[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO.]

The Synthesis of the 3′-Fluoro and 3′,5′-Difluoro Derivatives of 4-Amino-4-deoxy-N^{10}-methylpteroylglutamic Acid (Methotrexate)[1]

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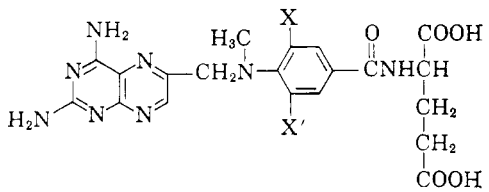
The 3′-fluoro (II) and 3′,5′-difluoro (III) derivatives of methotrexate (I) have been synthesized. Both compounds are potent folic acid antagonists and markedly inhibit the growth of the transplanted 6C3HED mouse lymphosarcoma.

Methotrexate (I) and its 3′-chloro (IV), 3′-bromo (V), 3′,5′-dichloro (VI), and 3′-bromo-5′-chloro (VII) derivatives[2] have been found to be



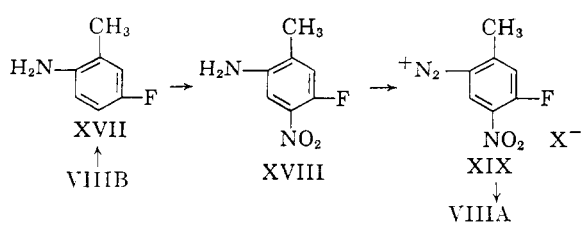
	X	X′		X	X′
I.	H	H	V.	Br	H
II.	F	H	VI.	Cl	Cl
III.	F	F	VII.	Br	Cl
IV.	Cl	H			

highly effective in prolonging the life span of mice with advanced leukemia (L1210).[3] In our laboratories, these compounds have been found to inhibit the growth of the transplanted 6C3HED mouse lymphosarcoma.[4] It was thus of interest to prepare the corresponding 3′-fluoro (II) and 3′,5′-difluoro (III) derivatives of methotrexate (I) for evaluation as antineoplastic agents.

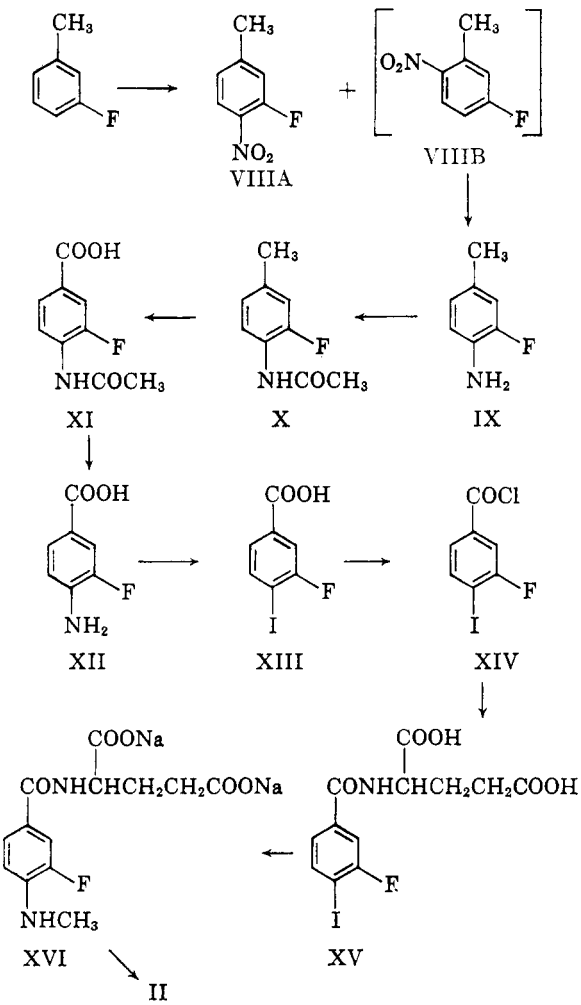
Since the direct introduction of fluorine into the benzene ring of methotrexate is not feasible, the preparation of 3′-fluoro- and 3′,5′-difluoromethotrexate was accomplished by the standard methotrexate synthesis procedure,[5] using the appropriately substituted fluoro derivatives of *N*-[4-methylaminobenzoyl]glutamic acid.

Synthesis of 3′-fluoromethotrexate (II)

Careful nitration[6] of *m*-fluorotoluene gave, in 67% yield, a readily separable mixture of the 4-nitro (VIIIA) and 6-nitro (VIIIB) derivatives, in the ratio of about 1:12. Attempts to convert VIIIB to VIIIA by the sequence



gave erratic results at the final step. In small scale (0.1 mole) runs wherein the nitroaniline (XVIII) was diazotized in concentrated sulfuric acid solution with



nitrosyl sulfuric acid, and the diazonium salt (XIX) (X = HSO_4) then reduced by the addition of an aqueous slurry of sodium hypophosphate and

(1) Methotrexate and amethopterin are generic names for 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid. Its full chemical name is *N*-{*p*-{[(2,4-diamino-6-pteridinyl)methyl]-methylamino}benzoyl}glutamic acid.

(2) R. B. Angier and W. V. Curran, *J. Am. Chem. Soc.*, **81**, 2814 (1959).

(3) A. Goldin, S. R. Humphreys, J. M. Venditti, and N. Mantel, *J. Natl. Cancer Inst.*, **22**, 811 (1959).

(4) A. E. Sloboda, *J. Pharmacol. Exptl. Therap.*, **128**, 419 (1960).

(5) D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist, *J. Am. Chem. Soc.*, **71**, 1756 (1949).

