1. The exact manner of preparation and degassing of the mobile phase should be left to the operator as long as the final solution has the proper pH.

2. The preparation and injection of folic acid solutions should be made more flexible to allow for smaller amounts of folic acid and larger injection volumes. The former change may be necessary to prevent overloading of some detectors while the latter change permits better reproducibility of manual injections.

3. The particle base may be nonspherical since its shape is not critical to the determination.

These recommendations have been incorporated in the latest USP supplement (4).

The majority of collaborators were able to achieve acceptable separation and precision with the chromatographic method in spite of the variety of instrumental and operating conditions used, indicating that the method should be widely applicable.

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Potential Antineoplastics I: Substituted 3,5-Dioxo- and 3-Thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines

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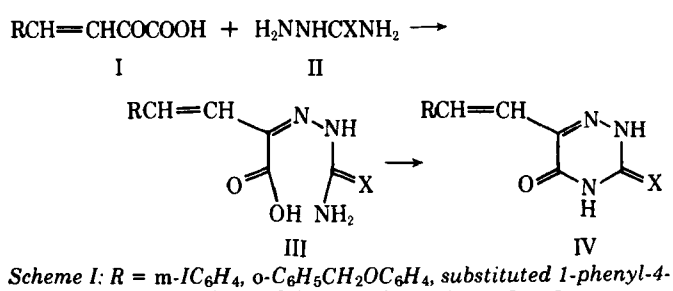
Received April 24, 1978, from the *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, A.R. Egypt.* Accepted for publication July 10, 1978.

Abstract □ The synthesis of some 6-substituted 3,5-dioxo- and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines for possible antineoplastic activity is reported. The assigned structures were substantiated by IR, NMR, and mass spectral studies of representative members of the series. Four compounds were tested against P-388 lymphocytic leukemia and were inactive.

Keyphrases □ 1,2,4-Triazines, substituted—synthesized, antineoplastic activity evaluated in mice □ Antineoplastic activity—various substituted 1,2,4-triazines evaluated in mice □ Structure–activity relationships—substituted 1,2,4-triazines evaluated for antineoplastic activity in mice

Of the different 6-aza analogs of pyrimidines screened for anticancer potency, 6-(3,4-methylenedioxystyryl)-3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazine (IV, R = 3,4-$CH_2O_2C_6H_3$ and X = S, Scheme I) was more active than mercaptopurine and fluorouracil in animals (1, 2).

In a search for novel antineoplastic agents, it was of interest to prepare 6-styryl-3,5-dioxo- and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines with new substituents



Scheme I: R = m-IC_6H_4, o-$C_6H_5CH_2OC_6H_4$, *substituted 1-phenyl-4-pyrazolyl, or* $C_6H_5CH{=}CH$ *and* X = *O or S*

on the phenyl ring (IV*a*–IV*c*, Table I). The diverse biological activities of the pyrazole nucleus suggested the synthesis of two new *as*-triazines in which a substituted 1-phenyl-4-pyrazolyl moiety replaced the phenyl ring: 6-[β-(3,5-dimethyl-1-phenyl-4-pyrazolyl)vinyl]- and 6-[β-(1,5-diphenyl-3-methyl-4-pyrazolyl)vinyl]-3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines (IV*d* and IV*e*, Table I). It was also of interest to prepare and screen *as*-triazines having the phenyl ring separated from position 6 by a 1,3-butadiene instead of the vinyl side chain: 6-(4-phenyl-1,3-butadienyl)-3,5-dioxo- and 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines (IV*f* and IV*g*, Table I).

RESULTS AND DISCUSSION

Chemistry—For the synthesis of the new triazines, the reactions shown in Scheme I were followed. Condensation of arylidenepyruvic acids (I) with semicarbazide (II, X = O) hydrochloride or thiosemicarbazide (II, X = S) yielded semicarbazono- or thiosemicarbazonoarylidenepyruvic acids (III*a*–III*g*, Table I). Subsequently, III*a*–III*g* were cyclized to the corresponding 1,2,4-triazines (IV*a*–IV*g*, Table I) in the presence of sodium hydroxide. Compound IV*g* previously was synthesized by heating thiosemicarbazonocinnamylidenepyruvic acid (III*g*) with aqueous sodium carbonate for 3 hr (3). Conversion of IV*g* to IV*f* was reported to take place when IV*g* was heated with aqueous alkaline potassium permanganate solution followed by acidification of the reaction mixture (3).

