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MICROBIAL TRANSFORMATIONS. 14. REGIOSELECTIVE HYDROXYLATION OF (1R)-CARYOLANOL BY *Aspergillus niger*. A REEXAMINATION OF THE ^{13}C NMR SPECTRUM OF CARYOLANOL

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Summary. Oxidation of caryolan-1-ol 1 by the fungus Aspergillus niger (MMP 521) led in fair yield (26%) to a single diol by regiospecific hydroxylation of the C(14) methyl group. ^{13}C NMR analysis led to modification of the previous attributions to caryolan-1-ol (1).

In the course of our work concerning the study of microbial hydroxylations of non activated carbon atoms (1), we have been interested in studying the bioconversion of caryolan-1-ol, a well known derivative of caryophyllene. Such studies are of interest for at least two reasons : - first, the regioselective hydroxylation of non-activated carbon atoms is a subject of high contemporary importance since these reactions are, up to now, almost impossible to achieve with acceptable regioselectivities and yields by chemical ways (2) - second, because it appears that the bioconversion of readily available monoterpenes or sesquiterpenes may constitue a straightforward access to new important fragrances or flavors (3).

We found previously six fungal strains that could transform cedrol (thus allowing the straightforward synthesis of cedrene-8-one-3, an odoriferous minor component of cedar wood essential oil (4)) four of which were also able to biohydroxylate caryolanol leading to the same single metabolite (as shown by comparison of their g.c. retention time). After 72 hr culture, the calculated yields in analytical experiments were respectively of 6% (with *Absidia coerulea*, MMP 1894); 5% (with *Beauveria sulfurescens*, ATCC 7159);

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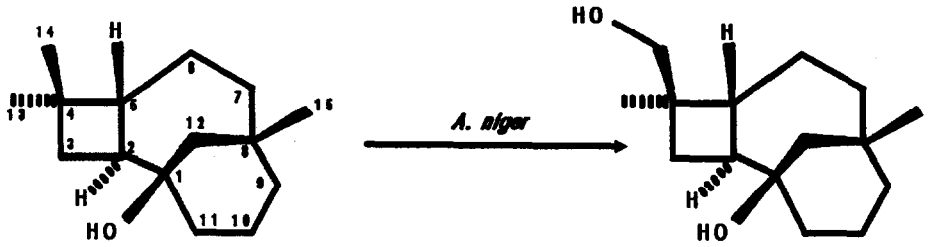
5% (with *Corynespora casiicola*, DSM 62475) and 37% (with *Aspergillus niger*, MMP 521). Using this last strain, preparative-scale experiments were conducted in order to isolate this metabolite, obtained with a 26% non-optimized yield after bulb to bulb vacuum distillation. The structure of this metabolite has been established by detailled ^{1}H and ^{13}C NMR spectroscopy as being 2. This result compares very favourably with those of Barton et al. using the Gif system, where yields of under 5% of two ketones were obtained (5).

The ^{13}C NMR spectra of caryolan-1-ol 1, as well as the ^{13}C and ^{1}H NMR spectra of its hydroxylated metabolite 2, are detailed on Table 1. For this latter compound, the results of a DEPT multiplicity experiment (6) indicate the presence of only two methyl groups and of a methylenic carbon atom bearing a hydroxyl moiety. Similar observations were found from the ^{1}H NMR spectrum, and show that hydroxylation obviously occured on one of the three methyl groups. The location of this hydroxyl group was unambiguously determined by careful analysis of the ^{13}C methyl shifts, showing that it is located on C(14). Indeed, examination of Dreiding models suggests that only the C(13) methyl group experiences a shielding (γ gauche steric interaction) (7) with C(6), allowing the assigment of both the gem methyl groups of 1. Therefore, the value of the remaining methyl shift of 2 (δ = 16.7 ppm) indicates that hydroxylation occured on C(14). Finally as the only invariant signal is at 33 ppm, it has to be attributed to the C(15) methyl group. As a consequence the previously reported chemical shifts of the methyl groups of caryolan-1-ol 1 have to be corrected (5, 8).

The C(14) regioselectivity of this hydroxylation is identical to the one observed for the main metabolite of (-)caryophyllene with rabbits (8). Furthermore, several C(14) hydroxylated products have been isolated from the Polish mushroom *Lactorius camphoratus*, and from *Inura spiraefolia* (Compositae) (9). These results interestingly indicate that the same regioselectivity for hydroxylation of this type of compound is observed in plants, microorganisms and mammals.

