The Preparation of the (6*R*)- and (6*S*)-Diastereoisomers of 5-Formyltetrahydrofolate (Leucovorin)

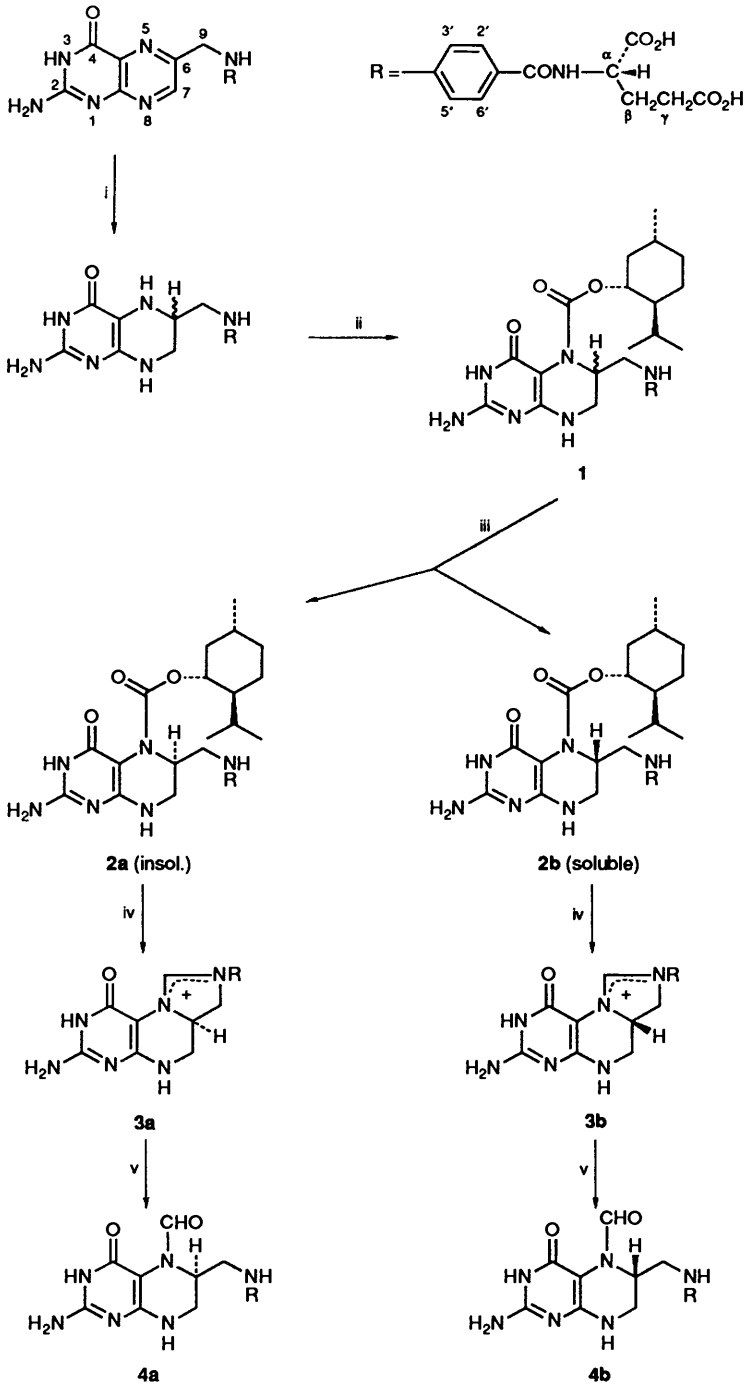
Jonathan Owens, Lilias Rees, Colin J. Suckling* and Hamish C. S. Wood*
Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK

The separation of the (6*R*)- and (6*S*)-diastereoisomers of tetrahydrofolic acid by derivatisation on N-5 with chiral auxiliary reagents followed by fractional crystallisation or extraction is described. Chloroformates of chiral alcohols were used as chiral auxiliaries and those derived from cyclic terpene alcohols were found to be most effective for the separation. The cleavage of the derivative and conversion *in situ* into 5,10-methenyltetrahydrofolic acid which was subsequently hydrolysed to afford 5-formyltetrahydrofolate (leucovorin) was investigated for the derivatised tetrahydrofolates; that from (−)-menthol was the only one that combined satisfactory separation with sufficient lability for efficient conversion into 5-formyltetrahydrofolate. The characterisation and optical purity of the products is described.

