Synthesis and Cytotoxic Evaluation of Some 6-Arylidene-2-(α-hydroxyamino-α-arylmethyl)cyclohexanone Oximes and Related Compounds

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Abstract □ Reaction of 2,6-bis-(phenylmethylene)cyclohexanone (**1**) with a 4-molar excess of hydroxylamine hydrochloride and sodium acetate to produce the corresponding oxime **2** gave rise to 2-(α-hydroxyamino-α-phenylmethyl)-6-phenylmethylenecyclohexanone oxime (**5a**), whose structure was deduced from high-resolution proton nuclear magnetic resonance spectroscopy and confirmed by X-ray analysis. Compound **2** was eventually prepared from **1** with hydroxylamine per se and not with a mixture of hydroxylamine hydrochloride and sodium acetate. Ten analogues of **5a**, namely **5b–5k**, were prepared and evaluated for cytotoxicity. Six of the 11 compounds in series **5**, as well as **1**, showed activity in the 240–950 μM range against murine mammary EMT6 cells. Series **5** was also examined for cytotoxicity in an in vitro screen conducted by the National Cancer Institute with ~54 cell lines, and four compounds demonstrated selective toxicity toward various groups of tumors.

2,6-bis-(Phenylmethylene)cyclohexanone (**1**) is cytotoxic toward two tumor cell lines in vitro,[1,2] and molecular modification to the corresponding oxime, **2**, was considered for the following reasons. First, the pH of a number of tumors is lower than that of normal cells,[3] and because oximes may serve as prodrugs[4] whereby the ketone may be released under acidic conditions,[5] selective release of cytotoxic ketones in tumor cells is possible. Second, previous work from these laboratories showed that certain oximes of acyclic conjugated styryl ketones had greater antileukemic activity than the corresponding ketones.[6,7] Hence, a synthetic route for the preparation of the oxime **2** was required initially.

Results and Discussion

Chemistry—The initial attempt to prepare **2**, which had not been reported previously, involved the heating of **1** with either an equimolar amount or a 1-molar excess of hydroxylamine hydrochloride and sodium acetate in aqueous ethanol. No reaction occurred; therefore, the ketone was treated with a 4-molar excess of the other reactants. The product formed had a proton nuclear magnetic resonance (^{1}H NMR) spectrum (60 MHz) and elemental analysis inconsistent with the structure of the oxime **2**. A review of the literature revealed that **1** had previously been reacted with an excess of hydroxylamine hydrochloride in the presence of sodium acetate in ethanol, and the product isolated was tentatively assigned the structure **3**[8]; subsequently formula **4** was suggested for this compound.[9] High-resolution ^{1}H NMR spectroscopy of the product in deuterated dimethyl sulfoxide revealed that the compound had 10 aryl protons and three peaks, each of which integrated for less than one proton, which were attributed to exchanges between the hydrogen atoms of hydroxyl and/or amino groups of the compound with the hydrogen atoms of water in the solvent. One olefinic proton and a single hydrogen atom at 4.10 ppm were also observed. Several peaks in the range 3.54–1.02 ppm, which integrated for seven protons, were thought to be due to cyclohexyl hydrogen atoms. However, the peaks overlapped considerably, and even with Gaussian multiplication, resolution was not achieved. To establish the structure of the product unequivocally, X-ray analysis was undertaken vide infra, and the results (Figure 1) indicated that the product is represented by the structure **5a**. A correlation spectroscopy (COSY) experiment revealed that the phenylmethylene proton at C8 was coupled to the hydrogen atoms at position 5 of the cyclohexyl ring and that the N7 hydrogen atom was coupled to the protons attached to the O7 and C7 atoms. The hydrogen at position 2 was at the highest frequency of all of the cyclohexyl protons, and it was coupled to the hydrogen atoms attached to the O7 and N7 atoms. The spectrum revealed the chemical shifts and couplings of the cyclohexyl protons at the highest frequency (details are given in *Experimental Section*).

