Lysosomotropic Agents. 4.[1] Carbobenzoxyglycylphenylalanyl, a New Protease-Sensitive Masking Group for Introduction into Cells

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Bioactive primary and secondary amines, when acylated with the Z-Gly-Phe group, are transported into pinocytic cells, such as macrophages, P-815 mastocytoma, SV-40 3T3, and leukemia 1210, much faster than the parent compounds. Amines, such as lysosomotropic detergents [R. A. Firestone, J. M. Pisano, and R. J. Bonney, *J. Med. Chem.*, **22**, 1130 (1979)] and nitrogen mustard, which are deactivated by acylation, are unmasked by enzymic action intracellularly, probably in lysosomes because an acidic pH maximum in activity exists which acts only on the L isomer. The added polarity and molecular weight brought about by acylation prevents the amines' normally facile entry into cells by simple diffusion, restricting it to an active-transport mechanism.

Lysosomotropic detergents are cytotoxic compounds, designed as anticancer agents, that act by selectively rupturing the membranes of lysosomes, causing cell death through the action of the lysosomal enzymes that are released.[3] Thus, their targets are cells containing many lysosomes, rather than (as with most other antineoplastics) those that are replicating. Since the compounds' molecular weights are about 250 and they are lipophilic, they can enter cells by direct permeation through the plasma membranes,[4] as well as by pinocytosis.

Although these compounds are useful in themselves, their utility would be increased if they could be made to select for another property of cells in addition to the possession of lysosomes. The high pinocytic rates reported for certain types of malignant cells[5-7] led us to try to restrict entry to that route, using the technique known as piggyback endocytosis.[8]

Background. Piggyback endocytosis is the introduction into cells of a substance that is either mixed or combined with a carrier, which if combined is then removed inside the cell. The carrier may do any or all of the following: (1) deactivate the compound of interest; (2) facilitate entry into the target cells; (3) prevent entry into other cells or body compartments; (4) prevent entry into cells by simple diffusion.

An ingenious application of this principle is the treatment of leukemia with adriamycin–DNA complex,[5,9] which has (1), (3), and (4) of the above properties and, possibly, (2). DNA masks the toxicity of the active drug, inhibits entry into cells in need of protection such as those of the heart[10] because they are much less pinocytic, inhibits nonspecific entry by simple diffusion into cells in general because of its high molecular weight, and may facilitate its entry into the highly pinocytic leukemia cells, although uptake of DNA–daunomycin into L1210 cells in vitro is slower than that of the free drug.[11] After pinocytic (or endocytic)[12] uptake of the DNA–adriamycin, the toxic drug is unmasked inside the target cells by intralysosomal digestion of the DNA. DNA–ellipticine has similar properties.[6]

Other applications of the principle are the association, by either chemical or physical means, of drugs with steroids,[13] liposomes,[14] nanocapsules,[15] sugars,[16] antibodies,[17] dextran,[18] etc.[19] This list, oriented toward the anticancer field, is by no means exhaustive.

Table I. Numbering of Compounds

1	Z-Gly-L-Phe-N(CH_2CF_3)$C_{12}H_{25}$
2	Z-Gly-D-Phe-N(CH_2CF_3)$C_{12}H_{25}$
3	Gly-L-Phe-N(CH_2CF_3)$C_{12}H_{25}$
4	HN(CH_2CF_3)$C_{12}H_{25}$ [a]
5	Gly-L-Phe-N$HC_{12}H_{25}$
6	Gly-D-Phe-N$HC_{12}H_{25}$
7	Z-Gly-L-Phe-N(CH_2CH_2Cl)$_2$
8	Z-Gly-D-Phe-N(CH_2CH_2Cl)$_2$
9	Gly-L-Phe-N(CH_2CH_2Cl)$_2$
10	HN(CH_2CH_2Cl)$_2$·HCl
11	Z-Gly-L-Phe-OH
12	Z-Gly-D-Phe-OH
13	Z-Gly-L-Phe-NH_2
14	Z-Gly-D-Phe-NH_2
15	BOC-Gly-L-Phe-N(CH_2CH_2Cl)$_2$
16	Z-Gly-L-Phe-N$HCH_2CH_2C_8F_{17}$
17	$C_8F_{17}CH_2CH_2NH_2$

[a] Reference 3.

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(12) Strictly speaking, pinocytosis refers to ingestion of liquid and endocytosis to solid particles. However, we use them interchangeably because the precise physical state of our compounds under test conditions is often not known.

