of action of these antimetabolites will be reported in subsequent communications.

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Isolation, Characterization, and Synthesis of Linatine. A Vitamin B_6 Antagonist from Flaxseed (*Linum usitatissimum*)*

H. J. Klosterman, G. L. Lamoureux, and J. L. Parsons

ABSTRACT: The name linatine (I) was assigned to a vitamin B_6 antagonist isolated from linseed meal. Compound I inhibited the growth of chickens and *Azotobacter vinelandii* O, the assay organism. An LD_{50} of 2-mg per week-old chick was counteracted by simultaneous injection of 1 mg of pyridoxine. Acid hydrolysis of I yielded L-glutamic acid and a toxic substance (II), LD_{50} = 0.5 mg in 1-week-old chicks, which was characterized as 1-amino-D-proline, mp 155° dec, $[\alpha]_D^{25}$ + 113° (*c* 2, 0.5 M HCl); 3-nitrobenzylidene derivative, mp 123°; 2-hydroxy-5-nitrobenzylidene derivative, mp 136°, $[\alpha]_D^{24}$ +151° (*c* 0.35, ethanol). Compound II was prepared in 60% yield by reduction of 1-nitroso-D-proline with zinc dust in acetic acid. *N*-Carbobenzyloxyglutamic acid γ-hydrazide was converted to the azide and treated with the benzyl ester of II. Hydrogenolysis of the product yielded 1-[(*N*-γ-L-glutamyl)amino]-D-proline that was identical with I, $[\alpha]_D^{24}$ +46.4° (*c* 2.75, water). 1-[(*N*-γ-Glutamyl)-amino]-L-proline prepared in the same manner showed $[\alpha]_D^{24}$ −34.6° (*c* 2, water).

Both I and the diastereoisomer were equally toxic to chicks, but I was about 50 times more toxic toward *A. vinelandii* O.

Chicks and turkey poults show poor growth and typical vitamin B deficiencies when reared on linseed meal. Kratzer *et al.* (1954) noted that the vitamin deficiency symptoms could be prevented by the addition of 20 ppm of pyridoxine to linseed-containing diets and proposed that linseed meal contained a substance that was able to counteract the vitamin B_6 activity in an unsupplemented diet.

Klosterman *et al.* (1960) showed that the addition of a 70% ethanol extract of linseed meal to a standard ration produced poor growth and a vitamin B_6 deficiency. They also found that the substance responsible for poor growth could be absorbed from flaxseed extracts by cation-exchange resins and recovered by ammonia elution. Large-scale extraction of the toxic material from linseed meal has been described by Evenstad *et al.* (1965). This paper describes the isolation, characterization, and synthesis of the toxic vitamin B_6 antagonist from flaxseed. The trivial name linatine is suggested for this antagonist because of its association with the flax plant, *Linum usitatissimum*.

Experimental Section

Materials. *N*-Carbobenzoyloxyglutamic acid-γ-methyl ester was purchased from Mann Research Laboratories, Inc. D- and L-proline were obtained from Calbiochem. Dowex 1-X8 (Cl^- form), 200–400 mesh, was obtained from the Dow Chemical Co. and converted to the acetate form. Amberlite IRA-400 (Cl^- form) and Amberlite CG-120 (H^+ form), 100–200 mesh, were purchased from Mallinckrodt Chemical

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Works. The Amberlite IRA-400 was converted to the acetate form before use. Linseed flakes were provided by the Minnesota Linseed Oil Co. The flakes were obtained after the crushed flaxseed had been extracted with hexane in the usual commercial process, but were removed from the system prior to the solvent stripping process. Hexane was removed from the extracted flakes by air drying.

Biological Assays. Chick assays were of two types. Initial studies were based on growth-rate depression of chicks fed a semisynthetic diet containing various fractions of extracts of linseed flakes. In later studies, a rapid chick injection procedure was used (T. M. Farley, 1960, unpublished data). Materials to be assayed were dissolved in physiological saline solution and injected intraperitoneally using chicks 7–10 days old (60–70 g each) that had been reared on a diet deficient in vitamin B_6. Toxic fractions caused mild to severe vitamin B_6 deficiency symptoms in the chicks and death at higher dosage levels. LD_{50} determinations were made 6 hr after injection of the test sample.

In the chick assays, a positive test was verified by the reversal of the poor growth or deficiency symptoms by administering pyridoxine. In the case of the injection assay, simultaneous injection of 1 mg of pyridoxine prevented the development of symptoms in chicks receiving an LD_{50} dose. When feeding trials were used, the addition of 40 ppm of pyridoxine to the diet counteracted the effects of the antagonist and prevented development of deficiency symptoms.

