Large-scale Chemoenzymic Synthesis of Calcium (6*S*)-5-Formyl-5,6,7,8-tetrahydrofolate [(−)-Leucovorin] using the NADPH Recycling Method

Yukihiro Kuge,*[,a] Kunimi Inoue,[a] Kyoji Ando,[a] Tamotsu Eguchi,[b] Takashi Oshiro,[b] Kenichi Mochida,[b] Takayuki Uwajima,[b] Toru Sugaya,[a] Junji Kanazawa,[c] Masami Okabe[c] and Shinji Tomioka[a]

[a] *Sakai Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Takasu-cho, Sakai-shi, Osaka 590, Japan*
[b] *Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Asahi-cho, Machida-shi, Tokyo 194, Japan*
[c] *Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Nagaizumi-cho, Shizuoka 411, Japan*

Chemoenzymic large-scale synthesis of the calcium salt of (6*S*)-5-formyltetrahydrofolic acid [(−)-leucovorin, (6*S*)-**5**] was achieved from folic acid **1** *via* (6*S*)-tetrahydrofolic acid [(6*S*)-**3**] by using dihydrofolate reductase (DHFR) produced by *Escherichia coli*, harbouring a high-expression plasmid, pTP64-1. On the other hand, for the diastereoselective reduction of 7,8-dihydrofolic acid **2** to tetrahydrofolate (6*S*)-**3**, a new NADPH recycling system was constructed by coupling with glucose dehydrogenase from *Gluconobacter scleroides*. Having these enzymic systems to hand, compound **1** was reduced by zinc powder in alkaline solution to give compound **2** which, without isolation, was reduced enzymatically to afford tetrahydrofolate (6*S*)-**3** (94% de). The pH adjustment of the reaction mixture containing dihydrofolate **2** was done with phosphoric acid in order to remove zinc ion which inhibited the following enzymic reduction. The formed tetrahydrofolate (6*S*)-**3** was converted into entirely optically pure *N*-formyl compound (6*S*)-**5** on a large scale. The specific rotation value of (−)-leucovorin was $[\alpha]_D^{20}$ −13.3 (*c* 1, water). For the comparison of pharmacological effects, a completely optically pure form of (+)-leucovorin [(6*R*)-**5**] was also prepared on a preparative scale. Compound (6*S*)-**5** was 300-fold more active compared with the (6*R*)-diastereoisomer.

Methotrexate (MTX), commonly used in cancer chemotherapy,[1] prevents the biosynthesis of DNA by inhibiting dihydrofolate reductase (DHFR) which catalyses the conversion of 7,8-dihydrofolic acid **2** into (6*S*)-tetrahydrofolic acid [(6*S*)-**3**], and hence the biosynthesis of N^5,N^{10}-methylenetetrahydrofolic acid which provides one carbon unit in the conversion of dUMP into dTMP by thymidylate synthase. However, it is toxic to normal cells as well as to cancerous cells. Because the administration of leucovorin [(6*RS*)-5-formyltetrahydrofolate, (6*RS*)-**5**] does not require the DHFR-catalysed conversion of compound **2** into compound **3**, it can restore the reduced folate which is indispensable for C-1 metabolism of normal cells and has been used as a rescue agent after treatment with a high dose of MTX. It is now of great interest that the combination of leucovorin (6*RS*)-**5** and 5-fluorouracil (5-FU) exhibits a better response rate toward advanced colorectal cancer compared with treatment using 5-FU alone.[2] The modulation of cytotoxity by leucovorin **5** is based mainly on the great stabilization of the covalent ternary complex consisting of fluorodeoxyuridilate (FdUMP), thymidilate synthase, and leucovorin **5**. In clinical tests, compound **5** had been used as the diastereoisomeric mixture, and there have been several reports of the synthesis of a diastereoisomeric mixture of (6*R*)- and (6*S*)-**5**.[3] However, only the natural, (−)-diastereoisomer [(6*S*)-diastereoisomer][4] is effective.[5,6] Therefore, entirely optically pure isomer (6*S*)-**5** has been needed. There have been many reports about a synthetic[7,8] and/or enzymic[8,9] preparation of (−)-isomer (6*S*)-**5**, but none of them were suitable for large-scale synthesis (*e.g.*, low yield, use of expensive enzymes and co-enzymes, and unsatisfactory optical purity).

