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Organic synthesis, antibacterial evaluation, and quantitative structure-activity relationships (QSAR) of cosmetic preservatives related to 5-bromo-5-nitro-1,3-dioxane. I. Aliphatic analogs

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Synopsis

An aliphatic series of 2-substituted 5-bromo-5-nitro-1,3-dioxane analogs has been designed systematically to explore electronic, lipophilic, and steric requirements for optimal antibacterial activity. The compounds were prepared via the acid-catalyzed condensation of 2-bromo-2-nitropropan-1,3-diol with the appropriate aldehyde or ketone. Although most compounds exhibited rather poor antibacterial activity against *Staphylococcus aureus*, a number of the derivatives exhibited faster rates of inactivation of *Pseudomonas aeruginosa* than either the 5-bromo-5-nitro-1,3-dioxane (Bronidox®) or 2-bromo-2-nitropropan-1,3-diol (Bronopol®) controls.

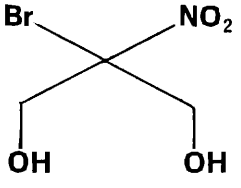
The 2-*n*-propyl and 2,2-diethyl analogs were the most active aliphatic derivatives tested *in vitro*. The joint application of quantitative antibacterial activity data (i.e., D-values) and computer-assisted multiparameter regression analysis (i.e., Hansch QSAR) demonstrated the importance of steric and/or lipophilic influences in determining antibacterial efficacy of the aliphatic analogs vs. *P. aeruginosa*. The aliphatic congener predicted by QSAR to possess optimal anti-*Pseudomonas* activity (i.e., the 2,2-dimethyl analog) was tested in a proprietary white lotion formula and found to possess a high order of preservative efficacy.

This work demonstrates the value of using QSAR to evaluate antibacterial activity data and to predict the member of a congeneric series possessing optimal levels of activity.

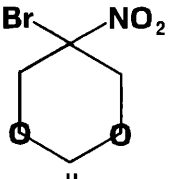
INTRODUCTION

Cosmetic manufacturers are required to demonstrate the safety and efficacy of their products. Preservative efficacy testing is performed to determine the type and concentration of preservative best suited to insure that a given product will not support microbial growth and become a health hazard (1,2). The variables that must be considered in preservative efficacy testing were reviewed in a recent article (3). Although each occurence of contamination of a finished product must be considered to determine the source of the problem, it is believed that most product contamination occurs when manufacturers fail to produce their products in accordance with good manufacturing practices or when the preservative system is inadequate.

The occurence of microorganisms in finished cosmetic products, either due to contamination during manufacture, adaptation of organisms to the preservative system, use of an inadequate preservative system, or contamination of a finished product by the consumer, has provided the impetus for chemists to continue their search for new, more effective preservatives. In the past few years, nitro- and halonitro-alcohols and their derivatives such as 2-bromo-2-nitro-1,3-propanediol (I) and 5-bromo-5-nitro-1,3-dioxane (II), have been investigated and, in some cases, used as cosmetic preservatives. Both I and II are reported to be quite effective against gram-negative bacteria, whereas only II exerts appreciable inhibitory activity against yeasts and molds (4,5). The solubility and partitioning properties of I and II are, in general, complementary to one another such that I is predominantly hydrophilic while II is predominantly hydrophobic.



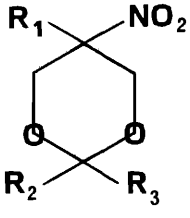
I



II

Fortunately for the commercial utility of I and II, their respective antimicrobial activities are fully manifest in most cosmetic preparations at concentrations of 0.1%–0.2%. One characteristic that limits the usefulness of I is that the observed antimicrobial activity decreases as the pH is increased from 6–8 (4). The antimicrobial activity of II is relatively constant over the pH range of 5–9 (5).

Lappas, Hirsch, and Winely (6) synthesized a number of structural analogs of II and evaluated their antimicrobial activity. Virtually all of the analogs possessing significant antimicrobial activity exhibited the gem-bromonitro moiety at position 5 (i.e., $-R_1=$ Br, III). They also found that monosubstituted aliphatic side-chain derivatives at position 2 (i.e., $R_2=$ C_nH_{2n+1} and $R_3=$ H) were more active than analogous disubstituted aliphatic derivatives. Although there seemed to be some indication that antibacterial activity falls off between the methyl (i.e., $R_1=$ Br, $R_2=$ CH_3 and $R_3=$ H) and the *n*-decyl (i.e., $R_1=$ Br, $R_2=$ *n*-$C_{10}H_{21}$ and $R_3=$ H) analogs, there was no indication as to what the most active analog in the series was, or whether the observed changes in biological activity were related to lipophilic or steric effects. The monosubstituted aromatic side-chain derivatives examined (i.e., $R_2=$ Ar, $R_3=$ H) exhibited antimicrobial activities which approached or surpassed the I controls. They also concluded that no relationship existed between observed levels of antimicrobial activity and percent water solubility. Unfortunately, too few derivatives were synthesized and evaluated to enable quantitative structure-activity correlations to be determined.



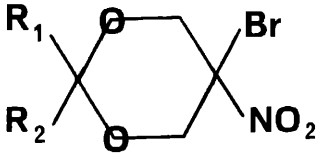
III

The purposes of the work reported here were twofold. Our primary goals were to synthesize a larger set of analogs of II, encompassing a wider range of hydrophobic, steric, and electronic properties than those previously prepared by Lappas *et al.* (6), to assess the quantitative rates of inactivation of selected test microorganisms, and to determine such structure-activity relationships (SAR) as might manifest themselves. A secondary goal was to ascertain whether the analog of II, predicted by SAR studies and found to be the most active compound in the congeneric series, would exhibit this enhanced level of antimicrobial activity in an actual cosmetic formulation (i.e., a lotion), relative to II itself. The present paper describes our results with a series of aliphatic II analogs; a future manuscript will discuss the structure-activity relationships in the corresponding aromatic series of II analogs.

MATERIALS AND METHODS

SERIES DESIGN

A series of II analogs, substituted in the 2-position by various aliphatic substituents were used. These analogs are listed below.



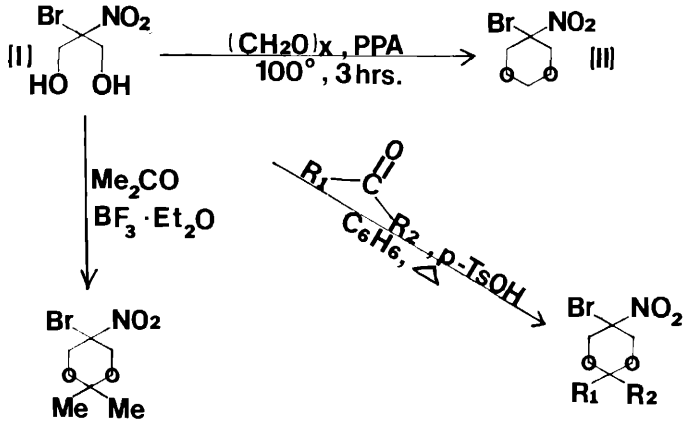
(R_1,R_2) = (H,H), (Me,H), (Et,H), (*n*-Pr), (*n*-Bu,H), (*n*-Am,H), ($c-C_6H_{11}$,H), (Me,Me), (Me,Et) (Et,Et), (*n*-Pr,Et), (-[CH_2]$_4$-), (-[CH_2]$_5$-)

ORGANIC SYNTHESIS

All starting materials and solvents were reagent grade and were obtained from Aldrich Chemical Co. or Eastman Kodak Co. Paraformaldehyde was purchased from Matheson, Coleman & Bell Manufacturing Chemists. Melting points were determined on a Mel-Temp® apparatus (Laboratory Devices, Inc.) and are uncorrected. The infrared (ir) spectra were determined as neat oils or as Nujol mulls using a Perkin-Elmer Model 700 spectrophotometer. The 60 MHz proton magnetic resonance (nmr) spectra were recorded on a Varian T-60 nuclear magnetic resonance spectrophotometer in benzene-d_6 or acetone-d_6, with Me_4Si as an internal standard. The 70 eV mass spectra were determined on an RMU-7 mass spectrophotometer. Microanalyses were performed by Midwest Microlab, Ltd. (Indianapolis) for all new compounds and were within $\pm 0.4\%$ of theoretical values, unless otherwise indicated.

