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Potential Antitumor α-Methylene-γ-butyrolactone-Bearing Nucleic Acid Bases. 2. Synthesis of 5'-Methyl-5'-[2-(5-substituted uracil-1-yl)ethyl]-2'-oxo-3'-methylenetetrahydrofurans

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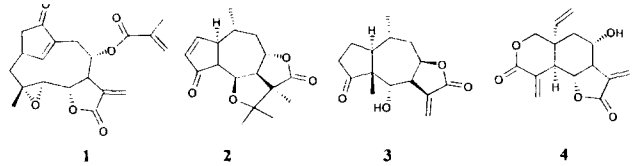
Ten, heretofore unreported, 5'-methyl-5'-[2-(5-substituted uracil-1-yl)ethyl)]-2'-oxo-3'-methylenetetrahydrofurans (H, F, Cl, Br, I, CH_3, CF_3, CH_2CH_3, $CH{=}CH_2$, SePh) (**7a-j**) were synthesized and evaluated against four cell lines (K-562, FM-3A, P-388 and U-937). For the preparation of α-methylene-γ-butyrolactone-linked to 5-substituted uracils (**7a-j**), the convenient Reformasky type reaction was employed which involves the treatment of ethyl α-(bromomethyl)acrylate and zinc with the respective 1-(5-substituted uracil-1-yl)-3-butanone (**6a-j**). The 5-substituted uracil ketones (**6a-j**) were directly obtained by the respective Michael type reaction of vinyl methyl ketone with the K_2CO_3 (or NaH)-treated 5-substituted uracils (**5a-j**) in the presence of acetic acid in the DMF solvent. The α-methylene-γ-butyrolactone compounds showing the most significant antitumor activity are **7e**, **7f**, **7h** and **7j** (inhibitory concentration (IC_{50}) ranging from 0.69 to 2.9 μg/ml), while **7b**, **7g** and **7i** have shown moderate to significant activity. The compounds **7a**, **7c** and **7d** were found to be inactive. The synthetic intermediate compounds **6a-j** were also screened and found marginal to moderate activity where compounds **6b** and **6g** showed significant activity (IC_{50} : 0.4~2.8 μg/ml).

Key words : 5'-Methyl-5'-[2-(5-substituted uracil-1-yl)ethyl)-2'-oxo-3'-methylenetetra-hydrofuran, 1-(5-Substituted uracil-1-yl)-3-butanone, Stille coupling reaction, Tris(dibenzylidenacetonyl)bispalladium (Pd_2dba_3), Tri(2-furyl)phosphine, Antitumor activity, IC_{50}, Reformatsky reaction, Human chronic myelogenous (K-562), Mouse lymphoid neoplasma (P-388), Mouse mammary carcinoma (FM-3A), Human histiocytic lymphoma (U-937)

INTRODUCTION

It has been recognized (Kupchan, *et al.*, 1969a,b; Lee, *et al.*, 1972) in the past years that a number of sesquiterpene lactones and other derivatives obtained from natural sources bearing α-methylene-γ-butyrolactone and related moieties have exhibited interesting biological activity and significant antitumor activity. Structure-activity relationships for these complex natural products have indicated that one of the structural requirements for significant cytotoxic antitumor activity is an $-CH_2{=}C{-}C{=}O$ system as part of an ester as well as a ketone, present in elephantophin(1) (Kupchan, *et al.*, 1969), tenulin(**2**) (Hall, *et al.*, 1977), helenalin(**3**) (Hall, *et al.*, 1977) and vernolephin(**4**) (Kupchan, *et al.*, 1969). The cytotoxic activity of α-methylene-γ-butyrolatones has been attributed to their ability of acting as alkylating agents by virture of a Michael-type addition with biological cellular nucleophiles such as L-cysteine, glutathione or thiol-rich enzymes (phosphofractokinase, glycogen synthetase and DNA polymerase), to the α-methylene-γ-butyrolactone moiety itself (Lee, *et al.*, 1976; Kupchan, *et al.*, 1970). It has been established that the α-methylene-γ-butyrolactone is the most reactive chemical functionality in both **1**, **3** and **4**, with no reaction being observed between L-cysteine and the epoxide 1, or the endocyclic α,β-unsaturated lactone in **1**. These views are in accord with the theory of tumor inhibition by the selective alkylation of biological macromolecules which have been advanced by Kupchan and co-workers.



