

Table I. Chromatographic Mobility of Some Iodinated Thyronines

Compd	Solvent		
	A	B	C
1	0.42	0.24	0.32
2		0.22	0.22
3		0.14	0.20
4	0.19	0.21	0.29
5	0.73	0.34	0.375

5 which was removed during the preparative TLC after iodination. If the product was not to undergo a chromatographic separation, the small amount of 5 could be removed by increasing the volume of HBr to 15.5 ml and the time of hydrolysis to 5.5 h. A second recrystallization of the hydrochloride from 2 N HCl before conversion to the free amino acid was required to eliminate the larger amount of 4 formed: overall yield, 55%. Purity was checked by paper chromatography (see Table I) (solvent A) (ascending, 18°): mp 253°; $[\alpha]^{21D} +24.8^\circ$ [c 1, 1 N HCl-EtOH (1:1)] (lit.⁷ +24.9°). Anal. (C₁₅H₁₄INO₄·H₂O) I.

3,3'-Diiodo-L-thyronine (2). 1 (0.834 mg) in 40 ml of concentrated NH₄OH was treated dropwise with 1 N iodine solution (4.8 ml, 20% excess). After standing 1 h the solvent was removed under vacuum, adding 1-propanol if necessary to control foaming. The residue was redissolved in water by a few drops of ammonia and separated from an insoluble brown material. On addition of acetic acid to the filtrate, the amino acids precipitated as an off-white solid: 930–990 mg (80–85%). Batches (90–100 mg) were dissolved in ethanol–2 N ammonia (3:1) about 1 ml, and the solutions were streaked onto 20 × 20 cm preparative layer plates and developed in solvent B. They were dried and redeveloped several times until sufficient separation between the two heavy stripes was achieved, as determined by minimum exposure to uv light. A faint stripe of highest *R_f*, identified as 5, was discarded. The middle stripe held 2; the stripe of lowest *R_f* contained the triiodothyronine (3). The silica was eluted with ethanolic ammonia and filtered and the filtrate evaporated under vacuum. The dried products were recovered either by adding water and filtering or by dissolving in water by addition of a few drops of ammonia, transferring to a centrifuge tube, precipitating with acetic acid, washing the precipitate repeatedly with water, and lyophilizing. About 50% of the theoretical amount of 2 was obtained (mp 196°), whereas the less soluble 3 recovery averaged 75% (mp 204°). Using analytical plates with solvent B gave a single spot, but since 1 was not separated from the other compounds, solvent C was used. This also produced a single spot. 2 also gave a single peak by gas chromatography after silylation. The technique was that of Cahnmann:²¹ column, 2 ft by 2.4 mm i.d. OV-1 (1%) on Supelcoport; oven programmed for 7.5°/min rise; nitrogen carrier (44 ml/min); starting temperature 178°. Under these conditions, compounds 4, 1, 2, and 3 emerged at 194, 210, 233, and 248°,

respectively: $[\alpha]^{20D} +19.6^\circ$ [c 1, 1 N HCl-EtOH (1:1)] (lit.⁷ +18.8°). Anal. (C₁₅H₁₃I₂NO₄·2H₂O) I. Anal. (C₁₅H₁₂I₃NO₄·H₂O) I.

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Antifungal Activity of 4-Substituted Crotonic Acid Esters

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Twenty-three 4-substituted crotonic acid esters were tested for antifungal activity against *Candida albicans*, *Aspergillus niger*, *Mucor mucedo*, and *Trichophyton mentagrophytes*. For the analogues of the methyl ester containing substituents in the 4 position, the following order of fungitoxicity was observed: I > Br > Cl > CH₃S > CH₃O > F = H. Of the homologues of the esters of the 4-iodo and 4-bromo compounds which included methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, and *n*-hexyl, ethyl 4-iodocrotonate was most toxic to the four fungi at pH 7.0 in the presence of 10% beef serum (*C. albicans*, 18 µg/ml, *A. niger*, 40 µg/ml, *M. mucedo*, 5 µg/ml, *T. mentagrophytes*, 4 µg/ml). It is believed that the mechanism of fungitoxicity is due, in part, to a nucleophilic reaction involving SH-containing compounds. This is based on the correlation of fungitoxicity with the order of leaving groups in the nucleophilic reaction and the protection against the toxicity of the test compounds to the fungi by cysteine and glutathione.

