Preparation of Optically Active γ- and δ-Lactones by Microbiological Reduction of the Corresponding Keto Acids

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ABSTRACT

TUYNENBURG MUYS, G. (Unilever Research Laboratory, Vlaardingen, The Netherlands), B. VAN DER VEN, AND A. P. DE JONGE. Preparation of optically active γ- and δ-lactones by microbiological reduction of the corresponding keto acids. Appl. Microbiol. **11**:389–393. 1963.—It was found, by means of simple screening methods, that several yeasts (*Saccharomyces* and *Candida*), molds (*Cladosporium*), and bacteria (*Sarcina*) are able to reduce keto acids to hydroxy acids, which are easily converted into lactones. Chemical analysis showed that some of the microorganisms (*Saccharomyces and Candida*) produce dextrorotatory lactones and others (*Cladosporium* and *Sarcina*) produce levorotatory lactones. High yields of dextrorotatory (both γ- and δ-lactones) were obtained by using *Saccharomyces cerevisiae*. The physical properties of the carefully distilled lactones obtained indicated high purity and high optical purity.

Optically active lactones, which occur in minute amounts in butterfat (Boldingh and Taylor, 1962), can be obtained in three ways.

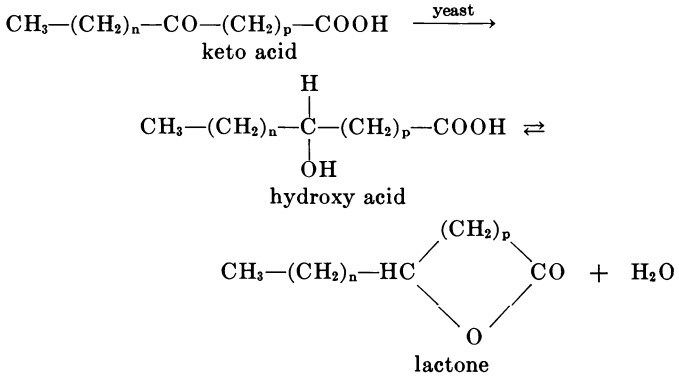
Isolation from natural sources. So far, little evidence has been found for the occurrence of optically active γ- or δ-lactones in nature. Parasorbic acid [(+)5-methylpentene-2-lactone] is present in rowan berries (Doebner, 1894; Kuhn and Jerchel, 1943; Diemair and Franzen, 1959), and massoia lactone [(−)5-pentyl-pentene-2-lactone] has been extracted from the bark of the New Guinea massoia tree, *Cryptocaria massoia* (Meyer, 1940). Recently, Winter et al. (1962) reported the isolation of a levorotatory unsaturated lactone, 5-(penten-2 cis-yl)-pentanelactone, from jasmine oil.

Dextrorotatory γ-lactones can be prepared from the antibiotics nemotin and odyssine (Bu'Lock, Jones, and Leeming, 1955, 1957; Bu'Lock et al., 1956) or from (+)4-dodecen-8-yl)-butanelactone (Fränkel, 1939), but a limiting factor is that these natural compounds can only be isolated in minute quantities.

Resolution of racemic mixtures. Following the conventional pattern of salt formation with optically active bases, racemic lactones can be resolved. For example, Levene and Haller (1926, 1928, 1929), Levene, Haller, and Walti (1927), and Levene and Mori (1928) studied the resolution of 4-methylbutanelactone. Since repeated crystallization is essential, the yields are low. For an economical process, racemization of the remaining mother liquors should be considered.

Microbiological reduction of keto acids. Various microorganisms appear to be able to reduce keto compounds into the corresponding hydroxy compounds. Applications of such microbiological reductions have been surveyed by Neuberg (1949) and by Vischer and Wettstein (1958). Optically active malic acid was formed from α-ketosuccinic acid by the action of yeasts (Neuberg and Gorr, 1925). Acetoacetic acid was reduced by yeasts to (+)β-hydroxybutyric acid (Friedmann, 1931*a*, *b*) and by animal cells to (−)β-hydroxybutyric acid (Heitzmann, 1942). Microbiological reductions of β-ketocaproic and β-ketocaprylic acids were reported by Lemieux and Giguere (1951).

We have now found that microbiological reduction of γ- and δ-keto acids can also be effected by the use of various microorganisms. No literature reference can be traced. The reactions occur as shown in the equation below where p equals either 2 (γ-series) or 3 (δ-series).



The enantiomorphic form of the corresponding hydroxy acid formed is dependent on the microorganism used, but sometimes a partially racemized mixture is obtained. Optically active γ- and δ-lactones can easily be obtained from the hydroxy acids. Microbiological reduction of γ- and δ-keto acids, therefore, constitutes a very convenient route to optically active γ- and δ-lactones, since a 100% yield is theoretically possible and a technical-scale manufacture is very well feasible.

