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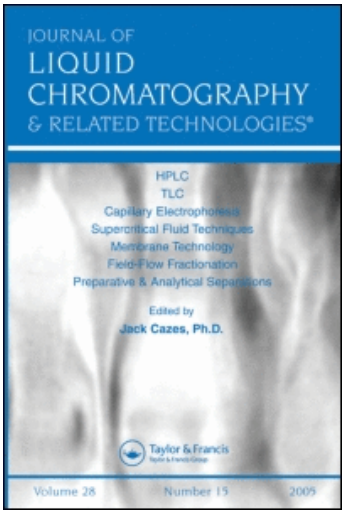

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EVALUATION OF HYDROPHOBICITY AND ANTITUMOR ACTIVITY OF A MOLECULE LIBRARY OF MANNICH KETONES OF CYCLOALKANONES

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EVALUATION OF HYDROPHOBICITY AND ANTITUMOR ACTIVITY OF A MOLECULE LIBRARY OF MANNICH KETONES OF CYCLOALKANONES

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ABSTRACT

A series of Mannich ketones of cycloalkanones were synthesized to study the relative importance of structure and specific substitutions in relation to their hydrophobicity and antitumor activity. Substitutions were carried out with morpholinyl, pirrolidinyl, piperidyl, and tetrahydro-isoquinolyl groups in various position on four different base structures. Hydrophobicity of Mannich ketones was characterized by chromatography data

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(log k′) and by software calculated parameters (c log P). Cell proliferation inhibitory activity of cycloalkanones was evaluated by MTT and apoptosis assays on A431 human adenocarcinoma cells. Our results suggest that the higher the hydrophobicity values (log k′ and c log P) the higher the antitumor- and apoptotic activity of Mannich ketones. Determination of hydrophobicity by measuring the log k′ or by calculating the c log P values of the compounds may help to predict their biological activities.

INTRODUCTION

Combinatorial chemistry provides drug discovery facilities with a growing number of biologically diverse active compounds. In recent years, high throughput screening, where collections of thousands of compounds are screened with the intention of finding relevant biological activity, has proven valuable in finding new lead compounds (1). It has been noted, that the synthesis of combinatorial libraries tends to result in compounds with higher molecular weights and higher lipophilicity, and presumably lower aqueous solubility, than with conventional synthetic strategies. For this reason, computational screens and experimental design have been suggested and used to select sublibraries with relevant physico-chemical properties of active drugs, such as lipophilicity and solubility (2). The analysis of the large data sets and exploiting their predicting power is an emerging area in the field of drug design. Hence, there is much interest in fast, reliable, and generally applicable structure-based methods for the prediction of aqueous solubility of new drugs before a promising drug candidate has even been synthesized.

One of the current approaches in rational drug design is to estimate hydrophobic nature of the molecules on the basis of certain experimentally determined physico-chemical parameters, because hydrophobic properties of the molecules play an important role in the mechanism of their biological action, as well as in the structure-biological activity relationships (3–5). Another possibility is the use of different softwares to calculate hydrophobicity on the basis of the structural moieties making up the molecule investigated (6).

Hydrophobicity is commonly considered as a measure of the relative tendency of the molecule to prefer a non-aqueous rather than an aqueous environment (7) and it was recognised as one of the most important parameters influencing the fate of a molecule within the body and it has a pivotal role in QSAR studies (6). Hydrophobicity is usually expressed as some kind of molecular parameter describing the partition of the molecule between a hydrophilic (aqueous) and lipophilic (non-aqueous or organic) phase. Since the proposal of Hantsch, the hydrophobicity of molecules has been typically

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characterised by the log 1-octanol/water partition coefficient (log P_{ow}) (8,9). In the last decades, different reasons promoted the displacement of the shake flask method conventionally used to determine log P_{ow}. One of them is the experimental difficulty of this method, other drawbacks aroused by the invention of the combinatorial molecular libraries in the rational drug design (10).

