vacuo removal of ethanol, the yellow oil was crystallized from ethyl acetate to afford **21** as yellow crystals in a yield of 2.57 g (91.5%): mp 161–163 °C; NMR δ 1.3 (t, 6 H, J = 4 Hz), 2.4 (s, 6 H), 4.1 (q, 4 H, J = 4 Hz), 5.1 (s, 1 H), 6.1 (br s, 1 H), 7.2–8.1 (m, 4 H); IR (KBr) 3346, 1645 cm^{-1}. Anal. ($C_{19}H_{22}N_2O_6$) C, H, N.

Octyl 2,6-Dimethyl-3-carbethoxy-4-(3-nitrophenyl)-1,4-dihydropyridine-5-carboxylate (22). A solution of 1 (5 g, 0.02 mol) and **20** (4.0 g, 0.02 mol) in 50 mL of ethanol was heated to reflux with stirring for 24 h. The ethanol was removed in vacuo, and the product was purified by column chromatography on silica gel with hexanes–ethyl acetate (5:1) as the eluent. Crystallization from ethyl acetate–hexanes afforded **22** as a yellow waxy solid in a yield of 1.77 g (19.3%): mp 70–72 °C; NMR δ 0.9–1.6 (m, 18 H), 2.4 (s, 6 H), 3.8–4.3 (m, 4 H), 5.1 (s, 1 H), 5.8 (br s, 1 H), 7.2–8.1 (m, 4 H); IR (KBr) 3330, 1695 cm^{-1}. Anal. ($C_{25}H_{34}N_2O_6$) C, H, N.

Pharmacology. Inhibition of [^{3}H]Nitrendipine Binding. The ability of the compounds to block voltage-dependent Ca^{2+} channels in smooth muscle was evaluated by inhibition of specific [^{3}H]nitrendipine binding as previously described by Bolger and co-workers.[16] Briefly, terminal ileum from male guinea pigs was removed and placed in ungassed physiologic saline at 37 °C of the following composition (mM): NaCl, 137; KCl, 2.68; $CaCl_2$, 1.8; $MgCl_2$, 1.05; NaH_2PO_4, 0.36; $NaHCO_3$, 11.9; dextrose 5.55. The longitudinal muscle layer was removed, and the isolated strips were placed in ice cold 50 mM Tris·HCl buffer, pH 7.2, at 4 °C, minced, and homogenized in 10–20 volumes/wet weight tissue in ice-cold Tris buffer. The homogenate was then centrifuged at 4 °C at 11000g for 20 min, and the supernatant was centrifuged at 10000g for 10 min and then recentrifuged at 45000g for 45 min. The pellet from the 45000g spin was used for the binding studies. Protein concentration determination was by the method of Bradford.[21]

Membrane protein (20–80 μg) was incubated in a total volume of 5 mL of 50 mM Tris·HCl (pH 7.2) for 60 min at 25 °C with 0.1 nM [^{3}H]nitrendipine (2,6-dimethyl-3-ethoxy-5-[^{3}H]methoxy-4-(3-nitrophenyl)-1,4-dihydropyridinedicarboxylate, specific activity 80 Ci/mmol) (New England Nuclear, Boston, MA), and varying concentrations of specific compounds (where indicated). Equilibrium binding is reached in this period.[14] Nonspecific binding is defined in duplicate tubes by the addition of unlabeled 10^{-7} M nitrendipine. Binding experiments were performed in subdued light. Analyses of data employed standard programs[22] implemented on an IBM computer.

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Registry No. 1, 39562-16-8; **2**, 15802-69-4; **3**, 113567-97-8; **4**, 113567-98-9; **5**, 113567-99-0; **6**, 113568-00-6; **7**, 113568-01-7; **8**, 113568-02-8; (±)-**9**, 113568-03-9; **9**, 113568-12-0; (±)-**10**, 113568-04-0; **10**, 113568-13-1; (±)-**11**, 113568-05-1; **11**, 113568-14-2; (±)-**12**, 113568-06-2; **12**, 113568-15-3; (±)-**13**, 113568-07-3; **13**, 113568-16-4; (±)-**14**, 113568-08-4; **14**, 113568-17-5; **15**, 33736-01-5; **16**, 113568-09-5; **17** (isomer 1), 113568-10-8; **17** (isomer 2), 113584-19-3; **18**, 113568-11-9; **19**, 16436-00-3; **20**, 27618-18-4; **21**, 21829-28-7; **22**, 88284-24-6; $H_3CCOCH_2CO_2Et$, 141-97-9; $H_3CCOCH_2CO_2Me$, 105-45-3; $HO(CH_2)_2OH$, 107-21-1; $HO(CH_2)_4OH$, 110-63-4; $HO(CH_2)_6OH$, 629-11-8; $HO(CH_2)_8OH$, 629-41-4; $HO(CH_2)_{10}OH$, 112-47-0; $HO(CH_2)_{12}OH$, 5675-51-4; $H_3C(CH_2)_7OH$, 111-87-5; diketene, 674-82-8; ethyl aminocrotonate, 7318-00-5; 3-nitrobenzaldehyde, 99-61-6; 4-nitrobenzaldehyde, 555-16-8.