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A large number of possible drug candidates bearing this functionality of the general structure of α-methylene-γ-butyrolactone moiety have been synthesized (Lee, *et al.*, 1975; Dehal, *et al.*, 1980; Heindel, *et al.*, 1981; Cassady, *et al.*, 1978; Rosowsky, *et al.*, 1974; Sanyal, *et al.*, 1986), with a view to develope effective clinical drugs since naturally found derivatives have therapeutic indices that prelude their clinical use. Several new synthetic approaches to the development of such a cytotoxic α-methylene-γ-butyrolactone moiety are excellently reviewed (Ohler, *et al.*, 1970; Grieco, 1975; Gammill, *et al.*, 1975).

As part of our effort to develop more useful antitumor agents (Kim, *et al.*, 1992; 1993a,b; 1994a-f; 1995; 1996), we were particularly interested in synthesizing suitably substituted nucleic acid base-bearing this moiety as a biological carrier. An extensive literature survey revealed that relatively scanty literature references are known. We have synthesized potential target-specific alkylating agents by introducing the antitumor cytotoxic moiety, α-methylene-γ-butyrolactone function into 5-substituted uracil nucleic acid bases **5a-j**, and evaluated these synthesized compounds **7a-j** against four cell lines (K-562/S, P-388/S, FM-3A/S and U-937/S).

MATERIALS AND METHODS

Melting points were determined on an electrothermal capillary melting point apparatus and uncorrected. TLC was performed on glass plates coated with silicone oxide (silica gel $60F_{254}$) and compounds were visualized using a UV lamp. Proton nuclear magnetic resonance and ^{13}C-NMR spectra were obtained with a Varian EM-360 spectrophotometer and Varian Gemini 200 MHz, Brucker AM 300 and DPX 200 (solution in dimethylsulfoxide-d_6 with tetramethylsilane as internal standard). The organic solvents and chemicals were obtained from commercial products and purified by the appropriate methods before use. Pertinent data for synthesized compounds (**6a-j, 7a-j**) are listed in Table I and II.

General Procedure for the Synthesis of 1-(5-Substituted uracil-1-yl)-3-Butanones (6a-j)

To a stirred solution of 5-substituted uracil (**5a-j**) (0.3 g, 2.68 mmol) in DMF (30 ml) was added K_2CO_3 (0.44 g, 3.21 mmol). The mixture was stirred by adding a dissolved mixture of methyl vinyl ketone (0.28 ml, 3.21 mmol) and acetic acid (0.18 ml, 3.21 mmol) in DMF (8 ml) in small portions during 1-2 hours period. The reaction mixture was continuously stirred for additional 5 hours, and evaporated in vaccuo. The oily residue were dissolved in $CHCl_3$, and washed with aq. NaCl solution and 10% $NaHCO_3$. The organic layers were dried over $MgSO_4$, filtered, and evaporated, which were applied to a column packed with silica gel, and eluted (CH_2Cl_2:MeOH=10:1) (See Table 1).

5-Phenylseleneyluracil (5j)

The dissolved uracil (1 g, 8.92 mmol) in dry pyridine (50 ml) was vigorously stirred at 80°C for one hour, and slowly added phenylseleneyl chloride (2.05 g, 10.71 mmol) in small portions under N_2 atmosphere. The reaction mixture was stirred for 30 hours. On the end of reaction, the reaction mixture was allowed to room temperature and quenched with benzene. The solution was concentrated in vacuo, and the benzene was added to the residue and the diphenylselenide was eliminated. Crystallization from ethanol gave **5j** (1.95 g, 82% yield). mp. 250-251°C ^{1}H-NMR 7.23~7.38 (m, 5H), 7.73 (s, 1H), 11.25 (brs, 2H).

