(8) A. A. Seida, A. D. Kinghorn, G. A. Cordell, and N. R. Farnsworth, *Lloydia*, in press.
(9) S. M. Kupchan, J. A. Lacadie, G. A. Howie, and B. R. Sickles, *J. Med. Chem.*, **19**, 1130 (1976).
(10) M. E. Wall and M. C. Wani, *Ann. Rev. Pharmacol. Toxicol.*, **17**, 117 (1977).
(11) One of the referees has suggested that the severe changes in functionality produced as a consequence of the cleavage of the hemiketal moiety in **3**, e.g., replacement of the C-11 hydroxyl by ketone, may play an important role in the antitumor activity requirements.

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Received July 24, 1978

Articles

Collagenase-Sensitive Peptidyl-Nitrogen Mustards as Potential Antitumor Agents

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Contribution No. 2124 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898. Received March 16, 1978

Attempts to design an agent which would release cytotoxic nitrogen mustards within collagenase-producing tumors led to the synthesis of Cbz-L-Pro-L-Leu-Gly-L-Pro-Gly-$NHC_6H_4N(CH_2CH_2Cl)_2$ (**10**). **10** was cleaved in vitro by bacterial and tumor-associated collagenase as expected at the peptide bond joining L-leucine and glycine to give Gly-L-Pro-Gly-$NHC_6H_4N(CH_2CH_2Cl)_2$ which was over six times more toxic, on a molar basis, than **10**. In vivo tests of **10** against well-advanced Sarcoma-180 gave disappointing results. The lack of specific antitumor activity may be accounted for by the presence of competing cleavage reactions by collagenases in certain normal tissues.

The usefulness of most antitumor agents has been severely limited by their lack of specificity. One approach to circumvent this involves administration of antitumor agents in inactive or latentiated forms.[1-3] Harper[4] has defined drug latentiation as the chemical modification of a biologically active compound to form a new compound which liberates this active compound on in vivo enzymatic attack.

In order to achieve the maximum benefit in cancer therapy, the enzyme which acts upon the latentiated agent should be associated with the targeted tumor tissue.[5] A proposal for using peptide-latentiated nitrogen mustards was originally made by Danielli in 1953.[5b] Unfortunately, most studies of such latentiated antitumor agents have failed to exploit qualitative or quantitative differences between tumor and normal tissue. This study attempts to exploit tumor-associated collagenase[6-12] by using a nitrogen mustard inactivated by a collagenase-sensitive peptide.

Collagenase is a highly specific[13,14] collagen-degrading enzyme[15] which is elaborated by many, if not all, slow-growing tumors.[6-12] It has been suggested that the enzyme aids tumor infiltration of healthy tissue by degrading the surrounding connective tissue.[7,8] This mechanism may be important in tumors of mesodermal origin since collagenase is absent from the normal striated muscle and fibrous connective tissue from which these collagenase-containing tumors arise.[11]

The foregoing suggests that a synthetic agent may have tumor-specific activity if it can be activated within the tumor by either endogenous or artificially introduced[16] collagenase. We therefore synthesized a nitrogen mustard peptide derivative designed to serve as a substrate for tumor-localized collagenase.

The mustard portion of this compound was derived from *N,N*-bis(2-chloroethyl)-*p*-phenylenediamine (**2**). This parent mustard was selected because it is highly cytotoxic but, as suggested initially by Danielli,[5b] can be rendered relatively nontoxic by acylation with groups which are not easily removable by normal mammalian enzymes. (Such acylating groups include alkyl- or arylcarboxylic acid residues[18,19] as well as amino acids[20,21] or serum proteins.[22])

In the current study we report on several of the properties of compound **10** of Table I. In this compound the acyl moiety is Cbz-L-Pro-L-Leu-Gly-L-Pro-Gly. The first four amino acids, starting at the N-terminus, have been sequentially arranged the same way as they are in the Wünsch–Heidrich substrate,[23] which has been shown to be cleaved by a tumor-associated collagenase.[8] The bond joining the L-leucyl and glycyl residues in this peptide is sensitive to cleavage by collagenase. The fifth amino acid, glycine, was added to move the sensitive bond further from the sterically bulky M. The Cbz moiety was attached to the N-terminus of the peptide to prevent premature chain degradation by aminopeptidases. We expected that collagenase cleavage of the acylated mustard would result in liberation of a tripeptide nitrogen mustard (Gly-L-Pro-Gly-M) which could then serve as a substrate for aminopeptidases. If such were the case, the removal of the remaining two glycyl residues and the prolyl residue would lead to the liberation of the highly cytotoxic HM (**2**, Table I).

Whole animal toxicity studies of model compounds in mice (Table I) indicated that this sequence of events should take place with **10**. The molar toxicities of peptidyl nitrogen mustard compounds fell into two distinct classes: those whose toxicities were similar to that of the parent mustard (HM) and those which showed significantly reduced toxicities. The factor which appears to determine the class into which a peptidyl nitrogen mustard falls is the ease of removal of the group attached to the N-terminus of the peptidyl chain by mammalian enzymes. Relatively resistant groups include Cbz, *t*-Boc, D-phenylalanine, and D-lysine while normal L-configuration

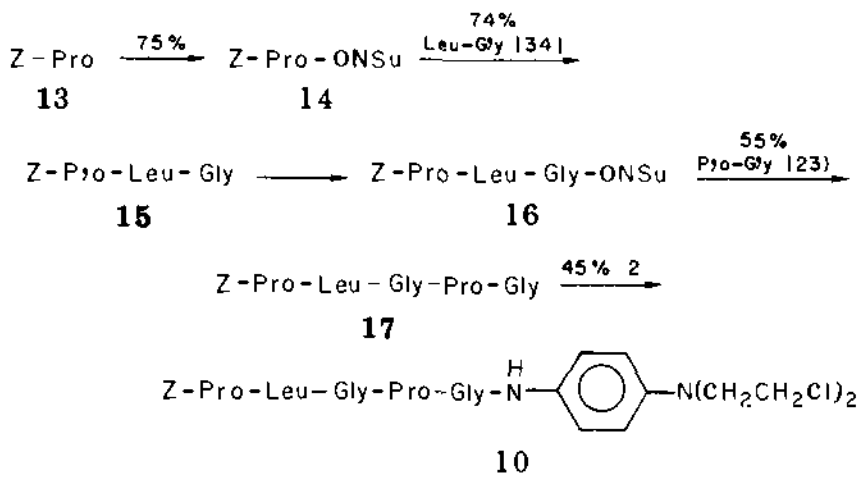
0022-2623/78/1821-1188$01.00/0

Table I. Toxicities of Nitrogen Mustards in Mice

no.	compd tested[a]	14-day LD_{50} in μmol/kg
1	L-Leu-Gly-L-Pro-Gly-M·F_3AcOH[b,c]	10.4
2	HM·HCl	10.8
3	Gly-L-Pro-Gly-M·F_3AcOH	19.7
4	D-Lys-L-Pro-L-Leu-Gly-L-Pro-Gly-M·2F_3AcOH	78
5	*t*-Boc-D-Ala-L-Pro-L-Leu-Gly-L-Pro-Gly-M[d]	94.5
6	bis(*t*-Boc)-D-Lys-L-Pro-L-Leu-Gly-L-Pro-Gly-M	100
7	*t*-Boc-D-Phe-L-Pro-L-Leu-Gly-L-Pro-Gly-M	105
8	D-Phe-L-Pro-L-Leu-Gly-L-Pro-Gly-M·F_3AcOH	106
9	Cbz-L-Pro-Gly-M[e]	119
10	Cbz-L-Pro-L-Leu-Gly-L-Pro-Gly-M	123