Compound II was prepared via the polyphosphoric acid (PPA)-catalyzed condensation of I and paraformaldehyde (6), while 5-bromo-5-nitro-2,2-dimethyl-1,3-dioxane was obtained by the boron trifluoride etherate-catalyzed condensation of I and acetone (6). All other analogs of II were prepared via the p-toluenesulfonic acid-catalyzed conden-

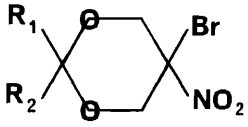
sation between I and the appropriate aliphatic aldehyde or ketone, allowing for azeotropic distillation and collection of evolved water by use of a Dean-Stark trap (9, 10). These reactions are shown below:



(1–3)

Physical properties and yields of the 2-substituted analogs of II are presented in Table I. A representative example of a synthetic procedure is given in the following:

Table I
2-Alkyl-5-Bromo-5-Nitro-1,3-Dioxanes: Physical Properties & Antibacterial Activity



No.#	R_1	R_2	% Yield	mp °C or bp °C (torr)	D-Values (hr)*	
					S. aureus	*P. aeruginosa*
1	H	H	55	57–60[a,b]	2.2	<0.6
2	CH_3	H	81	53.5–55 (0.030)[a]	4.3	<0.6
3	CH_3CH_2	H	48	56–59[a]	<3.2	<0.8
4	$CH_3(CH_2)_2$	H	63	44–47; 77–78 (0.04)[a]	2.2	<0.6
5	$CH_3(CH_2)_3$	H	56	81–84 (0.04)	3.3	<0.6
6	$CH_3(CH_2)_4$	H	100	91–93 (0.035)	6.0	3.5
7	c-C_6H_{11}	H	68	103–106	<5.8	3.5
8	CH_3	CH_3	71	80–83[a]	3.5	<0.6
9	CH_3	CH_3CH_2	89	70–72 (0.035)	5.2	<0.6
10	CH_3CH_2	CH_3CH_2	66	42–45	4.0	<0.6
11	CH_3CH_2	$CH_3(CH_2)_2$	84	81–83 (0.035)	<2.2	2.2
12	$-(CH_2)_4-$		72	76–79	5.5	<0.6
13	$-(CH_2)_5-$		71	88–91[a]	4.7	22.2
	Bronopol®				3.5	1.2
	Propylene Glycol				9.2	34.9
	Saline				21.2	45.6

All compounds had ir and nmr spectra consistent with assigned structures.
* 0.10% solutions in 10% propylene glycol/saline.
[a] mp 49–50°C, 58–59°C, 79–81°C, 86–89°C, and bp 73–75°C (0.005 torr), 72°C (0.005 torr) reported (66) for compounds 1, 3, 8, 13, 4, and 2, respectively; [b] mp 58–60°C reported (5) for compound 1.

5-BROMO-5-NITRO-2-CYCLOHEXYL-1,3-DIOXANE [7]

A three-neck round bottom flask, equipped with heating mantle-Variac®, magnetic stirrer, equilibrating side-arm addition funnel, and Friedrich Condenser/Dean-Stark trap combination was charged with 80 mL of sodium-dried benzene and the system was flushed with nitrogen. Stirring was initiated as 10.0g (0.050 mole) of I, and then 0.20g of p-toluenesulfonic acid monohydrate was added. After stirring this slurry for 15 min, a solution of 8.41g (0.075 mole) of cyclohexane carboxaldehyde in 20 mL of benzene was slowly added dropwise over 30 min. Once the addition was complete, the reaction mixture was stirred at room temperature for an additional 30 min and was then refluxed for 3 hr. The solution, initially water-white, turned yellow on heating and finally a light brown upon refluxing. A volume of 1.0 mL water was collected in the Dean-Stark trap. The reaction mixture was refluxed for 15 minutes with Norit, gravity filtered, decanted into a separatory funnel, and subjected sequentially to washings with 2 × 50mL water, 2 × 25 mL 5% $NaHCO_3$ solution, 2 × 25 mL 10% $NaHSO_3$ solution, and 2 × 50 mL water. The resulting benzene extract was dried overnight using anhydrous sodium sulfate, gravity filtered from the spent drying agent, and the solvent was removed by use of a rotary evaporator. The resulting oil solidified upon scratching and was recrystallized from (60–90°) Ligroin to yield 10.0 g (68%) of a white crystalline solid, m.p. 103–106°C. The identity of [7] was confirmed by the use of ir (see peak table below) and nmr spectroscopy, as well as by elemental microanalysis.

ir (KBr) : 2945,2925,2851,1442 cm^{-1} (aliphatic C-H), 1561,1334,914 cm^{-1} (NO_2), 1178,1138,1077,1036 cm^{-1} (acetal C-O-C-O-C), 550 cm^{-1} (C-Br)
Calculated for $C_{10}H_{16}O_4NBr$: N− 4.76%; Found : N− 4.60%

TEST ORGANISMS

The test organisms used in this study were taken from the Jergens culture collection and consisted of *Staphylococcus aureus* (FDA 209 strain) and *Pseudomonas aeruginosa* (PRD 10 strain). These organisms were grown and inoculated into the test samples as described in a previous report (10).

TEST SAMPLES

Screening studies were performed by dispersing or dissolving 100 mg of each analog in 9.0 mL of propylene glycol and pipetting 1 mL of this into 9 mL of 0.85% saline to give a final concentration of 0.1% of each test compound in 10% propylene glycol/saline solution. Propylene glycol was chosen as the solubilizing/dispersing agent because II is supplied commercially in propylene glycol (Henkel, Inc.).

Relative biological responses (RBR) studies were performed using *P. aeruginosa* in 10% polysorbate 80/saline solutions containing 0.1 mM of each analog. Lotion test samples were prepared using a proprietary formulation without preservatives or fragrance. The lotion was prepared for testing by dissolving the desired amount of either 5-bromo-5-nitro-2,2-dimethyl-1,3-dioxane [8] or 5-bromo-5-nitro-2,2-diethyl-1,3-dioxane [10] in the oil phase and heating to 70°C. Then, this oil phase was added to the water phase with continuous agitation at 70°C. Sufficient amounts of the test compound were used to give final concentrations of 0 (unpreserved control), 0.01, 0.1, and 0.2% in the lotion.

TEST PROCEDURE

Suspensions of the test organisms were prepared and introduced into the test samples, and aerobic plate counts (APC) were performed as described in a previous report (10). The APC values obtained at different times for each test compound were used to calculate the decimal reduction time (D-value), which is the time required for 90% inactivation of the population for each organism treated with the specified concentration of test compound.

The D-values for each test compound were determined using APC values from 0–24, 2–24, or 4–24 hr, depending on the time at which the APC values were maximum. The D-values obtained with different concentrations of [8] and [10] in the test lotion were used to construct a preservative death time curve so that the concentration of preservative necessary to provide satisfactory preservative efficacy could be calculated (11).

These data were then normalized relative to the activity of II, which was assigned a value of 100. The measure of RBR was defined as 1/D-value in order to denote that an intrinsically more active congener should exhibit a given level of biological activity at a lower molar concentration than a less active congener in the same series. Logarithmic transformation of the RBR values allowed the data set to be placed on a "linear free energy" (LFER) scale and utilized in the derivation of extrathermodynamic QSAR, correlating observed changes in antibacterial activity with changes in molecular structure.

