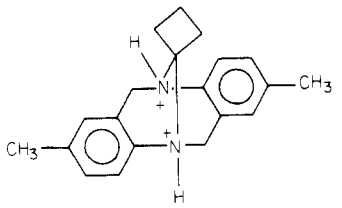
acid rather than 205 ppm found in neutral media.[19]

The 13,13-spirocyclobutyl derivative of Tröger's base (4), synthesized by the procedure used for 3, has also been studied. In dilute acid, this molecule readily loses cyclobutanone. However, in contrast to 3, a concentrated sulfuric acid solution of 4 indicates the presence of "closed" structure 10. This is clear from the single H_{AB} pattern



10

at 4.3 and 5.0 ppm and a spectrum fully consistent with C_2 symmetry. Unlike the dimethyl derivative, the spirocyclobutyl compound is stable in a vial unprotected from air.

In summary, dilute acidic solutions of Tröger's base contain the protonated amine in its closed form 7 with no detectable iminium ion 2. In concentrated acid, Tröger's base exists as the closed structure 5. In contrast, the 13,13-dimethyl derivative 3 rapidly loses acetone in dilute acid but forms the open iminium ion 8 in concentrated acid. One would therefore expect Tröger's acid to racemize in dilute acid only while the dimethyl derivative would racemize in concentrated acid. The tendency to form 8 undoubtedly reflects the added stability of a tertiary iminium ion relative to a primary iminium ion. The 13,13-spirocyclobutyl derivative 4, which also hydrolyzes rapidly in dilute acid, remains in the closed structure 10 in concentrated acid. Although a tertiary iminium carbon is present, the ring strain of the carbonium ion inhibits ring opening of the diprotonated ion.

Experimental Section

NMR spectra were obtained on a Varian 360L NMR spectrometer, a Bruker WM-360 widebore 8.5 Tesla Multi-Nuclear NMR spectrometer, or an IBM WP200SY Multinuclear Fourier Transform NMR spectrometer.

Tröger's base[1] (mp 135 °C, lit.[3] mp, 135–136 °C) and its monohydrochloride (mp 211–212 °C, lit.[31] mp 213 °C) were prepared by the published procedure.[3]

2,8,13,13-Tetramethyl-6*H*,12*H*-5,11-methanodibenzo[*b*,*f*][1,5]diazocine (3) was synthesized according to the published procedure, using chromatographic purification of 2,8-dimethyl-5*H*,6*H*,11*H*,12*H*-dibenzo[*b*,*f*][1,5]diazocine with basic silica gel eluted with benzene–methanol and a trace of *p*-toluenesulfonic acid to catalyze the reaction with acetone. The melting point agreed with the literture value:[16] ^{1}H NMR (acetone-d_6) δ 1.34 (s, 6 H), 2.16 (s, 6 H), 4.00 (d, 2 H), 4.64 (d, 2 H), 6.84 (br s, 2 H), 6.92 (br s, 4 H).

13,13-Spirocyclobutyl-2,8-dimethyl-6*H*,12*H*-5,11-methanodibenzo[*b*,*f*][1,5]diazocine (4) was synthesized by using cyclobutanone (Aldrich) in the manner of 3: mp 185–187 °C; ^{1}H NMR ($CDCl_3$) δ 1.91 (m, 4 H), 2.19 (s, 6 H), 2.31 (m, 2 H), 3.96 (d, 2 H), 4.69 (d, 2 H), 6.62 (s, 2 H), 6.89–7.31 (m, 4 H). Anal. Calcd C, 82.72; H, 7.64; N, 9.65. Found: C, 82.63; H, 7.67; N, 9.38.

Acknowledgment. We acknowledge Mr. Joseph Flisak and Drs. Frank Jordan, David Craik, and Maria Konienczny for obtaining some of the NMR spectra as well as Dr. David Kristol for obtaining some of the UV spectra used in this study. Helpful conversations with Drs. Joel Liebman and Howard Perlmutter are gratefully acknowledged. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

Registry No. 1, 529-81-7; 3, 88703-13-3; 4, 88703-14-4; 5, 88703-15-5; 7, 88703-16-6; 8, 88703-17-7; 10, 88703-18-8.

(19) From Table 5.6 of ref 15, p 143.

The Amino Blocking Reagent 1-Isopropyl-3-ethoxy-4-nitro-2-oxo-3-pyrroline and the *N*-Hydroxysuccinimide Esters of *N*-(1-Cyclohexyl- and 1-Isopropyl-4-nitro-2-oxo-3-pyrrolin-3-yl)glycine. Reagents for the Introduction of *N*-Glycyl Residues

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A previous paper[2] described the preparation of the reactive nitro enol ether 1-cyclohexyl-3-ethoxyl-4-nitro-2-oxo-3-pyrroline (**6a**), as well as some applications of this compound as a reagent for the introduction of a reversible amino protecting group (the 1-cyclohexyl-4-nitro-2-oxo-3-pyrrolin-3-yl or "NOPY" group). Applications of the NOPY blocking group in simple examples of peptide synthesis[2] and protein modification[3] have been described. Advantages of the NOPY group are its ease of introduction (short reaction times in partially aqueous media at ca. 25 °C and pH 7.5–9), ease of removal (treatment with ammonia or aqueous base at ca. 25 °C), and the convenience with which it is monitored through its high absorptivity at 367–385 nm and visibility on thin-layer chromatography plates with 254-nm fluorescent indicator. For many applications, however, it would be useful to have a new blocking reagent that would be more water soluble and which would introduce a blocking group that adds only minimally to the hydrophobic character of a resulting protected amino acid or peptide derivative. A reagent of the NOPY type with cyclohexyl replaced by a smaller group was thought likely to have such properties. The corresponding 1-isopropyl reagent has been obtained and found to meet these expectations. (Efforts to prepare methyl and ethyl reagents did not succeed.) The new reagent is quite water soluble, and has yielded an N-protected glycine active ester with solubility appropriate for reaction with both peptides and proteins. The acronym NOPYE (for nitrooxopyrrolinyl ethyl ether) had been applied to compound **6a**;[2] we propose henceforth to call this original cyclohexyl reagent "c-NOPYE" and **6b**, the new isopropyl-containing reagent, "i-NOPYE." The derived NOPY groups would similarly be distinguished as "c-NOPY" and "i-NOPY".

