# New 9,10-Secosteroids from Biotransformations of Bile Acids with *Rhodococcus ruber*

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The biotransformations of cholic, deoxycholic, and hyocholic acids with *Rhodococcus ruber* are reported. In all biotransformations, the $C_{17}$-side chain is partially degraded, and the new 9,10-secosteroids **4a** (54%) and **4b** (55%) are obtained from cholic and deoxycholic acids, respectively. The loss of $H_2O$ from C(11)–C(12) of secosteroids **4a** and **4b** affords the compounds **5a** (5%) and **5b** (20%), respectively. On the other hand, in the biotransformation of hyocholic acid with *R. ruber* the 9,10-secosteroid **4c** is not detected, but, rearranging to an intramolecular hemiacetal form, it evolves to the final furan derivative **6c** (35%) by easy elimination of two molecules of $H_2O$. The new secosteroids were characterized by IR, NMR, and 2D-NMR spectroscopy, and mass spectroscopy.

**Introduction.** – Aerobic bacterial degradation of bile salts was intensely investigated, and a general scheme based on the structure of isolated degradation intermediates have been proposed [1]. A recent review [2] suggests that the biotransformation pathway of bile acids (*Fig. 1*) starts with the oxidation of the OH group at C(3), followed by the dehydrogenation of the C(1)–C(2) and C(4)–C(5) bonds to the 3-oxo 1,4-diene structures.

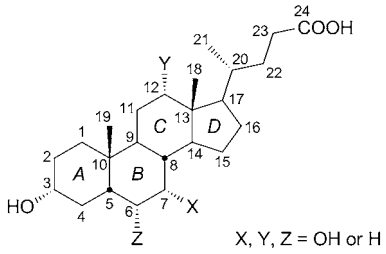



Fig. 1. *Structures of bile acids*

At the same time, the acidic side chain is removed by two consecutive $\beta$-oxidation steps [3] yielding an androsta-1,4-diene-3,17-dione derivative (ADD) bearing various OH groups depending on the starting bile salt (*Fig. 2*).

ADDs are subject to hydroxylation at C(9) by 3-ketosteroid 9$\alpha$-hydroxylase (KSH), a two-component iron-sulfur-containing monooxygenase [4–6]. This reaction leads to the spontaneous cleavage of the *B*-ring by a *retro*-aldol cleavage, and the

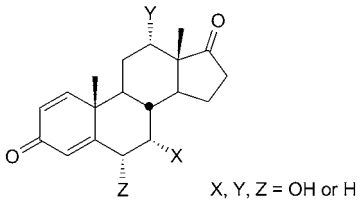



Fig. 2. *Structure of ADD*

aromatic 9,10-secosteroids are obtained [7]. This crucial step to 9,10-secosteroid derivatives occurs only with 3-oxo-1,4-diene bile acids, and the KSH activity appears to be affected by the hindrance of the side chain at C(17) and, if present, by the configuration of the HO–C(12) group.

Considering the large number of 17-oxo-secosteroids isolated from steroid bacterial degradations [2][8–14], the presence of a C(17)=O 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 a partially degraded (3 C-atoms) acidic side chain at C(17) (*i.e.*, *Mycobacterium fortuitum* degradation of cholesterol and phytosterols [15]). In addition, all 9,10-secosteroids obtained from bacterial degradation of 12$\alpha$-OH bile acids contain exclusively 12$\beta$-OH groups [2][16–18]. The gene, encoded for the enzyme that inverts the configuration of the HO–C(12) group, has been identified in *Comamonas testosteroni* [18], and this epimerization seems to facilitate the 9$\alpha$-hydroxylation.

In a recent work, we have pointed out that the complete degradation of the side chain at C(17) is not essential for KSH 9$\alpha$-hydroxylation, since two 9,10-secosteroids were obtained with a partial degradation of the side chain at C(17) by biotransformation of hyodeoxycholic acid with some *Rhodococcus* spp. [19].

Herein, we report the biotransformation of various bile acids (*i.e.*, cholic acid (**1a**), deoxycholic acid (**1b**), and hyocholic acid (**1c**)) with *Rhodococcus ruber*. A series of new 9,10-secosteroids with a partial degradation of the side chain at C(17) are obtained (*Scheme*) and characterized.

