IN VITRO STUDY OF A GROUP OF BLOCKED STEROIDS AS ANTI-MYCOTIC AGENTS*

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In 1947 and 1949 Reiss tested a series of androgenic and estrogenic steroids for fungistatic effect *in vitro* (2, 3). A number of the hormones tested were observed to have this property. Diethyl stilbesterol, ethynyl estradiol, testosterone, and methyl testosterone were among the most effective compounds.

The present paper concerns an *in vitro* study of sixteen steroid compounds prepared by Dr. Max N. Huffman (1) of the Oklahoma Medical Research Institute, as part of a project designed to test the assumption that a methyl group substitution at C_3 might render the steroid molecule more resistant to biochemical change without altering its pattern specificity. The compounds tested were: (1) 3β-methoxy-17β-hydroxy-Δ^5-androstene-17α-acetic acid; (2) Δ^5-pregnene-3β, 20α-diol-3-methyl ether; (3) pregnenolone-3-methyl ether; (4) Δ^{16}-pregnenolone-3-methyl ether; (5) 16, 17-epoxypregnenolone-3-methyl ether; (6) 21-acetoxypregnenolone-3-methyl ether; (7) 17α-hydroxypregnenolone-3-methyl ether; (8) 17α-ethylandrostene-3β, 17β-diol-3-methyl ether; (9) 17α-ethynyl-androstene-3β, 17β-diol-3-methyl ether; (10) 17α-methylandrostene-3β, 17β-diol-3-methyl ether; (11) $\Delta^{5,17}$-pregnadiene-3β, 21-diol dimethyl ether; (12) Δ^5-androstene-3β, 16α, 17β-triol-3-methyl ether; (13) dehydroisoandrosterone-3-methyl ether; (14) Δ^5-androstene-3β, 17β-diol-3-methyl ether; (15) Δ^5-pregnene-3β, 20β-diol-3-methyl ether; and (16) 17-iso-Δ^5-pregnene-3β, 17β, 21-triol-3-methyl ether.

METHODS

In testing each compound six or more flasks of Difco Sabourand Dextrose Agar, pH 5.6, were melted and placed in a water bath at 45° C. A solution (a suspension, in some instances) of 2.5 percent of the compound acetone was added to the first flask in the amount of 4.0 ml. per 100 ml. of medium. The same solution was added to a second flask in the amount of 0.40 ml. per 100 ml. of medium. This gave concentrations of 0.1 and 0.01 percent of the compound. 0.4 ml. of various dilutions of the foregoing acetone solution gave the other concentrations tested. Acetone in the amount of 4.0, 0.40, and 0 ml. per 100 ml. of medium was added to the remaining flasks to serve as controls. The flasks were left in the water bath for one hour to permit some of the acetone to evaporate from the medium. During this time they were shaken frequently to keep

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the compound in suspension. The inoculum of fungous cells was prepared by making volumetric dilutions of gauze-filtered saline suspensions of lightly ground mycelium or of yeast-like cells, calculated on the basis of the known behavior of the strains used to give from 100 to 300 colonies per 10 ml. of medium. Poured plates were made using 10 ml. of the agar test dilutions and 0.1 ml. of fungus inoculum. The plates were incubated at 30° C. and examined for fungous colonies

TABLE 1

Preparation and characteristics of the fungous inocula

TEST FUNGUS SPECIES	PREPARATION OF INOCULUM	NATURE OF INOCULUM	APPROXIMATE VOLUMETRIC DILUTION FOR 100–300 COLONIES PER PLATE	APPROXIMATE INCUBATION TIME BEFORE FINAL READING OF GROWTH IN POUR PLAT PLATES
T. mentagrophytes	mat of 3 week slant peeled off and ground lightly in mortar	chiefly microconidia	1/20,000	5 days
M. canis	mat of 3 week slant peeled off and ground lightly in mortar	hyphal fragments	1/1,000	6 days
C. albicans	suspension from one week slant	yeast cells	1/200,000	2 days
C. neoformans	suspension from one week slant	yeast cells	1/50,000	3 days
B. dermatitidis	suspension from one week cystine chocolate slant (incubated at 37° C.)	yeast-like cells	1/10,000	8 days
H. capsulatum	suspension from one week cystine chocolate slant (incubated at 37° C.). Dilutions made in supernatant of original suspensions, and in Sabouraud dextrose broth	yeast-like cells	1/20,000	12 days
C. immitis	mycelium from one week broth culture, ground lightly in mortar	hyphal fragments	1/100	4 days
N. asteroids	suspension from one week cystine chocolate slant, ground lightly in mortar	bacillary fragments (clumped)	1/20,000	10 days

every other day from two to fourteen days. Growth was graded as 0, +, ++, +++, and ++++, taking the growth in the 0% acetone control plate, once the colonies were clearly apparent, as ++++.

