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Polypyrroles as Antioxidants: Kinetic Studies on Reactions of Bilirubin and Biliverdin Dimethyl Esters and Synthetic Model Compounds with Peroxyl Radicals in Solution. Chemical Calculations on Selected Typical Structures

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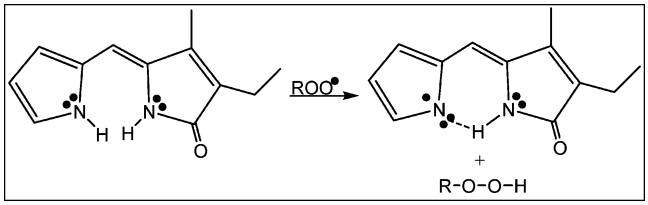

Rate constants for hydrogen-atom transfer (HAT) from bilirubin dimethyl ester (BRDE) and biliverdin dimethyl ester (BVDE) to peroxyl radicals during inhibited autoxidation of styrene initiated by azo-bisisobutyronitrile (AIBN) were k_{inh}(BRDE) = 22.5 × 10^4 and k_{inh}(BVDE) = 10.2 × 10^4 M^{-1} s^{-1}, and the stoichiometric factors (n) were 2.0 and 2.7, respectively. A synthetic tetrapyrrole (bis(dipyrromethene)) containing the α-central (2,2′) CH_2 linkage gave k_{inh} = 39.9 × 10^4 M^{-1} s^{-1} and n = 2.3, whereas the β-linked (3,3′) isomer was not an active antioxidant. Several dipyrrinones were synthesized as mimics of the two outer heterocyclic rings of bilirubin and biliverdin. The dipyrrinones containing N−H groups in each ring were active antioxidants, whereas those lacking two such "free" N−H groups, such as N−CH_3 dipyrrinones and dipyrromethenes, did not exhibit antioxidant activity. Overall, the relative k_{inh} values compared to those of phenolic antioxidants, 2,6-di-*tert*-butyl-4-methoxyphenol (DBHA) and 2,6-di-*tert*-butyl-4-methylphenol (BHT), were 2,2′-bis(dipyrromethene) > BRDE > DBHA > dipyrrinones > BVDE > BHT. This general trend in antioxidant activities was also observed for the inhibited autoxidation of cumene initiated by AIBN. Chemical calculations of the N−H bond dissociation enthalpies (BDEs) of the typical structures support a HAT mechanism from N−H groups to trap peroxyl radicals. Intramolecular hydrogen bonding of intermediate nitrogen radicals has a major influence on the antioxidant activities of all compounds studied. Indeed, chemical calculations showed that the initial nitrogen radical from a dipyrrinone is stabilized by 9.0 kcal/mol because of H-bonding between the N−H remaining on one ring and the ground-state pyrrolyl radical of the adjacent ring in the natural zusammen structure. The calculated minimum structure of bilirubin shows strong intramolecular H-bonding of the N−H groups with carbonyl groups resulting in the known "ridge-tile" structure which is not an active HAT antioxidant. The calculated minimum structure of biliverdin is planar. BRDE is readily converted into BVDE by reaction with the electron-deficient DPPH• radical under argon in chlorobenzene. An electron-transfer mechanism is proposed for the initiating step in this reaction, and this is supported by the relatively low ionizing potential of a model dipyrrole representing the two central rings of bilirubin.

Introduction

The bile pigments biliverdin (BV) and bilirubin (BR) are common products from heme catabolic pathways in which the conversions heme → biliverdin → bilirubin are generally understood.[1] Bilirubin is often regarded as cytotoxic because it is associated with neonatal jaundice, and it may cause brain damage at high concentrations. However, such damaging effects are unusual[1a] and BR has beneficial effects due to its antioxidant properties which were recognized some 50 years ago.[2] The bile pigments are of particular significance as antioxidants because they provide a continuous natural source of protection against

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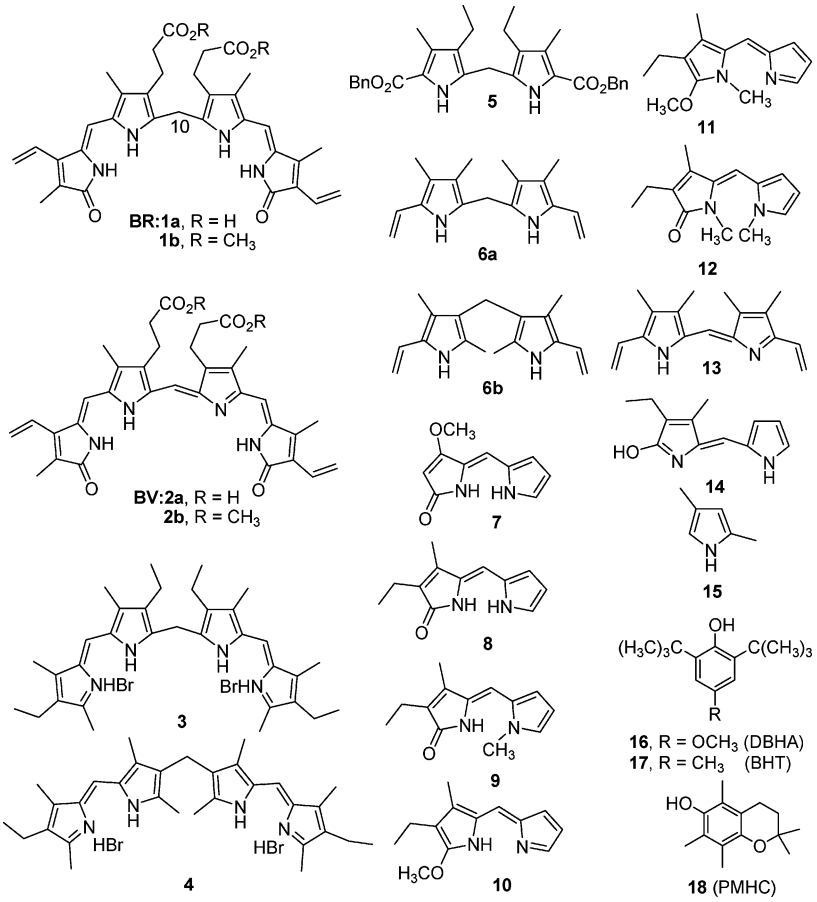

FIGURE 1. Pyrroles used in this study. **1**−**5**, **7**−**12**, and **15** are synthetic compounds. **6a**−**b**, **13**, and **14** are theoretical structures used in calculations. **16**−**18** are known antioxidants.

free-radical oxidation in human blood.[3] In the last two decades, interest in both BR and BV as antioxidants has been shown by several groups.[4] There is some evidence that the antioxidant properties of BR provide cytoprotection in biological systems[4b,5] and even contribute to the antioxidant capacity of jaundiced newborn infants.[5d] Most natural antioxidants act by deactivating damaging peroxyl radicals by H-atom transfer (HAT). For example, phenolic antioxidants, such as vitamin E, function by transferring a phenolic H-atom, and the mechanism and structural features which determine the activity of such phenolic antioxidants in solution are generally well understood.[6] On the other hand, BR possesses an entirely different structure compared to that of phenolic antioxidants (see Figure 1) so that both the antioxidant mechanism and the site of activity are of continuing interest.

