Chemical Modification of Peptides Containing γ-Carboxyglutamic Acid

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Received October 30, 1981

At acidic pH the γ-proton of the γ-carboxyglutamic acid (Gla) side chain undergoes rapid exchange. We have utilized the reactivity of the resulting enol form to develop a method for the chemical modification of peptide-bound Gla residues. Reaction of Gla peptides with a morpholine–formaldehyde mixture at pH 4.5 yields the Mannich base adduct. Fragmentation of the Mannich base occurs rapidly in 50% aqueous DMF to yield carbon dioxide, morpholine, and the corresponding γ-methyleneglutamyl residue.

In connection with our studies on γ-carboxyglutamic acid containing peptides and proteins[4] we have developed a potentially useful method of modifying the malonyl side chains of Gla residues. The earlier NMR observations of Stenflo et al.[5] and Marki et al.[6] indicated that the γ-proton of the Gla side chain exchanged readily with deuterons. Consequently, we examined the pH dependence of the exchange using the model peptide L-phenylalanyl-L-leucyl-L-γ-carboxyglutamyl-L-γ-carboxyglutamyl-L-leucine methyl ester. At 100 MHz the γ-proton resonance of the Gla residues of the pentapeptide falls under an envelope centered at about 3.7 ppm. At a given pH, the first-order loss of γ-protons can be determined from semilogarithmic plots as shown in Figure 1. The observed rates of γ-proton exchange for deuterium at several pH values are given in Table I. An approximate 10-fold increase in exchange rate is observed over a similar increase in hydronium ion concentration. This result suggests a linearly dependent γ-proton exchange, as expected for acid catalysis; however, since enolization at the γ-carbon depends on the ionization state of the γ-carboxyl groups, a linear dependence is not expected over a larger pH range. Indeed, the data in Table I indicate that γ-proton exchange is very slow under mildly basic conditions (pH 8.54, τ = 23 h) whereas pH values moving toward neutrality significantly increase the rate of exchange.

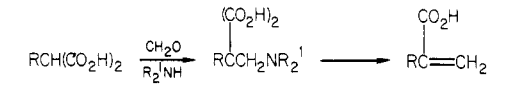
Price et al.[7] have recently made similar observations. They examined the stability of bone Gla protein containing γ-tritio Gla residues in the pH range 1.5–8.8. The exchange rates were essentially identical at pH 1.5 and 3.1, the region at which the first ionization occurs (pK = 2.0). A 10-fold decline in exchange rate as the pH was increased from 3.1 to 5.4 suggested that the second ionization (pK = 4.4) is accompanied by a decrease in γ-proton exchange. The 200-fold slower exchange at pH 7.4 and 8.8 indicated, as we observed, that the fully ionized side chain does not facilitate γ-proton exchange and in fact is sufficiently slow so that γ-tritiated Gla derivatives may be utilized for study in this pH region.

These studies suggested that the Gla side chain with the enolic center at the γ-carbon atom could function as a nucleophile in the pH range 2–5. Thus treatment of a Gla peptide with an appropriate electrophile should lead to modification of the malonyl side chain. Our choice of electrophilic reagents was influenced by the longer range goal of developing a Gla modification procedure which could be used for protein-bound Gla residues. Thus we were interested in electrophilic reagents that could be utilized in aqueous solution at pH 2–5.

Table I. γ-Proton Exchange Rates In H-Phe-Leu-Gla-Gla-Leu-OH

pH	10^3k, min^{-1}	τ, h
7.24	6.5	1.8
7.36	3.0	3.8
7.42	2.3	5.0
7.49	1.7	6.0
7.79	1.5	7.9
8.54	0.5	23

The early work of Mannich and Ganz[8] appeared to meet these requirements. Mannich and Ganz demonstrated that the electrophilic intermediate produced from formaldehyde and dialkylamines reacted with monosubstituted malonic acid derivatives **2** in aqueous solution to yield the corresponding Mannich base (**3**) in 70–90% yield (eq 1). When



(1)

2a, R = C_2H_5; **b**, R = $C_6H_5CH_2$

3a, R = C_2H_5; R^1 = CH_3; **b**, R = $C_6H_5CH_2$; R^1 = CH_3

4a, R = C_2H_5; **b**, R = $C_6H_5H_2$

an aqueous solution of **3** was heated, they reported that carbon dioxide and the dialkylamine were lost and that the α-substituted acrylic acid **4** was obtained in 65–85% yield. We repeated the experiments of Mannich and Ganz using ethyl- and benzylmalonic acids and demonstrated that these observations were correct; **3a** and **3b** were obtained 42% and 85% yields, respectively. Decarboxylation of either **3a** or **3b** by using the conditions described[8] yielded crude products whose NMR spectra were consistent with **4a,b**.

