New 9,10-Secosteroids from Biotransformations of Hyodeoxycholic Acid with *Rhodococcus* spp.

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The biotransformations of hyodeoxycholic acid with various *Rhodococcus* spp. are reported. Some strains (*i.e.*, *Rhodococcus zopfii*, *Rhodococcus ruber*, and *Rhodococcus aetherivorans*) are able to partially degrade the side chain at C(17) to afford 6α-hydroxy-3-oxo-23,24-dinor-5β-cholan-22-oic acid (**2**; 23%) and 6α-hydroxy-3-oxo-23,24-dinorchol-1,4-dien-22-oic acid (**3**; 23–30%), together with two new 9,10-secosteroids **4** and **5** (10–45%), still bearing the partial side chain at C(17) and adopting an intramolecular hemiacetal form. In addition, the 9,10-secosteroid **5** showed an unprecedented *C(4)*-hydroxylation. The new secosteroids were fully characterized by MS, IR, NMR, and 2D-NMR analyses.

Introduction. – Bile acid salts are surface-active steroid compounds [1] that occur in the digestive tracts of vertebrates [2]. Their main physiological function is to emulsify bile lipids and dietary lipids. In humans and many other mammals, bile acids are synthesized in the liver. The biosynthesis proceeds *via* modification of the steroid skeleton and shortening of the side chain of cholesterol [3]. Although the bacterial biosynthesis of steroids, and thus also of bile salts, are rarely reported [4–7], the ability of prokaryotic microorganisms to degrade and transform this class of compounds has been extensively documented [8–10]. Regarding the bile acids (*Fig. 1*), in addition to the well-known process of anaerobic transformation in the intestinal tract, several examples of aerobic bacterial degradation are reported [11][12].

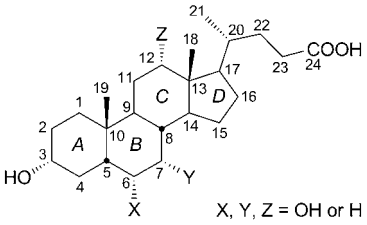


Fig. 1. *Structure of bile acids*

These degradations proceed through a pathway initial step of which is the HO–C(3) group oxidation to the corresponding 3-oxo derivative, followed by dehydrogenation of the C(1)–C(2) and C(4)–C(5) bonds to 3-oxo-1,4-diene structures.

At the same time, the acidic side chain is removed probably through two consecutive β-oxidation steps [13]. After *A*-ring oxidation, C(9) is subject to hydroxylation by 3-ketosteroid 9α-hydroxylase (KSH), a two-component iron-sulfur-containing monooxygenase [14–16]. This reaction leads to the spontaneous cleavage of the *B*-ring by a *retro*-aldol cleavage, and the aromatic 9,10-secosteroids are obtained [17]. Studies on the specificity of KSH purified from *Rhodococcus rhodochrous* DSM 43269 [16] showed that the enzyme accepts a variety of 3-keto steroids with different *A*-ring structures as substrates but the 9α-hydroxylation that induces the *B*-ring cleavage occurs only with 1,4-tetradehydro-3-oxo steroids. Moreover, the KSH activity appears to be affected by the hindrance of the side chain at C(17). Considering the large number of 17-oxo-secosteroids isolated from bacterial steroid degradations [4–11], the presence of a 17-oxo group seems to be a prerequisite for the KSH catalysis. There is only one example of a 9,10-secosteroid obtained from bacterial degradation with an acidic C_3 side chain at C(17) (*i.e.*, *Mycobacterium fortuitum* degradation of cholesterol and phytosterols [18]). In addition, *R. rhodochrous* KSH activity has been detected also with progesterone, a C(17)-acetylated 4-didehydro steroid [16], but the expected 9,10-secosteroid was not isolated.

Recently, some astrogorgols, a small group of secosterols from gorgonian genera of *Astrogorgia* [19], *Calicogorgia* [20], and *Muricella* [21][22] containing a 3-hydroxy-10-methyl aromatic ring, have been shown to possess cytotoxic, brine-shrimp lethal, antivirus, and anti-inflammatory activities, as well as the ability to inhibit the cell division of the fertilized starfish (*Asterina pectinifera*) eggs [19–23]. Finally the unusual structural patterns of 9,10-secosteroids have also attracted the attention of chemists for total synthesis [24–26].

On the basis of these considerations and exploiting our long experience in the synthesis [27][28] and biotransformation of bile acids [29–31], in this article, the biotransformations of hyodeoxycholic acid (=(3α,5β,6α)-3,6-dihydroxycholan-24-oic acid; **1**) with *Rhodococcus* spp. are reported. New metabolites, in particular two 9,10-secosteroids with a partial *C(17)*-side-chain degradation and an intramolecular hemiacetal function, were obtained (*Scheme*) and fully characterized.

