Synthesis, Biological Testing, and Stereochemical Assignment of an End Group Modified Retro-Inverso Bombesin C-Terminal Nonapeptide

Mark Cushman,*,† Jurjus Jurayj,† and James D. Moyer‡

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47906, and Laboratory of Biological Chemistry, National Institutes of Health, Bldg. 37, Bethesda, Maryland 20582

Received August 23, 1989

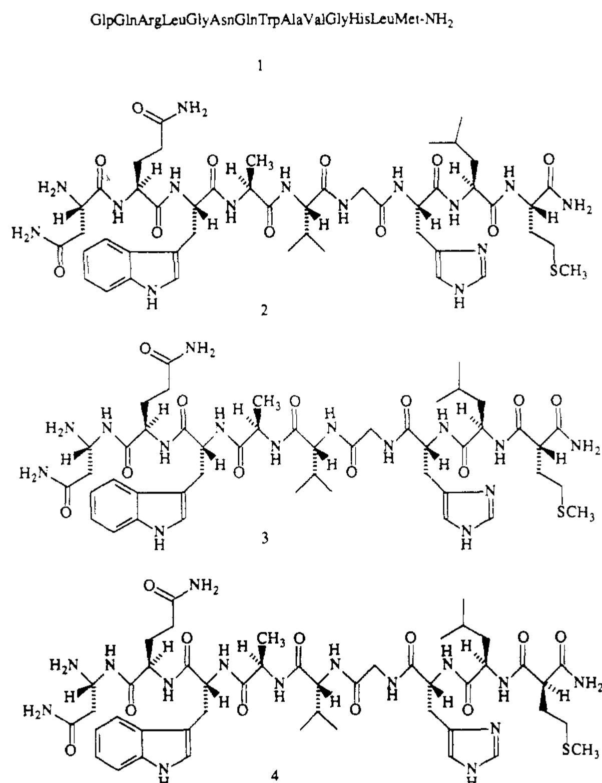
The end group modified retro-inverso bombesin C-terminal nonapeptide **3** and its diastereomer **4** have been synthesized. The absolute configurations at the substituted malonamic acid residues in **3** and **4** were determined by chemical correlation with D- and L-2-aminobutyric acids of known absolute configuration. Thus, the absolute configurations at the substituted malonamic acid residues in each diastereomer of **25a** were determined by Raney nickel desulfurization followed by Hofmann degradation to give the dipeptide derivatives **30** and **31** having established absolute configurations. Hydrolysis of **25a** then gave the diastereomeric acids **25b** having defined stereochemistries. Coupling of the diastereomer of **25b** having the *R* configuration at the substituted malonamic acid residue to the hexapeptide **27** then gave a stereochemically defined diastereomer of **19**, which was converted to **20b**. Deprotection of **20a** and **20b** gave **3** and **4**, respectively. As shown by an assay that measures the increase in inositol phosphates in GH3 rat pituitary cells stimulated by bombesin-like peptides, the retro-inverso peptide **3**, having an absolute configuration at the substituted malonamic acid residue corresponding to that of the methionine residue in bombesin, was essentially inactive as an agonist, whereas peptide **4**, having the opposite configuration at the substituted malonamic acid residue, had weak agonist activity when compared to that of bombesin. Neither **3** nor **4** had any bombesin antagonist activity.

Introduction

Bombesin (**1**)[1] and structurally related peptides display a wide spectrum of biological activities.[2,3] Recent interest has focused on the ability of bombesin-like peptides to stimulate the growth of small cell lung carcinoma (SCLC), possibly by an autocrine mechanism.[4-6] A monoclonal antibody to bombesin has been found to block its binding to cellular receptors and inhibit both the clonal growth of SCLC in vitro and SCLC xenografts in vivo.[5] Furthermore, several bombesin receptor antagonists have been found to inhibit the growth of SCLC and Swiss 3T3 cells.[7-9] Therefore, studies toward the development of new bombesin antagonists as potential therapeutic agents are warranted.

Structure–activity studies have indicated that the C-terminal nonapeptide **2** retains full agonist activity and that tryptophan in position 8 and histidine in position 12 are important for activity.[10] Thus it seemed logical that attention in any future studies could be focused on the nonapeptide **2**. Other structure–activity work has focused on amino acid deletions or D-amino acid substitutions.[11-13] At the onset of this study, no effort had been made to assess the importance of the amide backbone on the activity of bombesin. More recently it was shown that replacement of certain amide bonds in bombesin by a CH_2NH group dramatically affects its activity and leads to potent antagonists.[14]

A different approach to evaluate the importance of the backbone would be the use of the retro-inverso modification, in which the normal L-amino acid residues are replaced by the corresponding D-amino acids and the direction of the peptide backbone is reversed.[15] This results in maintenance of side-chain topochemistry and enhanced metabolic stability since peptides containing the "abnormal" D-amino acid residues are not substrates for peptidases. However, linear peptides present a problem in that the normal peptide and its retro-inverso isomer lack end group and charge complementarity.[16] Several suggestions have been presented to resolve this problem, but the use of "false" termini (a *gem*-diaminoalkyl analogue



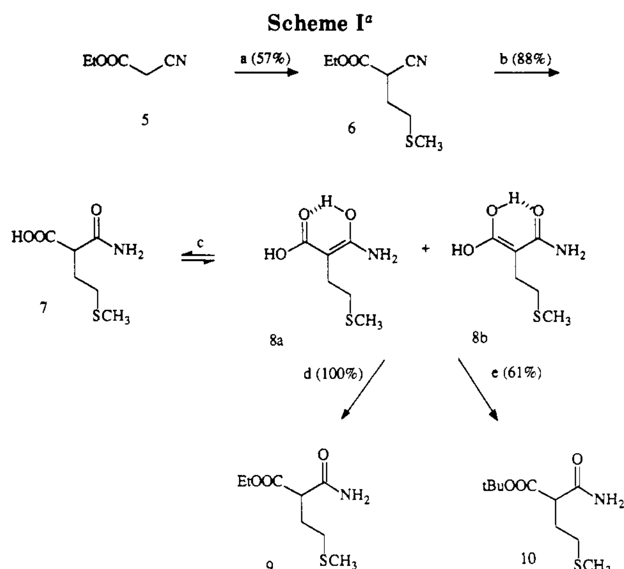
for the amino terminus and a C-2 substituted malonic or malonamic acid for the carboxy terminus) seems to be the

† Purdue University.
‡ NIH.

(1) Anastasi, A.; Erspamer, V.; Bucci, M. *Experientia* **1971**, *27*, 166.
(2) Walsh, J. H. In *Brain Peptides*; Krieger, D. T., Brownstein, M. J., Martin, J. B., Eds.; Wiley-Interscience: New York, 1983; pp 941–960.
(3) Erspamer, V.; Melchiorri, P. In *Neuroendocrine Perspectives*; Muller, E. E., McLeod, R. M., Eds.; Elsevier Science: Amsterdam, 1983; Vol. 2, pp 37–106.
(4) Carney, D. N.; Cuttitta, F.; Moody, T. W.; Minna, J. D. *Cancer Res.* **1987**, *47*, 821.
(5) Cuttitta, F.; Carney, D. N.; Mulshine, J.; Moody, T. W.; Fedorko, J.; Fischler, A.; Minna, J. D. *Nature* **1985**, *316*, 823.
(6) Woll, P. J.; Rozengurt, E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1859.
(7) Woll, P. J.; Coy, D. H.; Rozengurt, E. *Biochem. Biophys., Res. Commun.* **1988**, *155*, 359.

0022-3263/90/1955-3186$02.50/0 © 1990 American Chemical Society

Scheme I[a]



[a] (a) (1) KI, NaOEt, EtOH, room temperature (15 min); (2) $CH_3SCH_2CH_2Cl$, reflux (12 h). (b) KOH, *t*-BuOH, reflux (15 min). (c) $(CD_3)_2CO$, room temperature (7 days). (d) AcCl, EtOH, room temperature (21 h). (e) DCC, *t*-BuOH, 4-(dimethylamino)pyridine, CH_2Cl_2, DMF, 0 °C to room temperature (3.5 h).

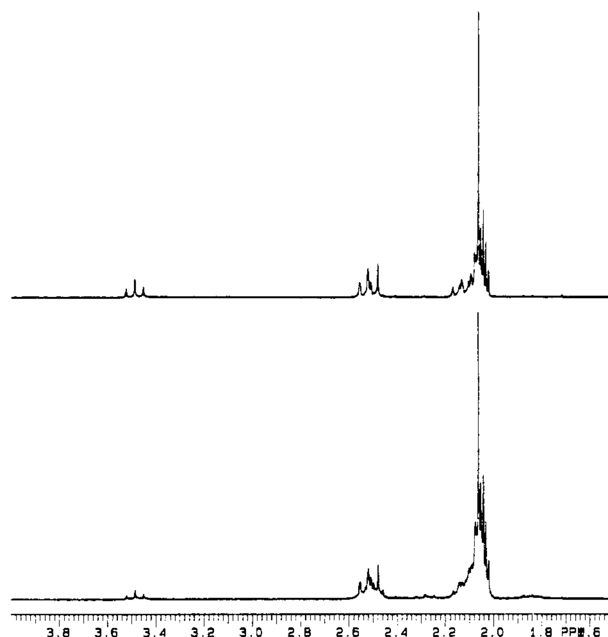


Figure 1. High-field part of the 200-MHz NMR spectrum of purified acid **7**. Top: 2 days after dissolution in acetone-d_6. Bottom: 7 days after dissolution.

most logical since it results in a retro-inverso isomer that most closely resembles the original peptide.[15] This can be visualized by comparing the structure of bombesin C-terminal nonapeptide **2** with that of its end group modified retro-inverso isomer **3**. In contrast, the diastereomer **4**, which arises from the use of a racemic malonamic acid, lacks the required complementarity. It is to be noted that for the sake of illustrating the complementarity concept, the direction of the peptide bonds (defined as that from the carbonyl carbon to the nitrogen atom) in **3** and **4** is reversed, i.e. from right to left, whereas the more conventional way of representing peptides, in which the amide bond direction is from left to right, is used for the rest of the structures. Although this particular retro-inverso modification seems to be the general solution to the end group problem, its use has been quite limited, with only three biologically inactive analogues being reported.[17,18]

The "partial retro-inverso modification", in which a retro-inverted segment is incorporated in the middle of a peptide sequence, constitutes a different but related solution to the end group problem[15] and has found wider applicability than the above mentioned modification.