The proposed mechanisms by which BR acts as an antioxidant include: (i) HAT from the methylene at position C-10 to the peroxyl radicals,[4i] (ii) peroxyl radical addition to the pyrroles,[4e] and (iii) single-electron transfer (SET) to peroxyl radicals.[4a] Our preliminary report, which showed that polar media have a remarkable enhancing effect on the antioxidant activity of BR, supported the SET mechanism.[4a]

We now report on a detailed study of the antioxidant properties of derivatives of BR and BV and of some structural

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models, including: (1) determination of absolute rate constants, k_{inh}, and stoichiometric factors, n, for trapping peroxyl radicals by their dimethyl esters, BRDE and BVDE; (2) comparison of these antioxidant properties with those of some synthetic di- and tetrapyrroles that mimic at least some of the structural features of BR and BV; and (3) calculations of the bond dissociation enthalpies (BDEs) and ionization potentials (IPs) of synthetic model compounds and theoretical structures selected to provide some clarification between the observed antioxidant properties and the "expected" HAT or SET activities of pyrroles related to BR and BV. The structures for the compounds studied are shown in Figure 1.[7,8]

The dimethyl esters, **1b** and **2b**, of BR and BV were selected for a detailed study of antioxidant properties because they contain the functional groups of the parent molecules, **1(a)** and **1(b)**, and the spectroscopic and related properties of these esters are generally well-known.[9] The tetrapyrrolic compounds, **3** (α-2,2′ C-10 linkage) and **4** (β-3,3′ C-10 linkage), contain some skeletal features of bilirubin, and model compound **5** and structure **6** contain the central dipyrrole linkages. The dipyrrinones, **7**−**9** and **12**, all contain the outer pyrrollic moiety common to both bilirubin and biliverdin, but some have different substituents, in particular, N−CH_3 groups in **9** and **12**. Structures **10** and **11** add extra diversity to the compounds studied. Structural models **6**, **13**, and **14** are "theoretical" models selected for calculations for comparison with the synthetic model compounds. 2,4-Dimethylpyrrole, **15**, was selected as a typical monopyrrole for calculations and the determination of antioxidant properties. Three common phenolic antioxidants, 2,6-di-*tert*-butyl-4-methoxyphenol, **16** (DBHA), 2,6-di-*tert*-butyl-4-methylphenol, **17** (BHT), and the vitamin E analogue, 6-hydroxy-2,2′,5,7,8-pentamethylchroman, **18** (PMHC), were used as the phenolic antioxidants in the kinetic studies for comparison with the pyrroles.

Results

1. Syntheses. Synthetic methods for the new pyrroles and dipyrrinones and analytical data for identification along with characterization purposes are given as Supporting Information.

2. Antioxidant Activities and Stoichiometric Factors. Common antioxidants, such as phenols, inhibit free-radical oxidation of organic substrates in nonpolar solvents by trapping chain-carrying peroxyl radicals, ROO•, by hydrogen-atom transfer (HAT)[6] (reaction 1):

$$ROO^{\bullet} + A{-}H \rightarrow ROOH + A^{\bullet} \quad (1)$$

The phenoxyl radical, A•, traps a second peroxyl radical by reaction 2:

$$ROO^{\bullet} + A^{\bullet} \rightarrow \text{nonradical products} \quad (2)$$

In this HAT pathway, the number of peroxyls trapped per molecule of phenolic antioxidant, the stoichiometric factor n, is normally 2.0.[6] The bile pigments might be expected to inhibit oxidation of substrates in a nonpolar medium by a similar HAT process. Quantitative kinetic methods of autoxidation are well-known to provide data on antioxidant activities of HAT antioxidants in various media.[6] The autoxidation of styrene in chlorobenzene, initiated by AIBN, has certain advantages for determining the rate constants for peroxyl-radical trapping by antioxidants.[6a] For example, the slow thermal decomposition of AIBN (half-life at 30 °C = 2.07×10^3 h), followed by the very rapid reactions of the carbon-centered radicals formed, ensures a controlled rate of chain initiation (R_i) throughout the experiments, an important requirement for such quantitative studies. The reactions under these conditions are given by eqs 3−6:

$$[(CH_3)_2C(CN)N{=}]_2 \xrightarrow{\Delta} \xrightarrow{O_2} ROO^{\bullet} \qquad (\text{rate} = R_i) \quad (3)$$

$$ROO^{\bullet} + CH_2{=}CHPh \rightarrow ROOCH_2C^{\bullet}HPh \quad (4)$$

$$ROOCH_2C^{\bullet}HPh + O_2 \rightarrow ROOCH_2CH(OO^{\bullet})Ph \quad (=R'OO^{\bullet}) \quad (5)$$

$$R'OO^{\bullet} + CH_2{=}CHPh \xrightarrow{k_p} R'OOCH_2C^{\bullet}HPh \quad (6)$$

The length of time the oxidation of styrene is suppressed, i.e., the induction period, is given by

$$\tau = \frac{n[\text{ArOH}]}{R_i} \quad (7)$$

where n is normally 2.0 for phenols.[6a] During the induction period, the rate of oxygen uptake is given by:

$$\left(\frac{-d[O_2]}{dt}\right)_{inh} = \frac{k_p[PhCH{=}CH_2]R_i}{nk_{inh}[ArOH]} \quad (8)$$

Values of k_{inh} are calculated using the integrated form of eq 8 in eq 9:

$$-\Delta[O_2]_t = k_p[\text{PhCH}{=}CH_2]\frac{\ln(1 - t/\tau)}{k_{inh}} \quad (9)$$

A plot of $\Delta[O_2]_t$ vs $\ln(1 - t/\tau)$ gives a straight line with a slope equal to $k_p[\text{PhCH}{=}CH_2]/k_{inh}$, and from the known k_p of styrene, the k_{inh} is evaluated.