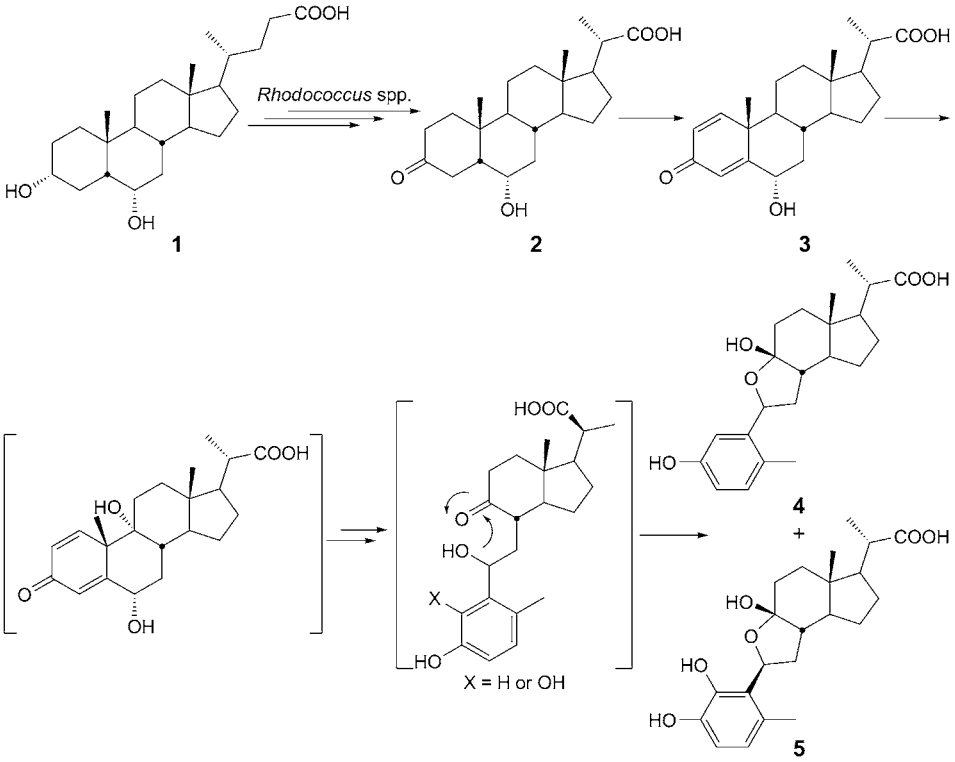
Results and Discussion. – Various *Rhodococcus* strains were tested for their ability to biotransform hyodeoxycholic acid (**1**). Interesting results were obtained with *R. aetherivorans*, *R. ruber*, and *R. zopfii* (*Table 1*).

Table 1. *Biotransformation of Hyodeoxycholic Acid* (**1**) *with* Rhodococcus *spp.*

Bacteria	Time [h]	Yield [%]			
		2	**3**	**4**	**5**
Rhodococcus ruber	24		27	32	10
Rhodococcus aetherivorans	24		30	45	
Rhodococcus zopfii	24	23	24		30

The biotransformation of **1** (1 g/l) with *R. ruber* afforded, after 24 h incubation at 28°, 6α-hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oic acid (**3**; 27%), previously

Scheme. *Biotransformation Pathway of Hydeoxycholic Acid* (**1**) *with* Rhodococcus *spp.*



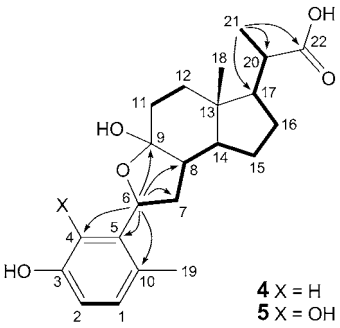
characterized as methyl ester [32], and the new 3,9β-dihydroxy-6ξ,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid (**4**) and 3,4,9β-trihydroxy-6β,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid (**5**; 32 and 10% yield, resp.). The compounds **3** and **4** were also obtained with *R. aetherivorans* (30 and 45% yield, resp.), while the biotransformation with *R. zopfii* furnished the 1,4-diene derivative **3** (24% yield), the catechol derivative **5** (30% yield), and also the new 3-oxo-23,24-dinorcholanoic acid **2** (23% yield). Compound **2** was formed by the partial degradation of the side chain at C(17) and HO–C(3) oxidation of hyodeoxycholic acid (**1**). The 3-oxoderivative **2** is successively oxidized at C(1)–C(2) and C(3)–C(4) to 3-oxo-1,4-diene derivative **3**, which is hydroxylated at C(9) by KSH. The *C(9)*-α-hydroxylation led to the spontaneous cleavage of the C(9)–C(10) bond, followed by HO–C(6) group attack at the C(9)=O function to give the 9,10-secosteroids **4** and **5** in hemiacetal form. This rearrangement accomplished after the spontaneous cleavage of the *B*-ring has been previously reported for degradation of hyodeoxycholic acid (**1**) but with the complete degradation of the side chain at C(17) to carbonyl group [17]. Furthermore, the hydroxylation at C(4) to form the catechol derivative **5** has never been reported for **1**. On the other hand, there is no evidence for the *C(4)*-hydroxylation of the *A*-ring to the catechol secosteroid **5** occuring on the opened hydroxy-keto intermediate or on the