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Table II. Experiments I and II. Toxicity to Mouse Peritoneal Macrophages (MPM)[a] of a Masked vs. Unmasked Lysosomotropic Detergent

compd	concn mM	concn μg/mL	appearance, 3 h: expt I	% LDH released,[b] 24 h: expt I	expt II
control[c]			OK	5	7
1	0.375	227	vacuoles, rounded	73	
	0.165	100			61
4	0.375	100	OK	52	64
5	0.541	210	rounded, dying	85	
	0.514	200			78
11	0.561	100			17

[a] The experimental procedure has already been described.[27] [b] LDH (lactate dehydrogenase) is used as a cytoplasmic marker enzyme. [c] Control = Me_2SO only, 1 μL/mL.

A very recent example[20] is daunorubicin (DNR) acylated on the amino group with Leu-Leu, Ala-Leu, and Leu, which meet criteria (1), (3), and (4) above, being 4–6 times lower in toxicity to mice than DNR itself; however, it does not meet property (2). In vivo, equitoxic doses are much more active than DNR, but only against L1210 injected subcutaneously and not intravenously, so that it is possible that they are unmasked by proteases on the tumor cell surface rather than intracellularly. This possibility does not reduce their potential therapeutic usefulness, however.

Results and Discussion

Our initial goal was to find a masking group for lysosomotropic detergents that prevented entry into cells by simple diffusion. For this purpose a lipophobic group that at least doubled the molecular weight was indicated.[4] It would also be advantageous if the detergents were deactivated until after intralysosomal processing to remove the masking group. For this, an amide linkage was indicated because the acylated amines would be too weak as bases to be protonated even inside lysosomes. The obvious choice, then, was a peptide.

A perusal of the literature uncovered several candidates, from which Gly-Phe was chosen for the initial study because it is readily cleaved from primary amines by Cathepsin C,[21] a common lysosomal protease. However, a simple peptide is not a satisfactory masking group for a lysosomotropic detergent, because although acylation of the detergent's amine abolishes it detergency, the peptide-amine molecule as a whole might be a lysosomotropic detergent in its own right since it possesses a lysosomotropic amine in the glycine moiety. For this reason, in the initial study the blocked precursor 1 was screened instead of 3.

Table III. Experiments III–V. Toxicity toward P-815 Mastocytoma, 5 h

compd	concn μg/mL	concn mM	expt:	% ^{51}Cr released[a] III	IV	V
control				13	16	11
4	200	0.750		15		
1	200	0.331		22		
2	200	0.331		11		
5	100	0.257		95		
6	100	0.257		93		
7	50	0.104			22	
	100	0.208		29	27	
	150	0.312			34	19
8	100	0.208		16		
10	37	0.208			16	
	50	0.281				10
11	100	0.281			16	
12	100	0.281			16	
13	100	0.282			17	
14	100	0.282			17	

[a] Percent ^{51}Cr released from labeled cells is a measure of cell death.[28]

Another problem was that lysosomal proteases had only been reported to hydrolyze peptides of primary amines,[21] while all our detergents at that time were secondary (or tertiary). However, we knew of no reported *failures* to hydrolyze peptides of secondary amines and so were not deterred.

In the event, 1 exhibited the desired cytotoxic activity (experiments I and II, Table II) with mouse peritoneal macrophages (MPM), causing vacuolization and cell death in the same manner as the parent amine 4.[3] Thus, it seemed likely that at least one of the three amide bonds was being broken. MPM were chosen for the first experiments because they are a good model for cancer cells, being highly pinocytic and rich in lysosomes.

Another important observation was that despite the increase in molecular weight and lipophobicity resulting from acylation, the peptide-amine 1 acted *faster* than the parent amine 4 at equimolar or lower concentrations. This accelerated action of Z-Gly-Phe amides turned out to be a characteristic found with all types of (pinocytic) cells that were studied and with all the Z-Gly-Phe amides.

The high activity of glycyl-L-phenylalanyldodecylamide (5) was not surprising, since it could act as a lysosomotropic detergent whether or not it was cleaved (vide supra), and subsequent experiments indeed suggested activity for the intact molecule. The carrier group 11 alone had little or no activity in these and subsequent experiments.