The naturally active diastereoisomer of tetrahydrofolate derivatives has been shown to have the 6*S* configuration.[1,2] A mixture of the (6*R*)- and (6*S*)-diastereoisomers of 5-formyltetrahydrofolate (leucovorin) is widely used in rescue therapy of patients undergoing treatment for cancer with methotrexate[3] and together with 5-fluorouacil in treatment of colorectal cancer.[4] It has, however, been shown that thymidylate synthase from *L. casei* is inhibited by the non-natural isomer of 5,10-methylenetetrahydrofolate[5] as is 5,10-methylenetetrahydrofolate dehydrogenase from *E. Coli*.[6] Moreover, the non-natural isomer of 10-formyltetrahydrofolate is a potent competitive inhibitor of glycinamide ribonucleotide formyl transferase.[7] In these cases the non-natural diastereoisomer cannot be regarded as biologically inert and there is, therefore, a potential clinical requirement for the natural (6*S*)-isomer of leucovorin. The preparation of single diastereoisomers of tetrahydrofolate derivatives has been reported by fractional crystallisation,[8] by chromatography,[9,10] and by enzyme (dihydrofolate reductase)-catalysed reduction.[11,12] Although samples of each diastereoisomer are available, in principle, by the first two methods, the quantity that can be produced is very small and insufficient for a thorough biological evaluation of the relative properties of the diastereoisomeric leucovorins in clinical practice. Enzyme-catalysed reaction, of course, affords only the natural isomer. We report here the first practical method for the preparation of substantial quantities of each diastereoisomer of leucovorin. A preliminary account of part of this work has been published,[13] and a patent on the process has been granted.[14] More recent patent applications claim processes for the preparation of the pure isomers by crystallisation of various salts of leucovorin.[15]

Separation of Diastereoisomeric Urethane Derivatives of Tetrahydrofolate.—In our studies using dihydrofolate reductase,[11] we used 1-naphthylethyl isocyanate as a chiral derivatising agent to aid characterisation of the diastereoisomeric purity of the products. This compound afforded crystalline derivatives that were readily separable by HPLC. In principle, the chromatographic separation of the urethane derivatives offered a method for the preparation of the required pure diastereoisomers of leucovorin provided that the urea could be cleaved without disruption of the remainder of the molecule. It was clearly unlikely that such selectivity could be achieved and we therefore investigated the preparation of more labile derivatives, in particular urethanes in which one of the amide linkages of the urea is replaced by the more labile ester link. We therefore prepared the urethane **1** by treating (6*RS*)-tetrahydrofolate, prepared by reduction of folic acid with sodium borohydride under nitrogen, *in situ*, with (−)-menthyl chloroformate which is available commercially (Scheme 1). The derivative **1** was obtained crystalline and characterised fully. The HPLC chromatogram showed two equal and well resolved peaks corresponding to each diastereoisomer. However, when purification by recrystallisation from ethanol was attempted, the recrystallised product showed an unequal proportion of diastereoisomers that was mirrored by the proportions in the mother liquors. Repeated recrystallisation improved the separation of diastereoisomers. It thus appeared that we had fortuitously discovered a simple method of separating the 6*R*- and 6*S*-diastereoisomers of the menthylurethanes of tetrahydrofolate.

There was no reason to believe that the separation obtained using (−)-menthylurethanes was optimal and we therefore undertook a survey of the potential of other chiral chloroformates to provide a more easily separable mixture. The need to cleave the urethane without racemisation in the following conversion into leucovorin was borne in mind when selecting the chloroformates for test. Also, we examined a range of alcohols to test their potential as recrystallisation solvents. The results are summarised in the Experimental section. The proportions of diastereoisomers of all the derivatives prepared could be monitored by HPLC but not all led to easy separations by recrystallisation or solvent extraction. In general, cyclic terpenoid urethanes performed best and higher alcohols were an improvement on ethanol. The best partners were found to be the (−)-menthylurethane **1** and butan-1-ol; the isomer **2a**, leading to the natural diastereoisomer of leucovorin, was found to be the less soluble on extraction at room temperature overnight. From this material, it was possible to obtain highly pure samples of the natural isomer of leucovorin by repeated extraction (see below). Similarly pure samples of the more soluble (−)-menthylurethane **2b** were, however, not obtained from the mother liquors. As described below, satisfactory conditions for the conversion of these urethanes into leucovorin were established and it became important to discover a method for the production of gram quantities of the non-natural isomer of leucovorin for biological evaluation. We therefore investigated (+)-menthyl chloroformate as a potential chiral auxiliary. Unfortunately, the resulting derivative was found to have very poor properties with regard to separation by solvent extraction. The best method for obtaining highly pure samples of the non-



Scheme 1 *Reagents and conditions:* i, $NaBH_4$, aq. NaOH; ii, (−)-menthyl chloroformate, pH 7; iii, butan-1-ol extraction; iv, HCO_2H, HBr at 52 °C; v, pH 6.5–7.0 (reflux) then $CaCl_2$.