The microbiological assay was based on zones of inhibition of *Azotobacter vinelandii* O in seeded agar plates on which were placed assay disks containing the sample to be tested. The inoculum was grown on slants of Ashby's mannitol agar (Jones, 1913) for 48 hr at 37°. The cells from one slant were suspended in 5 ml of sterile salt solution (medium less mannitol, agar, and calcium carbonate), and adjusted to a Klett reading of 30 with a no. 54 filter. This suspension (1 ml) was added to each 100 ml of the assay medium at 40°, and 10 ml was pipetted into 15 × 100 mm plastic Petri plates. The assay medium was a modification of Ashby's in that erythritol at 0.25% replaced mannitol, calcium carbonate was omitted, and Difco agar was used at 1.0% final concentration. The pH was adjusted to 6.75 before autoclaving at 15 lb for 15 min. Samples to be tested were pipetted onto 6.35-mm Schleicher and Schuell no. 540-E paper disks which were then placed on the seeded agar surface. The plates were incubated 2 days at 37° after which the diameters of the zones of inhibition were measured.

Analyses. Amino acid analyses were performed using the Technicon Autoanalyzer and the single column buffer gradient system described in Technicon research bulletin no. 10, except where otherwise noted. Molecular weights were determined with a Mechrolab vapor pressure osmometer. Melting points were determined microscopically with the stage temperature increased at the rate of 3–5°/min. Carboxyl carbon was determined by the method of Van Slyke *et al.* (1941).

The identity of the gases following treatment of hydrolyzed linatine with nitrous and periodic acids was determined with an Aerograph A-90-C gas chromatograph equipped with a thermal conductivity detector using helium as the carrier gas. Carbon dioxide and nitrous oxide were identified by use of a 0.6 × 25 cm column of acid-washed Norit A (100–200 mesh). Nitrogen was identified with a 0.6 × 90 cm column packed with 80–100 mesh Molecular Sieve 5A (Coast Engineering Laboratory, Redondo Beach, Calif.). Retention volumes were compared with known gas samples.

Ultraviolet absorption spectra were determined with a Beckman DK-2 spectrophotometer. Elemental analyses were performed by the Clark Microanalytical Laboratory, Urbana, Ill.

Thin layer chromatography was performed on plates coated with silica gel G (Brinkman Instruments, Inc.) using 70% aqueous ethanol as the developing solvent. The components were located with ninhydrin, the prussian blue test of Kahler *et al.* (1941), or the periodate–benzidene test (Cowgill and Pardee. 1957). Aqueous solutions were evaporated under reduced pressure using a rotating evaporator and a water bath heated to 40°.

Extraction of Linatine (I). Solvent-extracted linseed flakes were extracted with 70% isopropyl alcohol by the method of Evenstad *et al.* (1965). The extract was passed through a column of Amberlite IRA-400 (acetate). Elution of the resin with 1 M acetic acid, followed by evaporation under reduced pressure, gave 5.8 g of dry material/kg of flakes. The material was dissolved in a small volume of water and stored at −20°.

The extracts showed an LD_{50} of 50 g-equiv[1]/chick by the chick injection method. Microbiological assay showed that 0.015 g-equiv produced a 55-mm diameter zone of inhibition. All of the common amino acids were present in the extracts. Thin layer chromatography and bioassay demonstrated a zone of inhibition at R_F 0.12.

Fractionation of the Crude Extract. The extract from 25 kg of linseed flakes was applied to a 2.5 × 25 cm water-jacketed column of Amberlite CG-120 (H^+ form), and the column was washed with 1 l. of water and eluted with 3 l. of 0.15 M ammonia solution. The ammonia eluate was collected and evaporated to dryness to give 10 g of solids.

The amino acid analyzer showed that most of the common amino acids were still present in the extract. Thin layer chromatography revealed substances at R_F 0.15 and 0.50 that reduced periodate and gave a positive prussian blue test. An aliquot of the ammonia eluate corresponding to 0.02 g-equiv produced a 56-mm zone of inhibition in the microbiological assay. The LD_{50} by the chick injection method was 50–75 g-equiv/chick.

Chromatography on Dowex 1 (Acetate). A portion

[1] A gram-equivalent is the material obtained from 1 g of spent linseed flakes.

of the ammonia eluate corresponding to 2.5 kg of linseed flakes was applied to a 4.5 × 54 cm column of Dowex 1 (acetate) which had been equilibrated with 0.3 M acetic acid. The column was eluted with the same solvent and the eluate was collected in fractions of 19 ml each. Growth inhibition of *A. vinelandii* was found in tubes 55–65 (990–1170-ml effluent volume). These tubes also gave positive ninhydrin and prussian blue tests and reduced periodate. The contents of tubes 55–65 were collected and evaporated to dryness to give 47 mg of amorphous material with LD_{50} of 75 g-equiv/chick.

Thin layer chromatography showed components at R_F 0.11 and 0.60 that reacted with ninhydrin and the prussian blue reagent. Only the component at R_F 0.11 inhibited the growth of *A. vinelandii* O.

Chromatography on Silica Gel. ISOLATION OF LINATINE. The active fraction from the Dowex 1 (acetate) column (47 mg) was chromatographed on a 4.5 × 9 cm column of silica gel (Anasil S, Analytical Engineering Laboratory) with 50% aqueous ethanol as the solvent, using air pressure to give a flow rate of 2–3 ml/min. The effluent was collected in fractions of 16 ml each. Fractions 30–70 inhibited *A. vinelandii* O and gave positive ninhydrin and prussian blue tests. These fractions were combined and evaporated to dryness to give a residue that was contaminated by silica. Thin layer chromatography revealed a single component at R_F 0.11 that reduced periodate and gave positive tests with the ninhydrin and prussian blue reagents.

