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Article *in* Antimicrobial Agents and Chemotherapy · April 1972

DOI: 10.1128/AAC.1.3.263 · Source: PubMed

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Vol. 1, No. 3
Printed in U.S.A.

Antimicrobial Properties of Natural Phenols and Related Compounds: Obtusastyrene and Dihydro-Obtusastyrene

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Received for publication 26 November 1971

Factors influencing the antimicrobial properties of obtusastyrene and dihydro-obtusastyrene were studied. Both of these compounds were soluble in acetone, alcohol, and olive oil. In water, they were soluble at concentrations of 34 and 53 μg/ml, respectively. The minimal inhibitory concentrations against gram-positive bacteria and yeast were below 100 μg/ml. The compounds were not effective against gram-negative bacteria at 200 μg/ml or lower concentrations. With initial populations of cells greater than 10^6/ml, the concentrations of these compounds required to prevent growth were greater than with lower cell populations. Changing the *p*H of the growth medium did not decrease the effectiveness of these two compounds in the *p*H range of 3 through 8. Both obtusastyrene and dihydro-obtusastyrene were rapidly bactericidal to *Staphylococcus aureus* and *Bacillus cereus* at 25 μg/ml.

Obtusastyrene (4-cinnamylphenol) was originally extracted and identified from the heartwood of *Dalbergia obtusa* as a result of a phytochemical survey (1, 4). The co-occurrence in other *Dalbergia* species of obtusastyrene with other neoflavanoid compounds has been reported (4). In a search for the compounds responsible for the antimicrobial activity of *Dalbergia nigra*, two related neoflavanoid compounds, Dalbergione I and II, were found to be effective antimicrobials against gram-positive and -negative bacteria and acid-fast bacteria, plus some fungi (3).

These results prompted us to survey the antimicrobial activity of obtusastyrene and related compounds. A report of this survey, performed by use of a replica-plating technique, has appeared (8). Obtusastyrene was found to be an effective antimicrobial agent against gram-positive bacteria, yeasts, and molds in the range of 6 to 100 μg/ml. It was less effective against gram-negative bacteria. The minimal inhibitory concentration was not greatly influenced by the *p*H of the medium in the range from 3 to 7. This report describes further studies of obtusastyrene and its saturated side chain analogue dihydro-obtusastyrene (DH-obtusastyrene).

MATERIALS AND METHODS

The synthesis of obtusastyrene from phenol and cinnamyl alcohol has been reported (7, 8). DH-obtusastyrene is prepared from obtusastyrene by hydrogenation of the side chain in the presence of palladium (Fig. 1).

For culturing microorganisms, Trypticase Soy Broth or Agar with 2% added yeast extract (*p*H 7) was used except as noted below. This same broth was also used for serial dilutions. Routine incubation temperature was 28 C for 24 or 48 hr. Both antimicrobial agents were dissolved in acetone, and samples were added to the sterilized medium. Appropriate acetone controls were run in each experiment.

Solubility values for the two compounds were calculated from spectrophotometric absorption curves at 254λ for obtusastyrene and 274λ for DH-obtusastyrene. Solubility standards were prepared by dissolving known quantities in 50% alcohol. All samples were heated to 50 C and cooled to insure saturation before dilution and determination of the absorption.

Minimal inhibitory concentrations (MIC) were determined by plate counts. A 24-hr culture was inoculated into broth containing the appropriate concentration of one of the drugs. After 24 hr, samples were withdrawn, and the number of viable cells was determined by means of pour plates with dilutions as necessary.

To determine the bactericidal rate of 25 μg of obtusastyrene or DH-obtusastyrene per ml, the number of viable cells remaining in the broth culture containing *Bacillus cereus* or *Staphylococcus aureus* was estimated by use of the pour-plate technique and sampling at time intervals. The culture was maintained on a shaker between samplings. Diluting the broth inoculum with agar during pour-plating

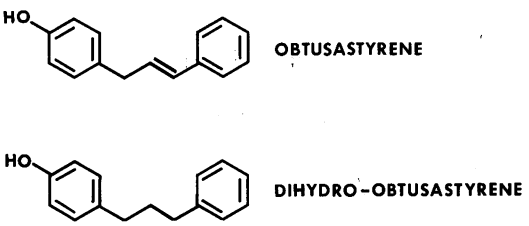

FIG. 1. *Structure of obtusastyrene and dihydro-obtusastyrene.*

reduced the total antimicrobial concentration to 1 μg/ml, which is well below the MIC of the two compounds.

The influence of the size of the initial population on the effectiveness of the antimicrobial compounds was determined by diluting a 48-hr culture to approximate levels of the organism into broth containing different concentrations of the drugs. After 24 hr of incubation on a shaker, the cultures were plated to determine surviving numbers of organisms.

