QSAR for the cytotoxicity of 2-alkyl or 2,6-dialkyl, 4-X-phenols: the nature of the radical reaction

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In a continuation of studies on the radical mediated toxicity of phenols to leukemia cells, a set of di- and tri-substituted phenols with mostly alkyl substituents in the *ortho* position were examined. These analogs are similar in structure to the commercial antioxidants BHA and BHT. A QSAR analysis of their growth inhibitory constants led to the formulation of this simple but unusual equation based on 18 congeners:

$$\log 1/C = -0.47E_{S\text{-}2} + 2.42E_R + 2.43; \qquad r^2 = 0.934$$

$E_{S\text{-}2}$ is the Taft steric parameter for the larger of the two *ortho* substituents while E_R is Otsu's radical parameter, which was originally defined to correlate radical reactions.

Introduction

In the last ten years, the phenolic moiety has come under increased scrutiny because of its bewildering array of biological activities *in vitro* and *in vivo*.[1] It appears to act as a potent antioxidant in flavonoids yet it wields considerable toxicity as in the case of *p*-cresol and diethylstilbestrol.[2–4] This unusual behavior of phenols has been attributed to its univalent oxidation to form aryloxyl free radicals. It has also led to numerous comprehensive studies on its biological activity at the molecular and cellular level.[5]

In a recent study on the cytotoxicity of a series of simple and complex mono-substituted phenols *versus* L1210 leukemia cells, the following QSAR 1 was obtained for electron-rich phenols.[5]

Inhibition of growth of L1210 cells by phenols with electron releasing substituents

$$\log 1/C = -0.19(\pm 0.02)\mathrm{BDE} + 0.21(\pm 0.03)\log P + 3.11(\pm 0.010) \quad (1)$$
$$n = 52, \qquad r^2 = 0.920, \qquad s = 0.202, \qquad q^2 = 0.909$$

In eqn. (1), BDE represents the homolytic O–H bond dissociation energy that strongly implicates a radical mechanism of toxicity, while C constitutes the molar concentration of X-phenol that induces 50% inhibition of growth in the cell line (n is the number in the sample, r^2 the correlation coefficient, s the standard deviation and q^2 the cross validated r^2). The BDE term can be effectively replaced by σ^+ as in QSAR 2, but the correlation is less robust ($r^2 = 0.895$). This may be attributed in part to the lack of accurate σ^+ values for complex, estrogenic phenols such as estradiol, estriol, equilin, equilenin, diethylstilbesterol and bisphenol A which required estimation.

$$\log 1/C = -1.35(\pm 0.15)\sigma_P{}^+ + 0.18(\pm 0.04)\log P + 3.31(\pm 0.11) \quad (2)$$
$$n = 51, \qquad r^2 = 0.895, \qquad s = 0.227, \qquad q^2 = 0.882$$

The fact that $\sigma_P{}^+$ cytotoxicity parallels the BDE toxicity can be interpreted in terms of the large radical stabilizing effects of electron donor substituents. Recent analyses of substituent effects on phenoxyl radical formation have shown that electron donating substituents such as OH and NH_2 have lower BDEs than predicted, indicating that they can stabilize the radical *via* spin delocalization.[6,7] Using *ab initio* and density functional theory (DFT), Brinck *et al.* have shown that the ΔBDEs of phenols with electron-withdrawing substituents are mostly determined by the polar stabilization of the parent molecule.[7] The polar effect is generally reduced for the electron-donating phenols and is also found to destabilize the phenol. In these cases, the spin delocalization effect predominates and leads to radical stabilization. Radical effects are smaller and more irregular for electron withdrawing substituents. The success of correlations between BDE values and σ^+ values is consistent with these conclusions. In a similar study, Tomiyama *et al.* demonstrated by a comparison of enthalpy (ΔH) and activation energies (E_a) that electron-donating substituents lower these energies and cause an increase in antioxidant activities whereas electron-withdrawing substituents lead to an increase in the transition state barrier and a decrease in antioxidant activity.[8] A recent extensive survey of radical reactions reveals that Brown's σ^+ parameter is useful in delineating the role of radicals in various reactions; numerous examples of phenoxyl radical formation in chemical as well as biological systems are well correlated by σ^+.[9] Radical formation of di-and poly-substituted phenols is also well defined by a summation of the σ^+ values.