Complete ^{13}C NMR assignments for 2 were deduced from the concerted use of heteronuclear and homonuclear chemical shift correlation diagrams (Figure 1). The establishment of the proton connectivity is easily available from the homonuclear correlation experiment (COSY) (10) while the relationships between all the carbon and hydrogen atoms were achieved from ^{13}C-^{1}H shift correlation spectrum with proton decoupling in F_1 dimension (XHCORRD) (11). Table 1 summarizes the various ^{1}H chemical shifts of 2 extracted from the cross-sections of the 2D-heteronuclear chemical shift diagram.



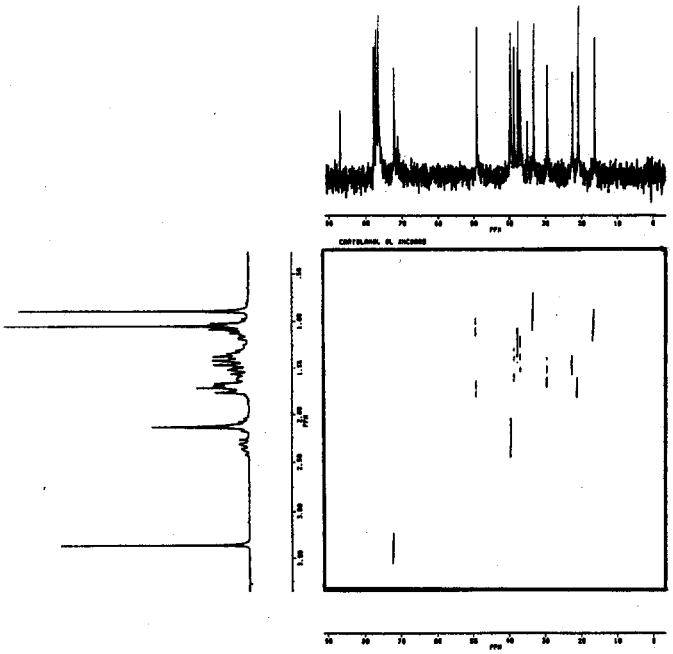
1 2

Table 1. ^{13}C and ^{1}H chemical shifts for 2.

Atom	1[a]		2[b]		
	δ ^{13}C	Multiplicity	δ ^{13}C	Multiplicity	δ ^{1}H[c]
1	70.9	C	71.10	C	-
2	39.7	CH	39.88	CH	2.42
3	34.5	CH_2	29.42	CH_2	1.44 and 1.71
4	34.8	C	39.61	C	-
5	44.9	CH	39.61	CH	2.18
6	22.1	CH_2	22.46	CH_2	1.47 and 1.55
7	37.5	CH_2	37.47	CH_2	(d)
8	34.9	C	34.86	C	-
9	36.6	CH_2	36.86	CH_2	1.33 and 1.62
10	20.7	CH_2	20.88	CH_2	1.77
11	38.7	CH_2	38.55	CH_2	1.38 and 1.70
12	48.6	CH_2	49.05	CH_2	1.08 and 1.75
13	20.7	CH_3	16.70	CH_3	1.05
14	30.5	CH_3	71.99	CH_2	3.43
15	33.3	CH_3	33.20	CH_3	0.93

(a) Values from Ref. 5 and 13 with revised assignments (see text)
(b) Assignments determined from 2D measurements.
(c) Values determined from the cross-sections of heteronuclear chemical shift correlation diagram.
(d) Unresolved signals.
(e) Determined from DEPT spectra.

Figure 1.



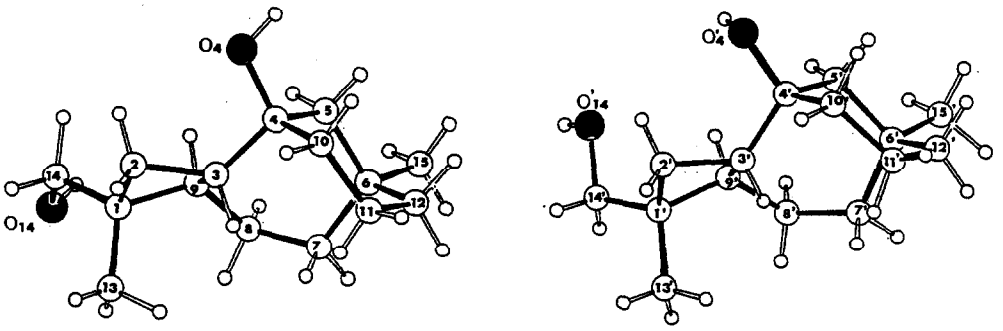
Two-dimentional ^{13}C-^{1}H shift correlated diagram of 2.