Synthesis of the i-NOPYE Reagent (6b). A synthetic sequence based on a different type of starting material was

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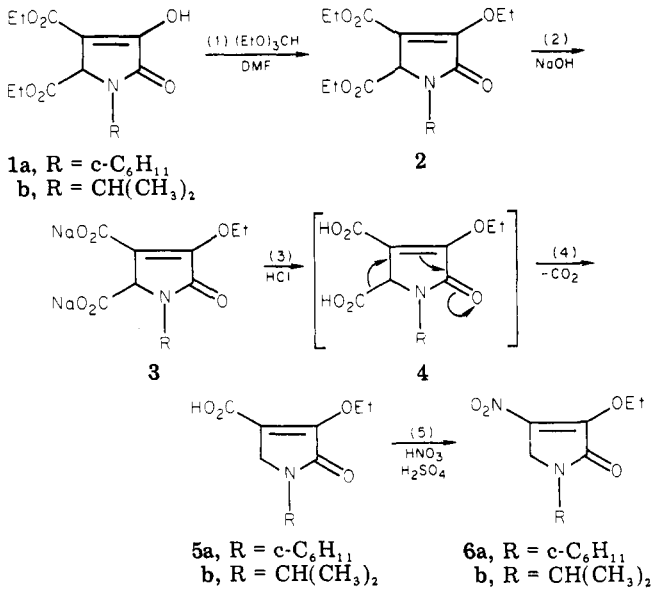
(2) Southwick, P. L.; Dufresne, R. F.; and Lindsey, J. J. *J. Org. Chem.* **1974**, *39*, 3351–3354.

(3) Negri, D. J.; Southwick, P. L; Brown, W. E. *Biochim. Biophys. Acta* **1979**, *579*, 31–39.

0022-3263/84/1949-1130$01.50/0

Scheme I



needed to obtain satisfactory and reproducible yields of the i-NOPYE reagent. Conversion of the enol of 4-carbethoxy-1-isopropyl-2,3-dioxopyrrolidine into the final intermediate, 3-ethoxy-1-isopropyl-2-oxo-3-pyrroline-4-carboxylic acid (**4b**) was attended by formation of excessive amounts of dark-colored byproducts in the saponification step.[2] The alternative synthetic route devised to circumvent this problem is shown in Scheme I. Starting from the readily available enolic 4,5-dicarbethoxy-1-isopropyl-2,3-dioxopyrrolidine (**1b**),[4] rather than the corresponding 4-monocarbethoxy derivative, ethylation of the enolic hydroxyl was carried out with triethyl orthoformate. The resulting crude enol ether **2b** was not purified but was saponified directly to yield a solution containing the disodium salt **3b**, which upon acidification underwent conversion to the diacid **4b**, followed by rapid decarboxylation with precipitation of the enol ether monocarboxylic acid **5b**. Direct nitration of **5b** accompanied by decarboxylation then produced the nitro enol ether **6b** as in the synthesis of **6a**.[2] The new sequence gave reliable yields of 60% to 65% in the overall conversion of the diester **1b** to the enol ether acid **5b**, and this transformation was reduced to an easily conducted one-pot process. It has since been found that the sequence of Scheme I, starting with the 4,5-diester, also gives more reliable results in the preparation of the c-NOPYE intermediate than did the previously published procedures starting from the 4-mono ester.[2] The 5-ester group serves as a removable blocking group against side reactions.

The new nitro enol ether **6b** showed a solubility in water in excess of 10 mg/mL at 25 °C; its solubility is more than sufficient for use in purely aqueous solutions to block amino functions of proteins. Proteins such as lysozyme and chymotrypsin, for example, underwent extensive reaction in water or 6 M guanidine hydrochloride with the i-NOPYE reagent **6b** under conditions previously used with the c-NOPYE reagent **6a**,[3] but without the need to add acetone or any other miscible organic solvent.

The c-NOPY- and i-NOPY-Gly-OSu Glycylating Reagents (8a and 8b). Since NOPY groups, like peptides and proteins themselves, have the character of amides, it was anticipated that solvent systems which are known to dissolve many proteins and/or polypeptides could be adjusted to dissolve *N*-NOPY amino acids and properly selected acylating derivatives of such protected amino acids. Such solubility properties should facilitate use of these derivatives for attachment of amino acid residues to peptides or proteins by the salt-coupling technique, in which free carboxylate groups of the substrate are not blocked, and the coupling reaction can be carried out in aqueous or mixed organic–aqueous solutions. As a first test of this possibility, *N*-hydroxysuccinimide esters of N-i-NOPY- and N-c-NOPY-Gly have been prepared and used to attach glycine residues to a dipeptide and a protein by the salt-coupling procedure. These new reagents for glycine attachment (**8a,b**) are stable crystalline compounds. The c- or i-NOPY-protected glycines were obtained by treatment of glycine at ca. 25 °C with a NOPYE reagent in water or a water–acetonitrile mixture at pH 8–9 (Scheme II). The *N*-hydroxysuccinimide active esters **8a** and **8b** were then produced from these products by treatment with dicyclohexylcarbodimide (DCC) and *N*-hydroxysuccinimide in tetrahydrofuran (THF).[5]