Studies on Gla derivatives were conducted by using *N*-(benzyloxycarbonyl)-D-γ-carboxyglutamic acid α-methyl ester (**5**) and *N*-(benzyloxycarbonyl)-L-γ-carboxyglutamyl-L-alanyl-L-leucine benzyl ester (**6**). Treatment of **5**, obtained from the corresponding γ,γ-di-*tert*-butyl ester (**7**, eq 2), in trideuterioacetonitrile–water with aqueous dimethylamine and formaldehyde at room temperature resulted in the evolution of CO_2. The ^{13}C NMR spectrum of the product, **8**, exhibited resonances at 125.6 and 141.7 ppm assigned as C=CH_2 on the basis of off-resonance decoupling studies; the elemental analysis and

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(4) H. C. Marsh, M. M. Sarasua, D. A. Madar, R. G. Hiskey, and K. A. Koehler, *J. Biol. Chem.*, **256**, 7863 (1981).
(5) J. Stenflo, P. Fernlund, W. Egan, and P. Rosepstoff, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 2730 (1974).
(6) W. Marki, M. Oppliger, and R. Schwyzer, *Helv. Chim. Acta*, **60**, 807 (1977); W. Marki, M. Oppliger, P. Thanei, and R. Schwyzer, *ibid.*, **60**, 798 (1977).
(7) P. A. Price, M. K. Williamson, and D. J. Epstein, *J. Biol. Chem.*, **256**, 1172 (1981).
(8) C. Mannich and E. Ganz, *Ber.*, **55**, 3486 (1922).

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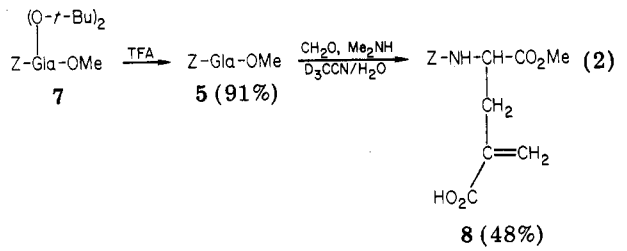
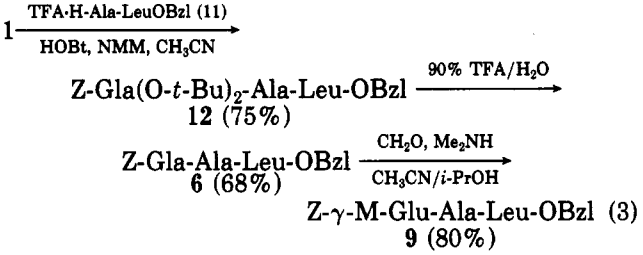
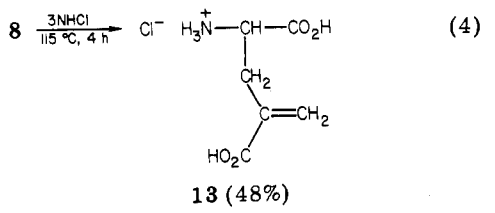


8 (48%)

^{1}H NMR spectrum [δ 5.71 (d, 1 H, C=CH_2), 6.21 (d, 1 H, C=CH_2)] were also consistent with the assigned structure of *N*-(benzyloxycarbonyl)-D-γ-methyleneglutamic acid α-methyl ester (8, Z-D-MGlu-OMe). Similar treatment of 6 with formaldehyde/dimethylamine provided *N*-(benzyloxycarbonyl)-L-γ-methyleneglutamyl-L-alanyl-L-leucine benzyl ester (9) in approximately 80% yield (eq 3).



In order to obtain a sample of D-γ-methyleneglutamic acid hydrochloride (γ-MGlu) 13 for comparison with the literature[9] and for use as a standard for the amino acid analyzer, 8 was converted to 13 with aqueous HCl in 48% yield (eq 4). The melting point of 13 was essentially the



13 (48%)

same as that previously reported[9] although the specific rotation of 13 obtained from 8 was somewhat lower. Elemental analysis was consistent with the structure 13. The purified sample of 13 eluted from the long column of the amino acid analyzer at 100 min as a single peak between Pro (97 min) and Gly (116 min); Glu elutes at 96 min. When the reaction mixture from the conversion of 8 → 13 was analyzed on the amino acid analyzer, a ninhydrin-positive peak which eluted at the same position as aspartic acid was noted; the area of the "Asp" peak increased as a function of hydrolysis time (Table II) at the expense of the MGlu peak. The identity of this byproduct is unknown at this point although we tentatively assume that the "Asp" peak is γ-(hydroxymethyl)glutamic acid resulting from ring opening of the corresponding δ-lactone. Smith et al.[9e] report that substitution of a hydroxyl group on the β- or γ-position of either glutamic acid or γ-methylglutamic acid results in markedly decreased chromatographic retention times. In their system, for example, (2*S*, 4*R*)-γ-hydroxy-γ-methylglutamic acid emerged just before aspartic acid. The proton NMR spectrum of the crude reaction mixture of the acid hydrolysis of 8, however, gave no indication of the γ-methyl signal expected if γ-hydroxy-γ-methylglut-

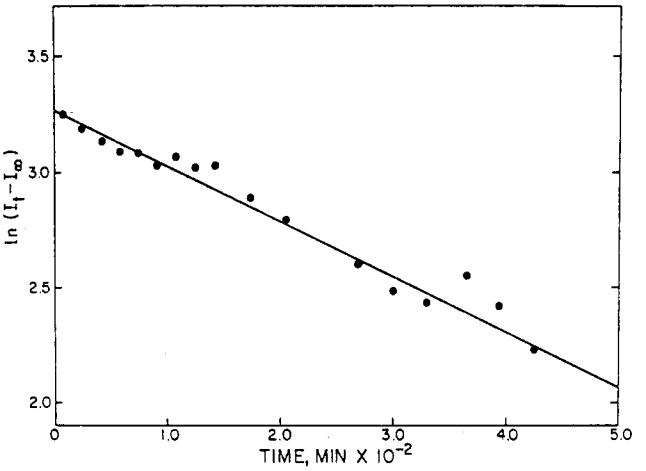


Figure 1. Semilogarithmic plot showing the loss of integrated signal intensity of the γ-hydrogen resonance of the Gla residues of L-phenylalanyl-L-leucyl-L-γ-carboxyglutamyl-L-γ-carboxyglutamyl-L-leucine methyl ester as a function of time.