hemiacetal **4**. Since there is only one example of a 9,10-secosteroid with a partial side chain at C(17) isolated by the degradation of the cholesterol [18], and it is reported that the *C(9)*-hydroxylation by KSH of bile acids occurs exclusively after the complete removal of the side chain at C(17) [11], this is the first example of 9,10-secosteroids from bile acids with a partial side chain at C(17). The characterization of new metabolites **2**, **4**, and **5** has been achieved through various and integrated spectroscopic methods such as MS, IR, NMR, and 2D-NMR.

The ^{1}H-NMR of 3-oxo-6α-hydoxy-23,24-dinor-5β-cholan-22-oic acid (**2**) shows three typical and distinctive signals of the bile acid: two *singlet*s (δ(H) 0.71 and 1.02) and a *doublet* (δ(H) 1.25) attributable to the Me(18) and Me(19), and Me(21), respectively [33]. The *multiplet* (δ(H) 4.05–4.18) was easily assigned to H–C(6), analogously to the homologous resonance of hyodeoxycholic acid (**1**). The ^{13}C-NMR spectra (broad band (BB) and distortionless enhancement by polarization transfer (DEPT)) exhibited 22 signals: for three Me, eight CH_2, seven CH groups, and four quaternary C-atoms, in agreement with a 23,24-dinor derivative. The signal shifted most downfield at δ(C) 215.5 was readily assigned to the C(3)=O group, whilst the resonance at δ(C) 181.1 was attributed to the C(22)OOH function. The most downfield CH signal (δ(C) 67.6) was assigned to C(6) on the basis of the analogy of the homologous resonance of hyodeoxycholic acid (**1**). Finally, the three upfield resonances (δ(C) 21.0, 17.0, and 12.2) were ascribed to Me(19), Me(21), and Me(18), respectively. The ESI-MS, exhibiting a negative molecular-ion peak at 361 *m*/*z* ($[M-H]^-$) confirmed the proposed structure.

Regarding the characterization of 3,9β-dihydroxy-6ξ,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid (**4**), a first step was a prominent ion peak at *m*/*z* 355 in the ESI-MS spectrum (negative-ion mode) attributable to the $[M-H_3O]^-$ ion. The ready loss of H_2O confirmed the presence of a non-aromatic OH group (*i.e.*, hemiacetal OH) in a position favorable for elimination. This structure was supported by the IR spectrum that showed only one CO absorption at 1715 cm^{-1}, attributable to the C(22)OOH. This was in agreement with the absence of a C(9)=O group [17]. ^{1}H- and ^{13}C-NMR data of compound **4** are compiled in *Tables 2* and *3*, respectively.

All the H-atom resonances were associated to those of the directly attached C-atoms through the DEPT and 2D-NMR HMQC experiments. The H-atom *multiplet*s are arranged in sequence through the COSY experiment, yielding the three spin systems evidenced in bold in *Fig. 2*.



4 X = H
5 X = OH

Fig. 2. *COSY and key HMB correlations of the proposed hemiacetals* **4** *and* **5**. Further HMBCs are omitted for clarity.

Table 2. *^{1}H-NMR Data of* **4** *and* **5**. At 400 and 300 MHz, respectively, in CD_3OD; δ in ppm; J in Hz.

