UDC 547.92[577.174.53]:576.851.132.095.3

46. Masao Uchibayashi: Studies on Steroids. XVII.*1 Formation of Prednisolone from Reichstein's Substance S by *Pseudomonas*.*2

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The previous papers[1,2] of this series reported the microbiological transformation of Reichstein's Substance S (I) by *Pseudomonas* species to 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II), hydrocortisone, 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione, and 17α,-20β,21-trihydroxy-4-pregnen-3-one, and thus revealed the presence in the *Pseudomonas* species of the enzyme systems capable of performing three reactions, 1-dehydrogenation, 11β-hydroxylation, and 20β-hydrogenation. In view of the fact that the 1-dehydrogenation and 11β-hydroxylation were observed independently of each other, it appeared logical then to contemplate the possibility that both reactions could be induced to occur simultaneously if proper conditions for the fermentation were applied. The present paper describes the realization of the above idea, demonstrating the microbiological formation of prednisolone (IV) by the action of *Pseudomonas* on Reichstein's Substance S (I).

A mutant*4 produced by irradiation of *Pseudomonas* sp. 109*5 with ultraviolet ray was incubated in the synthetic medium described previously[1] and the conversion of Reichstein's Substance S (I) was conducted. Filtration of the culture broth and extraction of the filtrate with ethyl acetate yielded a powdery steroid mixture. Paper chromatography showed the mixture to contain, besides the unchanged substrate, two conversion products which were supposedly 17α,20β,21-trihydroxy-1,4-pregnadien-3-one[1] (II) and prednisolone (IV), as indicated by their Rf values, coloration with the antimony trichloride reagent, and behavior towards ultraviolet ray.

In order to isolate the products the crude steroid mixture was acetylated with acetic anhydride and pyridine, and chromatographed on Florisil. Elution with a solvent mixture of ether and acetone afforded three compounds in the following order. The first compound was the acetate of the substrate and the second, melting at 178~179°, was 17α-hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one (III), identified by comparison of melting point and infrared spectrum with those of an authentic sample.[1] The third compound was obtained as colorless granules, m.p. 230°, after recrystallization from dioxane and ethyl acetate. Infrared spectrum of this product exhibited absorption bands indicative of a 1,4-dien-3-one structure, and the coloration with antimony trichloride and behavior towards ultraviolet ray were no different from those of prednisolone acetate. Direct comparison of the product was thus made with the acetate derived from authentic prednisolone in melting point and infrared spectrum, and their identity was rigorously established.

For the microbiological production of prednisolone, application of organisms effecting either 1-dehydrogenation or 11β-hydroxylation on appropriate substrates has been the method of choice so far. In contrast to these processes, the results now obtained offer

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*1 This paper constitutes a part of a series entitled "Studies on Steroids" by Hayao Nawa. Part XVI: This Bulletin, 8, 122(1960).

*2 For a preliminary report, see Tetrahedron, No. 18, 17(1959).

*3 Juso-nishino-cho, Higashiyodogawa-ku, Osaka (内林政夫).

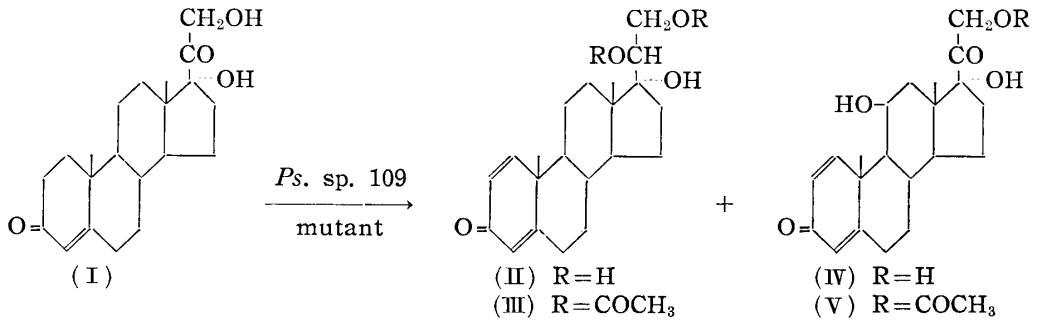
*4 An account of microbiology and fermentation of the mutant will be published elsewhere by J. Terumichi.