In general the effect of the retro-inverso modification on the biological activity of peptides has been rather unpredictable. In many cases biologically inactive materials have resulted. Examples include bradykinin,[19] α-melanotropin-(5–9)-pentapeptide,[20] tuftsin,[16] and desaminogastrin-tetrapeptide amide.[16] In other cases, agonists having low potencies have been obtained. These include retro-inverso analogues of luteinizing hormone-releasing hormone,[21,22] thyrotropin-releasing hormone,[21] angiotensin,[23] somatostatin,[24,25] substance P,[26,27] dermorphin,[28] and [Leu[5]]enkephalin.[29] Recently, a series of retro-inverso gastrin analogues were reported which actually functioned as potent gastrin antagonists.[30] In contrast to these results, work with retro-inverso enkephalinamides led to several compounds which were more potent than the parent peptide and were also longer acting,[31] and certain retro-inverso dermorphins have been prepared that are more potent than dermorphin itself.[32] Against this

(8) Layton, J. E.; Scanlon, D. B.; Soveny, C.; Morstyn, G. *Cancer Res.* **1988**, *48*, 4783.
(9) Trepel, J. B.; Moyer, J. D.; Cuttitta, F.; Frucht, H.; Coy, D. H.; Natale, R. B.; Mulshine, J. L.; Jensen, R. T.; Sausville, E. A. *Biochem. Biophys. Res. Commun.* **1988**, *156*, 1383.
(10) Broccardo, M.; Erspamer, G. F.; Melchiorri, P.; Negri, L.; De-Castiglioni, R. *Br. J. Pharmacol.* **1975**, *55*, 221.
(11) Rivier, J. E.; Brown, M. R. *Biochemistry* **1978**, *17*, 1766.
(12) Heinz-Erian, P.; Coy, D. H.; Tamura, M.; Jones, S. W.; Gardner, J. D.; Jensen, R. T. *Am. J. Physiol.* **1987**, *252*, G439.
(13) Saeed, Z. A.; Huang, S. C.; Coy, D. H.; Jiang, N.-Y.; Heinz-Erian, P.; Mantey, S.; Gardner, J. D.; Jensen, R. T. *Peptides* **1989**, *10*, 597.
(14) Coy, D. H.; Heinz-Erian, P.; Jiang, N.-Y.; Sasaki, Y.; Taylor, J.; Moreau, J.-P.; Wolfrey, W. T.; Gardner, J. D.; Jensen, R. T. *J. Biol. Chem.* **1988**, *263*, 5056.
(15) Goodman, M.; Chorev, M. *Acc. Chem. Res.* **1979**, *12*, 1.
(16) Hayward, C. F.; Morley, J. S. in *Peptides 1974, Proceedings of the 13th European Peptide Symposium*; Wolman, Y., Ed.; Wiley: New York, and Israel Universities Press: Jerusalem, 1975; pp 287–298.
(17) Chorev, M.; Willson, C. G.; Goodman, M. In *Peptides, Proceedings of the 5th American Peptide Symposium*; Goodman, M., Meienhofer, J., Eds.; Wiley: New York, 1977; pp 572–574.
(18) Willson, C. G.; Goodman, M.; Rivier, J. In *Peptides, Proceedings of the 5th American Peptide Symposium*; Goodman, M., Meinehofer, J., Eds.; Wiley: New York, 1977; pp 579–580.
(19) Stewart, J. M.; Wooley, D. W. In *Hypotensive Peptides*; Erdos, E. G., Back, N., Sicuteri, F., Eds.; Springer: Berlin, 1966; p 23. Vogler, K.; Lanz, P.; Lergier, W.; Haefely, W. *Helv. Chim. Acta* **1966**, *49*, 390.
(20) Chung, D.; Li, C. H. *Biochem. Biophys. Acta* **1967**, *136*, 570.
(21) Goodman, M.; Chorev, M. In *Perspectives in Peptide Chemistry*; Eberle, A. N., Geiger, R., Wieland, T., Eds.; Karger: Basel, 1981; pp 283–294.
(22) Chaturvedi, N.; Goodman, M.; Bowers, C. *Int. J. Peptide Protein Res.* **1981**, *17*, 72.
(23) Goissis, G.; Nouaihetas, V. L. A.; Paiva, A. C. M. *J. Med. Chem.* **1976**, *19*, 1287.
(24) Pallai, P.; Struthers, S.; Goodman, M.; Vale, W. *Biopolymers* **1983**, *22*, 2523.
(25) Pallai, P.; Struthers, R. S.; Goodman, M.; Moroder, L.; Wunsch, E.; Vale, W. *Biochemistry* **1985**, *24*, 1933.
(26) Chorev, M.; Rubini, E.; Gilon, C.; Wormser, U.; Selinger, Z. *J. Med. Chem.* **1983**, *26*, 129.
(27) Chorev, M.; Yaion, M.; Wormser, U.; Levian-Teitelbaum, D.; Gilon, C.; Selinger, Z. *Eur. J. Med. Chem.* **1986**, *21*, 96.
(28) Salvadori, S.; Marastoni, M.; Balboni, G.; Sarto, G.; Tomatis, R. *Int. J. Peptide Protein Res.* **1985**, *25*, 526.
(29) Chorev, M.; Gilon, N.; Roubini, E.; Ackerman, E.; Levian-Teitelbaum, D.; Rosin, M. *Coll. Czech. Chem. Commun.* **1988**, *53*, 2519.
(30) Rodriguez, M.; Dubreuil, P.; Bali, J.-P.; Martinez, J. *J. Med. Chem.* **1987**, *30*, 758.
(31) Chorev, M.; Shavitz, R.; Goodman, M.; Minick, S.; Guillemin, R. *Science* **1979**, *204*, 1210.

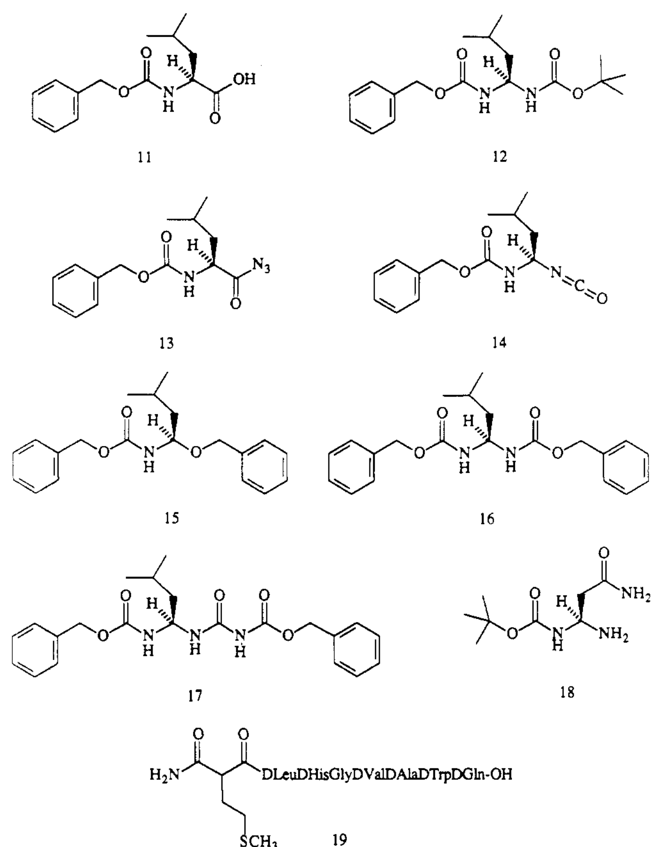
background, the present retro-inverso bombesin analogues 3 and 4 could only be tested for biological activity without any particular expectations about what might result.

Results

Synthesis of the Carboxy Terminus Analogue. The synthesis of the required substituted malonamic acid 7 is portrayed in Scheme I. The anion derived from ethyl cyanoacetate (5) was alkylated with 2-chloroethyl methyl sulfide[33] to give the product 6.[34] The ester and nitrile groups in 6 were then hydrolyzed to afford the desired malonamic acid derivative 7.[35] The crude acid 7 was observed by NMR to undergo complete enolization to give a mixture of 8a and 8b when dissolved in aprotic solvents. On the other hand, purified 7 underwent a slow enolization to give an equilibrium mixture of the three forms under the same conditions (Figure 1). The formation of the ester derivatives 9 and 10 from the mixture of 8a and 8b proved that 7 was indeed undergoing enolization instead of an undesired degradation, and it also indicated that enolization would probably not prevent amide bond formation during peptide synthesis.

Synthesis of the Amino Terminus Analogue. Our original plan was to construct the geminal amino amide unit at the end of the chain in 3 by performing a Curtius rearrangement on a peptide containing a C-terminal asparagine residue. Some model studies of this reaction on simple acylated asparagine derivatives therefore seemed appropriate. Attempts to rearrange the acyl azide derivative of Z-L-asparagine in trifluoroacetic acid or to react Z-L-asparagine with diphenyl phosphorazidate gave disappointing results. It was felt that the side chain amide group of asparagine was interfering with the reaction at the isocyanate stage, since asparagine could be cleanly incorporated into peptides with diphenyl phosphorazidate (an azide transfer agent). In order to test this idea, the desired transformation was tried on an amino acid derivative lacking a reactive side chain. The reaction of Z-L-leucine 11 with diphenyl phosphorazidate and triethylamine followed by heating at reflux in benzene containing *tert*-butyl alcohol also did not lead to the desired product 12. However, the acyl azide 13 was easily made by the mixed anhydride method, and it rearranged readily to the isocyanate 14. When this isocyanate was reacted with *tert*-butyl alcohol in refluxing benzene, it took 15 h before the complete decomposition of the starting material as evidenced by IR, and the desired product 12 was not detected. In contrast to *tert*-butyl alcohol, benzyl alcohol reacted readily with the isocyanate 14 to give the products 15, 16, and 17, in accordance with a literature precedent.[36] However, in our hands the product 15, which originates by the loss of the isocyanate ion from 14 and capture of the resulting cation with benzyl alcohol, was the major product. Having established the proper conditions for the Curtius rearrangement, the reaction of Z-L-asparagine under these conditions was reinvestigated without success. Therefore, it is clear that the Curtius rearrangement was not compatible with the side chain amino group of asparagine and that a new strategy for the construction of the geminal amino amide unit of 3 would have to be devised.