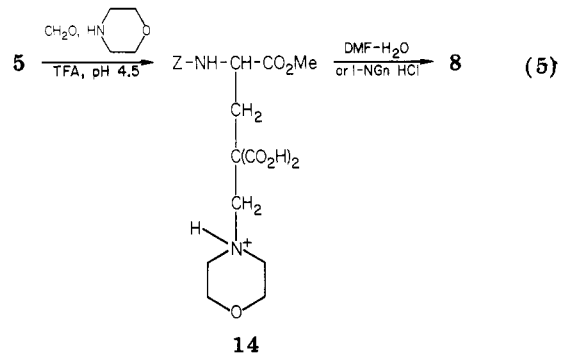
Table II. Amino Acid Analysis of *N*-(Benzyloxycarbonyl)-γ-methyleneglutamic Acid α-Methyl Ester (8) after Acid[a] Hydrolysis

time, h	amt, μmol		% "Asp"
	MGlu	"Asp"	
0	0.0808	0.0015	1.8
4	0.073	0.0066	8.3
24	0.0585	0.0143	19.6
48	0.0587	0.0158	21.2

[a] Hydrolysis experiments were conducted in evacuated, sealed tubes by using 4 N HCl at 115 °C.

amic acid were present. Marcus et al.[9c] suggested that δ-lactone formation of γ-methyleneglutamic acid might explain the facile reaction of this amino acid with hydroxylamine in the hydroxamic acid–ferric chloride color assay. These considerations would appear to support the suggestion that during the acid hydrolysis of 8 some γ-(hydroxymethyl)glutamic acid is also produced via a lactonization–lactone opening sequence.

The use of morpholine as the amine component in the Mannich reaction of Gla peptides was evaluated. An aqueous solution of 5 was treated with morpholine and the pH adjusted to ~4.5 with TFA; addition of a 37% formaldehyde solution provided an immediate precipitate, presumably the Mannich base 14 (eq 5). When the solid



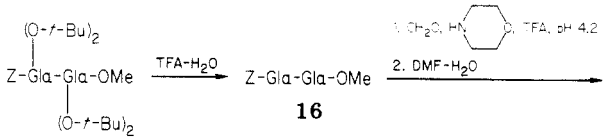
14

14 was isolated and redissolved in 50% (v/v) DMF–H_2O solution, immediate evolution of CO_2 was observed. The workup of the reaction mixture provided 8 in 83% yield from 14. Similar results were obtained when isolated 14 was dissolved in 1 N guanidine hydrochloride solution; 8 was obtained in 62% yield from 14. When 5 was treated with formaldehyde–morpholine (pH 4.5) and 14 was not

(9) (a) P. C. Wailes and M. C. Whiting, *J. Chem. Soc.*, 3636 (1955); (b) S. Nakayama and T. Kaneko, *Nippon Kagaku Kaishi*, 78, 1216 (1956); (c) A. Marcus, J. Feeley, and L. M. Shannon, *Arch. Biochem. Biophys.*, 100, 80 (1963); (d) G. K. Powell and E. E. Dekker, *Prep. Biochem.*, 11, 339 (1981); (e) A. Smith, P. J. Peterson, and L. Fowden, *J. Chromatogr.*, 62, 144 (1971).

isolated but redissolved in 50% DMF–H_2O, a 75% overall yield of 8 was obtained. Treatment of 14 with either 2,2,2-trifluoroethanol or with aqueous dimethylamine solution (pH ~10) provided no significant amounts of 8. These experiments suggest that the decarboxylative elimination reaction is accelerated by basic polar aprotic solvents perhaps by a pathway similar to that proposed by Corey and Fraenkel[10] in their studies on the pyridine-assisted decarboxylation of various 2-carboxycinnamic acids.

Since many of the Gla residues in the proteins of blood occur in juxtaposition, the Mannich reaction using formaldehyde–morpholine was applied to *N*-(benzyloxycarbonyl)-D-γ-carboxyglutamyl-D-γ-carboxyglutamic acid methyl ester (16) obtained from the corresponding fully protected dipeptide derivative 15 (eq 6). The objective



15 16

Z-γ-M-Glu-γ-M-Glu-OMe (6)

17 (72%)

of this experiment was to establish that the formation of the Mannich base and the subsequent fragmentation reaction would occur on adjacent Gla residues. Incubation of 16 with the formaldehyde–morpholine mixture at pH 4.2 followed by lyophilization and addition of DMF–H_2O provided a 72% yield of *N*-(benzyloxycarbonyl)-D-γ-methyleneglutamyl-D-γ-methyleneglutamic acid methyl ester (17). The structure of the product was established by elemental analysis, the nature of the 250-MHz NMR spectrum, and the appearance of only MGlu and "Asp" when the acid hydrolysate was subjected to amino acid analysis.