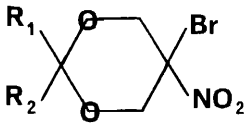
QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSAR)

The QSAR were derived utilizing the Hansch 3AA Multiparameter Regression Analysis program, available through the Southwestern Ohio Regional Computer Center (SWORCC) at the University of Cincinnati. Computer runs were performed using an Anderson-Jacobson Model 832 TTY/Model A242 Acoustic Data Coupler remote terminal on the Amdahl Systems Computer at SWORCC. Extrathermodynamic substituent parameter values were taken from the recent compilation of Hansch and Leo (12) or from the review article by Verloop (13).

RESULTS

The rates of inactivation of the test organisms in 10% propylene glycol/saline solutions containing 0.1% of each test compound were determined using *S. aureus* and *P. aeruginosa*. The D-values obtained in the screening tests with the aliphatic substituents are listed in Table I. It is apparent that *P. aeruginosa* was more susceptible to the analogs than was *S. aureus*, because many D-values for the former organisms were <0.6 hr. This value represents the lower limit of sensitivity of the D-value determination when performed using the time intervals selected in this experiment, because no organisms were recovered at the second APC determination. Therefore, the rates of inactivation (i.e., D-values) of *P. aeruginosa* alone were subsequently determined in 0.1 mM solutions of aliphatic analogs [1–13] in 10% polysorbate 80/saline and these values are given in Table II.

Table II
Structure-Activity Data for 2-Alkyl-5-Bromo-5-Nitro-1,3-Dioxane Analogs Using *P. aeruginosa* as Test Microorganism



No.	R_1	R_2	D-Value[a] (HR)	log 1/D (obsd.)	log 1/D (pred.)[b]	$\Sigma\pi^c$ R_1, R_2	Σ MR[c] R_1, R_2	$\Sigma\sigma^{*c}$ R_1, R_2
1	H	H	4.8	2.00	1.91	0.00	2.06	0.98
2	CH_3	H	5.0	1.98	2.05	0.56	6.68	0.49
3	CH_3CH_2	H	4.3	2.05	2.11	1.02	11.33	0.39
4	$CH_3(CH_2)_2$	H	3.8	2.10	2.08	1.55	15.99	0.37
5	$CH_3(CH_2)_3$	H	5.2	1.96	1.98	2.13	20.64	0.36
6	$CH_3(CH_2)_4$	H	9.2	1.72	1.79	2.53	25.27	0.33
7	c-C_6H_{11}	H	14.6	1.52	1.66	2.51	27.72	0.34
8	CH_3	CH_3	4.6	2.02	2.11	1.12	11.30	0.00
9	CH_3	CH_3CH_2	4.0	2.08	2.08	1.58	15.95	−0.10
10	CH_3CH_2	CH_3CH_2	3.7	2.11	1.98	2.04	20.60	−0.20
11	CH_3CH_2	$CH_3(CH_2)_2$	7.2	1.83	1.79	2.57	25.26	−0.22
12	$-(CH_2)_4-$		8.2	1.77	—[d]	1.39	18.59	−0.26
13	$-(CH_2)_5-$		4.9	1.99	1.84	1.77	24.25	−0.18
Bronopol®			4.5	—	—			

[a] 0.10 mM solutions in 10% polysorbate 80/saline.
[b] Predicted on the basis of eq 12.
[c] Extrathermodynamic parameter values taken from ref (12).
[d] Not included in the derivation of eq 12.

The D-values obtained with *S. aureus* in the presence of the mono-substituted aliphatic analogs showed an increase in the rate of inactivation as the substituent chain length increased from one to three carbons. Thus, the D-values decreased from 4.3 hr to 3.2 hr to 2.2 hr as the substituent chain length increased from methyl- to ethyl- to n-propyl-, respectively. Further increase in the carbon chain length resulted in a decrease in antibacterial activity of the test compounds (Figure 1). This observation seems to parallel that made by Kabara (14) several years ago regarding the activity of a series of octadecyl fatty acids in inhibiting the growth of Group A streptococci. The rates of inactivation of *P. aeruginosa* with the monosubstituted aliphatic analogs were so fast that no bacteria were recovered at the APC determination at 3 hr, and this resulted in most of the D-values being <0.6 hr. This did not allow discrimination of different rates of inactivation in the propylene glycol/saline test solutions.

The effect of 0–0.2% of analogs [8] and [10] gave D-values of >30, 4.4, 0.3, and 0.04 hr, and >30, 6.6, 0.9, and 0.3 hr, respectively, in test lotions inoculated with *P. aeruginosa*. In both cases, the lotions containing 0% of [8] or [10] were not preserved, as evidenced by the large D-values. Although the rates of inactivation increased (i.e., the D-values decreased) with increasing concentrations of [8] and [10], the D-values were lower for [8] than [10] at each concentration tested. The correlation coefficients for the linear regressions used in the D-value determinations ranged from −0.98 to −1.00, indicating that there was good fit of the data to the linear regressions.

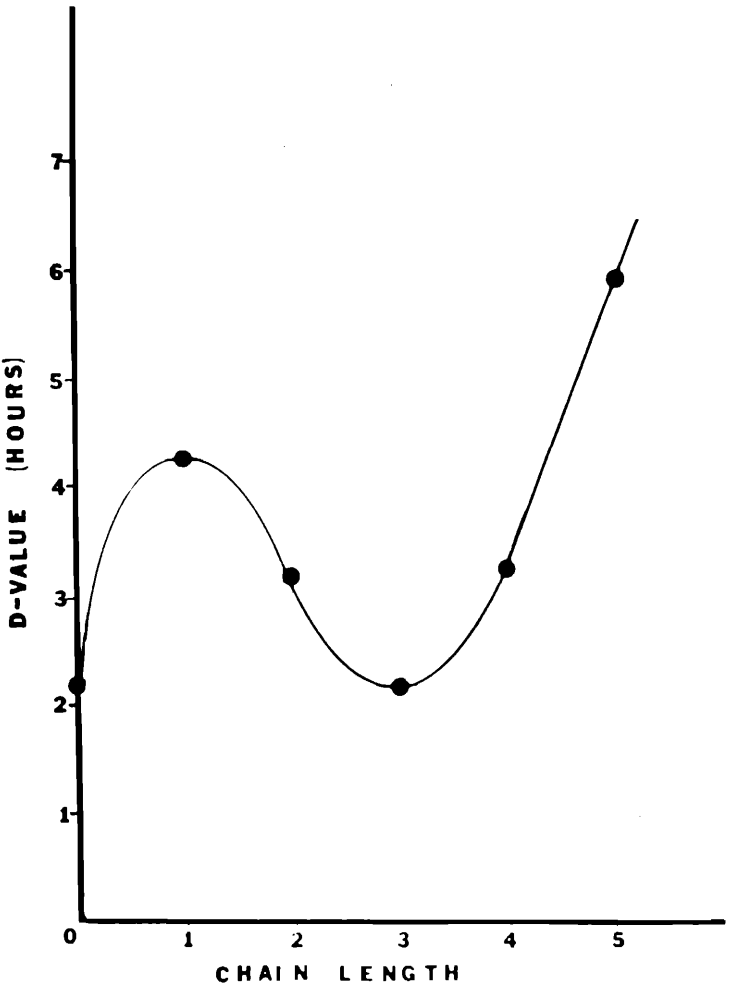


Figure 1. Effect of aliphatic substituent chain length in 2-alkyl-5-bromo-5-nitro-1,3-dioxane analogs on the rate of inactivation (i.e., D-Values) of *S. aureus* in propylene glycol/saline.

The D-values obtained with the different concentrations of [8] or [10] in lotion (Figure 2 illustrates this for analog [10]) were used to construct the preservative death time curve for *P. aeruginosa*. The preservative death time curve obtained in this manner for analog [10] is illustrated in Figure 3. From these curves, it was calculated that 0.04% and 0.06% of analog [8] and [10], respectively, would be required to obtain D-values of 4 hr in the test lotions. The correlation coefficients for these linear regressions were -0.88 and -0.89, which indicates a moderately good fit of the data to the linear regressions.

