STUDIES ON THE CHEMICOENZYMATIC PRODUCTION OF D-(−)-PANTOYL LACTONE

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INTRODUCTION

D-(-)-Pantoyl lactone is an important starting material for the chemical synthesis of D-(+)-pantothenic acid. The current world capacity of calcium pantothenate production and its demand are presumed to be about 4000 and 3600-4000 tons/year, respectively. It is mainly used as an additive of animal feed (about 3000 tons/year). Pantethine, the disulfide of pantetheine, and coenzyme A derived from D-(+)-pantothenic acid are also used as pharmaceutical products in several countries. They have been suggested to be effective in reducing cholesterol level, curing fatty liver and related diseases.

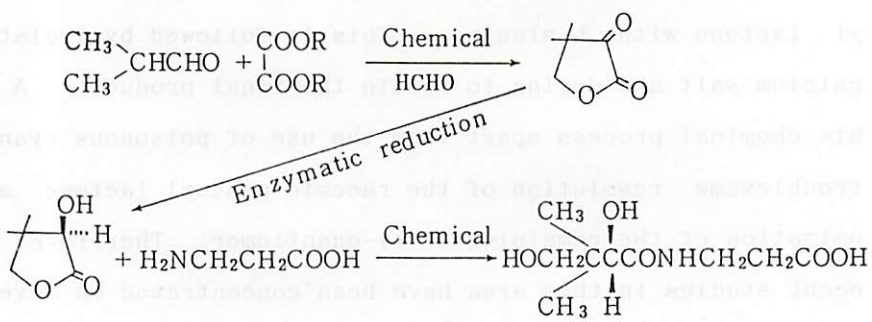
At present, commercial production of pantothenate depends exclusively on chemical synthesis. The conventional chemical process involves reactions yielding racemic pantoyl lactone from isobutyraldehyde, formaldehyde and cyanide, optical resolution of racemic pantoyl lactone to D-(-)-pantoyl lactone with quinine, quinidine, cinchonidine, brucine and so on and condensation of D-(-)-pantoyl lactone with β-alanine. This is followed by isolation as the calcium salt and drying to obtain the final product. A problem of this chemical process apart from the use of poisonous cyanide is the troublesome resolution of the racemic pantoyl lactone and the reracemization of the remaining L-(+)-enantiomer. Therefore, most of the recent studies in this area have been concentrated on development of an efficient method to obtain D-(-)-pantoyl lactone.

In recent years, a lot of microbial and enzymatic processes for the production of useful compounds have been developed. Great progress has been made in this field including application of biocatalyst to chemical synthetic routes, processing of foods, medical treatment, clinical analysis, environmental treatment, and so

on. Reactions catalyzed by enzymes and enzyme systems display far greater specificities than more conventional form of organic reactions, and of all the reactions available, enzyme and/or microbial synthesis and transformations have the great potential.[1)]

Therefore, it is expected that application of biological process to the field of synthetic chemistry will be further extended. In developing new biotransformation process, it is of importance that a new enzyme or enzymatic reaction would be found and its properties, reaction mechanism and regulation system would become clear.

D-(-)-Pantoyl lactone is an important starting material for the chemical synthesis of D-(+)-pantothenic acid as described above. In the course of study on the synthesis of D-(-)-pantoyl lactone, I established an efficient chemobiological process which involves one-pot chemical synthesis of ketopantoyl lactone followed by its stereoselective reduction to D-(-)-pantoyl lactone with microbial cells as the catalyst.



Chapter 1 describes an efficient one-pot synthesis method giving ketopantoyl lactone in a high yield (81%) from isobutyraldehyde, diethyl oxalate, formalin and sodium methoxide near room temperature.

Chapter 2 deals with microbial reduction of ketopantoyl lactone

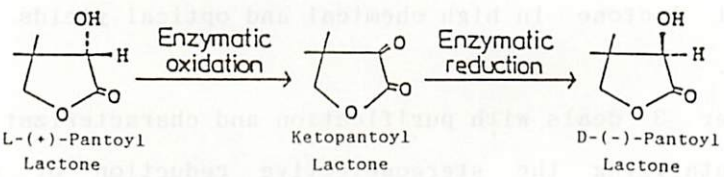
to D-(-)-pantoyl lactone. I surveyed the reducing ability of a variety of microorganisms for ketopantoyl lactone and found that variety of microorganisms exhibited the ability to reduce ketopantoyl lactone, the D-(-)- and L-(+)-enantiomer ratio varying. Among them, Rhodotorula minuta IFO 0920 and Candida parapsilosis IFO 0708 gave the D-(-)-enantiomer almost specifically in excellently high yields. The optimal reaction conditions for the production of D-(-)-pantoyl lactone in high chemical and optical yields were also determined.

Chapter 3 deals with purification and characterization of the enzymes catalyzing the stereoselective reduction of ketopantoyl lactone to D-(-)- and L-(+)-pantoyl lactone. The enzymes from C. parapsilosis and S. cerevisiae gave D-(-)-pantoyl lactone on the reduction of ketopantoyl lactone, on the contrary, the one from M. ambiguus gave L-(+)-pantoyl lactone. Substrate specificities, kinetic properties, inhibitors and several other properties of the enzymes were investigated. These enzymes are shown to be novel carbonyl reductases which are specific only to conjugated polyketones and require NADPH as a cofactor.

Chapter 4 deals with stereospecific reduction of α-diketones. Analogs of ketopantoyl lactone and isatin, and (R)- and (S)-camphorquinone gave corresponding (R)-alcohols through the action of the enzyme from C. parapsilosis. Reduction of several analogs of ketopantoyl lactone and isatin was also investigated.

Further, in the course of this study, I found that a stereospecific oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone is also a promising reaction for practical purpose for the production of D-(-)-pantoyl lactone, because racemic pantoyl lactone is still one of the cheap intermediates for D-(-)-pantoyl lactone

synthesis. If the stereospecific oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone in its racemic mixture might be possible without giving any modification of the remaining D-(-)-enantiomer, the above mentioned stereospecific reduction system might be also applicable to convert the formed ketopantoyl lactone to D-(-)-pantoyl lactone. Based on this idea, I screened for microorganisms capable



L-(+)-Pantoyl Lactone — Ketopantoyl Lactone — D-(-)-Pantoyl Lactone

of converting only the L-(+)-pantoyl lactone to ketopantoyl lactone, and found that Nocardia asteroides AKU 2103 and several other microbial strains can be used as potent catalysts for this conversion. In Chapter 5, optimization of reaction conditions for the improvement of ketopantoyl lactone production was investigated with N. asteroides. One-pot synthesis of D-(-)-pantoyl lactone from its racemic mixture with Rhodococcus erythropolis IFO 12540 was also investigated.

CHAPTER 1. One-Pot Synthesis of Ketopantoyl Lactone and Its Analogs from Diethyl Oxalate and Aldehydes through the Action of Sodium Methoxide[a,b)]

Ketopantoyl lactone (1a) is an important intermediate in calcium pantothenate synthesis. Stereoselective reduction of 1a[2-6)] gives D-(-)-pantoyl lactone (D-(-)-2), which reacts with β-alanine to give pantothenic acid. Many methods have been developed for the synthesis of 1a,[2,7-10)] of which two are typical. One is the oxidation of 2[7,8)] and the other is the condensation of dimethylpyruvic acid (3) with 35% formalin (HCHO).[2)] The most efficient process for the former reaction would be air oxidation of 2 catalyzed by rutenium catalysts,[8)] however, the method involves precious catalysts and a high reaction temperature. The latter method involves 3 as a substrate and gives 1a in a 70% yield, as reported by Kuhn and Wieland.[2)]

3 is also an important starting material for amino acids and other organic compounds, as well as for 1a. Many synthetic methods for 3 have been developed,[11-18)] however, none of them is satisfactory for industrial use. In recent years, double carbonization of aromatic halides appeared promising, however, the double carbonization of isopropyl halide is difficult and has not been reported.

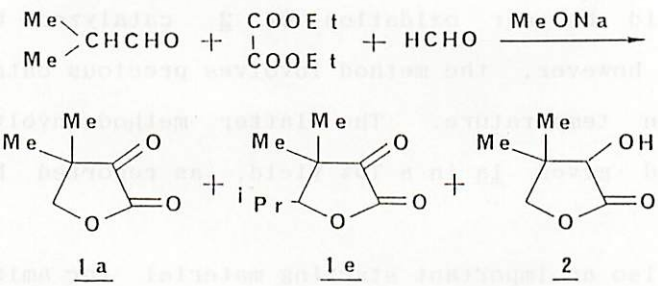
The fact that there was no efficient method for the synthesis of 1a prompted me to search for a new synthetic method for 1a. I tried to synthesize 1a under mild conditions with easily available reagents and found a simple and efficient one-pot synthesis of 1a. I report here the full scope of the one-pot synthesis of 1a. Further, I also report the syntheses of some ketopantoyl lactone

analogs in a similar manner.

RESULTS

Synthesis of 1a from isobutyraldehyde, diethyl oxalate and formaldehyde

1a was obtained in a 81% yield, as shown in the Materials and Methods. Dimethyl oxalate was formed through the reaction of diethyl oxalate with a methanolic solution of 28% sodium methoxide (28% MeONa). An intermediate, 5-isopropylketopantoyl lactone (1e), was



1a 1e 2

Scheme 1. Synthesis of Ketopantoyl Lactone from Methylpropanal, Diethyl Oxalate and Formaldehyde.

formed on the addition of isobutyraldehyde to the reaction mixture. When HCHO was added to the reaction mixture, 1a appeared. The yield of 1a increased, with a decrease in the amount of 1e, on incubation with aqueous NaOH. 2, a reduction product of 1a, was formed as a by-product in the latter part of the incubation. Between 20 min to 3 h incubation after the addition of HCHO is necessary to obtain a good yield of 1a. Because, when aqueous NaOH was added immediately after the addition of HCHO under the same conditions, the yields of 1a (37%) and 1e (21%) remained unchanged on further incubation and a

Table 1. Synthesis of 1a under Different Reaction Conditions

Run	Molar ratio[a]	Yields (%)[b]		
		1a	1e	2
1	1.0:1.0:1.0:1.0	56	4	0
2	1.5:1.0:1.0:1.0	70	16	0
3	2.0:1.0:1.0:1.0	16	84	0
4	1.0:1.0: 0 :1.0	32	20	0
5	1.0:1.0:0.5:1.0	37	18	0
6	1.0:1.0:1.2:1.0	60	0	8
7	1.0:1.0:1.5:1.0	42	0	14
8	1.2:1.2:1.1:1.0	81	3	3
9	0.8:1.2:1.1:1.0	56	12	6
10	1.5:1.2:1.4:1.0	54	0	22

[a] Molar ratio, isobutyraldehyde:HCHO:NaOH:diethyl oxalate.

[b] Analyzed after 1 h incubation after adding aqueous NaOH.

good yield of 1a could not be obtained. Table 1 shows the results under different reaction conditions. Reactions were carried out as described in Materials and Methods, unless otherwise stated. Runs 1-3 show the effect of the molar ratio of isobutyraldehyde to diethyl oxalate. The yield of 1a increased to 70% when the proportion of isobutyraldehyde was 1.5. However, the yield decreased the proportion of isobutyraldehyde was 2.0, with a concomitant increase in 1e. Runs 4-7 show the effect of the molar ratio of 40% aqueous NaOH to diethyl oxalate, the highest yield of 1a was obtained when the proportion of 40% aqueous NaOH was 1.2. A low quantity of 40% aqueous NaOH gave 1e and a high quantity of it gave 2 as a by-product. 2 was formed through the reduction of 1a due to the action of HCHO and aqueous NaOH as shown by Run 10. Further investigation led to elucidation of the optimum conditions, i.e., those in Run 8. In this investigation, a 28% yield of isobutyric acid based on diethyl oxalate was obtained. Run 8 shows that 1.48 mol of

isobutyraldehyde, 1.48 mol of HCHO and 1.12 mol of diethyl oxalate are necessary for the synthesis of 1 mol of 1a. Run 9 shows that an equimolar amount of 28% MeONa is necessary for the synthesis.

Intermediates in the synthesis

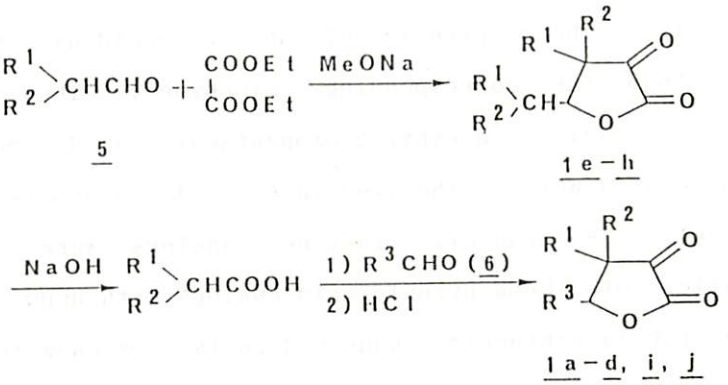
Isobutyraldol (4) was yielded through the aldol condensation of isobutyraldehyde under alkaline conditions.[19] The reaction of 4 with HCHO gave a 75% yield of 1a together with a 95% yield of methyl formate. 1e can be synthesized in a 92% isolated yield from isobutyraldehyde and diethyl oxalate. These results strongly suggest that 1e is one of intermediates in 1a synthesis. The hydrolysis of 1e under alkaline conditions gave 3, which gave 1a in a 67% yield on condensation with HCHO, as mentioned above. 3 was also synthesized in a 91% yield when no HCHO was used for 1a synthesis. These results support that 4, 1e and 3 are intermediates in the reaction.

The difficulty in decomposing 1a to 3 and HCHO shows that the reaction of 3 with HCHO only proceeds in one direction to give 1a. This was supported by that no 3 was detected in the reaction mixture.

Synthesis of dihydro-2,3-furanediones

It was found that 1e was decomposed under alkaline conditions to give isobutyraldehyde and 3, which gave 1a on condensation with HCHO. A mixture of 1e and HCHO gave 1a in a 65% yield, as reported by Kuhn and Wieland.[2] These results showed that if I used other aldehydes for 1a synthesis, other ketopantoyl lactone analogs could be synthesized.

When I used other sorts of aldehydes for 1e synthesis, 1f, 1g and 1h were obtained in 83, 30 and 79% isolated yields, respectively. Further, the hydrolysis of 1f, 1g and 1h under alkaline conditions gave the corresponding pyruvic acid analogs. Thus, 3-methyl-2-



Scheme 2. Proposed Reaction Pathway for 1 Synthesis.

Table 2. Syntheses of Some Ketopantoyl Lactone Analogs

Run	Substrates[a] 5 R^1	5 R^2	6 R^3	Molar ratio[b]	Isolated Yield (%)[c]
1	Me	Me	H	1.2:1.2:1.1:1.0	1a(76)
2	Me	Et	H	1.2:1.2:1.1:1.0	1b(39)
3	Et	Et	H	1.2:1.2:1.1:1.0	1c(32)
4	Me	Pr	H	1.2:1.2:1.1:1.0	1d(46)
5	Me	Me	-	2.0: 0 : 0 :1.0	1e(92)
6	Me	Et	-	2.0: 0 : 0 :1.0	1f(83)
7	Et	Et	-	2.0: 0 : 0 :1.0	1g(30)
8	Me	Pr	-	2.0: 0 : 0 :1.0	1h(79)
9	Me	Me	Et_2CH	1.0:1.4: 0 :1.0	1i(46)
10	Me	Me	MePrCH	1.0:1.2: 0 :1.0	1j(48)

[a] The substituent groups, R^1-R^3, are shown in Scheme 2.

[b] Molar ratio, 5:6:NaOH:diethyl oxalate.

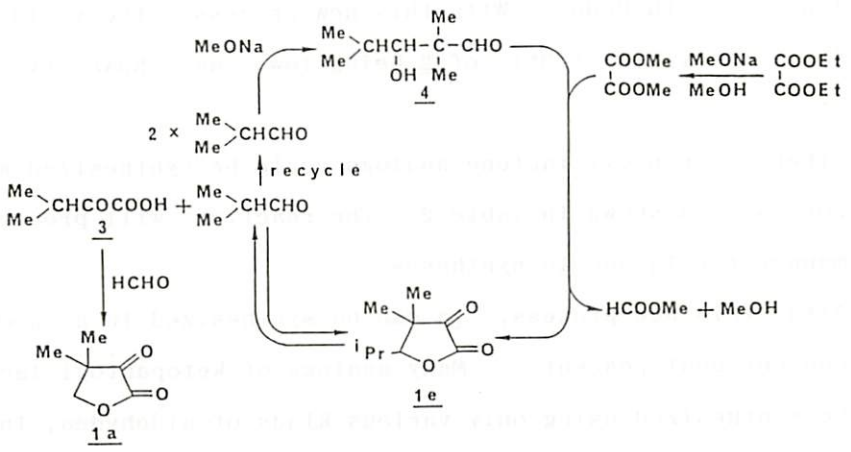
[c] The isolated yield was calculated on the basis of diethyl oxalate.

oxopentanoic acid, 3-ethyl-2-oxopentanoic acid and 3-methyl-2-oxohexanoic acid were obtained in 63, 23 and 50% isolated yields, respectively. These pyruvic acid analogs could also be synthesized directly from the corresponding aldehydes. Thus, 3-methyl-2-oxopentanoic acid, 3-ethyl-2-oxopentanoic acid and 3-methyl-2-oxohexanoic acid were synthesized in 85, 64 and 80% isolated yields, respectively. Ketopantoyl lactone analogs were obtained on condensation of these pyruvic acid analogs with HCHO in the same manner as for 1a synthesis. Runs 2-4 in Table 2 show that the yields of 1b, 1c and 1d were 39, 32 and 46%, respectively. Other ketopantoyl lactone analogs were synthesized, as shown by Runs 9-10 in Table 2.

DISCUSSION

The reaction seemed to proceed through a complicated reaction pathway, because 3,3-dimethyl-2,4-dioxobutanoate, the most probable intermediate if the reaction proceeds as described in the literature,[20,21] was not detected. A proposed pathway for this reaction is shown in Scheme 3. 4 is formed from isobutyraldehyde under alkaline conditions,[19] followed by the reaction of 4 with diethyl oxalate to 1e and methyl formate. Then, 1e decomposes under alkaline conditions to give 3 and isobutyraldehyde, and then the isobutyraldehyde formed is recycled.

To obtain a good yield (81%) of 1a, I tried to optimize the reaction conditions. The incubation time after the addition of HCHO is important for this purpose, because the yield of 1a did not increase with the incubation period with aqueous NaOH when the aqueous NaOH was added immediately after the addition of HCHO. The



Scheme 3. Proposed Reaction Pathway for 1a Synthesis.

reason for this might be the slow decomposition of 1e under the incubation conditions with HCHO. The recycling of isobutyraldehyde was supported by that one mol of 1a was derived from 1.48 mol of isobutyraldehyde, as shown by Run 8 in Table 1. 1e was obtained in a 92% isolated yield when a bimolar ratio of isobutyraldehyde to diethyl oxalate was used. An equimolar amount of MeONa is necessary because the analytical yield of 1e decreased to 50% when a half amount of 28% MeONa was used as to diethyl oxalate for 1e synthesis. Similarly, the amount of 28% MeONa is important for 1a synthesis, as shown by Run 9 in Table 1.

3 was formed in a good yield from 1e through the action of NaOH, and 1a can be synthesized from 3 and HCHO.[2] This also shows the possibility that the reaction from 1e to 1a may proceed through 3 as an intermediate. As I detected no 3 in the reaction mixture, the reaction of 3 and HCHO seems to proceed rapidly to give ketopantoic acid.

During the 1a synthesis, by-product 2 was formed in a 14% yield through the reaction of 1e with HCHO, and in a 11% yield through the

reaction of 3 with HCHO. With this new process, the yield of 1a is superior, the yield (<3%) of 2 being low, as shown by Run 8 in Table 1.

Other ketopantoyl lactone analogs could be synthesized with this new process, as shown in Table 2. The reaction will proceed in the same manner for 1a and 1e syntheses.

With this new process, 1a can be synthesized in a good yield with conventional reagents. Many analogs of ketopantoyl lactone can also be synthesized using only various kinds of aldehydes, instead of isobutyraldehyde and HCHO.

MATERIALS AND METHODS

Materials. An authentic sample of 1a was synthesized by oxidation of 2, as described below. 3 was synthesized by the method of Kuhn and Wieland.[2] 4 was prepared from isobutyraldehyde by a method based on that of Hagemeyer et al.[19] All other reagents were commercially available and used without further purification.

Analyses. Elemental analyses were carried out with a Hitachi 026 CHN analyzer. Mass spectral measurements were carried out with a Shimadzu GC-MS 7000 (GC-MS). Infrared and ^{1}H-NMR spectral measurements were carried out with a Shimadzu Infrared Spectrophotometer IR 430 and a Hitachi High Resolution NMR Spectrometer R-24B (60 Mc), respectively. The yields of the products were determined with a Shimadzu GC-7AF gas liquid chromatograph (GLC) equipped with a flame ionization detector. The column (1 m long, 3.0 mm inside diameter) was packed with 10% PEG 20M on 60 to 80 mesh Chromosorb W(AW-DMCS; Nishio Kogyo, Japan). The operation parameters were: injection port, 190°C; detector port, 190°C; column, 150°C;

helium carrier, 70 ml/min; hydrogen, 0.6 kg/cm^2; air, 0.6 kg/cm^2. For analysis of the products, the reaction mixture was acidified by adding a half volume of conc. HCl, and then the solution was incubated for 20 min at 50°C to lactonize the products. The extract with ethylene dichloride was injected into the GLC. The internal standard for quantitative analysis of the products was m-dimethoxybenzene. The retention times (min) were as follows: m-dimethoxybenzene, 2.7; diethyl oxalate, 2.6; 1a, 4.3; 1b, 6.3; 1c, 7.5; 1d, 8.1; 1e, 7.0; 1f, 10.2 and 11.3 (diastereomers); 1g, 16.3; 1h, 16.6; 1i, 11.2; 1j, 11.2; 2, 7.3; and 3, 3.3. Methyl formate was measured essentially in the same manner as described above: glass column (3 m long, 3 mm inside diameter); column, 70°C. The retention time was 2.6 min. The yields of the products are based on diethyl oxalate and, sometimes, shown in parentheses.

Authentic 1a. 31.0 g (0.19 mol) of Br_2 was added to a refluxing mixture of 26.0 g (0.20 mol) of 2, 600 ml of CCl_4 and 20 ml of water. After incubation for 2 h under refluxing conditions, the reaction mixture was separated into CCl_4 and aqueous layers. The CCl_4 layer was cooled to 10°C and the crystals formed were obtained by filtration. Vacuum drying of the crystals gave 8.8 g (34%) of 1a. Authentic 1a was obtained by further recrystallization from CCl_4. Anal. Calcd: C, 56.2%; H, 6.3%. Found: C, 56.3%; H, 6.4%; MS, m/Z 128 (M), 70, 56, 41 (Base); IR (KBr, cm^{-1}) 3510, 2970, 1770, 1455, 1390, 1275, 1170, 1040; ^{1}H-NMR ($CDCl_3$, δ) 1.03 (s, 6H), 4.27 (s, 2H); mp. 64-65°C.

1e from isobutyraldehyde. To a mixture of 29.2 g (0.20 mol) of diethyl oxalate and 38.6 g (0.20 mol) of 28% MeONa was added 28.8 g (0.40 mol) of isobutyraldehyde at 10°C. The mixture was stirred for 1 h. Methyl formate was formed in a 95% yield, as judged by GLC

analysis. The reaction mixture was acidified with 20 ml of conc. HCl and the NaCl formed was filtered off, followed by washing with 20 ml of MeOH. Distillation of the mixture combined with the filtrate and washed MeOH gave 31.1 g (92%) of 1e at 102-106°C/4 Torr. Anal. Calcd: C, 63.5%; H, 8.3%. Found: C, 63.3%; H, 8.2%; MS, m/Z 170 (M), 99, 98, 84, 83, 71, 70 (Base), 55, 43, 42, 41; IR (KBr, cm^{-1}) 3430, 2970, 1780, 1460, 1270, 1035; ^{1}H-NMR ($CDCl_3$, δ) 0.98, 1.07 (d, J = 6.4 Hz, each 3H), 1.20 (s, 3H), 1.31 (s, 3H), 2.01 (m, J = 6.4 Hz, 1H), 4.17 (d, J = 6.4 Hz, 1H); mp. 48-50°C.

1a. To a mixture of 77.1 g (0.40 mol) of 28% MeONa and 58.4 g (0.40 mol) of diethyl oxalate was added 34.6 g (0.48 mol) of isobutyraldehyde over a period of 5 min at 10°C, and then the mixture was stirred for a further 30 min at the same temperature. After the temperature of the reaction mixture had been raised to 40°C, 41.1 g (0.48 mol) of 35% formalin was added over a period of 5 min, and then stirring was continued for a further 1 h at 40°C. The addition of 44.0 g (0.44 mol) of aqueous NaOH and stirring for 1 h at 40°C gave 1a in a 81% analytical yield. 1e and 2 were formed as by-products, in 3.0% and 3.2% yields, respectively. To this solution was added 70 ml of conc. HCl and then the mixture was stirred for 1 h at the same temperature. The resulting reaction mixture was filtrated to remove NaCl and then the NaCl was washed with 100 ml of MeOH. The combined mixture of the filtrate and washed MeOH was distilled. 31.4 g (61% yield) of 1a was obtained at 85-90°C/4 Torr. Recrystallization of the 1a from 200 ml of CCl_4 gave 27.3 g (53%) of plate-like crystals with mp. 64-65°C. All the analytical data were the same as those of authentic 1a.

1e from 4. The reaction was carried out at 10°C. To a mixture comprising 14.6g (0.1 mol) of diethyl oxalate and 19.3 g (0.1 mol) of

28% MeONa was added 14.6 g (0.10 mol) of 4 over a period of 5 min. After stirring for 2 h, 21.0 g (0.20 mol) of conc. HCl was added to this solution and then stirring was continued for a further 1 h. After adding 100 ml of MeOH to the mixture, the NaCl was filtered off. The filtrate was extracted with 100 ml of CH_2Cl_2 after evaporating MeOH. Distillation of the CH_2Cl_2 layer after drying over Na_2SO_4 gave 12.8 g (75%) of 1e at 86-91°C/1.5 Torr; mp. 47-50°C.

3 from 1e. To 17.0 g (0.10 mol) of 1e in 50 ml of MeOH was added 40.0 g (0.20 mol) of 20% aqueous NaOH over a period of 10 min. The mixture was stirred for 2 h at the same temperature. The yield of 3 was determined after acidification of the reaction mixture with 30 ml of conc. HCl. 3 was formed in a 92% yield, and the products were identified by GLC and GC-MS analyses, in comparison with authentic 3.

3 from isobutyraldehyde. To a mixture of 146 g (1.00 mol) of diethyl oxalate and 193 g (1.00 mol) of 28% MeONa was added 86.6 g (1.20 mol) of isobutyraldehyde. After stirring for 1 h, 960 g (1.20 mol) of 5% aqueous NaOH was added over a period of 15 min and then the mixture was stirred for 1.5 h at 25°C. The yield of 3 in the reaction mixture was 91%. MeOH was evaporated at 18-19°C/30 Torr after acidification with conc. HCl (pH to 4). The residual aqueous solution was acidified with 80 ml of conc. HCl and then extracted 5 times with 600 ml of ethyl acetate. The combined organic layer was dried over Na_2SO_4. Distillation of this organic layer gave 80.0 g (67%) of 3 at 72°C/15 Torr. MS, m/Z 116 (M), 71 (Base), 45, 43, 42, 41, 39, 27; IR (KBr, cm^{-1}) 3250, 2980, 2940, 2880, 1730, 1470, 1390, 1370, 1330, 1280, 1180; ^{1}H-NMR ($CDCl_3$, δ) 1.21 (d, J = 6.9 Hz, 6H), 3.41 (m, J = 6.9 Hz, 1H), 10.06 (bs, 1H).

1a from 3. According to the method of Kuhn and Wieland,[2] 8.6 g

(67%) of 1a and 1.4 g (11%) of 2 were formed from 11.6 g (0.10 mol) of 3 and 9.4 g (0.11 mol) of HCHO.

1a from 1e. To 17.0 g (0.10 mol) of 1e was added 12.9 g (0.15 mol) of HCHO over a period of 5 min at 40°C. After the mixture had been stirred for 1 h at the same temperature, the mixture was cooled to 20°C. To the mixture was added 30.0 g (0.15 mol) of 20% aqueous NaOH and then the mixture was stirred for 1 h. When the mixture was incubated for 1 h at 50°C with 10 ml of conc. HCl, 8.3 g (65%) of 1a, 1.8 g (14%) of 2 and 0.9 g (6%) of 1e were formed.

Synthesis of 1b, 1c and 1d. To a mixture of 73.0 g (0.50 mol) of diethyl oxalate and 96.5 g (0.50 mol) of 28% MeONa was added 0.60 mol of aldehyde at 10°C, and then the mixture was stirred for 1 h. Then 51.1 g (0.60 mol) of HCHO was added to the mixture after the temperature had been raised to 40°C, and then the mixture was stirred for 1 h at the same temperature. The mixture was cooled to 20°C and then 55.0 g (0.55 mol) of 40% aqueous NaOH was added. After 1 h stirring, 100 ml of conc. HCl was added to the mixture, and then stirring was continued for a further 1 h. The NaCl formed was filtered off and the filtrate evaporated to remove the MeOH. The residual aqueous solution was extracted five times with 300 ml of ethyl acetate. Distillation of the combined organic layer after drying over Na_2SO_4 gave the product. 2-Methylbutanal, 2-ethylbutanal and 2-methylpentanal were used for 1b, 1c and 1d synthesis, respectively. 27.5 g (39%) of 1b was obtained at 83°C/2 Torr. Anal. Calcd: C, 59.2%; H, 7.1%. Found: C, 59.0%; H, 7.0%; MS, m/Z 142 (M), 84, 83, 70, 69, 55 (Base), 42, 41, 39; IR (KBr, cm^{-1}) 2970, 1780, 1455, 1240, 1170, 1150, 1110; ^{1}H-NMR ($CDCl_3$, δ) 0.95 (bt, 3H), 1.29 (s, 3H), 1.67 (m, 2H), 4.46, 4.60 (ABq, J_{AB} = 9.1 Hz, each 1H). 24.7 g (32%) of 1c was obtained at 120°C/6 Torr. Anal. Calcd: C; 61.5%,

H; 7.7%. Found: C, 61.6%; H, 7.7%; MS, m/Z 156 (M), 98, 84, 83, 69, 55, 41; IR (KBr, cm^{-1}) 2980, 1780, 1460, 1380, 1240, 1150, 1110; ^{1}H-NMR ($CDCl_3$, δ) 0.92 (t, J = 7.0 Hz, 6H), 1.74 (q, J = 7.0 Hz, 4H), 4.51 (s, 2H). 36.1 g (46%) of 1d was obtained at 90°C/2 Torr. Anal. Calcd: C, 61.5%; H,7.7%. Found: C, 61.6%; H, 7.6%; MS, m/Z 156 (M), 114, 113, 98, 84, 83, 69, 56 (Base), 55, 41; IR (KBr, cm^{-1}) 2950, 1780, 1460, 1385, 1230, 1145, 1110; ^{1}H-NMR ($CDCl_3$, δ) 0.95 (m, 3H), 1.14-1.95 (m, 4H), 1.29 (s, 3H), 4.47 and 4.63 (ABq, J = 9.2 Hz, each 1H).

