Substituted Salicylanilides II

Further Antimicrobial and Other Studies with Halonitrosalicylanilides

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A detailed examination of the general antimicrobial spectra of halonitrosalicylanilides previously reported upon is described together with some general observations. Some other biological data are also presented.

THE OBSERVATION of antimicrobial activity among certain halonitrosalicylanilides has been previously reported (1). These compounds can be represented by the general formula shown in Fig. 1.

Since that report, a more detailed examination of the general spectra of antimicrobial action of the series has been made. In addition, data have also been obtained upon some areas of the general biological activity of these compounds together with further toxicity data. These results are reported below, together with some general observations.

It was of interest to determine the potencies of one of the more active members of each of the 3-nitro and the 5-nitro series of compounds against a variety of fungi and bacteria. 4′-Chloro-3-nitrosalicylanilide (I) from the former series and 4′-bromo-5-nitrosalicylanilide (II) from the latter series were chosen (see Fig. 2).

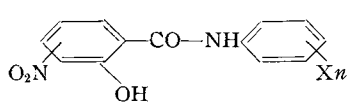
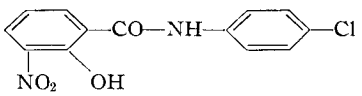
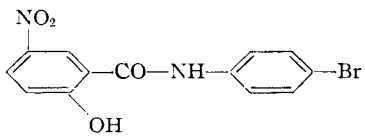


Fig. 1.—General formula for halonitrosalicylanides where X is Br, Cl, F or I and n = 1, 2, or 3.



(I)



(II)

Fig. 2.—Structural formulas for the two compounds studied.

These two compounds were evaluated against a number of interesting microorganisms by determining the minimum concentrations required to cause inhibition of their growth. The data are presented in Table I.

On the basis of these data, it can be seen as had already been observed in the preliminary study that with only a few exceptions, the 5-nitro compound is a more potent antimicrobial than the 3-nitro compound.

In vivo, activity of 4′-chloro-5-nitrosalicylanilide against *Mycobacterium leprae* was determined in mice. The results of the study with a streptomycin control are presented in Table II.

A degree of activity was clearly evident. However, the activity was of lesser magnitude than the control antimicrobial.

In a further study with two related organisms, *Mycobacterium tuberculosis* and *Mycobacterium avium*, and comparison with *Salcide*, employing the serial broth dilution method, threshold values of 5 mcg./ml. were obtained. *Salcide* is a commercial preparation containing approximately equal amounts of 3′-chloro-3-nitrosalicylanilide, 3′-chloro-5-nitrosalicylanilide, 4′-chloro-3-nitrosalicylanilide, and 4′-chloro-5-nitrosalicylanilide.

Thirteen of the halonitrosalicylanilides were then compared in activity against two Gram-positive and one Gram-negative bacteria and a fungus. A mixture of two of the anilides was also examined. These results are shown in Table III. Several interesting facts become apparent upon studying the data. In the first place, as had been initially observed to a limited degree, the compounds were more potent against the Gram-positive organisms than the particular strain of *E. coli* used.

Manifestation of synergism is seen upon comparison of the value against *Aspergillus niger* for the mixture with the values for the unmixed materials. Similar synergisms were encountered with other mixtures and are referred to below. Comparison of the values for the 3-nitro and 5-nitro compounds unequivocally demonstrates the consistently higher potencies of the latter. Finally, it was possible to select on the basis of these results, the most active and interesting members of the series for further study. These were 3′-chloro-5-nitrosalicylanilide, 4′-chloro-5-nitrosalicylanilide, and 4′-iodo-5-nitrosalicylanilide.