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Decomposition of *N*-(2-Chloroethyl)-*N*-nitrosocarbamoyl Amino Acid Amides

Helga Süli-Vargha,*† József Bódi,† Miomir Mészáros,‡ and Kálmán Medzihradszky‡

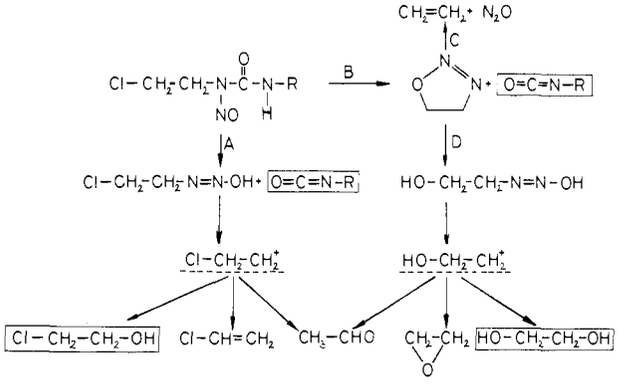
Research Group for Peptide Chemistry, Hungarian Academy of Sciences, H-1088 Budapest, Muzeum krt. 4/B, Hungary, and Institute of Organic Chemistry, Eötvös University, H-1088 Budapest, Muzeum krt. 4/B, Hungary. *Received December 7, 1987*

The chemical decomposition of *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl (Q(NO)) prolinamide and valinamide were studied under physiological conditions. The volatile products were identified with GC. Q(NO)-Pro-NH_2 gave twice the amount of ethylene glycol and only one-fifth of the 2-chloroethanol produced by Q(NO)-Val-NH_2 or BCNU, pointing to different pathways of their decomposition. The carbamoylating activity was also investigated in the presence of cyclohexylamine, and it was found to lead mainly to intramolecular carbamoylation with the formation of hydantoin derivatives.

The (2-chloroethyl)nitrosoureas (CENU-s) are highly active antitumor agents, and several of them are in clinical use as well. These compounds decompose rapidly under physiological conditions, producing alkylating and carbamoylating moieties (for a review, see ref 1).

The chemistry of BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] was perhaps the most thoroughly examined.[2-4] Brundrett has shown that BCNU decomposes at pH 7.4 through 2-chloroethanediazohydroxide and yields 2-chloroethanol and acetaldehyde as major products (Scheme I, path A), while at pH 5 it decomposes through 4,5-dihydro-1,2,3-oxadiazole with ethylene glycol and acetaldehyde as major products (Scheme I, paths B, D).[5] Previously, Colvin et al. observed that dMCNU (1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea), lacking in vitro antitumor activity, has a much higher half-life in aqueous solution at pH 7.4 than the other CENU-s, and its decomposition does not yield chloroethanol, but mainly acetaldehyde.[3] These differences were thought to be due to

Scheme I. Decomposition of (2-Chloroethyl)nitrosoureas in Aqueous Solution



the chemical structure of the molecule having a tertiary nitrogen atom in the N3 position of the urea, while other

† Hungarian Academy of Sciences.
‡ Eötvös University.

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Table I. Antitumor Activities of Q(NO) Amino Acid Amides on L1210 Leukemia in Mice[9]

compd	doses[a] mg/kg	doses[a] mmol/kg	% ILS[b]
Q(NO)-Pro-NH_2	100[c]	0.4	420
	200	0.8	tox
Q(NO)-Val-NH_2	20[c]	0.08	212
	40	0.16	tox
BCNU	20[c]	0.11	115
	38	0.21	tox

[a] Single ip dose on the first day after transplantation. [b] % ILS = (T − C)/C × 100 (T and C = median survival in days for treated and control groups, respectively). [c] The shown dosages were maximal effective ones.