TABLE I

Benzoic Acid Der.	λ_{max} (Mμ) 0.1N HCl	0.1N NaOH	CH_3OH	log ϵ CH_3OH	λ_{min} (Mμ) 0.1N HCl	0.1N NaOH	CH_3OH
p-Iodo	256	247	249	4.20	228	225	225
3-Fluoro-4-iodo (XIII)	255	245	246	4.16	224	221	220
3,5-Difluoro-4-iodo (XXIX)	253	245	245	4.15	218	217	217

cuprous oxide,[7] an 81% yield of 3-fluoro-4-nitrotoluene (VIIIA) was isolated. In larger scale runs, temperature control and effective stirring proved difficult to maintain, and the isolated product was chiefly 6-nitro-*m*-cresol.[8] When the diazotization of the nitroaniline (XVIII) was carried out in aqueous hydrochloric acid with sodium nitrite, and the diazonium salt (XIX) (X = Cl) then reduced with aqueous hypophosphorous acid,[9] 6-nitro-*m*-cresol was the sole product isolated. The lability of the fluorine in similar nitrobenzenediazonium salt derivatives has been reported.[10]

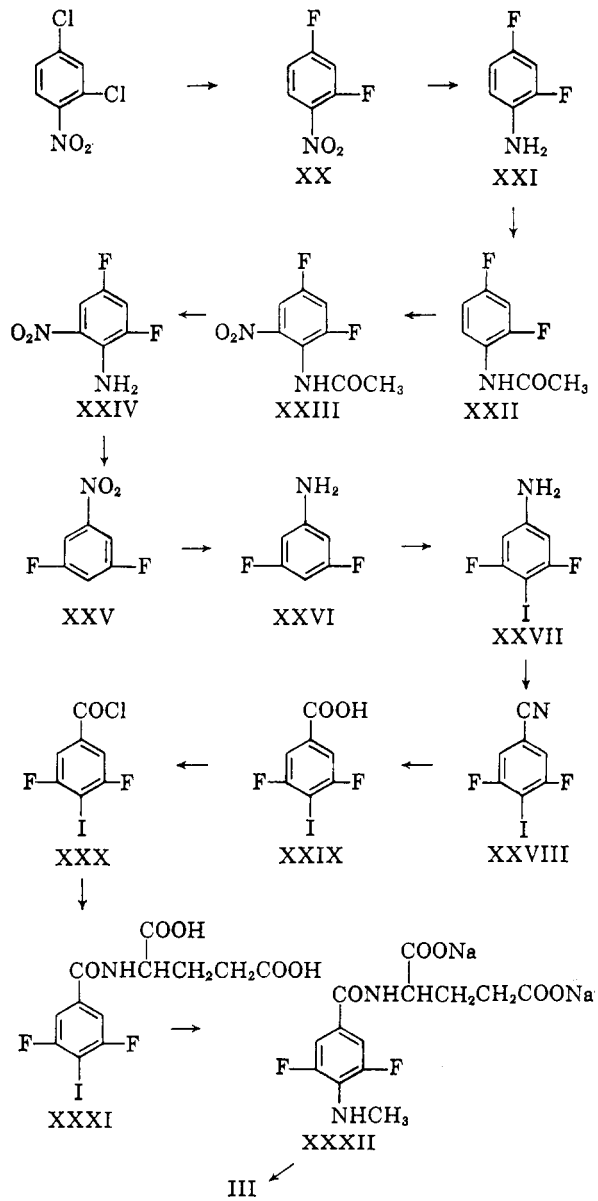
The remaining steps of the sequence VIIIA → XVI proceeded without difficulty. The purification of the crude 3′-fluoromethotrexate (II) obtained from the reaction of XVI, 2,3-dibromopropionaldehyde, and 2,4,5,6-tetraaminopyrimidine is described in detail in the Experimental part.

Synthesis of 3′,5′-difluoromethotrexate (III)

The portion of the sequence from XX → XXVI has been described by Finger and his co-workers.[7,11]

Jurd[12] has found that the action of iodine upon aniline in cold ethanol solution in the presence of excess yellow mercuric oxide gives *p*-iodoaniline in 80% yield. Application of his procedure to 3,5-difluoroaniline (XXVI) gave a 90% crude yield of a monoiodo derivative, whose *N*-acetyl derivative possessed an ultraviolet absorption spectrum ($\lambda_{max}^{CH_3OH}$ 254 mμ, log ϵ = 4.40; $\lambda_{min}^{CH_3OH}$ 221 mμ) very similar to that of p-iodoacetanilide ($\lambda_{max}^{CH_3OH}$ 253 mμ, log ϵ = 4.38; $\lambda_{min}^{CH_3OH}$ 222 mμ). Further evidence that the monoiodo derivative was 3,5-difluoro-4-iodoaniline-(XXVII) was its conversion, in two steps (XXVII → XXIX), to a difluoroiodobenzoic acid whose ultraviolet absorption spectrum was very similar to those of *p*-iodobenzoic acid and authentic 3-fluoro-4-iodobenzoic acid (XIII), as shown in Table I.

Except for weak "benzenoid" absorption, the ultraviolet absorption spectra of *o*- and *m*-iodobenzoic acids showed no characteristic maxima or minima between 200 and 400 mμ.



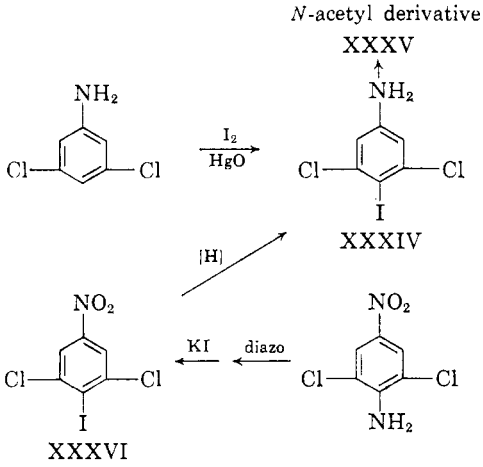
The remaining steps of the sequence XXIX → 3′,5′-difluoromethotrexate (III) were carried out in a manner similar to that discussed in the preparation of 3′-fluoromethotrexate (II).

