Pharmacological Testing Methods. Antagonism of *d*-amphetamine lethality in grouped mice was determined using a previously reported procedure.[7] Groups of 10 mice were treated with the test compound at graded doses and placed in wire mesh cages (20 × 13 × 13.5 cm). After 30 min, *d*-amphetamine sulfate in saline was administered at a dose of 15 mg/kg, which causes 90–100% death in untreated grouped mice. Deaths were measured after 24 h. ED_{50} values were determined and defined as the dose of compound that prevented death in 50% of the test animals.

Effects of the compounds on locomotor activity in rats were determined as previously described[7] by oral treatment of groups of five rats with graded doses of the test compounds. Locomotor activity was determined for each individual rat as measured over a 5-min interval at the time of peak effect (previously measured using a selected dose of the compound) utilizing an Animex® activity counter. The MDD_{50} was measured from a linear regression analysis and is defined as the dose that produces 50% reduction in motor activity as compared to the control animals.

Inhibition of tetrabenazine-induced depression of exploratory behavior in mice was determined in the reported manner.[7] Groups of five mice were treated with a dose of the test compound orally, and after 1 h were treated with tetrabenazine hexamate (aqueous) at a dose of 30 mg/kg ip. Treated mice were placed on a horizontal disk (18-in. diameter) after 30 min and exploratory behavior was measured within 10 s according to an observational response rating scale. The MED (minimum effective dose) was established by dosing initially at 25 mg/kg orally and halving the dose until the test compound is found inactive in the above procedure.

Induction of catalepsy was determined using groups of six to eight rats which were treated orally with graded doses of the test compound.[10] Each rat was tested for catalepsy at various times from 30 min to 18 h after drug administration. Catalepsy is defined as the failure of rats to move from an unnatural posture caused by placing their paws upon four separate raised pedestals within 10 s. Untreated animals remove one or more paws from the pedestals during this test in less than 10 s (usually immediately). ED_{50} values were determined and defined as the dose of compound that caused catalepsy in 50% of the test animals.

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Preparation and Purification of L-(±)-5-Formyl-5,6,7,8-tetrahydrofolic Acid

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Reinvestigation of the conversion of folic acid to leucovorin [L-(±)-5-CHO-THF] led to improved methods for the synthesis of this drug, which is suitable for clinical use. Also, methods were developed for the chromatographic and nonchromatographic purification of less pure samples of L-(±)-5-CHO-THF.

The active investigation of high-dose methotrexate with citrovorum factor [L-(−)-5-formyl-5,6,7,8-tetrahydrofolic acid, L-(−)-5-CHO-THF][1] rescue for the treatment of a number of solid tumors and hematologic malignancies requires large amounts of both methotrexate and leucovorin [L-(±)-5-CHO-THF].[2] Recently, our laboratory reported an improved method for the large-scale synthesis of methotrexate,[3] and in this paper we report improvements in the method for the large-scale preparation of L-(±)-5-CHO-THF.

The elegant work around 1950 by several research groups resulted in the synthesis of L-(±)-5-CHO-THF.[4,5] Although bioassay methods showed that mixtures of L-(±)-THF and reagents containing formate [e.g., HCO_2H, HCO_2Et, and $C_6H_5N(Me)CHO$] produced citrovorum factor activity, the method adopted for the isolation of the mixture of diastereomers involved the catalytic hydrogenation of 10-formylfolic acid (10-CHO-FA) in HCO_2H and treatment of the L-(±)-(5,10-CH-THF)$^+$ generated in situ with base at elevated temperatures to give crude L-(±)-5-CHO-THF, which was purified by column chromatography. Mechanisms involving the transformations of (5,10-CH-THF)$^+$ and 10-CHO-THF to 5-CHO-THF have been proposed.[6]