Iwakura and Tsuda[10] constructed *Escherichia coli* harbouring a high-expression plasmid (pTP64-1, obtained from pBR322) by using a gene-cloning technique for DHFR production.

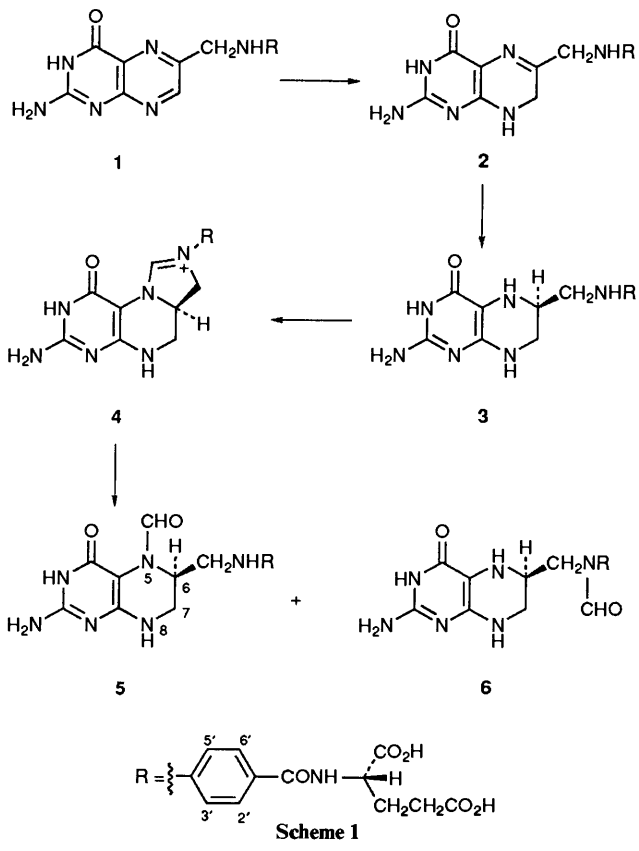
We have already reported the optimum expression conditions of the DHFR gene and have established a versatile purification method for DHFR as a single protein.[11] On the other hand, enzymic reduction of dihydrofolate **2** by DHFR required highly expensive dihydronicotinamide adenine dinucleotide phosphate (NADPH) as mentioned above. From this standpoint, we established a new and very effective recycling system of NADPH coupled with glucose dehydrogenase from *Gluconobacter scleroides*.[12] Based on these results, a large-scale diastereoselective reduction of dihydrofolate **2** to tetrahydrofolate (6*S*)-**3** was possible.

We described the chemoenzymic synthesis of (−)-leucovorin (6*S*)-**5** from folate **1** on a gram scale *via* diastereoselective reduction of dihydrofolate **2** with DHFR in 1990[13] (Scheme 1). Details of this study, including the scale-up synthesis, are presented here.

Results and Discussion

Purification of DHFR.—First, we studied the optimum expression conditions of the gene pTP64-1 in *E. coli* for overproduction of DHFR. The effect of various organic compounds on the production of DHFR by *E. coli* C600pTP64-1 was examined. We found that the concurrent addition of sorbitol and yeast extract to Bouillon medium stimulated the enzyme productivity to 17.2 U cm^{-3} of broth, which occupied 30% of the whole soluble protein and the productivity was 3000-times compared with the wild-type strain, although the productivity of our trimethoprim-resistant *E. coli* mutant induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was only 40-times that of the wild strain.[11]

Purification of DHFR was through DEAE-cellulofine column chromatography and Superose Prep 12 gel filtration to give a single protein in 54% total purification yield (Table 1).



Scheme 1

Table 1 Purification of DHFR from *E. coli* C600/pTP64-1[a]

	Total protein (mg)	Total units (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	21 400	28 100	1.31	100
DEAE-Cellulofine	21 230	16 960	13.7	60.4
Superose Prep 12	786	15 100	19.2	53.8

[a] Culture conditions: medium; 1.0% sorbitol, 0.8% peptone, 0.5% yeast extract, 0.3% NaCl, pH 7.0. Cultivation: 37 °C, 18 h.