Electron withdrawing substituents on the phenol ring also induced toxicity, albeit much less than their electron donating counterparts. The QSAR developed was considerably different from that of QSAR 1 and 2 as see in QSAR 3.[10]

Inhibition of growth of L1210 cells by phenols with electron withdrawing substituents

$$\log 1/C = 0.62(\pm 0.16)\log P + 2.35(\pm 0.31)$$
$$n = 14, \qquad r^2 = 0.936, \qquad s = 0.191, \qquad q^2 = 0.915 \quad (3)$$

In eqn. (3), C represents the concentration of X-phenol that inhibits cell growth by 50%. Mono-*ortho* substituted phenols

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were fitted well by eqn. (1) and (2).[5] When sequestered alone, these positional isomers showed a strong dependence on σ_P^+ and BDE but lacked any significant dependence on hydrophobic or steric parameters, suggesting that the formation of the *ortho* substituted aryloxyl radical may be mediated by a simple unencumbered radical or the orientation of the hydroxy groups may be such that the hydroxy group points "away" from the substituent and thus no repulsive interaction between the two groups is seen.[11] See eqn. (4) and (5).

$$\log 1/C = -1.50(\pm 0.45)\sigma_P^+ + 3.15(\pm 0.28) \quad (4)$$
$$n = 14, \quad r^2 = 0.816, \quad s = 0.323, \quad q^2 = 0.762$$

$$\log 1/C = -0.17(\pm 0.03)\mathrm{BDE} + 3.18(\pm 0.16) \quad (5)$$
$$n = 14, \quad r^2 = 0.936, \quad s = 0.191, \quad q^2 = 0.915$$

The addition of a steric parameter in QSAR 5 did not improve the correlation; the lack of a substituent in the other *ortho* (6-) position means that steric hindrance was not a factor in the reactivity of the unencumbered 2-X-phenoxyl radical.

Continued interest in the radical forming ability of substituted phenols has led to a study of polysubstituted phenols and in particular, analogs of the widely used antioxidants: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Fig. 1).

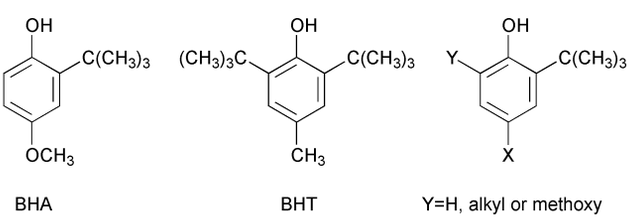

Fig. 1 Structures of BHA, BHT and analogs

BHA and BHT were generally recognized as safe additives in food by the FDA although these antioxidants have been implicated in a number of toxic and carcinogenic events in animals. BHT causes damage in alveolar and pulmonary endothelial cells in the murine lung.[12] It also induces hemorrhagic death and liver necrosis in rats as well as carcinogenesis in mice and rats.[13–15] The carcinogenicity of BHA has been well established in rodents and other experimental animals but there is no data on its carcinogenicity in man.[16–19] Although the toxic and carcinogenic effects of BHT and BHA have been well documented, their mechanisms of action at the molecular level have not been clearly elucidated, in that the role of various substituents on the phenolic ring have not been delineated. This study examines the cytotoxic effects of a series of 4-X-2-alkyl or 4-X-4,6-dialkylphenols *versus* L1210 leukemia cells in terms of their physico-chemical attributes.

Results

The cytotoxicity data in Table 1 have led to the formulation of QSAR 6.

$$\log 1/C = -0.47(\pm 0.10)E_{S-2} + 2.42(\pm 0.70)E_R + 2.43(\pm 0.21) \quad (6)$$
$$n = 18, \quad r^2 = 0.934, \quad s = 0.136, \quad q^2 = 0.904$$
outliers: 2,6-di-OMe; 2-Me-4-NO_2; 2-Me-4-COMe

E_{S-2} is Taft's steric parameter for the larger of the two *ortho* substituents. Since all substituent values of E_{S-2} are negative, the negative coefficient with the E_{S-2} term in QSAR 6 indicates that cytotoxicity is enhanced by larger substituents. Summation of E_{S-2} for the 2 and 6 positions did not yield a significant QSAR. There also appears to be no steric effect wielded by 4-substituents. Using σ^+ in place of E_R in the above QSAR yields a poorer result ($n = 18$, $r^2 = 0.797$, $\rho = 0.54$). If the 2-Me, 4-OMe congener is removed ($n = 17$, $r^2 = 0.862$, $\rho = 0.73$), the correlation is slightly better but the sign of the rho (ρ) value remains positive. The positive ρ associated with QSAR 6 is unusual and in stark contrast to QSAR 1. Otsu's E_R parameter was designed specifically for radical reactions and was based on hydrogen abstraction from substituted cumenes by a polystyryl radical.[20] On the other hand, sigma plus was defined from the solvolysis of X-cumyl chlorides. Nevertheless, its use in correlating radical reactions has been shown to be widespread and appropriate.[9]

The strong dependence of cytotoxicity on E_{S-2} of the mostly hydrophobic substituents on the phenyl ring, led to an examination of the role of hydrophobicity in this phenomenon. Despite the collinearity between calculated partition coefficients (*C*log *P*) and E_{S-2}, ($r^2 = 0.692$), utilization of *C*log *P* in lieu of E_{S-2} as in QSAR 6 gives rise to a much poorer correlation as seen in QSAR 8.