Sterilized melted agar was adjusted to *p*H 3 through 7 for yeasts or 5 through 8 for bacteria to determine the influence of *p*H on the activity of the two compounds. Either 1.0 M HCl or NaOH was used to adjust the *p*H of the medium. After adjusting the *p*H, 100, 50, 25, or 12.5 μg of obtusastyrene or DH-obtusastyrene per ml was added; the medium was inoculated with between 30 and 300 organisms and poured into plates. After incubation, the organisms on the plates were counted. Yeasts were cultured on potato-dextrose-agar.

RESULTS

To help predict the parameters of antimicrobial activity, the solubilities of obtusastyrene and DH-obtusastyrene were determined. Both compounds were very soluble in olive oil, acetone, and alcohol. A 4% solution of either compound could easily be made in 50% alcohol. The aqueous solubility of obtusastyrene was 34 μg/ml and that of DH-obtusastyrene was 53 μg/ml. Increasing the *p*H from 6.4 to 9.2 and 11.5 increased the solubility of obtusastyrene from 38 to 40 and 63 μg/ml and that of DH-obtusastyrene from 45 to 42 and 1,380 μg/ml, respectively. Increasing the alcohol content of aqueous solutions increased the solubility of these compounds.

The minimal concentrations required to prevent multiplication of the cells are shown in Table 1. The initial population of cells in these studies was 10^7 to 10^8/ml. Below the MIC, the cells multiplied, whereas at higher concentrations they were not able to reproduce and no viable count was observed. Obtusastyrene was effective at somewhat lower concentrations than DH-obtusastyrene. The gram-negative organisms *Alcaligenes faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Serratia marcescens* were most resistant to both compounds; more than 100 μg/ml was required to prevent growth. Gram-positive organisms were more susceptible than gram-negative organisms to these compounds, so that 50 to 100 μg/ml eliminated viable counts. Both compounds were effective against yeasts at concentrations lower than 100 μg/ml.

The influence of the size of the initial microbial population on the minimal amount of either compound required to eliminate viable organisms is shown in Table 2. Increasing the concentration of organisms from small populations (10^2/ml) to very large microbial populations (10^8/ml) generally increased the amount of compound required to sterilize the population after 18 to 24 hr. The susceptibility of *B. cereus* to obtusastyrene or DH-obtusastyrene was so great that 20 μg/ml was effective over the entire population range.

Both compounds at 25 μg/ml had a marked bactericidal influence on *B. cereus* and *S. aureus* when the compound was added to a broth culture (Fig. 2 and 3). Figure 2 shows the rapid loss of viability for *B. cereus* subcultured after both 5 and 24 hr. In the cultures that were

TABLE 1. *Minimal inhibitory concentration of obtusastyrene (OB) and dihydro-obtusastyrene (DHOB) against selected microorganisms*[a]

Microorganism	Minimal inhibitory concn (μg/ml)	
	OB	DHOB
Bacteria		
Alcaligenes faecalis B170	100	100
Bacillus cereus 2006	20	50
Escherichia coli ML30	>200	>200
Pseudomonas aeruginosa AR-45	>200	>200
Salmonella typhimurium Tm-1	>200	>200
Sarcina lutea	>50	50
Serratia marcescens	>200	>200
Staphylococcus aureus SG8A	50	50
Streptococcus lactis	50	50
Yeasts		
Candida tropicalis C147	20	20
Hansenula anomala	50	50
Geotrichum candidum	20	20
Pichia chodati fermentans C238	100	100
Saccharomyces cerevisiae 522	20	20
Torula utilis NRRL Y660	50	50
Zygosaccaromyces japonticus C124	10	10

[a] Initial concentration of microorganisms: bacteria, 10^7 to 10^8/ml; yeasts, 10^4 to 10^7 ml.

TABLE 2. *Minimal concentrations (μg,ml) of obtusastyrene and dihydro-obtusastyrene required to kill cells after 18 to 24 hr as influenced by the initial population*

Drug and organism	Initial microbe population/ml						
	10^2	10^3	10^4	10^5	10^6	10^7	10^8
Obtusastyrene							
B. cereus	20	—	20	—	20	—	—
S. aureus	—	10	20	—	20	—	—
E. coli	—	200	—	>400		>400	—
S. typhimurium	>400	—	>400	—	>400	—	>400
S. cerevisiae	10	20	20		>50	—	—
Dihydro-obtusastyrene							
B. cereus	20	—	20	—	20	20	—
S. aureus	20	20	20	20	20	100	—
E. coli	—	200	—	400	—	>400	—
S. typhimurium	>400	—	>400	—	>400	—	>400
S. cerevisiae	10	20	20	—	>50	—	—