Initially, our efforts were directed toward the preparation and isolation of L-(±)-(5,10-CH-THF)$^+$ via FA, 10-CHO-FA, and L-(±)-10-CHO-THF. Treatment of FA·2H_2O with HCO_2H gave a diformylated derivative (^1H NMR), which was monodeformylated on recrystallization from H_2O to give 10-CHO-FA·H_2O. Chemical reduction of 10-CHO-FA to L-(±)-10-CHO-THF with $Na_2S_2O_4$, $NaBH_4$, or $NaBH_3CN$ (pH 6.7) under a variety of conditions was unsatisfactory, these reactions leading to mixtures of folates and decomposition products. Although the catalytic hydrogenation of 10-CHO-FA with Pt in HCO_2H was reported to occur readily with rapid stirring

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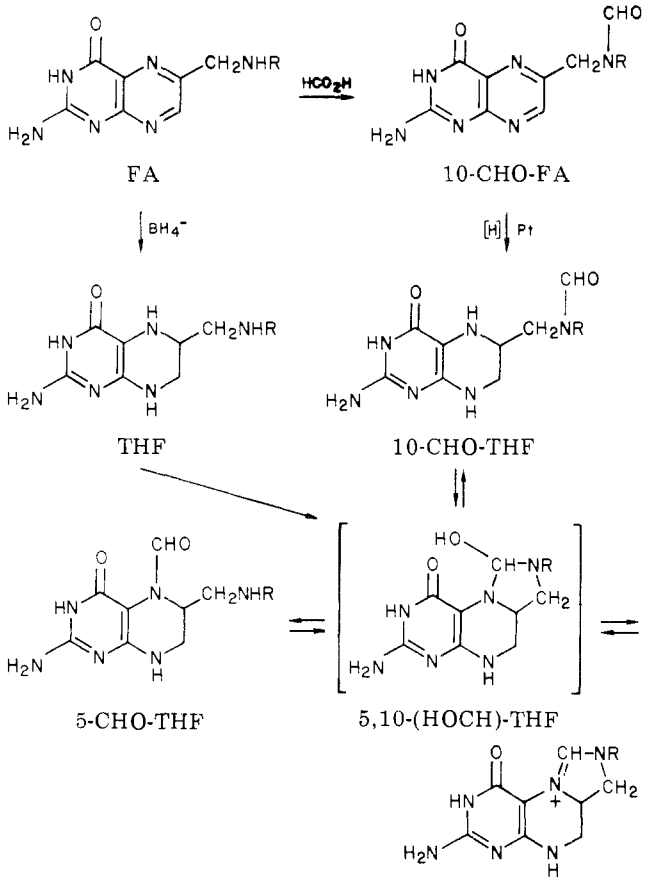
Scheme I



(1500 rpm),[5] this conversion was slow under normal stirring conditions. In contrast, this hydrogenation was easily effected in CF_3CO_2H.[7] In this regard, the rate of hydrogenation was depressed when HCO_2H was added to solutions of 10-CHO-FA in CF_3CO_2H. The initial product of hydrogenation, L-(±)-10-CHO-THF, was readily dehydrated by the acidic solvent to give L-(±)-(5,10-CH-THF)$^+$,[9,10] which was isolated as the chloride in near quantitative yield.

A more convenient route for the large-scale preparation of L-(±)-(5,10-CH-THF)$^+Cl^-$ resulted from the use of L-(±)-THF as an intermediate (Scheme I). Preliminary experiments indicated that about a 12-fold excess of $NaBH_4$ was required to ensure the complete reduction of FA to L-(±)-THF and that the use of a buffered reaction medium[11] was unnecessary. Previously, the isolation of L-(±)-THF (unstable) in the presence of 2-mercaptoethanol gave a mixture of FA, DHFA, and L-(±)-THF.[12,13] It is well documented that ascorbic acid stabilizes solutions of tetrahydrofolates, and the addition of ascorbic acid to the borohydride reaction mixture before complete acidification resulted in the isolation of L-(±)-THF as a boron complex in yields up to 96%. A solution of the isolated L-(±)-THF in 98:2 HCO_2H–CF_3CO_2H gave a good yield of L-(±)-(5,10-CH-THF)$^+Cl^-$ free of boron impurities. In addition, a procedure was developed that circumvented the isolation of L-(±)-THF. Treatment of an aqueous solution of L-(±)-THF, resulting from the $NaBH_4$ reduction of FA, with an equal volume of HCO_2H gave L-(±)-(5,10-CH-THF)$^+Cl^-$ essentially free of boron impurities.