H-Atom	**4**	**5**
H–C(1)	6.92 (*d*, *J* = 8.0)	6.43 (*dd*, *J* = 8.2, 0.8)
H–C(2)	6.66 (*dd*, *J* = 8.0, 2.7)	6.53 (*d*, *J* = 8.2)
H–C(4)	6.99 (*d*, *J* = 2.7)	–
H–C(6)	5.40 (*dd*, *J* = 9.1, 6.4)	5.26 (*d*, *J* = 7)
CH_2(7)	2.08–2.20 (*m*)[a])	1.98–2.04 (*m*, H_β), 2.11–2.17 (*m*, H_α)
H–C(8)	2.08–2.20 (*m*)[a])	2.36–2.44 (*m*)[b])
CH_2(11)	1.76–1.87 (*m*)[c]), 2.08–2.20 (*m*)[a])	2.21–2.28 (*m*, H_α), 2.29–2.35 (*m*, H_β)
CH_2(12)	1.33–1.42 (*m*), 1.76–1.87 (*m*)[c])	1.39–1.52 (*m*, H_α)[d]), 1.80–1.91(*m*, H_β)
H–C(14)	1.48–1.58 (*m*)	1.25–1.34 (*m*)
CH_2(15)	1.24–1.28 (*m*), 1.76–1.87 (*m*)[c])	1.15–1.20 (*m*), 1.65–1.75 (*m*)
CH_2(16)	1.42–1.54 (*m*), 1.76–1.87 (*m*)[c])	1.39–1.52 (*m*)[d]), 1.94–1.99 (*m*)
H–C(17)	1.61–1.70 (*m*)	1.54–1.65 (*m*)
Me(18)	0.81 (*s*)	0.87 (*s*)
Me(19)	2.22 (*s*)	2.12 (*s*)
H–C(20)	2.35–2.44 (*m*)	2.36–2.44 (*m*)[b])
Me(21)	1.22 (*d*, *J* = 6.8)	1.22 (*d*, *J* = 6.8)

[a])–[d]) Overlapping *multiplets*.

Table 3. *^{13}C-NMR Data of* **4** *and* **5**. At 100 and 75 MHz, respectively, in CD_3OD; δ in ppm.

C-Atom	**4**	**5**	C-Atom	**4**	**5**
C(1)	131.9	121.6	C(12)	37.0	37.0
C(2)	114.9	115.2	C(13)	42.9	43.2
C(3)	156.7	143.6	C(14)	50.9	58.1
C(4)	113.9	140.4	C(15)	26.0	25.5
C(5)	143.5	123.4	C(16)	28.6	28.5
C(6)	78.6	75.3	C(17)	53.4	53.1
C(7)	36.6	41.9	C(18)	10.9	11.1
C(8)	48.4	49.7	C(19)	18.6	17.1
C(9)	109.7	109.3	C(20)	43.8	43.8
C(10)	126.8	128.7	C(21)	17.8	17.7
C(11)	28.7	31.3	C(22)	180.6	180.6

The key HMBCs of Me(21) and H–C(6), indicated with the arrows in *Fig. 2*, define the shortened carboxylic side chain and the hemiacetal five-membered ring, which are the crucial features of compound **4**.

The ^{1}H-NMR showed, among other, signals of three aromatic H-atoms (δ(H) 6.66 (*dd*), 6.92 (*d*), and 6.99 (*d*)), which represent a typical pattern of the methylphenol ring in the 9,10-secosteroid [34].

The *doublet* at δ(H) 1.22 and the *singlet* at δ(H) 0.81 were easily assigned to Me(21) and Me(18), respectively, while the aromatization of the *A*-ring was confirmed by downfield shift of the Me(19) signal (δ(H) 2.22) compared with δ(H) 1.02 of the homologous in 6α-hydroxy-3-oxo-23,24-dinor-5β-cholan-22-oic acid (**2**). The config-

uration of H–C(8) was assumed to be unchanged with respect to the precursor **3** or hyodeoxycholic acid (**1**; *i.e.*, H_β–C(8)), since neither the 9α-hydroxylation nor the *retro*-aldol rearrangement, involved in formation of **4**, should require the change of the configuration at C(8) [17]. The hemiacetal HO–C(9) group should be in β-position to the *C* ring, since the HO–C(6) can readily attach only to the α-position of C(9) [17]. Moreover, the H-atom resonance (δ(H) 5.40) of **4**, assigned to the benzylic H-atom H–C(6), appeared as *dd* ($J = 9.1$, 6.4; *Table 2*), as expected for coupling to the two vicinal H-atoms at C(7). Nevertheless, these data were insufficient to assign the configuration at C(6), and further experiments (*i.e.*, NOE-DIFF, 2D-ROESY, or single-crystal X-ray) failed. In the absence of further evidences, the configuration at this position remained uncertain (*i.e.*, 6ξ). As for compound **2**, the ^{13}C-NMR spectra (BB and DEPT) of **4** exhibited 22 signals: for three Me, five CH_2, eight CH groups, and six quaternary C-atoms in agreement with a 23,24-dinor derivative. The most downfield signal (δ(C) 180.6) was easily attributable to C(22)OOH function, while the six signals (δ(C) between 156.7 and 113.9) were assigned to the aromatic C-atoms C(1) to C(5), and C(10). The signal at δ(C) 109.7 was attributed to the hemiacetal C(9). On the other hand, the H–C(6) signal (δ(C) 78.6) is *ca.* 11 ppm downfield with respect to the homologous H–C(6) of hyodeoxycholic acid (**1**): this downfield shift was consistent with the proposed tetrahydrofuran ring arrangement. Finally, the three upfield resonances at δ(C) 18.6, 17.8 and 10.9 were ascribed to Me(19), Me(21), and Me(18), respectively.