For any compound to be active as an antioxidant, its inhibition rate constant (k_{inh}) must be several orders of magnitude greater than the k_p of the substrate, which for styrene is 41 M^{-1} s^{-1}.[6] Under this condition, the oxygen uptake is suppressed until all of the antioxidant is used up and then the rate returns to the

(7) Bilirubin is well-known to have a tendency to form a secondary structure known as the ridge-tile structure,[8] which has important effects on its physical properties (e.g., solubility) and possibly its behavior as an antioxidant (vide infra). In biliverdin, the sp^2 connection at C-10 blocks the formation of the ridge-tile structure.[8d] The structures for bilirubin, biliverdin, and all of the model compounds are shown in their natural configuration about the unsaturated linkages for the pyrroles; that is, *Z,Z* for bilirubin, *Z,Z,Z* for biliverdin, *Z,Z* for **3** and **4**, and *Z* for all the other model compounds. These isomers were used in all of our experiments because the configurations may have important effects on the antioxidant properties. The importance of using the correct structural isomers for BR and BV has been emphasized before: Ritter, S. *C. E. Sci. Technol.* **2003**, *81*, 29.

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TABLE 1. Antioxidant Activities, k_{inh}, and Stoichiometric Factors, *n*, of Bilirubin Dimethyl Ester (BRDE), Biliverdin Dimethyl Ester (BVDE), and Synthetic Model Tetrapyrroles and Dipyrrinones Compared to Those of DBHA during Inhibited Peroxidation of Styrene (0.87 M) Initiated by AIBN (20−21 mM) in Chlorobenzene

compound, $M \times 10^6$	k_{inh}, $M^{-1}\,s^{-1} \times 10^{-4}$ [a]	n[b]
BRDE, 5.81−6.81	22.5	2.0
BVDE, 3.82−5.63	10.2	2.7
3, 4.35−7.30	39.9	2.3
4, 4.70	not measurable[c]	
8, 5.90−11.6	12.4	1.8
10 and **15**, 5.75 and 9.08	not measurable[c]	
DBHA, 5.19−7.76	16.1	(2.0)
BHT	1.4[6]	(2.0)

[a] Rate constants for reaction with peroxyl radicals determined by the inhibition of the oxygen uptake method at 30 °C. From plots of oxygen uptake vs $\ln(1 - t/\tau)$ (eq 9) and using $k_p = 41\ M^{-1}\,s^{-1}$ for styrene. Results from at least three determinations with error limits of less than 20%. [b] The stoichiometric factors calculated from $n = R_i \times \tau/[\text{compound}]$, where $R_i = 2 \times [\text{ArOH}]/\tau$ and ArOH is either DBHA or PMHC. The stoichiometric factor for phenols is normally 2. Results from at least three determinations with error limits of less than 12%. [c] Compounds **4**, **10**, and **15** did not give induction periods but only slight reductions in oxygen uptake.

TABLE 2. Antioxidant Activities, k_{inh}, and Stoichiometric Factors, *n*, of Bilirubin Dimethyl Ester (BRDE) and Biliverdin Dimethyl Ester (BVDE) Compared to the Antioxidant Properties of a Synthetic Tetra- and Dipyrrole, and Dipyrrinones during Inhibited Peroxidation of Cumene (5.35 M) Initiated by AIBN (22−23 mM) in Chlorobenzene

compound, $M \times 10^6$	k_{inh}, $M^{-1}\,s^{-1} \times 10^{-4}$ [a]	n[b]
BRDE, 2.55−5.13	1.27	2
BVDE, 2.53−5.14	0.77	2.3
4, 5.45	not measurable[c]	
5, 4.96	not measurable[c]	
7, 6.14−7.88	0.84	2
8, 3.83−7.29	0.62	1.7
9, **10**, **11, 12, 15**, 5.65−7.50	not measurable[c]	
BHT, 5.87−12.6	0.52	(2.0)

[a] Rate constants determined from plots of oxygen uptake vs $\ln(1 - t/\tau)$ (eq 9) and using $k_p = 0.18\ M^{-1}\,s^{-1}$ for cumene. [b] The stoichiometric factors calculated from $n = R_i \times \tau/[\text{compound}]$, where $R_i = 2 \times [\text{ArOH}]/\tau$ and ArOH is either DBHA or PMHC. The stoichiometric factor for phenols is normally 2. Results from at least three determinations with error limits of less than 12%. [c] Compounds **4** and **5**, **9**−**12**, and **15** did not give measurable induction periods (see text).

uninhibited rate. There are many compounds that react comparatively slowly with peroxy radicals, and during this time, chain termination mainly occurs by recombination of peroxyl radicals (eq 10). Under this condition, the oxygen uptake may

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \qquad (10)$$

be only slightly suppressed and the inhibitor will persist in the experiment well beyond the expected induction period based on the known R_i (eq 7), which is measured by an efficient antioxidant. Compounds which show this behavior are classified as "retarders" rather than as antioxidants.[10] The absolute rate constants, k_{inh}, and stoichiometric factors, *n*, for some model tetrapyrroles, BRDE, BVDE, **3**, and the dipyrrinone **8** are given in Table 1 compared with those for the commercial antioxidants, DBHA and 2,6-di-*tert*-butyl-4-methylphenol (BHT). The tetrapyrrole **3**, a 2,2′-bis(dipyrromethene), is the most effective antioxidant of those studied, and the order of antioxidant activity (k_{inh}) found is **3** > BRDE > DBHA > **8** ≥ BVDE > BHT. It was interesting to find that the tetrapyrrole with the unnatural β-3,3′ linkage, **4**, was inactive as an antioxidant, in contrast to the behavior of **3**, and the dipyrromethene, **10**, was also inactive, in contrast with the activity of dipyrrinone **8**.

The profile of suppressed oxygen uptake will depend on the inhibition rate constant, k_{inh}, of the antioxidant relative to the propagation rate constant, k_p, of the substrate (see eq 8) and also on the termination rate constant of the substrate peroxyls (eq 10). Therefore, some experiments were carried out in cumene which has a much lower k_p of $0.18\ M^{-1}\,s^{-1}$ at 30 °C,[11a] and tertiary cumenyl peroxyls exhibit lower terminating rate constants compared to those of primary and secondary (e.g., here, polystyryl peroxyl) radicals[11b] so that even relatively weak antioxidants are known to give well-defined induction periods in cumene.[11c] It was thought that some of the compounds that did not effectively suppress the peroxidation of styrene might exhibit induction periods in cumene. The results of these experiments, given in Table 2, show that this is NOT the case; the compounds that were not active inhibitors in styrene were also not effective in cumene.