The IR spectra of the triazines showed multiple bands in the 3500–2900-cm^{-1} region (NH stretching) and strong bands at 1720–1680 (C=O stretching) and 1560–1520 (amide II) cm^{-1}. In addition, the spectra of the 3-thio derivatives revealed a band of medium intensity between 1300 and 1270 cm^{-1} (C=S stretching) but lacked the band characteristic of the SH group of a thiol tautomer. These data indicate the existence of

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Table I—Semicarbazono- and Thiosemicarbazonoarylidenepyruvic Acids and 6-Substituted 3,5-Dioxo- and 3-Thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines

Compound	R	X	Melting Point	Recrystallization Solvent[a]	Molecular Formula	Analysis, %	Calc.	Found
IIIa	*m*-IC_6H_4	O	219–220°	EW	$C_{11}H_{10}IN_3O_3$	C	36.8	36.8
						H	2.8	2.8
						N	11.7	11.6
IIIb	*m*-IC_6H_4	S	194–195°	EW	$C_{11}H_{10}IN_3O_2S$	C	35.2	34.8
						H	2.7	3.1
						N	11.2	10.7
IIIc	*o*-$C_6H_5CH_2OC_6H_4$	S	175–177°	E	$C_{18}H_{17}N_3O_3S$	N	11.8	12.3
						S	9.0	9.2
IIId	3,5-Dimethyl-1-phenyl-4-pyrazolyl	S	193°	EB	$C_{16}H_{17}N_5O_2S$	C	55.95	55.8
						H	5.0	5.2
						S	9.3	8.9
IIIe	1,5-Diphenyl-3-methyl-4-pyrazolyl	S	216°	EW	$C_{21}H_{19}N_5O_2S$	C	62.2	62.5
						H	4.7	5.0
						S	7.9	7.9
IIIf	C_6H_5CH=CH	O	196–197°	E	$C_{13}H_{13}N_3O_3$	C	60.2	59.8
						H	5.05	5.5
						N	16.2	16.1
IIIg	C_6H_5CH=CH	S	193–194°	E	$C_{13}H_{13}N_3O_2S$ (3)		—	—
IVa	*m*-IC_6H_4	O	215–217°	E	$C_{11}H_8IN_3O_2$[b]	C	38.7	38.3
						H	2.4	2.5
						N	12.3	12.3
IVb	*m*-IC_6H_4	S	237–238°	E	$C_{11}H_8IN_3OS$[c]	C	37.0	37.0
						H	2.3	2.2
						N	11.8	11.6
IVc	*o*-$C_6H_5CH_2OC_6H_4$	S	222°	E	$C_{18}H_{15}N_3O_2S$	C	64.1	63.6
						H	4.5	4.3
						N	12.45	12.0
						S	9.5	9.8
IVd	3,5-Dimethyl-1-phenyl-4-pyrazolyl	S	302–305°	A	$C_{16}H_{15}N_5OS$	C	59.05	58.5
						H	4.65	5.0
						S	9.85	10.4
IVe	1,5-Diphenyl-3-methyl-4-pyrazolyl	S	290–292°	EW	$C_{21}H_{17}N_5OS \cdot H_2O$	C	62.2	62.4
						H	4.7	5.1
						S	7.9	7.9
IVf	C_6H_5CH=CH	O	300–303°	E	$C_{13}H_{11}N_3O_2$ (3)	C	64.7	64.8
						H	4.9	4.8
						N	17.2	16.8
IVg	C_6H_5CH=CH	S	257°	E	$C_{13}H_{11}N_3OS$ (3)		—	—

[a] A = acetic acid, E = ethanol, EB = ethanol–benzene, and EW = ethanol–water. [b] Mass spectrum: *m/e* (relative intensity) 341 (18) (M^+), 340 (9) (M − 1), 316 (14), 315 (25), 300 (11), 298 (5), 297 (11), 272 (13), 271 (21), 270 (14), 256 (29), 255 (100), 241 (7), 230 (7), 156 (11), 145 (7), 144 (26), 143 (8), 129 (18), 128 (50), 127 (7), 117 (16), 116 (14), 115 (38), 114 (19), 113 (32), 102 (16), 101 (21), 95 (21), 89 (11), and 77 (16). [c] Mass spectrum: *m/e* (relative intensity) 357 (100) (M^+), 314 (4), 281 (27), 269 (4), 254 (15), 253 (50), 240 (9), 229 (17), 128 (5), 127 (17), 114 (5), and 113 (8).

these triazines in the 3,5-dioxo- or 3-thioxo-5-oxo form (IV) in the solid state.

