The Chemistry of Antimycin A. XI. N-Substituted 3-Formamidosalicylic Amides[1]

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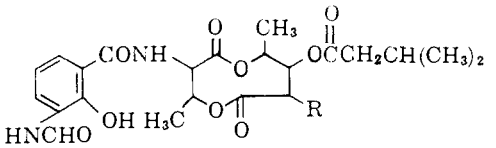
A series of antimycin analogs consisting of substituted amides of 3-formamidosalicylic acid was prepared by condensing 3-nitrosalicylic acid with suitable primary amines in the presence of *o*-phenylene phosphorochloridite, catalytically hydrogenating the nitro groups to primary amines, and formylating the products. The inhibitory power of the analogs was estimated by their effect on reduced coenzyme Q-cytochrome c reductase. The higher members of the homologous *n*-alkyl amides exhibited up to one-half the activity of antimycin A, while the longer chain 3-nitro intermediates showed appreciable but lower activity. Antibacterial, antiviral, and antifungal screening with the *n*-alkyl series showed no important activity. The results support the hypothesis that the localization of activity is centered around the aromatic portion of the antimycin molecule and that the remaining dilactone portion probably is important only insofar as it contributes the proper solubility characteristics to the molecule.

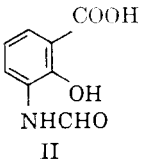
It has been demonstrated that the characteristic ability of Antimycin A (I)[2] to inhibit the electron-transport system of higher animals[3] is abolished by methylation of the phenolic hydroxyl, removal of the N-formyl group, or by alkaline cleavage of the dilactone ring.[2,4] However, both antimycin A_1 and A_3, which differ only in bearing *n*-hexyl or *n*-butyl side chains, respectively, are about equally active,[5] and the N-formal group may be replaced by an acetyl with only slight loss of activity.[2,4]

For these reasons, it appeared that the most essential portion of the antimycin structure (I) was the aromatic nucleus and the immediately adjacent substituent groups. The remainder of the structure might conceivably be of little importance other than to provide a certain degree of lipid solubility. As a start on the program of synthesizing analogs of antimycin A of a relatively simple structure, attention was therefore turned to substituted amides of 3-formamidosalicylic acid (II).



Antimycin A_1 R = *n*-hexyl
Antimycin A_3 R = *n*-butyl

(I)



II

These compounds were prepared by the route shown in the next column.

The intermediate 3-nitro compounds prepared are summarized in Table I and the corresponding 3-formamido derivatives in Table II. The inhibitory power of the analogs was estimated by noting their effect on the activity of reduced coenzyme Q-cytochrome c reductase as measured with a slight modification of the procedure described by Hatefi, *et al.*[6]

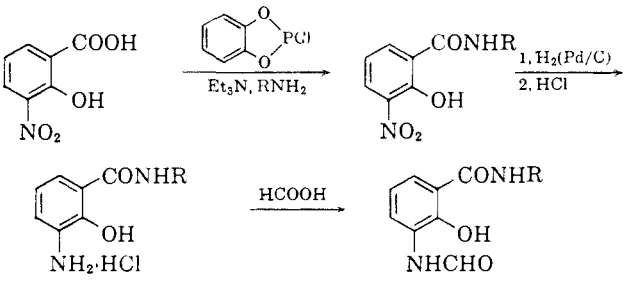
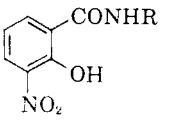


TABLE I

3-NITROSALICYLAMIDES



R[a]	M.p., °C	Yield, %	% Nitrogen Calcd.	% Nitrogen Found
n-Butyl	46–47	38	11.76	11.85
n-Hexyl	65–65.5	40	10.52	10.57
n-Octyl	60.5–62	46	9.52	9.43
n-Decyl	64.5–66	38.4	8.69	8.81
n-Dodecyl	74.5–75.5	42	7.99	8.05
n-Hexadecyl	84.5–85.5	43.2	6.89	7.04
n-Octadecyl	89–90	44.5	6.45	6.49
Phenyl	126–127	37	10.85	10.71
Benzyl	122–122.5	38	10.29	10.18
Phenethyl	130–131	34	9.79	9.59
Ethyl phenylalanyl[b]	100–100.5	41	7.82	7.73

[a] Crystallized from acetic acid–water. [b] Crystallized from ethanol–water.

Experimental

3-Nitrosalicyl-N-alkylamides.—These compounds were prepared by a modification of the method of Anderson, *et al.*[7] As an example, the preparation of the octadecyl amide is described in detail.

