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BIOTRANSFORMATION OF PROGESTERONE BY THE GREEN ALGA *CHLORELLA EMERSONII* C211-8H

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Key Word Index—*Chlorella emersonii*; C211-8h; progesterone; biotransformation; hydroxyprogesterones; testololactone.

Abstract—2β-Hydroxyprogesterone, 6β-hydroxyprogesterone, 9α-hydroxyprogesterone, 14α-hydroxyprogesterone, 16α-hydroxyprogesterone and 21-hydroxyprogesterone are the main bioproducts in the progesterone bioconversion by axenic cultures of *Chlorella emersonii* C211-8h. After 30 days 6β,9α-dihydroxyprogesterone, 6β,21-dihydroxyprogesterone and 16α,21-dihydroxyprogesterone are also formed. In acid conditions the reaction is slower and 2β-hydroxyprogesterone and 16α-hydroxyprogesterone have not been found among the bioproducts. In hyperhaline conditions the yields are generally lower and testololactone is also formed.

INTRODUCTION

The potential use of freshwater microalgae as bioreactors is being investigated by our group [1]. In a study of the effects of some Rhodophyta and Chlorophyta on progesterone (**1**) we found that C211-8h *Chlorella emersonii* induced hydroxylation of substrate yielding, as the main bioproducts, 2β-hydroxyprogesterone (**2**), 6β-hydroxyprogesterone (**3**), 9α-hydroxyprogesterone (**4**), 14α-hydroxyprogesterone (**5**), 16α-hydroxyprogesterone (**6**) and 21-hydroxy-progesterone (**7**) [2].

We have now examined the influence of the reaction time upon the reaction yields as well as the effects of the stress conditions such as the acidity and the hyperhalinity of the medium on the course of the bioconversion. Under stress, remote metabolic pathways might be activated, thus inducing different bioproduct syntheses.

RESULTS AND DISCUSSION

In all of the three procedures employed, sterile progesterone (**1**) was added to an axenic culture of *C. emersonii* in BBM during the exponential phase of growth of the strain, with an algal concentration about 6×10^5 cells ml^{-1}. Previous experiments showed that the extreme acid and hyperhaline conditions for this strain were at pH 3.5 by H_2SO_4 and at 3% NaCl. The reactions were monitored after 7, 15, 30, 45 and 60 days.

Under the conditions employed the alga induced bioconversion of progesterone, whereas the substrate was not significantly modified in a sterile medium control. In the standard procedure after 7 days 11% substrate was biotransformed and after 60 days only 20% was unreacted. In hyperhaline conditions the reaction was slower and at pH 3.5 started after about 3 weeks; at the end of the experiments 68 and 93% progesterone, respectively, were bioconverted (Fig. 1).

We also examined the bioproducts when running the biotransformations in semipreparative conditions. The products were isolated by HPLC and identified by ^1H NMR [3], ^{13}C NMR [4] and mass spectrometry. In the standard conditions (see Experimental) the major bioproducts were 2β-hydroxyprogesterone (**2**), 6β-hydroxyprogesterone (**3**), 9α-hydroxyprogesterone (**4**), 14α-hydroxyprogesterone (**5**), 16α-hydroxyprogesterone (**6**) and 21-hydroxyprogesterone (**7**). During the course of the experiment, the amounts of **2** and **3** increased progressively, giving 7.6% and 29.6% final yields; compounds **4**, **6**, and **7** were found at the highest concentrations between 15 and 30 days, whereas **5** remained almost constant (Fig. 2).

After the first three weeks, minor components were formed in the reaction mixture and their spectroscopic analysis showed the presence of dihydroxylated compounds. The main compounds were identified as 6β,9α-dihydroxyprogesterone (**9**), 6β,21-dihydroxyprogesterone (**10**) and 16α,21-dihydroxyprogesterone (**11**). These compounds could be formed by a further hydroxylation of bioproducts **4**, **6** and **7**, thus giving a possible explanation of the reduction of their amounts in the second month of the experiment.

In the culture of C211-8h grown under hyperhaline conditions the same bioproducts of the control were found but, in addition, a testololactone (**8**) [5] was isolated (Table 1). In these conditions the percentages of

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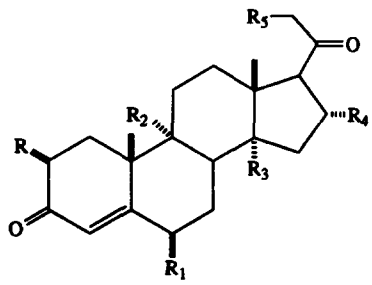
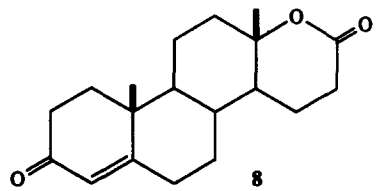


