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Transformation of steroids by *Bacillus* strains isolated from the foregut of water beetles (Coleoptera: Dytiscidae): II. Metabolism of 3β-hydroxypregn-5-en-20-one (pregnenolone)

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Abstract

The in vitro metabolism of pregnenolone by two *Bacillus* strains (HA-V6-3 and HA-V6-11) isolated from the foregut of the water beetle *Agabus affinis* (Payk.) was examined in the course of our studies about a possible participation of gut micro-organisms in the biosynthesis of prothoracic defensive steroids of dytiscids. The transformation products were identified by EI GC–MS of culture extracts after derivatization. The dominating reactions were hydroxylations, with 7α-hydroxypregnenolone as the major product. With considerably lower yields, 7β- and 15ξ-hydroxypregenolone were formed by both strains, while 11, 17 and 16α-hydroxypregnenolone were produced only by HA-V6-3. The occurrence of 7, 11α- and 7β, 11α-dihydroxypregnenolone as well as several minor products containing a 17α-OH group proved the capability of HA-V6-11 to hydroxylate pregenenolone at C_{11} and C_{17} as well. The monohydroxylated 7-OH-pregnenolones were partly oxidized to 7-oxopregnenolone by both strains. In trace amounts, HA-V6-3 performed 3β-acetylation of pregnenolone. © 2001 Published by Elsevier Science Ltd.

Keywords: Dytiscidae; Foregut; Steroid biotransformation; Pregnenolone; *Bacillus*; Gas chromatography–mass spectrometry

1. Introduction

Water beetles (Dytiscidae) are characterized from a chemical point of view by two complex gland systems, the pygidial glands and the prothoracic defensive glands. In many species, considerable amounts of steroids are stored in the prothoracic defensive glands. These are segregated in form of a milky fluid when the beetles are seized by a predator, an effect, which can also be evoked by slightly squeezing a beetle. It has been shown that the dytiscid steroids possess a strong antifeedant effect against fish [1–3].

Though dozens of different steroid compounds have been found in the prothoracic defensive glands of dytiscids so far (compilations in [3–5]), the biosynthesis of these steroids still remains a black box. It is common knowledge that insects are not capable to synthesize the steroid skeleton de novo [6]. Though several steroid converting enzymes have been found in tissues or in the hemolymph of insects [7], the steroid skeleton must be taken up with the food generally. Cholesterol as the major zoosterol is assumed to be the most important precursor of the dytiscid defensive steroids, although steroids of the vertebrate-hormone type like pregnenolone, progesterone or estradiol have also been found in small amounts in tissues of potential prey insects [7]. A participation of microorganisms in the steroid biogenesis has been discussed in the past due to the findings of Chapman et al. [8], coming to the conclusion that the mechanism of double bond formation in the $\Delta^{4,6}$-unsaturated steroid of the Dytiscid *Acilius sulcatus* is likely to be mediated by microorganisms. It has been shown that the foregut is the main site of cholesterol uptake in *Dytiscus marginalis* [9]. Provided that microorganisms play a role in the biosynthesis of Dytiscid steroids, it may, therefore, be concluded reasonably that they should be located in the foregut.

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Aerobic endospore-forming bacilli seem to be common and typical inhabitants of the crop of the water beetle *Agabus affinis* (Payk.). This was revealed by isolation and characterization of 30 bacterial strains from the foregut microflora of two water beetle species [10]. Selected isolates have been screened for their ability to transform a variety of steroid precursors in vitro.

In a short series of papers, we present results of experiments that contribute novel aspects of microbial steroid biotransformation. In a previous article, we reported the transformation of androst-4-en-3,17-dione (AD) by two *Bacillus* strains isolated from the digestive tract of water beetles [11]. AD was transformed with high efficiency. The present paper deals with the transformation of a C_{21} steroid substrate (pregnenolone) by the same isolates.

2. Materials and methods

2.1. Chemicals and reagents

All solvents used were of analytical grade (Fluka, Deisenhofen/Germany). 3β-Hydroxypregn-5-en-20-one (pregnenolone) was provided by Fluka (Deisenhofen). 7, 11 and 17α-hydroxypregnenolone as well as 7, 11α- and 7β, 11α-dihydroxypregnenolone were gifts of Schering AG, Berlin (Dr W. Boidol). 7β-Hydroxypregnenolone and 7-oxopregnenolone were purchased from Steraloids Inc. (Newport, RI, USA). 16α-Hydroxypregnenolone and pregnenolone acetate were obtained from Sigma (Deisenhofen/Germany).

N-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Fluka (Deisenhofen), MOX® reagent (2% methoxyamine-HCl in pyridine) from Pierce (Rockford, IL, USA). Ingredients of microbiological media were obtained from Merck (Darmstadt, Germany).

2.2. Transformation experiments, sample preparation and GC–MS analysis

Transformation and control experiments have been carried out using the methods and microbiological media described earlier [11]. Six hours after inoculation with 1 ml starting culture, pregnenolone (10 mg dissolved in 1 ml *N*,*N*′-dimethylformamide) was added to the culture broth to reach a starting substrate concentration of 0.2 g/l. One-milliliter samples were drawn after 24, 48 and 96 h, respectively.

Procedures for the extraction of the samples and derivatization of the steroids as their trimethysilyl ether (TMS) or *O*-methyloxim trimethylsilyl ether (MO-TMS) derivatives have been described in detail previously [11].

