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Comparison of free-radical inhibiting antioxidant properties of carvedilol and its phenolic metabolites†‡

Thomas C. Malig, Mitchell R. Ashkin, Austin L. Burman, Manuel Barday,
Belinda J. M. Heyne and Thomas G. Back*

Carvedilol is a widely prescribed drug for the treatment of heart failure and the prevention of associated ventricular arrhythmias. It has also been reported to function as a biological antioxidant *via* hydrogen atom transfer from its carbazole N–H moiety to chain-propagating radicals. Metabolites of the drug include phenolic derivatives, such as 3-hydroxy-, 4'-hydroxy- and 5'-hydroxycarvedilol, which are also potential antioxidants. A comparison of the radical-inhibiting activities of the parent drug and the three metabolites was carried out in two separate assays. In the first, hydrogen atom transfer from these four compounds to the stable radical DPPH was measured by the decrease in the UV-visible absorption at 515 nm of the latter. The known radical inhibitors BHT, 4-hydroxycarbazole and α -tocopherol were employed as benchmarks in parallel experiments. In the second assay, inhibition of the photoinduced free-radical 1,2-addition of Se-phenyl *p*-tolueneselenosulfonate to cyclopropylacetylene, along with competing ring-opening of the cyclopropane ring, was monitored by ^1H NMR spectroscopy in the presence of the carvedilol-based and benchmark antioxidants. In both assays, carvedilol displayed negligible antioxidant activity, while the three metabolites all proved superior radical inhibitors to BHT, with radical-quenching abilities in the order 3-hydroxy- > 5'-hydroxy > 4'-hydroxycarvedilol. Among the metabolites, 3-hydroxycarvedilol displayed even stronger activity in both assays than α -tocopherol, the best of the benchmark antioxidants. These results suggest that the radical-inhibiting antioxidant properties that have been attributed to carvedilol are largely or exclusively due to its metabolites and not to the parent drug itself.

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Introduction

Heart failure is a debilitating chronic disease and a leading cause of death, particularly in developed countries. For example, this disease affects *ca.* six million people in the United States, with 550 000 new cases diagnosed each year¹ and associated health expenditures estimated at over \$30 billion in 2012.² Furthermore, heart failure patients are vulnerable to ventricular arrhythmias (VAs) that can result in sudden death. While several therapies exist, treatment with beta-blockers is among the most common and carvedilol (1), a potent nonselective beta-blocker, is often the drug of choice.³ Furthermore, it was recently discovered that carvedilol is unique among the beta-blocker class of drugs in its ability to suppress store overload induced calcium release (SOICR) in cardiac myocytes through its ability to bind to the ryanodine re-

ceptor (RyR2).⁴ A defective RyR2 ion channel can result in irregular gating and release of abnormal Ca^{2+} waves from the sarcoplasmic reticulum into the cytosol of cardiomyocytes, ultimately triggering VAs. This finding led in turn to the design of several novel carvedilol analogues that retain the SOICR-suppressing activity of the parent compound while reducing beta-blockade by *ca.* 1000-fold.^{4,5} While carvedilol is generally administered as its racemate, it was also found that the (*R*)-enantiomer of carvedilol retains the SOICR-inhibiting properties, along with considerably diminished binding to the beta-adrenoreceptor.⁶ Since carvedilol is *ca.* nanomolar in its beta-blocking ability, but micromolar in its effect on SOICR, side effects stemming from excessive adrenoreceptor inhibition (*e.g.* bradycardia, hypotension), associated with the relatively high doses required for SOICR suppression by racemic carvedilol, can be significantly decreased by administration of the (*R*)-enantiomer, while the desired SOICR attenuation remains intact. These observations raise the possibility of new therapies for heart failure and associated VAs, based on the use of more selective carvedilol analogues.

Moreover, the heart and cardiovascular system in general are particularly prone to oxidative stress. A third beneficial

Department of Chemistry, University of Calgary, 2500 University Drive N.W.,
Calgary, Alberta, T2N 1N4, Canada. E-mail: tgback@ucalgary.ca

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function of carvedilol, in addition to beta-blockade and SOICR suppression, is its potential antioxidant activity. For example, one investigation of ferrous ion-mediated lipid peroxidation indicated that carvedilol serves as an antioxidant and free-radical trap,⁷ while a subsequent study concluded that the antioxidant activity of carvedilol was the result of its ability to sequester ferric ions and not of its radical scavenging behaviour.⁸ It has also been reported that carvedilol protects cardiomyocytes from free-radical damage and prevents leakage of calcium ions from the ryanodine receptor.⁹ In another investigation,¹⁰ production of the prostaglandin 8-isoPGF_{2α} was monitored as a measure of oxidative stress in an endothelial cell culture assay and carvedilol was observed to lower 8-isoPGF_{2α} levels. In contrast, however, no evidence was found that carvedilol inhibits oxidative stress in healthy human volunteers.¹⁰ Thus, the precise role of carvedilol as a biological antioxidant remains unclear.

