Bottromycin. Separation of Biologically Active Compounds and Preparation and Testing of Amide Derivatives

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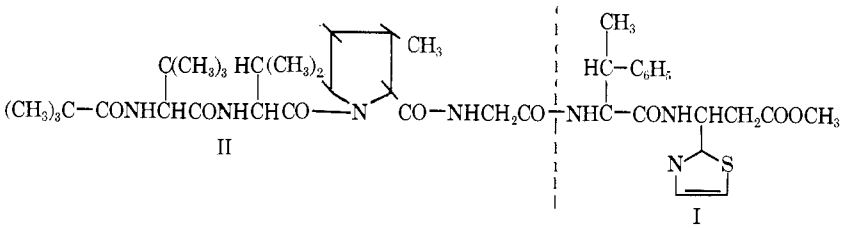
Five biologically active compounds were separated from the bottromycin complex. A number of derivatives of the terminal carboxyl group of bottromycin A_1 were prepared and tested. When tested against *Staphylococcus aureus* the simple amides are all more active *in vivo* and less active *in vitro* than the parent ester. Correlation of R_m value and bioactivity appears to give broad peaks which are different for *in vivo* and *in vitro* tests. Several of the compounds are active when tested against *Mycoplasma*.

Early workers with bottromycin[1,2] reported that the antibiotic contained a unique dipeptide, β-methylphenylalanyl-β-(2-thiazolyl)-β-alanine methyl ester (I). Mild hydrolysis of the antibiotic removed the terminal methyl group and produced the corresponding acid which had low antibiotic activity. More recently[3] the antibiotic was shown to consist of two substances, bottromycin A_1 which contains *cis*-3-methyl-*l*-proline and bottromycin B, which contains *l*-proline.



The sequence and identity of amino acids as indicated in II was suggested.[4] It was proposed that the basic property of the antibiotic is due to an amidine structure at one of the peptide bonds. Pivalic acid was obtained by alkaline hydrolysis of the compound. Subsequently it was reported[5] that bottromycin contained a third component, designated A_2 which yields 4-methyl-2-pentenoic acid instead of pivalic acid on alkaline hydrolysis.

Although certain similarities of the *Streptomyces* cultures producing the antibiotics were observed, the organisms apparently are not identical.[3] Recently, we have observed that a mixture of peptide antibiotics is produced by an unidentified streptomycete[6] which also produces the antibiotic, netropsin. The peptide antibiotics were shown to be of the bottromycin group based on the liberation of the acetyl derivative of I on treatment with acetic anhydride. Five biologically active components of the peptide mixture are discernible by paper chromatography. These have been designated A through E in order of decreasing polarity. Partition column chromatography yielded small quantities of A, D, and E and larger quantities of B and C. The properties of these compounds are listed in Table I. Most of the bioactivity is due to component C. This substance yields pivalic acid (identified by gas chromatography) and methylproline (identified by paper strip chromatography and Moore–Stein amino acid analysis) and is apparently identical with bottromycin A_1. Component B yields proline and is apparently identical with bottromycin B. The remaining compounds which account for only a trace of the original bioactivity have not been characterized chemically but were shown to possess antibiotic activity in both *in vitro* and *in vivo* tests.

Since excessive doses (based on *in vitro* potency) of bottromycin A_1 appeared to be required for an *in vivo* response, it seemed possible that the antibiotic is degraded *in vivo*. Attempted degradation of the molecule by exposure to a variety of proteases was unsuccessful. Liver brei likewise did not cause loss of antibiotic activity. However, since the acid is inactive, the possibility of stabilizing the antibiotic *in vivo* by preparing compounds resistant to esterases was investigated.

Amides were synthesized by treating the methyl ester in methanol or dimethylformamide solution with the desired amine. The reaction was followed readily and conveniently by paper chromatography of the reaction mixture. The disappearance of the parent compound and concurrent formation of a new bioactive zone was observed. At 50–60°, the reaction is complete in about 24 hr using primary amines. No appreciable reaction was obtained with secondary amines. Ir spectroscopy indicated loss of the ester band at 1250 cm^{-1} but no other major change was observed. Elemental analysis, obtained on a few compounds, was satisfactory. Although the ester was not crystalline, several amides were obtained crystalline. The properties of these compounds are listed in Table II.

Tertiary amides were obtained by reaction of secondary amines with the intermediate 2,4-dinitrophenyl ester. This ester was prepared from the free acid by

(1) J. M. Waisvisz, M. G. van der Horven, J. van Peppen, and W. C. M. Zwennis, *J. Am. Chem. Soc.*, **79**, 4520 (1957).

