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MICROBIOLOGICAL TRANSFORMATIONS—IX†

MICROBIOLOGICAL REDUCTION OF CAREN-3-DIONE-2,5 BY *RHODOTORULA MUCILAGINOSA*‡

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Abstract—The microbiological fermentation of 3-caren-2,5-dione (**1**) by means of *Rhodotorula mucilaginosa* strain yielded: caran-2,5-dione (**2**), (+)-2-caranon-5-ol (**3**), (−)-*trans*-3-caren-2-on-5-ol (**4**) and (+)-*trans*-3-caren-5-on-2-ol (**5**). The structures were established by spectral methods. All the alcohols obtained microbiologically possess the *S*-configuration.

The enzyme system of *Rhodotorula mucilaginosa* is capable of selective transformation of steroids, one example being the observed reduction of a ketone group on C-3 in testosterone preceded by the reduction of Δ-4,5-double bond.[1] These transformations produce androsterone and epiandrosterone in combined yield of 73%. Introduction of steric hindrance such as a Me group or Cl atom into the ring A of testosterone affects the course of transformation: the Me group inhibits the reduction of the conjugated system with the result that one alcohol (*S*) on C-3 is formed and the double bond is reduced to A/B *trans* system with a yield of about 20%.[2] On the other hand, the presence of a Cl atom helps to reduce the conjugated system of ring A of 4-chlorotestosterone to an allylic alcohol.[3] The sensitivity of *Rh. m.* species to structure differences in steroid substrates encouraged us to examine the transformations of a conjugated ketone of the terpene series using this strain. Microbiological reactions of the CO group or unsaturated bonds in terpenes are little known.

Earlier studies in this area are exemplified by stereospecific reduction of (+)- and (−)-carvone and (+)- and (−)-carvotanacetone to saturated ketones and alcohols by *Aspergillus niger* and *Pseudomonas ovalis* strains.[4,5]

3-Caren-2,5-dione (**1**) is an interesting substrate because it has two ketone groups conjugated with an unsaturated bond and also with a cyclopropane ring. This substrate was obtained by autoxidation of (+)-3-carene.[7] In spite of its chirality we were able to demonstrate the optical activity by CD measurements.§

The CD curve obtained shows two extrema at 340 nm (+) 0.02 and 290 nm (−) 0.02. The CD diagram suggests that the curve obtained might result from conjugation of two CO groups or might represent two different bands characteristic for ketone groups adjacent to CH_3—C≦ and ≧C—H groupings. Microbiological transformation of **1** was carried out as described for steroid substrates. The fermentation was carried out in one litre solutions containing *Rh. m.* cultured in a maltose medium. After 2 or 3 weeks of fermentation solutions containing about 100 mg/l of substrate (**1**), the product mixture was isolated by extraction with ether and was chromatographed on silica gel yielding 4 products of established structure: 2,5-carandione (**2**), (+)-2-caranon-5-ol (**3**), (−)-*trans*-3-caren-2-on-5-ol (**4**)[‖] and (+)-*trans*-3-caren-5-on-2-ol (**5**) (Fig. 1).

As the substrate and its transformation products were unstable the time of reaction had a distinct effect upon the yield. Prolonged fermentation produced comparatively large amounts of a mixture of more polar compounds which we were unable to separate and to identify (Table 1).

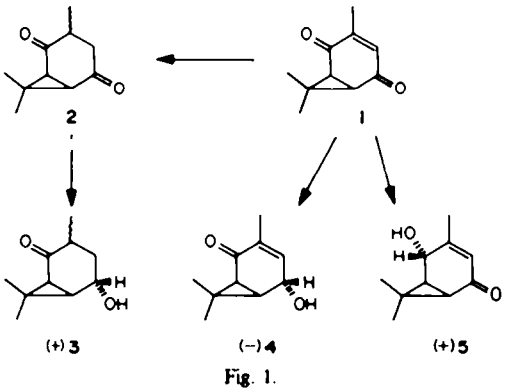


Fig. 1.

†Part VIII: *Acta Mycol.*, Poland (1975) in press.