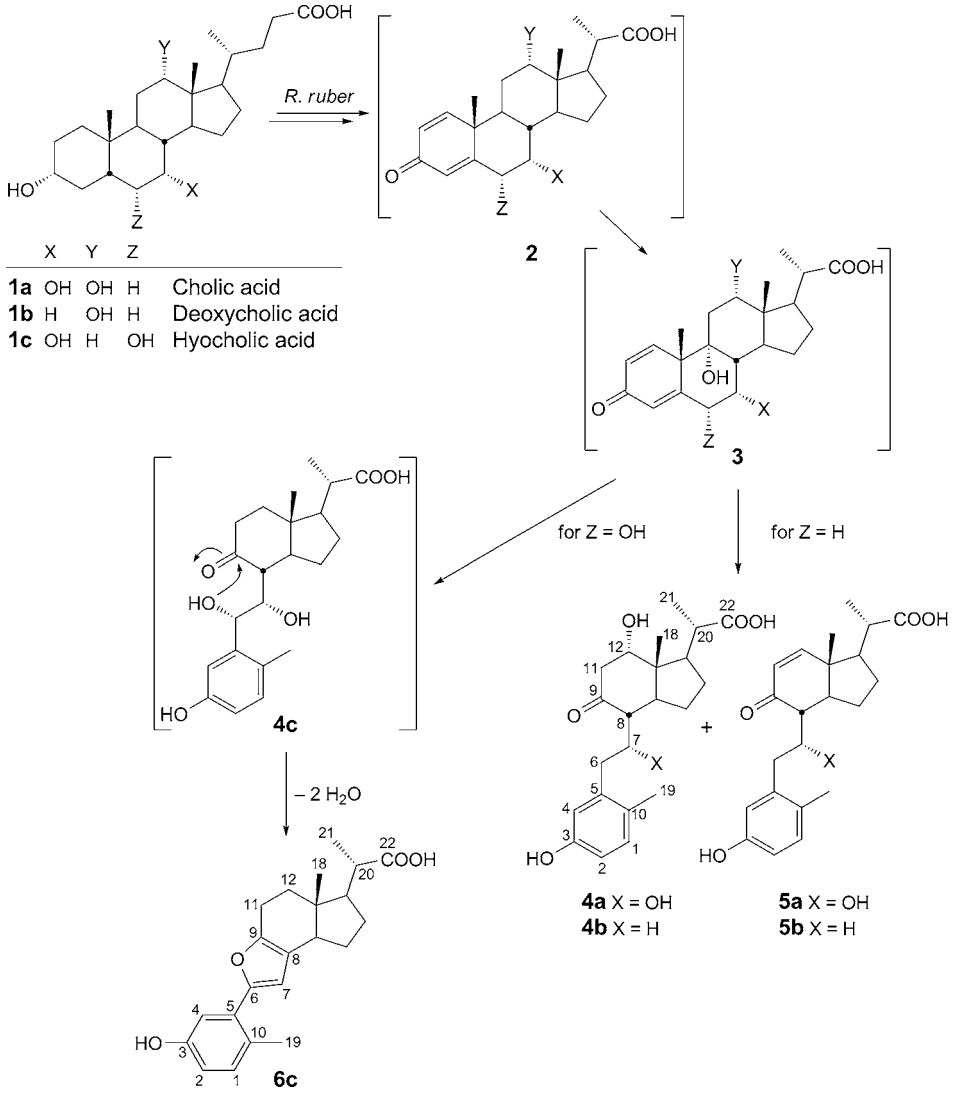
**Results and Discussion.** – *Rhodococcus ruber*, able to produce 9,10-secosteroids with hyodeoxycholic acid [19], was also employed in the biotransformations of cholic acid (**1a**), deoxycholic acid (**1b**), and hyocholic acid (**1c**) (*Table 1*).

Table 1. *Biotransformations of Bile Acids* **1a**–**1c** *with* Rhodococcus ruber

| Bile acids | Time [h] | **4** (Yield [%]) | **5** (Yield [%]) | **6** (Yield [%]) |
|---|---|---|---|---|
| **1a** | 24 | **4a** (54) | **5a** (6) | |
| **1b** | 24 | **4b** (55) | **5b** (20) | |
| **1c** | 24 | | | **6c** (35) |

The biotransformation of **1a** (1 g/l) with *R. ruber* afforded, after 24 h incubation at 28°, 3,7$\alpha$,12$\alpha$-trihydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid (**4a**; 54%) and 3,7$\alpha$-dihydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10),11-tetraen-

Scheme. *Biotransformation Pathway of Bile Acids* **1a**–**1c** *with* Rhodococcus ruber




22-oic acid (**5a**; 6%). Analogous biotransformation of **1b** furnished 3,12$\alpha$-dihydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic acid (**4b**; 55%) and 3-hydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10)-tetraen-22-oic acid (**5b**; 20%). In both cases, the starting material was completely consumed.