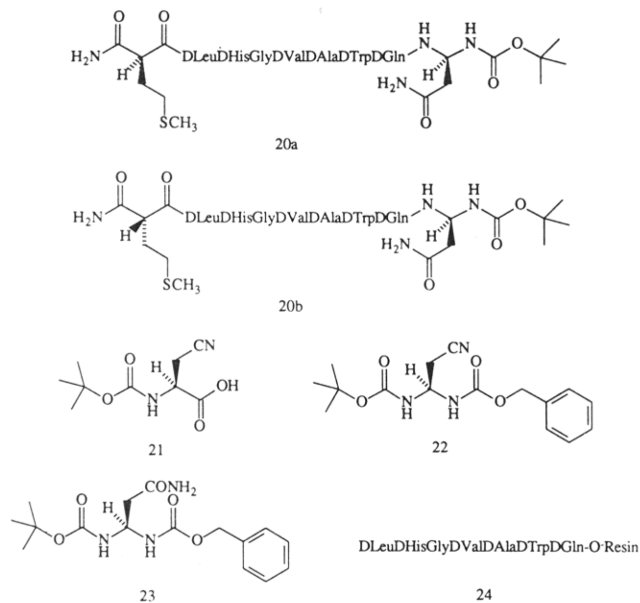
(32) Salvadori, S.; Marastoni, M.; Balboni, G.; Sarto, G. P.; Tomatis, R. *J. Med. Chem.* **1985**, *28*, 769.
(33) Kirner, W. R. *J. Am. Chem. Soc.* **1928**, *50*, 2446.
(34) Gagnon, P. E.; Savard, K.; Gaudry, R.; Richardson, E. M. *Can. J. Res.* **1947**, *25b*, 28.
(35) Hall, J. H.; Gisler, M. *J. Org. Chem.* **1976**, *41*, 3769.
(36) Chorev, M.; MacDonald, S. A.; Goodman, M. *J. Org. Chem.* **1984**, *49*, 821.



The new approach consisted of a manual coupling between compound 18 and a diastereomeric mixture of octapeptides 19 to give BOC protected retro-inverso peptides 20a and 20b, which could be deprotected under mild conditions to give a diastereomeric mixture of 3 and 4. The octapeptide 19 could be prepared by solid-phase technology, whereas 18 would have to be synthesized from the Curtius rearrangement of an asparagine derivative in which the amide is suitably protected. The possibility of masking the amide on the side chain of asparagine as a nitrile seemed like a reasonable choice, since amide–nitrile interconversions are well established[37] and it seemed likely that a nitrile would not interfere with the Curtius rearrangement. Therefore, BOC-L-asparagine was dehydrated with DCC in pyridine to BOC-L-β-cyanoalanine (21) in good yield.[38] Curtius rearrangement of the acyl azide derivative of 21 in refluxing benzene containing benzyl alcohol gave 22 in 25% yield. The nitrile group of 22 was hydrated using basic hydrogen peroxide in acetone to afford 23.[39] Hydrogenolysis of 23 with 10% Pd–C in methanol gave the desired fragment 18.

Synthesis of the Retro-Inverso Nonapeptides 3 and 4. Having the required end pieces 7 and 18 in hand, it was possible to start the synthesis of the desired peptide. The peptide-resin 24 was assembled and coupled to the acid 7 with DCC/HOBt in DMF, and the reaction product was cleaved with HF to give peptide 19 as a mixture of separable diastereomers. The coupling of 19 to 18 using isobutyl chloroformate and *N*-methylmorpholine in 10:1 THF/DMF did not give any of the desired product 20. The most likely reason for this failure is the insolubility of the starting peptide 19 in the reaction mixture. The

(37) Liberek, B. *Chem. Ind. (London)* **1961**, 978, and references cited therein.
(38) Ressler, C.; Ratzkin, H. *J. Org. Chem.* **1961**, *26*, 3356.
(39) Radziszewski, B. *Ber. Dtsh. Chem. Ges.* **1885**, *18*, 355. Noller, C. R. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. II, p 586.



coupling of **18** to **19** could not be affected with DCC/HOBt in DMF, and this could be due to the well-known side reactions that glutamine and asparagine undergo in the presence of DCC.[40] Finally, the desired coupling was achieved using BOP [(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate] as the condensing agent and triethylamine as the base, which gave **20** in 19% yield after purification.[41] Removal of the BOC group with 1:1:0.05 TFA–CH_2Cl_2–$HSCH_2CH_2SH$ afforded the diastereomeric peptides **3** and **4**. These two diastereomers could be separated by HPLC and were configurationally stable in cold unbuffered aqueous solution.

The diastereomer having the longer HPLC retention time (tested as the acetate salt) showed some bombesin agonist activity in an assay that determined the increase in inositol 4-monophosphate in GH3 rat pituitary cells, whereas the other diastereomer showed little if any activity (Figure 2).[42] Neither diastereomer was a bombesin antagonist (Figure 3). A kinetic study showed that both pure **3** and **4** were stereochemically stable under the test conditions (balanced salt solution[43] at 37 °C) during a period of 20 min, which is twice as long as the time period required for the assay. Complete equilibration of these two substances took about 24 h but was accompanied by partial decomposition.

Configurational Assignment. In view of the difference in agonist activity between the two diastereomers, an attempt was made to determine the absolute configurations of the malonamic acid residues in these two substances. The original plan was to synthesize the C-terminal dipeptides **25a**, separate the diastereomers by HPLC, and determine the stereochemistry of each diastereomer by stereospecific degradation to a methionine derivative **26** of known configuration. If this were achieved, a stereochemically defined diastereomer of the dipeptide **25a** could be saponified to the corresponding acid **25b**, which could then be coupled manually to the hexapeptide **27** to afford a stereochemically defined diastereomer of **19**. This might then be used to prepare either of the retro-inverso peptides **3** or **4** with established stereochemistry.

(40) Bodanszky, M.; Martinez, J. *Synthesis* **1981**, 333.
(41) Castro, B.; Dormoy, J.-R.; Dourtoglou, B.; Evin, G.; Selve, C.; Ziegler, J. C. *Synthesis* **1976**, 751.
(42) Moyer, J. D. Unpublished Results.
(43) Drummond, A. H.; Raeburn, C. A. *Biochem. J.* **1984**, *224*, 129.

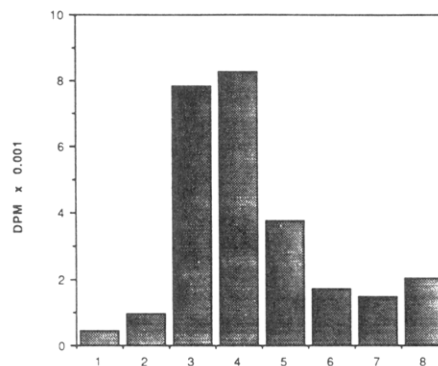


Figure 2. Agonist activity. GH_3 cells growing in RPMI 1640 medium without inositol, but with 5% horse serum, were prelabeled with 4 μC/mL of [^{3}H]myoinositol. The cells were removed by gently scraping and suspended in a balanced salt solution with 10 mM LiCl, then incubated at 37 °C for 10 min with the indicated peptide. The incubations were terminated by extraction with 5% trichloroacetic acid and I(4)P determined by HPLC.[42] Each value is the result of duplicates agreeing within an average of 14%. (1) Control, 0 min. (2) Control, 10 min. (3) Bombesin, 15 nM. (4) Bombesin, 30 nM. (5) Compound **4**, 50 μM. (6) Compound **3**, 50 μM. (7) *p*GluGlnArgLeuGlyAsnGlnTrpAlaValGlyDPheLeuMet-NH_2, 50 μM. (8) *p*GluGlnArgLeuGlyAsnGlnTrpAlaValGlyHisLeuψ(CH_2NH)Leu-NH_2, 50 μM.