General Procedure for the Synthesis of Ethyl α-(bromomethyl)acrylate

To a solution of dry, distilled ethylene glycol (12.6 g, 2.3 mol) in dry THF (43 ml) was added cautiously NaH (3.0 g, 0.12 mol). After the first vigorous reaction had subsided, the mixture was heated under reflux for 24 hours. The syrupy suspension of sodium ethylene glycolate resulting was cooled to room temperature and then added slowly to a solution of ethyl β,β'-dibromoisobutyrate (27.8 g, 0.10 mol) in THF (85

Table I. 1-(5-Substituted uracil-1-yl)-3-Butanones (**6a-j**)

Comp.	R	Mp (°C)	Yield (%)	NMR(DMSO-d_6)					IR (KBr), ν(c=o), cm^{-1}
				$COCH_3$	N-CH_2	CH_2CO	6-**H**	3-**H**	
6a	H	133-135	60	2.17	3.95	2.94	7.45	9.05	1709
6b	F	138-139	49	2.10	3.69	2.85	7.97	11.62	1710
6c	Cl	169-171	55	2.10	3.83	2.88	8.08	11.58	1703
6d	Br	164-165	71	2.10	3.83	2.86	8.13	11.75	1705
6e	I	192-194	43	2.17	3.95	2.93	7.88	11.67	1694
6f	CH_3	144-146	51	2.10	3.79	2.85	7.46	11.07	1712
6g	CF_3	166-168	36	2.20	4.01	2.98	8.04	8.43	1751
6h	CH_2CH_3	122-123	49	2.18	3.92	2.94	7.20	8.53	1710
6i	$CH=CH_2$								
6j	SePh	211-213	58	2.17	3.93	2.93	7.75	8.48	1698

Table II. 5'-Methyl-5'-[2-(5-Substituted uracil-1-yl)ethyl]-2'-oxo-3'- methylenetrahydrofurans (**7a-j**)

Comp.	R	Mp (°C)	Yield (%)	NMR (DMSO-d_6)					IR (KBr),
				5'-C**H**$_3$	N-C**H**$_2$	4'-**H**$_2$	**H**$_b$	**H**$_a$	$\bar{\nu}$ (c=o), cm^{-1}
7a	H	179-181	86	1.37	3.75	2.85	5.75	6.06	1718
7b	F	207-208	87	1.37	3.73	2.85	5.74	6.06	1742
7c	Cl	253-254	92	1.37	3.76	2.85	5.74	6.06	1742
7d	Br	253-255	80	1.37	3.76	2.85	5.74	6.06	1743
7e	I	212-214	93	1.37	3.76	2.86	5.73	6.06	1746
7f	CH_3	243-245	87	1.38	3.72	2.85	5.73	6.06	1749
7g	CF_3	192-194	78	1.49	3.95	2.85	5.62	6.31	1741
7h	CH_2CH_3	143-144	82	1.49	3.97	2.91	5.70	6.18	1753
7i	$CH=CH_2$	>300	43	1.48	3.88	2.85	5.69	6.31	1701
7j	SePh	164-166	89	1.45	3.82	2.82		6.29	1759

ml). The temperatue was kept below 45°C by controlling the rate of reactions when addition was complete, the mixture was stirred for 90 minutes and then poured into water (400 ml). The organic layer which separated was extracted into CH_2Cl_2 (100 ml × 3). The aqueous layer was acidified with aqueous 5N nitric acid and treated with silver nitrate. After drying, the silver bromide precipitate weighed 10 g (60%). The methylene chloride was evaporated under reduced pressure and the residue was fractionated through the colunm, bp 44~45°C (1.7 mmHg).

