MICROBIAL CHAIN DEGRADATION OF CHOLESTEROL BY ARTHROBACTER SIMPLEX

F. Naghibi, M. Tabatabai-Yazdi*, M.R. Noori-Daloii+, M.A. Faramarzi and F. Farnia

Department of Biotechnology, School of Pharmacy, Tehran University of Medical Science, Tehran, Islamic Republic of Iran
+ Department of Biochemistry, School of Medicine, Tehran University of Medical Science, Tehran, Islamic Republic of Iran

Abstract

Because of the importance of microbial metabolites of cholesterol as precursors in the synthesis of steroidal drugs, we decided to study the ability of *Arthrobacter simplex* to degrade cholesterol. *Arthrobacter simplex* cleaves the side chain of cholesterol in the presence of 2,2'-dipyridyl and produces 4-androstene-3,17- dione (AD) and androsta-1,4-diene-3,17-dione(ADD). Two other intermediates with intact side chain, 4-cholestene-3-one and cholesta-1,4-diene-3-one were also detected and purified in the culture of *A. simplex*.

Introduction

Biotransformations have been used for many years in the chemical synthesis of pharmaceutical products. Because of the structural similarity of cholesterol to steroidal drugs and its abundant occurrence at a relatively low price, it can be considered as a potential raw material for the production of precursors for synthesis of these drugs.

In order to utilize cholesterol as a raw material in the partial synthesis of steroidal drugs, the side chain of cholesterol must be cleaved, giving rise to a C-19 or C-21 structure [1,2]. Conventional chemical oxidation of the saturated aliphatic C-17 side chain results in very low yields [3]. As an alternative to chemical methods, the microbial removal of the aliphatic side chain of cholesterol offers a promising method for use of these sterols.

In 1913, Söhngen [3] reported that mycobacteria are capable of decomposing cholesterol when it is used as the sole source of carbon in medium. In systematic studies on the cholesterol decomposing activity of 1589 microbial strains, Arima *et al.* [4] observed complete degradation of cholesterol in the genera *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Bacillus*, *Protaminobacter*, *Serratia* and *Microbacterium*.

The complete degradation of cholesterol or other sterols is, however, of no commercial interest. An important goal of microbial side chain cleavage is to produce C-19 or C-22 structures with intact steroid nucleus [5]. It has been found that the sterol ring structure and the side chain were metabolized by different mechanisms. The enzymatic reactions did not follow a given order but occurred simultaneously and independently [1].

The key enzyme in sterol ring fission, 9 a-hydroxylase, is a monoxygenase containing Fe^{++} as essential metal ion. Removal or replacement of this ion results in the complete inactivation of the enzyme which in turn inhibits degradation of the sterol ring [6].

In this paper, we report the capability of *A. simplex* PTCC 1036 to convert cholesterol to androst-4-ene-3,17 dione (AD) and androsta-1,4-diene-3,17-dione (ADD) in the presence of some chelating agents and some metal ions. In this way it also produces 4-cholestene-3-one and cholesta-1,4 diene-3- one as intermediates with intact side chain.

Keywords: *Arthrobacter simplex*; Biodegradation; Cholesterol

Experimental Section

The microorganism *Arthrobacter simplex* PTCC 1036 was obtained from the Persian Type Culture Collection PTCC (ATCC 6946, NCTC 4215). *A. simplex* was precultured in flasks containing medium A consisting of 3.7% brain heart infusion (oxoid) at 30°C and 150 rounds per minute.

The growth cycle of *A. simplex* was studied and it was found that after six hours the culture was in the exponential phase. The production medium B was inoculated with 10% of medium A. The production medium B was selected from three media previously examined. One of these media was a synthetic medium in which *A. simplex* did not grow rapidly. The two others were complex media in which the growth rate of *A. simplex* did not differ very much.

Medium B consisted of nutrient broth 0.8 g, yeast extract 0.1 g, myoinositol 0.1 g, K_2HPO_4 0.2 g, water to 100 ml, pH was adjusted to 7.0 before sterilization. The flasks containing inoculated medium B were placed in an incubator-shaker at 30°C and 150 rounds per minute.

The substrate was added to the medium after 20 hours and the enzyme inhibitor after 24 hours, under sterile conditions. The substrate was 0.1% cholesterol and the enzyme inhibitors were three chelating agents and three metal ions as listed below:

2,2'-Dipyridyl	1 mM
0-Phenantroline	0.1 mM
8-Quinolinol	1 mM
Cobalt sulphate	10 mM
Nickel sulphate	1 mM
Copper sulphate	1 mM

Two weeks after inoculation the broth was extracted with chloroform and concentrated at 50°C in a rotary evaporator. The conditions in the other experiments were the same.