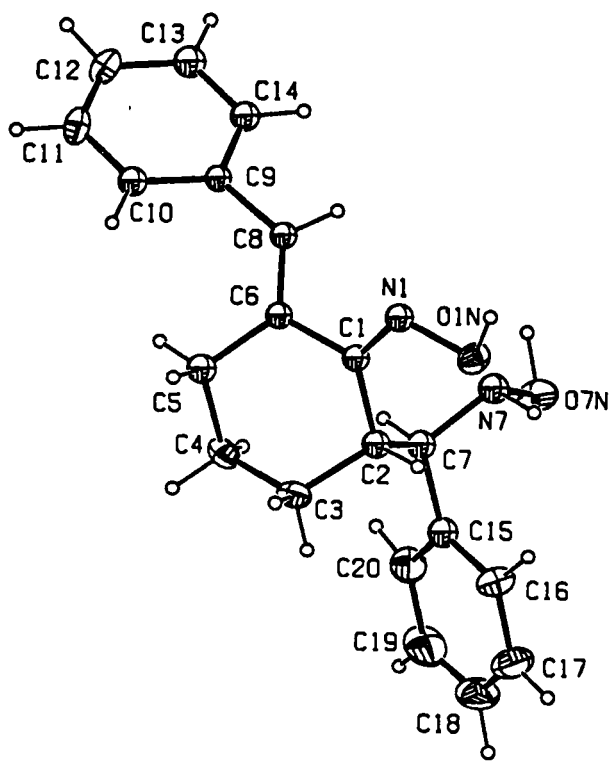

Figure 1—ORTEP diagram of the structure of **5a**.

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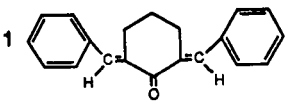

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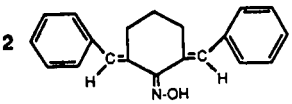

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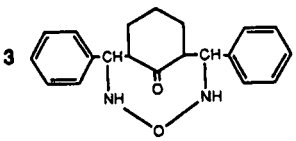

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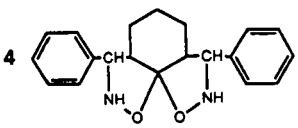

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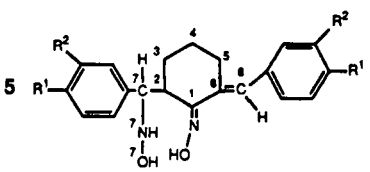


a: $R^1=R^2=H$ g: $R^1=OCH_3$; $R^2=H$

b: $R^1=Cl$; $R^2=H$ h: $R^1=OC_6H_5$; $R^2=H$

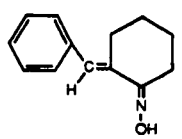
c: $R^1=R^2=Cl$ i: $R^1=OCH_2C_6H_5$; $R^2=H$

d: $R^1=F$; $R^2=H$ j: $R^1=R^2=OCH_2O$

e: $R^1=Br$; $R^2=H$ k: $R^1=R^2=OCH_2CH_2O$

f: $R^1=CH_3$; $R^2=H$

6



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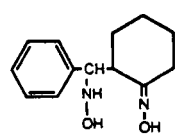

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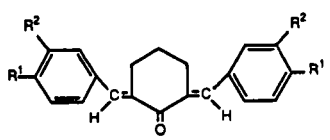

a: $R^1=Br$; $R^2=H$

b: $R^1=R^2=OCH_2CH_2O$

X-ray crystallography revealed that the central, six-membered ring of **5a** assumes a pseudochair conformation due to the two sp^2 carbon atoms at positions 1 and 6. The conjugated system, in which the phenyl group made angles of 35.7(3)° with the C6–C8–C9 plane and 76.8(2)° with the C1–N1–01N plane, is nonplanar. The angle between the two planes C6–C8–C9 and C1–N1–01N is 41.7(3)°. The configuration of the olefinic and carbimino double bonds is *E*, and in the case of the carbimino group, a *Z* configuration would have caused marked steric hindrance between the 01N atom and both the C8 and H8 atoms. The configurations at C2 and C7 carbon atoms are *R* and *S*, respectively. Whereas no intramolecular hydrogen bonding was observed, two intermolecular hydrogen bonds were noted: 01N–H70 ··· H7N(−z, 1 − x, 1 − y) [2.844(2) Å, < 0HN = 160(3)°]; 07H–H70 ··· N1(1 − y, 1 − z, − x)[2.795(2) Å, < 0HN = 159(4)°].

Attempts to observe the stability of **5a** at 37 °C in Sorenson's buffer (pH 7.4) were unsuccessful because of the very low solubility of this compound in the solvent. However, under more vigorous conditions, which departed from simulated physiological conditions (namely, the use of either acid or alkali in an organic solvent), **5b** was converted to 2,6-bis(4-chlorophenylmethylene)cyclohexanone in high yield (i.e., loss of hydroxylamine from the side chain and hydrolysis of the hydroxyimino group to a ketone function occurred). Whether compounds **5** will follow a similar path under the influence of biological macromolecules is conjecture, but it is conceivable that these compounds are prodrugs of the corresponding unsaturated ketones.