General Procedure for the Synthesis of 5'-Methyl-5'-[2-(5-substituted uracil-1-yl)ethyl]-2'-oxo-3'-methylene-tetrahydrofurans (7a-j)

A solution of ethyl α-(bromomethyl)acrylate (0.25 g, 1.3 mmol) in anhydrous THF (50 ml) was added dropwise with vigorous stirring under nitrogen to a mixture of granulated active Zn (0.09 g, 1.3 mmol), *p*-hydroquinone (0.003 g) and the 1-(5-substituted uracil-1-yl)-3-butanone (**6a-j**) (0.17 g, 1 mmol) in anhydrous THF (20 ml). Once the reaction has started, addition was adjusted such that the temperature does not rise above 40~50°C, The reaction mixture was stirred for about 4 hours at 50°C, cooled and poured into ice-cold 7% HCl (25 ml). The reaction mixture was extracted with chloroform (100 ml × 4), and the chloroform extractions were washed with aq $NaHCO_3$, followed by aq NaCl, dried over anhydrous $MgSO_4$. Filtration and evaporation gave residues, which were crystallized from an appropriate solvent (Table II).

5'-Methyl-5'-[2-(5-vinyluracil-1-yl)ethyl]-2'-oxo-3'-methylenetetrahydrofuran (7i)

A solution of 5'-methyl-5'-[2-(5-iodouracil-1-yl)ethyl]-2'-oxo-3'-methylenetetahydrofuran **7e** (0.2 g, 0.532 mmol) in dry NMP (2 ml, from calcium hydride) was treated with tri(2-furyl)phosphine (0.012 g, 0.053 mmol), tris (dibenzylidene acetonyl)bispalladium (0.034 g, 0.037 mml) and vinyltributyltin (0.2 g, 0.638 mmol), and the pale yellow solution was stirred under argon for 16 hours at room temperature. The solvent was removed in vacuo and the residue was chromatographed on silica gel and eluted with $CHCl_3$:MeOH=25:1. (0.074 g, 49% yield). See Table II.

MTT-Microculture Tetrazolium Assay

The antitumor effect of the synthesized compounds was determined by the modified methods (Mosmann, *et al.*, 1983; Carmichael, *et al.*, 1987; Kim, *et al.*, 1994b,c,d,f). The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co., St. Louis, M.O.) by the mitochondrial dehydrogenase of vial cells to a blue water-nonsoluble formazan crystal product which can be measured spectrophotometrically (Mosmann, *et al.*, 1983; Carmichael, *et al.*, 1987; Kim, *et al.*, 1994b,c,d,f). Following appropriate incubation of cells (K-562, P-388, FM-3A, U-937 cells) in the presence or absence of synthesized compounds, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, M.O.) was added to each well and incubated at 37°C for a further 4 hours before processing as described below.

For cell growth, serially increasing cell numbers were plated in different columns across 96-well microtiter plates. Well growing cell were harvested, counted and inoculated at the concentrations of 2×10^4 cells/ml into 96-well microtiter plates. After 24 hours, synthesized compounds (**7a-j** and **6a-j**) were applied to triplicate culture wells and the cultures were incubated at 37°C for 3 days. Following this incubation, 2 μl of MTT solution (5 mg/ml in phosphate buffer solution; KCl 0.2 g, KH_2PO_4 0.2 g, NaCl 8.0 g, Na_2HPO_4 1.15 g, $MgCl_2$ 0.101 g/ml, pH=7.4) was added to microculture wells. After 4 hours incubation at 37°C, the supernatant was removed from each well and 100 μl of 100% DMSO was added to solubilize the formazan crystals which were formed by the celluar reduction of MTT. After thorough mixing with mechanical plate mixer, absorbance spectra was read on ELISA Processor II microplate Reader (Behering Co.) at a wavelength of 570 nm and a reference wave-

Table III. IC_{50} values of 5'-Methyl-5'-[2-(5-Substituted uracil-1-yl)ethyl]-2'-oxo-3'-Methylenetetrahydro-furanes (**7a-j**) and related compounds (**6a-j**)