[a] Tests were carried out on Carworth Farms CF,S strain, female mice weighing 16-20 g. For standard dose levels of compound, a 78-mg sample was prepared at an initial concentration of 13 mg/mL in a 1% aqueous solution of Methocel (methylcellulose, viscosity 100 Hz, grade MC from Dow Chemical Co.; for compounds not soluble in Methocel solution, the mixture was ultrasonicated in a 28 × 59 mm specimen vial using a Blackstone ultrasonic probe system equipped with a 0.5-in. diameter probe). Dilutions of the stock solution were made with Methocel to give doses in the range of 4-324 mg/kg, based on an average mouse body weight of 18 g and a dose of 0.45 mL per mouse. Five mice were tested at each dosage by the intraperitoneal route, and the number of survivors was noted 14 days after the injection. We thank Dr. D. H. Smith of Du Pont Biochemicals Department for these studies. [b] F_3AcOH = trifluoroacetic acid. [c] M = -$NHC_6H_4N(CH_2CH_2Cl)_2$. [d] *t*-Boc = *tert*-butyloxycarbonyl. [e] Cbz = benzyloxycarbonyl.

Scheme I. Synthesis of Pentapeptide Mustard 10[a]



[a] -ONSu = *N*-hydroxysuccinimidyl ester.

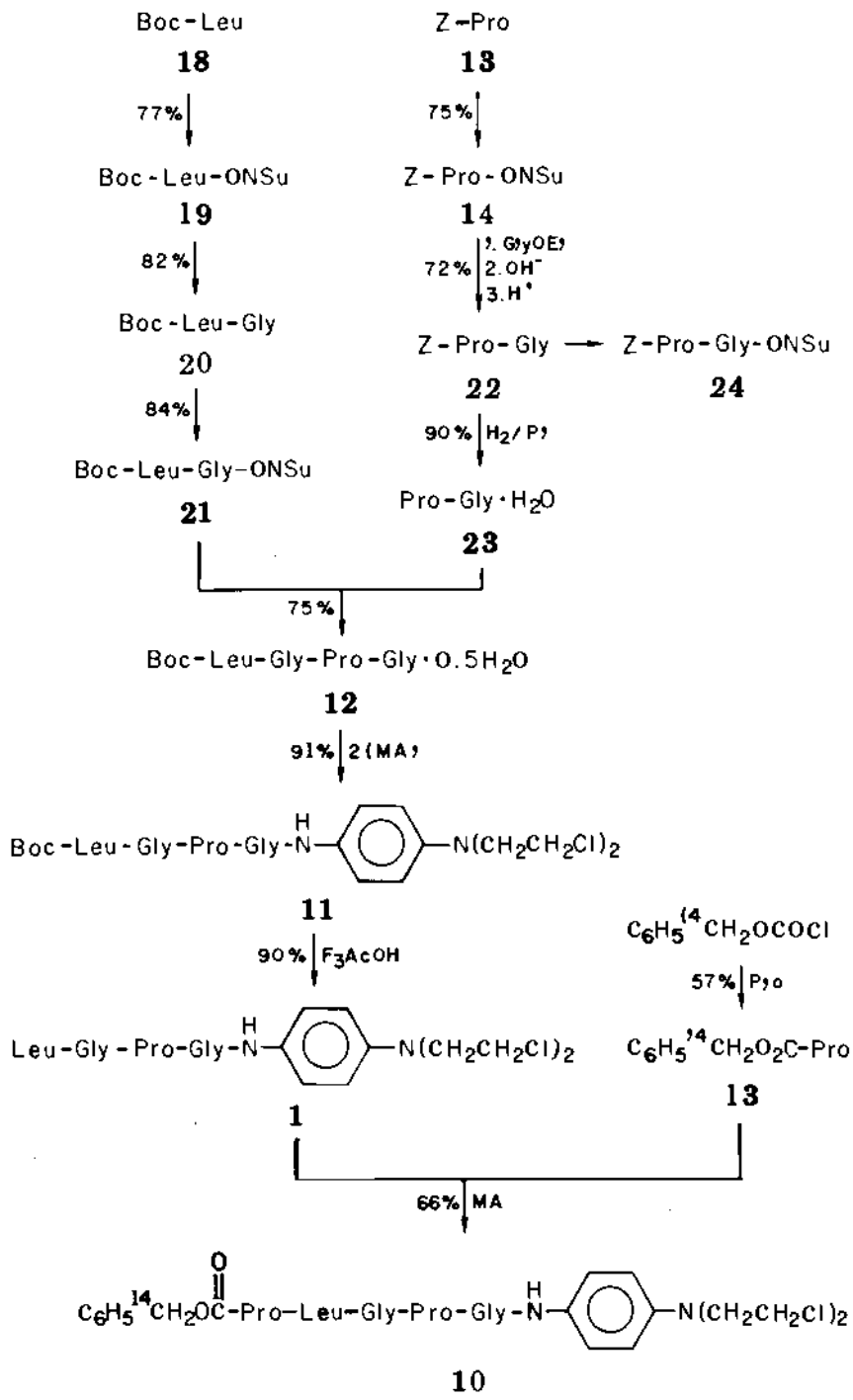
amino acids are easily removed (Table I).

To facilitate the study of the key reactions, **10** was synthesized in which the benzyloxycarbonyl methylene carbon was labeled with ^{14}C (3.8 mCi/mmol). The low aqueous solubility of the labeled compound necessitated the development of a collagenase assay system in which both enzyme and substrate were solubilized under conditions where the enzyme remained active. A pH 7.3 calcium acetate containing reaction mixture made 28% in dimethyl sulfoxide (Me_2SO) satisfied this requirement.

Results

In the presence of bacterial collagenase, or the tumor homogenate, the disappearance of **10** was accompanied by the simultaneous appearance of a unique radioactive substance, indicated by arrows in Figures 1 and 2 which depict TLC autoradiographs of the reaction mixtures.