CENU-s contain a secondary nitrogen atom in this position. This question was further investigated by Brundrett, who has shown that dMCNU decomposes at pH 7.4 via the oxadiazole intermediate, producing acetaldehyde and ethylene glycol, and therefore it is not cytotoxic to cells.[5] Later Brundrett et al. found dMCNU active against L1210 in mice, which they explained by an enzymatic demethylation process.[6]

While investigations for less toxic CENU-s among the amino acid derivatives were carried out, proline and sarcosine (chloroethyl)nitrosoureas were found to exert significant antitumor activity, both in vitro and in vivo, although they have a tertiary nitrogen atom in the N3 position, like dMCNU. For the investigation of their in vitro antitumor activity, Panasci et al.[7] used the human tumor stem cell assay, and in the case of the sarcosinamide congener, they observed a much higher antitumor activity ($P < 0.05$) against gliomas, than in that of BCNU. The in vivo antitumor activities of the (2-chloroethyl)nitrosourea derivatives of the amino acid amides were studied first by Suami et al.,[8] almost all of them showing excellent antitumor activity against L1210 leukemia in mice. It was of special interest that to achieve the same increase of life span, at least 4–8 times higher doses of the sarcosinamide derivative were required than in the case of the other amino acid derivatives. Besides, the contradiction seemed to arise that the other amino acid derivative, also possessing a tertiary nitrogen atom, the prolinamide congener, showed much less activity than the sarcosinamide congener. In order to study this discrepancy, we also synthesized and tested, among other amino acid derivatives, the *N*-(2-chloroethyl)-N-(nitrosocarbamoyl)prolinamide. Comparing the effective and the toxic doses, we found the proline derivative to have the same specific behavior as the sarcosine derivative had in the experiment of Suami et al. In Table I you can find several examples taken from our previous publication[9] to illustrate these differences: Q(NO)-Val-NH_2 and BCNU having both the effective and toxic doses in the same molar range, while in the case of the Q(NO)-Pro-NH_2 much higher doses were required (Q(NO):*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl). Recently we synthesized peptide CENU-s with proline terminus, and they also proved to be effective against L1210 leukemia in mice.[10]

Table II. Decomposition of Q(NO) Amino Acid Amides

compd	cc,[a] M × 10^{-3}	k (×10^2),[b] min^{-1}	$t_{1/2}$, min
Q(NO)-Val-NH_2	2.54	4.52 (4.35, 4.70)	15.5
Q(NO)-Pro-NH_2	2.28	0.274 (0.262, 0.288)	255.5

[a] 0.1 M phosphate buffer, 37 °C. [b] Figures in parentheses show 95% confidential limits.

Table III. Relative Yields of 2-Chloroethanol and Ethylene Glycol

compd (pH 7.4, 0.1 M phosphate buffer)	2-chloroethanol	ethylene glycol
BCNU	1 (51)[a]	1 (2)[a]
Q(NO)-Val-NH_2	1.17	0.97
Q(NO)-Pro-NH_2	0.23	1.77

[a] Product yields (mole percents of BCNU reacted; pH 7.4, 0.1 M phosphate buffer) were taken from the results of Brundrett.[5]

For the better understanding of the pharmacology of these compounds, we report the comparison between the aqueous decomposition at physiologic pH of Q(NO)-Val-NH_2 and Q(NO)-Pro-NH_2, the former possessing, the latter lacking an N3 hydrogen.

Results

The *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl amino acid amides were synthesized from the appropriate amino acid amides with the aid of *N*-(2-chloroethyl)-*N*-nitrosocarbamic acid succinimido ester.[8,10] Their decomposition was followed up under physiological conditions be measuring the decrease of the extinction at 400 nm, the λ_{max} for the *N*-nitroso bond in case of the valine derivative, and at 395 nm in case of the proline derivative. Rate constants and half-lives were calculated on the basis of apparent first-order kinetics (Table II).

The half-life data determined in this way differ to some extent from those calculated by Suami et al.,[8] presumably because of the different solution concentrations. Nevertheless we have also observed the especially long half-life (255.5 min) of the proline derivative, as compared to that (15.5 min) of the valine derivative.

To investigate the decomposition products, the amino acid derivatives were allowed to decompose at 37 °C in 0.1 M phosphate buffer at pH 7.4 in a sealed vial for 3 days. For the analysis of the volatile products, samples were taken from the liquid and gas phases for GC. First of all the presence of 2-chloroethanol and of ethylene glycol are to be examined from the decomposition products, because the presence or absence of either of them shows the pathway of the aqueous decomposition (see Scheme I).