In the ^{1}H-NMR spectra, the aromatic and vinyl protons were in the range of δ 6.4–8.0 ppm (multiplet) for IV*a* and IV*b* and the two NH protons were at δ 11.0 ppm (singlet) for IV*a* and at δ 12.8 (singlet) and 13.2 (singlet) ppm for IV*b*. The mass spectrum of IV*a* involved the sequential loss of two molecules of HNCO from the molecular ion (*m/e* 341, 18%), giving *m*-iodocinnamonitrile (*m/e* 255, 100%) (V). The spectrum of IV*b* was characterized by an intense molecular ion, which successively lost HNCO and SH, leading to *m/e* 314 (4%) (VI) and *m/e* 281 (27%) (VII), respectively. A loss of HCS from VI yielded the species *m/e* 269 (4%) (VIII).

Antineoplastic Activity—Compounds IV*a*, IV*b*, IV*f*, and IV*g*[1] were screened against P-388 lymphocytic leukemia in mice according to a standard protocol[2] (4). Median survival time was taken as the activity parameter for tumor evaluation. A compound is considered active in this system if the ratio of the median survival times for treated to control mice (T/C) is ≥125%. None of the compounds reached this value when tested by a three-dose assay at 400, 200, and 100 mg/kg/injection.

EXPERIMENTAL[3]

Arylidenepyruvic Acids (I)—The required acids were prepared by the base-catalyzed condensation of pyruvic acid with the appropriate aldehyde, as previously reported (5).

Semicarbazono- and Thiosemicarbazonoarylidenepyruvic Acids (IIIa–IIIg, Table I)—A solution of I (0.002 mole) in ethanol (20 ml) was heated for 15 min with a solution of semicarbazide (II, X = O) hydrochloride or thiosemicarbazide (II, X = S) (0.002 mole) in a mixture of water (10 ml) and acetic acid (0.5 ml). Most of the alcohol was evaporated; the yellow product was filtered, washed with water, dried, and recrystallized from the proper solvent. The yields were 80–90%.

6-Substituted 3,5-Dioxo- and 3-Thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines (IVa–IVg, Table I)—A solution of the appropriate IIIa–IIIg (0.01 mole) in 1 *N* NaOH (15 ml) and water (5 ml) was refluxed for 15 min. After cooling, the yellow clear solution was acidified with dilute hydrochloric acid to pH 6. Then the yellow product was filtered, washed with water, and dried. It was recrystallized from the appropriate solvent as yellow crystals in almost quantitative yields.

[1] NSC 275892, 275891, 275894, and 275893, respectively.

[2] Drug Evaluation Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, MD 20014.

[3] Melting points were determined in open glass capillaries and are uncorrected. IR spectra were recorded on a Perkin-Elmer 421 spectrometer with potassium bromide. ^{1}H-NMR spectra were determined on a Varian A60A spectrometer with deuterated dimethyl sulfoxide as the solvent. Mass spectra were obtained using an Organic MS 20 AEI (70 ev) instrument. Microanalysis, for samples dried over phosphorus pentoxide at 70° under reduced pressure, was carried out at the Microanalytical Unit, University of Cairo, Cairo, A.R. Egypt.

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Antihypercholesterolemic Studies with Sterols: β-Sitosterol and Stigmasterol

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Abstract □ Stigmasterol, which differs from β-sitosterol by unsaturation at C_{22}, was tested for antihypercholesterolemic activity under an experimental protocol that gave the results expected with β-sitosterol and cholestyramine. In terms of serum cholesterol, stigmasterol had a barely significant antihypercholesterolemic effect while exhibiting no obvious effect on the heart or liver. It was concluded that saturation of the side chain, at least at C_{22}, is important in conferring antihypercholesterolemic activity on a sterol.