All potentially meaningful QSAR, involving linear combinations of the Hansch hydrophobic parameter (π), the Taft-Hammett electronic parameter (σ^*), the Taft steric parameter (E_s), the molar refractivity parameter (MR), the Swain-Lupton field and resonance electronic parameters (F and R), and the Verloop steric parameters for mo-

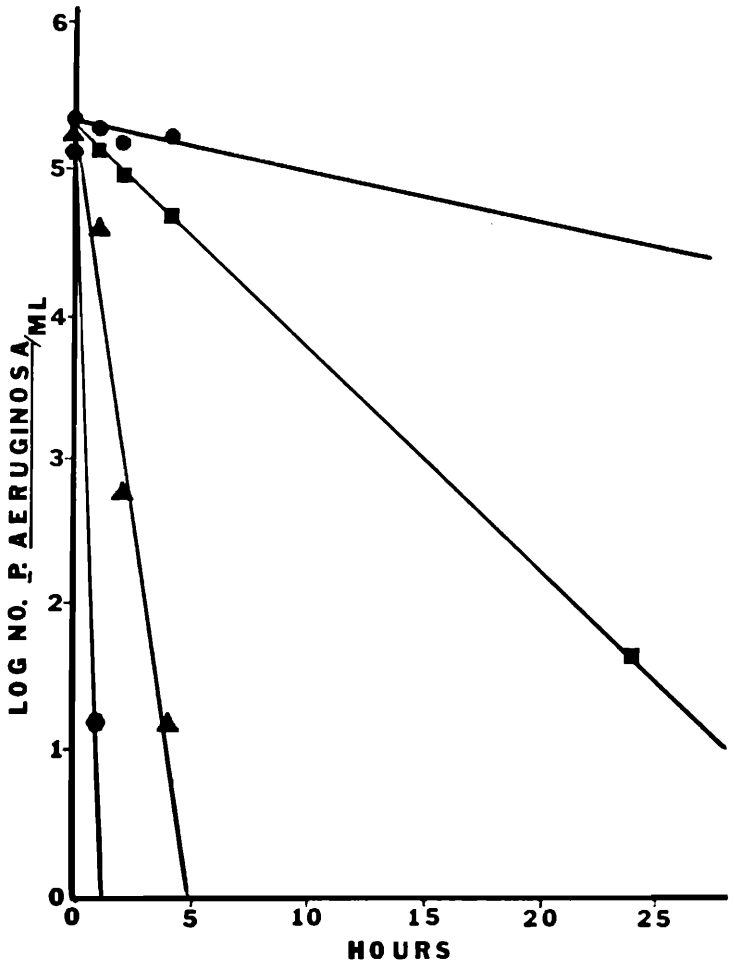


Figure 2. Effect of 5-bromo-5-nitro-2,2-diethyl-1,3-dioxane [10] concentration on the rate of inactivation of *P. aeruginosa* in lotion. Symbols: lotion containing 0% (control), ●—●; lotion containing 0.01%, ■—■; lotion containing 0.10%, ▲—▲; and lotion containing 0.2%, ⬢—⬢.

lecular length, minimum width, and maximum width (L_1, B_1, and B_4), respectively, were derived. A "forward step-wise" development of the QSAR for the aliphatic series of 2-substituted-II analogs is presented in Table III. In these equations, n is the number of data points upon which the regression equation is based, r is the multiple correlation coefficient for the regression, s is the standard deviation, and the numbers in parentheses are the 95% confidence intervals for the coefficients. In guarding against chance or fortuitous correlations, "outlier" data points (15) were not excluded from the data set if they were endpoints, and at least five data points were required per independent variable used in the final regression equations. The statistical significance of each successive regression "step" relative to the foregoing "step" was assessed by means of the F-statistic (16). Parameter values used in the derivation of the final set of regression equations (i.e., 11–14) are given in Table II, along with the observed vs predicted antibacterial activities on the basis of regression equation 12.

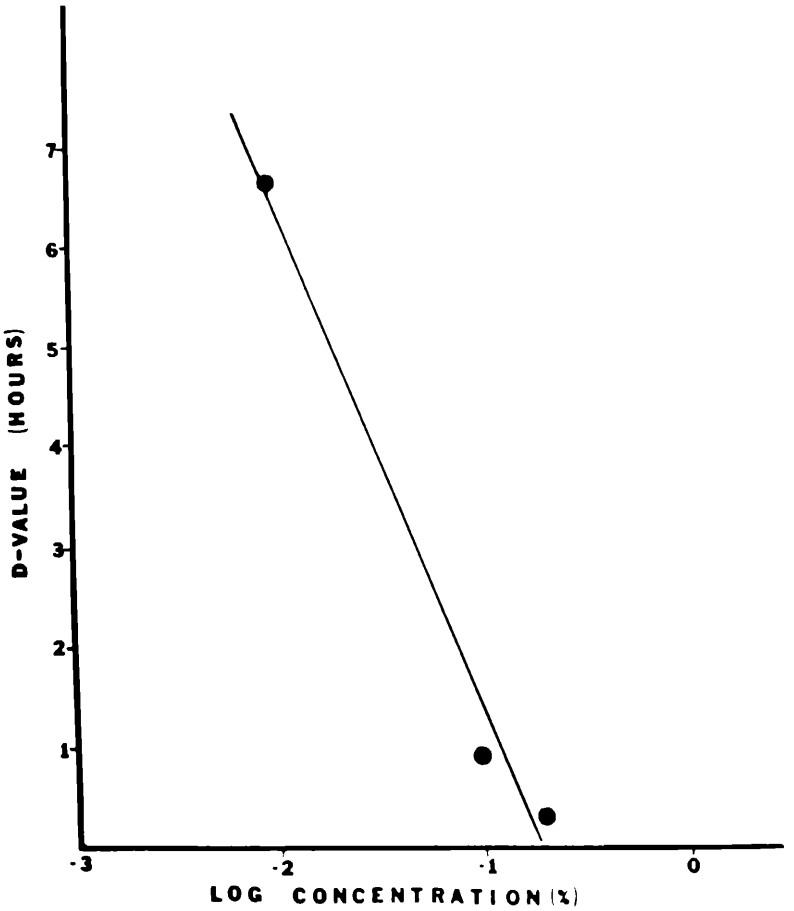


Figure 3. Preservative death time curve for *P. aeruginosa* in lotion containing 0.01–0.20% of 5-bromo-5-nitro-2,2-diethyl-1,3-dioxane [10].

DISCUSSION

MICROBIOLOGY

S. aureus and *P. aeruginosa* are recognized pathogens in the cosmetic industry (17). The rates of inactivation of these organisms by a series of 2-alkyl substituted 5-bromo-5-nitro-1,3-dioxane (II) analogs were determined by use of the linear regression method (10). This method provides a quantitative expression of the rate of death of a specific test organism in the presence of each test analog. The rate of inactivation of the test organism is given by the D-value. Note that faster rates of inactivation of the test organisms correspond to smaller D-values; consequently, the test compound that gives the smaller D-value has the greater antibacterial activity.