Questions immediately raised were: Was Z-Gly-Phe being hydrolyzed, or Z-Gly, or just Z? Did this happen inside or outside the cells? Was the hydrolysis enzymic? If so, what enzyme (since Cathepsin C hydrolyzes Gly-Phe but not Z-Gly-Phe amides[22] and, therefore, could act only if the Z were first removed by some other means)? Answers to all but the last question have been found.

Against P-815 mastocytoma, 1 showed the same characteristic speed of action vis-a-vis 4 (experiments III and

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Table IV. Experiments VI-XII. Toxicity of Masked vs. Unmasked Nitrogen Mustard

	Expts VI-VIII. SV40 3T3, 4 h							
	% inhib uptake of:	thymidine				leucine		
	compd:	7		10		7		10	
concn, M × 10^4	expt:	VI	VII	VI	VII	VII	VIII	VII	VIII
1		7	12			−1	5		
1.5				−7					
3		60	46	2	−2	10	33	5	−7
4			61		8				
6		93	70	18	8	36	83	3	−7
8					15				
10			92		30				
17					34			5	
28					44				

Expt IX. Time Course. SV40 3T3, 3 × 10^{-4} M

	% inhib uptake of:	thymidine	
time, h	compd:	7	10
3		76	−82
4		80	−11
6		83	12
8		86	15
24		88	39

	Expts X and XI. SV40 3T3, 4 h					
	% inhib uptake of:	thymidine				
	compd:	7		15	9	10	
concn, M × 10^4	expt:	X	XI	X	XI	X	XI
1		14	29	−4	5	−11	−14
3		46	36	33	16	−5	−11
6		90	75	52	60	6	4

Expt XII. Toxicity toward L1210, 4 h

	% inhib uptake of:	thymidine	
concn, M × 10^4	compd:	7	10
0.5		28	5
1.5		57	0
3		76	9

IV, Table III). During the time period of this assay, limited to 5 h because of leakage of the ^{51}Cr marker from healthy cells, **4** induced no significant ^{51}Cr release above control, while **1** showed significant toxicity at half the molar concentration.

The question of whether cleavage is enzymic or not was first addressed with **2**, the D isomer of **1**. That an enzyme is involved was indicated by the observation that **2** was inactive toward both MPM and P-815. The D isomer, in fact, turned out to be inactive with all Z-Gly-Phe and Gly-Phe derivatives and all cell types, with but one exception: both L (**5**) and D (**6**) forms of glycylphenylalanyldodecylamide were highly toxic to P-815. This exception is easily understood, however, as explained above. The carrier groups **11**–**14** were all inactive.

There was no apparent reason why the desirable properties of **1** should not extend to Z-Gly-Phe conjugates of bioactive amines other than lysosomotropic detergents. It seemed worthwhile to try incorporating into our cytotoxic peptides yet another mode of selectivity, i.e., for cells that are replicating. Consequently, the Z-Gly-Phe derivatives **7** and **8** of nitrogen mustard **10** were prepared.

They showed the same pattern of bioactivity with P-815 as those of amine **4** (experiments III–V, Table III). During the restricted time of this assay, the parent amine **10** induced no ^{51}Cr release above control, while the Z-Gly-L-Phe derivative **7** exhibited significant dose-related toxicity. The D isomer **8**, like **2**, was inactive. The doubling time of these cells is about 15 h, so that the percent of cell kill by a cycle-specific agent, such as nitrogen mustard, during a 5-h period is severely limited.

If both **1** and **7** undergo cleavage at the same point(s), two lines of evidence show that at least the Phe-amine bond is broken. First, **7** was found to be toxic to all dividing cells. Since nitrogen mustards act via intramolecular displacement of the β-chlorine atoms by the nitrogen,[23] the amide bond linking Phe and the parent amine **10** must be undergoing hydrolysis by the test cells.

The unmasking of **1** and **7** must be not only enzymic but also intracellular, because if it happened outside the cells, the accelerated action could not be explained. There must also be some form of active transport into cells of Z-Gly-Phe amides or (with the unlikely possibility of extracellular removal of Z) Gly-Phe amides, or both. The transport system is not known, but a reasonable hypothesis is receptor-mediated endocytosis, for which analogy exists.[24] Whether the Z–Gly and Gly–Phe bonds remain intact is not known.