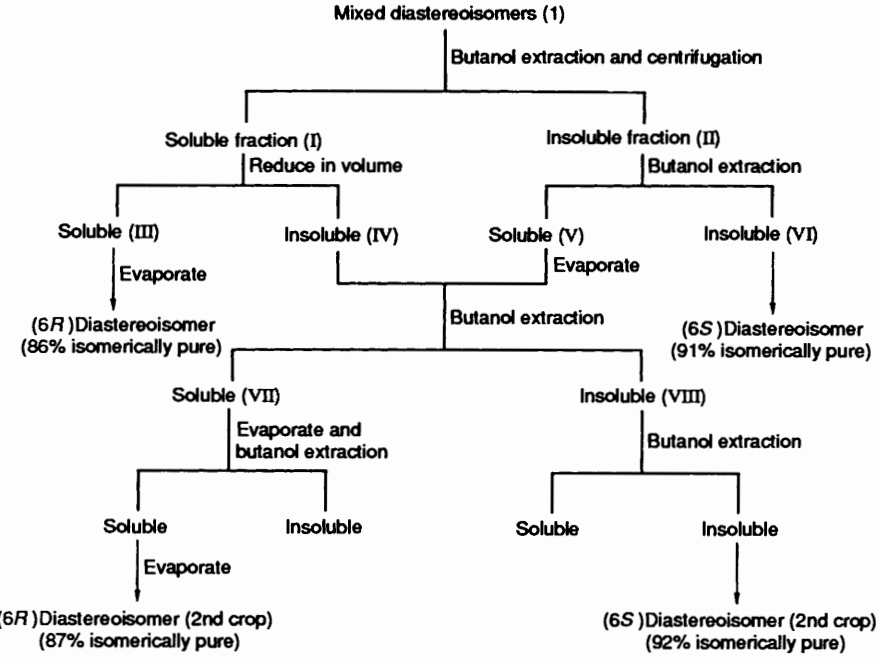
natural isomer therefore relied upon chromatography of the (−)-menthyl derivative.

Since complete resolution was not obtained in one cycle of extraction, it was necessary to develop a sequential process recycling portions of the mixtures. Scheme 2 shows a typical extraction sequence using butan-1-ol as solvent. It was found that, in order to obtain satisfactory extractions, the butanol must be substantially free of water and the mixed diastereoisomers must also be dried as described in the Experimental section. All the butanol used in these operations was, therefore, redistilled; recovered butanol was satisfactory provided that redistillation was carried out before it was used for extraction. The largest batches of (−)-menthylurethanes that have been separated in this way began from 17 g of mixture and led to 6.65 g of the natural diastereoisomer and 6.69 g of the unnatural diastereoisomer in purities of 91 and 86% respectively as shown by HPLC and NMR.

Conversion of Menthylurethanes into Leucovorin.—There are several problems to be considered in establishing conditions for the cleavage of the separated urethanes, namely, the avoidance of racemisation, the avoidance of cleavage of the glutamate-pteroate amide bond, and oxidation of the tetrahydrofolate system. With these problems in mind, we sought conditions under which the tetrahydrofolate liberated would be acylated *in situ* by formic acid to yield leucovorin which, being a 5-acyltetrahydrofolate, is stable to oxidation. A wide range of reagents was investigated following precedent in the literature

Table 1 Optical rotations of leucovorin $[\alpha]_D$

(6*R*) (unnatural)	(6*S*) (natural)	(6*RS*)	Ref
+28.3 (H_2O, Ca salt)	−15.1 (H_2O, Ca salt)	+14.26 (H_2O, Ca salt)	8
> +50 (buffer)	−33.5 (buffer)		9
	−25.2 (H_2O, Ca salt)	+16.0 (H_2O, Ca salt)	16
	−28.5 (H_2O)		2
	+2.1 (H_2O, Ca salt)		11
+22.9 (H_2O, Ca salt)	−12.5 (H_2O, Ca salt)		14
	−13.3 (H_2O, Ca salt)		12
+43.7 (Ca salt)	−15 (Ca salt)		15

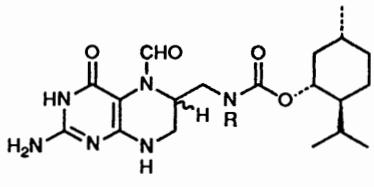


Scheme 2

of protecting groups. In our preliminary publication,[13] we reported the use of hydrogen bromide in a mixture of acetic and formic acids as an appropriate reagent. The success of this mixture depends upon careful temperature control; if the temperature was allowed to rise above 59 °C, significant racemisation at position 6 and cleavage of glutamate resulted. We have since found that the cleavage and formylation can be accomplished better using formic acid alone saturated with hydrogen bromide gas at 52 °C. This led directly to formation of the 5,10-methenyltetrahydrofolic acids **3a** and **3b**. No other acidic conditions have proved satisfactory.

We also considered the possibility of reductive cleavage of the urethane; if a suitable reducing agent could be found, the urethane could be converted directly into leucovorin with the alcohol as leaving group. Once again, many reagents were investigated but none was satisfactory. Severe problems were experienced with the solubility of the urethanes as substrates for this type of reaction; their extreme insolubility in solvents of low polarity virtually excluded the use of powerful nucleophilic reducing agents such as aluminium hydrides.