$$\log 1/C = 0.26(\pm 0.12)C\log P + 2.59(\pm 0.50) \quad (7)$$
$$n = 18, \quad r^2 = 0.593, \quad s = 0.326, \quad q^2 = 0.464$$

Hydrophobicity in tandem with E_R leads to the following QSAR:

$$\log 1/C = 0.21(\pm 0.09)C\log P + 2.30(\pm 1.24)E_R + 2.57(\pm 0.36) \quad (8)$$
$$n = 18, \quad r^2 = 0.800, \quad s = 0.236, \quad q^2 = 0.638$$

The significant differences in r^2 (0.134) and q^2 (0.266) for QSAR 6 and 8 clearly establish the significance of steric attributes in the generation of cytotoxicity.

Recently, Garg *et al.*[21,22] have examined the various types of toxicity wielded by X-phenols and sequestered them into three main groups: toxicities governed by hydrophobicity alone, by hydrophobicity and sigma minus parameters and by hydrophobicity and sigma plus parameters. Examination of this data using these approaches led to the formulation of QSAR 9 and 10 which again, in terms of statistical significance, pale by comparison to QSAR 6.

$$\log 1/C = 0.23(\pm 0.09)C\log P + 0.68(\pm 0.36)\sigma^- + 2.88(\pm 0.39) \quad (9)$$
$$n = 18, \quad r^2 = 0.806, \quad s = 0.233, \quad q^2 = 0.655$$

$$\log 1/C = 0.25(\pm 0.10)C\log P + 0.57(\pm 0.50)\sigma^+ + 2.98(\pm 0.56) \quad (10)$$
$$n = 18, \quad r^2 = 0.710, \quad s = 0.285, \quad q^2 = 0.400$$

From a study of these hydrophobicity and Hammett sigma based QSAR 7–10, it is clear that these particular *ortho* alkyl substituted phenols behave in a fashion that deviates from most other phenols.

The three outliers 2,6-$(OCH_3)_2$-phenol, 2-CH_3-4-NO_2-phenol and 2-CH_3-4-acetylphenol are mispredicted by a factor greater than four. The deviations of these three analogs are more than three times the standard deviation of the equation (QSAR 6). The 2,6-$(OCH_3)_2$ phenol is more active than predicted and is also the only substituted phenol with alkoxy groups in both *ortho* positions. This could be related to the conformations of these phenols.[11] Ingold *et al.* have shown *via* computational and experimental data that steric repulsion exerted by *ortho* methyl or *ortho tert*-butyl groups is not sufficient to force the OH bond

Table 1 Cytotoxicity data and physico-chemical parameters of 2-alkyl and 2,6-dialkyl-4-X-phenols

Compound number	Substituent	log 1/*C*							
		Observed	Predicted[a]	Deviation	E_R	E_{S-2}	σ^+	Relative BDE[b]/ kcal mol^{-1}	*C*log *P*[c]
1	2,6-di-Me	3.02	3.16	−0.14	0.06	−1.24	−0.62	−4.30	2.37
2	2,6-di-OMe[d]	3.86	3.22	0.64	0.22	−0.55	−1.56	−5.80	1.11
3	2,4,6-tri-Me	3.20	3.23	−0.03	0.09	−1.24	−0.93	−5.90	2.87
4	2,6-di-CMe_3	3.85	3.88	−0.03	0.06	−2.78	−0.52	−8.00	4.93
5	2,6-di-CMe_3-4-Me	4.04	3.95	0.09	0.09	−2.78	−0.83	−9.70	5.43
6	2,6-di-C_2H_5	3.26	3.19	0.07	0.06	−1.31	−0.60	−4.90	3.43
7	2,6-di-$CHMe_2$	3.25	3.38	−0.13	0.06	−1.71	−0.56	−5.80	3.93
8	2,4,6-tri-CMe_3	3.90	3.95	−0.05	0.09	−2.78	−0.78	−9.30	6.75
9	2-CMe_3-6-Me	3.73	3.88	−0.15	0.06	−2.78	−0.57	−5.40	3.65
10	2,6-di-CMe_3-4-NO_2	4.90	4.87	0.03	0.47	−2.78	0.27	−3.70	5.31
11	2,6-di-CMe_3-4-C_2H_5	3.91	3.95	−0.04	0.09	−2.78	−0.82	−9.50	5.96
12	2,6-di-CMe_3-4-Br	4.11	4.17	−0.06	0.18	−2.78	−0.37	−7.90	6.09
13	2,4-di-CMe_3	4.24	3.88	0.37	0.06	−2.78	−0.52	−4.20	5.03
14	2-CMe_3-4-Me	3.80	3.88	−0.08	0.06	−2.78	−0.57	−4.50	3.70
15	2,4-di-Me	3.04	3.16	−0.12	0.06	−1.24	−0.62	−3.50	2.42
16	2-Me-4-F	3.09	2.92	0.18	−0.04	−1.24	−0.38	−3.70	2.36
17	2-Me-4-NO_2[d]	3.49	4.08	−0.57	0.44	−1.24	0.48	2.40	2.30
18	2-Me-4-Br	3.46	3.37	0.09	0.15	−1.24	−0.16	−2.10	3.08
19	2-Me-4-OMe	3.39	3.35	0.04	0.14	−1.24	−1.09	−6.90	2.02
20	2-Me-4-COMe[d]	3.14	3.66	−0.52	0.27	−1.24	0.33	—	1.90
21	2-CMe_3-4-C_2H_5	3.80	3.88	−0.08	0.06	−2.78	0.16	−4.40	4.23