Diphenylamines (2, Fig. 1) are well-known industrial antioxidants because of their ability to inhibit undesired radical chain reactions in polymers, fuels, lubricants and other materials, generally by means of hydrogen donation from the N–H group to a chain-propagating radical species.¹¹ The structural similarity of diphenylamines to the carbazole moiety of carvedilol supports the expectation that carvedilol could similarly serve as a radical-inhibiting antioxidant. However, carbazole and 2-hydroxycarbazole were inactive in inhibiting oxidation of methyl linoleate, while several 3-hydroxycarbazoles proved more effective in this assay.¹² Several carbazole alkaloids such as carazostatin (3, Fig. 1),¹³ have also been shown to exhibit antioxidant activity in various assays, typically augmented by the presence of phenolic hydroxyl substituents. Furthermore, carvedilol is rapidly metabolized in mammals to a variety of products, including hydroxylated species, cleavage products and glucuronidates.¹⁴ In humans, radiolabelling experiments indicated that only 9% of the unchanged drug

remained in the plasma after 1.5 hours, while only 2% of the excreted labelled compounds consisted of the intact drug.^{14a} Among the metabolic pathways that have been reported are the CYP-mediated oxidations of the aromatic groups of carvedilol to their phenolic counterparts, including the 4'- and 5'-hydroxy derivatives 4 and 5, and 3-hydroxycarvedilol (6) (Fig. 1).¹⁵ Since various phenols are widely recognized as radical inhibitors and biological antioxidants (*e.g.* alpha-tocopherol), it is reasonable that phenolic carvedilol metabolites could contribute to, and perhaps exceed, any *in vivo* antioxidant properties displayed by the parent drug. Several earlier studies of the effects of 6 (also known as BM-910228 or SB211475) on pathologies where oxidative stress is implicated reported that this metabolite suppresses lipid peroxidation,¹⁶ mitigates post-ischemic injury¹⁷ and provides neuroprotective benefits.¹⁸ Moreover, 6 proved less effective than 1 in protecting against ischemic reperfusion injury,¹⁹ while both compounds prevented hydroxyl radical-mediated cardiac contractile dysfunction.²⁰ Neither drug was effective in preventing nephropathy in kidney transplants.²¹ It is possible that some of the above results are unrelated to the antioxidant activities of 1 and 6, while the antioxidant behaviour of metabolites 4 and 5, and of other hydroxylated metabolites of carvedilol, has been less studied. This uncertainty underscores the need for an improved mechanistic understanding of the relative abilities of carvedilol and its phenolic metabolites to function as radical-inhibiting antioxidants.

We recently reported the synthesis of phenols 4–6, in order to determine their SOICR-inhibiting properties, and found that they are comparable to those of 1.²² Thus, the metabolism of carvedilol to these products neither significantly activates nor deactivates its ability to suppress SOICR. With these compounds in hand, we now report the relative free radical inhibiting abilities of 1, 4–6 and several benchmark compounds used for comparison in two separate assays.

Fig. 1 Structures of carvedilol, three phenolic metabolites and some structurally related compounds.

One method for evaluating the activity of phenolic or other radical inhibitors is to measure their ability to transfer hydrogen to diphenylpicrylhydrazyl (DPPH, 7, Scheme 1). The latter is a stable free radical with a strong UV/visible absorption at 515 nm, while the corresponding hydrazine (DPPH-H, 8) is essentially transparent at that wavelength. Thus, by monitoring the intensity of the DPPH absorption with time, it is possible to measure the decrease in its concentration and therefore to estimate the radical-inhibiting ability of putative antioxidants (Scheme 1).²³

In Fig. 3–6, the results are shown at three different initial antioxidant concentrations (5 μM , 25 μM and 50 μM), representing 0.1, 0.5 and 1.0 equiv. relative to DPPH (50 μM). The time ($t_{1/2}$) required by each antioxidant to decrease the concentration of DPPH to 25 μM , (half of its initial concentration), was also measured for each antioxidant for the plots where 0.5 equiv. were added.²⁴ Moreover, some phenolic radical-inhibiting antioxidants can quench more than 1 equiv. of DPPH, as the phenoxy radicals derived from such species after hydrogen transfer are able to continue reacting with additional DPPH.^{25,26} These results are summarized in Table 1.

$$\text{ArOH} + \text{DPPH (7)} \rightleftharpoons \text{ArO}^\bullet + \text{DPPH-H (8)}$$

Scheme 1

Fig. 2 Structures of benchmark antioxidants **9–11**.

