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Regio- and stereoselective hydroxylation of bi- and tricyclic enones by fungal strain *Fusarium culmorum*

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Abstract

The strain of *Fusarium culmorum* AM282 was used to investigate hydroxylation of both enantiomers of 4a-methyl hexahydronaphtalenone and a related sesquiterpenoid enone: (1*S*)-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one. It was found that the position and stereochemistry of the introduced hydroxyl group depended on the structure of a substrate. The *S*-enantiomer of 4a-methyl-hexahydronaphtalenone was hydroxylated exclusively at the allylic, 8*R*-axial position, whereas the *R*-enantiomer mainly at the non-activated 6*S*-equatorial position (the mixture of (6*S*) and (8*S*) hydroxy derivatives in 6:1 ratio was formed). Tricyclic, sesquiterpenoid derivative of *R*-hexahydronaphtalenone was hydroxylated at the unique 11*S*-equatorial position. In a simple reaction, new hydroxylated chiral synthons: *trans*-(4a*S*,6*S*)-4,4a,5,6,7,8-hexahydro-6-hydroxy-4a-methyl-2(3*H*)-naphthalenone and (1*S*,11*S*)-11-hydroxy-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one were prepared.

Keywords: Biotransformation; Hydroxylation; Octalenone; Sesquiterpenoids; *Fusarium culmorum*

1. Introduction

Regio- and stereoselective introduction of a hydroxyl group into organic compounds of low molecular weight can provide optically pure derivatives, which are used as building blocks for further synthesis of various biologically active and commercially important natural products. The lack of specificity and the occurrence of undesired side reactions are a major drawback in the use of organic chemical methods for hydroxylation. Alternative to chemical methods are biotransformations using hydroxylating fungi [1].

Fusarium culmorum AM282 is a filamentous fungus that had been previously used for hydroxylation of 4-en-3-oxo steroid hormones and their derivatives [2,3]. We showed that the reactions were of significant regio- and stereoselectivity and that the position of the introduced hydroxyl group depended on the substrate structure. The hydroxylation took place at the equatorial positions of the steroid skeleton (C or D ring) whereas axial hydroxyl group was introduced mainly into the allylic position (B ring). Lack of a methyl group in the 1–3 *cis* position (at C-10) or an electrodonating or/and possessing free valence electrons substituents at C-4 promoted stereoelectronically favoured allylic hydroxylation [3].

In this study, in order to explore biocatalytic potential of this microorganism with respect to obtaining chiral (hydroxylated) synthons, conversion of (*R*)- and (*S*)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone **(1)** and derivative of its *R*-enantiomer: (1*S*)-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one **(4)** was investigated. On the other hand, we also deemed profitable to check how the alteration of configuration of methyl group at C-4a (this position is analogous to C-10 of steroid system) and the bulky cyclopropane moiety at the B ring influence on regio- and stereoselectivity of the hydroxylation by *F. culmorum*.

2. Experimental

2.1. Substrates

(*S*)-(+)- **(S-1)** and (*R*)-(−)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3*H*)-naphthalenone **(R-1)** were purchased from Merck. (1*S*)-1,4,4-Trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one **(4)** was obtained from Wrocław University of Technology, where it was prepared from (+)-carene.

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2.2. Microorganism

The microorganism *F. culmorum* AM282 used in this study was obtained from the collection of the Institute of Biology and Botany, Medical University of Wrocław.

2.3. Conditions of cultivation and transformation

The strain of *F. culmorum* was maintained on Sabouraud 4% dextrose agar slope and freshly subcultured before using in the transformation experiment.

Three hundred milliliters Erlenmeyer flasks, each containing 100 ml of medium consisting of 3% glucose and 1% peptone, were inoculated with a suspension of *F. culmorum* and incubated for 3 days at 20 °C in a rotary shaker. After this growth period, 25–30 mg of a substrate in 1 ml of acetone were added to each of the cultures, and the transformation was continued under the same conditions for 10–12 days (as long as the contents of substrate in the reaction mixture was changing; it was then checked that prolonged incubation time did not influence the extent of the substrate transformation). Conversion of the substrates was monitored by GC and TLC. Each experiment was performed with at least three parallels.