Synthesis of 1f, 1g and 1h. To a mixture of 73.0 g (0.50 mol) of diethyl oxalate and 96.5 g (0.50 mol) of 28% MeONa was added 1.00 mol of aldehyde at 10°C, and then the mixture was stirred for 2 h. To the mixture was added 60 ml of conc. HCl, followed by stirring for a further 1 h, and then the NaCl formed was filtered off and the filtrate evaporated to remove MeOH. The residual aqueous solution was extracted five times with 300 ml of ethyl acetate. Distillation of the combined organic layer after drying over Na_2SO_4 gave the product. 2-Methylbutanal, 2-ethylbutanal and 2-methylpentanal were used for 1f, 1g and 1h synthesis, respectively. 81.7 g (83%) of 1f was obtained at 125°C/3 Torr. Anal. Calcd: C, 66.6%; H, 9.2%. Found: C, 66.5%; H, 9.1%; MS, m/Z 198 (M), 126, 97, 84, 69, 57, 55, 43, 41 (Base); IR (KBr, cm^{-1}) 2900, 1770, 1450, 1375, 1325, 1300, 1225, 1190, 1150, 1030; ^{1}H-NMR ($CDCl_3$, δ) 0.74-1.17 (m, 9H), 1.28 (d, J = 4.8 Hz, 3H), 1.39-2.47 (m, 5H), 4.28, 4.40, 4.53 and 4.64 (each d, J = 8.5, 5.9, 4.9 and 2.5 Hz, 1H). 33.3 g (30%) of 3g was obtained at 125°C/3 Torr. Anal. Calcd: C, 69.0%; H, 9.8%. Found: C, 68.8%; H, 9.7%; MS, m/Z 226 (M), 154, 126, 125, 98, 83, 69, 55, 43, 41; IR (KBr, cm^{-1}) 2980, 1780, 1460, 1230, 1190, 1130; ^{1}H-NMR ($CDCl_3$, δ) 0.91-1.42 (m, 12H), 1.42-2.53 (m, 9H), 4.32 (d, J = 1.8

Hz, 1H). 89.0 g (79%) of 3h was obtained at 112°C/2 Torr. Anal. Calcd: C, 69.0%; H, 9.8%. Found: C, 68.8%; H, 9.7%; MS, m/Z 226 (M), 154, 139, 112, 111 (Base), 98, 83, 71, 70, 69, 67, 55, 43, 41; IR (KBr, cm^{-1}) 2950, 1775, 1460, 1385, 1225, 1160; ^{1}H-NMR ($CDCl_3$, δ) 0.76 (d, J = 5.8 Hz, 3H), 1.0 (m, 6H), 1.3 (m, 1H), 1.9 (m, 1H), 4.24, 4.35, 4.51 and 4.59 (each d, J = 8.4, 5.5, 4.0 and 2.4 Hz, 1H).

Synthesis of 1i. To a mixture of 73.0 g (0.50 mol) of diethyl oxalate and 96.5 g (0.50 mol) of 28% MeONa was added 36.0 g (0.10 mol) of isobutyraldehyde at 10°C, followed by stirring for 1 h. To the mixture was added 70.0 g (0.70 mol) of 2-ethylbutanal and then the mixture was stirred for a further 3 h. To the mixture was added 68.0 g of conc. HCl and then the mixture was stirred for a further 1 h. The NaCl formed was filtered off and then the MeOH was distilled off from the mixture. The residual aqueous solution was extracted five times with 300 ml of ethyl acetate. Distillation of the combined organic layer after drying over Na_2SO_4 gave 46.1 g (46%) of 1i at 102-106°C/2 Torr. Anal. Calcd: C, 66.6 %; H, 9.2%. Found: C, 66.4%; H, 9.1%; MS, m/Z 198 (M), 181, 171, 153, 126, 98, 97 (Base), 71, 70, 69, 55; IR (KBr, cm^{-1}) 2950, 1780, 1460, 1385, 1340, 1270, 1200, 1160, 1100, 1005; ^{1}H-NMR ($CDCl_3$, δ) 1.00 (m, 6H), 1.24 (s, 3H), 1.35 (s, 3H), 1.10-2.00 (m, 5H), 4.03 (d, J = 4.6 Hz, 1H).

1j. 1j was synthesized in a similar manner to 1i using 60.0 g (0.60 mol) of 2-methylpentanal in place of 2-ethylbutanal. Distillation of the organic layer gave 47.6 g (48%) of 1j at 102-104°C/3 Torr. Anal. Calcd: C, 66.6%; H, 9.2%. Found: C, 66.5%; H, 9.0%; MS, m/Z 198 (M), 181, 171, 153, 126, 113, 84, 83 (Base), 71, 70, 69, 55; IR (KBr, cm^{-1}) 2940, 1770, 1455, 1380, 1270, 1190, 1150; ^{1}H-NMR ($CDCl_3$, δ) 0.86 (d, J = 5.9 Hz, 3H), 0.91 (m, 3H), 1.23 (s, 3H), 1.33 (s, 3H), 1.30 (m, 4H), 1.89 (m, 1H), 4.25 and 4.38 (each d,

J = 6.6 and 4.3 Hz, 1H).

Synthesis of 3-methyl-2-oxobutanoic acid, 3-ethyl-2-oxobutanoic acid and 3-methyl-2-oxopentanoic acid from 1. In the same manner, 3-methyl-2-oxobutanoic acid, 3-ethyl-2-oxobutanoic acid and 3-methyl-2-oxopentanoic acid were synthesized in 63, 23 and 50% isolated yields, respectively, from 1f, 1g and 1h.

Synthesis of 3-methyl-2-oxopentanoic acid, 3-ethyl-2-oxopentanoic acid and 3-methyl-2-oxohexanoic acid from methylbutanal, ethylbutanal and 2-methylpentanal. Analogs of pyruvic acid were synthesized from the corresponding aldehydes in the same manner as in the case of synthesis of "3 from isobutyraldehyde". 3-Methyl-2-oxobutanoic acid, 3-ethyl-2-oxobutanoic acid and 3-methyl-2-oxohexanoic acid were obtained in 85, 80 and 64% isolated yields, respectively, at 75-80/12, 70-75/6 and 55-56^{o}C/10 Torr.

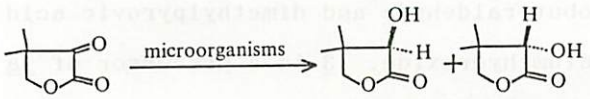
SUMMARY

A new synthetic method for ketopantoyl lactone (1a) was developed. 1a was synthesized in a 81% yield when isobutyraldehyde was added to a mixture of diethyl oxalate and a 28% sodium methoxide methanol solution, followed by the addition of formalin and sodium hydroxide to the reaction mixture at 10-40^{o}C. 1a was also synthesized from isobutyraldol (4). The reaction of 4 with diethyl oxalate gave 5-isopropylketopantoyl lactone (1e), which was decomposed to isobutyraldehyde and dimethylpyruvic acid (3) through the action of sodium hydroxide. 3 is a precursor of 1a and was also obtained, in a 91% yield, without formalin during the 1a synthesis. Analogs of 1a and 3 were synthesized in the same manner.

CHAPTER 2. Stereospecific Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by Microorganisms

Section 1. Distribution of ketopantoyl lactone-reducing activity in microorganisms[c)]

As described in Chapter 1, I have established a novel and efficient process for the synthesis of ketopantoyl lactone from diethyl oxalate, isobutyraldehyde and formalin by the action of sodium methoxide. If the stereoselective reduction of ketopantoyl lactone to D-(-)-pantoyl lactone is possible, ketopantoyl lactone might be a very promising starting material for the synthesis of D-(-)-pantoyl lactone. Microbial or enzymatic reduction of ketopantoyl lactone may be one of the possible ways of doing this, because biochemical reductions are usually enantiospecific compared with chemical reductions. Although basic studies on enzymatic reduction of ketopantoyl lactone have been reported by Wilken and coworkers,[4,22-25)] little has been done to obtain D-(-)-pantoyl lactone in a high yield. An early study by Lanzilotta et al.[3)] was the only one concerning conversion of ketopantoyl lactone to D-(-)-pantoyl lactone, but the yield was not satisfactory and the active strains were restricted to only Byssochlamys fulva. Thus, I surveyed the reducing ability of a variety of microorganisms for ketopantoyl



Ketopantoyl lactone | D-(−)-Pantoyl lactone | L-(+)-Pantoyl lactone (Eq. 1)

lactone. Here, I report that a variety of microorganisms exhibit the ability to reduce ketopantoyl lactone with a variety of D-(-)- to L-(+)-enantiomer ratios (Eq. 1). Among these, Candida parapsilosis, Rhodotorula minuta and several fungal strains were found to give almost specifically the D-(-)-enantiomer in excellently high yields. This suggests the possibility that D-(-)-pantoyl lactone might be produced efficiently by enzymatic stereospecific reduction of ketopantoyl lactone.

MATERIALS AND METHODS

Chemicals. Analytical samples of D-(-)- and L-(+)-pantoyl lactones were purchased from Tokyo Kasei, Japan. L-Menthyl chloroformate was synthesized by the method of Annett and Stumpf.[26] Ketopantoyl lactone was synthesized as described in Chapter 1. All other reagents of analytical grade were commercially available.

Microorganisms. All strains used from AKU culture collection (Faculty of Agriculture, Kyoto University, Kyoto).

Media and cultivation. The following media were used. Media GC for molds and yeasts contained 5% glucose and 5% corn steep liquor, pH 6.0. Medium GCC for bacteria was medium GC supplemented with 1% $CaCO_3$, pH 7.0. Medium GS for actinomycetes contained 2% glucose, 1% sucrose, 1% meat extract, 0.5% Polypepton, 0.2% yeast extract, 0.3% NaCl, 0.2% KH_2PO_4, 0.4% K_2HPO_4, 0.1% $MgSO_4.7H_2O$ and 0.1% corn steep liquor, pH 7.0. Medium GE for basidiomycetes contained 1% glucose and 0.5% Ebios (Ebios Pharmaceuticals, Japan), pH 5.0. Cultivations were carried out in test tubes (16.5 x 165 mm) containing 5 ml of medium at 28°C, usually for 48 h with shaking.

Reaction. To each 48 h-culture was added an aqueous solution

(90 μl) containing 50 mg of ketopantoyl lactone, and the incubation was continued for a further 48 h. After the reaction mixture was centrifuged or filtered to remove insoluble materials, the supernatant or filtrate was analyzed for total pantoyl lactone, the ratio of D-(-)- and L-(+)-enantiomers and remaining ketopantoyl lactone.

Analysis of pantoyl lactone and ketopantoyl lactone. All the pantoates in the supernatant or filtrate (1.0 ml) were converted to the corresponding lactone forms by adding 1.0 ml of 6 N HCl and heating at 80°C for 15 min. To the lactonized solution was added 1.0 ml of ethyl acetate and the mixture was vigorously shaken for 1 min. The resultant organic layer was analyzed for pantoyl lactone and ketopantoyl lactone with a Shimadzu GC-7AF gas liquid chromatography (GLC) equipped with a flame ionization detector. The column (1 m long, 3.0 mm inside diameter) was packed with PEG 20M on 60 to 80 mesh Chromosorb W (AW-DMCS, Nishio Kogyo, Japan). The operational parameters were: Injection port, 200°C: detector port, 200°C: column, 150°C: nitrogen carrier, 60 ml/min: hydrogen, 0.6 kg/cm^2: air, 0.6 kg/cm^2. Quantitation was performed by multiplication of the weight by the peak area. Unless otherwise stated, I use the term of pantoyl lactone for a mixture of pantoyl lactone and pantoic acid, because both are possible products from ketopantoyl lactone.

Determination of stereospecificity of pantoyl lactone. The method of Wilken and Dyar[25] was used. The supernatant or filtrate (usually 2.0 ml) was acidified with 1.0 ml of 6 N HCl to give a final volume of 3.0 ml. To this solution was added 3.0 ml of ether and the mixture was shaken vigorously for 1 min. The ethereal layer was pipetted out, dried over 10 mg of anhydrous magnesium sulfate for 30 min at room temperature and then put into a small tube. To the

residue obtained by heating the ethereal layer in the small tube on a hot plate was added 0.1 ml of pyridine/ether (1:4, v/v) and then 60 µl of 1 M L-menthyl chloroformate in benzene. After standing for 15 min, the mixture was diluted to 1.16 ml with ethyl acetate. The ratio of D-(-)- and L-(+)-pantoyl lactone as their diastereomers was determined by GLC. The conditions for the chromatography were essentially the same as those described above except for the following: Glass column (2 m long, 3 mm inside diameter) packed with Silicon DC QF1 on 60 to 80 mesh Chromosorb W (AW-DMCS, Nishio Kogyo, Japan): injection port, 250°C: detection port, 250°C: column, 205°C. The peak area of the L-(+)-pantoyl lactone derivative was 112% compared to that of the D-(-)-pantoyl lactone derivative.

Preparation of crude extract. C. parapsilosis IFO 0708 was grown in a 2-l flask containing 500 ml of medium GC at 28°C for 48 h with shaking. Cells were collected by centrifugation (12000 x g, 15 min, 4°C) and washed with 0.02 M potassium phosphate buffer, pH 7.0. The washed cells were suspended in 15 ml of the same buffer and disrupted with an ultrasonic oscillator for 20 min (19 kHz, 0-15°C). The resultant supernatant after centrifugation was used as the crude extract. Protein concentration by the method of Lowry et al.[27] using bovine serum albumin as a standard.

RESULTS

Survey of ketopantoyl lactone reducing ability in a variety of microorganisms

Using 48-h cultures of various strains as enzyme sources, I preliminarily surveyed the activity to reduce ketopantoyl lactone to pantoyl lactone as described under Materials and Methods. In all,

191 strains of bacteria (30 genera), 59 strains of actinomycetes (3 genera), 230 strains of yeasts (31 genera), 81 strains of molds (23 genera) and 42 strains of basidiomycetes (13 genera) were investigated in the survey. In general, the ability to convert added ketopantoyl lactone to pantoyl lactone was widely distributed in a variety of microorganisms. Strains which yielded pantoyl lactone with greater than 30% molar conversion were selected and the ratios of D-(-)- and L-(+)-enantiomers were determined. The ratios of D-(-)-and L-(+)-enantiomers of the yielded pantoyl lactone were randomly distributed as shown in Fig. 1. Stereospecificities shown

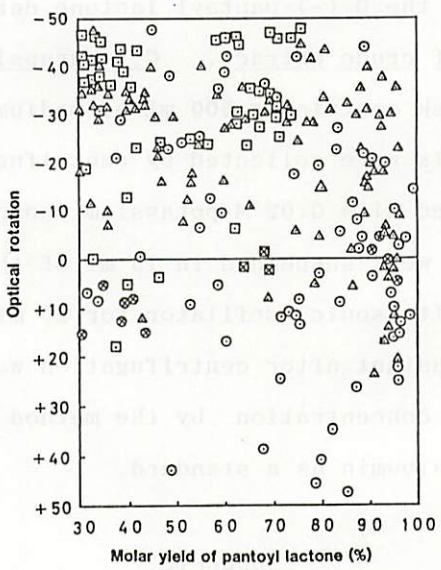


Fig. 1. Distribution of Ketopantoyl Lactone-reducing Abilities and Ratio of D-(-)- and L-(+)-Pantoyl Lactones Yielded

Optical rotations of the yielded pantoyl lactone were calculated from the ratio of D-(-)- and L-(+)-enantiomers by assuming $[\alpha]_D^{25}$ = -50.7 and +50.7° for the D-(-)- and L-(+)-enantiomers, respectively. △, yeasts; ⊙, molds; ⊡, bacteria; ⊠, actinomycetes; ⊗, basidiomycetes.

by the tested strains almost completely showed no relation to the genera or sources. This phenomenon was especially notable in molds. For example, Mucor racemosus IFO 4581 produced almost specifically the L-(+)-enantiomer with a more than 85% molar yield. On the contrary, Mucor javanicus IFO 4569 yielded racemic mixtures with a 59% molar yield. In the case of two strains of Rhizopus oryzae, AKU 3104 and IFO 5440, the former produced predominantly the L-(+)-enantiomer (50% enantiomeric excess (e.e.); molar yield of pantoyl lactone, 71%), but the latter produced almost specifically the D-(-)-enantiomer (96% e.e.; molar yield, 45%). Among 9 strains of Rhodotorula glutinis, which produced pantoyl lactone with greater than 90% molar yields, 5 gave racemic mixtures, 2 gave predominantly the L-(+)-enantiomer and 2 gave the D-(-)-enantiomer with more than 70% e.e. In contrast to these variations in stereospecificity of pantoyl lactone yielded by molds and yeasts, basidiomycetes strains usually produced racemic mixtures with high yields. Although many bacterial strains tested predominantly produced D-(-)-enantiomer, yields and ratios of D-(-)- and L-(+)-enantiomers again showed no relation to the genera or sources. Among these, Agrobacterium tumefacience AKU 300 almost specifically produced the D-(-)-enantiomer with high yield (96% e.e.; molar yield, 76%). When medium GCC was replaced by several other common bacterial media, the yield of pantoyl lactone by A. tumefacience markedly decreased, this phenomenon was also observed with other bacterial strains which produced pantoyl lactone in significantly high yields. Basidiomycetes usually gave higher yields in medium GE compared with other usual media. Production of pantoyl lactone by actinomycetes was generally poor in medium GS. I tried several media, but could not find any suitable media for this purpose. When the survey was

carried out with the strains which has predominantly produced the D-(-)-enantiomer at a concentration of ketopantoyl lactone of 25 mg/ml, many of the strains were repressed as to their productivities of pantoyl lactone.

Reduction of ketopantoyl lactone to D-(-)-pantoyl lactone by selected strains

Through the above survey, I selected the 9 strains listed in Table 1 as promising producers of D-(-)-pantoyl lactone. To increase the D-(-)-pantoyl lactone yield, I investigated the reducing ability of the selected strains at concentration of 30 and 60 mg of ketopantoyl lactone per ml. Medium GC was used in this experiment, because this medium always gave higher values for cell growth, pantoyl lactone yields and D-specificity among several media tested.

Table 1. Reduction of Ketopantoyl Lactone to Pantoyl Lactone by the Selected Straines

Reactions were carried out as described in the text except for the concentration of ketopantoyl lactone (KPL) as indicated.

Strain	Initial KPL concentration			
	30 mg/ml		60 mg/ml	
	Yield(%)[a]	%e.e.[b]	Yield(%)[a]	%e.e.[b]
Saccharomyces sake AKU 4022	20.0	65	0.2	nd[c]
Saccharomyces sake AKU 4029	30.9	72	0.3	nd
Shizosaccharomyces pombe IFO 0346	29.2	84	5.6	83
Sporobolomyces holsaticus IFO 1032	75.9	88	13.0	83
Candida parapsilosis IFO 0708	59.1	90	49.0	76
Candida parapsilosis IFO 0585	79.4	81	42.2	68
Rhodotorula minuta IFO 0920	87.3	71	0.6	nd
Aspergillus niger IFO 4415	78.2	78	22.8	41
Byssochlamys fulva IFO 7901	30.7	89	0.7	nd

[a] Molar yield.

[b] Optical purity of D-(-)-pantoyl lactone.

[c] nd, not determined.

As shown in Table 1, excellent conversion was found in the reaction mixtures with S. holsaticus, two strains of C. parapsilosis, R. minuta and Aspergillus niger, when the concentration of ketopantoyl lactone was 30 mg/ml. No significant change of the ratio of D-(-)-enantiomer to the yielded pantoyl lactone was observed with any strains. Even at the concentration of 60 mg of ketopantoyl lactone per ml, the two strains of C. parapsilosis produced D-(-)-pantoyl lactone in significantly high yields within 48 h. Although the total yield of pantoyl lactone reached more than 40 mg/ml with these two strains after 96 h, the optical purity of the D-(-)-enantiomer decreased to about 60% e.e.

Optimization of reaction conditions

From the results shown in Table 1, I selected R. minuta, C. parapsilosis IFO 0708 and A. niger for a further experiments. Firstly, I examined the composition of the reaction medium. The results with C. parapsilosis are shown in Table 2 and 3. Growing the yeast with glucose, fructose, sorbitol or xylose was found to be good for obtaining better D-specificity. Among these carbon sources, sorbitol was most suitable for enhancing both cell growth and the yield of pantoyl lactone. It should be noted that xylose was a poor carbon source for cell growth but the resultant whole broth exhibited markedly high ability to produce pantoyl lactone with a satisfactory D-(-)-enantiomer ratio. Replacement of corn steep liquor in medium GC by other nutrients or nitrogen sources generally decreased both cell growth and the pantoyl lactone yield. Among the tested nutrients or nitrogen sources, only meat extract as a substitute for corn steep liquor gave an excellent yield of D-(-)-pantoyl lactone. For the other two strains, R. minuta and A. niger, medium GC was most suitable for producing D-(-)-pantoyl lactone with high yields. Other

Table 2. Effect of Carbon Sources on the Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by C. parapsilosis

Reactions were carried out as described in the text except for the carbon source indicated and the initial concentration of ketopantoyl lactone (30 mg/ml).

Carbon source	Growth (OD_{610})	Molar yield of PL[a](%)	%e.e. of D-(-)-PL
Glucose	62	66.5	85
Sucrose	82	70.6	72
Glycerol	77	66.2	70
Maltose	69	54.8	68
Fructose	43	55.8	85
Sorbitol	87	76.4	82
Xylose	21	75.0	81

[a] PL, pantoyl lactone.

Table 3. Effect of Nitrogen Sources or Nutrients on the Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by C. parapsilosis

Reactions were carried out as described in the text except for the nitrogen source or nutrient indicated and the initial concentration of ketopantoyl lactone (30 mg/ml).

Nitrogen source or nutrient	Growth (OD_{610})	Molar yield of PL[a](%)	%e.e. of D-(-)-PL
Corn steep liquor	62	66.5	85
Polypepton	15	14.1	77
Meat extract	60	72.7	81
Yeast extract	44	19.2	66
Bactotryptone	38	54.1	86
Soybean hydrolyzate	42	65.5	74
Casamino acids	13	14.5	43

[a] PL, pantoyl lactone.

miscellaneous changes in the medium did not improve the yield of D-(-)-pantoyl lactone significantly with these three strains.

As an increase in the initial concentration of the substrate,

ketopantoyl lactone, gave a decrease in velocity of the reduction and resulted in incomplete conversion of the substrate to the product, ketopantoyl lactone was added in portions of 10 mg/ml at 24 h-intervals three times. The results with the above three strains are shown in Fig. 2. In every case, the reduction proceeded linearly and

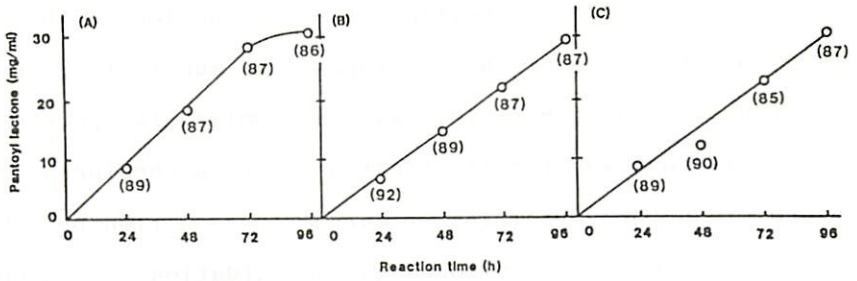


Fig. 2. Specific Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by R. minuta (A), C. parapsilosis (B) and A. niger (C)
Reactions were carried out as described in the text except that ketopantoyl lactone was added in portions of 10 mg/ml at 24 h-intervals three times. Numbers in parentheses show %e.e. of the D-(-)-enantiomers.

all the substrate added was converted to pantoyl lactone after 96 h. The optical purities of the D-(-)-enantiomer finally obtained were 86, 87 and 87% e.e. for R. minuta, A. niger and C. parapsilosis, respectively. In a similar manner, 45 mg of ketopantoyl lactone per ml was stoichiometrically converted to pantoyl lactone without a significant change in D-specificity in the reaction mixtures with these three strains after 120 h.

Identification of the true substrate for the reduction

The lactone ring of ketopantoyl lactone is known to be spontaneously hydrolyzed to ketopantoic acid, especially under alkaline conditions. Conversely, ketopantoic acid is lactonized under mild acidic conditions. Therefore, it is important to

determine which is the substrate for the reduction. When the pH of the reaction mixture was measured during the reaction, it was usually acidic (pH 2-4.5). This phenomenon suggests that ketopantoyl lactone is directly reduced to pantoyl lactone. In fact, no acid forms of the substrate or product were found in the reaction mixtures of the above three strains on analysis without acidification. To confirm this, we measured the velocities of the reduction in the reaction mixtures in which one of the two forms of substrate, ketopantoyl lactone and ketopantoic acid, was predominantly present. When ketopantoyl lactone was firstly treated for ring-opening at pH 6, 7 or 8 and then the velocities of enzymatic reduction by the crude extract at each pH were monitored as the oxidation of NADPH, they

Table 4. NADPH-dependent Reduction of Ketopantoyl Lactone or Ketopantoic Acid by Crude Extract of C. parapsilosis

Method 1. a reaction mixture (2.49 ml) containing 0.8 μmol NADPH, 500 μmol potassium phosphate, at the indicated pH, and 355 μg of the crude extract was incubated for 2 min at 30°C. The reaction was initiated by addition of 10 μl of 0.1 M ketopantoyl lactone (KPL) in 0.015 N HCl and the initial decrease in absorbance at 340 nm was followed.

Method 2. a reaction mixture (2.41 ml) containing 1 umol KPL in 0.015 N HCl and 500 umol potassium phosphate at the indicated pH, was incubated for 10 min at 30°C and then the reaction was initiated by addition of 0.8 μmol NADPH and 355 μg of the crude extract in a total volume of 2.50 ml.

pH of potassium phosphate	Method	Decrease in absorbance at 340 nm
6.0	1	2.43
	2	1.32
7.0	1	4.80
	2	0.33
8.0	1	4.44
	2	0.06

markedly decreased in comparison with direct reduction of ketopantoyl lactone at every pH tested (Table 4). This again suggests that ketopantoyl lactone is the substrate for the reduction.

Isolation and identification of D-(-)-pantoyl lactone from reaction mixtures

C. parapsilosis IFO 0708 was grown in a 2-l flask containing 500 ml of medium GC at 28°C. The reaction was initiated on the 2nd day by adding 5 g of ketopantoyl lactone. On the 4th day, a further 5 g of the substrate was added, and the reaction was continued for a further 4 days. The reaction was stopped by removing cells by centrifugation. To the supernatant (500 ml) containing 7.0 g of D-(-)-pantoyl lactone and 1.6 g of ketopantoyl lactone was added Na_2SO_4 (150 g). The lactonized compounds were extracted with 500 ml of methyl isobutyl ketone. The organic layer was evaporated in vacuo. The crude product (6.2 g) was crystallized from toluene (yield, 3.4 g; purity as pantoyl lactone based on weight, 95.6% (by GLC analysis); $[\alpha]_D^{25}$ (C = 1, H_2O), -46.8° (reported, -50.7°[28]); D-(-)-pantoyl lactone; 100% e.e.; ^{1}H-NMR (DMSO-d_6, δ) 1.00 (6H, s), 3.82 (2H, s), 3.96 (1H, d, J = 6 Hz), 5.78 (1H, d, J = 6 Hz); MS m/Z; 71, 57, 55, 53, 45, 43, 41, 39, 31, 29, 27).

DISCUSSION

In the present study, I demonstrated that the ability of microbial cultures to reduce ketopantoyl lactone to pantoyl lactone in the growth medium was widely distributed in a variety of microorganisms. It is not as yet clear why the reduction of an unnatural bifunctional ketone, ketopantoyl lactone, by microorganisms resulted in the formation of D-(-)- and L-(+)-enantiomers in varying

ratios as shown in Fig. 1. One of the possible explanations for this unique phenomenon is the existence of more than one carbonyl reductase able to reduce ketopantoyl lactone in microbial cells. For example, King, Jr. et al.[4] reported the occurrence of two kinds of ketopantoyl lactone reductase (EC 1.1.1.168) in baker's yeast. These enzymes give specifically the D-(-)-enantiomer, but their physiological roles have not been clarified yet. Other probable enzymes catalyzing this unique reduction may be several α-hydroxy acid dehydrogenases and α-keto acid reductases usually present in microbial cells. Alcohol dehydrogenase, α-glycerophosphate dehydrogenase and so on may also be possible enzymes catalyzing the reduction of ketopantoyl lactone.