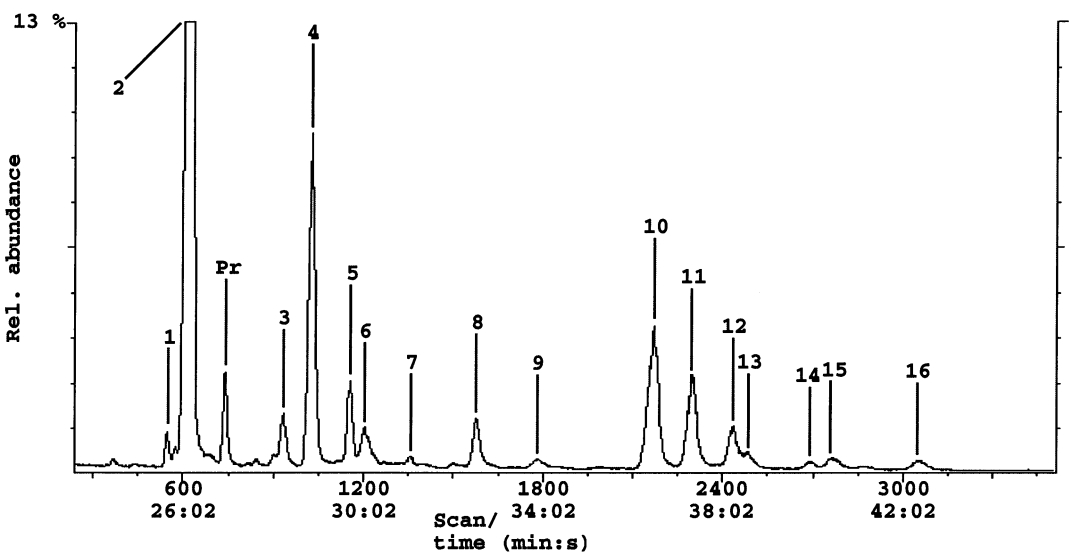

Fig. 1. Total ion current chromatogram of trimethylsilylated culture extract of strain HA-V6-11 after an incubation period of 48 h; enumeration of TMS derivatives according to compound numbers in Table 1. Pr, 3β-Hydroxypregn-5-en-20-one-TMS (pregnenolone; substrate), **1**, 17α-hydroxypregn-5-en-3,20-dione-*bis*-TMS, **2**, 3β, 7α-dihydroxypregn-5-en-20-one-*bis*-TMS (7α-hydroxypregnenolone), **3**, 3, 7β,11α-trihydroxypregn-5-en-20-one-*tris*-TMS (7β, 11α-dihydroxypregnenolone), **4**, 3, 7β, 11α-trihydroxypregn-5-en-20-one-*tris*-TMS (7β, 11α-dihydroxypregnenolone), **5**, 3, 7β-dihydroxypregn-5-en-20-one-*bis*-TMS (7β-hydroxypregnenolone), **6**, 3β, 7ξ, 17α-trihydroxypregn-5-en-20-one-*tris*-TMS, **7**, 3β, 15ξ-dihydroxypregn-5-en-20-one-*bis*-TMS, **8**, 3β, 14α, x-trihydroxypregn-5-en-20-one-*bis*-TMS, **9**, 3β, 7ξ, x-trihydroxypregn-5-en-20-one-*tris*-TMS, **10**, 3β, x-dihydroxypregn-5-en-y,20-dione-bis-TMS, **11**, 3β-hydroxypregn-5-en-7,20-dione-TMS (7-oxopregnenolone), **12**, 3β, 14α-dihydroxypregn-5-en-y,20-dione-TMS, **13**, 3β, 17α-dihydroxypregn-5-en-y,20-dione-*bis*-TMS, **14**, 3β, x-dihydroxypregn-5-en-y,20-dione-bis-TMS, **15**, 3β, 17α, 20ξ-trihydroxypregn-5-en-x-one-*tris*-TMS, **16**, 3β, x-dihydroxypregn-5-en-y,20-dione-*bis*-TMS; **1**, **6**–**10**, **12**–**16** tentative structures; TMS, trimethylsilyl ether.

Table 1
Products formed upon transformation of pregnenolone by strain HA-V6-11[a]

	Transformation product	Relative retention time (5α-cholestane = 1.00)				Percent peak area (TMS derivatives)
		TMS	*bis*-TMS	*tris*-TMS	MO-TMS	48 h
1	17α-Hydroxypregn-5-en-3,20-dione[a]	–	0.91	–	–	+
2	**3β, 7α-Dihydroxypregn-5-en-20-one**	–	**0.92**	–	**0.93**	++
3	3, 7β, 11α-Trihydroxypregn-5-en-20-one	–	–	1.00	–	+
4	3β, 7, 11α-Trihydroxypregn-5-en-20-one	–	–	1.02	–	+
5	3, 7β-Dihydroxypregn-5-en-20-one	–	1.05	–	1.03	+
6	3β, 7ξ, 17α-Trihydroxypregn-5-en-20-one[b]	–	–	1.06	–	*t*
7	3β, 15ξ-Dihydroxypregn-5-en-20-one[b]	–	1.09	–	–	*t*
8	3β, 14α, x-Trihydroxypregn-5-en-20-one[b]	–	1.15	–	–	+
9	3β, 7ξ, x-Trihydroxypregn-5-en-20-one[b]	–	–	1.20	–	*t*
10	3β, x-Dihydroxypregn-5-en-y,20-dione[b]	–	1.29	–	–	+
11	3β-Hydroxypregn-5-en-7,20-dione	1.31	–	–	1.25	+
12	3β, 14α-Dihydroxypregn-5-en-y,20-dione[b]	1.35	–	–	–	*t*
13	3β, 17α-Dihydroxypregn-5-en-y,20-dione[b]	–	1.36	–	–	*t*
14	3β, x-Dihydroxypregn-5-en-y,20-dione[b]	–	1.41	–	–	*t*
15	3β, 17α, 20ξ-Trihydroxypregn-5-en-x-one[b]	–	–	1.43	–	*t*
16	3β, x-Dihydroxypregn-5-en-y,20-dione[b]	–	1.50	–	–	*t*

[a] Main product printed in bold letters; numbers of compounds like in Fig. 1; TMS, trimethylsilyl ether, MO-TMS, *O*-methyloxim trimethylsilyl ether derivative (fully derivatized); ++, major product (relative peak area >50%; substrate not included), +, minor product (relative peak area 1–50%), *t*, traces (relative peak area <1%).
[b] Tentatively identified.

2.3. GC–MS analysis

EI mass spectra (70 eV) were recorded with a Finnigan GCQ™ GC–MS system equipped with a 25 m 25QC2/HT5 capillary column (i.d. 0.22 mm, film 0.1 μm). Detailed experimental conditions are given in [11]. All samples were injected in splitless mode (1 min). Depending on availability, compounds were identified by comparison of their mass spectra and retention times to those of authentic standards. In case reference compounds were not accessible, mass spectral characteristics were utilized to elucidate the structure as far as possible.

3. Results

The transformation of pregnenolone by the two *Bacillus* strains was completed largely after an incubation period of 48 h. Only slight quantitative alterations in the spectrum of products were detected after 96 h. Apart from compound **1**, none of the transformation products possessed a 3-oxo group. For that reason, formation and chromatographic separation of *syn*-/*anti*-isomeres, often causing problems after MO-TMS derivatization of 3-oxo steroids [12], did not occur. On the other hand, the relatively rude conditions necessary for MO-TMS derivatization caused a partial decomposition of the extracted steroids that was also observed for some reference compounds, resulting in decreased sensitivity. Therefore, many of the minor transformation products could not be detected after MO-TMS derivatization of the extracts.

As observed with AD [11], strain HA-V6-11 was metabolically more active than HA-V6-3. After an incubation period of 48 h, only a small residue of pregnenolone was found in the extract of HA-V6-11 (Fig. 1), while most of the substrate was left untouched by HA-V6-3 (note that the pregnenolone peak is cut off at 6.25% in Fig. 5).

3.1. Transformation of pregnenolone by strain HA-V6-11

The transformation experiments with HA-V6-11 yielded a very wide range of products (Table 1). Within 48 h, pregnenolone was metabolized for the most part (cf. Fig. 1). Due to the high metabolic activity of the strain, a variety of polyoxygenated minor products was

formed already after an incubation period of only 24 h. For the majority of these compounds, it was not possible to clarify the position or configuration of all functional groups by GC–MS because of the great number of possible configurational isomers, lack of data in the literature, insufficient mass spectrometric characteristics, and/or poor availability of reference steroids with higher polarity.

Overall, a total of 16 different transformation products were detected; however, compound **2** occupied more than 50% of the total peak area of all products. The mass spectrum of **2** as its *bis*-TMS ether (M^+ 476; Fig. 2) was dominated by the cleavage of trimethylsilanol, leading to an outstanding base peak at m/z 386 [M-90]. No other ion reached more than 10% relative intensity. The spectrum was largely concordant with published intensities of the most important peaks of 3β, 7α- and 3, 7β-dihydroxypregn-5-en-20-one-*bis*-TMS [13], except from the molecular ion, which was not recorded in the reference cited.

The spectra of compounds **2** and **5** after trimethylsilylation did not differ significantly from each other; neither did the spectra of the MO-*bis*-TMS derivatives (M^+ 505; cf. Figs. 2 and 3), which also showed poor fragmentation apart from the very intense [M-90] ion. Further fragments were derived from successive elimination of $-OCH_3$ [M-31] from the methoxyimino group and the two trimethylsilyloxy (TMSO) groups as trimethylsilanol [M-90]. By comparison of the retention times with the derivatized authentic steroids, the two compounds could be identified as 7α- (2) and 7β-hydroxypregnenolone (**5**).