Scheme II. Synthesis of ^{14}C-Labeled Pentapeptide Mustard 10[a]



[a] MA = mixed anhydride synthesis; F_3AcOH = trifluoroacetic acid.

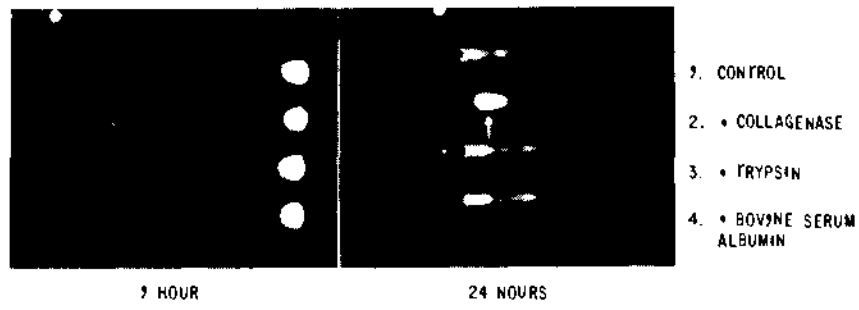


Figure 1. Autoradiograph of TLC plate: reaction mixtures containing proteins and ^{14}C-labeled 10.

This substance could be extracted with chloroform from the incubation mixtures which had been acidified with hydrochloric acid. The acidic components in the chloroform layer could then be extracted into 10% aqueous sodium carbonate. Upon acidification of the carbonate solution, followed by its chloroform extraction, a single radioactive substance was isolated which was isographic with an authentic sample of the expected cleavage product, carbobenzyloxy-L-prolyl-L-leucine. This acidic compound was not produced in collagenase-free controls or in controls in which trypsin or bovine serum albumin was added in place of collagenase.

The transitory appearance of additional spots in the autoradiographs (Figures 1 and 2) suggests that **10** participates in some nonenzymatic side reactions. The

Figure 2. Autoradiograph of TLC plate: reaction mixtures containing collagenase or tumor homogenate and ^{14}C-labeled **10**.

products of these side reactions were neutral and served as substrates for collagenolytic degradation. They may arise from the slow S_N1 hydrolysis of the chloroalkyl portion of the molecule.

Testing of compounds **4** and **10** in mice with well-advanced Sarcoma-180 tumors demonstrated no uniform antitumor activity when the compounds were administered by a single intraperitoneal dose over a range of ca. 10–50% of the LD_{50}.

Subsequently it was found that homogenates of mouse, pig, rat, and guinea pig liver produced cleavage of labeled compound **10** to release labeled carbobenzyloxy-L-prolyl-L-leucine. This result is consistent with recent reports of the existence of collagenase in certain normal mammalian tissues.[24-33]

Discussion

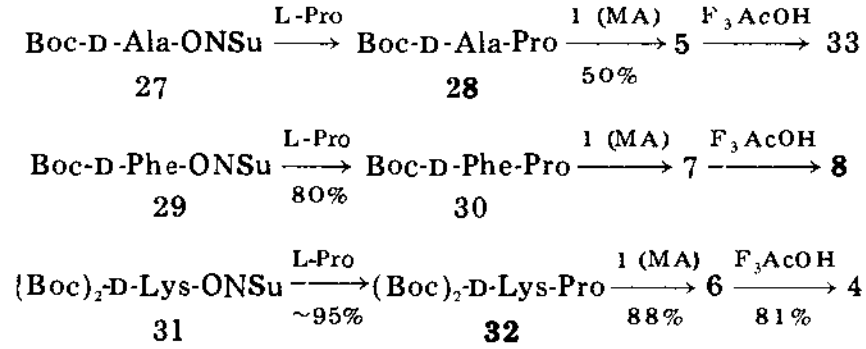
The pattern of toxicities of compounds **1–10** (Table I) is consistent with the masking of the nitrogen mustard **2** with a peptide residue. The results of the in vitro observations of the collagenase and tumor extract mediated cleavage of **10** indicate that the desired cleavage of the masking group occurs. In the whole animal, however, premature cleavage and/or chloroalkyl hydrolysis of **10** appears to compete successfully with the desired tumor-specific cleavage.

It is also possible that insufficient aminopeptidase activity in the tumor may limit the cleavage of the liberated Gly-Pro-Gly-M to HM (**2**) and prevent the achievement of a therapeutic concentration of the free nitrogen mustard. Such a retardation of hydrolysis has been reported with the Walker carcinosarcoma 256; cleavage of the Gly-M bond was slower than in normal tissue.[19,20] Dalton and Hebborn noted, however, that enzymatic cleavage of the acetyl–glycine bond of the acylated peptide mustard, Ac-Gly-M, was still rate limiting, and this latter compound exhibited a good therapeutic index in the Walker tumor.[21] Apparently, even in this tumor with depressed aminopeptidase activity, the liberation of the mustard HM (**2**) occurred sufficiently rapidly to produce a therapeutic effect.

Synthesis. The peptide intermediates were synthesized as indicated in the flow sheets of Schemes I–III by the reaction of N-blocked amino acid or peptide *N*-hydroxysuccinimide esters with amino acids or dipeptides or by the mixed anhydride synthesis. Activation of peptide carboxylates (other than those with C-terminal glycine or proline) was avoided in order to minimize racemization. Coupling of peptide segments to the *p*-phenylenediamine mustard **2** was generally not satisfactory using active ester syntheses but could readily be achieved using the mixed anhydride procedure.

The *tert*-butyloxycarbonyl tetrapeptide mustard **11** was deblocked with trifluoroacetic acid to give **1** which was used to synthesize ^{14}C-labeled **10** (Scheme II) as well as the D-amino acid terminated peptides **4–8** (Scheme III). An alternate route to **10** was provided by the mixed anhydride coupling of carbobenzyloxy-L-prolyl-L-leucyl-glycyl-L-prolylglycine (**17**) to **2** (Scheme I). The properties of new compounds are given in Tables II and III.

Scheme III. Synthesis of D-Amino Acid Blocked Hexapeptide Mustards[a]



[a] All amino acids are in the L configuration unless otherwise noted. See footnotes in Schemes I and II.

Experimental Section

Optical rotations were measured with a Hilger and Watts polarimeter (1-dm cell). Melting points are uncorrected. The amino acids used were all of the L configuration unless otherwise noted. Standard peptide nomenclature is used.[34]

***N,N*-Bis(2-chloroethyl)-*p*-phenylenediamine Hydrochloride (2).** *Warning: This compound is highly toxic and is a vesicant even in dilute aqueous solution.*[39] *The intermediate acetyl derivative is a skin irritant. These materials should be handled with rubber gloves in a hood.* The monohydrochloride **2** originally reported by Everett and Ross[39] was prepared by the method of Szekerke et al.[40] The pinkish crystalline monohydrochloride melted at 243–246 °C (lit.[40] mp 240 °C).