Previously, the L-(±)-(5,10-CH-THF)$^+$ prepared in situ was converted to L-(±)-5-CHO-THF in a hot, neutral, or alkaline medium with a reaction time of about 1 h.[5,9] Extensive investigations on the conversion of L-(±)-(5,10-CH-THF)$^+Cl^-$ to L-(±)-5-CHO-THF over the pH range of 5.5–11.4 revealed that the purity of the L-(±)-5-CHO-THF formed was greatest when the reaction was performed under neutral or slightly acidic conditions. For the large-scale synthesis of L-(±)-5-CHO-THF from L-(±)-(5,10-CH-THF)$^+Cl^-$, the pH of the reaction medium was maintained near 6.7 for about 11 h, which gave a product with a ratio of the intensity of absorption at $\lambda_{max}^{282}/\lambda_{min}^{242}$ of 3.4–3.9.[14,15] These samples of L-(±)-5-CHO-THF contained 10-CHO-DHF and PABGA as the major impurities and 10-CHO-FA and pterins as minor impurities.[16] The major portion of the 10-CHO-DHF impurity in L-(±)-5-CHO-THF samples was formed from unconverted L-(±)-(5,10-CH-THF)$^+$ and L-(±)-10-CHO-THF during the basic workup of the reaction. The obtainment of L-(±)-5-CHO-THF of high quality[17] resulted from the use of isolated (purified) samples of L-(±)-(5,10-CH-THF)$^+Cl^-$ and from the conversion of the latter under conditions in which the formation of decomposition products was minimized.

Further purification of L-(±)-5-CHO-THF was effected by column chromatography. Although the procedure was tedious, practically pure L-(±)-5-CHO-THF was obtained from less pure samples by column chromatography on Sephadex G-10 developed with aqueous $Ca(OH)_2$ (pH 8). A better method, however, was the column chromatography of these samples on Florisil developed with aqueous mercaptoethanol, which gave L-(±)-5-CHO-THF in 28–35% yield (from FA). In later work, a nonchromatographic procedure was developed for the removal of most of the impurities from L-(±)-5-CHO-THF samples. In this procedure, a mixture of crude L-(±)-5-CHO-THF, $MgCl_2$, and $Ca(OH)_2$ in H_2O at pH 12 gave a precipitate of the inorganic oxides, most of the impurities, and some 5-CHO-THF. From the filtrate, L-(±)-5-CHO-THF of good quality was recovered.

Experimental Section

The ultraviolet absorption spectra were determined with a Cary Model 17 spectrophotometer and LC chromatograms were determined as previously described.[16,18]

L-(±)-5,10-Methenyl-5,6,7,8-tetrahydrofolic Acid Chloride. To a suspension of FA·2H_2O (1673 g, 3.500 mol) in H_2O (35 L), which was under an atmosphere of N_2 and cooled to 8 °C in an ice bath, was added slowly with stirring 50% NaOH (370 mL). The resulting clear yellow solution (pH 8, meter) was treated over a 1-h period with a solution of $NaBH_4$ (1673 g) in H_2O (5 L). During the addition, the temperature increased to a maximum of 17 °C. The solution was stirred for an additional 30 min, followed by the decomposition of excess $NaBH_4$ with concentrated HCl. The large amount of H_2 generated was vented to a hood. During the first half of the decomposition step, efficient cooling was required to maintain the temperature of the solution below 24 °C. The decomposition of $NaBH_4$ was essentially complete after the addition of 2000 mL of concentrated HCl, which required a period of 3 h. The resulting solution (pH 8.3) was adjusted to pH 6.6 with 500 mL of concentrated HCl over a period of 30 min. After the addition of a solution of ascorbic acid (175 g) in H_2O (800 mL), the pH of the solution was then adjusted to 3.5 with an additional 1800 mL of concentrated HCl over a period of 1 h. The resulting cream-colored suspension of L-(±)-THF was pumped into a Buchner funnel (11-L capacity) fitted with a glass-fiber paper (Whatman GF/D) and under an atmosphere of N_2.[19] This filtration was carried out in two batches because of the large amount of solid. Near the end of the filtration, a small portion (~50 g) of the L-(±)-THF slurry was exposed to air and was discarded. Each batch of the wet precipitate was dissolved in a mixture of 98:2 HCO_2H (97%)–CF_3CO_2H and transferred under vacuum to a 24-L flask. A total volume of 12750 mL of the acid mixture was used. After standing at room temperature for 14 h, the dark red solution was evaporated to dryness in vacuo