The molecular ion of **11** as TMS ether was found at m/z 402 (base peak; Fig. 4a), which is equal to the molecular weight of a hydroxyprogesterone TMS ether; however, the intense ion at m/z 129 indicated the presence of a 3β-TMSO-Δ^5 structure [14], supplying evidence for a pregnenolone derivative with an additional oxo function. The conspicuous fragments at m/z 317 [M-85] and 227 [M-(90 + 85)], probably arising

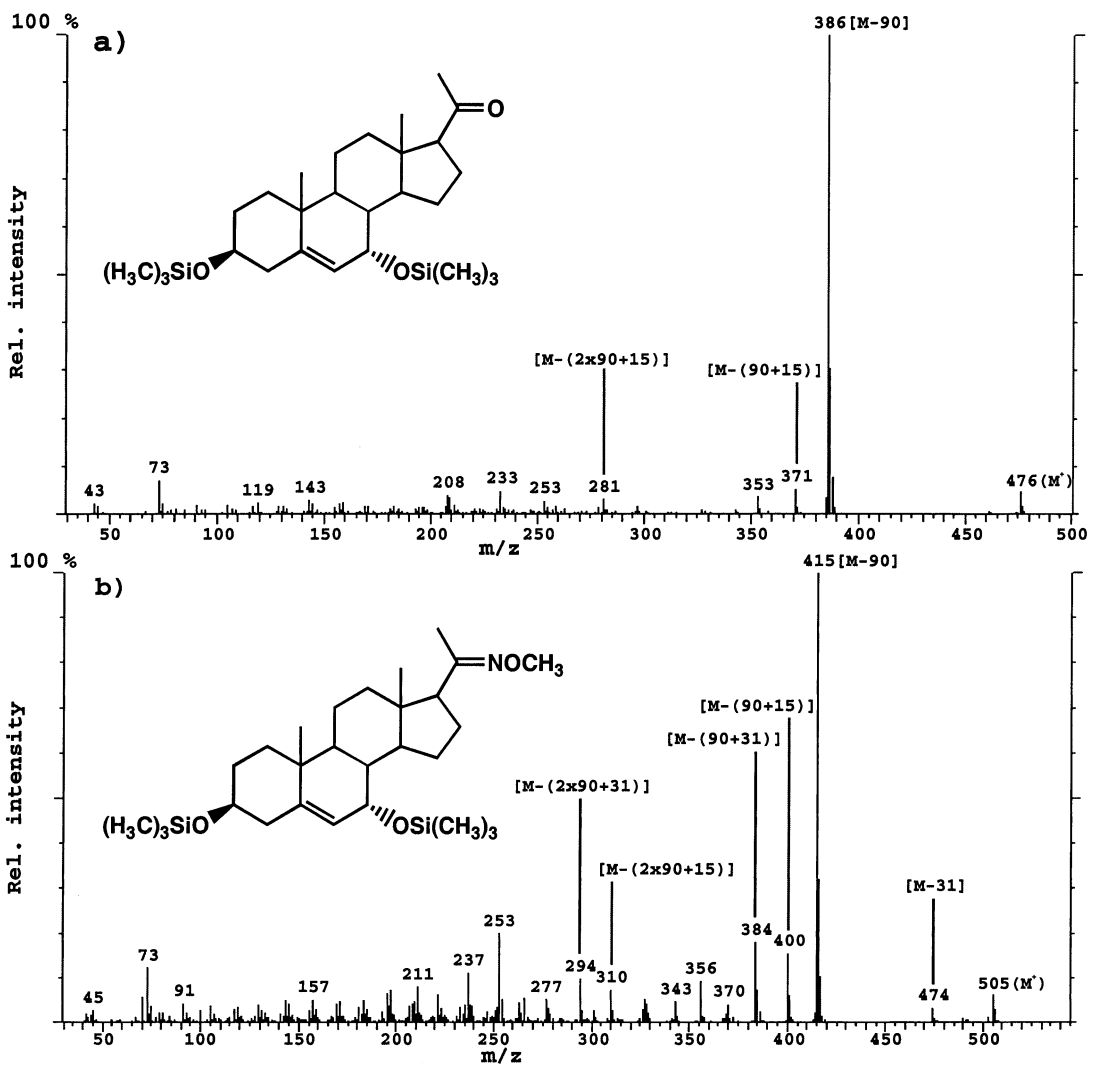

Fig. 2. Background subtracted mass spectra of 7α-hydroxypregnenolone (compound 2) — (a) *bis*-TMS, (b) MO-*bis*-TMS.

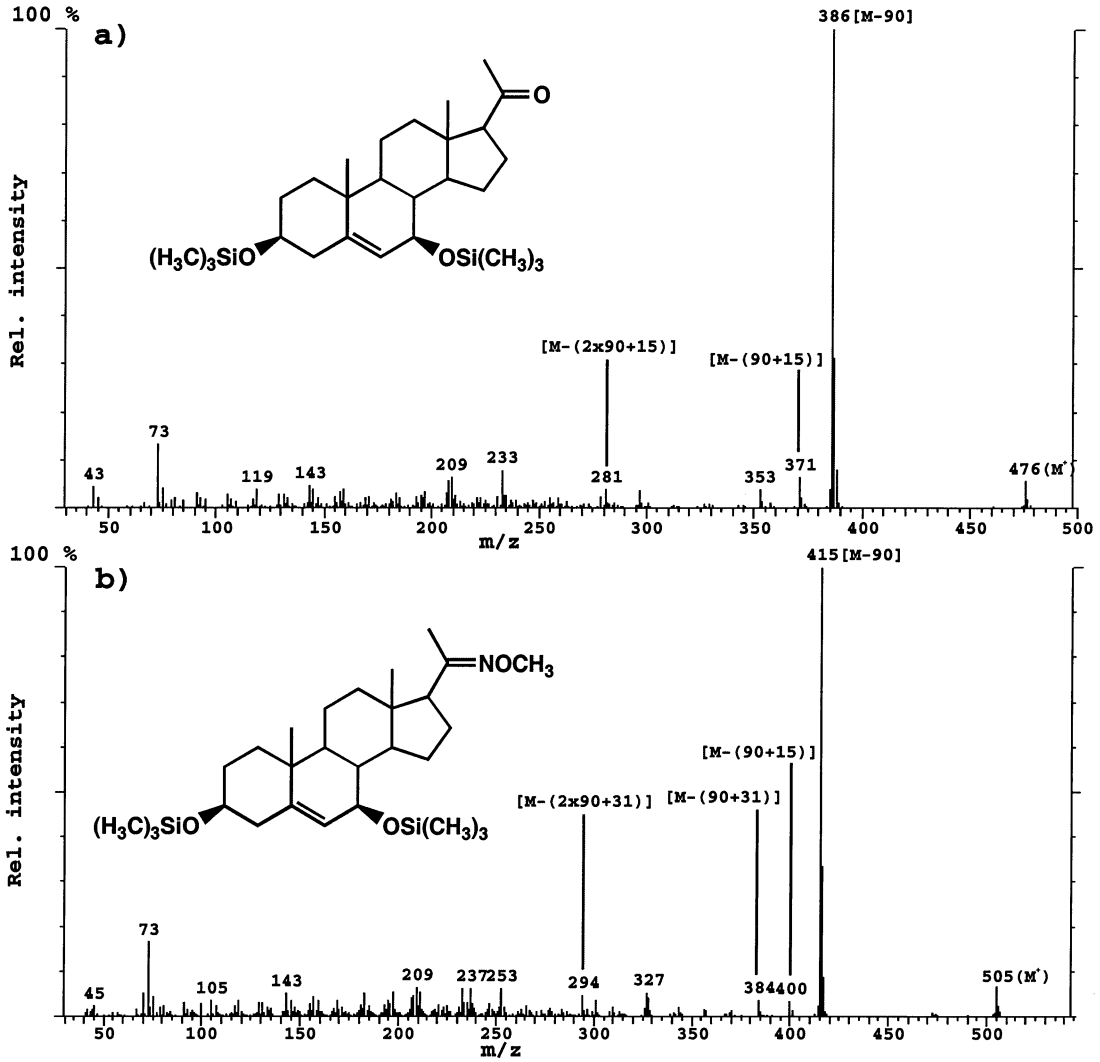

Fig. 3. Background subtracted mass spectra of 7β-hydroxypregnenolone (compound 5) — (a) *bis*-TMS, (b) MO-*bis*-TMS.

from cleavage of the $C_{13/17}$ and $C_{14/15}$ bonds (Fig. 4a) [15], indicated that no functional group was located in the D-ring. Peak **11** showed the same retention time and mass spectrum as authentic 3β-hydroxypregn-5-en-7,20-dione-TMS (7-oxopregnenolone). This structure was confirmed by the *bis*-MO-TMS derivative (M^+ 460; Fig. 4b). It cannot be deduced from our experiments if **11** is formed by oxidation of either 7α- or 7β-hydroxypregnenolone or from both compounds.