The results of the DPPH assay of carvedilol (1) and its three phenolic metabolites 4–6 under the same conditions as employed for the above benchmarks are shown in Fig. 7–10, respectively, and the results are summarized in Table 1. To our surprise, carvedilol itself displayed negligible antioxidant activity, where even with an equimolar concentration of the drug relative to DPPH, there was insignificant reduction in DPPH concentration, compared to a control reaction with no antioxidant present (Fig. 7). In striking contrast, 4'-

Fig. 3 DPPH concentration vs. time in the presence of varying amounts of BHT (**9**) in methanol.

Table 1 DPPH assay of carvedilol (1), its metabolites 4–6 and benchmark compounds 9–11

Antioxidant	$t_{1/2}^a$ (s)	DPPH consumed ^{b,c}
BHT (9)	840	2.6
4-Hydroxycarbazole (10)	180	3.0
α -Tocopherol (11)	26	2.0
Carvedilol (1)	No reaction	0.0
4'-Hydroxycarbazole (4)	23	2.6
5'-Hydroxycarbazole (5)	<10	5.6
3-Hydroxycarbazole (6)	Instant ^d	2.5

^a Time required for the decrease in DPPH concentration from 50 μ M to 25 μ M by 25 μ M (0.5 equiv.) of antioxidant. ^b Number of moles of DPPH consumed per mol of antioxidant at equilibrium. ^c Initial concentration of antioxidant was 5 μ M (0.1 equiv.). ^d The reaction was nearly complete by the time the first measurement could be made.

Fig. 4 DPPH concentration vs. time in the presence of varying amounts of 4-hydroxycarbazole (10) in methanol. **Fig. 5** DPPH concentration vs. time in the presence of varying amounts of α -tocopherol (11) in methanol.

hydroxycarvedilol (4) proved to be a potent radical-inhibiting antioxidant. Fig. 8 shows that 0.5 equiv. of 4 afforded a $t_{1/2}$ of 23 s, comparable to the results obtained with α -tocopherol. At the lowest initial concentration, each mol of compound 4 consumed 2.6 mol of DPPH. 5'-Hydroxycarvedilol (5) was even more effective than 4, with the two higher concentrations employing 0.5 and 1.0 equiv. reaching equilibrium within *ca.* 1 min (Fig. 9). Furthermore, when 0.1 equiv. of 5 was used, the antioxidant consumed *ca.* 5.8 mol of DPPH per mol of 5. Finally, the reaction of 3-hydroxycarvedilol (6) with DPPH was

Fig. 6 Summary of results using 0.5 equiv. of each benchmark antioxidant in methanol.

essentially instantaneous, as all three concentrations attained equilibrium within the time required to record the first data point (Fig. 10).²⁷ Metabolite 6, however, consumed only 2.5 equivalents of DPPH at its lowest concentration. An overlay of the plots of the three metabolites 4–6 at the medium 0.5 equiv. level is provided in Fig. 11 to facilitate comparison.

These observations clearly indicate that in the DPPH assay, carvedilol (1) has negligible radical-inhibiting antioxidant activity, while its three phenolic metabolites (4–6) are powerful antioxidants, even when compared to α -tocopherol, the best of the three benchmark compounds employed for comparison. Nevertheless, several limitations to this assay have been reported,^{11a} such as interference from single-electron transfers from the corresponding phenoxide anion to the radical DPPH, which independently affords DPPH-H (8) upon protonation. The relatively high acidity of phenols ensures the presence of significant concentrations of phenoxide ions in alkaline or even neutral media. Thus, in order to verify the above results, a second, different assay was devised, based on the ability of putative radical inhibitors to interrupt propagation steps in a radical chain reaction. Since compounds 4–6 appeared to be highly competent in the DPPH assay, a particularly fast and efficient radical chain process was desired for this purpose.