Contour plot of the homonuclear ^{1}H-^{1}H chemical shift correlation (COSY LR) for 2 (cross-correlation peak marked with arrow is discussed in the text).

Finally, unambiguous assignments of H(2) and H(5) protons can be established from the analysis of COSY diagram optimized for the observation of long-range couplings (COSY LR) (12). From the contour plot of the 2D-COSY LR spectrum (Figure 2), it is obvious that the methylene proton signal located at 3.43 ppm displays cross-peak via a long-range coupling (^4J) with the proton at 2.18 ppm which, therefore, is assigned to H(5). As a consequence, the previously reported shifts of the C(2) and C(5) methine pair must also be reversed (5, 13).

The structure of 2 we have proposed in this work has been ascertained by X-Ray crystallography, which confirms our NMR-deduced structure and, therefore, our various chemical shifts attributions. Compound 2 crystallizes with two molecules in the asymmetric unit. Perspective views of both molecules are reported on figure 2 with the numbering scheme.

Figure 2. Perspective views of both conformations of 2 present in the asymmetric unit.



2 2'

The two independent molecules are linked by intermolecular hydrogen-bridges of the type O-H...O, involving an important rotation of the hydroxyl group. The torsional angles are respectively $C_9-C_1-C_{14}-O_{14}$ 71.0° (0.3) and $C'_9-C'_1-C'_{14}-O'_{14}$ -48.6° (0.3). The hydrogen-bond scheme is listed in Table 2, and the molecular packing, with the hydrogen-bonding network is illustrated on figure 3.

CONCLUSION

Oxidation of caryolan-1-ol 1 by the fungus *Aspergillus niger* (MMP 521) leads to one single diol which results from regiospecific hydroxylation of the C(14) methyl group. This is obtained in fair yield (26%), a result which compares very favourably with the one previously described by Barton et al. who

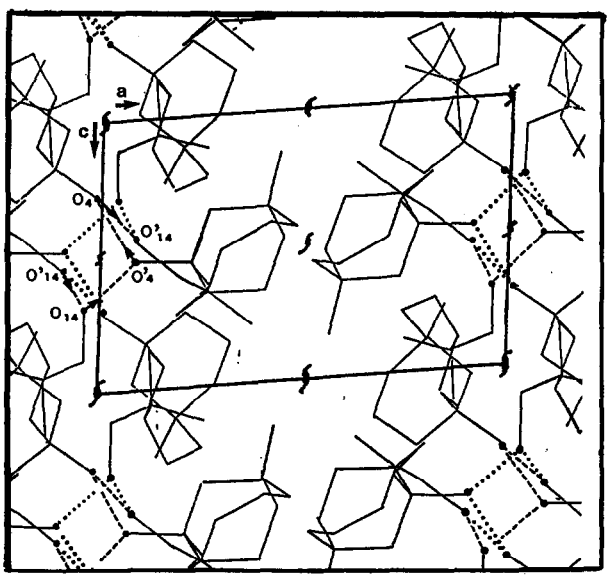
have obtained much lower yields of a mixture of two ketones using the Gif system oxydation (5). The thus obtained C(14) hydroxylation allowed us to perform accurate ^{13}C NMR analysis, leading to some modifications of the previously proposed attributions for 1. This result illustrates the large potential offered by the bioconversion of natural product, and in particular sesquiterpenoids, thus allowing rapid synthetic access to new molecules which could be of interest to the industry of perfumes and aromas.

Table 2. Hydrogen bonds.

D-H.....A	D.....A (Å)	H-D (Å)	H.....(Å)	< A.....H-D(°)
O'_{14}-H'O_{14}....O_{14}^{i}	2.705 (4)	0.74 (4)	1.96 (3)	173 (2)
O_{14} -H O_{14}....O'^{ii}_{4}	2.750 (4)	0.78 (4)	1.97 (3)	175 (3)
O'_4 -H'O_4.....O_4^{iii}	2.847 (3)	0.89 (3)	1.97 (2)	168 (3)
O_4 -H O_4.....O'^{iii}_{14}	2.861 (3)	0.91 (3)	1.97 (3)	166 (3)

Symmetry code : (i) -x, -0.5+y, -z ; (ii) x, y, z-1 ; (iii) x, y, z

Figure 3. Molecular packing of the crystal of 2 (Hydrogen bonds are indicated by dashed lines)



EXPERIMENTAL PART

General : Vapor phase chromatographic analyses have been performed using a Girdel 300 chromatograph equiped with a capillary column (OV1701) and a Shimadzu CR 5A integrator. IR spectrum were performed using a Perkin Elmer 1310 spectrometer. The 1D and 2D NMR spectra were recorded using a Bruker AM-200 spectrometer (Centre Interuniversitaire de RMN de Marseille). ^{13}C and ^{1}H measurements were carried out using $CDCl_3$ as solvent and tetramethylsilane as an internal standard. Chemical shifts are given in δ ppm. Melting points, uncorrected, were determined in capillary tubes using a Büchi 510 apparatus. Microanalysis were obtained from the "Service Central de Microanalyse" Vernaison (France). The bulb to bulb distillation apparatus is a Büchi GKR-50. The preparative bioconversions were performed using a Setric G.I. Set-2 type fermentation jar.