The reasons for the failure to obtain the desired compounds **2** were considered. First, reaction of 2-phenylmethylenecyclohexanone with a slight excess of hydroxylamine hydrochloride and sodium acetate produced **6**. Thus, the failure of **1** to react under these conditions may have been due to steric hindrance of a second phenylmethylene group to the attacking nucleophile. Second, because hydroxylamine has a pK_a of 7.97,[10] it is conceivable that under the reaction conditions it is mainly in the protonated form and its nucleophilicity will be reduced. Hydroxylamine per se was prepared and reacted with 2,6-bis(phenylmethylene)cyclohexanone (**1**) to give **2**, albeit in low yield. The question was raised, therefore, as to whether the use of a primary amine rather than a mixture of the amine salt and sodium acetate would be useful as a general synthetic route to carbimino derivatives of cyclohexanones containing one or two phenylmethylene groups adjacent to the keto function. 2-Phenylmethylenecyclohexanone phenylhydrazone was formed in comparable yields from the corresponding ketone by using either phenylhydrazine or a mixture of phenylhydrazine hydrochloride and sodium acetate. However, whereas **1** also reacted with both phenylhydrazine and a mixture of phenylhydrazine hydrochloride and sodium acetate mixture, use of the free base gave a higher yield of the hydrazone. Thus, the following observations may be made. For 2-arylidenecyclohexanones, it is likely that either the free amine or a mixture of the amine salt and sodium acetate may be used to give the corresponding product. However, for nucleophilic attack at the more hindered carbonyl group in 2,6-bis(arylidene)cyclohexanones, use of the free bases may be preferable or possibly the sole route to be used. If the primary amines are strongly basic, the use of free primary amines per se, rather than a mixture of the amine hydrochloride and sodium acetate, may give higher yields.

Cytotoxic Evaluations—A number of derivatives containing an arylmethylene function have shown activity against the murine mammary EMT6 tumor in vitro.[11–13] To develop structure–activity relationships with this cell line, various analogues of **5a** were prepared (namely, **5b–5k**), in which a variety of substituents with different electronic and hydrophobic properties were introduced into the aryl ring. In addition, compound **7** was prepared, which like **6**, contains part of the **5a** molecule and may indicate the pharmacophoric group of **5a** should this latter compound display cytotoxic properties. The cytotoxicity of **1**, **2**, and **5–7** against the EMT6 tumor was evaluated (Table I). Concentrations of 500 μM or more of **5h** caused precipitation of the oxime into the media, and hence, the cytotoxicity of **5h** could not be evaluated in the same way as that of the other compounds and **5h** was excluded from any discussion of structure–activity relationships. In addition, over the concentration range 400–600 μM, **5a** caused a 15–22% inhibition of EMT6 cells, whereas with **5a** at

Table I—Physical Data and IC_{50} and GI_{50} Values for 1, 2, and 5–7

Compound	mp, °C	Yield, %	IC_{50}, μM[a]	GI_{50}, M[b]	
				MG MID	Delta
1	116–117	90	283	NA[c]	NA
2	130–132	3	>1000	NA	NA
5a	172–174	43	>1000	$10^{-4.27}$	1.37
5b	170–171	39	240	$10^{-4.69}$	0.56
5c	174–176	35	312	$10^{-4.76}$	0.24
5d	176–177	45	661	$10^{-4.44}$	0.34
5e	177–178	37	244	$10^{-4.78}$	0.30
5f	175–176	35	260	$10^{-4.46}$	0.49
5g	168–169	38	950	$10^{-4.38}$	0.41
5h	166–167	41	>400[d]	$10^{-4.52}$	0.83
5i	169–170	44	>1000	$10^{-4.81}$	0.67
5j	163–164	48	>1000	$10^{-4.63}$	0.20
5k	102–104	31	>1000	$10^{-4.65}$	0.35
6	125–126	87	>1000	NA	NA
7	192–193	62	>1000	$10^{-4.03}$	0.59

[a] IC_{50} values are for inhibition of the growth of EMT6 cells (EMT6 cell plating efficiency, 0.70 ± 0.05). [b] GI_{50} values are the average cytotoxicities against ~54 human tumors. [c] NA, Result is not available. [d] Concentrations of 5h of 500 μM and above cause precipitation of the compound from the media.

800 and 1000 μM, no inhibition was noted. Poor solubility of 5a in the media may have caused this anomalous result, although no precipitation of drugs was observed. The other compounds listed in Table I (except for 2, 5i–5k, 6, and 7) inhibited EMT6 cell growth at a concentration of 100 μM.