Unlike **4**, the structure of 3,4,9β-trihydroxy-6β,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid (**5**) has been completely determined. Compound **5** showed spectroscopic properties very close to those of **4**. In fact, the ESI-MS spectrum (negative-ion mode) of **5** exhibited a prominent ion peak at *m*/*z* 371 attributable to the $[M - H_3O]^-$ ion and the ready loss of H_2O, as for compound **4**, confirming the presence of the hemiacetal OH group. The IR spectrum showed only one CO absorption attributable to the C(22)OOH.

As above, ^{1}H- (*Table 2*) and ^{13}C-NMR (*Table 3*), and DEPT, 2D-NMR HMQC, and COSY experiments defined the complete structure which was further confirmed by the key HMBCs shown in *Fig. 1*. The ^{1}H- and ^{13}C-NMR data of the compounds **4** and **5** were very similar with the only exception regarding the aromatic signals: both ^{1}H- and ^{13}C-NMR spectra indicated the presence of an additional OH group at C(4). In fact, the resonances of C(1) (δ(C) 121.6), C(3) (δ(C) 143.6), and C(5) (δ(C) 123.4) of compound **5** were shifted upfield with respect to C(1) (δ(C) 131.9), C(3) (δ(C) 156.7), and C(5) (δ(C) 143.5) of compound **4**. In addition, the C(4) signal of compound **5** is shifted downfield (*ca.* 37 ppm) with respect to C(4) signal of **4** (δ(C) 140.4 and δ(C) 113.9, resp.). Further, the aromatic H-atoms of compound **5** showed only two resonances at δ(H) 6.43 (H–C(1)) and 6.53 (H–C(2)), while the H–C(3) signal (δ(H) 6.99) of **4** was absent in the spectrum of **5** confirming the hydroxylation at C(4). An other interesting feature was the ^{1}H resonance assigned to the benzylic H-atom, H–C(6) (δ(H) 5.26), of the compound **5** that appeared as *doublet* ($J = 7$ Hz; *Table 2*) instead of *dd* as expected from coupling to CH_2(7). This implied that one of the two coupling constants H–C(6)/H–C(7) was close to zero (*Fig. 3*).

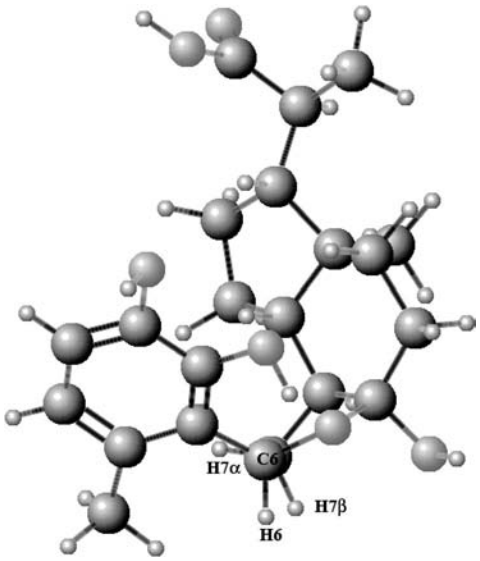


Fig. 3. *Three-dimensional stereomodel of* **5**. C(6) eclipses C(7), dihedral angles H(6)–H(7)α is *ca.* 90° and H(6)–H(7)β *ca.* 15°

A stereomodel of the proposed hemiacetal structure **5**[1]) shows that there exists only one configuration producing an H(6)–C(6)–C(7)–H(7) dihedral angle of *ca.* 90°. Such spatial arrangement is consistent with the observed coupling constants of H–C(6) signal at δ(H) 5.26 (*i.e.*, *ca.* 0 Hz for the H(6)–C(6)–C(7)–H_α(7) (dihedral angle of *ca.* 90°) and 7 Hz for the H(6)–C(6)–C(7)–H_β(7) (dihedral angle of *ca.* 15°)). This stereoisomer requires an inversion of the configuration at C(6) with respect to the original 6α-OH in hyodeoxycholic acid. The configurations at C(8) and C(9) of **5** are assumed to be the same as those proposed for **4**. The structure proposed for **5** is, therefore, 3,4,9β-trihydoxy-6β,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid, also supported by PM3 semiempirical calculations (lower heat of formation with respect to the opposite configuration at C(6)).