Against SV40-transformed 3T3 (experiments VI-XI, Table IV) and L-1210 cells (Experiment XII, Table IV), **7** showed the same accelerated transport and hydrolysis that it did toward P-815. It inhibited incorporation of radiolabeled thymidine (measures inhibition of cell divi-

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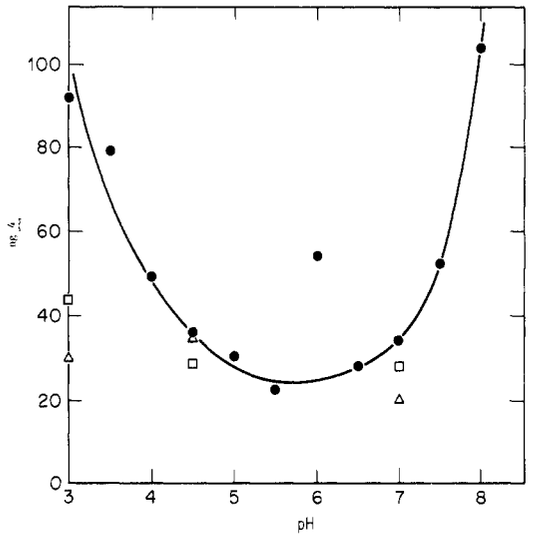

Figure 1. Plot of experiment XIV. pH profile for hydrolysis of 1 and 2 to 4 by MPM cell homogenate: (●) 1 + cells; (△) 1, no cells; (□) 2 + cells.

sion) and leucine (measures inhibition of protein synthesis, i.e., cell death) in a dose-dependent manner to a significant extent in 4 h (experiments VI–VIII), while the parent mustard **10** was almost without effect. In a time course experiment (IX), **7** acted with at least ten times the speed of **10**. Both **15** (experiment X) and **9** (experiment XI) also acted faster than **10** but not quite as fast as **7**. Apparently the phenomenon of accelerated action resides in the Gly-Phe moiety, whether the glycine's amino group is acylated or not. However, to date we have concentrated on Z-Gly-Phe rather than Gly-Phe because it seems more likely to withstand hydrolysis by extracellular proteases.

The second line of evidence that the Phe–amine bond is broken is the fact that treatment of **1** with MPM cell homogenate afforded isolable amine **4**. Several hydrolysis experiments were done on **1** and **2**, varying the pH of the reaction (experiments XIII–XV, Table V, and Figure 1). The hydrolysis product **4** was analyzed by GC–MS, generally monitoring only the pseudomolecular ion. However, positive identification of **4** from a full-scan mass spectrum was obtained from time to time. Improvements in technique with time allowed detection of **4** at progressively lower levels. Reproducibility with respect to both quantity of **4** liberated and pH profile was erratic because of variability of potency and enzyme spectrum from sample to sample of cell homogenate.

Experiment XIII showed the existence of an enzymic hydrolysis pathway for **1** at pH 4.5, since cell sap was required, and the D isomer **2** was almost unaffected. A small positive response with **2** is indeed expected because it was made by coupling amine **4** with Z-Gly-Phe using dicyclohexylcarbodiimide, which normally gives a small amount of racemization. Thus, our sample of **2** contained a few percent of the L isomer **1**.

Experiment XIV revealed two pH maxima, at 3 or below and at 8 or above, with a minimum at about 6. The acid maximum activity is heat labile, further supporting the enzymic nature of the process (experiment XV). It was also partially inactivated by 10 μM $HgCl_2$, 4 mM leupeptin, and 3 mM pepstatin, indicating possibly more than one enzyme.

We conclude that the hydrolysis of **1** to **4** is enzymic and intracellular, with at least one, acidic, pH maximum. A lysosomal locale for hydrolysis is most likely.

Another compound briefly examined was **16**, whose parent amine **17** is a powerful lysosomotropic detergent.

Table V. Experiments XIII–XV.
Hydrolysis of **1** and **2** to **4** by MPM Cell Homogenate, 37 °C, 18 h, 0.165 mM (100 μg/mL)

Expt XIII. pH 4.5		
compd	cell homogenate	4 isolated, ng
1	present	92
2	present	<20
1	absent	<14

Expt XIV. pH Profile			
pH	ng of 4 from: 1 + cells	1, no cells	2 + cells
3.0	92	30	44
3.5	79		
4.0	49		
4.5	36	36	29
5.0	30		
5.5	22		
6.0	54		
6.5	28		
7.0	34	20	28
7.5	52		
8.0	104		

Expt XV. Effect of Various Inactivators		
sample	ng of 4 at: pH 3	pH 8
1	53	24[a]
1 + 4 mM pepstatin	27	
1 + 3 mM leupeptin	23	
1 + 10 μM $HgCl_2$	28	
1, steam-treated 10 min	0	
2		20[a]
1, no cells		13[a]

[a] A maximum figure because of irregularities in the GC–MS reading.