2.4. Isolation and identification of the biotransformation products

All the fermentation media were extracted three times with 15–20 ml of chloroform. Transformation products were separated by column chromatography on silica gel. TLC was carried out using Merck Kieselgel 60 F_{254} plates with hexane/acetone/chloroform (1:0.7:0.3 v/v) for **1**, and hexane/acetone (2.5:1 v/v) for **4** as eluents. These eluents were also used for preparative column chromatography. Visualization of products was performed by spraying the plates with 2% solution of $H_3[P(Mo_3O_{10})_4]$ and 1% solution of $Ce(SO_4)_2$ in 10% sulphuric acid, followed by heating to 120 °C. GC analysis was performed using a Hewlett Packard 5890A Series II GC instrument (FID, carrier gas H_2 at flow rate of 2 ml min^{-1}), equipped with a HP-1 column (cross-linked Methyl Silicone Gum, 25 m × 0.32 mm × 0.52 μm film thickness). Temperature program for **1**: 120 °C/1 min, gradient 8 °C/min to 160 °C, gradient 50 °C/min to 300 °C/3 min, injector temperature 200 °C, detector temperature 300 °C, and for **4**: 150 °C/1 min, gradient 4 °C/min to 220 °C, gradient 10 °C/min to 300 °C/2 min; injector and detector temperature 300 °C. Optical rotations were measured on an Autopol IV automatic polarimeter (Rudolph). Melting points (uncorrected) were determined on a Boetius apparatus. MS analyses were performed on a Varian Chrompack GC CP-3800 Saturn 2000GC/MS/MS with an ionizing energy of 70 eV. Structures of the biotransformation products were determined by means of ^{1}H NMR and ^{13}C NMR (1D and 2D COSY). The spectra were recorded on a DRX 300 MHz Bruker spectrometer and measured in $CDCl_3$ with TMS as an internal standard).

2.5. Biotransformation

2.5.1. (S)-(+)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3H)-naphthalenone (S-1)

After incubation of **S-1** (100 mg) in the culture medium for 12 days, 20% of the unreacted substrate (RR_t 4.32 min), and 71% of **2** (RR_t 6.02 min) were detected.

(4a*S*,8*R*)-4,4a,5,6,7,8-Hexahydro-8-hydroxy-4a-methyl-2(3*H*)naphthalenone **(2)** (56 mg, 56% isolated yield): crystals, m.p. = 70–71 °C (lit. m.p. = 72 °C, [5]); $[\alpha]_D^{20}$ + 96 (c 0.902, $CHCl_3$) (lit. $[\alpha]_D^{21}$ + 96.5 (c 1.025, $CHCl_3$), [5]); ^{1}H NMR, δ ppm, *J* Hz, 1.31 (1H, dt, *J* = 13.5, 3.5, H-5_{ax}), 1.41 (3H, s, CH_3-4a), 1.5 (1H, m, H-6_{eq}), 1.6 (1H, m, H-7_{ax}), 1.68 (1H, m, H-5_{eq}), 1.73 (1H, m, H-4_{eq}), 1.83 (1H, ddd, *J* = 14.5, 13.7, 4.5, H-4_{ax}), 2.0 (1H, m, H-7_{eq}), 2.05 (1H, m, H-6_{ax}), 2.34 (1H, dddd, *J* = 17.4, 4.5, 2.7, 1.1, H-3_{eq}), 2.55 (1H, ddd, *J* = 17.4, 14.5, 5.3, H-3_{ax}), 4.30 (1H, t, *J* = 2.8, H-8_{eq}), 5.79 (1H, br s, H-1); ^{13}C NMR, δ ppm: 16.1 (C-6), 24.1 (CH_3-4a), 33.2 (C-7), 34.3 (C-3), 35.3 (C-4a), 39.4 (C-4), 41.1 (C-5), 72.6 (C-8), 126.5 (C-1), 167.5 (C-8a), 200.7 (C-2); EIMS 70 eV *m/z* (relative abundance): 180 $[M]^+$ (46), 151 (100), 137 (19), 123 (50).

