Industrialization of the Microbial Resolution of Chiral C_3 and C_4 Synthetic Units: From a Small Beginning to a Major Operation, a Personal Account

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Abstract: This account describes the research and development of the microbial resolution of chiral C_3 and C_4 synthetic units through to the production stage. These chiral C_3 and C_4 synthetic units are mainly used for the production of various pharmaceuticals, new materials such as liquid crystals, chiral polymers, and natural compounds as well as in basic chemical research. The research started in 1983 and the industrial plant was built in 1994. The development is still ongoing and is being broadened to include C_4 chiral units, chiral propylene glycol, and so on. This project started as simple research on the activated sludge from an epichlorohydrin plant and evolved through many events and much research to an industrial production. We describe the various implications and the flow of events in the research and development through to the production of these chiral C_3 and C_4 synthetic units.

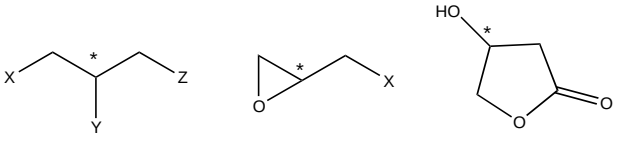
Keywords: butyronitrile; chiral C_3 and C_4 synthetic units; 4-chloro-3-hydroxybutyrate; 3-chloro-1,2-propanediol; epichlorohydrin; glycidol; halohydrin; 3-hydroxy-γ-butyrolactone; microbial resolution; propylene glycol

Abbreviation: Abbreviations: BN: butyronitrile; CHB: 4-chloro-3-hydroxybutyrate; EP: epichlorohydrin; DCP: 2,3-dichloro-1-propanol; 1,3-DCP: 1,3-dichloro-2-propanol; GLD: glycidol; HL: 3-hydroxy-γ-butyrolactone; PG: propylene glycol; compounds marked with * are optically active.

1 Introduction

We would like to describe our experiences leading to the production of chiral C_3 and C_4 synthetic units (Figure 1) in this review.

We have been involved in the production and development of chiral synthetic units for the past 10 to 20 years because we felt that chiral compounds would be quite important.[1] Now, looking back on the develop-



	a	b	c	d
X:	Cl,	Cl,	H,	Cl
Y:	Cl,	OH,	OH,	OH
Z:	OH,	OH,	OH,	CN or COOR

X: Cl, OH, OCOR

Figure 1. The basic chiral C_3 and C_4 synthetic units.

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Toshio Suzuki (right) is Manager and Group Leader of the Bio-Laboratory of Daiso Co. Ltd. where he has been working since 1988. He received his undergraduate education at the Biology Course of the Faculty of Science, Osaka City University in 1986, and then his MS from the graduate school of the Faculty of Agricultural Chemistry, Osaka Prefecture University in 1988. He received his Ph. D. from Osaka City University for the thesis "Study on microbial and enzymatic dehalogenation of haloalcohol" in 1994.

Drs. Kasai and Suzuki were awarded the Selection of Attractive Innovation Prize from the Science and Technology Agency in 1995, the Industrial Technology Prize in 1996 from the Society for Bioscience and Bioengineering, Japan, and the Invention Prize in the Kinki area from the Japan Institute of Invention and Innovation in 1996.

ment of our research, we can learn many things and, perhaps, provide some hints and tips for other researchers. Although the industrialization of our work may still be on a small scale, many important pharmaceuticals, liquid crystals, and various other chemicals have been made.[1] A characteristic of our work is not merely the simple production of optical active compounds, but rather to supply new starting chemicals for the preparation of novel optically active compounds. Therefore, it would be valuable to assess the implications of the work, to describe how this research and development started, and to supply some background information as well as our personal commentaries.

In the first part of the review, we deal mainly with the research, development and background of chiral C_3 synthetic units such as DCP*, EP*, CPD*, GLD*. In the second part, recent research and production results on chiral C_4 synthetic units such as BN*, CHB*, HL* and the C_3 chiral unit of PG* or 1,2-diols are addressed.

2 Outline of Chiral C_3 and C_4 Synthetic Units

Our chiral research started with DCP* and EP* and then progressed to the development of chiral C_3 synthetic units such as CPD*, GLD* PG*, 1,2-diols, chiral C_4 synthetic units such as BN*, CHB*, HL* and various derivatives thereof. The related background and the history of our study through to production are shown in Table 1. Other reports on the production of chiral C_3 and C_4 synthetic units are summarized in Table 2. It is clear that many events and reports were linked to each other.

3 The Beginning

In 1983 April, one of the authors (NK) began this study in the laboratory of Osaka Soda Co. Ltd. (presently Daiso Co., Ltd.) as the company's first true scientific researcher in the bio-field. Of course, there was no room and little research equipment. Methane fermentation using excess activated sludge was proposed by the company as my research topic. Some companies had already done preliminary trials of the fermentation so I clearly rejected the proposed topic on the first day. After some discussion with the head of the laboratories, Dr. Nose, I agreed to study the active sludge with regard to its bioreactions because I was interested in the production of the fine chemicals using enzymes or microorganisms, for example, propylene oxide and fructose production in the Cetus process[2] (Figure 2).

The Cetus process became known as the first joint project between the biotechnology and the petroleum fields. This project had a significant impact. Since Daiso is a major producer of sodium hydroxide, chlorine and epichlorohydrin in Japan, I thought that research on the preparation of halogenated compounds by bio-methods would be useful and interesting – that it would be worthwhile to investigate the reactions and search for potential uses of new chemicals from bio-methods. However, there was no plan for the production of EP* at that point in time.

Table 1. Milestones in the development of chiral C_3 and C_4 synthetic units.

Scale	Year	C_3 Chirals Topics and Remarks	C_4 Chirals
	1968	Castro et al.; DBP degradator	
	1978	Baldwin et al.; Synthesis of *(R)*- and *(S)*-EP from D-Mannitol	
	1979		
	1980	Cetus Process — Chiral EP generation, Stereoselective Lipases, Hydrolysis	
	1981		
	1982	Cambou and Klibanov et al. — Iriuchijima et al.; *(S)*-DCP-OAc (90%ee)	
	1983	Daiso started our study — *(R)*-DCP generation	
5 mL; EP* 0.1 g !	1984	*(R)*-EP from DCP — Ellis et al.; *(R)*-and *(S)*-EP via diastereomer of CPD	
	1985		
100 L	1986	Sharpless Oxidation; GLD 91%ee — EU PAT of DCP* — ICI S.C.Dr.Taylor visited Daiso Head Office — Chiral CPD generation	
1 KL	1987		
	1988	CHEMTECH and *(S)*-EP generation — Schurig; Complexation GC — Visiting EU	
5 KL	1989	FLC Dreaming — *(R)*-EP for EU — Janssen; Isolating of CPD degradator — *(R)*- *(S)*-CPD	
	1990	Kaneka CPD — assimulating bacteria/non-stereoselective	
5 KL/Fixed Reactor	1991	— Mutants isolated	
	1992	CPD USA PAT	
	1993	Merck Dreaming; Chiral Dep. Started — Glycidyl Tosylate; ANDENO, ARCO, KANEKA, Daiso — C_4 Chirals Generation BN — C_4 Chirals generation R-MH HL* formation	
94/12; 30 KL	1994	— Patent Selection	S-MH
	1995	— Academic Prize	
	1996	Amsterdam Colloquium — Patent Prize	
	1997		
	1998		
	1999	Jacobsen Catalyst	
	2000		
	2001		
	2002	Review writing	

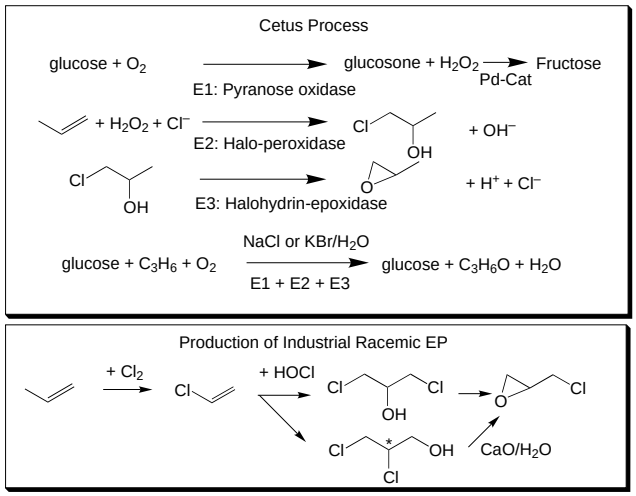


Figure 2. Reactions of the Cetus process and the industrial production of racemic epichlorohydrin.

4 EP*

Several comments and descriptions in books now describe the start of the production of optically active EP (EP*) as a bold step, a surprising, but realistic method. EP* was a real and desirable chiral C_3 synthetic unit, and the prototype of all other chiral C_3 synthetic units. In fact, much effort had already been devoted to this small, functionalized synthetic unit.

In 1978, a paper entitled "A synthesis of EP* from D-mannitol" published by Baldwin et al. of the Merck group[3] was the first report on the synthesis of pure EP*. Four years later, in 1982, papers entitled "The stereoselective hydrolysis using the lipase of 1-acetoxy-2,3-dichloropropane (DCP-OAc) and 1,2-diacetoxy-3-chloropropane (CPD-di-OAc)" were published by Iriuchijima et al.[4,5] DCP-OAc* of 90% ee was obtained, and *(S)*-propranolol (78% ee) was synthesized. Klibanov et al. also attempted the stereoselective ester conversion using lipases with DCP and tributyrin.[6] However, I did not know of these reports and found that half of the DCP was stereoselectively degraded. If I had been aware of the above papers, I would also have started screening for resolution of DCP-OAc, the same as these authors did. The complete resolution of DCP-OAc is difficult, but it seemed to be an attractive topic. I started to investigate the degradation activity of the activated sludge from a plant producing halohydrins and chlorinated low molecular aliphatic compounds. I thought that the degradation of halohydrins was easily done because the real activated sludge was working well to treat waste water.

Table 2. Chiral production of chiral C_3 and C_4 synthetic units.

Compound	Field	Method	Plant	Substrate	*R/S*	% ee	Loss of Enantiomer	Remarks
EP	Petrochemical	Organic synthesis	Yes	DCPs	–	0		
	Biological	Microbial resolution	Yes	DCP	*R/S*	> 99	Yes	
	Enzymatic	Lipase		DCP	*R/S*	> 99	Yes	Baldwin's halohydrins
		Lipase		Halohydrin	*R/S*	> 99	Yes	Baldwin's halohydrins
		Lipase		Glycidyl Ester	*R*	> 99	Yes	
		Epoxidation		AC	*S*	> 99		
		Epoxyhydrolase		EP	*R/S*	> 99		
	Asymmetric catalysis	Asymmetric hydration	Yes	EP	*R/S*	> 99	Yes	Jacobsen catalysis
	Organic chemistry	Organic synthesis		D-Mannitol	*R/S*	> 99		Multisteps, Baldwin's halohydrins
CPD	Petrochemical	Organic Chemistry		EP	–	–		
	Biological	Microbial resolution	Yes	CPD	*R/S*	> 99	Yes	
		Microbial resolution	Yes	CPD	*S*	> 99	Yes	
		Lipase		CPD	*R*	90	Yes	Ester
	Organic chemistry	Diastereomer isolation		CPD	*R/S*	100		
	Asymmetric catalysis	Asymmetric hydration	Yes	EP	*R/S*	> 99	Yes	Chiral EP* required/ Jacobsen catalysis
GLD	Asymmetric catalysis	Asymmetric oxidation	Yes	AA	*R/S*	91		Sharpless catalysis, peroxide and removal required
	Organic chemistry	Epoxide hydrolysis	Yes	CPD	–	0		
C_4	Biological	Esterase	Yes	CHB	*R*	> 99	Yes	Precursor synthesis required
		Microbial resolution	Yes	CHB	*R*	> 99	Yes	Precursor synthesis required/HL* formation
		Microbial/asymmetric reduction	Yes	COBE	*S*	> 99		Precursor synthesis required
	Organic chemistry	Organic chemistry	Yes	EP*	*R/S*	> 99		chiral EP* required

DCP: 2,3-dichloropropanol; DCPs: 2,3-dichloropropanol and 1,3-dichloro-2-propanol; AC: allyl chloride; EP: epichlorohydrin; CPD: 3-chloro-1,2-propanediol; CHB: 4-chloro-3-hydroxybutyrate; COBE: 4-chloro-3-oxobutyrate ethyl ester.