This was the first peptide of a primary amine in our study, and for this reason it was expected to enzymically hydrolyze with especially great efficiency. Apparently it did so, because the speed of vacuolization of MPM induced by **16** was equal to or greater than that of the parent **17**, but surprisingly there was little cell kill even after 24 h, although **17** was highly cytotoxic. Thus, while **17** caused >97% release of lactate dehydrogenase (LDH) from MPM in 24 h (a measure of cell death) at concentrations at or above 0.022 mM, **16**, despite heavy vacuolization within a few hours, at 0.11 mM caused no significant release of LDH above control after 24 h. Compare this with **1**, which is cytotoxic even though its parent amine **4** is very much less potent than **17**.

Our explanation for this anomaly is that hydrolysis of **16** to **17**, whose pK is about 8.3, raises the pH of the lysosomes[25] quickly to a point where the enzymic hydrolysis of the Z-Gly-Phe group stops[26] before **17** reaches its minimum concentration for detergency, while the less basic **4** (pK = 5.5) does not stop the hydrolysis of **1**, allowing **4** to build concentration ad libitum. The existence of threshhold concentrations for the onset of lysosomotropic detergency is well established.[3] This limitation does not exist, of course, for nitrogen mustard **10**.

The study of **7** in vivo has so far been hampered by its very low water solubility, which we are trying to increase by means of suitable modifications. We are also investigating Z-Gly-Phe derivatives of other cytotoxic compounds, as well as other peptides capable of being removed by malignant cells.

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Conclusions

Carbobenzoxyglycylphenylalanyl is an effective deactivating carrier into cells for primary and secondary amines. The peptides enter by an active-transport mechanism, possibly piggyback endocytosis, and are hydrolyzed intracellularly, probably in lysosomes, to the bioactive amines. They are, thus, selective for lysosome-bearing cells that have the right active-transport mechanism and, additionally, in the case of 7 are actively replicating. These properties are potentially useful in cancer chemotherapy.

Experimental Section

All reactions done under nitrogen employed a Firestone Valve (Ace Glass Co., catalog no. 8766-12). NMR spectra were run on Varian T-60 and SC-300 instruments and are reported in parts per million from Me_4Si in $CDCl_3$, except as noted. In experiments I–XI, Me_2SO solutions of the test compounds were added to the culture media such that the final concentration of Me_2SO was 0.1%. Control experiments also had 0.1% Me_2SO added.

Experiments I and II and Assays of 16 and 17. Toxicity of Masked vs. Unmasked Lysosomotropic Detergents. Mouse peritoneal macrophages (MPM) were cultured as described previously.[27] Lactate dehydrogenase (LDH), a cytoplasmic enzyme, was assayed in the spent media and cell homogenate. The percent of total activity leaked into the media was calculated, with a value greater than 10% indicating toxicity due to the compound. Morphology of cells was determined by examining the cultured cells with an inverted Zeiss microscope.

Experiments III–V. The cell culture and assay procedures have been published.[28]

Experiments VI–XII. Toxicity of Masked vs. Unmasked Nitrogen Mustard. Experiments VI–XI. SV40-transformed 3T3 cells were maintained as monolayers in Dulbecco's minimum essential medium containing 10% fetal calf serum. To measure incorporation of [^{3}H]thymidine and [^{3}H]leucine, we plated out cells in plastic dishes at 1.5×10^5 cells/dish and incubated them overnight. The following day, the cells were washed, and the medium was replaced with medium containing 1 μCi of [^{3}H]-thymidine (specific activity 6.7 Ci/mmol) or 1 μCi of [^{3}H]leucine (specific activity 46 Ci/mmol). After incubation for the indicated time at 37 °C, cells were washed and removed from the dish with 0.125% trypsin. Protein was precipitated by the addition of an equal volume of 5% trichloroacetic acid. The incorporation of tritium into the protein precipitate was determined by dissolving in Soluene (New England Nuclear Corp.) and counted in the liquid scintillation spectrophotometer. Compounds were added to the cell cultures at the time of addition of either [^{3}H]thymidine or [^{3}H]leucine.