From among the decomposition products of both amino acid derivatives we could confirm the presence of 2-chloroethanol, ethylene glycol, acetaldehyde, and vinyl chloride with the aid of control samples. A difference was found between the proportions of the ethylene glycol and the 2-chloroethanol; Q(NO)-Pro-NH_2 yielded two times more ethylene glycol than Q(NO)-Val-NH_2, and it produced one-fifth of the 2-chloroethanol produced from Q(NO)-Val-NH_2.

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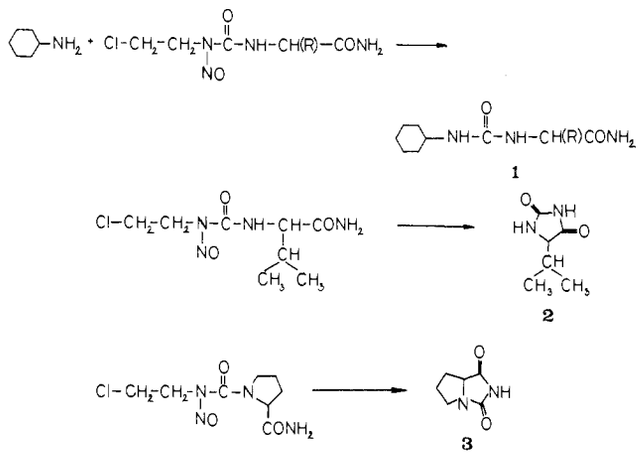
Scheme II. Carbamoylating Reactions of *N*-(2-Chloroethyl)-*N*-nitrosocarbamoyl Amino Acid Amides



To make these comparisons more concrete, we let BCNU decompose under the same conditions, and the products were submitted to the same GC analysis. It was found that the amounts of ethylene glycol and 2-chloroethanol produced from BCNU are nearly the same as those produced from Q(NO)-Val-NH_2. The relative yields of these decomposition products are shown in Table III, with the products of BCNU taken as references.

For the investigation of the nonvolatile products we used first thin-layer chromatography. Suami et al. reported that the aqueous decomposition of the (2-chloroethyl)nitrosourea congener of sarcosinamide yielded sarcosinamide.[8] In the case of Q(NO)-Pro-NH_2, on the chromatogram we have identified proline and, in accordance with the above-mentioned observation, prolinamide too. However, under the same experimental conditions in the buffered solution of Q(NO)-Val-NH_2, only a single ninhydrin-negative spot could be detected. This compound was extracted with ethyl acetate from the buffer solution and was found to possess a hydantoin structure according to the IR spectrum. This observation points to the occurrence of intramolecular carbamoylation. Since carbamoylation plays an important role in the pharmacological action of the CENU-s, we performed some further experiments to study this reaction.

Boivin and Boivin[11] described the formation of substituted ureas from nitrosomethylureas and amines in boiling water. As a model compound bearing a primary amino group, we chose cyclohexylamine (CHA) and thought that its reaction with a (2-chloroethyl)nitrosocarbamoyl amino acid would result in the formation of the appropriate substituted urea 1 (Scheme II). However, when Q(NO)-Val-NH_2 was boiled in water in the presence of CHA, the expected urea derivative (cyclohexylcarbamoyl)valinamide was only formed in a negligible amount. As a main product, the same compound could be isolated, as it was previously found under physiological conditions. According to IR and NMR spectra, this compound proved to be 5-isopropylhydantoin **2**. The carbamoylating activity of Q(NO)-Pro-NH_2 was investigated in the same way. In this case also a hydantoin derivative was isolated as main product, identified by IR and NMR spectra as 1,3-diazabicyclo[3.3.0]octane-2,4-dione **3**. Among the byproducts small amounts of (cyclohexylcarbamoyl)prolinamide were separated, while proline and polinamide were only identified on TLC. It should be mentioned that under alkaline conditions (2-chloroethyl)carbamoyl amino acids cyclize into hydantoin derivatives as well.[12]

Discussion

Many investigations have been carried out on the decomposition of the CENU-s to determine the role of alkylating and carbamoylating activity in the antitumor action of these compounds. It is now widely accepted that the main reason for their antitumor activity is the alkylation and the cross-linking of DNA strand pairs by the chloroethyl carbonium ion. Carbamoylation is thought to be caused by organic isocyanates on proteins, resulting in the inactivation of certain enzymes. The carbamoylating activity is greatly reduced in the case of compounds that readily undergo intramolecular cyclization reaction, e.g. several CENU-s of sugars and nucleosides.

