Microbial Aromatization of Steroids into Equilin

S. N. SEHGAL AND CLAUDE VÉZINA

Department of Microbiology, Ayerst Research Laboratories, Montreal, Quebec, Canada

Received for publication 27 August 1970

This report describes the bioconversion of 19-hydroxy-androsta-4,7-diene-3,17-dione into equilin with Nocardia rubra. Through mutation and improvement of medium and of conditions, the bioconversion could be improved to yield 40% equilin for a substrate concentration of 1 g/liter. Aromatization of several other 19-hydroxy and 19-nor substrates of the androstene series is reported, and the influence of various substitutions of the substrate molecule on aromatization is discussed.

Aromatization of 19-nor or 19-hydroxy-androstan-4-en-3-one steroids has been reported by several workers (2-4). Zedric et al. (5) were the first to report the bioconversion of 19-nor-androsta-4,7-diene-3,17-dione into equilin with Corynebacterium simplex. However, they reported that the reaction was erratic due to various amounts of Δ4-estrones in the bioconversion products. Bagli et al. (1) from this laboratory reported the bioconversion of 19-hydroxy-androsta-4,7-diene-3,17-dione into equilin with Nocardia restrictus. In continuation of their work, attempts were made to make the microbial bioconversion economically feasible by selection of more active species, superior mutants, and improvement of bioconversion conditions. This paper also deals with several other substrates which were transformed into equilin.

MATERIALS AND METHODS

Microorganisms. Microorganisms were maintained on nutrient agar slants at 4°C. A loopful of microbial growth was inoculated into 50 ml of sterile nutrient broth in 250-ml Erlenmeyer flasks to develop the inoculum. The inoculated flasks were incubated at 25°C for 24 hr on a rotary shaker (240 rev/min, 2-inch stroke). A 5-ml amount of the resulting culture served to inoculate 50 ml of bioconversion media in 250-ml Erlenmeyer flasks. A suitable enzyme inducer (usually progesterone), at a concentration between 0.5 and 5 mg dissolved in 0.5 ml of ethanol, was added to each bioconversion flask at the time of inoculation. The inoculated flasks were incubated for 48 hr at 25°C, at which time the required substrate dissolved in 0.5 ml of dimethylformamide was added with stirring, and incubation continued for another period of time (between 24 and 120 hr).

Extraction of transformation products. At the end of incubation, the whole fermentation broth was extracted twice with ethyl acetate-benzene (1:4). The extract was dried over sodium sulfate and evaporated to dryness under vacuum.

TLC. The dry residue was dissolved in 5 ml of methanol-benzene (1:1) and a measured volume spotted on thin-layer chromatography (TLC) plates coated with silica gel G. The plates were developed for 2.5 hr in 10% collidine in carbon tetrachloride, dried in air, and sprayed with a solution of 1% p-nitrobenzene diazonium fluoroborate in 50% acetic acid to reveal the red spots of equilin (Rf about 0.75). The density and area of the spots were compared with those of known standards of equilin spotted on the same plate.

GLC. The crude extract was partially purified before submitting to gas-liquid chromatography (GLC). A 3-mg amount of crude extract was dissolved in 0.2 ml of methanol-benzene (1:1) and spotted as a streak on a silica gel G thin-layer plate (2 by 3 inches; 5.08 by 7.62 cm). The plate was developed in benzene-ethyl acetate (70:30) for about 10 min, air dried, and observed under shortwave blackray ultraviolet (UV) lamp. Two well separated bands of phenolic ketones (estrone, equilin, equilenin, and Δ4-estrene) and phenolic alcohols (estradiol and dihydro-equilenin) were obtained. The bands were scraped and eluted with acetone. The elution efficiency of standards from thin-layer plates was 80%. The acetone extracts were dried and subjected to GLC. The phenolic ketones were transformed into trimethylsilyl ether derivatives and chromatographed on 2: 5% DEGS on GASCHROM Q at a column temperature of 200°C (G. Schilling, Ayerst Research Laboratories, personal communication).

Spectrophotometric assay of equilin. The assay of equilin is based on the specific blue color (635 nm) which develops when an alcoholic solution of equilin is heated in the presence of the equilin reagent (U.S. Pharmacopoeia, 1970, 18th ed.). A linear curve is obtained for concentrations between 5 and 10 μg/ml. Equilenin, estrone, and Δ4-estrene, the other most common phenolic products, do not react. This assay was used as a screening procedure.

