Biotransformation of progesterone to valuable steroids by isolated strain of *Streptomyces*

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Introduction and purpose
The possibility of using of novel marine actinomycetes as a source of production of some industrially important hydroxyl steroid derivatives in one-step biotransformation process of progesterone was investigated in this study.

Materials and methods
Genomic DNA of the actinomycetes isolate was extracted using GeneJET Genomic DNA purification kit. The actinomycetes isolate was identified by 16S rDNA. The molecular sizes of amplified regions were confirmed using agarose gel electrophoresis. The identified *Streptomyces* strain was inoculated into the transformation medium, which was supplemented with progesterone as a substrate (5 mg/50 ml medium). The transformation products were separated and characterized on the basis of their infrared/nuclear magnetic resonance/mass spectrometry analysis as pregnenolone (**1**), 3β-hydroxyandrost-4-ene-17-one (**2**), and testosterone (**3**).

Results and conclusion
Because of the excellent track record of actinomycetes in this regard, new actinomycetes strain isolated from marine sources was used in this investigation. The isolated strain was identified by 16S rDNA. The 16S rDNA region was amplified using PCR (about 500 bp) using universal primers. According to sequencing similarities and multiple alignments, the isolate was found to be closely related to *Streptomyces tunisiensis* strain CN-207 with 98% identity. The infrared spectrum, ^{1}H-nuclear magnetic resonance, and mass spectral database was used in the identification of the three isolated compounds 1, 2, and 3. The isolated steroid molecules from the total extract (0.3 g) are pregnenolone (**1**), 3β-hydroxyandrost-4-ene-17-one (**2**), and testosterone (**3**).

Keywords:
^{1}H-nuclear magnetic resonance, mass spectroscopy, microbial transformation, progesterone, *Streptomyces* spp

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Introduction

The importance in the production of steroid drugs and hormones was realized for the first time in 1952 when Murray and Peterson of Upjohn Company patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* spp. [1,2]. A variety of steroids are widely used as antirheumatic, anti-inflammatory, immunosuppressive, diuretic, sedative, and anabolic; recent applications of steroid compounds include the HIV infections, treatment of some forms of cancer, and treatment of declared AIDS [3]. In the last decade, there was a suggestion that some steroids such as pregnenolone and progesterone and their derivatives (e.g. allopregnenolone) may act as allosteric modulators of neuronal receptors [4,5]. Progesterone also plays an important role in the repair of myelin sheath of damaged nerves [6,7].

Introduction of a hydroxyl group to a steroid molecule is one of the most important steps in the preparation of various steroidal derivatives. In microbial hydroxylation, hydroxylase enzyme can introduce a hydroxyl group to various positions of the steroid molecule [8]. Hydroxyl derivatives were reported with high biological activity than its precursor substrate, about half of the discovered bioactive secondary metabolites are produced from them [9]. Many microorganisms have been widely discovered to be great steroid hydroxylators, mainly some *Streptomyces* spp. The conversion of 20-carbonyl to 20β-hydroxyl in yield averaging between 20 and 75% resulted from the incubation of corticosteroid with *Streptomyces griseus* [10]. As regards the use of *Streptomyces roseochromogenes*, there have been numerous reports for the transformation of a large variety of substrates. The production of 16α-hydroxyestrone and 17β-hydroxyestrone was a result of incubation of estrone [11]. As there is excellent track record of actinomycetes in this respect, a large number of research studies have been

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focused on the successful isolation of novel actinomycetes from marine sources to achieve the biotransformation of progesterone. Exogenous progesterone was converted to 16α-monohydroxy and 2β,16α-dihydroxyprogesterone by *S. roseochromogenes* NCIB 1098 using the site-selective cytochrome P450 that catalyze the hydroxylation of steroid progesterone [12].

In this study, we examined the new strain of *Streptomycetes* isolated from some marine algae and identified by 16S rDNA for biotransformation of progesterone, a pharmaceutically steroid substance, and the characterization of the encountered steroid derivatives was outlined.