at a maximum H_2O-bath temperature of 60 °C. The superficially dried residue was suspended in 0.5 N HCl (35 L) containing 2-mercaptoethanol (1 mL/L of acid) and warmed to 45 °C, and the whole was concentrated under aspirator pressure to remove formic and trifluoroacetic acids (~3 L). After standing at room temperature for 18 h, the L-(±)-(5,10-CH-THF)$^+Cl^-$ was collected by filtration on a glass-fiber paper, washed with 0.01 N HCl (6 L), and dried in vacuo over P_2O_5: yield 1119 g (63%). A boron analysis indicated the absence of boron salts. Anal. Calcd for $(C_{20}H_{22}N_7O_6)^+Cl^-·H_2O$: C, 47.11; H, 4.74; Cl, 6.75; N, 19.23. Found: C, 47.24; H, 4.65; Cl, 7.16; N, 19.28.

Concentration of the filtrate to about one-third the original volume deposited a second crop, which was less pure L-(±)-(5,10-CH-THF)$^+Cl^-$: yield 95 g (~5%); total yield 1214 g (~68%).

In a modification of the procedure described above, FA·$2H_2O$ (10.0 g, 20.9 mmol) was reduced to L-(±)-THF, and the resulting solution was adjusted to pH 7 and diluted with 95% HCO_2H (270 mL). During the addition of HCO_2H, a precipitate of L-(±)-THF formed and redissolved rapidly as the volume of HCO_2H increased (final pH, 1.1). After standing for 18 h at room temperature, an inorganic precipitate (5.6 g) was removed by filtration. The filtrate was treated with concentrated HCl (3.5 mL) and evaporated to dryness under reduced pressure at 40 °C. The resulting solid was washed by stirring with cold 1% ascorbic acid (100 mL), collected by filtration, washed with additional 1% ascorbic acid solution, and dried in vacuo over P_2O_5: yield 10.0 g. A boron analysis indicated that this sample contained boron. Anal. Calcd for $C_{20}H_{22}ClN_7O_6·0.3H_3BO_3·2H_2O$: C, 43.96; H, 4.96; B, 0.59; Cl, 6.49; N, 17.94. Found: C, 43.78; H, 4.89; B, 0.61; Cl, 6.59; N, 17.70.

The above solid was stirred for 1 h in cold 0.5 N HCl (100 mL) containing mercaptoethanol (0.1 mL), recollected by filtration under N_2 pressure, washed in the funnel with additional 0.01 N HCl (100 mL), and dried in vacuo over P_2O_5: yield 8.5 g (79%). A boron analysis indicated that this sample contained a trace amount of a boron impurity (found, 0.06%). Anal. Calcd for $C_{20}H_{22}ClN_7O_6·H_2O$: C, 47.11; H, 4.74; Cl, 6.95; N, 19.23. Found: C, 47.34; H, 4.73; Cl, 6.84; N, 19.29.