The residue was dispersed in 5 ml of water and filtered through an ultrafine, fritted glass filter to remove silica aggregates. The filtrate was applied to a 1 × 23 cm column of Dowex 1 (acetate) which had been equilibrated with distilled water. Most of the silica was washed through the column with 100 ml of water and the column was eluted with 250 ml of 1 M acetic acid. Evaporation of the eluate yielded 20 mg of linatine (I) as a slightly yellow amorphous solid that contained 5.09% ash (silica).

Anal. Found: (ash-free basis) C, 44.9; H, 6.82; N, 15.8; α-carboxyl carbon, 4.1; mol wt, 25 ; $[\alpha]_D^{25}$ +46° (*c* 2.75, water).

Linatine is very soluble in water but insoluble in anhydrous organic solvents. It resisted all attempts to obtain well-defined crystals and melted with decomposition over a wide temperature range.

Microbiological assay showed a 62-mm zone of inhibition from 1 μg of linatine which corresponded to 0.125 g-equiv of linseed flakes. The chick injection assay gave an LD_{50} of 2 mg which corresponded to 250 g-equiv. Both assays suggested that the amount of linatine recovered was only 10–20% of that found in the original extracts.

Linatine could not be completely separated from alanine by the amino acid analyzer with the standard buffer gradient. The ninhydrin color value for linatine was 0.65 of the norleucine standard (molar basis). Quantitative chromatographic analysis of linatine in the presence of other amino acids was more readily obtained by use of a 0.6 × 100 cm column of Dowex 1 (acetate), 200–400 mesh, using an acetic acid gradient elution and the Technicon Autoanalyzer. The gradient was obtained with a nine-chamber Autograd filled as follows: chambers 1 and 2, 75 ml of water each; chambers 3–6, 75 ml of 0.4 M acetic acid; chambers 7–9, 75 ml of 1.3 M acetic acid. With a pumping rate of 0.66 ml/min, the following retention times were noted: neutral and basic amino acids as a single peak at 65 min; linatine, 130 min; and glutamic acid, 280 min. With the use of this system and freshly prepared extracts of laboratory-prepared linseed meal, the linatine content of whole flaxseed was estimated to be 80–100 ppm.

Preparation and Hydrolysis of DNP-Linatine. The dinitrophenyl (DNP) derivative of linatine was prepared following the procedure described by Cowgill and Pardee (1957). A portion of the DNP-linatine was hydrolyzed in constant-boiling hydrochloric acid for 4 hr at 100°. The hydrolysate was evaporated to dryness, the residue was extracted with ether, and the ether layer was chromatographed on thin layer plates using two solvent systems. Solvent A was a mixture of chloroform–*t*-amyl alcohol–glacial acetic acid (70:30:3) and solvent B was composed of chloroform–benzyl alcohol–glacial acetic acid (70:30:3). Before hydrolysis DNP-linatine showed an R_F of 0.10 in solvent A. After hydrolysis the DNP derivative showed an R_F of 0.27 in solvent A as did DNP-glutamic acid. In solvent B the DNP derivative obtained after hydrolysis and DNP-glutamic acid both migrated at R_F 0.33.

Hydrolysis of Linatine. IDENTIFICATION OF L-GLUTAMIC ACID AND ISOLATION OF A TOXIC HYDROLYTIC PRODUCT (II). Linatine (88 mg) was hydrolyzed in 50 ml of 1 M hydrochloric acid at 100° for 4 hr. Thin layer chromatography showed a component at R_F 0.56 which gave a typical pink color with ninhydrin reagent and a second component at R_F 0.50 which gave a brown color with ninhydrin. This latter spot also reduced Tollen's reagent and periodic acid and gave a positive test with the prussian blue reagent.

The hydrolysate was evaporated to dryness, dissolved in 5 ml of water, applied to a 0.8 × 20 cm column of Dowex (acetate), and washed with 50 ml of water. The resin was then eluted with 50 ml of 1 M acetic acid. The water wash and the acetic acid eluate were processed separately.

The acetic acid eluate did not inhibit *A. vinelandii* O and on evaporation yielded 40 mg of white solid which was recrystallized from 6 M hydrochloric acid. The crystals melted with decomposition at 191–193°, $[\alpha]_D^{24}$ +24.9° (*c* 2.5, 5 M HCl).

With thin layer chromatography the crystals showed R_F 0.56, the same as glutamic acid, and gave a normal pink color with ninhydrin. An authentic sample of L-glutamic acid hydrochloride showed the same decomposition point and specific rotation as the isolated crystals. Quantitative amino acid analysis of the unfractionated hydrolysate showed that glutamic acid had been produced in 50% yield.

Evaporation of the effluent and water wash from the above Dowex 1 (acetate) column yielded 40 mg of a yellow syrup which resisted crystallization. Thin layer

chromatography showed a single component at R_F 0.50 that reduced Tollen's reagent, periodic acid, and gave a positive prussian blue test. The syrup (40 mg) was dissolved in 0.5 ml of absolute ethanol and crystals of II separated on standing, mp 155° dec. Thin layer chromatography showed a single component at R_F 0.50 that reduced Tollen's reagent and periodic acid and gave a positive prussian blue test.