Glycine residues were added to both the dipeptide L-phenyl-L-leucine (H-L-Phe-L-Leu-OH) and its ethyl ester (H-L-Phe-L-Leu-OEt) by use of **8a** and/or **8b**. Coupling with the ethyl ester was conducted in THF, whereas coupling with H-L-Phe-L-Leu-OH itself was carried out by the salt-coupling technique in a solvent mixture of water, acetonitrile, and dimethylformamide (DMF) in the presence of potassium carbonate.[6] The product of the latter coupling procedure, the protected tripeptide c-NOPY-L-Gly-L-Phe-L-Leu-OH (**10a**), was deblocked by brief treatment with methanolic ammonia to yield the free tripeptide **11**.[7] An advantage inherent in the use of NOPY protecting groups lies in the fact that the available ammonia deblocking procedure can yield deblocked peptide directly in the free form rather than as a hydrochloride or other salt. The heterocyclic amino derivatives c- or i-NOPY-NH_2 (**12**), formed from the NOPY groups in ammonia deblocking, are nonbasic compounds that are readily removed by virtue of their solubility in organic solvents such as acetone or methylene chloride.

A test of the possibility of using i-NOPY-Gly-OSu (**8b**) for introducing glycine residues into proteins or polypeptides was performed by using lysozyme as the substrate. There are seven free amino functions in lysozyme that might be subject to reaction with **8b**. These are located on the six lysine residues, one of which is the N terminal. To a solution of lysozyme in an aqueous 6 M guanidine hydrochloride solution was added compound **8b** (dissolved in a small volume of acetonitrile) in a 5:1 molar excess over protein amino functions. The pH of the solution was adjusted to 8–9, and the mixture was stirred at room temperature for 2 h. The modified protein isolated after this treatment was subjected to amino acid analysis, the

(4) Southwick, P. L.; Vida, J. A.; Fitzgerald, B. M.; and Lee, S. K., *J. Org. Chem.* **1968**, *33*, 2051–2056.

(5) Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem Soc.* **1964**, *86*, 1839–1842.

(6) In some of the experiments, removal of excess amounts of **8a** or **8b** was sought by addition of *N,N*-dimethylethylenediamine to form an acid-extractable basic amide (**13a** or **13b**). Cf.: (a) Shvachkin, Yu. P.; Guin, S. K.; Kaufman, K. D.; Doelling, R. *Zh. Obskch. Khim.* **1982**, *52*, 1664–1668; *Chem. Abstr.* **1982**, *97*, 145246*x*. (b) Schattenkerk, C.; Voskuyl-Holtkamp, I.; Bokhorst, R. *Recl. Trav. Chim. Pays-Bas* **1973**, *92*, 92. (c) Loew, M.; Kisfaludy, L. *Acta Chim. Acad. Sci. Hung.* **1965**, *44*, 61–66. In later experiments *N*-methylpiperazine was substituted as the quenching agent (to give **14a**), in order to minimize the possibility that NOPY protecting groups would be removed by nucleophilic exchange. Introduction of the quenching step, however, had little effect on results; apparently reagents **8a** and **8b** and their hydrolysis products were adequately removed by conventional workup procedures.

(7) Dewey, R. S.; Schoenewaldt, E. F.; Joshua, H.; Paleveda, W. J., Jr.; Schwan, H.; Barkemeyer, H.; Arison, B. H.; Veber, D. F.; Strachan, R. G.; Minkowski, J.; Denkewalter, R. G.; Hirschmann, R. *J. Org. Chem.* **1971**, *36*, 49–59.