1 R = R_1 = R_2 = R_3 = R_4 = R_5 = H
2 R = OH R_1 = R_2 = R_3 = R_4 = R_5 = H
3 R_1 = OH R = R_2 = R_3 = R_4 = R_5 = H
4 R_2 = OH R = R_1 = R_3 = R_4 = R_5 = H
5 R_3 = OH R = R_1 = R_2 = R_4 = R_5 = H
6 R_4 = OH R = R_1 = R_2 = R_3 = R_5 = H
7 R_5 = OH R = R_1 = R_2 = R_3 = R_4 = H
9 R_1 = R_2 = OH R = R_3 = R_4 = R_5 = H
10 R_1 = R_5 = OH R = R_2 = R_3 = R_4 = H
11 R_4 = R_5 = OH R = R_1 = R_2 = R_3 = H



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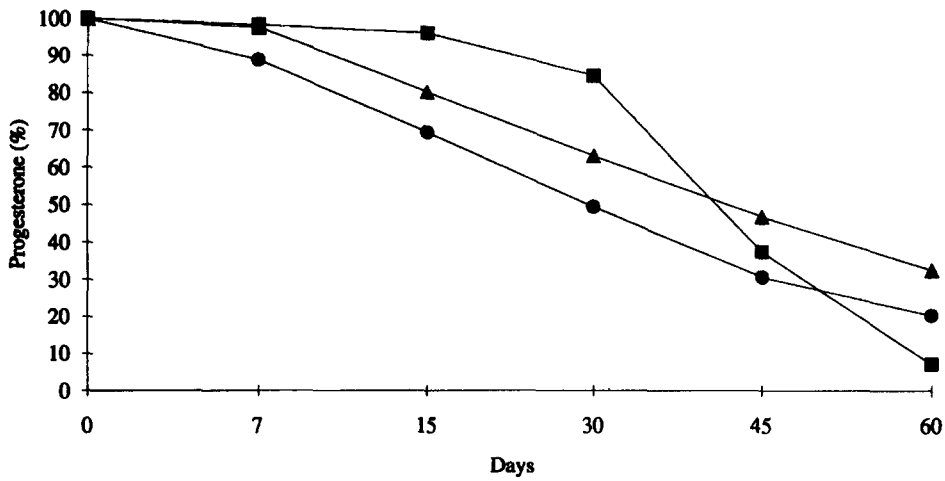

Fig. 1. Percentage bioconversion of progesterone in BBM (●), in BBM with 3% NaCl (▲) and in BBM at pH 3.5 (■).

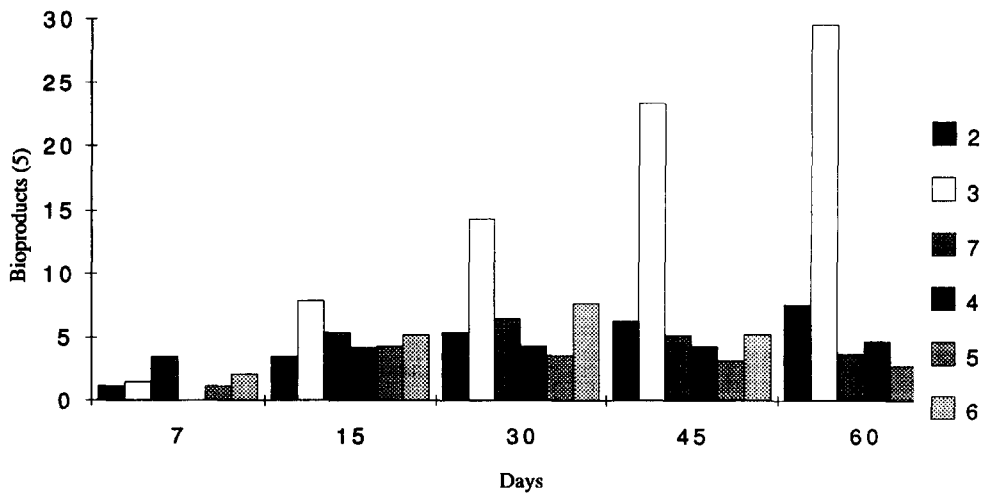

Fig. 2. Percentage of isomeric hydroxyprogesterones formed under standard conditions (see Experimental).