The iodination of other *m*-halogenated anilines by the Jurd[12] procedure was studied briefly. *m*-Fluoroaniline[13] gave a 40% yield of 3-fluoro-4-iodoaniline (XXXIII) (*N*-acetyl derivative $\lambda_{max}^{CH_3OH}$

(6) G. Schiemann, *Ber.*, **62B**, 1794 (1929).
(7) G. C. Finger, F. E. Reed, and J. L. Finnerty, *J. Am. Chem. Soc.*, **73**, 154 (1951).
(8) W. Stoedel, *Ann.*, **217**, 51 (1883).
(9) N. Kornblum, *Org. Reactions*, **2**, 277 (1944).
(10) G. C. Finger, F. H. Reed, D. M. Burness, D. M. Fort, and R. R. Blough, *J. Am. Chem. Soc.*, **73**, 145 (1951).
(11) G. C. Finger and C. W. Kruse, *J. Am. Chem. Soc.*, **78**, 6036 (1956).
(12) L. Jurd, *Australian J. Sci. Res.*, **2A**, 111 (1949).
(13) Pierce Chemical Co., Rockford, Ill., and Columbia Chemicals Co., Columbia, S. C.

251 mμ, log ε = 4.38; $\lambda_{min}^{CH_3OH}$ 223 mλ). The conversion of 3-fluoro-4-iodoaniline to 3-fluoro-4-iodobenzoic acid (XIII) is under study.

3,5-Dichloroaniline also yielded a monoiodo derivative in 34% yield, whose structure was shown to be 3,5-dichloro-4-iodoaniline by an independent synthesis from 2,6-dichloro-4-nitroaniline.



Biological activity. For the purpose of measuring folic acid antagonism, an arbitrary value of 100 is assigned to the antagonist activity of pure methotrexate for half-maximum inhibition of the growth of *Streptococcus faecalis* 8043. In terms of this standard, the antagonist activity of II is 80–100, while that of III is 10.

The antineoplastic activity of II and III against the transplanted 6C3HED mouse lymphosarcoma is similar to that for 3′,5′-dichloromethotrexate (VI),[4] but the toxicities are somewhat greater, especially in the case of II.

EXPERIMENTAL[14]

3-Fluoro-4-nitrotoluene (VIIIA) *and 5-fluoro-2-nitrotoluene* (VIIIB). The nitration of *m*-fluorotoluene (Eastman Kodak) and separation of the mononitro derivatives was accomplished by the method of Schiemann.[6] From 200 g. (1.82 moles) there was obtained 13.5 g. (5%) of 3-fluoro-4-nitrotoluene (VIIIA), m.p. 53–54° (lit.[6] m.p. 53.2°), and 227.5 g. (62%) of crude 5-fluoro-2-nitrotoluene (VIIIB), b.p. 71–81° (2.5 mm.).

2-Fluoro-p-toluidine (IX) *and 2′-fluoro-p-acetotoluidide* (X). A slurry of 220 g. (3.95 g.-atoms) of electrolytic iron powder in 1 l. of 0.78*N* ammonium chloride solution[7] was stirred at 90° as 163 g. (1.05 moles) of 3-fluoro-4-nitrotoluene (VIIIA) was added in small portions during 30 min. The mixture was stirred at reflux for 2 hr. and then steam distilled, a mobile water-white oil being obtained in the distillate. It was removed by extraction with 600 ml. of diethyl ether. After drying over magnesium sulfate and removal of solvent on the steam bath, the crude 2-fluoro-*p*-toluidine (IX) was dissolved in 250 ml. of dry benzene and treated slowly with 125 ml. of acetic anhydride. The reaction mixture was stirred overnight at room temperature. Addition of 500 ml. of petroleum ether (b.p. 35–65°) precipitated a shiny white crop of 2′-fluoro-*p*-acetotoluidide (X). Concentration of the mother liquor to a small volume gave a further yield of product; the total yield was 159.5 g. (91% based on starting 3-fluoro-4-nitrotoluene); m.p. 128–130°; lit.[15] m.p. 128–128.5°. Recrystallization from ethanol did not change the melting point.

4-Acetamido-3-fluorobenzoic acid (XI). Eighty grams (0.48 mole) of 2′-fluoro-*p*-acetotoluidide (IX) was slowly added to a stirred solution of 236 g. (1.71 moles) of potassium permanganate and 164 g. (1.37 moles) of magnesium sulfate in 8 l. of water at 70°. The suspension was then refluxed for 6 hr. and let stand overnight at room temperature. A 224-g. sample (2.12 moles) of solid sodium carbonate was dissolved in the mixture, the precipitated manganese dioxide filtered off and washed with 1500 ml. of hot water. Acidification of the combined filtrate and wash water with 400 ml. of concd. hydrochloric acid precipitated 4-acetamido-3-fluorobenzoic acid (XI), which was collected, washed neutral, and dried; yield 80 g. (85%), m.p. 275–280° dec. Recrystallization from 350 parts of boiling water gave an analytical sample, m.p. 282–283° dec.

Anal. Calcd. for $C_9H_8FNO_3$: C, 54.82; H, 4.09; F, 9.59; N, 7.10. Found: C, 54.70; H, 4.41; F, 9.80, 9.64; N, 7.03, 7.12.

