Synthesis and *In Vitro* Evaluation of the Antifungal Activity of a Series of Thiomalic Acid Derivatives

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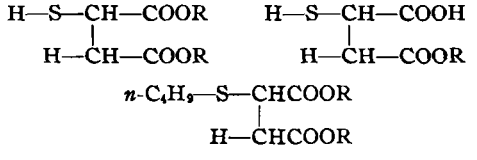
Mono and diesters of thiomalic acid with various alcohols were prepared, and the *S-n*-butyl derivatives of the diesters were prepared. These compounds were analyzed, various physical constants were determined, and the fungicidal and fungistatic activities were measured, using undecylenic acid as the standard for comparison against *Trichophyton mentagrophytes*. Several of the compounds under the conditions of the tests exhibited fungicidal and/or fungistatic activity.

THIOMALIC ACID (mercaptosuccinic acid, 2-mercapto-1,4-butanedioic acid) possesses two structural features which are present in the structures of some commercially available antifungal compounds—namely, the $-\overset{|}{\underset{|}{C}}-S-$ group and the carboxylic acid group. Barry, *et al.* (1), found that *S*-alkylthiomalic acids and their half esters strongly inhibited the growth of various bacteria. This paper reports the preparation of a series of esters of thiomalic acid and *S-n*-butylthiomalic acid; the general formulas for these compounds are



where R is n-C_3H_7—, n-C_4H_9—, and n-C_5H_{11}—. The fungistatic and fungicidal activities of these compounds are also reported.

EXPERIMENTAL

The esters listed in Table I were prepared by standard procedures. The following examples illustrate the methods and conditions used.

Diesters.—The dipropyl, dibutyl, and diamyl thiomalates were prepared as follows. A mixture of 15 Gm. (0.1 mole) of thiomalic acid, 45 ml. of the appropriate alcohol, and 4 ml. of concentrated sulfuric acid was refluxed for 3 hours. The excess alcohol was removed by distillation under reduced pressure, and the residual liquid was poured with stirring into 150 ml. of a saturated aqueous solution of sodium bicarbonate. When neutralization of the acid was complete, the crude esters were recovered by ether extraction.

Monoesters.—The monopropyl, monobutyl, and monoamyl thiomalates were prepared as follows. A mixture of 15 Gm. (0.1 mole) of thiomalic acid, 0.1 mole of the appropriate alcohol, 4 ml. of concentrated sulfuric acid, and 35 ml. of dioxane was refluxed for 12 hours. The volume of the reaction mixture was then reduced by distillation under reduced pressure. The residual liquid was poured with stirring into 150 ml. of a saturated aqueous solution of sodium bicarbonate. The alkaline mixture, which had assumed a wine color, was extracted with several portions of ether to remove any diester produced by the reaction. Concentrated hydrochloric acid was added until the aqueous mixture was acid to litmus. This discharged the color and caused the monoester to separate as an oil. Ether extraction of the acidified mixture resulted in the isolation of the crude monoesters.

S-n-Butyldiesters.—The dipropyl, dibutyl, and diamyl *S-n*-butyl thiomalates were prepared according to the method of Barry, *et al.* (1). Clean, finely divided sodium metal, 0.5 Gm. (22 mmoles), was dissolved in 22 ml. of dry ethanol. The appropriate thiomalic acid diester (22 mmoles) and *n*-butyl bromide (22 mmoles) were added to the sodium ethylate solution and refluxed for 3 hours. The mixture was cooled, and the liquid layer was filtered from the solid sodium bromide. After removing the excess ethanol by distillation under reduced pressure, the residual liquid was poured into 20 ml. of water, the aqueous mixture was extracted with ether to remove the ester. The crude dialkyl *S-n*-butyl thiomalates were obtained upon evaporating the ether.

The crude esters were all light yellow viscous liquids with the monoesters being most viscous and darkest in color. Distillation at pressures of 1–3 mm. of mercury produced distillates that were colorless. If prolonged exposure to high bath temperature occurred during the distillation of the esters, the distillate assumed a yellow color. This decomposition was prevented by flushing the apparatus with nitrogen before distillation and by furnishing nitrogen to the aspirator tube during the reduced pressure distillation and distilling as rapidly as possible. Distillation reduced the yield of ester materially except for dialkyl *S-n*-butyl thiomalates. The monoesters were particularly subject to decomposition during distillation. The pure esters were characterized (see Table I) and used for the antifungal tests.

Relative Solubilities of the Mono and Diesters.—The relative solubilities of the mono and diesters were determined in some common solvents. These data are recorded in Table II.