Conversion into Leucovorin and Characterisation of the Product.—The 5,10-methenyltetrahydrofolic acids were converted into the corresponding (6*S*)- and (6*R*)-leucovorins **4a** and **4b** by hydrolysis at pH 6.5–7.0 in the absence of air. The calcium salts were isolated. Previous workers[2,8,9] have relied heavily upon optical rotations as evidence for the diastereoisomeric purity of their products. We have surveyed the previously reported data in comparison with our own and find that the method is unreliable (Table 1). Therefore, we have used two alternative and independent methods to establish the diastereoisomeric purity of our products; the methods are HPLC on a chiral column,[17] and NMR spectroscopy on N-10 derivatives of leucovorin in which an additional chiral centre is introduced by the derivative. The chemical purity of the products can be established by HPLC on a standard reversed phase column. Chromatography of the leucovorin diastereoisomers on 'Resolvosil' columns shows that the purity of the natural (6*S*)-leucovorin was better than 90% and that of the (6*R*)-diastereoisomer *ca.* 82%. Leucovorin was acylated on N-10 with (−)-menthyl chloroformate; the resulting urethane **5** was examined by NMR (Table 2) and HPLC. The chemical shift of the formyl proton provided the clearest guide to isomeric purity. The (6*S*)-isomer **5a** showed a peak at δ 8.71 and the (6*R*)-isomer **5b** at δ 8.75. HPLC on reversed-phase columns confirmed the diastereoisomeric purity of the (6*S*)-isomer as



5a, 5b

R as in Scheme 1

Table 2 ^{1}H NMR chemical shifts of tetrahydrofolate derivatives[a]

Compound		Stereochemistry at C-6	Solvent	2'-, 6'-H	3'-, 5'-H	Substituent at C-5
5-(−)-Menthyloxycarbonyl-	**1**	6*RS*	0.1 mol dm^{-3} NaOD/D_2O	7.71 (bs), 7.77 (d)	6.83 (d), 6.91 (d)	—
	2a	6*S*	0.1 mol dm^{-3} NaOD/D_2O	7.71 (br s)	6.83 (d)	—
	2b	6*R*	0.1 mol dm^{-3} NaOD/D_2O	7.77 (d)	6.92 (d)	—
5,10-Methenyl-	**3a**	6*R*[b]	$(CD_3)_2SO$	8.00 (d)	7.51 (d)	9.65 (s)
[chloride salt]	**3b**	6*S*[c]	$(CD_3)_2SO$	8.00 (d)	7.51 (d)	9.65 (s)
5-Formyl-	**4a**	6*S*	0.1 mol dm^{-3} NaOD/D_2O	7.59 (d)[d]	6.65 (d)[d]	8.59 (s)[d]
[Ca salt]				7.65 (bs)[e]	6.70 (d)[e]	7.89 (s)[e]
	4b	6*R*	0.1 mol dm^{-3} NaOD/D_2O	7.58 (d)[d]	6.65 (d)[d]	8.59 (s)[d]
				7.63 (d)[e]	6.72 (bs)[e]	7.89 (s)[e]
5-Formyl-10-(−)-menthyl-		6*RS*	$(CD_3)_2SO$	7.81 (d), 7.84 (d)	7.32 (d), 7.36 (d)	8.71 (s), 8.75 (s)
oxycarbonyl-	**5a**	6*S*	$(CD_3)_2SO$	7.82 (d)	7.32 (d)	8.71 (s)[f]
	5b	6*R*	$(CD_3)_2SO$	7.83 (d)	7.35 (d)	8.75 (s)[g]

[a] Only signals characteristic of diastereoisomers are shown. [b] Natural diastereoisomer. [c] Unnatural diastereoisomer. [d] Major conformation (*cf.* ref. 20). [e] Minor conformation (*cf.* ref. 20). [f] Plus trace at δ 8.75. [g] Plus minor signal at δ 8.71.

around 95% and that of the (6*R*)-isomer as 82%. Of the methods of analysis, the quickest was undoubtedly HPLC on chiral columns but the limited lifetime of the columns and variable resolution militate against this method. In our experience, derivatisation on N-10 with (−)-menthyl chloroformate followed by NMR spectroscopy is the most robust method for the determination of diastereoisomeric purity.