The equilin reagent is prepared as follows. To 145 g of freshly distilled and cooled phenol, 90 ml of concentrated sulfuric acid are added and mixed until the phenol is liquefied. The mixture is allowed to stand in the dark for 16 to 24 hr. An 84-ml amount of the mixture is added to 280 ml of 50% sulfuric acid (v/v).
Table 1. Aromatization of 19-hydroxy-androsta-4,7-diene-3,17-dione (AY-14,022) into equilin by various microorganisms

| Microorganism and Ayerst collection no. | Equilin detected | Microorganism and Ayerst collection no. | Equilin detected |
|--------------------------------------|------------------|----------------------------------------|------------------|
| Arthrobacter simplex B-197           | +                | N. asteroides B-233                    | -                |
| A. simplex B-239                     | -                | N. asteroides B-236                    | -                |
| Arthrobacter sp. B-297               | +                | N. canicuria B-285                     | ++               |
| Bacillus sphaericus B-189            | +++              | N. convoluta B-229                     | +                |
| Bacterium cyclooxydans B-194         | ++               | N. corallina B-195                     | +++              |
| Corynebacterium hofmanni B-251       | +                | N. corallina B-230                     | ++               |
| C. simplex B-190                     | -                | N. corallina B-231                     | ++               |
| C. simplex B-191                     | +++              | N. corallina B-299                     | +                |
| Microminospora sp. B-119             | -                | N. erythropolis B-286                  | +                |
| Microminospora sp. B-203             | -                | N. erythropolis B-300                  | ++               |
| Microminospora sp. B-223             | -                | N. formica B-238                       | -                |
| Mycobacterium fortuitum B-302        | -                | N. leishmania B-232                    | -                |
| M. rhodocerus B-234                  | -                | N. opaca B-298                         | +++              |
| M. rhodocerus B-235                  | -                | N. restrictus B-226                    | +++              |
| M. rhodocerus B-287                  | -                | N. rubra B-118                         | +++              |
| M. rhodocerus B-301                  | +                | Nocardia sp. B-288                     | +                |
| Mycobacterium sp. B-241              | -                | Pseudomonas aeruginosa B-177           | -                |
| Mycobacterium sp. B-243              | -                | P. cruciviae B-282                     | +                |
| Nocardia asteroides B-227            | ++               | P. dacunhae B-281                      | ++               |
| N. asteroides B-228                  | ++               | Pseudomonas sp. B-283                  | -                |
|                                    |                  | Streptomyces lavendulae B-207          | -                |

* +, Equilin detected; -, equilin not detected.

Table 2. Media used for the aromatization of AY-14,022 into equilin with Nocardia rubra AY B-118

| Ingredients (g/liter)* | Medium no. |
|-----------------------|------------|
|                       | 1 2 3 4 5 6 7 8 9 10 11 12 13 |
| Beef extract (Difco)  | 3 3 4 3 4.0 5 3 |
| Peptone (Difco)       | 5 5 5 1 1 3 10 3 1 |
| Yeast extract (Difco) | 1 1 5 20 3 1 10 3 1 |
| Tryptone (Difco)      | 5 5 20 |
| Malt extract (Difco)  | 10 10 |
| Neopeptone (Difco)    | 5 20 5 20 |
| Edamine (Sheffield Chemical) | 5 5 10 6 |
| Corn steep liquor     | 10 10 |
| N-Z-Case (Sheffield Chemical) | 5 5 10 6 |
| Amber meat protein hydrolyzate (Amber Labs) | 5 5 10 6 |
| Sheffield soy powder  | 5 5 10 6 |
| Glucose               | 2.5 1 20 40 20 4.4 4.4 2.5 8.8 8.8 |
| NaCl                  | 2.5 2 40 80 80 40 80 80 |
| K2HPO4                | 4.4 4.4 2.5 2.5 2.5 2.5 |
| KH2PO4               | 8.8 8.8 8.8 8.8 8.8 8.8 |

* pH adjusted to 7.0 before sterilization.