Experiment XII. Compounds **7** and **10** were incubated with 10^6 freshly isolated ascites L1210 cells from mice and 2 μCi of [^{3}H]thymidine in Dulbecco's medium, containing 0.02% L-asparagine and Pen-Strep, and 10% heat-inactivated calf serum for 4 h at 37 °C (5% CO_2); final Me_2SO concentration was 0.05%.

Experiments XIII–XV. Hydrolysis of 1 and 2 to 4 by MPM Cell Homogenate. MPM, grown as described, were scraped from the dish, sonicated, and treated with 0.01% NaN_3. To 1 part (v/v) cell homogenate were added 1.25 parts 0.4 M buffer, water to make 5 parts, and 0.02 part of a 10% solution of **1** or **2** in EtOH. In a typical experiment, 0.1 mg (1.7×10^{-4} mmol) **1** or **2** was used. Acetate buffer was used in all experiments at pH 3–5.5. At pH 6.0–8.0, Tris buffer was used in experiments XIV and XV. The samples were vortexed for 1.5 min, sonicated for 5 min, incubated at 37 °C for 18 h, and extracted with hexane. The hexane was carefully removed by distillation at atmospheric pressure through a 4-in. helix-packed column, and **4** was analyzed on a Finnigan 4000 mass spectrometer with an Incos data system. The glass GC column, 2 mm i.d. × 150 cm, was packed with 3% SP-2100 on 80-100 mesh Supelcon AW-DMCS (Supelco, Inc.) and conditioned at 250 °C with helium flow overnight. The operating conditions were as follows: column temperature at 185 °C; injector port, separator oven, and transfer line at 250 °C. The instrument was operated in the chemical-ionization mode using methane as the carrier gas (30 mL/min). Amine **4** was detected and quantitated by single ion monitoring of m/e 268 (M + 1). Full-scan mass spectra, run from time to time, were identical with that of authentic **4**.

Carbobenzoxyglycylphenylalanyl-*N*-(2,2,2-trifluoroethyl)-*n*-dodecylamide (1 and 2). A solution of 1.42 g (5.3 mmol) of **4** in 10 mL of THF was added to a mixture of 1.96 g (5.5 mmol) of Z-Gly-L-Phe and 1.14 g (5.5 mmol) of DCC in 10 mL of THF. The reaction mixture was stirred at room temperature overnight, filtered, evaporated, taken up in $CHCl_3$, washed with aqueous H_3PO_4, 10% Na_2CO_3, and brine, dried with $MgSO_4$, filtered, evaporated, and chromatographed on 59 g of silica gel with 4:1 $CHCl_3$–EtOAc, affording 1.21 g (38%) of **1**: NMR 3.04 (d), 3.10 (d, J = 7 Hz, $CHCH_2Ph$), 3.82 (d, J = 6 Hz, $NHCH_2CO$), 4.65 (d, J = 7 Hz, $CHCH_2Ph$), 5.09 (s, $PhCH_2O$), 7.27 (m), 7.36 (s, Ph's) ppm; peaks for **4** as described,[3] except for CH_2CF_3 and $CH_2C_{11}H_{23}$, which are shifted downfield ~0.4 ppm. Compound **2** was prepared similarly.

Glycylphenylalanyldodecylamide (5 and 6). A solution of 924 mg (5.0 mmol) of *n*-dodecylamine in 20 mL of THF was added over 7 min to a solution of 1.78 g (5.0 mmol) of Z-Gly-L-Phe and 1.02 g (5.0 mmol) of DCC in 30 mL of THF at 0 °C under N_2. The mixture was stirred at 0 °C for 4 h, filtered, evaporated, and chromatographed on 100 g of silica gel with 2:1 $CHCl_3$–EtOAc, affording 890 mg (34%) of carbobenzoxyglycyl-L-phenylalanyldodecylamide: TLC R_f 0.3; NMR of Z-Gly-Phe as above, 3.2 (m, $NCH_2C_{11}H_{23}$) ppm.

The Z group was removed from 561 mg (1.1 mmol) of this compound by hydrogenation at 25 psi in 20 mL of EtOH with 500 mg of 10% Pd/C for 2 h, filtration, evaporation, and chromatography on two 20 × 20 cm PLC plates with 4:1 $CHCl_3$–EtOAc, affording 90 mg (22%) of **5**: NMR 1.0 and 1.25 ($C_{11}H_{23}$), 3.05–3.25 (m, CH_2Ph, CH_2CO, $CH_2C_{11}H_{23}$), 4.5 (m, $CHCH_2Ph$), 7.3 (s, Ph); mass spectrum, m/e 389. Compound **6** was prepared similarly.