(1) This work was supported in part by grant G-3527 from the National Science Foundation.

(2) E. E. van Tamelen, J. P. Dickie, M. E. Loomans, R. S. Dewey, and F. M. Strong, *J. Am. Chem. Soc.*, **83**, 1639 (1961).

(3) F. M. Strong, "Topics in Microbial Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1958, pp. 37–42.

(4) A. L. Tappel, *Biochem. Pharmacol.*, **3**, 289 (1960).

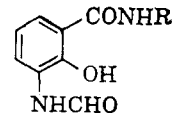
(5) W. C. Liu and F. M. Strong, *J. Am. Chem. Soc.*, **81**, 4387 (1959).

(6) Y. Hatefi, A. G. Haavik, and D. E. Griffiths, *J. Biol. Chem.*, **237**, 1681 (1962).

(7) G. W. Anderson and R. W. Young, *J. Am. Chem. Soc.*, **74**, 5307 (1952).

TABLE II

3-FORMAMIDOSALICYLAMIDES



R	M.p., °C.	Yield, %	% Carbon Calcd.	% Carbon Found	% Hydrogen Calcd.	% Hydrogen Found	% Nitrogen Calcd.	% Nitrogen Found
n-Butyl	100–101.5[a]	37	61.00	61.31	6.83	6.85	11.86	11.65
n-Octyl	77–78[a]	41	65.72	65.84	8.27	8.23	9.58	8.56
n-Decyl	71–72[a]	67	67.47	67.23	8.81	8.89	8.74	8.12
n-Dodecyl	76.5–77[b]	74	68.93	69.44	9.26	9.96	8.04	8.29
n-Hexadecyl	82.5–84[b]	85	71.24	71.80	9.97	10.26	6.92	7.12
n-Octadecyl	88.5–90.5[b]	52	72.18	73.05	10.25	10.50	6.48	7.04
Phenyl	175–176[c]	88	65.62	65.46	4.72	5.14	10.93	10.55
Benzyl	115–116[a]	49	66.65	66.75	5.22	5.37	10.37	10.65
Phenethyl	95–96[a]	58	67.59	67.60	5.67	5.73	9.85	9.87
Ethyl phenylalanyl	167–168[a]	45	64.03	64.11	5.66	5.59	7.86	8.13

[a] Recrystallized from benzene–Skellysolve B. [b] Recrystallized from ethanol–water. [c] Recrystallized from acetic acid–water.

To a solution of 2.11 g. (20.9 mmoles) of triethylamine and 4.02 g. (14.9 mmoles) of *n*-octadecylamine in 100 ml. of benzene[8] was added 3.34 g. (19.3 mmoles) of *o*-phenylene phosphorochloridite dissolved in 50 ml. of benzene. The *o*-phenylene phosphorochloridite, b.p. 80–81 (10 mm.), was prepared in 92% yield by the method of Anschütz, *et al.*[9] After 10 min. at room temperature the reaction mixture was filtered to remove the precipitate of triethylamine hydrochloride, which weighed 2.48 g. (18 mmoles). To the filtrate was added 2.68 g. (14.7 mmoles) of 3-nitrosalicylic acid; the mixture was refluxed 30 min. and allowed to stand for an additional 20 min. The reaction mixture was then shaken with 25 ml. of saturated aqueous sodium bicarbonate solution which caused the immediate precipitation of an orange solid. This was collected by filtration, triturated with an additional 10 ml. of the bicarbonate solution, washed thoroughly with water, and dried. The benzene layer was separated from the aqueous phase, washed twice with 10 ml. portions of water, and evaporated in a rotary evaporator. The orange solid which remained was combined with the other solids and the whole crystallized from acetic acid–water to yield 2.83 g. (6.54 mmoles, 44.5%) of a light yellow crystalline product, m.p. 89–90°. Recrystallization from acetic acid–water raised the m.p. to 91–92°.

Anal. Calcd. for $C_{25}H_{42}N_2O_4$: C, 69.09; H, 9.74; N, 6.45. Found: C, 69.11; H, 9.81; N, 6.43.

Other 3-Nitrosalicyl-N-(substituted)-amides.—Amides were also prepared from butyl-, hexyl-, octyl-, decyl-, dodecyl-, and hexadecylamines in the *n*-alkyl series, and from aniline, benzylamine, phenethylamine, and ethyl phenylalanate in the phenyl-substituted series. The procedure was analagous to that with octadecylamine, with slight modifications in the solvents used for recrystallization. The analyses and melting points of the compounds obtained are shown in Table I.

