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MICHAEL ADDITION OF THIOLS WITH 4-METHYLENEGLUTAMIC ACID: PREPARATION OF ADDUCTS, THEIR PROPERTIES AND PRESENCE IN PEANUTS

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The thioethers, S-(4-amino-2,4-dicarboxybutyl)cysteamine, S-(4-amino-2,4-dicarboxybutyl)cysteine and S-(4-amino-2,4-dicarboxybutyl)glutathione, were synthesized by a Michael addition between 4-methyleneglutamic acid and the respective thiol. In dilute aqueous solution, the reactions exhibit second order kinetics; glutathione reacts much slower than cysteine or cysteamine. The adducts were characterized chromatographically, electrophoretically, and by their infra-red and nuclear magnetic resonance spectra. None of these thioethers was detected in peanut plants (*Arachis hypogaea* L.), even though large amounts of 4-methyleneglutamic acid, its amide, and glutathione are synthesized during peanut germination.

INTRODUCTION

A variety of plants, primarily legumes, accumulate significant quantities of 4-MeGlu[1] and 4-MeGln(1-3). Large amounts are especially formed in germinating peanuts, wherein 4-MeGln is found as the predominant free amino acid in the xylem sap(4). For this reason, it is thought that the main function of such glutamate derivatives might be in nitrogen transport. No direct evidence, however, supports this role or eliminates other possibilities.

4-MeGlu and 4-MeGln are unusual amino acids in having an α,β-unsaturated carboxyl function which should readily undergo Michael additions(5). The thiol group is a good nucleophile for such additions; several cases in which a cysteinyl residue reacts with unsaturated compounds to form thioethers in biological systems have been reported(6-8). Since glutathione, cysteine and

[1]Abbreviations: 4-MeGlu, 4-methyleneglutamic acid; 4-MeGln, 4-methyleneglutamine; 4-MeGlu-cysteamine, S-(4-amino-2,4-dicarboxybutyl)cysteamine; 4-MeGlu-cys, S-(4-amino-2,4-dicarboxybutyl)cysteine; 4-MeGlu-GSH, S-(4-amino-2,4-dicarboxybutyl)glutathione.

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presumably other thiols are also present in peanut seedlings (9), adduct formation with 4-MeGlu and 4-MeGln in the plant seemed reasonable. We, therefore, synthesized and characterized the adducts of 4-MeGlu with cysteamine, cysteine and glutathione, and examined extracts of germinating peanuts for their presence.

EXPERIMENTAL

Materials: DL-4-MeGlu was synthesized as previously described (10). All other chemicals were commercially available and of analytical grade. IR spectra were recorded on a Perkin-Elmer Model 283 spectrophotometer and NMR spectra with a Varian T-60 spectrometer using 3-(trimethylsilyl)propionic acid as an internal reference. Melting points (uncorrected) were determined in sealed capillary tubes using a Thomas-Hoover melting point apparatus. Amino acid analyses were carried out on a Beckman Model 120B amino acid analyzer modified for three buffer operation (4). Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, MI.

Cysteamine, cysteine and glutathione conjugates of 4-MeGlu were prepared by modification of an earlier procedure (11). DL-4-MeGlu (795mg, 5 mmol) was dissolved in 20 ml of water with an equimolar quantity of either cysteamine (568mg), cysteine·HCl (878mg), or glutathione (1540mg). The reaction mixture was adjusted to pH 8.5 with 3N NaOH and incubated 48 hr at 25°C. The solution was then acidified to pH 2.5 with 4N HCl and applied to a column (1.5 x 20cm) of BioRad AG 50-X8 resin (200-400 mesh, H^+). The column was washed with several volumes of water and the adduct, along with any unreacted substrates, displaced with 1N NH_4OH. Ninhydrin-positive fractions were concentrated to an oil at 37°C *in vacuo*. The oil was dissolved in a small volume of water and applied to a column (1.5 x 20cm) of BioRad AG1 X8 resin (200-400 mesh, acetate phase). For the cysteamine adduct, the solution was adjusted to pH 12 with 3N NaOH. The column was washed with water and the acidic amino acids eluted with a 500-ml linear gradient (0 -> 1.0N) of acetic acid. Ninhydrin-positive fractions were pooled and concentrated to clear oils which were crystallized from water by adding acetone. The 4-MeGlu-cys conjugate was crystallized from water.

The yield of S-(4-amino-2,4-dicarboxybutyl)cysteamine was 0.55g (52%): mp=211-212°C (decomp.); NMR (D_2O-NaOD) 2.00(2H,m), 2.75(2H,s), 2.8(1H,buried), 2.85(2H,t), 3.20(2H,d), 3.60(1H,m). IR (KBr pellet, cm^{-1}) 3450, 2940, 1680, 1600, 1510, 1400, 1340, 1320.
Anal. Calcd for $C_8H_{16}N_2O_4S$: C,40.63, H,6.77, N,11.85. Found: C,40.54, H,6.79, N,11.53.