Construction of NADPH Recycling System and Chemoenzymic Conversion of Folate **1** *into Compound* (6R)-**4**.—Using this DHFR, the diastereoselective reduction of dihydrofolate **2** to tetrahydrofolate (6*S*)-**3** was studied. As a result, the addition of DHFR (1.5 U cm^{-3}) to a solution of substrate **2** (100 mmol dm^{-3}) and NADPH (100 mmol dm^{-3}) at pH 7 afforded compound (6*S*)-**3** in quantitative yield after the mixture had been stirred at 37 °C for 3 h. The optical purity of the product (6*S*)-**3** was determined by the method of Rees[8] and was estimated to be 94% de. This stereoselective reduction was surely the key step in the synthesis of (−)-leucovorin (6*S*)-**5**. However, for a large-scale synthesis of compound (6*S*)-**5**, there are several problems. First, NADPH is very expensive and is difficult to obtain in large quantities. Secondly, both compounds **2** and **3** are very air-sensitive and are easily oxidized.

For the first problem, we studied the construction of the NADPH recycling system. There are many merits to this not only because of the reduced requirement for NADPH, but also because of the substitution of cheaper and more stable nicotinamide adenine dinucleotide phosphate (NADP) for NADPH. Some systems had been reported as follows; isocitrate dehydrogenase (**2**: 3.4 mmol dm^{-3}, NADPH: 0.007 mol equiv., 168 h),[8] glucose-6-phosphate dehydrogenase/creatine kinase (**2**: 7.7 mmol dm^{-3}, NADPH: 0.0064 mol equiv., 120 h),[8] malic enzyme (**2**: 2.9 mmol dm^{-3}, NADPH: 0.2 mol equiv., 3 h),[14]

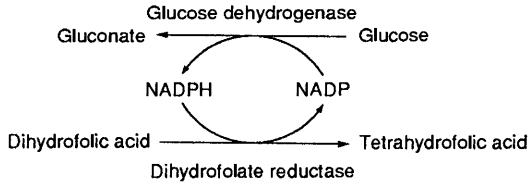


Fig. 1 NADPH-recycling system for the diastereoselective reduction of dihydrofolic acid

6-phosphogluconate dehydrogenase (**2**: 0.12 mmol dm^{-3}, NADPH: 0.33 mol equiv., 1 h).[15] However, most of them gave rise to several problems; *e.g.*, long reaction time, low concentration of substrate, low turnover number, and a complicated system including the use of several enzymes. From this standpoint, we searched for other counter-enzymes. As a result, glucose dehydrogenase (GluDH) from *Gluconobacter scleroides* was found to be the most suitable, and the stereoselective reduction was completed within 2.5 h (40 °C, pH 8) by adding a catalytic amount of NADP (0.01 mol equiv.; 2 mmol dm^{-3}) to a mixture of dihydrofolate **2** (200 mmol dm^{-3}), glucose (220 mmol dm^{-3}), DHFR (14.4 U cm^{-3}), and GluDH (14.4 U cm^{-3}) (Fig. 1).