***n*-Hexylamine Salt of 3-Nitrosalicyl-N-(*n*-hexyl)-amide.**—If the product obtained in the preparation of 3-nitrosalicyl-N-alkylamides by the previously described procedure was recrystallized from a non-acid solvent, a different type of substance was isolated. This situation was investigated in the case of the *n*-hexyl analog, and the product proved to be an *n*-hexylamine salt, presumably of the strongly acidic phenol group. In this experiment equimolar quantities (*ca.* 5.5 mmoles) of triethylamine, *n*-hexylamine, *o*-phenylene phosphorochloridite, and 3-nitrosalicylic acid were allowed to react as described above, but the crude solid product was crystallized from methanol–ethyl ether (1:16, v./v.). Recrystallization from ethanol–water yielded 0.63 g. (1.73 mmoles, 32.5%), of yellow plates, m.p. 115.5–117°. The infrared spectrum showed a wide band between 3.1 and 3.9 μ, characteristic of an amine salt.

Anal. Calcd. for $C_{19}H_{33}N_3O_4$: C, 62.10; H, 9.05; N, 11.44. Found: C, 61.99; H, 9.03; N, 10.66.

Recrystallization of the salt from acetic acid–water gave the free N-(*n*-hexyl)-amide as a light yellow crystalline product, m.p. 65.5–66.5° (Table I). The salt also readily yielded *n*-hexylamine by ether extraction of its solution in aqueous sodium hydroxide. The amine was isolated as the crystalline hydrochloride, m.p. 219–222° (reported[10] 219°).

3-Formamidosalicyl-N-alkylamides.—The following example is illustrative of the method used for preparing *n*-alkyl formamido compounds listed in Table II.

To 0.537 g. (1.24 mmole) of 3-nitrosalicyl-N-(*n*-octadecyl)-amide was added 150 ml. of methanol and 96 mg. of 10% palladium-on-carbon hydrogenation catalyst. The mixture was hydrogenated in a Paar bomb under 1.4–2.8 kg./cm.^{2} pressure until no further uptake was noted (1 hr.). The catalyst was filtered off and the filtrate evaporated in a rotary evaporator. During these operations the flasks were flushed with nitrogen in order to minimize oxidation of the aminophenol. To the residue was added 40 ml. of 88% formic acid and the mixture refluxed for 30 min. The addition of 60 ml. of water caused a solid to precipitate which was readily extracted with 75 ml. of ethyl ether followed by two additional 25 ml. portions. The combined ether extract was washed with 25 ml. of water, four 25-ml. portions of saturated aqueous sodium bicarbonate solution, and finally with 25 ml. of water. The bicarbonate served to remove the excess formic acid and to hydrolyze any phenolic formate which might have been formed during the reaction. The ether was evaporated, leaving a light brown solid which was crystallized from ethanol–water. The final product was decolorized with charcoal and yielded 0.28 g. (0.646 mmole, 52%) of a nearly white crystalline product, m.p. 88.5–90.5°.

Anal. Calcd. for $C_{26}H_{44}N_2O_3$: C, 72.18; H, 10.25; N, 6.48. Found: C, 73.05; H, 10.50; N, 7.04. $\lambda_{max}^{CHCl_3}$ 5.98 μ (N-formyl carbonyl); 6.07 μ (NH-acyl I); 6.51 μ (NH-acyl II).

3-Formamidosalicyl-N-(phenyl-substituted)-amides.—Modification of the above procedure prevented air oxidation and resulted in final products of much better purity. The following example illustrates the improved synthesis for the phenyl-substituted analogs in Table II.

The 3-nitrosalicyl-N-phenethylamide (5.0 g., 17.7 mmoles) was dissolved in 300 ml. of a 2:1 absolute ether–ethanol solution, and 500 mg. of 5% palladium-on-carbon was added. The mixture was hydrogenated for 2 hr. (2.8 kg./cm.^{2}), the catalyst removed, and dry hydrogen chloride bubbled through the solution. Precipitation of the amine hydrochloride salt was complete after 10 min. The crystals were filtered and washed with three 20 ml. portions of absolute ether, dried, and immediately dissolved in a solution containing 6 g. of sodium formate in 100 ml. of 88% formic acid. The mixture was heated in a boiling water bath for 1 hr., 100 ml. of distilled water added to the solution, and heating continued for 5 min. to hydrolyze any phenolic formate present. Cooling of the mixture resulted in formation of a brown oil which was dried by several azeotropic evaporations with benzene–ethanol. Crystallization from benzene–Skellysolve B gave 2.9 g. (58% yield) of 3-formamidosalicyl-N-phenethylamide, m.p. 93–95°. Recrystallization from the same solvents raised the m.p. to 95–96°.

