CHEMICAL STRUCTURE AND ANTIMICROBIAL ACTIVITY OF BIS-PHENOLS

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The marked antimicrobial properties of 2,2′-methylenebis [4-chlorophenol], (G-4®, dichlorophene) and of 2,2′-methylenebis-[3,4,6-trichlorophenol], (G-11®, hexachlorophene) reported from our laboratories (1–4) induced the senior author to prepare numerous bis-phenols during the past two decades. This was done with the hope of finding more potent antiseptics, especially as additives for soap, and of correlating biological activity with chemical structure. By establishing such a relationship, a pattern might evolve which would lead to the synthesis of compounds with superior qualities.

The behavior of various series of bis-phenols against fungi, such as *Chaetomium globosum* and *Aspergillus niger*, had been thoroughly investigated by Marsh and his co-workers (5, 6). We shall present here only the study of the activity of a selected group of bis-phenols against *Staphylococcus aureus* and *Escherichia coli*, and against a pathogenic fungus, *Trichophyton mentagrophytes*, and their comparison with hexachlorophene being chosen as a standard in view of its wide use as an antiseptic. It had been previously stated that bis-phenols, such as hexachlorophene or bithionol, do not kill bacteria rapidly whether they are on the skin or in any substrate, but exert a slow inhibitory action which in time will lead to the death of the organisms. For this reason, we determined the bacteriostatic levels of the compounds against the three microörganisms employing serial dilution technique. The results are shown in Tables 1 to 5. While, naturally, we have done work of this kind in previous years, the data presented here are derived from a recent study carried out under identical conditions. *S. aureus* would appear to be the most suitable organism for the evaluation of topically applied antiseptics; we felt that the tests with this organism should also be run in the presence of soap. The latter results provide a better indication of the utility of the compounds, especially as the principal use of active bis-phenols lies in the field of antiseptic and deodorant soaps and detergents. We are aware that the data obtained *in vitro*, while helpful for selecting the most promising compounds, do not constitute the final criterion of their value as skin anti-

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septics. This depends on *in vivo* studies of their ability to degerm the skin, as may be demonstrated by standard hand-washing techniques. There appears to be a fairly good relationship between *in vitro* and *in vivo* activity (7).

Experimental

Preparation of the Bis-Phenols

The compounds used in this study were prepared by standard methods. Many of the compounds listed in the tables have been described in the scientific literature and in patents;* for the substances which are new, the synthesis is largely evident from their structure and the procedure may be derived from the known preparation of analogs. This paper would not be the proper place to present experimental details; it may suffice to make a few general statements.

All compounds of Table 1 are derivatives of diphenylmethane and were obtained by condensation of the appropriate phenols with aqueous formaldehyde or paraformaldehyde in the presence of sulfuric acid of varying strength, the conditions depending on the reactivity of the phenol employed. The asymmetrical compounds Nos. 13 and 14 had to be made in two steps: alkaline condensation of 2,4-dichlorophenol with formaldehyde yielded 2,4-dichloro-6-hydroxymethylphenol which was brought to reaction with *p*-chlorophenol (No. 13) and 2,4,5-trichlorophenol (No. 14) under acid conditions. It should be mentioned that compound 9 was synthesized by debromination of compound 5 by means of zinc dust in potassium hydroxide solution.

Similarly, the compounds listed in Table 2 were prepared by condensation of the monophenols with acetaldehyde, chloral, benzaldehyde and substituted benzaldehydes. Bromination of compounds 15 and 18 resulted in compounds 16 and 19, respectively. The stilbenediols (Nos. 20 and 21) were obtained from the corresponding benzoins (8).

The reaction of phenols with sulfur dichloride led to the formation of the 2,2′-thiobis-phenols listed in Table 3. If the phenols are substituted by more than one chlorine, aluminum chloride is needed as a catalyst. Dechlorination of compound 30 led to compound 29, bromination to compound 33.

The bis-phenols listed in Tables 4 and 5 were generally prepared by the same methods as had been employed for the compounds of the preceding tables. We might add that compound 43 was obtained by first preparing octachlorodiphenylene dioxide from potassium pentachlorophenoxide at 300°C., and heating the dioxide with sodium hydroxide in aqueous methanol at 160°C. Compounds 45, 58 and 59 resulted from

* Many references are found in the review article "Ths Bis-phenols" (4).