(2) J. M. Waisvisz, M. G. van der Horven, and B. Te Nijenhuis, *ibid.*, **79**, 4524 (1957).

(3) S. Nakamura, T. Chikaike, K. Kavasawa, N. Tanaka, H. Yonehava, and H. Umezawa, *J. Antibiotics* (Tokyo), **A18**, 47 (1965).

(4) S. Nakamura, T. Chikaike, H. Yonehava, and H. Umezawa, *ibid.*, **A18**, 60 (1965).

(5) S. Nakamura, N. Tanaka, and H. Umezawa, *ibid.*, **A19**, 10 (1966).

(6) The streptomycete has been characterized and named *Streptomyces canadensis*: H. B. Woodruff, *et al.*, to be published.

TABLE I
SEPARATION OF BOTTROMYCINS

Component	R_m[a]	Wt, g	MIC, μg/ml[b]	Bioactivity % of total
A	4.0	0.130	1.0	0.10
B (bottromycin B)	1.6	0.800	0.04	12.4
C (bottromycin A_1)	1.0	1.410	0.01	87.0
D	0.89	0.016	0.04	0.25
E	0.82	0.016	0.04	0.25

[a] $R_m = \log [(1 - R_f)/R_f]$. Ratio of peak fraction number for bottromycin A_1 and other compound. [b] Minimum inhibitory concentration (MIC), an *in vitro* test performed using *Staphylococcus aureus* and a 6-hr incubation.

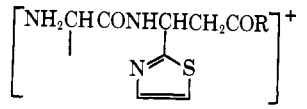
TABLE II
SUMMARY OF PROPERTIES AND TEST RESULTS OF BOTTROMYCINS AND AMIDE DERIVATIVES OF BOTTROMYCIN A_1

Component	R_f	*In vitro*[a] MIC, μg/ml	*In vivo*[b] ED_{50}, μg/dose	Ratio *in vivo*: *in vitro*
A	0.5	1.0	>500	
B[c]	0.28	0.04	200	5000
C[d]	0.19	0.01	50	5000
D	0.13	0.04	100	2500
E	0.11	0.04	100	2500
B methylamide	0.60	0.20	50	250
C ethyl ester	0.09	0.02	100	5000
C benzyl ester	0.01	0.04	*e*	
C amide[f]	0.62	0.10	25	250
C methylamide	0.52	0.05	10	200
C ethylamide	0.33	0.05	15	300
C *n*-propylamide	0.13	0.5	10	20
C isopropylamide	0.15	1.0	10	10
C *t*-butylamide	0.06	0.25	10	40
C cyclohexylamide	0.05	0.2	*e*	
C β-phenethylamide	0.02	*e*	*e*	
C α-naphthylmethylamide	0.02	*e*	*e*	
C benzylamide	0.02	0.5	18	36
C 2,3-propanediol-1-amide	0.79	1.0	25	25
C 2-ethanolamide	0.69	0.5	18	36

[a] Standard test condition using *Staphylococcus aureus* test organisms, 6-hr incubation. [b] Test conditions, intraperitoneal infection, intraperitoneal treatment, two doses 0 and 6 hr post-infection. [c] Bottromycin B. [d] Bottromycin A_1. [e] Not tested. [f] All compounds are terminal amides of the type RCONHR.

reaction with 2,4-dinitrophenyl carbonate in the presence of triethylamine.[7] The product amides[8] were isolated by preparative tlc and the purity was demonstrated by tlc. The properties of compounds prepared by this method are listed in Table III. The structure of the amides was verified in many cases by mass spectrometry.

At moderate temperatures a characteristic peak arises from the C-terminal dipeptide and corresponds to the structure



Since the R group is in this case the variable position, the presence of this fragment serves as a confirmation of structure.[9]

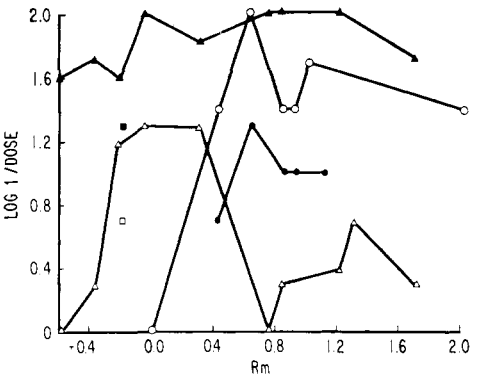
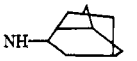
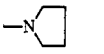
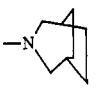


Figure 1.—Plots of the logarithm of the number of effective doses per unit weight *vs.* R_m for a series of compounds: ○, esters *in vitro;* ●, esters *in vivo;* △, amides *in vitro;* ▲, amides *in vivo* (these four are all bottromycin A_1 derivatives); □, bottromycin B methylamide *in vitro;* ■, bottromycin B methylamide *in vivo*.