Scheme II

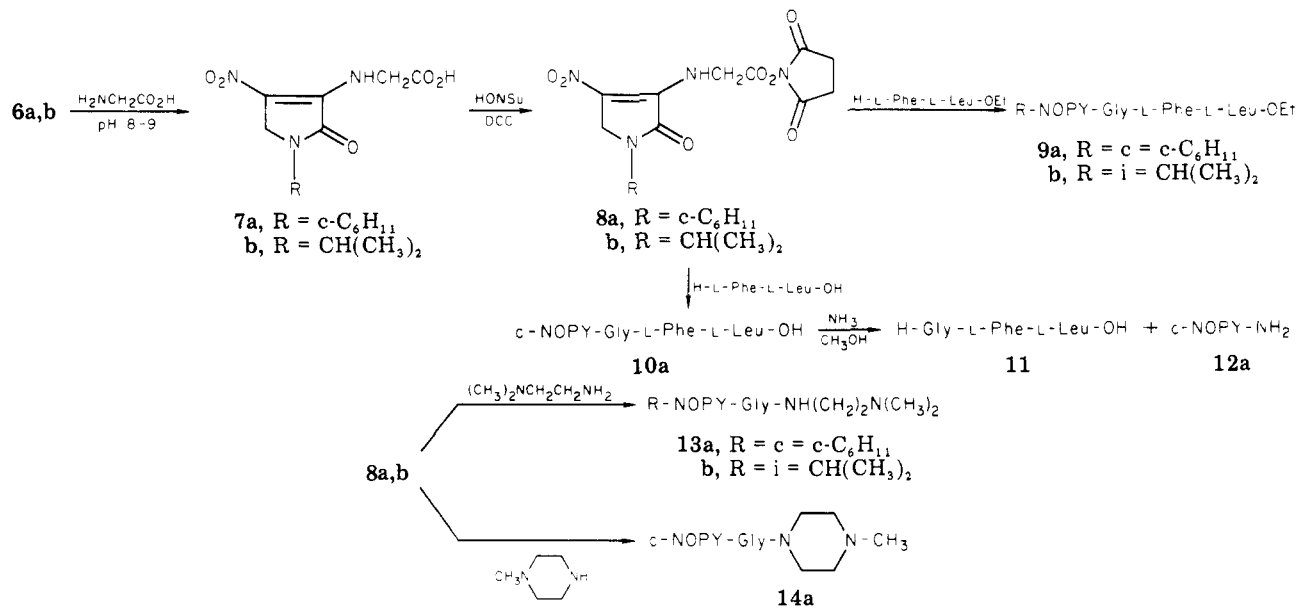


Table I. Recovery of Glycine and Lysine from Acid-Hydrolyzed NOPY-Gly-Modified and DNP-Modified Lysozyme

	lysozyme (lit.)	NOPY-Gly lysozyme	DNP lysozyme	DNP-Gly lysozyme
glycine	12	16.8	11.3	12.2
lysine	6	5.8	0.2	5.9

result of which indicated that 4.8 additional glycine residues had been introduced per lysozyme molecule (total glycine content was increased from 12 to 16.8).

The i-NOPY groups were removed from the modified protein by brief treatment with ammonium hydroxide at room temperature to verify this result, since the resulting deblocked glycylated lysozyme could be treated with excess 2,4-dinitrofluorobenzene (DNFB) to determine how many of the lysine residues were protected from reaction as a result of the presence of attached glycine residues. After complete acid hydrolysis of the DNFB-treated glycylated lysozyme, amino acid analysis of such samples indicated that from 4.8 to 5.9 of the six original lysines (Table I) had escaped reaction with DNFB under conditions which, in a control experiment with unmodified lysozyme, caused reaction of DNFB with 5.9 of the 6 lysine residues. It was evident that the active ester **8b** is an effective glycylating agent for use in water-containing solvent systems suitable for at least some proteins or polypeptides.

Experimental Section

Spectrophotometers used for the reported measurements were a Perkin-Elmer Model 237B (infrared spectra) and Perkin-Elmer Model 202 (ultraviolet spectra). Infrared bands in the range 2.5–9.25 μm are reported. Nuclear magnetic resonance measurements were made with a Perkin-Elmer Model R-24 instrument (60 MHz) and a Bruker Model WM-300 instrument (300 MHz). We are indebted to Professor Miguel Llinas for measurements with the latter instrument, which was acquired with support from NIH Grant GM-27390. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Amino acid analyses were performed on a Durrum D-500 amino acid analyzer. Samples for analysis were hydrolyzed in 6 N HCl for 24 h at 110 °C in vacuo. Elemental analyses were by M-H-W Laboratories, Phoenix, AZ. Thin-layer chromatography (TLC) on NOPY derivatives was carried out on Baker-flex silica 1B2-F sheets.

3-Ethoxy-1-isopropyl-2-oxo-3-pyrroline-4-carboxylic Acid (5b). A solution prepared from 22 g (0.078 mol) of **1b**,[4] 55 mL (48 g, 0.33 mol) of triethyl orthoformate, 75 mL of DMF, and 0.5 g of *p*-toluenesulfonic acid hydrate was heated and stirred with a magnetic stirrer in an insulated distillation apparatus of the Claisen type. The voltage to the heating mantle was regulated so that the temperature of the distilling vapors leaving the flask (mainly EtOH and HCO_2Et) did not exceed 78 °C, while the temperature registered by a thermometer placed in the boiling liquid rose gradually to 150 °C. After ca. 3 h at a constant voltage setting, the distillation almost ceased and the temperature at the still head fell to 40–45 °C. The reaction mixture was then transferred to a rotary evaporator for the removal of the DMF and unused triethyl orthoformate under reduced pressure.

The residual thick oil was dissolved in a mixture prepared from 100 mL of EtOH, 100 mL of H_2O, and 16 g of NaOH and heated on a steam bath for 45 min under a reflux condenser. The solution was transferred to a rotary evaporator, and the ethanol and water were removed under reduced pressure. The residue was dissolved in 100 mL of water, cooled in an ice bath, and acidified to pH 2 with hydrochloric acid. The product separated as tan crystals; mp ca. 127 °C, yield 10 g (60%). A sample was recrystallized from CH_2Cl_2–ether or successively from EtOH and from EtOAc to give a white product: mp 131–133 °C; ^1H NMR ($CDCl_3$) δ 11.05 (s, 1 H, CO_2H), 4.99–4.25 (q + septet, 3 H, overlap of OCH_2CH_3 and $NCH(CH_3)_2$, J = 7 Hz), 4.00 (s, 2 H, CH_2 of pyrroline), 1.54–1.20 (t + d, 9 H, overlap of CH_2CH_3 and $NCH(CH_3)_2$, J = 7 Hz); UV (95% EtOH) 252 nm (ε 13 400); IR (Nujol) 3.75, 3.85, 4.05, 5.90, 6.02, 6.15, 6.91, 7.06, 7.19, 7.29, 7.50, 7.98, 8.25, 8.55, 8.75, 9.03 μm.

Anal. Calcd for $C_{10}H_{15}NO_4$: C, 56.32; H, 7.09; N, 6.57. Found: C, 56.25; H, 7.16; N, 6.62.