To further our knowledge about the halonitrosalicylanilides and to learn more about the synergism between some of the members, several experiments were designed involving mixtures of the compounds. Because of the many combinations possible with all 13 of the active halonitrosalicylanilides, only those exhibiting the highest degree of activity were selected

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TABLE I.—QUANTITATIVE ANTIMICROBIAL DETERMINATIONS FOR TWO HALONITROSALICYLANILIDES BY SERIAL BROTH DILUTION

	Antifungal *in Vitro* Test Results[a] (Broth Dilution)					
	4′-Chloro-3-Nitrosalicylanilide			4′-Bromo-5-Nitrosalicylanilide		
	Inhibition			Inhibition		
	Complete	Partial	None	Complete	Partial	None
B. dermatitidis	>200	200	100	6.25	3.13	>1.56
C. albicans	100	...	50	200	...	100
C. neoformans	25	12.5	6.25	3.13	...	>1.56
H. capsulatum (yeast phase)	100	50	25	6.25	...	3.13
N. asteroides	3.13	...	1.56	1.56	...	>1.56
T. interdigitale	6.25	3.13	1.56	3.13	1.56	>1.56

	Antibacterial *in Vitro* Test Results[a] (Broth Dilution)	
	Turbidimetric, 50% Inhibition	Turbidimetric, 50% Inhibition
A. aerogenes	>20	15
A. tumefaciens	0.83	1.1
B. firmus	0.71	1.1
E. coli (2 strains)	12. ->20	3.8
K. pneumoniae (3 strains)	8. ->20	3.3
P. vulgaris (2 strains)	14. ->20	7.3
S. schottmuelleri	16	14
S. typhimurium	19	10
S. typhosa	>20	13
S. paradysenteriae	10	5
S. sonnei	>20	16
S. aureus (4 strains)	0.35–3.7	0.68–1.1
S. infrequens	9.7	2.1
V. comma	1.8	1.5

[a] All of the results are expressed as the concentration of antimicrobial in the microbial growth broth in mcg./ml. which produces inhibition.

TABLE II.

Treatment of the Animals	Dose	Leprosy Score in Mesentery[a]
Leprosy control untreated	...	4.7
Streptomycin	2 mg. subcutaneously	2.7
4′-Chloro-5-nitrosalicylanilide	0.1% in the diet	3.5

[a] The score is based upon a count of the number of infected cells in the mesentery (2).

for the synergistic studies. Quite by accident, a mixture exhibiting a very high degree of synergistic action was initially prepared consisting of 3 parts of 3′-chloro-5-nitrosalicylanilide and 1 part of 4′-iodo-5-nitrosalicylanilide. In an initial experiment, the threshold inhibition values were determined for each of the two unmixed materials and for the three-to-one mixture against six different microorganisms. The results of this study are summarized in Table IV. It can be seen that the synergism appears to be exhibited against fungi, but not against bacteria. A particularly resistant strain of *E. coli* had been used in these studies for the sake of detecting any synergistic action.

Other mixtures were examined in order to determine whether any generalizations about the types of structural combinations exhibiting synergism might be possible. The three-to-one mixture was adhered to rather arbitrarily since initially a high degree of synergism had been observed with that ratio. *Aspergillus niger* was used throughout as the test organism since it had exhibited the greatest response to the initial synergistic ratio tried in preliminary studies. The results are presented in Table V.

TABLE III.—*In Vitro* ANTIMICROBIAL ACTIVITY OF A GROUP OF SUBSTITUTED HALONITROSALICYLANILIDES[a]