The initial experiments with the test analogs were performed using propylene glycol as the solubilizing/dispersing agent, because II is supplied commercially in this medium. These screening experiments revealed that *P. aeruginosa* was more suceptible to the aliphatic II congeners than was *S. aureus;* therefore, *P. aeruginosa* was selected for use in the subsequent RBR studies. Several of the analogs did not exert their antibac-

Table III
QSAR Development for 2-Alkyl-5-Bromo-5-Nitro-1,3-Dioxane Analogs Using *P. aeruginosa* as Test Microorganism

Equation No.	n	r	s	$F_{x,y}$
4. $\log 1/D = 2.13\ (\pm 0.23) - 0.12\ (\pm 0.13)\ \Sigma\pi$	12	0.543	0.154	
5. $\log 1/D = 1.96\ (\pm 0.14) - 0.05\ (\pm 0.34)\ \Sigma\sigma^*$	12	0.114	0.182	
6. $\log 1/D = 2.16\ (\pm 0.24) - 0.01\ (\pm 0.01)\ \Sigma MR$	12	0.569	0.151	
7. $\log 1/D = 2.02\ (\pm 0.38) - 0.03\ (\pm 0.14)\ \Sigma B_1$	12	0.141	0.182	
8. $\log 1/D = 2.03\ (\pm 0.30) - 0.02\ (\pm 0.06)\ \Sigma B_4$	12	0.220	0.179	
9. $\log 1/D = 2.10\ (\pm 0.31) - 0.02\ (\pm 0.04)\ \Sigma L$	12	0.341	0.173	
10. $\log 1/D = 1.97\ (\pm 0.33) + 0.02\ (\pm 0.17)\ \Sigma E_s$	11[a]	0.073	0.192	
11. $\log 1/D = 2.38\ (\pm 0.26) - 0.02\ (\pm 0.01)\ \Sigma MR - 0.33\ (\pm 0.28)\ \Sigma\sigma^*$	12	0.794	0.118	$F_{1,9} = 7.46$ (11 *vs* 6)
12. $\log 1/D = 1.82\ (\pm 0.26) + 0.05\ (\pm 0.04)\ \Sigma MR - 0.002\ (\pm 0.001)\ (\Sigma MR)^2$	12	0.855	0.100	$F_{1,9} = 13.77$ (12 *vs* 6)
$(\Sigma MR)_o = 12.28\ (6.14 - 14.57)$				
13. $\log 1/D = 1.93\ (\pm 0.24) + 0.32\ (\pm 0.35)\ \Sigma\pi - 0.16\ (\pm 0.12)\ (\Sigma\pi)^2$	12	0.799	0.116	$F_{1,9} = 9.31$ (13 *vs* 4)
$(\Sigma\pi)_o = 1.03\ (-0.38 - 1.34)$				
14. $\log 1/D = 1.83\ (\pm 0.23) + 0.05\ (\pm 0.03)\ \Sigma MR - 0.002\ (\pm 0.001)\ (\Sigma MR)^2$	11[a]	0.902	0.088	
$(\Sigma MR)_o = 11.93\ (6.99 - 14.07)$				

[a] Data point for analog 13 not used in the derivation of this equation. See text.

terial effect against *P. aeruginosa* during the first few hours of the D-value determination study. Although the reason for this is not known, it is expected that this delay may have been due to a lag in the uptake of the analog by the bacteria, which were not provided with any nutrients in the test system (18).

The antibacterial effects of the 2-substituted aliphatic analogs against *P. aeruginosa* in 10% polysorbate 80/saline were similar to those observed in the propylene glycol/saline test system. In general, the D-values for *P. aeruginosa* were smaller than those observed for *S. aureus* in the presence of the same test compounds. This was not unexpected, since the parent compound of the series, II, is reported to possess greater antibacterial activity against gram-negative organisms than against gram-positive organisms (5). In the aliphatic series studies, the most active monosubstituted congener against *P. aeruginosa* in polysorbate 80/saline was the *n*-propyl analog [4], whereas di-ethyl analog [10] was the most active disubstituted compound tested.

QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSAR)

Mechanistic Implications

Within the last 15 years, a useful technique for analyzing structure-activity relationship, the Hansch QSAR Paradigm, has been developed and is finding increasing acceptance in pharmaceutical studies of biologically active compounds. Since the Hansch approach has been reviewed extensively (19), only a brief description will be given here. Essentially, the underlying concept of the "QSAR Paradigm" is that the biological activity of a given compound depends on the difference in hydrophobic, electronic, and steric factors between the derivative under consideration and the parent-active com-

pound in the series. The contribution of each effect to the overall biological activity is assumed to be independently additive. Typically, these relationships are of the mathematical form:

$$\log 1/C = K + \log P + b(\log P)^2 + c\sigma + dE_s + e\mu + \ldots \quad (15)$$

wherein K = a constant, C = molar concentration required to produce some standardized level of biological response (i.e., a minimum inhibitory concentration, or a D-value), P = 1-octanol/water partition coefficient, σ = Hammett electronic substituent parameter, E_s = Taft steric parameter, and μ = dipole moment. Corresponding to the use of n-independent variables, $2^n - 1$ QSAR correlation equations can be derived, and so these equations are usually obtained via computer-assisted multiparameter regression analysis.

If a QSAR equation is truly "significant" in a physical as well as in a statistical sense, it is often possible to discern: 1) the mechanism of action of a series of bioactive congeners on a cellular or subcellular level, and 2) the direction that a chemist ought to take in synthesizing the most active compound in the series.

The basis for substituent selection was the set of criteria set forth by Biel and Martin (20) for the design of congeneric series of bioactive materials. In general, these criteria are: 1) only compounds for which accurate physicochemical parameter values (i.e., π, σ, E_s, MR, etc.) are known should be included; 2) no two compounds should be made which essentially duplicate each other's physicochemical properties (i.e., steric, electronic, hydrophobic); 3) the series of congeners should be planned so that the independent variable sets of physicochemical parameters are not highly "cross-correlated" with each other; and 4) the widest possible "range" of physicochemical parameter values should be inherent in the set of congeners selected. Unfortunately, it was not possible to effectively avoid the almost total cross-correlation between π and MR for the set of simple aliphatic derivatives explored in the current work. Thus, it is seen that the covariance (r^2) for π and MR is 0.94 (Table IV). This is discussed in greater detail below.

Table IV
Cross-Correlation Matrix (r^2) for the Substituent Parameters of the 2-Alkyl-5-Bromo-5-Nitro-1,3-Dioxane Analogs

	$\Sigma\pi$	Σ MR	$\Sigma\sigma^*$	ΣE_s	Σ F	Σ R	Σ L	ΣB_1	ΣB_4
$\Sigma\pi$	1.00	0.94	0.33	0.07	0.40	0.02	0.31	0.04	0.35
Σ MR	0.94	1.00	0.35	0.10	0.00	0.00	0.12	0.00	0.14
$\Sigma\sigma^*$	0.33	0.35	1.00	0.00	0.13	0.27	0.01	0.00	0.07
ΣE_s	0.07	0.10	0.00	1.00	0.00	0.06	0.00	0.06	0.00
Σ F	0.40	0.30	0.13	0.00	1.00	0.06	0.51	0.52	0.46
Σ R	0.02	0.00	0.27	0.06	0.06	1.00	0.20	0.33	0.38
Σ L	0.31	0.12	0.01	0.00	0.51	0.20	1.00	0.74	0.94
ΣB_1	0.04	0.00	0.00	0.06	0.52	0.33	0.74	1.00	0.69
ΣB_4	0.35	0.14	0.07	0.00	0.46	0.38	0.94	0.69	1.00

Preliminary results in the case of an aromatic series of II analogs to be described in a future manuscript show that the Biel-Martin injunctions of "maximum variance/minimum covariance" with respect to physicochemical parameters are readily and successfully achieved by selecting aryl substituents based upon the hierarchical cluster analysis scheme outlined by Unger and Hansch (21).

The D-values obtained for *P. aeruginosa* in the presence of 0.10 mM concentrations of each aliphatic congener of II in 10% polysorbate 80/saline were used to develop the QSAR, which involved correlating variations in hydrophobic, electronic, and steric factors of the analogs with observed changes in antibacterial activity.