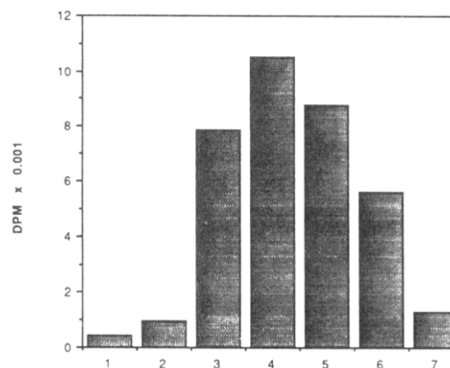
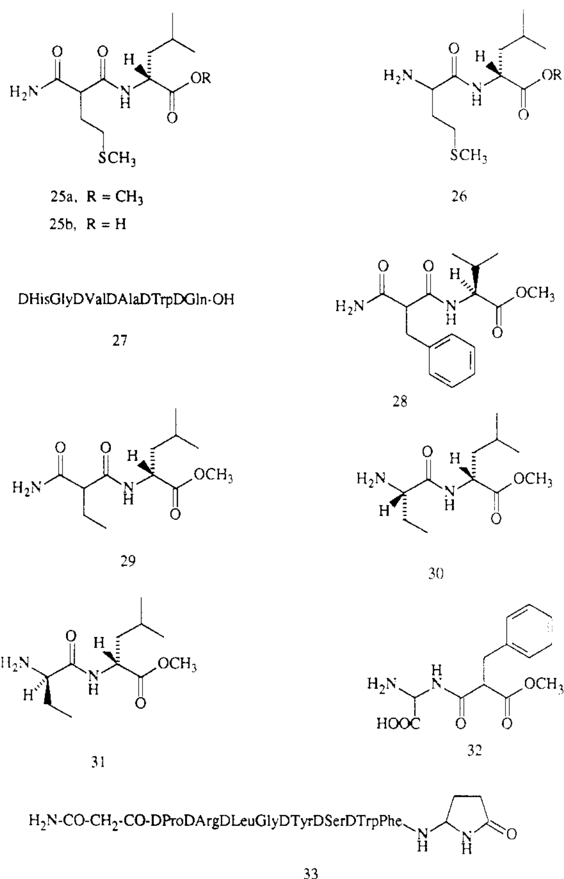


Figure 3. Antagonist activity. GH_3 cells growing in RPMI 1640 medium without inositol, but with 5% horse serum, were prelabeled with 4 μC/mL of [^{3}H]myoinositol. The cells were removed by gently scraping and suspended in a balanced salt solution with 10 mM LiCl and then incubated at 37 °C for 10 min with the indicated peptide. The incubations were terminated by extraction with 5% trichloroacetic acid and I(4)P determined by HPLC. Each value is the result of duplicates agreeing within an average of 14%. (1) Control, 0 min. (2) Control, 10 min. (3) Bombesin, 15 nM. (4) Bombesin, 15 nM + compound **4**, 50 μM. (5) Bombesin, 15 nM + compound **3**, 50 μM. (6) Bombesin, 15 nM + *p*GluGlnArgLeuGlyAsnGlnTrpAlaValGlyDPheLeuMet-NH_2, 50 μM. (7) Bombesin, 15 nM + *p*GluGlnArgLeuGlyAsnGlnTrpAlaValGlyHisLeuψ(CH_2NH)-Leu-NH_2, 50 μM.

The diastereomeric peptides **25a** were prepared by a dicyclohexylcarbodiimide- and *N*-hydroxysuccinimide-mediated coupling of the substituted malonamic acid **7** with D-leucine methyl ester hydrochloride using *N*-methylmorpholine as the base. The two diastereomers of **25a** were easily separated on a C-18 reverse-phase HPLC column. All attempts to affect the Hofmann rearrangement using the PIFA [*I*,*I*-bis(trifluoroacetoxy)iodobenzene] reagent[44] were unsuccessful. Use of 1 equiv of PIFA oxidized the sulfide to a sulfoxide as evidenced by mass spectroscopy. Use of a larger amount of the reagent did not result in the desired rearrangement. It seemed that the presence of the sulfur atom was complicating the re-

(44) Loudon, G. M.; Radhakrishna, A. S.; Almond, M. R.; Blodgett, J. K.; Boutin, R. H. *J. Org. Chem.* **1984**, *49*, 4272.

action since Goodman has reported the successful Hofmann rearrangement of **28** with PIFA[45] as a key step in the determination of the absolute configuration of its malonamyl residue.



It was thus necessary to modify the original scheme in order to eliminate the sulfur interference. An attractive option was to desulfurize peptide **25a** with Raney nickel, since the reaction conditions are mild and neutral. Furthermore, the compounds obtained from Hofmann rearrangement of the desulfurization products will be derivatives of D- and L-2-aminobutyric acids (Aba), which can be obtained commercially in resolved form. The desulfurization of a diastereomeric mixture of peptide **25a** was carried out successfully with Raney nickel in refluxing ethanol to give peptide **29** as a mixture of diastereomers that were separated. The reaction of peptide **29** with 1 equiv of PIFA in aqueous acetonitrile was also successful, as evidenced by FABMS and HPLC comparison to authentic samples of the products **30** and **31**, which were prepared by standard peptide synthesis methods. Having established the viability of the approach, the pure, individual diastereomers of **25a** had to be used as substrates. The diastereomers of **25a** were separated by semipreparative HPLC, and then each diastereomer was desulfurized with Raney nickel. HPLC analysis indicated that some epimerization did occur during this reaction, but for all practical purposes it was established that the diastereomer of **25a** having the longer HPLC retention time gives rise to the longer retained isomer of **29**, and the diastereomer of **25a** having the shorter retention time gives the isomer of **29** having the shorter retention time. Next, the longer retained isomer of **29** was treated with PIFA to mainly produce dipeptide **30**, which has an (*S*)-(+)-aminobutanoic acid residue. Therefore, it was concluded that the diastereomer of **25a** having the longer HPLC retention time has the *R* configuration at the substituted malonamic acid.

The ester groups of a diastereomeric mixture of peptides **25a** were saponified with 0.5 M sodium hydroxide to give peptide **25b** as a mixture of diastereomers that were separated by HPLC. Esterification of the longer retained isomer of **25b** gave rise to longer retained isomer of **25a**, thus proving that the longer retained isomer of **25b** also has an *R* stereocenter at the malonamic acid residue.

The longer retained isomer of **25b** was then coupled to the hexapeptide **27** by the mixed anhydride method. HPLC analysis showed that only the longer retained isomer of peptide **19** was formed as the product of this reaction. It was therefore concluded that this diastereomer also has the *R* configuration at the malonamic acid residue.

Next, the longer retained diastereomer of **19** was coupled to fragment **18** using BOP as the coupling agent and triethylamine as the base. HPLC analysis showed that when the *R* isomer of **19** was used, only the longer retained isomer of **20** was formed, indicating that this diastereomer also has the *R* stereocenter at the malonamic acid residue, as shown in structure **20b**.

When the BOC protecting group on the diastereomer of **20** having the shorter HPLC retention time (**20a**) was cleaved with $TFA-CH_2Cl_2-HSCH_2CH_2SH$ (1:1:0.05), some racemization at the malonamic acid residue occurred. However, the product having the shorter retention time was the major product. Similarly, when the longer retained isomer **20b** was deblocked under the same conditions, the longer retained product was formed. Therefore, it can be concluded that the longer retained isomer in the retro-inverso peptide is **4**, having the *R* configuration at the malonamic acid residue, and the shorter retained isomer is **3**.

Discussion

The chemistry involved in the preparation of retro-inverso peptides is well established, yet the synthesis of **3**, and its diastereomer **4**, still offered some challenges. To begin, the synthesis of malonamic acids has been achieved either by multistep routes starting with C-2 substituted malonates,[45] or by treating C-2 substituted cyanoacetate esters with harsh reagents such as hot concentrated sulfuric acid.[46] Obviously the presence of the oxidizable sulfur atom in the side chain of acid **7** precludes the use of the second method, hence the saponification with potassium hydroxide/*tert*-butyl alcohol may prove to be a direct and mild synthesis of certain malonamic acids. Also, the presence of the sulfur in dipeptide **25a** complicated its Hofmann rearrangement with PIFA, which necessitated its chemical modification prior to the rearrangement. Another consequence is that methionine, like tryptophan and tyrosine,[47] should be protected when the PIFA methodology is used to construct partially modified retro-inverso peptides.

The second and more important challenge was the incorporation of a monoacylated *gem*-diaminoalkyl analogue of asparagine into the retro-inverso peptide and the implications it has on the stability of the peptide. A retro-inverso isomer **32** of the dipeptide sweetener (*R*,*S*)-α-aminomalonyl-L-phenylalanine methyl ester has been reported to be rather unstable.[17] On the other hand, no

(45) Pallai, P. V.; Richman, S.; Goodman, M. In *Peptides, Synthesis-Structure-Function, Proceedings of the 7th American Peptide Symposium (1981)*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL, 1981; pp 85–88.

(46) Fisher, E.; Brauns, F. *Ber.* **1914**, *47*, 3181.

(47) Pallai, P. V.; Richman, S.; Struthers, R. S.; Goodman, M. *Int. J. Peptide Protein Res.* **1983**, *21*, 84.

problems of instability were reported for the retro-inverso analogue of [D-Phe[2]]-luteinizing hormone-releasing factor (**33**), which incorporates a *gem*-diaminoalkyl analogue of pyroglutamic acid.[18] Although monoacyl *gem*-diaminoalkyls are masked aldehydes, they have surprising stability towards hydrolysis.[48] It has been estimated that retro-inverso peptides containing these units should have a half life of 10–50 h at 25 °C and physiological pH.[48] When the acylating moiety is of the alkoxycarbonyl type, these compounds become more susceptible to hydrolysis by a factor of ca. 10 at 50 °C and pH 4.64.[48] In contrast, diacyl *gem*-diaminoalkyl molecules are very stable toward hydrolysis, as evidenced by the stability of **22**, **23**, **33**, and partially modified retro-inverso peptides. In the present case, both peptides **3** and **4** were stable enough to permit their synthesis, isolation, and biological testing without the need for any handling precautions. This pattern of relative stability seems to reflect on the commonly used methods of preparation of acylated *gem*-diaminoalkyl compounds, namely the Curtius[36] and Hofmann[47] rearrangements, where it was found that *N*-acyl amino acids were preferred over *N*-alkoxycarbonyl amino acids as substrates for these reactions. However, in the present case, the presence of an amide group in the side chains of asparagine and glutamine, and the need for mild final deprotection justifies the use of the urethane-containing intermediates **23** as a building block for the desired peptide.

The reportedly low nucleophilicity of the free amino group in **18** could have contributed to the difficulty experienced in its coupling to the octapeptide **19**. In fact, Goodman recommended that fragment couplings to *gem*-diaminoalkyl residues were to be avoided.[47] However, the successful use of BOP, which effectively generates the reactive benzotriazole esters, may serve to extend the scope of this coupling and permit more flexibility in the design of retro-inverso peptides.