Carbobenzoxyglycylphenylalanylbis(2-chloroethyl)amine (7 and 8). Bis(2-chloroethyl)amine hydrochloride, 1.347 g, (7.5 mmol), was treated with 100 mL of 25% aqueous NaOH, extracted into 100 mL of CH_2Cl_2, dried with K_2CO_3, filtered, and evaporated, giving 457 mg (43%) of free base. This was taken up in 15 mL of THF and added dropwise to 1.176 g (3.3 mmol) of Z-Gly-L-Phe and 681 mg (3.3 mmol) of DCC in 50 mL of THF. The mixture was stirred at 0 °C under N_2 for 5 h, filtered, evaporated, taken up in EtOAc, washed with pH 2 aqueous H_3PO_4, K_2HPO_4, and brine, dried with $MgSO_4$, filtered, and evaporated, leaving 1.483 g of **7**. PLC of 83 mg with 1:1 $CHCl_3$–EtOAc returned 23 mg (26%): TLC R_f 0.3; NMR of Z-Gly-Phe as above, 3.5 [br s, $N(CH_2CH_2Cl)_2$, NCH_2 appears at 3.0 ppm in unacylated nitrogen mustard free base]; mass spectrum, m/e 479 (Cl_2). Compound **8** was prepared similarly.

Glycyl-L-phenylalanylbis(2-chloroethyl)amine (9). To 4.79 g (10 mmol) of **7** in a little CH_2Cl_2 was added 25 mL of 30% HBr in AcOH. The mixture was stirred for 30 min at room temperature, added to 100 mL of cold ether, and kept for 2.5 h in the freezer. The solids were filtered, taken up in MeOH, evaporated, dissolved in 1 M HCl, and washed with CH_2Cl_2. The aqueous layer was basified with NaOH and extracted with EtOAc, which was then dried over K_2CO_3, filtered, and evaporated, leaving 1.1 g (32%) of product: NMR of Gly-Phe as above ($CHCH_2Ph$ at 5.1 ppm), 3.45–3.7 (m, mustard) ppm. It was stored as its hydrochloride.

***tert*-Butoxycarbonylglycylphenylalanylbis(2-chloroethyl)amine (15).** Boc-Gly-L-Phe was prepared by treating 2.22 g (10 mmol) of Gly-L-Phe with 2.32 g (10 mmol) of Boc_2O and 1.39 mL (10 mmol) of Et_3N in CH_2Cl_2 for 3 h at room temperature. The product was extracted into aqueous K_2HPO_4 and then back into CH_2Cl_2 after acidification. Evaporation afforded Boc-Gly-L-Phe as a glass: yield 1.132 g (35%); NMR 1.4 (s, Boc), Gly-Phe as before, 11.4 (s, OH) ppm.

A mixture of 1.34 g (11 mmol) of nitrogen mustard free base, 3.04 g (10 mmol) of Boc-Gly-L-Phe, and 2.01 g (10 mmol) of DCC in THF was stirred for 5 h at room temperature, filtered, evaporated, taken up in EtOAc, washed with water, aqueous H_3PO_4,

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K_2HPO_4, and brine, dried with K_2CO_3, filtered, evaporated, and chromatographed on 80 g of silica gel with 9:1 $CHCl_3$–MeOH, R_f ~0.5, affording 3.11 g (70%) of **15**: NMR Boc, Gly, Phe, and mustard as above ($CHCH_2Ph$ at 5.1 ppm).

2-(Perfluoro-*n*-octyl)ethyl Iodide.[29] *n*-Perfluorooctyl iodide, 19.5 g (36 mmol), was placed into a flask equipped with a gas inlet and water-cooled condenser topped with a dry ice condenser. Ethylene gas was admitted, 0.19 g (0.8 mmol) of benzoyl peroxide was added, and the mixture was heated for 5 h at 85 °C under ethylene. The product was obtained as a white powder: yield 20.33 g (99.3%); mp 54–55.5 °C; NMR 2.3–2.9 (m), 3.1–3.5 (m) ppm; mass spectrum, *m/e* 574.