These results indicate that the formation of the Mannich base adduct between the malonyl side chains of peptide-bound Gla residues is rapid and efficient. Fragmentation of the Mannich base adduct to yield γ-methyleneglutamyl residues, carbon dioxide, and morpholine is likewise rapid and nearly quantitative. We will report our work on the application of this method to Gla-containing proteins in a subsequent publication.

Experimental Section[11]

Deuterium-Exchange Experiments. L-Phenylalanyl-L-leucyl-L-γ-carboxyglutamyl-L-γ-carboxyglutamyl-L-leucine methyl ester[12] (28 mg, 0.03 mmol) was dissolved in a solution containing 7 mg (0.05 mmol) of disodium phosphate in 0.6 mL of water. The pH was adjusted to the desired value by addition of microliter quantities of HCl or NaOH. The solution was then lyophilized and reconstituted with 0.6 mL of D_2O at zero time. The first-order loss of the integrated of the γ-proton resonance was followed as a function of time.

NMR spectra were obtained on a Varian XL-100 instrument. Samples were run in 5-mm tubes at ambient temperature and internally locked on D_2O. Typically a sweep width of 1000 Hz was pulsed 100 times at 2-s intervals.

Exchange rates were detemined in the following manner. After D_2O addition, the entire spectrum was recorded and integrated. Then, at specified intervals, without further adjustment of the instrument, spectra were recorded, and the γ-proton integration was taken over a time period of 1–2τ. To determine a $t = \infty$ value, we recorded the entire spectrum and integration at a time greater than 10τ. This value was normalized with respect to the original set of integrations by using the high-field peak of $\delta = 1.3$ ppm. The estimation of a $t = \infty$ value represented the largest source of error in establishing the exchange rates.

***N*-(Benzyloxycarbonyl)-γ,γ-di-*tert*-butyl-D-γ-carboxyglutamic Acid (1).** The optically active enantiomer was obtained from the L-quinine salt of *N*-(benzyloxycarbonyl)-γ,γ-di-*tert*-butyl-D-γ-carboxyglutamic acid as previously described.[13] The quinine salt was partitioned between 20% citric acid/ethyl acetate, and the organic layer was evaporated in vacuo to leave 1, which was further purified by recrystallization from ethyl acetate/pentane to give optically pure 1: mp 89–90 °C; $[\alpha]^{23}_D$ +11.2° (*c* 1.0, MeOH).

2-Carboxy-2-ethyl-3-(dimethylamino)propionic Acid (3a). By use of the procedures of Mannich and Ganz,[8] 1.32 g (0.01 mol) of ethyl malonic acid was added to a solution containing 1.13 mL (0.01 mol) of 40% aqueous dimethylamine and 3.0 mL of water. The solution was treated with 0.81 mL (0.01 mol) of 37% aqueous formaldehyde and stirred at 0 °C. Recrystallization of the precipitated white solid from ethanol provided 3a: 0.8 g (42%); mp 101–103 °C dec (lit.[8] mp 101 °C).

Anal. Calcd for $C_8H_{15}O_4N$: C, 50.79; H, 7.99; N, 7.40. Found: C, 50.73; H, 8.01; N, 7.40.

2-Benzyl-2-carboxy-3-(dimethylamino)propionic Acid (3b). The procedure of Mannich and Ganz[8] provided an 85% yield of 3b, mp 82–82 °C (lit.[8] mp 88 °C).

Anal. Calcd for $C_{15}H_{17}O_4N$: C, 62.14; H, 6.82; N, 5.57. Found: C, 62.22; H, 6.86; N, 5.49.

***N*-(Benzyloxycarbonyl)-γ,γ-di-*tert*-butyl-D-γ-carboxyglutamic Acid α-Methyl Ester (7).** To a solution of 8.74 g (20 mmol) of 1 in 30 mL of methanol was added 3.26 g (10 mmol) of cesium carbonate. After 1 h, the solvent was removed in vacuo, the resulting oil was dissolved in ethyl acetate, and the solvent was again removed in vacuo to yield a white solid. The solid was dissolved in 30 mL of DMF, and 2.90 g (21 mmol) of methyl iodide was added. After 2 h, the solution was diluted with 100 mL of water and extracted three times with ether. The combined organic layers were extracted as follows: twice with water, twice with saturated sodium bicarbonate, twice with water, twice with 20% citric acid, twice with water, twice with saturated sodium bicarbonate, once with water, once with saturated sodium chloride. After the mixture was dried over anhydrous magnesium sulfate, the solvent was removed in vacuo, and the resulting solid was recrystallized from ether/pentane to give 7: 8.20 g (91%); mp 76–77 °C; $[\alpha]^{23}_D$ +16.0° (*c* 1.00 MeOH).

Anal. Calcd for $C_{23}H_{33}NO_8$: C, 61.18; H, 7.37; N, 3.10. Found: C, 61.21; H, 7.37; N, 3.11.