Epichlorohydrin: EP

Racemic EP is a C_3 compound derived from propylene and has a glycerol skeleton with a very reactive chlorine atom and an epoxy ring. The world output in 2001 was estimated to be over 1,200,000 tons, and it is mostly used as a cross-linking agent and a composition intermediate. For example, it is used for epoxy adhesives and as sealant for semiconductor or memory devices in electronics, in epoxy rubber (in applications such as fuel hoses in cars), or for various chemicals and medicines. Although it is being made wholesale, EP is epoxidized and distilled from 1,3-DCP and DCP by the reaction with alkali and water in industrial plants (see Figure 2). Considering this industrial continuous racemic reaction, the concentration of DCPs would not need to be so high. For the Cetus process, the reaction concentration was reported to be low. Batch-type production and continuous production are considered to be different.

EP, CPD and 1,3-DCP were easily and completely degraded in an inorganic medium. (Yes, the activated sludge is fundamentally a catalyst for degradation.) Many degrading bacteria and molds were easily isolated as the degrading microorganisms. In the latter, we had screened the stereoselective assimilating bacteria for CPD* production, but in the first CPD degradation experiment that year, CPD was easily and perfectly degraded and stereoselective degradation was not envisioned. I investigated the degradation routes, the

identification of the capability of the microorganisms and the effects of the concentration of halohydrins.

In November 1983, the degrading examination reached DCP. Checking for DCP degradation was not yet under study, because 1,3-DCP was the main halohydrin in industrial racemic EP production, and DCP was considered to be a minor compound in the actual industrial production of EP.[7] The degradation of 2,3-dibromo-1-propanol (DBP) had been reported in the literature.[8] This report was concerned with the degradation of DBP in polluted soil or water, the degradation route was postulated as DBP → epibromohydrin → 3-bromo-1,2-propanediol → glycidol → glycerol.[8] In the UK, Hardman and Slater had also studied the bacterial complete degradation of DCP from the aspect of environmental studies.[9,10]

DCP has some resistance to biodegradation; the enrichment culture was done for about two weeks. In December 1983, all the sealed test tubes become turbid and white, and some kinds of microorganisms were observed in the test tubes after the first week. Some strains grew well on the synthetic medium. I thought that DCP was also degraded, and this was also a reasonable result. However, I noted that all the degradation ratios of the test tubes were merely 50%. First, the neutralization was thought to be insufficient with the amount of added calcium carbonate, and then sufficient calcium carbonate was added and the degradation test was examined again. I carefully studied the above-mentioned papers by Iriuchijima et al., and found that they reported 90% ee of DCP-OAc by kinetic resolution using lipases, but the pure DCP* had not been obtained yet. The series of concentrations of DCP and excess washed cells were reacted together with enough calcium carbonate. I remember very clearly that I could not restrain my excitement for the analysis by gas chromatography. All the results showed a 50% degradation ratio at the stopping point, regardless of the added DCP concentration; the DCP degradation could be stereoselective. Analysis showed that *(R)*-DCP was degraded and the residual DCP was pure *(S)*-DCP. The first stereoselective resolution of DCP* had been achieved, and the microorganisms were isolated.[11] The stereoselective microorganisms were in a restricted area of soil around the plant. In the second screening for *(S)*-DCP assimilating bacterium, many completely degrading microorganisms were found from the same area. Anyway, this was the first preparation of pure *(S)*-DCP; this was the first time and consequently, the first example of a stereoselective microorganism for halohydrins[12,13].

In February 1984, a DCP assimilating bacterium was cultivated using a jar fermentor, and about 6 g of resolved *(S)*-DCP was obtained. The *(S)*-DCP was thought to be pure; however, *(S)*-DCP was often racemized in the reaction to give EP*. During the epoxidation, the liberated chlorine atom attached to the formed EP* to give the symmetrical 1,3-DCP. This 1,3-DCP was rapidly epoxidized 200 times faster than DCP to EP. Surprisingly, EP* was very easily racemized by mixing with simple sodium chloride solution. Synthetic researchers always said that a racemization was not supposed to occur so easily in the epoxidation reaction. Most people doubted if the production was even possible or a patent feasible. The practical production had been considered impossible for a long time in our research laboratory. Most of workers were synthetic and inorganic researchers, so this chain of thoughts might have been natural. I am a microbiologist and not well experienced in the synthesis of EP* from DCP*, however, I had been gradually establishing reaction conditions without racemization. One day in the spring of 1984, pure *(R)*-EP* (only 0.1 g) was obtained at last. This agreed well with the reported value of Baldwin's EP*[2] based on the specific rotation ($[\alpha]_D$: -33.3) and also NMR analysis.[3]

Chiral Analysis

Nowadays, the requirement for the chiral purity from the consumer is better than 99–99.5% ee. The general analysis of chiral purity is done by NMR and HPLC, which are easy and convenient; however, they are not sufficient because a highly sensitive detection is difficult. Complexation GC by Schurig,[14] and a chemically reacted cyclodextrin GC column[15] were recently developed, and the general analysis is now done quickly and with high performance. The minimum analysis sensibility is about 0.03% ee. In our chiral production, the analysis became easy and quick, and then the technology for the production and the guaranteed high chiral purity of the chiral substances were established. The production know-how was also efficiently obtained. In this connection, it is interesting that a recent Jacobsen catalyst is a Co-salen complex[16] and Co^{++} is most effective in the chelating complex for complexation GC.

The construction of an immobilized bioreactor was carried out for DCP* preparation,[12] because the amounts of available DCP* were not sufficient for study and our laboratory did not have a jar fermentor: the bacterium was cultivated using my old school equipment, and the harvested cells reused. In those days when most researchers still doubted the reality of our production of EP*, Mr. Nakata, vice-director, who was the chief of EP rubber, was interested in our EP*. He often said to me that you should do industrialization of EP*, and he supported us. Their group was interested in chiral polymers and the sequence of EP polymerization such as head-to-head or head-to-tail using EP*. The polymer derived from EP* was a crystalloid, whose melting point is observed to be in a narrow range. The EP* polymer would also be of some use in the future.

5 Turning Point

Changes always occur suddenly. In December 1986, after our patent had been granted, Dr. Taylor, the ICI biochemical group chief, visited our head office. He asked about our DCP stereoselective assimilation. According to his information, ICI already had used a stereoselectively assimilated halo-acid for their agrochemicals; however, the stereoselective degradation of halohydrin and its use was unique and potentially very interesting for chemical purposes. In April 1988, our patent was introduced in "TECHGRAM JAPAN " in "CHEMTECH"-1988, April: "2,3-Dichloro-1-propanol, the optically active precursor of epichlorohydrin, has been isolated by allowing a bacterium to consume the unwanted isomer. An optical purity in excess of 99% was obtained. Wasteful, but effective".[17]

Wasteful or Effective Method?

Many studies on the production of chiral units have been reported. As for the C_3 chiral units, racemic EP, CPD and propylene glycol are being produced on a huge scale. These can be produced at surprisingly low cost. Therefore, if one enantiomer is lost for the resolution, it is only a loss of about $ 1 at most. Even the cost of simple acetylation is over $ 2–3. Generally, getting a high chiral purity is the most important step. Repeating resolution or further purification after resolution to achieve $>99\%$ ee would lead to higher costs. Currently, the requirement for chiral purity from the user is above 99–99.5% ee. A chiral purity of $>99.8\%$ ee is necessary at least. Therefore, cost-performances of the catalyst, the starting material and catalyst specificity are very important. The interconversions of the C^3 and C^4 chiral units and related compounds are shown in Figure 3. Various routes are being developed to determine which is the best way.

In Japan, Professor Takano and Associate Professor Ogasawara of Tohoku University followed our work on DCP* and EP*. They studied the C_3 chiral unit of benzyl glycidyl ethers from D-mannitol and D-ascorbic acid.[18] They were interested in linking our EP* with their glycidyl ether, and developed EP* for the synthesis of natural products.

Furthermore, a pharmaceutical project using EP*, *(R)*-Levoprotiline, came from the Ciba-Geigy (Figure 4).[18] We were testing the 100-L bioreactor, and the 1000-L reactor was just being planned, but the 5000-L bioreactor was hurriedly started for the production of the EP* project. The 5000-L reactor was running while the 1000-L reactor remained in the planning stage. The project of *(R)*-Levoprotiline was stopped but the

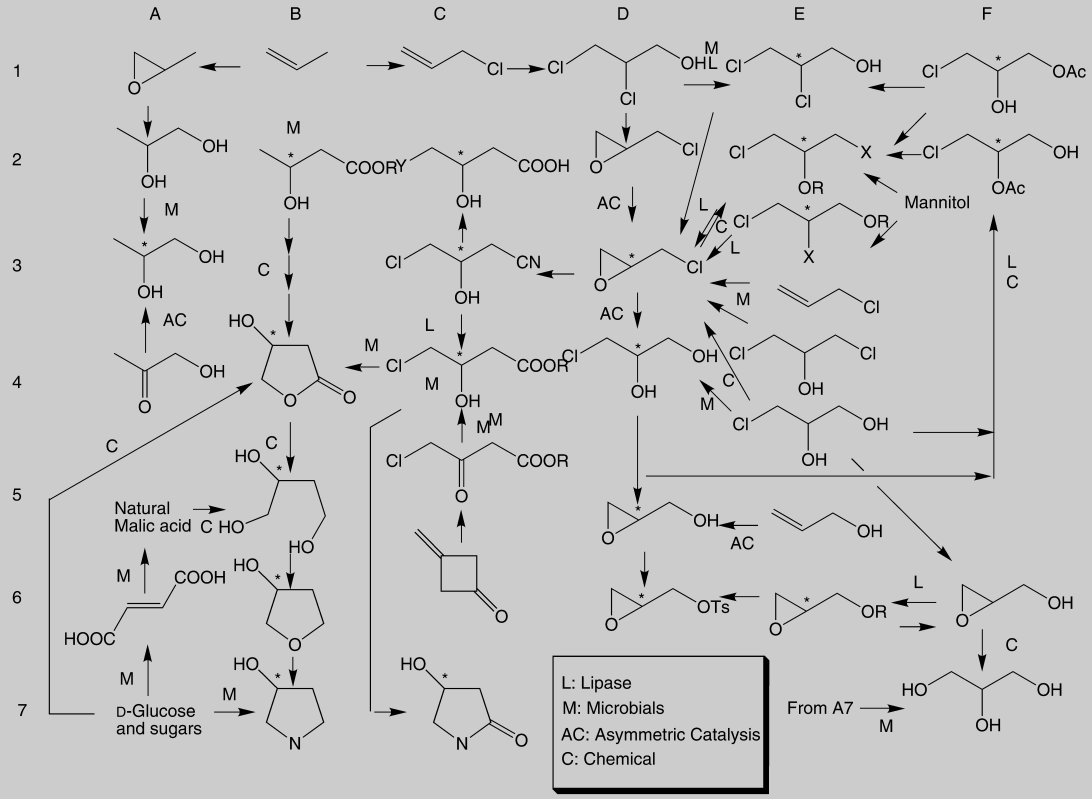


Figure 3. Linkages between C_3 and C_4 chiral units.