The material inhibited the growth of *A. vinelandii* O and was toxic to chickens, with an LD_{50} of 0.5 mg. It was stable in the crystalline state and in hydrochloric acid solution. Neutral aqueous solutions darkened and lost their toxicity on standing for 24 hr at room temperature.

Anal. Calcd for $C_5H_{10}N_2O_2$: C, 46.1; H, 7.76; N, 21.2. Found: C, 46.3; H, 7.89; N, 20.7; $[\alpha]_D^{23}$ +111° (*c* 0.4, 0.5 M HCl).

Characterization of the Toxic Hydrolytic Product (II). TREATMENT WITH NITROUS ACID. A solution of II (4.7 mg) in 2 ml of 1 M acetic acid was allowed to react with 1 ml of 1 M sodium nitrite at 50° in a closed system under helium. At the end of 90 min, an aliquot of the gases was fractionated by gas chromatography on Norit A and the Molecular Sieve 5A columns. Two components with elution times corresponding to known samples of carbon dioxide and nitrous oxide were detected on the Norit A column. There was no evidence for the production of elemental nitrogen. The gases normally produced on acidification of sodium nitrite did not interfere with the determinations. In control runs in which known glutamic acid was added, the production of nitrogen gas as a result of the action of nitrous acid was readily established by analysis of the gaseous reaction products with the Molecular Sieve 5A column.

REACTION WITH PERIODIC ACID. A 4-mg sample of linatine was hydrolyzed, evaporated to dryness, and the residue was dissolved in 1 ml of water. A solution of 12 mg of periodic acid in 1 ml of water was added and allowed to react at room temperature in a closed system purged with helium. After 30 min, aliquots of the reaction gases were injected into the gas chromatograph. The presence of carbon dioxide and nitrogen was established using the Norit A and the Molecular Sieve 5A columns, respectively. Control samples consisting of reagent blanks and glutamic acid showed neither carbon dioxide nor nitrogen.

PREPARATION OF BENZYLIDENE DERIVATIVES OF II. A solution of 47 mg of II was added to 51.4 mg of 3-nitrobenzaldehyde in 2 ml of methanol. After warming at 50° for 5 min, water was added until the solution became cloudy. On cooling, 22 mg of the 3-nitrobenzylidene derivative separated as bright yellow needles, mp 123–124°.

Anal. Calcd for $C_{12}H_{13}N_3O_4$: C, 54.8; H, 5.06; N, 15.9. Found: C, 55.1; H, 4.98; N, 15.9; $[\alpha]_D^{23}$ +113° (*c* 0.6, ethanol).

The 2-hydroxy-5-nitrobenyzlidene derivative was prepared in the same manner from 67 mg of II and 41 mg of 2-hydroxy-5-nitrobenzaldehyde. The yellow product was recrystallized from ethyl ether plus petroleum ether (bp 30–60°), melted at 136–137°, and showed $[\alpha]_D^{24}$ +151° (*c* 0.35, ethanol).

REACTION WITH PYRIDOXAL PHOSPHATE. An aliquot of hydrolyzed linatine in water corresponding to 0.14 mg of linatine was added to 4 ml of 0.1 M sodium phosphate buffer, pH 6, and 2 ml of pyridoxal phosphate (0.265 mg/ml). The yellow color of pyridoxal phosphate gradually faded. The absorption spectrum of the solution was measured at intervals and became constant after 75 min with λ_{max} 374 mμ when read against a reagent blank. The same absorption maximum was observed when pyridoxal was substituted for pyridoxal phosphate, but the reaction rate was much slower. The presence of glutamic acid did not interfere with complex formation.

Hydrogenolysis of Linatine. A solution of 9 mg of linatine in 1 ml of 80% ethanol was heated at 100° with 100 mg of Raney nickel catalyst for 6 hr in a sealed tube. Analysis of the supernatant with the amino acid analyzer disclosed the presence of three components with elution times that corresponded to glutamine, proline, and unchanged linatine. In this experiment and in tests with known samples of glutamine and proline, a substantial amount of both glutamine and proline were retained by the catalyst and quantitation of products of the hydrogenolysis of linatine was not possible.

Syntheses

1-Nitrosoproline. Both enantiomorphs of 1-nitrosoproline were prepared from the corresponding isomers of proline by a preparative modification of the method of Hamilton and Ortiz (1950). To 1 g of D- or L-proline and 6 g of sodium nitrite in 10 ml of water, 16 ml of 3 M sulfuric acid was added over a period of 10 min. The reaction mixture was extracted four times with 25-ml portions of ether, the ether extract was dried over calcium chloride and evaporated to dryness at 0° under reduced pressure. The crystals which separated were recrystallized from 1:1 ether–benzene to give 0.9 g of 1-nitroso-D- or L-proline, mp 109–110° dec, $[\alpha]_D^{24}$ −173 (for 1-nitroso-L-proline) and +175° (for 1-nitroso-D-proline) (*c* 2, water). The ultraviolet absorption spectrum gave λ_{max}^{water} 347 mμ (ε 13,800).