4-Amino-3-fluorobenzoic acid (XII). A suspension of 159.5 g. (0.81 mole) of 4-acetamido-3-fluorobenzoic acid (XI) in a mixture of 1600 ml. of ethanol, 80 ml. of water, and 250 ml. of concd. hydrochloric acid was refluxed for 5 hr. A clear solution resulted in 30 min. The ethanol was removed *in vacuo*, leaving a semisolid residue. This was dissolved in 1500 ml. of warm water and neutralized with solid sodium carbonate. Considerable oil (ethyl 4-amino-3-fluorobenzoate (?)) was present. A 60-g. sample (1.5 moles) of solid sodium hydroxide and 300 ml. of ethanol were added to the mixture, which was stirred at 90° until the oil had dissolved (20 min.). The solution was treated with activated charcoal, filtered, and the filtrate acidified with 200 ml. of glacial acetic acid. Cooling overnight at 4° gave a precipitate of 4-amino-3-fluorobenzoic acid (XII) which was collected, washed neutral, and dried. The yield was 73 g. (58%), m.p. 212–214°; lit.[16] m.p. 215–216°.

Schmelkes and Rubin[16] prepared 4-amino-3-fluorobenzoic acid (XII) by a two step procedure from 3-fluoro-4-nitrotoluene (VIIIA) (aqueous potassium permanganate oxidation to 3-fluoro-4-nitrobenzoic acid followed by ammoniacal ferrous hydroxide reduction). Their over-all yield was 19% as compared to the 45% over-all yield by the above three step procedure.

3-Fluoro-4-iodobenzoic acid (XIII) *and 3-fluoro-4-iodobenzoyl chloride* (XIV). A suspension of 25 g. (0.16 mole) of 4-amino-3-fluorobenzoic acid (XII) in 150 g. of 6 *N* sulfuric acid was mixed with 45 g. of crushed ice and stirred at 0–5° as a solution of 13.5 g. (0.20 mole) of sodium nitrite in 50 ml. of water was slowly added. When addition of the sodium nitrite solution was complete, 100 ml. of ice water was added, and the solution stirred at 0–5° for 30 min. A starch-KI test for nitrous acid was positive. The diazonium salt solution was added to a solution of 45 g. (0.27 mole) of potassium iodide in 150 g. of 6*N* sulfuric acid. After the initial violent gas evolution had subsided, the mixture was heated on the steam bath for 2.5 hr. The excess iodine was destroyed by the addition of solid sodium sulfite. The cooled suspension was filtered and the collected crude 3-fluoro-4-iodobenzoic acid (XIII) washed neutral and dried; weight 32.4 g. (75%). Repeated reprecipitations from alkaline solution or recrystallizations from aqueous ethanol failed to give a completely acceptable analytical sample, though the m.p. of 229–230° dec. remained constant.

Anal. Calcd. for $C_7H_4FIO_2$: C, 31.60; H, 1.52; F, 7.14; I, 47.71. Found: C, 32.38; H, 1.81; F, 7.15; I, 46.80.

(14) All melting points were determined in capillary tubes and are uncorrected. The ultraviolet absorption spectra were determined on a Cary recording spectrophotometer. Optical rotations were determined in a 1 dm. semimicro tube.

(15) A. Ostaszynski, *Bull. soc. sci. lettres Lódź, Classe III* (**15**), 7 (1952).

(16) F. C. Schmelkes and M. Rubin, *J. Am. Chem. Soc.*, **67**, 1631 (1944).

3-Fluoro-4-iodobenzoyl chloride (XIV) was prepared from the acid (XIII) by refluxing with excess thionyl chloride in benzene until a clear solution resulted. Removal of unchanged thionyl chloride and benzene *in vacuo* gave a solid residue which was taken up in benzene, decolorized with activated charcoal, and filtered. The benzene solution of 3-fluoro-4-iodobenzoyl chloride was used directly in the next step.

N-(3-Fluoro-4-iodobenzoyl)-L-glutamic aid (XV). Eleven grams (0.075 mole) of L-glutamic acid was dissolved in 500 ml. of water containing 7.0 g. (0.175 mole) of sodium hydroxide. Separate solutions of 4.0 g. (0.10 mole) of sodium hydroxide in 150 ml. of water and 3-fluoro-4-iodobenzoyl chloride (XIV) (from 20 g. (0.075 mole) of 3-fluoro-4-iodobenzoic acid) in 150 ml. of benzene were prepared. The solution of disodium L-glutamate was stirred at 15–20° (external cooling) as 25-ml. portions of the sodium hydroxide and 3-fluoro-4-iodobenzoyl chloride solutions were added every 5 min. When the additions were complete, stirring was continued for another 3 hr. as a stream of air was passed over the surface to remove the benzene. The aqueous solution was treated with activated charcoal and filtered. Acidification of the filtrate with 35 ml. of concd. hydrochloric acid gave a white precipitate of *N*-(3-fluoro-4-iodobenzoyl)-L-glutamic acid, which was collected, washed neutral, and dried; yield, 23.2 g. (78%). A sample recrystallized from aqueous ethanol, melted at 184–185° dec. and had a $[\alpha]_D^{25}$ of + 13.9° (*c*, 2.161 in 0.5% sodium carbonate).

Anal. Calcd. for $C_{12}H_{11}FINO_5$: C, 36.47; H, 2.81; F, 4.81; I, 32.12; N, 3.54. Found: C, 36.64, 36.69; H, 3.11, 2.97; 5.08; I, 31.57, 31.75; N, 3.55.