*5 An organism identified as closely akin to *Pseudomonas boreopolis*.[1]

1) Part XIV: This Bulletin, 8, 112(1960).

2) Part XV: *Ibid.*, 8, 117(1960).

a method entirely unprecedented in that both bioconversion reactions can be effected simultaneously by a single microörganism, particularly by an organism belonging to bacteria. Thus, with the one-step preparation of prednisolone from Reichstein's Substance S it was felt that the purpose of producing medicinally useful steroid hormones by the action of *Pseudomonas* on Reichstein's Substance S was accomplished.



(I)

(II) R=H
(III) R=$COCH_3$

(IV) R=H
(V) R=$COCH_3$

Experimental*6

Transformation of Reichstein's Substance S (I) (17α,21-Dihydroxy-4-pregnene-3,20-dione) by a Mutant of *Pseudomonas* sp. 109—Incubation of the mutant*4 of *Pseudomonas* sp. 109, obtaind by treatment with ultraviolet ray, was effected in a synthetic medium containing corn-steep liquor, K_2HPO_4, $MgSO_4$, $FeSO_4$, glycerol, and urea. EtOH solution of 12 g. of Reichstein's Substance S (I) was then added and the fermentation was continued for 24 hr. at 28°. The culture broth was filtered and the filtrate was extracted with AcOEt. Concentration of the extract under reduced pressure yielded 8.1 g. of a powdery crude steroid mixture, which was shown by paper chromatography to contain two new compounds as well as unchanged substrate.

Isolation of 17α-Hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one (III) and 11β,17α-Dihydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione (V)—A 4.0-g. sample of the crude steroid mixture was dissolved in 50 cc. of pyridine and 30 cc. of Ac_2O. The solution was allowed to stand at room temperature for 24 hr. and then warmed at 50° for 1 hr. After concentration of the solution *in vacuo*, the residue was dissolved in $CHCl_3$ and the solution was washed successively with dil. HCl, $NaHCO_3$ solution, and water, and dried over anhyd. $MgSO_4$. Removal of the solvent gave a residue which was dissolved in a small volume of $CHCl_3$-MeOH (1:1) and chromatographed over 400 g. of Florisil with a solvent mixture of Et_2O and Me_2CO as developer, with increasing content of Me_2CO.

The fractions eluted by Et_2O-Me_2CO (98:2) yielded 2.0 g. of the acetate of the starting material (I), m.p. 234~235°, which was identified by mixed m.p. determination and infrared spectrum.

The fractions from eluates of Et_2O-Me_2CO (92:8) was recystallized from Me_2CO to 0.3 g. of colorless prisms (III), m.p. 178~179°. This compound was found to be identical with 17α-hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one[1] (III) by comparison of infrared spectrum, m.p., and other properties.

Further fractions eluted by Et_2O-Me_2CO (85:5) afforded, after repeated recrystallizations from dioxane-AcOEt, 0.8 g. of colorless granules (V), m.p. 230°. IR ν_{max} cm^{-1} : 3344, 3257, 1742, 1718, 1647, 1587, 1222. *Anal.* Calcd. for $C_{23}H_{30}O_6$: C, 68.63; H, 7.51. Found : C, 68.37; H, 7.38.

Comparison of m.p., infrared spectrum, paper chromatogram, and mixed m.p. determination showed the unequivocal identity of the product with prednisolone acetate (V ; 11β,17α-dihydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione) derived from authentic prednisolone (v. i.).