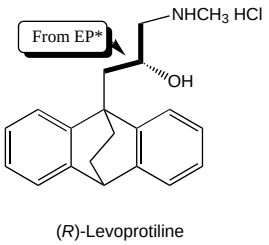


Figure 4. *(R)*-Levoprotiline pushed our *(R)*-EP* production forwards. Thick line is from EP*. This is a typical insertion of the EP* skeleton.

application using our EP* was a driving force for us to move on to the next stage.[19]

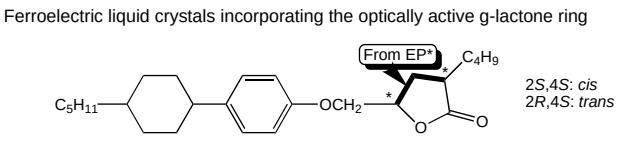
In the 5000-L scale-up, a bioreactor with beads of *(S)*-DCP producing bacterium immobilized in calcium alginate or acrylamide worked well without any problem. But, one day, various obstacles appeared; there was trouble due to mechanical damage and the appearance of the calcium alginate-degradation bacterium (alginate is a carbon source, the powerful assimilating bacterium had grown!).[20] The immobilized cell-beads were completely liquefied, and the reactor was stopped. We had to change our production method, however, a good result was found in the repeated production by the immobilized reactor. We had experience by which the *(R)*-DCP assimilating bacterium sometimes changed to the *(S)*-DCP assimilating bacterium type. The *(S)*-DCP, the opposite isomer, assimilating bacterium was hardly isolated although screening was repeated to use the obtained *(R)*-DCP. We confirmed the existence of the *(S)*-DCP assimilating bacterium type, the screening and the mutation were actively being continued.

The head of the laboratory, Dr. Nose, once quoted to me that Prof. Kenichi Fukui, a Nobel laureate and his former teacher, had said that "Both enantiomers are required, but it would be very difficult". Both isomers of EP* were desired, and *(S)*-EP was synthesized from *(R)*-EP.[1] However, the scale-up of the synthesis became tangled with the anti-catalyst in the hydrogenation. A synthesis researcher complained about the bad synthesis, we ourselves were compelled to do the synthesis and started to setting flasks, and on that day, the opposite type of bacterium, namely, the *(S)*-DCP assimilating bacterium was isolated at last.[13] We had succeeded to get both isomers in our hands (Figure 9).

In the same year, FLC (ferro-electric liquid crystals) were attracting attention for use in high-vision TVs and PC displays. FLC were generated in 1980 by Clark and Ragawal, Sweden. The FLC essentially needs asymmetric compounds. One day, the γ-lactone ring made from EP* was evaluated by an electronics company and showed very high-speed switching at the level of a world record (Figure 6).[21] A liquid crystals research group was rapidly establish, and the study of FLC using EP* was initiated. The use of the chiral unit began to be recognized as a profitable business project in our laboratories.

FLC and liquid crystals were considered to be in big demand for thin-liquid crystal TVs and PC displays in those days. The liquid crystal display has become the main stream with big markets: however, various types of displays such as the plasma display or a THF type are mainly produced for flat TVs and PCs now. FLC is a desirable form, but it is not yet the main type of liquid crystals. Further new chiral molecules would be desired.

The production of L-carnitine or *(R)*-GABOB (γ-aminobutyrate) using EP*[22] loomed large but sank elsewhere. In those days, the Kaneka group, a famous chemical and bio-chemical group, presented the production of CPD*.[23] They are famous for the production of hydantoin-hydantoinase, β-hydroxybutyrate, and they knew and studied well the production of chiral C_3 synthetic units. According to our information, *(R)*-CPD was studied for use as a chiral unit in the synthesis of antibiotics. Many persons switched their interest from EP* to CPD*.



Properties for mixtures of BSC-126 and optically active lactone compound

Content (wt %)		τ (μsec)
cis	10%	44
trans	10%	97

Figure 6. FLC molecule using EP*. Thick line is from EP*.

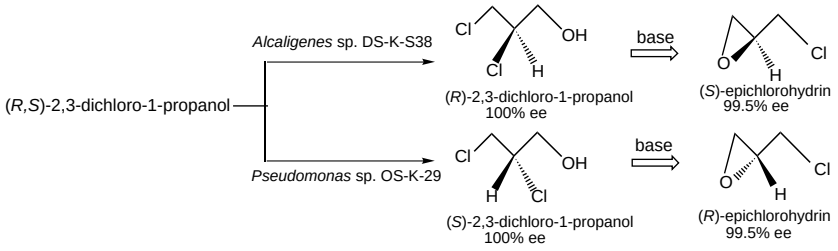


Figure 5. Microbial resolution of DCP.

6 CPD* Production

CPD* was not thought to be more important than EP*. EP* was considered to be equivalent to CPD* and to be more useful because of its reactivity. We also thought that CPD would be quickly and simply obtained from EP, but the answer was simple: racemization occurred in the ring opening. We had both EP* isomers, and both CPD* isomers (88% ee) were easily obtained from EP*. We then did screening with both CPDs* again, and the screening of our microbial resolution was considered easy. CPD is less toxic to the microorganisms, and many assimilating bacteria were isolated from the petroleum plant and tank yard of EP, but most of them were not stereoselective. The degradation test showed the same results as we had already found in the first year. We carried out many screenings using each isomer of CPD* and selected the desired strains. The desired microorganisms were found on medium containing each of the *(R)*-/*(S)*-CPD at last. Three excellent bacteria were isolated for the production of each isomer. The *(S)*-CPD assimilating bacteria all belonged to *Pseudomonas* sp. and the *(R)*-CPD ones were all *Alcaligenes* sp. CPD* and glycidol* (GLD*) were generated using the microorganisms (Figure 7).[24]

At about the same time in 1989, Professor Janssen of the Degradation of Halogenated Compounds Research Group, Groningen University, the Netherlands, also reported a CPD assimilating bacterium from soil.[25] Still, the degrading bacterium was not stereoselective. On the other hand, Kaneka's CPD* generation was a stereoselective metabolism using cells of microorganisms. We read their reports, presentations and studied the patent in detail. These researches and our bacteria for CPD* were seemingly very close to each other. Characteristic features of Kaneka's bacteria are that one optical isomer is metabolized *via* 3-chloro-2-keto-1-propanol. That is stereoselective, and the generated *(R)*-CPD* has highly optical purity, 100% ee; however, the cells of the microorganisms must be cultured in separate rich medium. The catalyst must be made and the cells must be harvested in another jar fermentor. The catalyst preparation costs would thus be high. Also, there was a difference in that our bacteria are all assimilators and could multiply with dehalogenation of CPD*: the resolution and the cell preparation (catalyst), is done at the same time. This means that the catalyst preparation cost is low. Surprisingly, the isolation of the stereoselective dehalogenating and assimilating microorganisms of CPD was the first report of that type, the same as for DCP.

In September 1997, in Amsterdam, a colloquium on "Halogenation and Dehalogenation" was held by the Royal Academy of Netherlands, where we met and discussed with Prof. Janssen and Dr. Hardman who studied CPD and DCP degrading bacteria for waste cleaners.[9] They did not think about stereoselective degrading. This was a parting of the ways, however, because each study was aimed at different applications and implications. The Cetus process also did not consider about stereoselectivity for propylene chlorohydrin.

7 Industrial Production of CPD* and EP*

The next status change also came suddenly. A big step-up for the C_3 chiral unit production was made for the HIV-1 protease inhibitor L-735 Indinavir by Merck with the possibility of really using the CPD* derivatives (glycidyl tosylate) (Figure 8).[26]

It was reported that glycidyl tosylate is necessary as a chiral synthetic unit. The producers of ANDENO (glycidyl butyrate by lipase), ARCO (chiral glycidol by Sharpless oxidation), Kaneka, and us, Daiso, had a chance to produce it. A chiral production group was established in our company. We had to scale-up,

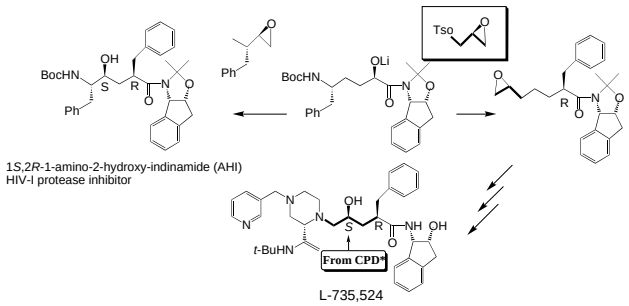


Figure 8. Report on Indinavir using glycidyl tosylate was another driving force. Thick line is from EP*.

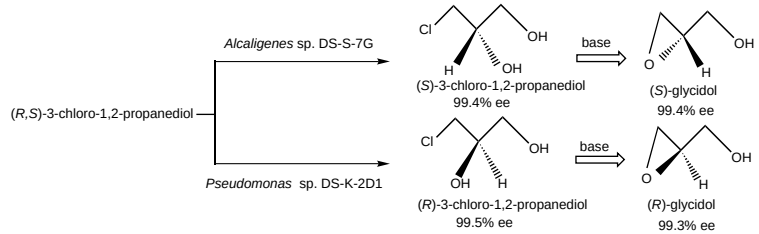


Figure 7. Microbial resolution of CPD.

although we had no experience on a larger microbial production scale.

The researchers in the chemical engineering field were gathered together, and worried about the black box of cultivation of microorganisms and the methods. Luckily, I had enough time to discuss this with Dr. Hasegawa of the Kaneka group at the symposium of the society for organic chemistry using enzymes. He was a famous researcher who had succeeded in the industrialization of chiral β-hydroxybutyrate, and showed me a very important point: his statement was that "Industrialization must never fail." This was well considered and understood, and we tried. In December 1994, the production of CPD* and EP* was established on a 30-kL scale. Of course, the production has never failed. The production has flourished since several mutants of the bacteria were obtained and the productivity was well improved. The production of C_4 chiral units has also been under development. The enzymatic study was also advanced.[27–31]

For Never Failing Production

The industrial production of C_3 and C_4 chiral compounds was carried out by a fed-batch system cultivation (treated amount of CPD, DCP becomes doubled). The control is somewhat difficult, but the cultivation is done using a controlling system and laboratory digital data. For the establishment of this controlling system, we did not have any experience and knowledge to run it on a large scale. We had to build it by ourselves; however, this finally produced a good result. We had gained some knowledge and hints for building it. Digital and continuous fermentation generated many mutants, and the production was well improved.[32]

Personal hints for the digital chiral production are as follows.