The second experiment was performed with five different concentrations of copper sulphate (0.5, 0.1, 0.05, 0.01, 0.005 mM). The third experiment was carried out with 2,2'-dipyridyl as inhibitor to determine the molecular structure of biotransformation products. The dried extract was fractionated by chromatography on a column of silica gel (35-70 mesh) (2.5 cm× 60 cm) and eluted with chloroform. Three fractions were obtained. The first and third fractions were separated on preparative thin layer plates. Separation of the first fraction into compound I and compound II was carried out on silica gel G plates (20 cm × 45 cm) and developed with chloroform: ethylacetate: carbon tetra chloride 4: 2: 4. Separation of the third fraction into compound III and compound IV was carried out on silica gel G plates (20 cm × 45 cm) and developed with chloroform: ethylacetate: benzene 1: 1:1.

Another experiment (No. 4) was designed to determine the best concentration of cholesterol as substrate for microbial biotransformation. Five media were prepared containing 0.1%, 0.2%, 0.3%, 0.5%, 1%, 2% cholesterol, respectively.

Mass spectra of the purified compounds were recorded with a Finningan mat TSQ-70. FTIR spectra were recorded with a Magna-IR spectrometer 550 (Nicolet Co.), HNMR and ^{13}CNMR spectra were recorded with V-NMR 400 (Varian). Melting points were taken on a Kofler hot stage apparatus and are uncorrected.

For analytical thin layer chromatography, 250 um thick silica gel pre-coated plates with a 254 nm fluorescent indicator from Sigma company were used. For preparative thin layer chromatography, we used Kieselgel 60 HF (Art. 7741) from the Merck company.

Results and Discussion

In the first experiment, chromatography of the concentrated extract on thin layer plates showed that cholesterol was degraded by *A. simplex* in five of the six media and that the degradation products in the first five media were similar. Different concentrations of copper sulphate were used as inhibitor in the next experiment but we could not detect any steroid-containing degradation product in any of the media. This is in contrast with a report from Shah *et al.* [7], who found that $CuSO_4$ could be used for selective side chain degradation of sterols.

In the fourth experiment we wanted to find out if higher concentrations of cholesterol would lead to better results or higher yields of C-19 metabolites. We obtained the highest concentration (by means of colour intensity in TLC) of ADD with 0.3% W/V cholesterol as substrate. Higher concentrations of substrate changes the conditions of the medium so that it produces lower yields.

In the third experiment, we isolated four metabolites. Their purity was tested by TLC. The physico-chemical properties of the isolated compounds are described below:

1- RF values of compounds I, III and IV in thin layer chromatography were identical with the authentic samples of cholesta-4-ene-3-one, androst-4-ene-3,17-dione and androsta-1, 4-diene-3,17-dione, respectively.

2- Mass spectrometry (EI):

Compound I: MS m/z 384(M, C H O), 342, 299, 260, 229, 124

Compound II: MS m/z 382 (M, C H O), 122

Compound III: MS m/z 286 (M, C H O), 244, 200, 148, 124

Compound IV: MS m/z 284 (M, C H O), 266, 172, 159, 122

The mass spectra of compounds I, III and IV were identical with those of authentic samples.

3- Nuclear Magnetic Resonance

Compound I: HNMR (400 MHz, $CDCL_3$) δ 5.708 (S, 1H, H_4) ^{13}CNMR (100 MHz, $CDCL_3$) δ 199.50 (C_3) δ 171.54 (C_5), δ 123.73 (C_4) δ 56.05, δ 55.84, δ 53.77, δ42.34, δ39.59, δ39.47, δ38.55, δ36.08, δ35.73, δ35.66, δ35.56, δ33.95, δ32.92, δ32.01, δ28.17, δ27.99, δ24.16, δ23.79, δ22.84, δ22.57, δ20.99, δ18.63, δ17.35, δ11.94

Compound II: HNMR* (80 MHz, $CDCL_3$) δ7.1 (d, 1H, H_1), δ6.3 (d, 1H, H_4) δ 6.1 (s, 1H, H_2)

Compound III: HNMR (400 MHz, $CDCL_3$) δ 5.73 (s, 1H, H_4) ^{13}CNMR (100 MHz, $CDCL_3$) δ 220.27(C_{17}), δ 199.05 (C_3), δ 170.16 (C_5) δ 123.92 (C_4), δ 53.60, δ 50.61, δ 34.92 (C_8, C_9, C_{14}), δ 47.30, δ 38.44 (C_{10}, C_{13}), δ35.56, δ35.49, δ33.73, δ32.37, δ31.08, δ30.54, δ21.56, δ 20.11, (C_1, C_2, C_6, C_7, C_{11}, C_{12}, C_{15}, C_{16}), δ17.19, 13.53 (C_{18}, C_{19}).