Disodium N-(3-fluoro-4-methylaminobenzoyl)-L-glutamate (XVI). A mixture of 22 g. (0.055 mole) of *N*-(3-fluoro-4-iodobenzoyl)-L-glutamic acid (XV), 22 ml. of water, 4.5 g. (0.11 mole) of sodium hydroxide, 35 ml. of 25% aqueous methylamine (large excess), and 200 mg. of fine copper dust was shaken in a stainless steel autoclave at 125° for 3 hr. The reaction mixture was evaporated to dryness *in vacuo*. The residue was dissolved in 80 ml. of warm water, treated with activated charcoal, and filtered. After addition of 800 ml. of ethanol, the mixture was evaporated to dryness *in vacuo*. Ethanol addition and evaporation to dryness were repeated twice more. The light brown residue[17] was extracted with two 150-ml. portions of boiling acetone to remove the sodium iodide present and then dried *in vacuo*. The yield of crude disodium *N*-(3-fluoro-4-methylaminobenzoyl)-L-glutamate was 14.1 g. (74%). It was used directly in the next step.

Anal. Calcd. for $C_{13}H_{13}FN_2O_5Na_2$: F, 5.55. Found: F, 5.23.

3′-Fluoromethotrexate (II). A mixture of 9.8 g. (8.3 g. real, 0.035 mole) of 2,4,5,6-tetraaminopyrimidine sulfate and 8.5 g. (0.035 mole) of barium chloride dihydrate in 175 ml. of water was stirred at 60° for 10 min., then cooled to 45°. Six grams (0.0175 mole) of disodium *N*-(3-fluoro-4-methylaminobenzoyl)-L-glutamate was added, and the *p*H was adjusted to 4. At 40–45° were added 11 ml. of acetic acid containing 0.035 mole of 2,3-dibromopropionaldehyde, 4.4 g. of iodine and 8.8 g. of potassium iodide in 30 ml. of water and sodium hydroxide solution as necessary to maintain *p*H 4. The resulting mixture was cooled well and filtered.

The crude product was extracted with aqueous ammonium carbonate. The extract was lyophylized, put on a Celite[18] column and developed with a butanol-methanol-0.5% aqueous ammonium carbonate mixture (6:1:4). The eluate was concentrated under reduced pressure, clarified, adjusted to *p*H 4, cooled well, and filtered. The product was chromatographed once more in the same way and then slurried (100 mg.) with 50 mg. of magnesium oxide and 50 mg. of activated charcoal in 5 ml. of water at 90°, clarified and cooled. The precipitated magnesium salt was centrifuged and reprecipitated twice from hot water. The free acid was obtained by dissolving the salt in water, acidifying to *p*H 4, cooling, and centrifuging.

Anal. Calcd. for $C_{20}H_{21}FN_8O_5 \cdot H_2O$: C, 49.0; H, 4.73; F, 3.87; N, 22.8. Found: C, 48.8; H, 4.64; F, 3.80; N, 22.7.

Ultraviolet spectra: in 0.1*N* sodium hydroxide λ_{max} 225 mμ (log ε 4.26), 259 mμ (log ε 4.46), 368 mμ (log ε 3.90), λ_{min} 237 mμ, 335 mμ; in 0.1*N* hydrochloric acid λ_{max} 243 mμ (log ε 4.25), 290 mμ (log ε 4.21), 330 mμ (inflection) (log ε 4.07), λ_{min} 231 mμ, 260 mμ.

4-Fluoro-o-toluidine (XVII). 5-Fluoro-2-nitrotoluene (VIIIB) was reduced to 4-fluoro-o-toluidine (XVII) by the iron−0.78*N* ammonium chloride procedure[7] employed in the preparation of 2-fluoro-*p*-toluidine (IX). The amine was steam-distilled into a receiver containing 6*N* sulfuric acid, yielding the sulfate of XVII in 80% yield, m.p. 235–238° dec.

4-Fluoro-5-nitro-o-toluidine (XVIII). A solution of 34 g. (0.195 mole) of 4-fluoro-*o*-toluidine sulfate in 147 ml. of concd. sulfuric acid was stirred at 0–5° as 12.1 ml. of fuming nitric acid (*d* = 1.5) was added dropwise during 20 min. Stirring at 0–5° was continued for another 30 min., the solution turning red in color. The reaction mixture was drowned on ice, the suspension made barely alkaline with ammonium hydroxide, and cooled overnight at 4°. The crude yield of 4-fluoro-5-nitro-*o*-toluidine (XVIII) was 32.4 g. (98%), m.p. 87–93.5°. Recrystallization from aqueous ethanol raised the m.p. to 93–95° (lit.[15] m.p. 96–97°).

4-Fluoro-5-nitro-o-tolydiazonium sulfate (XIX) *and its reduction to 3-fluoro-4-nitrotoluene* (VIIIA). A solution of 13.1 g. (0.079 mole) of 4-fluoro-5-nitro-*o*-toluidine (XVIII) in 80 ml. of concd. sulfuric acid was stirred at 0°. A solution of nitrosylsulfuric acid (from 5.6 g. (0.08 mole) of sodium nitrite in 60 ml. of concd. sulfuric acid) was then added during 20 min., followed immediately by the addition of 60 ml. of 85% phosphoric acid during 1 hr. The mixture was stirred for another 30 min., cooled to 0° and treated during 30 min. with a slurry of 50 g. (0.47 mole) of sodium hypophosphite monohydrate and 7 g. of cuprous oxide in 30 ml. of water. The temperature was held below 35° by external cooling. Vigorous gas evolution and severe foaming occurred. After stirring at room temperature for 30 min., the reaction mixture was drowned into 500 ml. of cold water and stored overnight at 4°. The precipitate was collected, washed neutral, and air-dried; weight 9.7 g. (81% crude yield) of 3-fluoro-4-nitrotoluene. Purification by steam distillation, followed by recrystallization from ethanol gave a product melting at 47–49°; lit.[6] m.p. for 3-fluoro-4-nitrotoluene 53°. Analysis indicated the presence of an impurity.