As medicine becomes more complex, iatrogenic diseases follow.¹ In recent years, this has been manifested by an

increase in the incidence of fungal infections.² Frequently, the attacking fungi are opportunistic invaders. They cause

infections that are secondary to debilitating diseases and to many types of medication which weaken the immune defenses of the host.³⁻⁷

Among the organisms implicated in deep fungal infections are *Candida* sp. (*C. albicans*, *C. tropicalis*, *C. parapsilosis* inter alia), *Cryptococcus neoformans*, *Aspergillus* sp. (*A. fumigatus*, *A. flavus* inter alia), species of the order Mucorales (*Rhizopus*, *Absidia*, and *Mucor* sp.), and *Histoplasma capsulatum*.⁸ Very few antifungal agents are available for the treatment of deep fungal infections, and the drug of choice is amphotericin B, in spite of its mammalian toxicity and side effects.^{7,8} Other agents that are of interest against *Candida* are 5-fluorocytosine and clotrimazole.⁷

As a result of our interest in this area,⁹ a study of the esters of 4-substituted crotonic acids was initiated. While this work was in progress, we became aware that others had reported the antifungal activity of ethyl 4-bromo-2-methylcrotonate and phenyl 4-bromocrotonate against plant pathogenic fungi.¹⁰

A systematic examination of the effect of structural alteration to antifungal activity of the 4-substituted crotonic acid esters was carried out. The alterations included replacement of the methyl group in the 4 position of methyl crotonate by hydrogen and substitution of a fluorine, chlorine, bromine, iodine, methoxyl, or thiomethyl substituent for hydrogen on carbon-4. The two most active derivatives were further modified by preparing the ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, and *n*-hexyl esters.

The unsaturated esters were obtained by esterification of crotonic acid with the appropriate alcohol by azeotropic distillation with benzene in the presence of sulfuric acid as the catalyst. The *n*-propyl, *n*-butyl, *n*-pentyl, and *n*-hexyl esters were prepared in this manner.^{11,12} The methyl esters of 4-methoxy-,¹³ 4-methylthio-,¹⁴ 4-fluoro-,¹⁵ and 4-chlorocrotonic acids¹⁶ were prepared by methods found in the literature. Bromination of the 4 position of the crotonic esters was achieved by means of *N*-bromosuccinimide and benzoyl peroxide in carbon tetrachloride.¹⁷ Of the six bromo esters prepared, the methyl,¹⁷ ethyl,¹⁸ and propyl¹⁹ esters were known.

The esters of 4-iodocrotonic acid were prepared from the corresponding bromo esters by treatment with sodium iodide in acetone at room temperature. Of the iodo compounds prepared, only ethyl 4-iodocrotonate was previously reported, and it was prepared from ethyl 4-chlorocrotonate.²⁰ It should be mentioned that the distillates were darker than the products prepared for distillation. This was also observed previously.²⁰ The data characterizing the crotonic acid esters are given in Table I.

All of the compounds were tested against *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 1004), *Mucor mucedo* (ATCC 7941), and *Trichophyton mentagrophytes* (ATCC 9129) in Sabouraud dextrose agar (Difco) at pH 5.6 and 7.0 in the presence and absence of 10% beef serum (Miles Labs.) according to published methods,^{9,21} and the results are listed in Table II. Since it was our desire to locate compounds which would be of potential medicinal interest, the highest level tested was 100 μ g/ml, and, for the purpose of comparison, the most valuable results were those obtained at pH 7.0 in the presence of beef serum.