[a] Predicted using QSAR 6. [b] Relative to phenol, BDE = 0. [c] Calculated using *C*log *P* Version 4.0. [d] Data points omitted in formulation of QSAR 6

out of the plane of the aromatic ring. Thus the phenolic OH hydrogen can "tuck" itself between two out of plane H or CH_3 groups while the third H or CH_3 group can lie in the plane of the aromatic ring. This manoeuvring destabilizes the phenol relative to the phenoxyl radical. In the case of the BHA/BHT analogs, compounds **1** and **3**–**21** would be subject to similar changes in geometry.

Compound **2** is an anomaly in terms of its conformational constraints and its biological activity. *o*-Methoxyphenols have two conformations—one with the methoxy group facing the phenolic group and one with it in the away position. The former position is stabilized by internal hydrogen bonding. For *o*,*o*-dimethoxyphenols in which internal hydrogen bonding must occur, one methoxy group would point away from and one towards the OH moiety. However, in the corresponding radical, both groups would point away from the phenoxyl radical's oxygen atom leading to its destabilization and perhaps its enhanced reactivity. The aberrant behavior of compounds **17** (2-Me-4-NO_2) and **20** (2-Me-4-$COCH_3$) cannot be explained; the nitro and acetyl groups may be subject to enhanced metabolism in the cells.

Further insight on the reactions of radicals with 2,6-di-*tert*-butylphenols with substituents in the 4-position is revealed by the following examples: QSARs 11–14.

Hydrogen abstraction from 4-X-2,6-di-tert-butylphenols by 5-butylperoxyl radicals in isopentane at −37 °C.[23]

$$\log k_2 = -1.09(\pm 0.32)\sigma^+ - 0.54(\pm 0.15)$$
$$n = 8,\quad r^2 = 0.992,\quad s = 0.166,\quad q^2 = 0.854\quad \textit{outlier:}\ \text{4-Cl} \qquad (11)$$

Hydrogen abstraction from 4-X-2,6-di-tert-butylphenols by styrene peroxide radicals at 65 °C.[24]

$$\log k_{rel} = -1.15(\pm 0.22)\sigma^+ + 0.21(\pm 0.11)$$
$$n = 7,\quad r^2 = 0.974,\quad s = 0.114,\quad q^2 = 0.952 \qquad (12)$$

Hydrogen abstraction from 4-X-2,6-di-tert-butylphenols by tetralin peroxide (1,2,3,4-tetrahydro-1-naphthyl hydroperoxide) radicals at 65 °C.[24]

$$\log k_{rel} = -1.34(\pm 0.23)\sigma^+ + 0.09(\pm 0.12) \qquad (13)$$
$$n = 7,\quad r^2 = 0.979,\quad s = 0.119,\quad q^2 = 0.950\quad \textit{outlier:}\ \text{4-}C_6H_5$$

Photooxidation quenching of singlet oxygen from 4-X-2,6-di-tert-butylphenols in methanol.[25]

$$\log k = -1.68(\pm 0.28)\sigma^+ + 6.12(\pm 0.07) \qquad (14)$$
$$n = 7,\quad r^2 = 0.979,\quad s = 0.076,\quad q^2 = 0.968\quad \textit{outlier:}\ \text{4-}C_6H_5$$

These examples illustrate that for a variety of radical reactions under differing conditions, σ^+ gives excellent correlations with negative ρ values. Note the lack of a steric parameter in QSARs 11–14. This is attributed to the constancy of the steric effect in the 2 and 6 positions where all the congeners bear bulky *tert*-butyl groups in these positions. Variation in the steric effect at the *para* position has no impact on phenoxyl radical formation. These ρ^+ values are contradictory to those observed with the BHA and BHT analogs. It is apparent that the most bulky multi-substituted phenols of QSAR 6 are behaving in a manner distinct from those supporting QSAR 1 or QSAR 11–14. These results suggest that the use of E_R is more appropriate in a sterically demanding environment.