***N*-(Benzyloxycarbonyl)-D-γ-carboxyglutamic Acid α-Methyl Ester (5).** A solution of 4.51 g (10 mmol) of 2 in 10 mL of 90% trifluoroacetic acid/water was stirred for 2 h at room temperature. The reaction was diluted with toluene, and the aqueous trifluoroacetic acid was removed by repeated azeotropic distillation. The resulting oil was dissolved in water, and the water was lyophillized to yield a solid. The solid was recrystallized from ethyl acetate/diisopropyl ether at −20 °C to yield 5: 2.72 g (80%); mp 119–120.5 °C; $[\alpha]^{23}_D$ +19.4° (*c* 1.0, MeOH).

Anal. Calcd for $C_{15}H_{17}NO_8$: C, 53.09; H, 5.05; N, 4.13. Found: C, 53.01; H, 5.09; N, 4.12.

***N*-(Benzyloxycarbonyl)-D-γ-methyleneglutamic Acid α-Methyl Ester (8). (A) Via Dimethylamine–Formaldehyde.** To a solution of 100 mg (295 μmol) of 5 in 0.37 mL of trideuteroiacetonitrile and 0.10 mL of water in an NMR tube were added sequentially 33 μL of a 40% aqueous dimethylamine and 24 μL of 37% aqueous formaldehyde. Both ^1H and ^{13}C NMR scans were obtained of the mixtures at three stages: solution in acetonitrile/water (D_2O in a separate sample); after addition of dimethylamine; after the reaction with formaldehyde was com-

(10) E. J. Corey and G. Fraenkel, *J. Am. Chem. Soc.*, **75**, 1168 (1953).

(11) Melting points are uncorrected. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA. NMR data were obtained on a Brucker WM-250 spectrometer unless otherwise indicated. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. Unless otherwise stated, products were dried in vacuo over P_2O_5 and sodium hydroxide pellets.

(12) The synthetic details for preparation of the pentapeptide are described by M. M. Sarasua, M. E. Scott, J. A. Helpern, P. B. W. ten Kortenaar, N. T. Boggs, III, L. G. Pedersen, K. A. Koehler, and R. G. Hiskey, *J. Am. Chem. Soc.*, **102**, 3404 (1980).

(13) N. T. Boggs, III, B. Goldsmith, R. E. Gawley, K. A. Koehler, and R. G. Hiskey, *J. Org. Chem.*, **44**, 2262 (1979).

pleted. After the addition of formaldehyde, the solution was allowed to stand for 6 h at room temperature then overnight at −20 °C. Evolution of carbon dioxide became obvious 1–2 h after addition of formaldehyde. The ^1H NMR spectra exhibited resonances at δ 5.71 (d, 1 H, C=CH_2) and 6.21 (d, 1 H, C=CH_2).

After the last spectrum was obtained, the solvent was removed in vacuo, and the resulting oil (108 mg) was dissolved in ethyl acetate. The organic solution was extracted twice with 20% citric acid and twice with saturated brine, and dried over magnesium sulfate. Evaporation of the solvent in vacuo gave 53.2 mg (64% crude) of a solid, mp 82–86 °C. Recrystallization from ether/pentane gave 43.8 mg (48%) of white powder, mp 84–85.5 °C. Repetition of the reaction on a 0.884-mmol scale provided 203 mg (74%) of **8**, mp 83–86.5 °C.

Anal. Calcd for $C_{15}H_{17}NO_6$: C, 58.62, H, 5.58, N. 4.56. Found: C, 58.48; H, 5.64; N, 4.51.

(B) Via Morpholine–Formaldehyde. A solution of 200 mg (590 μmol) of **5**, 7 mL of water, and 78 μL (750 μmol) of morpholine was adjusted to pH ~4.5 with trifluoroacetic acid. Two minutes after the addition of 60 μL (770 μmol) of 37% aqueous formaldehyde a white solid precipitated. After 20 min the solvent was removed in vacuo at room temperature, and 10 mL of 50% (*v*/*v*) DMF/water at room temperature was added. Immediate evolution of CO_2 was observed. After 1 h the solvents were removed in vacuo at room temperature, and the resulting solid was partitioned between 20% citric acid/ethyl acetate. The organic layer was extracted twice with 20% citric acid, once with water, and once with saturated sodium chloride and dried over magnesium sulfate. Evaporation of the solvent gave a solid that was recrystallized from ether/pentane to yield **8**: 136 mg (75%); mp 83–85 °C; $[\alpha]^{25}_D$ +23.8° (*c* 1.00 MeOH).

D-γ-Methyleneglutamic Acid Hydrochloride Salt (13). A 140-mg (455 μmol) sample of **8** was treated with 2 mL of 3 N HCl for 4 h at 115 °C in an evacuated, sealed tube. The solvent was evaporated in vacuo, and the resulting solid was recrystallized several times from ethanol/ether to yield **5**: 43.1 mg (48%); mp 196–199 °C; $[\alpha]^{23}_D$ −7.9° (*c* 1.00 3 N NCl) [lit.[9a] mp 196–198 °C; $[\alpha]^{20}_D$ −14.0° (11% HCl)].

Anal. Calcd for $C_6H_{10}NO_4Cl$: C, 36.84; H, 5.15; N, 7.16. Found: C, 36.87; H, 5.17; N, 7.17.

Mannich Base Adduct (14). A solution of 200 mg (590 μmol) of **5** in 7 mL of water and 78 μL (750 μmol) of morpholine was adjusted to pH 4.5. One hour after the addition of 60 μL (770 μmol) of 37% aqueous formaldehyde the solvent was removed at room temperature in vacuo. The solid was dissolved in water, and the water was lyophilized to give 214 mg (83%) of a solid. This unstable solid was immediately subjected to either procedure A or B.