When reduction of ketopantoyl lactone is concentrated on for practical use, stereospecific reduction with microorganisms or enzymes yielding D-(-)-pantoyl lactone will be very interesting. This has long been suggested to be one of the possible routes for the practical synthesis of D-(-)-pantoyl lactone. The main problems with this route were that chemically synthesized ketopantoyl lactone was rather expensive and that apparently there were no strains exhibiting the ability to reduce ketopantoyl lactone both in a high yield and with high stereospecificity. Therefore, our present results that several fungal strains such as R. minuta, C. parapsilosis, A. niger and so on stereospecifically reduce ketopantoyl lactone to D-(-)-pantoyl lactone in high yields suggest the possibility of these microorganisms being used as a practical catalyst for the stereospecific reduction.

SUMMARY

The ability to reduce ketopantoyl lactone added to the culture medium to pantoyl lactone was surveyed in a variety of microorganisms. Many of the microorganisms including molds, yeasts, bacteria, actinomycetes and basidiomycetes exhibited this ability. The ratio of D-(-)- and L-(+)-enantiomers of the yielded pantoyl lactone, however, showed no relation to the genera or sources of strains. Among them, Rhodotorula minuta IFO 0920, Candida parapsilosis IFO 0708 and Aspergillus niger IFO 4415 were found to convert ketopantoyl lactone (45 mg/ml) completely and almost specifically to D-(-)-pantoyl lactone. The main enzyme catalyzing this stereospecific reduction was suggested to be ketopantoyl lactone reductase (EC 1.1.1.168).

Section 2. Enzymatic production of D-(-)-pantoyl lactone from ketopantoyl lactone[a,d]

A variety of microorganisms exhibited the ability to reduce ketopantoyl lactone, the D-(-)- and L-(+)-enantiomer ratio varying as described in Section 1, Chapter 2. Among them, Rhodotorula minuta IFO 0920 and Candida parapsilosis IFO 0708 were found to give almost specifically the D-(-)-enantiomer in excellently high yields when ketopantoyl lactone was added to the culture broth. This suggested the possibility that D-(-)-pantoyl lactone might be produced efficiently through enzymatic stereospecific reduction of ketopantoyl lactone.

I further selected strains showing high activity as to this conversion using washed cells of several fungi as catalysts, and optimized the reaction conditions for the production of D-(-)-pantoyl lactone in high chemical and optical yields. I report here the optimization of the conditions for the production of D-(-)-pantoyl lactone using cells separated from the culture broth, and prove the efficiency of the production of D-(-)-pantoyl lactone from ketopantoyl lactone through microbial reduction.

MATERIALS AND METHODS

Chemicals and microorganisms. These were described in Section 1, Chapter 2.

Media and cultivation. Medium GC containing 5% glucose and 5% corn steep liquor, pH 6.0, was used. Cultivations in 5 ml of medium were carried out in test tubes (16.5 x 165 mm) at 28°C for 48 h with shaking (240 strokes/min). For large-scale cultivations, three of

the above mentioned 5 ml cultures were inoculated into 500 ml of medium in a 2-l shaking flask, followed by incubation for 3 days at 28°C with shaking (120 strokes/min). When necessary, the cells were harvested by centrifugation (for yeast) or suction filtration (for molds), washed with the same volume, as that of broth, of water and then used as described below.

Reactions. (i) Growing cell method. To 5 ml of a 48 h-culture was added 0.1-0.3 ml of the ketopantoyl lactone solution (0.5 g/ml), and then the incubation was continued for a further 48 h at the same temperature. After the reaction, insoluble materials were removed by centrifugation or suction filtration, and the supernatant was analyzed for total pantoyl lactone, the D-(-)- and L-(+)-enantiomer ratio and remaining ketopantoyl lactone (see Section 1, Chapter 2). (ii) Washed cell method. The cells were separated from the culture broth. The reaction mixture (5 ml) containing yeast cells of turbidity of 30 at 610 nm (OD) or 2% wet weight of mold filaments, 5% carbon compound as an energy source and 1-6% ketopantoyl lactone, was shaken in a test tube (16.5 x 165 mm) at 28°C and 240 strokes/min. The reaction time was 48 h unless otherwise stated. OD 30 corresponded to 15 mg/ml and 10 mg/ml dry cells for C. parapsilosis and R. minuta, respectively.

Optimization of the culture and reaction conditions. These experiments were carried out with C. parapsilosis IFO 0708 and R. minuta IFO 0920 throughout. The details are given in the legends to the respective Figures and Tables.

Analysis of pantoyl lactone and ketopantoyl lactone. Analyses of the yields of pantoyl lactone and ketopantoyl lactone and the enantiomeric excess of the formed D-(-)-pantoyl lactone was carried out as described in Section 1, Chapter 2.

RESULTS

Reaction system

Comparison of the washed cell and growing cell methods was carried out using *R. minuta*. The reducing velocity and the yield of pantoyl lactone with the washed cell method were superior to those with the growing cell method, as shown in Fig. 1. When a concentration of 3% of ketopantoyl lactone was added, the yield of pantoyl lactone reached a constant level after 24 h-incubation with the washed cell method, however, it did not reach a constant level within 48 h-incubation with the growing cell method. When a

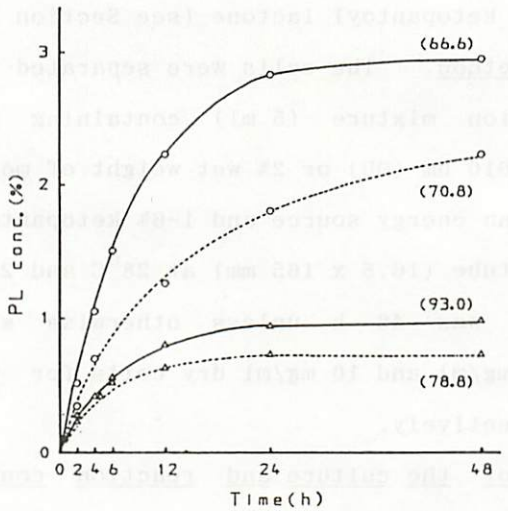


Fig. 1. Time Course of the Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by *R. minuta*

Reactions were carried out with 1 (△) or 3 (○)% of ketopantoyl lactone as described under Materials and Methods. ———, washed cell method; ------, growing cell method. The values in parentheses are %e.e. of the formed D-(-)-pantoyl lactone after 48 h-reaction. Abbreviation: PL, pantoyl lactone.

concentration of 1% of ketopantoyl lactone was added, the stereospecificity of the formed pantoyl lactone with the washed cell method was superior to that with the growing cell method.

Survey for an excellent strain for the production of D-(-)-pantoyl lactone by the washed cell method

The yields of pantoyl lactone with the washed cell method were determined using the 9 potential strains. The 5 yeast strains listed in Table 1 exhibited good reducing ability. The reduction by R. minuta was retarded with an initial concentration of 3% of

Table 1. Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by Washed Cells of Selected Stralnes

Strain	Initial KPL[a] Concentration(%)	Formed PL[b] Yield(%)	Formed PL[b] %e.e.[c]
Schizosaccharomyces	1	58.5	66.6
pombe	3	53.7	49.4
IFO 0346	5	44.6	41.4
Sporobolomyces	1	90.3	79.2
holsaticus	3	90.2	78.8
IFO 1032	5	62.0	78.0
Candida	1	92.3	85.4
parapsilosis	3	92.1	81.0
IFO 0708	5	90.8	80.0
Candida	1	92.3	84.4
parapsilosis	3	86.7	77.4
IFO 0585	5	43.3	86.6
Rhodotorula	1	90.7	93.0
minuta	3	47.8	66.6
IFO 0920	5	6.0	35.6

[a] KPL, ketopantoyl lactone.

[b] Molar yield of pantoyl lactone. PL, pantoyl lactone.

[c] Optical yield of D-(-)-pantoyl lactone.

ketopantoyl lactone. On the other hand, C. parapsilosis IFO 0708 was able to reduce more than 90% of the added ketopantoyl lactone (5%). In the case of Schizosaccharomyces pombe and C. parapsilosis IFO 0585, the stereospecificity of the formed D-(-)-pantoyl lactone significantly decreased with increasing initial ketopantoyl lactone concentration. In the case of R. minuta, good D-stereospecificity was obtained with a low concentration of ketopantoyl lactone, but the stereospecificity of the formed pantoyl lactone in the reaction mixture with both C. parapsilosis IFO 0708 and Sporobolomyces holsaticus was almost completely independent of the amount of ketopantoyl lactone initially added. Other strains reported in Section 1, Chapter 2 such as Byssochlamys fulva IFO 7901, Aspergillus niger IFO 4415 and IFO 4343, and Saccharomyces sake AKU 4022 and AKU 4029, gave low yields of pantoyl lactone (less than 5%) with a concentration of 5% of ketopantoyl lactone. Thus, I selected R. minuta for the highly stereoselective reduction of ketopantoyl lactone, and C. parapsilosis IFO 0708 for the production of a high concentration of D-(-)-pantoyl lactone, and used them in the following experiments.

Optimization of the cultivation conditions

I examined the composition of the culture medium for the cultivation of C. parapsilosis with high activity. The activity of the washed cells was assayed with an initial concentration of 6% of ketopantoyl lactone, as described in the text. Medium GC gave OD 62 and a 61% yield of D-(-)-pantoyl lactone with 80% e.e. Replacement of glucose in medium GC by other carbon sources, sucrose, glycerol, maltose or sorbitol, gave OD between 69 and 87 and pantoyl lactone yields between 63 and 71%. On the contrary, fructose and xylose gave low OD and low pantoyl lactone yields (Table 2). Replacement of corn

Table 2. Effects of Carbon Sources on the Reduction of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone by Washed Cells of C. parapsilosis IFO 0708

Reactions were carried out as described under Materials and Methods, except that the carbon source in the GC medium was changed as indicated in the table. The initial concentration of ketopantoyl lactone was 6%.

Carbon source	OD_{610}	Molar yield of PL[a](%)	%e.e. of D-(-)-PL
Glucose	62	60.8	79.2
Sucrose	82	62.7	76.8
Glycerol	77	71.1	71.8
Maltose	69	70.7	72.6
Fructose	43	43.8	82.0
Sorbitol	87	68.9	74.4
Xylose	21	19.5	82.6

[a] PL, pantoyl lactone.

Table 3. Effects on Energy Sources on the Reduction of Ketopantoyl Lactone by Washed Cells of C. parapsilosis IFO 0708

Reactions were carried out as described under Materials and Methods, except that the energy source was changed, as indicated in the table. The initial concentration of ketopantoyl lactone was 6%.

Energy source	Molar yield of PL[a](%)	%e.e. of D-(-)-PL
None	10.5	76.2
Sucrose	29.4	72.6
Maltose	11.7	68.2
Glucose	83.5	80.2
Sorbitol	78.7	78.6
Fructose	90.1	80.8
Xylose	64.4	75.6
Glycerol	31.4	77.8

[a] PL, pantoyl lactone.

steep liquor in medium GC by other nitrogen sources or nutrients generally decreased both cell growth and pantoyl lactone yield.

Among the tested nitrogen sources and nutrients, meat extract, Polypepton (Daigo Nutritional, Japan), yeast extract, bactotryptone, soybean meal hydrolyzate (Ajinomoto, Japan) and casamino acids, meat extract gave good OD and a good yield of D-(-)-pantoyl lactone, 60 and 83%, respectively. Other miscellaneous changes in the medium did not improve the OD of the culture broth or the yield of D-(-)-pantoyl lactone significantly. All the carbon sources and nitrogen sources tested in this investigation gave between 72 and 83% e.e. for D-(-)-pantoyl lactone. Medium GC was also the most suitable for the production of D-(-)-pantoyl lactone with R. minuta cells. I hereafter used GC medium, due to the OD, yield and % e.e. of the formed D-(-)-pantoyl lactone.

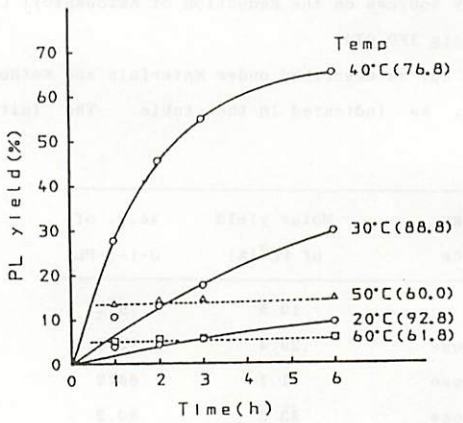


Fig. 2. Effect of the Reaction Temperature on the Conversion of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone with Washed Cells of C. parapsilosis
Reactions were carried out with 1% of ketopantoyl lactone as described under Materials and Methods, except that the reaction temperature was changed as indicated. The values in parentheses are %e.e. of the D-(-)-pantoyl lactone. Abbreviation: PL, pantoyl lactone.

Optimization of the reaction conditions

The energy source for the reduction was investigated. Table 3 shows the results with typical sugars as energy sources. Glucose, sorbitol and fructose gave high conversion yields with satisfactory optical purities of D-(-)-pantoyl lactone. I used glucose as the energy source for further investigation. The optimum concentration of glucose was determined to be 5%.

The yield of pantoyl lactone at the optimum pH, 3.5, was 87% with 1% ketopantoyl lactone under the conditions described under Materials and Methods. The reduction scarcely proceeded at pH 6.0. Figure 2 shows the optimum temperature for this reaction. The reduction of ketopantoyl lactone proceeded rapidly at 40°C, but the stereospecificity of the formed pantoyl lactone decreased with increasing reaction temperature. Under higher temperature conditions (50 and 60°C), the reaction proceeded rapidly only in the initial stage, after which the reaction soon stopped due to inactivation of the cells. From the basis of the data as to the reducing velocity and the stereospecificity of the formed pantoyl lactone, I employed 28°C as the optimal reaction temperature.

Since an increase in the initial concentration of the substrate, ketopantoyl lactone, decreased the reducing velocity and resulted in incomplete conversion of the substrate to the product, crystalline ketopantoyl lactone was added in small portions a few times. When 1 g each of ketopantoyl lactone was added, at 12 h-intervals, five times to the reaction mixture (100 ml) with *R. minuta* cells (1.0 g) as the catalyst and glucose (5 g) as the energy source, and the reaction was continued for a further 24 h, 99% of the added ketopantoyl lactone was converted to D-(-)-pantoyl lactone with 94.4% e.e. (Fig. 3). In the case of *C. parapsilosis*, when 3 g each of

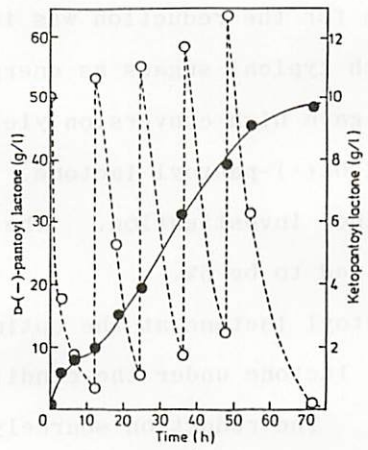


Fig. 3. Stereoselective Conversion of Ketopantoyl Lactone of D-(-)-Pantoyl Lactone by R. minuta Cells

See the text for details. ●, D-(-)-pantoyl lactone; ○, ketopantoyl lactone.

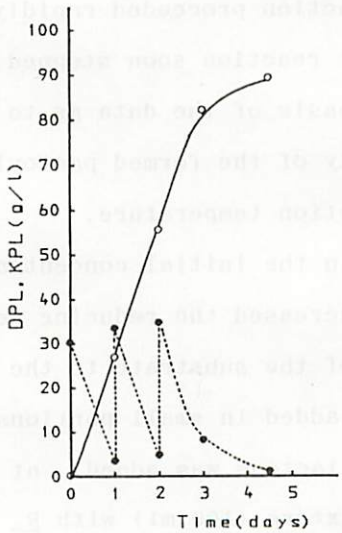


Fig. 4. Stereoselective Conversion of Ketopantoyl Lactone to D-(-)-Pantoyl Lactone with Washed Cells of C. parapsilosis

See the text for details. DPL, D-(-)-pantoyl lactone; KPL, ketopantoyl lactone.

ketopantoyl lactone was added at 24 h-intervals, three times and the reaction was continued for a further 36 h, 99% of the added ketopantoyl lactone was converted to D-(-)-pantoyl lactone with 80.4% e.e. (Fig. 4).

DISCUSSION

A high concentration of D-(-)-pantoyl lactone could be obtained through the reduction of ketopantoyl lactone with the washed cell method. Cells of R. minuta IFO 0920 and C. parapsilosis IFO 0708 gave excellent chemical and optical yields. The amount of the L-(+)-enantiomer formed as a by-product increased with increasing initial concentration of ketopantoyl lactone or with increasing reaction temperature. It seems that there are more than two enzymes that reduce ketopantoyl lactone to each enantiomer. King and Wilken isolated an enzyme, ketopantoyl lactone reductase (EC 1.1.1.168), which catalyzes this reaction, from Saccharomyces cerevisiae NRRL Y-2034.[22] However, a lyzate of this yeast gave a mixture of the D-(-)- and L-(+)-enantiomers of pantoyl lactone.[25] These facts indicate that there are plural enzymes that catalyze the reduction of ketopantoyl lactone, and consequently both enantiomers are obtained under catalysis by the microbial cells.

Ketopantoyl lactone may cause the inactivation of C. parapsilosis cells because more than 98% of the cells could not grow after treatment with a concentration of 8% of ketopantoyl lactone (pH 3.5) at 28°C for 2 h, and the cells after this treatment could not catalyze the reduction of ketopantoyl lactone (unpublished observation). Since a high concentration of ketopantoyl lactone showed such an inhibitory effect and the formed pantoyl lactone was

not so inhibitory, the present reaction system in which ketopantoyl lactone is added in small portions a few times to the reaction mixture may be efficient.

Figure 5 shows the proposed mechanism for this reaction. The coenzyme in this reaction is NADPH (see Section 1 and 2, Chapter 3), and the formed $NADP^+$ might be regenerated to NADPH in cells through the consumption of energy sources such as glucose, fructose and sorbitol, which are all 5 or 6 carbon sugars.

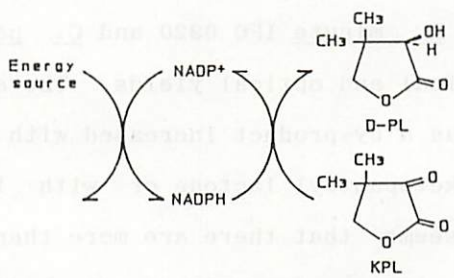


Fig. 5. The Proposed Reaction Mechanism for the Reduction of Ketopantoyl Lactone Coupled with the Regeneration of NADPH

D-PL, D-(-)-pantoyl lactone; KPL, ketopantoyl lactone.

SUMMARY

The efficient enzymatic conversion of ketopantoyl lactone to D-(-)-pantoyl lactone was found to take place on incubation with washed cells of Candida parapsilosis IFO 0708 or Rhodotorula minuta IFO 0920. They showed high conversion activity when grown with 5% corn steep liquor and 5% glucose, sucrose, maltose or glycerol. Under suitable reaction conditions, the amounts of D-(-)-pantoyl lactone reached 49.5 g/l (94.4% e.e.; molar yield, 99%) and 89.9 g/l (80.4% e.e.; molar yield, 99%) with cells of R. minuta and C. parapsilosis, respectively.

CHAPTER 3. Purification and Characterization of NADPH-dependent Conjugated Polyketone Reductases

Section 1. A novel fungal enzyme, NADPH-dependent carbonyl reductase, showing high specificity to conjugated polyketones: purification and characterization[e)]

There is a group of reductase that catalyzes the reduction of various aldehydes and ketones.[29,30)] They occur widely in living organisms, and are suggested to be involved in the metabolism of biogenic and xenobiotic carbonyl compounds. Among them, aldehyde reductase (EC 1.1.1.2) and aldose reductase (EC 1.1.1.21) have been characterized in some detail, and suggested to be implicated in the metabolism of aldehydes derived from biogenic amines, the reduction of sugar aldehydes and the biosynthesis of ascorbic acid. The occurrence of other members of reductase family, so-called carbonyl reductases, has also been found in a variety of tissues. These enzymes apparently mainly differ from aldehyde reductase and aldose reductase in their ability to reduce aromatic ketones and their sensitivity to specific reductase inhibitors. Some of them were purified from mammalian tissues and characterized.[31-33)] They are suggested to be the enzymes responsible for the metabolism of xenobiotic carbonyl compounds.

There have been several reports on microbial enzymes concerning the metabolism of carbonyl compounds.[22,34-39)] Most of these enzymes show rather strict specificity toward a single physiological metabolite or only a few carbonyl compounds; it has been demonstrated that they are closely related to the metabolism of their own substrates.[22,34-39)] On the other hand, only a little attention has

been paid to microbial reductases which show rather wide substrate specificities toward both natural and unnatural carbonyl compounds and quinones.

In Section 1, Chapter 2, I investigated the microbial distribution of reduction ability using ketopantoyl lactone as substrate. Through this survey, I found that Mucor ambiguus reduces ketopantoyl lactone to L-(+)-pantoyl lactone in good enantiomeric excess. So far as I know, strain with such activity has not been reported previously. This section describes the purification and characterization of the enzyme which reduce ketopantoyl lactone to L-(+)-pantoyl lactone.

MATERIALS AND METHODS

Materials. Ketopantoyl lactone and its analogs were synthesized as reported as described in Chapter 1. D-(-)- and L-(+)-Pantoyl lactone were purchased from Tokyo Kasei (Japan). Coenzymes and marker proteins for relative molecular mass determination by high-performance gel-permeation liquid chromatography, yeast glucose-6-phosphate dehydrogenase, yeast hexokinase, pig heart isocitrate dehydrogenase and yeast glutathione reductase used for stereochemical analysis of hydride transfer from NADPH were obtained from Oriental Yeast (Japan). [1-^{3}H]Glucose was obtained from New England Nuclear (USA). Standard proteins for relative molecular mass determination by Sephadex G-100 were purchased from Boehringer Mannheim (FRG). DEAE-Sephacel, phenyl-Sepharose CL-4B, Sephadex G-100 and the low-Mr standard protein kit for sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis were purchased from Pharmacia (Sweden). Blue-Sepharose CL-4B were prepared as described.[40] The Ampholytes for

isoelectric focusing were obtained from LKB-Produktor AB (Sweden). Substrates and inhibitors were obtained from Aldrich (USA), Nakarai Chemicals (Japan), Wako Pure Chemicals (Japan) and Tokyo Kasei (Japan). All other reagents were commercially available and analytical grade.

Microorganisms and cultivation. M. ambiguus AKU 3006 was obtained from AKU culture collection (Faculty of Agriculture, Kyoto University). The organism was grown on a 2% agar slant containing 5% malt extract and 0.3% yeast extract, pH 6.0, for 4 days at 28°C, from which mycelia were transferred to a test tube (16.5 x 165 mm) containing 5-ml subculture medium composed of 5% maltose, 5% meat extract and 0.3% ketopantoyl lactone, pH 5.6; it was shaken for 3 days at 28°C and 240 strokes/min. Three of 5-ml cultures were then inoculated into 500 ml of the same medium in a 2-l flask, followed by incubation for 7 days at 28°C with shaking (120 strokes/min).

Enzyme assay. The standard assay mixture contained, in 2.5 ml, 1.0 μmol of isatin (or ketopantoyl lactone), 0.80 μmol of NADPH, 500 μmol of potassium phosphate buffer, pH 7.0, and the enzyme. After a 2-min preincubation at 30°C, the reaction was initiated by the addition of the substrate; then the decrease in absorbance at 414 nm was monitored spectrophotometrically at 30°C. A blank without NADPH and that without the enzyme were routinely assayed. A molar absorption coefficient of 849/M/cm for isatin was used for the calculation of the activity. The decrease in absorbance at 340 nm was monitored in the case of ketopantoyl lactone. A molar absorption coefficient of 6220/M/cm for NADPH was used. One unit of the enzyme was defined as the amount catalyzing a change in absorbance at 414 nm corresponding to the reduction of 1 μmol isatin/min under the standard assay conditions.

Purification of the carbonyl reductase of Mucor ambiguus. Wet cells (1665 g) of M. ambiguus obtained from eighty-500-ml cultures were suspended in 0.01 M Tris/HCl buffer, pH 7.4, containing 0.1 mM dithiothreitol (1600 ml), followed by disruption with an ultrasonic oscillator (19 kHz, 3 h, 2-5°C; Kaijo Denki 4280, Japan). All subsequent procedures were carried out at 0-5°C; and 0.01 M Tris/HCl buffer, pH 7.4, containing 0.1 mM dithiothreitol was used unless otherwise stated.

The cell debris was removed by centrifugation. The supernatant was fractionated with ammonium sulfate (70-100%), followed by dialysis against the buffer (8 l). The dialyzed solution was applied to a DEAE-Sephacel column (2.3 x 32 cm) equilibrated with the buffer. After washing the column with the buffer (250 ml), the elution was carried out with a 2-l linear gradient of 0-0.5 M KCl in the buffer. The active fractions were combined and then fractionated with ammonium sulfate. The precipitate appearing between 60-80% saturation was dissolved in a minimum volume of 0.01 M Tris/HCl buffer, pH 7.4, containing 0.1 mM dithiothreitol and 4 M NaCl. The enzyme solution was placed on a column (0.8 x 11 cm) of phenyl-Sepharose CL-4B equilibrated with the same buffer. After washing the column with the same buffer (100 ml), the enzyme was eluted by lowering the ionic strength of NaCl linearly, from 4 to 0 M, in the same buffer (100 ml). The active fractions were combined and lyophilized. The lyophilizate was dissolved in 5 ml of the buffer. The enzyme solution was placed on a column (1.8 x 100 cm) of Sephadex G-100 equilibrated with the buffer. The rate of sample loading and column elution were maintained at 5 ml/h by a peristaltic pump (LKB 2121, Sweden). The active fractions were combined and placed on a column (0.6 x 6 cm) of blue-Sepharose CL-4B equilibrated with the

buffer. After washing the column with the buffer (15 ml), the enzyme was eluted with a 30-ml linear salt gradient of 0-1 M NaCl in the buffer. The active fractions were combined and then concentrated by lyophilization. The lyophilizate was dissolved in 1.0 ml buffer and then dialyzed against 300 ml buffer.

Analytical methods for purified carbonyl reductase. Analytical electrophoresis in a 7.5% polyacrylamide gel was carried out as described by Davis.[41] SDS-polyacrylamide gel electrophoresis was performed in a 12% polyacrylamide slab gel using a Tris/glycine buffer system, as described by King and Laemmli.[42] The relative molecular mass of the subunit of the enzyme was determined from the relative mobility to the standard proteins. The isoelectric point of the enzyme was determined as described by Winter and Karlsson[43] using a density gradient, with a pH range of 3 to 11, containing 2% Ampholyte.

Analytical gel filtration for molecular mass determination on a calibrated column (1.8 x 100 cm) packed with Sephadex G-100 was carried out according to Andrews.[44] The gel bed was equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.2 M KCl and 0.1 mM dithiothreitol. A constant flow (5 ml/h) of the equilibrating buffer was maintained with a peristaltic pump (LKB 2121, Sweden) at 4°C.

The enzyme solution (100 µl, 0.12 mg protein) was subjected to high-performance gel-permeation liquid chromatography (Hitachi 655, Japan) on a TSK G-3000 SW column (0.75 x 60 cm, Toyo Soda, Japan) at room temperature. Potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM dithiothreitol and 0.2 M NaCl was used as the mobile phase at a flow rate of 0.3 ml/min. The absorbance of the effluent was monitored at 280 nm with a data module (Hewlett Pakard

3390A, USA) automatically. The relative molecular mass of the enzyme was determined from the relative mobility compared to the standard proteins.

Protein concentration determination. The protein concentrations were determined by a Bio-Rad protein assay kit with bovine serum albumin as a standard, as described in Bio-Rad Protein Assay Instruction Manual essentially according to the method of Bradford.[45] The effluent from the chromatographic columns were analyzed spectrophotometrically at 280 nm.

Stoichiometry. The stoichiometry of the reaction between ketopantoyl lactone and NADPH was followed. The 1-ml reaction mixture containing, in 1 ml, 200 μmol of potassium phosphate buffer, pH 7.0, 40 μg enzyme, 40 μmol NADPH, 57.7 μmol ketopantoyl lactone, was incubated for 1 h at 30°C. Aliquots (1-5 μl) were withdrawn at 15-min intervals and the consumption of NADPH was followed spectrophotometrically at 340 nm. The formation of pantoyl lactone and its enantiomeric purity were determined as described below.

Substrate specificity. The composition of the reaction mixture for investigation of the substrate specificity was the same as that for the standard assay mixture, except that the carbonyl compound of interest was used as the substrate instead of isatin. Unless otherwise stated, the decrease in absorbance at 340 nm due to oxidation of NADPH was monitored under the same conditions as described under Enzyme assay. The blank values obtained on autooxidation of NADPH by p-benzoquinone, ninhidrin and alloxane were subtracted.

Stereochemical analysis of hydride transfer from NADPH. [4B-^{3}H]NADPH was prepared with the B-specific enzyme, yeast glucose-6-phosphate dehydrogenase and [1-^{3}H]glucose-6-phosphate which was

generated by hexokinase from [1-^{3}H]NADP^{+} with A-specific isocitrate dehydrogenase. This was then reduced with B-specific glucose-6-phosphate dehydrogenase to [4-^{3}H]NADPH. The methods used were essentially the same as those described by Wilken et al.[23)]

Reaction mixtures for determination of stereospecificity of ketopantoyl lactone reduction by the carbonyl reductase from M. ambiguus contained 50 µmol potassium phosphate buffer, pH 6.5, 60 nmol [4A-^{3}H]NADPH (approximately 1.5 x 10^{5} dpm) or 35 nmol [4B-^{3}H]NADPH (approximately 2.0 x 10^{5} dpm), 1.0 µmol ketopantoyl lactone and the enzyme (about 5 units) in a final volume of 1 ml. After incubation at 30°C for 10 min, the reaction mixture was analyzed by a chromatographic system similar to that described by Wilken et al.[23)] The radioactivity was measured with a Packard Tri-Carb 300C liquid scintillation system.

Other methods. Pantoyl lactone was determined by GLC as described in Section 1, Chapter 2. The enantiomeric purity of the pantoyl lactone formed was determined by GLC after derivatization with L-menthyl chloroformate (see Section 1, Chapter 2).