Synthesis of *N*-Hydroxysuccinimide Esters[35] (Table II). A solution of 0.25 mol of the protected amino acid or peptide and 0.25 mol of *N*-hydroxysuccinimide in 1 L of dry 1,2-dimethoxyethane or chloroform was cooled quickly in an ice bath, and a chilled solution of 0.26 mol of dicyclohexylcarbodiimide (*warning: causes skin sensitization, avoid contact*) in 200 mL of 1,2-dimethoxyethane was added and mixed. The resulting solution was held at 0–5 °C overnight. The solvent was stripped and the residue was recrystallized quickly from boiling ethanol unless otherwise noted in Table II. Excessive heating with ethanol results in contamination by the ethyl ester. Many of these compounds have limited stability and should be used promptly.

Peptide Couplings with *N*-Hydroxysuccinimide Esters.[35] A solution of 5 mmol of N-protected amino acid or peptide *N*-hydroxysuccinimide ester in 25–100 mL of 1,2-dimethoxyethane (depending on solubility) or ethanol (see Table II) was chilled in ice. (Where indicated, 0.5 vol of methanol was added.) Then a solution of 5 mmol of amino acid or dipeptide and 10 mmol of sodium bicarbonate in 15 mL of water was added in one portion. The solution was stirred overnight at 4 °C and was then concentrated to a volume of 5–10 mL. The solution was filtered, and the filtrate was extracted with ether (discarded). The aqueous layer was acidified to pH 1.5–2 (HCl) and twice extracted with chloroform. The chloroform extracts were dried ($MgSO_4$) and concentrated. The residue was crystallized as indicated in Table II.

Coupling of Peptides to *N,N*-Bis(2-chloroethyl)-*p*-phenylenediamines. The general procedure is described for a 7-mmol preparation. A solution of 7.00 mmol of the N-protected peptide and 0.708 g of purified (distilled from CaH_2, stored under N_2) *N*-methylmorpholine in a mixture of 20 mL of dry dioxane and 20 mL of dry tetrahydrofuran in a 250-mL flask was cooled below 0 °C (methanol-ice), and a solution of 0.956 g of isobutyl chloroformate in 5 mL of tetrahydrofuran was added. Two minutes later was added a solution prepared by the addition of 0.708 g of *N*-methylmorpholine to a stirred solution of 1.887 g of *N,N*-bis(2-chloroethyl)-*p*-phenylenediamine hydrochloride (*caution: vesicant*) in 6 mL of dioxane and 3 mL of water. The mixture was stirred 30 min and then poured under nitrogen into 500 mL of a deaerated ice-water mixture. A curdy, whitish solid or heavy oil separated and adhered to the flask walls as the mixture was warmed to room temperature. The solvent was decanted and fresh, deaerated water was added, and the mixture was allowed to stand overnight at 5 °C under nitrogen. The water was decanted and the solid was purified as indicated in Table III.

Table II. Peptide Intermediates

no.	compd	mp, °C	$[\alpha]^{25}_D$, deg (*c*, solvent)	yield, % (recry-std)	synth method (solvent)[a]	reagents employed	purificn method[b]	sch-eme	formula	analyses
12	Boc-Leu-Gly-Pro-Gly·0.5H_2O	87-102	−63 (1.02, $CHCl_3$)	75	NSu (E)	21 + 23[c]	ES	II	$C_{20}H_{34}N_4O_7$·0.5H_2O	C, H, N
15	Z-Pro-Leu-Gly	163.8-164.4[d]	−81[e] (1.07, EtOH)	~80	NSu (D + M)	14 + 34[f]	W	I	$C_{21}H_{28}N_3O_6$	
16	Z-Pro-Leu-Gly-ONSu	153.2-156.8	−75 (1.01, $CHCl_3$)		DCCI (C)	15 + HONSu	M/P	I	$C_{30}H_{39}N_5O_9$	C, H
17	Z-Pro-Leu-Gly-Pro-Gly·H_2O	90 (softens)	−109 (1.01, $CHCl_3$)	55	NSu (D + M)	16 + 23	CE	I	$C_{28}H_{39}N_5O_8$·H_2O	C, H, N
20	Boc-Leu-Gly	122-123	−18 (1.00, $CHCl_3$)	82	NSu (E)	18 + Gly	EAH	II	$C_{13}H_{24}N_2O_5$	C, H, N
21	Boc-Leu-Gly-ONSu	117-118	−29 (1.02, $CHCl_3$)	82	DCCI (D)	20 + HONSu	E	II	$C_{17}H_{27}N_3O_3$	C, H, N
22	Z-Pro-Gly	124-128[g]	−61 (5.0, MeOH)	74	NSu (E)	14 + Gly	EH	II	$C_{15}H_{18}N_2O_5$	
24	Z-Pro-Gly-ONSu	141-143.2	−71 (1.01, $CHCl_3$)	43	DCCI (D)	22 + HONSu	A	II	$C_{19}H_{21}N_3O_7$	C, H, N
26	Boc-Gly-Pro-Gly	178.2-179.6	−92 (1.01, MeOH)	58	NSu (D + M)	Boc-Gly-ONSu[h] + 23[c]	EA/A		$C_{14}H_{23}N_3O_6$	C, H, N
27	Boc-D-Ala-ONSu	159.6-162.8	+35 (1.03, $CHCl_3$)	89	DCCI (D)	Boc-D-Ala[i] + HONSu	DH	III	$C_{12}H_{18}N_2O_6$	N
28	Boc-D-Ala-Pro	136.2-139.2	−75 (1.08, $CHCl_3$)	~60	NSu (D)	27 + Pro	*j*	III	$C_{13}H_{22}N_2O_5$	C, H, N
29	Boc-D-Phe-ONSu	150.1-153.1	+13 (1.03, $CHCl_3$)	84	DCCI (D)	Boc-D-Phe[i] + HONSu	H	III	$C_{18}H_{22}N_2O_6$	C, H, N
30	Boc-D-Phe-Pro	179.2-180	−112 (1.01, $CHCl_3$)	80	NSu (D)	30 + Pro	EAH	III	$C_{19}H_{26}N_2O_5$	C, H, N
31	$(Boc)_2$-D-Lys-ONSu	100.5-105.2	+16 (1.03, $CHCl_3$)	42	DCCI (D)	$(Boc)_2$-D-Lys[k] + HONSu	E	III	$C_{20}H_{33}N_3O_8$	C, H, N
32	$(Boc)_2$-D-Lys-Pro·0.5H_2O	glass		95	NSu (D)	31 + Pro	*l*	III	$C_{21}H_{37}N_3O_7$·0.5H_2O	C, H, N