Experimental

^{1}H NMR spectra were recorded using a Bruker WM-250 (250 MHz) spectrometer. Chemical shifts were measured relative to solvent or *tert*-butyl alcohol as internal standards. HPLC was carried out using ODS-2 reversed-phase columns (Hichrom) or 'Resolvosil' chiral columns (Mackerey-Nagel) at a flow rate of 40–50 cm^3 h^{-1}. The system comprised an LDC model 396 micropump and Cecil Instruments CE 212 and CE 2012 variable wavelength monitors. The solvent systems used were: (1) 5:95 acetonitrile–TRIS hydrochloride buffer (50 mmol, dm^{-3}, pH 7), (2) 25:75 acetonitrile–TRIS hydrochloride buffer (50 mmol dm^{-3}, pH 7) and (3) 5:95 acetonitrile–TRIS hydrochloride buffer (50 mmol dm^{-3}, pH 7) plus 2-mercaptoethanol (10 mmol dm^{-3}). In each case the flow rate was 40–50 cm^3 h^{-1}. Optical rotations, measured using a Perkin-Elmer 241 polarimeter with a 1-dm path-length cell, are recorded in units of 10^{-1} deg cm^2 g^{-1}.

Mixed Diastereoisomers (6RS) *of* 5-(−)-*Menthyloxycarbonyltetrahydrofolate.*—Folic acid (50 g) was suspended in distilled water (1050 cm^3) in a 10 dm^3 3-necked flask equipped with a mechanical stirrer, a gas inlet and bubbler for oxygen-free nitrogen, and a pressure-equalising dropping funnel. The procedure which follows was carried out under nitrogen. The flask was placed in an ice–water bath and aqueous sodium hydroxide (50%; 21 cm^3) was added; this was followed by sodium borohydride (50 g) in water (150 cm^3) which was added dropwise over 30 min. The reaction mixture was stirred for 4.5 h at 0–5 °C and then further sodium borohydride (50 g) in distilled water (50 cm^3) was added over 30 min. The mixture was stirred under nitrogen overnight. Examination of the reaction mixture at this stage by HPLC [solvent system (1), λ_{max} 285 nm] showed a single peak for the tetrahydrofolic acid (R_t 7.5 min). Under these conditions folic acid has a longer R_t value (10 min).

The reaction mixture was placed in an ice–water bath and the excess of sodium borohydride was destroyed by dropwise addition of concentrated hydrochloric acid (175 cm^3) added over 45 min. TRIS hydrochloride buffer (50 mmol dm^{-3}, pH 7; 1 dm^3) which had been degassed and saturated with nitrogen was added and the pH of the reaction mixture adjusted to 7. A solution of (−)-menthyl chloroformate (30 cm^3) in ethanol (2.5 dm^3, degassed and saturated with nitrogen) was added in a single portion immediately after preparation of the solution, and the whole reaction was stirred at room temperature for 21.5 h. Examination of the reaction mixture at this stage by HPLC [solvent system (2), λ_{max} 285 nm] showed two peaks of equal area with R_t values of 11.5 and 14 min. respectively.

Using a rotary evaporator the reaction mixture was reduced to half volume and filtered. The filtrate was cooled in an ice–water bath and a solid precipitated by slow addition of concentrated hydrochloric acid until the pH was 2.5–3. The crude product which separated was collected by centrifugation and purified by dissolution in aqueous sodium hydroxide (0.5 mol dm^{-3}; 1.5 dm^3), filtering of the solution and slow reprecipitation as before. The slurry was centrifuged and the solid washed with a little water and filtered off. The solid was sucked dry and finally dried *in vacuo* over phosphorus pentoxide to give the mixed diastereoisomers of 5-(−)*menthyloxycarbonyl*-(6RS)-*tetrahydrofolic acid* (85 g), m.p. 190–207 °C (decomp.) (Found: C, 55.5; H, 6.4; N, 14.9. $C_{30}H_{41}N_7O_8 \cdot H_2O$ requires C, 55.8; H, 6.7; N, 15.2%). HPLC [solvent system (2), λ_{max} 285 nm] showed two peaks of equal area at 11.5 and 14 min.

Prepared similarly were the mixed diastereoisomers of the corresponding (+)-menthyloxycarbonyl derivatives, m.p. 204–220 °C (decomp.) (Found: C, 56.8; H, 6.7; N, 15.4. $C_{30}H_{41}N_7O_8$ requires C, 57.4; H, 6.6; N, 15.6%). HPLC [solvent system (2), λ_{max} 285 nm] showed two peaks of equal area at 12 and 14 min.

Separation of the Mixed Diastereoisomers (6RS) *of* 5-(−)-*Menthyloxycarbonyltetrahydrofolic Acid* **1**.—The dry mixture of the diastereoisomers (17 g) was stirred overnight with dry butan-1-ol (1.5 dm^3). The mixture was centrifuged to give a soluble fraction (I) and an insoluble fraction (II). Using a rotary evaporator the soluble fraction (I) was reduced in volume to 400 cm^3 at 50–60 °C. This resulted in formation of a new precipitate (IV) which was collected by centrifugation. The supernatant (III) was evaporated to dryness to give the menthyloxycarbonyl derivative of (6*R*)-tetrahydrofolic acid (5.5 g). The precipitate (IV) was combined with a soluble fraction (V) obtained as described below.