The products **4** and **5** are obtained through a common pathway (*Scheme*) that proceeds with the partial degradation of the side chain at C(17), the oxidation of the HO–C(3), the dehydrogenation of the C(1)–C(2) and C(3)–C(4) bonds to 3-oxo-1,4-diene intermediates **2** that are finally hydroxylated at C(9) by KSH to give the

intermediates **3**. The $\alpha$-hydroxylation at C(9) leads to the spontaneous cleavage of the C(9)–C(10) bond to the 9-oxo-9,10-secosteroids **4**. Subsequently, the compounds **4a** and **4b** (from **1a** and **1b**, resp.) are partially dehydrated to the corresponding enones **5a** and **5b**.

The high-resolution mass spectrum (HR-ESI-MS) of **4a** revealed a molecular formula $C_{22}H_{30}O_6$ while the low-resolution (LR) ESI-MS spectrum (positive-ion mode) showed a distinctive pattern of intense peaks at *m/z* 391 ($[M+H]^+$), 373 ($[M+H-H_2O]^+$), and 355 ($[M+H-2\ H_2O]^+$). The ready loss of two molecules of $H_2O$ confirmed the presence of two non-aromatic OH groups in position favorable for elimination (*i.e.*, HO–C(7) and HO–C(12)). The most significant $^1$H- and $^{13}$C-NMR data for compound **4a** are collected in *Tables 2* and *3*, respectively.

Table 2. *Selected $^1$H-NMR Data of* **4a** *and* **4b**, *and* **5a** *and* **5b**. At 400 MHz in $CD_3OD$; $\delta$ in ppm; *J* in Hz. Atom numbering as indicated in the *Scheme*.

| H-Atom | **4a** | **4b** | **5a** | **5b** |
|---|---|---|---|---|
| H–C(1) | 6.93 (*d*, *J* = 8.2) | 6.89 (*d*, *J* = 8.2) | 6.93 (*d*, *J* = 8.2) | 6.90 (*d*, *J* = 8.2) |
| H–C(2) | 6.52 (*dd*, *J* = 8.0, 2.7) | 6.48 (*dd*, *J* = 8.2, 2.7) | 6.52 (*dd*, *J* = 8.0, 2.7) | 6.50 (*dd*, *J* = 8.0, 2.7) |
| H–C(4) | 6.62 (*d*, *J* = 2.7) | 6.56 (*d*, *J* = 2.7) | 6.68 (*d*, *J* = 2.7) | 6.58 (*d*, *J* = 2.7) |
| H–C(7) or $CH_2$(7) | 3.90–3.96 (*m*) | n.a.[a]) | 4.14–4.20 (*m*) | n.a. |
| $CH_2$(11) or H–C(11) | n.a. | n.a. | 5.80 (*d*, *J* = 9.4) | 5.85 (*d*, *J* = 9.4) |
| H–C(12) | 4.06–4.10 (*m*) | 4.13 (*m*) | 7.39 (*d*, *J* = 9.4) | 7.45 (*d*, *J* = 9.4) |
| Me(18) | 0.91 (*s*) | 1.03 (*s*) | 0.90 (*s*) | 0.98 (*s*) |
| Me(19) | 2.20 (*s*) | 2.20 (*s*) | 2.20 (*s*) | 2.20 (*s*) |
| Me(21) | 1.25 (*d*, *J* = 6.5) | 1.25 (*d*, *J* = 6.5) | 1.35 (*d*, *J* = 6.5) | 1.35 (*d*, *J* = 6.5) |

[a]) n.a.: Not assigned.