Ten of the compounds were analyzed by mass spectrometry.[10] The N-methylhydroxamate and the hydrazide did not give the indicated parent peak, presumably because of the low volatility (and stability?) of the resulting dipeptide fragment.

TABLE III
PROPERTIES OF ADDITIONAL CARBOXYL DERIVATIVES OF BOTTROMYCIN A_1

Compd (RCO–)	R_f tlc[a]	*In vivo* act. ED_{50}, μg/dose
$NHC(CH_3)_3$	1.0	39
$NHCH(CH_3)_2$	0.82	...
NH(α-naphthyl)	1.1	>100
$NHCH_2C_6H_5$	0.88	...
NHC_6H_5	0.65	60
NHC_6H_4F-*p*	1.75	>100
	0.95	...
$NHCH_2CH_2N(C_2H_5)_2$	<0.5	>100
$N(CH_3)_2$	0.85	...
$N(C_2H_5)_2$	1.1	...
$N[CH(CH_3)_2]_2$	1.1	82
	1.0	95
	1.0	...
$NHNH_2$	0.25	46[b]
$NHN(CH_3)_2$	0.48	35
$N(OH)CH_3$	0.24	28

[a] Due to some variation in absolute movement the R_f relative to bottromycin A_1 is listed. Tlc was carried out using silica gel G plates and developed with MeOH–$CHCl_3$ (6:94). The R_f of bottromycin A_1 is usually about 0.45. The zones were located by spraying with 0.01% bromophenol blue or by placing the dried plate in an iodine vapor chamber. [b] N. Fanaka, T. Nishimura, S. Nakamura, and H. Umezawa, *J. Antibiotics* (Tokyo), **19,** 149 (1966).

Biological Tests.—The new compounds were tested for inhibition of multiplication of *Staphylococcus aureus* both *in vitro* and *in vivo*. The *in vitro* tests were carried out using a rapid tube dilution test.[11] Mouse *in vivo*

(7) R. Glatthard and M. Matter, *Helv. Chim. Acta*, **40**, 795 (1963).
(8) We are indebted to Mr. K. W. Kelly for technical assistance.
(9) G. Albers-Schonberg (submitted for publication) has further data on the mass spectrum of bottromycin A_1.
(10) We are indebted to G. Albers-Schonberg, J. Beck, and N. R. Trenner for running and interpreting the mass spectra.
(11) We are indebted to E. O. Stapley and Rose M. Miller for *in vitro* tests.

tests were carried out using a standard procedure.[12] The compounds were administered intraperitoneally tested once. Under these test conditions differences in relative potency values greater than a factor of 4 are highly significant.

The parent esters have been reported to protect chick embryos against two strains of *Mycoplasma*.[13] Several of the new compounds were also found to have high potency in this test and were also active in tests with chicks infected with *Mycoplasma*.[14]

Results and Discussion

All compounds were active in the test systems employed. When compared by *in vitro* tests the amide derivatives were all less active than the esters. However, by *in vivo* tests all amides obtained from primary amines were more active than the esters.

Although significantly different specific *in vitro* activity is obtained with the individual compounds, including esters, only slight differences in the *in vivo* tests were observed. As a group, the amides are about ten times as active *in vivo* as the esters while specific activity differences by the *in vitro* tests range from $^1/_5$ to $^1/_{100}$ as active as the esters.

No satisfactory explanation for these results can be offered. Possibly some controlling factor which is not influenced by differences in polarity at the carboxyl terminal of the molecule but which may reflect an inherent difference between ester and amide groups overrides relatively minor differences between the amides themselves.

The general hypothesis[15] that activity relationships of a series of compounds can be correlated with the logarithm of the partition ratio of the individual compounds can be tested using the potency and the R_m value (log $[(1 - R_f)/R_f]$) in the paper chromatography system for esters and amides of primary amines.