For the second problem, a one-pot conversion of folate **1** into tetrahydrofolate (6*S*)-**3** without isolation of dihydrofolate **2** under anaerobic conditions was needed. At this stage, the method of preparation of compound **2** was important. At first, we tried to prepare compound **2** by partial reduction of folate **1** by using sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$) according to the known method[16] and estimated the components of the reaction mixture. Because the selectivity of the reduction with this reagent was not so good, the diastereoisomeric tetrahydrofolate (6*RS*)-**3** was formed in 5% yield as the result of over-reduction (Table 2). This over-reduction lowered the optical purity of the required product (−)-leucovorin (6*S*)-**3** in the following enzymic reduction. Furthermore, *p*-aminobenzoyl glutamate (PABGA, 7%) was formed as determined by comparison with a commercially available authentic sample by HPLC and TLC.[17] On the other hand, the reduction using zinc powder in alkaline solution[18] was much better. Thus compound **1** was converted quantitatively into dihydrofolate **2** by the known method, and both tetrahydrofolate **3** and PABGA were not detected at this stage as the result of our estimation. Therefore, this reaction mixture, after excess of zinc had been removed under anaerobic conditions, could serve as the substrate solution of DHFR reduction without further purification. However, reduction by zinc could proceed only at pH > 13.0 (Fig. 2). Therefore, the pH had to be adjusted to 8.0 before the enzymic reduction. When the pH was adjusted by HCl, stoichiometrically formed zinc ion inhibited the following enzymic reduction (Table 3). This could be overcome by substituting H_3PO_4 for HCl in the pH adjustment. Thus, water-insoluble zinc phosphate could be removed from the mixture only by filtration. Using such a simple procedure, the activity could be entirely recovered (Table 3). After the reduction, the formed tetrahydrofolate (6*S*)-**3** was more unstable than dihydrofolate **2** to air oxidation and was difficult to store as an intermediate. So compound **3**, after filtration under anaerobic conditions, was immediately treated with formic acid in HCl to give the rather stable (6*R*)-5,10-methylylidenetetrahydrofolic acid [(6*R*)-**4**] after concentration of the reaction mixture and crystallization by the addition of 1 mol dm^{-3} HCl.

Hydrolysis of (6R)-**4** *to* (6*S*)-**5**.— In the following hydrolysis of compound (6*R*)-**4** to *N*-formyl compound (6*S*)-**5**, the effect of pH and temperature was very important. Thus, the lower the reaction temperature, the more 10-formyltetrahydrofolic acid **6**

Table 2 Chemical reduction of folate **1** to dihydrofolate **2**

Reagent (mol equiv.)	pH	Temp. (T/°C)	Time (t/h)	Conversion rate (%)	**1** (%)	(6*RS*)-**3** (%)	PABGA[a] (%)
$Na_2S_2O_4$ (4.6)	6	25	3	77	4	5	7
Zn (5.5)	14	25	2	100	0	0	0

[a] PABGA = *p*-aminobenzoyl glutamate.

Table 3 Effect of preparation of compound **2** on the following enzymic reduction

Preparation method of **2**		DHFR reduction	
Reagent	pH Adjustment[a]	Reaction time (t/h)	Conversion rate (%)[b]
$Na_2S_2O_4$	none (isolated)	2.5	100
Zn	HCl	5.0	51
Zn	H_3PO_4	2.5	100

[a] Adjusted to pH 8. [b] Based on initial amount of **2**.

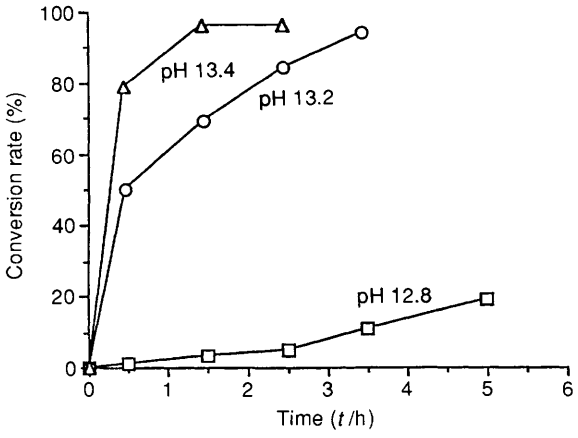


Fig. 2 Effect of pH on Zn reduction of compound **1**. The alkaline solution (100 cm^3) of folate **1** (15 g) was adjusted to each pH with 10 mol dm^{-3}, and zinc powder (2.2 g) was added. The solution was stirred at room temperature under N_2. The assay of compound **2** was the same as described in the Experimental section.