Compound	*Staphylococcus aureus* (MGH #1)[b]	*Escherichia coli* (ATCC10740)	*Bacillus subtilis* var. *globigii*	*Aspergillus niger*[c]
3′-Chloro-5-nitrosalicylanilide	5.2–10	>300	30–40	>100
2′-Chloro-5-nitrosalicylanilide	20–25	>300	10–15	>100
4′-Iodo-5-nitrosalicylanilide	3.4–5.2	>300	>10	...
4′-Bromo-5-nitrosalicylanilide	5–10	...	10–15	...
4′-Fluoro-5-nitrosalicylanilide	>10	200–300	5.2–10	>100
2′,4′-Dichloro-3-nitrosalicylanilide	40–50	>300	>70	>100
4′-Fluoro-3-nitrosalicylanilide	>60	...	...	...
2′-Chloro-3-nitrosalicylanilide	5.2–10	...	...	>100
3′-Chloro-3-nitrosalicylanilide	>60	>300	>70	>100
4′-Chloro-3-nitrosalicylanilide	20–25	...	>70	...
4′-Bromo-3-nitrosalicylanilide	20–25	>300	15–20	>100
4′-Chloro-5-nitrosalicylanilide	5.2–10	...	30–40	>100
4′-Chloro-5-nitrosalicylanilide and 4′-Chloro-3-nitrosalicylanilide (3:1)	>10	>300	50–60	50–60
2′,4′-Dichloro-5-nitrosalicylanilide	>10	>300	10–15	>100

[a] Tube dilutions are plated on nutrient agar following 72 hours' incubation at 37° and antimicrobial activity is expressed as the lowest concentration (mcg./ml.) which completely inhibits the growth of a specific test organism. [b] Penicillin resistant strain of *Staphylococcus aureus* obtained from Metropolitan General Hospital, Cleveland, Ohio. [c] Plate-agar dilution assay.

TABLE IV.—THE ANTIMICROBIAL ACTIVITY OF UNMIXED AND MIXED 3′-CHLORO-5-NITROSALICYLANILIDE AND 4′-IODO-5-NITROSALICYLANILIDE[a]

Organism	Activity, mcg./ml. Unmixed 3′-Chloro-5-nitrosalicylanilide	Unmixed 4′-Iodo-5-nitrosalicylanilide	3:1 Mixture of the Chloro and Iodo Compounds
Streptococcus pyogenes (C203)	>2-<10	>10-<20	>10-<20
Streptococcus pyogenes	10–15	5–10	10–15
Escherichia coli	>600	>700	>600-<700
Aspergillus niger	>600	400-500	15–20
Memnoniella echinata	>600	>600	10–20
Aspergillus terreus	>600	>600	600

[a] Minimum-plate dilution for fungi and minimum-tube dilution for bacteria.

TABLE V.—ACTIVITIES OF VARIOUS 3:1 MIXTURES OF HALONITROSALICYLANILIDES *vs. Aspergillus niger*[a]

Mixtures	Parts	Minimum Threshold Concentration for Inhibition mcg./ml.
2′-Chloro-5-nitrosalicylanilide	3	>600
4′-Iodo-5-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	>600
4′-Iodo-3-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	400
4′-Fluoro-5-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	20–30
4′-Chloro-5-nitrosalicylanilide	1	
3′-Chloro-5-nitrosalicylanilide	3	15–20
4′-Iodo-5-nitrosalicylanilide	1	
4′-Chloro-5-nitrosalicylanilide	3	>600
4′-Iodo-5-nitrosalicylanilide	1	

[a] In all cases, the unmixed components had values of over 600 mcg./ml.

The most interesting conclusion obtained from Table V is the great specificity of the various components of the mixtures for synergism. In this limited current study of synergism, it can be generalized that only mixtures of halo-5-nitrosalicylanilides where one component has a halogen in the 3′-position and the other in the 4′-position exhibit any appreciable degree of synergistic activity.

Our investigations with the halonitrosalicylanilides then took another direction in an attempt to discover some more general biological characteristics of the materials. It was of interest to determine the fate of the material when introduced into an animal by the oral route. The rabbit was selected as a suitable animal and suspensions of 3′-chloro-5-nitrosalicylanilide in gum acacia were administered orally. In these studies, gross symptoms of toxicity such as emesis, diarrhea, or weight loss were not detected at daily dosages of 1 Gm. per Kg. for a 1-week period.

Pooled feces of the rabbits were extracted with dimethylformamide, a solvent in which 3′-chloro-5-nitrosalicylanilide is extremely soluble. The extractions were filtered and evaporated to dryness and tested for antimicrobial activity *vs. Staphylococcus aureus*, one of the organisms very sensitive to this series. No trace of antimicrobial activity was detected for the residue. Thus the conclusion is reached that either the substituted salicylanilide is chemically modified in the gastrointestinal tract or else is very slowly absorbed into the system at sublethal levels.