Anal. Calcd. for $C_7H_6FNO_2$: C, 54.2; H, 3.90; F, 12.25; N, 9.03. Found: C, 55.0, 55.1, 55.2; H, 4.20, 4.22, 4.14; F, 11.51, 11.65, 11.88; N, 9.11.

Attempts to carry out the above reaction on a scale above 0.1 mole proved impracticable due to the severe foaming and difficulty in maintaining the temperature below 35°.

Diazotization of 4-fluoro-5-nitro-*o*-toluidine (XVIII) in dilute hydrochloric acid solution with aqueous sodium nitrite solution, followed by reduction with 50% hypophosphorous acid gave a fluorine-free compound melting at 54–55° which upon reduction with iron-0.78*N* ammonium chloride and subsequent acetylation with acetic anhydride, gave a compound melting at 169–170°.

Anal. Calcd. for 2′-hydroxy-*p*-acetotoluidide ($C_9H_{11}NO$): C, 65.44; H, 6.71; N, 8.62. Found: C, 65.77; H, 7.11; N, 8.62; F, 0.0.

(17) A sample of the solid dissolved in dilute nitric acid gave a heavy precipitate of silver iodide on treatment with silver nitrate solution.

(18) Celite is Johns-Manville's registered trademark for diatomaceous silica products.

6-Nitro-*m*-cresol is reported[8] to melt at 56°, and can be reduced and then acetylated to 2′-hydroxy-*p*-acetotoluidide, m.p. 171°[19].

3,5-Difluoroaniline (XXVI). The seven step reaction sequence from 1,3-dichloro-4-nitrobenzene (Aldrich Chemical Co.) to 3,5-difluoroaniline (XXVI) was carried out by the procedures of Finger and co-workers.[7,11] For all intermediates, the observed yields and physical constants checked the literature values.

3,5-Difluoro-4-iodoaniline (XXVII) *and 3′,5′-difluoro-4′-iodoacetanilide.* Thirty-three grams (0.15 mole) of freshly prepared dry yellow mercuric oxide was added to a solution of 25.8 g. (0.20 mole) of 3,5-difluoroaniline (XXVI) in 500 ml. of absolute ethanol. The suspension was stirred vigorously at room temperature as 5-g. portions of iodine were added at 10-min. intervals until 51 g. (0.20 mole) had been added. The reaction mixture was stirred for an additional 5 hr. and then allowed to stand overnight at room temperature. The solids were removed by filtration through Celite.[18] Concentrated sodium bisulfite solution was added to the filtrate to destroy unchanged iodine. It was then evaporated to dryness *in vacuo*. The dark residue was triturated with 125 ml. of 25% potassium iodide solution, the crude 3,5-difluoro-4-iodoaniline (XXVII) collected, washed with 200 ml. of cold water and dried; yield, 46.5 g. (90%). Recrystallization from 40 parts of 25% ethanol gave shiny white plates, m.p. 112–114°.

Anal. Calcd. for $C_6H_4F_2IN$: C, 28.26; H, 1.58; F, 14.90; I, 49.77; N, 5.49. Found: C, 28.11; H, 1.70; F, 14.26; I, 50.52; N, 5.35.

A mixture of 5 g. (0.02 mole) of 3,5-difluoro-4-iodoaniline (XXVII), 5 ml. of acetic anhydride, and 25 ml. of benzene was refluxed for 45 min. After cooling to room temperature, the precipitate was collected and recrystallized from 200 ml. of 25% ethanol, yielding 1.5 g. (26%) of pure 3′,5′-difluoro-4′-iodoacetanilide, m.p. 158–159°, $\lambda_{max}^{CH_3OH}$ 254 mμ (log ε = 4.40); $\lambda_{min}^{CH_3OH}$ 221 mμ.

Anal. Calcd. for $C_8H_6F_2INO$: C, 32.34; H, 2.04; F, 12.83; I, 42.73; N, 4.72. Found: C, 32.66; H, 2.27; F, 12.55; I, 42.67; N, 4.70.

3,5-Difluoro-4-iodobenzonitrile (XXVIII) *and 3,5-difluoro-4-iodobenzoic acid* (XXIX). Forty-two grams (0.165 mole) of 3,5-difluoro-4-iodoaniline (XXVII) was dissolved in a mixture of 225 ml. of glacial acetic acid and 225 ml. of dry dioxane. The solution was stirred at 5° as 18 ml. of coned. sulfuric acid was slowly added. The suspension of the sulfate salt of XXVII was cooled below −10° and held there by an external Dry Ice–acetone bath as a solution of 24 ml. (slight excess) of isobutyl nitrite in 90 ml. of dry dioxane was slowly added. The suspended salt slowly dissolved. After a further 30 min. at −10° to −5°, 2 liters of dry ethyl ether was added to precipitate the solid diazonium sulfate. This was collected and washed with another liter of dry ethyl ether. It was then dissolved in 1250 ml. of ice water.[20] Meanwhile a solution of 60 g. (0.38 mole) of cupric sulfate in 750 ml. of water was treated with 100 g. (1.45 moles) of potassium cyanide. The brown suspension was warmed to 65° and then cooled below 40°. It was stirred vigorously as the diazonium salt solution was slowly added. Heavy gas evolution and foaming resulted. When the reaction had subsided, the mixture was stirred at 70–75° for 2 hr. After cooling to room temperature, the brown precipitate was collected, washed neutral, and dried. It was triturated with six 100-ml. portions of ether. The ether extracts were combined, and the ether removed *in vacuo*, leaving a residue of crude 3,5-difluoro-4-iodobenzonitrile (XXVIII).