The synthesis of retro-inverso peptides always involves the incorporation of racemic 2-substituted malonic acid derivatives, resulting in the production of diastereomers. Since only one of these isomers possesses topological similarity to the parent peptide, it is important for the interpretation of biological activity to separate the diastereomers and assign absolute configurations.[21] However, this has rarely been accomplished[49] due to the additional work required to separate the diastereomers and determine their absolute configurations. Moreover, the configurational stabilities of the malonyl residues after the diastereomers are separated may present a problem.[27] In addition, a decision has sometimes been made not to separate the diastereomers because the lack of activity does not justify the labor required.[26] It is known that the half esters of 2-substituted malonic acids are subject to rapid racemization that precludes their resolution by classical methods.[49,50] In contrast, the 2-substituted malonamic acids are more configurationally stable and can be resolved.[51] The configurational stabilities of 2-substituted malonic acid derivatives after incorporation into peptides are dependent on both the nature of the substituent and the amino acid sequence surrounding the incorporation site, with the times required for establishment of equilibrium being on the order of minutes,[27] hours,[29,52] or days.[47]

(48) Loudon, G. M.; Almond, M. R.; Jacob, J. N. *J. Am. Chem. Soc.* **1981**, *103*, 4508.
(49) For a notable exception, see: Fournié-Zaluski, M. C.; Lucas-Soroca, E.; Devin, J.; Roques, B. P. *J. Med. Chem.* **1986**, *29*, 751.
(50) Kitazume, T.; Sato, T.; Kobayashi, T.; Lin, J. T. *J. Org. Chem.* **1986**, *51*, 1003.
(51) Fischer, E.; Brauns, F. *Sitzungsber, Preuss. Akad. Wiss., Phys.-Math. Kl.* **1914**, 714.

In the present case of retro-inverso bombesin C-terminal nonapeptide (**3**), the configuration of the malonyl residue was unusually stable in cold unbuffered aqueous solution. The two isomers, **3** and **4**, were also stable in the buffered medium used for biological testing, for a period that was twice as long as the time period required to conduct the tests. It can be concluded that the observed activities reflect the "real" effects exhibited by each pure diastereomer, and that the effort to separate the diastereomers was warranted. Also, when attempting to assign the configurations of **3** and **4**, the configurational stability of the malonamyl residue allowed the stereospecific synthesis of the structurally defined peptide **20b** from the *R* diastereomer of **25b**, contrary to the belief that preparation of a single configurationally characterized analog from optically pure intermediates may be a futile effort.[27]

As it turned out, peptide **3** lacked both agonist and antagonist activities. Peptide **4** was a week bombesin agonist with potency less than 0.1% that of bombesin, but lacked antagonist activity. Although the difference in activity between the inactive diastereomer **3** and the weak agonist **4** may not be of great significance, these results clearly are in disagreement with the common belief[49,52] that the retro-inverso diastereomer that more closely resembles the parent peptide should display higher activity. They are also not harmonious with the observation that [D-Met[14]]bombesin is 10% as active as bombesin in lowering the temperature of cold exposed rats following intracisternal administration.[11] Nonetheless, the inactivity of **3** seems to indicate that the interaction of the receptor with the peptide backbone is essential for the activity of bombesin. However, this interpretation should be advanced with caution, since it is believed that some differences in side chain topology exist between retroenantiomers and the parent peptide.[53] In addition, the transformation of a peptide to its retro-inverso counterpart may introduce new intramolecular interactions that alter the conformation.

Experimental Section

Melting points were determined on a Thomas-Hoover Unimelt or Fisher-Johns melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian FT-80, Varian XL-200, Varian VXR-500, Perkin-Elmer R-32, Chemagnetics A-200, and GE QE 300 spectrometers. IR spectra were recorded on a Beckman IR-33 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter using the sodium D line at room temperature. Low-resolution chemical-ionization mass spectra (CIMS) were determined on a Finnigan 4000 spectrometer using ammonia or 2-methylpropane as the reagent gas. High-resolution CIMS, low-resolution fast atom bombardment mass spectra (FABMS), and peak match FABMS were obtained on a Kratos MS50 spectrometer. Microanalyses were performed by the Purdue Microanalytical Laboratory.

Analytical thin-layer chromatography was done on Baker-flex silica gel 1B2-F plastic coated sheets and Merck silica 60 F_{254} glass coated plates. Column chromatography was performed using Sigma 70–230-mesh silica gel. Analytical reverse-phase HPLC was on a 4.6 × 250 mm, 10 μm, C-18, Vydac 218TP column. Semipreparative HPLC was accomplished on a 10 × 250 mm, 10 μm, C-18, Vydac 218TP column. Centrifugal countercurrent chromatography was performed on a P.C. Inc. Ito multilayer coil separator–extractor instrument.

Distilled, deionized water, Fisher Scientific HPLC grade acetonitrile, and Chemical Dynamics Corporation's sequalog grade trifluoroacetic acid were used in all HPLC work. Reagent grade 1-butanol was distilled before use for counter current chroma-

(52) Richman, S. J.; Goodman, M.; Nguyen, T. M.-D.; Schiller, P. W. *Int. J. Peptide Protein Res.* **1985**, *25*, 648.
(53) Freidinger, R. M.; Veber, D. F. *J. Am. Chem. Soc.* **1979**, *101*, 6129.

tography. Acetone was stored over anhydrous potassium carbonate. Benzyl alcohol was distilled from potassium carbonate. Benzene was dried by azeotropic distillation. *N*,*N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. Triethylamine, *N*-methylmorpholine, and pyridine were distilled from calcium hydride and stored over potassium hydroxide pellets.

2-(Aminocarbonyl)-4-(methylthio)butyric Acid (7 and 8).[35] A solution of ethyl 2-cyano-4-(methylthio)butyrate[34] (6, 250 mg, 1.33 mmol) in *tert*-butyl alcohol (2.4 mL) was stirred at reflux with powdered potassium hydroxide (500 mg) for 25 min. After cooling, water was added and the layers were separated. The aqueous phase was washed with chloroform (2 × 15 mL), acidified to pH 1 with concentrated hydrochloric acid, and concentrated to dryness. The residue was triturated with acetone and filtered. The filtrate was concentrated, and the residue was recrystallized from a mixture of acetone and carbon tetrachloride to give the product 7 (210 mg, 88%) as white plates: mp 114–115 °C; IR (KBr) 3345, 3200, 2900, 2580, 1720, 1660, 1590, 1430, 1400, 1280, 1220, 1115, 950, 770 cm^{-1}; NMR [200 MHz, $(CD_3)_2CO$, recorded immediately after dissolution] δ 7.20 (br, 1 H), 6.70 (br, 1 H), 3.48 (t, 1 H, J = 7.1 Hz), 2.55–2.48 (m, 2 H), 2.16–2.07 (m, 2 H), 2.06 (s, 3 H); CIMS (NH_3 ionizing gas) m/e (relative intensity) 195 (M^+ + 18, 51), 178 (MH^+, 15), 151 (100), 134 (47).

Ethyl 2-(Aminocarbonyl)-4-(methylthio)butyrate (9). Acetyl chloride (10 drops) was added to a solution of 2-(aminocarbonyl)-4-(methylthio)butyric acid (8, 20 mg, 0.13 mmol) in absolute ethanol (2 mL), and the mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated on a rotary evaporator, and the residue was chromatographed on silica gel, eluting with ethyl acetate–hexane (7:20). Concentration of the fractions containing the product yielded a white solid (23 mg, 100%): mp 72–72.5 °C; IR (KBr) 3385, 3180, 2995, 2975, 2955, 2910, 1735, 1650, 1440, 1300, 1185, 1135, 1010 cm^{-1}; NMR (80 MHz, $CDCl_3$) δ 6.47 (br, 1 H), 6.02 (br, 1 H), 4.22 (q, 2 H, J = 7 Hz), 3.46 (t, 1 H, J = 7 Hz), 2.55 (m, 2 H), 2.24 (m, 2 H), 2.09 (s, 3 H), 1.29 (t, 3 H, J = 7 Hz).

***tert*-Butyl 2-(Aminocarbonyl)-4-(methylthio)butyrate (10).**[54] Dicyclohexylcarbodiimide (64 mg, 0.31 mmol) was added to an ice-cold solution of 2-(aminocarbonyl)-4-(methylthio)butyric acid (8, 50 mg, 0.28 mmol), *tert*-butyl alcohol (62 mg, 0.85 mmol), and 4-(dimethylamino)pyridine (5 mg) in methylene chloride–dimethylformamide (3 mL, 2:1). After 5 min, the cooling bath was removed and the mixture was stirred at room temperature for 3.5 h. The precipitated 1,3-dicyclohexylurea was then filtered, and the filter cake was washed with a small volume of carbon tetrachloride. The filtrate was concentrated on a rotary evaporator, and the residual dimethylformamide was removed under high vacuum. The solid residue was triturated with cold carbon tetrachloride (2 mL) and filtered. The filtrate was concentrated, and the residue was chromatographed on silica gel, eluting with ethyl acetate–hexane (7:20) to give the product 10 (35 mg, 61%) as a white solid. The analytical sample was obtained by fractional crystallization from aqueous methanol: mp 104–106 °C; IR (KBr) 3410, 3330, 3260, 2995, 2925, 2850, 1715, 1670, 1655, 1635, 1570, 1535, 1450, 1400, 1360, 1305, 1225, 1150, 1135, 1080, 880, 830, 795, 730, 700 cm^{-1}; NMR (80 MHz, $CDCl_3$) δ 6.44 (br, 1 H), 5.83 (br, 1 H), 3.34 (t, 1 H, J = 7 Hz), 2.53 (m, 2 H), 2.29–2.09 (m, 2 H), 2.09 (s, 3 H), 1.47 (s, 9 H).