FUNGICIDAL AND FUNGISTATIC STUDIES

Fungicidal Test.—The method of testing the esters of thiomalic acid for fungicidal activity was, with

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TABLE I.—DERIVATIVES OF THIOMALIC ACID

$$R_2\text{—}S\text{—}CH(COOR_1)\ (\alpha)$$
$$H\text{—}CH(COOR)\ (\beta)$$

R	R_1	R_2	Formula	Yield, %	Boiling Range, °C./mm. Hg	Sulfur, %[a] Calcd.	Sulfur, %[a] Found	$d_4^{20°}$	$n_D^{20°}$	$(R_L)D$ Calcd.	$(R_L)D$ Found
n-C_3H_7—	*n*-C_3H_7—	H—	$C_{10}H_{18}O_4S$	60	135–139/1	13.69	13.59	1.0700	1.4579	59.37	59.72
n-C_4H_9—	*n*-C_4H_9—	H—	$C_{12}H_{22}O_4S$	58	150–155/1	12.23	12.28	1.0332	1.4573	68.61	69.18
n-C_5H_{11}—	*n*-C_5H_{11}—	H—	$C_{14}H_{26}O_4S$	57	165–175/1	11.04	10.95	1.0074	1.4564	77.93	78.40
n-C_3H_7—	H—	H—	$C_7H_{12}O_4S$	46	125–132/2	16.69	16.60	1.2298	1.4858	45.43	44.84
n-C_4H_9—	H—	H—	$C_8H_{14}O_4S$	44	145–152/2	15.55	14.89	1.1857	1.4811	50.05	49.49
n-C_5H_{11}—	H—	H—	$C_9H_{16}O_4S$	41	164–170/3	14.56	14.67	1.1500	1.4799	54.71	54.38
n-C_3H_7—	*n*-C_3H_7—	*n*-C_4H_9—	$C_{14}H_{26}O_4S$	55	124–127/1	11.04	11.13	1.0285	1.4602	78.13	77.34
n-C_4H_9—	*n*-C_4H_9—	*n*-C_4H_9—	$C_{16}H_{30}O_4S$	52	140–144/1	10.07	10.01	1.0011	1.4590	87.37	86.95
n-C_5H_{11}—	*n*-C_5H_{11}—	*n*-C_4H_9—	$C_{18}H_{34}O_4S$	50	153–158/1	9.26	9.40	0.9811	1.4585	96.69	96.45

[a] Analytical method (2).

TABLE II.—RELATIVE SOLUBILITY OF THE MONO AND DIESTERS[a]

Thiomalate Ester	Water	Benzene	Acetone	Pet. Ether	Methyl Alcohol	Chloroform	Tetrahydrofuran
Monopropyl	−	+++	+++	−	++	+++	+
Dipropyl	−	+++	+++	+	++	+++	+
Monobutyl	−	+++	+++	−	++	+++	+++
Dibutyl	−	+++	+++	+	+++	+++	+++
Monoamyl	−	+++	+++	−	+++	+++	+++
Diamyl	−	+++	+++	+++	+++	+++	+++

[a] Insoluble in ten parts (volume) of solvent, −; soluble in three to four parts, +; soluble in equal part, ++; soluble in less than equal part, +++.

minor modifications, the procedure described by Burlingame and Reddish (3) and as adapted and modified by Golden and Oster (4) for use in testing alcohol soluble compounds.

The culture medium used was Sabouraud's dextrose agar containing 10% horse serum and maintained at a pH of 5.6 to prevent bacterial contamination. Petri dishes of this medium were streaked with a culture of *T. mentagrophytes* (No. 640 Emmons) and incubated at 28 ± 1° for 10 to 15 days. Cultures of this age were cut into disks of 1-cm. diameter with a sterile cork borer and transferred aseptically to seeding tubes containing 10 ml. of 0.5%, 1.0%, and 2.0% acetone solutions of each compound listed in Table III. After a 2-minute contact with the test compound, each disk was placed in 10 ml. of sterile broth and shaken gently for 3 minutes. This was done to free the culture of water soluble material. The disk was then removed from the broth and washed with 10 ml. of acetone in the same manner for 5 minutes, thereby removing the test compound from the culture. Once again each disk was immersed separately in sterile broth for 3 minutes to remove the acetone and then was spread culture side down over the surface of a sterile slant of Sabouraud's agar. These slants were incubated at 28 ± 1° for 14 days and then observed for growth. The results are outlined in Table III.

Undecylenic acid was used as a control in the same concentrations as the test compounds and exhibited 100% fungicidal activity at each concentration. Acetone which was used as a solvent for the test compounds was also used as a control and, as with other investigators (4, 5), had no effect upon the test fungus. All tests were performed in triplicate.

Fungistatic Test.—The method of testing for the presence of fungistatic activity was a modification of the procedure described by Kligman and Rosenweig (6) and Collins and Wiese (7). Sabouraud's dextrose agar was the culture medium used and was maintained at a pH of 5.6 to prevent bacterial contamination. Ten compounds, each in concentrations of 0.5%, 1%, and 2.0%, were tested in triplicate.

Petri dishes of Sabouraud's medium were streaked with a culture of *T. mentagrophytes* (No. 640 Emmons). A sterile filter paper disk, 13 mm. in diameter, was placed in each acetone solution of the test compounds and the controls. Each disk was picked up with sterile forceps, the excess liquid drained off by touching the disk to the wall of the container, and then immediately dropped onto the agar surface of a Petri dish which had been streaked with the test organism. These Petri dishes were then incubated at 37° for 7 days, at which time the zones of inhibition were measured. These data are recorded in Table IV and represent the results from two separate determinations.