MATERIALS AND METHODS

Screening of keto acid-reducing microorganisms. A number of strains of yeasts, molds, and bacteria were investigated for their ability to reduce keto acids to their corresponding hydroxy acids. The microorganisms examined were cultivated in pure culture at 30 C in 300-ml conical flasks containing 100 ml of nutrient solution (20% malt extract, 1% peptone, and 2% glucose) at pH 5.7. After 3 days of incubation, sufficient cell material had formed to start the reduction test, which consisted of adding 100 ml of the same nutrient solution, enriched with 0.02% δ-ketocapric acid, to the grown cultures.

TABLE 1. *Microorganisms producing lactone odor from keto acids*

Microorganism	Medium*	Reduction of keto acids†	
		γ-C_{11}	δ-C_{12}
Molds			
Byssochlamys fulva	DMA	±	−
Circinella umbellata	DMA	+	+
Phialophora richardsiae	DMA	+	−
P. lagerbergii	DMA	+	−
P. obscura	DMA	+	+
P. fastigiata	DMA	+	−
Cladosporium suaveolens	DMA	++	−
C. butyri	DMA + 1% peptone	++	−
Eremothecium ashbyii	DMA	+	−
Fusarium moniliforme	DMA	±	±
Margarinomyces bubaki 1	DMA	++	+
M. bubaki 2	DMA	+	+
Oospora lactis	DMA	++	+
Penicillium notatum	DMA + 1% peptone	+	−
Verticillium dahliae	DMA	+	−
Cephalosporium sp	DMA	+	+
Yeasts			
Saccharomyces cerevisiae	DMA	++	++
Candida pseudotropicalis	DMA	++	++
C. pseudotropicalis var. *lactosa*	DMA	++	++
S. fragilis	DMA	++	++
S. lactis	DMA	+	+
C. monosa	DMA	++	+
C. dattila	DMA	+	+
C. globiformis	DMA	++	++
Pichia membranaefaciens	DMA	++	++
Bacteria			
Bacillis macerans	MEA (pH 7)	±	±
Escherichia coli	MEA (pH 7)	±	+
E. freundii	MEA (pH 7)	+	++
Lactobacillus buchneri	Yeast + TEA	±	+
Staphylococcus aureus var. B_2	WA + 1% $CaCO_3$	±	+
Micrococcus conglomeratus	MEA (pH 7)	±	±
Sarcina lutea	DMA + 1% peptone (pH 7)	++	++

* Abbreviations: DMA = dilute malt agar; MEA = meat extract agar; TEA = tomato extract agar; WA = whey agar. Except where indicated, the pH values were 6.

† Symbols: − = negative; ± = scarcely noticeable; + = clearly noticeable; ++ = strong odor.

The mixtures were incubated for another 3 days at 30 C. Then the pH of the liquid was adjusted to 2 by the addition of sulfuric acid, and the mixtures were heated at 80 C for 15 min. After cooling, the liquids were extracted with 40 ml of diethyl ether. In addition, a culture to which no keto-acid had been added was also extracted with diethyl ether. The formation of any lactone from a keto acid could be ascertained by its highly characteristic smell. To this end, a filter paper strip was dipped into the extract, and its smell was assessed after evaporation of the ether.

The following method was found to be more suitable, however, for a great number of experiments. The microorganism investigated was cultivated in four petri dishes, usually on a medium of malt agar (Table 1). After 5 days at 30 C, the cell material (about 1 g) was suspended in 10 ml of physiological saline. With this material, the following three experiments were carried out. A 3-ml amount of the suspension was added to 20 ml of water agar, containing: 20 mg of glucose (blank), 20 mg of glucose and 2 mg of 4-keto-undecanoic acid, or 20 mg of glucose and 2 mg of 5-keto-dodecanoic acid. The mixtures were poured into petri dishes, and after incubation for 24 and 48 hr at 30 C the odor of the plates was assessed by five people.

RESULTS

A total of 60 species of molds were investigated, 16 of which yielded positive results; 9 of 16 yeast species were positive; 7 of 46 species of bacteria produced lactone odor from keto acids (Table 1). Only experiments yielding positive results were tabulated.

Reduction of δ-ketocapric acid using various microorganisms. After the experiments in which microorganisms were screened for their ability to reduce keto acids to lactones, a number of microorganisms were selected for the investigation into the optical activity of the lactones formed.

Preliminary experiments showed that an 80% yield of lactone can be obtained when 40 g of baker's yeast are used for the reduction of 1000 mg of δ-ketocapric acid in 1000 ml of 10% yeast extract with 40 g of glucose at 30 C. The lactone formed was dextrorotatory. The reduction of δ-ketocapric acid by other microorganisms was carried out with an amount of cell material which was equal (on a dry-weight basis) to that of the baker's yeast.