As expected, any compound which exhibited induction periods in styrene gave well-defined induction periods in cumene. For the two esters BRDE and BVDE, the relative k_{inh} of BRDE/BVDE in cumene of 1.7 was similar to that of 2.2 found in styrene (Table 1). The 3,3′-bis(dipyrromethene), **4**, did not give an induction period in cumene. The two dipyrrinones **7** and **8** both gave induction periods and measurable k_{inh} values. Compound **10**, which was not an active antioxidant in styrene, similarly was inactive in cumene. Also, it is important to note that any model compound containing a $N-CH_3$ group, compounds **9**, **11**, and **12**, failed to exhibit antioxidant activity in cumene. The lack of antioxidant activity in compounds **9**−**12** implies that a simple monopyrrole would also lack antioxidant activity. This was confirmed by finding that 2,4-dimethylpyrrole, **15**, lacked activity under these conditions in both styrene and cumene (Tables 1 and 2). The structural requirements for antioxidant activity in the di- and tetrapyrrolic systems are of particular importance and provide insight into the antioxidant mechanism of the active compounds (see Discussion).[12]

The stoichiometric factors, $n \leq 2$, given by the active di- and tetrapyrrolic structures (Tables 1 and 2) indicate that they are capable of trapping at least two peroxyl radicals per molecule of antioxidant. Nonintegral values of *n* were frequently found; for example, for BVDE, $n = 2.3-2.7$. Such behavior is well-known for weak antioxidants and can be attributed to some chain termination by peroxyl radicals (reaction 10) which extends the induction period of the antioxidant. In addition, most of these pyrroles exhibited rather unique behavior compared to phenolic

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(12) A reviewer suggests that the higher k_{inh} for these polypyrroles in styrene compared to cumene, by as much as 20-fold for compound **8** (see Tables 1 and 2), means a different mechanism in these hydrocarbons. We have no evidence for this at this time. Steric effects may account for some difference because tertiary peroxyl radicals (e.g, cumenyl peroxyl) are 3−5 times less reactive than primary and secondary peroxyls (e.g., polystyryl peroxyl) in H-atom abstraction (Ingold, K. U. *Acc. Chem. Res.* **1969**, *2*, 1−9); so, BHT is about 3 times more active in styrene than in cumene.

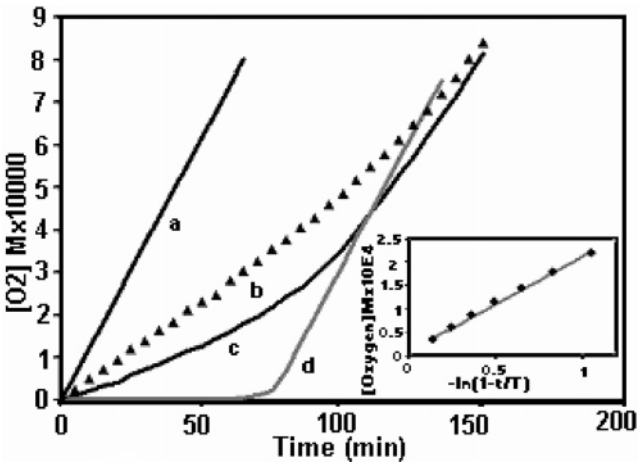


FIGURE 2. Oxygen uptake profile of styrene, 0.870 M, initiated by AIBN, 20.5 mM, at 30 °C. (a) Uninhibited. (b) Inhibited by DBHA, 5.21 μM, giving $k_{inh} = 14.4 \times 10^{-4}$ M^{-1} s^{-1}. (c) Inhibited by BRDE, 5.81 μM, giving $k_{inh} = 25.6 \times 10^{-4}$ M^{-1} s^{-1}. (d) Inhibited by PMHC, 5.54 μM. Inset: incremental oxygen uptake plot of curve c. The k_{inh} for BRDE was calculated from this plot (eq 9).

antioxidants in that the oxygen uptake did not return to its uninhibited rate at the end of the measurable induction period. This effect is shown in Figure 2 where the oxygen uptake profiles for BRDE and the phenolic antioxidant PMHC are shown. The latter shows ideal behavior where the rate returns to the uninhibited rate at the end of the induction period. This is not the case for BRDE, where the rate continues to be slightly suppressed long after the initial break in the curve. However, the plot of eq 9 from which k_{inh} is calculated is linear (see inset).

This behavior of BRDE is most probably due to the formation of the secondary, weaker antioxidant, BVDE, in the experiment which acts to extend the induction period. This was confirmed by showing that BRDE is concerted to BVDE under these conditions, as detailed below.

3. Conversion of Bilirubin (BRDE) into Biliverdin (BVDE). (i) Aerobic Conditions. Although the natural enzymatic conversion is from biliverdin to bilirubin, in the laboratory, the opposite oxidation process is quite common.[1a,b,4i] It was desirable to confirm that this reaction occurs under our controlled conditions (e.g., constant R_i and the absence of light). For this purpose, BRDE was reacted with peroxyl radicals generated from AIBN under oxygen in toluene at an elevated temperature of 68 °C to permit a build up of products. Samples taken for thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) (see Supporting Information) analyses showed the formation of BVDE during the course of the reaction.

(ii) Anaerobic Conditions. Because in vivo partial pressures of oxygen are much lower than those under laboratory conditions,[3b] it was of interest to determine if the conversion of BRDE to BVDE occurs in the *absence* of oxygen. For this purpose, the diphenylpicrylhydrazyl (DPPH$^\bullet$) radical was used as the attacking radical. This radical is very often used to determine the hydrogen atom donating ability of antioxidants by observing the decay of the DPPH$^\bullet$ color in the visible spectrum.[13] Because the color of the reactants interferes with this method, we used the decay of the ESR spectrum of DPPH$^\bullet$ to monitor the reaction in the presence of excess BRDE under argon at 25 °C in the ESR probe. The reaction of BRDE to BVDE involves an overall stoichiometry of 1:2, BRDE/DPPH$^\bullet$, and the reaction was conveniently monitored by using a ratio of 2:1, BRDE/DPPH$^\bullet$, so that the reaction terminated in minutes when the typical DPPH$^\bullet$ ESR signals decayed leaving a simple mixture of unreacted BRDE and BVDE product, as shown by TLC and HPLC analysis (shown in Supporting Information).