These demands contribute to the development and application of those separation methods of high performance, which beyond the fast separation of the analysed components, are simultaneously able to provide data characterizing physico-chemical properties (e.g., distribution coefficient, retention factor, hydrophobicity) of the compounds analysed. High-performance liquid chromatography in reversed-phase separation mode (RP-HPLC) has long been recognized as a potential method for lipophilicity determination (11,12,30). Various approaches have been described which employ octanol in the chromatographic system or just use conventional octadecyl silica column and hydroorganic mobile phase (3,11,12). The properties of the compounds are characterised directly from the chromatographic retention determined by the interaction of solutes with the stationary and mobile phases. When highly efficient reversed-phase stationary phases were used with hydroorganic mobile phases, the correlation between the chromatographic partition data and the octanol/water partition data was strong when structurally related compounds were investigated (13). The aim in this respect is to estimate hydrophobic character of the molecules on the basis of their retention factors (k') determined in various separation processes. Expected biological activity of the constituents of a molecule library can be evaluated on the basis of their retention factors (3). This may prove to be especially useful for pre-screening of thousands of molecules synthesized by combinatorial chemistry methods. The number of molecules to be biologically tested can be considerably reduced if biological activity of the compounds can reliably be predicted on the basis of those data. In that case, there is no need to perform biological characterisation of every single member of the library (7).

Among the various biological parameters investigated, the knowledge of cytotoxicity of the compounds is one of the most important data. Many different methods are available to assess cytotoxicity in culture, including the microculture tetrazolium assay (e.g. MTT) (14,15). Since measurements of *in vitro* growth in microculture wells by cell mediated reduction of tetrazolium salt to water insoluble formazan crystals showed excellent correlation with measurements of cellular protein in adherent cell line, as well as viable cell count in suspension cell cultures (16), this assay provides sensitive and reproducible indices of growth as well as drug sensitivity in individual cell lines. Thus, this colorimetric assay based on enzyme acitivity of various dehydrogenases of the living cells is suitable for cytotoxicity testing of antitumor drugs *in vitro* (16,17).

However, as it was observed in many cases, cytotoxic agents like antitumor compounds can reduce not only the number of tumor cells as a measure of

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decreased MTT reduction but they can induce various types of cell death, including apoptosis (18). Apoptosis is a physiological phenomenon, which means apoptotic or programmed cell death where the dying cell plays an active part in its own destruction relying on the new protein synthesis, and among other morphologic hallmarks (19). Furthermore, it is becoming increasingly clear that apoptosis is a significant mode of cell death in the response of tumor cells to various anticancer treatment. Therefore, new apoptosis-inducer molecules provide new targets for drug design (20).

In our previous work, we reported on the antifungal effect of *E*-2-arylidene-1-tetralones, *E*-3-arylidenechroman-4-ones, and *E*-3-arylidene-1-thiochroman-4-ones-homoisoflavones (21). These compounds were screened against human pathogenic yeasts showing marked antifungal activity (22). Furthermore, several Mannich ketones are described having antibacterial, antifungal, and cytotoxic activity (23–28). To search for new types of antiproliferative compounds possessing apoptosis-inducing activity, a library of Mannich ketones of cycloalkanones was designed. Twenty-one compounds were synthesized with different base structures and substituents.

The aim of the present work was to separate the members of the Mannich ketone library by a suitable RP-HPLC method, to characterize their hydrophobicity by experimental parameter obtained from the separation process (retention factor, k'), and by a parameter obtained by computer prediction method (c log P calculation). Furthermore, to determine the antitumor activity of Mannich ketones by MTT test and apoptosis assay. Our measurements may be suitable for further characterization of the relationship between the hydrophobicity parameters and antitumor activity of the drugs. Furthermore, such a system may help to predict the biological activity of the element of a molecule library and it offers help to compose a more rational library.

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EXPERIMENTAL

Chemicals

Mannich ketones of cycloalkanones were synthesized in our laboratory. Purity of the compounds was better than 95% in each case as shown by HPLC analysis. Triethylamin and phosphoric acid were purchased from Fluka (Buchs, Switzerland); acetonitrile (ACN) from Chemolab (Budapest, Hungary). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and bovine serum albumin were obtained from Sigma. DMSO was purchased from Merck (Darmstadt, Germany). Foetal calf serum and RPMI-1640 medium were obtained from GIBCO (Grand Island, N.Y., USA). Formic acid was extra pure grade (27001) from Riedel-deHaën.

