LITERATURE CITED

1. P. Naito, J. Morito, and K. Yeno, Kagaku Kogyo, 22, 137-143 (1971).
2. US Patent No. 2422195; Chem. Abs., 41, p. 5555 (1947).
3. F. Cuiban and S. Ciliani, Rev. Chim. (Bucur.), 10, 74 (1959).
4. U. K. Patent No. 804236; Chem. Zent., 131, 6920 (1960).
5. S. A. Harris, J. Am. Chem. Soc., 63, 3363 (1941).
6. G. Bekker, An Introduction to the Electronic Theory of Organic Reactions [in Russian], Moscow (1965), p. 164.
7. The Chemistry of the Ether Linkage, S. Patai, ed., New York (1967), pp. 21-81.
8. S. A. Harris, J. Am. Chem. Soc., 62, 3203 (1940).
9. K. Leidler, The Kinetics of Organic Reactions [in Russian], Moscow (1966), pp. 18, 31.
10. H. D. Gibbs, J. Biol. Chem., 72, 649 (1927).
11. I. V. Scudi, W. A. Bastedo, and T. I. Wibb, J. Biol. Chem., 154, 63 (1944).
12. V. A. Devyatnin, Methods of Chemical Analysis in the Production of Vitamins [in Russian], Moscow (1964), p. 184.

CLEAVAGE OF THE SIDE CHAIN OF SITOSTEROL BY MICROORGANISMS

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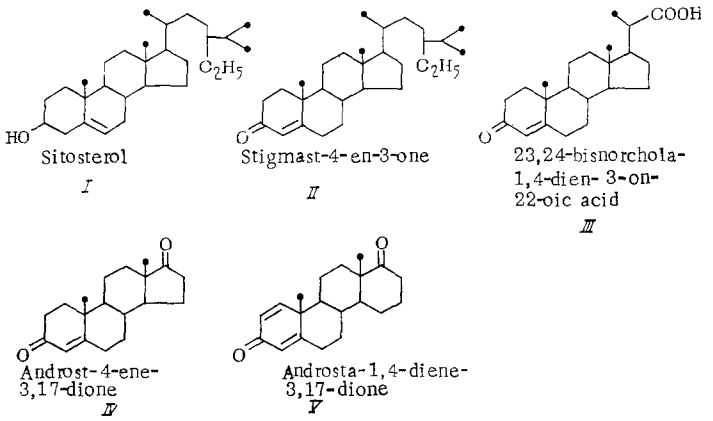
The use of sitosterol (I) as a raw material for the production of steroid hormones requires the microbiological cleavage of the side chain at C^{17} [1].

Cleavage of the side chain from I by the enzymes of microorganisms is accompanied by degradation of the steroid nucleus to carbon dioxide and water. In order to avoid this undesirable outcome, inhibitors of this degradation such as cobalt and nickel salts, 8-hydroxyquinoline, and $\alpha\alpha'$-dipyridyl are added to the culture fluid [2]. The microbiological cleavage of the side chain from I in the presence of an inhibitor may result in the following sequence of products [3]:



The object of this investigation was to assess the ability of some microorganisms to effect cleavage of the side chain of I to form III and 17-ketosteroids (17-KS), which can be used for the subsequent synthesis of steroid hormones.

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TABLE 1. Degradative Capacity of Some Microorganisms

Microorganism	No. of strains	No. of strains which had degraded sitosterol after			No. of inactive strains
		24 h	48 h	72 h	
Nocardia	35	5	6	21	3
Actinomadura	13	—	1	4	8
Streptomyces	47	6	15	12	14

Ninety-five cultures of actinomycetes from the collection of the Scientific-Research Institute for the Search for New Antibiotics, Academy of Medical Sciences of the USSR, were examined. These comprised cultures of the genera Nocardia (35), Actinomadura (13), and Streptomyces (47).

A sample containing 97% of sitosterol was obtained from the Leningrad Academy of Wood Technology.

Transformation products II-V were identified by thin-layer chromatography (TLC) using the appropriate visualizing reagents obtained from the Laboratory for the Chemistry of Steroid Hormones of the All-Union Scientific-Research Pharmaceutical Chemistry Institute.

Data on the ability of actinomycetes to degrade I are presented in Table 1. It will be seen that the ability to degrade I is displayed by quite a high proportion of the actinomycete cultures, and is most marked in the Nocardia cultures. The Streptomyces cultures were somewhat less active, and the representatives of the Actinomadura family showed little activity. The active cultures degraded I almost completely. Following alkaline and acid hydrolysis of the biomass [4] of two active strains of Nocardia after 48 hours' transformation, no I was found in the hydrolysates. Only a few produced II (not more than 3% of the sterol added). In subsequent work using inhibitors of the degradation of the steroid nucleus, cultures which cleaved I were employed.