RESULTS

Purification of the carbonyl reductase

Through the purification procedures described under Materials and Methods, the enzyme was purified 1340-fold with a yield of 12% from the cell-free extract, with isatin as the substrate (Table 1). The enzyme was adsorbed tightly on membrane filters such as PM-10 and YM-10 (Amicon, USA); therefore I concentrated the enzyme solution by lyophilization.

The amount of protein extracted from mycelia varied

Table 1. Purification of Carbonyl Reductase from M. ambiguus AKU 3006

Enzyme activity was measured as described under Materials and Methods using isatin and ketopantoyl lactone as substrates.

Step	Protein	Total activity units		Specific activity units/mg		Ratio of the specific	%e.e. of L-(+)-PL[b]
	mg	Isatin	KPL[a]	Isatin(I)	KPL	activity I/KPL	formed
Cell-free extract	1716	601	652	0.35	0.38	0.92	74
Ammonium sulfate	480	566	576	1.18	1.20	0.98	77
DEAE-Sephacel	15.3	444	352	29	23	1.28	100
Phenyl-Sepharose CL-4B	2.2	268	202	122	92	1.33	100
Sephadex G-100	0.49	100	76	205	155	1.32	100
Blue-Sepharose CL-4B	0.15	71	53	470	354	1.33	100

[a] KPL, ketopantoyl lactone.

[b] PL, pantoyl lactone.

significantly with the disruption method used. For example, ultrasonication yielded only about 1 mg protein from 1 g wet mycelia. This value was more than seven times lower than that obtained on mechanical disruption with a Dyno-mill (Willy A. Bachofen Maschinenfabrik, Switzerland). On the other hand, 93% of the activity extracted on mechanical disruption was recovered on ultrasonication. This was highly advantageous for obtaining a starting crude extract with high specific activity.

The crude extract showed lower specific activity toward isatin than toward ketopantoyl lactone. After the DEAD-Sephacel step, however, the isatin activity was about 1.3 times higher than the ketopantoyl lactone activity; this ratio did not change significantly throughout the following steps. The enantiomeric excess for the L-(+)-enantiomer of pantoyl lactone also reached a constant value (100%) after the same step. These results indicate that there were more than two enzymes which reduce ketopantoyl lactone in the crude

extract. They were separated on the DEAE-Sephacel chromatography.

Criteria for purity

On polyacrylamide gel electrophoresis, the purified enzyme migrated as a single species, as found on staining for protein on the 7.5% gel. The purified enzyme also gave only one band on SDS/polyacrylamide gel electrophoresis (Fig. 1). Ampholyte electrofocusing also gave only one absorption peak (pH 6.4) of protein, which coincided with the carbonyl reductase activity toward both isatin and ketopantoyl lactone. Further evidence of the purity of the enzyme preparation was the result of high-performance gel-permeation liquid chromatography, which gave quite a symmetrical single protein peak (Fig. 2a).

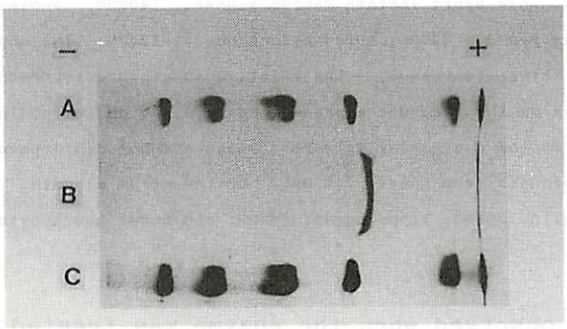


Fig. 1. SDS Slab Gel Electrophoresis of the Enzyme

The conditions for electrophoresis were given under Materials and Methods. (A, C) Marker proteins; Mr: phosphorylase b, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; and soybean trypsin inhibitor, 20000. (B) The enzyme, 30 μg. The direction of migration is from the cathode (left) to the anode (right).

Molecular mass and subunit structure

On the calibrated column of Sephadex G-100, the relative molecular mass of the enzyme was estimated to be 54000 ± 2000 (Fig. 2b). The relative molecular mass of the enzyme on high-performance gel-permeation liquid chromatography was 44000 ± 1000 (Fig. 2a). A

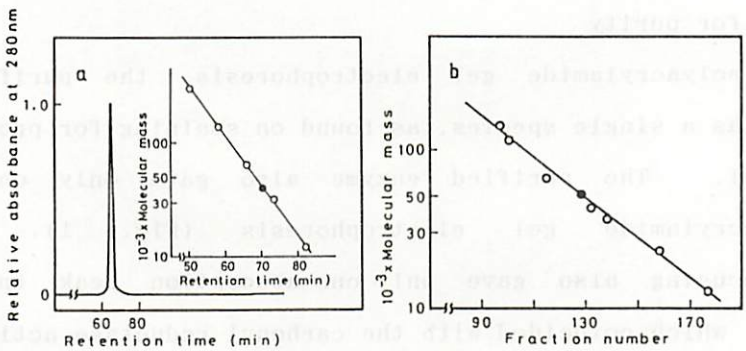


Fig. 2. High-performance Gel-permeation Liquid Chromatography (a) and Gel-filtration on a Sephadex G-100 column (b) of the Enzyme

(a) The elution profile to the enzyme and determination of the relative molecular mass of the enzyme. The protein standard (○), in order of decreasing Mr, were: glutamate dehydrogenase from yeast, 290000; pig heart lactate dehydrogenase, 140000; enolase from yeast, 67000; adenylate kinase from yeast, 32000; horse cytochrome c, 12400. The absorption at 280 nm was expressed as the relative absorbance. The relative absorbance represents the percentage of full-scale deflection on the recorder expressed as 0 to 1.0 on the ordinate. (b) The protein standards (○) in order of decreasing Mr were: yeast alcohol dehydrogenase, 150000; glucose-6-phosphate dehydrogenase from yeast, 125000; bovine serum albumin, 68000; egg albumin, 45000; β-bactoglobulin, 36800; trypsinogen, 24000; and horse heart cytochrome c, 12500.

single band was observed when the enzyme was treated with 1 % SDS and 10 mM 2-mercaptoethanol and electrophoresed on a gel containing 10% SDS (Fig. 1). The relative molecular mass corresponding to this band was estimated to be 27500 from its mobility relative to those of reference proteins. The enzyme appears to consist of two identical subunits in molecular mass.

Spectral properties

The absorption spectrum of the enzyme showed a maximum at 276 nm. No absorbance was detectable above 320 nm. Thus, the enzyme does not contain flavin, which is the coenzyme in most quinone reductases.[29,30]

Substrate specificity and kinetic properties

A broad range of carbonyl compound was tested to investigate the substrate specificity of the enzyme (Table 2). Quinones such as p-benzoquinone, p-toruquinone, α-naphthoquinone, β-naphthoquinone and phenanthraquinone were effective substrate. Several polyketones, e.g., isatin and its analogs, ninhidrin, alloxan and ketopantoyl lactone, were also readily reduced. These are all conjugated polyketones. However, polyketones having analogous ring structure such as 1,2-cyclohexadione and parabanic acid, were not reduced. Ketopantoic acid, delactonized form of ketopantoyl lactone, was not reduced at all. Dehydroascorbic acid was reduced, but only at 3% the rate of ketopantoyl lactone. Cyclohexanone and acetone, which are substrates for mammalian carbonyl reductases, were not reduced.[26,46,47] D-Glycelaldehyde and p-nitrobenzaldehyde, typical substrates for aldehyde reductase, and xylose, a typical substrate for aldose reductase,[46-49] were also not reduced. No activity was detected either with menadione and phylloquinone, potent substrates for mammalian[50] and plant[51] quinone reductases, respectively.

Table 2. Substrate Specificity, Michaelis Constants and Maximum Velocity of the Enzyme

Enzyme activities were measured as described under Materials and Methods. The wavelengths (λ) and absorption coefficients (ε) used for measurement of the enzyme activity are given for some substrates. Abbreviations used: 1c, dihydro-4,4-diethyl-2,3-furanedione; 1d, dihydro-4-methyl-4-propyl-2,3-furanedione; 1e, dihydro-5-isopropyl-4,4-dimethyl-2,3-furanedione; 1f, dihydro-5-(2-butyl)-4-methyl-4-ethyl-2,3-furanedione; 1g, dihydro-5-(3-pentyl)-4,4-diethyl-2,3-furanedione; 1h, dihydro-5-(2-pentyl)-4-methyl-4-propyl-2,3-furanedione; 1i, dihydro-5-(3-pentyl)-4,4-dimethyl-2,3-furanedione; and 1j, dihydro-5-(2-pentyl)-4,4-dimethyl-2,3-furanedione. Relative activity was calculated with isatin was taken as 100%. The Km and Vmax values were estimated from Lineweaver-Burk double-reciprocal plots. For the Km values the concentration of NADPH was held constant at 0.32 mM. The following compounds (0.4 mM) were not reduced: ketopantoic acid, diacetyl, 1,2-cyclohexanedione,

(continued)

parabanic acid, D-glyceraldehyde, menadione, glyoxalate, pyruvate, 2-oxobutylate, 2-oxovalerate, 2-oxoisovalerate, 2-oxohexanoate, 2-oxo-4-methylpentanoate, 2-oxo-3-methylvalerate, 2-oxo-3-ethylvalerate, 2-oxo-3-methylhexanoate, 2-oxo-3-phenylbutyrate, 2-oxomalonate, oxaloacetate, 2-oxoglutarate, 2-oxoadipate, oxalosuccinate, 3-hydroxypyruvate, benzoylformate, 3-indolpyruvate, 3-indolglyoxalate, 2-oxo-D-gluconate, acetoacetate, levulinate, glyoxal, methylglyoxal, acetone, hydroxyacetone, acetylacetone, dihydroxyacetone, dihydroxyacetone phosphate, methyl ethyl ketone, methyl isobutyl ketone, acetoin, benzoin, benzaldehyde, formaldehyde, acetoaldehyde, propionaldehyde, n-butylaldehyde, p-nitrobenzaldehyde, glutaraldehyde, chloroacetoaldehyde, betainaldehyde and crotonaldehyde.

Substrate	λ	ε	Concentration	Relative velocity	Km	Vmax
	nm	1/M/cm	mM	%	μM	μmol/min/mg
Isatin	414	849	0.4	100	50	910
1-Methylisatin	425	553	0.4	63	12	710
5-Nitroisatin	415	645	0.08	19		
5-Bromoisatin	435	743	0.08	68		
5-Methylisatin	441	656	0.08	35		
5-Isatinsulfonic acid	408	710	0.08	22		
Ketopantoyl lactone			0.4	77	710	540
1c			0.4	2		
1d			0.4	45	3300	2140
1e			0.4	48	4170	21400
1f			0.4	22	1610	5340
1g			0.4	2		
1h			0.4	21	240	1380
1i			0.4	50	580	590
1j			0.4	62	380	560
Ninhydrin			0.4	22		
Alloxan			0.4	66	2500	590
Dehydroascorbic acid			0.4	2		
p-Benzoquinone			0.4	169		
p-Toruquinone			0.4	60	410	600
α-Naphthoquinone			0.2	34		
β-Naphthoquinone			0.2	42	53	350
Camphorquinone			0.4	18		
Acenaphthequinone			0.08	10	210	140
Phenanthraquinone			0.08	52		

In addition to ketopantoyl lactone, several of its analogs (see Table 2) were also tested as substrates for the carbonyl reductase. Dihydro-5-isopropyl-4,4-dimethyl-2,3-furanedione (1e), dihydro-5-(3-pentyl)-4,4-dimethyl-2,3-furanedione (1i) and dihydro-5-(2-pentyl)-4,4-dimethyl-2,3-furanedione (1j), analogs carrying an aliphatic substituent only at the 5 position, were reduced at 60-80% the rate in the case of ketopantoyl lactone. On the other hand, modification at both of the 4 and 5 positions markedly reduced the reaction velocity.

Michaelis-Menten type kinetics were observed with all the substrate tested (see Table 2). Among these carbonyl compounds, isatin (Km = 49.9 μM, Vm = 909 units/mg) and 1-methylisatin (Km = 12.3 μM, Vm = 712 units/mg) seem to be the most favorable substrate for the enzyme. Quinones were more effectively utilized than ketopantoyl lactone, as judged from the estimated pseudo-second-order rate constants (Vmax/Km).

Double-reciprocal plots of v against ketopantoyl lactone concentration gave parallel lines for various NADPH concentrations (Fig. 3a). Parallel lines were also observed for various ketopantoyl lactone concentrations in double reciprocal plots of v against NADPH concentration (Fig. 3b). These results suggest that the reaction proceeds via a ping-pong mechanism, as designated by Cleland.[52] Km values of 714 μM for ketopantoyl lactone and 135 μM for NADPH were obtained. The Vmax was 541 units/mg protein.

NADPH was almost absolutely required for the reduction of all the substrates listed in Table 2. Only 1-2% of the rates, compared to those with NADPH, was observed when NADPH was replaced with an equimolar amount of NADH in the assay mixture.

Stoichiometry

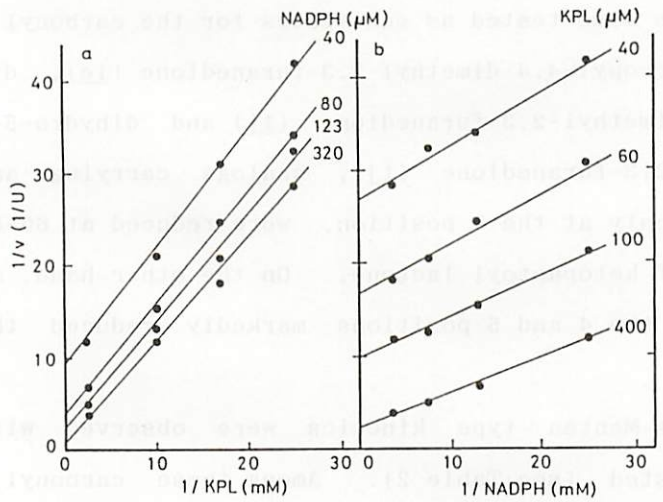


Fig. 3. Kinetic Analysis of the Enzyme

Double-reciprocal plots of v against concentration of (a) ketopantoyl lactone (KPL) and (b) NADPH. The enzyme activity was assayed under the standard conditions, except that the concentrations of ketopantoyl lactone and NADPH were changed.

Using ketopantoyl lactone as the substrate, the stoichiometry of the enzymatic reaction was investigated under the conditions given described under Materials and Methods. After the reaction, 30.5 μmol NADPH had been consumed and 35.3 μmol pantoyl lactone formed. The pantoyl lactone formed was confirmed to be the L-(+)-enantiomer in 100% enantiomeric excess, on analysis after derivatization with L-menthyl chloroformate. Thus, for every mol of NADPH oxidized, one mol of L-(+)-pantoyl lactone was produced. The reversibility of the reduction was investigated with L-(+)-pantoyl lactone and $NADP^+$. No formation of ketopantoyl lactone and NADPH was observed at pH 7 and 9.

Stereospecificity of hydrogen transfer from NADPH

When [4A-^{3}H]NADPH was oxidized enzymatically in the presence of

ketopantoyl lactone, the tritium was mostly incorporated into pantoyl lactone (95%) but not retained substantially in $NADP^+$ (6%). On the other hand, no incorporation of tritium into pantoyl lactone was detected when reacted with [4B-^{3}H]NADPH. More than 95% of the label was found in $NADP^+$. These results demonstrate that the Mucor enzyme is a A-specific enzyme.

Inhibitors

Various compounds were investigated as to their inhibitory effects on the enzyme activity with ketopantoyl lactone as the substrate (Table 3). Quercetin, a nonspecific inhibitor of mammalian oxidoreductases,[53] caused 83% inhibition at 0.01 mM. Dicoumarol, which is a potent inhibitor of NAD(P)H dehydrogenase (quinone reductase)[50] and an inhibitor of carbonyl reductase from human brain,[54] inhibited the enzyme by 48% at a concentration of 0.1 mM. No effect on the enzyme activity was observed in the presence of barbiturates or hydantoins which are inhibitors of aldehyde reductases,[55-57] or pyrazole which is an inhibitor of alcohol dehydrogenase.[58] Essentially the same inhibition pattern was observed when isatin was used as the substrate.

Three polyketones which have analogous ring structures to that of isatin, 3-methyl-1,2-cyclopentanedione, 1,3-cyclohexanedione and parabanic acid, were found to inhibit the reduction of ketopantoyl lactone. None of them served as substrates, as shown in Table 2.

A kinetic study of the inhibition by these polyketones revealed that the inhibition proceeded uncompetitively. The Ki values for the inhibition by 3-methyl-1,2-cyclohexadione estimated by Cornish-Bowden[54] were 80.9 μM and 114 μM as for ketopantoyl lactone and NADPH, respectively. 1,3-Cyclohexanedione and parabanic acid showed Ki values of 64.5 μM and 3.14 mM, respectively, for ketopantoyl

Table 3. Effects of Various Compounds on the Activity of the Enzyme

Enzyme activities were measured as described under Materials and Methods, except for addition of the indicated compounds. Ketopantoyl lactone was used as the substrate. The activity without an inhibitor was taken as 100%. Abbreviations used: ClHgBzOH, p-chloromercuribenzoate; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid).

Inhibitor	Concentration mM	Activity %
Quercetin	0.01	17
Dicoumarol	0.1	52
Barbital	1	98
Diphenylhydantoin	1	88
Cyclohexanedione	1	17
3-Methyl-1,2-cyclohexanedione	1	16
1,3-Cyclohexanedione	1	17
Parabanic acid	1	83
Na_2 EDTA	1	98
8-Hydroxyquinoline	0.1	42
o-Phenanthroline	1	61
α,α'-Dipyridyl	1	101
ClHgBzOH	0.1	85
Nbs_2	0.1	15
Iodoacetic acid	1	85
NaF	1	98
Semicarbazide	1	98

lactone.

Neither D-(-)- nor L-(+)-pantoyl lactone (1-10 mM) had any effect. $NADP^+$ (1 mM) caused 42% inhibition. The enzyme was competitively inhibited compared to NADPH by $NADP^+$; the Ki value was determined to be 210 μM by the method of Cornish-Borden.[54)]

Some chelating agents, such as 8-hydroxyquinoline and o-phenanthroline, were inhibitory toward the enzyme, but EDTA and α,α'-dipyridil showed no effect at all. The enzyme was inhibited by p-

chloromercuribenzoate and 5,5'-dithiobis(2-nitrobenzoic acid) at 0.1 mM, but the inhibition by iodoacetic acid and N-ethylmaleimide were not so strong at millimolar concentrations. Among the metal salts tested, $FeCl_2$ (1.0 mM) and $CdCl_2$ (1.0 mM) inhibited the enzyme activity by 69.8 and 39.8%, respectively.

Effects of pH and temperature

The effect of pH on the activity of the enzyme was investigated using both isatin and ketopantoyl lactone as substrates. As shown in Fig. 4a, the enzyme showed maximum activity as to isatin reduction between pH 6 and 7. The optimum for the reduction of ketopantoyl lactone was found to be approximately pH 6. This difference may be

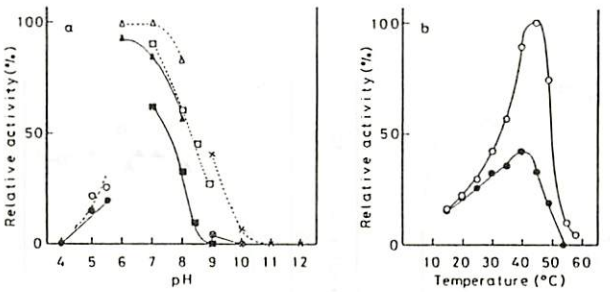


Fig. 4. pH Dependency (a) and Temperature Dependency (b) of the Activity of the Enzyme, with Isatin and Ketopantoyl Lactone as Substrates

(a) The enzyme activity was assayed under the standard assay conditions except that different buffers were used to adjust the pH. Acetic acid/sodium acetate buffer was used for pH 4-5.6 (isatin, ○ ; ketopantoyl lactone, ●), potassium phosphate buffer for pH 6-8 (isatin, △ ; ketopantoyl lactone, ▲), Tris/HCl buffer for pH 7-9 (isatin, □ ; ketopantoyl lactone, ■) and glycine/NaOH buffer for pH 9-11 (isatin, ⊗ ; ketopantoyl lactone, ×). (b) Assay were performed at various temperatures with isatin (○) or ketopantoyl lactone (●) as substrate. Other conditions were the same as those of the standard assay conditions. The relative activity is expressed as the percentage of the maximum activity observed under the experimental conditions.

due to the rapid and spontaneous hydrolysis of ketopantoyl lactone to ketopantoic acid under mild alkaline conditions.

The activity of the enzyme was measured at various temperature between 15-55°C at pH 7 with ketopantoyl lactone and isatin as substrates. The results are shown in Fig. 4b. The optimum temperature for the reduction of isatin was found to be 45°C. When ketopantoyl lactone was used as a substrate, the optimum temperature was 40°C. This difference may also be due to accerelation of the spontaneous hydrolysis of ketopantoyl lactone at high temperatures.

Stability of the enzyme

The enzyme was incubated at various temperatures for 10 min at pH 7.0, and then the remaining activity was measured. As shown in Fig. 5a, the enzyme was stable below 40°C; 80% and 15% of its

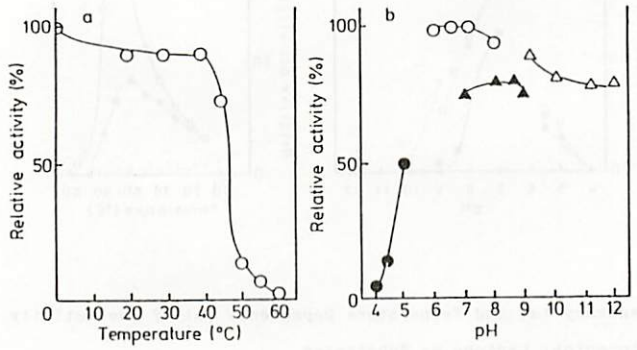


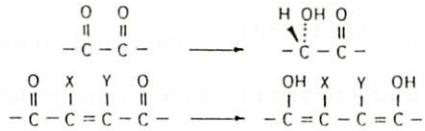
Fig. 5. Effects of Temperature (a) and pH (b) on the Stability of the Enzyme
The enzyme (0.4 μg), in a total volume of 2.5 ml, was incubated in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol at various temperatures for 10 min. Then the remaining activity was measured using 0.25 ml of each solution as the enzyme source under the standard conditions. Isatin was used as the substrate. (b) The enzyme (0.4 μg), in a total volume of 2.5 ml, was incubated at 30°C for 30 min in 50 mM buffers containing 0.1 mM dithiothreitol: acetate/HCl, pH 4-5 (●); potassium phosphate, pH 6-8 (○); Tris/HCl, pH 7-9 (▲); and glycine/NaOH, pH 9-12 (△). Then the remaining activity was measured as described above.

original activity after heating 45°C and 50°C, respectively.

No loss of the enzyme activity was observed in a pH range 6-7 on incubation for 30 min at 30°C in 200 mM potassium phosphate buffer, as shown in Fig. 5b; 91% and 79% of its initial activity was retained after incubation in 200 mM glycine/NaOH buffer at pH 9 and 12, respectively. The enzyme completely lost its activity at pH 4.

DISCUSSION

The NADPH-dependent carbonyl reductase of M. ambiguus catalyzes only the reduction of conjugated polyketones as follows:



The aldehyde, aldose and monoketones so far tested, all common substrates for mammalian carbonyl reductases, did not serve as substrates. The Mucor carbonyl reductase is unique also in its molecular form. It is composed of two apparently identical subunits of Mr = 27500, while most carbonyl reductases have been reported to be monomers with Mr of nearly 30000.[22,58,59]

In comparison with common carbonyl reductases, the Mucor carbonyl reductase shows some similarity to human brain carbonyl reductase,[53] in that it is not a flavoprotein and that it is inhibited by both with quercetin and dicoumarol. However, the human brain carbonyl reductase reduces not only simple quinones, such as phenanthraquinone and p-benzoquinone, but also aldehydes, monoketones and potassium ferricyanide, which cannot be utilized by the Mucor carbonyl reductase.

Another group of NADPH-dependent carbonyl reductases showing some similarity to the Mucor enzyme may be the DT-diaphorases. Enzymes of this group are widely distributed in living organisms. They can catalyze the reduction of simple quinones, such as p-benzoquinone, β-naphthoquinone and phenanthraquinone, as well as the Mucor enzyme does. However, they are flavoprotein and require FMN and FAD for full activity, that can use both NADH and NADPH as coenzymes, and they act as menaquinone reductase.[60]

There have been several reports on microbial enzymes which reduce carbonyl compounds. However, most of the enzymes show rather strict specificity toward a single normal metabolite or only a few carbonyl compounds, and have been well characterized as to their physiological functions.[22,34-39] Jones and Beck[61] and Robinson[62] reviewed microbial and enzymatic reactions concerning reduction of various natural and unnatural carbonyl compounds. Some enzymes which show wide substrate specificity were also reported in their reviews. The enzymes, however, reduce mainly monoketones and some of conjugated ketones. In comparison with these microbial enzymes, the Mucor carbonyl reductase is again unique in its substrate specificity, as described above. Among these microbial enzymes, the ketopantoyl lactone reductase (EC 1.1.1.168) of Saccharomyces cerevisiae[4] may be the only enzyme that resembles the Mucor carbonyl reductase, in that it can also reduce ketopantoyl lactone well. However Mucor enzyme is quite different from ketopantoyl lactone reductase in that the former is much larger (Mr = ca. 54000) than the latter (Mr = ca. 29000). The Mucor enzyme is composed of two identical subunits with Mr of 27000, while ketopantoyl lactone reductase is a monomeric enzyme. Another clear difference is that the former produce only the L-(+)-enantiomer but the latter gives

only the D-(-)-enantiomer of pantoyl lactone as the reaction product. The Km value (714 μM) of the Mucor enzyme for ketopantoyl lactone is about 50 times higher than that of the ketopantoyl lactone reductase.

Considering all of the facts described above, the Mucor enzyme seems to be a new type of carbonyl reductase. The strict specificity of the enzyme for only conjugated polyketones suggests that the name "conjugated polyketone reductase" may be given to it. It will be of great interest to determine how the enzyme functions under physiological conditions.

SUMMARY

A novel enzyme which specifically catalyzes the reduction of conjugated polyketones was purified to homogeneity from cells of Mucor ambiguus AKU 3006. The enzyme has a strict requirement for NADPH and irreversively reduces a number of quinones such as p-benzoquinone, β-naphthoquinone, acenaphthoquinone. The enzyme also reduces polyketones such as isatin and ketopantoyl lactone, and their analogs. The apparent Km values for isatin and ketopantoyl lactone are 49.9 and 714 μM, respectively. The reduction of ketopantoyl lactone proceeds stereospecifically to yield L-(+)-pantoyl lactone. The pro-S(A) hydrogen at C-4 of NADPH is transferred to the substrate. The enzyme is not a flavoprotein and consists of two polypeptide chains with an identical relative molecular mass of 27500. Quercetin, dicoumarol and some SH-reagents inhibit the enzyme activity. 3-Methyl-1,2-cyclopentanedione and 1,3-cyclohexanedione are uncompetitive inhibitors with Ki values of 80.9 and 64.5 μM, respectively, with respect to ketopantoyl lactone.

Section 2. Ketopantoyl lactone reductase from Candida parapsilosis: purification and characterization as a conjugated polyketone reductase[f)]

The group of the enzymes which reduce ketopantoyl lactone to yield pantoyl lactone is classified ketopantoyl lactone reductase (EC 1.1.1.168). These enzymes seems to exist widely in nature. In fact, my studies (see Section 1, Chapter 2) and Lanzilotta et al.[16)] showed that the ability to reduce ketopantoyl lactone to pantoyl lactone widely distributed in a variety of microorganisms. Purification and characterization of the enzyme however has been carried out only from Saccharomyces cerevisiae NRRL Y-2034.[4)] The name of ketopantoyl lactone reductase is therefore derived from the enzyme. Other than ketopantoyl lactone, only keto-ω-methylpantoyl lactone is reported to be a substrate for the enzyme.[4)]

Purification of ketopantoyl lactone reductase of S. cerevisiae resulted in the separation of two nearly homogeneous forms of the enzyme.[4)] Both forms have nearly the same molecular mass (27000), exhibit similar kinetic properties and require only NADPH as a cofactor, consist of one subunit and reduce ketopantoyl lactone to D-(-)-pantoyl lactone. If I can characterize another ketopantoyl lactone reductase other than the S. cerevisiae enzyme, the wide existence of enzymes of this type in nature would be strongly supported. To clarify this point I attempted to purify another enzyme from Candida parapsilosis of which cells showed a strong activity to reduce ketopantoyl lactone to D-(-)-pantoyl lactone.

MATERIALS AND METHODS

Materials. These were described in Section 1, Chapter 3.

Microorganism and cultivation. C. parapsilosis IFO 0708 was used. The organism was cultivated in 2-l flasks containing 500 ml of a medium containing 25 g of maltose and 25 g of meat extract, pH 5.6. Cultivation was carried out at 28°C for 3 days with shaking.

Enzyme assay. The same assay conditions and unit definition used for Mucor conjugated polyketone reductase were used (see Section 1, Chapter 3).

Purification of the enzyme. Wet cells (2170 g) of C. parapsilosis obtained from eighty-500 ml-cultures were suspended in 0.01 M Tris/HCl buffer, pH 7.4, containing 0.1 mM dithiothreitol (1630 ml), and then disrupted with an ultrasonic oscillator (19 kHz, 2 h, 2-15°C; Kaijo Denki 4280, Japan). All subsequent procedures were carried out at between 0 and 5°C, and 0.01 M Tris/HCl buffer, pH 7.4 containing 0.1 mM dithiothreitol was used unless otherwise stated.