1. The optimization of methyl alcohol utilizing bacterium cultivation described in a lecture by Professor Emeritus Shimizu of Nagoya University provided an important strategy in fermentation. The cultivation of the bacterium was very difficult, but it grew well under a very limited ammonium concentration if the concentration was precisely controlled.

2. Cultivation is a chemical reaction and a reproducible result is maintained by digital data. In 1988, there was an opportunity to see the equipment in the research facilities of Professor Wandrey at Bonn University and KFA. Several sensors were placed in the cultivation vessel, and a personal computer was used to control it.

These facts were very impressive. Fortunately, I was interested in personal computers and programming, and some others had been trained. We began to collect as much digital data as much as possible from the black box.

CPD is stereoselectively assimilated and the bacterium (catalyst) multiplies using CPD* and the ammonium. A multiplication reaction for CPD* is shown in Figure 9.

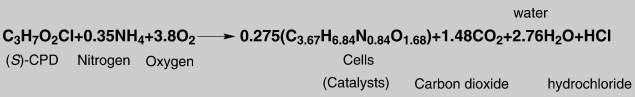


Figure 9. Reaction from CPD to cell.

Considering the chemical reaction, it is a very slow oxidation process. The heat is radiated as respiratory heat. This response was detected and controlled by the computer, and the multiplication of the catalyst and the deterioration were continuously monitored. The controlling system is of a fuzzy control form. The method is not the usual ON/OFF, but rather control imitating the skill of the advanced knowledge of an expert person. For the practical 30-kL industry plant, digital data and logic were used; the mechanical data of the 5000-L reactor and the many simulation data which were collected from several 1–2-L mini-jars were input to a PC. As for the cultivation types, all were done by fed-batch cultivation. The actual plant correlated very well with the collected data from the 1–2-L jar, and it became possible for duplication to be well produced, and the simulation was also well done.[32] As for the chemical reaction, such data digitization and control are thought to be very important.

Synthetic or Enzymatic Catalysts?

Enantiomer distinction of a halo-acid by an enzyme or microbial system has already been studied.[33,34] However, there were no resolutions of the halohydrin, and the stereoselective enzyme was not known yet. Janssen et al. clarified the mechanism of epoxyhydrolase aggressively,[35–37] while de Bond et al. reported the use of the enzyme.[38] It is said that the selectivity of the enzyme is too severe, and it was also stated that there is a problem with stability. However, does the same hold for the chiral synthetic catalysts?

Generally, the catalyst for chiral systems would be the same. As much as possible, both broad substrate specificity and sharp stereospecificity are desired. However, in principle, a catalyst is not able to satisfy both requisites. Recently, progress on biocatalyst engineering has been very rapid, mainly due to the development of directed evolution strategies. Gene shuffling and new high-throughput screening methods play an important role. Successful examples of the improvements in biocatalytic performance have

been reported in the literature, for example, the enhancement of thermostability, increased activity at extreme pH values, and improved enantioselectivity.

On the other hand, a synthetic catalyst can also be improved in specificity and stability by computer designing. Principally, a chiral surface only recognizes a chiral surface, which is ultimately a natural phenomenon, and the same is true whether the catalyst is an enzyme or synthetic. In the case of the enzyme, it is generally composed of chiral amino acids and is occasionally suitable for the proposed compounds, and then the targeted resolution is achieved. A microorganism is considered as a catalyst pool. This$_8$is because there are microorganisms of beyond 10 /g cells in the soil. The desired one must exist; the problem is how to select and isolate it.

8 Introduction of Chiral C_4 Synthetic Units

Next, we would like to focus on the optically active C_4 synthetic units, because they are very important as chiral building units for the syntheses of pharmaceuticals, agrochemicals, natural products and new materials just like the C_3 chiral building blocks.

It was believed that there were different uses and respective markets for the C_3 and C_4 chiral units for the synthesis of pharmaceutical intermediates in 1994 when we started our investigation. For example, optically active EP (EP*) and optically active CPD (CPD*) as C_3 chiral building blocks were applicable to several pharmaceutical intermediates through oxazolidinone derivatives; EP* was used for *(S)*-atenolol,[39] L-carnitine,[22,40] a nucleoside type of antiviral drug,[41] and ferroelectric liquid crystals.[21] On the other hand, optically active CPD* and its glycidyl derivatives could be used for the antibacterial agent Linezolid by Pharmacia,[42] the anti-HIV agent by Merck[26] and the protein kinase C-beta inhibitor by Eli-Lilly[43] and so on (Figure 10).

With respect to the C_4 chiral compounds, they can be moieties of many antibacterial agents represented by the carvapenem antibiotics and so on, for example, β-hydroxybutyrate and 1,3-butanediol. As shown in Figure 11, optically active 4-chloro-3-hydroxybutyrate (CHB*) and 4-chloro-3-hydroxybutyronitrile (BN*) are key compounds as C_4 chiral building blocks for the syntheses of L-carnitine,[40] L-GABOB,[44] β-hydroxybutyric acid, 3-hydroxy-γ-butyrolactone, and 4-hydroxy-2-pyrrolidone. From this point of view, we made the decision to mainly develop the production of BN* and CHB* as C_4 chiral units.

In Figure 3, the relationship between the C_3 and C_4 chiral building blocks is shown, and the methods for generating C_4 chiral units from C_3 chiral compounds are summarized. The basic route begins with EP* which is converted to CHB *via* BN by the addition of KCN and by alcoholysis; also CPD* is a key compound for conversion into these C_3 and C_4 derivatives.

Although the kinetic resolution of EP by the Jacobsen catalyst has been developed in the recent generation of EP*,[16] our self-developed microbial method based on the asymmetric assimilation of racemic DCP was known as a practical method at the beginning of the 1990's.[11–13]

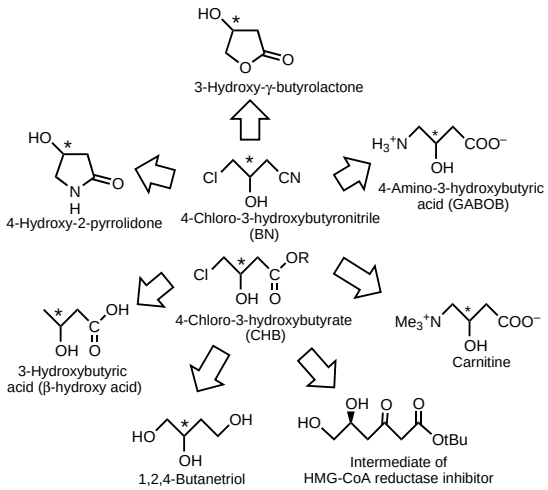


Figure 11. Potential of C_4 chiral chlorohydrins.

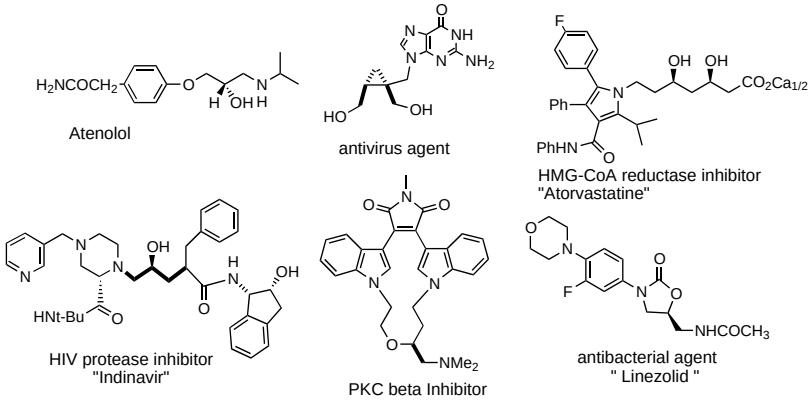


Figure 10. Synthetic applications of C_3 and C_4 chiral units.

Also, generation of BN* by the enzyme, halohydrin halide lyase[45] and CHB* by reduction technologies using the BINAP[46] catalyst and several reductase enzymes from several yeasts[47,48] were reported. But, some critical problems exist in these methods, i.e., only a single enantiomer can be produced and the obtained enantiomers often have a low enantiomeric excess. Moreover, expensive catalysts and co-enzymes are needed for the reaction.

Therefore, in the practical production of C_4 chiral building blocks, we have investigated unique methods for generating C_4 chiral units by haloalcohol dehalogenating enzymes so that these developed methods could be used for the production of both CHB* and BN*. In addition, BN and CHB have secondary alcohol and carboxylic acid functions, respectively. We used stereoselective ester degradation methods for the generation of these C_4 chiral materials. In those days, we obtained the interesting information that a Japanese pharmaceutical company was ready to generate a new antibiotic using *(R)*-4-hydroxy-2-pyrrolidone and we should mainly try the production of *(R)*-CHB.

In the following sections, we would like to focus on our developments for the generation of the C_4 chiral building blocks, BN, CHB and their derivatives, by haloalcohol dehalogenating enzymes and ester degradation enzymes. In particular, we would like to introduce the dual production of *(R)*-CHB and *(S)*-3-hydroxy-γ-butyrolactone (HL) in a one-pot reaction.

9 New Generation of Chiral C_4 Building Units

We carried out further screenings in order to prepare our desired C_4 chiral building blocks by more efficient methods, and these results are shown in Table 3. We developed the preparation of ethyl *(S)*-CHB (CHBE) and *(S)*-BN ($>98\%$ ee) in a yield of 30–40% using the resting cells of *Pseudomonas* sp. OS-K-29, which are capable of stereoselectively assimilating DCP.[49] These activities depend on the stereoselective dehalogenation of haloalcohols.

On the other hand, these newly isolated strains, belonging to the genera *Pseudomonas, Enterobacter, Citrobacter* and *Bacillus*, stereoselectively degraded ester compounds such as 4-chloro-3-acetoxybutyronitrile (BNOAc) and CHB. As a consequence, we developed the optical resolution of BNOAc and CHB as C_4 chiral building blocks. Thus, the BN*, BNOAc*, CHB*, and HL* that we developed as chiral C_4 building units have versatile potential for conversion into chiral C_4 compounds. For example, optically active GABOB, carnitine, 4-hydroxy-2-pyrrolidone, and 1,2,4-butanetriol were easily synthesized from these chiral C_4 synthons (see Figure 11).

In particular, the dual production of *(R)*-CHB ($>99\%$ ee) and *(S)*-HL ($>95\%$ ee) in a one-pot manner became possible on an industrial scale. This reaction mode attracted much interest and is important. We will describe these technologies in detail later.

Table 3. Generation of chiral C_4 building blocks.