Curtius Rearrangement of *N*-Carbobenzoxy-L-leucine. Triethylamine (70 μL, 0.5 mmol) was added to a solution of *N*-carbobenzoxy-L-leucine (11, 132 mg, 0.5 mmol) in acetone (3 mL). The solution was cooled in an ice–salt bath, and ethyl chloroformate (47.7 μL, 0.5 mmol) was added. The mixture was stirred for 15 min. A cold solution of sodium azide (65 mg, 1 mmol) in water (1 mL) was added to the stirred reaction mixture. After 30 min, the reaction mixture was poured into ice water (30 mL) and extracted with methylene chloride (3 × 5 mL). The combined extracts were dried ($MgSO_4$) in the freezer and filtered, and the solvent was evaporated to give a yellow oil. The oil was dissolved in dry benzene (4 mL), benzyl alcohol (58 μL, 0.6 mmol) was added, and the mixture was heated at reflux for 2 h. The solvent was removed on a rotary evaporator, and the residue was chromatographed on silica gel (15 g, 2 × 12 cm, 200 mL of 8:92 ethyl acetate–hexane, 250 mL of 25:75 ethyl acetate–hexane) to give the three products, 15, 16, and 17.

***N*-Carbobenzoxy-1-amino-1-(benzyloxy)-3-methylbutane (15):** yield 42.8%; oil; R_f 0.35 (15:85 ethyl acetate–hexane); $[\alpha]_D$ 0° (c 2.0, MeOH); IR (neat) 3380, 3300, 3040, 3010, 2930, 2850, 1700, 1520, 1455, 1440, 1385, 1370, 1355, 1320, 1270, 1240, 1210, 1105, 1025, 1010, 970, 890, 800, 755, 715, 680 cm^{-1}; NMR (500 MHz, $CDCl_3$) δ 7.40–7.26 (m, 10 H), 5.19–5.11 (m and s, 3 H), 5.04 (d, 1 H, J = 9.7 Hz), 4.66 and 4.54 (q_{AB}, 2 H, J = 11.7 Hz), 1.81–1.71 (m, 1 H), 1.67–1.60 (m, 1 H), 1.42 (ddd, 1 H, J = 13.7, 7.5, 6.1 Hz), 0.91 (d, 3 H, J = 6.6 Hz), 0.87 (d, 3 H, J = 6.6 Hz); low-resolution FABMS m/e (relative intensity) 220 (MH^+ − 108, 100).

Anal. Calcd for $C_{20}H_{25}NO_3$: C, 73.35; H, 7.70; N, 4.28. Found: C, 73.19; H, 8.01; N, 4.28.

***N*,*N'*-Dicarbobenzoxy-1,1-diamino-3-methylbutane (16):** yield 13.5%, recrystallized from EtOH–H_2O; R_f 0.12 (15:85 ethyl acetate–hexane); mp 125–126 °C; IR (KBr) 3330, 3080, 3060, 3020, 2950, 2860, 1705, 1550, 1510, 1445, 1375, 1340, 1285, 1235, 1145, 1090, 1040, 1020, 1000, 960, 890, 760, 730, 710, 680 cm^{-1}; NMR (500 MHz, $CDCl_3$) δ 7.35–7.28 (m, 10 H), 5.52 (br, 2 H), 5.07 and 5.04 (s and br, 5 H), 1.72 (br, 2 H), 1.67–1.58 (m, 1 H), 0.90 (d, 6 H, J = 6.5 Hz); low-resolution FABMS m/e (relative intensity) 393 (MH^+ + 22, 59), 371 (MH^+, 27), 220 (MH^+ − 151, 100); high-resolution FABMS m/e calcd MH^+ 371.1970, found 371.1972.

Benzyl *N*-(*N*-carbobenzoxy-1-amino-3-methylbutyl)-allophanate (17): yield 14%, recrystallized from ethyl acetate–hexane; R_f 0.06 (15:85 ethyl acetate–hexane); mp 150–151 °C; IR (KBr) 3345, 3310, 3080, 3060, 3030, 2950, 2860, 1710, 1695, 1540, 1520, 1490, 1450, 1380, 1360, 1320, 1280, 1255, 1240, 1140, 1120, 1075, 1040, 985, 960, 925, 900, 730, 690 cm^{-1}; NMR (500 MHz, $CDCl_3$) δ 8.23 (br, 1 H), 7.40–7.30 (m, 10 H), 6.95 (s, 1 H), 5.51 (br, 1 H), 5.32–5.24 (m, 1 H), 5.16 (s, 2 H), 5.14–5.06 (m, 2 H), 1.80–1.72 (br, 2 H), 1.70–1.61 (m, 1 H), 0.93 (d, 6 H, J = 6.5 Hz); low-resolution FABMS m/e (relative intensity) 436 (MH^+ + 22, 99.9), 414 (MH^+, 24), 263 (MH^+ − 151, 100), 220 (MH^+ − 194, 50); high-resolution FABMS m/e calcd MH^+ 414.2028, found 414.2019.

***N*-(*tert*-Butoxycarbonyl)-L-β-cyanoalanine (21).** A solution of *N*-(*tert*-butoxycarbonyl)-L-asparagine[55] (100 mg, 0.431 mmol) in redistilled pyridine (2 mL) was cooled in a 16–20 °C water bath. Dicyclohexylcarbodiimide (93.5 mg, 0.454 mmol) was added, and the mixture was stirred for 3 h. The precipitated dicyclohexylurea was filtered off and washed with pyridine (1 mL). The pyridine was removed at room temperature under vacuum, and the syrupy residue was triturated with water (5 mL). After cooling for 1 h, the mixture was filtered and the filtrate was concentrated to about 1 mL and then acidified with 1 N HCl to pH 2.1 and extracted with ethyl acetate (2 × 5 mL). The extracts were dried ($MgSO_4$), filtered, and concentrated to give a yellow oil. This oil was stirred with hexane (4 mL) for 20 h, and the solution was cooled in the freezer overnight. The white solid (58 mg, 63%) was filtered and air-dried: mp 80–81 °C dec; $[\alpha]_D$ −6° (c 0.5, EtOH); IR (KBr) 3600–2700, 3310, 2260, 2240, 1740, 1695, 1645, 1550, 1520, 1455, 1410, 1390, 1360, 1340, 1330, 1320, 1300, 1265, 1250, 1220, 1180, 1150, 1050, 1020, 980, 960, 900, 870, 840, 810, 770, 740, 695 cm^{-1}; NMR (80 MHz, $CDCl_3$) δ 8.50 (br, 1 H), 5.70 (br, 1 H), 4.50 (m, 1 H), 3.00 (br d, 2 H, J = 7 Hz), 1.47 (s, 9 H).

(*S*)-*N*-(*tert*-Butoxycarbonyl)-*N'*-carbobenzoxy-3,3-diaminopropionitrile (22). Triethylamine (32.5 μL, 0.234 mmol) was added to a solution of *N*-(*tert*-butoxycarbonyl)-L-β-cyanoalanine (21, 50 mg, 0.23 mmol) in acetone (2 mL). The mixture was cooled and stirred in an ice–salt bath, and then ethyl chloroformate (22.3 μL, 0.234 mmol) was added. The mixture was stirred in the ice–salt bath for 15 min. An ice-cold solution of sodium azide (30.4 mg, 0.367 mmol) in water (0.5 mL) was added, and the stirring was continued for 30 min. The mixture was diluted with ice-cold water (15 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The extracts were dried ($MgSO_4$), filtered, and evaporated to give a clear oil that was dissolved in dry benzene (2 mL). Benzyl alcohol (31.4 μL, 0.304 mmol) was added, and the mixture was heated at reflux under an atmosphere of nitrogen for 2.5 h. The solvent was removed on a rotary evaporator, and the residue was purified by chromatography on a silica gel column

(54) Neisis, B.; Steglich, W. *Org. Synth.* **1984**, *63*, 183.

(55) Itoh, M.; Hagiwara, D.; Kamiya, T. *Tetrahedron Lett.* **1975**, 4393.

(6 g, 1.5 × 30 cm), eluting with EtOAc–hexane–triethylamine (20:79.5:0.5) to give the product as a white solid (20 mg, 27%). The analytical sample was recrystallized from CH_2Cl_2–CCl_4: mp 168–169 °C; $[\alpha]_D$ -3.8° (*c* 1.0, EtOH); IR (KBr) 3325, 3060, 3040, 3020, 2990, 2960, 2930, 2220, 1690, 1670, 1530, 1490, 1450, 1400, 1380, 1355, 1345, 1320, 1290, 1220, 1150, 1130, 1065, 1025, 1000, 940, 920, 890, 855, 835, 760, 730, 710, 675, 630 cm^{-1}; NMR (200 MHz, $CDCl_3$) δ 7.35 (s, 5 H), 5.80 (br, 1 H), 5.54 (br, 1 H), 5.24 (quint, 1 H, *J* = 8 Hz), 5.12 (s, 2 H), 3.07 (br d, 2 H, *J* = 8 Hz), 1.44 (s, 9 H); high-resolution CIMS *m/e* calcd MH^+ 320.1610, found 320.1613.

(*S*)-*N*-(*tert*-Butoxycarbonyl)-*N'*-carbobenzoxy-3,3-diaminopropionamide (23). An aqueous solution of 1 N Na_2CO_3 (60 μL, 0.03 mmol) and 30% H_2O_2 (200 μL, 1.76 mmol) were added successively to a solution of **22** (19 mg, 0.059 mmol) in acetone (0.5 mL). The mixture was stirred at room temperature for 24 h and then filtered. The filter cake was washed with water (2 mL) and acetone (1 mL) and air-dried to give a white solid (12.5 mg, 63%). The analytical sample was recrystallized from acetonitrile: mp 205–207 °C; $[\alpha]_D$ -2.9° (*c* 0.8, DMF); IR (KBr) 3450, 3400, 3320, 3180, 3010, 2970, 2920, 1690, 1675, 1650, 1545, 1500, 1400, 1345, 1320, 1290, 1235, 1160, 1140, 1030, 1010, 850 cm^{-1}; NMR (200 MHz, CD_3CN) δ 7.46 (s, 5 H), 6.34 (br, 2 H), 6.12 (br, 1 H), 5.86 (br, 1 H), 5.45 (m, 1 H), 5.16 (s, 2 H), 2.68 (d, 2 H, *J* = 6 Hz), 1.50 (s, 9 H).

Anal. Calcd for $C_{16}H_{23}N_3O_5$: C, 56.95; H, 6.87; N, 12.46. Found: C, 56.92; H, 6.84; N, 12.72.