The yield of S-(4-amino-2,4-dicarboxybutyl)cysteine was 1.02g (69%).
Anal. Calcd for $C_9H_{16}N_2O_6S \cdot H_2O$: C,36.21, H,6.03, N,9.39. Found: C,36.45, H,6.01, N,9.04.

The diastereoisomers of 4-MeGlu-cys were resolved as follows. After the BioRad AG 50-X8 resin was eluted with 1N NH_4OH, the ninhydrin-positive fractions were pooled and concentrated *in vacuo* at 37°C. The residue was dissolved in boiling water and applied to a column (1.6 x 48cm) of BioRad AG1 X8 resin. The column was then washed with water and eluted with a linear gradient of acetic acid (0 -> 0.6N; 500 ml water + 500 ml of 0.6N acetic acid). Those fractions containing the resolved diastereoisomers (designated form A and form B) were pooled, concentrated to dryness, and recrystallized from boiling water.

TABLE 1: Relative Mobilities of 4-MeGlu-thiol Adducts.

	Thin-Layer Chromatography R_f[a]			Thin-Layer Electrophoresis R_m[a,b]			Amino Acid Analysis Retention Time[a] (min)
	1	2	3	1	2	3	
4-MeGlu	0.46	0.30	0.47	0.59	-0.42	-0.81	82
Cysteamine	0.58	0.73	0.57	ND[c]	ND	ND	ND
Cystamine	0.42	0.73	0.39	ND	ND	ND	ND
4-MeGlu-cysteamine	0.17	0.18	0.46	1.14	0.22	0.17	172
Cysteine	0.41	0.26	0.54	0.56	0.09	0.10	ND
Cystine	0.08	0.22	0.24	0.56	ND	0.07	112
4-MeGlu-cysteine	0.10	0.06	0.19	0.65	-0.36	-0.55	85
Glutathione(red)	0.41	0.10	0.58	0.40	-0.36	-0.65	40
Glutathione(ox)	0.10	0.03	0.38	ND	ND	-0.72	42
4-MeGlu-glutathione	0.09	0.04	0.32	0.51	-0.56	-0.88	50

a) See "EXPERIMENTAL" section for solvent composition.
b) Mobilities are expressed relative to methyl green indicator dye.
c) ND = not determined.

The yield of form A was 0.39g (26%): mp=201-203°C (decomp.); NMR (D_2O-NaOD) 1.90(2H,m), 2.70(3H,s), 2.90(2H,d), 3.45(2H,m). IR (KBr pellet, cm^{-1}) 3520, 3400, 3110, 1695, 1600, 1500, 1425, 1360, 1300, 1230, 870.

The yield of form B was 0.32g (22%): mp=226-227°C (decomp.); NMR (D_2O-NaOD) 1.95(2H,m), 2.70(3H,s), 2.90(2H,d), 3.55(2H,m). IR (KBr pellet, cm^{-1}) 3450, 3120, 1695, 1600, 1420, 1300, 1240, 870.

The yield of S-(4-amino-2,4-dicarboxybutyl)glutathione was 1.70g (75%): mp=129-135°C (decomp.); NMR (D_2O-NaOD) 2.25(4H,m), 2.50(2H,d), 3.90(3H,s), 3.95(2H,s), 4.55(1H,t). IR (KBr pellet, cm^{-1}) 3420, 3100, 1730, 1650, 1540, 1410, 1230.
Anal. Calcd for $C_{14}H_{26}N_4O_{10}S$: C,41.18, H,5.58, N,12.01, S,6.88. Anal. Calcd for $C_{19}H_{34}N_4O_{12}S$: C,42.07, H,6.27, N,10.33, S,5.90 (including one molecule each of water and acetone). Found: C,42.18, H,6.14, N,10.96, S,6.25.

Thin-layer chromatography of reaction mixtures and purified products was carried out on microcrystalline cellulose plates (Analtech) in the following solvents: (1) isopropanol-formic acid-water (40:2:10), (2) ethanol-ammonium hydroxide-water (80:5:15), (3) phenol-water (75:25). Thin-layer electrophoresis was performed on the same support using a flat bed apparatus in the following systems: (1) buffer pH 1.9 (acetic acid-formic acid-water) (4:1:45), 2 hr at 20 V/cm and 7 mA; (2) buffer pH 4.5 (pyridine-acetic acid-water) (10:15:200), 1.5 hr at 20 V/cm and 40 mA; (3) buffer pH 6.5 (pyridine-acetic acid-water) (25:1:235), 1.5 hr at 30 V/cm and 15 mA.