2-(Perfluoro-*n*-octyl)ethylamine (17).[30] The previous compound, 5 g (8.7 mmol), was placed into a bomb with 15 mL of *n*-BuOH and 1.33 g (89 mmol) of NH_3, heated to 80 °C for 3.5 h, cooled, and vented. Aqueous 10% NaOH was added, and the mixture was extracted 3 times with ether. The combined ether extracts were washed with brine, dried with K_2CO_3, filtered, and treated with anhydrous HCl. Solvents were evaporated, with the BuOH taken off at reduced pressure at 60–70 °C. The residue was washed with hexane, affording 1.06 g (24.5%) of **17**·HCl as a white powder. Owing to the volatility of the free amine, **17** was stored as its hydrochloride. NMR (CD_3OD) 2.68 (t of t, J = 18 and 7 Hz, CH_2CF_2), 3.36 (t, J = 7 Hz, $CH_2NH_3^+$) ppm; mass spectrum, *m/e* 462 (free base). Anal. ($C_{10}F_{17}H_7NCl$) C, H, N; C: calcd, 24.05; found, 23.53.

Carbobenzoxyglycylphenylalanyl Derivative (16) of 2-(Perfluorooctyl)ethylamine. Compound **17**·HCl, 0.75 g, (1.9 mmol), was converted to the free base by stirring with 20 mL of CH_2Cl_2, 10 mL of 50% NaOH, and 5 mL of H_2O for 30 min. The aqueous layer was saturated with K_2CO_3. The organic layer was decanted, dried with K_2CO_3, filtered, and added at −10 °C to a stirred solution of 0.84 g (2.4 mmol) of Z-Gly-Phe, 0.21 mL (1.5 mmol) of Et_3N, and 0.140 mL (1.5 mmol) of ethyl chloroformate in CH_2Cl_2. The reaction mixture was stirred under N_2 for 3 h at −10 °C and for 28 h at room temperature. The solvent was evaporated and replaced with EtOAc. The solution was washed with aqueous H_3PO_4, water, K_2HPO_4, and brine, dried with $MgSO_4$, filtered, and evaporated. PLC using 1:1 $CHCl_3$–EtOAc, R_f ~0.5, afforded 491 mg (41%) of **16**: NMR 3.5 (m, $C_8F_{17}CH_2CH_2$) ppm; NMR for Z-Gly-Phe as before; mass spectrum, *m/e* 801.

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DL-*threo*-β-Fluoroaspartate and DL-*threo*-β-Fluoroasparagine: Selective Cytotoxic Agents for Mammalian Cells in Culture

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Absolute configuration assignments have been made for the diastereomers of DL-β-fluoroaspartate by X-ray analysis. The cytotoxicity of these isomers against various mammalian cells was examined. DL-*threo*-β-Fluoroaspartate shows selective cytotoxicity. Growth of the most sensitive cells is completely inhibited by 13 μM DL-*threo*-β-fluoroaspartate in the presence of 100 μM L-aspartate, a component of the culture medium. A difference in the rate of transport of DL-β-fluoroaspartate among the cells studied is an important factor determining cell specificity. For those cells that are sensitive to DL-β-fluoroaspartate, the threo isomer is, in all cases, more potent than the erythro isomer. Radioactivity derived from L-*threo*-β-fluoro[^{14}C]aspartate is incorporated into proteins at a rate comparable to the rate of incorporation from L-[^{14}C]aspartate. We synthesized DL-*threo*-β-fluoroasparagine. This compound is also cytotoxic but less specific and less potent than DL-*threo*-β-fluoroaspartate. However, the cell specificity can be enhanced in the presence of 1 mM L-aspartate, which can protect some cells but not others from the cytotoxic effects of DL-*threo*-β-fluoroasparagine. Jensen sarcoma cells, which require asparagine, are not protected by L-aspartate. Therefore, a combination of L-aspartate and DL-*threo*-β-fluoroasparagine can be used to inhibit specifically the growth of asparagine-requiring tumors.

The synthesis of analogues of biological substrates represents one of the important approaches to the preparation of pharmacologically useful compounds. In designing analogues of biologically active molecules, one would like to introduce minimal structural changes and still affect the chemical properties of the molecule. Substitution of fluorine for hydrogen leads to compounds which meet these criteria. Fluorine is not much larger than hydrogen but can significantly affect the chemical properties of the molecule. For instance, the substitution of one of the α-hydrogens of acetic acid by fluorine lowers the pK of the carboxyl group by 2 units. The biological properties of many fluorine-containing compounds have been explored.[1-14] However, the properties of polyfunctional amino acids[15] in which fluorine is next to a functional group have not been extensively studied.[16,17] These amino

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