Materials and methods

Samples of some marine algae were collected from the coastal region in Sharm El-Sheikh, Egypt. All samples were collected in sterilized plastic bags. After surface sterilization, fragments of ~1 cm^3 from the inner part of each macro-organism were inoculated on Petri dishes using three different procedures. The first was by spreading the fragments on different recipes of agar plate's media. The second was by placing the fragments without spreading on agar plates containing the same media recipes. The third procedure was performed using inoculation of homogenized fragments in agar plates. Homogenized fragments were placed in test tubes with 10 ml of sterilized sea water. Thereafter, serial dilutions were obtained to 10^{-2}, and 100 µl of each dilution was inoculated. Petri dishes were incubated at 25°C.

Microorganism and cultivation conditions

The marine actinomycetes strain *Streptomyces* isolated from some algal samples was used in the present study. The organism was maintained on starch casein agar slants and incubated for 7–10 days at 28°C, and then maintained at 4°C until further use. Inoculum was prepared from 7-day-old culture of the strain. Five milliliters of sterile distilled water was added to the slants having optimum growth. Cells were scratched with sterilized inoculating needle and the tube was gently shaken and transferred to 50 ml of inoculum medium, which was previously sterilized in an autoclave. The flasks were then incubated at 28°C with shaking at 180 rpm for 10 days [13].

Polymerase chain reaction 16S rDNA and electrophoresis

Genomic DNA of the actinomycetes isolate was extracted using GeneJET Genomic DNA purification kit (#K0721; Thermo Scientific, Waltham, USA). The 16S rDNA was amplified with PCR using primers designed to amplify the 1500 bp fragment of the 16S rDNA region. The forward primer was 5′-AGA GTT TGA TCC TGG CTC-3′and the reverse primer was 5′-GGT TAC CTT GTT ACG ACT T-3′. PCR was performed using Maxima Hot Start PCR Master Mix (#K1051; Thermo Scientific), and the mixture consisted of 20 µmol/l of each primer, 5 µl of template DNA, and 25 µl Maxima Hot Start PCR Master Mix (2×) in total volume 50 µl water (nuclease free). The PCR was carried out for one cycle of DNA denaturation at 95°C for 10 min, 35 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 1 min and 30 s, and one additional cycle of a final extension was performed at 72°C for 10 min. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis. Sequencing to the PCR product has been performed on GATC Company using ABI 3730xl DNA sequencer (ABI, Dusseldorf, Germany). The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Faraday Ave, Carlsbad).

DNA sequencing

PCR products were purified using Qiagen extraction kit (Qiagen, Zurich, Switzerland) according to the manufacturer's instructions before applying to DNA sequencer. Sequencing was performed using the automated fluorescent dye terminator sequencing method originally developed (DYEnamicET Terminator Cycle Sequencing kit, ABI 3130; Amersham Pharmacia Biotech).