The most statistically significant single parameter correlations involved the π and the MR parameters (Table III). Interpreting the algebraic signs of the coefficients in the language of physical organic chemistry (22), equations 4 and 6 reveal that the antibacterial efficacy against *Pseudomonas* is promoted by sterically small, relatively hydrophilic substituents at the 2-position. The slope of equation 4 is <0.4, which Hansch and Dunn (23) refer to as hydrophobically "insensitive". This implies that the biological test system did not produce large changes in activity as a result of small changes in substituent lipophilicity. Equations 4 and 6 are useful in "explaining" only about 25% of the r^2 inherent in the raw biological data. In addition, the 95% confidence intervals for the coefficients in equations 4 and 6 are larger than desired, implying that some refinement in these correlations is possible. These improvements are represented by equations 11–13. Equation 11, which is more statistically significant at the 95% confidence limit than equation 6 (i.e., $F_{1,9} = 7.46$; $F_{1,9\alpha 0.025} = 7.21$), implies that sterically small, electron-donating aliphatic substituents promote antibacterial activity. Comparing equations 13 and 4 (i.e., $F_{1,9} = 9.31$; $F_{1,9\alpha 0.025} = 7.21$), we see that a statistically significant parabolic relationship with respect to lipophilicity requirements for antibacterial activity exists at the 95% confidence limit (Figure 4). Similarly, a

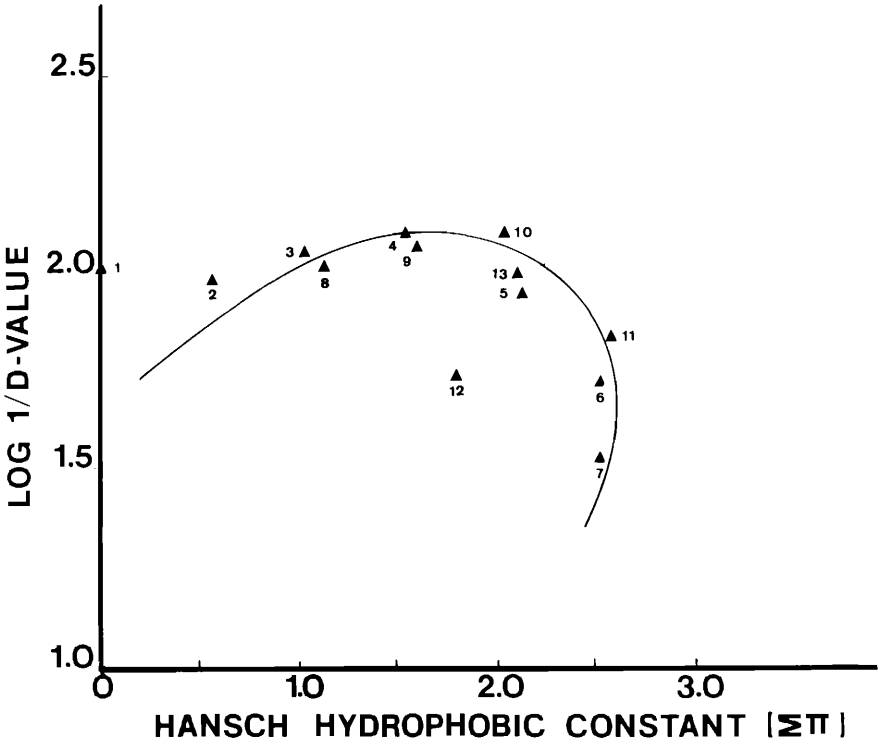


Figure 4. Quantitative structure-activity relationship (QSAR) profile, in which the relative biological response (i.e., 1/D-value) is plotted as a function of the Hansch hydrophobic parameter ($\Sigma\pi$).

comparison of equations 6 and 12 reveals a statistically significant parabolic relationship with respect to steric size requirements for substituents at position 2 of compound II (i.e., $F_{1,9} = 13.77$; $F_{1,9\alpha 0.005} = 13.61$) at the 99% confidence limit (Figure 5). A

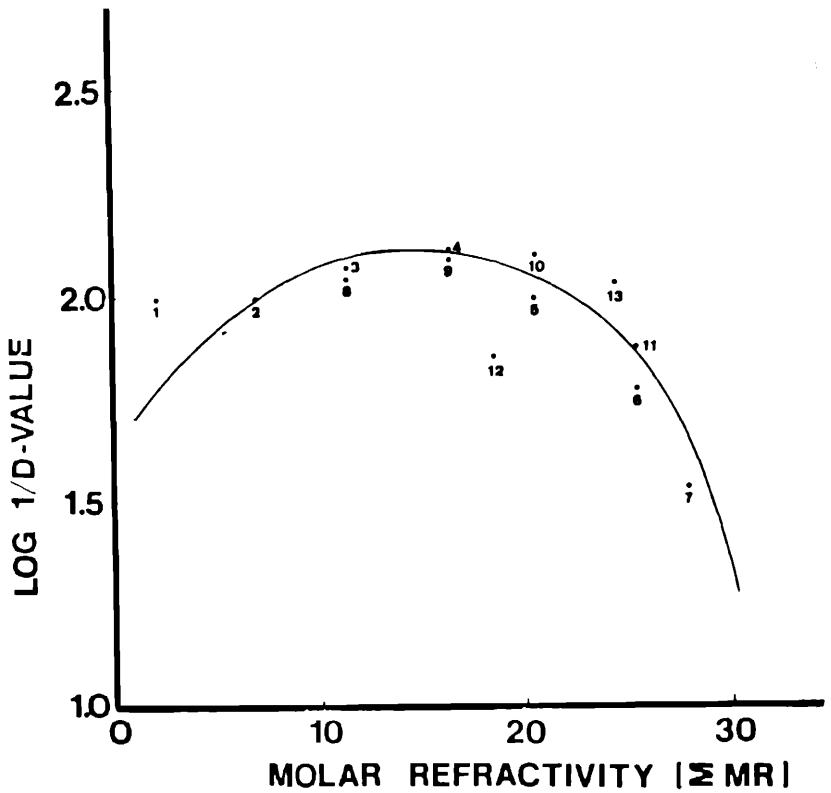


Figure 5. Quantitative structure-activity relationship (QSAR) profile, in which the relative biological response (i.e., 1/D-value) is plotted as a function of the molar refractivity parameter (ΣMR).

final refinement in equation 12 is achieved by deleting the data point for the "spiro" analog [13], because it fits the regression equation poorly. It is believed that "spiro" ring systems, such as are found in analogs [12] and [13] may represent sterically hindering moieties with respect to the ability of 2-substituted II analogs to engage sulfhydryl groups located in the bacterial cell membrane, due to restricted conformational access (see below). The resulting regression 14 exhibits "tighter" 95% confidence limits on its coefficients and "explains" successfully approximately 81% of the r^2 inherent in the raw biological data.

By optimizing the values of the independent variables, π and MR, in regression equations 12 and 13 (24), we were able to calculate which substituent(s) were predicted to produce the optimal antibacterial activity against *P. aeruginosa*. Taking the partial derivative of the RBR variable with respect to the independent variable in question and setting this algebraic expression equal to zero allowed us to solve for the inflection point (i.e., apex) of the parabola in question. When this was done for equation 12, we

found the optimum sum of the MR (i.e., Σ MR_0) was 12.28. This corresponds to a set of substituents of steric bulk intermediate between the 2,2-dimethyl- (or 2-ethyl-) and 2-methyl-2-ethyl- (or 2-*n*-propyl-) substituent pairs. Operating analogously with respect to equation 13, the lipophilicity optimum (i.e., $\Sigma\pi_0$) of the 2-substituents is found to be 1.03. This corresponds to the 2,2-dimethyl- (or 2-ethyl-) analogs. Lappas *et al.* (6) concluded that the 2,2-dimethyl- analog was the most active disubstituted congener of II. They also reported that the 2-methyl- analog [2] was the most active monosubstituted aliphatic congener; this was not corroborated by our experimental data or by QSAR, both of which point towards a C_2-C_3 analog as the most active monosubstituted aliphatic analog in the series (c.f., Figures 4–5).