The following observations based on the EMT6 screen may be made. First, conversion of 1 into the oximes 2 and 5a lowers or possibly abolishes activity. Second, in the series of 2-arylidene-6-(α-hydroxyamino-α-arylmethyl)cyclohexanone oximes, 5b, 5c, 5e, and 5f had comparable activities, followed by the activities of 5d and 5g. Third, the lack of activity of 6 and 7 at 1000 μM suggests that these fragments of the molecule 5a have little or no cytotoxic properties towards EMT6 cells. Exponential and linear plots were made of the concentrations of 5b–5g required to inhibit 50% of the growth of the cells (IC_{50} values) versus the molar refractivity and the sigma and pi values of the aryl substituents. The highest correlation coefficients (r) were obtained when the pi values were considered [i.e., r = 0.742 (exponential) and r = 0.768 (linear)], but according to the test for zero correlation,[14] these correlations are not significant ($p < 0.10$).

Recently, the National Cancer Institute (NCI) introduced a new in vitro screen in which compounds are evaluated initially against ~60 human tumors obtained from different diseases (namely, melanoma, leukemia, and non-small cell lung, small cell lung, colon, central nervous system, ovarian, and renal cancers).[15] Compounds are generally evaluated at five concentrations (namely, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M). The concentrations of compounds that cause 50, 0, and −50% of growth of the tumors compared with untreated cells (GI_{50}, TGI, and LC_{50} values, respectively) indicate the growth-inhibiting, cytostatic, and cytotoxic properties, respectively, of the compound. The data are presented in several ways, including mean graphs[16] in which the sensitivity of a particular cell line to the candidate drug is compared with the average sensitivity of all the cell lines tested. The bar scale is logarithmic. Hence, a bar that is one unit to the right of the main axis indicates that a particular cell line is 10 times more sensitive to the compound than the mean. A bar that is two units to the left of the axis, for example, means that the cell line has 1% sensitivity to the compound compared with the average sensitivity of all cell lines. The delta value is an indication, in a logarithmic scale, of the increased sensitivity among the cell lines to the compound compared with the average cytotoxicity of all the cell lines. A delta value of one or more is considered significant (i.e., the compound displays selective toxicity for one or more tumors). If for any reason, such as low potency of the compound, the GI_{50}, TGI, and LC_{50} concentrations for each cell line are not obtained, then the highest concentration used is preceded by a > sign (e.g., $>10^{-4}$ M). However, because this value is used to determine the mean value for all cell lines, if one or more of these values with a > sign are present, the average GI_{50}, TGI, or LC_{50} values are not, strictly speaking, mean values. Hence, the term MG MID (mean graph midpoint) is used. The principal objective of this new screen is to find disease-specific compounds rather than chemicals that are lethal to all living cells. Subpanel specificities (e.g., against leukemia or colon cancer) are recorded and reviewed, and where appropriate, in vivo experimentation is implemented.

Evaluation of compounds 5 in this screen showed that the compounds were, in general, insufficiently active for determination of TGI and LC_{50} values for the majority of the tumor cell lines. However, GI_{50} values for most tumors were obtained, and the MG MID values are given in Table I, along with the delta values. The average MG MID value in the GI_{50} screen was $10^{-4.58}$ M. A reference compound, melphalan, had an average GI_{50} value of $10^{-4.58}$ M and a delta value of 1.38. Hence, most of the compounds are as potent as melphalan, and the delta value of 5a is similar to that of melphalan. In addition, 5a, 5e, and 5h (leukemia) and 5g (central nervous system, colon, and renal tumors) demonstrated subpanel specificity. In contrast to compounds 5, 7 at a maximum concentration of $10^{-4.00}$ M yielded GI_{50} values for only nine of the 55 cell lines. Hence, 7 is by far the least active of the oximes evaluated in the NCI screen. However, a subpanel specificity of 7 to leukemia was noted.

Two representative ketones (namely, 8a and 8b) had average GI_{50} values of $10^{-5.08}$ M (delta, 0.69) and $10^{-4.65}$ M (delta, 0.95), respectively. Compound 8b demonstrated subpanel specificity towards small cell lung cancers. Thus, compared with oximes with similar aryl substitution patterns, 8a is more potent than 5e, and 8b has some subpanel specificity, in contrast to 5k.

