An Improved Synthesis of Lysinoalanine Monohydrochloride

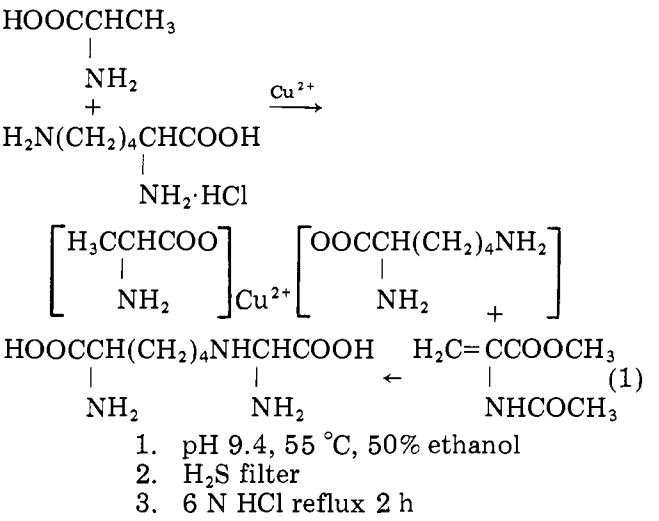
Lysinoalanine (N,α-(DL-2-amino-2-carboxyethyl)-L-lysine) monohydrochloride was synthesized in 56% yield from methyl 2-acetamidoacrylate and L-lysine hydrochloride. The reaction was carried out under alkaline conditions in 50% ethanol with lysine being introduced as the lysyl–alanyl–cupric complex. Copper was subsequently removed from the system with hydrogen sulfide. Acid hydrolysis afforded lysinoalanine monohydrochloride which was purified by ion-exchange chromatography and crystallization from pyridine–ethanol–water and ethanol–water mixtures.

Lysinoalanine (N,α-(DL-2-amino-2-carboxyethyl)-L-lysine, (I) is an unnatural amino acid which is found in alkaline-treated proteins and in protein foods which have received intense levels of heating (Sternberg et al., 1975; DeGroot and Slump, 1969). Alkaline treatment of proteins and lye peeling of fruits and vegetables are currently widely practiced in the food industry. It is anticipated that the use of alkali will certainly become more important as world demand increases for isolated proteins to be used in textured foods, milk-like products, and other specialized protein isolates.

The pharmacological significance of lysinoalanine and its analogues has recently taken on greater importance. Woodard et al. (1975) reported that lysinoalanine causes kidney lesions when injected intraperitoneally into rats. It is not yet known to what extent these results can be extrapolated to feeding studies or to higher animals and man. One problem that we encountered in undertaking these studies was the difficulty of acquiring lysinoalanine in substantial quantities, at reasonable cost. Bohak (1964) and Patchornik and Sokolovsky (1964) first reported lysinoalanine in alkaline-treated proteins. Bohak (1964) also chemically synthesized lysinoalanine from α-carbobenzyloxy-L-lysine and N-phenylacetyldehydroalanine. Unfortunately, we have found the reaction of the (unesterified) dehydroalanine carboxylic acid with the amino group of lysine to be extremely slow (Snow et al., 1976), and the yields of the adduct are so low that large quantities of starting materials would be required to produce enough material to conduct a long-term feeding study. Okuda and Zahn (1965) prepared lysinoalanine synthetically with the ethyl ester of acetamidoacrylic acid and N,α-acetyllysine; this method greatly improved the addition reaction rates and improved the overall yield. Garcia-Dominguez et al. (1973) have reported a further improved synthetic procedure using methyl α-acetamidoacrylate.

Our attempts to employ these previously reported routes for the synthesis of lysinoalanine found these procedures to be extremely tedious and time-consuming, affording, at best, milligram quantities of material. We also found that the reaction sequences employed were generally accompanied by undesirable side reactions and/or required the use of substantial quantities of N,α-acetyllysine or N-α-carbobenzyloxylysine, both expensive materials. We have now found that the alanine-lysine mixed amino acid–copper complex, previously reported (Finley et al., 1972) for making ϵ-dinitrophenyllysine, can be substituted for either N,α-acetyl- or N-α-carbobenzyloxylysine, and reacts readily with methyl 2-acetamidoacrylate in 50% aqueous ethanol solutions at 55 °C (pH 9.4). This affords the intermediate lysinoalanine adduct which, upon acid hydrolysis, yields lysinoalanine monohydrochloride, as shown in sequence 1.

We have found that improved yields of lysinoalanine are obtained when the mixed amino acid (alanine-lysine)–copper complex, rather than the pure lysine–copper complex, is used to add lysine to dehydroalanine. Undoubtedly, the lysine α-amino group is better protected



in the mixed amino acid–copper complex. This can be attributed to a decreased steric factor which allows enhanced coordination between the α-amino groups, the carboxyl groups, and the copper, leaving the ϵ-amino group freer to react and better protecting the α-amino group. The use of 50% aqueous ethanol as the reaction medium is required to retard the saponification of methyl 2-acetamidoacrylate which occurs rapidly in aqueous solutions, particularly at elevated pH (6).

Lysinoalanine monohydrochloride is a crystalline white solid which precipitates from water–ethanol–pyridine mixtures as a tan precipitate and as amorphous white crystals from ethanol–water at room temperature. Chromatography according to Spackman (1969) on short basic column showed our synthetic material to form a single peak with a retention time of 23 min (lysine, 27 min), the same retention time as the natural product. Lysinoalanine monohydrochloride has a ninhydrin color constant 1.59 times larger than that of leucine. The synthetic material contained from 5–6% unreacted lysine. TLC analysis on cellulose using 1-butanol–acetone–diethylamine–H_2O (10:10:2:5 v/v) developed with ninhydrin gave a single spot with R_f 0.18.