Calcium L-(±)-5-Formyl-5,6,7,8-tetrahydrofolate. Solid L-(±)-(5,10-CH-THF)$^+Cl^-$ (1212 g) was added with stirring under a N_2 atmosphere to boiling H_2O (30 L) over a period of 20 min. During the addition and thereafter for 1 h, hot, oxygen-free 3.7 N NaOH (~2 L) was added at a rate to maintain an acidic reaction medium. At this point, complete dissolution of the solid was obtained with oxygen-free 1 N NaOH. The resulting solution was refluxed for 11 h while maintaining the pH between 6.5 and 6.9 (meter) with 1 N NaOH (total, ~450 mL). The progress of the reaction was followed by LC. After standing for an additional 8 h without heat, the solution (56 °C, pH 7.7) was treated with a clarified solution (1200 mL) of $CaCl_2$ (600 g), which lowered the pH to 7.2. The solution was diluted with EtOH (3.2 L) and transferred through tygon tubing with a peristaltic pump to a flask cooled in an ice–salt mixture. When the temperature of the mixture was less than 10 °C, the bright yellow solid that deposited was removed by filtration. On exposure to air, this solid darkened to a brown color and became gummy. TLC of the semidried residue (~400 g) showed that it contained L-(±)-5-CHO-THF contaminated with numerous impurities (see purification below).

The clear yellow filtrate from above was pumped into a large container and diluted with EtOH (total, 102 L). The resulting slurry of cream-colored precipitate of L-(±)-5-CHO-THF·Ca was cooled (<10 °C) in an ice bath for 18 h, the solid was collected by filtration, washed with EtOH (7 L), and dried in vacuo over P_2O_5: yield 897 g (~45% from FA);[17] UV λ_{max} (0.1 N NaOH) 282 nm (ϵ 28.8 × 10^{-3}) [$\lambda_{max}^{282}/\lambda_{min}^{242}$ (0.1 N NaOH) = 3.6[14]]; TLC [Avicel, 0.1 M phosphate buffer (pH 7)] R_f 0.76 (10-CHO-DHF), 0.85 (CF, absorbing), 0.93 (10-CHO-FA).[20] Anal. Calcd for $C_{20}H_{21}N_7O_7·0.5C_2H_6O·1.8H_2O$: C, 44.49; H, 4.91; N, 17.29; Ca, 7.07; ash (CaO), 9.89. Found: C, 44.49; H, 4.97; N, 17.29; Ca, 7.18; ash (CaO), 9.65.

LC assay of this sample indicated the presence of L-(±)-5-CHO-THF·Ca (78%) and, excluding ethanol and water, the following impurities: PABGA·Ca (3.1%), 10-CHO-DHF (4.6%), 10-CHO-FA (<0.5%), pterins (1.0%), and unidentified and undetected material (3.0%).[16]

Purification of Calcium L-(±)-5-Formyl-5,6,7,8-tetrahydrofolate. Florisil (350 g, 100–200 mesh) was suspended in H_2O (three times), and the fines were removed by decantation. The defined slurry of florisil was poured into a glass column (3.8 × 67 cm) and washed with H_2O until the effluent was clear and then with 0.2% aqueous mercaptoethanol (4000 mL). After a solution of impure L-(±)-5-CHO-THF·Ca (3.0 g) in 0.2% aqueous mercaptoethanol (10 mL) was applied, the column was developed with 0.2% aqueous mercaptoethanol at a rate of 0.3 mL/min. After 17.5 h, *p*-aminobenzoylglutamic acid was eluted from the column over the next 8 h. At this time (25.5 h), L-(±)-5-CHO-THF began to elute and three fractions were collected: I, 0.6 mL/min, 6.5 h; II, 0.6 mL/min, 10 h; III, 3 mL/min, 8 h.[21] Each fraction was concentrated to about one-fifth volume in vacuo (oil pump) at ~45 °C when an off-white flocculent solid began to precipitate. Each of the resulting mixtures was adjusted to pH 12 (meter) with 1 N NaOH and filtered through a thin Celite pad to remove the somewhat gelatinous insoluble material (MgO). The clear filtrates were adjusted to pH 7 (meter) with dilute HCl, treated with 25% aqueous $CaCl_2$ (clarified by filtration), readjusted to pH 7.5 (meter), and diluted slowly with 5 volumes of cold EtOH. The white precipitates were collected by filtration and dried in vacuo over P_2O_5.