2.5.2. (R)-(−)-4,4a,5,6,7,8-hexahydro-4a-methyl-2(3H)-naphthalenone (R-1)

After incubation of **R-1** (120 mg) in the culture medium for 12 days, 10% of the unreacted substrate (RR_t 4.32 min) was detected. The main metabolite was **3** (70%) (RR_t 7.08 min). The minor product of transformation was **ent-2** (12%) (RR_t 6.02 min).

(4a*S*,6*S*)-4,4a,5,6,7,8-Hexahydro-6-hydroxy-4a-methyl-2(3*H*)naphthalenone **(3)** (77 mg, 54% isolated yield): crystals, m.p. = 96–97 °C; $[\alpha]_D^{20}$ − 195 (c 0.700, $CHCl_3$); ^{1}H NMR, δ ppm, *J* Hz: 1.24 (3H, s, CH_3-4a), 1.33 (1H, t, *J* = 12, H-5_{ax}), 1.40 (1H, dm, *J* = 14, H-7_{ax}), 1.80 (1H, ddd, *J* = 13.4, 5.5, 2.8, H-4_{eq}), 1.88 (1H, ddd, *J* = 14.2, 13.4, 4.5, H-4_{ax}), 1.96 (1H, dm, *J* = 12, H-5_{eq}), 2.14 (1H, dm, *J* = 14, H-7_{eq}), 2.34 (2H, m, H-3_{eq} and H-8_{eq}), 2.46 (2H, m, H-3_{ax} and H-8_{ax}), 4.04 (1H, tt, *J* = 11.2, 4.2, H-6_{ax}), 5.76 (1H, br s, H-1); ^{13}C NMR, δ ppm: 23.0 (CH_3-4a), 31.0 (C-8), 33.4 (C-3), 35.7 (C-7), 36.6 (C-4a), 38.0 (C-4), 49.8 (C-5), 66.5 (C-6), 124.7 (C-1), 167.3 (C-8a), 199.4 (C-2); EIMS 70 eV, *m/z* (relative abundance): 181 (59) $[M+H]^+$, 162 (100) $[M-H_2O]$, 152 (31) $[M-CO]$, 134 (81), 119 (47), 108 (47).

(4a*R*,8*S*)-4,4a,5,6,7,8-Hexahydro-8-hydroxy-4a-methyl-2(3*H*)naphthalenone **(ent-2)** (5 mg, 4% isolated yield): crystals, m.p. = 67 °C (lit. m.p. = 68–69 °C, [5]); $[\alpha]_D^{20}$ − 95 (c 0.511, $CHCl_3$) (lit. $[\alpha]_D^{21}$ − 95 (c 1.04, $CHCl_3$), [5]); ^{1}H NMR, δ ppm, *J* Hz, 1.31 (1H, dt, *J* = 13.5, 3.5, H-5_{ax}), 1.41 (3H, s, CH_3-4a), 1.5 (1H, m, H-6_{eq}), 1.6 (1H, m, H-7_{ax}), 1.68 (1H, m, H-5_{eq}), 1.73 (1H, m, H-4_{eq}), 1.83 (1H, ddd, *J* = 14.5, 13.7, 4.5, H-4_{ax}), 2.0 (1H, m, H-7_{eq}), 2.05 (1H, m, H-6_{ax}), 2.34 (1H, dddd, *J* = 17.4, 4.5, 2.7, 1.1, H-3_{eq}), 2.55 (1H, ddd, *J* = 17.4, 14.5, 5.3, H-3_{ax}), 4.30 (1H, t, *J* = 2.8, H-8_{eq}), 5.79 (1H, br s, H-1); ^{13}C NMR, δ ppm: 16.1 (C-6), 24.1 (CH_3-4a), 33.2 (C-7), 34.3 (C-3), 35.3 (C-4a), 39.4 (C-4), 41.1 (C-5), 72.6 (C-8), 126.5 (C-1), 167.5 (C-8a), 200.7 (C-2); EIMS 70 eV

m/z (relative abundance): 180 $[M]^+$ (42), 151 (100), 137 (20), 123 (51).