Method A. 1. Formation of Pregnenecarboxylic Acid and 17-KS. Transformation of I in the presence of nickel and cobalt ions. The effect of these inhibitors on the transformation was assessed in experiments with three strains of Nocardia. The inhibitors were initially employed in a concentration of 70 mg% [2], but since at this concentration of cobalt sulfate a considerable amount of unreacted sterol was present even after 72 h, the inhibitor concentration was reduced to 30-60 mg%. Compound III was found in the culture fluid from all three cultures. When cobalt salts (sulfate and chloride) were used, the amounts of III were greater than those found in experiments with nickel salts. We subsequently studied the ability of 70 strains of actinomycetes to produce III and 17-KS on the presence of cobalt sulfate, when it was found that 40 of the cultures failed to produce the required compounds under these conditions. Four strains of Nocardia, 12 strains of Streptomyces, and three strains of Actinomadura produced between 1 and 3% of these products. The greatest quantity of III (16%) was obtained using N. caviae No. 8, N. corallina No. 9, and N. polychromogenes No. 24, which in addition to III also afforded up to 4% of V (at an inhibitor concentration of 40 mg%). Particularly interesting results were obtained using N. albaparaffinae No. 11, when V was the main product (11%), and only a small amount (4%) of III was formed.

Method B. Transformation of I in the presence of $\alpha\alpha'$-Dipyridyl. The effect of this inhibitor was studied in 13 strains of Nocardia, two strains of Actinomadura, and six strains of Actinomyces. The inhibitor was introduced, in concentrations of 2.5 to 15 mg%, into the medium at the same time as the sterol, and at concentrations of 20 to 70 mg%, in three equal portions (at the same time as the sterol was added, and after transformation had proceeded for 7 and 24 h). For three strains of Nocardia, a change in the concentration of the inhibitor from 12.5 to 60 mg% had no effect on the amounts of reaction products. All the cultures, with the exception of a single strain of Nocardia, two strains of Actinomadura, and five strains of Streptomyces, yielded III as the main product, in amounts greater than 10%. The greatest amounts (27-33%) of this compound were obtained using six strains of Nocardia (20 mg% of dipyridyl), whereas V was formed in only small amounts by these cultures (2-10%). An exception was N. albaparaffinae No. 11, which afforded V as the main product in 22% yield, together with 2-4% of III (16 mg% of inhibitor). N. uniformis No. 30 was the only culture to give IV (11%).

Method C. Transformation of I in the presence of 8-hydroxyquinoline was investigated in experiments using nine strains of Nocardia, two strains of Actinomadura, and six strains of Streptomyces. Inhibitor concentrations were 5-12 mg%. At the 12 mg% concentration, the inhibitor was introduced into the medium in three

portions, as in the experiments with higher concentrations of αα'-dipyridyl. The greatest amount of III (10%) was obtained in experiments using Actinomadura madurae No. 22 at an inhibitor concentration of 10 mg%. Three strains of Nocardia, and Streptomyces fumosus, gave 2% of V.

These experiments show that different inhibitors affect the product ratios and the amounts produced in any given culture. Thus, N. uniformis No. 30 with the inhibitors cobalt sulfate, αα'-dipyridyl, and 8-hydroxyquinoline, formed III in 2, 10, and 6% yields respectively; 22 and 2% of V were found with the last two inhibitors, and IV (10%) was only obtained when αα'-dipyridyl was used.

The best yields of III and 17-KS were obtained using αα'-dipyridyl as inhibitor, in a concentration of 20 mg%.

2. Effect of Conditions of Culture and Transformation on the Ability of Microorganisms to Produce Pregnenecarboxylic Acid and Androstadiendione. In studying the effect of the composition of the nutrient medium on the activity of nine strains of Nocardia, media were employed which differed with respect to carbon and nitrogen sources, and in the ratios of these components. When cobalt sulfate, αα'dipyridyl, and 8-hydroxyquinoline were used as the inhibitors, the best or equivalent medium with seven strains of Nocardia was one containing soluble starch 1%, yeast extract 0.01%, and sodium chloride 0.2% (pH 7.0). This medium was optimal for the study of the ability of various microorganisms to effect cleavage of the side chain I.

The effect of changes in temperature (over the range 27-32°C), both on the growth and on the transformation of I were examined in experiments with three strains of Nocardia. These experiments showed that changes in temperature within these limits had no effect on the activity of the cultures.