[a] DCCI = dicyclohexylcarbodiimide method; NSu = *N*-hydroxysuccinimide method.[35] Reaction solvents: E = ethanol, D = 1,2-dimethoxyethane, M = methanol, C = chloroform. [b] Recrystallization solvents: A = ethanol; CE = dissolved in chloroform, ether added; DH = 1,2-dimethoxyethane-hexane; M/P = dichloromethane-hexane and then 2-propanol (compound reacts rapidly with primary alcohols); E = ether; EA/A = ethyl acetate and then acetone; EAH = ethyl acetate-hexane; ES = stirred 3 days with ether, crystals filtered off and dried; H = hexane; W = water. [c] Pro-Gly·H_2O in 90% yield by hydrogenation of 15 g of Z-Pro-Gly in 55 mL of H_2O + 55 mL of CH_3OH + 1.5 mL of CH_3COOH; 0.4 g of 5% Pd/C; 3 atm of H_2.[37] [d] Lit.[36] mp 163.5–164 °C; $[\alpha]^{23.5}_D$ −85.2° (*c* 2, ethanol). [e] $[\alpha]_D$ at 24°. [f] Leu-Gly hemihydrate from Cyclo Chemical Co., now Vega Biochemicals, Tucson, Ariz. [g] Lit.[37] mp 123–124 °C; $[\alpha]^{21}_D$ −63.2° (*c* 5, methanol). [h] Boc-Gly-ONSu from Fluka, Tridom Chemical Inc., Hauppauge, N.Y. [i] Boc-D-Ala, Boc-D-Phe, and D-Lys from Vega Biochemicals. [j] Crystallized when triturated with 2:1 hexane-ether. [k] $(Boc)_2$-D-Lys dicyclohexylamine salt (from D-lysine[1,38]) in chloroform was twice extracted with 0.4% HCl. The chloroform solution was stripped, and the oily residue was reacted with equimolar portions of dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in dimethoxyethane. [l] Obtained as a glassy solid; used without further purification.

Table III. Peptide Nitrogen Mustards

no.	compd[a]	mp, °C	$[\alpha]^{25}_D$, deg (c, solvent)	yield, % crude	yield, % recrystd	synth method[b]	reagents employed[b]	purifcn method[c]	formula	analyses
1	Leu-Gly-Pro-Gly-M·F_3AcOH	63 (softens)	+20 (1.01, $CHCl_3$)	90		F_3AcOH	11 + F_3AcOH	T	$C_{27}H_{39}Cl_2F_3N_6O_9$	C, H, N, Cl, F
3	Gly-Pro-Gly-M·F_3AcOH	112 dec	+40 (1.00, $CHCl_3$)	87		F_3AcOH	25 + F_3AcOH	T	$C_{21}H_{28}Cl_2F_3N_5O_5$	C, H, N, Cl, F
4	D-Lys-Pro-Leu-Gly-Pro-Gly-M·$2F_3$AcOH	142-146		81		F_3AcOH	6 + F_3AcOH	T	$C_{40}H_{59}Cl_2F_6N_9O_{10}$	N
5	Boc-D-Ala-Pro-Leu-Gly-Pro-Gly-M	115-125	-20 (1.02, $CHCl_3$)	50		MA	28 + 1	T	$C_{38}H_{58}Cl_2N_8O_8$	C, H, N
6	Boc-D-Lys-(Boc)-Pro-Leu-Gly-Pro-Gly-M	115-118		88		MA	32 + 1	TW	$C_{46}H_{73}Cl_2N_9O_{10}$	Cl, N
7	Boc-D-Phe-Pro-Leu-Gly-Pro-Gly-M·H_2O	122-130	-39 (1.01, $CHCl_3$)	88		MA	30 + 1	W	$C_{44}H_{62}Cl_2N_8O_8$	C, H, N
8	D-Phe-Pro-Leu-Gly-Pro-Gly-M·F_3AcOH	134 dec	-12 (1.02, $CHCl_3$)	79		F_3AcOH	7 + F_3AcOH	T	$C_{41}H_{55}Cl_2F_3N_8O_8$	C, H, N, Cl, F
9	Z-Pro-Gly-M	162.8-163.2	+20 (1.01, $CHCl_3$)		54	MA	22 + 2	CE	$C_{25}H_{30}Cl_2N_4O_4$	C, H, Cl, N
					26	NSu	24 + 2	CE		
10	Z-Pro-Leu-Gly-Pro-Gly-M	168.9-169.9	-53 (1.02, $CHCl_3$)		45	MA	17 + 2	CA	$C_{38}H_{51}Cl_2N_7O_7$	C, H, Cl, N
		167.0-169.5			63	MA	13 + 1	CA		
11	Boc-Leu-Gly-Pro-Gly-M	85 (softens)	-8 (1.03, $CHCl_3$)	91	65	MA	12 + 2	AH/(TW)	$C_{30}H_{46}Cl_2N_6O_6$	C, H, N, Cl
25	Boc-Gly-Pro-Gly-M[d]	149-153	+35 (1.02, $CHCl_3$)	89	44	MA	26 + 2	CH	$C_{24}H_{35}Cl_2N_5O_3$	C, H, N
33	D-Ala-Pro-Leu-Gly-Pro-Gly-M·F_3AcOH	138	-25 (1.03, $CHCl_3$)	87	87		5 + F_3AcOH	T	$C_{35}H_{51}Cl_2F_3N_8O_8$	Cl, N, F[e]

[a] M = NH-*p*-C_6H_4-N(CH_2CH_2Cl)$_2$; F_3AcOH = trifluoroacetic acid. [b] F_3AcOH = trifluoroacetic acid removal of Boc group (see Experimental Section for procedure); MA = mixed anhydride method (see Experimental Section for procedure); NSu = *N*-hydroxysuccinimide ester method (see Experimental Section for procedure). [c] T = crude product triturated with ether overnight, then filtered, and washed with ether. W = dioxane-tetrahydrofuran reaction mixture crystallized when poured into 15 vol of water. AH = crude product dissolved in ethanol, hexane added to incipient cloud-point, and then activated charcoal added, followed by filtration. TW = crystallized by dissolving in tetrahydrofuran and adding ~15 vol of water; oily precipitate slowly crystallizes. CA = dissolved in chloroform, solvent stripped quickly, oily residue dissolved in ethanol, treated with activated charcoal, filtered, and seeded. CE = crude product dissolved in chloroform, 3 vol of ether added, treated with activated charcoal, filtered, and seeded. CH = recrystallized from chloroform, hexane added. [d] Product darkened in daylight. [e] Anal. Calcd: C, 50.06; H, 6.12. Found: C, 49.26; H, 6.26. Other analyses satisfactory.

Deprotection of Boc-peptide-*p*-phenylenediamine Mustards with Trifluoroacetic Acid. The use of hydrobromic acid in acetic acid was found to cause some replacement of chlorine by bromine. The following procedure gave satisfactory deblocking in all cases.