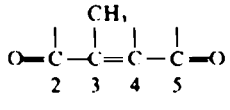
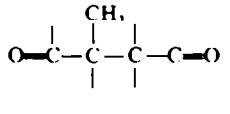
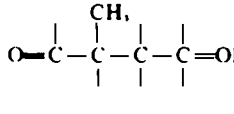
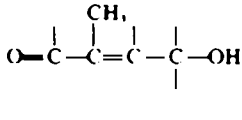
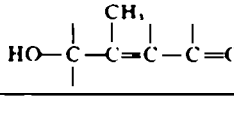
‡The strain was obtained from a collection of Dr. A. Nespiak, Department of Botany of the Medical Academy, Wroclaw, Poland

§We are much indebted to Prof. G. Snatzke of Bochum University, FRG for measurements and their interpretation.

‖*trans*: relative position of OH group and cyclopropane ring.

The main transformation products were the saturated ketoalcohol (3) and the allylic ketoalcohol (5). After shorter fermentation periods the product mixtures contained in addition the saturated diketone (2), absent in products of 3-week fermentation. This suggests that 2 represents an intermediate product reduced further to the ketoalcohol (3). The ketoalcohol (4) with a 3,4-double bond, isomeric with 5, was formed in amounts independent of fermentation time with a yield of about 9%. These results indicate that two pathways are possible for carendione transformation. The first pathway involves first the reduction of the $\Delta^{3,4}$-bond with the formation of 2 which is further reduced to the keto-alcohol (3). This order of reduction is typical for microbiological reactions of α,β-unsaturated steroid ketones.[8] The second pathway yields directly the allylic alcohol on C-2 or C-5 without prior reduction of a double bond. The products (4 and 5), containing an α,β-unsaturated ketone and of an allylic alcohol HO—C—C=C—C=O probably do not readily react further. Small variations of yields of both products with reduced $\Delta^{3,4}$-bond and of products containing this bond intact suggest that there is not much preference for either of the two pathways.

The deviation from the typical sequence of microbiological reduction (unsaturated bond—ketone), making possible the appearance of the second type of transformation, is due, in addition to other factors, to the interaction of the carbonyl groups on each side of the double bond in carendione. Such interaction brings about a destabilization of ketone on C-2 and C-5 and makes possible its reduction to an allylic alcohol. On the other hand, the α,β-unsaturated ketone in 4 and 5 is more stable and, therefore, its further reduction is more difficult. This is reflected in the frequencies of valence vibrations of C=O of ketones in question:

Compound	bond system	IR (cm $^{-1}$)
1		1670
2		1705
3		1700
4		1665
5		1660

The frequencies indicate that the ketoalcohols (4 and 5) are more stable than both the substrate and saturated products (2 and 3). Similar rationalization of structural effects in microbiological reductions is reported by Ringold,[9] concerning the C-3 ketone in 6β and 6α-fluorotestosterone. The C=O bands of these compounds are at 1687 and 1679 cm $^{-1}$, resp. and the reduction yields two alcohols, saturated and allylic. On the other hand, the reduction of testosterone (1660 cm $^{-1}$) produces exclusively and in good yields the saturated alcohol. The author explains these differences by a destabilization of the first two substrates by a halogen atom on C-6. A similar effect is to be expected in carendione because of the presence of a CO group at the end of a conjugated system.

Table 1. Yields of the products

Time of transformation (days)	Starting substance (1)	Products in % 2	3	4	5	Mixture of more polar compounds
14	7%	5	35	9	35	10
21	—	—	26	8	31	35

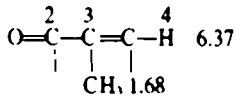
The structures of substrate and of the four products of its microbiological reduction were established by UV, IR and NMR spectroscopy.

The 3-caren-2,5-dione (1) was obtained by autoxidation of natural (+)-3-carene of known absolute configuration—1*S*,6*R*.

The IR spectrum of 1 contains bands corresponding to valence vibrations of the CO groups and of C=C bond at 1670 and 1620 cm $^{-1}$, respectively. The molar extinctions of the conjugated system are 10,200 and 8730 for maxima at 243 and 228 nm.