A solution consisting of 5.6 ml of concentrated hydrochloric acid and 2.4 ml of a 0.1% cobalt nitrate solution, brought to 28 ml with distilled water, is added to the above mixture. The reagent is heated to 95°C in a water bath and maintained at that temperature for 30 min. The reagent is then cooled and stored in amber bottles. Samples of unknown solutions were prepared by pipetting 5 ml of bioconversion broth into a glass centrifuge tube, shaking the mixture with 2 ml of methylene chloride, and centrifuging. The supernatant fluid was removed by aspiration. The methylene chloride extract was evaporated to dryness under nitrogen. The residue was dissolved in 2.5 ml of freshly distilled ethanol, mixed well and centrifuged; 0.5 ml of the ethanol solution was used for spectrophotometric assay. The blank consisted of the same broth free of substrate and similarly extracted.
The test was run as follows. A standard solution of equilin containing 20 to 30 µg and an unknown extract containing about the same quantity of equilin were added separately to clean glass tubes. The volume in each tube was brought to 0.5 ml with ethanol. The culture broth extract was used as a blank. To each tube, 5.0 ml of the equilin reagent was added; all tubes were kept in an ice bath during this manipulation. After addition of the equilin reagent, all tubes were transferred to a boiling water bath for 9 min and then cooled rapidly. The absorbance was read at 635 nm by using a Beckman DK-IA spectrophotometer.

Preparation of mutants. The growth of N. rubra on a large nutrient agar slant was suspended in 20 ml of saline, the suspension was poured into a 250-ml Erlenmeyer flask containing glass beads, and the flask was agitated at 28 C for 30 min on a rotary shaker operating at 240 rev/min (2-inch stroke) to break clumps and chains of cells. The suspension was filtered through glasswool to remove remaining clumps, and the resulting filtered suspension was diluted to read 50% transmittance at 660 nm (1-cm diameter cuvette) in a Coleman model Junior 6-A spectrophotometer; this corresponded to about 10^6 cells per ml. A petri plate containing 15 ml of the adjusted suspension was placed on a rotary shaker (80 rev/min) 20 inches below a GE Mineralite UV lamp in dimmed light. Samples were taken at 0, 2.5, 5, 10, 15, 20, and 30 min, diluted, and plated on Emerson's agar; viable counts were determined after 72 hr of incubation at 28 C. The dilutions corresponding to >99% kill were then replated to obtain 400 to 500 isolated colonies which were transferred to Emerson's agar slants and incubated for 48 hr at 28 C. After incubation, all slants were kept at 4 C.

RESULTS

Some 40 organisms belonging to the genera Arthrobacter, Bacillus, Bacterium, Corynebacterium, Micromonospora, Mycobacterium, Nocardia, Pseudomonas and Streptomyces were screened for their ability to aromatize 19-hydroxy-androsta-4,7-diene-3,17-dione (AY-14,022) into equilin. TLC and visual observation of the equilin spot were used as criteria. The best bioconversion was obtained with N. rubra AY B-118. The transformation yield as determined by GLC was 15%. The results are presented in Table 1.

Effect of the medium. Thirteen different media (Table 2) were used to evaluate the bioconversion of AY-14,022 with N. rubra. The best bioconversion (20%) was obtained in medium 11 which was used for subsequent studies.

Transformation by survivors of the mutagenic treatment. About 400 strains of N. rubra which survived the mutagenic treatment were screened for their ability to transform AY-14,022 into equilin. Some typical results are given in Table 3. The best mutant (AY B-329) transformed 30 to

| TABLE 3. Bioconversion of AY-14,022 into equilin by survivors of Nocardia rubra AY B-118 |
|---------------------------------|---------------------------------|---------------------------------|
| Experiment no. | No. of survivors tested | No. of mutants more active than parent | Activity ratio (mutant to parent) |
|----------------|------------------------|------------------------------------|-------------------------------|
| 1              | 30                     | 4                                  | 1.72 to 1.95                  |
| 2              | 31                     | 5                                  | 1.18 to 1.73                  |
| 3              | 38                     | 5                                  | 1.25 to 1.75                  |
| 4              | 27                     | 8                                  | 1.25 to 1.5                   |
| 5              | 28                     | 10                                 | 1.1 to 1.9                    |
| 6              | 29                     | 5                                  | 1.3                           |
| 7              | 30                     | 5                                  | 1.1 to 1.5                    |
| 8              | 30                     | 5                                  | 1.5                           |
| 9              | 30                     | 1                                  | 1.5                           |