Some time ago, we reported that the 1,2-addition of *Se*-phenyl *p*-tolueneselenosulfonate (12) to acetylenes proceeds *via* a free-radical addition when initiated by its thermolysis in the presence of AIBN²⁸ or by photolysis.²⁹

Fig. 7 DPPH concentration vs. time in the presence and absence of 1 equiv. of carvedilol in methanol.

Fig. 8 DPPH concentration vs. time in the presence of varying amounts of 4'-hydroxycarvedilol (4) in methanol.

Fig. 9 DPPH concentration vs. time in the presence of varying amounts of 5'-hydroxycarvedilol (5) in methanol.

Fig. 10 DPPH concentration vs. time in the presence of varying amounts of 3-hydroxycarvedilol (6) in methanol.

Among such selenosulfonation reactions, we observed that cyclopropylacetylene undergoes both 1,2-addition to afford **14** as the dominant product, along with ring-opening of the cyclopropane moiety to produce the allenic minor product **16** *via* radical **15** (Scheme 2).³⁰ The ring-opening of cyclopropyl-substituted radicals³¹ and the group transfer of the phenylseleno moiety from various sources to chain-propagating radicals^{32,33} have been previously investigated, while authentic samples of the two products **14** and **16** were easily prepared *via* our previous method.³⁰ Thus, the ability of carvedilol, its metabolites and corresponding benchmarks to inhibit the process shown in Scheme 2 was expected to

Fig. 11 Summary of results using 0.5 equiv. of carvedilol metabolites 4–6 in methanol.

provide additional insight into their relative radical-inhibiting antioxidant properties.

The assay was performed by initiating the process with UV light in a photoreactor equipped with 254 nm lamps and a turntable. The reactions were monitored *via* ¹H NMR spectroscopy in CDCl₃ solution, in which the disappearance of the aromatic signals from the *p*-toluenesulfonyl group of **12** at δ 7.2 ppm and the appearance of the well-separated corresponding signals from **14** and **16** at δ 7.7 and δ 7.8 ppm, respectively, was measured by integration (see Fig. 12). The disappearance of the acetylenic proton of cyclopropylacetylene at δ 1.8 ppm was employed to follow its consumption. Overlap of the vinylic and allenic signals from **14** and **16** at *ca.* δ 5.9 ppm made these less desirable for monitoring the progress of the reaction. Dimethyl sulfone was employed as an internal standard. Each assay was performed under argon after degassing the solution by several freeze–pump–thaw cycles. The kinetic plot for the control selenosulfonation of cyclopropylacetylene in the absence of an antioxidant is shown in Fig. 13, where the disappearance of the selenosulfonate is accompanied by the simultaneous formation of **14**

Fig. 12 ^1H NMR spectra of the reaction of selenosulfonate **12** with cyclopropylacetylene. A) At start ($t = 0$ min); B) at $t = 5$ min; C) at end ($t = 30$ min).

Fig. 13 Kinetic plot for the selenosulfonation of cyclopropylacetylene with no inhibitor (control).

and **16**, produced in the final ratio of *ca.* 7.0:1. The consumption of cyclopropylacetylene tracked that of the selenosulfonate closely, but is not shown for the sake of clarity. The reaction was 50% complete, based on the mol fraction of remaining **12**, in 4.6 min ($t_{1/2}$) and the selenosulfonate was fully consumed within 30 min. The disappearance of the selenosulfonate followed first order kinetics (see Fig. S2 in the ESI †), consistent with the homolysis of **12** as the expected rate-determining step in Scheme 2.

Fig. 14 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of BHT (**9**).

Table 2 Radical inhibition of the selenosulfonation of cyclopropylacetylene by carvedilol (**1**), its metabolites **4–6** and benchmark compounds **9–11**

Compound	$t_{1/2}$ (min)	Time to completion (min)
No inhibitor	4.6	30
BHT (9)	7.9	45
4-Hydroxycarbazole (10)	32.4	90
α -Tocopherol (11)	27.8	60
Carvedilol (1)	4.6	30
4'-Hydroxycarvedilol (4)	8.7	45
5'-Hydroxycarvedilol (5)	13.8	90
3-Hydroxycarvedilol (6)	64.6	180

The $t_{1/2}$ represents the time required for consumption of half of the selenosulfonate **12**. Time to completion represents time required to consume >95% of the selenosulfonate.