Our present investigations on the carbamoylating activity of two structurally different representatives of another class of CENU-s, of (2-chloroethyl)nitrosourea congeners of amino acid amides show that during the decomposition of these compounds mainly intramolecular carbamoylation occurs.

The reaction was studied in model experiments, in the course of which we let decompose Q(NO)-Val-NH_2, where the Q(NO) group was coupled to a secondary nitrogen atom, and Q(NO)-Pro-NH_2, where the same group was attached to a tertiary nitrogen atom in the presence of a primary amine (CHA) in boiling water. Under such conditions, usually substituted urea derivatives are formed, but in the case of the (2-chloroethyl)nitrosourea congeners of the amino acid amides these compounds were produced only in a negligible amount. As main products we isolated in both cases hydantoin derivatives **2** and **3**. It is worth mentioning that, in the presence of CHA, even Q(NO)-Pro-NH_2 got transformed into the bicyclic hydantoin **3** rather than into the appropriate urea derivative. This observation shows that carbamoylation also occurs when the (2-chloroethyl)nitrosocarbamoyl group is coupled to a tertiary nitrogen atom and the formation of an isocyanate is impossible. Similar observation was made in the case of 1-(2-chloroethyl)-3,3-disubstituted-1-nitrosoureas having a hydroxyl group at the β-position of the substituents, when the intramolecular carbamoylation caused the formation of oxazolidinones.[13] However, under physiological conditions without CHA, only Q(NO)-Val-NH_2 was transformed to the hydantoin **2** while Q(NO)-Pro-NH_2 generated proline and prolinamide. To make it sure that no hydantoin derivative was formed as an intermediate through the decomposition of Q(NO)-Pro-NH_2 at physiological conditions, we performed a control experiment and found that **3**, after standing for 3 days in phosphate buffer solution at pH 7.4, remained practically unchanged according to TLC.

A comparison of the amounts of 2-chloroethanol and ethylene glycol produced during the decomposition of Q(NO)-Val-NH_2 and BCNU shows that the pathway of their chemical decomposition is very similar, following mainly path A in Scheme I. Q(NO)-Pro-NH_2 produced two times more ethylene glycol than BCNU or Q(NO)-Val-NH_2 did and only one-fifth of the 2-chloroethanol produced by BCNU or Q(NO)-Val-NH_2. Certainly there can be no direct quantitative correlation between the amount of the generated 2-chloroethanol and the antitumor activity of the CENU-s, since, beside acetaldehyde and vinylchloride, this is only one of the major end products of the alkylating

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chloroethyl carbonium ion. However, the significantly less amount of 2-chloroethanol produced during the chemical decomposition of Q(NO)-Pro-NH_2 indicates that this compound decomposes simultaneously on paths A and B, with path B through the oxadiazole intermediate playing a greater role here than in the case of BCNU or Q-(NO)-Val-NH_2. This observation may explain why the effective and toxic doses of sarcosinamide and prolinamide are several times higher than those of the other amino acid derivatives.

Experimental Section

NMR spectra were recorded on Bruker WM 250 spectrometer; IR and UV spectra were recorded on Specord IR 75 and Specord UV–vis spectrometers (Karl Zeiss, Jena), respectively.

Gas chromatography was performed on a Hewlett-Packard 5700 A GC glass column. Detection was carried out with F 10, with N_2 as a carrier gas. Acetaldehyde and vinyl chloride were detected on a Chromosorb 101 column (3 × 1000 mm, 90 °C). 2-Chloroethanol was determined on a column of 2% Carbowax 20 M on Gaschrom Q 100–200 mesh at 70 °C; diacetyl ethylene glycol was determined on a column of 2% OV 225 on Gaschrom Q 100–120 mesh at 80 °C. TLC was carried out on Merck DC-Alufolien Kieselgel 60. Column chromatography was performed on silica gel (Merck, Kieselgel 60, Art 10832). Solvent systems for chromatography were the following (v/v): A, butanol–acetic acid–water, 4:1:1; B, butanol–pyridine–acetic acid–water, 4:1:1:1; C, chloroform–methanol, 9:1.

Detection was made by ninhydrin and Cl_2–tolidine.

Decomposition Studies. A 0.05-mmol portion of the nitrosourea (Q(NO)-Val-NH_2, Q(NO)-Pro-NH_2, BCNU) was allowed to decompose in 1.5 mL of 0.1 M sodium phosphate buffer at 37 °C in a sealed vial under nitrogen atmosphere for 3 days.