Procedure A. To 132.4 mg (302 μmol) of **14** was added 3 mL of 1 N guanidine hydrochloride salt solution. After 2 h the solution was acidified with citric acid and extracted with ether. The organic layer was evaporated in vacuo, and the resulting solid was recrystallized from anhydrous ether/pentane to yield **8**: 57.5 mg (62%, 51% overall); mp 85–86.5 °C.

Procedure B. To 181.6 mg (414 μmol) of **14** was added 3 mL of 50% DMF/water at room temperature. After 2 h the solvents were evaporated in vacuo at room temperature, and the resulting oil was partitioned between 20% citric acid/ether. The organic layer was evaporated in vacuo, and the resulting solid was recrystallized from ether/pentane to yield **8**: 114.50 mg (89%, 74% overall); mp 85–86.5 °C.

***N*-(*tert*-Butoxycarbonyl)-L-alanyl-L-leucine Benzyl Ester (10).** To a solution of 4.84 g (12.30 mmol) of the tosylate salt of leucine benzyl ester in 60 mL of acetonitrile at room temperature was added 1.38 mL (1.25 g) of *N*-methylmorpholine. After the mixture was stirred for 10 min, 3.35 g (12.30 mmol) of *N*-(*tert*-butoxycarbonyl)-L-alanine *N*-hydroxysuccinimide ester was added in one portion, and the reaction mixture was stirred for 48 h. Removal of the acetonitrile in vacuo yielded an oil which was taken up in 10 mL of ethyl acetate. After the ethyl acetate solution was warmed to 40 °C, 75 mL of pentane was added, and the organic layer was decanted from the resulting oil. The oil was similarly treated twice more, and the combined organic layers were concentrated in vacuo. The resulting oil was dissolved in 10 mL of methanol, and 30 mL of water was added. After removal of the supernatant liquid by decantation, the oil was dissolved in ethyl acetate and dried over magnesium sulfate. Removal of the solvent in vacuo gave 4.17 g (86%) of an oil, $[\alpha]^{26}_D$ −53.8° (*c* 1, MeOH).

L-Alanyl-L-leucine Benzyl Ester Trifluoroacetate Salt (11). To 4.00 g (10.20 mmol) of **10** in a dry nitrogen atmosphere at room temperature was added 5 mL of trifluoroacetic acid. After the mixture was stirred for 105 min, 50 mL of anhydrous ether, 50 mL of cyclohexane, and 60 mL of pentane were added. The solution was cooled overnight at 0 °C to yield a solid. After filtration the solid was dissolved in 50 mL of cyclohexane and 60 mL of pentane, warmed on a stream bath, and cooled again to 0 °C. Filtration gave a white solid: 3.5 g (87%); mp 132–133 °C; $[\alpha]^{23}_D$ −26.4° (*c* 1.0, MeOH). The analytical sample was recrystallized from an acetonitrile–ether mixture.

Anal. Calcd for $C_{18}H_{25}N_2O_5F_3$: C, 53.19; H, 6.20; N, 6.89. Found: C, 53.23; H, 6.20; N, 6.91.

***N*-(Benzyloxycarbonyl)-γ,γ-di-*tert*-butyl-L-γ-carboxyglutamyl-L-alanyl-L-leucine Benzyl Ester (12).** A solution of 4.70 g (11.60 mmol) of the trifluoroacetate salt **11**, 5.06 g (11.60 mmol) of the L enantiomer of **1**, and 1.56 (11.60 mmol) of *N*-hydroxybenztriazole in 100 mL of acetonitrile was cooled to 0 °C. Five minutes after the addition of 1.29 mL (11.60 mmol) of *N*-methylmorpholine, 2.62 g (12.70 mmol) of *N*,*N*-dicyclohexylcarbodiimide was added in one portion, and the solution was stirred at 0 °C for 75 h. After removal of the solvent, ethyl acetate was added, and the mixture was filtered. The organic layer was washed with 20% citric acid, water, 5% sodium bicarbonate, water, and saturated brine and dried over magnesium sulfate. Removal of solvent gave 6.62 g of crude product which was recrystallized from an ethyl acetate–pentane mixture to give **12**: 6.13 g (75%); mp 116.5–118 °C; $[\alpha]^{25}_D$ −23.7° (*c* 1.0 MeOH).

Anal. Calcd for $C_{38}H_{53}N_{10}O_3$: C, 64.11; H, 7.50; N, 5.90. Found: C, 64.10; H, 7.52; N, 5.91.

***N*-(Benzyloxycarbonyl)-L-γ-carboxyglutamyl-L-alanyl-L-leucine Benzyl Ester (6).** To 10 mL of 90% trifluoroacetic acid/water at 0 °C was added 1.00 g (1.41 mmol) of **12**. After 2 h toluene was added, and the aqueous trifluoroacetic acid was removed by azeotropic distillation in vacuo to yield a solid. The crude product was dissolved in water, and the water was lyophillized to yield 0.83 g (98%) of crude peptide. The solid was recrystallized from ethyl acetate–pentane mixture to yield **6**: 0.58 (69%); mp 139–140 °C; $[\alpha]^{24}_D$ −48.2° (*c* 1.02, MeOH).