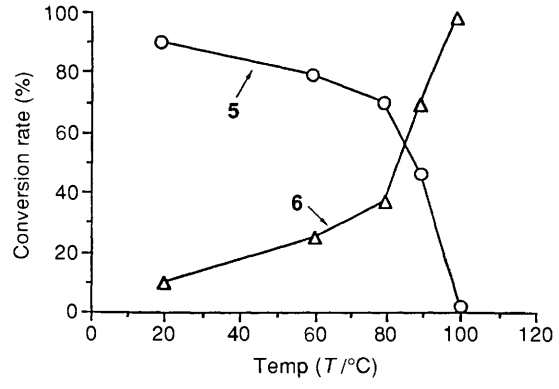
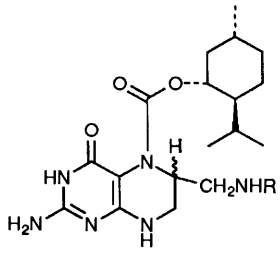


Fig. 3 Effect of reaction temperature on hydrolysis of compound **4**. The pH of the reaction mixture was kept between 6.5 and 6.9.

was formed (Fig. 3). The stability of (−)-leucovorin (6*S*)-**5** was best between pH 6 and 7. Therefore, this hydrolysis was done by refluxing compound **4** under neutral conditions. The product (6*S*)-**5** was purified by the method of Temple.[13] The optical purity of compound (6*S*)-**5** was 100% de which was estimated by HPLC[19] (using a Resolvosil column), whereas the optical purity of tetrahydrofolate (6*S*)-**3** prepared in the NADPH recycling system was also 94% de. It might be said that the enhancement of the optical purity could be attributed to the crystallization of compound (6*R*)-**4**. In spite of Owens' demonstration[7] of the unreliability of the previously reported value, the $[\alpha]_D^{20}$-value of the entirely optically pure (−)-leucovorin (6*S*)-**5** we prepared was -13.3×10^{-1} deg cm^2 g^{-1} (*c* 1, water, as the tetrahydrate).

This method is quite applicable to the large-scale synthesis, and now it is possible to prepare compound (6*S*)-**5** on more than a 20 kg scale.

Synthesis of (+)-*Leucovorin*.—We next studied the preparation of a completely diastereoisomerically pure form of (+)-leucovorin [(6*R*)-**5**] for pharmacological studies. Enzymic selective consumption of the natural (6*S*)-diastereoisomer in compound (6*RS*)-**3** by *Lactobacillus casei* thymidylate synthase[6] had been reported to give a non-natural (6*R*)-diastereoisomer with 99.8% de, but it could be said that the method was not suitable for obtaining it on a gram scale because of low enzyme productivity and inhibition of the enzymic consumption of the (6*S*)-diastereoisomer by the (6*R*)-diastereoisomer.[20] There have been some reports[7] on the optical resolution of the menthyloxycarbonyl derivative of tetrahydrofolate (6*RS*)-**3** [(6*RS*)-**7**], but the optical purities were not satisfactory [86–92% de for the (6*R*)- and (6*S*)-isomer].



(6*RS*)-**7**

We could prepare completely optically pure (+)-leucovorin (6*R*)-**5** by modifying the resolution method of (6*RS*)-5-menthyloxycarbonyltetrahydrofolic acid [(6*RS*)-**7**] by recrystallization. First, compound (6*RS*)-**7** was prepared by the known method[7] and the (6*R*)-diastereoisomer was extracted from the (6*RS*)-mixture at 60% de. This was chromatographed on HPLC to afford a small amount of the entirely optically pure form of compound (6*R*)-**7**. Using this material as the seed for crystallization, the (6*R*)-enriched (60% de) derivative was recrystallized from methanol to afford 99% de of the (6*R*)-diastereoisomer. This was converted into compound (6*S*)-**4** by treatment with formic acid in 30% HBr in acetic acid and then entirely optically pure (+)-leucovorin was obtained by hydrolysis on a gram scale.

Biological Studies of (−)- *and* (+)-*Leucovorin*.—The comparison of biological activities between leucovorins (6*S*)- and (6*R*)-**5** is shown in Fig. 4. Although 0.01 μmol dm^{-3} of (−)-leucovorin (6*S*)-**5** effected the growth promotion of folate-deficient cells, more than 3 μmol dm^{-3} of *d*-leucovorin (6*R*)-**5** was needed for an equal effect. As many reports had shown,[5,6]