Proton magnetic resonance is comparatively simple† (Table 2). The olefinic proton signal is at $\delta = 6.67$ ppm. It is a quartet ($J = 1$ Hz) resulting from a long-range coupling with protons of a Me-10 group. Both protons directly bound with the cyclopropane ring are magnetically equivalent and are shown as a two-proton singlet at $\delta = 2.45$. The protons of the Me-10 group, produce a doublet at $\delta = 2.19$ ($J = 1$ Hz) because of coupling with the olefinic proton. The H atoms of both geminal Me-8 groups are almost equivalent magnetically. Their chemical shifts are 1.65 and 1.62 ppm respectively.

The structure and conformation of the optically active ketoalcohol (4) was determined by UV, IR and NMR spectroscopy. The IR spectrum shows the CO group (1665 cm $^{-1}$) and OH group (3620 cm $^{-1}$). The presence of an α,β-unsaturated ketone is shown by the UV max of 222 nm, molar extinction 9100. The NMR spectrum confirmed these features and yielded also additional information on the structure and conformation of 4 (Fig. 2). Because the absolute configuration of the molecule was known it was possible to deduce the sign of chirality on C-5 where the reduction took place. The structure of the allylic ketoalcohol is proved by the chemical shift values of vinyl proton H-4 ($\delta = 6.37$ ppm) and of the Me-10 protons ($\delta = 1.68$ ppm). The value of $\delta = 6.37$ is characteristic for olefinic protons present at the end of a conjugated system composed of a double bond and a CO group[9]



†HMDS was as the reference.

Table 2. Chemical shifts (δ ppm) in NMR spectra

Number of atom C	Substrate 1	Product 4	Product 5	Product 3
Me-10	2.19 (3H, d)	1.68 (3H, m)	1.93 (3H, d)	1.03 (3H, d)
Me-8	1.65 (3H, S)	1.18 (3H, S)	1.17 (3H, S)	1.20 (3H, S)
Me-9	1.62 (3H, S)	1.24 (3H, S)	1.20 (3H, S)	1.28 (3H, S)
H-4	6.67 (1H, k)	6.37 (1H, m)	5.60 (1H, m)	
H-5		4.88 (1H, m)		4.54 (1H, m)
H-2			4.70 (1H, m)	
H-0		2.90 (1H, S)	2.82 (1H, S)	
H-1, H-6	2.45 (2H, S)			

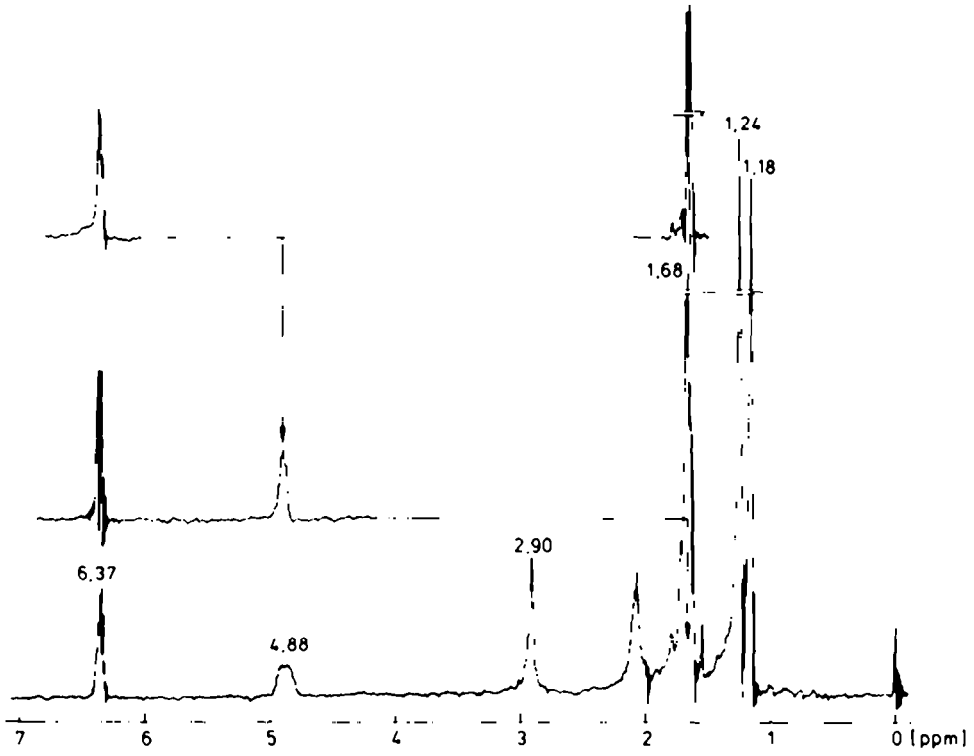
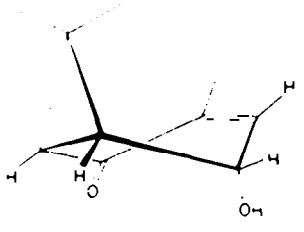