MATERIALS AND METHODS

Methyl 2-acetamidoacrylate was prepared via a two-step sequence from N-acetyl-L-cysteine (Calbiochem) in an overall yield of 62% (Gravel et al., 1972). L-Lysine monohydrochloride (Merck), DL-alanine (Calbiochem) basic cupric carbonate (Baker), and Dowex 50W-X8 (H^+), 200–400 mesh (Bio-Rad) ion-exchange resin were used as received. The NMR spectrum was determined on a Varian HA100 spectrometer. Chemical shifts are expressed in parts per million from Me_4Si calculated from internal HOD.

The alanine-lysine–cupric complex was prepared by boiling 4.6 g (0.025 mol) of L-lysine·HCl, 2.2 g (0.025 mol) of DL-alanine, 5.5 g (0.025 mol) of basic $CuCO_3$, and 50 mL of aqueous 0.1 M sodium borate for 0.25 h, cooling to 25

°C, and filtering. Absolute ethanol (50 mL) was then added to the filtrate, followed by 1.3 g (0.013 mol) of methyl 2-acetamidoacrylate. The homogeneous solution was then heated for 24 h at 55 °C in a sealed vessel. After concentration in vacuo, the oil was redissolved in 100 mL of water and H_2S bubbled through the solution for 3 h. After treatment with charcoal and filtration, 100 mL of 12 N HCl was added to the colorless filtrate and the solution refluxed for 2 h. The crude lysinoalanine hydrochloride was then concentrated in vacuo and the resulting oil dissolved in 25 mL of H_2O and chromatographed on Dowex 50W-X8 (200–400 mesh, 2.0 × 42 cm). The column was washed, successively, with 4 L of water, 2 L of 1 N HCl, and 1 L of 2 N HCl, and the lysinoalanine hydrochloride eluted with 4 L of 4 N HCl. After concentration in vacuo, the resulting oil was redissolved and redried in vacuo with 2 × 50 mL of H_2O to assure removal of maximum HCl. The final oil, dissolved in 10 mL of H_2O, was treated with 1 g of activated carbon, filtered, and 10 mL of 1 M pyridine in 95% ethanol added. The solution was then diluted with 480 mL of 95% ethanol and cooled at −20 °C for 48 h. The lysinoalanine monohydrochloride was collected by filtration and recrystallized from ethanol–water at room temperature, collected by filtration, and dried in vacuo over P_2O_5 to yield 1.97 g (56%) of lysinoalanine monohydrochloride: mp 156 °C; ^{1}H NMR (D_2O) 2.45 (m, 6 H), 3.95 (t, 2 H, J = 4.0 Hz), 4.25 (d, 2 H, J = 3.5 Hz), 4.55 (t, 1 H, J = 3.5 Hz), 4.80 (t, 1 H, J = 3.5 Hz).

Anal. Calcd for $C_9H_{20}N_3O_4Cl$: C, 40.01; H, 7.52; N, 15.58; Cl, 13.14. Found: C, 40.06; H, 7.78; N, 15.67; Cl, 12.89.

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Native Iranian Peanut Resistance to Seed Infection by *Aspergillus*

The presence of aflatoxin in three varieties of peanuts that are currently cultivated in Iran has been examined by two methods. The results indicated that two varieties of the peanuts were contaminated with this toxin, but the native Iranian seeds, Local Gilan Iran, in comparison with standard, showed no contamination. The finding of this peanut which resists seed infection by *Aspergillus* is important from nutritional and economical points of view.

Aflatoxins, the possible etiological agents in certain human diseases (FAO–WHO–UNICEF, 1972; Newbern and Butler, 1969; Wogan, 1968; Shank et al., 1972; Austwick and Ayerst, 1963), have been detected in peanuts grown in various countries (Natarajan et al., 1975; Barnes, 1970). There are also several reports that showed peanut resistance to seed infection by *Aspergillus flavus* and aflatoxin production (Kulkarni et al., 1967; Roa and Tupuli, 1967). Further plantings of these two varieties mentioned by these authors and rehydration of the seeds cultivated in 1969 indicated very little resistance to *A. flavus* (Mixon and Rogers, 1973). The following studies were conducted to determine the presence of aflatoxin in peanuts which are currently cultivated in Iran.

EXPERIMENTAL SECTION

Peanuts. From 14 varieties of peanuts (mostly with foreign origin) that are currently cultivated in Iran, three varieties, namely, Local Gilan Iran (peanut I), Flori-Spanish 334-A (peanut II), and Georgia 119–20 (peanut III) were randomly chosen. The product of two reported years of 1974 and 1975 was obtained from the Seed and Plant Improvement Institute, Karaj, Iran, and used for this study. Samples were kept in the cold room (4 °C) during experiments, though all three varieties were stored in the same condition in warm humid weather of the northern part of Iran before starting the experiments, without any special care to prevent fungal growth. As far as history goes, peanut II and III were brought from the United States in about 1965 and were grown in Iran since that time. Peanut I is locally grown in Gilan Iran.

Examination and Concentration. Holaday and Barnes (1973) and AOAC (1970) methods were used to examine aflatoxin in peanuts. These experiments were repeated more than 20 times for each variety and product. To measure the concentration of aflatoxin in the samples, precoated plastic sheet Polygram Sil G (Machery Nagel and Co., Duren) plates and developing solution of methanol–chloroform (97:3 v/v) were used for TLC. Aliquots of 1-mL of the extract of peanut II and 60 μL