Table 3. *Selected $^{13}$C-NMR Data of* **4a** *and* **4b**, *and* **5a** *and* **5b**[a]). At 400 MHz in $CD_3OD$; $\delta$ in ppm. Atom numbering as indicated in the *Scheme*.

| C-Atom | **4a** | **4b** | **5a** | **5b** |
|---|---|---|---|---|
| 1 | 132.0 | 131.8 | 131.9 | 131.9 |
| 2 | 114.1 | 113.6 | 114.1 | 113.7 |
| 3 | 156.2 | 156.3 | 156.1 | 156.4 |
| 4 | 117.9 | 116.7 | 118.0 | 116.7 |
| 5 | 140.2 | 143.3 | 139.9 | 143.0 |
| 7 | 71.8 | n.a.[b]) | 72.3 | n.a. |
| 9 | 213.2 | 213.6 | 202.8 | 204.5 |
| 10 | 128.4 | 127.7 | 128.5 | 127.6 |
| 11 | n.a. | n.a. | 130.4 | 129.5 |
| 12 | 73.6 | 73.9 | 159.0 | 159.0 |
| 18 | 12.6 | 12.6 | 14.2 | 13.9 |
| 19 | 18.8 | 18.5 | 18.7 | 18.5 |
| 21 | 16.8 | 16.9 | 17.7 | 17.9 |
| 22 | 180.6 | 180.6 | 179.9 | 180.6 |

[a]) All signals are reported in the *Exper. Part.* [b]) n.a.: Not assigned.

The $^1$H-NMR showed, among others, signals of three aromatic H-atoms ($\delta$(H) 6.52 (*dd*), 6.62 (*d*), and 6.93 (*d*)), which represents a typical pattern of the methylphenolic ring in 9,10-secosteroid [20]. The *doublet* ($\delta$(H) 1.25) and the *singlet* ($\delta$(H) 0.91) 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 ($\delta$(H) 2.20) compared with the corresponding signal ($\delta$(H) 0.92) of cholic acid (**1a**) [21]. The configuration of H–C(8) was assumed to be unchanged with respect to the cholic acid **1a** (*i.e.*, $H_\beta$–C(8)), since neither the 9$\alpha$-hydroxylation nor the *retro*-aldol rearrangement, involved in formation of **4a**, should require change of the configuration at C(8) [7]. It is noteworthy that the signal at $\delta$(H) 4.06–4.10 ppm (see *Table 2*), attributable to H–C(12), showed a profile typical of a H-atom with a $\beta$-configuration [22]. In addition, when the same H–C(12) resonating at $\delta$(H) 4.06–4.10 ppm was saturated, and the NOE-difference spectrum was acquired, an enhancement of *ca.* 3% of the Me(18) signal at $\delta$(H) 0.91 was observed. This experiment confirmed the $\beta$ orientation of the H-atom at C(12) and, consequently, the $\alpha$-configuration of the OH group [23]. This is, to the best of our knowledge, the first example of 9,10-secosteroid derived from bacterial degradation of cholic acid with the12-OH group in $\alpha$-position [2][16–18].

The $^{13}$C-NMR spectra (BB and DEPT) of **4a** exhibited 22 signals (see *Exper. Part*) in agreement with a 23,24-dinor derivative. The most downfield signals (see *Table 3*) ($\delta$(C) 213.2) and ($\delta$(C) 180.6) were easily attributable to the C(9) and the C(22)OOH respectively, while the six signals (from $\delta$(C) 156.2 to 114.1) were assigned to the C-atoms C(1)–C(5) and C(10).

The signals at $\delta$(C) 71.8 and 73.6 were attributed to the H–C(7) and H–C(12), respectively, and, finally, the upfield resonances at $\delta$(C) 18.8, 16.8, and 12.6 were ascribed to the Me(19), Me(21) and Me(18), respectively.

The HR-ESI-MS of **5a**, derived from **4a** by loss of $H_2O$ from H–C(11) and HO–C(12), indicated the molecular formula $C_{22}H_{28}O_5$, while the LR-ESI-MS (negative-ion mode) exhibited three intense peaks at *m*/*z* 371, 743, and 1115, attributable to the monomer, dimer, and trimer, respectively. The IR spectrum showed two distinctive stretchings at 1651 and 1609 cm$^{-1}$, attributable to an $\alpha,\beta$-unsaturated ketone. The most significant $^1$H-NMR signals (*Table 2*) of **5a** were very similar to those of **4a** (*i.e.*, three aromatic H-atoms ($\delta$(H) 6.52 (*dd*), 6.68 (*d*) and 6.93 (*d*)), the *doublet* $\delta$(H) 1.35 and the *singlet*s $\delta$(H) 0.90 and 2.20 assigned to Me(21), Me(18), and Me(19), resp.). The configuration at C(8) was assumed to be unchanged with respect to the cholic acid **1a** (*i.e.*, $H_\beta$–C(8)). The *doublet* at $\delta$(H) 7.39 assigned to the H–C(12) was in agreement with the C(11)=C(12) bond. Of course, the 'twin' H–C(11) *doublet* was also present at $\delta$(H) 5.80. The $^{13}$C-NMR spectra (BB and DEPT) of **5a** (very similar to **4a**) were in accordance with a 23,24-dinor structure (see *Exper. Part*). The most downfield signals (*cf. Table 3*; $\delta$(C) 202.8 and 179.9) were assigned to the C(9) and C(22), respectively, while the signals at $\delta$(C) 130.4 and 159.0 were attributable to the C(11)=C(12) bond.

