Note

Reduction of α,β-Unsaturated Ketones with *Tetrahymena pyriformis*—A Detoxication Reaction

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In previous work, we have shown that α,β-unsaturated ketones could be reduced by numerous microorganisms, particularly fungi[1,2] aerobic bacteria[3] and anaerobic bacteria.[4] These reactions generally lead to a mixture of the corresponding saturated alcohol and saturated ketone.

These results suggested that this bioreduction reaction might be very general. Accordingly, we extended its study to a protozoan, *Tetrahymena pyriformis*. We report here the results of the reaction of this microorganism with several α,β-unsaturated ketones.

Tetrahymena pyriformis strain GL was grown under axenic conditions. The culture medium was PPY composed of 0.75% Difco proteose peptone, 0.75% Difco yeast extract and various salts according to Plessner *et al.*[5] The culture was maintained in its exponential growth phase by daily reseeding at 28°C, the optimal growth temperature.

The lethal dose of all the compounds involved was determined by adding increasing amounts of substrate to 100 ml of the culture medium and 1 ml of *T. pyriformis* in its exponential growth phase, added just before the compound being tested, in 500 ml conical flasks. For every amount of substrate, the condition of *T. pyriformis* was observed for 48 hr. The population evolution for each sample was determined by cell counting with a Coulter Counter ZM Coultronic Model.

Bioconversions were run out by the following procedure: 500 ml of a culture of *T. pyriformis* were placed in a 5 liters conical flask containing 1 liter of the culture medium. The compound to be reduced was added after 24 hr of culture, when a plateau had been reached (2.5 to 3.5×10^5 cells/ml). The quantity of compound added was less than the lethal dose as previously determined. After the reaction time, the mixture was centrifuged, and the supernatant was saturated with ammonium sulfate and extracted four times with ether. After drying and evaporating the solvent, the residue was analyzed by GPC. Separation of the products was carried out by column chromatography (Silicagel, pentane–ether at 90 : 10, v/v as the eluant).

The first compound studied was 2-cyclohexen-1-one at a concentration of 0.017 g/liter, *i.e.*, slightly less than the lethal dose of 0.023 g/liter. The results obtained are set out in Table I. After 2 hr of reaction, only 46% of the starting material was left, all the starting material being consumed after 10 hr. Concomitantly, cyclohexanone was formed, which was then progressively reduced to cyclohexanol. After 48 hr of reaction the mixture contained 88% cyclohexanol. The lethal doses obtained for the starting material and the two products are also given in Table I. 2-Cyclohexen-1-one is evidently highly toxic towards *T. pyriformis* (about 20 mg/liter), whereas cyclohexanone and cyclohexanol are respectively 48 and 74 times less toxic. The reduction of 2-cyclohexen-1-one may thus be regarded as a detoxification of the medium. This reaction first occurs rapidly in order to eliminate the most toxic compound, subsequent conversion of the cyclohexanone to cyclohexanol taking place more slowly as it represents a lesser degree of detoxification.

The yields were high (of the order of 80%) as shown using an internal standard in GPC analysis.

The reduction reaction was shown to be intracellular, the reduction of 2-cyclohexen-1-one being carried out as previously described. The reaction mixture was then centrifuged after 24 hr of reaction, and a further amount of 2-cyclohexen-1-one (0.017 g/liter) was added to the resulting supernatant. After 48 hr, the starting material was recovered unchanged, so that no enzymes presumably, involved in the reduction had been released into the culture medium.

In order to determine the stereochemistry of the reaction, we studied the reaction of (−)-carvone at a concentration below the toxic level (0.10 g/liter), with the results shown in Table II. The reaction was found to be slower than that with 2-cyclohexen-1-one; 10% of (−)-carvone was still present after 48 hr of reaction. However, reduction did occur, leading after 4 days of reaction to a mixture of three products, which were separated by column chromatography. The first product obtained was (+)-dihydrocarvone, this product being identified by comparing its ^{1}H NMR spectrum (60 MHz, $CDCl_3$, Me_4Si as the internal standard) with that of an authentic sample obtained by a microbiological reduction of (−)-carvone.[1] The second product was (+)-neodihydrocarveol, and it was similarly identified by comparing its ^{1}H NMR spectrum with that of an authentic sample prepared from (−)-carvone.[1] The third and last product was a dihydrocarveol

TABLE I. REDUCTION OF 2-CYCLOHEXEN-1-ONE BY *T. pyriformis*: LETHAL DOSE AND YIELD OF PRODUCTS *versus* REACTION TIME

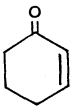
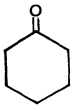
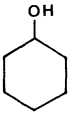
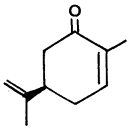
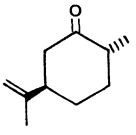
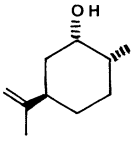
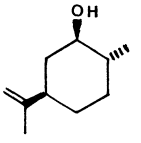
Lethal dose (g/liter)	0.023	1.1	1.7
			
	2-Cyclohexen-1-one	Cyclohexanone	Cyclohexanol
Reaction time 2 hr	46%	54%	0%
Reaction time 10 hr	0%	64%	36%
Reaction time 24 hr	0%	38%	62%
Reaction time 48 hr	0%	12%	88%