The crude XXVIII was added to a mixture of 150 ml. of glacial acetic acid, 15 ml. of coned. sulfuric acid, and 15 ml. of water. The suspension was refluxed for 17 hr. and drowned into 2 liters of water. The brown precipitate was collected, washed neutral, and dissolved in one liter of hot water containing 45 g. of sodium carbonate. The alkaline solution was treated with activated charcoal, filtered, and the filtrate acidified with coned. hydrochloric acid. The precipitated 3,5-difluoro-4-iodobenzoic acid (XXIX) was collected, washed neutral, and dried, yield 18.6 g. (40%). A second reprecipitation from dilute sodium hydroxide solution gave an analytically pure sample, m.p. 223–235° dec. The ultraviolet absorption data are given in Table I.

Anal. Calcd. for $C_7H_3F_2IO_2$: C, 29.60, H, 1.06, F, 13.38, I, 44.69. Found: C, 29.58, H, 1.28, F, 13.01, I, 44.77.

When the diazotization of 3,5-difluoro-4-iodoaniline was carried out in dilute hydrochloric acid solution with aqueous sodium nitrite, and the acidic diazonium salt solution was added directly to the potassium cuprous cyanide solution,[21] the yield of 3,5-difluoro-4-iodobenzoic acid was only 10%.

3,5-Difluoro-4-iodobenzoyl chloride (XXX) *and N-(3,5-difluoro-4-iodobenzoyl)-L-glutamic acid* (XXXI). XXX and XXXI were prepared in a manner identical to that described for the corresponding monofluoro derivatives XIV and XV, respectively. The crude yield of XXXI was 84%. Recrystallization from 33% ethanol gave an analytically pure product, m.p. 194–196°, $[\alpha]_D^{25}$ = +13.8° (*c* 1.997, 0.5% Na_2CO_3).

Anal. Calcd. for $C_{12}H_{10}F_2INO_5$: C, 34.88, H, 2.44, F, 9.20, I, 30.72, N, 3.39. Found: C, 35.13, H, 2.56, F, 9.39, I, 31.08, N, 3.40.

Disodium N-(3,5-difluoro-4-methylaminobenzoyl)-L-glutamate XXXII. The preparation of XXXII was carried out, on a 0.055-mole scale, exactly as described for the monofluoro derivative (XVI). The isolated crude XXXII was used directly in the synthesis of 3′,5′-difluoromethotrexate (III).

3′,5′-Difluoromethotrexate (III). The preparation of III followed the procedure described for the monofluoro derivative (II). The crude material obtained from an 0.03 mole run was stirred at 60° for 0.5 hr. in 600 ml. of water containing 3 g. of lime. The insoluble material was filtered and washed with 300 ml. of hot water. The filtrate was adjusted to *p*H 10.8 with aqueous zinc chloride, clarified, acidified to *p*H 4, and filtered. This cake was slurried at 60° with 500 ml. of water and enough sodium hydroxide to give *p*H 11–12. The *p*H was then adjusted to 7 while cooling to 20°. After clarification the filtrate was acidified to *p*H 4. The product was filtered and purified further through the magnesium salt as described for the monofluoro derivative (II).

Anal. Calcd. for $C_{20}H_{20}F_2N_8O_5$: F, 7.75, N, 22.8. Found: F, 8.01, N, 23.0.

Ultraviolet spectra: in 0.1*N* sodium hydroxide λ_{max} 225 mμ (log ε 4.33), 259 mμ (log ε 4.48), 370 mμ (log ε 3.91), λ_{min} 238 mμ, 328 mμ, in 0.1*N* hydrochloric aid, λ_{max} 243 mμ (log ε 4.32), 290 mμ (log ε 4.16), 334 mμ (log ε 4.10), λ_{min} 233 mμ, 263 mμ, 325 mμ.

3-Fluoro-4-iodoaniline (XXXIII) *and 3′-fluoro-4′-iodoacetanilide.* The monoiodination of *m*-fluoroaniline (22.2 g., 0.20 mole) was carried out in the same manner as described for 3,5-difluoroaniline (XXVI). The crude 3-fluoro-4-iodoaniline (XXXIII) was recrystallized from 50% ethanol, giving 19.1 g. (40%) of pure product, m.p. 76–77°.

Anal. Calcd. for C_6H_5FIN: C, 30.40, H, 2.13, F, 8.02, I, 53.55, N, 5.91. Found: C, 30.16, H, 2.26, F, 8.40, I, 53.62, N, 5.93.

Acetylation of XXXIII with acetic anhydride in refluxing ether, and recrystallization of the crude product from 50% ethanol gave pure 3′-fluoro-4′-iodoacetanilide, m.p. 151–152°, $\lambda_{max}^{CH_3OH}$ 251 mμ log ε = 4.39, $\lambda_{min}^{CH_3OH}$ 223 mμ.

Anal. Calcd. for C_8H_7FINO: C, 34.43, H, 2.53, N, 5.02. Found: C, 34.34, H, 2.72, N, 4.98.

3,5-Dichloro-4-iodoaniline (XXXIV) *and 3′,5′-dichloro-4′-iodoacetanilide* (XXXV) (*by the iodination of 3,5-dichloroaniline*). A solution of 16.2 g. (0.10 mole) of 3,5-dichloroaniline (Aldrich) in 250 ml. of ethanol was slurried with 16 g

(19) A. Proskouriakoff and R. J. Titherington, *J. Am. Chem. Soc.*, 52, 3978 (1930).