1-Cyclohexyl-3-ethoxy-2-oxo-3-pyrroline-4-carboxylic Acid (5a). The 1-cyclohexyl derivative **5a** was prepared in the current investigation from the enolic 4,5-dicarbethoxy-1-cyclohexyl-2,3-dioxopyrrolidine (**1a**)[4] by a similar procedure conducted on nearly the same scale (ca. 0.088 mol of **1a** used in many experiments). Both the 4,5-dicarbethoxy derivative **1a** and the 4,5-dimethoxy derivative (mp 160 °C) have been used as starting materials with similar results. The procedure was identical with that described above for **5b**, except that is was not necessary to conduct an evaporation to remove ethanol from the saponification mixture, which was instead filtered and poured into a mixture of 200 mL of ice and 50 mL of ethanol before acidification to precipitate the product. The typical yield of **5a**, mp 154–155 °C, from 0.088 mol of starting material was 16 g (72%), a result equivalent to the best obtained from the more complex two-step conversion of 4-carbethoxy-1-cyclohexyl-2,3-dioxopyrrolidine described previously.[2]

1-Isopropyl-4-nitro-3-ethoxy-2-oxo-3-pyrroline (i-NOPYE, 6b). 3-Ethoxy-1-isopropyl-2-oxo-3-pyrroline-4-carboxylic acid (**5b**, 8 g, 0.0376 mol) in powdered form was added in small portions

through a seive to a stirred mixture of 70 mL of concentrated sulfuric acid and 4.7 mL of white fuming nitric acid (90%) that had been cooled to −5 °C prior to the start of the addition of **5b**. The rate of addition of **5b** was controlled so as to keep the temperature of the reaction mixture from rising above −3 °C and to minimize accumulation of undissolved lumps of starting material in the mixture. Stirring was continued for 3 h at −3 °C. Effervescence caused by carbon dioxide evolution began after the mixture had been stirred for a few minutes and virtually ceased after an hour or two. When the mixture was poured slowly into a stirred suspension of 1 L of crushed ice the product separated as a white crystalline precipitate. It was collected by filtration as soon as the ice was melted and washed on the filter with a small amount of cold water. The yield was 4.9 g (61%), mp 47–48 °C. Recrystallization from acetone or benzene and petroleum ether (bp 30–60 °C) raised the melting point to 49–50 °C: ^{1}H NMR ($CDCl_3$) δ 4.90 (q, 2 H, OCH_2CH_3, J = 7 Hz), 4.40 (septet, 1 H, $NCH(CH_3)_2$, J = 7 Hz), 4.15 (s, 2 H, CH_2 of pyrroline), 1.55–1.20 (t + d, 9 H, overlap of $-OCH_2CH_3$ and $NCH(CH_3)_2$, J = 7 Hz); IR (thin film) 3.34, 3.39, 3.47, 5.87, 6.08, 6.69, 6.92, 7.17, 7.3, 7.30, 7.38, 7.67, 7.93, 8.10, 8.40, 8.55, 8.75, 8.87, 9.05 μm; UV (95% EtOH) 293 nm (ε 9800).

Anal. Calcd for $C_9H_{14}O_4N_2$: C, 50.46; H, 6.59; N, 13.08. Found: 50.32; N, 6.46; N, 13.12.

***N*-(1-Isopropyl-4-nitro-2-oxo-3-pyrrolin-3-yl)glycine (7b, i-NOPY-Gly-OH).** Glycine (0.75 g, 0.01 mol) and 0.7 g (0.005 mol) of potassium carbonate were dissolved in 10 mL of water. A solution of 1.07 g (0.005 mol) of i-NOPYE (**6b**) in 5 mL of acetonitrile was added, and the mixture was stirred for 1 h at room temperature and then acidified to pH 2 by addition of 6 N hydrochloric acid to the stirred mixture. Stirring was continued while the mixture was cooled in an ice bath to complete separation of the product as a finely divided crystalline precipitate, which was collected by filtration. The yield was 1.0 g (82%); mp 180–185 °C. Recrystallization from acetic acid–water gave pale yellow crystals; mp 185–186 °C; ^{1}H NMR ($CDCl_3$ and CF_3CO_2H) δ 8.35 (br, 1 H, NH), 4.79 (d, 2 H, CH_2 of Gly, J = 6 Hz), 4.41 (septet, 1 H, $NCH(CH_3)_2$, J = 7 Hz, partly obscured by adjacent signals), 4.31 (s, 2 H, CH_2 of pyrroline ring) 1.34 (d, 6 H, $NCH(CH_3)$, J = 7 Hz); IR (Nujol) 3.01, 5.85, 5.90, 6.06, 6.92, 7.05, 7.25, 7.90, 8.20, 8.90, 9.08 μm; UV (EtOH) 262 nm (ε 3900), 369 (ε 14 400).

Anal. Calcd for $C_9H_{13}N_3O_5$: C, 44.44; H, 5.39; N, 17.28. Found: C, 44.22; H, 5.59; N, 16.95.

***N*-Hydroxysuccinimide Ester of *N*-(1-Cyclohexyl-4-nitro-2-oxo-3-pyrrolin-3-yl)glycine (8a, c-NOPY-Gly-OSu).** To a solution of 0.432 g (3.75 mmol) of *N*-hydroxysuccinimide (HOSu) and 0.71 g (2.5 mmol) of *N*-(c-NOPY)-Gly (**7a**) in 10 mL of tetrahydrofuran (THF) was added 0.6 g (2.9 mmol) of dicyclohexylcarbodiimide (DCC), and the mixture was stirred overnight at room temperature.[6] The crystalline precipitate that separated was removed by filtration and extracted with 20 mL of boiling THF to recover a quantity of the *N*-hydroxysuccinimide ester that separated in the precipitate along with *N*,*N*′-dicyclohexylurea (DCU). After cooling and filtering to remove DCU, the extract was combined with the first filtrate, and the solution was taken to dryness under reduced pressure in a rotary evaporator. The residue was treated with 5 mL of hot methylene chloride, and the resulting solution was diluted with 10 mL of petroleum ether (bp 30–60 °C). A light tan crystalline precipitate separated; mp 180–181 °C. The yield was 0.723 g (76%). Recrystallization from methylene chloride–petroleum ether (bp 30–60 °C) raised the melting point to 192–194 °C; IR (Nujol) 3.04, 5.42, 5.60, 5.76, 5.92, 6.06, 6.96, 7.03, 7.15, 7.28, 7.81, 7.93, 8.05, 8.23, 8.40, 9.23 μm.