The cell debris was removed by centrifugation (10000 x g, 30 min). The supernatant was fractionated with ammonium sulfate (60-80%), followed by dialysis against the buffer (8 l). The dialyzed solution was applied to a DEAE-Sephacel column (5.0 x 37 cm) equilibrated with the buffer. After washing the column with the buffer (900 ml), the enzyme was eluted with a 2-l linear gradient of KCl (from 0 to 0.5 M in the buffer). The active fractions were combined and then fractionated with ammonium sulfate. The precipitate appearing between 70 and 100% saturation was dissolved in a minimum volume of the buffer containing 4 M NaCl. The enzyme solution was placed on a column (0.9 x 10 cm) of phenyl-Sepharose CL-4B equilibrated with the buffer containing 4 M NaCl, from 4 to 0 M, in the buffer (140 ml). The active fractions were combined and then

concentrated by salting out with ammonium sulfate at 90% saturation. The precipitate was dissolved in 1.8 ml of the buffer. The enzyme solution was placed on a column (2.4 x 74 cm) of Sephadex G-75 equilibrated with the buffer containing 0.2 M KCl. The rate of sample loading and column elution was maintained at 15 ml/h. The active fractions were combined and then lyophilized. The lyophilizate was dissolved in 1.5 ml of the buffer. The enzyme was again applied to the Sephadex G-75 column. The conditions for the second filtration were the same as for the first. The active fractions were combined and then concentrated by lyophilization. The lyophilizate was dissolved in 1.0 ml of the buffer and then dialyzed against 500 ml of the buffer to remove salts.

Analytical methods for the purified enzyme. Analytical electrophoresis in a 7.5% polyacrylamide gel was carried out as described by Davis.[41] SDS-polyacrylamide gel electrophoresis was performed in a 12% polyacrylamide slab gel with a Tris/glycine buffer system, as described by King and Laemmli.[42] The relative molecular mass of the subunit of the enzyme was determined from its mobility relative to those of the standard proteins. The isoelectric point of the enzyme was determined as described in Section 1, Chapter 3.

Analytical gel filtration for molecular mass determination was carried out on a calibrated column (1.5 x 100 cm) packed with Sephadex G-100 according to Andrews.[44] The gel bed was equilibrated, at a constant flow rate (4 ml/h), with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.2 M KCl and 0.1 mM dithiothreitol at 4°C. Analytical gel-permeation chromatography was carried out as follows: 100 µl of the enzyme solution was subjected to high-performance gel-permeation liquid chromatography (Hitachi 638-30, Japan) on a G-3000 SW column (0.75 x 60 cm; Toyo Soda, Japan)

or GS-520 column (0.76 x 50 cm; Asahi Kasei, Japan) at 0.3 ml/min with 50 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM dithiothreitol and 0.2 M NaCl as the mobile phase, at room temperature. The absorbance of the effluent was monitored at 280 nm with a data module (Shimadzu C-R1B; Japan) automatically. The relative molecular mass of the enzyme was determined from the mobility relative to those of the standard proteins.

Analytical ultracentrifugation was carried out with a Hitachi 282 ultracentrifuge at 25°C. The sedimentation velocity experiments were performed at 60000 rpm, and the progress of the sedimentation boundary was followed at 280 nm using a Hitachi absorption scanner at 6-min intervals. A Hitachi RA60HC rotor and a double sector cell were used. The relative molecular mass of the enzyme was determined by the sedimentation equilibrium method, according to Van Holde and Baldwin,[63] using a Hitachi absorption optical system at 280 nm. Multicell operations were carried out for 3 samples with different initial concentrations, ranging from 0.151 mg/ml to 0.302 mg/ml, using a Hitachi RAM18SC rotor. The rotor was centrifuged at 13000 rpm for 20 h. The calculations were carried out as described by Schachman.[64]

Determination of protein concentrations. Protein concentrations were determined as described by Lowry et al.[27] with bovine serum albumin as a standard. For the purified enzyme and the effluent from the chromatographic columns, protein concentrations were determined by measuring the absorbance at 280 nm. An absorbance value of 0.83 for 1 mg/ml and a 1-cm light path, as determined by absorbance and dry-weight measurement, was used.

Stoichiometry. The stoichiometry of the reaction between ketopantoyl lactone and NADPH was as described in Section 1,

Chapter 3.

Substrate specificity. The composition of the reaction mixture for investigating the substrate specificity was the same as that of the standard assay mixture except that the carbonyl compound of interest was used as the substrate instead of isatin. Unless otherwise stated, the decrease in absorbance at 340 nm due to oxidation of NADPH was monitored. The blank values obtained on autooxidation of NADPH by ninhydrin and alloxane were subtracted.

Effects of inhibitors. The composition of the reaction mixture for investigating the effects of inhibitors was the same as that of the standard assay mixture except for the first addition of the inhibitors to the reaction mixture. The Ki value was determined by changing the concentrations of the inhibitors.

Other methods. Pantoyl lactone and ketopantoyl lactone were determined by GLC as described previously (see Section 1, Chapter 2). The stereospecificity of the formed pantoyl lactone was determined by GLC after derivatization with L-menthyl chloroformate (see Section 1, Chapter 2).

RESULTS

Purification of ketopantoyl lactone reductase of C. parapsilosis

Through the purification procedures described under Materials and Methods, the enzyme was purified 1476-fold with respect to isatin with a yield of 7% from the cell-free extract, as shown in Table 1. The enantiomeric excess of the formed D-(-)-pantoyl lactone with the cell-free extract was lower (86.3%) than that of the purified enzyme (100%). After the DEAE-Sephacel step the enantiomeric excess of the formed D-(-)-pantoyl lactone was nearly 100%. The specific activity

Table 1. Purification of Ketopantoyl Lactone Reductase from C. parapsilosis IFO 0708

Enzyme activity was measured as described under Materials and Methods with isatin and ketopantoyl lactone as substrates.

Step	Protein	Total activity units		Specific activity units/mg		Ratio of the specific	%e.e. of the formed
		isatin	KPL[a]	isatin(I)	KPL	activity I/KPL	D-(-)-PL[b]
Cell-free extract	77688	13210	11980	0.170	0.154	1.10	86.3
Ammonium sulfate	14357	19090	15240	1.33	1.06	1.25	73.8
DEAE-Sephacel	881	17530	12618	19.9	14.4	1.38	100
Phenyl-Sepharose CL-4B	44.4	6700	4700	151	106	1.42	100
Sephadex G-75	9.0	2220	1570	247	175	1.41	100
Sephadex G-75	3.7	930	640	251	173	1.45	100

[a] KPL, ketopantoyl lactone.

[b] PL, pantoyl lactone.

ratio of isatin to ketopantoyl lactone of the crude extract was 1.1. After DEAE-Sephacel step, however, the isatin activity was about 1.4-times higher than the ketopantoyl lactone activity, and this ratio did not change significantly in the following steps. The enantiomeric excess for the D-(-)-enantiomer of pantoyl lactone also reached a constant value (100%) at the same step. This suggests that there was another enzyme which reduces ketopantoyl lactone to the other enantiomer, L-(+)-pantoyl lactone, in the crude extract. However, no other activities which catalyze the reduction of ketopantoyl lactone to D-(-)-pantoyl lactone as reported in S. cerevisiae[4] were found throughout the purification. The enzyme was absorbed tightly on a membrane filter, such as a PM-10 or YM-10 (Amicon, USA), and therefore, I concentrated the enzyme solution by lyophilization.

Crystallization of the enzyme

The enzyme can be crystallized by the addition of solid ammonium

sulfate, little by little, to the concentrated enzyme solution (about 10 mg/ml) at the second Sephadex G-75 gel filtration step. Fine needle crystals appeared at about 75% ammonium sulfate saturation after standing for 1 day at 4°C. The specific activity of the crystalline enzyme was 253 units/mg for isatin, which was essentially the same as that of the purified enzyme at the second gel filtration step. A photograph of the crystals is presented in Fig. 1.

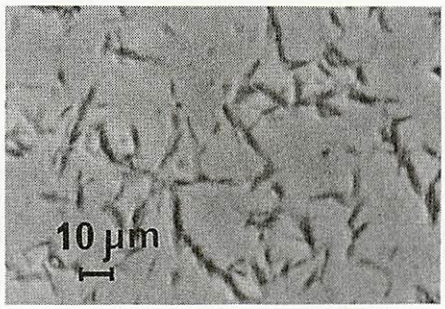


Fig. 1. Photograph of the Crystalline Candida Ketopantoyl Lactone Reductase

Criteria for purity

Polyacrylamide gel electrophoresis of the purified enzyme (preparation at the second Sephadex G-75 gel filtration step) showed that it migrated as single species, as judged by staining for protein on 7.5% gel. The purified enzyme gave only one band on SDS-polyacrylamide gel electrophoresis. It sedimented as a single symmetrical absorption peak of protein in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol on analytical ultracentrifugation. Ampholyte electrofocussing also gave only one absorption peak (pH 6.3) of protein, which coincided with the ketopantoyl lactone reductase activity. Further evidence for the

purity of the enzyme preparation was the result of high-performance gel-permeation liquid chromatography, which gave a quite symmetrical single protein peak.

Molecular mass and subunit structure

The sedimentation coefficient ($S_{20,w}$) of the enzyme dissolved in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 mM dithiothreitol was determined to be 4.8 S. A relative molecular mass of 39000 was determined by the sedimentation equilibrium method for the enzyme dissolved in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 mM dithiothreitol, assuming a partial specific volume of 0.74 cm^3/ml. On a calibrated column of Sephadex G-100, the molecular mass of the enzyme was estimated to be 31000, and those determined by high-performance gel-permeation liquid chromatography on G-3000SW and GS-520 columns were 29800 and 37000, respectively. By varying the gel concentration for the polyacrylamide gel electrophoresis between 5 and 12% and then replotting its mobility according to Hedrick and Smith,[65] its molecular mass was determined to be approximately 40000. A single band was observed when the enzyme was treated with 1% SDS and 10 mM 2-mercaptoethanol, and then electrophoresed on a gel containing 10% SDS. The relative molecular mass of the material in the band was estimated to be 41600, based on its mobility relative to those of the reference proteins. This difference in molecular mass may be due to the affinity of the enzyme to the permeation gel, i.e., Sephadex G-100, G-3000 SW or GS-520. The enzyme thus appears to be a monomer with Mr of about 40000.

Spectral properties

The absorption spectrum of the carbonyl reductase showed a maximum at 278 nm. The enzyme contained no substance showing absorption in the visual region of 320-600 nm. Therefore the enzyme

does not contain flavin, which is the coenzyme for most quinone reductases.

Substrate specificity of the enzyme

A broad range of carbonyl compounds including 2-, 3- and 4-ketoacids, aliphatic and aromatic ketones and aldehydes, and quinones was tested to investigate the substrate specificity of the enzyme (Table 2). The enzyme did not catalyze reduction of any monoketones and aldehydes tested. Quinones such as β-naphthoquinone, camphorquinone, acenaphthequinone and phenanthraquinone were efficiently reduced, and polyketones (isatin and its analogs, ketopantoyl lactone, ninhydrin and alloxan) were also readily reduced. The latter are all conjugated polyketones. Among these polyketones, isatin and 5-methylisatin were rapidly reduced. Ketopantoic acid, the delactonized form of ketopantoyl lactone, and dehydroascorbic acid were not reduced. Menadione and phylloquinone, potent substrate for mammalian[50] and plant quinone reductase,[51] were also inert as substrates. D-Glyceraldehyde and *p*-nitrobenzaldehyde, and xylose, typical substrate for aldehyde and aldose reductase, respectively,[46-49] were also not reduced.

Kinetic properties

Michaelis-Menten type kinetics were observes when isatin (Km = 14 μM, Vmax = 306 units/mg), ketopantoyl lactone (Km = 333 μM, Vmax = 481 units/mg) and camphorquinone (Km = 63 μM, Vmax = 323 units/mg) were tested as substrates.

Double-reciprocal plots of v against the ketopantoyl lactone concentration (5-1000 μM) gave parallel straight lines for various NADPH concentrations (33, 50, 100 and 320 μM). Parallel straight lines were also observed for various concentrations of ketopantoyl lactone (5, 6.7, 20, 100 and 1,000 μM) in double-reciprocal plots of

Table 2. Substrate Specificities of the Enzyme

Enzyme activities were measured as described under Materials and Methods. The activity for isatin was set as 100%. The following compounds were not reduced: ketopantoic acid, diacetyl, diacetyl monoxime, pyruvic acid, dimethylpyruvic acid, methylethylpyruvic acid, 3-hydroxypyruvic acid, 3-indolepyruvic acid, methyl pyruvate, ethyl pyruvate, 2-ketobutyric acid, 2-ketovaleric acid, 2-ketoisovaleric acid, 2-ketocaproic acid, 2-ketoisocaproic acid, 2-keto-3-methylvaleric acid, 2-keto-3-methylvaleric acid, 2-keto-3-ethylvaleric acid, 2-keto-3-methylcaproic acid, 2-keto-3-phenylbutyric acid, 2-ketomalonic acid, oxaloacetic acid, 3-indoleglyoxalic acid, glyoxilic acid, 2-ketoglutaric acid, 2-ketoadipic acid, oxalosuccinic acid, benzoylformic acid, 2-keto-D-gluconic acid, acetoacetic acid, levulinic acid, glyoxal, methylglyoxal, acetone, hydroxyacetone, dihydroxyacetone, acetylacetone, dihydroxyacetone phosphate, methyl ethyl ketone, isobutyl ketone, acetoin, benzaldehyde, formaldehyde, acetoaldehyde, propionaldehyde, n-butylaldehyde, chloroacetoaldehyde, chloral, betainaldehyde, crotonaldehyde, 1,3-cyclohexanedione, anthrone, D-glyceraldehyde, menadione, hydantoin, barbituric acid, parabanic acid, uracil, hypoxanthine, allantoin, nicotinamide, cystine and dimethyl fumarate. The wavelengths (nm) and absorption coefficients (1/M/cm) for enzyme activity measurements were as follows: isatin, 414, 849; 1-methylisatin, 425, 553; 5-nitroisatin, 415, 645; 5-bromoisatin, 435, 743; and 5-methylisatin, 441,656.

Substrate	Concentration μM	Relative velocity %	Km μM
Isatin	0.4	100	14
1-Methylisatin	0.4	86	85
5-Nitroisatin	0.4	29	
5-Methylisatin	0.4	108	16
Ketopantoyl lactone	0.4	69	333
Ninhydrin	0.4	8	
Alloxan	0.4	30	
Dehydroascorbic acid	0.4	1	
α-Naphthoquinone	0.4	1	
β-Naphthoquinone	0.08	34	
Camphorquinone	0.4	97	
Acenaphthequinone	0.08	43	
Phenanthraquinone	0.08	32	
1,2-Cyclohexanedione	0.4	4	

v against the NADPH concentration (20-320 μM). These results suggest that the reaction proceeds by a ping-pong mechanism, as designated by Cleland.[52] The enzyme showed a Km of 333 μM for ketopantoyl lactone and one of 156 μM for NADPH. The Vmax was 481 units per mg protein.

NADPH was almost absolutely required for the reduction of ketopantoyl lactone, only 2% of the rate in the case with NADPH being observed when NADPH was replaced with an equimolar amount of NADH in the assay system. The Km value for NADH obtained under the assay conditions with ketopantoyl lactone as the substrate was 2 mM (Vmax = 41.5 units/mg).

Stoichiometry

Using ketopantoyl lactone as the substrate, the stoichiometry of the reduction catalyzed by the enzyme was investigated under the conditions given under Materials and Methods. During the reaction, 35.4 μmol NADPH was consumed and 32.9 μmol pantoyl lactone was formed. The pantoyl lactone formed was confirmed to be only the D-(-)-enantiomer on analysis according to the L-menthyl chloroformate method. The amount of ketopantoyl lactone remaining unreacted in the reaction mixture was estimated to be 24.1 μmol. Thus, for every mol of NADPH oxidized, 1 mol of D-(-)-pantoyl lactone was formed. The reversibility of the reduction was investigated with various concentration of D-(-)-pantoyl lactone (0.1-30 mM) and $NADP^+$ (0.1-15 mM). No formation of ketopantoyl lactone and NADPH was observed at pH 7, 8 and 9.

Inhibitors

The effects of several compounds known to be inhibitors of some mammalian dehydrogenases or reductases were examined. Of the compounds tested, quercetin (0.1 mM), a nonspecific inhibitor of mammalian oxidoreductases,[53] inhibited 94% of the activity of the

Table 3. Effects of Polyketones, Polyols and Organic Acids as Inhibitors on Enzyme Activity

Enzyme activity was measured as described under Materials and Methods with the indicated compounds (1 mM), using isatin as the substrate. The activity without an inhibitor was taken as 100%.

Compound	Relative velocity(%)	Ki(μM) isatin	Ki(μM) NADPH	Type of inhibition
None	100			
Ketopantoyl lactone	38	190		competitive
3-Methyl-1,2-cyclopentadione	41			
3,4-Dihydroxy-3-cyclobutene-1,2-dione	28	1.4	0.5	uncompetitive
Cyclobutenediol-1,2,3,4-tetraone	4	0.2		uncompetitive
Parabanic acid	79	3140		uncompetitive
Dehydroascorbic acid	79			
L-Ascorbic acid	61			
Tartaric acid	88			
Pyrogallol	88			

enzyme. Dicoumarol, which inhibits mammalian NAD(P)H dehydrogenase (quinone reductase),[50] showed no effect on the enzyme activity. The following compounds also showed no effect at 1 mM: barbital and 5,5-diphenylhydantoin, inhibitors of aldehyde reductase,[55,56,66] pyrazole, an inhibitor of alcohol dehydrogenase,[58] and oxamidic acid, an inhibitor of lactate dehydrogenase.[67]

Several polyketones which have analogous structures to isatin were effective as inhibitors of isatin reduction, as shown in Table 3. With the exception of ketopantoyl lactone, none of them served as substrates. Ketopantoyl lactone inhibited the reduction of isatin. Both compounds seemed to attack the same active center of the enzyme competitively (Fig. 2a). On the other hand, 3,4-dihydro-3-

cyclobutene-1,2-dione was found to exhibit uncompetitive inhibition with respect to both isatin and NADPH (Fig. 2a, b). Uncompetitive inhibition with respect to isatin was also observed with cyclohexanediol-1,2,3,4-tetraone (Fig. 2a) and parabanic acid. Pyrogallol and some organic acids, such as tartaric acid and L-ascorbic acid, were also inhibitory for the enzyme.

The effect of the metal ions at 1 mM were investigated using 0.1 M maleic acid buffer instead of potassium buffer under the standard assay conditions. The activity of the enzyme in reducing ketopantoyl lactone was completely inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. Cd^{2+} and Al^{3+} also showed 97% and 83% inhibition, respectively.

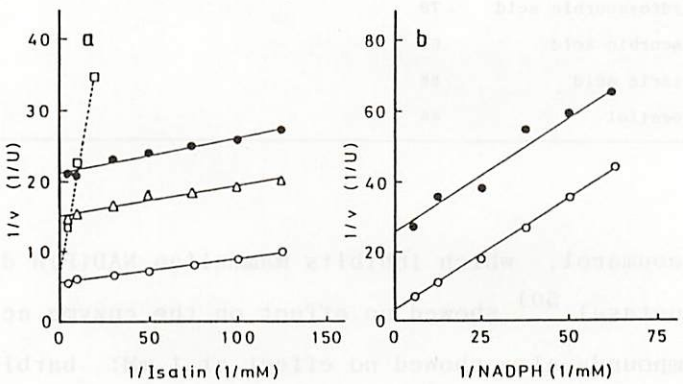


Fig. 2. Inhibition of the Enzyme
Double-reciprocal plots of v against isatin (a) and NADPH (b). The enzyme activity was assayed under the standard assay conditions using 0.5 μg of the enzyme, except that the concentrations of NADPH and isatin were changed. (a) Addition of no inhibitor (○), 4 μM of 3,4-dihydroxy-3-cyclobutene-1,2-dione (●), 0.4 μM of cyclohexenediol-1,2,3,4-tetraone (△) and 8 mM of ketopantoyl lactone (□). (b) Addition of no inhibitor (○) and 4 μM of 3,4-dihydroxy-3-cyclobutene-1,2-dione (●).

The enzyme activity was markedly inhibited by SH-reagents such as p-chloromercuribenzoate (0.1 mM, 72% inhibition) and iodoacetic acid

(1 mM, 49% inhibition). Chelating agents (8-hydroxyquinoline, o-phenanthroline and α,α'-dipyridyl) and carbonyl reagents (semicarbazide and phenyl hydrazine) had no effect on the enzyme activity.

Effects of pH and temperature on the enzyme activity

The effect of pH on the activity of the enzyme was investigated as shown in Section 1, Chapter 3. The enzyme showed maximum activities of 175 units/mg at pH 7.0. The activity of the enzyme was measured at various temperatures at pH 7.0 with ketopantoyl lactone as a substrate as shown in Section 1, Chapter 3. The maximum activity of the enzyme was observed at 40°C.

Stability of the enzyme

When the enzyme was incubated in 200 mM potassium phosphate buffer, pH 7.0, at various temperatures for 10 min, 58 and 77% of the initial enzyme activity was lost at 40 and 60°C, respectively. The enzyme was relatively stable between pH 6.0 and 10.0 in 50 mM potassium phosphate buffer and 50 mM glycine/NaOH buffer, but 33% of the initial activity was lost at pH 4.0 when the enzyme solution was incubated in 50 mM citrate/HCl buffer for 30 min at 30°C.

DISCUSSION

Carbonyl reductases can reduce a wide variety of carbonyl compounds such as aldehydes, ketones and ferricyanide. They are not flavoproteins and some of them require NADPH preferentially as an electron donor.[46-49,53] They are assumed to take part in the metabolism of xenobiotic carbonyl compounds and in the maintenance of the level of simple quinones and NADPH in cells.[46-49,68] The present Candida ketopantoyl lactone reductase is similar to human

brain carbonyl reductase[53] in that (1) it is not a flavoprotein, (2) it utilizes several quinones and carbonyl compounds as substrates, and (3) it requires NADPH almost specifically as a cofactor. These result suggest that the enzyme could be classified as a carbonyl reductase. However, the Candida enzyme is quite different from the common carbonyl reductases purified from mammalian and plant sources,[46-49,51,53,68] in that it reduces only conjugated polyketones.

I have reported that the enzyme purified from cells of Mucor ambiguus exhibits a broad substrate specificity toward conjugated polyketone compounds (see Section 1, Chapter 3). The enzyme is composed of two identical subunits (Mr = 27000) and can reduce ketopantoyl lactone, but the product of the reaction is the L-(+)-form of pantoyl lactone. The present enzyme purified from cells of C. parapsilosis also exhibited a broad substrate specificity toward a variety of conjugated polyketone compounds. The enzyme, however, is a monomer (Mr = 40000) and gives the other enantiomer, D-(-)-pantoyl lactone, on the reduction of ketopantoyl lactone. Dicoumarol did not inhibit the Candida enzyme, but it did inhibit the Mucor carbonyl reductase.

King et al.[4] reported that ketopantoyl lactone reductase (EC 1.1.1.168) from S. cerevisiae catalyzes the formation of the D-(-)-enantiomer of pantoyl lactone from ketopantoyl lactone. Because ketopantoic acid did not serve as a substrate, they suggested that a ring structure is required in the substrate carbonyl compounds for the enzyme. This seems to be the case because the ketopantoyl lactone reductase of C. parapsilosis purified here shows a broad substrate specificity for polyketones and quinones (see Table 2). However, the properties of the two enzymes are different from each

other; molecular mass, Vmax and Km values (for ketopantoyl lactone and NADPH) of the enzyme from Candida are considerably larger than those of the Saccharomyces enzyme. Results described here strongly suggest that Candida ketopantoyl lactone reductase is a conjugated polyketone reductase.

SUMMARY

Ketopantoyl lactone reductase was purified and crystallized from cells of Candida parapsilosis IFO 0708. The enzyme was found to be homogeneous on ultracentrifugation, high-performance gel-permeation liquid chromatography and SDS-polyacrylamide gel electrophoresis. The relative molecular mass of the native and SDS-treated enzyme is approximately 40000. The isoelectric point of the enzyme is 6.3. The enzyme was found to catalyze specifically the reduction of a variety of natural and unnatural polyketones and quinones other than ketopantoyl lactone in the presence of NADPH. Isatin and 5-methylisatin are rapidly reduced by the enzyme, the Km and Vmax values for isatin being 14 μM and 306 μmol/min per mg protein, respectively. Ketopantoyl lactone is also a good substrate (Km = 333 μM and Vmax = 481 μmol/min/mg protein). Reverse reaction was not detected with pantoyl lactone and $NADP^+$. The enzyme is inhibited by quercetin, several polyketones and SH-reagents. 3,4-Dihydroxy-3-cyclobutene-1,2-dione, cyclohexenediol-1,2,3,4-tetraone and parabanic acid are uncompetitive inhibitors for the enzyme, the Ki values being 1.4, 0.2 and 3140 μM, respectively, with isatin as substrate. The results suggested that ketopantoyl lactone reductase is a kind of conjugated polyketone reductase.

Section 3. Ketopantoyl lactone reductase is a conjugated polyketone reductase[g)]

I have found that both Mucor ambiguus AKU 3006 (see Section 1, Chapter 3) and Candida parapsilosis IFO 0708 (see Section 2, Chapter 3) produces a novel carbonyl reductase which uses only conjugated polyketones as substrates. The reduction of ketopantoyl lactone by the Candida enzyme proceeds stereospecifically to yield D-(-)-pantoyl lactone and contrary one by the Mucor enzyme gave L-(+)-pantoyl lactone. These enzymes show a wide substrate specificity toward both natural and unnatural carbonyl compounds such as isatin and ketopantoyl lactone and quinones. As described in Section 1, Chapter 2, the ability to reduce ketopantoyl lactone to pantoyl lactone is widely distributed in a variety of microorganisms. These results indicate the possibility that this sort of enzyme exists widely in nature. I have suggested that ketopantoyl lactone reductase (EC 1.1.1.168), which was purified from Saccharomyces cerevisiae NRRL Y-2034 and was shown to catalyze reduction of ketopantoyl lactone,[4)] might also be a conjugated polyketone reductase (see Section 1 and 2, Chapter 3). However, it is not clear whether or not ketopantoyl lactone reductase attacks a variety of conjugated polyketone compounds and quinones. Therefore, the substrate specificity, kinetic properties, inhibitors and several other properties of the enzyme were investigated in the present study. The results obtained here clearly indicate that ketopantoyl lactone reductase should be included in the class of conjugated polyketone reductase.

MATERIALS AND METHODS

Ketopantoyl lactone was synthesized as shown in Chapter 1. All other reagents were commercially available and of analytical grade. Ketopantoyl lactone reductase was assayed as described in Chapter 2. One unit of the enzyme was defined as the amount catalyzing a change corresponding to the reduction of 1 μmol of ketopantoyl lactone per min under the assay conditions. Specific activity was expressed as units of enzyme activity per min per mg protein. Protein concentrations were determined as described by Lowry et al.[27] with bovine serum albumin as a standard.

The purification procedures of ketopantoyl lactone reductase of S. cerevisiae NRRL Y-2034 used were essentially the same as those described by King et al.[4] The enzyme was homogeneous, as judged on SDS/polyacrylamide gel electrophoresis. The enzyme preparation was confirmed to produce only the D-(-)-enantiomer of pantoyl lactone on reduction of ketopantoyl lactone as judged by GLC after derivatization with L-menthyl chloroformate.[26] It showed a specific activity of 120 μmol/min/mg protein for ketopantoyl lactone when assayed according to the procedure of King et al.[4] The Km value (17 μM for ketopantoyl lactone) indicates that this enzyme is the Form A enzyme (Km = 14 ± 2 μM for ketopantoyl lactone) reported by King et al.[4] Substrate specificities and the effect of inhibitors were investigated as described in Section 1, Chapter 3. Ki values were determined by changing the concentrations of the inhibitors.[54]

RESULTS

A broad range of carbonyl compounds including 2, 3 and 4-ketoacids, aliphatic and aromatic ketones and aldehydes, and quinones was tested to investigate the substrate specificity of the enzyme

(Table 1). Quinones such as camphorquinone and β-naphthoquinone were efficiently reduced, and polyketones (ketopantoyl lactone and its analogs, isatin and its analogs, and ninhydrin) were also readily

Table 1. Substrate Specificity of Ketopantoyl Lactone Reductase

The wavelength (nm) and extinction coefficients (1/M/cm) for the enzyme activity measurements were as follows: isatin, 414, 849; 5-methylisatin, 441, 656; 5-nitroisatin, 415, 645; 5-bromoisatin, 435, 743; and 1-methylisatin, 425, 553, respectively. For other substances, decrease in 340 nm due to NADPH oxidation was monitored. For detail of assay, see the text. Abbreviations used: 1c, dihydro-4,4-diethyl-2,3-furanedione; 1d, dihydro-4-methyl-4-propyl-2,3-furanedione; 1e, dihydro-5-isopropyl-4,4-dimethyl-2,3-furanedione; 1f, dihydro-5-(2-butyl)-4-methyl-4-ethyl-2,3-furanedione; 1g, dihydro-5-(3-pentyl)-4,4-diethyl-2,3-furanedione; 1h, dihydro-5-(2-pentyl)-4-methyl-4-propyl-2,3-furanedione; 1i, dihydro-5-(3-pentyl)-4,4-dimethyl-2,3-furanedione; and 1j, dihydro-5-(2-pentyl)-4,4-dimethyl-2,3-furanedione.

Substrate	Concentration mM	Relative rate of reduction (%)	Km μM
Ketopantoyl lactone	0.4	100	17
1c	0.4	16	420
1d	0.4	26	983
1e	0.4	96	9.3
1f	0.4	333	43
1g	0.4	7	
1h	0.4	52	90
1i	0.4	112	9.8
1j	0.4	107	13
Isatin	0.4	125	6.6
5-Nitroisatin	0.08	64	
5-Bromoisatin	0.08	279	3.1
5-Methylisatin	0.08	130	8.3
Ninhydrin	0.4	181	520
Alloxan	0.4	40	830
β-Naphthoquinone	0.4	95	93
Camphorquinone	0.4	144	64

reduced. The latter are all conjugated polyketones. Among these polyketones, 5-bromoisatin was the most rapidly reduced. Ketopantoyl lactone analogs carrying an aliphatic substituent at 5 position, e.g., dihydro-5-isopropyl-4,4-dimethyl-2,3-furanedione (1e), dihydro-5-(3-pentyl)-4,4-dimethyl-2,3-furanedione (1i) and dihydro-5-(2-pentyl)-4,4-dimethyl-2,3-furanedione (1j), showed higher affinity than ketopantoyl lactone. On the other hand, modification at 4 position brought about remarkable elevation of Km value and reduction of the reaction velocity. Menadione and phylloquinone, potent substrates for mammalian[50] and plant quinone reductases,[51] were inert as substrates. D-Glyceraldehyde and p-nitrobenzaldehyde, and xylose, typical substrates for aldehyde and aldose reductases, respectively,[46-49], were not reduced. Any other monoketones and aldehydes so far tested were also inert as substrates.