By GC–MS, it was not possible to identify completely the structure of more than two from probably five different dihydroxypregnenolones that were formed by HA-V6-11. Upon trimethylsilylation, compounds **3** and **4** both showed a molecular ion at *m*/*z* 564, pointing to a dihydroxypregnenolone-*tris*-TMS ether, and a very similar fragmentation pattern. Analogous to **2** and **5**, the outstanding [M-90] fragment could have indicated the presence of a 7-TMSO group. By comparison with the authentic steroids, the compounds were identified as 7β, 11α-dihydroxypregnenolone (**3**) and the corresponding 7α, 11α-isomer (**4**). According to the proportion of products **2** and **5**, much more 7α, 11α- than 7β, 11α-dihydroxypregnenolone was formed. MS data — 3β, 7β, 11α-Trihydroxypregn-5-en-20-one-*tris*-TMS (**3**), 564 [M^+] (4%), 549 [M-15] (14), 474 [M-90] (100), 459 [M-(90 + 15)] (10), 384 [M-(2 × 90] (11), 369 [M-(2 × 90 + 15)] (19), 341 [M-(2 × 90 + 43)] (5), 296 (11), 277 (10), 257 (11), 251 (13), 233 (9), 213 (12), 209 (12), 195 (10), 183 (10), 169 (10), 157 (10), 143 (9), 129 (11), 119 (13), 73 (30), 43 (6); 3β, 7, 11α-Trihydroxypregn-5-en-20-one-*tris*-TMS (**4**), 564 [M^+] (3), 474 [M-90] (100), 459 [M-(90 + 15)] (10), 384 [M-2 × 90] (72), 369 [M-(2 × 90 + 15)] (86), 341 [M-(2 × 90 + 43)] (10), 295 (15), 270 (20), 259 (20), 251 (34), 233 (13), 209 (15), 172 (13), 157 (10), 143 (11), 129 (17), 73 (41), 43 (7).

The spectrum of compound **1** (M^+ 474) showed a prominent series of ions including *m*/*z* 431 [M-43], 341 [M-(90 + 43)] and 251 [M-(2 × 90 + 43)]. By recording the mass spectra of several trimethylsilylated 17α-hy-

droxy-20-oxo C_{21} reference steroids, we were able to confirm that the cleavage of a 17β-side chain (C_{20+21}) (i.e. [M-43]) is favored highly by the presence of a 17α-TMSO group (unpublished results; cf. also spectrum of 17α-hydroxypregn-4-en-3,20-dione-*bis*-TMS in [16]). The ion at m/z 129, normally indicating the presence of a 3β-TMSO-Δ^5 structure [14], is only weak in the spectrum of **1**. Though this needs to be confirmed by analysis of the authentic compound, we suggest that **1** has the structure 17α-hydroxypregn-5-en-3,20-dione. MS-data (**1**), *bis*-TMS, 474 [M^+] (20%), 459 [M-15] (69), 443 (8), 431 [M-43] (16), 384 [M-90] (22), 369 [M(90 + 15)] (33), 341 [M-(90 + 43)] (30), 294 [M-(2 × 90)] (37), 279 [M-(2 × 90 + 15)] (46), 261 (27), 251 [M-(2 × 90 + 43)] (100), 235 (31), 221 (21), 209 (61), 195 (44), 171 (48), 157 (24), 143 (27), 129 (13), 91 (12), 73 (44), 43 (13).

Peak **6** (M^+ 564) might represent another dihydroxypregnenolone carrying a 7-hydroxygroup because of the very intense [M-90] ion (cf. **2** and **5**). The series of fragments at m/z 521 [M-43], 431 [M-(90 + 43)], 341 [M-(2 × 90 + 43)] and 251 [M-(3 × 90 + 43)] probably indicated the presence of a 17α-TMSO group (cf. **1**). Hence, the structure of **6** is probably 3β, 7ξ, 17α-trihydroxypregn-5-en-20-one. MS data (**6**), *tris*-TMS, 564 [M^+] (4%), 521 [M-43] (40), 474 [M-90] (83), 431 [M-(90 + 43)] (42), 390 (28), 369 [M-(2 × 90 + 15)] (12), 341[M-(2 × 90 + 43)] (47), 285 (16), 259 (23), 251 [M-(3 × 90 + 43)] (100), 209 (29), 195 (17), 183 (9), 169 (12), 143 (11), 133 (16), 117 (22), 73 (45).

The same clue about a dihydroxypregnenolone with a 7-OH group was shown by the spectrum of peak **9**; however, the spectrum did not allow structural assignment of the third OH group. MS data, 3β, 7ξ,x-trihydroxypregn-5-en-20-one-*tris*-TMS (**9**; with 'x' denoting the position and configuration of the third OH function), 564 [M^+] (6%), 549 [M-15] (2), 474 [M-90] (100), 459 [M-(90 + 15)] (6), 384 [M-2 × 90] (14), 369 [M-(2 × 90 + 15)] (26), 341 [M-(2 × 90 + 43)] (4), 299 (6), 270 (16), 251 [M-(3 × 90 + 43)] (13), 233 (9), 209 (7), 157 (6), 143 (6), 129 (6), 105 (5), 73 (21), 43 (2).

The molecular ion (M^+ 476) and elimination of two

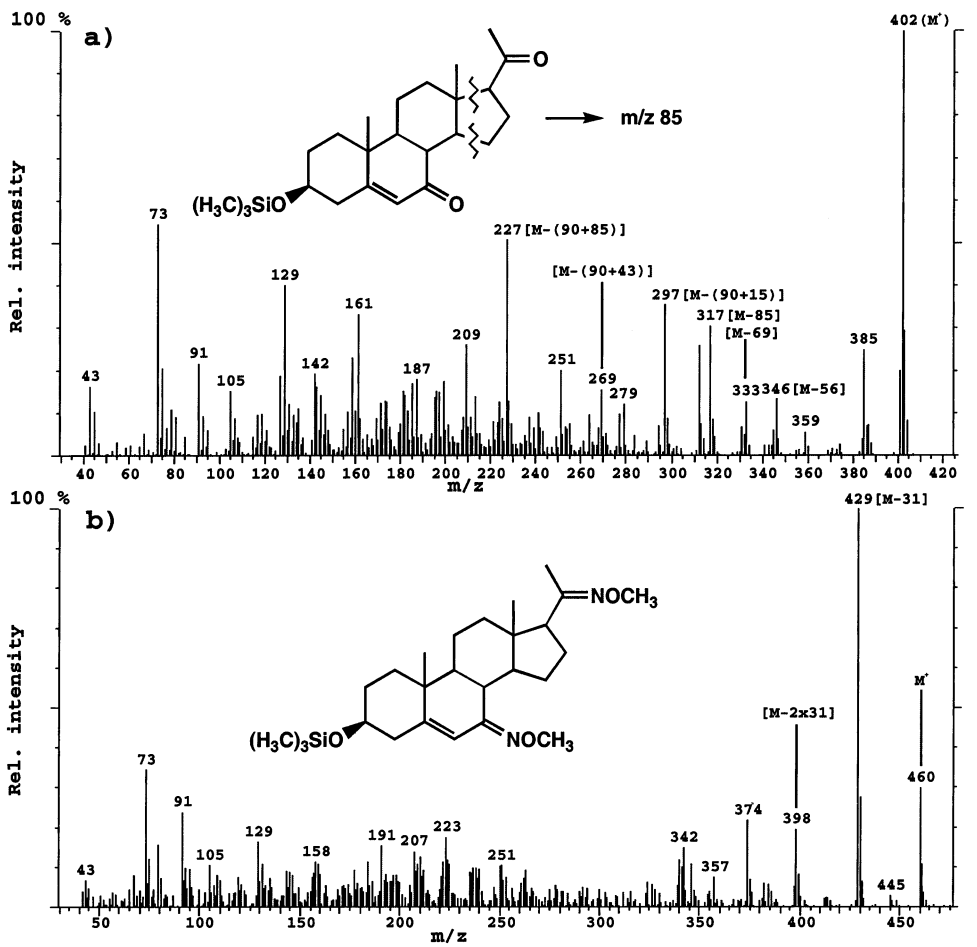

Fig. 4. Background subtracted mass spectra of 7-oxopregnenolone (compound **11**) — (a) TMS ether, (b) *bis*-MO-TMS derivative.