Compound	IC_{50} (μg/ml)[a]			
	K-562/S[b]	P-388/S[c]	EM-3A/S[d]	U-937/S[e]
7a		23.0	25.0	29.0
7b		7.9	6.9	4.6
7c		20.0	2.2	25.0
7d		15.0	3.8	24.0
7e		2.0	1.0	2.9
7f		2.9	2.9	2.2
7g	1.7	4.4	2.0	
7h	1.5	2.5	1.0	
7i	3.0	2.8	3.2	
7j	0.74	0.78	0.69	
6a		10.0	3.5	9.2
6b		0.4	1.2	0.41
6c		17.0	10.0	12.5
6d		9.0	5.1	17.5
6e		22.0	3.1	25.0
6f		52.0	31.0	68.0
6g	1.4	2.8	1.6	
6h	30	60	18	
6i				
6j	16.0	27.0	22.0	

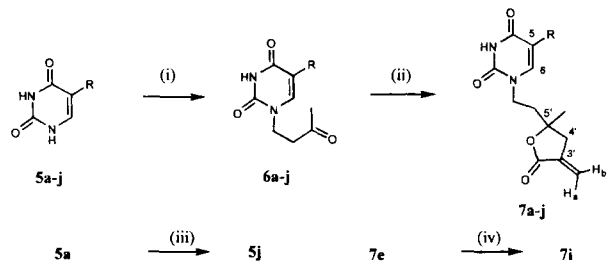
[a]mean values of triplicate runs. The comcentration of synthesized compounds required to reduce cell numbers to 50% of controls in a growth inhibition assay.
[b]Human chronic myelogenous neoplasma cell.
[c]Mouse leukemia cell.
dMouse mammary carcinoma cell.
[e]Human histioticytic lymphoma cell.

length of 650 nm (absorbance peak for DMSO). All measurements were carried out in triplicate. There was good reproducibility between replicate wells with standard errors $\leq \pm 10\%$ (Carmichael, *et al.*, 1987) (Table III).

RESULTS AND DISCUSSION

Chemistry

The synthesis of these uracil α-methylene-γ-butyrolactone (**7a-j**) and their related derivatives (**6a-j**) was carried out using the method similar to our previous work and outlined in Scheme. For the preparation of α-methylene-γ-butyrolactone bearing 5-substituted uracils, 5'-methyl-5'-[2-(5-substituted uracil-1-yl)ethyl]-2'-oxo-3'-methylene-tetrahydrofurans (**7a-j**), the convenient Reformatsky type reaction (Ohler, *et al.*, 1970) was employed which involves the treatment of ethyl α-(bromomethyl)acrylate (Ferris, 1995), and zinc with the respective 1-(5-substituted uracil-1-yl)-3-butanone (**6a-j**). The 5-substituted uracil ketones (**6a-j**), uracil-1-yl-3-butanone (**6a**), 1-(5-fluorouracil-1-yl)-3-butanone (**6b**), 1-(5-chlorouracil-1-yl)-3-butanone (**6c**), 1-(5-bromouracil-



R : **a** = H, **b** = F, **c** = Cl, **d** = Br, **e** = I, **f** = CH_3, **g** = CF_3, **h** = CH_2CH_3, **i** = $CH{=}CH_2$, **j** = SePh

[a]**Reagents** : (i) $CH_2{=}CHCOCH_3$, DMF, K_2CO_3(or NaH), acetic acid. (ii) $BrCH_2C(=CH_2)COOEt$, Zn, N_2, THF, HCl : Reformatsky reaction, (iii) PhSeCl, pyridine. (iv) NMP, rt, tri(2-furyl)phosphine, vinyltributyltin, Pd_2dba_3 = tris(dibenzylidene acetonyl)bispalladium.