The same reaction was then repeated in the presence of 1 mol% of each of the benchmark antioxidants **9–11**, carvedilol (**1**) and its metabolites **4–6**, as shown in Fig. 14–20, respectively, along with the plot for the disappearance of selenosulfonate **12** under control conditions (dashed line) in each assay for comparison. The times required for 50% and full completion of each reaction are summarized in Table 2. The results in the presence of BHT (**9**) (Fig. 14) indicate only a slight retardation of the process compared to the control reaction, with $t_{1/2} = 7.9$ min and the time required for completion of *ca.* 45 min. 4-Hydroxycarbazole (**10**) proved a more effective radical inhibitor (Fig. 15), with $t_{1/2} = 32.4$ min and complete consumption of **12** reached at *ca.* 90 min. While α -tocopherol (**11**) displayed a comparable $t_{1/2} = 27.8$ min compared to that of **10**, it is noteworthy that the reaction was almost completely inhibited for the first 20 min, following which the disappearance of **12** and appearance of the products **14** and **16** proceeded at a much faster pace. This lag time is consistent with the powerful radical-inhibiting properties of α -tocopherol, which persisted until it was fully consumed (only 1 mol% was present initially), following which the reaction resumed a more normal course.

When carvedilol (**1**) was subjected to this assay under identical conditions, it had no discernible effect on the rate of the selenosulfonation reaction (Fig. 17). The plot for the

Fig. 15 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of 4-hydroxycarbazole (**10**).

Fig. 16 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of α -tocopherol (11).

Fig. 17 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of carvedilol (1).

Fig. 18 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of 4'-hydroxycarvedilol (4).

Fig. 19 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of 5'-hydroxycarvedilol (5).

Fig. 20 Kinetic plot for the selenosulfonation of cyclopropylacetylene in the presence of 1 mol% of 3-hydroxycarvedilol (6).

consumption of 12 superimposed closely on that of the control reaction. This confirms the finding from the DPPH assay, where carvedilol displayed essentially no antioxidant activity. The 4'-hydroxy metabolite 4 (Fig. 18) revealed weak activity, comparable to that of BHT (Fig. 14), with $t_{1/2} = 8.7$ min and a completion time of 45 min. Inhibition of the selenosulfonation reaction was more effective with the 5'-hydroxy derivative 5 (Fig. 19), where a longer $t_{1/2}$ of 13.8 min and a completion time of *ca.* 90 min were observed. Finally, 3-hydroxycarvedilol (6) (Fig. 20) resembled α -tocopherol (Fig. 16) in that it displayed a similar lag time, where it very effectively quenched the radical chain process for the first 20 minutes, followed by a more rapid reaction rate. However, a longer $t_{1/2}$ of 64.6 min and overall completion time of >180 min indicate that 6 is even more effective than α -tocopherol in this assay.

It is also noteworthy that the ratio of the amount of 1,2-addition product 14 to that of the allene 16 was relatively constant throughout these experiments, within the range 5.94:1 to 7.02:1. Moreover, the rate of ring-opening of the cyclopropylcarbinyl radical 17 (Fig. 21) was reported^{31a} to be $1.3 \times 10^8 \text{ s}^{-1}$ at 25 °C and this process has been used as a radical clock reaction to measure the rates of competing processes.³⁴ While cyclopropylvinyl radicals such as 13 in Scheme 2 have been less thoroughly studied, Baines *et al.*³⁵ reported that radical 18 (Fig. 21) undergoes ring-opening with an even faster rate constant of $1.6 \pm 0.2 \times 10^{10} \text{ s}^{-1}$ at 20 °C. If it is assumed that the ring-opening of radical 13 is roughly comparable to that of 18,³⁶ then it can be concluded that the phenylseleno group transfer from selenosulfonate 12 to radical 13 in the 1,2-addition to cyclopropylacetylene is an exceptionally facile process, as the corresponding product 14 consistently dominated over the ring-opened allene 16.

The relatively slow initial homolysis of the selenosulfonate 12 and its unimolecular decomposition suggest that the resulting *p*-toluenesulfonyl radical is the principal target for hydrogen-donating antioxidants, resulting in the expected formation of the corresponding sulfinic acid 19 (Fig. 21).³⁷ However, we also considered the possibility that the vinyl radical 13 might react to some extent with hydrogen atom donors. This possibility was excluded through the failure to

Fig. 21 Structures 17–20.

observe, by either GC-MS or HPLC analysis, the expected product 20 (Fig. 21) in the reaction mixture, even when the quantity of the antioxidant was increased from 1% to 50%. An authentic sample of 20 was prepared (see ESI†) and could be detected under these conditions even at very low concentrations. We were also unable to detect the sulfinic acid 19 (Fig. 21) in the reaction mixture by these methods, but that does not rule out its formation, as sulfinic acids are known to undergo conjugate additions to other vinyl sulfones,³⁸ similar to those produced in the present process, as well as self-condensation to afford α -sulfinyl sulfones, that in turn decompose readily to a variety of other products.³⁹ We therefore postulate that hydrogen atom transfer to the initially-formed sulfonyl radical is indeed the principal pathway for inhibition of the overall process shown in Scheme 2.