Discussion

In QSAR 6 there are two nitro containing congeners one of which is well fitted, while the other having only one small *ortho* substituent (2-Me, 4-NO_2) is poorly fitted. This latter compound behaves more like those of QSAR 1 where the cytotoxicities of simple phenols having only one *ortho* substituent are not well predicted unless log *P* is set to zero (QSAR 4). An attempt to combine the simple mono-substituted *ortho* phenols of QSAR 4 with the polysubstituted phenols in QSAR 6 resulted in a poor correlation. In QSAR 6, E_{S-2} is the most significant parameter accounting for 70% of the variance while E_R accounts for 23%. There are other radical parameters, but none that cover as wide a range as E_R.[26] Use of $\sigma^\bullet$ defined by Cleary *et al.*[27] reduces the data set to 14 data points because of the paucity of established $\sigma^\bullet$ values. Use of $\sigma^\bullet$ in lieu of E_R yields r^2 of 0.902, with $\rho = +1.54$, which is essentially the same quality of fit as E_R. The strong dependence on Otsu's E_R and Cleary's $\sigma^\bullet$ suggests that these reactions may be radical mediated. The *ortho*-substituted complex phenols in the current study behave quite differently from the phenols that have been studied previously (QSAR 1 and 2), and even from the small set of *ortho*-substituted electron-rich phenols that were examined

later (QSAR 4 and 5). This difference in behavior is difficult to explain, but it is not unexpected. Radical-mediated phenolic toxicity is a multi-step process and large structural variations may alter the nature of the rate-determining step and may even lead to a change in mechanism. It is possible that different biological radicals are responsible for activation of sterically hindered phenols, and the resulting hindered phenoxyl radicals may interact with a different set of cellular targets than their less hindered congeners.

An example of how *ortho* substituents affect the magnitude, if not the type, of substituent effect is given by phenol ionization (QSAR 15–17).

Ionization of 4-X-2,6-di-tert-butyl phenols in 50 aqueous methanol.[28]

$$\begin{aligned} &\mathrm{p}K_a = -4.76(\pm 0.57)\sigma^- + 14.3(\pm 0.42) \\ &n = 10, \quad r^2 = 0.979, \quad s = 0.385, \quad q^2 = 0.961 \quad (15) \\ &\textit{outliers:}\ \ \mathrm{COO^-},\ \mathrm{SO_3^-},\ \mathrm{N(Me)_3^+},\ \mathrm{NO} \end{aligned}$$

Ionization of simple X-phenols in 51 aqueous ethanol.[29]

$$\begin{aligned} &\mathrm{p}K_a = -2.44(\pm 0.28)\sigma^- + 11.15(\pm 0.13) \\ &n = 15, \quad r^2 = 0.964, \quad s = 0.202, \quad q^2 = 0.924 \quad (16) \\ &\textit{outlier:}\ \ \mathrm{4\text{-}I} \end{aligned}$$

Ionization of simple phenols in aqueous solution.[30]

$$\begin{aligned} &\mathrm{p}K_a = -2.15(\pm 0.22)\sigma^- + 9.81(\pm 0.19) \\ &n = 9, \quad r^2 = 0.987, \quad s = 0.145, \quad q^2 = 0.981 \quad (17) \end{aligned}$$

The intercepts of these equations show that 2,6-disubstituted phenols are considerably less acidic [eqn. (15)] than less hindered phenols [eqn. (16), (17)] even after solvent effects on pK_a are taken into account. The loss of acidity can be attributed to steric hindrance causing loss of solvation (loss of hydrogen bonding) in the 2,6-disubstituted phenoxide anions. The same factor also explains the enhanced effect that substituents have on the ionization of 2,6-disubstituted phenols [compare slopes for eqn. (15)–(17)]. Loss of hydrogen bonding promotes charge delocalization in the phenoxide anion, making this anion more susceptible to substituent effects. QSAR 15–17 highlight the difficulty of proton loss in ionization. A similar problem may occur in H$^\bullet$ abstraction.

It has been well established by Bradbury and Lipnick that a large number of simple organic molecules may be classified as narcotics.[31] Narcosis may be classified into two types: Type 1, nonpolar narcosis and Type 2 designated as polar narcosis.[32] Nonspecific toxicity which is generally exhibited by nonpolar narcotics is directly proportional to the concentration at the site of action which is usually the membrane. This toxicity can be directly predicted from their hydrophobicities by QSAR. This is seen in QSAR 7. However, in this case the toxicity is greater than this baseline approach since only 59% of the variance in the data can be explained by hydrophobicity. Thus it is clear that the phenols in this case are not acting *via* a Type 1 narcosis. Extensive studies of phenols acting *via* a Type 2 polar narcosis mechanism of action have been carried out by Cronin and Schultz who developed a two-parameter approach (log P and E_{LUMO}) to deal with the "excess" toxicity of phenols.[33,34] This model provides a mechanistic basis for toxicity in terms of partitioning and bioreactivity. A similar approach to the results in Table 1 resulted in the formulation of QSAR 9, which only explains 80% of the variance in the data. This suggests that other interactions are at play as indicated by QSAR 6 where steric and radical reactivity parameters are significant.