Strain and substrate	Residual ratio [%]	Optical purity [% ee]	Products
Pseudomonas sp. OS-K-29			
4-Chloro-3-hydroxybutyronitrile	40	94.5 (*S*)	(*R*)-3,4-Dihydroxybutyronitrile
Methyl 4-chloro-3-hydroxybutyrate	56	56.0 (*S*)	(*R*)-Methyl 3,4-dihydroxybutyrate
Ethyl 4-chloro-3-hydoroxybutyrate	35	98.5 (*S*)	(*R*)-Ethyl 3,4-dihydroxybutyrate
Pseudomonas sp. DS-K-19			
4-Chloro-3-acetoxybutyronitrile	45	99.2 (*S*)	(*R*)-4-Chloro-3-hydroxybutyronitrile
Pseudomonas sp. DS-mk3			
4-Chloro-3-acetoxybutyronitrile	27	99.0 (*R*)	(*S*)-4-Chloro-3-hydroxybutyronitrile
Pseudomonas sp. DS-K-717			
4-Chloro-3-acetoxybutyronitrile	43	99.4 (*R*)	(*S*)-4-Chloro-3-hydroxybutyronitrile
Bacillus sp. DS-ID-819			
Methyl 4-chloro-3-hydroxybutyrate	43	91.9 (*S*)	(*R*)-3-hydroxy-γ-butyrolactone
Ethyl 4-chloro-3-hydoroxybutyrate	37	99.0 (*S*)	(*R*)-3-hydroxy-γ-butyrolactone
Pseudomonas sp. DS-K-NR818			
Methyl 4-chloro-3-hydroxybutyrate	40	98.5 (*R*)	(*S*)-3-hydroxy-γ-butyrolactone
Ethyl 4-chloro-3-hydoroxybutyrate	42	98.4 (*R*)	(*S*)-3-hydroxy-γ-butyrolactone
Enterobacter sp. DS-S-75			
Methyl 4-chloro-3-hydroxybutyrate	48	99.5 (*R*)	(*S*)-3-hydroxy-γ-butyrolactone
Ethyl 4-chloro-3-hydoroxybutyrate	43	99.8 (*R*)	(*S*)-3-hydroxy-γ-butyrolactone

Application of Haloalcohol Dehalogenase from *Pseudomonas* sp.

Based on the structure of the C_4 chiral compounds, BN and CHB that we are developing, we noted the following strategies; both compounds have a 4-chloro-3-hydroxy function, and therefore, we planned their new generation with an enzyme, haloalcohol dehalogenase from the strain *Pseudomonas* sp. OS-K-29, which is capable of stereoselectively assimilating DCP. The characterization and substrate specificity of the haloalcohol dehalogenase from *Pseudomonas* sp. OS-K-29 are shown in Figure 12. This enzyme was found to show a unique reaction mode, that is, this enzyme catalyzed the dechlorination reaction, thus stereoselectively converting the haloalcohol into the corresponding diol.

We then tried to apply the optical resolution of racemic BN using the resting cells, with the result that this resolution reaction gave (*S*)-BN at 94.5% ee in a yield of 35–40%. Upon further examination, use of the resting cells of *Pseudomonas* sp. OS-K-29 allowed us to produce ethyl (*S*)-CHB, as well as (*S*)-BN from the racemate (Table 3). For the 1% (v/v) racemic BN, ten cycles of the resolution reaction were carried out, and the activity was stable for at least 700 h on a 5-L vessel scale (Figure 13).

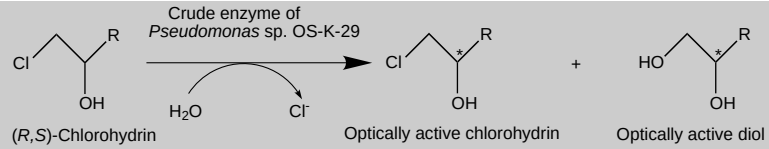


Figure 12. Reaction mode of haloalcohol dehalogenase from *Pseudomonas* sp. OS-K-29.

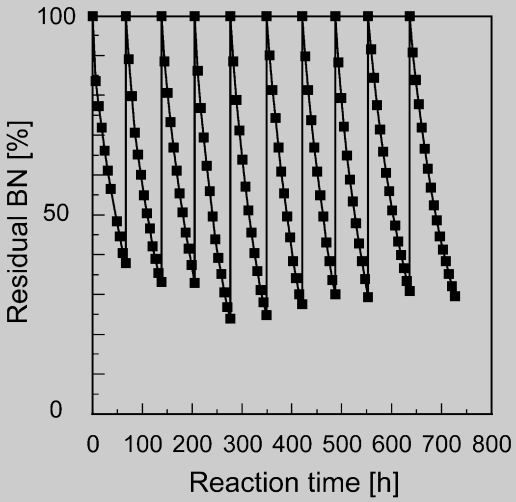


Figure 13. Repeated production of (*S*)-BN by *Pseudomonas* sp. OS-K-29.

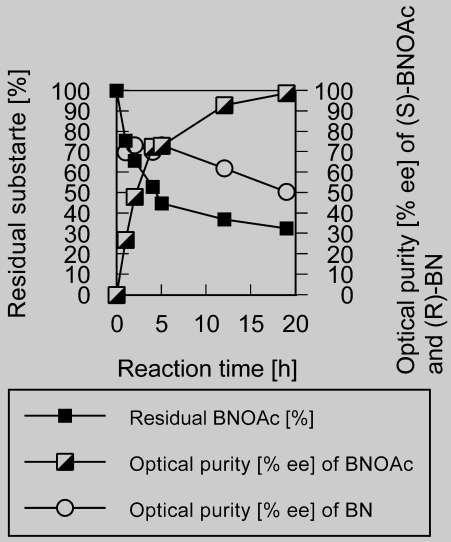


Figure 14. Production of (*S*)-BNOAc on a 1-L scale.

Production of BNOAc, a Secondary Alcohol Ester, by an Ester-Degrading Enzyme

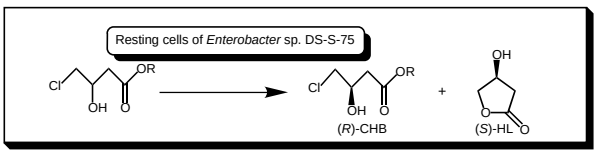
The preparation of optically active BN and its derivatives using our haloalcohol dehalogenases and the halohydrin halide-hydrogen lyase of Nakamura et al. is known.[45] We then tried to resolve (*R,S*)-BNOAc by a secondary alcohol ester-degrading enzyme. Several kinds of bacteria capable of stereoselective ester degradation were discovered. Above all, *Pseudomonas* sp. DS-K-19, which left (*S*)-BNOAc, was unique in its characterization, and we would like to mention it here.[50] In particular, BNOAc was the best inducer for the ester-degrading enzyme, but this compound had harmful effects on the growth of this strain. As a consequence, a 0.2% (v/v) concentration was found to be suitable for enzyme induction and cell growth. Finally, this activity was increased 20 times over that without inducers. This enhanced activity was also observed in the reverse stereoselective type of the strain, *Pseudomonas* sp. DS-K-717 (4 times).

We then carried out the optical resolution of (*R,S*)-BNOAc (10%, v/v) on a 1-L scale to produce (*S*)-BNOAc (Figure 14), and (*S*)-BNOAc was recovered at more than 98% ee in a yield of 32% in 24 h. This resolution reaction was reproduced up to a 1-kL scale, so that this technology could be scaled-up to an industrial production. Moreover, in order to utilize this method more efficiently, if the formed (*R*)-BN with a lower optical purity (45% ee) was converted to (*R*)-BNOAc again after the extraction of (*S*)-BNOAc and treated by the reverse type of strain, *Pseudomonas* sp. DS-K-717, the total yield would be substantially increased.

10 Production of *(R)*-CHB and *(S)*-HL by *Enterobacter* sp.

CHB* was considered to be a very important compound as a C_4 chiral building block together with BN*; *(S)*-CHB is considered to be a very important compound, which can be easily converted to an intermediate for the HMG-CoA reductase inhibitor,[51] called a "statin drug" for hyperlipidemia, the demand for which was estimated to be several hundred tons (see Figure 11). Also, *(R)*-CHB could be used for the synthesis of L-carnitine and *(R)*-4-hydroxy-2-pyrrolidone as the moiety of several antibiotics. Already, several production methods have been reported based on the asymmetric reduction by chemical and enzymatic catalysts, but some problems existed, namely, regeneration of electron acceptors[52] and imperfect stereospecificity by BINAP.[46]

On the other hand, we carried out screening trials for bacteria capable of dehalogenating either enantiomer from the racemate (see Table 3). We have isolated a desirable strain, *Enterobacter* sp. DS-S-75, from many



Substrate	Specific activity [mU/mg of wet cells]	CHB Residual ratio [%]	CHB Optical purity [% ee]	HL Conversion ratio [mol %]	HL Optical purity [% ee]
Methyl CHB	19.5	48.0	99.5 (*R*)	48.0	95.9 (*S*)
Ethyl CHB	18.0	42.7	99.8 (*R*)	45.1	92.4 (*S*)
i-Propyl CHB	17.1	50.0	99.4 (*R*)	47.5	96.8 (*S*)
Propyl CHB	16.3	49.6	96.6 (*R*)	47.0	96.6 (*S*)
Butyl CHB	13.9	48.9	99.7 (*R*)	47.1	96.7 (*S*)

Figure 15. Stereoselectivity for various CHB by *Enterobacter* sp. DS-S-75.

soil samples, which exhibited *(S)*-CHB-converting activity to *(S)*-HL with high stereoselectivity; especially, the *(R)*-isomer and *(S)*-HL were simultaneously produced with excellent optical purities in a high molar yield of 48% from racemic CHB (Figure 15). The resting cells of the strain DS-S-75 exhibited no dechlorinating activity for *(R)*-CHB, indicating that this resolution reaction is carried out with complete stereoselectivity. In addition, this resolution reaction showed a high stereoselectivity for ethyl, methyl, isopropyl, propyl and butyl esters of CHB, and most of all, methyl CHB (CHBM) was preferred over the others. Interestingly, the preparation of *(R)*- and *(S)*-HL from D- and L-hexose was known,[53,54] but we have developed a potential production method for *(S)*-HL by our isolated *Enterobacter* sp. having a novel dechlorinating activity.

The dehalogenating activity of the cell-free extracts of the strain DS-S-75 showed a high specificity for CHB but did not show this for other halohydrins such as BN, DCP, CPD and butylene chlorohydrin. Halo acids, chloroacetone and EP were not degraded, either. Thus, the *(S)*-CHB dechlorinating enzyme from the strain DS-S-75 would be a novel type of dehalogenase as compared with the haloalcohol dehalogenase[10,55–57] and the halohydrin dehydro-dehalogenase (HDDase).[27,28] In addition, this resolution was a very interesting reaction and was found to involve a carboxy esterase with high stereoselectivity in accordance with our recent enzyme research.

With respect to HDDase, its characteristics are introduced in Section 15 below.

11 Scale-Up of Production of *(R)*-CHB and *(S)*-HL

In order to perform a scale-up to ton levels, the optimal conditions were investigated concerning the influence of pH, temperature and substrate concentration on the activity. The optimal pH and temperature were estimated to be 6.7 and 30 °C, respectively. A concentration of over 8% (w/w) of CHBM had a detrimental effect on the activity.

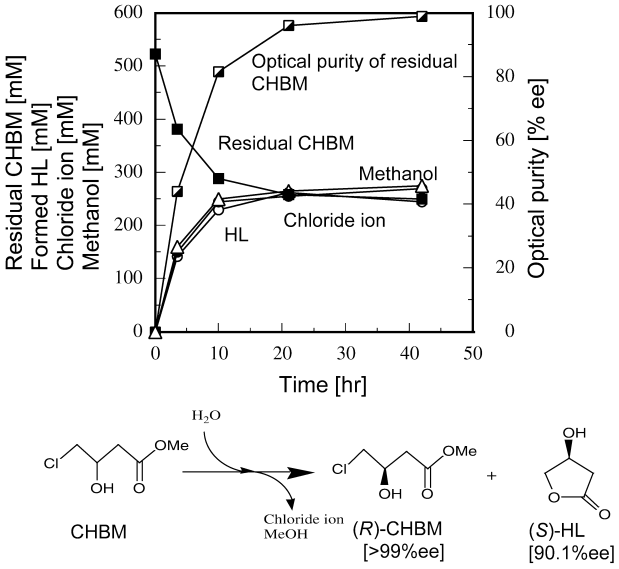


Figure 16. Resolution reaction of *(R,S)*-CHBM with *Enterobacter* sp.DS-S-75.

