Tetrahedron Vol. 48, No. 40, pp. 8601-8614, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00

Approaches to the Synthesis of Endothiopeptides: Synthesis of a Thioamide-Containing C-Terminal Bombesin Nonapeptide

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(*Received in USA* 13 *July* 1992)

Key Words: Dithioester, epimerization, thioacylation, endothiopeptide, thioamide, bombesin.

Abstract: *Several approaches have been investigated for the synthesis of a bombesin C-terminal nonapeptide analogue AsnGlnTrpAlaValGlyHisLeu-ψCSNHMet-NH_2. A new activated dithioester **16** has been synthesized. Thioacylation of methionine methyl ester with **16** was always accompanied by racemization, resulting in the formation of diastereomeric mixtures of reaction products.*

Bombesin (**1**)[1] and the structurally related gastrin releasing peptide (GRP) act as autocrine growth factors for small cell lung carcinoma (SCLC).[2] Structure-activity studies have indicated that the C-terminal nonapeptide of bombesin is the shortest fragment that retains full agonist activity.[3] It has been demonstrated that bombesin receptor antagonists inhibit the growth of SCLC and Swiss 3T3 cells.[4] Therefore, bombesin antagonists have potential for development as therapeutically useful anticancer agents. Several bombesin antagonists were obtained by reducing the 13-14 amide bond in [Leu[14]] bombesin[5a] and shorter fragments,[5b] or by replacing this bond with a methyleneoxy group in GRP analogues.[5c] The most likely reason for the success of this approach is a change in the conformation of the modified peptide, which results in retention of the affinity of the ligand for the receptor, but abolishes its intrinsic biological activity.

The thioamide group represents a minimal modification of the peptide backbone, yet the larger size and lower electronegativity of the sulfur atom, as compared to oxygen, are expected to induce some conformational distortions in the modified peptide.[6] Thioamide analogues of several biologically active peptides have been prepared but the results were highly variable.[7] We decided to prepare a bombesin C-terminal nonapeptide analogue **2** with a thioamide group replacing the amide linkage between Leu[8] and Met.[9]

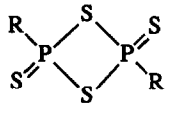
GlpGlnArgLeuGlyAsnGlnTrpAlaValGlyHisLeuMet-NH_2

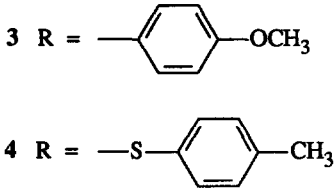
1

AsnGlnTrpAlaValGlyHisLeu-ψCSNH-Met-NH_2

2

The synthesis of endothiopeptides has been achieved by a variety of methods,[8] but the real breakthrough came with the development of Lawesson's reagent **3**, which thionates peptides without racemization.[9] Later, other more potent and selective reagents were introduced,[10] e.g. **4**. Methyl dithioester derivatives of amino acids have also been used by Clausen[8] and others[11] as thioacylating agents, with variable results. However, the question of racemization was not fully addressed. To help clarify the latter point, the reactions of dithioester **16** with methionine methyl ester and its hydrochloride were studied under a variety of conditions.

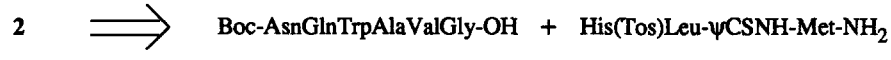




RESULTS AND DISCUSSION

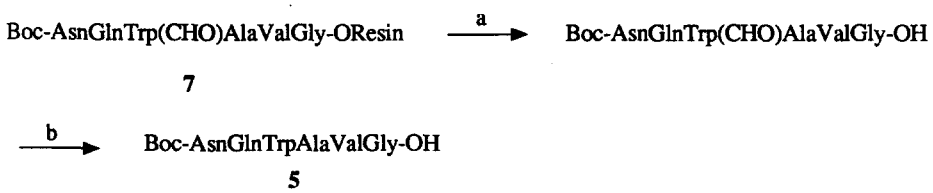
Bearing in mind that endothiopeptides are more successfully extended from the amino terminus,[10a, 12] the initially adopted strategy proposed a coupling between hexapeptide **5** and the endothiotripeptide **6**, as shown in Scheme 1.