Table 2
Products formed upon transformation of pregnenolone by strain HA-V6-3; main product printed in bold letters[a]

	Transformation product	Relative retention time (5α-cholestane = 1.00)				% Peak area (TMS derivatives)
		TMS	*bis*-TMS	*tris*-TMS	MO-TMS	48 h
2	**3β, 7α-Dihydroxypregn-5-en-20-one**	–	**0.92**	–	**0.93**	++
5	3, 7β-Dihydroxypregn-5-en-20-one	–	1.05	–	1.03	+
7	3β, 15ξ-Dihydroxypregn-5-en-20-one[b]	–	1.09	–	–	*t*
11	3β-Hydroxypregn-5-en-7,20-dione	1.31	–	–	1.25	*t*
17	3β-Hydroxypregn-5-en-20-one (17α side chain?)[b]	–	0.90	–	–	*t*
18	3β, 11α-Dihydroxypregn-5-en-20-one	–	–	1.00	1.00	*t*
19	3β, 17α-Dihydroxypregn-5-en-20-one	–	–	1.00	1.04	*t*
20	3β, 16α-Dihydroxypregn-5-en-20-one[b]	–	–	1.04	–	*t*
21	3β-Acetoxypregn-5-en-20-one	1.08	–	–	–	*t*

[a] Numbers of compounds like in Fig. 5; TMS, trimethylsilyl ether, MO-TMS, *O*-methyloxim trimethylsilyl ether derivative (fully derivatized); ++, major product (relative peak area >50%; substrate not included), +, minor product (relative peak area 1–50%), *t*, traces (relative peak area <1%).
[b] Tentatively identified.

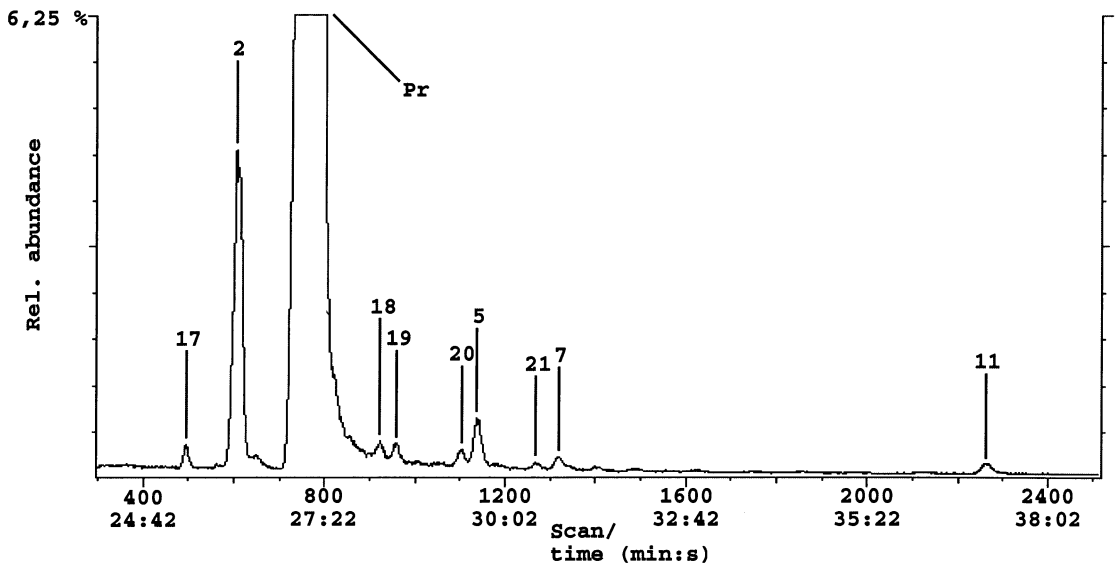

Fig. 5. Total ion current chromatogram of trimethylsilylated culture extracts of strain HA-V6-3 after an incubation period of 48 h; enumeration of TMS derivatives according to compound numbers in Table 2; GC–MS conditions see text. Pr, 3β-Hydroxypregn-5-en-20-one-TMS (pregnenolone, 17β-configuration of side chain; substrate), **2**, 3β, 7α-dihydroxypregn-5-en-20-one-*bis*-TMS (7α-hydroxypregnenolone), **5**, 3β, 7β-dihydroxypregn-5-en-20-one-*bis*-TMS (7β-hydroxypregnenolone), **7**, 3β, 15ξ-dihydroxypregn-5-en-20-one-*bis*-TMS, **11**, 3β-hydroxypregn-5-en-7,20-dione-TMS (7-oxopregnenolone), **17**, 3β-hydroxypregn-5-en-20-one-TMS (pregnenolon, 17α-configuration of side chain), **18**, 3β, 11α-dihydroxypregn-5-en-20-one-*bis*-TMS (11α-hydroxypregnenolone), **19**, 3β, 17α-dihydroxypregn-5-en-20-one-*bis*-TMS (17α-hydroxypregnenolone), **20**, 3β, 16α-dihydroxypregn-5-en-20-one-*bis*-TMS (16α-hydroxypregnenolone), **21**, 3β-acetoxypregn-5-en-20-one (pregnenolone acetate); **7**, **17** and **20** tentative structures; TMS, trimethylsilyl ether.

TMSO groups in the spectrum of **7** pointed to another hydroxypregnenolone. It showed a fragmentation pattern similar to 3β, 15α-dihydroxypregn-5-en-20-one-*bis*-TMS in [17]. The base peak in the spectrum of 15α- as well as 15β-hydroxypregnenolone is formed by m/z 281 [M-(2 × 90 + 15)]. However, the fragment at m/z 172 (comprising C_{15-17}, C_{20} and C_{21}), which is typical for 15-TMSO-20-oxo C_{21} steroids [18,19], is of significantly lower intensity for the 15α-hydroxypregnenolone in contrast to the 15β-isomer. Thus, we believe that **7** is 15α-hydroxypregnenolone, though this needs confirmation by comparison with the authentic compound. MS data, 3β, 15ξ-dihydroxypregn-5-en-20-one-*bis*-TMS (**7**), 476 [M^+] (1%), 461 [M-15] (4), 386 [M-90] (20), 371

[M-(90 + 15)] (3), 296 [M-(2 × 90)] (53), 281 [M-(2 × 90 + 15)] (100), 263 (14), 255 (31), 253 (57), 237 (11), 226 (34), 211 (35), 197 (10), 183 (13), 172 (9), 158 (16), 157 (12), 143 (16), 129 (11), 73 (21).

The mass spectrum of **8** was characterized by elimination of H_2O [M-18] from the molecular ion (M^+ 492) and from further fragments, giving evidence for the presence of an underivatized OH group and a *bis*-TMS derivative of a dihydroxypregnenolone. During our studies, we often observed this effect upon silylation of 14α-hydroxysteroids with MSTFA, the 14α-hydroxy group mostly remaining underivatized because of sterical hindrance. However, our knowledge about **8** is not sufficient to denote the position of the functional groups. MS data, 3β, 14α (?), x-trihydroxypregn-5-en-20-one-*bis*-TMS (**8**), 492 [M^+] (5%), 474 [M-18] (23), 402 [M-90] (44), 384 [M-(90 + 18)] (51), 369 [M-(90 + 18 + 15)] (31), 341 [M-(90 + 43 + 18)] (24), 312 [M-2 × 90] (48), 297 [M-(2 × 90 + 15)] (69), 294 [M-(2 × 90 + 18)] (43), 279 [M-(2 × 90 + 18 + 15)] (48), 270 (58), 251 [M-(2 × 90 + 43 + 18)] (100), 227 (55), 211 (45), 199 (54), 186 (61), 171 (62), 157 (51), 145 (75), 129 (69), 119 (26), 105 (25), 91 (23), 73 (86), 43 (33).