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the oxidation of the corresponding 2,2′-thiobis-phenols with hydrogen peroxide. Bromination of compound 46 led to compound 47, chlorination of compound 48 to compound 49.

Antimicrobial In Vitro Study

Bacteriostatic and fungistatic levels were ascertained for *Staphylococcus aureus* ATCC 6358, *Escherichia coli* ATCC 11229, and *Trichophyton mentagrophytes* ATCC 9129 by serial dilution technique. Stock solutions of the compounds to be tested were made in acetone or N,N-dimethylformamide followed by twofold serial dilutions in alcohol (S.D.A. 30). Aliquots from the solvent serial dilutions were added to A.O.A.C. agar tubes for bacterial tests and Sabouraud's Dextrose Agar (Difco) tubes for fungal tests. The tube contents were mixed and poured into sterile plates which were then spot-inoculated with the test organisms. Twenty-four hour A.O.A.C. broth cultures of *S. aureus* and *E. coli* were employed, the inoculum being diluted 1–100 in A.O.A.C. broth. Spores and mycelia fragments harvested from a seven-day Sabouraud's dextrose agar slant with water served as the Trichophyton inoculum. Inhibitory levels were recorded at the end of forty-eight hours at 37° C., and seven days at 30°C., for the bacteria and mold respectively. Controls were included to demonstrate lack of inhibition by the solvents employed. Replicate tests were conducted on different days in an attempt to minimize the day-to-day variation in the test procedure.

Bacteriostatic levels of the compounds in the presence of soap were ascertained by a similar technique except that aliquots of the stock solutions were added to a Maxine (Swift & Co.) soap solution. Twofold

TABLE 1—ANTIMICROBIAL ACTIVITY OF 2,2′-METHYLENEBIS-PHENOLS

No.	Compound Substituents	—Minimum Inhibitory Level, μg./ml.— *S. aureus*	*S. aureus* (in Presence of Soap)	*E. coli*	*T. mentagrophytes*
1	3,4,6-Trichloro,hexachlorophene, G-11®	0.39	1.56	25	3.12
2	4,5,6-Trichloro	0.39	0.78	25	6.12
3	3,4,5-Trichloro	0.20	0.39	6.25	3.12
4	4-Bromo-3,6-dichloro	1.56	6.25	50	6.25
5	4-Bromo-5,6-dichloro	1.56	6.25	>50	12.5
6	4-Chloro, dichlorophene, G-4	3.12	12.5	25	6.25
7	4-Methyl	25	>12.5	>50	50
8	4,6-Dichloro	0.78	1.56	>50	1.56
9	5,6-Dichloro	3.12	6.25	>50	3.12
10	4,6-Dibromo	0.78	3.12	>50	3.12
11	6-Bromo-4-chloro	0.78	1.56	>50	6.25
12	4-Chloro-6-methyl	1.56	3.12	>50	6.25
13	2,2′-Dihydroxy-3,5,5′-trichlorodiphenylmethane	1.56	3.12	50	3.12
14	2,2′-Dihydroxy-3,5,3′,5′,6′-pentachlorodiphenylmethane	0.78	1.56	50	3.12

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serial dilutions were made in sterile tap water and aliquots of the dilution series added to A.O.A.C. agar. Plates were streaked with *S. aureus* as previously mentioned, incubated at 37°C., and results recorded at the end of forty-eight hours. Controls demonstrating lack of inhibition due to the soap were employed. All bacteriostatic tests in the presence of soap were conducted at a soap/compound ratio of 50/1.

The values listed in Tables 1 to 5 are the higher numerical figures (μg./ml.) demonstrating inhibition in the twofold serial dilution series. If, e.g., one test showed inhibition at 3.12 μg./ml. and a second one at 6.25 μg./ml., the latter figure was chosen.