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Synthesis of Mannich Ketones

Mannich ketones have been prepared from the corresponding fused ketones as tetralones, indanones, etc. The method was the classical Mannich reaction applying ethanol as a solvent and HCl as a catalist (22). The products were purified through recrystallization and Mannich bases were liberated and purified using very mild conditions. Then they were precipitated with methanolic HCl. The NMR measurements were done on the Mannich bases. All structures were validated by a Waters LC/MS system equipped with a Waters 996 DAD UV detector and a Micromass ZMD MS detector. Mannich ketones with different base structures and substituents (R2) are summarized in Table 1. R1 substituents may be H- or OMe-, and their name and position are given in the Table 2.

Table 1. Basic Skeletons of Mannich Ketones of Cycloalkanones Serve as "Core" Structures

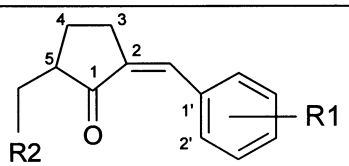
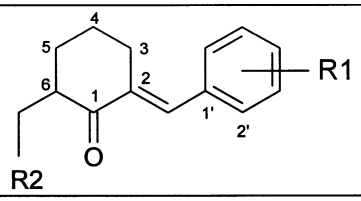
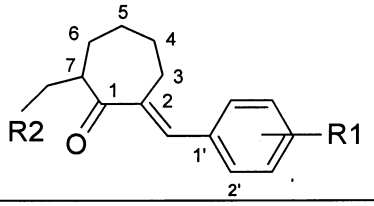
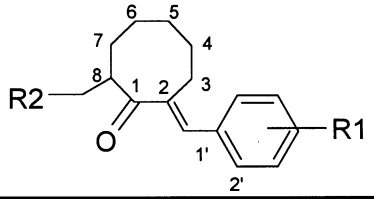
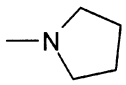
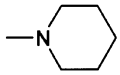
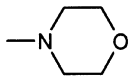
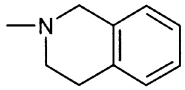
Type of structure	Structures
A	
B	
C	
D	

Table 2. R2 Substituents of the "Core" Skeletons of Mannich Ketones

Substituents	R2
1-pyrrolidinyl	
1-piperidyl	
4-morpholinyl	
2-(1,2,3,4-tetrahydro)-isoquinolinyl	

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HPLC Measurements

For chromatographic analysis stock solutions of 1.0 mg/mL of the samples in acetonitril : water (1 : 1) were prepared and filtered through a 0.2 μm Millipore filter unit. These solutions were kept in Eppendorf tubes at 4°C.

HPLC analysis of the samples were performed with a Varian (Basel, Switzerland) 9012 Solvent Delivery System, Varian 9065 Polychrom Diode Array Detector; column: Hypersil 5 MOS 5 μm, 300 × 4.6 mm (BST, Hungary); injector: Rheodyne; eluents: A, 0.25 N triethyl ammonium phosphate (TEAP), pH 2.25; B, 80% ACN + 20% A. Isocratic runs were performed in an eluent of 24 v/v% ACN in A eluent. Flow-rate: 1 mL/min. Temperature: 20°C. 20 μL portion from the stock solution was injected into loop of 50 μL volume and four parallel injections were analyzed. Retention factors (k′) of the samples were calculated from the experimentally determined retention data: $k' = (t_R - to)/to$.

Calculation of C log P Data

Software-predicted hydrophobicity of the compounds was calculated with the computer program c log P, which predicts this parameter with the so-called "fragment constant" method on the basis of the chemical structure of the compound processed. Briefly, the c log P program is based on Hansch-Leo's log P calculation method. It divides molecules into fragments and uses the constants of these fragments and correction factors taken from its database for log P

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Table 3. Groups in the Library of Mannich Ketones. Groups Were Created on the Basis of Core Structures and/or R1 and R2 Substituents