The insoluble fraction (II) was stirred overnight with butan-1-ol (1 dm^3) in a second butanol extraction. This gave a soluble fraction (V) which was evaporated to dryness and used as described above, and an insoluble material (VI) which was dried over phosphorus pentoxide *in vacuo* to give the menthyloxycarbonyl derivative of (6*S*)-tetrahydrofolic acid (5.47 g).

Fractions (IV) and (V) were combined and stirred with butan-1-ol (330 cm^3) for 48 h in a third butanol extraction. This again gave a soluble fraction (VII) and an insoluble fraction (VIII). The soluble fraction (VII) was evaporated to dryness using a rotary evaporator and the residue stirred overnight with butan-1-ol (35 cm^3). The soluble material was again evaporated to dryness to give a second crop of the menthyloxycarbonyl derivative of (6*R*)-tetrahydrofolic acid (1.2 g).

The insoluble fraction (VIII) was stirred overnight with butan-1-ol (200 cm^3) and the resulting insoluble material was dried as above to give a second crop of the menthyloxycarbonyl derivative of (6*S*)-tetrahydrofolic acid (1.2 g).

Obtained thus were 5-(−)*menthyloxycarbonyl*-(6S)-*tetrahydrofolic acid* (6.67 g), m.p. 212 °C (decomp.), $[\alpha]_D^{20}$ −161 (Found: C, 56.0; H, 6.65; N, 14.6. $C_{30}H_{41}N_7O_8$. H_2O requires C, 55.8; H, 6.7; N, 15.2%), HPLC [solvent system (2)]; single peak at 14 min and 5-(−)-*menthyloxycarbonyl*-(6R)-*tetrahydrofolic acid* (6.7 g), m.p. 225 °C (decomp.), $[\alpha]_D^{20}$ +168 (Found: C, 58.9; H, 7.35; N, 13.7. $C_{30}H_{41}N_7O_8 \cdot C_4H_9OH$ requires C, 58.2; H, 7.35; N, 14.0%), HPLC; single peak at 11.5 min.

Attempted separation of the (+)-menthyloxycarbonyl derivatives was not satisfactory.

Acid Cleavage of the 5-(−)-*Menthyloxycarbonyl Group.*—(a) Hydrogen bromide gas was bubbled into a well stirred solution of 5-(−)-menthyloxycarbonyl-(6*S*)-tetrahydrofolate (96% isomerically pure; 10 g) in formic acid (98%; 100 cm^3) containing 3 drops of water. This mixture was placed in an oil-bath stabilised at 52 °C. The reaction was followed by UV spectroscopy. The λ_{max} 285 nm disappeared while a new λ_{max} at 360 nm appeared.

Examination of the reaction mixture by HPLC [solvent system (2) λ_{max} 295 nm] every 30 min showed the disappearance of the starting menthyloxycarbonyl derivative. The reaction was complete within 2.5 h. 2-Mercaptoethanol (0.1 cm^3) was added and the reaction mixture reduced to dryness on a rotary evaporator at 40 °C. Hydrochloric acid (2 mol dm^{-3}; 70 cm^3, containing 0.1% 2-mercaptoethanol) was added and the mixture was placed in an ultrasound bath for 1 min to disperse the solid. The solid product was filtered off and washed with the hydrochloric acid solution (30 cm^3), using ultrasound to disperse it and then filtered off again. The solid was then dried *in vacuo* over phosphorus pentoxide to give the 5,10-methenyl-(6*R*)-tetrahydrofolic acid chloride (*NB.* Natural diastereoisomer) (6.2 g), $[\alpha]_D^{20}$ +17.0 (conc. HCl).

Examination of the product by HPLC [solvent system (3), λ_{max} 285 and 360 nm] gave a single peak retention time of 9.5 min and co-injected with an authentic sample of 5,10-methenyl-(6*RS*)-tetrahydrofolic acid chloride. The hydrochloric acid washings were reduced on a rotary evaporator at 40 °C to give a second crop (0.7 g) of the methenyl salt.

(b) Similar treatment of 5-(−)-menthyloxycarbonyl-(6*R*)-tetrahydrofolic acid gave 5,10-methenyl-(6*S*)-tetrahydrofolic acid chloride (*NB.* Unnatural diastereoisomer), $[\alpha]_D^{20}$ −17.6. HPLC; single peak at 9.5 min and co-injects with authentic (6*RS*) material.