Compound **4b** showed spectral properties very similar to those of **4a**. The HR-MS indicated a molecular formula $C_{22}H_{30}O_5$, while the LR-ESI-MS (negative-ion mode) showed three intense peaks at *m*/*z* 373, 747, and 1121, attributable to the monomer, dimer, and trimer, respectively. The most significant $^1$H- and $^{13}$C-NMR data of **4b** are compiled in *Tables 2 and 3*, respectively. The $^1$H-NMR spectrum of **4b**, as observed for

**4a**, exhibited signals of three aromatic H-atoms ($\delta$(H) 6.48 (*dd*), 6.56 (*d*), and 6.89 (*d*)), and a *doublet* ($\delta$(H) 1.25) and two *singlet*s ($\delta$(H) 1.03 and 2.20) that were easily assigned to Me(21), Me(18) and Me(19), respectively. The configuration at C(8) was assumed to be unchanged with respect to the deoxycholic acid **1b** (*i.e.*, $H_\beta$–C(8)), and the resonance at $\delta$(H) 4.13 was attributable to the H-atom at C(12) with a $\beta$-configuration for the same reasons as for **4a**. As observed for **4a**, the $^{13}$C-NMR spectra (BB and DEPT) of **4b** exhibited the 22 signals (see *Exper. Part*) in accordance with a 23,24-dinor derivative. The most downfield signals (see *Table 2*) ($\delta$(C) 213.6 and 180.6) were assigned to C(9) and C(22), respectively, while the resonances at $\delta$(C) 73.9 was ascribed to C(12).

Finally the HR-ESI-MS of **5b**, derived from **4b** by loss of $H_2O$ from H–C(11) and HO–C(12)), indicated the molecular formula $C_{22}H_{28}O_4$, while LR-ESI-MS (negative-ion mode) displayed an intense peak at *m*/*z* 355 attributable to the $[M-H]^-$ ion. The IR spectrum showed two distinctive stretchings at 1654 and 1609 cm$^{-1}$, attributable to an $\alpha,\beta$-unsaturated ketone. The $^1$H-NMR spectrum was very similar to that of **5a** (see *Table 2*). Also in this case, the configuration at C(8) was assumed to be unchanged with respect to the deoxycholic acid **1b** (*i.e.*, $H_\beta$–C(8)). The *doublet*s ($\delta$(H) 7.45 and $\delta$(H) 5.85) were assigned to the olefinic H-atoms H–C(12) and H–C(11), respectively. The $^{13}$C-NMR spectra (BB and DEPT) of **5b** exhibited 22 signals (see *Exper. Part*). The most downfield signals (*cf. Table 3*; $\delta$(C) 204.5 and 180.6) were easily attributable to C(9) and C(22), respectively, while the signals at $\delta$(C) 129.5 and 159.0 were ascribed to the C(11)=C(12) bond.

An analogous pathway is followed by the biotransformation of hyocholic acid **1c** with *R. ruber* that afforded the 3-hydroxy-6,9-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10),6,8-pentaen-22-oic acid (**6c** *cf. Scheme*) in 35% yield. In this case, however, the 9,10-secosteroid **4c** is not detected but evolves to the furan derivative **6c** by the attack of OH at C(6) to the C(9)=O function to give the corresponding hemiacetal (*Fig. 3*) that spontaneously underwent elimination of two molecules of $H_2O$. This hypothesis is supported by the stable hemiacetal derivatives obtained from biotransformation of hyodeoxycholic acid [19].

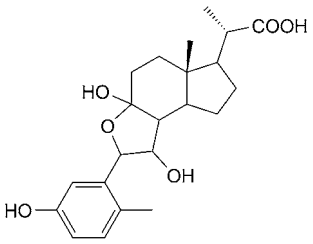



Fig. 3. *Hemiacetal structure of* **1c**

The HR-ESI-MS of **6c** provided the molecular formula $C_{22}H_{26}O_4$, while LR-ESI-MS (positive-ion mode) showed an ion peak at *m*/*z* 355 attributable to the $[M+H]^+$. The IR spectrum showed only one carbonyl absorption bond at 1715 cm$^{-1}$ attributable to the C(22)OOH group. $^1$H- and $^{13}$C-NMR data of compound **6c**, recorded in $CD_3OD$, are compiled in *Table 4*.