Group	Type of Structure	Number of Compounds	R1	R2	k' (*)	c log P	MTT (**)
I	A	1	H	1-piperidyl	2.13	3.29	23.1 ± 5
	B	2	H	4-methyl-piperidyl	4.65	4.23	–
	C	3	H	1-piperidyl	6.35	4.41	–
	D	4	H	1-piperidyl	8.92	5.32	39.3 ± 7
II	A	5	H	4-morpholinyl	1.13	2.63	38.7 ± 5
	A	6	H	1-pirrolidinyl	1.99	3.17	–
	A	7	H	1-piperidyl	2.13	3.29	–
	A	8	H	2-(1,2,3,4-tetrahydro)-isoquinolinyl	6.27	4.51	43.4 ± 6
III	A	5	H	4-morpholinyl	1.13	2.63	38.7 ± 5
	B	9	H	4-morpholinyl	1.88	3.19	–
	C	10	H	4-morpholinyl	3.45	3.75	–
	D	11	H	4-morpholinyl	5.6	4.31	42.7 ± 2
IV	A	12	2-OMe	1-piperidyl	2.87	3.21	44.1 ± 3
	A	13	3-OMe	1-piperidyl	3.09	3.21	–
	A	15	4-OMe	1-piperidyl	2.59	3.21	50.8 ± 4
V	A	16	2-OMe	4-morpholinyl	1.53	2.55	38.3 ± 6
	A	17	3-OMe	4-morpholinyl	1.63	2.55	–
	A	18	4-OMe	4-morpholinyl	1.3	2.55	40.4 ± 5
VI	B	9	H	4-morpholinyl	1.88	3.19	40.7 ± 6
	B	19	H	1-pirrolidinyl	3.02	3.73	–
	B	2	H	4-methyl-piperidyl	4.65	4.23	43.8 ± 3
VII	B	9	H	4-morpholinyl	1.88	3.19	40.4 ± 3
	B	20	4-OMe	4-morpholinyl	2.06	3.11	–
	B	21	4-Me	4-morpholinyl	3.66	3.69	44.4 ± 2

(*) Values are means of three parallel measurements where the standard error was less than 0.1 percent.
(**) Values are means of three different experiments with six wells per dose and time point in each cases and ±SD.

calculation (9,29). A c log P database was prepared with the computer program accessible via internet (www.daylight.com/daycgi/clogp). Hydrophobicity values were computed for the 21 Mannich ketones and are designed in the following as c log P values (see Table 3).

MTT Assay

To evaluate the antiproliferative effect of Mannich ketones on A431 cells (American Type Culture Collection No. CRL-1555), the MTT colorimetric assay was performed as described in References (16) and (17). The amount of formazan could be determined by photometer at 570 nm. Cells were plated into 96-well flat bottomed culture plates (Greiner, Germany) at a concentration of 10^4 cells per well in complete RPMI 1640 culture medium. Twenty-four hours after plating, fetal calf serum containing medium was removed and test solution of Mannich ketones was given to cells in various final concentrations, such as 200, 100, 50, 25, and 10 μg/mL. After incubation with drugs for 24 hours, MTT solution was added to the wells and plates were incubated at 37°C for 4 hours. Then sodium dodecyl sulphate (10 w/v% in 0.01 M HCl) was added and the amount of formazan formed was measured. Six wells per dose and time points were counted in three different experiments. The MTT assay has been performed only on selected members of the library. Compounds with highly hydrophobic nature and those with low hydrophobicity were selected. Percent of inhibition was calculated from the values of triplicate experiments and the results are expressed as percent of controls.

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Apoptosis Assay

To evaluate the apoptosis-inducing effect of Mannich ketones of cycloalkanones, 7×10^4/mL of A431 cells were cultured over glass coverslips in 6-well flasks. Treatment was performed 24 hours after plating with the following compounds: No. 1, 2, 3, and 4. Apoptosis-inducing activity was investigated at the final concentration of 100 μg/mL. Three cover slips per time point of both control and treated cells were fixed in ethanol/acetic acid (3 : 1) and stained with Haematoxylin and Eosin. At least 2000 cells per coverslip were investigated and counted. The cell pellets from control and treated cultures were resuspended at 1×10^7 cells/mL in fresh medium, and 25 μL of a solution of acridin orange and ethidium bromide was added. The mixture was immediately placed on the stage of an Olympus Fluorescence microscope and the image was analyzed and photographed. Apoptotic cells were characterized by morphological changes, including shrinkage of the cell and the nucleus; condensation and fragmentation of

the nuclear chromatin; membran blebbing; and the segmentation of the cell into apoptotic bodies. Apoptotic, necrotic, and live cells were compared, scored, and counted under microscope according to our earlier work (17).