1-Aminoproline. 1-Nitroso-D-proline or 1-nitroso-L-proline (1 g) was dissolved in 50 ml of 50% acetic acid and the solution was cooled in an ice bath. Zinc dust (4 g) was gradually added over a 15-min period while the reaction mixture was stirred vigorously and maintained below 10° by an ice bath. The course of the reaction was followed by measuring the rate of disappearance of the nitrosoproline absorption at 347 mμ and was complete after 15 min. The unreacted zinc dust was removed by filtration and the zinc was precipitated from solution by hydrogen sulfide. The precipitated zinc sulfide was removed by filtration and the filtrate was evaporated to dryness. The residual syrup was dissolved in 3 ml of absolute ethanol and 1-amino-D-proline or 1-amino-L-proline was separated as slightly yellow crystals, mp 155–156°.

Anal. Calcd for $C_5H_{10}N_2O_2$: C, 46.1; H, 7.76; N,

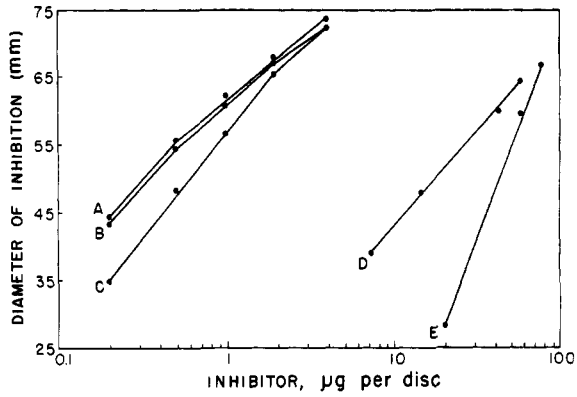


FIGURE 1: Inhibition of the growth of *A. vinelandii* O by linatine (A), 1-[(*N*-γ-L-glutamyl)amino]-D-proline (B), 1-amino-D-proline (C), 1-[(*N*-γ-L-glutamyl)amino]-L-proline (D), and 1-amino-L-proline (E).

21.2. Found for the D isomer: C, 46.3; H, 7.75; N, 20.8; $[\alpha]_D^{24}$ +113° (*c* 2, 0.5 M HCl). Found for the L isomer: $[\alpha]_D^{24}$ −111° (*c* 1.6, 0.5 M HCl).

The D isomer showed the same growth inhibition toward *A. vinelandii* O as the toxic hydrolytic product II. The L isomer was considerably less inhibitory as shown in Figure 1.

The derivative prepared by reacting 1-amino-D-proline with 3-nitrobenzaldehyde melted at 123–124°, $[\alpha]_D^{23}$ +112 (*c* 0.6, ethanol). The 2-hydroxy-5-nitrobenzylidene derivative of 1-amino-D-proline melted at 136–137° and showed $[\alpha]_D^{24}$ +154° (*c* 0.35, ethanol). The corresponding derivatives prepared from 1-amino-L-proline showed the same melting points as those obtained from the D isomer and the specific rotations were identical in magnitude, but of opposite sign.

1-Aminoproline Benzyl Ester. The general procedure of Neuman and Smith (1951) was followed to give the benzyl esters of 1-amino-D-proline and 1-amino-L-proline as viscous oils. Each ester chromatographed as a single spot R_F 0.63 and gave a positive prussian blue test. The L-benzyl ester showed $[\alpha]_D^{23}$ −67° and the D ester showed $[\alpha]_D^{23}$ +66° in ethanol. The esters were not characterized further.

1-[(N-γ-L-Glutamyl)amino]-D-proline (Linatine) (I). The procedure of Fodor *et al.* (1953) for the preparation of peptides by the azide method was modified for the synthesis of linatine. A solution of 6.84 g of *N*-carbobenzyloxy-L-glutamic acid γ-hydrazide in 240 ml of 0.75 M hydrochloric acid and 15 ml of glacial acetic acid was cooled to −5°, 225 ml of ether was added, followed by the addition of 20 ml of 10% sodium nitrite solution. The mixture was immediately extracted in the cold by ether, and the ether extract which contained *N*-carbobenzyloxy-L-glutamic acid γ-azide was dried over sodium sulfate and added to 1.77 g of 1-amino-D-proline benzyl ester in 100 ml of chloroform cooled to −5°. The resulting cloudy mixture was concentrated under reduced pressure at 0° until a clear solution resulted and was allowed to stand for 2 hr at 0° followed by 3.5 hr at room temperature. The course of the reaction was followed by observing the decrease in the concentration of 1-amino-D-proline benzyl ester. To accomplish this, 0.1-ml aliquots of the reaction mixture were withdrawn at intervals of 45 min and added to a solution of 0.5 mg of pyridoxal phosphate in 4 ml of 0.01 M phosphate buffer, pH 6.0. After 45 min the absorption of this complex was read at 367 mμ, the absorption maximum for the phosphopyridoxylidene derivative of 1-aminoproline benzyl ester. It was found that the aminoproline benzyl ester had essentially disappeared 3 hr after addition of the azide.