An increase in the concentration of I in the medium for transformation, from 0.2 to 0.5 g/liter, using three strains of Nocardia showed that the absolute amount of III remained constant, but that as a percentage of the initial substrate concentration it decreased sharply. The amount of unreacted I also decreased, and it therefore appears that as the concentration of the sterol in the medium is increased, the rate of degradation is accelerated.

The enzymes responsible for the cleavage of the side chain from I are known to be inducible [5]. With this in mind, we used the starting material I as an inducer, introducing it into the medium at the same time as the inoculum in a concentration of 0.05 g/liter. This enabled the amount of III produced to be increased from 22 to 37% at an αα'-dipyridyl concentration of 30 mg%, using N. corallina No. 10. When four strains of Nocardia were grown on an agar medium containing 0.05 g/liter of I, the activity of the cultures remained unchanged.

Addition of 0.1 mg% of Tween 80 to the medium containing the sterol [5, 6] retarded the formation of III by N. caviae No. 8, the amount decreasing from 19 to 10%.

For comparison, the method of introduction of I into the medium as an aqueous colloidal solution or as a solution in DMF showed that the solvent reduced the formation of III by N. polychromogenes No. 24 from 19 to 12%.

This investigation has shown that of the 95 cultures of actinomycetes examined, 70 were able to cleave I after 24-72 h. Of these cultures, only seven strains (N. caviae No. 8, N. corallina Nos. 9, 10, and 11, N. polychromogenes No. 24, N. mucosum No. 11, and Actinomadura madurae No. 22) produced around 30% of III after 48 hours' transformation at a concentration of I of 0.2 g/liter, using αα'-dipyridyl as inhibitor. Two strains, N. albaparaffinae No. 11 and N. uniformis No. 30, were found to produce up to 20% of V under the same conditions, and N. uniformis No. 30 also formed 10% of IV. Preliminary induction of the enzymes increased the amounts of products formed.

EXPERIMENTAL METHOD

Growth of Cultures and Procedure for Transformations. Cultures were maintained on a starch-yeast medium. Erlenmeyer flasks of 250 ml capacity containing 20 ml of liquid medium were inoculated with the cultures to be examined from agar slopes. Growth of the cultures, and subsequent transformations of I were carried out on a shaker (200 rpm) at 27°C. Into the 24-h culture of the microorganism was introduced an aqueous colloidal solution of I [7] in a concentration of 0.2 g/liter, together with a solution of the inhibitor (cobalt and nickel salts in distilled water, αα'-dipyridyl in ethanol, and 8-hydroxyquinoline in DMF).

The course of the transformation was followed, and the products determined, by TLC. After 24, 48, and 72 h, 2 ml samples of the culture fluid were withdrawn and extracted with 0.5 ml of chloroform. Samples from experiments in which $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline were used were acidified before extraction with 20% hydrochloric acid, to pH 2.0; 0.1 ml of the extract was applied to a Silufol UV-254 plate (Czechoslovak SSSR). The transformation products were separated using the solvent system chloroform–ether (4 : 1). Semiquantitative estimations were carried out by comparison with accurate reference concentrations. For quantitative analysis, 15 ml of culture fluid was extracted four times with an equal volume of chloroform. The solvent was evaporated, and the residue dissolved in methanol–chloroform (1 : 1) and applied to a plate. Quantitative estimation was carried out spectrophotometrically using an SF-10 instrument at $\lambda = 241$ nm. Unreacted I was identified by developing the chromatograms with a solution of valine and perchloric acid, followed by heating.

LITERATURE CITED

1. N. E. Voishvillo and A. V. Kamernitskii, Nauchn. Dokl. Vyssh. Shk., Biol. Nauki, 9, 7-19 (1976).
2. Zh. D. Lebedeva, O. B. Tikhomirova, L. M. Kogan, et al., Izv. Akad. Nauk SSSR, Ser. Biol., 4, 529-534 (1972).
3. C. Sih, K. Wang, and H. Tai, J. Am. Chem. Soc., 89, 1956-1958 (1967).
4. E. F. Efimochkina, T. S. Nazarova, and L. E. Nikitin, Mikrobiologiya, 4, 716-720 (1970).
5. C. K. A. Martin and F. Wagner, Eur. J. Appl. Microbiol., 2, 243-255 (1976).
6. W. J. Marsheck, S. Kraychy, and R. D. Muiz, Appl. Microbiol., 23, 72-77 (1972).
7. A. A. Imshenetskii, E. F. Efimochkina, V. A. Zanin, et al., Mikrobiologiya, 1, 31-37 (1968).