1-yl)-3-butanone (**6d**), 1-(5-iodouracil-1-yl)-3-butanone (**6e**), thymine-1-yl-3-butanone (**6f**), 1-(5-trifluoromethyluracil-1-yl)-3-butanone (**6g**), 1-(5-ethyluracil-1-yl)-3-butanone (**6h**) and 1-(5- vinyluracil-1-yl)-3-butanone (**6i**) were directly obtained by the respective reaction of K_2CO_3-treated (or NaH-treated) 5-substituted uracil (**5a-j**) with vinyl methyl ketone in the presence of acetic acid in the DMF solvent. It has been reported (Montgomery, 1961) that uracil was alkylated at the N-1 position. It was also reported that under similar conditions, 5-fluorouracil furnished N-3-substituted derivatives. Recently we have shown (Kim, *et al.*, 1994c,f) that the direct alkylation of 5-fluorouracil with ω-chloroalkyl nitrile afforded moderate yields of the isomeric mixture of N-1-substituted 1-(ω-cyanoalkyl)-5-fluorouracils and N,N-1,3-disubstituted 1,3-bis-(ω-cyanoalkyl)-5-fluorouracils with the N-1-substituted product as the predominant. The direct electrophilic addition of phenylselenenyl chloride to its uracil in dry pyridine and dimethyl formamide, has been reported previously failing to yield the directed 5-phenyl selenyluracil (**5j**) (Schinazi, *et al.*, 1986), and therefore 5-phenylselenenyluracil (**5j**) was prepared starting from 5-bromouracil in 5 steps (Goudgaon, *et al.*, 1993). However, we obtained its starting material (**5j**) from the direct electrophilic addition of phenylselenenyl chloride to the uracil under anhydrous pyridine in good yields. The 5-substituted vinyl lactone, 5'-methyl-5'-[2-(5-vinyluracil-1-yl)ethyl]-2'-oxo-3'-methylene-tetrahydrofuran (**7i**) was prepared from the Stille coupling reaction (Stille, 1986) between the synthesized 5-substituted iodo derivative, 5'-methyl-5'-[2-(5-iodouracil-1-yl)ethyl]-2'-oxo-3'-methylenetetrahydrofuran (**7e**) and unsaturated stannane, vinyltributyltin, with tri(2-furyl)phoshine and tris(dibenzylidene acetonyl)bispalladium. The physicochemical data of the 1-(5-substituted uracil-1-yl)-3-butanones (**6a-j**) and 5'-methyl-5'-[2-(5-substituted uracil-1-yl)ethyl]-2'-oxo'-3'-methylenetetrahydrofurans (**7a-j**) have been described in Table 1 and 2.

Antitumor activity

As illustrated in Table 3, the α-methylene-γ-butyrolactone compounds, 5'-methyl-5'-[2-(5-substituted uracil-1-yl)ethyl]-2'-oxo-3'-methylenetetrahydrofurans **7a-j** were screened for their *in vitro* antitumor activity against four cell lines; a) human chromic myelogenous leukemia cell (K-562), b) mouse lymphoid neoplasma cell (P-388), c) mouse mammary carcinoma cell (FM-3A) and d) human histiocytic lymphoma cell (U-937). The interesting α-methylene-γ-butrolactone compounds showing the most significant antitumor activity are **7e**, **7f**, **7h** and **7j** (inhibitory concentration, (IC_{50}) ranging from 0.69 to 2.9 μg/ml), while **7b**, **7g** and **7i** have shown moderate to significant activity. The compounds **7a**, **7c** and **7d** were found to be inactive. The synthetic intermediate compounds (**6a-j**) were also screened against the above four cell lines and found marginal to moderate activity where compounds (**6b**) and (**6g**) showed significant activity (inhibitory concentration (IC_{50}) ranging from 0.4 to 2.8 μg/ml). It has been found that structive-activity relationship of the compounds (**7a-j**) do not vary marginally in their effective chain length, being substitituted methyl and ethyl chain between the nucleic acid base and α-methylene-γ-butyrolactone (Kim, *et al.*, 1996).

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