Within each series of compounds and with each type of test the results are in general agreement with the above hypothesis. For each series a broad maxima of activity is obtained by plotting the logarithm of the number of effective doses per unit weight *vs.* R_m. These data are shown in Figure 1. Although the *in vivo* and *in vitro* potencies are maximum in the same R_m region for the esters, examples are limited. It appears that different maxima for the two tests are obtained with the amides, although the *in vivo* tests are all within a factor of 4. With the biological tests reported, the prediction of *in vivo* response based on the *in vitro* potency does not appear positive.

The large difference in the *in vivo/in vitro* ratio between the ester series and the amide series can be due to instability of the ester *in vivo* or to different tissue distribution. Because the distribution properties would be affected by the partition ratio and since ester and amide derivatives with nearly the same partition ratio were tested, it seems likely that the greater *in vivo* potency is in fact due to greater stability of the amides.

(12) A. K. Miller, "Antimicrobial Agents and Chemotherapy, 1963," American Society for Microbiology, Ann Arbor, Mich., 1964, p 33.

(13) B. M. Miller, E. O. Stapley, H. B. Woodruff, 7th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 1967.

(14) These tests were carried out by Dr. L. Sorensen, Merck Institute for Therapeutic Research, Rahway, N. J.

(15) C. B. C. Boyce and B. V. Milborrow, *Nature*, **208**, 537 (1965).

Experimental Section

Crude Bottromycin.—The weakly basic, solvent-soluble crude bottromycin was prepared by solvent extraction of fermentation broths at pH 8.5 with $CHCl_3$. The $CHCl_3$ extracts were concentrated and percolated through a column of Florosil using a 30:1 ratio of adsorbent to solvent-soluble solids. The column was then eluted with Me_2CO–$CHCl_3$ (1:1). The active cuts were combined and concentrated to dryness. The hydrochloride salt of the antibiotic mixture was prepared by addition of methanolic HCl to an ethereal solution of the extract. Alternately, the antibiotic mixture can be adsorbed from acidified broth on Dowex 50-X2 and eluted with a mixture of 70% MeOH and 30% 1 *N* NH_4OH. After removal of the MeOH, the activity was extracted into $CHCl_3$ followed by chromatography as above. The activity may be followed by antibacterial disk bioassay and paper chromatography.

Paper Strip Chromatography.—The paper strip system used is reversed phase. Whatman no. 1 paper was spotted with 1–5 μg of antibiotic and dipped in a 20% methanolic capryl alcohol solution up to the origin, blotted on absorbent paper, air dried about 5 min, and developed with 0.01 *M*, pH 6.0, phosphate buffer. The strips are removed when the solvent front reaches the end of the strip, dried in a vacuum oven to remove the capryl alcohol, and bioautographed on agar seeded with *Bacillus subtilis*.

Separation of Components.—To 5.5 kg of acid-washed Celite 545 (Johns Manville) in a Patterson-Kelly double-cone blender was added 6 l. of a 20% capryl alcohol solution in Me_2CO. After thorough mixing, the Celite was dried *in vacuo* to remove the acetone. The capryl alcohol impregnated Celite was then packed dry in a column (15 × 120 cm) under slight vacuum in thin layers with tamping. The column was charged with 2.8 g of crude bottromycin in 1800 ml of a 0.10 *M*, pH 6.0, phosphate buffer. The column was developed with pH 6.0 buffer. Fractions of 500 ml were collected. The results are shown in Table I.

Component C (Bottromycin A_1).—Fractions 190–215 from the above partition column were combined and concentrated fivefold. The concentrate was adjusted to pH 8.5 and extracted successively with three equal volumes of $CHCl_3$. The extracts were evaporated to dryness *in vacuo* and the residue was dissolved in Et_2O (300 ml). Methanolic HCl was added to precipitate the component C hydrochloride (1.40 g). *Anal.* Calcd for $C_{42}H_{62}N_8O_7S \cdot HCl \cdot 2H_2O$: C, 57.6; H, 7.6; N, 12.70; S, 3.57; Cl, 4.02. Found: C, 56.7; H, 7.25; N, 12.16; S, 3.89; Cl, 3.72.

The hydrochloride of component C was dissolved in 0.1% DL-tartaric acid at a concentration of 10 mg/ml and the pH carefully was adjusted to 8.7 with NaOH. The free base precipitated in 90% yield; mp 157–170° dec, $[\alpha]^{25}D$ −15° (*c* 0.5, 96% EtOH), mol wt (by titration) 808 (pK_a 8.2). *Anal.* Calcd for $C_{42}H_{62}N_8O_7S \cdot H_2O$: C, 59.9; H, 7.6; N, 13.32; S, 3.80. Found: C, 59.8; H, 7.6; N, 12.8; S, 4.3.