Table 4. *$^1$H- and $^{13}$C-NMR Data of* **6c**. At 400 MHz in $CD_3OD$; $\delta$ in ppm; $J$ in Hz.

| Position | $\delta$(H) | $\delta$(C) |
|---|---|---|
| 1 | 7.00 (*d*, *J* = 8.3) | 133.1 |
| 2 | 6.57 (*dd*, *J* = 8.3, 2.7) | 114.8 |
| 3 | – | 152.9 |
| 4 | 7.08 (*d*, *J* = 2.7) | 113.6 |
| 5 | – | 125.8[a]) |
| 6 | – | 156.4 |
| 7 | 6.33 (*s*) | 109.1 |
| 8 | – | 122.6[b]) |
| 9 | – | 150.2[b]) |
| 10 | – | 129.4[a]) |
| 11 | 2.7–2.77 (*m*) | 22.0 |
| 12 | 1.67–1.76 (*m*), 2.21–2.29 (*m*) | 36.4 |
| 13 | – | 45.0 |
| 14 | 2.61–2.69 (*m*) | 49.8 |
| 15 | 1.41–1.53 (*m*), 1.91–2.00 (*m*) | 24.5 |
| 16 | 1.56–1.66 (*m*), 2.00–2.00 (*m*) | 29.7 |
| 17 | 1.77–1.85 (*m*) | 51.6 |
| 18 | 0.69 (*s*) | 11.3 |
| 19 | 2.34 (*s*) | 21.4 |
| 20 | 2.40–2.51 (*m*) | 44.2 |
| 21 | 1.27 (*d*, *J* = 6.8) | 18.0 |
| 22 | – | 180.7 |

[a]), [b]) Assignments may be interchanged.

All the H-atom resonances were correlated with those of the directly attached C-atoms by the DEPT and 2D-NMR HMQC experiments, and the H-atom spin systems were established through the COSY experiment. In particular, the *singlet* ($\delta$(H) 2.34) of Me(19), and signals of the three aromatic H-atoms ($\delta$(H) 6.57 (*dd*), 7.00 (*d*), and 7.08 (*d*)) in the $^1$H-NMR formed the characteristic pattern consistent with a seco-phenol moiety [20] and derived by a well-known degradation pathway of bile acids (9$\alpha$-hydroxylation and *B*-ring cleavage *via retro*-aldol rearrangement). Moreover, the presence of a *singlet* ($\delta$(H) 0.69) and of a *doublet* ($\delta$(H) 1.27) indicated that Me(18) and Me(21) were still present in the molecule. On the other hand, the $^{13}$C-NMR spectrum displayed ten resonances (from $\delta$(C) 109.1 to 156.4) attributable to the aromatic C-atoms: six for the methylphenol moiety (*i.e.*, C(1)–C(5) and C(10)) and four for the furan ring (*i.e.*, C(6)–C(9)).

**Conclusions.** – The biotransformations of cholic, deoxycholic, and hyocholic acids (**1a**, **1b**, and **1c**, resp.) with *Rhodococcus ruber* produced five new metabolites (**4a** and **4b**, **5a** and **5b**, and **6c**) with a partial degradation of the side chain at C(17), confirming the 3-oxo-23,24-dinorchola-1,4-dien-22-oic acids **2a**–**2c** as substrates for KSH catalyzed *C(9)*-hydroxylation. The structure of these compounds supports the hypothesis that the side-chain degradation and the *A* ring-aromatization are not necessarily consecutive processes. In addition, the new secosteroids **4a** and **4b** are the first examples of 9,10-secosteroids isolated from bacterial degradation of bile acids,

possessing still the 12$\alpha$-OH group so the inversion of the configuration at C(12) does not appear necessary for the subsequent *B*-ring cleavage. Finally, the 9,10-secosteroid **6c** is derived from **4c**, by rearrangement to hemiacetalic form and losing two molecules of $H_2O$ to afford the furan derivative.