Anal. Calcd for $C_{16}H_{20}N_4O_7$: C, 50.52; H, 5.30; N, 14.73. Found: C, 50.42; H, 5.39; N, 14.58.

***N*-Hydroxysuccinimide Ester of *N*-(1-Isopropyl-4-nitro-2-oxo-3-pyrrolin-3-yl)glycine (8b, i-NOPY-Gly-OSu).** To a filtered solution of 1.15 g (10 mmol) of HOSu and 2.2 g (9 mmol) of N-(i-NOPY)-Gly (**7b**) in 40 mL of THF was added a solution of 2.06 g (10 mmol) of DCC in 10 mL of THF, and the mixture was kept at room temperature overnight.[5] After removal of the precipitated DCU by filtration, the filtrate was taken to dryness at reduced pressure in a rotary evaporator. The solid residue was dissolved in 10 mL of THF and crystallization was induced by addition of 20 mL of petroleum ether (bp 30–60 °C). The yield was 2.4 g (78%) of pale yellow crystals, mp 163–165 °C. An analytical sample of the same melting point was obtained by recrystallization from the same solvents: ^{1}H NMR ($CDCl_3$ and CF_3CO_2H) δ 8.3 (br, 1 H, N-H), 5.13 (d, 2 H, CH_2 of Gly, J = 7 Hz), 4.4 (septet, 1 H, $NCH(CH_3)_2$, J = 7 Hz, overlapped by adjacent signal at 4.21), 4.21 (s, 2 H, CH_2 of pyrroline ring), 2.95 (s, 4 H, CH_2 of succinimide ring), 1.36 (d, 6 H, $NCH(CH_3)_2$, J = 7 Hz); IR (Nujol) 2.98, 5.50, 5.60, 5.77, 5.89, 6.04, 6.07, 6.91, 7.03, 7.18, 7.28, 7.89, 7.92, 8.13, 8.56, 9.25 μm; UV (95% EtOH) 367 nm (ε 15 400).

Anal. Calcd for $C_{13}H_{16}O_7N_4$: C, 45.88; H, 4.94; N, 16.47. Found: C, 45.75; H, 4.92; N, 16.20.

***N*-(i-NOPY)-glycyl-L-phenylalanyl-L-leucine Ethyl Ester (9b, i-NOPY-Gly-L-Phe-L-Leu-OEt).** To a solution of 0.342 g (1 mmol) of L-phenylalanyl-L-leucine ethyl ester hydrochloride and 0.23 mL of *N*-methylmorpholine in 10 mL of THF was added **8b** (0.64 g; 1.9 mmol) with stirring after the solution had been cooled in an ice bath. Cooling was continued for 30 min, and then the mixture was allowed to warm to room temperature and stirring was continued overnight. After a small amount of insoluble material had been removed by filtration, the filtrate was concentrated under reduced pressure in a rotary evaporator. The residual oil was dissolved in 20 mL of methylene chloride, and 0.08 mL of *N*,*N*-dimethylethylenediamine was added. The mixture was stirred for 10 min at room temperature, then extracted twice with 15-mL portions of 2 N hydrochloride acid, once with 15 mL of 2% sodium bicarbonate, and once with 15 mL of water. Concentration of the methylene chloride solution under reduced pressure in a rotary evaporator left an orange-red oil, which yielded white crystals when mixed with ca. 5 mL of absolute ethanol. The yield was 0.24 g (45%): mp 220–222 °C; homogeneous by TLC, R_f 0.79 ($CHCl_3$:CH_2Cl_2:CH_3OH, 5:5:1); ^{1}H NMR ($CDCl_3$) δ 8.43 (br, 1 H, NH of Gly), 7.29–7.19 (m, 5 H, Ph of Phe), 4.76–4.69 (q, 1 H, α-H of Phe, J = 7 Hz), 4.60–4.57 (d, 2 H, CH_2 of Gly, J = 6.5 Hz), 4.53–4.39 (m, 2 H, overlap of α-H of Leu with $NCH(CH_3)_2$), 4.19–4.12 (s + q, 4 H, overlap of CH_2 of pyrroline and $-OCH_2CH_3$), 3.13–3.08 (m, 2 H, CH_2 of Phe), 1.71 (s, 2 H, NH of Phe and Leu), 1.61–1.45 (m, 3 H, $-CH_2CH(CH_3)_2$ of Leu), 1.29–1.23 (t + d, 9 H, overlap of $-OCH_2CH_3$ and $NCH(CH_3)_2$), 0.90–0.88 (d, 6 H, $-CH(CH_3)_2$ of Leu, J = 6 Hz); UV (EtOH) 369 nm (ε 13 700); $[\alpha]^{25}_{589}$ −2.8° (*c* 1.06, HOAc).