Component B (Bottromycin B).—Cuts 110–143 from the partition column were combined. The free base, $[\alpha]^{25}D$ −25° (*c* 1, 96% EtOH), mp 154–163° dec, showed the following analysis. *Anal.* Calcd for $C_{41}H_{60}N_8O_7S \cdot 2H_2O$: C, 58.2; H, 7.58; N, 13.25; S, 3.79. Found: C, 58.35; H, 7.35; N, 13.47; S, 3.93.

Bottromycin A_1 Methylamide.—Bottromycin A_1 (2.5 g) was dissolved in 30 ml of 10% MeOH–$MeNH_2$, sealed in a Carius tube, and heated at 50° for 20 hr. The reaction mixture was evaporated to dryness and the last trace of $MeNH_2$ was removed. The residue was crystallized from EtOAc, yield 1.9 g, mp 225–228° dec, $[\alpha]^{25}D$ −23° (*c* 1, 95% EtOH). *Anal.* Calcd for $C_{42}H_{63}N_9O_6S$: C, 61.3; H, 7.68; N, 15.33. Found: C, 61.0; H, 7.68; N, 15.39.

The hydrochloride was prepared by precipitation from ether by the addition of methanolic HCl. *Anal.* Calcd for $C_{42}H_{63}N_9O_6S \cdot 2H_2O$: C, 56.5; H, 7.7; N, 14.15. Found: C, 56.20; H, 7.6; N, 14.00.

Bottromycin A_1 Amide.—Bottromycin A_1 (200 mg) was dissolved in 10 ml of 10% MeOH–NH_3 and heated in a sealed tube for 20 hr at 50°. The amide was isolated by partition chromatography in the capryl alcohol–pH 6.0 buffer system, yield 120 mg, mp 173–179° dec, $[\alpha]^{25}D$ −32° (*c* 1, 95% EtOH). *Anal.* Calcd for $C_{41}H_{61}N_9O_6S \cdot H_2O$: C, 59.5; H, 7.6; N, 15.2; S, 3.9. Found: C, 59.0; H, 7.7; N, 15.0; S, 4.1.

Bottromycin A_1 Ethylamide.—Bottromycin A_1 (0.5 g) was prepared in the same way as the methylamide; mp 174–180° dec, $[\alpha]^{25}D$ −16° (*c* 0.1, 95% EtOH). *Anal.* Calcd for $C_{43}H_{65}N_9O_6S \cdot 2H_2O$: C, 59.20; H, 7.9; N, 14.41; S, 3.7. Found: C, 59.20; H, 7.63; N, 14.1; S, 4.00.

Bottromycin A_1 benzylamide was prepared similarly using benzylamine crystallized from EtOAc; mp 160–168°, $[\alpha]^{25}D$ −56° (*c* 1, 95% EtOH). *Anal.* Calcd for $C_{48}H_{67}N_9O_6S \cdot H_2O$: C, 62.9; H, 7.5; N, 13.7; S, 3.5. Found: C, 63.0, H, 7.6; N, 13.4; S, 3.5.

Bottromycin A_1 Ethyl Ester.—A 10-mg/ml solution of bottromycin A_1 free base in anhydrous EtOH containing 15% Et_3N was heated at 50° in a sealed tube for 20 hr. The resultant mixture of bottromycin A_1 and the ethyl ester of bottromycin A_1 was separated by partition chromatography. *Anal.* Calcd for $C_{43}H_{64}N_8O_7S \cdot H_2O$; C, 60.4; H, 7.5; N, 13.1; S, 3.7. Found: C, 60.1; H, 7.4; N, 12.7; S, 3.4.