2.5.3. *(1S)-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one* ***(4)***

After incubation of **4** (90 mg) in the culture medium for 10 days, 34% of the unreacted substrate (RR_t 3.48 min) and 58% of **5** (RR_t 5.72 min) were detected.

(1*S*,11*S*)-11-Hydroxy-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]-undec-7-en-9-one **(5)** (30 mg, 33% isolated yield): amorphous material, $[\alpha]_D^{20}$ − 183 (c 0.633, $CHCl_3$); ^{1}H NMR, δ ppm, *J* Hz, 0.66 (1H, m, H-5), 0.82 (1H, m, H-3), 1.02 (3H, s, CH_3-14), 1.04 (3H, s, CH_3-12), 1.10 (3H, s, CH_3-13), 1.69 (2H, m, H-2_{ax} and H-6_{eq}), 2.30 (1H, dd, *J* = 15.2, 7.4, H-2_{eq}), 2.39 (1H, dd, *J* = 13.3, 7.2, H-6_{ax}), 2.51 (1H, dd, *J* = 17.6, 11.7, H-10_{ax}), 2.71 (1H, dd, *J* = 17.6, 5.8, H-10_{eq}), 4.05 (1H, dd, *J* = 11.7, 5.8, H-11_{ax}), 5.80 (1H, s, H-8); ^{13}C NMR, δ ppm: 14.6 (C-13), 19.2 (C-14), 19.4 (C-3), 19.6 (C-4), 24.8 (C-5), 28.0 (C-12), 28.8 (C-6), 29.6 (C-2), 42.2 (C-1), 42.8 (C-10), 70.7 (C-11), 124.2 (C-8), 173.2 (C-7), 197.9 (C-9); EIMS 70 eV, *m/z* (relative abundance): 221 (59) $[M+H]^+$, 203 (100) $[(M+H)-H_2O]^+$, 176 (20), 161 (15), 133 (22), 105 (28).

3. Results and discussion

The strain of *F. culmorum* AM282, which had already been used for hydroxylation of 4-en-3-oxo steroids [2,3] was also found to be active biocatalyst in hydroxylation of both enantiomers of 4a-methyl-hexahydronaphtalenone **1** and the related sesquiterpenoid enone **4**. However, consumption of these substrates was slower than steroids. What is more, in all cases some amount of unreacted substrate was detected in the reaction mixture. The results of transformation are presented in Table 1. All the products obtained proved to be secondary alcohols. The structures of substrates and their metabolites are showed in Fig. 1.

After 12 days of incubation of **S-1** a single product was obtained, along with 20% of the recovered substrate. The structure of this metabolite was identified by its ^{1}H NMR spectrum which shows a C*H*OH signal (triplet at 4.30 ppm, *J* = 2.8 Hz) typical of an equatorial proton. Simultaneously, the downfield shift of the 4a-methyl signal of **2** with respect to substrate ($\Delta\delta$ = +0.2 ppm as a result of a 1,3-diaxial interaction with a *cis*-OH group) confirmed the presence of 8-axial hydroxyl group. Moreover, compound **2** was found to be identical in every respect (m.p., specific rotation, ^{1}H NMR, ^{13}C NMR, MS) to *cis*-(4a*S*,8*R*)-4,4a,5,6,7,8-hexahydro-8-hydroxy-4a-methyl-2(3*H*)-naphthalenone reported in the literatures [4,5]. Thus, in cause of transformation of **S-1** allylic hydroxylation at C-8 was the only pathway.