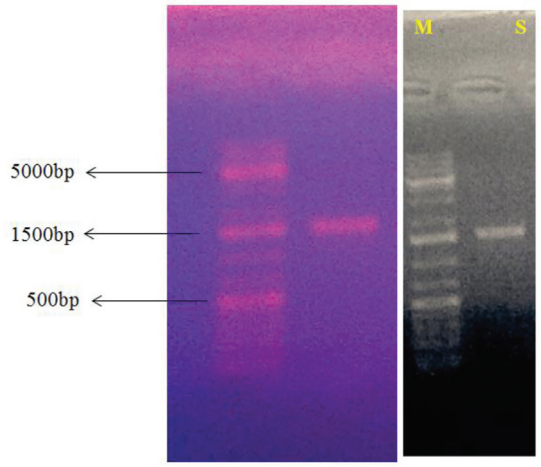
Bioconversion

The experiments were performed in 250 ml Erlenmeyer flasks, containing 50 ml of starch casein broth (contained in g/l: starch, 10; casein, 0.3; KNO_3, 2; NaCl, 2; K_2HPO_4, 2; $MgSO_4$, 0.7; H_2O, 0.05; $CaCO_3$, 0.02; $FeSO_4$, 0.7; H_2O, 0.01; pH, 7), supplemented with 5 mg of Progesterone in 1 ml ethanol with free (2 g wet cell paste) and extended to 5 days, in a rotary shaker (180 rpm) at 30°C. At the end of the transformation period, the medium was extracted with twice its volume with chloroform. The extraction was repeated three times. The organic phase was collected and evaporated to dryness. The dried solids (test material) were then dissolved in chloroform and fractionated using preparative thin layer chromatography (TLC) using solvent system of benzene/ethyl acetate/acetone (4 : 1 : 1, v/v/v) and detected under ultraviolet (UV) lamp [7,14]. After detection under UV light, the fractions were marked and cut from the plates. The metabolites were eluted from the adsorbent using chloroform.

Chromatographic fractionation

The dried chloroform extract (0.3 g) was chromatographed on silica gel 60 column with step gradient elution using chloroform/methanol (9 : 1, v/v) with increasing percentage of methanol. Fractions (25 ml) were collected while the fractionation processes were monitored by means of TLC analysis. The plates were examined under UV (short and long wave length) and p-anisaldehyde/H_2SO_4 spray reagent. Methanol containing desired steroid substance was identified by means of ^{1}H-nuclear magnetic resonance (NMR)/infrared (IR) and mass spectrometry (MS) for through matching of compounds on database [15].

Figure 1



Electrophoresis of PCR products for 16S rRNA of selected isolate. Lane M (molecular weight marker 1 kb DNA ladder); lane S is the PCR product of the selected isolate.

Infrared and ^{1}H-nuclear magnetic resonance spectroscopy

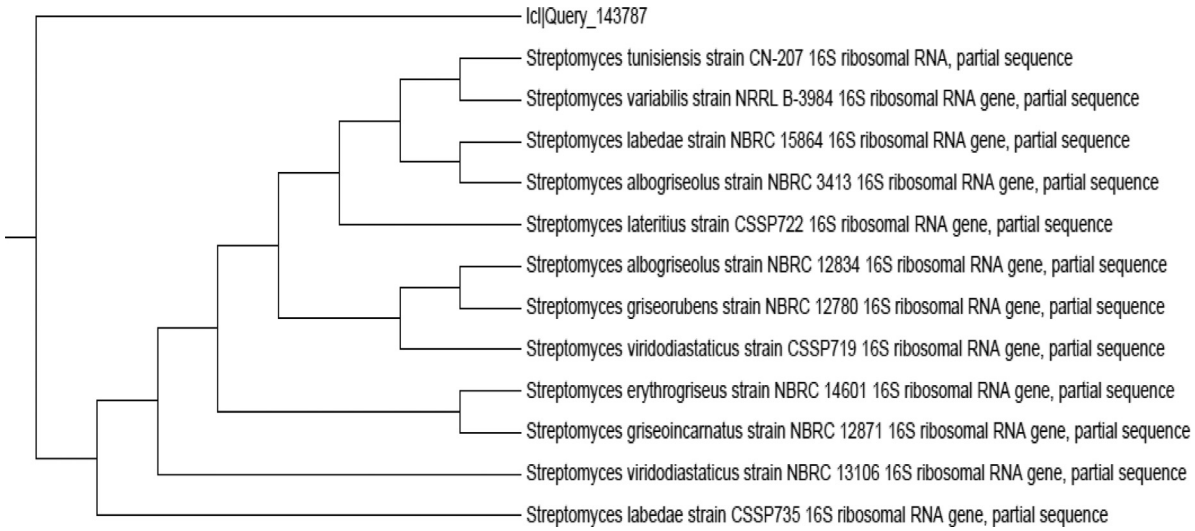
The IR spectra were recorded (KBr) on a Perkin-Elmer 1430 spectrometer (National Research Center, Dokki, Giza, Egypt). The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in δ ppm scale on a JEOL ECA 500 spectrometer (JEOL), run at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR (National Research Centre), with TMS ($SiMe_4$) as internal standard. The coupling constant values are reported in Hz. Mass spectra were recorded on GCMS-QP 1000 EX (Shimadzu, Tokyo, Japan) (gas chromatography-MS). All reactions were followed and the purity of the newly synthesized compounds were assessed using TLC Merck Alufolien Kieselgel Silica Gel 60 F254 (aluminum sheets, 20 cm×20 cm; Merck, Darmstadt, Germany) using $CHCl_3/CH_3OH$ (9 : 1, v/v) as eluent and detected using UV absorption at 254 nm.