The effects of several compounds known to be inhibitors of some mammalian dehydrogenases or reductases were examined. Of the compounds tested, quercetin (0.1 mM), a nonspecific inhibitor of mammalian oxidoreductases,[53] and dicoumarol (0.2 mM), which inhibits mammalian NAD(P)H dehydrogenase (quinone reductase)[54] inhibited 60 and 76% of the enzyme activity, respectively. The following compounds showed no effect at 1 mM, barbital and 5,5-diphenylhydantoin, inhibitors of aldehyde reductase,[55,56,66] and pyrazole, an inhibitor of alcohol dehydrogenase.[58]

Several polyketones which have analogous structures to isatin were effective as inhibitors of isatin reduction, as shown in Table 2. With the exception of ketopantoyl lactone, all of them did not serve as substrates. Ketopantoyl lactone inhibited the reduction of isatin. Both compounds seemed to attack the same active-center of the enzyme competitively. A kinetic study on the inhibition with

Table 2. Inhibition of Isatin Reduction Activity of the Enzyme by Polyketones[a]

Polyketone compound 0.4 mM	Relative rate of isatin reduction %	Ki μM	Manner of inhibition
None	100		
Ketopantoyl lactone	25	8.6	competitive
Parabanic acid	37	450	uncompetitive
Cyclohexanediol-1,2,3,4-tetraone	11	0.3	uncompetitive
3-Methyl-1,2-cyclopentadione	73	120	uncompetitive

[a] Decrease in 414 nm in the presence of each indicated polyketone compound was monitored. For detail of assay, see the text.

ketopantoyl lactone also revealed that the inhibition proceeded competitively (Ki = 8.6 μM). Cyclohexenediol-1,2,3,4-tetraone was found to exhibit uncompetitive inhibition with respect to both isatin (Ki = 0.3 μM) and NADPH (Ki = 0.2 μM). Parabanic acid and 3-methyl-1,2-cyclopentadione also showed uncompetitive inhibition with respect to isatin.

DISCUSSION

I have reported that the enzymes purified from cells of M. ambiguus and C. parapsilosis exhibit broad substrate specificity toward conjugated polyketone compounds (see Section 1 and 2, Chapter 3). The former enzyme reduces ketopantoyl lactone to give L-(+)-pantoyl lactone but the latter gives D-(-)-pantoyl lactone. The reaction of these enzymes proceed by a ping-pong mechanism, and the

enzyme is inhibited by quercetin and some SH-reagents. The ketopantoyl lactone reductase of S. cerevisiae described here also exhibit a broad substrate specificity toward a variety of conjugated polyketone compounds. The enzyme rather resembles the Candida enzyme in that it is a monomer[4] and produces D-(-)-pantoyl lactone on the reduction of ketopantoyl lactone. The both enzymes were not inhibited by dicoumarol, but it did inhibit the Mucor carbonyl reductase. Though there are some differences among these three enzymes, they resemble each other very well as a whole. This indicates that they belong to the same class. The strict specificity of the enzymes for only conjugated polyketones suggests that the name "conjugated polyketone reductase" should be given to them.

SUMMARY

Ketopantoyl lactone reductase (EC 1.1.1.168) of Saccharomyces cerevisiae was found to catalyze the reduction of a variety of natural and unnatural conjugated polyketone compounds and quinones, such as isatin, ninhydrin, camphorquinone and β-naphthoquinone in the presence of NADPH. 5-Bromoisatin is the best substrate for the enzyme (Km = 3.1 mM; Vm = 650 μmol/min/mg). The enzyme is inhibited by quercetin, and several polyketones. These results suggest that ketopantoyl lactone reductase is a carbonyl reductase which specifically catalyzes the reduction of conjugated polyketones.

CHAPTER 4. Stereospecific Reduction of Diketones by a Novel Carbonyl Reductase from Candida parapsilosis[h)]

The stereospecific reduction of prochiral α-diketones is of synthetic importance. Several enzymatic reactions have been shown to be promising in giving good enantiomeric excesses of the reduction products. The reduction of ketopantoyl lactone (1a) has been reported to give (R)-(-)-pantoyl lactone,[5)] which is a key intermediate in the synthesis of D-(+)-pantothenic acid, and the reduction of 2-(6-carbomethoxyhexyl)cyclopentane-1,3,4-trione gives important intermediates of (-)-prostaglandin E_1 and (-)-prostaglandin E_2.[69)] Furthermore, this type of reduction is involved in the metabolism of C_{18}-steroid hormones, such as in the reduction of 16-oxoesteron.[70)] Many kind of ketols exist in nature and play important physiological roles.[71)] In Section 1, Chapter 3, I reported that a fungus, Mucor ambiguus produces a new type of carbonyl reductase showing strict specificity for only conjugated polyketone compounds. The following studies demonstrated that similar enzymes are widely distributed in a variety of microorganisms (see Section 1, Chapter 2). For example, the carbonyl reductases of Candida parapsilosis and Saccharomyces cerevisiae also exhibit broad substrate specificities toward only conjugated polyketones. These results suggest that they can be grouped into a new carbonyl reductase family, which has not been reported previously. However, the two yeast enzymes (i.e., Candida and Saccharomyces carbonyl reductases) seemed to be different from the mold enzyme (i.e., Mucor carbonyl reductase) in their stereospecificities. The former enzymes gave the (R)-(-)-enantiomer of pantoyl lactone on reduction of 1a, but the latter gave the (S)-(+)-enantiomer. Because of their broad

substrate specificities for various prochiral α-diketone compounds, these enzymes seem to be highly suitable as tools for general synthetic studies. Here, I report the stereoselective reduction of several diketones, such as analogs of 3 and 1a, and (R)- and (S)-2 by the Candida enzyme, with emphasis on identification of the carbonyl group which can be reduced, demonstration of the absolute configurations of the reduction products and the effects of the insertion of several aliphatic substituents into the substrate molecules on the kinetics of the enzyme reaction.

RESULTS

Substrate specificity

In addition to 1a, several of its analogs were also examined as substrates (Fig. 1). The results are schematically presented in Fig. 2. Most of the analogs carrying bulky substituents at the 4 or 5 position of the lactone ring were reduced at lower rates than 1a, although they showed higher affinities for the enzyme than 1a. Among them, 1e (5-isopropylketopantoyl lactone) was the best substrate, as judged from that it showed the highest pseudo-second order rate constant ratio (Vmax/Km). On the other hand, 1b and 1d, which carry bulkier substituents than 1f, showed higher Vmax but lower Km values than 1a.

The reduction velocities for analogs of 3 increased when the substituent group was electron-donating. The Km (μM) and Vmax (units/mg) are as follows: 5-NO_2, 21, 211; 5-Br, 9, 244; no substituent group (i.e., isatin), 12, 306; 5-Me, 35, 353; 1-Me, 19, 302.

Identification of the products

1

	R^1	R^2	R^3
a	Me	Me	H
b	Me	Et	H
c	Et	Et	H
d	Me	Pr	H
e	Me	Me	Me_2CH
f	Me	Et	MeEtCH
g	Et	Et	Et_2CH
h	Me	Pr	MePrCH
i	Me	Me	Et_2CH
j	Me	Me	MePrCH

Fig. 1. Reduction of Analogs of Dihydro-4,4-dimethyl-2,3-furanedione by Candida Reductase

Table 1 and Fig. 3 show the results of the reduction of α-diketones. In the case of compounds 6 and 7, a and b indicate the order of elution. The ^{1}H-NMR data for the products showed that they were pure, with the exceptions of 8 and 9, and that they were derived through the reduction of one carbonyl group. 7a and 7b were estimated to be 100% e.e. from the ^{1}H-NMR data of non-bridge-headed methyn proton. 8 and 9 included small amounts of 3 and 4, respectively, which were formed through the air oxidation of 8 and 9, respectively, during concentration of aliquots of the respective column chromatography fractions. The derivatives of 5, 6a, 6b and 9 with L-menthyl chloroformate[26] each gave a single peak on GLC, with retention times of 13.1, 13.1, 15.2 and 6.3 min, respectively, indicating that the products were optically pure. All the alcohols

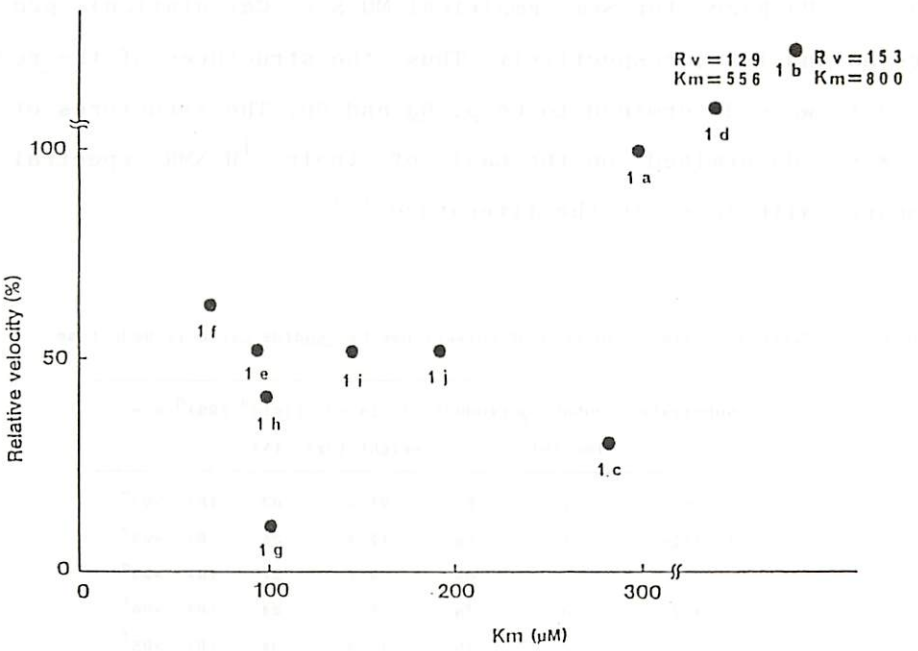


Fig. 2. Substrate Specificity for Analogs of Dihydro-4,4-dimethyl-2,3-furanedione of Candida Reductase

Enzyme activity was measured as described under Materials and Methods. The ordinate represents the Vmax, and the Vmax with dihydro-4,4-dimethyl-2,3-furanedione (1a, 481 units/mg enzyme) was taken as 100%.

formed were determined to be of the (R)-configuration by Horeau's method,[72,73] because in all cases the ratio of the areas of the two peaks, 8.1 min (retention time) vs 8.8 min, on the gas liquid chromatograms was greater than that in the case of cyclohexanol (standard compound). Determination of the configuration of 8 and 9 were based on that of (-)-mandelic acid.[73] Furthermore, 6a and 6b were determined to have (3R,5R)- and (3R,5S)-form structures, respectively, from the coupling constant of the hydrogen at the 5 position carbon, because the dihedral angles for H-C(5)-C(methylethyl)-H calculated with the MOPAC (A General Molecular

Orbital Package for Semi-Empirical MO-SCF Calculation) program[74] were 56 and 160°, respectively. Thus, the structures of the reduction products were determined to be 5, 6a and 6b. The structures of 7a and 7b were determined on the basis of their ^{1}H-NMR spectral data, compared with those in the literature.[75]

Table 1. Stereoselective Reduction of Polyketones by Candida Carbonyl Reductase

Substrate	Incubation time (h)	Product	Isolated weight (mg)	Yield[a] (%)	(RS)[d]	e.e.
1c	1	5	21.3	63	(R)	>99[e]
(RS)-1e	1	6a	12.9	38	(R)	>99[e]
		6b	9.1	27	(R)	>99[e]
(RS)-2	3	7a	8.0	24	(R)	>98[f]
		7b	11.3	34	(R)	>98[f]
3	1	8	13.5[b]	40[b]	(R)	
4	1	9	9.3[c]	28[c]	(R)	>99[e]

[a] Molar yields based on added NADPH. Molar conversions of each substrate to the respective products were 1c, 42%; 1e, 44%; 2, 38%; 3, 27% and 4, 18%.

[b] The values corrected by subtraction of the isatin formed through oxidation of the product during evaporation in the isolation step.

[c] The values corrected by subtraction of the 1-methylisatin formed through oxidation of the product during evaporation in the isolation step.

[d] The configurations of the products were determined according to the method of Horeau[72,73] using cyclohexanol as a standard compound. (R)-(-)-Dihydrobe of the (R) configuration.

[e] The values are based on the GLC analysis of the derivatives of the product with chloroformic acid (1R, 2S,5R)-(-)-5-methyl-2-(1-methylethyl)cyclohexanol ester.[26]

[f] The values are based on ^{1}H-NMR analysis.

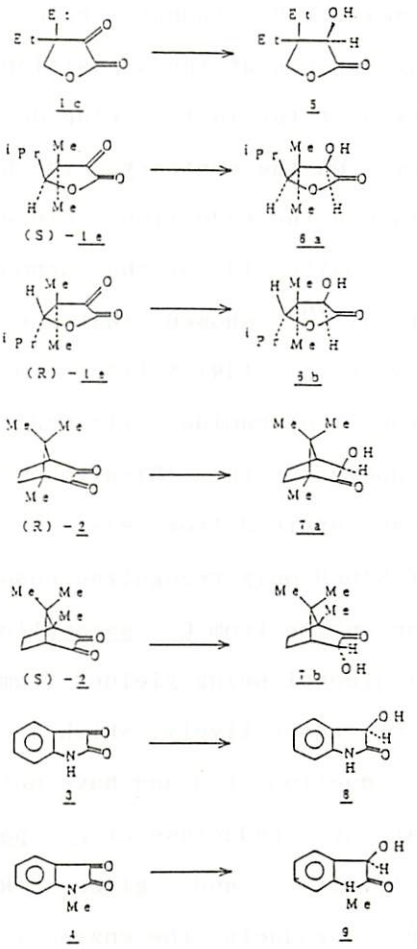


Fig. 3. Stereospecific Reduction of Conjugated Diketones by Candida Reductase

DISCUSSION

The results described here indicate that the polyketone reductase of C. parapsilosis catalyzes the reduction of a variety of diketones, including analogs of 1a and 3, and 2, to give (R)-alcohols. An (R)-alcohol 5 derived from 1c has negative optical rotation, as has (R)-(-)-pantoyl lactone. The reduction of (RS)-1e

also proceeded well, (R)-alcohols being yielded. These results suggest that ethyl groups at the 4 position and a methylethyl group at the 5 position of the lactone ring do not greatly affect the reduction velocity. On the contrary, the bridge-headed methyl group of 2 seemed to disturb the reduction, since, the reduction of 2 only occurred on the opposite side of the carbonyl group from the methyl group. Ohno et al.[76] showed that the reduction with a model compound of NADPH, (4R)-N-[(R)- α-methylbenzyl]-1-propyl-2,4-dimethyl-1,4-dihydronicotinamide, yielded (R)-7b from (S)-(+)-2 as a major product, however, the (S)-alcohol of 7a, but not the (R)-alcohol of 7a, was obtained from (R)-(-)-2. These results indicate that the model of NADPH only recognized endo- and exo-sites of 2. On the contrary, the enzyme from C. parapsilosis recognized the whole molecule, an (R)-alcohol being yielded from each enantiomer of 2. 3 and 4 gave 8 and 9, respectively, which are difficult to synthesize through chemical reduction of 3 and have not been reported.

As the carbonyl reductase of C. parapsilosis has a broad substrate specificity and gives (R)-alcohols with full stereospecificity as products, the enzyme is thought to be useful for the general synthesis of chiral α-hydroxy ketones.

MATERIALS AND METHODS

Materials. 1a and its analogs were synthesized as described in Chapter 1. NADPH was from Oriental Yeast (Japan). All other reagents, of analytical grade, were commercially available.

Preparation of the enzyme. A homogeneous preparation of conjugated polyketone reductase (ketopantoyl lactone reductase) was prepared from C. parapsilosis IFO 0708 as described in Section 2,

Chapter 3. The enzyme showed a specific activity of 175 μmol/min/mg protein for 1a. The enzyme assay was carried out as described in Section 1, Chapter 3.

Substrate specificity. The substrate specificity was determined, adding various substrates instead of 1a, in the same manner as for the Enzyme assay as described in Section 1, Chapter 3. The Km and Vmax values were determined from Lineweaver-Burk double reciprocal plots.

Isolation of the enzymatically synthesized products. Reaction method. A 6-ml mixture, containing 50 mg of substrate, NADPH (0.67 molar ratio, to the substrate), 3 mmol of potassium phosphate buffer (pH 7.0) and 170 μg of the enzyme, was incubated for a prescribed period, as shown in Table 2, at 30°C. When the substrates were 1c and 1e, 10 μl of a 16.7 w/v% substrate solution in 0.1 N HClaq was added every min 30 times to prevent hydrolysis of the lactone ring, with incubation for a prescribed period, as mentioned above.

Isolation of the products. The resultant mixture was extracted with five portions of 5 ml of acetic acid ethyl ester after heating with 1.0 ml of 6 N HCl for 10 min in boiling water. The combined organic layer was evaporated in vacuo after drying over anhydrous sodium sulfate. The residual mass was dissolved in a minimal amount of benzene and then applied on a column of silica gel (Wacogel C-200, 2.5 x 20 cm, Wako Pure Chemicals, Japan). A linear gradient of benzene (150 ml)/ethyl acetate (150 ml) was used for elution. Products in the fractions were monitored with a UV-lamp (254 nm) after developing on a silica gel sheet (Kieselgel $60F_{254}$, Merck, FRG) with a solvent system of benzene/ethyl acetate (volume ratio, 3/1). Fractions containing a product were combined and dried in vacuo.

Characterization of the products. Quantitative analysis of the products was carried out with a Shimadzu GC-7AF gas liquid chromatograph equipped with a flame ionization detector. A column (1 m long, 3.0 mm inside diameter) packed with Silicone OV-1 (3%) was used. The operation conditions were: injection port, 160°C; column, 150°C for isatins and their reduced products, and 110°C for other substrates and their reduced products; helium as a carrier, 70 ml/min; hydrogen, 0.6 kg/cm^2; air, 0.6 kg/cm^2. The retention times (min) of the substrates and products were as follows: 1c, 3.5; 5, 4.0; 1e, 2.2; 6a, 2.5; 6b, 2.2; 2, 3.4; 7a, 2.3; 7b, 2.3; 3, 4.0; 8, 3.5; 4, 2.7; 9, 2.1. The purity of the products, 5, 6a, 6b, 7a and 7b was judged to be larger than 95% by this GLC determination, and 8 and 9 were found to include only 3 and 4, respectively, as a impurity. ^{1}H-NMR spectra were obtained at 360 MHz. The purities of 8 and 9 were determined to be 88 and 92%, respectively, based on the intensity of their 3-H signal in the ^{1}H-NMR spectra.

The optical purities of the reduction products were determined by GLC after derivatization with L-menthyl chloroformate.[26] The absolute configurations of the formed alcohols were determined according to the method of Horeau.[72,73] The method is as follows: after heating a mixture containing 1 μmol of a compound to be assayed, 4 μl of dry pyridine and 2 μl of (RS)-2-phenylbutanoic acid anhydride for 90 min at 40°C, 4 μmol of (R)-1-phenylethylamine was added to the mixture. After heating for 15 min at 40°C, 60 μl of ethyl acetate was added and the resulting solution was analyzed by GLC as to the area ratio of the formed (S)-1-phenylethylamide of (R)-(-)-2-phenylbutyric acid and the (S)-1-phenylethylamide of (S)-(+)-2-phenylbutyric acid. The analytical conditions were the same as described above except for the following changes: silicone OV-17 (1%)

on Gas Chrom Q (100-120 mesh); carrier N_2, 50 ml/min; injection port, 250°C; detector port, 250°C; column, 215°C. The retention times of the (S)-1-phenylethylamide of R-(-)-2-phenylbutyric acid and the (S)-1-phenylethylamide of S-(+)-2-phenylbutyric acid were 8.1 and 8.8 min, respectively.

Determination of the molecular shapes of 6a and 6b derived from (RS)-1e was carried out by calculation with the MOPAC program (QCPE (Quantum Chemistry Program Exchange, Indiana University) #455) using the AM1 (Austin Model 1) option.[74]

The analytical data for the reduction products were as follows.

5. Anal. Calcd: C, 60.74%; H, 8.92%. Found: C, 60.7%; H, 8.9%; ^{1}H-NMR ($CDCl_3$, δ) 0.83 (Me, t, J = 7.6 Hz, 3H), 0.91 (Me, t, J = 7.6 Hz, 3H), 1.48 (CH_2, m, J = 7.2 Hz, 4H), 3.80 and 4.08 (CH_2, each d, J = 9.4 Hz, 2H), 2.8 (OH, bs, 1H); IR (film) 3450 (bd), 2980, 1785, 1465, 1195, 1120, 1010, 885 cm^{-1}; MS, m/Z 156, 86, 85 (Base), 71, 55, 43, 40; optical rotation $[\alpha]_D^{25}$ -13° (C = 1, MeOH).

6a. ^{1}H-NMR ($CDCl_3$, δ) 0.95 (Me, d, J = 6.8 Hz, 3H), 0.99 (Me, d, J = 6.8 Hz, 3H), 1.09 (Me, s, 3H), 1.10 (Me, s, 3H), 1.95 (H, m, 1H), 3.95 (H, d, J = 6.5 Hz, 1H), 4.05 (H, s, 1H), 2.7 (OH, bs, 1H); IR (film) 3450 (bd), 2975, 2940, 1775, 1475, 1225, 1120, 995, 975 cm^{-1}; MS, m/Z 101, 86, 85 (Base), 83, 73, 72, 57, 55, 44, 43, 40, 39; optical rotation $[\alpha]_D^{25}$ +48° (C = 1, MeOH).

6b. Anal. Calcd: C, 62.77%; H, 9.36%. Found C, 62.6%; H, 9.3%; ^{1}H-NMR ($CDCl_3$, δ) 0.88 (Me, s, 3H), 0.92 (Me, d, J=6.7 Hz, 3H), 1.03 (Me, d, J = 6.7 Hz, 3H), 1.21 (Me, s, 3H), 1.87 (H, m, 1H), 3.57 (H, d, J = 10.4 Hz, 1H), 4.01 (H, s, 1H), 2.5 (OH, bs, 1H); IR (KBr) 3410 (bd), 2980, 2930, 2890, 1760, 1485-1440, 1400-1200, 1180, 1150, 1020, 985 cm^{-1}; MS, m/Z 101, 86, 85 (Base), 83, 73, 72, 57, 55, 44, 43, 40, 39; mp., 121-2°C; optical rotation $[\alpha]_D^{25}$ -25° (C = 1, MeOH).

7a. Anal. Calcd: C, 71.39%; H, 9.59%. Found: C, 71.2%; H, 9.4%; ^{1}H-NMR ($CDCl_3$, δ) 0.85 (Me, s, 3H), 0.88 (Me, s, 3H), 0.92 (Me, s, 3H), 2.02 (H, d, J = 4.3 Hz, 1H), 3.65 (H, s, 1H), 2.4 (OH, bs, 1H), 1.9, 1.6 and 1.3 (m, 4H); IR (KBr) 3450 (bs), 2960, 1775, 1460, 1400, 1380, 1320, 1295, 1105, 1080, 1020, 835 cm^{-1}; mp., 207-9^oC; MS, m/Z 168, 125, 95, 84, 83 (Base), 69, 55, 43, 40, 39; optical rotation $[\alpha]_D^{25}$ +85^o (C = 1, MeOH).

7b. Anal.Calcd: C, 71.39%; H, 9.59%. Found: C, 71.4%; H, 9.6%; ^{1}H-NMR ($CDCl_3$, δ) 0.82 (Me, s, 3H), 0.97 (Me, s, 3H), 0.95 (Me, s, 3H), 2.20 (H, dd, J_1 = 4.7 Hz, J_2 = 4.0 Hz, 1H), 4.13 (H, d, J = 4.0 Hz, 1H), 2.4 (OH, bs, 1H), 1.9, 1.7 and 1.3 (m, 4H); IR (KBr) 3460 (bs), 2960, 1745, 1480, 1460, 1400, 1380, 1110, 1085, 1005, 980 cm^{-1}; MS, m/Z 168, 125, 95, 84, 83 (Base), 69, 55, 43, 40, 39; mp., 109-10^oC; optical rotation $[\alpha]_D^{25}$ -2^o (C = 1, MeOH).

8. ^{1}H-NMR (acetone-d_6, δ) 3.63 (H, s, 1H), 5.03 (OH, bd, J=6.1 Hz, 1H), 7.25-6.75 (aromatic H, 4H), 9.07 (NH, bs, 1H); IR (KBr) 3440 (bd), 1710, 1635, 1480, 1360, 1270, 1200, 1180, 1115, 750 cm^{-1}; MS, m/Z 147, 119 (Base), 93, 92, 64, 40; optical rotation $[\alpha]_D^{25}$ +7^o (C = 1, MeOH).

9 ^{1}H-NMR ($CDCl_3$, δ) 3.18 (Me, s, 3H), 3.52 (H, s, 1H), 5.01 (OH, s, 1H), 6.67-7.56 (aromatic H, 4H); IR (KBr) 3420 (bd), 1720, 1620, 1500, 1215, 760 cm^{-1}; MS, m/Z 161, 133, 105, 104 (Base), 92, 78, 63, 50, 44, 39; optical rotation $[\alpha]_D^{25}$ +3^o (C = 1, MeOH).

SUMMARY

An NADPH-linked carbonyl reductase purified from Candida parapsilosis IFO 0708 can reduce a variety of diketone compounds, such as analogs of isatin (3), ketopantoyl lactone (1a) and 1,7,7-

trimethylbicyclo [2,2,1]heptane-2,3-dione (2). Electron-donating substituents at the 5 position on the ring of 3 increased the reduction velocity, however, a 1-methyl group had no effect on it. Analogs of 1a carrying bulky substituents at the 4 or 5 position of the lactone ring were reduced at lower rates than 1a, although they showed higher affinities for the enzyme. Ones carrying less bulky substituents were reduced at higher rates, but had lower Km values. On reduction, 4,4-diethyldihydro-2,3-furanedione (1c), (R)- and (S)-5-isopropylketopantoyl lactone (1e), and (R)-(-)- and (S)-(+)-2, 3 and 1-methylisatin (4) all gave (R)-alcohols.

Chapter 5. Production of D-(-)-Pantoyl Lactone from DL-Pantoyl Lactone by Enzymatic Processes

Section 1. Stereoselective enzymatic oxidation and reduction system for the production of D-(-)-pantoyl lactone from a racemic mixture of pantoyl lactone[1)]

Pantoic acid and pantoyl lactone are important intermediates in the synthesis of D-(+)-pantothenic acid. The chemical process for producing pantoic acid or pantoyl lactone yields a racemic mixture of the D-(-)- and L-(+)-enantiomers. The racemic mixture must thus be resolved to obtain the D-(-)-enantiomer, while the L-(+)-enantiomer must be either discarded or reracemized. Hence, it is practically desirable to develop a stereoselective process giving high yield of the D-(-)-enantiomer from appropriate starting materials.

Several enzymatic and chemical processes using ketopantoyl lactone as a starting material have been proposed or investigated. Lanzilotta et al.[3)] reported that ketopantoyl lactone is stereoselectively reduced to D-(-)-pantoyl lactone with Byssochlamys fulva cells as the catalyst. The same reduction was reported to occur chemically under catalysis by the rhodium complex.[77,78)]

In previous Chapters, I described an efficient chemomicrobiological process which involves one-pot chemical synthesis of ketopantoyl lactone from isobutyraldehyde, diethyl oxalate, sodium methoxide and formalin, followed by its stereoselective reduction to D-(-)-pantoyl lactone with microbial cells as the catalyst. In the course of these studies, I found that the stereospecific oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone is also a promising reaction for the practical production of

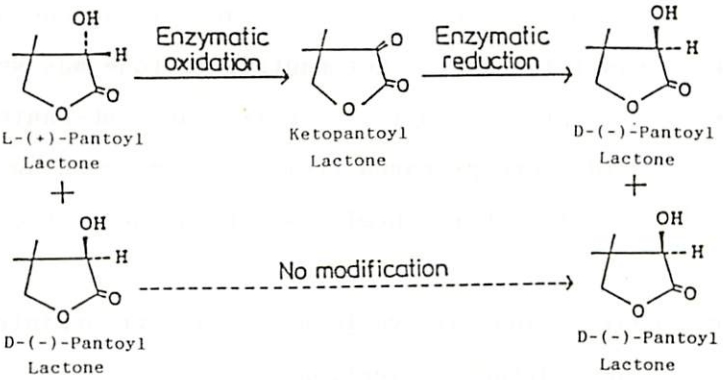


Fig. 1. Schematic Representation of the Enzymatic Oxidation-reduction System for the Production of D-(-)-Pantoyl Lactone from a Racemic Mixture of Pantoyl Lactone

D-(-)-pantoyl lactone, because racemic pantoyl lactone is still one of the most readily available intermediates for D-(-)-pantoyl lactone synthesis. If the stereospecific oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone in a racemic mixture of pantoyl lactone becomes possible without any modification of the remaining D-(-)-enantiomer, the above mentioned stereoselective reduction system[3,79] might also be applicable to the conversion of the ketopantoyl lactone formed to D-(-)-pantoyl lactone, as shown in Fig. 1. Based on this idea, I screened microorganisms for the ability to convert only the L-(+)-enantiomer of pantoyl lactone to ketopantoyl lactone. As a result of the screening, Nocardia asteroides AKU 2103 and several other microbial strains were found to be potent catalysts for the conversion. The data presented here show that a racemic mixture of pantoyl lactone can be converted efficiently to D-(-)-pantoyl lactone with a high yield through the use of such a stereoselective enzymatic oxidation and reduction system.