Conclusions

First, in an attempt to prepare 2 from the analogous ketone by using excess hydroxylamine hydrochloride and sodium acetate, 5a was obtained. The structure of 5a was deduced by 300-MHz ^1H NMR spectroscopy and confirmed by X-ray analysis. A series of these novel compounds was prepared successfully. Second, the desired oxime 2 was synthesized from 1 by using hydroxylamine as the free base; hence, this procedure may be a synthetic route to follow in the future in the preparation of 2,6-bis(arylmethylene)cyclohexanone oximes. Third, evaluation of 1, 2, and 5–7 against EMT6 cells revealed that the oxime 2 was less active than 1. Also, in compounds 5, four compounds had ~1% of the activity of melphalan. Fourth, examination of 5 in the NCI in vitro screen against a large number of tumors revealed that, in general, these compounds had similar overall toxicity to melphalan. Furthermore, the specific toxicity towards leukemia demonstrated by 5a, 5e, and 5h, and the three groups of cancers that were especially sensitive to 5g indicate that these four compounds are templates for future molecular modification. The facts that 2 is less potent than 1 in the EMT6 screen and that 8a and 8b had improved activities compared with the related oximes 5e and 5k in the NCI screen suggest that oximation of α,β-unsaturated cyclohexanones may be a detrimental molecular change that reduces cytotoxicity.

Experimental Section

Elemental analyses (C,H,N) of 2, 5–7, 2-phenylmethylenecyclohexanone phenylhydrazone, 2,6-bis(phenylmethylene)cyclohexanone phenylhydrazone, and 2,6-bis(phenylmethylene)cyclohexanone hydrazone and for carbon and hydrogen on 1 and all of the 2,6-bis(arylidene)cyclohexanones required in the synthesis of compounds 5 (performed by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada) were within 0.4% of the calculated values. Thin-layer chromatography (TLC) with silica gel plates and a fluorescent indicator was used. Melting points are uncorrected. ^{1}H NMR spectroscopy was performed with a Varian T60 spectrometer (60 MHz) and a Bruker AM300 FT instrument (300 MHz). The letters s, d, dd, m, and br mean singlet, doublet, double doublet, multiplet, and broad, respectively.

Chemistry—*Preparation of 2,6-bis(Phenylmethylene)cyclohexanone (1) and the Corresponding Oxime (2)*—Compound 1 was prepared by a literature method.[17] Initially, the following procedure was used to produce 2. A solution of sodium acetate (0.29 g, 0.0036 mol) and hydroxylamine hydrochloride (0.25 g, 0.0036 mol) in water (~4 mL) was added to a solution of 1 (0.987 g, 0.0036 mol) in ethanol (100 mL), and the mixture was heated under reflux for 48 h. After the mixture had cooled, water (~150 mL) was added, and the reaction mixture was refrigerated at −20 °C overnight. The solid deposited was unreacted ketone (0.85 g).

A solution of hydroxylamine (0.01 mol), which was prepared by a reported procedure,[18] and 1 (0.01 mol) in ethanol (95%, 100 mL) was heated under reflux for 4 h. The volume of the reaction mixture was increased to 150 mL with water. After the mixture had cooled, the gummy precipitate was collected by filtration. Trituration with petroleum ether (bp, 80–100 °C) gave colorless crystals, which, on recrystallization from ethanol (95%), produced 2.

Preparation of 6-Arylidene-2-(α-aryl-α-hydroxylaminomethyl)cyclohexanone Oximes (5)—The 2,6-bis(arylidene)cyclohexanones required in the synthesis of 5 were prepared in 60–90% yields with a literature method for the preparation of 1.[17] The crude products were recrystallized from ethanol. The melting points of the ketones that were precursors of 5a–5g and 5j were similar to literature values. The melting points (yields) of 2,6-bis(4-phenoxyphenylmethylene)cyclohexanone, 2,6-bis(4-benzyloxyphenylmethylene)cyclohexanone, and 2,6-bis(3,4-ethylenedioxyphenylmethylene)cyclohexanone were 159–160 (67%), 190–191 (64%), and 192–193 °C (60%), respectively. The ^{1}H NMR spectra (60 MHz) were in accord with the proposed structures.

A solution of sodium acetate (1.18 g, 0.0144 mol) and hydroxylamine hydrochloride (1.0 g, 0.0144 mol) in water (~10 mL) was added to a solution of the appropriate 2,6-bis(arylidene)cyclohexanones (1.0 g, 0.0021–0.0036 mol) in ethanol (100 mL). The reaction mixture was heated until the yellow color disappeared (4–7 h). After the mixture had cooled, water (~50 mL) was added to dissolve the colorless solid that had precipitated, and the solution was refrigerated overnight at −20 °C. The precipitate was collected, dried, and recrystallized from *n*-propyl alcohol [5k was recrystallized from ethanol (70%, v/v)]. TLC with benzene:ethyl acetate (3:1) revealed one spot.

Preparation of 2-Phenylmethylenecyclohexanone Oxime (6)—A solution of 2-phenylmethylenecyclohexanone (31.66 g, 0.17 mol) in ethanol (100 mL) was added to a solution of hydroxylamine hydrochloride (13.20 g, 0.19 mol) and sodium acetate (14.76 g, 0.18 mol) in water (40 mL), and the reaction mixture was heated at 60 °C with stirring for 1 h. Water was added to increase the volume to 250 mL and, after the mixture had cooled, the precipitate was removed by filtration, washed with water, and recrystallized from acetone to give 6; mp, 125–126 °C (literature value,[19] 125–126 °C).