Hansch and Leo (12) noted that log P (or π) and MR often turn out to be so highly collinear that either parameter will give about the same quality correlation. In the present study, a clear statistical preference for equation 12 over 13 cannot be made. Although the 95% confidence limits are "tighter" and the multiple correlation coefficient for equation 12 is superficially superior to that for equation 13, the apparent improvement is significantly influenced by the high cross-correlation between the parameters π and MR (i.e., $t_{11\ d.f.} = 1.348$; $t_{11\ d.f.\alpha 0.05} = 1.796$) (25). In vector terms, the angle between the π and MR unit vectors is given by arccos (0.94) = 19°, so that there is a large vector component of π built into MR. This, of course, renders difficult an unequivocal interpretation of any QSAR involving either parameter unless additional, more esoteric analogs of II are used in order to remove the collinearity between π and MR.

Nevertheless, circumstantial evidence leads us to favor a steric interpretation (i.e., eq 12) for the data described here. This is explained in the following paragraphs. The MR parameter is usually defined in terms of the Lorentz-Lorenz equation (eq 16):

$$MR = \frac{n^2 - 1}{n^2 + 1}\frac{MW}{d} = 4/3\pi N\alpha \qquad (16)$$

where n = the index of refraction, d = the density, MW = the molecular weight of a compound (usually taken as a liquid), N = Avogadro's Number, π = 3.14159, and α = the substituent polarizability.

Dietrich *et al.* (26) described MR as an "ambivalent" parameter in that it is both a measure of substituent volume and polarizability. Thus, it is a "corrected molar volume", which relates to how loosely the electrons in an atomic or functional group electron cloud are held as measured by n (27).

The work of Moriguchi, Kanaba, and Komatsu (28) showed that other measures of substituent bulk, such as the Van der Waals volume (V_w), are also often highly collinear with substituent lipophilicity, as modeled by log P (eq 17). Kier (29) demonstrated that the reason may be that measures of steric bulk and lipophilicity are both linearly correlated to a yet more fundamental topological index, the "molecular connectivity" (X), which can roughly be defined as a calculated measure of the way in which constituent atoms of a molecule are bonded to one another (eqs 18–21).

$$\log P = 2.51\ (\pm 0.13)\ V_w + 0.23\ (\pm 0.15).$$
$$n = 60,\ r = 0.980,\ s = 0.23. \qquad (17)$$
$$\alpha\ (\text{Polarizability}) = 9.26\ X + 1.60\ (\text{no limits given}).$$
$$n = 36,\ r = 0.99,\ s = 3.60. \qquad (18)$$

$$\log P = 0.95\ (\pm 0.01)\ X - 1.48\ (\pm 0.04).$$
$$n = 138,\ r = 0.99,\ s = 0.15. \quad (19)$$

$$CSA\ (\text{Hydrocarbon cavity surface area}) = 133.4 + 58.24\ X.$$
$$n = 69,\ r = 0.98,\ s = 11.2. \quad (20)$$

$$MR(\text{Alkanes}) = 1.37 + 10.44\ X.$$
$$n = 46,\ r = 0.95,\ s = 1.81. \quad (21)$$

Thus, the ambiguity between the operation of lipophilic and steric effects in antibacterial testing is not unique to this study. Hansch and coworkers (30) demonstrated the importance of lipophilic effects in the antibacterial activity of the parabens and other antibacterials for both gram-positive and gram-negative organisms. Kabara (31) suggested the importance of steric factors in the antimicrobial activity of butylated hydroxytoluene analogs against *Streptococcus mutans*. Paris *et al.* (32) recently found that Van der Waals radii, another measure of steric bulk, afforded the best QSAR, accounting for the rates of oxidation of phenols to the corresponding catechols by *P. putida U*.

We believe that steric factors play the primary role in determining the antibacterial activity (i.e., Table II) in the aliphatic series of II congeners explored in the present study (i.e., compounds 1–13). The reasons for this are: *first,* if steric bulk at the 2-position is detrimental to antimicrobial activity, we can rationalize the greater activity of analog [14] against *P. aeruginosa* (*vide infra*) in 10% polysorbate 80/saline, for which a D-value of 5.9 hr was observed, as compared with the activity of analog [7], for which a D-value of 14.6 hr was obtained. The former analog possesses a planar phenyl ring at position 2, which probably offers minimum steric interaction in the approach of [14] to the "receptor" at which the antibacterial mechanism is triggered. Compound [7], on the other hand, is a totally saturated analog of [14], possessing a cyclohexyl

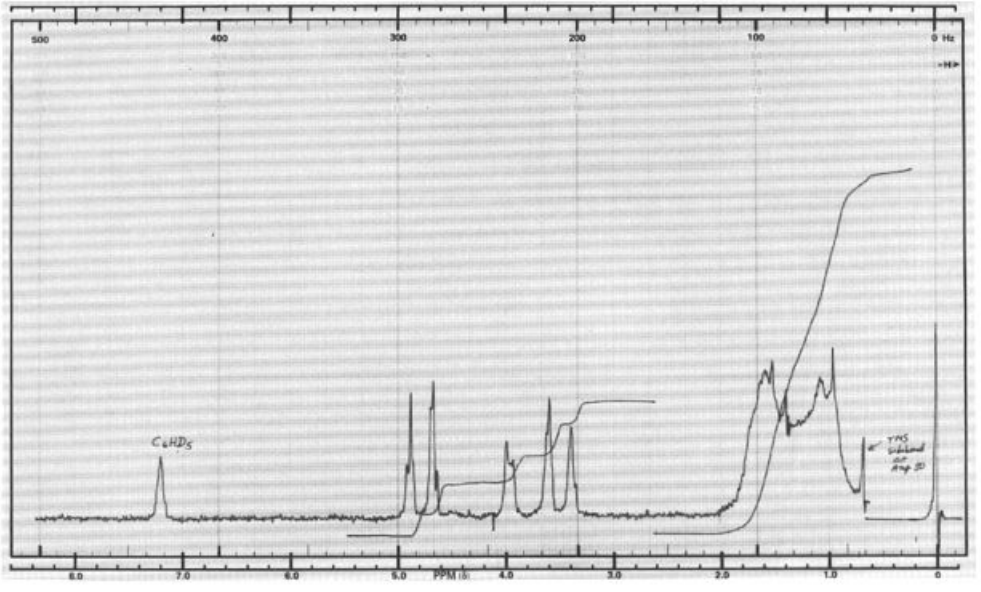
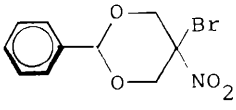
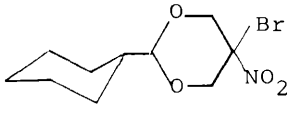


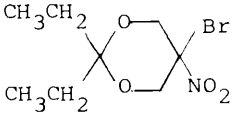
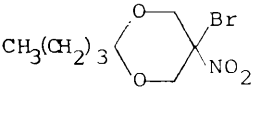
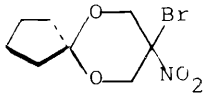
Figure 6. 60 MHz ^{1}H-nmr spectrum of 2-cyclohexyl-5-bromo-5-nitro-1,3-Dioxane [7] in C_6D_6 (TMS Internal Standard).

ring at position 2, which would be expected to exist primarily in the more sterically demanding, puckered "chair" conformation. In the room temperature nmr spectrum (Figure 6), the cyclohexyl ring axial and equatorial ring protons appear as a pair of partially resolved multiplets at 0.75-2.0δ, which are most likely due to a reduced conformational flexibility in the ring's ability to undergo inversion (i.e., "ring-flipping"), a phenomenon which is observed in cyclohexane itself only at reduced temperature (i.e., −65°C or below) (9). *Second,* the much reduced conformational flexibility of the spiro-cyclic analog [12] compared with its isolipophilic open-chain isomers [5] and [10] also probably represents a much greater steric demand on the approach of congener [12] to the bacterial "receptor", and this is reflected in lower observed antibacterial activity (i.e., D-value = 8.2 hr), compared with those of [10] and [5] which have D-values of 3.7 and 5.2 hr, respectively. The lower activity of [5] compared with [10] may also be related to steric considerations, in that the longer hydrocarbon side chain of *n*-butyl-analog [5] probably sweeps out a larger "excluded volume" than does that of the diethyl analog [10] and thus represents a substituent of greater apparent steric hindrance from the perspective of the bacterial "receptor".