The following fungi were used:

Trichophyton mentagrophytes, strain 1A3 & (C.D.C. ⁎A-280, isolated 1952);
Microsporum canis, strain 2A4 (C.D.C. ⁎M-1426-51, isolated 1951);
Candida albicans, strain 10A1 (C.D.C. ⁎146, isolated 1948);
Cryptococcus neoformans, strain 6A7 (Emmons ⁎3715, isolated 1947);

TABLE 2

Fungistatic effect of Δ^5-pregnene-3β,20α-diol-3-methyl ether

	TEST DILUTIONS % COMPOUND			CONTROL DILUTIONS % ACETONE		
	0.1	0.01	0.001	4.0*	0.4	0
T. mentagrophytes (1A3)	++++	++++	++++	++++	++++	++++
M. canis (2A4)	++++	++++	++++	++++	++++	++++
C. albicans (10A1)	++++	++++	++++	++++	++++	++++
C. neoformans (6A7)	++++	++++	++++	++++	++++	++++
B. dermatitidis (7A2)	0	++++	++++	++++	++++	++++
H. capsulatum (9A12)	0	0	++++	++++	++++	++++
C. immitis (11A2)	0	+++	+++	++	++++	++++
N. asteroids (4A4)	++++	++++	++++	++++	++++	++++

* 4.0% acetone dilution serves as control for 0.1% test dilution only.

TABLE 3

Fungistatic effect of 3β-methoxy-17β-hydroxy-Δ^5-androstene-17α-acetic acid

	TEST DILUTIONS % COMPOUND					CONTROL DILUTIONS % ACETONE		
	0.1	0.01	0.005	0.001	0.00075	4.0*	0.4	0
1A3	+++	+++		++++		++++	++++	++++
2A4	+	++		++++		+++	++++	++++
10A1	++++	++++		++++		++++	++++	++++
6A7	++++	++++		++++		++++	++++	++++
7A2	0	+++	++++	++++	++++	++++	++++	++++
7A3	0	++	+++	++++	++++	++++	++++	++++
9A11	0	0	0	++++	++++	++++	++++	++++
9A12	0	0	+	++++	++++	++++	++++	++++
11A2	0	+++	++++	++++	++++	++++	++++	++++
11A4	0	+++	++++	++++	++++	++++	++++	++++
4A2	0	0	0	0	++++	++++	++++	++++
4A4	0	++++	++++	++++	++++	++++	++++	++++

* 4.0% acetone dilution serves as control for 0.1% test dilution only.

Blastomyces dermatitidis, strain 7A2 (C.D.C. #S+A-373, isolated 1950); and strain 7A3 (Emmons 6053, isolated 1951);

Histoplasma capsulatum, strain 9A11 (Kligman #7A6); and strain 9A12 (Kligman #7A8);

Coccidioides immitis, strain 11A2 (C. A. Smith, Renner, isolated 1951), and strain 11A4 (C. A. Smith, Euphrate, isolated 1951);

Nocardia asteroides, strain 4A2 (Koons); strain 4A3 (Kligman #14A2), and strain 4A4 (Mycetoma, isolated 1952).

These eight species were selected as representative of the major fungus pathogens of man. The details of preparation and characteristics of the inoculum of each species are presented in Table 1.

RESULTS

Of the 16 steroids tested, two had fungistatic effect, see Tables 2 and 3. The other compounds had slight or no effect against the spectrum of fungi at 0.1% concentration.

SUMMARY

Sixteen steroid compounds with position 3 blocked by the substitution of a methyl group for the hydroxyl hydrogen were tested for fungistatic effect *in vitro*, using an agar pour plate technic. Two of the compounds produced significant fungistasis.

REFERENCES

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DISCUSSION

Dr. J. R. Frey, *Rio de Janiero, Brazil*: I see the importance of this paper in the fact that a new group of substances for the treatment of dermatomycosis is presented. As these substances are present in the human body, they may be better tolerated than the substances used so far in the treatment of these diseases. We feel that the most important target for the treatment of dermatomycosis is the systemic application of the drugs. As the described substances are probably well tolerated, experiments *in vivo* must be undertaken.

Dr. R. L. Mayer, Summit, New Jersey: The antifungal activity of certain steroids, especially those possessing hormonal activity, is very intriguing, and the question is still unsolved as to whether antifungal and hormonal activities are associated. I personally do not believe that such is the case. It seems to me that one may consider the steroids as long-chain aliphatic compounds—at least it is believed that they are broken down to such substances during metabolism. It is known that long-chain fatty acids and amines are good antifungal agents—undecylenic acid is one of the best anti-mycotic remedies. It is reasonable to assume that the action of these steroids is due to their chemical resemblance to the long-chain aliphatic acids. Stilbestrol is a strong antifungal agent, in fact, as strong as testosterone. There are among the antifungal steroids many which entirely lack hormonal activity. We have studied a number of amino steroids with antifungal properties.

DR. STEPHEN ROTHMAN, *Chicago, Illinois*: I would like to ask whether blank controls were done with acetone. It is difficult to remove this solvent from the medium and it may have fungistatic action.

MR. GERBERT REBELL (in closing): I want to thank the individuals who discussed the paper. I think I agree with Dr. Mayer that the hormonal activity perhaps also the steroid nature of these compounds may not be fundamental to their fungistatic activity. As he knows, we are investigating this thoroughly under the guidance of Dr. Huffman. In some cases we are unable to get compounds more active than those having hormonal effects.