Keyphrases □ β-Sitosterol—antihypercholesterolemic activity evaluated in chickens □ Stigmasterol—antihypercholesterolemic activity evaluated in chickens □ Antihypercholesterolemic activity—β-sitosterol and stigmasterol evaluated in chickens □ Structure–activity relationships—β-sitosterol and stigmasterol evaluated for antihypercholesterolemic activity in chickens

There is no conclusive evidence that lowering plasma cholesterol levels can either reverse or prevent certain cardiovascular diseases. Nevertheless, many antiatherosclerotic studies have been performed with compounds that reduce plasma cholesterol.

Currently, cholestyramine is the only antihypercholesterolemic agent that is relatively free of adverse reactions and reasonably effective in reducing plasma cholesterol levels in type II hyperlipemia (1–3). Unfortunately, it is not always effective (4); therefore, more toxic agents must often be employed.

Niacin and clofibrate, the agents most widely used, have been generally considered effective in reducing cholesterol and triglyceride levels (5). Although their effect on blood triglyceride levels is pronounced, they produce only modest reductions in blood cholesterol levels (6). Both are associated with unpleasant or hazardous side effects.

β-Sitosterol also lowers cholesterol levels in humans (1, 7–11). Reductions in plasma cholesterol levels of the same order of magnitude as those with cholestyramine have been observed (7, 12–14); however, the drug has little effect on plasma triglycerides (10). β-Sitosterol is free of detectable toxicity, and there is no evidence that it accumulates in tissues (2, 15, 16).

The value of these nontoxic agents in the treatment and prevention of such diseases as atherosclerosis, myocardial infarction, and stroke could be significant. Consequently, it was decided to study the structure–activity relationships of sterols related to cholesterol for their antihypercholesterolemic activity. The selection of the White Leghorn cockerel as the animal model was discussed previously (17), as was the use of GLC for sterol analysis (18).

EXPERIMENTAL

General—Day-old White Leghorn cockerels were fed *ad libitum* for 14 days. They were then weighed, banded, and randomly assigned to one of five groups: pretest group, sacrificed on the 1st day of the experiment; Group A, basic diet; and Groups B, C, and D, basic diet supplemented with 1% cholesterol, 1% cholesterol plus 1% test compound, and 1% test compound, respectively. Drinking water was always present.

Serum cholesterol levels were determined (blood collected *via* the brachial vein) at least once during the 4-week experiment. On the final day of the experiment, the chicks were weighed and killed; the hearts and livers were weighed, photographed, and frozen until analyzed. Serum samples also were collected.

Assays were performed by GLC with cholestane as an internal standard (18). Separation of β-sitosterol, campesterol, and stigmasterol from cholesterol was accomplished on columns packed with 3% SE-30 or 3% OV-17 on 100–120-mesh Gas Chrom Q (18). Commercially available laboratory control samples were used routinely to ensure day-to-day reproducibility (18).

Cholestyramine Experiment—In this experiment, the "test compound" was the anion-exchange resin cholestyramine. Only 35 chicks were employed (seven per group); otherwise, the details followed the general procedure described.

β-Sitosterol Experiment—Day-old chicks (White Leghorn cockerels) were fed *ad libitum* for 25 days. On Day 25, blood samples were collected from each chick in the four groups (11 chicks each) of weighed chicks. The chicks were then put on the special diets for 19 days; during this time, blood was collected every 4th day. On Day 19, they were killed.

Stigmasterol Experiment—In this experiment, 44 chicks were used (nine per group, except the pretest group which consisted of eight). The experiment was terminated on Day 24, following the general procedure.

RESULTS AND DISCUSSION

The selection of agents for these experiments was based largely on the knowledge that β-sitosterol and cholestyramine are antihypercholesterolemic agents. These agents were used as standards for comparison of relative activities. Since stigmasterol differs from β-sitosterol only by unsaturation at C_{22}, the results of these experiments should allow the influence of that function on antihypercholesterolemic activity to be determined.

In all experiments, the chicks tolerated their diets well, as indicated by their healthy appearance and consistent weight gain (Table I). The

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