MATERIALS AND METHODS

Chemicals. D-(-)-, L-(+)- and DL-pantoyl lactone were purchased from Tokyo Kasei (Japan). Ketopantoyl lactone was synthesized as described in Chapter 1. D-(-)-, L-(+)- and DL-Pantoic acid and ketopantoic acid were prepared from the corresponding lactones as described.[23] All other chemicals were as described in previous Chapters.

Microorganisms and cultivations. All microorganisms used were obtained from AKU culture collection.

The following media supplemented with 0.5% L-(+)-pantoyl lactone were used for cultivations. Medium A for molds and yeasts contained 5% malt extract (Difco, USA) and 0.3% yeast extract (Oriental Yeast, Japan), pH 5.6. Medium B for actinomycetes contained 1% Polypepton (Daigo Nutritional, Japan), 0.5% fish (bonito) extract (Mikuni, Japan), 0.1% yeast extract and 0.5% NaCl, pH 7.0. Medium B1, which contained 1.2% 1,2-propanediol, 1.5% vitamin-free casitone (Difco, USA), 0.5% fish extract, 0.1% yeast extract and 0.5% NaCl, pH 7.0, was also used for the cultivation of actinomycetes. Medium C for bacteria contained 1% glycerol, 1.5% Polypepton, 0.3% yeast extract, 0.3% K_2HPO_4 and 0.2% NaCl, pH 7.0. Medium D for basidiomycetes contained 1% glucose and 0.5% Ebios (Ebios Pharmaceuticals, Japan), pH 5.0. In the screening experiment, each strain was inoculated into 5 ml medium in a test tube (16.5 x 165 mm) and then incubated at 28°C with reciprocal shaking (240 strokes/min) for 2 to 5 days. For a large-scale cultivation, a 2-l shaking flask contained 500 ml medium was used. The cells were collected by centrifugation and then used for the reaction described below.

Screening for microorganisms capable of oxidizing L-(+)-pantoyl lactone to ketopantoyl lactone. First screening. Each reaction mixture containing cells from 5-ml culture and 15 mg (0.12 mmol) L-

(+)-pantoyl lactone in 3.0 ml 200 mM potassium phosphate buffer, pH 7.0, was incubated in a test tube (16.5 x 165 mm) at 28°C for 48 h with reciprocal shaking (240 strokes/min). After removal of the cells by centrifugation, the supernatant was analyzed for pantoyl lactone and ketopantoyl lactone, and the ratio of D-(-)- and L-(+)-pantoyl lactone was determined, as described below.

Second screening. This was performed for the microorganisms selected in the first screening. The microorganisms were grown in medium B supplemented with 1.5% 1,2-propanediol for 2 days as described above. Each reaction mixture containing cells from 5 ml culture, 45 mg $CaCO_3$ and either of 90 mg L-(+)-pantoyl lactone or 180 mg DL-pantoyl lactone in 3.0 ml 200 mM potassium phosphate buffer, pH 7.0, was incubated as described above. When the conversion of D-(-)-pantoyl lactone was being investigated, L-(+)-pantoyl lactone was replaced by an equimolar amount of D-(-)-pantoyl lactone.

Optimization of the culture and reaction conditions for the oxidation of L-(+)-pantoyl lactone. The experiments were carried out with washed cells of Nocardia asteroides AKU 2103 throughout. The details are given in the legends to the respective Figures and Tables.

Preparative scale reaction for the conversion of DL-pantoyl lactone to D-(-)-pantoyl lactone. The oxidation reaction was carried out with cells of N. asteroides. The microorganisms was cultivated in 500 ml medium B1 in a 2-l flask. Three 5 ml 2 day precultures in the same medium were inoculated followed by incubation for 2 days at 28°C with reciprocal shaking (120 strokes/min). The cells were harvested by centrifugation and the resulting wet cells (0.7 g as dry matter) were then incubated with 5.0 g DL-pantoyl lactone and 1.5 g $CaCO_3$ in 100 ml 200 mM potassium phosphate buffer, pH 7.0, at 28°C

with shaking (150 strokes/min). On the fourth day, the mixture was adjusted to pH 1.0 with 6 N H_2SO_4, heated at 80°C for 15 min and then centrifuged. The supernatant after adjustment of the pH to 4.0 with 6 N NaOH was used for the reduction reaction.

The reduction reaction was carried out with cells of Candida parapsilosis IFO 0784. The yeast was cultivated in 500 ml medium A for 3 days. The other cultivation conditions were the same as those for N. asteroides. To the supernatant (ca. 100 ml) 5.0 g glucose and wet cell paste of C. parapsilosis (1.5 g as dry weight) was added. The mixture was incubated for 2 days at 28°C with shaking (150 strokes/min). The reaction was stopped by removal of the cells by centrifugation.

Analysis of pantoyl lactone and ketopantoyl lactone. All the pantoates in the supernatant (1.0 ml) were converted to the corresponding lactone forms by adding 1.0 ml 6 N HCl followed by heating at 80°C for 15 min. To the lactonized solution 1.0 ml ethyl acetate was added, and then the mixture was vigorously shaken for 1 min. The resultant organic layer was analysed for pantoyl lactone and ketopantoyl lactone by GLC as described previously (see Section 1, Chapter 2). Unless otherwise stated, I used the terms pantoyl lactone and ketopantoyl lactone for a mixture of pantoyl lactone and pantoic acid and one of ketopantoyl lactone and ketopantoic acid, respectively, because the analytical method cannot distinguish lactone forms from the corresponding acid forms. The ratio of D-(-)- and L-(+)-pantoyl lactone was determined by diastereomer conversion with L-menthyl chloroformate (see Section 1, Chapter 2).

Other methods. Isolation of D-(-)-pantoyl lactone from the reaction mixture was carried out as described in Section 2, Chapter 2. Mass and ^{1}H-NMR spectra were measured with a Hitachi H-80 and

Nicolet NT-360 apparatus, respectively. Dry cell weight was read from a calibration curve of the absorbance of the culture broth at 610 nm against the dry cell weight, determined by drying the cells in an oven at 110°C overnight.

RESULTS

Selection of microorganisms showing high ketopantoyl lactone productivity

The ability to oxidize L-(+)-pantoyl lactone to ketopantoyl lactone was screened for in 606 type culture strains including 165 strains of bacteria (28 genera), 42 strains of actinomycetes (4 genera), 196 strains of yeasts (27 genera), 162 strains of molds (40 genera) and 41 strains of basidiomycetes (13 genera). Corynebacterium equi IAM 12443, Gluconobacter suboxydance IFO 3172, Nocardia asteroides AKU 2103, N. corallina AKU 2105, Rhodococcus erythropolis AKU 2106 and Streptosporangium roseum JCM 3005 were

Table 1. Oxidation of Pantoyl Lactone to Ketopantoyl Lactone by the Selected Microorganisms
Conditions for cultivation and reaction were as in Materials and Methods (second screening).

Microorganism	Ketopantoyl lactone formed (mg/ml) from			
	L-(+)-Pantoyl lactone 30 mg/ml		DL-Pantoyl lactone 60 mg/ml	D-(-)-Pantoyl lactone 30 mg/ml
Nocardia asteroides AKU 2103	25.5	11.3[a]	21.8	0.01
Nocardia corallina AKU 2105	18.3	6.1[a]	6.7	0.01
Rhodococcus erythropolis AKU 2106	22.3	9.5[a]	17.9	0.01
Corynebacterium equi IAM 12443	15.4	5.2[a]	5.8	0.01

[a] Values on incubation without $CaCO_3$.

found to produce ketopantoyl lactone with a greater than 20% molar conversion. Through this screening, I selected four strains listed in Table 1 for further investigation.

In order to increase the ketopantoyl lactone yield, the oxidizing activity of the selected strains was assayed under the conditions of 30 mg/ml of L-(+)-pantoyl lactone and 90 mg/ml of DL-pantoyl lactone as substrates. Excellent oxidation was found in the reaction mixtures with N. asteroides and R. erythropolis (Table 1). In all cases, the addition of $CaCO_3$ to the reaction mixture was necessary for conversion with high yields (see also Fig. 3b). The yields of ketopantoyl lactone from DL-pantoyl lactone were significantly low in all strains tested, when compared with those from the corresponding amount of L-(+)-pantoyl lactone alone. When the cells were incubated with D-(-)-pantoyl lactone in place of L-(+)-pantoyl lactone, oxidation did not occur, the D-(-)-pantoyl lactone remaining unmodified throughout the reaction. Since N. asteroides showed the most potent activity, I used it in the following experiments.

Optimization of the culture conditions for the preparation of N. asteroides cells showing high producibility

Inducers, which might raise the L-(+)-pantoyl lactone oxidizing activity of N. asteroides cells, were screened for. Supplementation L-(+)- or D-(-)-pantoyl lactone or the corresponding acids (0.1-1.0%) to medium B was ineffective for increasing enzyme formation and the addition of ketopantoyl lactone (0.25%) prevented growth of the cells. Various compounds having a polyol structure were also examined. 1,2-Propanediol (1.0%) markedly increased the oxidizing activity as well as the cell growth (Fig. 2). The maximum production of ketopantoyl lactone was attained with cells grown with 1.2% 1,2-

propanediol. Above this concentration, the activity markedly decreased although the cell growth still increased.

The effects of carbon sources, nitrogen sources and organic nutrients on the activity were also investigated using medium B supplemented with 1.0% 1,2-propanediol. The addition of vitamin-free casitone (1.5%) in place of Polypepton or increase in the Polypepton concentration to 1.5% caused a further increase in the activity of cells. The addition of glucose (1.0%) or fructose (1.0%) repressed the enzyme formation. Soybean hydrolysate (0.5%; Ajinomoto, Tokyo) and yeast extract (0.5%) were found to be effective as fish extract, when each of them was used in place of it. The addition of inorganic nutrients was generally ineffective.

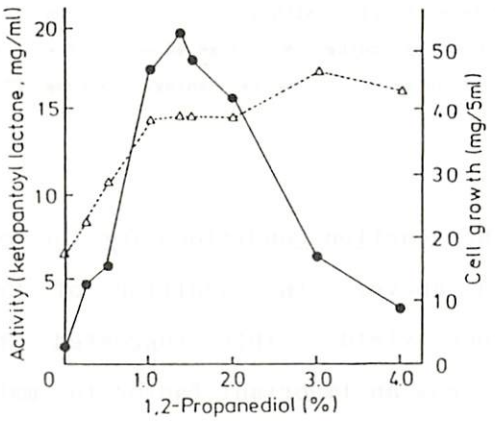


Fig. 2. Effects of the 1,2-Propanediol Concentration on the Growth of N. asteroides and Its L-(+)-Pantoyl Lactone Oxidizing Activity

N. asteroides was cultivated under the conditions given in Materials and Methods, in medium B supplemented with 1,2-propanediol as indicated. The reactions were carried out with 30 mg/ml of L-(+)-pantoyl lactone under the same conditions as those for the second screening. The oxidizing activity was expressed as ketopantoyl lactone formed in mg/ml under the above mentioned conditions. ●, Activity; △, growth.

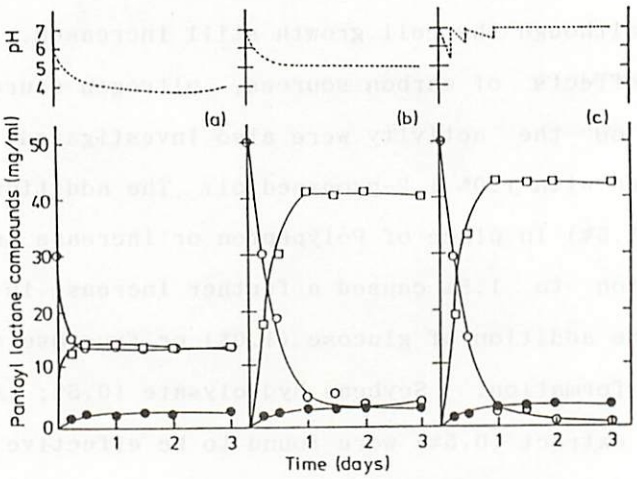


Fig. 3. Tome Course of L-(+)-Pantoyl Lactone Oxidation by N. asteroides Cells
The cultivation was carried out in a modified medium B1, which contained 1.5% Polypepton in place of vitamin-free casitone. The reaction was carried out with 30 (a) or 50 (b, c) mg/ml of L-(+)-pantoyl lactone with (b) or without (a,c) $CaCO_3$. In (c), the pH of the reaction mixture was adjusted to 7.0 by adding 1 N NaOH as shown. Other conditions were the same as those for the second screening. ○, L-(+)-Pantoyl lactone; □, ketopantoyl lactone; ●, D-(-)-pantoyl lactone.

Optimization of the reaction conditions for the oxidation

As mentioned above, the addition of $CaCO_3$ improved the ketopantoyl lactone yield. This suggested that the pH of the reaction mixture was an important factor in making the oxidation reaction more efficient. To investigate this further, I measured the change in pH during the oxidation with L-(+)-pantoyl lactone as a substrate. As shown in Fig. 3a, in parallel with the consumption of L-(+)-pantoyl lactone, the corresponding ketopantoyl lactone appeared with a gradual shift of the pH to the acidic side. During the first 10 h, ca. 15 mg/ml ketopantoyl lactone was accumulated and the pH of the mixture dropped to 4.5, after which there was no increase in the

ketopantoyl lactone yield. On the other hand, the reaction initiated by the addition of $CaCO_3$ gave larger than 40 mg/ml ketopantoyl lactone with a molar conversion larger than 90%. In this case, the pH change was more gradual and the pH of the mixture never reached 5.0 (Fig. 3b). Similarly, adjustment of the pH to 7.0 with 1 N NaOH during the reaction gave a ketopantoyl lactone yield of 44 mg/ml (Fig. 3c). In all cases, the accumulation of a small amount of D-(-)-pantoyl lactone occurred, suggesting that a part of the ketopantoyl lactone formed was further converted to D-(-)-pantoyl lactone.

Table 2 shows that the molar conversion to ketopantoyl lactone

Table 2. Effects of Substrate Concentration on the Formation of Ketopantoyl Lactone by N. asteroides Cells

Conditions for cultivation and reaction were as in Materials and Methods (second screening), except for the indicated concentrations of substrates.

Substrate (mg/ml)	Compounds found (mg/ml)			Ratio[a]
	L-(+)-Pantoyl lactone	Ketopantoyl lactone	D-(-)-Pantoyl lactone	
L-(+)-Pantoyl lactone				
30	0.6	24.7	4.7	0.98
50	4.3	42.1	3.6	0.91
60	5.8	47.3	6.8	0.90
100	35.3	61.5	3.1	0.65
DL-Pantoyl lactone				
50	3.6	19.5	26.7	0.92
60	6.2	21.7	32.2	0.90
70	10.2	22.6	37.2	0.85
80	16.2	24.0	41.8	0.82
90	21.6	22.9	45.5	0.76
100	26.2	23.3	50.5	0.74

[a] Molar ratio of ketopantoyl lactone plus D-(-)-pantoyl lactone to added substrate.

(plus D-(-)-pantoyl lactone) after 2 days was >90% with concentrations of L-(+)-pantoyl lactone of up to 60 mg/ml. In contrast, significant amounts of L-(+)-pantoyl lactone remained when the equimolar amount of DL-pantoyl lactone was used as a substrate in place of L-(+)-pantoyl lactone. For example, about 15% of the added L-(+)-pantoyl lactone (25 mg/ml) remained unchanged after the reaction with 50 mg/ml DL-pantoyl lactone as a substrate, while 20 to 30 mg/ml of the L-(+)-pantoyl lactone in the reaction mixture from which D-(-)-pantoyl lactone had been omitted was almost completely oxidized. Under the conditions given in Table 2, 19 to 25 mg/ml ketopantoyl lactone was produced regardless of the concentration of DL-pantoyl lactone up to 100 mg/ml. Since the data in Table 2 strongly suggested the D-(-)-enantiomer interfered with the oxidation of the L-(+)-enantiomer, I measured the rate of the oxidation of the L-(+)-enantiomer (initial concentration, 30 mg/ml) under the conditions with pH adjustment with NaOH given in Fig. 3c. The oxidation proceeded almost linearly for 2 days at a rate of 0.041 mg L-(+)-pantoyl lactone oxidized /h/mg cells. The same amount of cells oxidized the substrate at rates of 0.27 and 0.29 mg/h/mg when L-(+)-pantoyl lactone was used as a substrate at concentration of 30 and 60 mg/ml, respectively. Figure 4 shows the changes in the initial rate of oxidation with various concentrations of L-(+)-pantoyl lactone. The addition of D-(-)-pantoyl lactone (40 mg/ml) to each reaction mixture strongly repressed the rate of oxidation. Double reciprocal plots of the data suggested that the inhibition by the D-(-)-enantiomer was competitive. Considering these results, one possible way of overcoming this interference by the D-(-)-enantiomer would be to increase the activity per unit cells or more simply to increase the cell mass used as the catalyst.

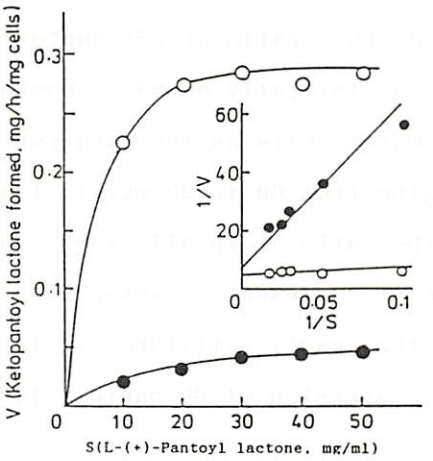


Fig. 4. Effect of D-(-)-Pantoyl Lactone on the Initial Rate of Oxidation of L-(+)-Pantoyl Lactone by N. asteroides Cells

The cells (13 mg/ml) were incubated for 4 h with the indicated concentration of L-(+)-pantoyl lactone with (●) or without (○) D-(-)-pantoyl lactone (40 mg/ml). Other conditions were the same as those for the second screening. The insert represents double reciprocal plots of the data.

Table 3. Effect of Cell Concentration on the Formation of Ketopantoyl Lactone

Conditions for cultivation and reaction were as in Materials and Methods (second screening), except for the indicated concentrations of cells and DL-pantoyl lactone.

Cell added	DL-Pantoyl lactone added	Compound found			Ratio[a]
		L-(+)-Pantoyl lactone	Ketopantoyl lactone	D-(-)-Pantoyl lactone	
	mg/ml	mg/ml	mg/ml	mg/ml	
14.7	60	8.0	20.7	31.3	0.87
16.7	60	5.0	22.9	32.1	0.92
20.3	60	3.6	22.4	34.0	0.94
24.3	60	3.0	23.9	33.1	0.95
24.3	70	4.3	27.8	37.9	0.94
24.3	80	7.3	30.2	42.6	0.91
24.3	90	10.6	31.2	48.2	0.88

[a] Molar ratio of ketopantoyl lactone plus D-(-)-pantoyl lactone to DL-pantoyl lactone added.

As shown in Table 3, increasing the cell mass as the catalyst remarkably improved the ratio of ketopantoyl lactone plus D-(-)-pantoyl lactone to the initially added DL-pantoyl lactone. Under the conditions of 24.3 mg/ml cells as the catalyst and DL-pantoyl lactone concentrations ranging from 60 to 90 mg/ml, I assayed the ketopantoyl lactone yield and the ratio. In all cases, nearly 90% of the added DL-pantoyl lactone was present as ketopantoyl lactone plus D-(-)-pantoyl lactone in the reaction mixture (Table 3).

Preparative scale conversion of DL-pantoyl lactone to D-(-)-pantoyl lactone

Firstly, I assayed the conversion of the formed ketopantoyl lactone to D-(-)-pantoyl lactone using several fungal strains previously shown to have potent ketopantoyl lactone reduction activity (see Section 1, Chapter 2). For this purpose, reaction mixtures, each of which (3.0 ml) had been incubated with N. asteroides cells (24.3 mg/ml) and DL-pantoyl lactone (80 mg/ml) under the conditions given in Table 3, were prepared. They contained ketopantoyl lactone and D-(-)-pantoyl lactone at concentrations in the ranges 28-33 and 41-44 mg/ml, respectively. To the lactonized supernatant (ca. 3 ml) 150 mg glucose and cell past of each fungus grown in 5 ml medium A was added under the conditions given in Materials and Methods and then the mixture was shaken in a test tube (16.5 x 165 mm) for 2 days at 28^{o}C and 240 strokes/min. The highest accumulation of D-(-)-pantoyl lactone (72 mg/ml) was found in the mixture with C. parapsilosis IFO 0784.

Preparative scale conversion was carried out as described under Materials and methods. The supernatant after the oxidation reaction with N. asteroides cells contained ketopantoyl lactone, D-(-)-pantoyl lactone and L-(+)-pantoyl lactone at concentrations of 20.0, 27.4 and

1.5 mg/ml, respectively. After the reduction with C. parapsilosis cells, the concentrations of those lactone compounds were 1.7, 43.7 and 3.5 mg/ml, respectively. From this mixture, 2.1 g pantoyl lactone was isolated. The sample showed 98.0% purity (based on weight), as judged on GLC. Other analytical data were as follows: optical purity as D-(-)-pantoyl lactone, 100% e.e.; mp. 91°C; $[\alpha]_D^{25}$ (C = 1, H_2O) -48.0°; ^{1}H-NMR (DMSO-d_6, δ), 1.00 (s, 6H), 3.82 (s, 2H), 3.96 (d, J = 6 Hz, 1H), 5.78 (d, J = 6 Hz, 1H); MS, m/Z 71, 57, 55, 53, 45, 43, 41, 39, 31, 29, 27.

DISCUSSION

Several microbial reactions for discriminating chiral or prochiral positions in molecules of pantoyl lactone or related compounds have been proposed to be applicable to the preparation of D-(-)-pantoyl lactone, since conventional optical resolution of this compound is very troublesome. D-(-)-Pantoyl lactone was obtained by degradation of the L-(+)-enantiomer in a racemic pantoyl lactone mixture.[80] In this case, however, the L-(+)-enantiomer unavoidably has to be discarded. Therefore, the molar yield of the D-(-)-enantiomer from DL-pantoyl lactone is never reached larger than 50%. Hydrolysis of L-(+)-pantoyl lactone to L-(+)-pantoic acid by means of a hydrolase was also shown to be useful for recovering the D-(-)-enantiomer from a racemic mixture of pantoyl lactone.[81] However, the process involving this reaction requires the lactonization of the L-(-)-pantoic acid followed by reracemization. As a result, the overall process is very complicated.

The stereoselective microbial reduction of ketopantoyl lactone seems to be one of the promising methods for the practical

preparation of D-(-)-pantoyl lactone, because it requires no optical resolution step and allows a high product yield without any interfering side reactions.[3,79] At present, however, DL-pantoyl lactone is still more readily available than ketopantoyl lactone. The conventional chemical oxidation of pantoyl lactone with bromine to yield ketopantoyl lactone is still costly for the practical purposes because of cost of bromine. Furthermore, the chemical oxidation allows the oxidation of the useful D-(-)-enantiomer. On the other hand, the specific microbial oxidation of L-(+)-pantoyl lactone reported here easily rules out the use of ketopantoyl lactone and can be connected to the above mentioned reduction reaction. Furthermore, cost for preparation of cells as the catalysts is not so high. Therefore, it may be useful for the practical preparation.

The L-(+)-pantoyl lactone oxidizing activity in N. asteroides cells was highly enhanced when they were cultivated in a medium containing 1,2-propanediol, but not L-(+)-pantoyl lactone. This suggests that 1,2-propanediol itself and/or its metabolites such as acetol and lactaldehyde may be inducers of the oxidation enzyme. The relatively limited distribution of the activity, i.e., most frequently in certain coryneform bacteria, may be also explained in terms of the metabolism of propanediol and related secondary alcohols, since they frequently show the ability to oxidize them and/or utilize them for growth.[82-86]

SUMMARY

A novel enzymatic process for the synthesis of D-(-)-pantoyl lactone from a racemic mixture of pantoyl lactone is described. The process involves the stereospecific oxidation of the L-(+)-enantiomer

of pantoyl lactone to ketopantoyl lactone followed by its stereoselective reduction to the D-(-)-enantiomer. The oxidation is carried out with cells of Nocardia asteroides AKU 2103 as the catalyst, which convert only the L-(+)-enantiomer of pantoyl lactone to ketopantoyl lactone without any modification of the remaining D-(-)-enantiomer. With 80 g/l of DL-pantoyl lactone as the substrate, larger than 90% of the added L-(+)-enantiomer was converted to ketopantoyl lactone under the optimum reaction conditions. The ketopantoyl lactone that accumulated in the reaction mixture was almost specifically converted to the D-(-)-enantiomer of pantoyl lactone on incubation with cells of Candida parapsilosis IFO 0784. Since this process is simple and requires no reracemization step, which is necessary for conventional chemical resolution, it is highly advantageous for the practical synthesis of D-(-)-pantoyl lactone.

Section 2. One step microbial conversion of a racemic mixture of pantoyl lactone to optically active D-(-)-pantoyl lactone[j)]

D-(-)-pantoyl lactone is an important intermediate for the chemical synthesis of D-(+)-pantothenic acid. The conventional synthetic process for D-(-)-pantoyl lactone involves reactions that yield racemic pantoyl lactone from isobutyraldehyde, formaldehyde, and cyanide and optical resolution of the racemic pantoyl lactone to D-(-)-pantoyl lactone. A drawback of this process is the troublesome resolution of the racemic pantoyl lactone followed by reracemization of the remaining enantiomer.

Several methods to omit the resolution-reracemization step have been reported. Lanzilotta et al.[3)] reported that ketopantoyl lactone is stereoselectively reduced to D-(-)-pantoyl lactone when Byssochlamys fulva cells are used as a catalyst. Candida parapsilosis, Rhodotorula minuta, and several fungal strains also were shown to be useful for this enzymatic reduction (see Section 1, Chapter 2). Achiwa et al.[77)] and Ojima et al.[78)] demonstrated that the same reduction also occurred with catalysis by the rhodium complex.

On the other hand, there has been no report of the utilization of racemic pantoyl lactone for the preparation of D-(-)-pantoyl lactone, other than the cases involving a reracemization step. Because racemic pantoyl lactone is still more readily available than ketopantoyl lactone, I attempted to use it for such a microbial process that would be free from reracemization. For this purpose I screened microorganisms that are capable of catalyzing such a conversion. I found that washed cells of Rhodococcus erythropolis convert the L-(+)-enantiomer in the racemic pantoyl lactone mixture

to the D-(-)-enantiomer without any modification of the coexisting D-(-)-enantiomer. Based on this finding, I report here a novel and efficient one-step conversion method for the preparation of D-(-)-pantoyl lactone from a racemic mixture of pantoyl lactone. The reactions involved in this conversion are also elucidated. Because this method is simple and requires no reracemization step, which is necessary for the conventional chemical resolution, it is highly advantageous for the practical preparation of D-(-)-pantoyl lactone.

MATERIALS AND METHODS

Chemicals. Analytical samples of D-(-)-, L-(+)-, and DL-pantoyl lactone were purchased from Tokyo Kasei (Japan). Ketopantoyl lactone was synthesized as shown in Chapter 1. Ketopantoic acid was prepared from ketopantoyl lactone by alkaline hydrolysis.[4] All other chemicals have been described previously (see Section 1, Chapter 5).

Microorganisms and cultivation. All bacterial strains used in this study were cultures that are preserved in AKU culture collection. Each organism were grown in a medium containing 1.2% 1,2-propanediol, 1% Polypepton (Daigo Nutritional, Japan), 1.5% soybean hydrolyzate (Ajinomoto, Japan), 0.15% yeast extract (Oriental Yeast, Japan), and 0.4% K_2HPO_4, pH 7.5. Usually, cultivation were carried out in test tubes (16.5 x 165 mm) containing 5 ml of the medium at 28°C for 3 days with shaking (240 strokes/min). For large-scale cultivations, a 2-l shaking flask containing 500 ml of the medium was used. The cells were collected by centrifugation and then used for the reaction, as described below.

Screening for microorganisms capable of converting L-(+)-pantoyl lactone to D-(-)-pantoyl lactone. Cells from a 5-ml culture were

suspended in 3 ml of 200 mM potassium phosphate buffer, pH 7.0, containing 90 mg (0.69 mmol) of L-(+)-pantoyl lactone and 45 mg of $CaCO_3$, and then the mixture was shaken in a test tube (16.5 x 165 mm) for 2 days at 28°C and 240 strokes/min. After the cells were removed by centrifugation, the supernatant was analyzed for pantoyl lactone and ketopantoyl lactone, and the ratio of D-(-)- and L-(+)-pantoyl lactone was determined, as described below.

Reactions with washed cells of R. erythropolis. R. erythropolis IFO 12540, which was selected as the most promising catalyst for the conversion through the screening procedure mentioned above, was grown for 3 days, as described above. The cells were collected by centrifugation, washed with deionized water, and then used for the reaction described below.

(i) Reaction with L-(+)-pantoyl lactone. The washed cells (35 mg as dry weight; usually obtained from a 5-ml culture) were shaken in 3 ml of 200 mM potassium phosphate buffer, pH 7.0, containing 50 mg of $CaCO_3$ and 30 mg (0.23 mmol) of L-(+)-pantoyl lactone for 10 h, as described above. When the conversion of ketopantoyl lactone or ketopantoic acid to D-(-)-pantoyl lactone was being investigated, L-(+)-pantoyl lactone was replaced by an equimolar amount of a respective ketopantoyl compound.

(ii) Reaction with DL-pantoyl lactone. The washed cells (35 to 70 mg) were shaken in 3 ml of 200 mM potassium phosphate buffer, pH 7.0, containing 50 mg of $CaCO_3$ and 60 mg (0.46 mmol) of DL-pantoyl lactone for 2 days as described above. On day 3, 150 mg of glucose and 0.1 mg of $CoCl_2$ were added, and then the mixture was shaken for an additional 2 days.