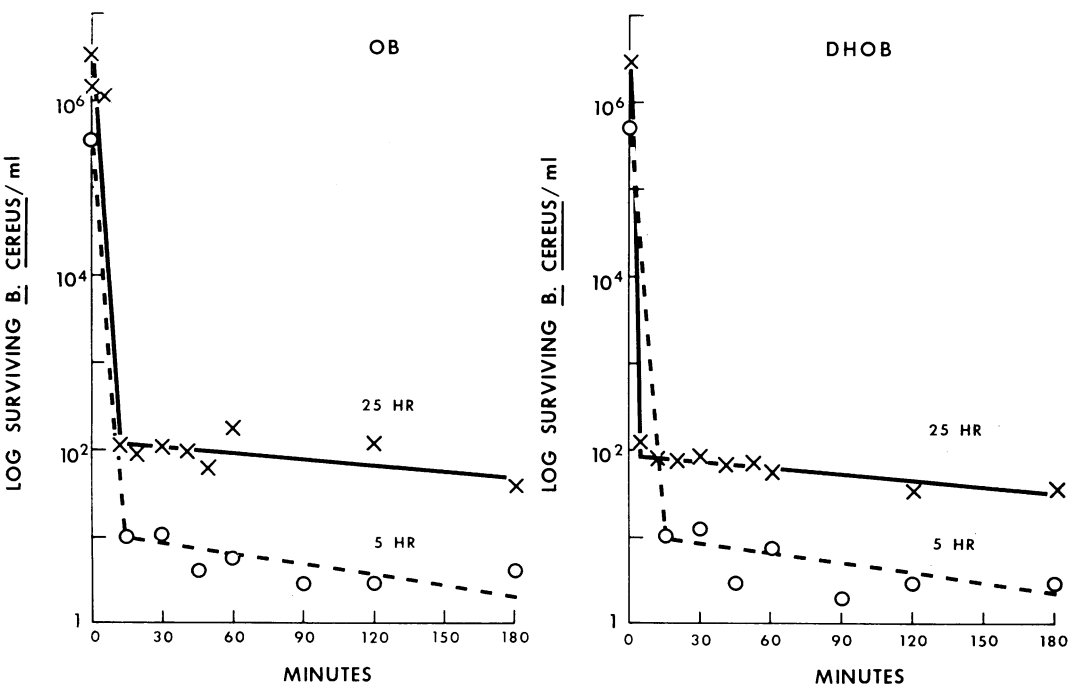

FIG. 2. *Death rate of B. cereus in Trypticase Soy Broth plus 2% yeast extract in shaker culture at 28 C with 25 μg of obtusastyrene (OB) or dihydro-obtusastyrene (DHOB) per ml. Results are shown for young (5-hr) and old (24-hr) cultures.*

treated 5 hr after subculturing, a few intracellular spores were seen microscopically but no external spores were observed. The 24-hr culture contained spores in virtually every cell, and some extracellular spores were evident. The lack of complete destruction was probably due either to resistant spores within the subcultured cells or to free spores. In several tests, no viable *B. cereus* cells were recovered in 24 hr. Similar results are shown in Fig. 3 for *S. aureus*, which rapidly lost viability down to a level of presumably more resistant organisms. The initial death rate was slower for *S. aureus* than for *B. cereus*. Results of similar experiments showed that obtusastyrene was rapidly destructive to *Streptococcus lactis* and *Saccharomyces cerevisiae*

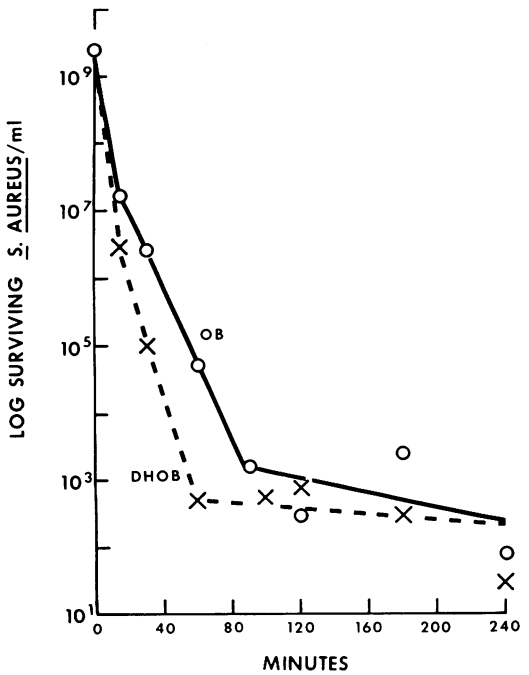

FIG. 3. *Death rate of a 24-hr culture of S. aureus in Trypticase Soy Broth plus 2% yeast extract in shaker culture at 28 C with 25 μg of obtusastyrene (OB) or dihydro-obtusastyrene (DHOB) per ml.*

at 25 μg/ml, to *Pichia chodati* at 12.5 μg/ml, and to *Candida tropicalis* at 50 μg/ml. It was not destructive to *E. coli* at 50 μg/ml or lower at 48 hr.