A comparison of the results obtained with the methyl 4-halocrotonates (Table II) shows the following order of antifungal activity: I > Br > Cl > F, with the iodo-compound being about twice as active as the bromo analogue on a molecular basis. To examine the effect on

Table I. Crotonic Acid Esters ($\text{XCH}_2\text{CH}=\text{CHCOOR}$)

X	R	Yield, %	Bp (mm), °C		n _D	ν_{neat} , cm ⁻¹		Formula	Analyses
			Obsd	Lit.		25°	C=O		
H	CH ₃	74	64 (7.5)	Purchased	1.4454	1722	1661	C ₆ H ₁₀ O ₃	C, H
CH ₃ O ^a	CH ₃	54	111-112 (19)	54-57.5 (8.0) ^e	1.4542	1722	1664		
CH ₃ S	CH ₃	15	127-131	95-97 (1.2) ^e	1.4019	1724	1652		
F	CH ₃	77	68-71 (8.5)	46 (16) ^f	1.4609	1730	1672		
Cl ^b	CH ₃	70	86-88 (7.0)	68.5 (11) ^c	1.4986	1728	1663		
Br	CH ₃	48	86-89 (5.5)	78-82 (8.0) ^d	1.4907	1725	1658		
Br	C ₂ H ₅	37	102-105 (5.5)	66-67 (0.3) ^g	1.4870	1723	1658		
Br	<i>n</i> -C ₃ H ₇	45	116-117 (5.5)	108-109 (15) ^h	1.4827	1725	1659		
Br	<i>n</i> -C ₄ H ₉	33	127-130 (5.0)		1.4814	1720	1656	C ₈ H ₁₄ BrO ₂	C, H, Br
Br	<i>n</i> -C ₅ H ₁₁	60	125-129 (2.7)		1.4807	1719	1652	C ₉ H ₁₆ BrO ₂	C, H, Br
Br	<i>n</i> -C ₆ H ₁₃	83	55-57 (0.2)		1.5574	1720	1642	C ₁₀ H ₁₇ BrO ₂	C, H, Br
I	CH ₃	70	55-58 (0.1)	90-92 (2.0) ^j	1.5423	1728	1645	C ₈ H ₁₄ IO ₂	C, H, I
I	C ₂ H ₅	75	68-71 (0.1)		1.5331	1710	1648	C ₉ H ₁₆ IO ₂	C, H, I
I	<i>n</i> -C ₃ H ₇	65	77-81 (0.2)		1.5426	1725	1642	C ₁₀ H ₁₈ IO ₂	C, H, I
I	<i>n</i> -C ₄ H ₉	53	91-94 (0.2)		1.5190	1721	1642	C ₁₁ H ₂₀ IO ₂	C, H, I
I	<i>n</i> -C ₅ H ₁₁		100-102 (0.05)		1.5126	1720	1645	C ₁₂ H ₂₂ IO ₂	C, H, I

^a Prepared by method in ref 13; product not fully characterized. ^b Prepared by method in ref 16. ^c H. O. House, V. K. Jones, and G. A. Frank, *J. Org. Chem.*, **29**, 3327 (1964). ^d L. N. Owen and M. U. S. Sultanbawa, *J. Chem. Soc.*, 3098 (1949). ^e See ref 14. ^f See ref 15. ^g See ref 18. ^h See ref 19. ⁱ See ref 20.

Table II. Antifungal Activity of Crotonic Acid Esters and Derivatives at pH 5.6 and 7.0 in Sabouraud Dextrose Agar in the Absence and Presence of Beef Serum^a