The production of *(R)*-CHBM and *(S)*-HL was carried out in a 5-L jar fermentor. The pH was controlled at pH 6.7 with 25% (w/w) aqueous NaOH during the resolution reaction. Figure 16 shows a profile of the resolution reaction of racemic CHBM with the resting cells of the strain DS-S-75. As the degradation of CHBM proceeded, the formation of *(S)*-HL, methanol and chloride ion was detected. The resolution was completely finished after 40 h. The residual amount of *(R)*-CHBM and its optical purity were estimated to be 48% and 99.1% ee, respectively. Also, *(S)*-HL was simultaneously obtained in a conversion yield of 48% at 90.1% ee. When $CaCO_3$ was used as a neutralizing agent instead of aqueous NaOH, *(S)*-HL with an optical purity of over 95% ee was obtained, suggesting that the scaled-up production of *(R)*-CHBM and *(S)*-HL in a one-pot manner is theoretically possible, but further optimization of the resolution reaction was needed due to the lower optical purity of *(S)*-HL on a larger scale.

12 Effect of Base for Stereoselectivity of *Enterobacter* sp.

As described above, the formed *(S)*-HL was obtained in high optical purity (95% ee) using $CaCO_3$ as a base, but when NaOH solution [25% (w/w)] was used, the formed *(S)*-HL showed lower optical purity, suggesting that the stereoselectivity would depend on the kind of base. Therefore, several kinds of bases (alkali) that we investigated were used for the resolution of CHMB in order to maintain a high stereoselectivity (Table 4). As a result, a weak base such as sodium carbonate or sodium bicarbonate gave a higher optical purity for the formed *(S)*-HL than a strong base such as sodium hydroxide

(E value 49.4). Most of all, ammonium solution [14% (w/w)] gave the best result not only in optical purity but also in degradation activity (E value 300). The rate was estimated to be twice those of other weak bases. From now on, we have decided on the use of an ammonium solution as the base.

13 Preparation of Resting Cells with High Degradation Activity

How the cells with high activity are cultured and obtained are very important and most critical subjects. In our experience of preparing cells of *Enterobacter* sp. DS-S-75, the following factors were investigated: 1) medium composition and concentration, 2) growth temperature, and 3) concentration of oxygen demand.

Base	(*R*)-CHBM (% ee)	(*S*)-HL (% ee)	E value
NaOH 25% (w/w)	87.6	89.0	49.4
K_2CO_3 10% (w/w)	98.3	95.5	207.1
KH_2CO_3 20%	98.0	95.7	209.9
Na_2CO_3 10% (w/w)	98.6	95.5	215.3
NaH_2CO_3 10%	95.6	95.6	171.5
$(NH_4)_2CO_3$ 10% (w/w)	98.1	95.6	207.2
$CaCO_3$ 5% (w/w)	98.1	95.1	185.4
NH_4OH 7% (w/w)	99.9	95.1	300.0

$$\text{E Value} = \frac{\ln \dfrac{\text{ee of product (1-ee of substrate)}}{\text{ee of product + ee of substrate}}}{\ln \dfrac{\text{ee of product (1+ ee of substrate)}}{\text{ee of product + ee of substrate}}}$$

Table 4. Effect of base on stereoselectivity.

First of all, we generally used a nutrient medium composed of 1% each of peptone, yeast extracts, and glycerol in culturing several of the bacteria we isolated. Most of all, the yeast extract was considered as a main factor, namely its type and the stability of its properties from lot to lot, so that we needed to select a stable variety (Figure 17).

Second, it often occurred that the optimal temperature for the growth of cells and the temperature of the highest activity were different. Because the total activity of the resting cells can be shown to be a product of cell amounts and activity per unit volume, both a high specific activity and high concentration of the cells were required in culturing the resting cells (Figure 18).

Third, dissolved oxygen most likely influenced the specific activity based on our experience. Thus, we investigated the relationship between generation numbers and specific activity under both excess and poor dissolved oxygen (DO) conditions (Figure 19). Although this strain showed lower specific activity in excess dissolved oxygen, in contrast, a higher specific activity (more than 2 times) was shown for a poor DO concentration. We have examined some relationships

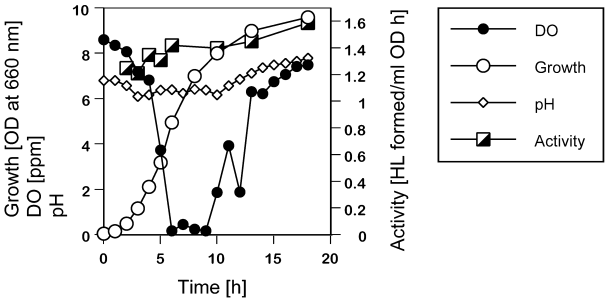


Figure 18. Cultivation profile of *Enterobacter* sp. DS-S-75.

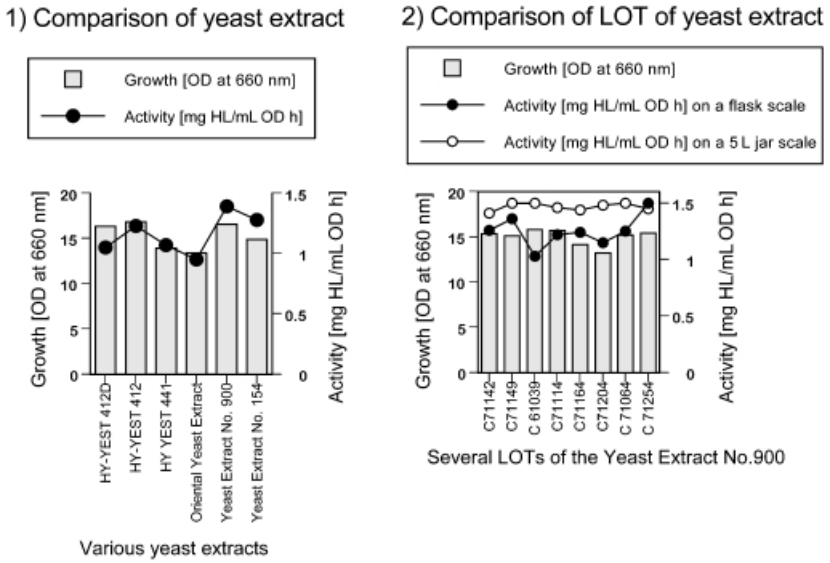


Figure 17. Selection of cultivation medium.

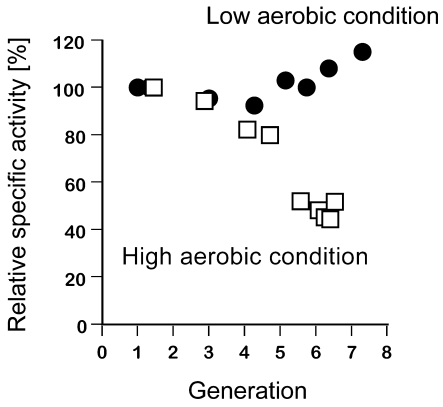


Figure 19. Effect of dissolved oxygen concentration for specific activity in cultivation.

between cell growth and DO concentration or specific activity. The relationships were very interesting; the results indicated that, for obtaining a higher specific activity, it is necessary to culture the cells under a poor oxygen concentration in the log phase. This theory could be reproduced on a scale from several liters to several tens of tons. In this manner, we established a cultivation method for producing resting cells with high specific activity.

14 Intelligent Production of *(R)*-CHBM and *(S)*-HL with a Fed-Batch System of Substrate

In order to produce *(R)*-CHBM and *(S)*-HL as C_4 chiral building blocks more efficiently on a practical scale, we are adopting the fed-batch system for the pH-dependent controlled production of C_3 chiral compounds based on the bacterial multiplication. The limiting concentration of CHBM was determined to be 8% (w/w), so that we started this reaction with a lower CHBM concentration [2% (w/w)] and fed additional substrate [up to 12% (w/w)] in order to avoid rate inhibition by higher concentrations of the substrate. Taking advantage of the released chloride ion when *(S)*-CHBM as a substrate was degraded and dechlorinated, our original fed-batch system was constructed. Additional substrate was fed, the amount of which was equal to the amount of consumed ammonium solution for pH control.

We then tried to produce *(R)*-CHBM and *(S)*-HL using this fed-batch system controlled by a computer. As a result, we succeeded in an industrial production of *(R)*-CHBM (> 99% ee) and *(S)*-HL (95% ee). Figure 20 shows the profile of the resolution reaction of 12% racemate between 2-L and 15-kL runs.

The microbial resolution of racemic CHBM using the resting cells of the strain DS-S-75 made possible the production of highly optically pure *(R)*-CHBM and *(S)*-HL in a one-pot reaction. Racemic CHBM as a substrate

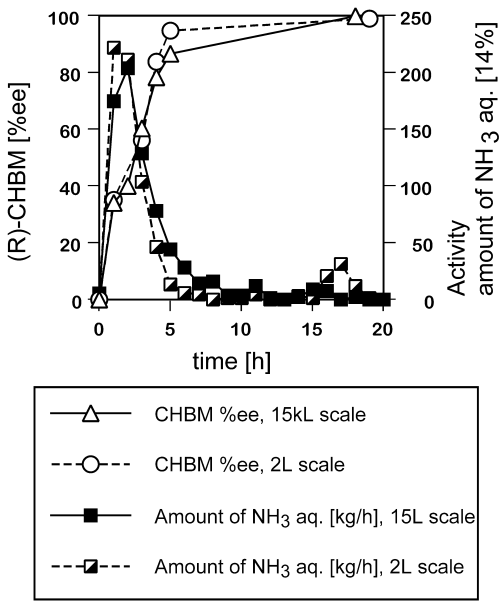


Figure 20. Comparison of activities between 5-kL and 15-kL scales on CHBM resolution.

can be easily obtained from EP *via* BN using petroleum chemicals. Thus, *(R)*-CHBM and *(S)*-HL as useful C_4 chiral synthons can be economically produced by this method. More recently, we have just succeeded in the purification and characterization of this enzyme. This enzyme was found to have the catalytic activity of a stereoselective carboxylate esterase (MW 75kDa, homodimer 27.5 kDa). In addition, this enzyme resolved 3-hydroxybutyrate (3HB), 2-hydroxybutyrate (2HB) and tetrahydrofuran-2-carboxylate with high stereoselectivity (Figure 21). More detailed studies of this enzyme and its gene for industrial production are now in progress.

15 Practical Generation of *(R)*-1,2-Diols by the Novel Dehalogenating Enzyme, HDDase

Besides success in the production of BN* and CHB* as C_4 chiral compounds, we would like to introduce an additional optical resolution, namely the production of *(R)*-1,2-diols with the unique dehalogenating enzyme, HDDase from *Alcaligenes* sp. DS-S-7G, capable of stereoselectively assimilating *(R)*-CPD.