Conclusions. – The biotransformations of hyodeoxycholic acid (**1**) with various *Rhodococcus* strains have furnished new metabolites, **2**, **4**, and **5**, with only a partial degradation of the side chain at C(17). On the other hand, 3-oxo-23,24-dinorchola-1,4-dien-22-oic acid (**3**), previously characterized as methyl ester, has never been proposed as substrate for KSH-catalyzed *C(9)*-hydroxylation. The new secosteroid **4** and **5**, rearranged in hemiacetal form, were fully characterized, and their structures suggest that the side-chain degradation and the *A*-ring aromatization are not necessarily consecutive processes. The new 9,10-secosteroids **4** and **5** are very promising as biologically active metabolites, so this preliminary biosynthetic approach will be applied to other bile acids.

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[1]) Calculated using [35].

Experimental Part

General. Sodium salt of hyodeoxycholic acid has been supplied by *ICE Industry* [36]. The *Rhodococcus* strains (*i.e.*, *R. aetherivorans*, *R. baikurensis*, *R. coprophilus*, *R. equi*, *R. fasciens*, *R. opacus*, *R. rhodnii*, *R. rhodococcus*, *R. rodochrous*, *R. ruber*, and *R. zopfii*) were from the *Cambrex-IEP GmbH* collection [37]. TLC: precoated silica-gel plates (thickness 0.25 mm; *Merck*) with cyclohexane/AcOEt/AcOH 60:40:1. M.p.: *510 Büchi* melting-point instrument; uncorrected. UV Spectra: *Jasco V630* specrometer. IR Spectra: in $CHCl_3$ soln.; *Perkin-Elmer 1310* grating infrared spectrometer. ^{1}H-, ^{13}C-, and DEPT-NMR Spectra: *Varian Gemini 300*, at 300 (^{1}H) and 75 MHz (^{13}C), resp.; chemical shifts, δ, referenced to the residual solvent signal ($CDCl_3$: δ(H) 7.26, δ(C) 77.0; CD_3OD: δ(H) 3.34, δ(C) 49.0); 2D-NMR experiments (COSY, HMQC, HMBC): *Varian Mercury Plus 400*, at 400 (^{1}H) and 100 MHz (^{13}C); processed using the manufacturer's software. MS: *LCQ Duo* (*TermoQuest*, San Jose, CA, USA), equipped with an electrospray ionization (ESI) source. The biotransformation sample (1 ml) was centrifuged (6000 rpm, 15 min) to remove the cells. The supernatant was acidified to pH 2 with 1N HCl and extracted with AcOEt (1 ml), and then analyzed, and phosphomolybdic acid soln. was used as the spray reagent to visualize the steroid spots.

Biotransformation of Hyodeoxycholic Acid (**1**) *with* Rhodococcus *spp. General Procedure.* A loopful of the selected *Rhodococcus* from a culture on Plate Count Agar (PCA) was inoculated in Plate Count Broth (PCB; 10 ml) containing glucose (1 g/l), yeast extract (2.5 g/l), and tryptone (5 g/l). After 48 h at 28° and 100 rpm, a portion of this culture (1 ml) was added to the same medium (100 ml) in a 250-ml flask. The growing was continued for 48 h under the same conditions, until the absorbance (660 nm) was 5 OD. To this culture **1** as sodium salt (0.1 g) was added, and the incubation was monitored either by TLC to check the substrate disappearance or measuring the absorbance at 280 nm in order to verify the formation of aromatic derivatives [16]. After 24 h, the broth was centrifuged (6000 rpm, 15 min), and the supernatant was acidified to pH 2 with 1M HCl and extracted with AcOEt (2 × 50 ml). The org. layer was dried (Na_2SO_4), and the solvent was evaporated. The crude products were purified by column chromatography (CC; SiO_2; cyclohexane/AcOEt/AcOH 60:40:1). The results are compiled in *Table 1*. For the purification and the complete characterization of the metabolites **2**–**5**, the biotransformations with *R. ruber*, *R. zopfiii*, and *R. aetherivorans* were repeated on prep. scale starting from **1** as sodium salt (0.5 g) added to the preinoculated medium (500 ml) in a 2-l flask, and the reactions were carried out as reported above.

6α-Hydroxy-3-oxo-23,24-dinor-5β-cholan-22-oic Acid (=*(2*S*)-2-[(5*R*,6*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*R*)-Hexadecahydro-6-hydroxy-10,13-dimethyl-3-oxo-1*H*-cyclopenta[*a*]phenanthren-17-yl]propanoic Acid*; **2**). White crystals (MeOH/H_2O). M.p. 180–182°. IR ($CHCl_3$, as methyl ester): 3360, 1720. ^{1}H-NMR (300 MHz, $CDCl_3$; selected peaks): 0.71 (*s*, Me(18)); 1.02 (*s*, Me(19)); 1.25 (*d*, *J* = 6.2, Me(21)); 4.05–4.18 (*m*, H_β–C(6)). ^{13}C-NMR (75 MHz, $CDCl_3$): 212.5 (C(3)); 181.1 (C(22)); 67.6 (C(6)); 55.7; 52.5; 50.1; 42.9; 42.2; 40.2; 39.6; 37.0; 37.0; 36.2; 36.0; 34.5; 34.3; 27.2; 24.2; 22.8; 21.0 (C(19)); 17.0 (C(21)); 12.2 (C(18)). ESI-MS: 361.4 (100, $[M-H]^-$).