Anal. Calcd for $C_{30}H_{37}N_3O_{10}\cdot H_2O$: C, 58.33; H, 6.37; N, 6.80. Found: C, 58.38; H, 6.36; N, 6.80.

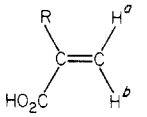
***N*-(Benzyloxycarbonyl)-L-γ-methyleneglutamyl-L-alanyl-L-leucine Benzyl Ester (9).** To a mixture of 284 mg (474 μmol) of **6**, 53 μL of 40% aqueous dimethylamine, and 0.5 mL of water was added enough 2-propanol for complete solution (0.29 mL). Thirty minutes after a solution of 38 μL of 37% aqueous formaldehyde was added evolution of carbon dioxide began. Within an additional 15 min the reaction mixture was solid. After the cake was broken up and 0.15 mL of 2-propanol and 0.35 mL of water were added, the mixture was stirred for an additional 150 min at room temperature. After addition of sufficient 2-propanol to dissolve the solid, the solution was transferred to a larger vessel, and all of the solvent was removed in vacuo leaving a semisolid which was dried in vacuo over phosphorus pentoxide. The resulting 266 mg of crude solid was recrystallized from twice from 2-propanol/water to give **9**: 94.5 mg (35%); mp 160–162.5 °C; $[\alpha]^{25}_D$ −50.2° (*c* 1.0, MeOH).

Anal. Calcd for $C_{30}H_{37}N_3O_8$: C, 63.47; H, 6.57; N, 7.40. Found: C, 63.34; H, 6.65; N, 7.41.

When a 250-mg (417 μmol) sample of **6** in 0.75 mL of acetonitrile and 50 μL of 2-propanol was treated with 47 μL of 40% aqueous dimethylamine and 34 μL of 37% aqueous formaldehyde, no precipitated solid was observed, and no carbon dioxide evolution was noted after 4 h at 25 °C. The solution was treated with 200 μL of water and stirred for 20 h at 25 °C. A workup in the usual manner provided 191 mg of crude product, mp 159–162.5 °C. Recrystallization from 2-propanol/water gave **9**: 189 mg (80%); mp 160–162.5 °C.

***N*-(Benzyloxycarbonyl)-D-γ-methyleneglutamyl-D-γ-methyleneglutamic Acid Methyl Ester (17).** The ester (2.32 g, 3.15 mmol) **15**[12] was converted to **16**[12] by treatment with 25 mL of 90% aqueous TFA for 2 h at room temperature. The acid **16** (1.75 g, 3.09 mmol) was stirred at room temperature in a

solution prepared from 37% aqueous formaldehyde (1.39 mL, 18.5 mmol), morpholine (2.39 mL, 27.4 mmol), water (100 mL), and TFA (1.38 mL, used to adjust the pH to 4.2). After 20 min the solution became turbid. The solvents were removed in vacuo, and the residue was dissolved in 50 mL of 50% aqueous DMF. After 2 h the solvent was removed in vacuo, and the resulting oil was triturated with 20 mL of 5% citric acid solution. The resulting powder was washed with an additional 5 mL of citric acid and with water and dried to yield 17: 1.02 g (72.4% based on 15); mp 215-217.5 °C dec; $[\alpha]^{25}_D$ +21.4° (c 1.0 MeOH); ^{1}H NMR (Me_2SO-d_6) δ 5.62 (s, 1 H, H^a of I), 5.64 (s, 1 H, H^a of I), 6.08 (s,



I

2 H^b of I); ^{13}C NMR (Me_2SO-d_6) δ 136.03 (s, C=CH_2), 136.64 (s, C=CH_2), off-resonance decoupled spectrum. Amino acid analysis of an acid hydrolysate of 17 indicated MGlu and "Asp" as the only ninhydrin-positive components.

Anal. Calcd for $C_{21}H_{24}O_9N_2$: C, 56.25; H, 5.39; N, 6.25. Found: C, 56.03; H, 5.37; N, 6.19.

Acknowledgment. This study was supported in part by grants from the National Institutes of Health (HL-20161 and HL-23881). Purchase of the NMR instrument was made possible by NSF Instrument Grants GU-2059 and GP-37602 and by NIH Grant 5S05RR07072. H.D.B. gratefully acknowledges the support of NIH Grant AM-07386-02; N.T.B., NIH Grant T32-HL-07255.

Registry No. D-1, 60686-52-4; L-1, 60686-50-2; **2a**, 601-75-2; **2b**, 616-75-1; **3a**, 80954-38-7; **3b**, 24643-58-1; **5**, 80954-39-8; **6**, 80954-40-1; **7**, 66513-60-8; **8**, 80954-41-2; **9**, 80954-42-3; **10**, 56618-30-5; **11**, 78746-71-1; **12**, 80954-43-4; **13**, 81024-46-6; **14**, 80954-44-5; **15**, 80963-05-9; **16**, 74201-25-5; **17**, 80954-45-6; tosylate leucine benzyl ester, 1738-77-8; *N*-(*tert*-butoxycarbonyl)-L-alanine *N*-hydroxysuccinimide, 3401-36-3.