The molecular ions of compounds **10**, **13**, **14** and **16** (M^+ 490) as well as the occurrence of [M-90] and [M-2 × 90] fragments speak in favor of pregnenolone

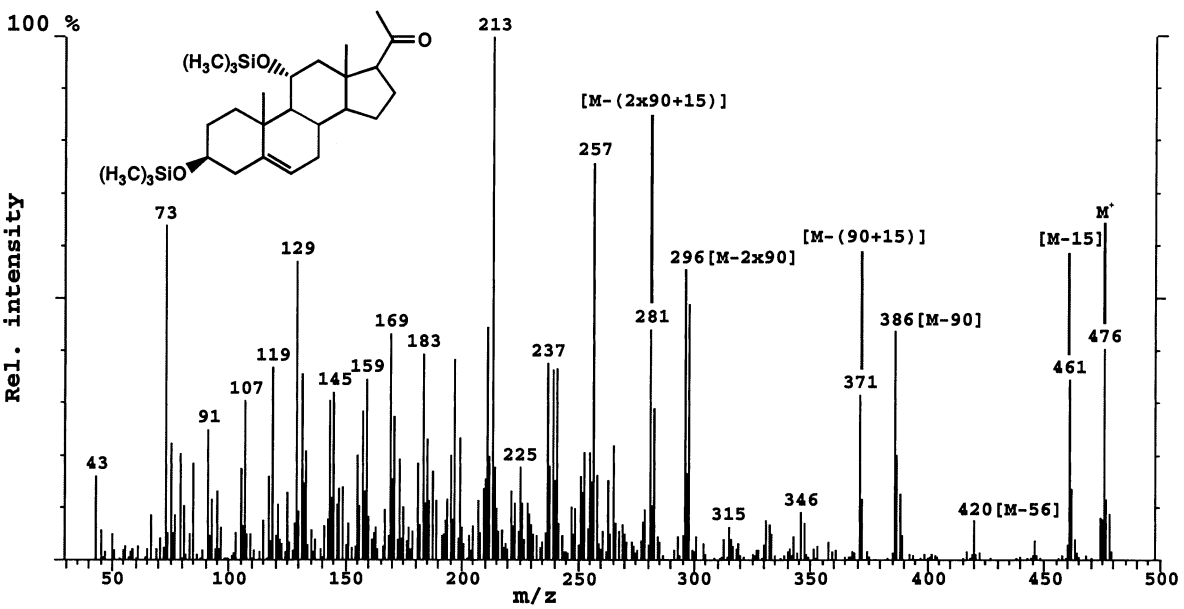

Fig. 6. Background subtracted mass spectrum of 11α-hydroxypregnenolone (compound **18**) as *bis*-TMS ether.

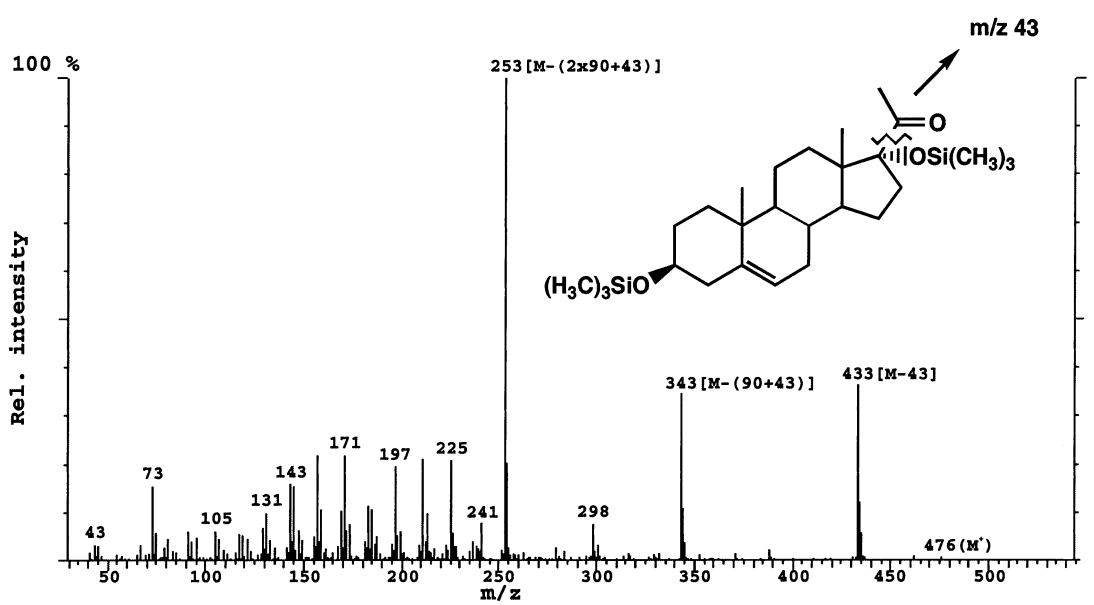

Fig. 7. Background subtracted mass spectrum of 17α-hydroxypregnenolone (compound **19**) as *bis*-TMS ether.

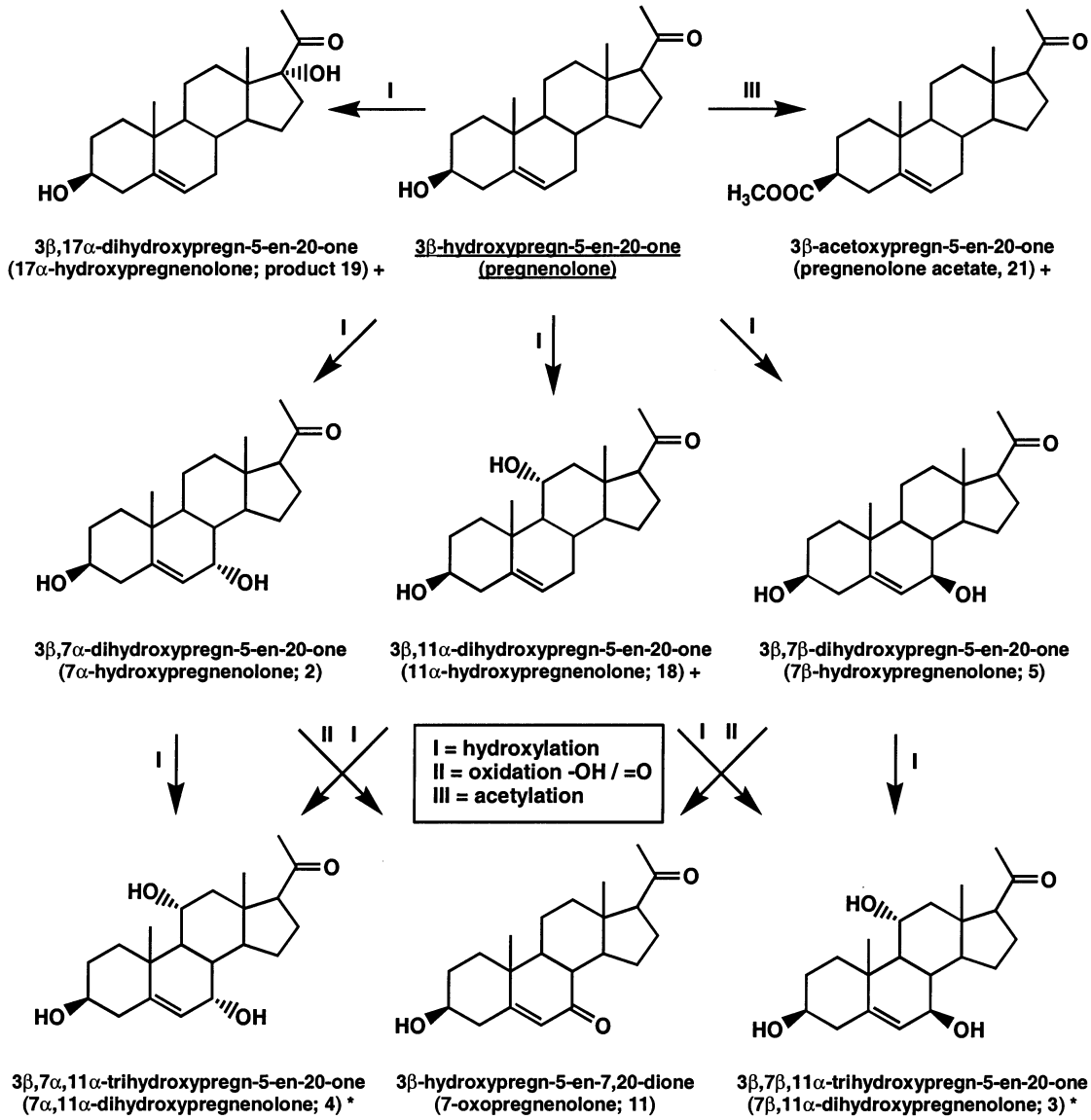

Fig. 8. Postulated pathway for transformation of pregnenolone by strains HA-V6-3 and HA-V6-11 (*Bacillus* sp.) isolated from *A. affinis* (only products with certainly identified structures are shown); compound numbers corresponding to Table 1 Table 2; + detected only for HA-V6-3, *, detected only for HA-V6-11.