Activity:  > 

(D = 5.9 HR) (D = 14.6 HR)

[14] [7]

Activity:  >  > 

(D = 3.7 HR) (D = 5.2 HR) (D = 8.2 HR)

[10] [5] [12]

Stretton and Manson (33) suggested that the bacterial "receptor that is involved in the action of geminal bromonitro compounds, such as I and II, may actually be constituted by the thiol moieties of mercaptoamino acids (i.e., cysteine) in some membrane-bound microbial protein. According to their proposal, the nitro moiety acts as a one-electron acceptor, thereby serving as a "free radical initiator". The thiol radicals thus formed may enter into a typical chain reaction, some ultimately undergoing dimerization to a disulfide. This, in turn, may result in a conformational change with concomitant alteration of function of the protein (Scheme I).

Scheme I

$$R - SH \longrightarrow R - S^- + H^+$$

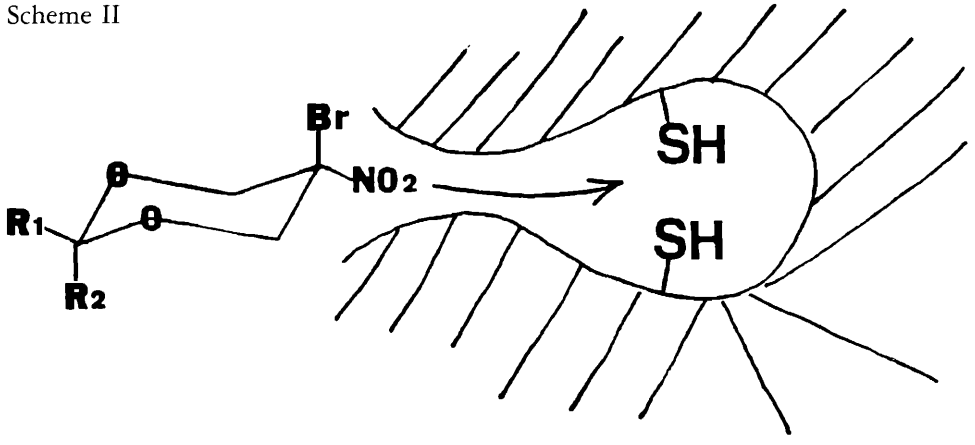
$$R - S^- + R_2C(Br)(NO_2) \rightleftharpoons R - S^{\bullet} + \left[R_2C(Br)(NO_2)\right]^{\pm}$$

$$R - S^{\bullet} + HS - R \rightleftarrows R - S - S - R + H^{\bullet}$$

$$R - S^{\bullet} + R - S^{\bullet} \longrightarrow R - S - S - R$$

If we envision the crucial -SH groups as existing in a hydrophobic cavity of restricted volume, such that only 2-substituted II analogs of defined steric dimensions can successfully enter the cavity, the MR dependence of antibacterial activity of these preservatives against *P. aeruginosa* can be explained (Scheme II).

Scheme II



This line of reasoning leads us to conclude that the optimal anti-*Pseudomonas* activity of monosubstituted analog [4] and disubstituted analogs [8] and [10] is due primarily to the steric dimensions conferred on II by these substituents and, secondarily, to electronic and hydrophobic factors.

Lotion Test

The ultimate test of the usefulness of the foregoing QSAR depends on the success with which the most active preservative-congener in the aliphatic series explored is predicted. In addition, it is most desirable that the enhanced level of preservative efficacy is manifested in an actual cosmetic formulation, because it is recognized that some components of cosmetic formulations inactivate preservatives. Although the 2,2-diethyl-II analog [10] gave the lowest experimental D-value against *P. aeruginosa in vitro* (Table III), QSAR equation 12 clearly predicted that the 2,2-dimethyl-II analog [8] would be more active. Accordingly, both [8] and [10] were tested as preservatives in a proprietary white lotion formulation devoid of fragrance or other preservatives. The aqueous phase of this lotion contained distilled water, SD Alcohol 40-B, glycerin, and sodium carbomer −941, while the oil phase of the lotion contained synthetic spermacet, glyceryl dilaurate, cetearyl alcohol, mineral oil, myristyl alcohol, ceteareth-20, cetyl alcohol, lanolin oil, and dimethicone.

Comparison of the D-values obtained with different concentrations of [8] and [10] in the test lotion showed quite nicely that the compound with the greater antibacterial activity in the test lotion was congener [8], exactly as predicted by QSAR. The amount of analog [8] or [10] required to provide a D-value of 4 hr (10) was found by calculation to be 0.04% and 0.06%, respectively. Thus, it is apparent that compound [8] was slightly more active in the lotion against *P. aeruginosa* PRD 10 than compound [10].

In an actual testing situation, the concentration of preservative predicted to be adequate from the preservative death time curve would be prepared and evaluated. This was not

done here. In addition, no attempt was made to compare the preservative efficacy of the 2-substituted-II analogs with other commercially available preservatives. It is expected that an investigator would include this as part of the testing in the development of a product formulation. It is also recognized that determination of preservative adequacy of a given compound would require additional testing. This testing would include the use of other test organisms, including gram-positive cocci, spore formers, other gram-negative rods, and representative yeasts and molds.

CONCLUSIONS

This study illustrates the value of using a "rational" approach to synthesizing bioactive compounds, selected on the basis of hydrophobic, electronic, and steric factors combined with a rapid, quantitative method of evaluating their biological activity. The results reported herein indicate clearly that QSAR can be used to evaluate quantitative antibacterial data such as D-values *in vitro* and to predict which analog(s) in a congeneric series have the greatest antibacterial activity. Most significantly, the QSAR prediction has led to a cosmetic preservative (i.e., [8]) fully active at 0.04% in a white lotion formulation, which compares favorably with the 0.1% concentration requirement claimed for the commercially available unsubstituted parent congener II itself.

The work reported herein also demonstrates that statistically significant correlations between antimicrobial activity of the II congeners and appropriate physicochemical parameters (i.e., log P pr MR) do exist. The failure by Lappas *et al.* (6) to find a significant relationship with percent water solubility may be attributed to a need to recognize the complex sequence of events which occurs when a compound such as II or any of its substituted analogs undergoes partitioning between solvent phases. Factors such as the free energy of solvation in water, 1-octanol (or other lipophilic solvent) and entropy of fusion (as measured macroscopically by mp) must all be taken into account (34 c,d). In the present case, there is apparently an important role played by partitioning of II congeners between the aqueous exobiophase and that portion of the lipoprotein bacterial membrane system wherein the hypothesized invaginated -SH moieties referred to earlier may be found; this is not successfully modelled by a water solubility parameter alone.

The question of cosmetic preservative design via QSAR was first addressed by Hansch and his coworkers (35) twelve years ago. Cosmetic preservatives constitute one class of bioactive materials that may be evaluated by use of quantitative antibacterial data and QSAR. It is hoped that the work reported here will stimulate interest in QSAR as an adjunct to the evaluation and development not only of cosmetic preservatives, but of other classes of bioactive raw materials as well. Also, this rational approach may have merit in developing alternatives to animal testing, wherein the biological response (i.e., killing of selected tissue culture cells, as represented by 1/D-value) could be explained by the use of the QSAR Paradigm.

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