6α-Hydroxy-3-oxo-23,24-dinorchol-1,4-dien-22-oic Acid (=*(2*S*)-2-[(6*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*R*)-6,7,8,9,10,11,12,13,14,15,16,17-Dodecahydro-6-hydroxy-10,13-dimethyl-3-oxo-3*H*-cyclopenta[*a*]phenanthren-17-yl]propanoic Acid*; **3**) [14]. White crystals (MeOH/H_2O). M.p. 270–272°. IR ($CHCl_3$, as methyl ester): 3360, 1720, 1650, 1610. ^{1}H-NMR (300 MHz, CD_3OD; selected peaks): 0.80 (*s*, Me(18)); 1.19 (*d*, *J* = 6.5, Me(21)); 1.27 (*s*, Me(19)); 4.42–4.53 (*m*, H_β–C(6)); 6.22 (*dd*, *J* = 10, 2.1, H–C(2)); 6.43 (*s*, slight splitting, H–C(4)); 7.26 (*d*, *J* = 10, H–C(1)). ^{13}C-NMR (75 MHz, CD_3OD): 186.6 (C(3)); 180.7 (C(22)); 174.6 (C(5)); 159.2 (C(1)); 127.6; 119.9; 68.8 (C(6)); 56.4; 54.0; 53.9; 45.4; 43.9; 43.8; 43.7; 40.5; 35.2; 28.3; 25.4; 23.8; 19.5 (C(19)); 17.6 (C(21)); 12.5 (C(18)). ESI-MS: 357.27 (100, $[M-H]^-$).

3,9β-Dihydroxy-6ξ,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic Acid (=*(2*S*)-2-[(3*a*R,5*a*S,8*b*S)-Decahydro-3a-hydroxy-2-(5-hydroxy-2-methylphenyl)-5a-methyl-2*H*-indeno[5,4-*b*]furan-6-yl]propanoic Acid*; **4**). White crystals (MeOH/H_2O). M.p. 150–153°. IR ($CHCl_3$, as methyl ester): 3655, 3480, 1715, 1595. ^{1}H-NMR (400 MHz, CD_3OD): see *Table 2*. ^{13}C-NMR (100 MHz, CD_3OD): see *Table 3*. ESI-MS: 355.25 (100, $[M-H_3O]^-$).

3,4,9β-Trihydroxy-6β,9α-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic Acid (=*(2*S*)-2-[(2*S*,3*a*R,5*a*S,8*b*S)-2-(2,3-Dihydroxy-6-methylphenyl)-decahydro-3a-hydroxy-5a-methyl-2*H*-indeno[5,4-*

b*]furan-6-yl]propanoic Acid*; **5**). White crystals (MeOH/H_2O). M.p. 160–162°. IR ($CHCl_3$, as methyl ester): 3660, 3480, 1715, 1595. ^{1}H-NMR (300 MHz, CD_3OD): see *Table 2*. ^{13}C-NMR (75 MHz, CD_3OD): see *Table 3*. ESI-MS: 371.27 (100, $[M - H_3O]^-$).