Mass spectrometry

Analyses

Gas chromatograph equipped with a mass spectrometric detector (Agilent 6890) with a direct capillary interface and fused silica capillary column PAS-ms (30 m×0.25 mm internal diameter×0.25 µm film thickness) was used. The samples were injected under the following conditions: helium was used as a carrier gas at ~1 ml/min pulsed splitless mode. The solvent delay was 3 min and the injection size was 1.0 µl. The mass spectrophotometric detector was operated in electron impact ionization mode at an ionizing energy of 70 eV scanning *m/z* 50–550. The ion source temperature was 250°C and the quadruple temperature was 150°C. The electron multiplier voltage was maintained at 1550 V above autotune. The instrument

Figure 2



The phylogenetic tree based on PCR product sequencing of DNA isolated from the selected isolate.

was manually tuned using perfluorotributylamine. Wiley mass spectral database was used in the identification of the separated peaks [16].

Results

Identification and phylogenetic analysis

The isolate producing the maximum yield was selected as the promising producer. Molecular identification of this isolate based on 16S rDNA sequencing was carried out. A fraction of the PCR mixture was examined using agarose gel electrophoresis (Fig. 1). According to sequencing similarities and multiple alignments, the isolate was found to be closely related to *Streptomyces tunisiensis* strain CN-207 with 98% identity. The phylogenetic tree was displayed using the Tree View program (Tree view company, Wiltshire, UK) (Fig. 2).

Characterization of the different transformation products

The enzymatic activities of *S. tunisiensis* strain CN-207 on progesterone in 7 days led to the formation of the three products, **1** and other two hydroxy-derived progesterone compounds (compounds **2** and **3**). Three progesterone derivatives were characterized through the application of MS technique (Table 1). MS was used for the identification of a large number of steroids due to its high chromatographic resolution capacity and reproducible ionization efficiency of the derivatization procedures used before injection of the sample. Bowden *et al.* [17] reported that derivatization can be time consuming; it permits the profiling of additional compounds by allowing both polar and nonpolar steroid substances to be successfully and easily separated. From the following results we found that the chemical structures of the isolated steroids **1**, **2**, and **3** were achieved using different spectral data.The IR spectrum of compound **1** displayed broad band at υ 3435 cm^{-1} corresponding to –OH group, υ 2929 cm^{-1} and υ 2860 cm^{-1} corresponding to aliphatic $-CH_3$ and $-CH_2$ groups, υ 1728 cm^{-1}–υ 1702 cm^{-1} for –CO groups, and υ 1648 for C=C group. Its ^{1}H-NMR reveals bands at δ 1 ppm, δ 1.3 ppm, and δ 2.3 ppm, three singlet signals attributed to three methyl groups, and 1.02–1.3 ppm, two multiplet signals corresponding to 16 protons for eight (CH_2) groups.