About 3 g of the *t*-Boc-peptide was stirred with 10 mL of trifluoroacetic acid. The solid dissolved slowly with copious evolution of gas. After 30 min at 25 °C, the solution was added dropwise to 1 L of ether while stirring vigorously. The resulting white crystalline precipitate was allowed to settle, and solvent was removed with a filter stick while maintaining an atmosphere of dry nitrogen. The solid was washed three times by stirring with fresh portions of ether. It was collected and dried with a stream of dry nitrogen.

Z-Pro-Leu-Gly-Pro-Gly (17). Z-Pro-Leu-Gly was prepared from Z-Pro-ONSu and Leu-Gly in 80% yield and converted to Z-Pro-Leu-Gly-ONSu which was recrystallized from methylene chloride–hexane. (Avoid recrystallization from alcohols.) A solution of 14.67 g (35 mmol) of this ester in 600 mL of warm glyme was cooled in ice, and 250 mL of methanol was added, followed by a solution of 6.72 g of Pro-Gly hydrate and 2.94 g of $NaHCO_3$ in 300 mL of water. The solution was held at 4 °C overnight and then stripped to a 200-mL volume. Water (200 mL) was added, and the mixture was filtered (some Z-Pro-Leu-Gly-OMe separates). The filtrate was acidified (pH 2) and extracted with chloroform (3 × 150 mL). The extract was stripped, and the residue (14.7 g) in 50 mL of $CHCl_3$ was treated with 500 mL of ether and stirred to produce 11.0 g (55%) of pentapeptide monohydrate.

Boc-Leu-Gly (20). Glycine (3.00 g) and 6.72 g of $NaHCO_3$ in 120 mL of water was added in one portion to 13.12 g of Boc-Leu-ONSu in 200 mL of ethanol. The mixture was stirred overnight, stripped to 20 mL with ether, diluted with 60 mL of water, and extracted with ether (discarded). The aqueous layer was acidified (pH 1.5). The oil produced 9.42 g (82%) of white crystalline Boc-Leu-Gly which was recrystallized from ethyl acetate–hexane.

Boc-Leu-Gly-Pro-Gly Hemihydrate (12). Boc-Leu-Gly was converted to Boc-Leu-Gly-ONSu in 76–84% yield (ether recrystallization). A solution of 24.6 g of this ester in 300 mL of ethanol was cooled rapidly, and a solution of 12.15 g of Pro-Gly hydrate and 10.73 g of sodium bicarbonate in 150 mL of water was added and stirred overnight. Solvent was stripped, water was added, and the mixture was filtered. The filtrate was extracted with chloroform (discarded), then acidified (HCl), and reextracted with chloroform. Extracts were dried ($MgSO_4$) and stripped, and 500 mL of ether was added. The residual sticky solid was dried to produce 21.6 g of white, powdery hemihydrate which gave a single spot on TLC (R_f 0.5, 90:10 v/v $CHCl_3$–CH_3OH; Merck F-254 analytical silica gel plates).

***N*-(Boc-Leu-Gly-Pro-Gly)-*N'*,*N'*-bis(2-chloroethyl)-*p*-phenylenediamine (11).** Detailed experimental directions are given for this key intermediate. Deaerated water was used throughout.

A solution of 19.61 g of **12** and 4.40 g of *N*-methylmorpholine in 120 mL of dioxane and 120 mL of tetrahydrofuran under nitrogen was cooled in a methanol–ice bath, and a solution of 5.94 g of isobutyl chloroformate in tetrahydrofuran was added. Two minutes later a solution of 11.72 g of **2** in 36 mL of dioxane and 18 mL of water was added (*caution: avoid skin contact*). The mixture was stirred for 30 min and was poured into 2 L of ice–water. The resulting sticky solid was washed with water by decantation and was allowed to stand in water overnight at 5 °C. The solid was separated by filtration and dissolved in chloroform. The solution was dried ($MgSO_4$), treated with activated charcoal, filtered, and stripped to give a purple solid which was decolorized by dissolving in 200 mL of ethanol, diluting the resulting solution with 800 mL of hexane, adding activated charcoal, and filtering. Solvent was stripped to give 26.0 g of white solid (91%) which was sufficiently pure for synthetic purposes. It could be crystallized by dissolving 0.4 g in 3 mL of tetrahydrofuran and precipitating with 50 mL of water. The oily precipitate was washed several times with water by decantation. It slowly crystallized on standing to give 0.28 g of white solid.

***N*-(Leu-Gly-Pro-Gly)-*N'*,*N'*-bis(2-chloroethyl)-*p*-phenylenediamine Trifluoroacetate (1).** A solution of 24.5 g of unrecrystallized **11** in 250 mL of trifluoroacetic acid was

allowed to stand at room temperature for 30 min. The excess acid was stripped under reduced pressure on a rotary evaporator and water pump protected by a CO_2 trap. The residual oil was diluted with 1 L of ether. The slurry was washed three times with ether by decantation, filtered, and dried under nitrogen to give 23.1 g (90%) of white crystals, mp 63 °C (softening).

***N*-[(7-^{14}C)Benzyloxycarbonyl]-L-proline (13).** A solution of 223 mg of (7-^{14}C)benzyl chloroformate [prepared from (α-^{14}C)benzyl alcohol and phosgene by the New England Nuclear Co.] was added over 30 s to a stirred, ice-cold solution of 200 mg of L-proline in 5.78 mL of 0.301 N aqueous NaOH. Simultaneously, 5.21 mL of 0.300 N NaOH was added at the same rate. The resulting mixture was stirred vigorously overnight. The organic layer was separated, and the water layer was washed twice with ether. (The ether and toluene layers were combined for disposal as radioactive waste.) The water layer was acidified to pH 1.5 (HCl) and was extracted (3 × EtOAc). The combined extracts were dried ($MgSO_4$), filtered, and concentrated. The residue (193 mg) was dissolved in 2.5 mL of EtOAc; 8 mL of hexane was added followed by activated charcoal. The mixture was filtered, and the filtrate was seeded. Hexane portions were added as crystallization proceeded. The crystalline product (114 mg) was filtered off and 121 mg of unlabeled **13** was added to the filtrate as a carrier. The recrystallization was repeated. The second crop weighed 119 mg.