RESULTS AND DISCUSSION

Results of the HPLC Measurements

A suitable isocratic ACN/TEAP eluent system has been developed for the separation of structurally relative compounds (Table 3) of the Mannich ketone library containing 21 compounds bearing different substituents on the same core (Table 1), and compounds having the same substituents (Table 2) on different cores (rings with different carbon number). Based on their structural features, these Group II cycloalkanones were successfully separated within 35 minutes, as shown by Figure 1, and their retention factors were calculated as indicated in Table 3. In order to compare retention data, c log P values, and cytotoxic activity of cycloalkanones in an easier way, we made 7 groups based on their structural features. Analyzing the retention factors of the groups, we found that retention factor of Mannich ketones, having the same R2 substituents, increased with the increase in carbon number of the cycloalkanon ring (Group I and III). Mannich ketones of eight-membered ring showed the highest values. When the influence of bases (R2) was investigated in the case of the same type of cycloalkanon ring, a clear increase could be measured in retention data, which was followed by the order of morpholinyl, pirrolidinyl, piperidyl, and tetrahydroisoquinolinyl moieties (Group II).

In that case, when both the type of structure and the R2 substituents were the same, we could investigate the effects of R1 substituents on the changes of retention parameters. As it can be seen in group VII, methyl- and methoxy groups slightly elevated the retention times.

Compounds with piperidyl groups on the core structure of "C and D" (Number 3, 4) and tetrahydro-isoquinolyl group on cycloalkanon structure "A" (Number 8), respectively showed the highest retention; while Mannich ketones containing morpholinyl groups had lower retention factors (Numbers 5, 9, 16, 17, and 18). When the morpholinyl group was replaced by the pirrolidinyl or piperidyl group the retention factors increased (Group I, II, and VI).

Considering the phenomena of isomeria library of Mannich ketones contains compounds which are structural isomers (Group IV, V). During the course of HPLC investigation, the stationary phase applied (Hypersil 5 MOS) was sensitive enough to separate the methoxy-substituated isomers (Figure 3). Moreover, the discrepancies within the retention factors of the isomers were found to be significant.

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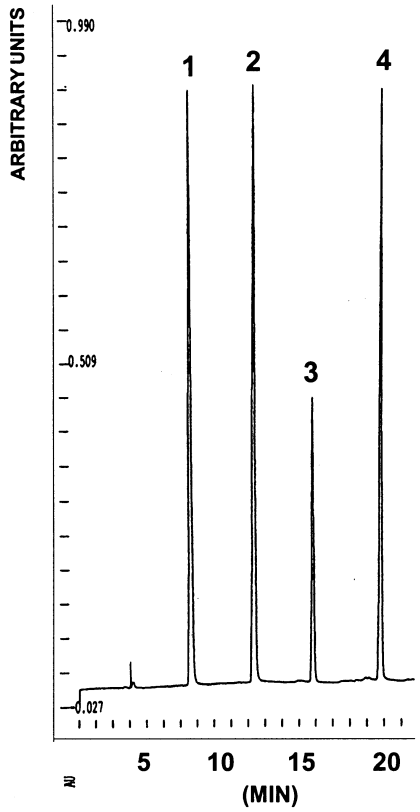

Figure 1. HPLC chromatograms of Mannich ketones of Group II. Isocratic run was performed in an eluent of 24 v/v% ACN in eluent A.

The Results of the C log P Calculation

To study further the hydrophobicity of our compounds, log P parameters have been calculated solely from molecular structure (c log P). These calculated c log P values were compared with the experimentally determined retention factors of the same compounds (Table 3). This comparison revealed that good linear correlation exists between the experimentally determined hydrophobicity ($\log k'$) and the calculated (c log P) one: $R = 0.9539$, $SD = 0.238$ (Figure 2).