Diastereomeric Peptide Derivatives 19. The coupling yields during the solid-phase synthesis of **24**, as evidenced by ninhydrin monitoring, were DLeu(99.3%)DHis(99.2%)Gly(99.6%)DVal-(99.3%)DAla(99.8%)DTrp(99.8%)DGlu-Resin. The peptide resin **24** (1.2195 g, 0.335 mequiv/g, 0.4329 mmol) was stirred for 24 h under a nitrogen atmosphere in a solution containing (*R*,*S*)-2-(aminocarbonyl)-4-(methylthio)butyric acid (**7**, 153.2 mg, 0.866 mmol), dicyclohexylcarbodiimide (179 mg, 0.866 mmol), and anhydrous hydroxybenzotriazole (117 mg, 0.866 mmol) in dry DMF (10 mL). The resin was filtered off, washed successively with DMF (10 mL), methanol (3 × 15 mL), and methylene chloride (2 × 15 mL), and air-dried. A ninhydrin test indicated that the coupling was 99% complete. The peptide was cleaved from the resin with a solution of dimethyl sulfide (0.85 mL) and anisole (0.85 mL) in HF (8.3 mL). Peptide **19** (150 mg) was purified by centrifugal countercurrent chromatography using a 4:0.5:5.5 butanol–acetic acid–water system with the upper phase as the stationary phase. The mobile phase was pumped in at a rate of 1 mL/min. A total of 50 mg of pure peptide was recovered (20 mg of each diastereomer and 10 mg of a mixed fraction): low-resolution FABMS calcd *m/e* 969.45 (MH^+), found 969.40.

Peptide Derivatives 20a and 20b. A suspension of (*S*)-*N*-(*tert*-butoxycarbonyl)-*N'*-carbobenzoxy-3,3-diaminopropionamide (**23**, 10 mg, 0.0296 mmol) and 10% Pd/C (2 mg) in methanol (2 mL) was stirred under a hydrogen atmosphere at room temperature for 1 h. The catalyst was filtered off, and the filtrate was concentrated by evaporation at room temperature. The crude product **8** was dissolved in chloroform (2 mL), and the solution was concentrated and dried under vacuum for 15 min in order to remove the last traces of methanol. The purified peptides **19** (20 mg, 0.0206 mmol) and the BOP reagent (9.2 mg, 0.0208 mmol) were added, followed by dry DMF (1.5 mL). Dry triethylamine (2.87 μL, 0.0206 mmol) was added, and the mixture was stirred at room temperature for 7 h. The DMF was then removed under high vacuum. The residue was dissolved in 0.3 M acetic acid (4 mL) and filtered. The filtrate was chromatographed on a Vydac 218 TP, C-18, 10 μm, 10 × 150 mm column. A gradient of 9% aqueous CH_3CN–0.1% TFA to 42% CH_3CN–0.1% TFA in 33 min was used at a flow rate of 3 mL/min. The desired fractions were lyophilized to give the diastereomer **20a** (1.5 mg) having the shorter retention time (FABMS calcd 1154.5778, found 1154.5880) and the diastereomer **20b** (3 mg) having the longer retention time (FABMS calcd 1154.5778, found 1154.5880).

Retro-Inverso Bombesin C-Terminal Nonapeptides (3 and 4). These were obtained by the deprotection of peptides **20a** and **20b** with an excess of trifluoroacetic acid/methylene chloride/1,2-ethanedithiol (1:1:0.05) under nitrogen. The crude peptides were chromatographed on a Vydac 218TP, C-18, 10 μ, 4.6 × 250 mm column. A gradient of 9% CH_3CN–0.1% TFA to 33% CH_3CN–0.1% TFA in 34 min was used at a flow rate of 1 mL/min.

Fractions containing peptide **3**, obtained from deprotection of peptides **20a** and **20b**, were combined and lyophilized, as were fractions containing peptide **4**. Each peptide was dissolved in 0.3 M acetic acid and filtered separately through an Amberlite IRA-400 (AcO^-) ion exchange resin. The eluents were lyophilized to give the diastereomers **3** (1.2 mg), having the shorter HPLC retention time, and the diastereomer **4** (1.5 mg), having a longer HPLC retention time. FABMS for **3**, calcd MH^+ 1054.5256, found 1054.5486. FABMS of **4**, calcd MH^+ 1054.5256, found 1054.5449.

Diastereomeric Peptide Derivatives 25a. Under an atmosphere of nitrogen, dry *N*-methylmorpholine (82.5 μL, 0.75 mmol) was added to a solution of 2-(aminocarbonyl)-4-(methylthio)butanoic acid (**7**, 133 mg, 0.75 mmol), D-leucine methyl ester hydrochloride (136 mg, 0.75 mmol), dicyclohexylcarbodiimide (186 mg, 0.90 mmol), and *N*-hydroxysuccinimide (103.7 mg, 0.90 mmol) in anhydrous DMF (2 mL). The mixture was stirred at room temperature for 24 h, and the precipitated dicyclohexylurea was filtered and washed with DMF (1 mL). The solvent was removed under high vacuum, and the residue was dissolved in ethyl acetate (25 mL), washed with 3 N hydrochloric acid (5 mL), saturated sodium bicarbonate (5 mL), and saturated sodium chloride (5 mL), and dried ($MgSO_4$). The drying agent was filtered off, and the filtrate was concentrated on a rotary evaporator. The residue was chromatographed on silica gel (10 g, 2 × 8 cm, 3% MeOH–CH_2Cl_2) to give a yellowish solid (169.9 mg, 74%). The diastereomeric components of the product were separated on a Vydac 218TP, C-18, 10 μm, 10 × 250 mm column using a gradient of 9% CH_3CN–0.1% TFA to 42% CH_3CN–0.1% TFA over 33 min at a flow rate of 3 mL/min, followed by lyophilization. For **25a** (shorter retention time diastereomer with the *S* configuration at the substituted malonamic acid residue): mp 113–115 °C; NMR (200 MHz, $CDCl_3$) δ 6.85 (br, 1 H), 6.72 (br d, 1 H, *J* = 9.3 Hz), 5.43 (br, 1 H), 4.58 (m, 1 H), 3.74 (s, 3 H), 3.33 (t, 1 H, *J* = 7.4 Hz) 2.61 (t, 2 H, *J* = 6.8 Hz), 2.19 (q, 2 H, *J* = 7.2 Hz), 2.10 (s, 3 H), 1.66–1.54 (m overlapping water peak), 0.950 and 0.938 (2 d, 6 H, *J* = 6 Hz). For **25a** (longer retention time diastereomer with the R configuration at the substituted malonamic acid residue: mp 112–113 °C; low-resolution FABMS *m/e* (relative intensity) 305 (MH^+, 100); NMR (200 MHz, $CDCl_3$) δ 7.05 (br d, 1 H, *J* = 6.6 Hz), 6.77 (br, 1 H), 5.64 (br, 1 H), 4.57 (m, 1 H), 3.74 (s, 3 H), 3.35 (t, 1 H, *J* = 7.4 Hz), 2.70–2.45 (m, 2 H), 2.20 (q, 2 H, *J* = 7.2 Hz), 2.09 (s, 3 H), 1.70–1.60 (m, 3 H), 0.95 and 0.88 (2 d, 6 H, *J* = 6.2 Hz).

Diastereomeric Peptide Derivatives 29. A vigorously stirred solution of a diastereomeric mixture of peptides **25a** (10 mg, 0.03 mmol) in ethanol (2 mL) was heated at reflux with W-2 Raney nickel (266 mg) for 1.25 h. After cooling, the supernatant liquid was decanted and the catalyst was washed with ethanol (3 × 2 mL). The solvent was removed on a rotary evaporator, and the residue was triturated with acetonitrile (5 mL), filtered, and concentrated to give a white solid (9.5 mg). This solid was dissolved in acetonitrile (2 mL) and purified by HPLC on a Vydac 218TP, C-18, 10 μm, 10 × 250 mm column using 21% CH_3CN–0.1% TFA as the eluting solvent at a flow rate of 3 mL/min. This purification gave 2.3 mg of the shorter retained isomer and 2.9 mg of the longer retained one (61% total yield). For **29** (mixture of diastereomers): low-resolution FABMS *m/e* (relative intensity) 259 (MH^+, 100). For **29** (shorter retention time): mp 99–101 °C; $[\alpha]_D$ +53.8° (*c* 0.21, MeOH); NMR (500 MHz, $CDCl_3$) δ 6.70 (br, 2 H), 5.47 (br, 1 H), 4.59 (dt, 1 H, *J* = 4.8, 8.5 Hz), 3.74 (s, 3 H), 2.96 (t, 1 H, *J* = 7.4 Hz), 1.95 (quint, 2 H, *J* = 7.4 Hz), 1.70–1.55 (m, 3 H), 1.02 (t, 3 H, *J* = 7.4 Hz), 0.945 and 0.937 (2 d, 6 H, *J* = 6.2, 6.1 Hz). For **29** (longer retention time): mp 112–114 °C; $[\alpha]_D$ +4.03° (*c* 0.31, CH_2Cl_2); NMR (200 MHz, $CDCl_3$) δ 6.85 (br, 1 H), 6.63 (br, 1 H), 5.50 (br, 1 H), 4.63–4.53 (m, 1 H), 3.73 (s, 3 H), 2.98 (br t, 1 H), 2.04–1.88 (m, 2 H), 1.72–1.49 (m, 3 H), 1.035 and 0.940 and 0.935 (t and 2 d, 9 H, *J* = 7.2, 6.0, 5.8 Hz).

Reaction of a Diastereomeric Mixture of Peptides 29 with PIFA. A solution of peptides **29** (8.5 mg, 0.033 mmol) in 3:1 acetonitrile–water (200 μL) was added to a solution of PIFA (14.1 mg, 0.033 mmol) in acetonitrile–water (1:1, 300 μL), and the mixture was stirred at room temperature for 6 h. The acetonitrile solvent was removed on a rotary evaporator, and the residue was diluted with water (1.5 mL). After addition of concentrated hydrochloric acid (3 drops), the solution was washed with ether

(3 × 4 mL). The aqueous solution was degassed under vacuum and used directly for HPLC analysis: low-resolution FABMS m/e (relative intensity) 231 (MH^+, 100).