4. Calculations. For calculation of the BDE, we used the lowest-level method (LLM) described by DiLabio et al.[14] The N−H or C−H BDE corresponds to the standard gas-phase enthalpy change at 298 K ($\Delta H°_{298}$) for X−H(g) → X$^\bullet$(g) + H$^\bullet$(g). Briefly, the LLM method uses the AM1 procedure to obtain the optimized geometry and frequencies, where T = 298.15 K, P = 1.00 atm, and the AM1 frequencies are scaled by the factor 0.973. At the geometry minimum, a single-point calculation was done using density functional theory where the method/basis set is (RO)B3LYP/6-311+G(2d,2p); here, RO indicates that for the radical the restricted open-shell B3LYP method was used. All electronic energies were then corrected by the thermal contribution to the enthalpy to obtain $\Delta H°_{298}$, the standard gas-phase enthalpy, at 298 K. To complete the specification of the method, we set the electronic energy of the H-atom to its exact value, −0.50000 hartree, and obtained its enthalpy, $\Delta H°_{298} = -0.50000 + (5/2)RT = -0.49764$ hartree. Starting geometries were obtained with the Spartan 02 builder module (WaveFunction, Inc.: Irvine, CA) using AM1; coordinates were then sent to the Gaussian 98 program for all subsequent calculations (Gaussian, Inc.: Pittsburgh PA).[15]

IPs were also calculated by the LLM method by using the method/basis set B3LYP/6-311+G(2d,2p)//B3LYP/6-31G(d) for the parent structure and ROB3LYP/6-311+G(2d,2p)//B3LYP/6-31G(d) for the radical. The calculation used $E°_0$, the internal energy at 0 K, where $E°_0$ is the sum of the total electronic energy plus the zero-point energy for each species and the IP is the difference in $E°_0$ between the product (cation) and the parent molecule. This LLM method is known to underestimate the IPs of aromatic compounds by about 4 kcal/mol.[16]

As pointed out (vide supra), bilirubin is well-known to have a tendency to form a ridge-tile structure containing intramolecular hydrogen bonds. This structure was calculated here, as was the optimum conformer of biliverdin; these structures are shown in Figure 3 for qualitative comparison. Bilirubin, Figure 3a, possesses the somewhat closed structure attributed to strong H-bonding. This strong tendency was confirmed by rotation within the molecule around the connecting CH_2 group which raised the energy by 14.4 kcal/mol, attributed to the loss of H-bonds. A similar result was obtained by Shelver et al. who

(13) Litwinienko, G.; Ingold, K. U. *J. Org. Chem.* **2003**, *68*, 3433−3488.

(14) DiLabio, G. A.; Pratt, D. A.; LoFaro, A. D.; Wright, J. S. *J. Phys. Chem. A* **1999**, *103*, 1653−1661.

(15) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*, revision A.9; Gaussian, Inc.: Pittsburgh, PA, 1998.

(16) DiLabio, G. A.; Pratt, D. A.; Wright, J. S. *Chem. Phys. Lett.* **1999**, *311*, 215−220.

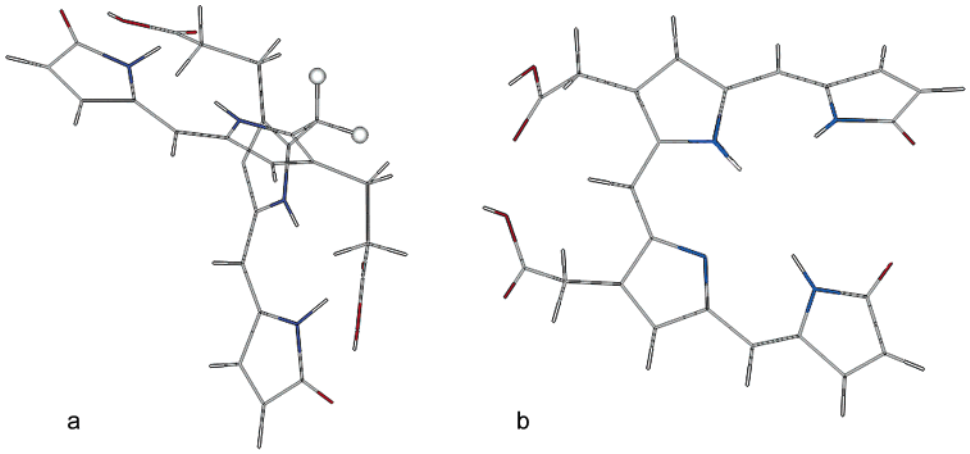


FIGURE 3. (a) Ridge-tile structure of BR. (b) Structure of BV. In **3a** and **3b**, the ethenyl groups of **1a** and **2a** in the terminal rings are replaced by methyls for determination of the minimum structures.

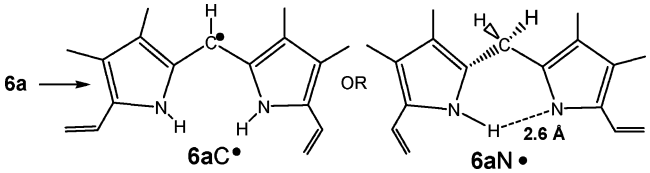


FIGURE 4.

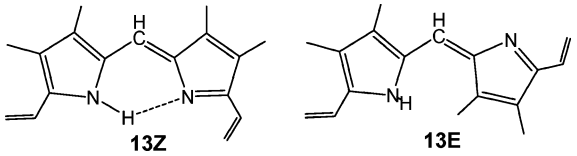


FIGURE 5.

reported a barrier of approximately 20 kcal/mol from rotation of the molecule about the CH_2 group.[17] In contrast, biliverdin, lacking the intramolecular H-bonding, is more open with three of the pyrrole rings in one plane (Figure 3b).

The theoretical model structures **6a** and **6b** (Figure 1) contain the basic central dipyrroles of bilirubin and of the experimental compounds **3** and **4**, respectively. The calculated BDE of the central C−H of **6a** is 70.1 kcal/mol and that of N−H is 84.7 kcal/mol. The calculated IP of 152.9 kcal/mol is about 50 kcal/mol below benzene and 4 kcal/mol below that of α-tocopherol.[18] In comparison, the BDE values for **6b** are C−H = 80.2 and N−H = 83.4 kcal/mol. It is also useful to compare the conformers of the carbon radicals derived from **6a** and **6b**. The angle between the planes of the pyrrole rings in the central carbon radical derived, **6a**C•, (Figure 4) is only 18.4° compared to an angle of 53.8° for the radical derived from **6b**. The methyl groups at 2 and 2′ of **6b** are probably responsible for the nonplanarity of **6b** and derived radicals. It was thought that stabilization of the nitrogen radical, **6a**N•, derived from **6a** might be provided by hydrogen bonding with the N−H of the remaining pyrrole. However, this proved not to be the case: when the N• and N−H of the two rings approach, they adopt out-of-plane conformations of 51.2° at a distance of 2.6 Å, a distance considered too long for effective hydrogen bonding (Figure 4).