Identification of the Volatile Products. A sample was taken from the gas phase and injected into the gas chromatograph for detection of acetaldehyde and vinyl chloride. Then, the buffer solution was divided, half of it was used for the detection of 2-chloroethanol, the other half for the detection of ethylene glycol.

2-Chloroethanol. The buffer solution was saturated with sodium chloride and extracted with ether, and the ether solution was injected into the GC.

Ethylene Glycol. The buffer solution was concentrated in vacuo, and acetic acid anhydride was added to the residue and evaporated again. Once more acetic acid anhydride was added to the residue, and the solution was allowed to stand for 2 h at 98 °C in a sealed flask. After evaporation of the acetic acid anhydride, 0.5 mL of water was added to the residue, and it was extracted twice with 0.5 mL of chloroform. The combined chloroform solutions were dried on Na_2SO_4 and injected into the GC.

Identification of the Nonvolatile Products. TLC was performed from the buffer solution in solvent system A and B with the appropriate amino acid amide and amino acid as controls.

Reaction with CHA. (a) Q(NO)-Val-NH_2 (0.5 g, 2 mmol) was boiled in 4 mL of water in the presence of 0.24 mL (2 mmol) of CHA for 45 min. After the reaction mixture was cooled, 28 mg (6%) of (cyclohexylcarbamoyl)valinamide was filtered off. R_f: 0.81 (A). IR: ν 3370, 3348, and 3195 (NH_2, NH), 2932 and 2857 (CH_2), 1672 (amide I), 1616 (amide II, NHCONH), 1577 (NH_2) cm^{-1}.

The filtrate was saturated with sodium chloride and extracted once with chloroform and twice with ethyl acetate. The combined ethyl acetate solutions were dried on Na_2SO_4 and concentrated in vacuo. The residue (240 mg, 91%) was crystallized from ethyl acetate–cyclohexane to give 5-isopropylhydantoin (160 mg, 60%). Anal. ($C_6H_{10}N_2O_2$) C, H, N. R_f: 0.72 (A), 0.38 (C). Mp: 140–141 °C. IR: ν 3295 and 3400–2700 (NH), 1765 and 1724 (CO) cm^{-1}. (NH), 1765 and 1724 (CO) cm^{-1}. ^{13}C NMR (DMSO-d_6): δ 17.7 and 20.0 (CH_3), 31.3 (CCHC), 64.6 (NCH), 159.5 (NCON), 176.9 (NCO).

(b) Q(NO)-Pro-NH_2 (1 g, 4 mmol) was boiled in 10 mL of water in the presence of 0.48 mL (4 mmol) of CHA for 45 min. Then, the reaction mixture was neutralized with 1 N HCl solution and evaporated in vacuo. The residue was separated on a silica gel column in solvent system C. (Cyclohexylcarbamoyl)prolinamide was isolated with 8% yield (7.8 mg). R_f: 0.38 (C), 0.73 (A). IR: ν 3370 and 3205 (CH_3), 1675 (amide I), 1630 and 1545 (amide II), 2965, 2935, and 2858 (CH_2). The main product 1,3-diazabicyclo[3.3.0]octane-2,4-dione was isolated in pure form in 45% yield (252 mg). A sample was crystallized from absolute benzol for analysis. Anal. ($C_6H_8N_2O_2$) C, H, N. R_f: 0.47 (C), 0.65 (A). Mp 134–135 °C. IR: ν 3185 (br, NH), 1757, 1715 (CO) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.7–2.3 (CCH_2CH_2C, several m 4 H), 3.22 and 3.72 (NCH_2, 2 m, 2 H), 4.14 (CH, dd, J = 7.5, 9.7 Hz), 9.2 (NH, br s, 1 H). ^{13}C NMR ($CDCl_3$): 26.9 and 27.3 (CCH_2CH_2C), 45.2 (NCH), 64.6 (NCH), 160.5 (NCON), 174.6 (CON).

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Registry No. 1 (R = $CH(CH_3)_2$), 114200-02-1; 2, 16935-34-5; 3, 40856-87-9; Q(NO)-Pro-NH_2, 81965-44-8; Q(NO)-Val-NH_2, 81965-27-7; 2-chloroethanol, 107-07-3; ethylene glycol, 107-21-1; acetaldehyde, 75-07-0; vinyl chloride, 75-01-4; cyclohexylamine, 108-91-8; (cyclohexylcarbamoyl)-Pro-NH_2, 114200-03-2.