Neither compound would be expected to be ionized in the *p*H range where microbial growth commonly occurs, so one would not expect *p*H changes to alter the effectiveness of those antimicrobial compounds. Table 3 shows the results obtained with several microorganisms inoculated into plates at different *p*H values containing obtusastyrene or DH-obtusastyrene compared with controls. Changing the *p*H of the medium 4 units did not decrease the effectiveness of 12.5 μg of obtusastyrene per ml enough to permit growth of *B. cereus*, *S. aureus*, *C. tropicalis*, or *S. cerevisiae*. At *p*H 7 and 8, obtusastyrene seemed less effective in controlling *E. coli* and *S. typhimurium*, indicating a possible *p*H effect on the antimicrobial properties. Similarly, DH-obtusastyrene effectiveness was not influenced by changes in *p*H at the lowest concentration tested. With *S. typhimurium*, 100 μg of DH-obtusastyrene per ml did not prevent growth, and change in *p*H had virtually no effect on the number of colonies. We conclude that the antimicrobial properties of these compounds were not influenced by changes in *p*H in the range tested.

DISCUSSION

The MIC values of obtusastyrene were slightly higher than those of a number of chlorinated phenol compounds (5), and the comparable MIC values of DH-obtusastyrene were somewhat higher than those of obtusastyrene. However, these two compounds do not contain any halogen atoms, which usually increase the antimicrobial activity and oral toxicity (5). Higher concentrations of these two compounds were required to inhibit gram-negative than gram-positive bacteria. Although these compounds vary in effectiveness against the genera tested, they had a spectrum of inhibitory activity that included several yeasts as well as the bacterial species.

Previously reported MIC values for obtusastyrene were higher than those reported here because of the difference in technique used and different growth medium (8). In the earlier report, a replica-plating technique on plate count agar was used. In this study, a rich medium was used to provide the maximal challenge for observing the antimicrobial properties of

TABLE 3. *Influence of pH on plate counts with medium containing obtusastyrene or dihydro-obtusastyrene*

Drug and organism	Drug concn (μg/ml)	No. of organisms					
		*p*H 3	*p*H 4	*p*H 5	*p*H 6	*p*H 7	*p*H 8
Obtusastyrene							
B. cereus	0	—	—	0	38	48	100
	12.5	—	—	0	0	0	0
S. aureus	0	—	—	0	220	59	51
	12.5	—	—	0	0	0	0
E. coli	0	—	—	0	680	680	760
	25	—	—	0	0	10	53
S. typhimurium	0	—	—	0	340	314	357
	25	—	—	0	0	29	26
C. tropicalis	0	0	300	140	140	—	—
	12.5	0	0	0	0	—	—
S. cerevisiae	0	0	210	190	230	—	—
	12.5	0	0	0	0	—	—
Dihydro-obtusastyrene							
B. cereus	0	—	—	20	24	25	24
	25	—	—	0	0	0	0
S. aureus	0	—	—	0	159	165	168
	25	—	—	0	0	0	0
E. coli	0	—	—	17	18	45	33
	100	—	—	0	0	0	0
S. typhimurium	0	—	—	0	54	52	160
	100	—	—	0	47	37	35
C. tropicalis	0	0	65	45	43	—	—
	25	0	0	0	2	—	—
S. cerevisiae	0	0	7	9	9	—	—
	25	0	0	0	9	—	—

these compounds. It is known that organic matter and microbial by-products decrease the antimicrobial effectiveness of phenol and halogen-substituted phenols (2).

Bennett (2) in a review article summarized the work of several workers in stating that the concentration of microorganisms influences the amount of phenol required to inhibit the inoculum. The size of the microbial population from 100 to 100 million per ml influenced the amount of obtusastyrene or DH-obtusastyrene required to inhibit the cells.

The very rapid loss of viability when cells were exposed to these compounds resembles that induced by the rapid leakage of cell constituents from cells exposed to *p*-chloro-*m*-xylenol (6). Thus, the action of these compounds is thought to be a physical damage to the cell permeability barriers, thus altering permeability and causing loss of viability as postulated by Judis (6). The difference in MIC observed with gram-negative and gram-positive cells tends to support this idea because of the different wall constituents of these two types of bacteria.

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