The ambiguity of the different roles of σ^+ and E_R in the correlation of radicals warrants further extensive investigation. A number of instances where E_R is a better measure of substituent effects have been identified (ref. 9, Table 7) but no clear guidelines emerge to establish what structural features select for one parameter over the other. The current study suggests that transition state geometry, especially steric hindrance, may be important. Crowding around the phenolic OH affects the ease of H$^\bullet$ removal, the lifetime of the phenoxyl radical, and the ease of phenoxyl attack on cellular targets, and any one or more of these factors may alter the nature of substituent effects.

The properties of the OH bond appear to be playing a significant role in the reactivities of phenolic compounds and their subsequent cytotoxicities. Despite the large number of newer experimental techniques and the design of computational tools that have addressed the kinetic behavior and thermodynamic stabilities of mono, di, tri and multisubstituted phenols, there are still derivatives that defy characterization such as 2,6-dimethoxyphenol.[35] In our cytotoxicity study, this phenol is also an outlier. Perhaps its electronic/steric attributes have not been adequately delineated.

Attempts to derive meaningful QSAR of multisubstituted phenols with BDE or σ^+ yielded poor results even after removing all but five of nine such compounds. The fact that 2,4-disubstituted phenols fitted QSAR 6 while 2-substituted phenols did not, suggests that E_R is better suited for sterically hindered 2,4,6-trisubstituted phenols. Clearly the toxicity of phenols is a multifaceted problem where the dependent variable log 1/C may not be uniform. Until this can be established one cannot gather all phenols together to obtain a single meaningful equation. However, QSAR 1 and 6 provide considerable insight into a complex problem and will provide valuable guidance for future studies.

Experimental

Melting points were determined on an electrothermal melting point apparatus (MEL–TEMP II with digital thermometer). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX 400 MHz NMR spectrometer with TMS as the internal standard; chemical shifts are given in δ (ppm) and coupling constants, J, in MHz. Infrared (IR) spectra were recorded on a Perkin Elmer 1600 series FTIR and only principal, sharply defined IR peaks are reported. Mass spectra were obtained from GC/MS data from a Hewlett Packard GC/MS system HP 6890 series with a mass selective detector. Thin-layer chromatography was performed on silica gel plates (silica gel IB-F Baker). Data for chemical elemental analysis were obtained from Desert Analytics (Tucson, AZ).

Materials

Most of the phenols are commercially available compounds except: 2,6-di-*tert*-butylhydroquinone (2,6-di-*tert*-butyl-4-hydroxyphenol), 2,6-di-*tert*-butyl-4-acetoxyphenol; 2,6-di-*tert*-butyl-4-nitrophenol; 2,6-diethylphenol; 2-methyl-4-nitrophenol; 4-methoxy-2-methylphenol and 4-bromo-2-methylphenol, which were synthesized as follows.

Syntheses

2,6-Di-*tert*-butylhydroquinone. 2,6-Di-*tert*-butylhydroquinone was synthesized by the oxidation of 4-hydroxy-3,5-di-*tert*-butylbenzaldehyde with hydrogen peroxide. Commercially available 4-hydroxy-3,5-di-*tert*-butylbenzaldehyde (1.93g; 8.25 mmol) was dissolved in pyridine (7 ml) and treated with aqueous potassium hydroxide (500 mg of KOH dissolved in 1 ml of H_2O) under nitrogen. Hydrogen peroxide (1.5 ml, 30% v/v) was added dropwise to the reaction mixture over a period of 30 min with constant stirring under nitrogen. After the addition of H_2O_2, the reaction mixture was refluxed with constant stirring

for 40 min under nitrogen and then cooled to room temperature. The reaction mixture was poured into 6 M HCl (18 ml) and extracted with ether. The ether layer was washed with water twice and dried over $MgSO_4$ (anhyd.). The solvent was removed and the crude product was crystallized from petroleum ether (bp 35–60 °C) After three consecutive crystallizations, the pure product of 2,6-di-*tert*-butylhydroquinone (1.1 g; 60.11%) was obtained, mp 104–105 °C;[36–38] δ_H (400 MHz; DMSO-d_6) 1.35 (18H, s, 6 CH_3), 6.3 (1H, s, OH), 6.5 (2H, s, Ph), 8.55 (1H, s, OH); δ_C (400 MHz, DMSO-d_6) 30.775 (6CH_3), 34.934 (2-C<), 111.362 (C3 & C5), 141.558 (C2 & C6), 146.344 (C1), 150.726 (C4); ν_{max}/cm^{-1} 3343.5 (OH), 3632.8 (OH); *m*/*z* 223 (M^+ + 1, 6%), 222 (M^+, 39), 207 (100), 82 (21), 57 (68) (Calc. $C_{14}H_{22}O_2$; C, 75.68; H, 9.91. Found: C, 75.39; H, 9.88%).