Scheme 1



The synthesis of the hexapeptide **5** was achieved as shown in Scheme 2. The resin-linked Boc-protected hexapeptide **7** was assembled on an ABI automatic peptide synthesizer and cleaved by catalytic transfer hydrogenation,[13] which permits the retention of the Boc protecting group. The crude peptide was treated with 1 M hydroxylamine of pH 9 to remove the tryptophan *N*-formyl protecting group.[14] The resulting peptide was purified by reverse phase HPLC.

Scheme 2

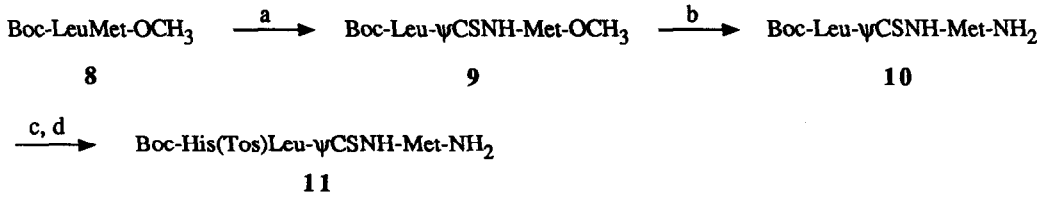


[a] $Pd(OCOCH_3)_2$, DMF, aq. HCO_2NH_4; [b] 1 M NH_2OH.

The synthesis of the Boc protected endothiotripeptide **11** proceeded from protected dipeptide **8**,[15] which was thionated with Lawesson's reagent in anhydrous benzene under reflux (Scheme 3). Under these conditions, no competing thionation of the carbamate or ester carbonyls was observed. Ammonolysis of the ester group was

achieved with saturated methanolic ammonia[12b] to give dipeptide **10** in moderate yield, contaminated by a closely eluting impurity. This impurity could only be removed by HPLC. Deprotection of the endothiodipeptide **10** with 1 N hydrogen chloride in acetic acid or 30% trifluoroacetic acid (TFA) in methylene chloride containing 5% 1,2-ethanedithiol (EDT) as scavenger, led to a complex mixture of products as evidenced by HPLC analysis. The crude deprotected dipeptide was coupled to Boc-His(Tos)-OH using dicyclohexylcarbodiimide (DCC) and *N*-methylmorpholine (NMM) in methylene chloride to give a very poor yield of **11**. Being impractical, this route was abandoned.

Scheme 3



[a] Lawesson's reagent, C_6H_6, Δ; [b] NH_3, CH_3OH; [c] 1 M HCl-CH_3COOH; [d] Boc-His(Tos)-OH, DCC, NMM, CH_2Cl_2.