Table 1
The results of hydroxylation of **1** and **4** by *F. culmorum*

Product of transformation (%)[a]						
S-1		**R-1**			**4**	
S-1	**2**	**R-1**	**ent-2**	**3**	**4**	**5**
20	71	10	12	70	34	58

[a] Determined by GC analysis of the crude chloroform extracts.

In contrast, 12-day incubation of **R-1** yielded two alcohols, along with 10% of the unreacted starting material. The main metabolite (70%) was *trans*-(4a*S*,6*S*)-4,4a,5,6,7,8-hexahydro-6-hydroxy-4a-methyl-2(3*H*)-naphthalenone **(3)**. The structure of this product was deduced from its ^{1}H NMR spectrum, which showed C*H*OH signal at 4.04 ppm (triplet of triplets), which could be ascribed to the axial either 6- or 7-proton. Finally, the 6-position of the newly introduced hydroxyl group was proved by 2D ^{1}H COSY spectrum, which shows no coupling between C*H*OH and 8-H signals. The equatorial (β) location of the hydroxyl group was elucidated by the observation of the magnitude of the coupling constants which were $^3J_{6ax,5ax} = {}^3J_{6ax,7ax} = 11.2$ Hz and $^3J_{6ax,5eq} = {}^3J_{6ax,7eq} = 4.2$ Hz. Additionally, such a stereochemistry of the hydroxyl group was supported by the low-field chemical shift of C*H*OH signal compared to 7α-H signal (δ = 3.56 ppm) (this position is analogous to C-7_{ax} of decalin system) of known 7β-hydroxyandrostenedione [6]. The spectroscopic data (^{13}C NMR, MS) of compound **3** corresponded to those described by Lafont and co-workers [7]. Since the authors assigned only the regions of resonating proton groups, detailed comparative analysis of ^{1}H NMR data was not possible. Nevertheless, the values of the chemical shift (4 ppm) and the coupling constants of the proton attached to the carbon atom bearing a hydroxyl group were comparable.

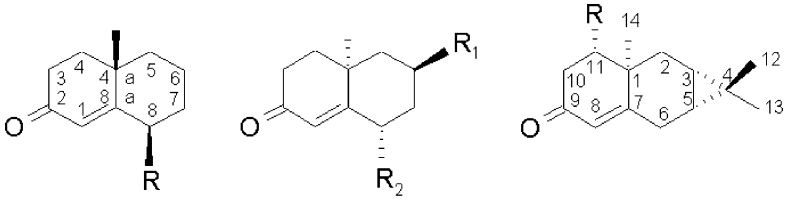

Fig. 1.

Spectroscopic and physical properties of **ent-2**, minor metabolite of **R-1**, were found to be identical in every respect (m.p., specific rotation, ^{1}H NMR, ^{13}C NMR, MS) to *cis*-(4a*R*, 8*S*)-4,4a,5,6,7,8-hexahydro-8-hydroxy-4a-methyl-2(3*H*)-naphthalenone reported in the literatures [4,5].

In the light of results described above, it was interesting to test how the hydroxylation pattern will be modified by the cyclopropyl moiety in **4**.

