperoxide. Isotopic labeling studies have shown that transfer of a proton from the solvent to the incipient hydroperoxide anion has precedent in the reaction of peroxyl radicals with a variety of organic reducing agents without transferable H atoms, and that the electron transfer and solvent proton transfer may be concerted.[30] In solvents with increased polarity and proton-donating ability, reactions of peroxyl radicals with organic reducing agents will increasingly involve an electron-transfer mechanism.[30] In the reaction of STAZN with AIBN, the bis-hydroxamic acid **13** is not observed in the nonpolar solvent system (80% cumene/20% benzene). This solvent system is neither expected to stabilize charged transition states nor readily donate a proton. The reduction potential of cumene peroxyl radical has, to our knowledge, never been measured but an estimation can be made based on the calculated reduction potential of the benzyl peroxyl radical (0.538 V vs SCE).[31] Therefore only compounds with

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sufficiently low oxidation potentials will be oxidized by the cumene peroxyl radical and enter into the reaction shown in Scheme 3. The low oxidation potential of STAZN ($E_{1/2} = 0.33$ V vs SCE) as measured by cyclic voltammetry (Figure 5) and high stability of the STAZN radical cation, as indicated by the voltammogram and based on analogy with compound **6** studied by Hunig,[14] should increase the rate of reaction with cumene peroxyl radicals, thereby breaking the radical chain and promoting more effective antioxidant activity in the more polar medium.

An additional factor to consider when comparing the relative efficacy of STAZN to the classical phenolic antioxidants is that the antioxidant activity of such phenols is known to be impaired by protic solvents. This has in fact been the subject of considerable research. The consensus seems to be that although the aforementioned argument holds that solvents with high dielectric constants are likely to facilitate electron transfer from phenol to peroxyl radical, hydrogen bonding between the phenol (hydrogen bond donor HBD) and the solvent (hydrogen bond acceptor HBA) inhibits the hydrogen atom transfer reaction with peroxyl radicals.[32] STAZN is clearly not susceptible to this type of inhibition.

As alluded to above, an examination of the oxidation potentials of STAZN vs those of PBN and S-PBN can also provide perspective in attempts to understand the basis for the improved antioxidant behavior of STAZN compared to that of the three α-phenyl nitrones PBN, S-PBN, and NXY-059 as observed in our NMR assay. Thus, with the oxidation potential of STAZN more than a full volt lower than that of PBN and S-PBN, it is perhaps not surprising to find that STAZN conspicuously outperforms the previously discussed α-phenyl nitrones in antioxidant function. Indeed, one is hard pressed to identify any efficient chain-breaking antioxidants (biological or otherwise) possessing oxidation potentials as high as those of the aforementioned α-phenyl nitrones. A known correlation between decreased oxidation potential and increased antioxidant activity has been elegantly put forth by Buettner in what is termed "The Pecking Order of Free Radicals and Antioxidants".[11] With the oxidation potential of STAZN within a tenth of a volt of that of Vitamin E, it is indeed reasonable that these two structurally diverse molecules can display similar activity in retarding free radical-induced lipid peroxidation—despite their differing antioxidant mechanisms. A number of reports in the recent literature demonstrate that, in apparent accord with their close oxidation potentials, there is little difference in the antioxidant efficacy of PBN and S-PBN.[33,34,35] By extrapolation of this trend observed for PBN vs S-PBN to NXY-059, it is unlikely that NXY-059, with its additional sodium sulfonate moiety, will exhibit drastically different antioxidant potency from that observed for PBN and S-PBN. This is borne out in the data in Figure 3, where there is no discernible difference in the antioxidant potency of NXY-059 vs PBN. In stark contrast, we find that to fully abrogate the oxidation of cumene (in the nonpolar solvent system), it takes a concentration of 20 μM STAZN vs 6000 μM PBN to do so. Thus, as with NXY-059, it appears that STAZN is approximately 300 times more potent than PBN in our antioxidant assay. Another factor that is important to mention is that the hydrophilic nature of NXY-059 precludes its penetration of the blood brain barrier,[7] whereas the high lipophilicity of STAZN and congeners should serve to render these new azulenyl nitrones readily brain permeable.

Conclusion

It is clear from the preceding data that the azulene derivative STAZN constitutes a novel breed of nitrone-based free radical scavengers with sharply increased antioxidant activity in our ^{1}H NMR assay over the standard α-phenyl nitrone derivatives (PBN, S-PBN, NXY-059) which have been and continue to be widely studied. The stability of radical cation **6** inspired the synthesis of STAZN. Experimental evidence suggests that this rationale has led to a compound (STAZN) that can trap free radicals via a mechanism involving one-electron oxidation, and that STAZN can rival Vitamin E in its ability to inhibit peroxidation. What is not yet clear is to what extent STAZN's antioxidant activity as seen in our NMR assay will translate to model systems more biomimetic in character and to experiments performed in vivo. In biological systems, in contrast to Vitamin E concentration, the concentration of STAZN is not expected to be regulated by specific carriers such as tocopherol binding proteins.[36] It is also interesting to note that hydroxamic acids themselves can inhibit lipid peroxidation by donating hydrogen atoms to oxidizing radicals or by chelating Fenton-active metals such as iron as do the hydroxamate-containing siderophore natural products (e.g. deferoxamine).[37] Thus, compound **13**, if formed in vivo, may act downstream of STAZN to inhibit oxidative stress as well. Biological testing of STAZN in a number of animal models of neurodegeneration is underway and the results of such studies will be reported in due course.

Experimemtal

See Supporting Information.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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