## Experimental Part

*General.* Sodium salt of cholic, deoxycholic, and hyocholic acids were supplied by *ICE industry* [24]. *Rhodococcus ruber* belongs to *Cambrex-IEP GmbH* collection [25]. TLC: Precoated silica-gel plates ($SiO_2$; thickness 0.25 mm; *Merck*) with cyclohexane/AcOEt/AcOH 60:40:1, withdrawing periodically aliquots of the biotransformation broth (1 ml). The broth was centrifuged (6000 rpm, 15 min) to remove the cells and the supernatant was acidified to pH 2 with 1M HCl, extracted with AcOEt (1 ml) and then analyzed. Phosphomolybdic acid soln. was used as spray reagent to visualize the steroid spots. M.p.: uncorrected; *510 Büchi* melting point instrument. UV Spectra: *Jasco V630* specrometer. IR Spectra: *Perkin-Elmer 1310* grating infrared spectrometer ($CHCl_3$ soln.) or *Perkin-Elmer Spectrum 100* FT-IR (ATR mode). The $^1$H- and $^{13}$C-, DEPT-NMR, and 2D-NMR spectra (COSY, HMQC) were recorded in $CD_3OD$ soln. in 5-mm tubes, at r.t., with a *Varian Mercury Plus 400*, operating at 400 ($^1$H) and 100 MHz ($^{13}$C), resp; the $^1$H chemical shifts referenced to the residual solvent signal ($CD_3OD$: $\delta$ (H) 3.34 ppm); the $^{13}$C chemical shifts with reference to $CD_3OD$ ($\delta$(C) 49.0 ppm). ESI-MS: *LCQ Duo* (*TermoQuest*, San Jose, CA, USA). HR-ESI-MS: *ESI-Q-TOF 6520 Agilent Technologies* instrument.

*Biotransformation of Bile Acids* **1a**–**1c** *with* Rhodococcus ruber. *General Procedure.* A loopful of *Rhodococcus ruber* 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 (5 ml) was added to the same medium (500 ml) in a 2-l flask. The growing was continued for 48 h under the same conditions until the absorbance (660 nm) was 5 OD. To this culture, the proper bile acid **1** as sodium salt (0.5 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 [4]. 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 × 100 ml). The org. layer was dried ($Na_2SO_4$), and the solvent was evaporated. The crude products were purified by CC ($SiO_2$; cyclohexane/AcOEt/AcOH 60:40:1). The results are compiled in *Table 1.*

*3,7α,12α-Trihydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic Acid* (=*(α*S*,1*R*, 3a*S*,4*R*,7*S*,7a*S*)-Octahydro-7-hydroxy-4-[1-hydroxy-2-(5-hydroxy-2-methylphenyl)ethyl]-α,7a-dimethyl-5-oxo-1*H*-indene-1-acetic Acid*; **4a**). Yield: 0.24 g (54%). Colorless gum. $[\alpha]_D^{20} = +30$ ($c$ = 0.8, MeOH). IR (ATR): 3356, 2947, 1694. $^1$H-NMR: see *Table 2*. $^{13}$C-NMR (100 MHz, $CD_3OD$): 213.2 (C(9)); 180.6 (C(22)); 156.2 (C(3)); 140.2 (C(5)); 132.0 (C(1)); 128.4 (C(10)); 117.9 (C(4)); 114.1 (C(2)); 73.6; 71.8; 55.7; 48.6; 48.2; 45.0; 44.2; 43.7; 39.5; 28.4; 25.6; 18.8 (C(19); 16.8 (C(21)); 12.6 (C(18)). ESI-MS (pos.): 391 (65, $[M+H]^+$), 373 ($[M+H-H_2O]^+$), 355 ($[M+H-2\,H_2O]^+$). HR-ESI-MS (neg.): 389.1973 ($[M-H]^-$, $C_{22}H_{29}O_6^-$; calc. 389.2042).

*3,7α-Dihydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10),11-tetraen-22-oic Acid* (=*(αS,4R,7aS)-2,3,3a,4,5,7a-Hexahydro-4-[(1*R*)-1-hydroxy-2-(5-hydroxy-2-methylphenyl)ethyl]-α,7a-dimethyl-5-oxo-1*H*-indene-1-acetic Acid*; **5a**). Yield: 26 mg (6%). Colorless gum. $[\alpha]_D^{20} = -20.1$ ($c$ = 1.0, MeOH). IR (ATR): 3247, 2928, 1704, 1651, 1609. $^1$H-NMR: see *Table 2*. $^{13}$C-NMR (100 MHz, $CD_3OD$): 202.8 (C(9)); 179.9 (C(22)); 159.0 (C(12)); 156.1 (C(3)); 139.9 (C(5)); 131.9 (C(1)); 130.4 (C(11)); 128.5 (C(10)); 118.0 (C(4)); 114.1 (C(2)); 72.3 (C(7)); 53.3; 49.6; 48.8; 46.1; 44.1; 39.7; 28.4; 25.6; 18.7 (C(19)); 17.7 (C(21)); 14.2 (C(18)). ESI-MS (neg.): 371 ($[M-H]^-$), 743 ($[2M-H]^-$), 1115 ($[3M-H]^-$). HR-ESI-MS (neg.): 371.1838 ($[M-H]^-$, $C_{22}H_{27}O_5^-$; calc. 371.1937).