(1*S*)-1,4,4-Trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one **(4)** was hydroxylated by *F. culmorum* to only one product with moderate yield. The MS data of this metabolite indicated incorporation of one additional hydroxyl group into substrate ($[M+H]^+$ *m/z* 221, $[(M+H)-H_2O]^+$ *m/z* 203). The regio- and stereochemistry of the hydroxyl group were established by analyzing differences between ^{1}H NMR and ^{13}C NMR spectra of the substrate and product reaction. The ^{1}H NMR spectrum of **5** showed that C*H*OH signal was a doublet of doublets (δ 4.05 ppm) and ^{13}C NMR showed new oxygenated methine signal at 70.7 ppm. Because the signals of C-3 to C-9 and C-12 to C-13 remain unchanged in ^{13}C NMR spectrum, we concluded that the new hydroxyl group was situated either at C-10 or C-11. For reason of downfield shifts of the following signals: C-1 (+6.0 ppm), C-10 (+8.1 ppm), and up-field shift of C-14 signal (−5.8 ppm) with respect to the substrate, the location of OH group in metabolite **5** becomes apparent. The equatorial disposition of 11-OH group was indicated by coupling constants which were $^3J_{11ax,10ax} = 11.7$ and $^3J_{11ax,10eq} = 5.8$ Hz. Based on these observation, the metabolite **5** was tentatively assigned the structure (1*S*,11*S*)-11-hydroxy-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one. The analogous change in chemical shifts of the respective signals in comparison with the substrate for 1β(eq) hydroxy derivatives of steroids [8] (1β position is analogous to 11eq of compound **5**), confirmed the presence of (11*S*)-hydroxyl group in **5**.

To sum up, all of the described reactions were highly stereoselective, leading to single diastereomers of the hydroxylated products. The general tendency observed for the strain of *F. culmorum* is the hydroxylation of the equatorial or the quasi-equatorial C–H bonds. An axial hydroxyl group was introduced only into an allylic position.

A significant difference in the regioselectivity of hydroxylation of both enantiomers of **1** was observed. Transformations of the **S-1** by *F. culmorum* proceeded with regio- and stereoselectivity such as that observed for 4-en-3-ketosteroids [2,3]. This result is in contrast to described in the literature for *Rhizopus arrhizus* ATCC 11145. That microorganism gave the effective regio- and stereoselective hydroxylation at axial allylic position of ketosteroids [9], but with (*S*)-4a-methylhexahydronaphthalenone no stereoselectivity was obtained [10].

Unexpectedly, in case of **R-1** the manifold higher (6:1) formation of product with hydroxyl group at non-activated 6*S*-equatorial rather than allylic 8*S*-axial position was observed. The stereochemistry of C-4a methyl group has a marked impact on the regioselectivity of hydroxylations by means of *F. culmorum*. Probably, it may affect the affinity of the substrate to the enzyme responsible for the hydroxylation.

It is remarkable that the regioselectivity of reaction and stereoselectivity of hydroxylation at C-6 differ from these described in literature [4,5,10,11]. For most of the strains used, the *S*-enantiomer led selectively to 8*R*-hydroxyenone, which is a product of allylic hydroxylation whereas *R*-enantiomer gave 8*S*-hydroxyl derivative as a main product, along with 6*R*-hydroxyenone. It is worth noting that all the compounds obtained in those studies were of *cis* configuration (in relation to 4a-methyl group).

The (4a*S*,6*S*)-4,4a,5,6,7,8-hexahydro-6-hydroxy-4a-methyl-2(3*H*)-naphthalenone **(2)** resulting from a *trans* hydroxylation of **R-1** by *F. culmorum* had not been obtained by means of microbial hydroxylation until our current study. It was previously prepared by a biomimetic manganese/porphyrin/imidazole catalytic system, but with poor yield (4.6%) as one of seven oxidation products [7].

The derivative of **R-1** with cyclopropyl moiety—substrate **4**, was hydroxylated in the A ring giving a unique oxygenation at C-11. During the literature studies we found the microorganisms previously used for transformation **4** exhibited highly preferential attack at one of the geminal methyl groups on the cyclopropane ring [12]. Only one of the tested biocatalysts, *Absidia blakesleeana* ATCC 10148, promoted hydroxylation in the A-ring (at C-10 position), but this transformation was neither regio- nor stereospecific. (1*S*,11*S*)-11-Hydroxy-1,4,4-trimethyltricyclo[5.4.0.0^{3,5}]undec-7-en-9-one **(5)** is a new compound which had not been obtained previously by other oxidation methods.

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