The theoretical model structure **13**, representing the two central rings of biliverdin (shown in Figure 5), gave a calculated N−H BDE of 93.7 kcal/mol and an IP of 145.9 for the zusammen structure, **13*Z***. In **13*Z***, the high BDE of the N−H bond is attributed to the formation of a strong in-plane hydrogen bond because the total BDE would include the N−H bond plus hydrogen bonding. The difference of 9.0 kcal/mol between the BDE of **13*Z*** and that of **6a** (84.7 kcal/mol) is therefore attributed to hydrogen bonding in **13*Z***, which is improbable in **6a**, and suggests a H-bond energy of approximately 9 kcal/mol in structures such as **13*Z***. Such H-bonding is obviously not possible in the entgagen structure, **13*E***.

Compound **8** was significant for both an experimental model (Table 1) and calculations as a model for the two outer dipyrrinone rings of both bilirubin and biliverdin, especially because **8** exhibits a peroxyl-radical trapping activity similar to that of biliverdin. Relevant calculations of the natural *Z* form and of the *E* isomer are shown in Figure 6.[19]

The somewhat higher BDE of the N−H bonds in the B-ring of both isomers of **8** is attributed to the electron-attracting carbonyl group which would be expected to destabilize the N• radical on H-atom removal. The much larger difference in the N−H BDE of the N−H bond of ring B in the *E* isomer compared to this bond in **8*Z*** is attributed to increased stabilization of the radical formed upon H-atom removal from **8*Z***, resulting from strong intramolecular H-bonding that lowers the energy of the transition state, forming the radical (Figure 6, **8*ZR***). This effect is obviously absent in **8*E***. The intermediate showed a calculated N•···H distance of 2.1 Å in the planar radical. An out-of-plane rotation of the radical gave a barrier of 9.0 kcal/mol which represents the BDE of this hydrogen bond.

It is also of interest to find that the enol form of **8*Z*** (Figure 1, structure **14**) gave a calculated N−H BDE of 96.9 kcal/mol and an O−H BDE of 76.6 kcal/mol. In this case, the high value for the N−H bond is attributed to the sum of the BDE of breaking the intramolecular hydrogen bond (N, ring B, to N−H, ring A) plus the BDE of a typical pyrrole N−H bond of approximately 87 kcal/mol.

(17) Shelver, W. L.; Rosenberg, H.; Shelver, W. H. *Int. J. Quantum Chem.* **1992**, *44*, 141−163.

(18) DiLabio, G. A.; Pratt, D. A.; Wright, J. S. *J. Org. Chem.* **2000**, *65*, 2195−2203.

(19) Although it might be expected that the N−H bond in A would be broken preferentially, there are two possibilities shown for the hydrogen-bonded intermediate, **8*ZR***.

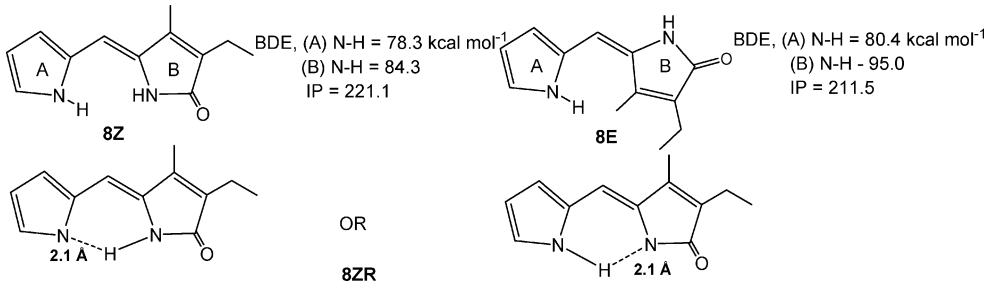


FIGURE 6.

TABLE 3. Energetics of a Second Hydrogen-Atom Abstraction

process	energetics kcal/mol
N−H BDE of a preformed carbon radical at connecting CH_2 to form **13*Z***	55.5
C−H BDE of a preformed nitrogen radical to form **13*Z***	40.9

Because the reaction that forms biliverdin from bilirubin requires the loss of two hydrogens overall, it is of interest to compare the energetics of bond breaking from intermediates resulting from the initially "preformed" carbon radical and the "preformed" nitrogen radical of the model structure **6a**. The results of these processes are shown in the calculations of Table 3. Finally, the relevant calculated BDE and IP of the 2,4-dimethylpyrrole, **15**, are N−H = 89.3 kcal/mol and IP = 169.1 kcal/mol.

Discussion

The interplay of various factors in polypyrroles, including conformational and substituent effects, and hydrogen bonding in parent molecules and in derived radicals make specific conclusions about reactivities and antioxidant mechanisms somewhat complicated. This is especially true for bilirubin where strong intramolecular hydrogen bonding interferes with HAT reactions to peroxyl radicals from N−H groups,[20] and the ridge-tile structure hinders HAT reaction from the connecting $-CH_2-$ group because the required stabilization of a planar $-C^{\bullet}-H$ radical could not occur without a large energy cost (e.g., 15−20 kcal/mol to break several H-bonds). As a result, bilirubin lacks antioxidant activity in a nonpolar organic solvent.[4a] This contrasts with reports on its activity in aqueous media when complexed with a protein or lipid membranes,[4] where the ridge-tile structure may be altered.[21] The use of dimethyl esters of bilirubin and biliverdin (BRDE and BVDE) avoids this complication because one can assume that the structures are more open. In addition, the various model compounds used provide a useful way of targeting definite reactive sites potentially responsible for antioxidant activities in the natural compounds themselves.

The relative antioxidant activities of the tetrapyrroles (**3** > BRDE > BVDE; Table 1) are attributed to the electron-attracting carbonyl groups at the ends of the conjugated system of the latter two compounds, which in the case of BVDE is conjugated throughout. This deactivating effect would tend to destabilize intermediate substrate radicals formed from reaction with peroxyls. In addition, BRDE and BVDE are known to form intermolecular dimers due to hydrogen bonding between the N−H groups and the ester carbonyl functions.[9a,c,d] It is well-known that intermolecular H-bonds inhibit the H-atom donating properties of antioxidants,[22] assuming that "free" N−H functions are required for these compounds to exhibit significant antioxidant activity (vide infra). As would be expected, the dipyrrinone, **8**, lies between BRDE and BVDE in antioxidant activity. The possibility that **8** might react with peroxyl radicals through its enolic form, **14**, was considered. However, there was no evidence for such an enol in carbon tetrachloride solutions from infrared spectra nor from NMR spectra in chlorobenzene (C_6D_5Cl).