derivatives with one additional hydroxy and oxo group. Product **12** had a molecular ion at m/z 418 and a [M-18] fragment, indicating the presence of an underivatized OH group (cf. **8**). Compound **13** probably includes an 17α-OH group (cf. **1**). Unfortunately, none of these mass spectra can be related to known steroids or allows further elucidation of the structures so far. MS data, 3β, x-dihydroxypregn-5-en-y,20-dione-*bis*-TMS (**10**), 490 [M^+] (47%), 475 [M-15] (12), 434 [M-56] (7), 400 [M-90] (31), 385 [M-(90 + 15)] (19), 372 [M-(90 + 28)] (7), 357 [M-(90 + 43)] (12), 331 (12), 324 (12), 310 [M-2 × 90] (94), 295 [M-(2 × 90 + 15)] (12), 282 [M-(2 × 90 + 28)] (20), 267 [M-(2 × 90 + 43)] (25), 252 [M-(2 × 90 + 43 + 15)] (36), 238 (100), 225 (24), 211 (21), 195 (45), 162 (39), 147 (35), 134 (25), 129 (25), 119 (17), 105 (18), 91 (16), 73 (90), 43 (25); 3β, 14α (?)-dihydroxypregn-5-en-y,20-dione-TMS (**12**), 418 [M^+] (10%), 400 [M-18] (100), 385 [M-(18 + 15)] (9), 357 [M-(43 + 18)] (8), 334 [M-(56 + 18)] (73), 310 [M-(90 + 18)] (20), 295 [M-(90 + 18 + 15)] (24), 277 (29), 264 (29), 243 (56), 227 (23), 214 (31), 187 (39), 173 (20), 161 (71), 143 (16), 129 (21), 105 (21), 91 (30), 73 (49), 43 (22); 3β, 17α-dihydroxypregn-5-en-y,20-dione-*bis*-TMS (**13**), 490 [M^+] (6%), 475 [M-15] (49), 447 [M-43] (34), 400 [M-90] (17), 385 [M-(90 + 15)] (34), 357 [M-(90 + 43)] (100), 343 (15), 310 [M-2 × 90] (10), 267 [M-(2 × 90 + 43)], 249 (32), 225 (54), 211 (12), 187 (14), 161 (17), 133 (71), 129 (16), 117 (13), 91 (14), 73 (53), 43 (11). 3β, x-dihydroxypregn-5-en-y,20-dione-*bis*-TMS (**14**), 490 [M^+] (25%), 475 [M-15] (9), 434 [M-56] (2), 400 [M-90] (19), 385

[M-(90 + 15)] (10), 361 [M-129] (9), 310 [M-2 × 90] (33), 295 [M-(2 × 90 + 15)] (7), 282 [M-(2 × 90 + 28)] (21), 268 (17), 225 (12), 195 (100), 181 (18), 147 (23), 133 (12), 129 (14), 105 (14), 91 (6), 73 (53), 43 (10); 3β, x-dihydroxypregn-5-en-y,20-dione-*bis*-TMS (**16**), 490 [M^+] (6%), 475 [M-15] (100), 432 [M-56] (7), 400 [M-90] (27), 385 [M-(90 + 15)] (13), 357 [M-(90 + 43)] (7), 343 (17), 295 [M-(2 × 90 + 15)] (11), 277 (8), 265 (10), 249 (14), 225 (12), 161 (18), 129 (9), 91 (11), 73 (24).

Product **15**, only found in traces, again showed evidence for a 17α-TMSO group by occurrence of a conspicuous series of ions including cleavage of the side chain. However, [M-43] in **1**, **6** or **13** was exchanged to [M-117], together with the ion at *m/z* 117 indicative of a side chain carrying a 20-TMSO group (e.g. [20,21]). MS data, 3β, 17α, 20ξ-trihydroxypregn-5-en-20-one-*tris*-TMS (**15**), 564 [M^+] (3%), 549 [M-15] (1), 474 [M-90] (19), 459 [M-(90 + 15)] (1), 447 [M-117] (64), 391 (10), 369 [M-(2 × 90 + 15)] (6), 357 [M-(117 + 90)] (100), 328 (5), 315 (8), 267 [M-(117 + 2 × 90)] (22), 249 (16), 239 (12), 225 (48), 197 (11), 147 (13), 133 (50), 117 (10), 73 (32), 45 (6).

3.2. Transformation of pregnenolone by strain HA-V6-3

Pregnenolone was metabolized to a total of nine different products (Table 2), though only 7α-hydroxypregnenolone (**2**) was formed in considerable yields (Fig. 5). 7β-hydroxypregnenolone (**5**) as well as 7-oxopregnenolone (**11**) were only produced in traces by HA-V6-3. Another product common with HA-V6-11 was 7 (15ξ-hydroxypregnenolone). A couple of minor products were found exclusively in the extracts of HA-V6-3.

For peak **17**, a mass spectrum was recorded with a molecular ion (M^+ 388) and fragments identical to pregnenolone-TMS. However, in addition to the different retention time, the intensity of the fragment at *m/z* 213 [M-(90 + 85)], formed by elimination of the 3-TMSO group and the C-atoms 15–17, 20 and 21, was increased significantly. This is a characteristic property of pregnenolone with 17α-configuration of the side chain (cf. spectra of 17α-pregnenolone in [22,23] and 17β-pregnenolone in [24]). MS data, 3β-hydroxy-17α-pregn-5-en-20-one-TMS (**17**), 388 [M^+] (26%), 373 [M-15] (3), 332 [M-56] (7), 298 [M-90] (59), 283 (34), 265 (25), 258 (21), 255 (14), 241 (36), 227 (11), 213 [M-(90 + 85)] (100), 199 (14), 185 (21), 171 (22), 159 (19), 145 (20), 129 (24), 119 (13), 105 (12), 85 (18), 73 (23), 43 (18).

Further evidence for the 17α-configuration of **17** was provided by compound **20**. In the spectrum of its TMS-derivative (M^+ 476) the typical combination of the fragments at *m/z* 157, 159, 172 and 186 indicated the presence of a 16-TMSO-20-oxo C_{21} steroid [25]. A conspicuous ion at *m/z* 172 is seen normally in spectra of 20-oxo C_{21} steroids with a TMSO group located in the D ring (C_{15} or C_{16}; C_{17} with less intensity), while *m/z* 157 = [172–15] is accompanied by an intense *m/z* 159 fragment only when a 16-TMSO group is present. The fragmentation pattern of **20** was identical to the spectra of 16α-hydroxypregnenolone-*bis*-TMS in [25,26]. Though the authentic steroid was not available, we presume that **20** has the structure 3β, 16α-dihydroxypregn-5-en-20-one. MS data, *bis*-TMS (**20**), 476 [M^+] (9%), 461 [M-15] (34), 433 [M-43] (19), 386 [M-90] (67), 371 [M-(90 + 15)] (41), 343 [M-(90 + 43)] (22), 330 (30), 296 [M-2 × 90] (73), 281 [M-(2 × 90 + 15)] (50), 253 [M-(2 × 90 + 43)] (66), 239 (31), 223 (21), 213 (28), 197 (35), 186 (25), 183 (46), 172 (58), 159 (61), 157 (100), 143 (58), 129 (42), 119 (15), 105 (23), 91 (20), 73 (64), 43 (20).