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REFERENCES

[1] A. F. Hofmann, K. J. Mysels, *Colloids Surf.* **1988**, *30*, 145.
[2] A. Moschetta, F. Xu, L. R. Hagey, G. P. van Berge-Henegouwen, K. J. van Erpercum, J. F. Brouvers, J. C. Cohen, H. H. Hobbs, J. H. Steinbach, A. F. Hofmann, *J. Lipid Res.* **2005**, *46*, 2221.
[3] D. W. Russel, *Ann. Rev. Biochem.* **2003**, *72*, 137.
[4] D. Kim, J. S. Lee, J. Kim, S. J. Kang, J. H. Yoon, W. G. Kim, C. H. Lee, *J. Microbiol. Biotechnol.* **2007**, *17*, 403.
[5] S. Maneerat, T. Nitoda, H. Kanzaki, F. Kawai, *Appl. Microbiol. Biotechnol.* **2005**, *67*, 679.
[6] H. B. Bode, B. Zeggel, B. Silakowski, S. C. Wenzel, H. Reichenbach, R. Müller, *Mol. Microbiol.* **2003**, *47*, 471.
[7] C. W. Bird, J. M. Lynch, F. J. Pirt, W. W. Reid, *Nature* **1971**, *230*, 473.
[8] M. V. Donova, *Appl. Biochem. Microbiol.* **2007**, *43*, 1.
[9] P. Fernandes, A. Cruz, B. Angelova, H. M. Pinheiro, J. M. S. Cabral, *Enzyme Microb. Technol.* **2003**, *32*, 688.
[10] S. B. Mahato, S. Garai, *Steroids* **1997**, *62*, 332.
[11] B. Philipp, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 903.
[12] S. Hayakawa, *Z. Allg. Mikrobiol.* **1982**, *22*, 309.
[13] A. Birkenmaier, J. Holert, H. Erdbrink, H. M. Moeller, A. Friemel, R. Schoenenberger, M. J. Suter, J. Klebensberger, B. Philipp, *J. Bacteriol.* **2007**, *189*, 7165.
[14] Y. Hu, R. van der Geize, G. S. Besra, S. S. Gurcha, A. Liu, M. Rohde, M. Singh, A Coates, *Mol. Microbiol.* **2010**, *75*, 107.
[15] J. K. Capyk, I. D'Angelo, N. C. Strynadka, L. D. Eltis, *J. Biol. Chem.* **2009**, *284*, 9937.
[16] M. Petrusma, L. Dijkhuizen, R. van der Geize, *Appl. Environ. Microbiol.* **2009**, *75*, 5300.
[17] R. J. Park, *Steroids* **1984**, *44*, 175.
[18] J. C. Knight, M. C. Novcha, *Steroids* **1985**, *46*, 789.
[19] N. Fusetani, H. Nagata, H. Hirota, T. Tsuyuki, *Tetrahedron Lett.* **1989**, *30*, 7079.
[20] M. Ochi, K. Yamada, H. Kotsuki, K. Shibata, *Chem. Lett.* **1991**, *20*, 427.
[21] Y. Seo, K. W. Cho, H. Chung, H.-S. Lee, J. Shin, *J. Nat. Prod.* **1998**, *61*, 1441.
[22] Y. Seo, J. Shin, J. I. Song, *J. Nat. Prod.* **1995**, *58*, 1291.
[23] M. Ochi, K. Yamada, Y. Kume, H. Kotsuki, K. Shibata, *Tennen Yuki Kagobutsu Toronkai* **1990**, *32*, 49.
[24] G. Della Sala, I. Izzo, F. De Riccardis, G. Sodano, *Tetrahedron Lett.* **1998**, *39*, 4741.
[25] D. F. Taber, S. C. Malcolm, *J. Org. Chem.* **2001**, *66*, 944.
[26] D. F. Taber, Q. Jiang, B. Chen, W. Zhang, C. L. Campbell, *J. Org. Chem.* **2002**, *67*,4821.
[27] G. Fantin, M. Fogagnolo, A. Medici, P. Pedrini, U. Cova, *Steroids* **1993**, *58*, 524.
[28] A. Medici, P. Pedrini, A. De Battisti, G. Fantin, M. Fogagnolo, A. Guerrini, *Steroids* **2001**, *66*, 63.
[29] G. Fantin, S. Ferrarini, A. Medici, P. Pedrini, S. Poli, *Tetrahedron* **1998**, *54*, 1937.
[30] P. Pedrini, E. Andreotti, A. Guerrini, M. Dean, G. Fantin, P. P. Giovannini, *Steroids* **2006**, *71*, 189.
[31] P. P. Giovannini, A. Grandini, D. Perrone, P. Pedrini, G. Fantin, M. Foganolo, *Steroids* **2008**, *73*, 1385.
[32] M. E. Tenneson, J. D. Baty, R. F. Bilton, A. N. Mason, *J. Steroid Biochem.* **1979**, *11*, 1227.
[33] S. Barnes, D. Kirk, '*Nuclear Magnetic Resonance Spectroscopy of Bile Acids*', in 'The Bile Acids – Chemistry, Physiology and Metabolism', Eds. K. D. R. Setchell, P. P. Nair, D. Kritchevsky, Plenum Press, New York-London, 1988, Vol. 4: 'Methods and Applications', Chapt. 3, p. 65.

[34] M. Horinouchia, T. Hayashia, T. Kudo, *J. Steroid Biochem. Mol. Biol.* **2004**, *92*, 143.
[35] MarvinSketch software ver. 5.4.1.1 (http://www.chemaxon.com).
[36] ICE (Industria Chimica Emiliana): http://www.iceitaly.com/
[37] Cambrex-IEP GmbH (Wiesbaden, Germany): http://www.biocatalysis.com/

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