Opening of the 5,10-*Methenyl Salt to Leucovorin.*—(a) 5,10-Methenyl-(6*R*)-tetrahydrofolic acid chloride (1.0 g) was suspended in water (25 cm^3 degassed and nitrogen saturated) and stirred under oxygen-free nitrogen. Aqueous sodium hydroxide (1 mol dm^{-3} degassed and nitrogen saturated) was added slowly. The rate of addition was such that the pH was kept within the range 7–9. After 3 h when the methenyl salt had all dissolved the pH was adjusted to 6.5–7 and the reaction mixture was refluxed under oxygen-free nitrogen for 5 h, the pH being kept at 6.5–7; the reaction was followed by HPLC [solvent system (1), λ_{max} 285]. The reaction mixture was allowed to cool overnight, after which the pH was adjusted to 8 and aqueous calcium chloride (1.5 cm^3 of a filtered solution of 10 g in 25 cm^3 water) was added followed by ethanol (30 cm^3). The mixture was filtered and the solid was washed with ethanol–water (1:1), ethanol and finally ether. This gave calcium 5-formyl-(6*S*)-tetrahydrofolate (0.57 g). Further addition of ethanol gave a second crop (0.38 g), $[\alpha]_D^{21}$ −17.3 (H_2O) (Found: C, 45.7; H, 4.2; N, 18.5. $C_{20}H_{21}N_7O_7Ca \cdot H_2O$ requires C, 45.4; H, 4.4; N, 18.5%). Examination of the product by HPLC [solvent system (3), λ_{max} 285] gave a single major peak (R_t 6 min) and which co-injected with authentic calcium 5-formyl-(6*RS*)-tetrahydrofolate.

(b) Similar treatment of 5,10-methenyl-(6*S*)-tetrahydrofolic acid chloride gave calcium 5-formyl-(6*R*)-tetrahydrofolate, $[\alpha]_D^{21}$ +25.1 (Found: C, 42.4; H, 4.3; N, 17.7. $C_{20}H_{21}N_7O_7Ca \cdot 3H_2O$ requires C, 42.5; H, 4.8; N, 17.3%). HPLC on a reversed-phase column [solvent system (3), λ_{max} 285] gave a single peak at 6 min.

Mixed Diastereoisomers (6RS) *of* 5-(−)-*Bornyloxycarbonyltetrahydrofolic Acid.*—Folic acid (2.0 g) was reduced to tetrahydrofolic acid by the procedure used above for the preparation of menthyloxycarbonyltetrahydrofolic acid. The reaction mixture was placed in an ice–water bath and the excess of sodium borohydride was destroyed by dropwise addition of concentrated hydrochloric acid (6 cm^3). TRIS hydrochloride buffer (50 mmol dm^{-3}, pH 7; 150 cm^3) which had been degassed and saturated with nitrogen, was added and the pH adjusted to 7. A solution of (−)-bornyl chloroformate[18] (1.2 g) in ethanol (degassed and saturated with nitrogen; 200 cm^3) was added in a single portion immediately after preparation of the solution, and the whole stirred at room temperature overnight. Using a rotatory evaporator, the reaction mixture was reduced to half volume and filtered. The filtrate was kept cool in an ice–water bath and adjusted to pH 3. The crude product which separated was collected by centrifugation and purified by dissolution in aqueous sodium hydroxide, filtering of the solution and reprecipitation by adjusting the solution to pH 3 as before. The slurry was centrifuged and the solid was washed with water (100 cm^3) and filtered off. The solid was sucked dry and finally dried *in vacuo* over phosphorus pentoxide to give the mixed diastereoisomers of 5-(−)-*bornyloxycarbonyl*-(6RS)-*tetrahydrofolic acid* (2.42 g), m.p. 204–220 °C (decomp.) (Found: C, 55.8; H, 6.4; N, 14.7. $C_{30}H_{39}N_7O_8 \cdot H_2O$ requires C, 56.0; H, 6.4; N, 15.2%). HPLC [solvent system (2), λ_{max} 285 nm] showed two peaks of equal area at 11.5 and 12.5 min.

The diastereoisomers were separated as described above for the menthyloxycarbonyl derivatives using either butan-1-ol or butan-2-ol. The separation was not so effective as that of the menthyloxycarbonyl derivatives. The separated diastereoisomers were converted into the separate diastereoisomers of calcium 5-formyltetrahydrofolate as described for the menthyloxycarbonyl analogues.

Mixed Diastereoisomers (6RS) *of* 5-(−)-*Isobornyloxycarbonyltetrahydrofolic Acid.*—Folic acid (2.0 g) was reduced to tetrahydrofolic acid and the excess of sodium borohydride was destroyed by the same procedure as used for the menthyloxycarbonyltetrahydrofolic acid preparation. TRIS hydrochloride buffer (50 mmol dm^{-3}, pH 7; 150 cm^3) which had been degassed and saturated with nitrogen was added and the pH adjusted to 7. A solution of (−)-isobornyl chloroformate[19] (1.2 cm^3) in ethanol (degassed and saturated with nitrogen; 200 cm^3) was added in a single portion immediately after preparation of the solution and the whole was stirred at room temperature.