Anal. Calcd for $C_{26}H_{37}O_7N_5 \cdot H_2O$: C, 56.81; H, 7.15; N, 12.75. Found: C, 56.78, 57.03; H, 7.02, 6.95; N, 12.60, 12.51. Amino acid analysis: Gly, 0.95; Phe, 1.00; Leu, 1.02.

***N*-(c-NOPY)-glycyl-L-phenylalanyl-L-leucine Ethyl Ester (9a, c-NOPY-Gly-L-Phe-L-Leu-OEt).** The procedure was the same as that described above for i-NOPY-Gly-L-Phe-L-Leu-OEt (**9b**). A 1-mmol quantity of H-L-Phe-Leu-OEt·HCl treated with 2 mmol of **8a** in this manner yielded a yellow solid when the final methylene chloride solution was evaporated. The product was dissolved in methylene chloride and crystallization induced by addition of an equal volume of ether. Pale yellow crystals were obtained; mp 212–213 °C, yield 0.40 g (70%). Recrystallization from ethanol gave nearly white crystals; mp 222–223 °C, homogeneous by TLC, R_f 0.82 ($CHCl_3$:CH_2Cl_2:CH_3OH, 5:5:1); UV (EtOH) 369 nm (ε 15 700); IR (Nujol) 2.93, 3.09, 5.74, 5.84, 5.93, 6.09, 6.52, 6.96, 7.21, 7.29, 7.41, 7.91, 7.98, 8.31, 8.71, 9.10 μm; $[\alpha]^{25}_{589}$ −5.77° (*c* 1.08, HOAc).

Anal. Calcd for $C_{29}H_{41}N_5O_7 \cdot {}^1/_2H_2O$; C, 59.98; H, 7.29; N, 12.06. Found: C, 59.84; H, 7.25; N, 12.11.

***N*-(c-NOPY)-glycyl-L-phenylalanyl-L-leucine (10, c-NOPY-Gly-L-Phe-L-Leu-OH).** To a solution of 0.570 g (1.5 mmol) of **8a** in 10 mL of acetonitrile and 2 mL of DMF was added a solution of 0.278 g (1 mmol) of L-phenylalanyl-L-leucine (Sigma) and 0.138 g (1 mmol) of potassium carbonate in 5 mL of water. After the mixture had been stirred at room temperature for 2 h, 0.11 mL of *N*-methylpiperazine was added and stirring was continued for 30 min. The solution was acidified to pH 2 with 6 N hydrochloric acid, and 15 mL of water was added. Scratching of the walls of the flask induced crystallization of the product as light-yellow needles; partial melting (transition) 132–136 °C, final melting with decomposition 165–175 °C; yield 0.389 g (71%); homogeneous by TLC, R_f 0.32 ($CHCl_3$:CH_2Cl_2:CH_3OH:HOAc, 36:4:2:1); IR (Nujol) 2.94, 3.02, 5.78, 5.84, 6.08, 6.52, 6.93, 7.22, 7.28, 7.40, 7.89, 7.97, 8.32, 8.72, 9.12 μm; UV (EtOH) 369 nm (ε 14 700); $[\alpha]^{25}_{589}$ −4.4° (*c* 1.03, HOAc).

Anal. Calcd for $C_{27}H_{37}H_5O_7$: C, 59.65; H, 6.86; N, 12.89. Found: C, 59.74; H, 7.00; N, 12.63. Amino acid analysis: Gly, 1.01; Phe, 0.99; Leu, 1.00.

Glycyl-L-phenylalanyl-L-leucine (11, H-Gly-L-Phe-L-Leu-OH). c-NOPY-Gly-Phe-Leu-OH (**10**, 0.545 g, 1 mmol) was dissolved in 40 mL of methanol, which had been saturated with anhydrous ammonia. The solution was stirred for 2 h at room temperature, and then concentrated to volume of ca. 5 mL under reduced pressure in a rotary evaporator. Pyridine (20 mL) was added, and the solution was again concentrated to ca. 5 mL. Upon addition of a small amount of acetone and 30 mL of ether, the product separated as a light buff-colored precipitate and was collected by filtration, yield 0.297 g. Colored impurities were removed by extraction with 5 mL of hot ethanol to yield 0.187 g (56%) of white product, mp 220–223 °C. The tripeptide separated as clumps of slender white needles, mp 225–227 °C dec, when its solutions in ammonium hydroxide were maintained for a time under reduced pressure. The product was homogeneous by TLC, R_f 0.44 (1-butanol:acetic acid:water, 10:1:3) on Analtech silica gel G plates (lit.[7] R_f 0.45): IR (Nujol) 3.03, 6.00, 6.04, 6.13, 6.47, 7.26, 7.48, 7.58, 7.69, 7.93, 8.10, 8.73 μm; $[\alpha]^{25}_{589}$ −12.9° (*c* 1.06, HOAc) [lit.[7] $[\alpha]^{25}_{589}$ −12.5° (*c* 1.04, HOAc).

Anal. Calcd for $C_{17}H_{25}N_3O_4$: C, 60.87, H, 7.51; N, 12.53. Found: C, 60.68; H, 7.80; N, 12.53.

i-NOPY Glycylation of Lysozyme. To a solution of 120 mg of lysozyme (Sigma) in 2.2 mL of 6 M guanidine hydrochloride was added 85 mg of **8b** in 0.2 mL of acetonitrile. The mixture was made alkaline (pH 8–9) by addition of 0.03 mL of *N,N,-N',N'*-tetramethylethylenediamine (TMEDA) and stirred at room temperature for 2 h and then acidified to pH 2 by addition of 6 N hydrochloric acid. Additional water was added (ca. 12 mL), and the precipitate of modified lysozyme was centrifuged down and then washed twice with 10 mL of water, three times with 10 mL of warm absolute ethanol, and once with 10 mL of acetonitrile. The final ethanol and acetonitrile supernatants did not show the presence of the *N*-NOPY chromophore (λ_{max} 380 nm), indicating that excess *N*-NOPY-Gly containing reagent had been removed. The resulting modified lysozyme was obtained in the form of a tan powder. The material gave an ultraviolet spectrum in acetic acid–water (1:1 v/v) in which the absorbance of 0.9 at 380 mm from the *N*-NOPY-glycine residue exceeded that at 280 nm (0.7). Amino acid analysis of this material gave the results expected for lysozyme, except for an increased amount of glycine amounting to 4.8 residues.