TABLE 2—ANTIMICROBIAL ACTIVITY OF 2,2′-ETHYLIDENE AND 2,2′-BENZYLIDENEBIS-PHENOLS, AND OF 2,2′-STILBENEDIOLS

No.	Compound	Minimum Inhibitory Level, μg./ml. *S. aureus*	*S. aureus* (in Presence of Soap)	*E. coli*	*T. mentagrophytes*
	2,2′-Ethylidenebis (. . . phenol) Substituent				
15	4-Chloro	3.12	12.5	50	3.12
16	6-Bromo-4-chloro	3.12	6.25	50	3.12
17	4-Methyl	12.5	>12.5	>50	50
18	2,2′-(2,2,2-Trichloroethylidene)-bis(4-chlorophenol)	0.78	12.5	>50	3.12
19	2,2′-(2,2,2-Trichloroethylidene)-bis(6-bromo-4-chlorophenol)	0.78	12.5	>50	3.12
20	5,5′-Dichloro-2,2′-stilbenediol	3.12	12.5	>50	3.12
21	3,5,3′,5′-Tetrachloro-2,2′-stilbenediol	0.39	3.12	>50	6.25
22	2,2′-Benzylidenebis(4-chlorophenol)	3.12	>12.5	>50	>50
23	2,2′-Benzylidenebis(6-bromo-4-chlorophenol)	6.25	>12.5	>50	>50
24	2,2′-Benzylidenebis(4,6-dimethylphenol)	6.25	>12.5	>50	>50
25	4,4′-Benzylidenedithymol	1.56	12.5	>50	>50
26	4,4′-(2-Chlorobenzylidene)dithymol	0.78	6.25	>50	>50
27	4,4′-(4-Chlorobenzylidene)dithymol	0.78	6.25	>50	50
28	4,4′-(4-Hydroxy-3-methoxybenzylidene)-dithymol	3.12	6.25	>50	50

The relationship of the antimicrobial activity of bis-phenols to their structure had been studied previously. Bechhold and Ehrlich (9) were the first ones to demonstrate that halogenation of *o,o′*-diphenol enhanced its antibacterial potency. From the figures presented in our tables, it may be seen that halogen substitution in the rings is essential. Marsh and Butler (5) and Marsh, Butler and Clark (6), in their investigations on the fungicidal properties of bis-phenols, have discussed the dependence on certain structural features for high activity. Corey and Shirk (10) reported on the influences of chemical structure of a number of bis-phenols with an unsubstituted methylene bridge upon antifungal potency. From these and other papers, it is apparent that there is no correlation between bacteriostatic and fungistatic properties. Pfleger and his associates (11)

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TABLE 3—ANTIMICROBIAL ACTIVITY OF 2,2′-THIOBIS-PHENOLS

No.	Compound	Minimum Inhibitory Level, μg./ml. *S. aureus*	*S. aureus* (in Presence of Soap)	*E. coli*	*T. mentagrophytes*
	Substituents				
29	...	>50	...	>50	25
30	4-Chloro	3.12	12.5	25	3.12
31	4-Bromo	3.12	12.5	25	3.12
32	4,6-Dichloro,(bithionol)	1.56	3.12	>50	0.78
33	6-Bromo-4-chloro	1.56	3.12	>50	1.56
34	3,4,6-Trichloro	0.78	6.25	>50	50
35	Tetrachloro	0.78	12.5	>50	>50
36	4-Chloro-6-isopropyl-3-methyl	3.12	>12.5	>50	>50
37	4-Chloro-3,5-dimethyl	6.25	>12.5	>50	>50
38	2-Hydroxy-2-methoxy-5,5′-dichloro-diphenylsulfide	6.25	>12.5	>50	12.5