Preparation of 2-(α-Phenyl-α-hydroxylaminomethyl)cyclohexanone Oxime (7)—A solution of 2-phenylmethylenecyclohexanone (1.86 g, 0.01 mol) in ethanol (100 mL) was added to a solution of hydroxylamine hydrochloride (2.78 g, 0.04 mol) and sodium acetate (3.25 g, 0.04 mol) in water (8 mL), and the mixture was heated under reflux for 3 h. Water was added to the mixture to increase the volume to 250 mL and, after the mixture had cooled, the precipitate was collected, washed with water, and recrystallized from ethanol to give 7; mp, 192–193 °C (literature value,[20] 192 °C).

Preparation of 2-Phenylmethylenecyclohexanone Phenylhydrazone—A solution of 2-phenylmethylenecyclohexanone (3.0 g, 0.016 mol) and phenylhydrazine (1.94 g, 0.018 mol) in ethanol (50 mL) was heated at 60 °C with stirring for 1 h. After the mixture had cooled, the reaction mixture was refrigerated at −20 °C overnight, and the precipitate was collected, dried, and recrystallized from ethanol to give 2-phenylmethylenecyclohexanone phenylhydrazone in 89% yield; mp, 119–120 °C (literature value,[21] 120 °C).

A solution of 2-phenylmethylenecyclohexanone (0.987 g, 0.0036 mol) in ethanol (100 mL) was added to a solution of phenylhydrazine hydrochloride (0.52 g, 0.0036 mol) and sodium acetate (0.29 g, 0.0036 mol) in water (~5 mL). The reaction was heated at 60 °C with stirring for 0.75 h. Water was added to increase the volume to 150 mL and, after the mixture had cooled, the precipitate was collected and washed with water. Recrystallization from ethanol gave 2-phenylmethylenecyclohexanone phenylhydrazone in 82% yield; mp, 119–120 °C.

Preparation of 2,6-bis-(Phenylmethylene)cyclohexanone Phenylhydrazone—A solution of 1 (2.74 g, 0.01 mol) and phenylhydrazine (1.62 g, 0.015 mol) in ethanol (100 mL) was heated under reflux for 4 h. After the mixture had cooled, the reaction mixture was refrigerated overnight at −20 °C, and the crude product was collected. Recrystallization of the precipitate from ethanol afforded the desired product in 85% yield; mp, 138 °C.

Solutions of 1 (0.987 g, 0.0036 mol) in ethanol (100 mL) and phenylhydrazine hydrochloride (0.723 g, 0.005 mol) and sodium acetate (0.41 g, 0.005 mol) in water (6 mL) were mixed and heated under reflux for 12 h. Sufficient water was added to make a clear solution and, after the solution had cooled, the precipitate was collected and washed with water. Recrystallization from ethanol (95%, v/v) gave 2,6-bis(phenylmethylene)cyclohexanone phenylhydrazone in 51% yield; mp, 138 °C.

Preparation of 2,6-bis-(Phenylmethylene)cyclohexanone Hydrazone—A solution of 1 (2.74 g, 0.01 mol) and hydrazine hydrate (0.75 g, 0.015 mol) in ethanol (100 mL) was heated under reflux for 4 h. Water was added to increase the volume of the reaction mixture to 200 mL. After the mixture had cooled, the precipitate was collected and recrystallized from ethanol to give the photosensitive hydrazone in 65% yield; mp, 106 °C.

Stability of 5b—A solution of 5b (0.5 g, 0.0013 mol) in ethanol (80 mL) containing hydrochloric acid (0.1 mL) was heated under reflux during which time the reaction was monitored by TLC with benzene:ethyl acetate (3:1). After ~2 h, TLC revealed the formation of a new compound. Evaporation of the solvent gave a residue that was recrystallized from ethanol to give 2,6-bis(4-chlorophenylmethylene) cyclohexanone (0.382 g, 85%). The TLC (benzene:ethyl acetate, 3:1) and ^{1}H NMR (60 MHz) characteristics of the compound were identical to those of an authentic specimen of the unsaturated ketone.

The experiment was repeated except that in place of hydrochloric acid, a solution of sodium hydroxide (1 N, 2 mL) was used. After ~2 h, the solvent was removed to give a crude product that was recrystallized from ethanol to give 2,6-bis(4-chlorophenylmethylene) cyclohexanone in 85% yield.