***N*-[(7-^{14}C)-Z-Pro-Leu-Gly-Pro-Gly]-*N'*,*N'*-bis(2-chloroethyl)-*p*-phenylenediamine (10) (Scheme II).** The 114- and 119-mg portions of (^{14}C)-**13** were combined with 16 mg of unlabeled **13**, 101 mg of *N*-methylmorpholine, 5 mL of tetrahydrofuran, and 5 mL of 1,2-dimethoxyethane in a 50-mL flask cooled to −10 °C. A solution of 137 mg of isobutyl chloroformate was added and after 2 min a solution of 672 mg of **1** and 101 mg of *N*-methylmorpholine in 5 mL of dioxane was added. The mixture was stirred for 30 min and was poured into 800 mL of a deaerated ice–water mixture. The white emulsion gradually crystallized. The liquid was decanted, and the residue was washed with water and extracted with chloroform. The organic layer was dried ($MgSO_4$) and filtered; the solvent was stripped. The oily residue (819 mg) was dissolved in 6 mL of ethanol. Hexane (14 mL) and activated charcoal (100 mg) were added, and the mixture was filtered into a tared bottle. A few micrograms of unlabeled **10** was added. As crystallization proceeded, 5 mL of hexane was added. Solvent was decanted, the solid which adhered to the walls of the bottle was washed with 1:10 ethanol–hexane, and the residue was vacuum dried to give 624 mg of solid. A second such recrystallization gave 354 mg of white crystalline **10** with a specific activity of 3.86 mCi/mmol.

A second crop of 182 mg was obtained by concentration of the filtrates and recrystallization from 4 mL of ethanol and 20 mL of hexane.

In a similar cold run the yield of recrystallized product was 66%. The infrared spectrum was identical with that of the product obtained by the method of Scheme I.

Collagenase Assays with ^{14}C-Labeled 10. Two types of experiments were carried out with the labeled compound. The first was designed to determine if **10** was susceptible to cleavage by bacterial collagenase (Figure 1).

A pH 7.3 buffer was made by dissolving 0.582 g of sodium acetate, 1.47 g of sodium diethylbarbiturate, 1.7 g of sodium chloride, 55 cm^3 of 0.1 N HCl, 169.4 mg of calcium acetate dihydrate, 48.1 mg of actidione (added to prevent possible collagenase synthesis in mammalian tissue homogenates), and 19.23 mL of dimethyl sulfoxide in 346.15 cm^3 of water.

The substrate solution was made by dissolving 2 mg of ^{14}C-labeled Cbz-L-Pro-L-Leu-Gly-L-Pro-Gly-M per milliliter of dimethyl sulfoxide.

The individual assay mixtures contained 0.3 mL of substrate solution plus 0.95 mL of buffer containing (a) nothing, (b) 5 mg of collagenase (375 units/mg) (Worthington Biochemical Corp., Freehold, N.J.), (c) 5 mg of trypsin (192 units/mg) (Worthington Biochemical Corp.), and (d) 5 mg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.).

The mixtures were incubated at 37 °C and 10-μL aliquots were withdrawn at various time intervals. The aliquots were chromatographed on 20 × 20 cm silica gel F-254 precoated preparative thin-layer chromatography plates (EM Laboratories Inc., Elmsford, N.Y.). The plates were developed with a benzene–ethyl acetate–diethylamine–methanol system (50:40:10:8), respectively. The plates were autoradiographed by placing them in contact with No Screen Medical X-Ray Film (Eastman Kodak Co., Rochester, N.Y.) for 4 days at room temperature. The round white circle in the upper left corner of each autoradiograph indicates a point on the line serving as the origin.

The second experiment demonstrated the cleavage of the collagenase-sensitive bond of the labeled compound with a homogenate of murine Sarcoma, S-180 (Figure 2).

A tumor homogenate was prepared by taking 0.85 g of the S-180 tissue and homogenizing it with 0.85 mL of cold buffer in a Tenbroeck tissue grinder of 15-mL capacity. An additional 0.5 mL of cold buffer was used to rinse the homogenate into a 15-mL centrifuge tube. The homogenate was centrifuged at 5000 rpm for 10 min in a refrigerated centrifuge. The supernatant was tested for collagenase activity on the radioactive substrate by using it as a "protein solution" in an assay similar to that described for the bacterial collagenase assay.

References and Notes

(1) W. C. J. Ross, "Biological Alkylating Agents", Butterworths, London, 1962.
(2) T. Higuchi and V. Stella, Eds., "Pro-drugs as Novel Drug Delivery Systems", ACS Symposium Series Vol. 14, American Chemical Society, Washington, D.C., 1975.
(3) G. T. Atwell, B. F. Cain, and William A. Denny, *J. Med. Chem.*, **20**, 520 (1977).
(4) N. J. Harper, *J. Med. Pharm. Chem.*, **1**, 467 (1959).
(5) (a) H. Druckrey and S. Raabe, *Klin. Wochenschr.*, **30**, 882 (1952); (b) J. F. Danielli, *Leuk. Res., Ciba Found. Symp., 1953*, 263 (1954).
(6) *J. Am. Med. Assoc.*, **216**, 1424 (1971).
(7) E. Keiditsch and L. Strauch, *Verh. Dtsch. Ges. Pathol.*, **54**, 438 (1970).
(8) E. Langer, E. Keiditsch, L. Strauch, and K. Hannig, *Verh. Dtsch. Ges. Pathol.*, **52**, 438 (1968).
(9) D. M. Robertson and D. C. Williams, *Nature (London)*, **221**, 259 (1969).
(10) R. Rohrbach, C. Thomas, R. Rümpler, and M. Lau, *Verh. Dtsch. Ges. Pathol.*, **54**, 442 (1970).
(11) A. C. Taylor, B. M. Levy, and J. W. Simpson, *Nature (London)*, **228**, 366 (1970).
(12) E. Keiditsch and L. Strauch, *Protides Biol. Fluids, Proc. Colloq.*, **22**, 405 (1975).
(13) Y. Nagai, S. Sakakibara, H. Noda, and S. Akabari, *Biochim. Biophys. Acta*, **37**, 567 (1960).
(14) S. Sakakibara, S. Akabari, Y. Nagai, and H. Noda, *Koso Kagaku Shimpojiumu*, **14**, 267 (1960).
(15) A. Nordwig, *Leder*, **13**, 10 (1962).
(16) (a) The possibility that collagenase can be artificially introduced into tumor tissue is suggested by our finding that the vegetative cells produced from the germination of the spores of certain tumor-localizing Clostridia (e.g., *Clostridium butyricum* Möse, ATCC 13732[16b]) produce collagenase. The use of these organisms in the clinical treatment of cancer has already been reported.[17] (b) J. R. Möse and G. Möse, German Patent 1 118 403 (1961).
(17) (a) R. W. Carey, J. F. Holland, H. Y. Whang, E. Neter, and B. Bryant, *Eur. J. Cancer*, **3**, 37 (1967); (b) D. Gericke, *Naturwissenschaften*, **52**, 629 (1965); (c) F. Heppner and J. K. Möse, *Zentralbl. Neurochirurgie (Leipzig)*, **27**, 183 (1966); (d) J. R. Möse, A. Propst, and F. Heppner, *Med. Klin. (Munich)*, **62**, 189 (1967); (e) A. Propst and J. K. Möse, *Verh. Dtsch. Ges. Pathol.*, **50**, 322 (1966).
(18) W. C. J. Ross, G. P. Warwick, and J. J. Roberts, *J. Chem. Soc.*, 3110 (1955).
(19) P. Hebborn and J. F. Danielli, *Biochem. Pharmacol.*, **1**, 19 (1958).
(20) F. Bergel and J. A. Stock, *J. Chem. Soc.*, 97 (1959).
(21) C. Dalton and P. Hebborn, *Biochem. Pharmacol.*, **14**, 1567 (1965).
(22) R. Wade, M. E. Whisson, and M. Szekerke, *Nature (London)*, **215**, 1303 (1967).
(23) E. Wünsch and H. G. Heidrich, *Hoppe-Seyler's Z. Physiol. Chem.*, **332**, 300 (1963).