In our original publication and in subsequent studies, we had found that generally the LD_{50} in the rat, for the halo-3-nitrosalicylanilides by the intraperitoneal route was in the order of 35 mg. per Kg. and that this value was 125 mg. per Kg. for the halo-5-nitrosalicylanilides. The oral feeding study mentioned above was carried out upon only one animal. Therefore, it cannot by any means, be considered a toxicity study. However, it does serve as an indication that the oral doses which are tolerated are many times greater than the intraperitoneal dose.

Because antimicrobial materials, particularly antibiotic broths, have often exhibited antitumor activity, all of the halonitrosalicylanilides prepared in our studies were submitted to the National Cancer Screening Service for a primary antitumor screening against four tumor systems in mice.

Some of the members exhibited varying degrees of antitumor activity in these tests. 4′-Iodo-5-nitrosalicylanilide qualified for further examinations but its activity was not considered consistent enough for more extensive studies. The results of that study are shown in Table VI.

TABLE VI.—REDUCTION OF TUMOR WEIGHT BY 4′-IODO-5-NITROSALICYLANILIDE[a]

Experiment	Daily Dose, mg./Kg.	Survivors	Tumor Weight—Medicated Animals	Tumor Weight—Non-medicated Animals	Reduction, %
I	113	6/6	5.5	8.2	67
II	113	6/6	1.2	7.7	15
III	113	4/6	8.2	7.8	105
IV	113	5/6	1.5	6.4	23
V	113	5/6	4.3	8.6	50
VI	113	3/6	4.5	8.8	51
VII	113	8/10	7.7	7.6	101

[a] The Erlich Ascites Tumor was used throughout.

DISCUSSION AND SUMMARY

The spectra of certain halonitrosalicylanilides have been investigated to show that they possess bactericidal and fungicidal activity against a large variety of microorganisms.

Upon comparing the members among themselves against five organisms, 3′-chloro-5-nitrosalicylanilide (I), 4′-chloro-5-nitrosalicylanilide (II), and 4′-iodo-5-nitrosalicylanilide (III) emerged as the three most active compounds. It was further of interest, to discover that mixtures of I and III and of II and III had activities as great as 30 times that of the unmixed materials against *Aspergillus niger* and *Memnoniella echinata*. The synergism was observed against fungi in general, but not bacteria. It was,

however, exhibited by several other mixtures to some degree, but only the above-mentioned combinations had a very high degree of activity in this respect.

In further studies upon general biological activity, it was found that the oral toxicity of 3′-chloro-5-nitrosalicylanilide may be many times less than by intraperitoneal administration.

An attempt to correlate pKa and antimicrobial activity for several different salicylanilides has been made (3). The hypothesis put forth was that pKa would be a measure of chelating ability and that, in effect, a correlation of pKa and activity was a correlation of chelating power and activity. In our own studies it was demonstrated that indeed the halonitrosalicylanilides were powerful chelating agents. Upon taking a solution of 5-nitrosalicylic acid and adding ferric chloride solution, a brilliant blood-red chelate complex is formed. However, when EDTA is added to the solution, the color is immediately discharged indicating the transferral of Fe^{+++} to form an EDTA-Fe^{+++} relatively colorless complex. However, when this was repeated with 4′-chloro-5-nitrosalicylanilide, the red color that formed could not be discharged by means of EDTA indicating the former to be a better chelater of iron than EDTA. It must be remembered, on the other hand, that excellent chelating agents such as EDTA do not exhibit general antimicrobial activity and that chelation alone does not give rise to antimicrobial activity. Furthermore, among some of the original halonitrosalicylanilides prepared by ourselves were a series of halo-3,5-dinitrosalicylanilides whose phenolic hydrogens would have a higher pKa than those of the mono-nitrated products. However, these compounds were completely devoid of activity against bacteria or fungi. Thus it is suggested that the requirements for a substituted salicylanilide to possess activity could logically involve its chemical functionality, general solubility, and size and shape of the molecule. These factors could affect its migration across physiological barriers and its ability to seat in enzyme sites which in addition to chelating ability, could determine or influence antimicrobial activity.