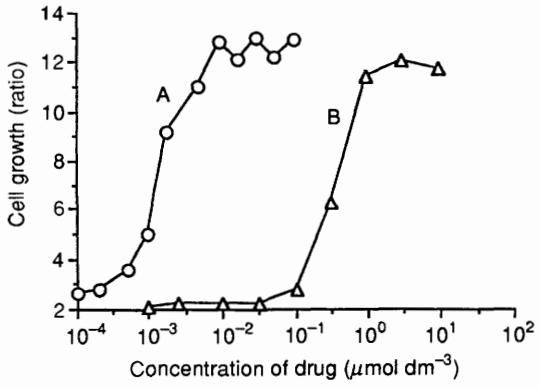


Fig. 4 Growth-promoting activity of (−)- and (+)-leucovorin, A and B respectively, on folate-deficient FM3A cells. FM3A cells were preincubated on RPMI1640 FA(−) medium (folic acid-deficient) for 24 h.* Each concentration of (−)- or (+)-leucovorin was added to this cell suspension (20 000 cells cm^{-3}) and the entire suspension was incubated. The cell numbers after incubation for 72 h were counted.

compound (6*R*)-**5** did not have any effect on the cell-growth inhibition activity of 5-FU. Other pharmacological studies, including toxicity, are now under investigation.

In summary, entirely optically pure (−)-leucovorin was prepared on a large scale by a combination of chemical and enzymic processes using the following techniques; (i) high production of DHFR, (ii) construction of an effective NADPH recycling system, (iii) an effective Zn^{2+}-removal method. To our knowledge, application of the mass production of DHFR based on a gene-cloning technique to the preparation of such a bioactive compound had not previously been known. Because the (6*S*)-diastereoisomer of compound **5** is essential as described above and is needed in large quantities in a completely diastereoisomerically pure form, such a process is quite suitable for this purpose.

Experimental

Materials.—Folic acid **1** was purchased from Takeda Pharmaceutical Co., Ltd. NADP was purchased from Oriental Yeast Co., Ltd. DHFR for the large-scale synthesis of tetrahydrofolate (6*S*)-**3** was produced as a yellow powder by the method reported by Oshiro,[11] at the Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd. GluDH, commercially available for clinical analysis, was a gift from Kyowa Medex Co., Ltd. All other chemicals were commercially available. ^1H NMR spectra were obtained with a Brucker AC-300 spectrometer, and the signals are given in ppm with tetramethylsilane as internal standard. Mass spectra were determined on a Hitachi M-80B mass spectrometer. IR spectra were recorded on a Shimadzu IR-435 spectrometer. Optical rotations were measured with a Horiba SEPA-200 polarimeter, and are given in units of 10^{-1} deg cm^2 g^{-1}. Microanalyses were measured with a Yanaco MT-3 CHN corder. Calcium contents were measured using a Hitachi Z-8100 polarized Zeeman Atomic Absorption Spectrometer. pH Values were determined with a Horiba F-15 pH meter. The activities of DHFR and GluDH were measured by a spectrophotometric method,[11,12,21] using a Shimadzu spectrometer UV-2100 system, based on the increase and decrease in absorbance at 340 nm caused by the oxidation of NADPH and the reduction of NADP, respectively. All the reactions were monitored by HPLC [column: YMC Pack A-312 ODS (6 × 150 mm); mobile phase: 50 mmol dm^{-3} citrate buffer–dioxane (94:6) (pH 3.5); flow rate: 1 cm^3 min^{-1}; detection: UV 285 nm]. The optical purities of tetrahydrofolate **3** were measured based on Rees' method[8] by converting it into the HPLC-separable diastereoisomeric urethane by using (*R*)-1-(1-naphthylethyl) isocyanate. The optical purities of compound **5** were measured based on HPLC[19] using a BSA (bovine serum albumin)-bound ODS column [column: Resolvosil (4 × 150 mm, two columns were linked), mobile phase: 50 mmol dm^{-3} phosphate buffer (pH 7.5), flow rate: 0.2 cm^3 min^{-1}; detection: UV 290 nm]. The optical purities of compound **7** were also measured by HPLC [column: YMC Pack A-312 ODS (6 × 150 mm), mobile phase: 50 mmol dm^{-3} Tris buffer–MeCN (3:1) (pH 7.0), flow rate: 1 cm^3 min^{-1}; detection: UV 285 nm].