XCH=CHCOOR		Levels of inhibition ^b															
		<i>C. albicans</i>				<i>A. niger</i>				<i>M. mucedo</i>				<i>T. mentagrophytes</i>			
		pH 5.6		pH 7.0		pH 5.6		pH 7.0		pH 5.6		pH 7.0		pH 5.6		pH 7.0	
X	R	- ^c	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
FCH ₂	CH ₃	NA ^{d,e}	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ClCH ₂	CH ₃	NA	NA	NA	NA	NA	NA	NA	NA	60	80	80	90	4	40	60	60
BrCH ₂	CH ₃	30	30	30	30	50	50	50	60	6	8	10	14	3	5	5	6
BrCH ₂	C ₂ H ₅	20	30	30	40	80	90	70	80	5	7	6	7	<1	2	2	4
BrCH ₂	<i>n</i> -C ₃ H ₇	18	30	30	40	80	90	60	80	4	5	4	7	<1	3	3	7
BrCH ₂	<i>n</i> -C ₄ H ₉	16	30	30	40	60	100	50	80	4	4	4	7	<1	4	3	8
BrCH ₂	<i>n</i> -C ₅ H ₁₁	20	50	30	50	70	NA	70	NA	3	14	14	30	<1	4	2	10
BrCH ₂	<i>n</i> -C ₆ H ₁₃	20	70	20	90	NA	NA	NA	NA	14	40	16	50	<1	14	4	30
ICH ₂	CH ₃	16	30	20	18	30	40	30	40	9	10	9	8	<1	3	2	4
ICH ₂	C ₂ H ₅	8	18	10	18	40	50	40	40	<1	4	3	5	<1	2	2	4
ICH ₂	<i>n</i> -C ₃ H ₇	10	16	12	20	30	60	30	60	2	5	3	6	<1	3	2	7
ICH ₂	<i>n</i> -C ₄ H ₉	10	20	12	30	30	70	20	70	2	5	4	7	<1	3	<1	7
ICH ₂	<i>n</i> -C ₅ H ₁₁	5	40	12	50	40	NA	30	NA	4	9	3	12	<1	4	2	10
ICH ₂	<i>n</i> -C ₆ H ₁₃	16	80	30	90	NA	NA	NA	NA	14	40	14	50	<1	8	3	20
Candididin		<1	<1	<1	2	2	5	5	7	<1	<1	<1	<1	<1	<1	3	9
Amphotericin B		<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

^a *C. albicans* and *M. mucedo* were incubated at 37° for 20 h and *T. mentagrophytes* and *A. niger* at 28° for 5 days.

^b Minimal concentrations of compound in μg/ml causing 100% inhibition of test organisms. Activity to 10 μg/ml was obtained in increments of 1 μg/ml, from 10 to 20 μg/ml in increments of 2 μg/ml, and from 20 to 100 μg/ml, the highest level tested, increments of 10 μg/ml were used. All tests were carried out in "I" plate Petri dishes. ^c - and + = absence or presence of 10% beef serum. ^d NA = not inhibitory below 100 μg/ml. ^e Methyl acrylate and the methyl, ethyl, propyl, butyl, pentyl, and hexyl esters of crotonic acid were inactive against all four fungi. Methyl 4-methoxycrotonate was also inactive, but methyl 4-methylthiocrotonate was inhibitory to *M. mucedo* (70 μg/ml) and *T. mentagrophytes* (90 μg/ml) at pH 5.6 in the absence of beef serum.

fungitoxicity of the chain length of the alkyl group in the ester function, the 4-bromo and 4-iodocrotonic esters from methyl to *n*-hexyl were tested. The order of antifungal activity against the four fungi of the esters of the bromocrotonate series was methyl > butyl > propyl > ethyl > pentyl > hexyl and of the iodocrotonate series was ethyl > methyl > propyl > butyl > pentyl > hexyl.

All of the iodocrotonates were more fungitoxic than the corresponding bromocrotonates, and the most active of all the compounds tested in this study was ethyl 4-iodocrotonate. The minimum inhibiting concentration for the four test organisms was as follows: *C. albicans*, 18 μg/ml; *A. niger*, 40 μg/ml; *M. mucedo*, 5 μg/ml; *T. mentagrophytes*, 4 μg/ml.