TABLE II. REDUCTION OF (−)-CARVONE BY *T. pyriformis*: LETHAL DOSE, OPTICAL ROTATION AND YIELD OF PRODUCTS *versus* REACTION TIME

Lethal dose (g/liter)	0.153	1.1	1.5	
				
	(−)-Carvone	(+)-Dihydrocarvone	(+)-Neodihydrocarveol	(−)-Dihydrocarveol
$[\alpha]_{578}^{25}$ $CHCl_3$	−62°	+16°	+34.8°	−27°
$[\alpha]_{578}^{25}$ lit.		+14°[10]	+33°[11]	−29°[6b]
Reaction time 48 hr	10%	41%	21%	28%
Reaction time 96 hr	0%	10%	58%	32%

with an optical activity $[\alpha]_J^{25°} = -27°$. Of the four possible isomers of dihydrocarveol derived from (−)-carvone, only one has a negative value of optical activity,[6a] namely (−)-dihydrocarveol with $[\alpha]_J^{25°} = -29°$.[6b] In addition, the ^1H NMR spectrum of this product showed a very wide multiplet (25 Hz) for the proton next to the hydroxyl, which is consistent with the proposed structure.[7] For further confirmation, this alcohol was oxidized by pyridinium chlorochromate (PCC) to give a saturated ketone with $[\alpha]_J^{25°} = +15°$ and a ^1H NMR spectrum identical to that of (+)-dihydrocarvone. This result confirms the stereochemistry of the methyl group α to the hydroxyl and thereby the structure of the alcohol, which can only be (−)-dihydrocarveol with the hydroxyl *trans*

with regard to the α methyl group. The isomer with the hydroxyl *cis* had already been characterized ((+)-neodihydrocarveol). These results show that the reduction of the double bond of α,β-unsaturated ketones by *T. pyriformis* occurred with the same stereochemistry as that observed with other microorganisms, particularly fungi.[1] On the other hand, the reduction of the carbonyl took place differently. Although, as observed with other microorganisms, an (*S*)-alcohol was obtained by reduction in accordance with Prelog's rule, reduction contrary to Prelog's rule also occurred giving an (*R*)-alcohol.

The values of the lethal dose of (−)-carvone, (+)-dihydrocarvone and (+)-neodihydrocarveol are given in Table II. (−)-Carvone was much less toxic towards *T. pyriformis* than 2-cyclohexen-1-one, though its reduction products were about as toxic as cyclohexanone and cyclohexanol. The reduction of (−)-carvone was slower than that of 2-cyclohexen-1-one which suggests a relationship between the toxicity of the unsaturated ketone and the reaction rate. The rate increased with the substrate toxicity.

The reduction of 3-methyl-2-cyclohexen-1-one was finally attempted. Even after 8 days exposure to *T. pyriformis*, no reaction occurred. The lethal dose of this unsaturated ketone was 1.6 g/liter *i.e.*, similar in value to those of the final reaction products in the two previous tests. The lethal dose of the expected product, 3-methylcyclohexanone, was determined and found to be 2.1 g/liter. This result thus supports the suggestion that the reduction of α,β-unsaturated ketones by *T. pyriformis* is a detoxification reaction. When the starting material is only weakly toxic, the reaction is correspondingly very slow.

These examples show that the reduction of α,β-unsaturated ketones can be achieved not only with fungi and bacteria but also with a protozoan. This reaction thus seems rather general in biological systems, and work underway at present shows that it can occur with plant cells[8] and rat-liver extracts.[9]

Finally, the results reported here show that the reaction of *T. pyriformis* is above all a detoxification reaction, and that there is a relationship between the toxicity of the substrate towards the protozoan and the rate of the reduction reaction, which yields products which are less toxic.

REFERENCES

1) A. Kergomard, M. F. Renard and H. Veschambre, *J. Org. Chem.*, **25**, 792 (1982).
2) A. Kergomard, M. F. Renard and H. Veschambre, *Agric. Biol. Chem.*, **46**, 97 (1982).
3) A. Kergomard, M. F. Renard and H. Veschambre, *Agric. Biol. Chem.*, **49**, 1497 (1985).
4) M. Desrut, A. Kergomard, M. F. Renard and H. Veschambre, *Biochem. Biophys. Res. Commun.*, **110**, 908 (1983).
5) P. Plessner, L. Rasmussen and E. Zeuthen, "Techniques Used in the Study of Synchronous *Tetrahymena* in Synchrony in Cell Division on Growth, Interscience, New York, 1964.
6) a) Beilsteins handbuch des Organischen Chemie, III, Supplement, Tome 6, 255, Springer-Verlag, Berlin, 1965. b) T. Nagazawa, *Repts. Osaka. Imp. Ind. Research. Inst.*, **4**, 19 (1938) (*Chem. Abstr.*, **214**, 1940).
7) Y. Noma, S. Nonomura, H. Ueda and C. Tatsumi, *Agric. Biol. Chem.*, **38**, 735 (1974).
8) A. Kergomard, V. Petiard, M. F. Renard and H. Veschambre, unpublished data.
9) A. Fauve, unpublished data.
10) T. Nagasawa, *Osaka Kogyo Gijutsu Shikensho Hokoku*, **19**(4), 1 (1938).
11) G. V. Pigulewski, S. A. Koshin and V. G. Kosstenko, *Zh. Obshch. Khim.*, **28**, 1413 (1958).