The sample (0.36 g) from fraction 1 contained a water-insoluble material and was reprecipitated from an aqueous solution with EtOH: yield, 0.29 g (6.5% from FA); $\lambda_{max}^{282}/\lambda_{min}^{242}$ = 4.37.[14] LC indicated that this sample contained only trace amounts of UV-absorbing impurities. Anal. Calcd for $C_{20}H_{21}N_7O_7·3.8H_2O·Ca$: C, 41.42; H, 4.97; N, 16.91; Ca, 6.91; ash (CaO), 9.67. Found: C, 41.36; H, 4.91; N, 16.74; Ca, 7.04; ash (CaO), 9.78.

The sample from fraction 2 (1.2 g, 27% from FA) contained only trace amounts of UV-absorbing impurities (LC)[16] and was analyzed without further purification. Anal. Calcd for $C_{20}H_{21}N_7O_7·3.5H_2O·Ca$: C, 41.81; H, 4.91; N, 17.07; Ca, 6.98; ash (CaO), 9.77. Found: C, 41.85; H, 4.85; N, 16.90; Ca, 6.93; ash (CaO), 10.17. The total yield of practically pure L-(±)-5-CHO-THF was 1.49 g (33.5% from FA).

Fraction 3 (0.50 g) gave a sample that was shown by LC to contain the following UV-absorbing components: *p*-aminobenzoylglutamic acid (<1%), 10-CHO-DHF (<5.5%), 10-CHO-FA (~1%), and L-(±)-5-CHO-THF (~92.5% by difference).

In a large-scale synthesis, the L-(±)-5-CHO-THF·Ca resulting from FA·$2H_2O$ (600 g, 1.26 moL) was purified in portions (24–30 g) on a glass column (8 × 120 cm) containing Florisil (2.6 kg): yield 213 g (28% from FA). The LC chromatogram showed only trace amounts of *p*-aminobenzoylglutamic acid and 10-CHO-DHF: UV λ_{max} (pH 7) 287 nm (ϵ 31.5 × 10^{-3}), λ_{max} (0.1 N NaOH) 282 (30.8), $\lambda_{max}^{282}/\lambda_{min}^{242}$ = 4.8.[14]

Nonchromatographic Purification of L-(±)-5-CHO-THF. A. The hygroscopic, brown, gummy residue (~400 g) containing glass-fiber filter pads obtained in the conversion of L-(±)-(5,10-CH-THF)$^+Cl^-$ to L-(±)-5-CHO-THF·Ca was stirred under N_2 in deaerated H_2O (4 L), and the pH of the medium was adjusted to 7.5 with solid $Ca(OH)_2$ (~1 g). The glass fibers were removed by filtration, and the residue was washed with portions of deaerated H_2O (2 L) until the washings were colorless. The clear filtrate was cooled in an ice bath and diluted with EtOH (600 mL). The dark, hygroscopic, yellow-brown precipitate of L-(±)-5-CHO-THF was collected by filtration, washed with EtOH, and dried in vacuo over P_2O_5: yield 199 g; UV $\lambda_{max}^{282}/\lambda_{min}^{242}$ = 3.7.[14]

The filtrate was diluted with additional EtOH (total, 3 L), and the pale yellow precipitate was collected by filtration, washed with EtOH, and dried in vacuo over P_2O_5: yield 17 g; UV $\lambda_{max}^{282}/\lambda_{min}^{242}$ = 3.90.[14]

The clear, yellow filtrate was diluted with additional EtOH (total, 15 L); after cooling, the resulting cream-colored precipitate was collected by filtration, washed with EtOH, and dried in vacuo over P_2O_5: yield 75 g; UV $\lambda_{max}^{282}/\lambda_{min}^{242}$ (0.1 N NaOH) = 4.19.[14] LC assay of this sample indicated the presence of L-(±)-5-CHO-THF·Ca (85%), PABGA·Ca (~2%), 10-CHO-DHF·Ca (~2%), 10-CHO-FA·Ca (trace), and pterins (~1%).[16] No doubt this sample also contained EtOH and H_2O (<10%).