Fig. 2.

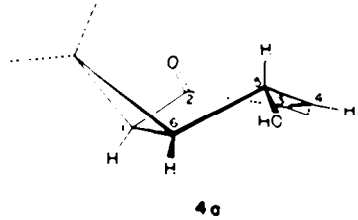
The value of δ = 1.68 for Me-10 protons is possible only when they are located in the middle of a conjugated center.[10] According to Dreiding models there are two possible conformations of the ketoalcohol (4: **4a**) and **4b** (Fig. 3). However, the conformation **4b** is excluded by the values of the chemical shift of Me-9 protons as in this conformation there should be visible a distinct shielding effect of the double bond and a value below 1 ppm.[10]

The orientation of the OH group was determined considering the long-range coupling of the H-5 hydrogen with Me-10 protons (homoallylic coupling). This coupling is possible only at certain exactly specified mutual positions of the atoms in question. In principle the homoallylic coupling is observed only when the angles θ and θ' are 90° (Fig. 4).[10,11]

For compound **4** this is possible only with an equatorial OH group. This conformation is also logically justified because of small steric interactions. According to the principles determining the chirality of centers, the *S*-sign should be ascribed to the C-5 atom.[12] The formation of an alcohol of absolute *S*-configuration on C-5 is also



4b



4a

Fig. 3.

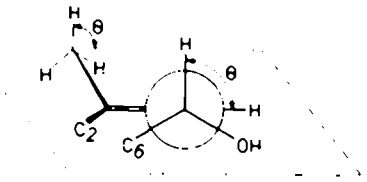


Fig. 4.

consistent with the properties of the enzyme system present in *Rh. m.*[3] It follows that compound **4** has the structure (−)-*trans*-3-caren-2-on-5-ol and conformation as shown by the formula **4a**.

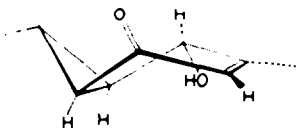
The ketoalcohol **5**, isomeric with **4**, contains a CO group and an OH group, shown in its IR spectrum by bands at 1660 and 3620 cm^{-1} UV absorption at 223 nm (molar extinction 11,200) indicates the α,β-unsaturated ketone. An analysis of the NMR spectrum and, in particular, a comparison of spectra for **4** and **5** made it possible to determine the structure **5**. The spectrum of **5** exhibits two 3-proton singlets of geminal Me groups at the cyclopropane ring at δ = 1.17 and 1.20 ppm, a 3-proton doublet of Me-10 protons at δ = 1.93 ppm (J = 1 Hz), resulting from allylic coupling with olefinic hydrogen H-4. This hydrogen is visible at δ = 5.60 ppm as a multiplet. There is also a second multiplet for H-2 at δ = 4.70 ppm, deshielded by the adjacent O atom from the OH group present on the same C atom C-2. The structural proof of this compound is similar to that of **4**. The value of δ = 5.60 is characteristic for an olefinic proton located in the center of a conjugated system of double bonds with a CO group.[10] Also the value of the chemical shift of Me-10 doublet (δ = 1.93 ppm) indicates that this group is located at the end of a conjugated system: O=C(5)−C(4)=C(3)−CH$_3$ (H at 4, 5.60) 1.93. Both shifts prove the structure of **5**. These assignments become still more distinct when compared with those for product **4** in which H-4 and Me-10 occupy reverse positions in the conjugated system. As in the case of **4** there are two possible conformations, **5a** and **5b** for caren-5-on-5-ol (Fig. 5). Because the chemical shifts of the geminal groups Me-8 and Me-9 are almost identical it is possible to assume, considering the arguments presented above, that the conformation **5b** can be excluded. The chemical shift value of the Me-9 group (δ = 1.20) also indicates that there are no shielding effects of the double bond.