DISCUSSION

Analysis of the diesters by standard methods for the mercapto group (8) showed them to be between 98–100% pure. The monoesters were submitted to the same analytical procedures, but the free carboxyl group interfered in these assay methods. Since the *S*-alkyl thiomalates lacked a free mercapto group, the sulfur contents of all of the derivatives of thiomalic acid prepared for this study were determined by the Parr sodium peroxide bomb method described by Smith and Shriner (2).

The structure indicated for the monoesters in Table I assumes that the ester linkage involves the β-carboxyl group, the group farthest removed from the carbon bearing the mercapto group. No experimental evidence for this structure verifies this assumption; however, further study of this problem is underway in this laboratory. Mention may be made that Heilbron (9) indicates β-carboxyl involvement in the structure for thiomalic acid monamide.

TABLE III.—NUMBER OF CULTURES UPON WHICH THIOMALIC ACID ESTERS EXERTED FUNGICIDAL ACTION[a]

	Concn., %		
Thiomalate	0.5	1.0	2.0
Free acid	0	0	0
Dipropyl	0	3	4
Dibutyl	0	0	4
Diamyl	0	5	6
Monopropyl	2	6	6
Monobutyl	0	1	3
Monoamyl	6	6	6
Dipropyl *S*-butyl	0	6	6
Dibutyl *S*-butyl	0	0	3
Diamyl *S*-butyl	6	6	6

[a] Each figure represents the results from a total of six determinations.

TABLE IV.—FUNGISTATIC ACTIVITIES OF THIOMALIC ACID ESTERS[a]

	Concn., %		
Thiomalate	0.5	1.0	2.0
Free acid	0	0	0
Dipropyl	0	2	5
Dibutyl	0	0	0
Diamyl	0	0	3
Monopropyl	5	7	15
Monobutyl	6	10	18
Monoamyl	8	15	29
Dipropyl *S*-butyl	3	7	40
Dibutyl *S*-butyl	0	0	0
Diamyl *S*-butyl	6	10	28

[a] Zones of inhibition measured in millimeters.

The method by which the monoesters were prepared yielded diesters as a side product to the extent of 10–15%. An attempt to increase the yield of monoester and to reduce the amount of discoloration during preparation by using polyphosphoric acid as the catalytic agent resulted in comparable yields but less discoloration during the reflux period.

An attempt has been made to prepare salts of the monoesters with various metal compounds. While some precipitates were obtained, these have not been properly characterized and were not tested for antifungal activity. The product produced with cupric ion was dark violet and probably represented a mixture of compounds in which the copper exists in both valence states as has been suggested for free thiomalic acid (10). Since metallic ions can form mercaptides and some can cause the oxidation of the mercapto group, additional study of the monoester salts will be undertaken.

Oster and Golden (11) found that the fungistatic values obtained by using several dermatophytes, *T. mentagrophytes*, *E. inguinale*, and *T. purpureum*, ran closely parallel. We decided (as they did) to use one organism, *T. mentagrophytes*, as the representative test organism to save time and reduce the number of tests. Because of its known antifungal activity, undecylenic acid was used as the standard in these comparative tests. Complete inhibition of growth was produced by undecylenic acid at all concentrations. Triplicate tests of the acetone solvent showed no antifungal effect.

The inhibition of growth around the filter paper disks was measured in millimeters and represents an average of three measurements.

SUMMARY

Mono and diesters of thiomalic acid with several alcohols and diesters of *S-n*-butyl thiomalic acid with the same alcohols were prepared. These compounds were analyzed for their sulfur content, various physical constants were determined, and the fungicidal and fungistatic activities were determined using *T. mentagrophytes* as the test organism. Undecylenic acid was used as the standard for comparisons.

In the fungicidal tests, disks of an agar culture of the test organism were exposed to various concentrations of the thiomalic acid derivatives. After washing in broth and in acetone to remove the test compounds, the disks were placed culture side down on the surface of a sterile slant of Sabouraud's agar. The slants were observed for growth after incubation. The absence of growth indicated that the compounds exerted a fungicidal action. Both monoamyl thiomalate and diamyl *S*-butyl thiomalates were fungicidal at each concentration in all tests. Diamyl, monopropyl, and dipropyl *S*-butyl thiomalates exhibited fungicidal activity at 1 and 2% concentrations. Thiomalic acid exhibited no fungicidal activity.

In the fungistatic test method sterile filter paper disks impregnated with acetone solutions of the test compounds were placed on Petri dishes of Sabouraud's agar streaked with the test organism. Inhibition of growth after incubation was indicative of fungistatic activity. Thiomalic acid was inactive, dipropyl and diamyl thiomalate showed slight activity at the highest concentration used, and monopropyl, monobutyl, monoamyl, dipropyl, and diamyl *S*-butyl thiomalates showed activity at all concentrations.

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