In accordance with the measured retention factors, c log P values increased with the increase of carbon number in the cycloalkanone ring (Group I and III). The same results were obtained with the bases (R2) bounded for the same type of cycloalkanone ring as we observed of the retention factors (Group II).

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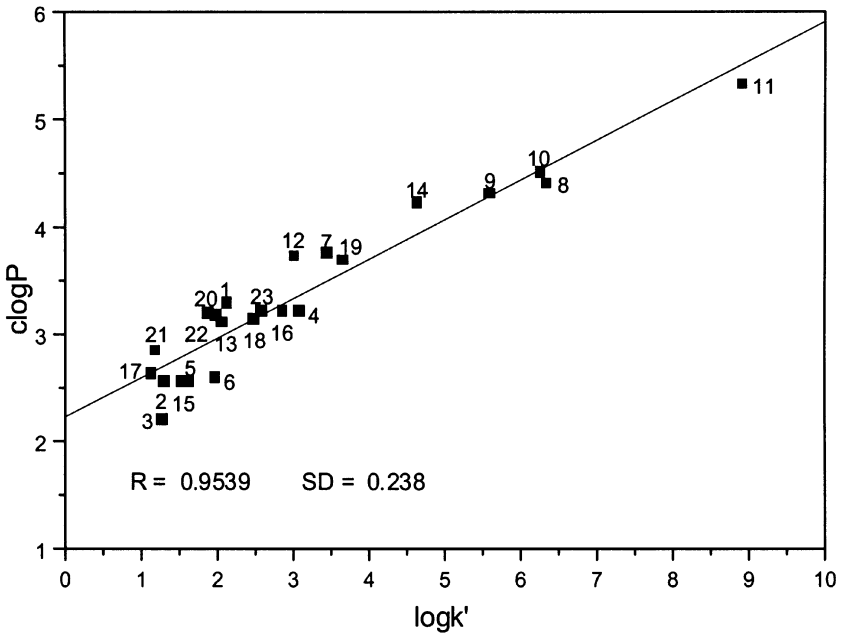

Figure 2. Relationship between log k′ and calculated hydrophobicity values (c log P) of the 21 cycloalkanones.

Piperidyl and tetrahydro-isoquinolinyl groups caused the strongest impact on c log P values. The highest c log P values were found in the case of compounds number 2, 3, 4, 8, and 11, respectively. In contrast, Mannich ketones with morpholinyl substituent had lower c log P values (Numbers 5, 16, 17, and 18). The c log P values became greater when the morpholinyl group was replaced by pyrrolidinyl, piperidyl, or methyl piperidyl moieties (Group I, II, and VI). The main differences between the measured and the calculated values could be observed in Group IV and V. As we expected, software calculation of c log P values could not differentiate among the methoxy isomers of Mannich ketones having the same core structure and the same bases as well. This means that software calculation is "blind" in some cases and could not realize the differences, which might be important factors for the prediction of biological activity. Contrary to this, our chromatographic system was sensitive enough to make small discrepancies among the various isomeric forms and to separate them. In Group VII, c log P value grew when hydrogen atom was substituted for methyl group, however, it slightly decreased by methoxy substitution.

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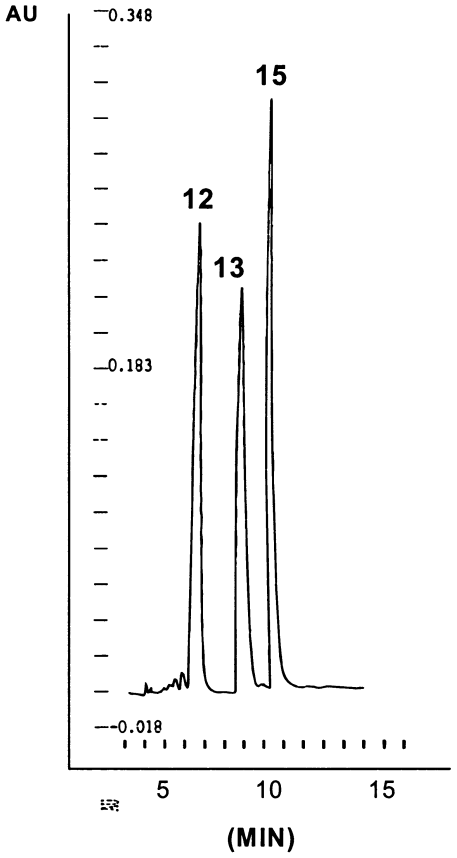

Figure 3. HPLC chromatograms of cycloalkanones of Group IV. Isocratic run was performed in an eluent of 24 v/v% ACN in eluent A.