Since allylic compounds are known to undergo nucleophilic substitution,²² it was desired to determine whether this played any part in the mode of fungitoxic action of the 4-substituted crotonic esters. Methyl 4-bromocrotonate was added to the growth medium at pH 7.0 at a concentration of one increment above the minimal inhibitory level for each organism (Table II). Cystine, cysteine, and glutathione, respectively, were added to the medium in molar ratios of 1:1 to 10:1 in increments of 1 equiv to one with respect to the toxicant, and the Petri dishes were inoculated and incubated as described above. Beef serum was omitted in order to avoid the complications which might be caused by the presence of a multitude of additional metabolites being added to the medium. Cystine had no effect on the toxicity of methyl 4-bromocrotonate. Cysteine and glutathione allowed all four fungi to grow in the presence of the toxicant. The ratio of SH-containing compound to toxicant for each fungus which prevented the inhibition of growth is shown in Table III. Methyl 4-methylthiocrotonate was studied in the same manner as methyl 4-bromocrotonate, against *M. mucedo* and *T. mentagrophytes*. The results were similar for both compounds (Table III).

It appears that the mechanism of fungitoxicity of the 4-substituted crotonic acid esters may possibly be based,

Table III. Protection by SH-Containing Metabolites against the Fungitoxicity of 4-Substituted Methyl Crotonates

Fungus	Inhibitory level of toxicant, $\mu\text{g/ml}$	Molar ratio of protectant to inhibitor		
		Cys-tine	Cys-te-ine	Glu-ta-thi-one
$\text{BrCH}_2\text{CH}=\text{CHCOOCH}_3$				
<i>C. albicans</i>	40	$>10^a$	2	3
<i>A. niger</i>	60	>10	<1	<1
<i>M. mucedo</i>	12	>10	4	8
<i>T. mentagrophytes</i>	6	>10	10	9
$\text{CH}_3\text{SCH}_2\text{CH}=\text{CHCOOCH}_3$				
<i>M. mucedo</i>	80	>10	<1	<1
<i>T. mentagrophytes</i>	70	>10	<1	<1

^a A molar ratio of cystine to toxicant of 10:1, the highest ratio tested, allowed all cultures to remain inhibited.

at least in part, on nucleophilic substitution. The reduction in toxicity of the compounds by beef serum, protection of the fungi by SH-containing metabolites, and the order of fungitoxicity due to the different substituents in the 4 position are consistent with the order of leaving groups in the nucleophilic reaction.²² That the size of the ester affects fungitoxicity in the homologous series indicates that chain length of the molecule affects its activity, as is the case with the fatty acids and their methyl esters.²¹

Experimental Section¹²

Butyl 4-Bromocrotonate. A mixture of butyl crotonate¹¹ (50 g, 0.35 mol), *N*-bromosuccinimide (63 g, 0.35 mol), and benzoyl peroxide (0.5 g, 0.002 mol) in 500 ml of dry CCl₄ was heated under reflux with stirring and exclusion of moisture for 10 h. The reaction mixture was cooled to room temperature with continued stirring, and the succinimide was removed by filtration. The filtrate was subsequently washed with H₂O (3 × 100 ml) and dried (Na₂SO₄). After removing the solvent in the rotary evaporator,

the residue was distilled under vacuum. The yield of product was 35 g (45%), bp 129–135° (16 mm).

Butyl 4-Iodocrotonate. To a solution of NaI (19.3 g, 0.133 mol) in $(\text{CH}_3)_2\text{CO}$ (150 ml) at room temperature was added butyl 4-bromocrotonate (22.1 g, 0.1 mol). After stirring for 1 h, the NaBr formed was removed by filtration. The solids were washed with $(\text{CH}_3)_2\text{CO}$ and the combined filtrates were poured into 600 ml of Et_2O . The ether solution was washed with 5% $\text{Na}_2\text{S}_2\text{O}_3$ solution (100 ml), followed by H_2O (2×200 ml), and dried (Na_2SO_4). The solvent was removed in the rotary evaporator, and the residue was distilled under reduced pressure. The yield of product was 18.9 g (70%), bp 100–110° (0.1 mm).