The reaction mixture was evaporated to dryness and dissolved in 140 ml of glacial acetic acid and 20 ml of ethanol. Palladium black from the hydrogenation of 3 g of palladium oxide was added and hydrogen was bubbled through at room temperature to effect cleavage of the carbobenzyloxy and benzyl ester groups. Thin layer chromatography showed the gradual development of a component with R_F 0.12. After 6 hr the catalyst was removed by filtration, washed, and the filtrate and washings were evaporated to dryness to give a clear syrup.

The desired product was separated from by-products by gradient elution chromatography on a 4.5 × 45 cm column of Dowex 1 (acetate). The elution gradient was produced by use of a Buchler varigrad filled in the following manner: chambers 1–3 each contained 500 ml of distilled water, chambers 4–7 each contained 500 ml of 0.4 M acetic acid; and chambers 8–9 each contained 500 ml of 1.3 M acetic acid. The flow rate was adjusted to 5.2 ml/min. The effluent was collected in tubes of 20 ml each which were assayed by spot tests and thin layer chromatography. Tubes 111–129 contained a single component with R_F 0.12 which corresponded to the R_F of natural linatine. Other reduction products which were well separated from this band were detected in the effluent. Tubes 111–129 were combined and evaporated to dryness, washed with hot absolute ethanol, and dried under vacuum at room temperature to give 0.75 g of linatine (I) as an amorphous white powder.

Anal. Calcd for $C_{10}H_{17}N_3O_5$: C, 46.3; H, 6.61; N, 16.2; α-carboxyl C, 4.63. Found: C, 46.7; H, 6.64; N, 15.8; α-carboxyl C, 4.2; $[\alpha]_D^{25}$ +46.4° (*c* 2.8, water).

The synthetic product was cochromatographed with natural linatine on the amino acid analyzer and on thin layer and paper strips. It also showed the same level of inhibition to *A. vinelandii* O as natural linatine and on hydrolysis it yielded glutamic acid plus a product that was identical with 1-amino-D-proline.

1-[(N-γ-L-Glutamyl)amino]-L-proline. The methods used for the preparation of linatine were followed except that 1-amino-L-proline benzyl ester was used in place of the D isomer. Evaporation of the same fractions from the effluent of the Dowex 1 column gave 1-[(*N*-γ-L-glutamyl)amino]-L-proline.

Anal. Calcd for $C_{10}H_{17}N_3O_5$: C, 46.3; H, 6.61; N, 16.2; α-carboxyl C, 4.63. Found: C, 47.0; H, 6.48; N, 16.3. α-carboxyl C, 4.2; $[\alpha]_D^{24}$ −34.6° (*c* 2, water).

Both synthetic linatine and the diastereoisomer resisted crystallization and were apparently amorphous solids, as was natural linatine. Synthetic linatine showed the same growth inhibition as natural linatine in the microbiological assay as shown in Figure 1. The diastereoisomer was only about 2% as inhibitory as natural linatine. With the chick injection assay, natural and synthetic linatine as well as the synthetic diastereoisomer all showed an LD_{50} = 2 mg/chick.

Discussion

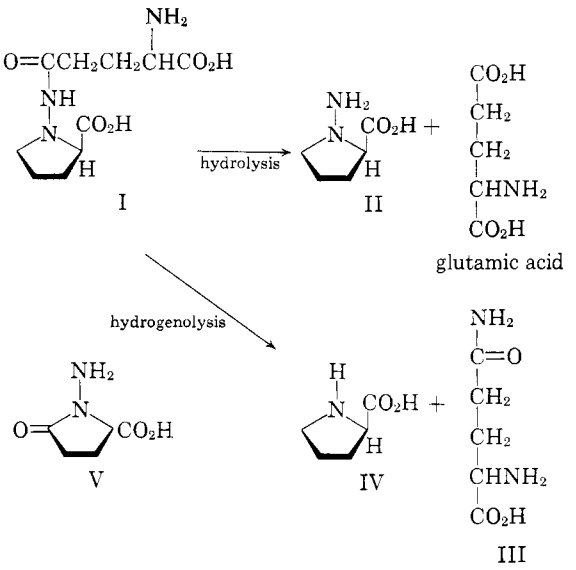
The isolation of linatine (I) confirms the earlier proposal of Kratzer *et al.* (1954) that linseed meal contains a vitamin B_6 antagonist. Upon hydrolysis of linatine, toxic 1-amino-D-proline is produced which is capable of forming a stable derivative of pyridoxal phosphate.

Linatine is present in flaxseed and linseed meal in relatively low concentration (*ca.* 100 ppm). Preparative ion-exchange chromatography gave highly active but impure preparations of linatine. These preparations lost most of their toxicity upon acid hydrolysis. A variety of procedures was tried to effect purification, with chromatography on Anasil S being the most effective. Although these linatine preparations always contained some silica, the amounts of silica were low enough to allow meaningful elemental analyses and chemical studies. Furthermore, acid hydrolysates of these preparations showed enhanced toxicity to chicks and *A. vinelandii*, the assay organism.