Compound IV: HNMR (400 MHz, $CDCL_3$) δ6.970 (d, 1H, H_1), δ 6.128 (d, 1H, H_2), δ 5.978 (s, 1H, H_4) ^{13}CNMR (100MHz, $CDCL_3$) δ219.79 (C_{17}), δ 186.01 (C_3), δ 168.26 (C_5), δ155.25, δ127.52, δ123.93, δ52.11, δ50.23, δ34.92 (C_1, C_2, C_4, C_8, C_9, C_{14}), δ47.54, δ43.29 (C_{10}, C_{13}) δ35.51, δ32.42, δ32.17, δ31.05, δ21.95, δ21.78 (C_6, C_7, C_{11}, C_{12}, C_{15}, C_{16}), δ 18.62, δ 13.70 (C_{18}, C_{19})

The HNMR and ^{13}CNMR spectra of compounds I, III and IV were identical with those of authentic samples.

4- Infrared spectroscopy:
Compound I: FTIR(KBr) λ_{max} 1680 cm^{-1} (S, C=0)
Compound III: FTIR(KBr) λ_{max} 1670 cm^{-1} (S, C=0)
λ_{max} 1730 cm^{-1}(S, C=0)

Compound IV: FTRI (KBr) λ_{max}1640 cm^{-1} (S, C=0)
λ_{max} 1720 cm^{-1} (S_1 C=0)
The IR-spectra of compounds I, III and IV were identical with those of authentic samples.

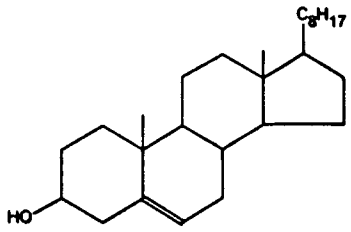
5- Melting points of three microbial metabolites were:
Compound I: 84-87°C
Compound III: 176-178°C
Compound IV: 143-145°C

Spectral and chromatographical data of three compounds, I, III, and IV were identical with the three substances named above (Scheme I).

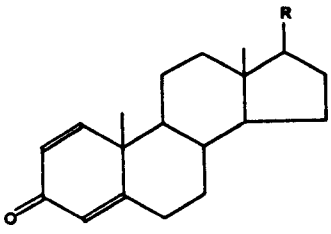
Compound I: cholest-4-ene-3-one

" III: androst-4-ene-3,17-dione
" IV: androsta-1,4-diene-3,17 dione

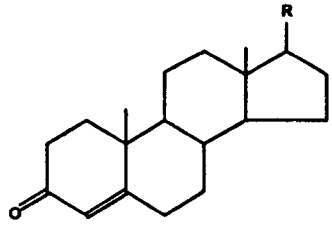
HNMR and mass spectrum of compound II isolated



Cholesterol



R= -C_8H_{17}(II) Cholesta-1,4-diene-3,17-dione
R= -C=0 (IV) Androsta-1,4-diene-3,17-dione



R= -C_8H_{17} (I) Cholest-4-ene-3-one
R= -C=0 (III) Androst-4-ene-3,17-dione

Scheme I

* Spectrum was recorded with a Bruker AC-80.

from microbial broth together with the information reported by previous workers [6] confirmed for us that II is cholesta-1,4-diene-3,17-dione (Scheme I).

Conclusion

Arthrobacter simplex PTCC 1036 is a potent microorganism for degrading cholesterol and can degrade its side chain selectively to produce C-19 metabolites which can be utilized as precursors in the synthesis of steroidal drugs. Two steroidal intermediates with intact side chains were also isolated from the microbial broth.

Acknowledgements

The authors would like to thank the National Research Center for Genetic Engineering and Biotechnology for its financial support of this study.

References

1. Martin, C.K.A. Sterols. In *Biotechnology*, Vol. 6a, (ed. J. Rehm and G. Reed), p. 79, Biotransformations (1986).
2. Nagasawa, M., Bae, M., Tamura, G. and Arima, K. Microbial transformation of sterols-part II. *Agr. Biol. Chem.*, **33**, 1644, (1969).
3. Martin, C.K.A. Microbial cleavage of sterol side chain. *Adv. in Appl. Microbiol.*, **22**, 29, (1977).
4. Arima, K., Nagasawa, M., Bae, M. and Tamura, G. Microbial transformation of sterols-part I. *Agr. Biol. Chem.*, **33**, 1636, (1969).
5. Ahmad, S., Garg, S.K. and Johri, B.N. Biotransformation of sterols: selective cleavage of the side chain. *Biotech. Adv.*, **10**, 1-67, (1992).
6. Sebek, O.K. and Perlman, D. Microbiol transformation of steroids and sterols. In *Microbiol technology-microbiol processes*, Vol. 1, (ed. Peppler-Perlman), p. 483, (1979).
7. Shah, K., Mehdi, I., Khan, A.W. and Vova, V.C. *Eur. J. Appl. Microbiol. Biotech.*, **10**, 167, (1980).
8. Nagasawa, M., Hashiba, H., Watanabe, N., Bae, M., Tamura, G. and Arima, K. Microbial biotransformation of sterols-part IV. *Agr. Biol. Chem*; **34**, 801, (1970).