Chemistry of Four-Membered Cyclic Nitrones. 1. Synthesis and Thermal Isomerization of 2,3-Dihydroazete 1-Oxides[1]

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Received October 13, 1981

Nitroalkenes (1) react with ynamines (1-aminoacetylenes, 2) to yield nitrocyclobutenes 3 and four-membered cyclic nitrones (2,3-dihydroazete 1-oxides, 5); in one case the open-chain isomer (4) of a four-membered cyclic nitrone was isolated. Nitrones 5 isomerize thermally to yield the corresponding *N*-vinylnitrones 4. Kinetic studies and X-ray analysis indicate that this reaction is a concerted conrotatory ring opening analogous to the ring opening of cyclobutenes. Only in the reaction of 1-nitrocyclopentene (**1d**) with **2b** has the initially formed nitronic ester **6** been isolated. The thermal ring contraction of **6** does not yield the corresponding four-membered cyclic nitrone but the isoxazoline derivative **7**. Compound **6** was further characterized by reaction with DMAD and with methyl propiolate to give the tricyclic products **8a** and **8b**, respectively. The mechanism of the stereospecific formation of the nitrones is discussed in terms of a concerted 1,3 sigmatropic shift.

As part of our work on [2 + 2] cycloadditions,[2,3] we have reported the reactions of electron-rich acetylenes (ynamines) with 3-nitrobenzo[*b*]thiophenes.[4] These reactions gave two products, cyclobutenes and *N*-(heteroaryl)-*C*-carbamoyl nitrones. The formation of the [2 + 2] cycloadducts, although it involves the reaction of the aromatic thiophene nucleus as a 2-π-electron moiety, is not surprizing since ynamines generally react with electron-deficient alkenes.[5] Moreover, nitroalkenes and nitroacetylenes react with electron-rich alkenes (enamines) to form nitrocyclobutanes[6] and nitrocyclobutenes,[7] respectively. The formation of the nitrones was the more surprizing result because it requires the participation of the nitro group and a transfer of oxygen from the nitro group to the C-1 carbon atom of the acetylene. An investigation into the scope of this reaction with nitro (hetero) aromatics revealed that several (hetero) aromatic compounds did not react and also that 3-nitrobenzofuran reacted in a completely different fashion. Mixtures of 1-benzoxepin, benzofuran[3,2-*c*]isoxazole, and quinoline *N*-oxide derivatives were obtained.[8,9]

These results, indicating that a reactive "nitroalkene" moiety is needed, have prompted us to investigate reactions of simple nitroalkenes with ynamines, although Nielsen and Archibald have reported that (*E*)-2-nitro-1-phenylpropene fails to react with 1-(diethylamino)-2-phenylacetylene.[10] The present paper describes the results of this investigation.[11]

Results

The acyclic 1-nitroalkenes **1a-c**, which are the thermodynamically most stable isomers, prepared by condensation

(1) Part of the forthcoming thesis of M.L.M.P.
(2) (a) Reinhoudt, D. N.; Volger, H. C.; Kouwenhoven, C. G.; Wijnberg, H.; Helder, R. *Tetrahedron Lett.* **1972**, 5269. (b) Hall, R. H.; den Hertog, H. J., Jr.; Reinhoudt, D. N., submitted for publication.
(3) (a) Reinhoudt, D. N.; Okay, G.; Trompenaars, W. P.; Harkema, S.; van den Ham, D. M. W.; van Hummel, G. J. *Tetrahedron Lett.* **1979**, 1529. (b) Reinhoudt, D. N.; Geevers, J.; Trompenaars, W. P.; Harkema, S.; van Hummel, G. J. *J. Org. Chem.* **1981**, *46*, 424.
(4) Reinhoudt, D. N.; Kouwenhoven, C. G. *Recl. Trav. Chim. Pays-Bas* **1976**, *95*, 67.
(5) For a recent review on ynamines see: Ficini, J. *Tetrahedron* **1976**, *32*, 1449.
(6) Kuehne, M. E.; Foley, L. *J. Org. Chem.* **1965**, *30*, 4280.
(7) Jaeger, V.; Viehe, H. G. *Angew. Chem.* **1970**, *82*, 836.
(8) de Wit, A. D.; Trompenaars, W. P.; Reinhoudt, D. N.; Harkema, S.; van Hummel, G. J. *Tetrahedron Lett.* **1980**, 1779.
(9) de Wit, A. D.; Trompenaars, W. P.; Pennings, M. L. M.; Reinhoudt, D. N.; Harkema, S.; van Hummel, G. J. *J. Org. Chem.* **1981**, *46*, 172.
(10) Nielsen, A. T.; Archibald, T. G. *Tetrahedron* **1970**, *26*, 3475.
(11) Some of these results have been described in preliminary communications: (a) de Wit, A. D.; Pennings, M. L. M.; Trompenaars, W. P.; Reinhoudt, D. N.; Harkema, S.; Nevestveit, O. *J. Chem. Soc., Chem. Commun.* **1979**, 993. (b) Pennings, M. L. M.; Reinhoudt, D. N. *Tetrahedron Lett.* **1980**, 1781. (c) Pennings, M. L. M.; Reinhoudt, D. N.; Harkema, S.; van Hummel, G. J. *J. Am. Chem. Soc.* **1980**, *102*, 7570.

0022-3263/82/1947-1816$01.25/0