High-Resolution ^{1}H NMR Spectrum of 5a—The ^{1}H NMR spectrum of 5a in dimethyl sulfoxide-d_6 was acquired at 300.134 MHz with a 4201-Hz spectral window. The data set size was set to 16 kilowords (TD = 16 384), requiring an acquisition time of 1.95 s and using one order of zero-filling to yield a resolution of 0.257 Hz/point. The spectrum was acquired with a pulse width of 30° and with a relaxation delay of 2.0 s. Typically, 512 free inductive decays were collected.

Spectra were initially processed without the use of a window function to ensure comparability between integrals of resonances. The spectrum was then reprocessed with the Gaussian resolution enhancement function. Chemical shifts were referenced to the methyl resonance of the internal standard tetramethylsilane (TMS) and are quoted in parts per million versus TMS. Coupling constants are given in hertz.

A two-dimensional spectrum of 5a was acquired by using a COSY 90 sequence.[22] Salient features were a data set of 1024 words × 256 files, with an order of zero-filling in the F1 direction. The resolution of the resulting spectrum was 4.5 Hz/point. The data for 5a are as follows: ^{1}H NMR: δ 1.02 (m, 1H, C5-H), 1.15 (m, 1H, C4-H), 1.45 (br m, 1H, C3-H), 1.47 (br m, 1H, C4-H), 2.26 (br m, 1H, C5-H), 2.66 (br d, 1H, C5-H, $J_{C5\text{-}H/C5\text{-}H}$ = 15.5 Hz), 3.54 (dd, 1H, C2-H, $J_{C2\text{-}H/C7\text{-}H}$ = 9.3 Hz; $J_{C2\text{-}H/NHOH}$ = 5.1 Hz), 4.10 (d, 1H, C7-H, $J_{C2\text{-}H/C7\text{-}H}$ = 9.0 Hz), 5.52 (br s, < 1H, N*H*OH), 6.55 (s, 1H, C8-H), 7.04 (s, < 1H, NHO*H*), 7.34–7.19 (m, 10H, aryl H), 11.07 (s, < 1H, NOH).

X-ray Analysis of 5a—The plate-shaped crystal belongs to the rhombohedral system, with dimensions of 0.05 × 0.25 × 0.33 mm, the

Table II—Cytotoxicity of Compounds against EMT6 Cells[a]

Compound	Cell Survival (%) at Indicated Compound Concentration (μM)									
	1000	800	700	600	500	400	300	250	200	100
1	12 ± 5	19 ± 17	—	21 ± 11	25 ± 16	33 ± 18	47 ± 14	—	74 ± 6	—
2	88 ± 7	96 ± 5	—	96 ± 5	99 ± 2	99 ± 1	—	—	—	—
5a	93 ± 33	94 ± 21	—	79 ± 10	78 ± 11	85 ± 6	—	—	—	—
5b	—	—	—	—	—	0	1 ± 1	39 ± 9	90 ± 17	100 ± 14
5c	—	—	—	—	0	1	15 ± 3	—	58 ± 10	84 ± 14
5d	17 ± 6	28 ± 14	45 ± 12	59 ± 18	60 ± 9	87 ± 11	—	—	—	—
5e	—	—	—	—	0	0	12 ± 10	—	77 ± 18	95 ± 8
5f	—	—	—	—	1 ± 1	4 ± 4	18	—	90 ± 7	97 ± 9
5g	43 ± 8	56 ± 9	—	73 ± 11	82 ± 5	84 ± 2	—	—	—	—
5h	86 ± 15	84 ± 12	—	85 ± 17	91 ± 9	77 ± 10	84 ± 1	—	89 ± 6	—
5i	84 ± 11	85 ± 10	91 ± 16	86 ± 7	86 ± 10	94 ± 4	—	—	—	—
5j	102 ± 19	98 ± 20	—	105 ± 14	105 ± 26	97 ± 13	—	—	—	—
5k	54 ± 25	59 ± 12	—	69 ± 13	80 ± 14	92 ± 20	—	—	—	—
6	82 ± 27	89 ± 10	—	98 ± 6	100 ± 7	100 ± 3	—	—	—	—
7	93 ± 7	94 ± 5	—	95 ± 8	96 ± 6	94 ± 6	—	—	—	—

[a] Values are means ± standard deviations; —, not tested.