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- (12) Methyl acrylate, crotonic acid, and methyl and ethyl crotonate were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis., and amphotericin B was purchased from Calbiochem, La Jolla, Calif. The synthetic procedures are general. Infrared spectra were obtained with a Perkin-Elmer Model 221 spectrophotometer on neat samples. Refractive indices were taken with an Abbe-3L, B & L refractometer. Gas chromatography was performed on a Varian Aerograph Model 1200 gas chromatograph with a flame ionization detector to which is attached a Varian Aerograph Model 20 recorder. The purity of the compounds was established by gas chromatography on a column of 3% Dexsil 400 on Anachrom A (90–100 mesh) purchased from Analabs, New Haven, Conn. All samples tested microbiologically were at least 95% pure.
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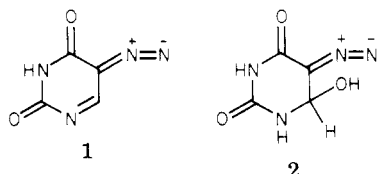
Synthesis and Antimicrobial Evaluation of Substituted 5,6-Dihydro-5-nitouracils

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Reaction of 5-nitouracil derivatives with sodium borohydride in methanol–water, followed by neutralization of the product with acid, has produced 5,6-dihydro-5-nitouracil (5), 5,6-dihydro-6-methyl-5-nitouracil (7), 5,6-dihydro-5-nitro-1-(4-nitrophenyl)uracil (10), and 5,6-dihydro-5-nitro-1-(β -D-ribofuranuronic acid ethyl ester)uracil (12). In assays for antimicrobial activity using strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Trichophyton mentagrophytes*, significant inhibition of growth was not found.

It has been demonstrated that 5-diazouracil (1) inhibits cell division,¹ causes an increase in the mean cell volume,² and has a broad-spectrum in vitro activity against pathogenic bacteria.³ The antimicrobial activity of 1 is, however, accompanied by severe mammalian toxicity. Since assigned structures⁴ for the 5-diazouracils and 5-diazouridines indicate the presence of a substituent at C-6, as in the hydrated form of 5-diazouracil (2), we sought to prepare other uracil derivatives containing a 5,6-dihydro structure with a strongly electronegative group at C-5. The synthesis of 5,6-dihydro-5-nitouracil and related deriv-



atives by reduction of the corresponding 5-nitouracil with sodium borohydride plus the evaluation for antimicrobial activity is herein reported.

Chemistry. In an early investigation⁵ of 5-nitopyrimidines, treatment of 5-nitro-1-(β -D-ribofuranuronic

acid isopropyl ester)uracil with sodium borohydride and aluminum chloride or with potassium borohydride and lithium chloride yielded a new product which was identified as the 5,6-dihydro-5-nitro derivative. Reduction of the 5,6 bond in *N*⁴-acetylcytidine⁶ and 5-acetyluracil⁷ has been reported using sodium borohydride. More recently it was found⁸ that reduction of 6-nitroquinoxaline to give 6-nitro-1,2,3,4-tetrahydroquinoxaline occurred upon treatment with sodium borohydride in methanol. No reduction of quinoxaline itself under the same conditions was found, indicating the necessity of the nitro group for reaction with the hydride.

When a solution of 5-nitouracil (3) in methanol–water was treated with sodium borohydride, a precipitate formed. This was identified as the sodium salt of 5,6-dihydro-5-nitouracil (4). Treatment of a solution of 4 in water to pH 3 with 1 N hydrochloric acid gave 5,6-dihydro-5-nitouracil (5). Similarly, 6-methyl-5-nitouracil (6) yielded 5,6-dihydro-6-methyl-5-nitouracil (7) (Scheme I). The ¹H NMR spectra of 7 in $\text{Me}_2\text{SO}-d_6$ revealed a mixture of two products, presumed to be the cis and trans isomers. The signal for the C_5H (δ 5.81 $J_{5,6}$ = 9.5 Hz) seems to indicate a predominance of the trans isomer,⁹ which could be expected with the bulky methyl and nitro groups