HDDase showed broad substrate specificities for alcohols but not for acids. In particular, halohydrins such as *(R)*-3-halo-1,2-propanediol and *(S)*-2,3-dihalo-1-propanol were dehalogenated with high dehydrodehalogenating activities. Also, 1,2-diols such as 1,2-propanediol, 1,2-butanediol, 1,2-pentanediol, 1,2-hexanediol and 3-phenyl-1,2-propanediol were transformed by

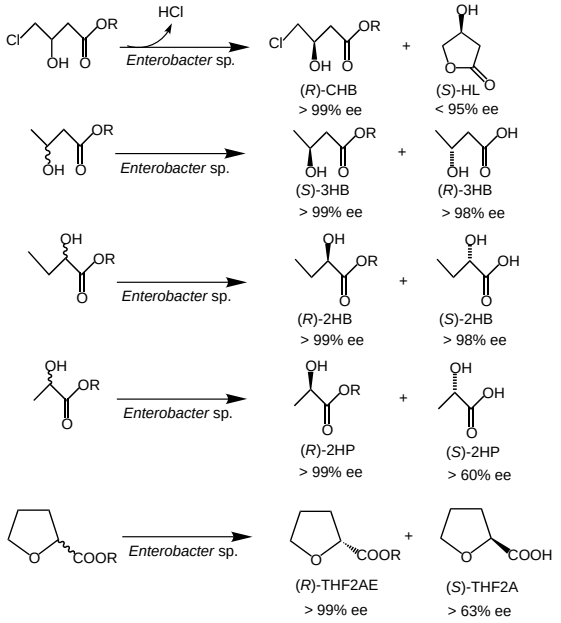
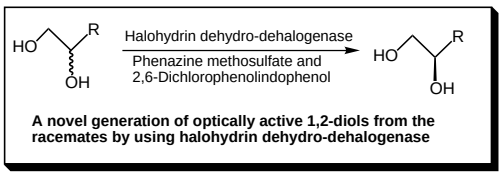


Figure 21. Novel generation of α- and β-hydroxybutyrates.

the dehydrogenating activities so that alkyl- and aryl-1,2-diols were converted into the corresponding aldehydes and formic acid *via* the corresponding 2-hydroxy-1-aldehydes. These results suggest that HDDase may have stereoselective dehydrogenating activity for non-halogenated alcohols, as well as for halogenated alcohols. In conclusion, HDDase has unique substrate specificities and high stereoselectivities for halohydrins and 1,2-diols; these features were applicable to the preparation of optically active 1,2-diols (Figure 22).

As described above, HDDase was confirmed to be a new type of dehalogenase based on the substrate specificities and the stereoselectivities. This generation of chiral 1,2-diols by HDDase will become more practical when an effective recycling of NAD^+ becomes more possible through recombinant gene technology. Jacobsen et al. and Noyori et al. have devel oped chemical catalysts for the kinetic hydrolytic resolution of epoxides[16] and the asymmetric reduction of ketones,[46] respectively. However, these technologies have the same problems; that is, use of expensive catalysts and low enantiomeric excess (94% ee by BINAP).

On the other hand, we also developed the asymmetric assimilation of *(R,S)*-1,2-propanediol to obtain *(R)*-PG.[58] Especially, the development of a novel type of anti-AIDS drugs, quinolone-type antibiotics such as Levofloxacine and oxazolidinone-type antibiotics such as Linezolid using *(R)*-1,2-PG, *(S)*-CPD and their derivatives have been actively pursued by pharmaceutical companies.[42,59–61] The demand for *(R)*-PG has attained more and more interest all around the world.



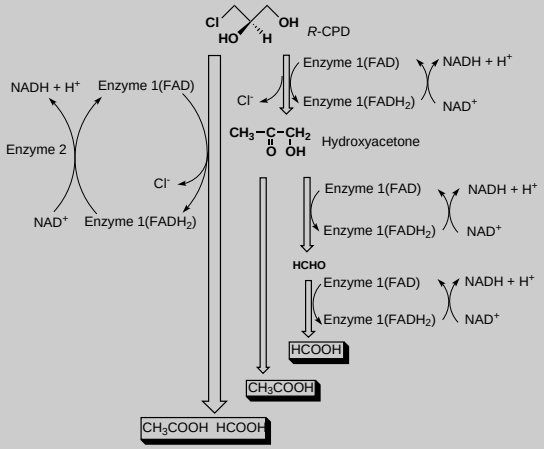
Substrate[a]	% ee	Residual substrate [%][b]
1,2 Propanediol	98.5 *(R)*	34.3
1,2-Butanediol	97.5 *(R)*	48.2
1,2-Pentanediol	98.2 *(R)*	50.2
1,2-Hexanediol	98.2 *(R)*	50.0
1,2-Dihydroxy-3-butene	98.0 *(R)*	49.1
1,2-Dihydroxy-5-hexene	98.3 *(R)*	40.1
1-Phenyl-1,2-ethanediol	95.1 *(R)*	39.5
CPD	98.5 *(S)*	50.2
3-Bromo-1,2-propanediol	98.5 *(S)*	49.3
DCP	99.0 *(R)*	46.2
2,3-Dibromo-1-propanol	99.0 *(R)*	48.1

[a] Racemic forms were used as a substrates.
[b] At initial time, each reaction solution contained 0.2% (v/v) of substrate.

Figure 22. Production of *(R)*-1,2-diols by HDDase.

Characterization of a Novel Type of Dehalogenation of (*R*)-CPD by the (*S*)-CPD-Assimilating Bacterium, *Alcaligenes* sp. DS-S-7G

The mechanism of the enzymatic dehalogenation of (*R*)-CPD was investigated.[27,28] The (*R*)-CPD-dechlorinating enzyme system of the strain DS-S-7G was composed of two components (Enzyme 1 and Enzyme 2). Enzyme 1 was a flavo-protein with a relative molecular mass (M_r) of 70,000 and was composed of



	Enzyme 1 (HDDase)	Enzyme 2
Molecular Weight		
PAGE	70,000	
HPLC		86,000
SDS-PAGE	58,000 16,000	53,000 33,000
Characters		
Flavin	FAD (1 mole/mole)	-
Co-factor	NAD^+	-
K_m (PMS as co-factor)	322 mM	-
V_{max} (PMS as co-factor)	3.34 mmole/min mg	-

Figure 23. Proposed degradation routes of (*R*)-CPD.

two kinds of polypeptides (58,000 and 16,000). The enzyme exhibited activity for converting (*R*)-CPD to hydroxyacetone with the liberation of chloride ions under aerobic conditions. On the other hand, Enzyme 2 with an M_r of 86,000, which was also composed of two kinds of polypeptides (33,000 and 53,000), showed no dechlorinating activity for (*R*)-CPD. (*R*)-CPD is oxidatively dechlorinated by these two enzymes in the presence of NAD^+. Based on the products of the enzymatic reaction and the activities, we proposed two degradation routes of (*R*)-CPD by the strain DS-S-7G (Figure 23).

Also, DCIP (2,6-dichlorophenolindophenol) and PMS (phenazine methosulfate) were found to be utilized as electron acceptors instead of NAD^+, so that the activity was increased by about one-thousand-fold. No enzyme catalyzing the stereoselective dehydrodehalogenation of halogenated alcohols is known. These facts indicate that Enzyme 1 is a new type of haloalcohol dehydrogenase. From these results, we designated Enzyme 1 as a halohydrin dehydro-dehalogenase (HDDase).

Castro et al. and Janssen et al. have already reported their haloalcohol dehalogenases-catalyzed epoxide formation from the corresponding haloalcohols releasing chloride ions.[8,25] This result suggested that the haloalcohol dehalogenase from *Alcaligenes* sp. was a quite novel type of haloalcohol dehalogenase.

16 Further Development and the Linkage

EP*, CPD*, the related halohydrins* and their numerous derivatives, are supplied in ton amounts around Europe, North America and Japan. These chiral materials have been used for chiral pharmaceuticals, agrochemicals, chiral polymers, liquid crystals and natural products, in physical chemistry and for research such as the elucidation of organic chemical reaction mechanisms and so on.

These tiny microorganisms, about 1 micrometer in length, have linked together fields such as petrochemistry, biotechnology, organic chemistry, physical chemistry and electronics. We further expect new developments and the connection of biochemistry, organic chemistry and physical chemistry.

17 Good Innovation and Results

We are very happy to produce these useful chiral substances. Their industrial production will stimulate the creation of more useful compounds. Our industrial production of chiral synthetic unit has been recognized by award of the Selection of Attractive Innovation Prize from the Science and Technology Agency in 1995, the Industrial Technology Prize in 1996 from the Society for Bioscience and Bioengineering, Japan, and the Invention Prize in the Kinki area from the Japan Institute of Invention and Innovation in 1996.

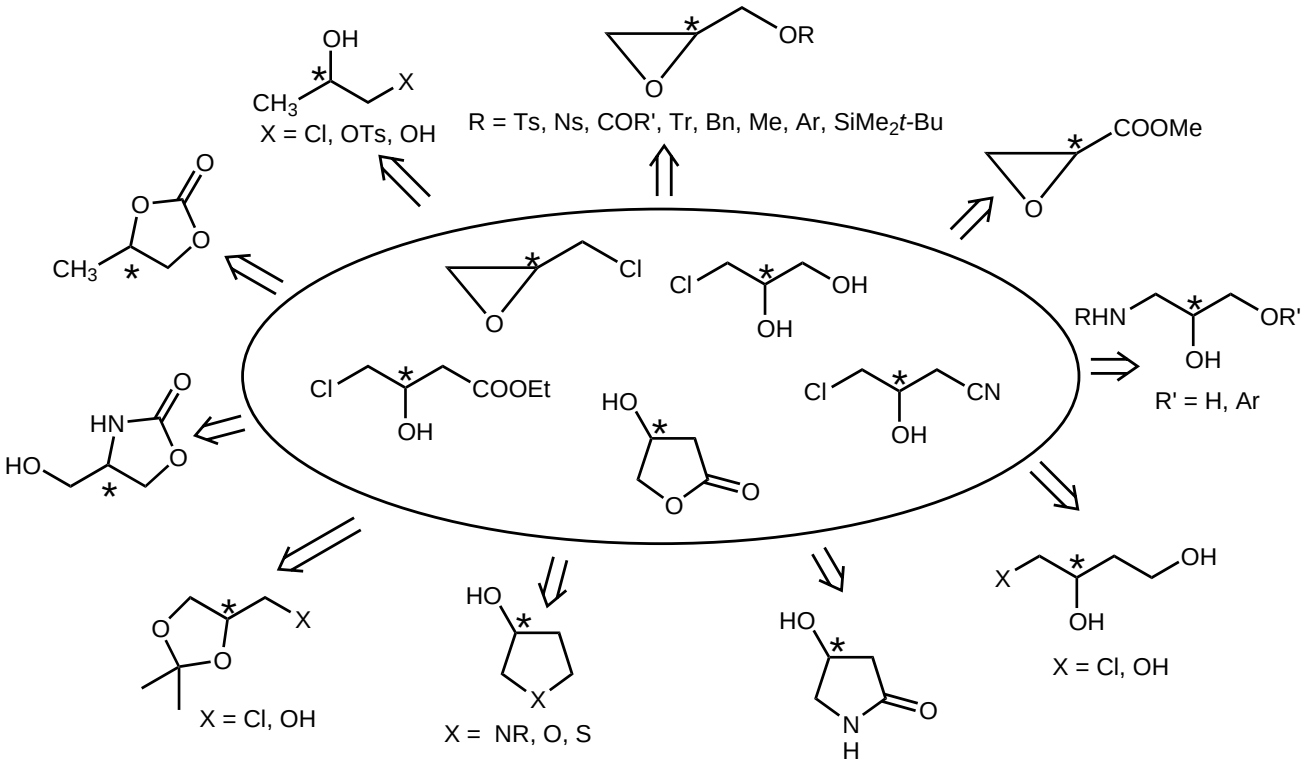


Figure 24. Development of derivatives from chiral C_3 and C_4 units

18 Concluding Remarks

In this account we have reviewed the generation of chiral C_3 and C_4 synthetic units of DCP*, EP*, CPD*, GLD*, CHB*, HL*, and PG*. We can supply more than 70 kinds of chiral building blocks based on these chiral C_3 and C_4 synthetic units that we introduce in Figure 24. Especially, we have described when and how our research and development started and the background. Overviewing these events, we see a narrow, zigzag but continuous path, and along the path we find various subjects and many persons. We are very glad to find that our products are small but useful chiral synthetic units and are widely used for many important pharmaceuticals and new materials. Various chemicals are being developed by many chemists all over the world. In Daiso, the synthetic group has grown up on the basis of our biological chiral production. Each group is connected and linked around the world. In the chemical business, the bio- and chemical methods are competing, and reasonable production is determined by itself. Many further studies are being carried out in research fields such as pharmacy, agrochemistry, organic chemistry, physiology, electronics, and the next new applications are also in progress.