The orientation of the OH group can be determined from NMR data. The convincing proof is presented by the multiplet of olefinic proton H-4 at δ = 5.60 ppm.

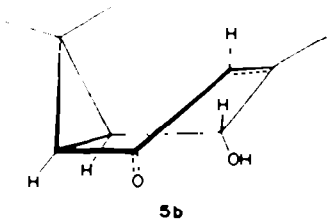
A more detailed analysis of this multiplet showed it to be a quartet resulting from a coupling with Me-10 protons with additional splitting by H-2 hydrogen (J = 1.6 Hz). Such allylic coupling is possible only for an axially oriented hydrogen H-2[ax] (Fig. 6). It follows that the signal of H-2 proton is a doublet (allylic coupling with H-4) split in addition to a doublet by the neighboring hydrogen H-1 (J = 5 Hz). The value of J = 5 Hz is consistent with the J value resulting from the Karplus correlation. With known absolute configuration of **1** it was possible to determine the chirality sign on the C-2 atom. This sign is "*S*" and it follows that a characteristic selectivity of reduction by *Rh. m.* is observed also in this case.

The spectrum of **3** shows the presence of a CO group at 1700 cm^{-1} (shifted towards higher frequencies in comparison to previously discussed products) and an alcohol group (3620 cm^{-1}). There is no conjugation of the ketone group because the molar extinction of UV max at 213 nm is of the order of 200. Also there is no olefinic $\Delta^{1,4}$-bond. The NMR spectrum of this compound is not as evident as in the case of **4** and **5** because only small insufficiently pure amounts could be isolated.

The doublet of Me-10 at δ = 1.03 ppm (J = 6 Hz) indicates a hydrogenated double bond. None of the geminal Me groups is either shielded or deshielded by OH or CO groups as indicated by singlets at δ = 1.20 and 1.28 ppm, i.e. in the same positions as observed for analogous Me groups in **4** and **5**. The positions of the OH and CO groups are suggested by the analysis of H-5 multiplet (geminal to OH). This multiplet is shown at δ = 4.54, i.e. at exceptionally low field. Its multiplicity and "width" (24 Hz) indicate the corresponding axially oriented proton and must be coupled with one of the neighboring protons (also axial) and with two vicinal equatorial protons. The resulting "width" is 24 Hz. It may be assumed, therefore that the OH group occupies position C-5 (Fig. 7), because only in this case the observed coupling is possible. However, a final proof of structure and conformation will be possible with additional CD and ORD data on hand and after the absolute configuration has been determined. At present the configuration *S* may be assumed because it is



5a



5b

Fig. 5.

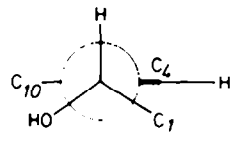
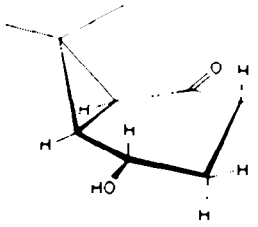


Fig. 6.



3

Fig. 7.

known that this is the usual configuration of products of reductions performed by *Rh.m.*

With high probability the structure of (+)-*trans*-caren-2-on-5-ol can be assigned to the optically active **3**.

The final product is the diketone **2**, present in mixtures only after shorter periods of fermentation. This compound, obtained in very small amounts, was identified by IR and UV. The IR spectrum shows an isolated ketone group at 1705 cm $^{-1}$. This high frequency and the absence of lower frequencies indicates that the conjugated system has disappeared. This is confirmed by the absence of absorption at 200 nm. These data support the structure of **2** as caran-3,5-dione.