* A total of 338 mutants of N. rubra (AY B-118) were screened, and a mutant labeled 7.5-30 (AY B-329) in experiment no. 8 was selected for further work.

| TABLE 4. Effect of preincubation of Nocardia rubra mutant AY B-340 in the presence of various inducers on the bioconversion of AY-14,022 into equilin |
|---------------------------------|---------------------------------|---------------------------------|
| Inducer                        | Per cent transformation into equilin at various concn of inducer |
|--------------------------------|------------------------|-----------------|-----------------|
|                                | 10 µg/ml | 25 µg/ml | 50 µg/ml | 100 µg/ml |
| None                           | 5     | 19.5    | 28.0    | 34.0        |
| Progesterone                   | 21.5  | 19.5    | 26.0    | 31.0        |
| Testosterone                   | 19.5  | 23.5    | 26.0    | 32.0        |
| Androst-4-ene-3,17-dione       | 19.5  | 23.0    | 26.0    | 32.0        |
| Reichstein's compound S.       | 17.5  | 23.0    | 23.0    | 28.0        |

| TABLE 5. Effect of substrate concentration and incubation on bioconversion of AY-14,022 into equilin with Nocardia rubra AY B-340 |
|---------------------------------|---------------------------------|---------------------------------|
| Substrate (mg/ml) | Incubation time (hr) | Transformation into equilin (%) |
|-------------------|----------------------|--------------------------------|
| 0.1               | 24                   | 45                             |
| 0.1 to 0.5*       | 120                  | 44                             |
| 0.4               | 24                   | 29                             |
| 1.0               | 24                   | 12                             |
|                   | 48                   | 17                             |
|                   | 72                   | 12                             |
|                   | 96                   | 30                             |
|                   | 120                  | 38                             |

* A 0.1-mg amount was added every 24 hr until 120 hr to give a final concentration of 0.5 mg/ml
35% of the substrate into equilin. Upon further irradiation of strain AY B-329 and selection of the survivors, a mutant (AY B-340) was isolated which in 6 hr consistently transformed AY-14,022 into equilin in yields higher than 50% at a substrate charge of 0.4 mg/ml.

**Effect of various inducers.** Progesterone, testosterone, androst-4-ene-3,17-dione, and Rechstein’s compound S were compared as possible inducers of the bioconversion system (Table 4). Although all the compounds tried induced the aromatization enzyme system, progesterone at 0.1 mg/ml was found to be the most effective inducer.

**Effect of substrate concentration and incubation time.** Substrate was added at a single charge of 0.1, 0.4, and 1.0 mg/ml and in successive addition of 0.1 mg/ml every 24 hr to a final concentration of 0.5 mg/ml. The results are presented in Table 5. About 40% of the substrate charged at 1 mg/ml could be transformed into equilin in 120 hr. Traces of estrone, Δ⁴-estrone, equilenin, and dihydroequilin were also produced.

**Transformation of other substrates.** Several other 19-nor and 19-hydroxy derivatives of the androstane series were subjected to bioconversion with *N. restrictus*, *N. rubra*, and *N. rubra* mutant AY B-340. The results are presented in Table 6. Most of the substrates had a double bond in position 7 and, if transformed, yielded mainly equilin with traces of equilenin, estrone, and Δ⁸-estrone. The substrates saturated in position 7 yielded estrone and Δ⁸-estrone. All 7-dehydro-19-hydroxy and 7-dehydro-19-nor substrates bearing a ketonic or hydroxyl group in position 3 could be transformed in fair yields into equilin, with traces of estrone, Δ⁴-estrone, equilenin, dihydroequilin, and dihydroequilenin. When the hydroxy group in position 3 was replaced by the corresponding methyl ether, in one case (AY-12,156) no bioconversion was obtained, and in the other case (AY-12,153) only traces of equilin were noticed on TLC. The presence of an acetoxy group in position 3 or 19 of the molecule reduced the bioconversion rates and yields significantly.