It is of interest to find that the tetrapyrrole, **4**, with a β-3,3′ linkage joining the pyrroles did not exhibit antioxidant activity but only slightly reduced the oxygen uptake. This is probably due to the steric effects of the methyl groups at positions 2 and 2′ which are expected to give the molecule a twisted structure, thereby preventing optimum stabilization of an intermediate substrate radical. This was confirmed by estimation of the geometry of the central carbon radicals ($CH_2 \rightarrow C^{\bullet}H$) derived from the connecting model compounds **6a** and **6b**, where that from **6a** was nearly planar and the radical from **6b** was decidedly nonplanar, giving an angle of 54° between the rings. The dipyrrole **5** contains the skeletal structure of the central rings of BRDE but replaces the vinyl groups at positions 5 and 5′ with ester groups. The effect of these strongly electron-attracting groups at both ends of the conjugation is to make this compound a very poor inhibitor which does not exhibit an induction period in styrene. Unfortunately, compound **6a** was not available for experimental studies.

Hydrogen bonding is a most important property affecting the antioxidant properties of these various pyrroles. In general, the effect can act to either reduce antioxidant activity when it acts on the parent molecule or increase activity when it operates to stabilize the intermediate radical. Intramolecular H-bonded hydrogens can be abstracted by peroxyls but only at diminished rates.[20] The reduction in activity is predicted to operate in any structure which has a N−H bond in one pyrrole and an available nitrogen lone pair in a second heterocyclic ring when the rings are essentially coplanar. The lack of activity observed for the dipyrromethene **10** is attributed to strong intramolecular hy-

(20) de Heer, M. I.; Mulder, P.; Korth, H.-G.; Ingold, K. U. *J. Am. Chem. Soc.* **2001**, *123*, 469−477.

(21) Spectroscopic studies of bilirubin in buffer or complexed with a bile salt or albumin indicate the formation of various aggregates with apparent tendencies to preserve the intramolecular H-bonded structure: Lee, K.-S.; Gartner, L. M. *Pediatr. Res.* **1976**, *10*, 782−788. Kurtin, W. E.; Heo, R.; Beimeir, D. J.; Tran, N. T.-V.; Elizondo, E.; Salas, R. E.; Morales, M.; Huang, L.; Frank, B. *J. Chem. Soc., Perkin Trans. 2* **1998**, 1677−1682. Honoré, B. *J. Biol. Chem.* **1987**, *262*, 14930−14944. However, bilirubin-IXα and its dimethyl ester undergo rapid reversible isomerization on exposure to visible light: McDonagh, A. G.; Lightner, D. D.; Wooldridge, T. A. *J. Chem. Soc., Chem. Commun.* **1971**, 110−112.

(22) Snelgrove, D. W.; Lusztyk, J.; Banks, J. T.; Mulder, P.; Ingold, K. U. *J. Am. Chem. Soc.* **2001**, *23*, 469−477.

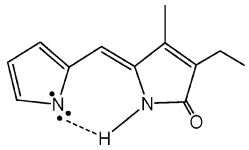


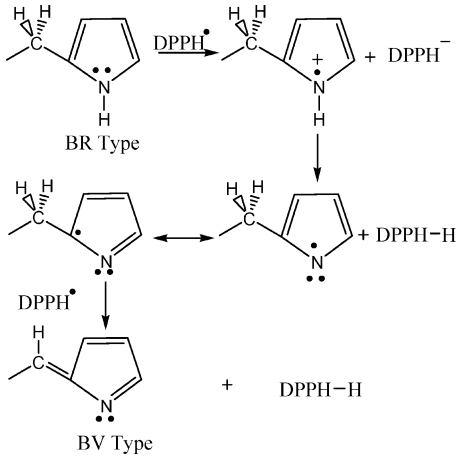
FIGURE 7. Radical derived from **8**.

drogen bonding of the N−H from one ring with a nitrogen lone pair from the other ring. Similarly, structures such as **13** and **14** would also not be active antioxidants. In such structures, a HAT reaction requires breaking of a strong hydrogen bond plus the N−H BDE. On the other hand, stabilization of a radical intermediate from a HAT reaction will increase antioxidant activity. For example, stabilization of the nitrogen radical, N•, derived from compounds with structures such as **7** and **8** accounts for their observed HAT activities. The H-bond energy in such structures appears to be rather strong (9 kcal/mol). This can be attributed to the actual electronic structure of the pyrrolyl radical. Loss of a hydrogen atom from the nitrogen atom of pyrroles may be expected to yield the radical with the odd electron occupying an in-plane σ-orbital, leaving six electrons in the π-molecular orbitals. However, studies of the pyrrolyl radical by theoretical methods[23] and photoelectron spectroscopy[24] revealed that the ground-state structure is $^2A_2$ corresponding to five π-electrons in the five-membered ring and a lone pair in an in-plane σ-type molecular orbital. Consequently, the hydrogen bonds stabilizing the so-called nitrogen radicals herein are strong because they involve a N−H group with a nitrogen lone pair N, as shown in Figure 7 for the radical derived from **8**.

It follows that only structures that possess N−H groups in adjacent rings joined by sp^2 connecting groups will be effective N−H HAT antioxidants. As such, dipyrroles, which possess one or more N−CH_3 groups, will not be antioxidants. Thus, compounds **9**, **11**, **12**, and **15** are not expected to be antioxidants, as was observed. This implies that reaction mechanisms that involve the addition of peroxyls to the pyrrole ring[4e] are very improbable because if this mechanism were active then all of these structures would possess antioxidant activities by this pathway. Although the importance of hydrogen bonding for the antioxidant activities of pyrroles has not been recognized previously, there is precedent for its significance in catechols and naphthalene diols. For example, the antioxidant activity of 1,8-naphthalene diol with peroxyl radicals is greater than that of PMHC because of enhanced hydrogen bonding of the intermediate aryloxyl radical over that of the parent compound.[25]

The combination of chemical calculations and kinetic data on model compounds provides some insight into the reactive sites and antioxidant mechanisms of the ester derivatives, **1b** and **2b**, of the natural bile pigments. The measured antioxidant activity, k_{inh}, of **8** is similar to that of **2b** (Table 1), and this together with the calculations of hydrogen bonding in the radical derived from a HAT reaction from a N−H indicate clearly that the antioxidant site of **2b** (and presumably of biliverdin) is in the two terminal rings containing N−H groups (Figure 1). Applications of our results to the reactive site and antioxidant