Elemental analyses suggested the empirical formula $C_{10}H_{18}\pm_1N_3O_5$ for linatine which was consistent with an observed molecular weight of 258. The isolation of L-glutamic acid and a toxic product (II) upon hydrolysis of linatine suggested that the glutamic acid moiety was linked to II through a peptide linkage. The formation of DNP-glutamic acid upon treatment of linatine with Sanger's reagent, followed by hydrolysis, indicated a free amino group on the glutamic acid portion of the linatine molecule. The evolution of approximately 1 mole of carbon dioxide upon treatment with ninhydrin according to the method of Van Slyke *et al.* (1941) suggested that the α-carboxyl of the glutamyl residue was free and that the linkage was formed through the γ-carboxyl of glutamic acid.

The gaseous products obtained by the treatment of II with nitrous acid and periodic acid indicated the presence of a hydrazine type of structure in II. The release of elemental nitrogen by treatment with periodic acid is unique for hydrazines and the release of nitrous oxide upon treatment of II with nitrous acid is characteristic of unsymmetrical secondary hydrazines (Veibel, 1954).

Hydrogenolysis of linatine with Raney nickel catalyst gave glutamine III and proline IV, showing that the glutamic acid portion of linatine is linked through the γ-carboxyl function to a 1-aminoproline residue. Synthetic 1-amino-D-proline was prepared by the reduction of 1-nitroso-D-proline with zinc and acetic acid and formed 3-nitrobenzylidene and 3-hydroxy-5-nitro-



benzylidene derivatives which were identical with those obtained from II. The physical properties of II and the two 1-aminoprolines are shown in Table I.

Synthetic 1-[(*N*-γ-L-glutamyl)amino]-D-proline (I), prepared by the azide method, was identical with linatine in its physical, chemical, and biological properties. The physical properties of natural and synthetic linatine are shown in Table II and the growth inhibition curves for the microbiological assay in Figure 1. The LD_{50} value of 2 mg/chick shown for linatine corresponds to an LD_{50} of approximately 30 mg/kg body weight. By comparison, the two 1-aminoprolines were about four times as toxic.

TABLE I: Properties of the Toxic Hydrolytic Product II and the 1-Aminoprolines.

Property	II	1-Amino-D-proline	1-Amino-L-proline
Specific rotation (deg)	+111	+113	−111
Melting point (°C)	155	155–156	155
3-Nitrobenzylidene Derivative			
Melting point (°C)	123–124	123–124	124
Specific rotation (deg)	+113	+112	−112
2-Hydroxy-5-nitrobenzylidene Derivative			
Melting point (°C)	136–137	136–137	136
Specific rotation (deg)	+151	+154	−154

The synthetic glutamyl peptides obtained with the two antipodes of aminoproline showed almost theoretical free α-carboxyl carbon, suggesting that the linkage

TABLE II: Properties of Natural and Synthetic Linatine.

Property	Natural	Synthetic
Specific rotation (deg)	+46	+46.4
R_F (tlc)[a]	0.11	0.12
LD_{50} (mg)[b]	2	2
Microbiological assay (mm/μg)[c]	62	60

[a] Thin layer chromatography on silica gel G. [b] Chick injection assay. [c] Diameter of zone of inhibition of *A. vinelandii* O.

was through the γ-carboxyl of the glutamyl residue in both cases. If some of the α-linked peptide was produced in the coupling reaction, as may sometimes be the case with the azide method (Sachs and Brand, 1954), the fractionation of the crude synthetic products by ion-exchange chromatography must have separated the α isomer from the desired γ isomer.

Linatine is the principal if not the only vitamin B_6 antagonist in flaxseed and linseed meal. It will induce characteristic vitamin B_6 deficiency symptoms in chickens which can be prevented or alleviated by timely administration of pyridoxine. Numerous efforts were made to locate other vitamin B_6 antagonists in extracts of flaxseed, but none could be demonstrated by the microbiological or the chick injection assay, or in earlier studies, by observing depressed growth rates of chicks. It is possible that smaller quantities of 1-amino-D-proline may exist in conjugation with other amino acids and are lost as the result of different chromatographic behavior. For example, 1-[(*N*-alanyl)amino]-D-proline would probably not have been retained by the Amberlite IRA-400 (acetate) resin in the initial isolation step. In view of the widespread occurrence of the γ-glutamyl derivatives of uncommon amino acids and other nitrogenous compounds, 1-amino-D-proline probably occurs in flaxseed only as the γ-glutamyl derivative.

The complex formed between 1-aminoproline and pyridoxal phosphate with the absorption maximum at 374 mμ at pH 6.0 provides a convenient means for the quantitative determination of the free 1-aminoproline content of various solutions. Most hydrazone derivatives of pyridoxal phosphate show absorption maxima at pH 6 at wavelengths shorter than 374 mμ. The increased absorbancy at 374 mμ was found to correlate directly with the size of the zone of growth inhibition in the microbiological assay when hydrolyzed linatine preparations were assayed. The same reaction was used to demonstrate that the reduction of 1-nitrosoproline proceeded smoothly in good yield at 0–10°.

When the reduction of 1-nitrosoproline was run at room temperature, proline was the main product. The crystallization of aminoproline from absolute ethanol proceeded readily after seed crystals were first obtained.