The dry matter of microorganisms grown in a petri dish (area 140 cm^2) was estimated by harvesting the cell material and drying it at 110 C for 1 hr. The amounts of cell material (calculated as dry matter) of the different microorganisms used in the reduction are given in Table 2.

The reductions were carried out as follows. A 500-ml bottle was filled with 200 ml of sterile 10% yeast extract. The cell material was suspended in 50 ml of 10% yeast extract and put into the bottle. After the temperature had been raised to 30 C, 2.5 ml of 10% keto acid solution (as sodium salt) and 21 ml of 30% glucose solution were added.

The initial pH was 6.5. Fermentation soon began. After 1 hr, another 2.5 ml of 10% keto acid solution and 21 ml of 30% glucose solution were added. After incubation for 24 hr at 30 C, the liquid was filtered over a Büchner funnel, using a filter aid (e.g., Hyflo, Johns-Manville, New York, N. Y.), with the residue on the filter being washed with water. The filtrate was acidified with sulfuric acid to pH 1 to 2, and then extracted four times with 250 ml of diethyl ether. The combined ether extracts were washed with 25-ml portions of water to remove any sulfuric acid.

The ether was evaporated, and the residue was dissolved in 1 N potassium hydroxide. Subsequent extraction with ether removed the unsaponifiable material. The hydroxy acid was liberated by the addition of sulfuric acid and taken up in ether. The ethereal solution was washed with small portions of water until free from sulfuric acid, and then dried on anhydrous sodium or magnesium sulfate. The ether was distilled off, and the hydroxy acid was lactonized by heating in vacuo in a round-bottom flask, fitted with an air condenser, for 1 hr at 130 to 140 C. (The vacuum connection should be at the top of the condenser.) The crude lactone was deacidified by dissolving in 50 ml of light petroleum and extracting the acids with 0.5 g of anhydrous triethanolamine while shaking vigorously. The light petroleum solution was filtered, using a small amount of Hyflo filter aid, and the solvent was evaporated, with the last traces being removed by heating in vacuo at 30 C. The rotation was measured after dissolving the lactone (without distilling) in 10 ml of methanol.

The results (Table 2) show that various yeasts of the genus *Candida* are able to give high yields of the dextrorotatory lactone from δ-ketocapric acid. The genus *Saccharomyces* is represented by two types.

Both the mold *Cladosporium butyri* and the bacterium *Sarcina lutea* gave the levorotatory antipode. With the latter organism, a larger amount of (−)-lactone was prepared. The rotatory power was lower than expected. No appreciable difference in the odor of the two enantiomorphic forms could be detected. *Pichia membranaefaciens* gave only a small amount of lactone (14 and 40 mg).

Reduction of various keto acids using Saccharomyces cerevisiae on a larger scale. To obtain a greater amount of

TABLE 2. *Reductions of δ-ketocapric acid using various microorganisms and optical activity of lactones obtained*

Microorganism	Medium	Incubation		Amount of cell material (g of dry matter)		Lactone from 500 mg of δ-ketocapric acid	Optical rotation
		Time	Temp	Per 140 cm² of culture medium	Used for reduction		
		hr	*C*			*mg*	
Molds							
Cladosporium butyri	Malt agar + 1% peptone	96	25	0.50	11.0	254	−43°[a]
C. butyri	Malt agar + 1% peptone	120	25	0.50	5.0	71	−15°
Penicillium notatum	Malt agar + 1% peptone	120	25	0.03	0.9	6	
Yeasts							
Saccharomyces cerevisiae	Malt agar	48	25	0.26	5.3	355	+48.2°
S. fragilis	Malt agar	48	30	0.30	4.7	229	+48.5°
Candida pseudotropicalis	Malt agar	48	30	0.26	5.3	252	+48.2°
C. dattila[b]	Malt agar	72	30	0.33	5.0	250	+12.2°
C. pseudotropicalis var. *lactosa*	Malt agar	48	30	0.21	4.4	240	+54.6°
C. globiformis	Malt agar	48	25	0.29	5.2	233	+55.8°
C. monosa	Malt agar	72	25	0.20	4.4	43	
C. lactis	Malt agar	96	30	0.35	4.6	7	
C. dattila[c]	Malt agar	72	30	0.33	5.0	199	+29.8°
Pichia membranaefaciens (from salad dressing)	Malt agar	48	25	0.30	4.6	14	
P. membranaefaciens (from salad dressing), control[d]	Malt agar	48	25	0.30	4.6	0	
P. membranaefaciens (from margarine)	Malt agar	48	25	0.25	5.1	40	
P. membranaefaciens (from margarine), control[d]	Malt agar	48	25	0.25	5.1	0	
Bacteria							
Escherichia freundii	Malt agar + 1% peptone	72	37	0.19	4.5	Trace	
Sarcina lutea	Malt agar + 1% peptone	48	25	0.35	4.6	302	−29.2°
S. lutea[e]	Malt agar + 1% peptone	48	25	0.35	22.1[e]	1400[e]	−36.8°
S. lutea	Malt agar + 1% peptone	96	25	0.35	7.3	279	−42.3°[a]

[a] Purified.
[b] Culture appeared to be infected.
[c] Pure culture.
[d] Experiment without keto acid.
[e] Experiment during which 2400 mg of δ-ketocapric acid were reduced.

various optically active lactones for the determination of their characteristics, the homologous series of γ-keto acids from C_9 up to and including C_{12} and the homologous series of δ-keto acids from C_8 up to and including C_{12} were reduced with fresh baker's yeast.