Eriksson et al. [22] isolated 17α-pregnenolone as well as further C_{21} steroids with a 17α-side chain from the faeces of conventionally reared rats, whereas such compounds were not detectable in the faeces of germfree rats. The incubation of gut contents or microflora isolated from the gut of conventional rats with 16α-hydroxy- or Δ^{16}-C_{21} steroids again resulted in occurrence of transformation products with 17α-side chain. Thus, it was concluded that gut microorganisms are responsible for the synthesis of 17α-C_{21} steroids by transformation of 16α-hydroxylated precursors, with 16-enes occurring as intermediates [23]. As a consequence, the detection of **17** and **20** in the culture extracts can be judged as a hint that the same metabolic steps might be performed by HA-V6-3. No 16α-hydroxypregnenolone was detected for HA-V6-11; accordingly, no formation of 17α-pregnenolone was observed.

Compound **18** was identified as 3β, 11α-hydroxypregn-5-en-20-one (11α-hydroxypregnenolone) by comparison with the reference steroid. The *bis*-TMS ether (M^+ 476) displayed a base peak at *m/z* 213 (Fig. 6), originating from cleavage of the two TMSO groups plus the D ring (C_{15-17}) and side chain (C_{20+21}) under migration of 1 H. The ion at *m/z* 420 [M-56] arises probably from McLafferty rearrangement and cleavage of the C-atoms 1–3 [27]. The MO-*bis*-TMS derivative (M^+ 505) was only detected after derivatization of the authentic compound.

The *bis*-TMS derivative of **19** showed the typical fragmentation of a 17α-TMSO-20-oxo C_{21} steroid with a series of prominent ions resulting from cleavage of the side chain ([M-43]; cf. **1**), whereas the molecular ion (M^+ 476) was only of weak intensity (Fig. 7). Mass spectrum and retention time were identical to authentic 17α-hydroxypregnenolone-*bis*-TMS. A pure spectrum of the MO-*bis*-TMS derivative of **19** (M^+ 505; cf. [28]) could be recorded with the reference steroid only, though traces were also detected upon MO-TMS derivatization of the extracts. The spectrum is charac-

terized by distinct ions at m/z 188 (side chain + $C_{16/17}$ + 1 H) and 156 (side chain + C_{15-17} + 1 H) [29]. MS data, 3β, 17α-dihydroxypregn-5-en-20-one-MO-*bis*-TMS (reference compound), 505 [M^+] (22), 490 [M-15] (10), 474 [M-31] (100), 415 [M-90] (11), 384 [M-(90 + 31)] (17), 362 (20), 325 [M-2 × 90] (8), 312 (11), 294 [M-(2 × 90 + 31)] (24), 279 [M-(2 × 90 + 31 + 15)] (12), 253 (14), 239 (33), 223 (14), 197 (23), 188 (18), 156 (25), 143 (16), 129 (11), 105 (17), 91 (15), 73 (50), 43 (10).

The dominating [M-60] fragment (m/z 298) in the spectrum of **21** (M^+ 358) indicated the presence of an acetate. The spectrum was in accordance with published MS data of pregnenolone acetate [30], and the structure was proved by comparison with the standard. MS data, 3β-acetoxypregn-5-en-20-one (**21**), 358 [M^+] (0.5%), 298 [M-60] (100), 283 [M-(60 + 15)] (31), 265 (15), 255 [M-(60 + 43)] (18), 213 [M-(85 + 60)] (36), 185 (15), 145 (23), 131 (12), 121 (10), 105 (15), 91 (19), 79 (17), 43 (14).

4. Discussion

The biotransformation of steroids by microorganisms has been a matter of extensive research over the past decades. Hundreds of publications are listed by the reviewing literature [31–37]. There is hardly one atom of the steroid skeleton that has not been found to be attacked by at least a few microbial strains. Nevertheless, if the huge microbial biodiversity is considered (varying estimations say that the number of bacterial species we know today is not more than 0.1–1% of the truth) it is not wishful thinking to expect that there is still much left to learn about microbial transformation of steroids.

Regarding the importance of *Bacillus* in all aspects of microbiology, amazingly little work has been carried out on steroid transformation by members of this genus. In most cases, when steroid substrates were incubated with *Bacillus* strains hydroxylations at different sites of the steroid skeleton were observed. These reactions are known to be catalyzed by site- and stereospecific cytochrome P-450 monooxygenases, whose molecular biology has been scrutinized only in a few cases so far [38–40]. The range and configuration of products depends on the organism or strain that is involved. The most frequently and efficiently oxidized carbon atoms are C_6, C_{11}, C_{14} and C_{15} (overview in [11]). In addition, several minor reactions have been observed with *Bacillus* isolates, including the reduction of Δ^4-double bonds or interconversion of 17-OH-/oxo-groups in C_{19} steroids.

A *Bacillus cereus* strain was reported to transform deoxycorticosterone (21-hydroxyprogesterone) yielding 6β-, 14, 15α- and 15β-hydroxylated products [41]. Several Δ^4-C_{21} steroids have been tested as substrates with a *Bacillus* isolate; the experiments yielded products with 6β-, 11, 14 and 15α-OH groups [42–45]. However, to our knowledge, C_{21} steroids with a Δ^5-double bond have not been utilized as substrates for *Bacillus* so far.

As expected, hydroxylations were the major metabolic activities that were observed for the *Bacillus* isolates from the gut of *A. affinis* (Fig. 8). The major difference between the utilization of androst-4-en-3,17-dione (AD) and pregnenolone by these isolates is the shift of the main reaction site from C_6 to C_7, resulting in formation of 7α-hydroxypregnenolone in contrast to 6β-hydroxy-AD as the main product (cf. [11]). Additionally, 7β-hydroxypregnenolone was found in minor amounts. Though small amounts of 7α-hydroxy-AD were also formed from AD [11], the major site of B-ring hydroxylation is determined by the position of the double bond, which has to be located in α-position to the functional group. This was also observed for many fungi upon incubation with Δ^5-unsaturated steroids [31–37].

According to AD, the main monohydroxylated product was partly further oxidized to the corresponding ketone. Furthermore, pregnenolone was also transformed to an 11α-hydroxylated product by HA-V6-3. Though 11α-hydroxypregnenolone was not detected as a product of HA-V6-11, the capability of the strain to perform 11α-hydroxylation is shown by the presence of 7, 11α- and 7β, 11α-dihydroxypregnenolone. No 14-monohydroxylated product was formed, though some evidence was found that two of the more polar products (**8**, **12**) might carry a 14α-OH group. Furthermore, D-ring hydroxylations were observed, leading to the formation of 15ξ-(both strains) as well as 16- and 17α-hydroxypregnenolone (only HA-V6-3). The latter monohydroxylated derivatives were not found for HA-V6-11, which is probably due to its high metabolic activity, resulting in formation of a variety of more polar unidentified products already after 24 h (including 17α-OH groups in **1**, **6**, **13** and **15**). 16- and 17α-Hydroxylation of C_{21} steroids has not been reported for *Bacillus* before.

An unusual metabolic step for bacteria is the 3β-acetylation that was performed by HA-V6-3, although this reaction was only carried out in tiny amounts. In contrast, the interconversion of C_{21} steroids with 17β-side chain to their corresponding 17α-configured isomers via 16-OH intermediates is not exceptional (e.g. [22,23]).

As we observed before with AD as a substrate, strain HA-V6-11 showed a remarkable metabolic activity, transforming pregnenolone with high overall efficiency. Though 7α-hydroxypregnenolone was formed with high yields, both strains will not be taken into consideration for industrial scale production and commercial purposes because of the great number of by-products that would require expensive purification procedures. On the

other hand, it should not be underestimated that microbial strains isolated from 'exotic' sources might represent a valuable resource for the future development of new biocatalysts based on modern molecular biology.

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