Peanuts (*Arachis hypogaea* L., Valencia cv Tennessee Red, G.W. Park Seed Co., Greenwood, S.C.) were germinated on damp sand for varying time periods. Amino acids were extracted with 70% boiling ethanol and acidic components purified by anion-exchange chromatography. Samples were hydrolyzed with 6N HCl at 110°C for 24 hr in evacuated sealed tubes.

Kinetic studies: Rates of thiol addition to 4-MeGlu were determined by removing aliquots of the reaction at timed intervals and adding to a solution of 4,4'-dithiodipyridine (12). Reactions were run in stoppered 20-ml serum bottles flushed with argon. Absorption measurements at 324 nm were made on a Cary 219 spectrophotometer.

RESULTS AND DISCUSSION

The three thiol compounds tested readily formed Michael addition products with 4-MeGlu; chromatographic and electrophoretic properties of the purified adducts are shown in Table 1. The product of each synthesis was shown to be

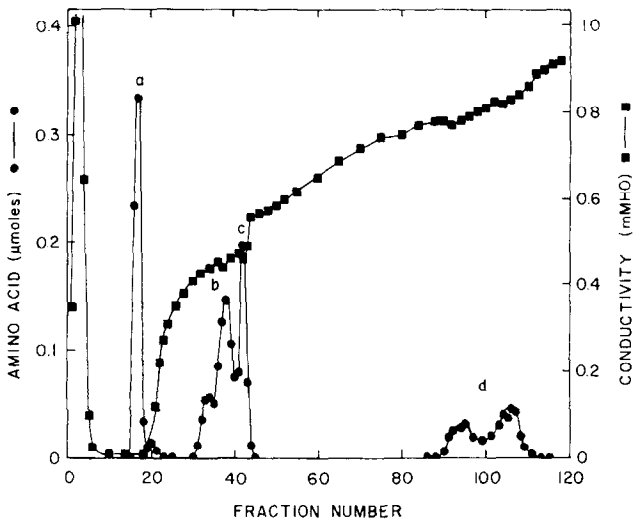

Fig.1. Column chromatography of 4-MeGlu-thioethers on BioRad AG1 X8 resin (acetate phase). The sample contained 20 mg each of DL-4-MeGlu(c), DL-4-MeGlu-cysteamine(a), DL-4-MeGlu-cys(b), and DL-4-MeGlu-GSH(d). The pH of the solution was adjusted to 11.0 with 1N NaOH just before it was applied to the column (1.0 x 18cm). The thioethers were eluted with a 0 -> 1.0N linear gradient of acetic acid (500 ml, total volume); fractions of 3.5 ml were collected.

distinct from the starting materials and their respective disulfide forms. In the case of 4-MeGlu-GSH, a small contamination by unreacted glutathione was found. Crystallization of 4-MeGlu-GSH from aqueous acetone also could result in retention of tightly bound acetone molecules; such has been observed in the case of oxidized glutathione(5). If true, this could explain the results of the elemental analysis. When hydrolysates of 4-MeGlu-GSH were subjected to automated amino acid analysis, glycine, glutamic acid, and 4-MeGlu-cys (the sum of both diastereoisomers) were found in approximately equal quantities. Column chromatographic elution patterns of the thioethers under consideration are shown in Figure 1.

Proton NMR assignments are based on comparison with known resonance frequencies for 4-MeGlu and the individual thiols. The chemical shifts observed with the three thioethers exhibit the differences expected due to the reaction of 4-MeGlu with each of the thiols.

Since addition to the double bond results in formation of a new chiral center, the adducts should be a mixture of diastereoisomers. When DL-4-MeGlu