Conclusions

The relative antioxidant abilities of carvedilol and three of its hydroxylated metabolites, along with three common phenolic radical-inhibiting antioxidants employed for comparison, were measured in two separate assays. The first assay revealed that the three benchmark antioxidants were effective in quenching the DPPH radical in the order BHT (9) < 4-hydroxycarbazole (10) < α -tocopherol (11). The three phenolic carvedilol derivatives displayed activities in the order 4'-hydroxycarvedilol (4) < 5'-hydroxycarvedilol (5) $\ll$ 3-hydroxycarvedilol (6). All three compounds were comparable to (in the case of 4) or more active (in the case of 5 and 6) than even the best benchmark oxidant 11.

In the second assay, based on inhibition of the selenosulfonation of cyclopropylacetylene, BHT exhibited a relatively modest effect on this free-radical chain reaction, while both 4-hydroxycarbazole and α -tocopherol proved to be potent inhibitors of this process. The three metabolites displayed the same order of antioxidant activity as in the DPPH assay, with 3-hydroxycarvedilol affording by far the strongest inhibitory effect, with nearly complete suppression of the process during an initial lag time of *ca.* 20 min.

Although several reports have adumbrated the antioxidant properties of carvedilol (1), to our surprise, the parent drug showed essentially no discernible antioxidant activity in either of the two assays described here. While our *in vitro* studies may not reflect the antioxidant properties of these compounds in a more complex *in vivo* environment, they strongly suggest that it is not carvedilol itself, but rather its phenolic metabolites that provide most or all of the protective antioxidant activity associated with the drug.

Experimental

BHT, 4-hydroxycarbazole, α -tocopherol, DPPH, cyclopropylacetylene and carvedilol were obtained from commercial sources. Selenosulfonate 12,⁴⁰ dimethyl sulfone⁴¹ and metabolites 4–6²² were prepared as described previously. The preparation and characterization of authentic samples of the 1,2-addition product 14, the allenic sulfone 16 and the cyclopropyl vinyl sulfone 20 are provided in the ESI.†

DPPH assay

A calibration curve of absorbance at $\lambda = 515$ nm vs. DPPH concentration was employed for the measurement of DPPH concentrations during the assays (see ESI†). The molar extinction coefficient of DPPH in methanol under these conditions was calculated to be 1.06×10^4 M⁻¹ cm⁻¹ (lit.⁴² 1.09×10^4 M⁻¹ cm⁻¹). A control experiment in the absence of an antioxidant showed a decrease in concentration of DPPH from 50.2 μ M to 49.0 μ M after 1 h, considered to be negligible. In a typical experiment 2.50 mL of a 70 μ M DPPH solution in methanol was added to a 3.5 mL cuvette at 30 °C. To the cuvette containing the DPPH solution, 1.0 mL of the antioxidant solution in methanol at 30 °C was then added in order to make up a final solution containing 50 μ M DPPH and an initial molar ratio of either 10:1, 2:1, or 1:1 of DPPH: antioxidant. The temperature was maintained at 30 °C throughout the experiment and absorbance readings began immediately at a rate of 4 scans per minute. Each assay was performed in triplicate.

Selenosulfonation inhibition assay

A stock solution containing selenosulfonate 12 (150 mM), cyclopropylacetylene (150 mM) and dimethyl sulfone (50 mM) as the internal standard was prepared in CDCl₃. The solution was degassed by several freeze–pump–thaw cycles with the introduction of argon. Aliquots of 50 μ L were measured into a series of NMR tubes and diluted with 450 μ L of CDCl₃. The NMR tubes were placed in a Rayonet UV reactor equipped with eight 254 nm lamps, a rotating carousel, and a cooling fan that maintained the temperature at 28 °C. An NMR tube was withdrawn at each of the required times and analyzed by ¹H NMR integration. Each assay was performed in triplicate. Error bars in Fig. 14–20 indicate ± 1 standard deviation.

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