TABLE 4—ANTIMICROBIAL ACTIVITY OF RELATED BIS-PHENOLS

No.	Compound	Minimum Inhibitory Level, μg./ml. *S. aureus*	*S. aureus* (in Presence of Soap)	*E. coli*	*T. mentagrophytes*
39	1,1′-Methylenebis(2-naphthol)	6.25	12.5	>50	6.25
40	3,3′-Methylenebis(2,4,6-trichlorophenol)	6.25	12.5	>50	3.12
41	3,3′-Methylenebis(4-bromo-2,6-dichlorophenol)	12.5	>12.5	>50	12.5
42	4,4′-Methylenebis(2,3,6-trichlorophenol)	12.5	>12.5	>50	3.12
43	2,2′-Oxybis(tetrachlorophenol)	0.78	1.56	>50	50
44	4,4′-Thiodiphenol	>50	...	>50	25
45	2,2′-Sulfinylbis(3,4,6-trichlorophenol)	12.5	>12.5	>50	25
46	*o,o′*-Diphenol	>50	...	>50	50
47	4,4′,6,6′-Tetrabromo-*o,o′*-diphenol	1.56	3.12	>50	6.25
48	Salicil(2,2′-dihydroxybenzil)	>50	...	>50	50
49	5,5′-Dichlorosalicil	3.12	12.5	>50	1.56

TABLE 5—BIS-PHENOLS SHOWING INHIBITORY LEVELS OF 50 μG./ML. AGAINST THE THREE TEST ORGANISMS

No.	Compound
50	2,2′-Methylenediphenol
51	2,2′-Methylenebis(4,6-dimethylphenol)
52	2,2′-Methylenebis(6-bromo-4-methylphenol)
53	2,2′-Methylenebis(4-nitrophenol)
54	2,2′-Methylenebis(4,6-dichlororesorcinol)
55	2,2′-Methylenebis(3,5,6-trichloroanisole)
56	4,4′-Methylenebis(2,6-dibromophenol)
57	2,2′-Thiobis(4-chloroanisole)
58	2,2′-Sulfinylbis(4-chlorophenol)
59	2,2′-Sulfonylbis(4-chlorophenol)
60	2,2′-Dihydroxy-5,5′-dichlorobenzophenone
61	1,1′-Methylenebis(6-bromo-2-naphthol)
62	1,1′-Methylenebis(3,6-dibromo-2-naphthol)
63	4,4′-(3,4-Dimethoxybenzylidene)dithymol
64	Bis(3,5-dichloro-2-hydroxybenzyl)amine

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investigated the *in vitro* antimicrobial activity of a series of bis-phenols with —S—, —SO—, —SO_2—, —S—S—, —S_3— and —S_4— linkages mostly in the ortho positions to the hydroxyl groups. The organisms employed were *Staphylococcus aureus*, *Salmonella paratyphi*, *Trichophyton gypseum* and *Torulopsis minor*. The authors stated, as we and others have also observed, that the 2,2′-thiobis-phenols are more bacteriostatic and fungistatic than the 4,4′-thiobis-phenols, and that the introduction of chlorine or bromine increased the antimicrobial activity considerably. None of the compounds tested inhibited *S. paratyphi* at a concentration lower than 12 μg./ml., whereas a number of compounds were active against *T. gypseum* and *S. aureus* at a level of 1 μg./ml. and less. The most potent of these compounds against these two organisms and against *Torulopsis minor* was 2,2′-thiobis(4,6-dichlorophenol). A series of 2,2′-methylenebis-phenols, 2,2′-ethylidene and 2,2′-benzylidenebis-phenols, and a number of other compounds were examined by Florestano and Bahler (12) for antibacterial activity *in vitro* against a variety of organisms. They found that the compounds showed more or less specificity against gram-positive bacteria, being distinctly more active against them than against the gram-negative species tested. Halogen substituents were essential for potency; bromine rather than chlorine substitution resulted in greater activity against gram-positive organisms, while antibacterial action on the gram-negatives remained the same. Florestano and Bahler also stressed the significance of the position of the hydroxy groups in the rings.

Wendel (13) examined five bis-phenols, Nos. 1, 6 and 8 of Table 1, No. 40 of Table 4 and 4,4′-methylenebis (3,5-dichlorophenol), for their antibacterial potency against *S. aureus*, *S. albus* and a Streptococcus. He confirmed previous findings, namely, that the bacterial activity is connected with the number of chlorine atoms in the rings, an increase being shown from two to four to six chlorines; and that this enhancement is only evident if the hydroxyl groups are adjacent to the methylene bridge.