**DISCUSSION**

In an earlier paper (1), it was reported from this laboratory that 19-hydroxy-androsta-4,7-diene-3,17-dione (AY-14,022) could be trans-

| Name and no. | Organism | Results |
|--------------|----------|---------|
| **19 nor series** | | |
| Estra-4,7-diene-3,17-dione (AY-12,158) | *Nocardia rubra* mutant | Up to 50% into equilin |
| Estra-5,7-diene-3,17-dione (AY-12,154) | *N. rubra* mutant | Bioconversion into equilin (30-40%) |
| Estra-5(10),7-diene-3,17-dione (AY-12,130) | *N. restrictus* | Good bioconversion into equilin |
| 17β-Hydroxyestra-4,7-dien-3-one acetate (AY-3,867) | *N. restrictus* | Good bioconversion into equilin |
| 17β-Hydroxyestra-5(10),7-dien-3-one (AY-9,054) | *N. restrictus* | Good bioconversion into equilin |
| 3-Hydroxyestra-3,5,7-trien-17-one acetate (AY-14,636) | *N. restrictus* | Traces of equilin |
| 3-Methoxyestra-3,5,7-trien-17-one (AY-12,156) | *N. restrictus* | No bioconversion |
| Estra-3,5,7-triene-3,17β-diol diacetate (AY-3,868) | *N. restrictus* | Traces of equilin |
| **19-hydroxy series** | | |
| 19-Hydroxyandrost-4,7-diene-3,17-dione (AY-14,022) | *N. rubra* mutant | Up to 50% bioconversion into equilin |
| Androsta-4,7-diene-3β,17β,19-triol (AY-14,028) | *N. rubra* | Bioconversion into equilin (40%) |
| Androsta-5,7-diene-3β,17β,19-triol (AY-14,021) | *N. restrictus* | Good bioconversion into equilin |
| 19-Hydroxyandrost-4,7-diene-3,17-dione acetate (AY-14,638) | *N. restrictus* | Traces of equilin formed |
| 3,19-Dihydroxy-androst-3,5,7-trien-17-one diacetate (AY-14,026) | *N. rubra* | Little or no bioconversion |
| 19-Hydroxy-3-methoxyandrost-3,5,7-trien-17-one (AY-12,153) | *N. restrictus* | Traces of equilin |
| 3,19-Dihydroxy-androst-3,5,7-trien-17-one 3-acetate (AY-12,124) | *N. restrictus* | Traces of equilin |
| 19-Hydroxy-androst-4,6-diene-3,17-dione (AY-14,040) | *N. restrictus* | Estrone and Δ⁸-estrone only |
formed to equilin with *N. restrictus* in yields of about 20%. However, the substrate charge was only 0.1 g/liter. Screening of other nocardiae revealed that *N. rubra* was superior to *N. restrictus*. Irradiation of *N. rubra* led to the isolation of a survivor which under optimum conditions transformed 40% of the substrate charged at 1 g/liter; these improvements make the process economically feasible. Bioconversion of various substituted substrates showed that 19-hydroxy- and 19-nor-3-hydroxylated substrates were transformed equally well although 19-nor compounds were transformed at a faster rate. The bioconversion yields were low when either position 3 or 19 or both were acetylated. When 3-methylether derivatives were used, no bioconversion was observed. It seems that acetate in position 3 or 19 is hydrolyzed to free alcohol before the substrate is aromatized; consequently, the yields of bioconversion are limited by the rate and yield of hydrolysis. However, when a stable group such as a methyl ether was introduced, the bioconversion was blocked.

ACKNOWLEDGMENTS

The technical assistance of Cécile Bolduc, Ching Yu Liang-Matula, and Evelyn Straja is gratefully acknowledged. We are thankful to G. Schilling and his group for gas-liquid chromatography analysis.

LITERATURE CITED

1. Bagli, J. F., P. F. Morand, K. Wiesner, and R. Gaudry. 1964. A simple synthesis of equilin. Tetrahedron Lett. No. 8:387–389.
2. Bowers, A., J. S. Mills, C. Casas-Campillo, and C. Djerassi. 1962. New approaches to C-11 oxygenated-19-nor-pregnanes. J. Org. Chem. 27:361–365.
3. Dodson, R. M., and R. D. Muir. 1961. The microbial aromatization of steroids. J. Amer. Chem. Soc. 83:4627–4631.
4. Gual, C., R. I. Dorfman, and S. R. Stitch. 1961. Aromatization of ring A, cleavage of ring D and oxidation of side chain in certain 19-nor-steroids. Biochim. Biophys. Acta 49:387–389.
5. Zedric, J. A., H. Carpio, A. Bowers, and C. Djerassi. 1963. The synthesis of equilin. Steroid 1:233–249.