***N*-(*tert*-Butoxycarbonyl)-L-2-aminobutanoic Acid and *N*-(*tert*-Butoxycarbonyl)-D-2-aminobutanoic Acid.**[55] Triethylamine (0.471 mL, 3 mmol) was added to a suspension of L-2-aminobutanoic acid (206 mg, 2 mmol) and 2-(((*tert*-butoxycarbonyl)oxy)imino)-2-phenylacetonitrile (BOC-ON, 542 mg, 2.2 mmol) in 1:1 acetone–water (8 mL). The mixture was stirred at room temperature for 4 h, and then the acetone was removed on a rotary evaporator. The residue was diluted with water (20 mL) and then washed with ether (4 × 20 mL). The aqueous phase was acidified to pH 2 with 3 N hydrochloric acid and extracted with methylene chloride (3 × 25 mL). The extracts were dried ($MgSO_4$), filtered, and concentrated on a rotary evaporator. The last traces of solvent were removed under high vacuum to give the product (0.41 g, 100%) as a colorless oil: $[\alpha]_D$ −9.4° (c 2.5, CH_2Cl_2); NMR (200 MHz, $CDCl_3$) δ 6.81 (br, 1 H), 6.21 and 5.04 (br and br d, 1 H, J = 7.8 Hz), 4.29–4.26 (2 m, 1 H), 1.92–1.70 (m, 2 H), 1.45 (s, 9 H), 0.98 (t, 3 H, J = 7.5 Hz).

The BOC derivative of D-2-aminobutanoic acid was prepared identically in quantitative yield as a colorless oil: $[\alpha]_D$ +9.5° (c 2.2, CH_2Cl_2); NMR (200 MHz, $CDCl_3$) δ 6.16–5.26 (br, 1.7 H), 5.03 (br d, 0.6 H, J = 7 Hz), 4.30–4.25 (m, 1 H), 1.95–1.66 (m, 2 H), 1.45 (s, 9 H), 0.98 (t, 3 H, J = 7 Hz).

***N*-*t*-BOC-LAbaDLeu-OMe and *N*-*t*-BOC-DAbaDLeu-OMe.** Triethylamine (27.8 mL, 0.2 mmol) was added to a solution of *N*-*t*-BOC-L-2-aminobutanoic acid (20.3 mg, 0.1 mmol), D-leucine methyl ester hydrochloride (18.1 mg, 0.1 mmol), and BOP (44.2 mg, 0.1 mmol) in acetonitrile (1 mL). The mixture was stirred at room temperature for 5 h, and then the solvent was removed on a rotary evaporator. The residue was dissolved in ether (25 mL), washed with 3 N hydrochloric acid (3 × 5 mL), saturated sodium bicarbonate (2 × 5 mL), and saturated sodium chloride (5 mL), and dried ($MgSO_4$). The drying agent was filtered off, and the filtrate was concentrated on a rotary evaporator. The last traces of solvent were removed under high vacuum, upon which the residue (31.5 mg, 95%) solidified: NMR (200 MHz, $CDCl_3$) δ 6.52 (d, 1 H, J = 9.6 Hz), 5.00 (br, 1 H), 4.62 (m, 1 H), 4.04 (m, 1 H), 3.72 (s, 3 H), 1.95–1.51 (m, 5 H), 1.45 (s, 9 H), 0.948 and 0.931 (d overlapping t, 9 H, J = 7 Hz).

The other diastereomer was prepared identically in 92% yield from *N*-*t*-BOC-D-2-aminobutanoic acid as an oil which solidified slowly: NMR (200 MHz, $CDCl_3$) δ 6.42 (br d, 1 H, J = 7.9 Hz), 5.03 (br, 1 H), 4.63 (m, 1 H), 4.02 (m, 1 H), 3.72 (s, 3 H), 1.97–1.54 (m, 5 H), 1.44 (s, 9 H), 0.95 and 0.93 (d overlapping t, 9 H, J_d = 5.9 Hz, J_t = 7.4 Hz).

LAbaDLeu-OMe and DAbaDLeu-OMe (30 and 31). These peptides were obtained from the BOC-protected precursors by reaction with excess 1:1 trifluoroacetic acid/methylene chloride under nitrogen. The crude products were used directly for HPLC analysis.

Peptide Derivative 25b. Aqueous 5% sodium hydroxide (0.225 mL, 0.293 mmol) was added to an ice-cold solution of compound **25a** (81 mg, 0.266 mmol) in 10:1 methanol–water (2.25 mL), and the mixture was stirred at 0 °C for 20 h. The resulting clear solution was acidified to pH 1 with 3 N hydrochloric acid, and then the solvent was removed under vacuum. The residue was triturated with hot acetone (2 × 5 mL) and filtered. The filtrate was concentrated on a rotary evaporator, and the residue was chromatographed on silica gel (5 g, 1 × 8 cm, CH_2Cl_2–MeOH–HOAc, 9:0.9:0.1) to give a yellowish solid (65.7 mg), which was still contaminated with some starting material. This solid was dissolved in methanol (3 mL) and further purified by reverse-phase HPLC on a Vydac 218TP, C-18, 10 μm, 10 × 250 mm column using a gradient of 9% CH_3CN–0.1% TFA to 42% CH_3CN–0.1% TFA in 34 min at a flow rate of 3 mL/min. The fractions containing the purified diastereomeric products were lyophilized separately to give 12.5 mg of a shorter retained isomer and 13 mg of the longer retained one (33% overall yield). For **25b** (mixture of diastereomers): IR (KBr) 3600–2700, 3440, 2960, 2920, 1705, 1655, 1640, 1545, 1530, 1400, 1270, 1230, 1195, 1150 cm^{-1}; low-resolution FABMS m/e (relative intensity) 291 (MH^+, 100), 274 (MH^+ − 17, 28). For **25b** (shorter retention time isomer with the S configuration at the substituted malonamide residue): mp 168–170 °C; $[\alpha]_D$ +22.7° (c 0.95, MeOH); NMR (300 MHz, pyridine-d_5–$CDCl_3$) δ 7.90 (d, 1 H, J = 7.7 Hz), 7.52 (br, 1 H), 6.04 (br), 4.68 (m, 1 H), 3.40 (t, 1 H, J = 7.4 Hz), 2.60 (t, 2 H, J = 6.9 Hz), 2.27–2.21 (m, 2 H), 2.05 (s, 3 H), 1.85–1.60 (m, 3 H), 0.96 and 0.93 (2 d, 6 H, J = 5.8 and 5.9 Hz). For **25b** (longer retention time isomer with the R configuration at the substituted malonamic acid residue): mp 124–125 °C; $[\alpha]_D$ +39.1° (c 1, MeOH); NMR (300 MHz, pyridine-d_5–$CDCl_3$) δ 8.05 (d, 1 H, J = 7.3 Hz), 7.56 (br, 1 H), 6.00–5.20 (br), 4.70 (m, 1 H), 3.47 (t, 1 H, J = 7.2 Hz), 2.70–2.50 (m, 2 H), 2.28 (q, 2 H, J = 7.2 Hz), 2.08 (s, 3 H), 1.86–1.60 (m, 3 H), 0.98 and 0.95 (2 d, 6 H, J = 6.2 Hz).

Esterification of 25b. A methanolic solution of **25b** was esterified with excess cold ethereal diazomethane. The volatiles were removed on a rotary evaporator, and the residue was dissolved in acetonitrile (1 mL) and analyzed by HPLC.

Purification of Peptide 27. The coupling yields during the solid-phase synthesis of the peptide-resin as evidenced by ninhydrin monitoring were DHis(99.0%)Gly(99.3%)DVal(99.3%)-DAla(99.2%)DTrp(99.4%)DGlu-Resin. The crude peptide (32 mg) was purified in 8-mg portions on a 10 × 250 mm, 10 μm, Vydac 218TP column. A gradient of 9% CH_3CN–0.1% TFA to 33% CH_3CN–0.1% TFA in 34 min was used at a flow rate of 3 mL/min. The fractions containing the desired peptide were lyophilized to give a white fluffy solid (10.5 mg); low-resolution FABMS m/e (relative intensity) 697 (MH^+, 100), 719 (MH^+ + 22, 62).

Peptide Derivative 19 (Long Retention Time Diastereomer with the *R* Configuration at the Substituted Malonamide Residue). Under an atmosphere of nitrogen, a solution of **25b** (long retention time isomer with the R configuration at the substituted malonamide residue, 1 mg, 3.45×10^{-3} mmol) in anhydrous THF (0.2 mL) was cooled to −12 °C in an ice–salt bath. Dry *N*-methylmorpholine (0.38 μL, 3.45×10^{-3} mmol) was added, followed by isobutyl chloroformate (0.45 μL, 3.45×10^{-3} mmol). After stirring for 10 min, a solution of peptide **27** (2.3 mg, 3.45×10^{-3} mmol) and *N*-methylmorpholine (0.76 μL, 6.90×10^{-3} mmol) in dry DMF (0.4 mL) was added by syringe, and stirring in the ice–salt bath was continued for 1 h. The solvents were removed under high vacuum, and the residue was dissolved in 2:3:2 MeOH–H_2O–DMF (0.7 mL) and analyzed by HPLC.

Peptide Derivative 20b (Long Retention Time Diastereomer with the *R* Configuration at the Substituted Malonamide Residue). The preparation was analogous to the previous preparation of the diastereomeric mixture of **20a** and **20b** but starting with the long retention time isomer of peptide **19** having the R configuration at the substituted malonamic acid residue.

Acknowledgment. This investigation was supported by Contract NO1-CM-67699, awarded by the National Cancer Institute, DHEW. We appreciate the technical assistance of Nancy Malinowski. We are also greatful to Dr. Rudiger Haugwitz, NIH, for arranging the collaboration between our two research groups.