2,6-Di-*tert*-butyl-4-acetoxyphenol. Commercially available 2,6-di-*tert*-butyl-*p*-benzoquinone (550 mg, 2.5 mmol) was dissolved in acetic anhydride (3 ml) and Zn dust (150 mg) was added followed by pyridine (0.5 ml). The reaction mixture was stirred at room temperature for 3 hours and filtered. To the filtrate, water (5 ml) was added and stirred for 30 minutes. The crude product precipitated, and was washed with water and dried. It was crystallized from petroleum ether (bp 35–60°) to give 2,6-di-*tert*-butyl-4-acetoxyphenol (400 mg, 60.5%), mp 90–95.5 °C;[39,40] δ_H (400 MHz; DMSO-d_6) 1.4 (18H, s, 6CH_3), 2.25 (3H, s, OCOCH_3), 6.82 (2H, s, Ph), 7.05 (IH, s, OH); δ_C (400 MHz; DMSO-d_6) 19.195 (CH_3), 28.465 (6CH_3), 32.954 (2-C<), 115.799 (C3 & C5), 139.084 (C2 & C6), 141.865 (C4), 149.626 (Cl), 167.967 (CO); ν_{max}/cm^{-1} 3587.1 (OH); *m*/*z* 265 (M^+ + 1, 3%), 264 (M^+, 12), 222 (94), 207 (100), 57 (54) (Calc. $C_{16}H_{24}O_3$; C, 72.73; H, 9.09. Found: C, 72.75; H, 9.07%).

2,6-Di-*tert*-butyl-4-nitrophenol. 2,6-Di-*tert*-butyl-4-nitrophenol was synthesized by the nitration of 2,6-di-*tert*-butylphenol with CH_3COOH–HNO_3 in cyclohexane. A mixture of CH_3COOH–HNO_3 (3 ml; 1 : 1 v/v) was added dropwise to a stirred solution of 2,6-di-*tert*-butylphenol (5.15 g; 25 mmol) in cyclohexane (15 ml) by maintaining the temperature at 10–20 °C. The reaction mixture was further stirred at this temperature for 10 min and the precipitate obtained was filtered, washed with water and dried. The crude product was crystallized from petroleum ether and gave the purified product as white needles (3.74 g; 59.6%); mp 155–156 °C;[41] δ_H (400 MHz; DMSO-d_6) 1.33 (18H, s, 6CH_3), 7.9 (2H, s, Ph), 8.45 (1H, s, OH); δ_C (400 MHz; DMSO-d_6) 29.911 (6CH_3), 35.033 (2-C<), 120.907 (C3 & C5), 139.384 (C2 & C6), 142.82 (C4), 157.53 (Cl); ν_{max}/cm^{-1} 3626.5 (OH); *m*/*z* 252 (M^+ + 1, 3.08%), 251 (M^+, 17), 237 (14), 236 (100), 208 (29.3) (Calc. $C_{14}H_{21}NO_3$; C, 66.93; H, 8.36; N, 5.57. Found: C, 67.17; H, 8.21; N, 5.66%).

2,6-Diethylphenol. 2,6-Diethylphenol was synthesized from commercially available 2,6-diethylaniline by a diazo reaction.[42] The product was obtained in 45.3% yield, mp 38 °C;[43] δ_H (400 MHz; DMSO-d_6) 1.115 (t, *J* = 7.75, 6H, 2CH_3), 2.574 (q, 4H, 2CH_2), 6.70 (t, *J* = 7.73, 1H, Ph), 6.95 (d, *J* = 6.97, 2H, Ph), 8.07 (s, 1H, OH); δ_C (400 MHz; DMSO-d_6) 14.773 (2CH_3), 23.284 (2CH_2), 119.843 (C4), 126.741 (C3 & C5), 130.76 (C2 & C6), 152.373 (C1); ν_{max}/cm^{-1} 3362 (OH); *m*/*z* 151 (M^+ + 1, 4.6%), 150 (M^+, 41.58), 135 (100), 121 (35.44), 91 (33.9); (Calc. $C_{10}H_{14}O$; C, 80; H, 9.33. Found: C, 79.43; H, 9.27%).