space group is $R3$, and the unit cell dimensions are a = 16.701(2) Å, α = 114.04(1)° (with V = 2820.7 Å^3), and Z = 6. Other relevant parameters were M_r = 322.38, D_m = 1.15 mg · m^{-3}, D_x = 1.139 mg · m^{-3} (no solvent), λ(Cu, Kα) = 1.5418 Å, μ = 0.60 cm^{-1}, F(100) = 496, and absolute temperature (T) = 287 K. The cell parameters were obtained by least-squares analysis of 25 reflections with 14.93 < θ < 22.03°. A CAD-4 Enraf-Nonius diffractometer was used for data collection, and a total of 12 422 reflections were collected with 3871 unique reflections: $-20 \le h \le 20$, $-20 \le k \le 20$, $-20 \le l \le 0$, $[(\sin\theta)/\lambda]_{max}$ = 0.62653 Å^{-1}. There were three monitoring reflections, and intensity variation was within 2.93%. No absorption, extinction, or anomalous dispersion corrections were made. The merging R value of 0.0153 was based on intensities for 8551 replicate reflections. The structure was solved by direct methods using *XTAL*-2.6.[23] All non-hydrogen atoms found on the E map were refined anisotropically. All hydrogen atoms were found on ΔF maps and were refined isotropically. A difference map calculated at this point had a number of peaks of ~1 e Å^{-3}, which were attributed to disordered ethanol solvent. The excess electron density could be refined into three oxygen atoms (01S, 02S, and 03S) with partial populations. These three sites and their symmetry equivalents were in close proximity and could not all be simultaneously occupied. A total of 3211 reflections with $I > 2\sigma(I)$ were used in the refinement of 332 parameters to give final R = 0.048, wR = 0.045 [$w = 1/\sigma^2(F)$], and S = 3.135. The F values were used in the least-square refinement in which the final $(\Delta/\sigma)_{av}$ was 0.009, $(\Delta/\sigma)_{max}$ was 0.11, and the maximum and minimum $\Delta\rho$ in the final ΔF map were +0.23 and −0.21 e Å^{-3}, respectively. The atomic-scattering factors were taken from the literature.[24] All calculations were performed on a VAX6330 computer at the University of Saskatchewan.

Cytotoxicity Evaluations—*Evaluation of 1, 2, and 5–7 against EMT6 Cells*—Details of this procedure have been described earlier.[11] In brief, various concentrations of the compound were dissolved in dimethyl sulfoxide, and 100 μL of the solution was added to the media. After 2 h, the cells were removed from the media and a colony-forming assay enabled the surviving fraction of cells to be calculated. Each compound was evaluated in duplicate on 4–6 separate occasions, and the IC_{50} values (Table I) were obtained from graphs of pooled data. In this assay, the IC_{50} value of the reference compound, melphalan, was 3 μM. The cytotoxicities of 1, 2, and 5–7 expressed as percent cell survival are given in Table II.

A one-way analysis of variance enabled comparisons of the potencies of certain compounds. At 400 μM, the potency of 1 is different from those of 5b, 5c, 5e, and 5f (p = 0.001), whereas the cytotoxicities of the latter four compounds are not significantly different from each other (p = 0.16). At 1000 μM, compounds 5d and 5g had significantly different activities from the other compounds 5 (p < 0.001), the cytotoxicities of 2, 5a, 5i, 5j, 5k, and 7 were not significantly different (p = 0.2135), and the potencies of 5d, 5g, and 6 were significantly different from each other (p = 0.001). The t test revealed that, at 1000 μM, the activity of 5g was statistically significantly different from that of 5d (p = 0.0195) and that of 6 (p = 0.002), whereas the activities of 5d and 7 were not significantly different from each other (p = 0.133). At 800 μM, 5d and 5g were significantly different in potency (p = 0.003).

The correlation coefficients of the exponential plots of the IC_{50} values versus molar refractivity and sigma and pi values of the aryl substituents in 5b–5g were 0.131, 0.512, and 0.742, respectively. The correlation coefficients of the linear plots of the molar refractivity and sigma and pi values of the aryl substituents in 5b–5g were 0.268, 0.570, and 0.768, respectively.

Evaluation of 5, 7, and 8 against Human Tumors—This assay was carried out by the NCI according to their protocols.[15] Evaluation of the GI_{50} values of 5b, 5c, 5j, 5k, 8a, and 8b indicated that these compounds had IC_{50} values of $>10^{-4.00}$ M against all cell lines. Hence, the MG MID values in Table I are true mean values. On the other hand, the following compounds had GI_{50} values of $>10^{4.00}$ M for some of the cell lines (the numbers of the cell lines are given in parentheses): 5a (20; one cell line had a GI_{50} of $>10^{-5.00}$ M and another had a GI_{50} of $>10^{-6.00}$ M), 5d (5), 5e (2; three cell lines had GI_{50} values of $>10^{-6.00}$ M), 5f (4), 5g (1), 5h (4), and 5i (6). Thus, the figures in Table I for these compounds reflect MG MID data.

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