Bottromycin *t*-Butylamide.—Bottromycin carboxylate[16] (480 mg) was dissolved in 5 ml of anhydrous DMF and was treated successively at 0° with 219 mg of bis(2,4-dinitrophenyl) carbonate and 0.14 ml of Et_3N. The solution was stirred at 0° for 75 min and then at room temperature for 10 min. To this mixture was added 2 ml of *t*-$BuNH_2$. A precipitate formed in a few minutes. The mixture was heated at 50–60° for 30 min, diluted with 20 ml of $CHCl_3$, and filtered to remove the yellow by-product. The filtrate was concentrated to near dryness under reduced pressure. The residue was dissolved in $CHCl_3$ and then washed (saturated NaCl containing 5% NH_3) until the aqueous layer was colorless. The organic solution was dried (Na_2SO_4) and concentrated to dryness. A yellow glass (390 mg) was obtained. Tlc (10% MeOH in $CHCl_3$, silica gel) gave two zones when sprayed with bromophenol blue. The desired amide was located at R_f 0.70, and a slower moving substance R_f 0.15 was observed. These components were separated by preparative tlc yielding 197 mg of the *t*-butylamide as an off-white glass.

This general procedure was used to prepare the derivatives listed in Table III. In some cases the thin layer chromatograms were developed with MeOH–$CHCl_3$ of different composition to improve resolution of the components. The zones were sometimes developed by staining with iodine vapor or located by spraying the dried plate with H_2O.

(16) J. M. Waisvisz and M. G. van der Horven, *J. Am. Chem. Soc.*, **80**, 383 (1958).

5,5-Diarylpenta-2,4-dienoic Acid Amides as Potential Antimalarial Agents[1]

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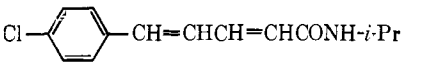
A series of 5,5-diarylpenta-2,4-dienoic acids and their amides have been synthesized and evaluated as antimalarial agents. The acids were prepared from the corresponding diaryl ketones either directly by a Reformatsky procedure or through acetylenic alcohol and acrolein intermediates. The preparation of a series of 3,3-bis(4-chlorophenyl)acrylic acid amides is also reported. One compound, N,N-diethyl-5,5-bis(4-chlorophenyl)-penta-2,4-dienoic acid amide, provided significant antiplasmodial activity.

Among the more novel compounds revealed by the World War II malaria program to have interesting antiplasmodial action was N-isopropyl-5-(*p*-chlorophenyl)penta-2,4-dienoic acid amide (**1**).[2] This compound was four times more active than quinine in the chick *Plasmodium gallinaceum* assay employed in that work and had a therapeutic index of 12.5. Two other amides, obtained by coupling the same acid with guanidine and cyanoguanidine, were inactive.[3] Because of the development of resistance to chloroquine in many parts of the world by *Plasmodium falciparum*, there is an increasing need for new antimalarial drugs of novel structural type.[4,5] This need suggested that examination of additional chemical structures related to **1** for antiplasmodial properties would be of value.



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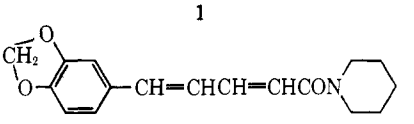

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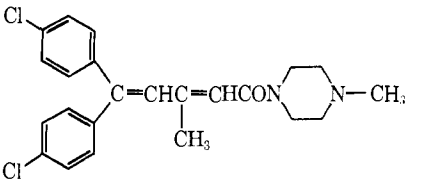

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In addition to antimalarial activity, other biological properties have been associated with pentadienoic acid derivatives. The hydrazide of 5-phenylpenta-2,4-dienoic acid has *in vitro* antituberculous activity,[6] and a series of the free acids shows inhibitory action against bacteria, yeast, and fungi.[7] Sorbic acid (2,4-hexadienoic acid) is widely used for its antifungal properties[8] and piperine (**2**, the pungent element of pepper) has insecticidal properties.[9] The use of derivatives of 5-(5-nitro-2-furyl)penta-2,4-dienoic acid as antibacterial agents has been patented.[10]

Of more pertinence to parasitic disease chemotherapy, it has been reported that a series of 5,5-diarylpenta-2,4-dienoic acid derivatives of piperazine (*e.g.*, **3**) possess marked activity against *Dicrocoelium dendriticum*, a liver fluke of considerable veterinary importance.[11,12] The closely related fluke, *Fasciola hepatica*, which infests both animals and man, is affected by these

(1) This work was supported by the U. S. Army Medical Research and Development Command under Contract No. DA-49-193-MO-2750. This contribution is no. 331 from the Army Research Program on Malaria.

(2) G. R. Coatney, W. C. Cooper, N. B. Eddy, and J. Greenberg, "Survey of Antimalarial Agents," Public Health Service Publication No. 193, Washington, D. C., 1953, p 139.

(3) Reference 2, p 137.

(4) *World Health Organ. Tech. Rept. Ser.*, **No. 296**, 1 (1965).

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