Table 1. Percentages of bioproducts produced in the bioconversion of progesterone in hyperhaline conditions

Days	2	3	4	5	6	7	8
7	—	1.1	—	—	1.0	—	1.0
15	—	3.3	1.4	1.2	2.5	—	1.7
30	1.1	4.9	2.4	2.3	5.8	1.4	4.2
45	1.3	13.4	2.7	4.7	6.2	3.0	4.8
60	2.0	17.1	3.1	9.8	7.6	4.8	5.2

Table 2. Percentages of bioproducts produced in the bioconversion of progesterone in acid conditions

Days	2	3	4	5	6	7
7	—	—	—	—	—	—
15	—	—	—	—	—	—
30	—	4.1	1.4	1.3	—	1.6
45	—	4.6	6.3	2.7	—	3.1
60	—	7.4	20.8	3.8	—	4.9

all the products progressively increased during the experiment and the overall presence of dihydroxylated products resulting was significantly lower.

The analysis of bioconversion products of strain C211-8h grown at pH 3.5 showed significant modifications and detectable amounts of the bioproducts were present after only 3 weeks. Compounds **2** and **6** were not found, **3** and **5** occurred in amounts lower than those observed in the standard conditions whereas the percentage of compound **4** after 60 days was considerably higher (Table 2).

The idea of utilizing microalgae for the production of fine chemicals is fascinating [6], but much remains to be done. It is evident that one of the major problems is the low yields of bioproducts. Moreover, in the standard conditions (see Experimental) a further conversion of the products occurs, thus leading to the formation of a large number of compounds present in very reduced amounts. As these results show, the management of environmental conditions can influence the quality of the algal bioconversions, limiting the number of the products obtained. In addition, low pH and high salinity of the medium strongly reduce the growth of bacteria and the predation by small invertebrates, which are the main constraints of the mass culture of microalgae both in open ponds and in closed bioreactors [7, 8].

EXPERIMENTAL

The strain C211-8h *C. emersonii* was supplied by The Culture Collection of Algae and Protozoan, Ambleside, Cumbria, U.K. In the standard procedure progesterone (**1**) (100 mg sterilized at 100° for 1 hr), dissolved in dioxane (0.7 ml) was added to the axenic culture of *C. emersonii* in BBM (50 ml) [9] during the exponential phase of growth of the strain. The inoculum was about 1.7×10^5 cells ml^{-1} and after 5 days the algal concn was 6×10^5 cells ml^{-1}. To obtain the acid conditions, a sterile soln of 1 M H_2SO_4 was added to the medium until pH 3.5. The desired NaCl concentration was obtained by adding NaCl (1.5 g) to the medium. A control of progesterone in sterile BBM was used in each experiment. The suspensions were stirred at room temp. with a photoperiod of 16 hr light/8 hr dark and samples (4 ml) were collected after 7, 15, 30, 45 and 60 days. The samples were extracted with EtOAc (2 × 10 ml) and *n*-butanol (2 × 10 ml) and the organic layers after evapn of the solvent were GC monitored on a Fractovap 4160 chromatograph (Carlo Erba) equipped with a 30 m glass capillary column coated with OV-101 at 280°C. In semiprep. conditions progesterone (500 mg) was added to the cultures (500 ml). After 60 days the suspensions were extracted with EtOAc (2 × 200 ml) and *n*-butanol (2 × 200 ml). The acetate extract from the standard procedure was evapd and the residue was chromatographed on a silica gel column eluting with benzene–Et_2O to give frs A–E. Fr. A (4:1) consisted of progesterone (**1**) and 9α-hydroxyprogesterone (**4**) which were separated by RP-HPLC on a Varian VISTA 5500 apparatus equipped with a Hibar LiChrosorb RP-8 column using MeOH–H_2O (4:1) as eluent. Fr. B (7:3) consisted of 2β-hydroxyprogesterone (**2**) which was purified by RP-HPLC with MeOH–H_2O (3:2). Fr. C (1:1) consisted of 21-hydroxyprogesterone (**7**) which was purified by prep. TLC (benzene–EtOAc, 3:2). Fr. D (3:7) was rechromatographed on RP-HPLC with MeOH–H_2O (3:2) to give 14α-hydroxyprogesterone (**5**). Fr. E (Et_2O) consisted of 6β-hydroxyprogesterone (**3**) and 16α-hydroxyprogesterone (**6**) which were sepd by RP-HPLC (MeOH–H_2O, 11:9). The butanolic extract was chromatographed on RP-HPLC (MeOH–H_2O, 7:3) to give 6β,9α-dihydroxyprogesterone (**9**), 6β,21-dihydroxyprogesterone (**10**) and 16α,21-dihydroxyprogesterone (**11**). From the reaction mixt. in hyperhaline conditions RP-HPLC (MeOH–H_2O, 3:2) of fr. A of the silica gel CC gave testololactone (**8**). EI-MS of the bioproducts were performed on a Kratos MS 50 apparatus. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AM 400 spectrometer.

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