Analytic experiments. These were performed as follows : The selected microorganism is inoculated into several 500 ml bottom baffled erlenmeyer flasks containing 100 ml culture medium (Corn Steep Liquor : 20 g/l, glucose : 10 g/l, sterilisation in an autoclave 20 mn at 115°C) and is cultured at 28°C on a reciprocal shaker (100 rpm). After 48 hrs growth, 1 ml of a 50 g/l solution of caryolan-1-ol in ethanol is added to the culture. The bioconversion is achieved for 72 hrs, and the entire culture medium is continuously extracted for 24 hrs using dichloromethane. The extracts are dried over anhydrous magnesium sulfate, and analysed by v.p.c. using n-hexadecane as an internal standard.

Preparative scale experiments : They were performed in a 2 l fermentor jar, using one liter of the same medium in which 10 ml of vaseline and 50 µl of Rhodorsil (silicone 426) were added before culture. This medium was inoculated with *Aspergillus niger* spores. The growth was followed using the O_2 consumption. After 30 hrs culture (29°C, 700 r.p.m., 0.4 v.v.m. aeration) one adds 10 ml of 50 g/l ethanolic caryolan-1-ol 1 solution (500 mg). The bioconversion is followed using 1 ml aliquots which are extracted with ethyl acetate and analysed by vapor phase chromatography. Once a 30% yield of 2 is obtained (about 70 hrs), the culture is filtered and the aqueous phase is continuously extracted with dichloromethane. After drying over magnesium sulfate, the organic phase is stripped off and the crude product is purified twice by bulb to bulb distillation (190°C, 0.3 mm Hg). This yields 140 mg of diol 2 which crystallizes in thin white needles. mp : 154°C; IR ($CDCl_3$) : 3400 cm^{-1};

Analysis : calculated for M = 238 : C 75.48; H 10.98; O 13.54; Found C 75.12, H 11.16, O 13.63.

NMR experiments. Resonance multiplicities for ^{13}C were established via the acquisition of DEPT spectra obtained for proton pulses Pθ = 90° (CH only) and Pθ = 135° (CH and CH_3 differentiated from CH_2). For the DEPT sequence the width of a ^{13}C 90° pulse was 13 µs, the width of a ^{1}H 90° pulse was 29 µs and the $(2J)^{-1}$ delay was set equal to 3.7 ms.

The homonuclear ^{1}H-^{1}H chemical shift correlated two-dimensional diagrams was obtained using the COSY-45 pulse sequence (COSY in the operating Bruker software). The spectral widths were F_2 = 1800 Hz and F_1 = ± 900 Hz allowing a digital resolution of 1.76 Hz per point. This spectrum was collected as 2048 x 1024 blocks of data and was processed using sinusoidal multiplication in each dimention followed by symmetrization of the final data matrix. Other parameters were as follows : number of increments in t_1, 256; scans, 16; phase cycling, 16 and relaxation delay, 1s. The COSY long-range (COSY LR) was applied using the same parameters and D2 was set to 0.2 s.

Heteronuclear two-dimensional ^{1}H-^{13}C chemical shift correlation experiment was obtained with proton decoupling in the F_1 dimension (XHCORRD). The spectrum was acquired with 4K x 256 data points and a data acquisition of 512 x 128 increments in t_1 and a zero filling in the F_1 dimension. Spectal widths of 4760 and ± 360 Hz were employed in the F_2 (^{13}C) and F_1 (^{1}H) domains respectively. Data were processed using unshifted sine bell functions for weighting in both dimensions. This provided a digital resolution of 2.32 Hz in F_2 and 2.81 Hz in F_1. The refocusing delay was 1.85 ms, the mixing delay 3.7 ms, the relaxation delay, 1s and sixteen phase cycling steps were employed.