Deblocking of a 23 mg. sample of this N-(i-NOPY)-glycylated lysozyme was carried out by suspending the material in a solution prepared from 2 mL of water, 2 mL of DMF, and 2 mL of concentrated ammonium hydroxide and stirring the mixture for 64 h. A small amount of solid that remained undissolved was centrifuged down. The ultraviolet spectrum of a sample of the supernatant solution diluted with ethanol indicated that it contained the deblocking product, i-NOPY-NH_2 (λ_{max} 355 nm). The residue obtained by evaporation of the supernatant was combined with the very small centrifuged pellet and mixed with a solution containing 6 mL of water, 6 mL of DMF, and 5 drops of 2,4-dinitrofluorobenzene (DNFB). The mixture was adjusted to a pH of 8–9 by addition of 6 drops of TMEDA and stirred overnight. Concentrated ammonium hydroxide (2 mL) was added to destroy any remaining DNFB and stirring was continued for 2 h. The mixture was dialyzed against distilled water (1 L) for ca. 2 h with two changes of water and then for 64 h with two additional changes. Samples of the resulting solution of DNFB-treated glycylated lysozyme were taken for amino acid analysis. Results of amino acid analyses are recorded in Table I.

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Registry No. **1a**, 16206-17-0; **1b**, 16206-02-3; **2a**, 88767-00-4; **2b**, 88767-01-5; **5a**, 52555-23-4; **5b**, 88767-02-6; **6b**, 88767-03-7; **7a**, 52555-24-5; **7b**, 88767-04-8; **8a**, 88767-05-9; **8b**, 88767-06-0; **9a**, 88767-07-1; **9b**, 88767-08-2; **10a**, 88767-09-3; **11**, 15373-56-5; $H_2NCH_2CO_2H$, 56-40-6; H-L-Phe-L-Leu-OEt·HCl, 88767-10-6; H-L-Phe-L-Leu-OH, 3303-55-7; lysozyme, 9001-63-2.

On Charge Distribution in Diazenium Salts

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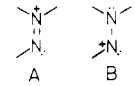
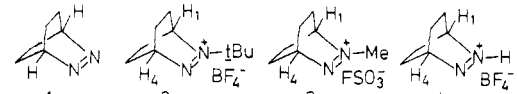
McBride and Bens[1] pointed out in 1959 that diazenium cations should be resonance hybrids of structures A and B and that the all-octet structure A should be preferred.



A B

Nelsen and Landis[2] reported that both bridgehead hydrogens for *tert*-butylated bicyclic azo compounds are shifted substantially downfield of the bridgehead hydrogens of the azo compounds. Bridgehead hydrogen chemical shifts (all in CD_3CN, relative to residual CD_2HCN at δ 1.93) are reported below for 2,3-diazabicyclo[2.2.2]oct-2-ene (**1**) and three cations derived from it. The upfield



1	2	3	4
$\delta(H_{1,4})$ 5.00	5.64, 5.84	5.27, 5.72	5.58(ave)

bridgehead hydrogens of both **2** and **3** appear as broadened triplets, and the downfield peaks are broadened singlets, suggesting that they correspond to the same hydrogen in both compounds. Replacement of the *tert*-butyl group of **2** by methyl as in **3** should principally affect the shift of H_1, causing it to move upfield because of a decrease in steric compression. This suggested the assignment of H_1 as the δ 5.64 peak in **2**, and the δ 5.27 peak in **3**. An NOE experiment verified this assignment of **3**, because decoupling the methyl group caused an 8.7% enhancement of the δ 5.27 peak compared to a 0.3% enhancement of the δ 5.72 peak. The result that H_1, on a carbon attached to the formally positive nitrogen, comes δ 0.45 upfield of H_4 surprised us and made us wonder about the charge distribution in these molecules.

Geometry-minimized MNDO calculations[3] were run on $(H_2N{=}NH)^+$ (**5**) and $(Me_2N{=}NMe)^+$ (**6**) as models. Both were constrained to be planar and the methyl groups were idealized by being held tetrahedral, with all CH distances the same (1.112 Å was obtained). **6** is calculated as being stablest in the geometry shown in Figure 1, with one CH bond of each methyl group in the molecular plane. The total charge densities at each atom are also shown in Figure 1; 76% of the charge is calculated to be at the hydrogens of **5** and 99% at the methyl groups of **6**. The charge density at the trisubstituted nitrogen (N_2) is calculated to be more negative than that at the disubstituted nitrogen (N_3) in both cases. This is principally a result of N=N π-bond polarization. The calculated p_z electron densities at N_2 and N_3 are given at the bottom of Figure 1, where it may be seen that the π-electron distributions are similar for **5** and **6**, with greater electron density at the formally positive nitrogen, as expected. The π-electron distributions at N_2:N_3 are calculated as 62:38 in **5**, and 60:40 in **6**. The calculated charges at the hydrogens of **5** and the carbons

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0022-3263/84/1949-1134$01.50/0