11β,17α-Dihydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione (V) from Prednisolone (IV)—A solution of 100 mg. of prednisolone (IV) in 1.25 cc. of pyridine and 0.75 cc. of Ac_2O was allowed to stand at room temperature for 36 hr. and then warmed at 50° for 1 hr. Treatment of the reaction mixture in the usual manner and recrystallization from dioxane-AcOEt furnished 80 mg. of colorless granules, m.p. 227~230°.

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*6 All m.p.s are uncorrected and the infrared spectra were measured in Nujol mulls.

Summary

Transformation of Reichstein's Substance S (I) by a mutant obtained by ultraviolet ray irradiation of *Pseudomonas* sp. 109 resulted in the formation of 17α.20β,21-trihydroxy-1,4-pregnadien-3-one (II) and prednisolone (IV; 11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), and thus the aim of the work presented in this and preceding three papers, namely the preraration of therapeutically valuable steroid hormones by the action of *Pseudomonas*, was successfully attained.

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47. Kyosuke Tsuda,[*1] Masaaki Kawamura,[*2] und Ryoichi Hayatsu[*2]: Untersuchungen der Eierstockextrakte von Kugelfischen. XI.[1),*3] Über Tetrodotoxin. (2).

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In der 6., 7. und 8. Mitteilung dieser Reihe[2)] haben wir die Isolierung des Tetrodotoxins aus dem Eierstockextrakt bei Kugelfischen[*4] und die allgemeinen Eigenschaften dieses Giftstoffes beschrieben. Tetrodotoxin[3)] befindet sich allgemein in den Organen von Fischen der *Spheroides*-Arten (*Tetraodontidae*) und kristallisiert sich in Essigsäure-Äthanol-Äther zu Prismen aus, die keinen scharfen Schmelzpunkt aufweisen und sich über ca. 220° unter Schwarzfärbung langsam zersetzen; $[\alpha]_D^{25}$ −8.64° (c=8.55 in verd. Essigsäure). Auf Grund der Analysen- und Titrationsresultate[2b)] kann man dem Tetrodotoxin eine Bruttoformel von $C_{12}H_{19}O_9N_3$ geben, wenn man es als eine monoacidische Base ansieht. Es ist in Wasser, Äthanol und Äther nicht löslich. Aber es löst sich in verd. Salzsäure und scheidet sich durch Neutralisation wieder kristallinisch ab.

Es bildet ein kristallinisches Chlorhydrat bzw. Tartrat, aber beide Salze sind äusserst hygroskopisch. Aus einer Lösung von Tetrodotoxin in Essigsäure-Äthanol ergibt sich eine freie Base beim Zugeben von Äther, so dass diese Methode auf die Umkristallisation des Stoffes angewandt werden kann. Das IR-Spektrum und die verschiedenen Farbenreaktionen dieses Stoffes sind bereits beschrieben worden.[2b)]

Die vorliegende Arbeit berichtet über allgemeine Reaktionen, speziell über ein Abbauprodukt des Tetrodotoxins.

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*3 Vorgetragen bei dem 3. Symposium über Naturstoff-Chemie in Tokio, am 17. Oktober, 1959. Vgl. Referatenband 3. Symposium über Naturstoff-Chemie, 94(1959).
*4 Man nennt sie in Japan Fugu oder Fuku und in Amerika Puffer. Als Ausgangsmaterial für die Herstellung von Tetrodotoxin wurden die Eierstöcke von *Spheroides rubripes* (Torafugu) und *S. porphyreus* (Mafugu) gebraucht.

1) X. Mitteil. B. Umezawa: Yakugaku Zasshi, **75**, 496(1955).
2) a) K. Tsuda, M. Kawamura: Yakugaku Zasshi, **72**, 187, 771(1952). b) K. Tsuda, M. Kawamura: Dieses Bulletin, **1**, 112(1953).
3) Siehe die Übersicht von D. A. Courville, B. W. Halstead, D. W. Hessel: Chem. Revs., **58**, 241 (1958). Vgl. auch die Angaben von H. Arakawa (Nippon Kagaku Zasshi, **77**, 1295(1956)) und E. A. Murtha (Exptl. Therap., **112**, 246(1958)).