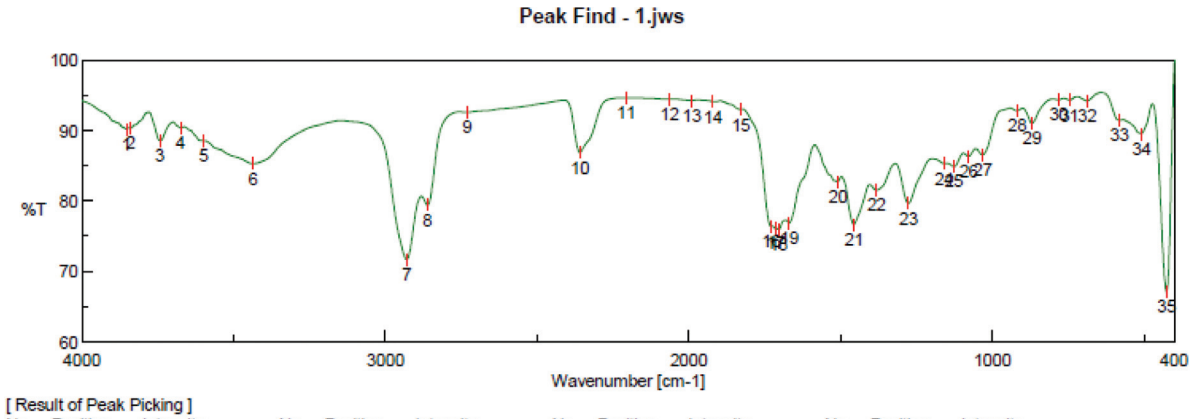
Table 1 Mass spectrometry analysis of the isolated compounds

Isolated compounds	R_f (min)	Molecular weight	Molecular formula
1	7.25	316.24	$C_{21}H_{32}O_2$
2	5.13	288.21	$C_{19}H_{28}O_2$
3	4.22	288.21	$C_{19}H_{28}O_2$

Infrared and ^{1}H-nuclear magnetic resonance spectra

The IR spectrum of compound **1** displayed broad band at υ 3435 cm^{-1} corresponding to –OH group, υ 2929 cm^{-1} and υ 2860 cm^{-1} corresponding to aliphatic $-CH_3$ and $-CH_2$ groups, υ 1728 cm^{-1}–υ 1702 cm^{-1} for –CO groups, and υ 1648 for C=C group. Its ^{1}H-NMR spectrum reveals bands at δ 1 ppm, δ 1.3 ppm, and δ 2.3 ppm, three singlet signals attributed to three methyl groups, δ 1.02–1.3 ppm, two multiplet signals corresponding to

Figure 3



[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3853.08	90.1282	2	3839.58	90.2929	3	3742.19	88.5249	4	3674.69	90.3103
5	3597.56	88.4697	6	3435.56	85.2647	7	2929.34	71.6234	8	2860.88	79.3603
9	2730.71	92.5164	10	2358.52	86.8017	11	2206.17	94.579	12	2063.46	94.3828
13	1991.14	94.1921	14	1920.75	94.0406	15	1829.15	92.8968	16	1728.87	76.3418
17	1714.41	76.0496	18	1703.8	75.9093	19	1669.09	76.7952	20	1509.03	82.6668
21	1455.99	76.5859	22	1381.75	81.4771	23	1275.68	79.5938	24	1159.01	85.2602
25	1126.22	84.827	26	1078.01	86.2775	27	1031.73	86.5105	28	917.95	92.7672
29	868.774	90.9779	30	781.029	94.2989	31	745.352	94.2632	32	687.498	94.1195
33	580.469	91.4311	34	508.151	89.5121	35	425.227	67.0017			