(iii) Preparative scale reaction and isolation of D-(-)-pantoyl lactone. A 500-ml flask with baffle plates containing 100 ml of a

reaction mixture, which contained 2.0 g of DL-pantoyl lactone, 1.5 g of $CaCO_3$, 20 mmol of potassium phosphate buffer, pH 7.0, and 150 mg of cells, was shaken on a rotary shaker for 2 days at 28°C and 260 rpm. On day 3, 5 g of glucose and 33 mg of $CoCl_2$ were added, and the mixture was shaken for an additional 4 days and then the mixture was centrifuged. The supernatant was adjusted to pH 1.0 with 6 N H_2SO_4 and heated at 80°C for 15 min. The lactonized compound in the supernatant were extracted with 100 ml of methyl isobutyl ketone after Na_2SO_4 (35g) was added. The organic layer was evaporated under reduced pressure. The pantoyl lactone in the residue was crystallized from toluene (yield 1.41 g). The analytical data were as follows: purity as pantoyl lactone as judged by GLC (see Section 1, Chapter 2), 96.0% (based on weight); optical purity, 100% e.e.; mp., 91°C; ^{1}H-NMR (DMSO-d_6, δ), 1.00 (s, 6H), 3.82 (s, 2H), 3.96 (d, J = 6 Hz, 1H), 5.78 (d, J = 6 Hz, 1H); MS, m/Z 71, 57, 55, 53, 45, 43, 41, 39, 31, 29, 27.

Analyses. All the pantoates in the supernatant (1.0 ml) were converted to the corresponding lactone forms by adding 1.0 ml of 6 N HCl and heating 80°C for 15 min. To the lactonized solution was added 1.0 ml of ethyl acetate, and then the mixture was vigorously shaken for 1 min. The resultant organic layer was analyzed for pantoyl lactone and ketopantoyl lactone with a gas liquid chromatograph (GC-7A; Shimadzu) equipped with a flame ionization detector. Details of the measurement have been described previously (see Section 1, Chapter 2). Unless otherwise stated, I used the terms pantoyl lactone and ketopantoyl lactone for a mixture of pantoyl lactone and pantoic acid and for a mixture of ketopantoyl lactone and ketopantoic acid, respectively. For measurement of the actual amount of the lactone forms, the supernatant without

lactonization was analyzed for pantoyl lactone and ketopantoyl lactone. The actual amount for pantoic acid was calculated by subtracting the pantoyl lactone value before lactonization from that after lactonization. Similarly, the actual amount for ketopantoic acid was determined as the difference between the ketopantoyl lactone values with and without lactonization. The ratio of D-(-)- and L-(+)-pantoyl lactone was determined by the diastereomer conversion method with L-menthyl chloroformate (see Section 1, Chapter 2).

Other methods. Cell dry weight was read from a calibration curve of the optical density of the culture broth at 610 nm against the cell dry weight, determined by drying the cells in an oven at 110°C overnight. Mass and ^{1}H-NMR spectra were measured with Hitachi M-80 and Nicolet NT-360 apparatuses, respectively.

RESULTS AND DISCUSSION

Formation of D-(-)-pantoyl lactone from L-(+)-pantoyl lactone

Using various bacterial strains, I preliminarily assayed the activity of the conversion of L-(+)-pantoyl lactone to D-(-)-pantoyl lactone, as described above. This activity was widely found in actinomycetes belonging to the genera Rhodococcus, Nocardia, Actinomadura, Nocardioides, Rothia, and Pseudonocardia (Table 1). In all cases ketopantoyl lactone was also detected as another reaction product. Conversely, there was no detectable conversion of the D-(-)-enantiomer to the L-(+)-enantiomer, to ketopantoyl lactone, or both (data not shown). As R. erythropolis IFO 12540 showed the most potent activity, I used it in the following experiments.

Reactions involved in the conversion of L-(+)-pantoyl lactone to D-(-)-pantoyl lactone

Table 1. Formation of D-(-)-Pantoyl Lactone from L-(+)-Pantoyl Lactone by Selected Actinomycetes

Strain	Concentration of the products found (mg/ml)	
	D-(-)-Pantoyl lactone	Ketopantoyl lactone
Nocardia sulphurea IFO 13270	4.06	10.2
Nocardia rugosa JCM 3193	1.78	17.1
Nocardia opaca IAM 12123	1.18	9.70
Rhodococcus erythropolis IFO 12540	7.05	7.37
Rhodococcus rhodochrous JCM 3202	1.21	19.0
Nocardioides albus IFO 13917	0.784	0.912
Rothia dentocariosa IFO 12531	0.579	0.716
Actinomadura cremea IFO 14182	0.175	0.606
Pseudonocardia fastidiosa IFO 14105	0.912	0.655

As the results mentioned above suggest that the conversion proceeded through stereospecific oxidation-reduction reactions with ketopantoyl lactone as an intermediate, I investigated the mechanism of the conversion in some detail. Surprisingly, neither ketopantoyl lactone nor D-(-)-pantoyl lactone was detected in the reaction mixture with L-(+)-pantoyl lactone as the substrate throughout the reaction, when the mixture was analyzed without lactonization. Only a decrease in the L-(+)-pantoyl lactone concentration with reaction time was detected. Both ketopantoyl lactone and D-(-)-pantoyl lactone were detected only after lactonization, and the sum of their amounts in the reaction mixture corresponded well to that of the consumed L-(+)-pantoyl lactone (Fig. 1a). Similarly, no ketopantoyl lactone or D-(-)-pantoyl lactone was detected when ketopantoyl lactone was used as the substrate in place of L-(+)-pantoyl lactone. Again, the ketopantoyl lactone that was initially added to the

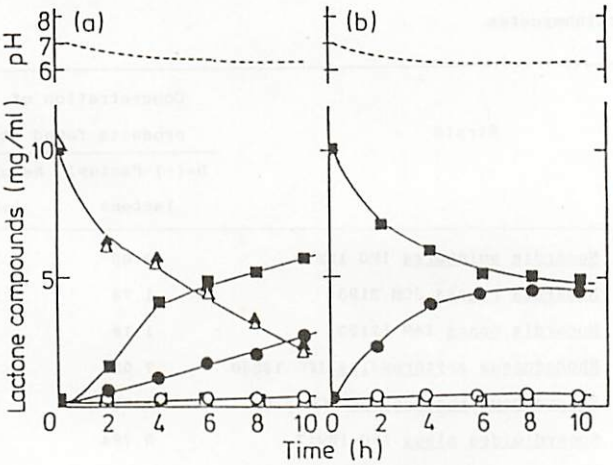


Fig. 1. Formation of D-(-)-Pantoyl Lactone from L-(+)-Pantoyl Lactone (a) or Ketopantoyl Lactone (b)

Reactions in six tubes were started at the same time under the conditions described in the text. The supernatant from one test tube was analyzed every 2 h. Symbols: ○ and ●, D-(-)-pantoyl lactone; □ and ■, ketopantoyl lactone; △ and ▲, L-(+)-pantoyl lactone. Open and closed symbols indicate the values before and after lactonization, respectively.

reaction mixture was completely recovered as a mixture of ketopantoyl lactone and D-(-)-pantoyl lactone only after lactonization (Fig. 1b). These results suggest that both the ketopantoyl and D-(-)-pantoyl compounds are present as the corresponding acid forms, i.e., ketopantoic acid and D-(+)-pantoic acid, respectively; and there were no side reactions other than those yielding ketopantoic acid and D-(-)-pantoic acid. Ketopantoyl lactone is known to be spontaneously hydrolyzed to ketopantoic acid under mild neutral to alkaline conditions similar to those used for the experiments shown in Fig. 1.[4] On the other hand, pantoyl lactone is hardly hydrolyzed to pantoic acid at all.[4] Therefore, the formation of ketopantoic acid was suggested to be the result of hydrolysis of the ketopantoyl

lactone formed from L-(-)-pantoyl lactone. In fact, hydrolysis was observed when ketopantoyl lactone was incubated without cells as the catalyst. Furthermore, the rate of hydrolysis was almost the same as that with cells (more than 1 mg/ml/min under the same conditions for the experiments shown in Fig. 1b), suggesting that the major part of hydrolysis is nonenzymatic. Other evidence supporting ketopantoic acid formation is the rapid acidification of the reaction mixture on incubation without $CaCO_3$ (data not shown). From these results it can be concluded that ketopantoic acid is produced through the enzymatic oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone followed by its spontaneous hydrolysis. When ketopantoic acid was used instead of ketopantoyl lactone, the reduction proceeded at essentially the same rate as that for the experiment shown in Fig. 1b, which shows that the substrate for the reduction to pantoic acid is ketopantoic acid and not ketopantoyl lactone. The reduction also proceeded stereospecifically to yield the D-(-)-enantiomer. Therefore, this reduction is distinguishable from that of ketopantoyl lactone to D-(-)-pantoyl lactone reported previously (see Section 1, Chapter 2). D-(-)-Pantoic acid might be formed through the action of some kind of carbonyl reductase such as ketopantoic acid reductase (EC 1.1.1.169). The D-(-)-pantoic acid formed could be easily lactonized by means of acid treatment.

Maintenance of the pH of the reaction mixture near neutrality seemed to be important for the effective conversion of L-(+)-pantoyl lactone to D-(-)-pantoic acid for the following reasons. The optimum pH for the enzymatic oxidation was 7.0 to 8.0 (data not shown). Such conditions were maintained by the addition of $CaCO_3$. Through which ketopantoyl lactone was continuously removed from the reaction mixture and the enzyme for the oxidation was freed from inhibition by

the reaction product. The reduction of ketopantoic acid also proceeded effectively under such conditions (data not shown).

Considering all of these facts, the sequence of the reactions involved in this conversion is summarized in Fig. 2.

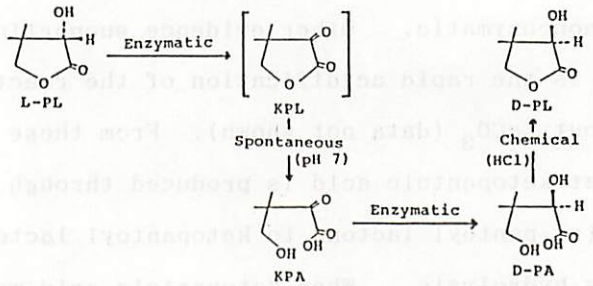


Fig. 2. The Reaction Sequence of the Stereospecific Oxidation-reduction System for the Conversion of Racemic Pantoyl Lactone to D-(-)-Pantoyl Lactone

Abbreviations: L-(+)-PL, L-(+)-pantoyl lactone; KPL, ketopantoyl lactone; KPA, ketopantoic acid; D-(+)-PA, D-(+)-pantoic acid; and D-(-)-PL, D-(-)-pantoyl lactone.

Production of D-(-)-pantoyl lactone from racemic pantoyl lactone mixture

The reaction sequence mentioned above would be applicable to the selective conversion of the L-(+)-enantiomer, in a racemic mixture of pantoyl lactone, to the D-(-)-enantiomer. First, I determined whether or not the rate of the oxidation of the L-(+)-enantiomer is influenced by the D-(-)-enantiomer. The amount of the unreacted L-(+)-enantiomer increasing initial concentration of the D-(-)-enantiomer (Table 2). With 10 mg of the D-(-)-enantiomer per ml, the rate of the conversion was 47.0%, whereas it was 82.2% on incubation without the D-(-)-enantiomer (Table 2). On incubation for an additional 38 h with 10 mg of the D-(-)-enantiomer per ml, the molar conversion of the added L-(+)-Pantoyl lactone (10 mg/ml) to

Table 2. Inhibition of the Oxidation of L-(+)-Pantoyl Lactone by D-(-)-Pantoyl Lactone[a]

Initial concentration of D-(-)-pantoyl lactone (mg/ml)	Concentration of the compounds found (mg/ml)			Rate of conversion %[b]
	L-(+)-Pantoyl lactone	Ketopantoyl lactone	D-(-)-Pantoyl lactone	
0	1.81	6.15	2.13	82.8
2.0	2.33	5.84	3.85	76.2
4.0	3.30	5.01	5.64	66.5
6.0	4.25	4.18	7.64	58.2
8.0	5.05	3.51	9.45	49.6
10.0	5.29	3.22	11.48	47.0

[a] Reaction conditions were the same as those for reactions with L-(+)-pantoyl lactone (10 mg/ml) given in the text, except for the addition of D-(-)-pantoyl lactone as indicated.

[b] Rate of conversion of the added L-(+)-pantoyl lactone to ketopantoyl lactone and D-(-)-pantoyl lactone.

ketopantoyl lactone and D-(-)-pantoyl lactone was 51 and 18%, respectively. In contrast, 96% of the added L-(+)-pantoyl lactone was present as a mixture of ketopantoyl lactone and D-(-)-pantoyl lactone, in a ratio of 69:27, in the reaction mixture without the D-(-)-enantiomer under the same reaction conditions. The inhibition of the oxidation reaction by D-(-)-pantoyl lactone was remarkably abolished by increasing the concentration of the cells. In the reaction mixture, however, the dominant product was ketopantoyl lactone, and further conversion to D-(-)-pantoyl lactone was still small (data not shown). To improve this the reaction conditions for the reduction were separately optimized by using ketopantoyl lactone as the substrate. The addition of glucose and $CoCl_2$ was found to promote this conversion. When 50 mg of cells was incubated in 3 ml of 200 mM potassium phosphate buffer, pH 7.0, containing 50 mg of $CaCO_3$, 60 mg (0.46 mmol) of ketopantoyl lactone, 150 mg of glucose,

and 0.1 mg of $CoCl_2$ for 4 days as described above, 88% of the added ketopantoyl lactone was recovered as D-(-)-pantoyl lactone. Based on these results, I determined the optimum reaction conditions to be those given in Materials and Methods. Table 3 shows the effect of cell concentration on the conversion of DL-pantoyl lactone to D-(-)-pantoyl lactone. In each case with more than 40 mg of cells, nearly

Table 3. Effect of Cell Concentration of Conversion of DL-Pantoyl Lactone to D-(-)-Pantoyl Lactone

Cells added mg/tube	Concentration of the compounds found (mg/ml)			%e.e. of D-(-)-pantoyl lactone
	L-(+)-Pantoyl lactone	Ketopantoyl lactone	D-(-)-Pantoyl lactone	
35	4.48	1.30	14.2	68.4
41	3.92	0.03	16.2	75.8
52	2.12	0.01	16.7	88.3
56	3.28	0.02	17.3	81.0
60	3.25	0.01	17.3	81.2
68	1.01	0.91	18.1	94.4

80% of the added DL-pantoyl lactone was present as D-(-)-pantoyl lactone in the reaction mixture. With 68 mg of cells, 18.1 mg of D-(-)-pantoyl lactone per ml was produced with a molar yield of 90.5% (94.4% e.e.).

A preparative scale conversion was carried out with 2.0 g of DL-pantoyl lactone as the substrate, as described above. After removal of the cells by centrifugation, the supernatant contained 1.78 g of D-(-)-pantoyl lactone, 0.11 g of ketopantoyl lactone, and 0.05 g of L-(+)-pantoyl lactone, from which 1.41 g of D-(-)-pantoyl lactone was obtained (yield 70.5%).

Because the present one-pot stereospecific oxidation-reduction system is simple and requires no reracemization step, it will be useful for the practical preparation of D-(-)-pantoyl lactone.

SUMMARY

Washed cells of Rhodococcus erythropolis IFO 12540 were found to convert only the L-(+)-enantiomer of pantoyl lactone to the D-(-)-enantiomer in a racemic mixture of pantoyl lactone. Under suitable reaction conditions, the amount of D-(-)-pantoyl lactone synthesized was 18.2 mg/ml (94.4% e.e.; molar yield, 90.5%). This conversion was suggested to proceed through the following successive reactions: first, the enzymatic oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone; second, the rapid and spontaneous hydrolysis of the ketopantoyl lactone to ketopantoic acid; and then, the enzymatic reduction of the ketopantoic acid to D-(-)-pantoic acid. After the reaction D-(-)-pantoic acid could be lactonized by means of acid treatment. During the conversion, the D-(-)-enantiomer, which was initially present in the reaction mixture, did not undergo any modification.

CONCLUSION

D-(-)-Pantoyl lactone is an important starting material for the chemical synthesis of D-(+)-pantothenic acid. The conventional synthetic process for D-(-)-pantoyl lactone involves reactions yielding racemic pantoyl lactone from isobutyraldehyde, formaldehyde and cyanide, and optical resolution of the racemic pantoyl lactone to D-(-)-pantoyl lactone. A disadvantage of this process in the troublesome resolution of racemic pantoyl lactone.

I have developed an efficient combined chemicoenzymatic method, which involves an efficient one-pot synthesis of ketopantoyl lactone as a substrate, followed by stereospecific reduction of it to D-(-)-pantoyl lactone using microbial cells as a catalyst. Ketopantoyl lactone is synthesized from isobutyraldehyde, diethyl oxalate, formalin and sodium methoxide. The reaction is performed in one-pot at room temperature with a yield of 81%. Stereoselective reduction of ketopantoyl lactone to D-(-)-pantoyl lactone is carried out with washed cells of Rhodotorula minuta IFO 0920 or Candida parapsilosis IFO 0708 as a catalyst and glucose as energy for the reduction. About 50 or 90 g/l of optically pure D-(-)-pantoyl lactone can be produced with a molar yield of nearly 100% by R. minuta or C. parapsilosis, respectively. The enzyme catalyzing this conversion was isolated as a crystalline form from C. parapsilosis cells and characterized. The enzyme catalyzing the reduction of ketopantoyl lactone to L-(+)-pantoyl lactone has been also isolated from Mucor ambiguus and characterized. It is a novel carbonyl reductase which specifically catalyzes the reduction of conjugated polyketone compounds.

Racemic pantoyl lactone can also be used as a starting

substrate. Nocardia asteroides AKU 2103 cells specifically oxidized the L-(+)-enantiomer in a racemic mixture of pantoyl lactone, which is then converted to D-(-)-pantoyl pantoyl lactone by the reduction with C. parapsilosis cells as described above. In these two enzymatic steps, the coexisting D-(-)-enantiomer remains without any modification. Under suitable conditions, 72 g/l of D-(-)-pantoyl lactone was obtained from 80 g/l of DL-pantoyl lactone. Similar specific oxidation and reduction reactions can also be carried out with a single microorganism as catalyst. On incubation with washed cells of Rhodococcus erythropolis IFO 12540, D-(-)-pantoyl lactone in the reaction mixture reached 18.2 g/l with a molar yield of 90.5% (optical purity, 94.4% e.e.). This unique conversion proceeds through the successive reactions as follows: (1) the enzymatic oxidation of L-(+)-pantoyl lactone to ketopantoyl lactone (the same enzyme as that in the N. asteroides has been suggested to be the responsible enzyme for this oxidation); (2) the rapid and spontaneous hydrolysis of ketopantoyl lactone to ketopantoic acid, and (3) the enzymatic reduction of the ketopantoic acid to D-(+)-pantoic acid. The enzyme catalyzing this reduction seemed to be ketopantoic acid reductase, because R. erythropolis cells could not utilize ketopantoyl lactone as substrate, different from C. parapsilosis.

Three types of microbial and enzymatic processes for the production of D-(-)-pantoyl lactone have been developed. Since this process is simple and requires no racemization process which has been operated in the conventional chemical resolution, it is highly advantageous for practical synthesis of D-(-)-pantoyl lactone.

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REFERENCES

1. H. Yamada and S. Shimizu, Angew. Chem. Int. Ed. Engl., 27, 622 (1988).
2. R. Kuhn and T. Wieland, Chem. Ber., 75B, 121 (1942).
3. R. P. Lanzilotta, D. G. Bladley and K. M. McDonald, Applied Microbiol., 130 (1974).
4. H. L. King, Jr, R. E. Dyar and D. R. Wilken, J. Biol. Chem., 249, 4689 (1974).
5. I. Ojima and T. Kogure, J. Organomet. Chem., 195, 239 (1980).
6. M. Seki, N. Baba, J. Oka and Y. Inoue, J. Org. Chem., 48, 1370 (1983).
7. O. Nagase, Y. Hosokawa and M. Shimizu, Chem. Pharm. Bull., 17, 398 (1969).
8. M. Matsumoto and S. Ito, Synth. Commun., 1, 697 (1984).
9. S. H. Lipton and F. M. Strong, J. Am. Chem. Soc., 71, 2364 (1949).
10. C. Broquet and J. Bedin, Bull. Soc. Chim. Fr., 1909 (1967).
11. F. Weygand, W. Steglich and H. Tanner, Ann.,658, 128 (1962).
12. J. Anatol and A. Medete, Bull. Soc. Chim. Fr., 189 (1972).
13. E. L. Eliel and A. A. Hartmann, J. Org. Chem., 37, 505 (1972).
14. D. Seebach, Synthesis, 1, 17 (1969).
15. C. W. Jefford and G. Barchietto, Tetrahedron Letters, 48, 4809 (1978).
16. L. Bassignani, A. Brandt, V. Caciagli and L. Re, J. Org. Chem., 43, 4245 (1978).
17. P. A. Manis and M. W. Rathke, J. Org. Chem., 45, 4952 (1980).
18. R. Fisher and T. Wieland, Chem. Ber., 93, 1387 (1960).

19. H. J. Hagemeyer, G. V. Hudson, S. H. Johnson, M. B. Edwards and H. N. Wright, Am. Chem. Soc., Div. Petrol. Chem., Preprints-Symposia 1, No. 2 (Chemicals from Petrol.), 63 (1956).

20. J. Schreiber, J. Bull. Soc. Chim. Fr., 61 (1952).

21. E. Gells and R. W. Hay, J. Chem. Soc., 3673 (1958).

22. H. L. King, Jr. and D. R. Wilken, J. Biol. Chem., 247, 4096 (1972).

23. D. R. Wilken, H. L. King, Jr. and R. E. Dyar, J. Biol. Chem., 250, 2311 (1975).

24. D. R. Wilken and R. E. Dyar, Arch. Biochem. Biophys., 189, 251 (1978).

25. D. R. Wilken and R. E. Dyar, Anal. Biochem., 112, 9 (1981).

26. R. G. Annett and P. K. Stumpf, Anal. Biochem., 47, 638 (1972).

27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).

28. E. T. Stiller, S. A. Harris, J. Finkelsteine, J. C. Kerenztesy and K. Folkers, J. Am. Chem. Soc., 62, 1785 (1940).

29. L. Ernster, Method Enzymol., 10, 309 (1975).

30. C. Martius, in The Enzymes (Boyer, P. D., ed.) 2nd. Ed, Vol. 7, pp. 517-532, Academic Press, New York (1963).

31. B. O. Hojberg, K. Blomberg, S. Stenberg and Ch. Lind, Arch. Biochem. Biophys., 207, 205 (1981).

32. Ch. Lind and B. Hojberg, Arch. Biochem. Biophys., 207, 217 (1981).

33. S. Hosoda, W. Nakamura and K. Hayashi, J. Biol. Chem., 249, 6416 (1974).

34. K. Murata, Y. Fukuda, K. Watanabe, M. Shimosaka, T. Saikusa and A. Kimura, Eur. J. Biochem., 151, 631 (1985).

35. P. Silber, H. Chung, P. Gargiulo and H. Schulz, J. Bacteriol., 118, 919 (1974).

36. O. Adachi, E. Shinagawa, K. Matsushita and M. Ameyama, Agric. Biol. Chem., 43, 75 (1979).

37. O. Adachi, T. Chiyonobu, E. Shinagawa, K. Mitsushita and M. Ameyama, Agric. Biol. Chem., 42, 2057 (1978).

38. J. M. Utting and L. D. Kohn, J. Biol. Chem., 250, 5233 (1975).

39. S. M. Arffin and H. E. Umbarger, J. Biol. Chem., 244, 1118 (1969).

40. N. Itoh, Agric. Biol. Chem., 46, 3029 (1982).

41. B. J. Davis, Ann. N. Y. Acad. Sci., 121, 404 (1964).

42. J. King and U. K. Laemmli, U. K., J. Mol. Biol., 62, 465 (1971).

43. A. Winter and C. Karlsson, LKB Application Note 219, LKB-Produkter AB, Sweden (1976).

44. P. Andrews, Biochem. J., 96, 595 (1965).

45. M. Bradford, Anal. Biochem., 72, 248 (1976).

46. H. Sawada, A. Hara, F. Kato and T. Nakayama, J. Biochem., 86, 871 (1979).

47. T. Nakayama, A. Hara and H. Sawada, Arch. Biochem. Biophys., 217, 564 (1982).

48. H. Sawada, A. Hara, T. Nakayama and F. Kato, J. Biochem., 87, 1153 (1980).

49. P. L. Hoffman, B. Wermuth and J. P. Von Warburg, Adv. Exp. Med. Biol., 132, 189 (1980).

50. L. Ernster, L. Danielson and M. Ljunggren, Biochem. Biophys. Acta, 58, 171 (1962).

51. V. L. Spitsberg and C. J. Coscia, Eur. J. Biochem., 127, 67 (1982).

52. W. W. Cleland, Ann. Rev. Biochem., 36, 77 (1967).

53. B. Wermuth, J. Biol. Chem., 256, 1206 (1981).

54. A. Cornish-Bowden, Biochem. J., 137, 143 (1974).

55. E. V. Ervin, B. Tabakoff and R. L. Bronaugh, Mol. Pharmacol., 7, 169 (1970).

56. A. J. Turner and K. F. Tipton, Biochem. J., 130, 765 (1972).

57. F. Hucho, U. Markau and H. Sund, Eur. J. Biochem., 37, 69 (1973).

58. Y.-M. Lin and J. Jarabak, Biochem. Biophys. Res. Commun., 81, 1227 (1978).

59. H. Thaler-Dao, B. Descomps, M. Saintot and A. Crastes de Paulet, Biochimie (Paris), 54, 83 (1972).

60. W. Daune and J. W. Hastings, Mol. Cell Biochem., 6, 53 (1975).

61. J. B. Jones and J. F. Beck, in Applications of biochemical systems in organic chemistry, part I (Jones, J. B., Sih, C. J. & Perlman, D., eds) pp. 107-401, J. Wiley, New York (1976).

62. J. Rètey and J. A. Robinson, Stereospecificity in organic chemistry and enzymology, p. 20, Verlag Chemie, Weinheim/Bergstr (1982).

63. K. E. Van Holde and R. L. Baldwin, R. L., J. Physic. Chem., 62, (1958).

64. H. K. Schachman, Methods Enzymol., 4, 32 (1957).

65. J. L. Hedrick and A. J. Smith, Arch. Biochem. Biophys., 126, 155 (1968).

66. M. M. Ris and J. P. Von Warburg, Eur. Biochem. 37, 69 (1973).

67. E. M. Tarmy and N. O. Kaplan, J. Biol. Chem., 243, 2579 (1968).

68. R. L. Felsted, M. Gee and N. R. Bachur, J. Biol. Chem., 249, 3672 (1974).

69. C. J. Sih, J. B. Heather, R. Sood, P. Price, G. Peruzzotti, L. F. Hsu Lee and S. S. Lee, J. Am. Chem. Soc., 97, 865 (1975).

70. J. A. Breuer, F. Breuer and H. Breuer, Acta Endocrinol., 58, 673 (1968).

71. H. Weiner and T. G. Flynn (eds.), Enzymology and Molecular Biology of Carbonyl Metabolism III, Aldehyde Dehydrogenase, Aldo-Keto Reductase and Alcohol Dehydrogenase, Alan R. Liss, New York (1987).

72. A. Horeau, Tetrahedron Lett., 15, 506 (1961).

73. J. D. Gilbert, C. J. W. Brooks, Anal. Lett., 6, 639 (1973).

74. M. S. J. Dewar, E. G. Zoebisch, E. F. Healy and J. J. P. Stewart, J. Am. Chem. Soc., 107, 3902 (1985).

75. B. Pfrunder and C. Tamm, Helv. Chim. Acta, 52, 1630 (1969).

76. A. Ohno, T. Goto, J. Nakai and S. Oda, Bull. Chem. Soc. Jpn., 54, 3478 (1981).

77. K. Achiwa, T. Kogure and I. Ojima, Chem. Lett., 297 (1978).

78. I. Ojima, T. Kogure, T. Terasaki and K. Achiwa, J. Org. Chem., 43, 3444 (1978).

79. H. Yamada and S. Shimizu, Biocatalysts in organic synthesis (Tramper, J., van der Plas, H.C. and Linko, P., eds) pp. 19-37, Elsevier, Amsterdam (1985).

80. Japanese patent 47-19745 (1972).

81. Japanese patent 57-152895 (1982).

82. O. Yagi and K. Yamada, Agric. Biol. Chem., 33, 1587 (1969).

83. S. Takao and M. Tanida, Nippon Nougeikagaku Kaishi, 51, 239 (1977).

84. K. R. Dhariwal and T. A. Venkitasubaramanian, J. Gen. Microbiol., 104, 123 (1978).

85. H. Yamada, A. Nagao, H. Nishise and Y. Tani, Agric. Biol. Chem., 46, 2333 (1982).

86. L. G. Lee and G. M. Whitesides, J. Org. Chem., 51, 25 (1986).

PUBLICATIONS

a. S. Shimizu, H. Yamada, H. Hata, T. Morishita, S. Akutsu and M. Kawamura (1987) Agric. Biol. Chem., 51, 289-290.

b. H. Hata, T. Morishita, S. Akutsu and M. Kawamura, submitted.

c. S. Shimizu, H. Hata and H. Yamada (1984) Agric. Biol. Chem., 48, 2285-2291.

d. H. Hata, S. Shimizu and H. Yamada (1987) Agric. Biol. Chem., 51, 3011-3016.

e. S. Shimizu, S. Hattori, H. Hata and H. Yamada (1988) Eur. J. Biochem., 174, 37-44.

f. H. Hata, S. Shimizu, S. Hattori and H. Yamada (1989) Biochim. Biophis. Acta, 990, 175-181.

g. H. Hata, S. Shimizu, S. Hattori and H. Yamada (1989) FEMS Microbial. Lett., 58, 87-90.

h. H. Hata, S. Shimizu, S. Hattori and H. Yamada, J. Org. Chem., in press.

i. S. Shimizu, S. Hattori, H. Hata and H. Yamada (1987) Enzyme Microb. Technol., 411-416.

j. S. Shimizu, S. Hattori, H. Hata and H. Yamada (1987) Appl. Environ. Microbiol., 53, 519-522.