X-Ray crystallographic study of compound 2. Crystal data : $C_{15}H_{26}O_2$, M = 238.36, monoclinic, space group $P2_1$, a = 10.915 (2), b = 17.544 (3), c = 7.303 (2) Å, β = 96.20(1)°, V = 1390.29 Å^3, Z = 4, d_x = 1.14, 2 molecules per asymtric unit. A crystal of dimensions 0.8x0.4x0.2 mm was mounted on a Philips PW1100 4-circle diffractometer, with graphite monochromated CuKα radiation (λ = 1.5418 Å). From 3814 measured independent reflections, 2809 were significant [I > 3σ (I)]. The reflections were corrected for Lorentz, polarisation and absorption effects (14). The structure was solved by direct methods (SHELX S86) (15). The atomic coordinates and anisotropic thermal parameters were refined by large blocks to a discrepancy factor of R = 4.80% and Rw = 5.83%. The minimized function in the refinement was $\Sigma w||Fo|-|Fc||^2$ with a final weighting scheme $w = 1.0188 / [\sigma^2(Fo) + 0.0022\ Fo^2]$, using σ from counting statistics.

All the hydrogen atoms were located on a difference-Fourier maps. They were assigned the equivalent isotropic thermal parameter of the bonded atom and their atomic coordinates were refined. The highest residue on the final electronic density map is 0.11 e/Å^3. Atomic coordinates, anisotropic thermal parameters, bond lengths and angles, with their estimated standard deviations are available from the Director of the Cambridge crystallographic Data Centre, University Chemical Laboratory Lensfield Road, Cambridge CB2 1EW. Any request should be accompagnied by the full literature citation.

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BIBLIOGRAPHY

(1) Preceeding reference see : Archelas, A.; Fourneron, J.D.; Furstoss, R. *J. Org. Chem.* **1988**, *53*, 1797.
(2) See for instance : "*Oxidation in Organic Chemistry*" Academic Press (New York).
(3) See for instance : a) Bock, G.; Benda, I.; Schreier, P. *Appl. Microbiol. Biotechnol.* **1988**, *27*, 351. b) Abraham, W.R.; Stumpf, B.; Kieslich, K.; Reif, S.; Hoffmann, H.M.R. *ibid.* **1986**, *24*, 31 ; c) Schindler, J.; Schmid, R.D. *Process Biochem.* **1982**, p. 2 ; d) Abraham, W.R.; Washausen, P.; Kieslich, K. *Z. Naturforsch.* **1987**, *42c*, 414.
(4) Lamare, V.; Fourneron, J.D.; Furstoss, R. *Tetrahedron Lett.* **1987**, *28*, 6269.
(5) Barton, D.H.R.; Beloeil, J.C.; Billion, A.; Boivin, J.; Lallemand, J.Y.; Lelandais, P.; Mergui S.; Morellet, N. *Nouv. J. Chim.* **1986**, *10*, 439.
(6) Doddrell, D.M.; Pegg, D.T.; Bendall, M.R. *J. Mag. Reson.* **1982**, *48*, 323.
(7) Grant D.M.; Cheney, B.V. *J. Am. Chem. Soc.* **1967**, *89*, 5315.
(8) Asakawa, Y.; Ishida, T.; Toyota, M.; Takemoto, T. *Xenobiotica* **1986**, *16*, 753.
(9) a) Daniewski, W.M.; Grieco, P.A.; Huffman, J.C.; Rymkiewicz, A.; Wawrzun A. *Phytochemistry.* **1981**, *20*, 2733-2734. b) Jeremic, D.; Milosavljevic, S.; Vaj, V. *Tetrahedron Lett.* **1982**, *23*, 1009.
(10) a) Aue, W.P.; Bartholdi, E.; Ernst, R.P. *J. Chem. Phys.* **1976**, *64*, 2229; b) Nagayama, K.; Kumar, A.; Wüthrich K.; Ernest, R.R. *J. Magn. Reson.* **1980**, *40*, 321 ; c) Freeman, R.; Morris, G.A.; Bax, A. *J. Magn. Reson.* **1981**, *42*, 164.
(11) a) Bax, A. *J. Magn. Reson.* **1983**, *53*, 517 ; b) Rutar, V. *J. Magn. Reson.* **1984**, *58*, 306.
(12) a) Bax A.; Freeman, R. *J. Magn. Reson.* **1981**, *44*, 542 ; b) Benn, R.; Günther, H. *Angew. Chem. Int. Ed. Engl.* **1983**, *22*, 390.
(13) Bohlmann, B.; Ziesche, J. *Phytochemistry* **1981**, *20*, 469.
(14) Walker, N.; Stuart, D. *Acta Cryst.* **1983**, *A39*, 158-166.
(15) Sheldrick, G.M. *Program for crystal structure solution*, Univ. of Göttingen, Federal Republic of Germany. **1986**.