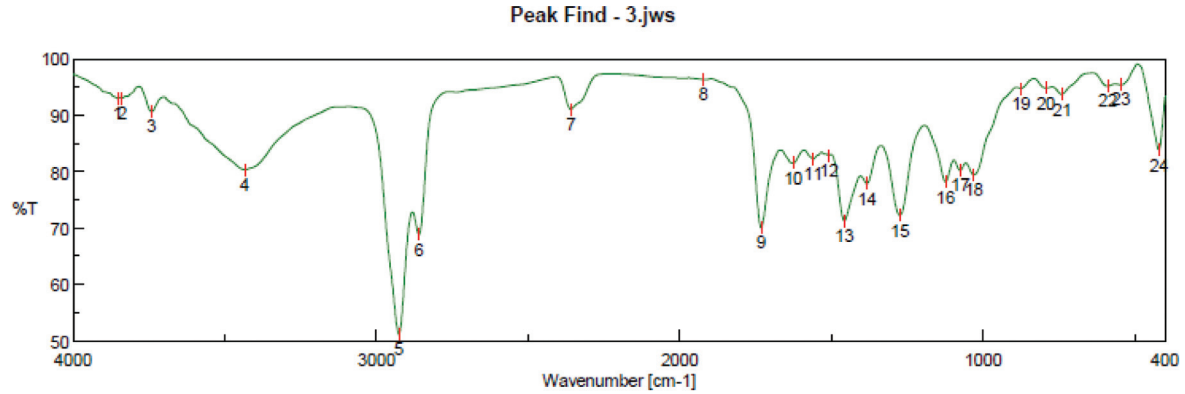
The infrared spectrum of compound **1**.

nineteen protons for eight CH_2 groups and three methine protons (SP^3), δ 2.16 ppm, triplet signal corresponding to one methane proton, δ 2.35 ppm, broad singlet signal for hydroxyl group, and δ 5.27 ppm, for methane proton (SP^2). Its mass spectra (*m/z*, %) was 316 (100%) (Fig. 3).

The IR spectrum of compound **2** displayed broad band at υ 3437 cm^{-1} corresponding to –OH group, υ 2928 cm^{-1} and υ 2861 cm^{-1} corresponding to aliphatic $-CH_3$ and $-CH_2$ groups, υ 1730 cm^{-1} for –CO group, and υ 1648 for C=C group. Its ^{1}H-NMR spectrum reveals bands at δ 1.02 ppm, δ 1.03 ppm, and δ 2.03 ppm, two singlet signals attributed to two methyl groups (six protons), δ 1.33–1.4 ppm and δ 2.0 ppm, three multiplet signals corresponding to 19 protons for eight (CH_2) groups and three methine protons (SP^3), δ 3.48 ppm, broad singlet signals corresponding to OH group, δ 4.20 ppm for methine proton(SP^3), and δ 5.30 ppm, multiplet signal for methane proton (SP^2) broad. Its mass spectra was (*m/z*, %) 288 (30%) (Fig. 4).

The IR spectrum of compound **3** displayed broad band at υ 3435 cm^{-1} corresponding to –OH group, υ 2925 cm^{-1} and υ 2858 cm^{-1} corresponding to aliphatic $-CH_3$ and