HPLC analysis showed that reaction was *ca.* 50% complete after 30 min. After a further 1.5 h HPLC analysis showed little change. A further addition of (−)-isobornyl chloroformate (1.2

cm^3) was made at this time followed by another an hour later. The whole was stirred at room temperature overnight. The product, an off-white precipitate, was filtered off and purified by dissolution in aqueous sodium hydroxide, filtering of the solution, and reprecipitation by adjusting the solution to pH 3. The slurry was centrifuged and the solid was washed with a little water and filtered off to give the mixed diastereoisomers of 5-(−)-*isobornyloxycarbonyl*-(6RS)-*tetrahydrofolic acid* (1.45 g), m.p. 204–220 °C (decomp.) (Found: C, 56.7; H, 6.4; N, 15.5. $C_{30}H_{39}N_7O_8 \cdot H_2O$ requires C, 56.0; H, 6.4; N, 15.2%). HPLC [solvent system (2), λ_{max} 285 nm] showed two peaks of equal area at 15 and 16.5 min.

These diastereoisomers could not be separated by solvent extraction. They were, therefore, separated by chromatography using preparative HPLC on a reversed-phase column and the purity of the separated diastereoisomers confirmed by analytical HPLC. The separated diastereoisomers were converted into calcium 5-formyltetrahydrofolate as described above.

Studies of the Diastereoisomeric Purity of Calcium 5-Formyltetrahydrofolate.—(1) *By chiral HPLC.*[17] Samples of calcium 5-formyltetrahydrofolate were examined on 'Resolvosil' columns using as the mobile phase, phosphate buffer (5 mmol dm^{-3}, pH 7.4), a flow rate of 0.5 cm^3 min^{-1} and an analytical wavelength of 285 nm with the following results: (6*RS*)-leucovorin (Wellcovorin): two peaks of equal area with R_t values of 12 min (6*S*) and 18 min (6*R*); (6*S*)-leucovorin: a major peak with a retention time of 11 min (6*S*) and a minor peak at 18 min (6*R*) in a ratio of 92:8; (6*R*)-leucovorin: a minor peak with a retention time of 11 min (6*S*) and a major peak at 18 min (6*R*) in a ratio of 18:82.

(2) *By derivatisation.* 10-(−)-*menthyloxycarbonyl derivatives* (**5**). Calcium 5-formyl-(6*RS*)-tetrahydrofolate (Wellcovorin; 10 g) was stirred in TRIS buffer (50 mmol dm^{-3}, pH 7; 400 cm^3) and ethanol (400 cm^3) and (−)-menthyl chloroformate (50 cm^3) was added. The reaction was stirred at room temperature for a total of 120 h, the progress of the reaction being followed by HPLC. The pH of the solution was adjusted to *ca.* 8 every 12 h by the addition of 2 mol dm^{-3} aqueous sodium hydroxide and additional quantities (50 cm^3) of (−)-menthyl chloroformate were added after 24, 48 and 96 h. After 110 h, HPLC showed that no leucovorin remained.

The pH was adjusted to 8 and the reaction mixture was evaporated under reduced pressure at < 30 °C to *ca.* 450 cm^3 and filtered. The filtrate was extracted with ether (6 × 500 cm^3), and the pH adjusted to 2.5 by the slow addition of 2 mol dm^{-3} hydrochloric acid at 0 °C. The resulting precipitate of 5-*formyl*-10-(−)-*menthyloxycarbonyl*-(6RS)-*tetrahydrofolic acid* (9.5 g) was collected, washed with a little water, and dried *in vacuo*, m.p. 204–220 °C (decomp.) (Found: C, 55.7; H, 5.8; N, 14.6. $C_{31}H_{41}N_7O_9 \cdot H_2O$ requires C, 55.3; H, 6.4; N, 14.6%). HPLC [solvent system (2), λ_{max} 285 nm] showed two peaks of equal area at 8 and 10 min.

Prepared similarly were: (a) 5-*formyl*-10-(−)-*menthyloxycarbonyl*-(6S)-*tetrahydrofolate*, m.p. 200–210 °C (decomp.), $[\alpha]_D^{20}$ −23 (Found: C, 55.8; H, 6.6; N, 13.8. $C_{31}H_{41}N_7O_9 \cdot H_2O$ requires C, 55.3; H, 6.4; N, 14.6%). HPLC [solvent system (2)] major peak at 10 min (95%) and minor peak at 8 min (5%).

(b) 5-*Formyl*-10-(−)-*menthyloxycarbonyl*-(6R)-*tetrahydrofolate*, m.p. 215–220 °C (decomp.), $[\alpha]_D^{20}$ −40.4 (Found: C, 53.3; H, 6.4; N, 13.8. $C_{31}H_{41}N_7O_9 \cdot 2H_2O$ requires C, 53.8; H, 6.5; N, 14.2%). HPLC [solvent system (2)] major peak at 8 min (82%) and minor peak at 10 min (18%).

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