From the date presented in Tables 1 to 5 we are able to draw certain general conclusions (some of them in conformity with the findings of others) with regard to the relationship of the antimicrobial properties of the bis-phenols and their chemical structure. However, before doing so, we would like to call attention to the two compounds most closely related to hexachlorophene, namely Nos. 2 and 3. No. 2, 2,2′-methylenebis-(4,5,6-trichlorophenol), demonstrated approximately the same activity as hexachlorophene being perhaps slightly more inhibitory against *S. aureus* in the presence of soap. No. 3, 2,2′-methylenebis (3,4,5-trichlorophenol), was outstanding, not only on account of its activity against *S. aureus*, but also because it was the only bis-phenol which showed a fair degree of bacteriostatis against gram-negative organisms. We have given its activity against *E. coli* as 6.25 μg./ml. This value resulted from two

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tests; in two other ones, the inhibitory level was found to be as low as 3.12 μg./ml. *Proteus vulgaris* and *S. typhosa* were inhibited at 6.25 μg./ml., *Pseudomonas aeruginosa* at 12.5 μg./ml. The question might be raised as to why compound 3, being definitely more active than hexachlorophene, at least *in vitro*, is not being studied *in vivo* and perhaps used commercially? The reason lies in the difficulty of economically making 3,4,5-trichlorophenol which is obtained in a multistep operation from *p*-nitroaniline (14, 15) and which is needed for the preparation of compound 3.

The following general statements may be made:

1. In general the antibacterial activity of the bis-phenols is rather specific, being stronger against gram-positive organisms than against gram-negative ones. However, compounds 1, 2, 3, 6, 30 and 31 are exceptions and do show good activity against *E. coli*. The presence of soap always lowers the inhibitory level against *S. aureus*. The potency against *Trichophyton mentagrophytes* is not striking, only three compounds (Nos. 8, 32 and 33) which have a similar chemical structure are fungistatic at a level lower than 2 μg./ml.

2. Linkage in the 2,2′-positions is essential for maximum activity. Previous observations (4) that bis-phenols linked in the 4,4′-positions are weak are generally correct; however, we observed that compounds obtained by the condensation of thymol with benzaldehyde and particularly with *o*- and *p*-chlorobenzaldehyde are quite active against *S. aureus*. However, in the presence of soap, the decrease in potency is more pronounced than with the 2,2′-methylenebis-phenols.

3. The methylene-bridged phenols are superior to the ones with ethylidene, benzylidene and thio-linkages in regard to activity against *S. aureus* in the presence of soap.

4. No compound without halogen in the phenol rings is active, with the exception of bis-phenols derived from thymol (Nos. 25, 26, 27), and one halogen must be in the para position. Chlorine substitution is usually preferable to bromine which is contrary to Florestano's findings (12).

5. Oxygen-containing linkages, such as —CO—, —SO—, —SO_2—, are detrimental.

6. Activity is lost if the hydroxyl groups are etherified (Nos. 55, 57). Esters, such as the diacetates, have been reported by Pfleger *et al.* (11) to be active. We found this to be true, and believe, in agreement with Pfleger, that the esters are hydrolyzed to a large degree to the free phenols during the tests.

7. Replacement of the phenols by naphthols (Nos. 39, 61, 62) leads to inactive compounds. The bis-compound from dichloresorcinol (No. 54) was also inactive at a level of 50 μg./ml.

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Summary

Bacteriostatic and fungistatic data on a number of bis-phenols have been presented and the relationship between chemical structure and antimicrobial activity has been discussed. The standard of reference was hexachlorophene which was not surpassed in bacteriostatic activity against *S. aureus* and *E. coli*, except by one of its isomers, 2,2′-methylenebis (3,4,5-trichlorophenol). The latter compound is distinctly more potent, but its manufacture would be difficult and costly.

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CHICAGO CHAPTER NEWS

Dr. Morris V. Shelanski spoke on April 12. The subject was a very timely one in the general category of toxicological testing of cosmetic products.

Dr. Shelanski attended Harvard University, 1939–1943, and continued his education in the Faculty of Medicine, McGill University and received degree M.D., C.M., in 1947. His present position is director, Industrial Biology Research and Testing Laboratories, Inc., Philadelphia, Pa.

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