Figure 4

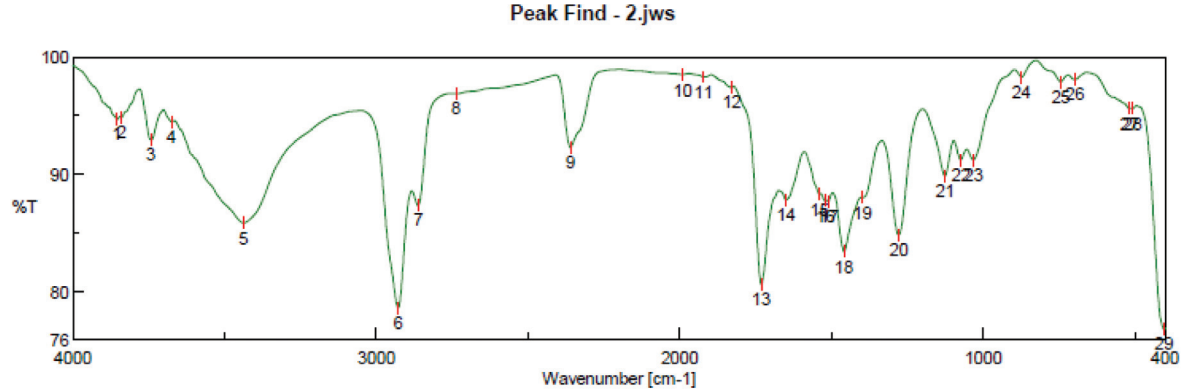


[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3853.08	92.9616	2	3837.65	93.0415	3	3741.23	90.6477	4	3434.6	80.3402
5	2925.48	51.1983	6	2858.95	68.8185	7	2358.52	91.0362	8	1921.72	96.3049
9	1731.76	70.0507	10	1626.66	81.5225	11	1559.17	82.2263	12	1508.06	82.8687
13	1456.96	71.2308	14	1382.71	77.8838	15	1273.75	72.2326	16	1123.33	78.1359
17	1075.12	80.2131	18	1029.8	79.4583	19	875.524	94.7256	20	792.6	94.7777
21	740.531	93.6768	22	589.147	95.155	23	545.756	95.4107	24	419.442	83.7982

The infrared spectrum of compound **2**.

Figure 5

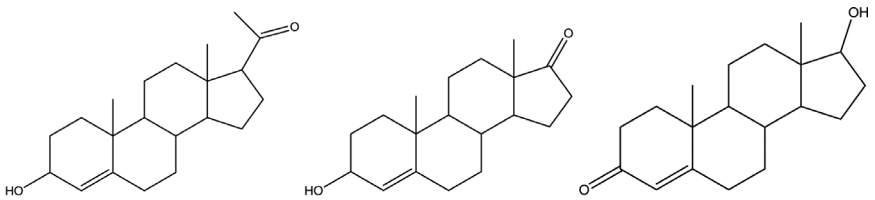


[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3854.04	94.6977	2	3839.58	94.8673	3	3742.19	92.9247	4	3674.69	94.4905
5	3437.49	85.8834	6	2928.38	78.6248	7	2861.84	87.3611	8	2737.46	96.8679
9	2359.48	92.2816	10	1991.14	98.4856	11	1921.72	98.3011	12	1829.15	97.3835
13	1730.8	80.6287	14	1648.84	87.8392	15	1539.88	88.3064	16	1520.6	87.766
17	1509.03	87.715	18	1457.92	83.4203	19	1397.17	88.0403	20	1277.61	84.8291
21	1125.26	89.8943	22	1073.19	91.2422	23	1031.73	91.2542	24	873.596	98.2672
25	743.424	97.8933	26	698.105	98.079	27	516.829	95.623	28	507.187	95.6062
29	403.05	76.8145									

The infrared spectrum of compound **3**.

Figure 6



Pregnenolone **1** 3β-hydroxyandrost-4-ene-3-one **2** Teststerone **3**

The isolated steroid molecules from the total extract (0.3 g).

$-CH_2$ groups, υ 1731cm^{-1} for –CO group, and υ 1626 for C=C group. Its ^{1}H-NMR spectrum reveals bands at δ 1 ppm and δ 1.03 ppm, two singlet signals attributed to two methyl groups (six protons), δ 1.33–1.45 ppm, δ 1.48–1.6 ppm, and 1.95, three multiplet signals corresponding to 17 protons for seven (CH_2) groups and three methine protons (SP^3), δ 2.95 ppm, multiplet signals corresponding to methine proton (SP^3), δ 3.30 ppm, triplet signals for methine proton (SP^3), δ 3.60 ppm, broad singlet signals corresponding to OH proton, and δ 5.55 ppm, multiplet signal for methane proton (SP^2). Its mass spectra (*m/z*, %) was 288 (30%) (Fig. 5).

Conclusion

We can conclude that the transformation of progesterone can be carried out using *S. tunisiensis* strain CN-207 actinomycetes isolated from sediment samples and identified by 16S rRNA. The whole cells yielded valuable transformation products. These products were separated and characterized on the basis of their MS analysis. The isolated steroid molecules from the total extract (0.3 g) are pregnenolone (**1**), 3β-hydroxyandrost-4-ene-3-one (**2**), and testosterone (**3**). These results may be of considerable industrial importance in view of the formation of important derivatives from a single step biotransformation process of progesterone (Fig. 6).

Financial support and sponsorship

Nil.

Conflicts of interest

None declared.

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