More polar compounds are also formed by microbiological transformations of **1** but we were not able to accomplish their separation. It is to be noted however, that these products should include the dialcohol from reduction of both CO groups.

EXPERIMENTAL

The following spectral determination were carried out: IR: UR—20 Carl Jena Zeiss Jena and Unicam SP-200; UV: VSU-1 and specord UV VIS Carl Zeiss Jena; NMR: 80 Mc Tesla 478 and JEOL 100 Mc; GC: GCHF 183 Giede GDR; CD: Circulardichromatograph (Bonn, University).

Rhodotorula mucilaginosa cultures were grown on maltose "Malto" nutrient manufactured by "Warta"-Srem. The m.ps were determined on Koffler's block (uncorrected). Preparative chromatography was carried out on silica gel (Woelm and Merck). Eluent: petroleum ether : ethyl acetate = 10 : 1. The purity of the substances was determined by thin layer chromatography on silica gel. Eluent: petroleum : ether : acetone = 1 : 1 : 1.

Preparative transformation of 3-caren-2,5-dione (**1**). The strain of *Rh. m.* was cultured in 2 l. flasks shaken at 27° of 1 l. maltose medium. 100 mg of substrate (**1**) dissolved in 2 ml acetone was added to 4-days culture. The fermentation was carried out for 14 or 21 days and after this time the products were extracted with ethyl ether. The mixture of products was separated chromatographically.

Fractions of chromatograph:

220 mg of products mixture after 14-days fermentation yielded:

Compound **2**	10 mg 5%
Substrate **1**	14 mg 7%
Compound **4**	18 mg 9%
Mixture of **3** and **5**	140 mg 70%
Unidentified more polar products	20 mg 10%

Analysis of mixture **3** and **5** in gas-chromatogram (10% SE-30) suprasorb AW-HDMS 60 8 mesh steel col. 100 0.4 cm/τ = 5.50 and 6.92 respectively. Calculated and valued composition of **3** and **5** was about 1 : 1.

540 mg of product mixture after 21-days fermentation yielded:

Compound **2**	Traces
Substrate (**1**)	Traces
Compound **4**	40 mg 8%
Compound **3**	25 mg 5%
Compound **5**	50 mg 10%
Mixture of **3** and **4**	210 mg 42%
Unidentified more polar products	175 mg 35%

Analysis of g.c. of mixture of **3** and **5** showed 1 : 1.

Properties of substrate and products. (The NMR spectra are collected in Table 2). 3-Caren-2,5-dione (**1**): m.p. 100–101°; IR: $\nu_{C=O}$ 1670, 1620 cm $^{-1}$; UV: $\epsilon_{\lambda 241}$ 10,200; $\epsilon_{\lambda 220}$ 8790; CD: $\Delta_{\lambda 290}$ 0.02, $\Delta_{\lambda 340}$ + 0.02; R_f 0.76. Caran-2,5-dione (**2**): m.p. 60–71°; $(\alpha)_D^{20}$ 0° (c – 1.2 in acetone) IR: $\nu_{C=O}$ 1705 cm $^{-1}$; R_f 0.90. (−)-*trans*-3-caren-2-one-5-ol (**4**): m.p. 84–86°; $[\alpha]_D^{20}$ −170° (c = 1 in acetone); UV: $\epsilon_{\lambda 222}$ 9100; IR: $\nu_{C=O}$ 1665, 3620 cm $^{-1}$; R_f 0.33. (+)-caran-2-on-5-ol (**3**): $[\alpha]_D^{20}$ + 14° (c – in acetone); UV: $\epsilon_{\lambda 213}$ 222; IR: $\nu_{C=O}$ 1700, 3620 cm $^{-1}$; R_f 0.25. (+)-*trans*-3-caren-5-on-2-ol (**5**); m.p. 104–106°; $[\alpha]_D^{20}$ + 230° (c – 11 in acetone); UV: $\epsilon_{\lambda 221}$ 11,200; IR: $\nu_{C=O}$ 1660, 3620 cm $^{-1}$; R_f 0.23.

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