(20) A portion of the solution gave a deep red precipitate when added to alkaline 2-naphthol.

(21) D. T. Mowry, *Chem. Rev.*, 42, 213 (1948).

(0.075 mole) of freshly prepared dry yellow mercuric oxide at room temperature. 25.5 g. (0.10 mole) of iodine was added in one portion, and the mixture stirred for 7 hr., then let stand overnight. The insolubles were removed by filtration through Celite,[18] and the filtrate concentrated to dryness *in vacuo*. Recrystallization from 200 ml. of 75% ethanol gave 9.8 g. (34%) of 3,5-dichloro-4-iodoaniline (XXXIV). It was converted directly to the *N*-acetyl derivative (XXXV) by refluxing with excess acetic anhydride in benzene solution. The crude XXXV was purified by recrystallization from ethanol, m.p. 223–224°, yield, 13.6 g. (41% based on starting 3,5-dichloroaniline).

Anal. Calcd. for $C_8H_6Cl_2INO$: C, 29.12, H, 1.83, Cl, 21.49, I, 38.46, N, 4.25. Found: C, 29.03, H, 2.04, Cl, 21.91, 21.67, I, 38.63, N, 4.17.

Preparation of XXXIV *and* XXXV *from 2,6-dichloro-4-nitroaniline.* 1,3-Dichloro-2-iodo-5-nitrobenzene (XXXVI) was obtained in 50% yield from 2,6-dichloro-4-nitroaniline by the method of Körner and Contardi.[22]

The reduction of XXXVI to XXXIV was carried out by the method of West.[23] 15.9 g. (0.05 mole) of 1,3-dichloro-2-iodo-5-nitrobenzene (XXXVI) was dissolved in 250 ml. of methanol containing 2 ml. of concd. hydrochloric acid. The solution was stirred at reflux as 10 g. (0.17 g.-atom) of iron powder was added in small portions. After addition was complete, the mixture was stirred at reflux for another 2 hr. A 1.5-g. sample of solid sodium hydroxide was added to neutralize the acid. The hot suspension was filtered and the residue washed with 100 ml. of hot methanol. The combined filtrate and wash was concentrated *in vacuo* to a dark semi-solid residue. Attempts to isolate pure XXXIV were unsuccessful, so the residue was refluxed with 15 ml. of acetic anhydride in 150 ml. of benzene. The reaction mixture was cooled, the precipitate collected and recrystallized from 50% ethanol to give 3.2 g. (10%) of authentic 3′,5′-dichloro-4′-iodoacetanilide XXXV, m.p. 223–224°. A mixed melting point determination on the 3′-5′-dichloro-4′-iodoacetanilide samples prepared by the above two reaction sequences showed no depression.

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(22) G. Körner and Contardi, *Atti accad. Lincei*, 22I, 823; *Chem. Abstr.*, **8**, 73 (1914).

(23) R. West, *J. Chem. Soc.*, **127**, 494 (1925).

[CONTRIBUTION FROM THE DIVISION OF PROTEIN CHEMISTRY, THE INSTITUTE FOR MUSCLE DISEASE, INC.]

Synthesis of β-Cyano-L-alanine and γ-Cyano-α-L-aminobutyric Acid, Dehydration Products of L-Asparagine and L-Glutamine; a New Synthesis of Amino Acid Nitriles

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Syntheses of two amino acids, β-cyano-L-alanine and γ-cyano-α-L-aminobutyric acid, are presented. These compounds correspond to L-asparagine and L-glutamine in which the β- and γ-carboxamides are replaced by nitrile groups. Carbobenzoxy-L-asparagine and carbobenzoxy-L-glutamine which served as starting materials were smoothly dehydrated to carbobenzoxy-β-cyano-L-alanine (II) and carbobenzoxy-γ-cyano-α-L-aminobutyric acid with *N,N′*-dicyclohexylcarbodiimide in a new route which had been suggested by a side reaction previously encountered in the synthesis of asparagine peptides. Selective removal of the carbobenzoxy group without reduction of the nitrile group was effected with sodium in anhydrous ammonia to yield β-cyano-L-alanine and γ-cyano-α-L-aminobutyric acid. In contrast, treatment of II with sodium in ammonia containing methanol yielded the reduction product α,γ-diaminobutyric acid, and with excess sodium in ammonia an unidentified less basic substance was obtained in addition. Characteristic color reactions with ninhydrin are presented for these amino acids along with other data.

During the synthesis of an asparagine-containing cyclic pentapeptide structurally related to the ring moiety of oxytocin an unusual side reaction was observed.[1] On countercurrent distribution of the cyclic pentapeptide product an additional pentapeptide was found. Examination of this showed that in place of the asparagine moiety a residue of α,γ-diaminobutyric acid was present. It appeared that the asparagine moiety had been partially converted by the coupling agent[2] tetraethyl pyrophosphite into a reducible form, so that after a further synthetic step involving sodium in liquid ammonia α,γ-diaminobutyric acid was formed after hydrolysis in place of aspartic acid and ammonia. The reactivities of the altered asparagine moiety, namely, its hydrolysis to aspartic acid and its reducibility, at least in part, to a basic grouping and the dehydrating nature of the coupling reagent that had been employed suggested that the asparagine-β-carboxamide had been dehydrated to a cyano group although other explanations were possible.

In order to gain further knowledge on this interesting side reaction of peptide synthesis it was de-

(1) C. Ressler, *J. Am. Chem. Soc.*, **78**, 5956 (1956).

(2) Anhydro products of unestablished structure were isolated from similar coupling reactions employing tetraethyl pyrophosphite or *N,N*-dicyclohexylcarbodiimide (D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *J. Am. Chem. Soc.*, **78**, 5954 (1956)).