B. A turbid solution of a portion (3.00 g) of the large crop of impure L-(±)-5-CHO-THF·Ca described in A was dissolved in H_2O (100 mL) containing about an equimolar amount of $MgCl_2·6H_2O$ (1 g) and treated portionwise with solid $Ca(OH)_2$. The pH increased rapidly to ~10.5 and then remained between 10.5 and

10.8. During this period a yellow-brown granular precipitate separated from the mixture, after which the pH increased rapidly to 12 as more $Ca(OH)_2$ was added. After stirring the mixture for an additional 30 min, the solid was removed by filtration under N_2 pressure and dried in vacuo over P_2O_5: yield 1.40 g. The major portion of this residue is probably composed of CaO and MgO; however, the LC chromatogram showed the presence of a number of impurities and some 5-CHO-THF.

The clear filtrate was adjusted to pH 7.5 with dilute HCl followed by the dropwise addition of EtOH (~20 mL) until permanent turbidity was reached. This mixture was cooled to 10 °C, and the yellow solid was collected by filtration and dried in vacuo over P_2O_5: yield 0.65 g; UV $\lambda_{max}^{282}/\lambda_{min}^{242} = 3.71$.[14] The LC chromatogram showed that this sample contained only small amounts of the usual impurities but increased amounts of FA and an unidentified material with a longer retention time. A magnesium analysis indicated the presence of a trace amount of magnesium (0.003%). Anal. Calcd for $C_{20}H_{21}N_7O_7 \cdot Ca \cdot 0.5C_2H_6O \cdot 1.2H_2O$: C, 45.35; H, 4.78; N, 17.63; Ca, 7.21; ash (CaO), 10.08. Found: C, 45.11; H, 5.01; N, 17.38; Ca, 7.20; ash (CaO), 10.07.

The filtrate was diluted with 3 volumes of EtOH (300 mL), and the resulting mixture was cooled in an ice bath. The white solid was collected by filtration, washed with EtOH, and dried in vacuo over P_2O_5: yield 1.33 g; UV $\lambda_{max}^{282}/\lambda_{min}^{242} = 4.78$.[14] LC assay and elemental analysis indicated the presence of L-(±)-5-CHO-THF·Ca (86%), PABGA·Ca (1%), 10-CHO-DHF (<1%), 10-CHO-FA (<1%), pterins (<1%), EtOH (4.2%), H_2O (3.3%), and unidentified material (~2.5%).[16] Anal. Calcd for $C_{20}H_{21}N_7O_7 \cdot Ca \cdot 0.5C_2H_6O \cdot H_2O$: C, 45.65; H, 4.74; N, 17.75; Ca, 7.25; ash (CaO), 10.14. Found: C, 45.59; H, 5.06; N, 17.69; Ca, 7.51; ash (CaO), 10.51.

When the above experiment was repeated using twice the weight of $MgCl_2$, a smaller amount (0.77g) of purified L-(±)-5-CHO-THF was recovered. These results indicated that further development of this procedure will be required to maximize the yield and purity of L-(±)-5-CHO-THF.

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(20) On TLC L-(±)-(5,10-CH-THF)$^+$, 10-CHO-DHF, and 10-CHO-FA were detectable at less than 5% the concentration of L-(±)-5-CHO-THF.
(21) At faster flow rates (e.g., fraction III), 10-CHO-DHF and 10-CHO-FA eluted before and during the elution of L-(±)-5-CHO-THF. At the slower rates, these impurities are decomposed under the basic (pH ~8.5) conditions of the mobile phase.