Currently, new substituted salicylanilides are being prepared with the idea of exploring some of the molecular requirements for activity. In these studies, particular emphasis is being placed upon investigating the relationship between chelating ability and activity together with some of the other considerations mentioned above. Also, because of the very specific type of synergism observed, a further investigation is underway in attempting to further determine its limitations and to theorize upon its origin. It is hoped that from these investigations more information may be obtained regarding the mechanistic and theoretical aspects of the antimicrobial action of variously substituted salicylanilides.

EXPERIMENTAL

Quantitative *in Vitro* Antibacterial Assays.—These were conducted turbidimetrically by the serial broth dilution method. Inhibition of growth was reported as complete, 50%, and incomplete and measured as the mcg./ml. concentration to cause the inhibition. In some instances absolute bactericidal end points were determined visually but confirmed by streaking replicate sections of nutrient agar plates with several dilutions from the test series.

Stock solutions of halonitrosalicylanilides were prepared by dissolving 100 mg. in 100 ml. of distilled water and adjusted to pH 7.3 with 0.1 *N* sodium hydroxide with some compounds requiring heating to 50° to obtain complete solubilization. The solutions were then sterilized in serum vials at 15 pounds pressure for 15 minutes.

One milliliter aliquots were removed with tuberculin syringes equipped with #22 gauge needles and aseptically transferred to vials containing 9.0 ml. of fluid thioglycollate (BBL). Subsequent dilutions were made in a similar manner. Each test series included viability and media controls.

The inoculum was prepared by making a minimum of three transfers of the test organism in fluid thioglycollate and inoculating 0.05 ml. of the test organism into each vial following 18 hours incubation at 37°. Readings were made at the conclusion of 24 and 72 hours of incubation.

Quantitative *in Vitro* Antifungal Assays.—These were conducted by the broth dilution turbidimetric and agar-dilution linear-growth methods. The former assay was performed in a manner similar to that described for determining antibacterial activity, but with the exception of using fluid dextrose Sabouraud throughout.

In the linear-growth technique, dilutions of the appropriate stock solution (0.001 Gm./ml.) were added to sterile plastic Petri dishes (100 x 15 mm.), and the final volume adjusted to 10 ml. with Sabouraud dextrose agar.

Following solidification, one drop of a concentrated spore suspension was placed in the center of each plate with a 1.0-ml. serological pipet. Controls consisted of untreated Sabouraud agar, though inoculated with the test fungus.

Diameter of the fungus on untreated plates was compared for 7 days with those treated with varying concentrations of a halonitrosalicylanilide. All plates were incubated at room temperature with complete antifungal activity expressed in micrograms per milliter.

Oral Fate of 3′-Chloro-5-nitrosalicylanilide.—A 2.9 Gm. quantity of 3′-chloro-5-nitrosalicylanilide was suspended in 40 ml. of a 10% suspension of gum acacia. This was slowly administered to a 2.9 Kg. rabbit by gastric intubation and was repeated on 5 consecutive days. Side effects or other indications of acute toxicity were not observed.

All of the feces collected during this period, approximately 200 Gm. were extracted by a total of 1.0 L. of dimethylformamide. Portions were combined, vacuum filtered, and evaporated to dryness in a small glass still at 60° and 5 mm. A 30% concentration of the residue was prepared in distilled water and screened against *Staphylococcus aureus* by the cup-plate method. No antimicrobial activity was observed. Controls of 3′-chloro-5-nitrosalicylanilide (0.001 Gm./ml.) elicited conspicuous zones of inhibition.

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