Last, we always quote the phrase: although biological and organic synthetic studies compete in making optically active compounds, they should principally join and support each other based on common chemistry.

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References

[1] N. Kasai, T. Suzuki, Y. Furukawa, *J. Mol. Catal. B: Enzymatic* **1998**, *4*, 237.
[2] S. L. Neidleman, *Hydrocarbon Processing* **1980**, 135.
[3] J. J. Baldwin, A. W. Raab, K. Mensler, B. H. Arso, D. E. McClure, *J. Org. Chem.* **1978**, *43*, 4876.
[4] S. Iriuchijima, A. Keiyu, N. Kojima, *Agric. Biol. Chem.* **1982**, *46*, 1593.
[5] S. Iriuchijima, N. Kojima, *Agric. Biol. Chem*. **1982**, *46*, 1153.
[6] B. Cambou, A. M. Klibanov, *J. Am. Chem. Soc*. **1982**, *104*, 2687.
[7] K. Weissermel, H. J. Arpe, *Industrialle Organische Chemie*, Verlag Chemie GmbH, Weinheim, **1976**.
[8] C. E. Castro, E. W. Bartnicki, *Biochemistry* **1968**, *7*, 3213.
[9] D. J. Hardman, J. H. Slater, *J. Gen. Microbiol*. **1981**, *123*, 117.
[10] H. M. S. Assis, P. J. Sallis, A. T. Bull, D. J. Hardman, *Enzyme Microb. Technol.* **1998**, *22*, 568.
[11] N. Kasai, K. Tsuiimura, K. Unoura, T. Suzuki, *Agric. Biol. Chem*. **1990**, *54*, 3185.
[12] N. Kasai, K. Tsuiimura, K. Unoura, T. Suzuki, *J. Indust. Microbiol*. **1992**, *9*, 97.
[13] N. Kasai, K. Tsuiimura, K. Unoura, T. Suzuki, *J. Indust. Microbiol*. **1992**, *10*, 37.
[14] V. Schurig, *J. Chromatography* **1988**, *441*, 135.
[15] A. Geerlof, J. B. A. van Tol, J. A. Jongejan, J. A. Duine, *J. Chromatography* **1993**, *648*, 119.
[16] L. E. Martinez, J. L. Leighton, D. H. Carsten, E. N. Jacobsen, *J. Am. Chem. Soc*. **1995**, *117*, 5897.
[17] TECHGRAM JAPAN, in *CHEMTECH*, **1988**, April, 226.
[18] S. Takano, K. Ogasawara, *J. Synth. Org. Chem. Jpn.* **1982**, *40*, 1038.
[19] H.-U. Blaser, et al., in *Process Chemistry in the Pharmaceutical Industry*, pp. 189-199, (Ed.: K. G. Gadamasetti), Marcel Dekker Inc., New York, **1999**.
[20] S. Kinoshita, Y. Kumoi, A. Ohsima, T. Yosida, N. Kasai, *J. Ferment. Bioeng*. **1991**, *72*, 74.
[21] M. Koden, T. Kuratake, F. Funada, K. Awane, K. Sakaguchi, Y. Shiomi, T. Kitamura, *Jpn. J. Appl. Phys.* **1990**, *29*, L981.
[22] N. Kasai, K. Sakaguchi, *Tetrahedron Lett*. **1992**, *33* , 1211.
[23] J. Hasegawa, M. Ogura, S. Tsuda, S. Maemoto, H. Kutsuki, T. Ohashi, *Agric. Biol. Chem*. **1990**, *54*, 1819.
[24] T. Suzuki, N. Kasai, *Bioorg. Med. Chem. Lett*. **1991**, *1*, 343.
[25] D. B. Janssen, D. Jager, B. Witholt, *Appl Environ Microbiol*. **1987**, *53*, 561.
[26] D. Askin, K. K. Eng, K. Rossen, R. M. Purick, K. M. Wells, R. P. Volante, P. J. Reider, *Tetrahedron Lett.* **1994**, *35*, 673.
[27] T. Suzuki, N. Kasai, R. Yamamoto, *Appl. Microbiol. Biotechnol*. **1994**, *42* . 270.
[28] T. Suzuki, N. Kasai, N. Minamiura, *Tetrahedron: Asymmetry* **1994**, *5* , 239.
[29] T. Suzuki, N. Kasai, N. Minamiura, *J. Ferment. Bioeng*. **1994**, *78* , 194.
[30] T. Suzuki, N. Kasai, R. Yamamoto, N. Minamiura, *Appl. Microbiol. Biotechnol*. **1993**, *40*, 273.
[31] T. Suzuki, N. Kasai, R. Yamamoto, N. Minamiura, *J. Ferment. Bioeng*. **1992**, *73* , 443.
[32] *Large-Scale Asymmetric Catalysis*, (Eds.: H.-U. Blaser, E. Schmidt), Wiley, in press.
[33] K. Soda, N. Esaki, T. Kurihara, J.-Q. Liu, V.Nardi-Dei, M. Nishihara, Y. Hata, T. Fujii, T. Hisano, S. Tsunasawa,

M. Miyagi, in *Mechanisms of biohalogenation and dehalogenation*, (Eds.: D. B. Janssen, K. Soda, R. Wever), Elsevier, Amsterdam, **1997**, p. 157.
[34] H. Kawasaki, in *Mechanisms of biohalogenation and dehalogenation*, (Eds.: D. B. Janssen, K. Soda, R. Wever), Elsevier, Amsterdam, **1997**, p. 175.
[35] R, Rink, M. Fennema, M. Smids, U. Dehmel, D. B. Janssen, *J. Biol. Chem*. **1997**, *272*, 14650.
[36] L. Tang, J. E. T. van Hylckama Vlieg, J. H. L. Spelberg, M. W. Fraaije, D. B. Janssen, *Enzyme and Microbial Technology* **2002**, *30*, 251.
[37] J. H. L. Sperberg, J. E. T. van Hylckama Vlieg, T. Bosoma, R. M. Kellogg, D. B. Janssen, *Tetrahedron: Asymmetry* **1999**, *10*, 2863.
[38] C. A. G. M. Weijers, J. A. M. de Bont, *J. Mol. Catal. B: Enzymatic* **1999**, *6*, 199.
[39] K. Kitaori, Y. Takehira, Y. Furukawa, H. Yoshimoto, J. Otera, *Chem. Pharm. Bull*. **1997**, *45*, 412.
[40] B. Zhou, A. S. Gopalan, F. Van Middlesworth, W.-R. Shieh, C. Sih, *J. Am. Chem. Soc*. **1983**, *105*, 5925.
[41] T. Onishi, T. Matsuzaka, S. Nishi, T. Tsuji, *Tetrahedron Lett*. **1999**, *40*, 8845.
[42] B. A. Pearlman, W. R. Perrault, M. R. Barbachyn, P. R. Manninen, D. S. Toops, D. J. Houser, T. J. Fleck, *US Patent* 5,837,870, **1998**.
[43] M. M. Faul, L. L. Winneroski, C. A. Krumrich, K. A. Sullivan, J. R. Gillig, D. A. Neel, C. J. Rito, M. R. Jirousek, *J. Org. Chem*. **1998**, *63*, 1961.
[44] B. E. Rossiter, K. B. Sharpless, *J. Org. Chem*. **1984**, *49*, 3707.
[45] T. Nakamura, T. Nagasawa, F. Yu, I. Watanabe, H. Yamada, *J. Bacteriol*. **1992**, *174*, 7613.
[46] M. Kitamura, M. Tokunaga, T. Ohkuma, R. Noyori, *Tetrahedron Lett*. **1991**, *32*, 4163.
[47] A. Matsuyama, H. Yamamoto, Y. Kobayashi, *Org. Proc. Res. Develop*. **2002**, *6*, 558.
[48] M. Kataoka, K. Yamamoto, H. Kawabata, M. Wada, K. Kita, H. Yanase, S. Shimizu, *Appl. Microbiol. Biotechnol*. **1999**, *51*, 486.
[49] T. Suzuki, H. Idogaki, N. Kasai, *Enzyme Microb. Technol*. **1998**, *24*, 13.
[50] H. Idogaki, N. Kasai, M. Takeuchi, M. Hatada, T. Suzuki, *Tetrahedron: Asymmetry* **2001**, *12*, 369.
[51] M. Wolberg, W. Hummel, C. Wandrey, M. Muller, *Angew. Chem. Int. Ed*. **2000**, *39*, 4306.
[52] H. Yamamoto, A. Matsuyama, Y. Kobayashi, *Biosci. Biotechnol. Biochem*. **2002**, *66*, 481.
[53] R. I. Hollingsworth, *J. Org. Chem*. **1999**, *64*, 7633.
[54] R. I. Hollingsworth, *US Patent* 6,239,311, **2001**.
[55] T. Nakamura, F. Yu, W. Mizunashi, I. Watanabe, *Agric. Biol. Chem*. **1991**, *55*, 1931.
[56] A. J. van den Wijngaard, D. B. Janssen, B. Witholt, *J. Gen. Microbiol*. **1989**, *135*, 2199.
[57] A. J. van den Wijngaard, P. T. W. Reuvekamp, D. B. Janssen, *J. Bacteriol*. **1991**, *173*, 124.
[58] T. Suzuki, H. Idogaki, A. Nakagawa, M. Ueda, *Jpn. Patent* JP2002-253295, **2002**.
[59] S. Atarai, S. Yokoyama, K. Yamazaki, S. Sakano, M. Imamura, M. Furukawa, I. Hayakawa, *Chem. Pharm*. **1987**, *29*, 1986.
[60] L. M. Schultze, H. H. Chapman, N. J. P. Dubree, R. J. Jones, K. M. Kent, T. T. Lee, M. S. Louie, M. J. Postich, E. J. Prisbe, J. C. Rohloff, R. H. Yu, *Tetrahedron Lett*. **1998**, *39*, 1853.
[61] P. R. Brondfuehrer, H. G. Howell, C. Sapino Jr., P. Vemishetti, *Tetrahedron Lett.* **1994**, *35*, 3243.