(8) Dried by distillation.

(9) L. Anschütz, W. Brocher, R. Nehir, and A. Ohrbeem, *Ber.*, **76**, 222 (1943).

(10) I. Heilbron, "Dictionary of Organic Compounds," Vol. I, Oxford University Press, New York, N. Y. 1953, p. 96.

Table III

Inhibition of Reduced Coenzyme Q-Cytochrome c Reductase by Antimycin Analogs Preincubated with the Enzyme Preparation[a]

Compound[b]	Incubation time,[c] hr.	Inhibition %
n-Butyl	0.25	0
n-Butyl	16	0
n-Octyl	0.25	0
n-Octyl	16	0
n-Decyl	0.25	0
n-Decyl	16	5
n-Dodecyl	0.25	0
n-Dodecyl	16	0
n-Hexadecyl	0.25	30
n-Hexadecyl	16	7
n-Octadecyl	0.25	30
n-Octadecyl	16	36
Antimycin A_3	0.25	99
Antimycin A_3	0.50	81
Antimycin A_3	16	50

[a] Final concentration of inhibitor, 0.5 mμg./ml., enzyme protein 0.32 μg./ml.; inhibitor/protein 1.56 μg./mg. [b] 3-Formamido analogs. [c] At 4°.

Anal. Calcd. for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.60; H, 5.73; N, 9.87.

Assay for Inhibition of Reduced Coenzyme Q-Cytochrome c Reductase.[11]—This procedure is a slight modification of that described by Hatefi, *et al.*[6] A 0.1-ml. sample of the enzyme solution (32 mg. of protein/ml.) was preincubated with 5–10 μg. of the test compound (5 mg./ml.) at 4° for a given period of time. The mixture was then diluted 1:1000 with an aqueous deoxycholate solution (0.5 mg./ml., pH 7.5), and a 0.01-ml. aliquot of the diluted solution was added to 0.99 ml. of the assay mixture which contained 0.022 μmole of EDTA, 45 μmoles of phosphate buffer (pH 7.4), 1 mg. of bovine serum albumin, 50 μg. (0.156 μmole) of CoQ_2H_2, and 1.66 mg. of cytochrome c. The reaction was stopped after 10 sec. by the addition of 2.0 ml. of a solution prepared from 9 vol. of 2% Triton X-100, 1 vol. of *M* tris-chloride buffer (pH 8.0) and 5 vol. of absolute ethanol. The optical density of the solution at 550 mμ was measured and compared with that of a standard solution which contained no enzyme. The results are shown in Tables III and IV and in Fig. 1.

In one experiment the procedure was modified in that instead of being preincubated with the enzyme, the inhibitor was added directly to the assay mixture prior to enzyme addition (Table V). In this case much higher inhibitor:protein ratios were used than in the previous experiments.

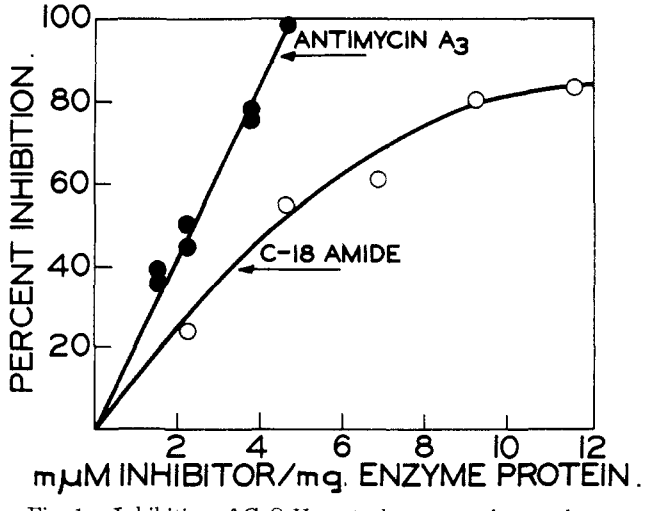


Fig. 1.—Inhibition of CoQ_2H_2–cytochrome c reductase by antimycin A_3 and by 3-formamidosalicyl-N-(*n*-octadecyl)-amide after 30 min. preincubation at 4°.

Table IV

Inhibition of Reduced Coenzyme Q-Cytochrome c Reductase by 3-Nitrosalicyl-N-alkylamides Preincubated with the Enzyme Preparation[a]

Compound	Inhibitor concentration,[b] mμg./ml.	Inhibitor/protein, μg./mg.	Incubation time,[c] min.	Inhibition, %
n-Hexadecyl	1	1.33	1	17
n-Hexadecyl	1	1.33	15	29
n-Hexadecyl	2	1.33	55	31
n-Octadecyl	1	1.33	1	13
n-Octadecyl	1	1.33	10	41
n-Octadecyl	1	1.33	30	44
n-Octadecyl	1	1.33	60	54
n-Octadecyl	2	2.66	2	34
n-Octadecyl	2	2.66	5	40
n-Octadecyl	2	2.66	15	49
n-Octadecyl	2	2.66	30	57
n-Octadecyl	2	2.66	60	60
Antimycin A_3	1	1.33	1	51
Antimycin A_3	1	1.33	10	57
Antimycin A_3	2	2.66	1	97

[a] Final concentration of enzyme protein 0.75 μg./ml. [b] Final concentration in assay mixture. [c] At 4°.

Table V

Inhibition of Reduced Coenzyme Q-Cytochrome c Reductase by Antimycin Analogs Added Directly to the Assay Mixture

Compound[a]	Inhibitor concentration,[b] μg./ml.	Protein concentration,[b] μg./ml.	Inhibitor/protein, μg./mg.	Inhibition, %
n-Butyl	5	0.32	15,600	11
n-Octyl	5	.32	15,600	60
n-Decyl	5	.64	7,800	85
n-Dodecyl	1	.32	3,110	57
n-Dodecyl	2	.32	6,220	73
n-Hexadecyl	1	.64	1,560	84
n-Octadecyl	1	.64	1,560	50

[a] 3-Formamido analogs. [b] Concentrations are expressed as the total concentration in the standard assay mixture.

Discussion

The preparation of amides of salicylic acid by use of *o*-phenylene phosphorochloridite as a condensing agent has been well described.[7] Employment of this method for the preparation of amides of 3-nitrosalicylic acid produced instead of the free phenol its primary amine salt. Since the method of preparation presumably converts the primary amine to the amidophosphite in quantitative yields, any mechanism which explains the condensation reaction must explain the presence of free amine in the final product. It would appear likely that the carboxyl group displaces the amine from the amidophosphite to form a mixed anhydride which then undergoes nucleophilic attack by the amine. Anderson, *et al.*,[7] have demonstrated that esters of phosphorochloridous acid form mixed anhydrides with carboxylic acids which readily react with amines to produce amides. However, in the case of the 3-nitrosalicylic acid, salt formation with the acidic phenol evidently competes with amide formation for the free amine. Whereas yields of over 90% are reported for amides of salicylic

(11) The authors wish to thank Dr. John Rieske, Institute for Enzyme Research, Madison, Wisconsin, for performing the enzyme inhibition studies on the antimycin analogs.

acid by this method, no yields over 50% were obtained in the present work with 3-nitrosalicyclic acid as might be predicted from this mechanism.

The 3-formamido-N-alkyl amides proved to be difficult to obtain in pure form, presumably due to the presence of oxidation products. Isolation of the intermediate aminophenol in the form of the hydrochloride salt prior to formylation greatly enhanced the purity of the crude 3-formamido product and hence of the analytical samples.

The activity of the analogs in the enzyme inhibition test varied considerably with the way the assay was carried out. If relatively large quantities of the *n*-alkyl analogs were added directly to the assay mixture, all members of the series showed some inhibition of the electron transport system. As shown in Table V, the greatest activity was exhibited by the hexadecyl compound, followed by the octadecyl analog, and decreasing activity was observed as the number of carbon atoms in the side chain decreased. When the compounds were added to the enzyme preparation, incubated for a given period of time, and diluted before being added to the assay mixture, the final concentration of inhibitor in the assay mixture was less than one thousandth that in the former method. Under these conditions only the hexadecyl- and octadecylamides showed inhibition, as shown in Table III. A more extensive comparison of the inhibitory action of antimycin A_3 and the C-18 formamido analog is shown in Fig. 1. The phenyl substituted amides showed no significant activity when tested at concentrations up to 2.5 times the concentration at which antimycin caused 85% inhibition.

Since the hexadecyl and octadecyl analogs showed appreciable activity, the corresponding nitro compounds were assayed (Table IV). Although the conditions were not exactly identical, it appears that substitution of the nitro group for the formamido group does not greatly alter the activity.

From these studies it would appear that the analogs inhibit electron transport similarly to antimycin but not as effectively, that the binding of the inhibitor to the enzyme complex is increased with increasing lipid solubility, and that the analogs are not as rapidly equilibrated with the enzyme as is antimycin. Although these model compounds are not perfect substitutes for antimycin, the fact that considerable inhibition was achieved tends to support the original hypothesis that the localization of activity resides in the aromatic moiety of the antimycin molecule. The localization of activity to the aromatic group also makes more attractive the idea of an antimycin–metal interaction *in vivo*.

In addition to the above activity studies, antibacterial, antiviral, and antifungal screening was carried out with the *n*-alkyl series of compounds.[12] Tests against *Trichomonas vaginalis* are summarized in Table VI. Although no important activity against this organism was found, it is noteworthy that of the compounds tested only those with intermediate length of side chain (10 carbon atoms) showed any effect at all, and that the nitro compound was somewhat more active than its formamido analog. These tests were carried out as described by Thompson, *et al.*[13]

(12) The authors wish to thank Parke, Davis and Company for carrying out the antibacterial and antiviral screening.

(13) P. E. Thompson, A. Bayles, S. F. Herbst, B. Olszewski, and J. E. Meisenhelder, *Antibiot. Chemotherapy*, **9**, 618 (1959).

TABLE VI

ACTIVITY OF ANTIMYCIN ANALOGS AGAINST *Trichomonas Vaginalis*

Compound	Concentration, μg./ml.	Effect
n-Butyl[a]	25	Inactive
n-Decyl[a]	25	Static
n-Decyl[a]	6.25	Inactive
n-Hexadecyl[a]	25	Inactive
n-Butyl[b]	25	Inactive
n-Decyl[b]	25	Cidal
n-Decyl[b]	6.25	Static
n-Decyl[b]	1.56	Inactive
n-Octadecyl[b]	25	Inactive

[a] 3-Formamido analogs. [b] 3-Nitro analogs.

Bacterial and tuberculosis screening was carried out with *Streptococcus pyogenes* strain C203, *Staphylococcus aureus* strain UC-76, *Proteus mirabilis* strain MGH-1, *Pseudomonas aeruginosa* strain 28, *Salmonella typhimurium* strain V-21, and *Mycobacterium tuberculosis* strain H37-rV. Tests for possible growth inhibition were carried out in test tubes by a standard broth dilution procedure with trypticase plus 10% serum as the growth medium. The inoculum was 0.1 ml. of a 10^{-6} dilution of a 24-hr. broth culture of the test organism. None of these organisms were inhibited by 20 μg./ml. of the *n*-alkyl compounds, either of the 3-nitro or 3-formamido series. One compound, 3-nitrosalicyl-*n*-octylamide, was tested for possible antiviral activity in tissue cultures of KB cells against measles, herpes simplex, HA_1 (parainfluenza 3), and poliovirus. The general testing procedure used was that described by Rightsel, *et al.*[14] No activity was noted at concentrations below those at which the test compound was itself toxic to the KB cultures.

Antifungal activity was tested against *Glomerella cingulata* as perviously described[15] (Table VII). The activity was negligible, but in contrast to the results of other tests the shortest chain (*n*-butyl) compound was more effective than intermediate chain length analogs.

TABLE VII

ANTIFUNGAL ACTIVITY OF ANTIMYCIN ANALOGS AS TESTED AGAINST *Glomerella Cingulata*[a]

R	Inhibition zone produced by 3-formamido compound, mm.	Inhibition zone produced by 3-nitro compound, mm.
n-Butyl	9	1
n-Hexyl		2
n-Octyl	5	2
n-Decyl	4	2
n-Dodecyl	2	1
n-Hexadecyl	1	1
n-Octadecyl	3	1
Phenyl		3
Benzyl		2
Phenethyl		1
Antimycin[b]	26	

[a] Tested at 1000 μg./ml. [b] Tested at 0.25 μg./ml.

(14) W. A. Rightsel, J. R. Dice, and R. Pittillo, *Univ. Mich. Med. Bull.* **24**, 222 (1958).

(15) B. R. Dunshee, C. Leben, G. W. Keitt, and F. M. Strong, *J. Am. Chem. Soc.*, **71**, 2436 (1949). Antifungal testing was conducted in the laboratories of the Wisconsin Alumni Research Foundation by R. A. Kubista to whom thanks are gratefully extended.