Free 1-aminoproline could not be detected in freshly prepared extracts by the pyridoxal phosphate assay, suggesting that 1-aminoproline occurs in the seed only in the combined form. The amino acid analyzer failed to show an identifiable peak when amounts up to 2 mg of 1-aminoproline were used. It appeared that the resin either absorbed the material firmly or it was decomposed while passing through the resin column.

The natural occurrence of the 1-aminoprolines or other α-hydrazino acids has not been reported previously, nor has the synthesis of the 1-aminoprolines been described. They represent a unique type of secondary unsymmetrical hydrazine with enantiomorphic forms that may act as selective inhibitors in nitrogen metabolism.

The bacteriostatic effect of aminoproline is not a general one. Of 25 organisms tested in the development of the microbiological assay, the growth of only *A. vinelandii* O was appreciably inhibited. In this case, 1-amino-D-proline was 50 times as toxic as the L isomer as shown by curves C and E of Figure 1. The same effect was noted when linatine (curve A) was compared with the diastereoisomer obtained from L-glutamic acid and 1-amino-L-proline (curve B) further confirming the expected stereochemical effect.

The mechanism by which aminoproline exerts its toxic effects is not known. Since the D and L forms show different levels of toxicity toward *A. vinelandii* O, the effect in this case is not merely one of complexing with pyridoxal phosphate. Both enantiomorphs will complex equally with pyridoxal phosphate. On the other hand, the prevention of toxicity in the chick by the administration of pyridoxine suggests a competitive relationship. The use of pyridoxine in the prevention and treatment of hydrazine poisoning has been reported by Uchida and O'Brien (1964).

The metabolic role of linatine in flax seed is not known. Examination of leaves of young plants failed to disclose any linatine. A related compound, 1-amino-5-oxoproline (V), has been suggested by Bach (1957) as a metabolic intermediate in nitrogen reduction in plants. The occurrence in mushrooms of a γ-glutamyl derivative of a substituted phenylhydrazine has been reported by Levenberg (1961) and it is possible that hydrazine derivatives may be rather widely distributed in nature. The metabolism of linatine in certain plants, animals, and microorganisms is being studied.

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8-(*N*-2-Fluorenylacetamido)guanosine, an Arylamidation Reaction Product of Guanosine and the Carcinogen *N*-Acetoxy-*N*-2-fluorenylacetamide in Neutral Solution*

Erik Kriek,† James A. Miller, Ursula Juhl, and Elizabeth C. Miller

ABSTRACT: The carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide and guanosine reacted readily at neutrality to yield a compound which was identified as 8-(*N*-2-fluorenylacetamido)guanosine. This compound was hydrolyzed by weak alkali at 37° to 8-(*N*-2-fluorenylamino)guanosine and by 1 N hydrochloric acid at 100° to 8-(*N*-2-fluorenylacetamido)guanine and 8-(*N*-2-fluorenylamino)guanine. The latter compound was synthesized by acid hydrolysis of the condensation product of 8-bromoguanosine 2′,3′,5′-triacetate and 2-fluorenamine.

N-Acetoxy-*N*-2-fluorenylacetamide also reacted with deoxyguanosine 5′-phosphate at neutrality to yield 8-(*N*-2-fluorenylacetamido)deoxyguanosine 5′-phosphate which was stable for 6 days at pH 7.2 and 37° and was hydrolyzed by weak acid at 37° to 8-(*N*-2-fluorenylacetamido)guanine. Guanine in soluble ribonucleic acid (sRNA) and deoxyribonucleic acid (DNA) reacted in the same fashion with *N*-acetoxy-*N*-2-fluorenylacetamide; acid hydrolysis of these nucleic acids after this reaction yielded a product identical with 8-(*N*-2-fluorenylamino)guanine.

It is generally considered that interaction of a chemical carcinogen or metabolite thereof with critical tissue constituents is necessary for carcinogenesis by these substances (reviewed in Miller and Miller, 1966). The central role of nucleic acids in replication and transfer of genetic information has thus made interactions of carcinogens with nucleic acid constituents of particular interest. Administration to rats of the general carcinogen *N*-2-fluorenylacetamide (FAA)[1] (Weisburger and Weisburger, 1958) or its more carcinogenic *N*-hydroxy metabolite (*N*-hydroxy-FAA) (Miller *et al.*, 1961) labeled with ^{14}C in the 9 position were shown by Farber and his associates (Farber *et al.*, 1962; Marroquin and Farber, 1962, 1965), Williard and Irving (1964), Miller *et al.* (1964), and Sporn and Dingman (1966) to result in the incorporation of ^{14}C into hepatic DNA and RNA. Since neither FAA nor *N*-hydroxy-FAA reacts with nucleic acids *in vitro* (Miller *et al.*, 1966), some further metabolite was implicated in the reaction. Kriek (1965, 1966), showed that *N*-hydroxy-2-fluorenamine, a probable

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† On leave of absence (March–July, 1966) from the Department of Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands. Requests for reprints should be sent to Dr. Kriek in Amsterdam or to Dr. Miller in Madison.

[1] Abbreviation used in this paper: FAA, *N*-2-fluorenylacetamide (alternative nomenclature: AAF, 2-acetylaminofluorene).