Assays for Enzymic Activities.—The activities of DHFR were measured[11,21] by using the standard reaction mixture in a volume of 1 cm^3 containing compound **2** (0.3 mmol dm^{-3}), NADPH (0.3 mmol dm^{-3}), dithiothreitol (DTT, 1 mmol dm^{-3}), 50 mmol dm^{-3} potassium buffer (pH 7.4), and the enzyme at 30 °C. The activities of GluDH were measured[12] using the standard reaction mixture in a volume of 1 cm^3 containing glucose (170 mmol dm^{-3}), NADP (0.44 mmol dm^{-3}), 0.1 mmol dm^{-3} potassium buffer (pH 7.0) and the enzyme at 30 °C.

(6R)-5,10-*Methylylidenetetrahydrofolic Acid Chloride* [(6R)-**4**].—Compound **1** (10.0 kg, 20.9 mol) was dissolved in 1 mol dm^{-3} NaOH (68 dm^3) and the pH of the soluton was adjusted to pH 13.5 with 10 mol dm^{-3} NaOH at 40 °C. Under N_2, zinc powder (7.5 kg, 114.7 mol) was added to the solution and the mixture was stirred at 40 °C for 2 h. The pH of the mixture was adjusted to 7.5 with H_3PO_4, then excess of zinc and zinc phosphate were filtered off under N_2. To the filtrate were added glucose (4.5 kg, 25.0 mol), GluDH (1 500 000 U), DHFR (750 000 U) and NADP (86.7 g, 0.21 mol) under N_2 and the mixture was stirred at pH 7.5, 40 °C for 2 h. Ascorbic acid (4.0 kg, 0.23 mol) was then added and the pH of the mixture was adjusted to 3.5. The mixture was cooled to 10 °C and the precipitate [(6*S*)-**3**] was filtered off under N_2. This was immediately dissolved in a mixture of formic acid (60 dm^3) and 12 mol dm^{-3} HCl (1.7 dm^3), and the solution was stirred at 25 °C for 12 h under N_2. The mixture was concentrated under reduced pressure, then to the residue was added 0.5 mol dm^{-3} HCl (60 dm^3) to afford tricycle (6*R*)-**4** (9.0 kg, 84% from **1**); ν_{max}(KBr)/cm^{-1} 1640, 1556, 1510, 1411, 1322, 1248 and 765; $\delta_H(CF_3CO_2D)$ 2.40–2.84 (4 H, m, β- and γ-H_2), 3.91 (1 H, m, α-H), 4.43–5.08 (5 H, m, 6-H and 7- and 9-H_2), 7.56 (2 H, d, *J* 7.9, 3′,5′-protons in *p*-phenylene group), 8.09 (2 H, d, *J* 7.9, 2′,6′-protons in *p*-phenylene group) and 9.58 (1 H, s, CH=N); *m/z* (SIMS) 456 (M^+ − Cl).

(6S)-5-*Formyltetrahydrofolic Acid* [(6S)-**5**, (−)-*Leucovorin*].—Compound (6*R*)-**4** (9.0 kg, 17.7 mol) and triethylamine (10 dm^3) were dissolved in water (pH 6.5–6.9) (45 dm^3) and the mixture was heated at 100 °C for 3 h under N_2. The mixture was filtered and the pH of the filtrate was adjusted to 7.0 with triethylamine. An aqueous solution of calcium chloride (6 dm^3; 7.2 kg, 64.9 mol) was added to the filtrate and the mixture was stirred for 2 h at 10 °C to afford the calcium salt of (−)-leucovorin (6*S*)-**5**. This solid, after filtration, was dissolved in a solution of magnesium chloride (1.36 kg, 14.3 mol) in water (240 dm^3), and the solution was adjusted to pH 12.0 by addition of calcium hydroxide (~1.25 kg, 16.9 mol). The mixture was filtered and the pH of the filtrate was adjusted to 7.0 with 1 mol dm^{-3} HCl, then the mixture was concentrated under reduced pressure to 120 dm^3. The mixture was charged onto a Florisil column (100 kg), eluted with water, and the fraction containing only (−)-leucovorin (6*S*)-**5** (400 dm^3) was concentrated under

* At this stage, they had a slight growth ability for further 72 h cultivation.