(24) E. A. Bauer, A. Z. Eisen, and J. J. Jeffrey, *Biochim. Biophys. Acta*, **206**, 152 (1970).
(25) L. L. Espey and P. Rondell, *J. Appl. Physiol.*, **23**, 757 (1967).
(26) G. Gries, H. Buresch, and L. Strauch, *Experientia*, **26**, 31 (1970).
(27) H. G. Heidrich, D. Prokopova, and K. Hannig, *Hoppe-Seyler's Z. Physiol. Chem.*, **350**, 1430 (1969).
(28) E. Koren and S. Milkovic, *J. Reprod. Fertil.*, **32**, 349 (1973).
(29) G. A. Levidikova and V. N. Orekhovich, *Clin. Chim. Acta*, **42**, 205 (1972).
(30) P. B. Robertson, R. B. Ryel, R. E. Taylor, and K. W. Shyu, *Science*, **177**, 64 (1972).
(31) L. Strauch, H. Vencelj, and K. Hannig, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 171 (1968).
(32) L. Strauch and H. Vencelj, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 465 (1967).
(33) E. Wojtecka-Lukasik, *Reumatologia*, **9**, 194 (1971).
(34) *J. Biol. Chem.*, **247**, 977 (1972).
(35) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
(36) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3103 (1954).
(37) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 449 (1958).
(38) L. Bernardi, G. Bosisio, R. DeCastiglione, O. Goffredo, and F. Chillami, *Gazz. Chim. Ital.*, **94**, 853 (1964).
(39) J. L. Everett and W. C. J. Ross, *J. Chem. Soc.*, 1972 (1949).
(40) M. Szekerke, R. Wade, and F. Bergel, *J. Chem. Soc.*, 1907 (1965).

Stereoselective Effects of *cis*- and *trans*-Cyclopropylbis(dioxopiperazines) Related to ICRF-159 on Metastases of a Hamster Lung Adenocarcinoma[1]

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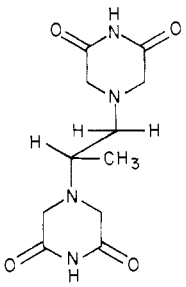
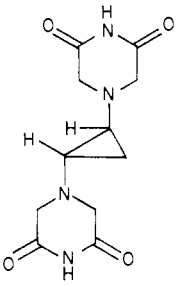
The synthesis for *cis*-4,4′-(1,2-cyclopropanediyl)bis(2,6-piperazinedione) (*cis*-**3**) is discussed. Stereoselective effects on metastases of *cis*-**3** and the previously reported *trans*-**2** isomer were compared to conformationally mobile ICRF-159 using a Syrian hamster lung adenocarcinoma (LG1002). Whereas ICRF-159 and *cis*-**3** significantly inhibited lung metastases, the *trans*-**2** isomer significantly increased the number of metastatic nodules in the lung. Thus, these studies have revealed that, at least in one tumor model, antimetastatic activity can be separated from metastatic potentiating activity by controlling drug geometry.

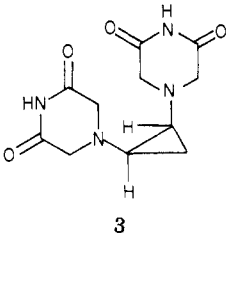
A previous report from these laboratories described the comparative effects of ICRF-159 (**1**), the *trans*-cyclopropyl analogue **2**, and certain related tetraacids and esters on cytotoxicity, mutagenicity, and scheduled and unscheduled DNA synthesis in tissue culture.[2] However, perhaps of greater significance is the observation that ICRF-159 inhibits metastases in the Lewis lung tumor (3LL) animal model without impeding the growth of the primary implant.[3-13] Histological examination of blood, lungs, and primary tumors indicated that antimetastatic activity is likely due to normalization of the developing blood vessels at the invading margins of the primary tumors.[4,9] Whereas this angiometamorphic effect is not unique to 3LL infected animals,[8,14,15] histological features suggested that ICRF-159 antimetastatic effects in an experimental transplanted murine squamous carcinoma did not depend upon morphological changes in vascularity.[16] Although ICRF-159 does not reduce metastases in all tumor models,[17] it is particularly interesting to note the results of Lazo et al.[18] These investigators have observed that incubation of exponentially growing B16 melanoma cells with ICRF-159 significantly increased their in vivo lung colony-forming efficiency.[18] Concurrently, we have been investigating the antimetastatic effects of ICRF-159 and the *trans*- and *cis*-cyclopropyl analogues (**2** and **3**, respectively) in the allogeneic hamster lung adenocarcinoma model. A priori we discuss the synthesis of *cis*-**3** and our preliminary biological results revealing the stereoselective actions of **2** and **3** on metastases in this animal model.



1

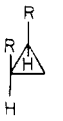


2

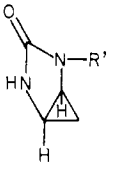


3

Synthetic Aspects. The synthesis for *cis*-**3** from *cis*-1,2-cyclopropanedicarboxylic acid (**4**) is similar, but not identical, to the reported[2] preparation of *trans*-**2** from the corresponding *trans*-dicarboxylic acid. Starting *cis*-**4** was prepared according to the method of Payne[19a] and McCoy[19b] and readily converted to the diacid chloride **5** by treatment with PCl_5.[20] Reaction of **5** with NaN_3 in aqueous acetone afforded the white crystalline diazide, **6**, which underwent Curtius rearrangement upon heating in toluene affording crude diisocyanate **7**. Treatment of **7** with *tert*-butyl alcohol, unlike the *trans*-isocyanate,[2] gave less than 5% of the desired dicarbamate **8** and produced diazabicyclohexanes **9** and **10** as major products.



4, R = CO_2H
5, R = COCl
6, R = CON_3
7, R = NCO
8, R = $NHCO_2$-*t*-Bu



9, R′ = CO_2-*t*-Bu
10, R′ = H

Dicarbamate **8** was rapidly hydrolyzed under acidic conditions to diamine **11**. Although carbamate **9** was easily converted to **10**, various attempts (both hydrolytic and reductive) to transform **10** to *cis*-diamine **11** failed. However, reaction of diisocyanate **7** with benzyl alcohol

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