*3,12α-Dihydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10)-trien-22-oic Acid* (=*(α*S*,1*R*, 3a*S*,4*S*,7*S*,7a*S*)-Octahydro-7-hydroxy-4-[2-(5-hydroxy-2-methylphenyl)ethyl]-α,7a-dimethyl-5-oxo-1*H*-indene-1-acetic Acid*; **4b**). Yield: 0.25 g (55%). Colorless gum. $[\alpha]_D^{20} = +12.1$ ($c$ = 0.8, MeOH). IR (ATR): 3350, 2953, 1702. $^1$H-NMR: see *Table 2*. $^{13}$C-NMR (75 MHz, $CD_3OD$): 213.6 (C(9)); 180.6 (C(22)); 156.3 (C(3)); 143.3 (C(5)); 131.8 (C(1)); 127.7 (C(10)); 116.7 (C(4)); 113.6 (C(2)); 73.9 (C(12));

51.6; 48.1; 48.0; 47.9; 44.5; 43.7; 31.9; 28.8; 28.3; 25.6; 18.5 (C(19)); 16.9 (C(21)); 12.6 (C(18)). ESI-MS (neg.): 373 ($[M-H]^-$), 747 ($[2M-H]^-$), 1121 ($[3M-H]^-$). HR-ESI-MS (neg.): 373.2024 ($[M-H]^-$, $C_{22}H_{29}O_5^-$; calc. 373.2093).

*3-Hydroxy-9-oxo-9,10-seco-23,24-dinorchola-1,3,5(10),11-tetraen-22-oic Acid* (=*(α*S,*4*S,*7a*S)-*2,3,3a,4,5,7a-Hexahydro-4-[2-(5-hydroxy-2-methylphenyl)ethyl]-α,7a-dimethyl-5-oxo-1 H-indene-1-acetic Acid*; **5b**). Yield: 86 mg (20%). Colorless gum. $[\alpha]_D^{20} = -7.1$ ($c = 1.6$, MeOH). IR (ATR): 3304, 2942, 1704, 1654, 1609. $^1$H-NMR: see *Table 2*. $^{13}$C-NMR (100 MHz, $CD_3OD$): 204.5 (C(9)); 180.6 (C(22)); 159.9 (C(12)); 156.4 (C(3)); 143.0 (C(5)); 131.9 (C(1)); 129.5 (C(11)); 127.6 (C(10)); 116.7 (C(4)); 113.7 (C(2)); 56.4; 51.8; 48 : 1; 46.3; 44.6; 31.1; 29.7; 28.1; 25.5; 18.5 (C(19)); 17.9 (C(21)); 13.9 (C(18)). ESI-MS (neg.): 355 ($[M-H]^-$). HR-ESI-MS (pos.): 357.2057 ($[M+H]^+$, $C_{22}H_{29}O_4^+$; calc. 357.1988).

*3-Hydroxy-6,9-epoxy-9,10-seco-23,24-dinorchola-1,3,5(10),6,8-pentaen-22-oic Acid* (=*(α*S,*5a*R)-*5,5a,6,7,8,8a-Hexahydro-2-(5-hydroxy-2-methylphenyl)-α,5a-dimethyl-4*H-*indeno[5,4-*b*]furan-6-acetic Acid*; **6c**). Yield: 150 mg (35%). White crystals ($MeOH/H_2O$). M.p. 175–177°. $[\alpha]_D^{20} = +14$ ($c = 0.8$, MeOH). IR ($CHCl_3$, as methyl ester derivative): 3400, 1715, 1595. $^1$H- and $^{13}$C-NMR: see *Table 4*. ESI-MS (pos.): 355 (100, $[M+H]^+$). HR-ESI-MS (neg.): 353.1759 ($[M-H]^-$, $C_{22}H_{25}O_4^-$; calc. 353.1831).

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