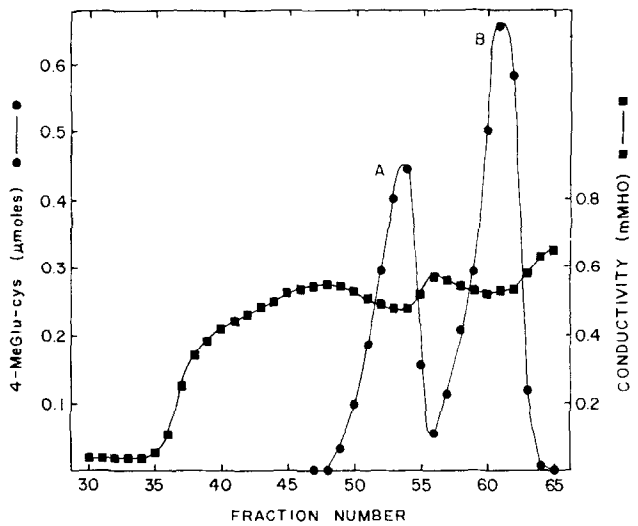

Fig.2. Elution pattern of the diastereoisomers of DL-4-MeGlu-cys from a column (1.6 x 48cm) of BioRad AG1 X8 resin (acetate phase). The sample contained 250 mg (0.84 mmol) of the combined diastereoisomers. Fractions (7.0 ml) were collected; the diastereoisomers were located and quantitated by reacting a 20 μl aliquot with ninhydrin. Recovery, based on the total DL-4-MeGlu-cys applied to the column, was > 95% (40% form A, 60% form B).

and L-cysteine were reacted, two diastereomeric forms of 4-MeGlu-cys were separated by column chromatography (Figure 2). The L-4-MeGlu-L-cys adduct was also synthesized and resolved into two diastereoisomers, confirming that a new chiral center had been formed.

When 4-MeGlu and thiol were present in equimolar concentrations, second order kinetics were observed. The disappearance of free thiol, as a function of reaction time, is shown by reciprocal second order rate plots in Figure 3. Reactions were carried out under argon to minimize aerobic oxidation of the thiols. Cysteine and cysteamine react more rapidly than glutathione, reflecting the catalytic function of the nearby β-amino group which is absent in glutathione (13).

No detectable amounts of 4-MeGlu or 4-MeGln-thiol adducts were found in ethanolic extracts of peanut plants (at ages of 4 to 14 days and mature plants). Although synthetic sulfhydryl adducts with 4-MeGln have not themselves been characterized, acid hydrolysis of adducts formed with either cysteine or glutathione and 4-MeGln will yield 4-MeGlu-cys. However, no

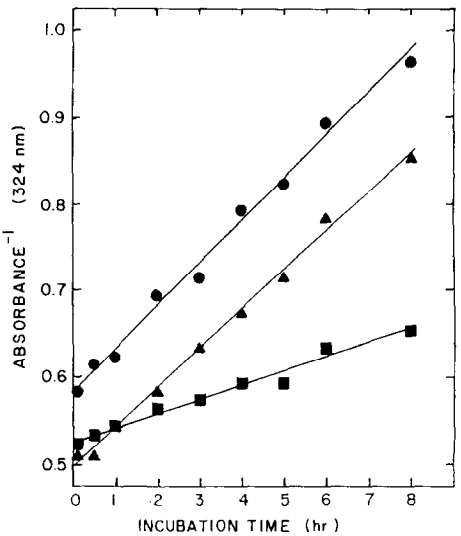

Fig.3. Kinetics of the reaction of DL-4-MeGlu (1.0 mmol) with thiols (1.0 mmol) at 37°C in 10 ml of deionized water (pH adjusted to 8.5 with 3N NaOH). The reaction flasks were maintained under an atmosphere of argon. In each case, 50 μl of the reaction mixture were diluted to 1.0 ml with water and a 20 μl aliquot then added to 0.98 ml of 2.0mM 4,4'-dithiodipyridine in 50mM sodium phosphate buffer (pH 7.4). The decrease in absorbance at 324 nm measured the quantity of free thiol remaining in the reaction mixture. (▲) cysteamine; (●) cysteine; (■) glutathione.

4-MeGlu-cys was seen in acid hydrolyzed extracts even though normal quantities of 4-MeGln were present. This finding might be due to the acidity of peanut extracts (*i.e.* pH 6) as the sulfhydryl group of cysteine is a poor nucleophile under acidic conditions. Alternatively, free thiols and 4-MeGlu/4-MeGln may not exist in the same subcellular compartment of intact peanut plants. In other plants, localized conditions of higher pH or different compartmentation might allow for such conjugations to occur.

In addition to reacting with free thiol compounds, 4-MeGlu or 4-MeGln might also act as an inhibitor of enzymes which contain active-site sulfhydryl groups; this possibility remains to be explored. The finding by Fitzpatrick *et al.* (14) that β-methylene-DL-aspartic acid inhibits rat brain glutamate-aspartate aminotransferase activity might be due to a Michael addition between this aspartate analog and an active-site thiol or amino group. If this proves correct, similar reactions between 4-MeGlu and a variety of enzymes would seem reasonable. Such interactions might provide a means for combating fungal attack during the early stages of germination when peanuts are most

susceptible to infection. Exploration and establishment of the various reactions 4-MeGlu or 4-MeGln undergo with other biological components of plants may ultimately lead to a better understanding of the role of these non-protein amino acids in the plant kingdom.

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