2-Methyl-4-nitrophenol. 2-Methyl-4-nitroaniline was converted into 2-methyl-4-nitrophenol by the diazonium method.[42] The crude product was crystallized from a 1 : 1 mixture of CCl_4–petroleum ether in 65.4% yield; mp 94 °C;[44,45] δ_H (400 MHz; DMSO-d_6) 2.1 (s, 3H, CH_3), 6.9 (d, *J* = 6.7, 1H, Ph), 7.92 (d, *J* = 6.7, 1H, Ph), 8.1 (s, 1H, Ph), 11.0 (br s, 1H, OH); δ_C (400 MHz; DMSO-d_6) 16.068 (CH_3), 114.915 (C6), 124.042 (C5), 125.794 (C3), 126.64 (C2), 139.576 (C4), 162.52 (C1); ν_{max}/cm^{-1} 3461.4 (OH); *m*/*z* 154 (M^+ + 1, 9.5%), 153 (M^+, 100), 123 (35), 77 (84).

4-Methoxy-2-methylphenol. 4-Methoxy-2-methylphenol was obtained from the diazotization of 4-methoxy-2-methylaniline.[42] The crude product was crystallized from a 1 : 1 mixture of CH_2Cl_2–petroleum ether in 58% yield; mp 64–65 °C;[44] δ_H (400 MHz; DMSO-d_6) 2.0 (s, 3H, CH_3), 3.55 (s, 3H, OCH_3), 6.47 (d, *J* = 2.8, 1H, Ph), 6.58 (d, *J* = 8.5, 2H, Ph), 8.67 (s, 1H, OH); δ_C (400 MHz; DMSO-d_6) 17.155 (CH_3), 54.817 (OCH_3), 112.281 (C5), 114.772 (C3), 115.941 (C6), 124.971 (C2), 149.446 (C1), 152.234 (C4); ν_{max}/cm^{-1} 3280 (OH); *m*/*z* 139 (M^+ + 1, 9.5%), 138 (M^+, 90.5), 123 (100), 95 (15), 77 (19), 67 (24).

4-Bromo-2-methylphenol. 4-Bromo-2-methylphenol was also synthesized by a diazonium reaction of 4-bromo-2-methylaniline and the crude product was crystallized from petroleum ether in 32.1% yield.[42] Mp 61 °C;[44] δ_H (400 MHz; DMSO-d_6) 2.09 (s, 3H, CH_3), 6.72 (d, *J* = 8.4, 1H, Ph), 7.13 (d, *J* = 8.4, 1H, Ph), 7.22 (s, 1H, Ph), 9.62 (s, 1H, OH); δ_C (400 MHz; DMSO-d_6) 16.942 (CH_3), 110.93 (C4), 117.758 (C6), 128.136 (C2), 130.387 (C5), 133.993 (C3), 156.057 (C1); ν_{max}/cm^{-1} 3260.8 (OH); *m*/*z* 188 (M^+ + 1, 81.8%), 187 (M^+, 15.2), 186 (87.9), 107 (100), 77 (72.8).

Cytotoxicity evaluation

L1210 cells were maintained in asynchronous logarithmic growth at 37 °C in RPMI medium with L-glutamine supplemented with 10% (v/v) FBS. All stock solutions and dilutions were made in unsupplemented RPMI medium.

Cell cultures were seeded at 2–5 × 10^4 cells ml^{-1} in duplicate for each inhibitor concentration in a 96 well microtiter plate (180 µl per well). The test compounds (20 µl) were then added to the cell cultures in 1 : 10 dilution in order to achieve the desired concentration. Each inhibitor was tested at a minimum of 8 concentrations. After 48 hours of continuous drug exposure, the cells were counted by using the CyQUANT GR assay kit from Molecular Probes. For this purpose, the medium was removed from the plates which were then frozen at −80 °C for a minimum of 1 h. The cells were thawed at 37 °C and 200 µl of CyQUANT GR dye/cell lysis buffer was added to each well. The plates were incubated for 5 minutes at 37 °C and their fluorescence was measured using a Cytofluor II multiwell fluorescence plate reader. The excitation maximum was 485 nm and the emission maximum was 530 nm. From the data, a dose response curve was drawn and the IC_{50} determined. The CyQUANT GR assay measures the ability of CyQUANT GR dye to bind to the cellular nucleic acids of viable cells. Cytotoxicity is expressed as the concentration of the phenol (IC_{50}) that causes a 50% reduction in fluorescence as compared with the controls.[46] Physicochemical constants were then utilized to formulate a quantitative structure–activity relationship (QSAR) for these compounds.

Computational methods

BDE was defined as the energy of the following reaction: X–PhOH + PhO$^\bullet$ ⟶ X–PhO$^\bullet$ + PhOH using RB3LYP/6-31G** energies for the phenols and UB3LYP/6-31G** energies for the radicals (obtained using Jaguar 4.1, "accurate" pseudospectral grids, numerical errors <0.2 kcal mol^{-1}).[47,48] Initial phenol geometries were obtained from an automatic conformer search (MMFF force field[48] using PC Spartan Pro, v. 1.0.5)[49] followed by RB3LYP/6-31G** geometry optimization of each conformer. The lowest energy conformer (after RB3LYP optimization) was used to calculate BDE and to generate the initial radical geometry.

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