Results of the MTT Assay

Molecules with slightly or highly hydrophobic properties were chosen from all groups of the library to analyze the biological significance of hydrophobicity and the influence of the substituents on cytotoxic activity. Comparison of the inhibitory effect of Mannich ketones on A431 cell proliferation (MTT activity) and hydrophobicity of the selected compounds, showed that the highly hydrophobic compounds were more active in all groups than their less hydrophobic member (Table 3). The highest cytotoxic activity values were measured in group IV where more than the fifty percent of the cells died after the treatment with compound No.15.

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Table 4.

Group	Type of Structure	Number of Compounds	Induction of Apoptosis
II	A	5	27.2 ± 5.7
	A	6	35.3 ± 4.8
	A	7	42.8 ± 8.4
	A	8	51.7 ± 7.7

The Results of the Apoptosis Experiments

Mannich ketones having the same cycloalkanone core structure but containing different bases as R2 substituents (Group II) were selected for the apoptosis assay (Table 4). In this assay, apoptosis-inducing ability of Mannich ketones was evaluated on the basis of cytomorphological criteria at the same concentration as was applied at the MTT test (100 μg/mL). Our findings showed that all of the compounds investigated were active apoptosis-inducers at that concentration. According to the values of Table 4, compounds having tetrahydroisoquinolinyl moiety were nearly two times more active than the compound having morpholinyl group. Furthermore, apoptosis-inducing ability of the compounds investigated was compared to their measured/predicted hydrophobicity values and to their MTT activities. These comparisons showed that the higher the hydrophobicity (either the experimentally measured values or the predicted/calculated ones) and the MTT activity (cell proliferation inhibition) the greater the apoptosis-inducing capability of the molecules. This suggests that lipophilicity of the molecules has an ifluence on the apoptosis-inducing activity of Mannich ketones of cycloalkanones.

CONCLUSIONS

Reversed phase HPLC method applied in this work proved to be applicable for fast analysis of Mannich ketone library of cycloalkanones. Separation of 21 Mannich ketones possessing similar chemical structure could be obtained within 35 minutes. An advantage of the method applied is that beyond the check of the chromatographic purity of the compounds synthesized, an experimental physicochemical parameter can be obtained during the course of the analysis, which characterises the hydrophobicity of the compounds (retention factor, k'). Furthermore, good correlation was found between the experimentally determined hydrophobicity parameter ($\log k'$) and the computer predicted hydrophobicity parameter ($c \log P$). This correlation confirms that both parameters can well be

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used for the characterization of hydrophobicity. However, in some cases, and especially in the case of isomers, only the chromatographic measurements provide real and acceptable data for the evaluation of hydrophobicity.

The influence of hydrophobicity on antiproliferative activity and on apoptotic activity of Mannich ketones shows that the higher the hydrophobicity the higher the cytotoxic- and apoptotic activity of Mannich ketones. Hydrophobicity values of Mannich ketones show similar behaviour to the results of the cell proliferation inhibition and apoptosis assays, clearly indicating that hydrophobicity of the compounds investigated plays a role in their anti-tumor activity. In other words, the measured physico-chemical parameters ($\log k'$) characterizing the hydrophobicity may provide useful data sets for the prediction or pre-screening the biological activity. Pre-selection of the molecules can easily be performed on this basis. There is no need to perform sample, time, chemical, and labor-demanding biological tests on each member of the library; only the molecules pre-selected in this way must be processed. The utilization of this method is possible in cases when high throughput characterization of hydrophobicity of a great number of molecules with similar chemical structure (e.g., members of a combinatorial library) is needed.

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