SCHEME 1



mechanism of the bilirubin derivative, **1b**, are a more complex problem. Not only could this compound possess antioxidant activity due to HAT from N−H groups of two sets of terminal dipyrrinones but also the central CH_2 group might be an active H-atom donor according to calculations on the model compound **6a**. The relatively low BDE of the connecting C−H bond (70.1 kcal/mol) of **6a** compared to the −N−H bonds (84.7 kcal/mol) indicates that the −CH_2− could be a H-atom donor.[26]

The reactions for the dipyrrinones **7** and **8** with peroxyl radicals and the lack of antioxidant activity of other compounds (e.g., **9**−**12**) described above are attributed to hydrogen-atom transfer (HAT) processes of the N−H groups. Alternatively, the initial step may occur by electron transfer in particular cases. Electron-rich pyrroles are known to participate in electron-transfer reactions, for example, in the electrochemistry of bilirubin, biliverdin, and dipyrrinones in polar solvents[27] and in the formation of a radical cation from 1,2,3-trimethylpyrrole by electron transfer to oxygen in organic solvents.[28] The increased antioxidant activity of bilirubin in the polar media of aqueous micelles and bilayers was attributed to a single-electron transfer (SET) reaction.[4a] The lower calculated IP for the model dipyrrole, **6a**, compared to that of the dipyrrinone, **8**, indicates that BR may also be susceptible to electron transfer even in a nonpolar solvent. The rapid conversion of the diester BRDE to BVDE by the electron-deficient DPPH• radical in an organic solvent is most likely initiated by electron transfer, as shown in Scheme 1. In this process, electron transfer from the pyrroles π-electron system forms the radical cation (or ion pair) followed by proton transfer leading directly to the ground-state pyrrolyl radical without the distortion that is associated with the direct breaking of the N−H bond.[23] On the basis of the low C−H BDE of the CH_2 group of this radical (40.9 kcal/mol, Table 3), a HAT reaction to the attacking radical is expected to take place readily to form BVDE as observed. As proposed earlier,[4a] this

(23) Bacskay, G. B.; Martoprawiro, J. C. M. *Chem. Phys. Lett.* **1998**, *290*, 391−398.

(24) Gianola, A. J.; Ichino, T.; Hoenigman, R. L.; Kato, S.; Bierbaum, V. M.; Lineberger, W. C. *J. Phys. Chem. A* **2004**, *108*, 10326−10335.

(25) Foti, M.; Johnson, E. R.; Vinquist, M. R.; Wright, J. S.; Barclay, L. R. C.; Ingold, K. U. *J. Org. Chem.* **2002**, *67*, 5190−5196.

(26) We are aware that comparison of BDEs involving different connecting atoms (X−H and Y−H) without data on the activation energies may *not* provide a valid prediction of the relative H-atom donor activity of the compounds (see: Zavitsas, A. A.; Chatgilialoglu, C. *J. Am. Chem. Soc.* **1995**, *117*, 10645−10654). Furthermore, the relatively low BDE for this C−H bond in **6a** may not be applicable to bilirubin in the ridge-tile form because it cannot readily extract a H-atom from its central CH_2 without energy costs in bond rotations.

(27) (a) Ribo, J. M.; Farrera, J.-A.; Claret, J.; Grubmayr, K. *Bioelectrochem. Bioenerg.* **1992**, *29*, 1−17. (b) Slifstein, C. H.; Ariel, M. *Electroanal. Chem. Interface Electrochem.* **1973**, *48*, 447−463.

(28) Beaver, B.; Teng, Y.; Guiree, P.; Hapiot, P.; Neta, P. *J. Phys. Chem. A.* **1998**, *102*, 6121−6128.

pathway may also be applicable to the reaction of BR with peroxyl radicals, especially in polar media.

Experimental Section

Materials. Bilirubin dimethyl ester and biliverdin dimethyl ester were obtained from a commercial supplier and used as received. The commercial inhibitors, 2,2′,5,7,8-pentamethyl-6-hydroxychroman (PMHC), 2,6-di-*tert*-butyl-4-methoxyphenol (DBHA), and 2,6-di-*tert*-butyl-4-methylphenol (BHT), were recrystallized from methanol before use. AIBN was recrystallized from methanol from a solution prepared at room temperature and cooled. Commercial styrene was separated from added stabilizer by bulb−bulb vacuum distillation at room temperature. The distilled styrene was passed through chromatographic silica gel immediately before use. Cumene, purest grade, was passed through silica gel before use. Solvents used were of HPLC purity. The diphenylpicrylhydrazyl radical (98%) was used as received.

Analyses. HPLC analyses were carried out on a 25 cm × 4.6 mm 5 μm silica adsorption column with a solvent mixture of 60:40 (v:v) hexane/ethyl acetate at a flow rate of 1.0 and 1.5 mL/min, and the components were detected by a variable wavelength detector. ESR spectra were determined using a power level of 2.5 mW and a modulation amplitude of 4 G. ESR spectra of chlorobenzene solutions were measured in 1.0 mm (ID) capillary tubes under argon.

Autoxidation/Inhibition Procedures. Autoxidations were carried out at 30 °C under 760 Torr of air in a dual-channel oxygen uptake apparatus equipped with a sensitive pressure transducer described previously.[29] The procedures for conditioning the apparatus and conducting a inhibition experiment have been described previously.[30]

Acknowledgment. The authors acknowledge discovery grants from the Natural Sciences and Engineering Research Council of Canada in support of this research. C.S.B. acknowledges the Sumner Foundation for financial support.

Supporting Information Available: (A) General experimental information, procedures, and data for the syntheses of **3**−**5** and **7**−**12**. (B) Traces of ^{13}C NMR spectra of new compounds. (C) Tables S1 and S2 with more details of the experimental kinetic data of reactions with peroxyl radicals. (D) HPLC traces for free-radical oxidations of bilirubin dimethyl ester. This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) Wayner, D. D. M.; Burton, G. W. *Handbook of Free Radicals and Antioxidants in Biomedicine*; CRC Press: Boca Raton, FL, 1989; Vol. 3, pp 223−232.

(30) Barclay, L. R. C.; Vinquist, M. R.; Mukai, K.; Itoh, S.; Morimoto, H. *J. Org. Chem.* **1993**, 5*8*, 7416−7420.