reduced pressure to 200 dm^3. The pH of the residue was adjusted to 12 with calcium hydroxide. The mixture was filtered and the pH of the filtrate was adjusted to 7.0 with 1 mol dm^{-3} HCl, then the solution was concentrated under reduced pressure to 150 dm^3. After the addition of calcium chloride (0.88 kg, 7.9 mol), a portion of EtOH (450 dm^3) was added to afford pure (−)-*leucovorin tetrahydrate* as the *calcium salt* (4.1 kg, 40%) (Found: C, 41.2; H, 5.2; N, 16.6; Ca, 5.7. $C_{20}H_{21}CaN_7O_7 \cdot 4H_2O$ requires C, 41.16; H, 5.01; N, 16.80; Ca, 6.87%), $[\alpha]_D^{20}$ −13.3 (*c* 1, water); Optical purity 100% de; ν_{max}(KBr)/cm^{-1} 1605, 1410, 1290 and 765; $\delta_H(D_2O)$ 1.81–2.12 (4 H, m, β- and γ-H_2), 2.97–3.51 (5 H, m, 6-H and 7- and 9-H_2), 4.09 (1 H, m, α-H), 6.45 (2 H, d, *J* 8.7, 3′,5′-protons in *p*-phenylene group), 7.40 (2 H, d, *J* 8.7, 2′,6′-protons in *p*-phenylene group) and 7.68 and 8.38 (1 H, 2 s, HCON); *m/z* (SIMS) 512 ($MH^+ - 4H_2O$).

(6R)-5-*Formyltetrahydrofolic Acid* [(6R)-**5**, (+)-*Leucovorin*].—To a suspension of compound **1** (100 g, 209 mmol) in water (2.1 dm^3) was added 50% aq. NaOH (42 cm^{-3}). Under N_2 aq. $NaBH_4$ (100 g, 2.64 mol in 300 cm^3) was added to the mixture at 5 °C. After stirring of the mixture for 2 h, 50 mmol dm^{-3} Tris buffer (2 dm^3) was poured into the mixture, and then a solution of (−)-menthyl chloroformate (60 cm^3, 279 mmol) in EtOH (5 dm^3) was added. The reaction mixture was stirred for 12 h, then was concentrated under reduced pressure to half volume, and the pH of the residue was adjusted to 3.0 to afford compound (6*RS*)-**7** (240 g). This compound was suspended in Pr^iOH (4 dm^3). The suspension was stirred at room temperature for 5 h and the precipitate was filtered off to afford the (6*R*)-diastereoisomer (60% de) (78 g). This (6*R*)-enriched diastereoisomer (4.4 g) was charged onto an HPLC column (ODS, 70 × 10 cm diam., mobile phase: 50 mmol dm^{-3} Tris buffer (pH 5.4)–MeCN (7:3), flow rate: 150 cm^3 min^{-1}) to afford the entirely optically pure (+)-diastereoisomer (500 mg). Using this (+)-diastereoisomer as a seed for crystallization, optical resolution on a large scale was carried out as follows; the (6*R*)-diastereoisomer (60 g, 60% de) was dissolved in MeOH (150 cm^3). After inoculation with a seed, the solution was kept at room temperature for 20 h. The precipitate was filtered off to afford 96% de of (+)-diastereoisomer. This procedure was repeated again to give 99% de of the (+)-diastereoisomer (19.8 g). The optically pure (+)-diastereoisomer was dissolved in a mixture of formic acid (200 cm^3) and 30% HBr in acetic acid. The solution was stirred for 5 h at 60 °C, then was concentrated under reduced pressure until the solution became a suspension. After the addition of 0.5 mol dm^{-3} HCl (400 cm^3), the suspension was evaporated to dryness. To the residue was added 0.5 mol dm^{-3} HCl (400 cm^3) and the precipitate was filtered off to afford compound (6*S*)-**4**. This was hydrolysed by the same procedure as described in the preparation of (−)-leucovorin to afford 6.2 g of (+)-leucovorin as a solid (6.2 g, 6.6% from **1**); $[\alpha]_D^{28}$ +30.0 (*c* 1, water); optical purity 100 % de.

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