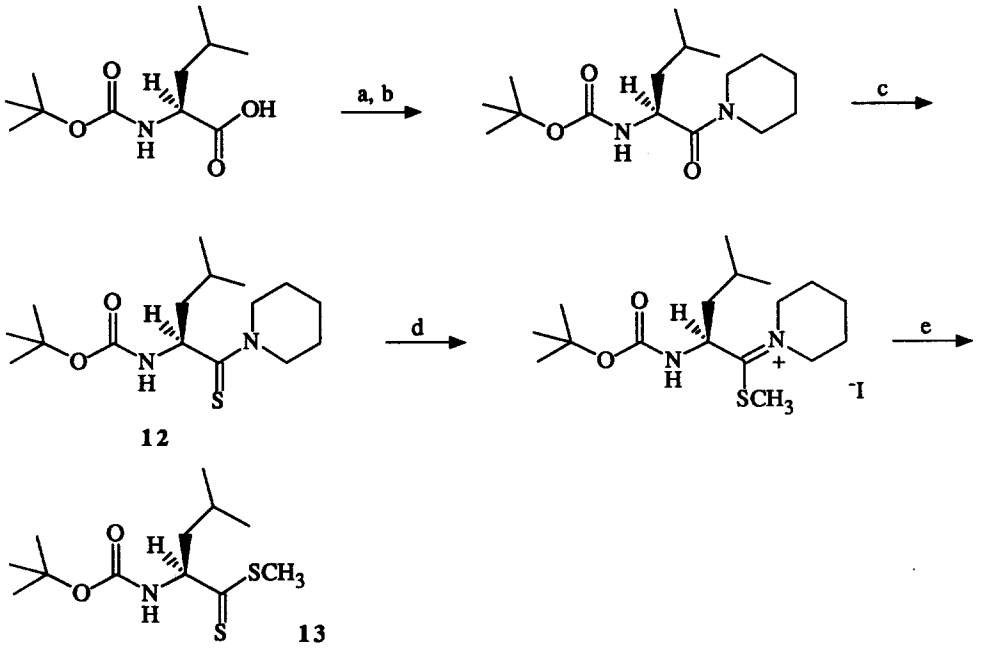
The reaction of dithioester **13** with methioninamide seemed like a viable approach to the synthesis of **10**. This particular dithioester has been reported to be unreactive as it failed to thioacylate glycinamide hydrochloride in methylene chloride.[16] Similarly, the *S*-benzyl dithioester derivative of Cbz-leucine was not very effective in thioacylating AspPhe-NH_2 in a mixture of THF and saturated sodium bicarbonate.[17] Nonetheless, it was hoped that useful thioacylation rates could be obtained by proper manipulation of the reaction conditions. The dithioester **13** was synthesized by a modification of the method of Thorsen,[16] as depicted in Scheme 4.

The reaction of **13** with methionine methyl ester hydrochloride was studied under a variety of conditions [ethyl acetate, NMM; DMF, NMM; DMF, hydroxybenzotriazole (HOBt), NMM; CH_3CN, triethylamine (TEA); CH_3CN, 2 eq. HOBt, 1 eq. TEA; CH_3CN, 2 eq. HOBt, 3 eq. TEA]. The reaction in ethyl acetate proceeded cleanly but very slowly, while decomposition of the dithioester was observed in DMF. The reaction in acetonitrile proceeded cleanly and at a faster rate than in ethyl acetate. As judged by TLC analysis, the reaction progressed to an appreciable extent after one week. Surprisingly, the addition of HOBt significantly retarded the reaction. In contrast, triethylammonium hydroxybenzotriazolate markedly accelerated the reaction and caused it to be complete in 2 days. Because the reaction conditions in the latter case are strongly basic, the possibility of racemization had to be considered. Indeed, the isolated product was found to be a 43:57 mixture of **9** and Boc-DLeu-ψCSNH-Met-OCH_3 (**14**) by HPLC[18] analysis on a BakerBond DNPG (covalent) chiral column using 3% 2-propanol in hexane as eluent. Under these conditions, base line separation of the stereochemically pure diastereomers **9** and **14** (prepared by thionation with Lawesson's reagent) could be achieved. To the best of our knowledge, this is the first chromatographic evidence that Lawesson's reagent effects racemization free thionation.

The reaction of excess **13** (5 equivalents) with methionine bound to a Merrifield resin in ethyl acetate was complete after 2 weeks as determined by a quantitative ninhydrin test.[19] A portion of the resulting resin was transesterified with methanol and TEA to give a mixture of **9** and Boc-DLeu-ψCSNH-Met-OCH_3 (**14**) in a

58:42 ratio, as determined by HPLC. The recovered **13** was totally racemized. When such long reaction times were used, racemization could not be avoided under the mildly basic conditions of solid phase synthesis.

Scheme 4



[a] NMM, *i*-BuOCOCl, THF; [b] piperidine; [c] Lawesson's reagent, C_6H_6, