ANTIMICROBIAL ACTION OF NITROGEN-CONTAINING STEROIDS

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Received for publication 21 January 1963

ABSTRACT

SMITH, RODNEY F. (University of Maryland, Baltimore), DONALD E. SHAY, AND NORMAN J. DOORENBOS. Antimicrobial action of nitrogen-containing steroids. *J. Bacteriol.* **85**:1295-1299. 1963.—A new group of 16 synthetic nitrogen-containing steroids have been tested against a variety of microorganisms for antimicrobial properties. The gradient plate screening method, serial dilution, and dry weight techniques were used in the studies. The organisms tested consisted of 14 gram-negative bacteria, 10 gram-positive bacteria, 2 actinomycetes, 7 yeasts, and 8 molds. Inhibitory properties were found to be specific and potent in four compounds, with inhibitory concentrations as low as 0.37 $\mu\text{g/ml}$. Three of the active steroids are 4-aza cholestanes and one is a 4-nor-3,5-secocholestane amide. Sensitivity to the compounds was greatest in the gram-positive bacteria, followed by the yeasts and molds. The gram-negative bacteria were not inhibited. All 16 steroids interfered to some extent with pigmentation in *Serratia marcescens* but not with pigment production in *Pseudomonas aeruginosa*. In a few instances, some of the molds were stimulated by the steroids at a concentration of 250 $\mu\text{g/ml}$.

During the last two decades, several papers have appeared in the literature describing the antimicrobial effects of various hormones and related steroid compounds. Raab (1946) showed that vitamin D and cholesterol were inhibitory in vitro to *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Aerobacter aerogenes*. Squire and Squire (1948) observed that cholestadiene, cholesterol, and cholesteryl chloride were inhibitory to some enteric bacilli. The antifungal activity of deoxycorticosterone was reported by Reiss (1947). Kull, Castellano, and Mayer (1953) indicated that a group of amino steroids exhibited an inhibitory effect on a num-

ber of fungi and bacteria. Lester and Hechter (1958) reported on the inhibitory effects of deoxycorticosterone, and indicated that the action of the compound was specific since any slight change in the molecular structure reduced its inhibitory activity. Their work points to a possible correlation between gram positive reaction and steroid sensitivity, since only gram-positive bacteria, yeasts, and molds were inhibited.

Recent work by Casas-Campillo, Balandrano, and Galarza (1961) on the effects of 21,21-dimethoxy progesterone and certain of its analogues again points to the sensitivity of the gram-positive microorganisms but not the gram-negative species. Yeasts were not inhibited by the compounds tested in the study. Maxwell, McGuire, and Tomkins (1960), however, reported the inhibitory effects of a group of androsterone derivatives on *Saccharomyces fragilis*, which indicate the specific and curious nature of the influence of steroids on microorganisms.

MATERIALS AND METHODS

Steroids. The nomenclature and structure of each steroid are given in Fig. 1. The steroids are designated by the letters ND, indicating all were prepared in the Pharmaceutical Chemistry Laboratory under the direction of Norman J. Doorenbos.

Media. Bacteria were cultured on nutrient agar and Eugonagar (BBL, 01-265), depending upon their growth requirements. Yeasts and molds were grown on Sabouraud agar, and the clostridia were tested on anaerobic agar using the Brewer anaerobic petri dish. Heat-sensitive steroids were sterilized by filtration and added to the culture media. Aqueous stock solutions of the steroids were prepared by using 95% ethanol or dilute HCl.

Screening technique. Because of the limited quantity of each compound that was tested (100 mg), the gradient plate technique described by Szybalski and Bryson (1952) was employed. The

on their sides and incubated at 32 C for 8 days. The mycelial mass was then separated on a filter pad by suction and dried in a hot-air oven at 80 C for 24 hr. All tests were run in triplicate.

RESULTS

Of the 16 steroid compounds evaluated (Fig. 1) significant antimicrobial activity was limited to a group of closely related aza-steroids, ND-307, -354, and -326 (Table 1). ND-276, a 4-nor-3,5-secocholestane amide, was inhibitory to some of the bacteria but not the fungi. Under the pH conditions of the tests, ND-276 is in an un-ionized form, whereas ND-307 is largely in the ionized form and ND-326 and -354 are completely ionized. ND-307 had the broadest spectrum of activity, but the closely related steroid ND-323 was inactive. The latter has a low water-solubility and was in suspension rather than solution, a fact that might explain its lack of activity. ND-354 and -326, both cholestane quaternary salts, were active, whereas ND-104, an androstene quaternary salt, had no inhibitory effects. The steroids ND-2, -3, -165, and -328 were partially ionized but showed no activity.

With the exception of *Brucella abortus*, none of the gram-negative bacteria showed any sensitivity to the steroids. The insensitive group included *Escherichia coli*, *A. aerogenes*, *Salmonella typhosa*, *S. paratyphi*, *S. choleraesuis*, *Shigella dysenteriae*, *P. mirabilis*, *Klebsiella pneumoniae*, *Serratia marcescens*, *P. aeruginosa*, *Neisseria catarrhalis*, and *Pasteurella multocida*.

Since the active compounds were related to cholesterol in that they possess the eight-carbon side chain at position 17, cholesterol was run as a control, but no inhibition was observed. Although none of the steroids inhibited the gram-negative bacteria, the 16 test compounds and cholesterol interfered to some extent with the production of the lipid-soluble pigment of *S. marcescens* when compared with those of the control plates. The water-soluble pigment of *P. aeruginosa* developed readily in the presence of the steroids.

The results of the screening tests (Table 1) show that inhibition was specific, since not all of the gram-positive bacteria or fungi were sensitive. The results indicate that the active steroids were effective in concentrations close to 1 μ g/ml. To confirm these findings, the conventional serial dilution method was employed (Table 2). In general, there was good agreement between the two

TABLE 1. Nitrogen-containing steroids effective against microorganisms when tested by the gradient plate method*

Microorganism	Steroid			
	307	354	326	276
Gram-negative bacteria				
<i>Brucella abortus</i>		+		
Gram-positive bacteria				
<i>Staphylococcus aureus</i>	+	+		
<i>S. epidermidis</i>	+	+		
<i>Sarcina lutea</i>	+	+	+	+
<i>Streptococcus pyogenes</i>	+	+	+	+
<i>Corynebacterium diphtheriae</i>				
type <i>mitis</i>	+	+	+	+
<i>Lactobacillus acidophilus</i>	+	+		+
<i>Leuconostoc mesenteroides</i>	+	+	+	
<i>Clostridium tetani</i>	+	+	+	+
<i>C. perfringens</i>	+	+	+	+
Actinomycetes				
<i>Mycobacterium smegmatis</i>	+		+	
<i>Nocardia asteroides</i>	+			
Yeasts				
<i>Candida albicans</i>	+	+		
<i>C. lipolytica</i>	+	+	+	
<i>Torulopsis glabrata</i>	+			
<i>Saccharomyces cerevisiae</i>	+			
<i>Cryptococcus neoformans</i>	+	+	+	
<i>Rhodotorula</i> spp.	+	+	+	
<i>Geotrichum candidum</i>	+			
Molds				
<i>Sporotrichum schenckii</i>		+	+	
<i>Penicillium</i> spp.			+	
<i>Microsporum canis</i>	+	+	+	
<i>Trichophyton mentagrophytes</i>	+		+	
<i>Allescheria boydii</i>	+		+	
<i>Rhizopus oryzae</i>	+			

* Symbols: + = inhibition of growth at 1 μ g/ml; a blank indicates the organism was insensitive to the compound. (All steroids were tested against all organisms.) Steroids not listed had no antimicrobial activity.

methods when comparing their inhibitory effects against bacteria and yeasts. The active steroids were effective at concentrations equivalent to those of many existing antibiotics. *Cryptococcus neoformans* was the most sensitive yeast, being inhibited by the smallest amount of each 4-aza-steroid. The two sporeforming anaerobic bacilli

were sensitive to the four active steroids, whereas the aerobic sporeformer *Bacillus subtilis* was not inhibited by any of the compounds. *Aspergillus fumigatus* and *Mucor racemosus* showed no sensitivity to the compounds in the initial screening tests.

Those molds that were sensitive when tested by the gradient plate technique were further evaluated by the dry weight procedure, employing varying amounts of the inhibitory steroid in concentrations of 1 to 100 $\mu\text{g/ml}$ (Table 3).

Those molds that were not sensitive were tested in the same manner but only in a concentration of 250 $\mu\text{g/ml}$ of the steroids (Table 4).

Microsporium canis was completely inhibited by 100 $\mu\text{g/ml}$ of ND-307, whereas *Allescheria boydii* and *Trichophyton mentagrophytes* were completely inhibited by 50 $\mu\text{g/ml}$ of ND-307. *M. canis* and *A. boydii* were the most sensitive to ND-326.

TABLE 2. Minimal inhibitory concentrations of steroids determined by the serial dilution method

Microorganism	Minimal inhibitory concn ($\mu\text{g/ml}$)			
	Steroid			
	307	354	326	276
<i>Staphylococcus aureus</i> ...	125	3.1	—*	—
<i>S. epidermidis</i>	125	3.1	—	—
<i>Sarcina lutea</i>	0.75	0.75	0.37	0.37
<i>Mycobacterium smegmatis</i>	125	—	125	—
<i>Streptococcus pyogenes</i> ...	0.75	3.1	6.2	25
<i>Corynebacterium diphtheriae</i> type mitis....	0.75	6.2	3.1	3.1
<i>Lactobacillus acidophilus</i>	0.75	1.5	—	6.2
<i>Leuconostoc mesenteroides</i>	0.75	1.5	1.5	—
<i>Brucella abortus</i>	—	12.5	—	—
<i>Clostridium tetani</i>	3.1	50	50	12.5
<i>C. perfringens</i>	6.2	25	100	6.2
<i>Candida albicans</i>	6.1	12.5	—	—
<i>C. lipolytica</i>	100	6.1	25	—
<i>Torulopsis glabrata</i>	100	—	—	—
<i>Saccharomyces cerevisiae</i> ...	3.1	—	—	—
<i>Cryptococcus neoformans</i> ...	0.75	3.1	1.5	—
<i>Rhodotorula</i> spp.....	0.75	3.1	6.1	—
<i>Geotrichum candidum</i>	3.1	—	—	—
<i>Nocardia asteroides</i>	1.5	—	—	—

* Organisms were not inhibited.

TABLE 3. Effects of ND-307, ND-354, and ND-326 on the germination and growth of mold spores using dry weight analysis

Steroid	Organism	Avg wt of control	Concn of steroid (in $\mu\text{g/ml}$)				
			1	10	25	50	100
ND-307	<i>Microsporium canis</i>	mg 109	7*	0	0	6	99
	<i>Allescheria boydii</i>	86	3	53	50	99	—†
	<i>Trichophyton mentagrophytes</i>	54	26	63	69	99	—
	<i>Rhizopus oryzae</i>	67	5	5	15	15	53
ND-326	<i>M. canis</i>	140	18	19	56	67	99
	<i>Penicillium</i> spp.	91	8	4	24	49	69
	<i>Sporotrichum schenckii</i>	50	13	56	56	68	68
	<i>T. mentagrophytes</i>	140	12‡	52	60	67	74
	<i>A. boydii</i>	86	33	99	—	—	—
ND-354	<i>M. canis</i>	140	12	3	6	10	29
	<i>S. schenckii</i>	50	5	29	43	58	58

* All data are given as per cent decrease in dry weight.

† Complete inhibition.

‡ Increase over the weight of the control.

TABLE 4. Effects of ND-307, -326, -354, and -276 on the germination and growth of mold spores using dry weight analysis

Organism	Steroid ^a			
	307	326	354	276
<i>Sporotrichum schenckii</i>	38 ^b	— ^c	—	59
<i>Penicillium</i> spp.....	2 ^d	—	5	36 ^d
<i>Aspergillus fumigatus</i>	24	5	30 ^d	17 ^d
<i>Microsporium canis</i>	—	—	—	65
<i>Trichophyton mentagrophytes</i>	—	—	7	58
<i>Allescheria boydii</i>	—	—	57	31 ^d
<i>Mucor racemosus</i>	10	47	38	22
<i>Rhizopus oryzae</i>	—	18	46 ^d	23

^a Concentration of steroid was 250 $\mu\text{g/ml}$.

^b All data are given as per cent decrease in dry weight.

^c This organism-steroid combination is presented in Table 3.

^d Increase over control.

DISCUSSION

This is the first report of a group of steroids containing nitrogen in the nucleus that show anti-

microbial properties. Kull et al. (1953) and Tarbet, Oura, and Sternberg (1953) indicated that certain steroids containing nitrogen in the form of amino groups attached to the steroid nucleus are most effective when tested against microorganisms.

The results of this work are in agreement with those of Lester and Hechter (1958) and Casas-Campillo et al. (1961), who found that sensitivity to steroids was limited essentially to the gram-positive microorganisms. The reason for this specificity of reaction is not clear. It is well known that bile salts containing the steroid sodium deoxycholate have widespread use in culture media for inhibiting gram-positive bacteria but have no value as an inhibitor of yeasts or molds.

The three most active steroids, ND-307, -354, and -326, which are classified as quaternary salts, are largely or completely present as positively charged ions. They do not appear to have properties similar to those of quaternary salt disinfectants since the latter are effective against gram-positive and gram-negative bacteria (Glassman, 1948). These steroids represent a new class of specific and potent antimicrobial compounds. They show promise as a new type of weapon in the treatment of infection. Work is under way to study the relation of structure to activity and to investigate the mechanism of action of these compounds. A report of the lethal effects of the steroids found active in this report has been published elsewhere.

ACKNOWLEDGMENT

This study was supported in part by research grant CY-4132 from the National Cancer Institute, U.S. Public Health Service.

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