The cell material used for the following experiments was baker's yeast supplied as fresh as possible. In addition to *S. cerevisiae*, this cell material contained small bacterial impurities such as *Lactobacillus delbrueckii*, a homofermentative lactobacillus, and strains of *L. fermenti*, heterofermentative lactic acid bacteria which are used in the souring of the mash in which the baker's yeast is cultivated.

Comparative experiments with pure cultures of *S. cerevisiae* and with baker's yeast in which δ-ketocapric acid had been reduced, showed that these bacterial impurities had no measurable influence upon the yield or on the rotation of the lactone obtained. The reductions were carried out at 30 C. The general procedure was as follows.

TABLE 3. *Reductions of various keto acids carried out with baker's yeast (Saccharomyces cerevisiae) at 30 C for 24 hr*

Reduction conditions					Product obtained		
Keto acid	Amt of acid	Yeast	Yeast extract (10%)	Glucose	Lactone yield	Optical rotation in methanol (+)	Measuring concn
	g	*g*	*liters*	*g*	%		*g/liter*
γ-C_9	10	1800	18	450	77	50.4°	81
γ-C_{10}	10	680	10	680	85	46.1°	49
γ-C_{10}	5	200	3	200	18	47.2°	35
γ-C_{11}	10	1800	18	450	72	44.1°	80
γ-C_{12}	10	1330	20	1330	60	40.4°	50
γ-C_{12}	5	200	3	200	8	41.1°	
δ-C_8	1	60	0.35	60	54	54.1°	22
δ-C_8	50	3000	40	3000	38	56.9°	
δ-C_8	10	1000	6	300	13	58.4°	22
δ-C_9	8.8	1800	18	1800	55	58.2°	
δ-C_{10}	10	600	3.5	600	75	51.5°	54
δ-C_{10}	200	12,000	102	12,000	47	55.6°	
δ-C_{11}	3	600	6	600	63	47.9°	
δ-C_{12}	9.6	600	3.5	600	17	42.2°	
δ-C_{12}	15	1800	18	900	56	44.0°*	52

* Purified.

TABLE 4. *Physical properties of dextrorotatory lactones*

Lactone	Optical rotation (+)	Refractive index*
Pentanelactones		
5-Propyl	58.4°	1.4552
5-Butyl	58.2°	
5-Pentyl	56.0°	1.4581
5-Hexyl	47.9°	
5-Heptyl	48.8°	1.4598
Butanelactones		
4-Pentyl	50.4°	1.4469
4-Hexyl	47.2°	1.4497
4-Heptyl	44.1°	1.4509
4-Octyl	41.1°	1.4523

* At 20 C (D-line).

The yeast was suspended in 10% sterile yeast extract. The temperature was raised to 30 C. Portions of 10% keto acid solution (dissolved as sodium salt) and of 30% glucose solutions were added in such a way that, after about 3 hr, all reactants had been added. The initial pH was about 6.5. At the end of the reduction, the pH had dropped to 4.5 to 4.8. The reduction time allowed was 24 hr, although reduction was usually complete after 18 hr.

The lactones were isolated from the fermentation mixture as indicated in the previous section. The conditions under which the reductions were made, the yields of lactone obtained, and their rotations (measured at about 20 C) are given in Table 3.

Physical constants of optically active lactones. The lactones obtained from various experiments were carefully distilled, and the optical rotation was measured in methanolic solutions. The specific rotations (measured at about 20 C) and the refractive indexes are shown in Table 4.

The δ-lactones appeared to have a slightly higher $[\alpha]_D$ than did the γ-lactones. Winter et al. (1962) reported that (−)5-pentyl-pentanelactone obtained from the unsaturated lactone isolated from jasmine oil by hydrogenation had $[\alpha]_D^{20} = -49.7°$ (without solvent), and n_D^{25} 1.4544.

DISCUSSION

This basic research led to an economical process for producing optically active lactones and for which patents have been granted. No attempts were made to investigate which enzymes are responsible for the reduction of keto acids to hydroxy acids. However, A. Francke (1963) of this laboratory has examined the enzymology of this reduction by *S. cerevisiae*, and reference may be made to his publication.

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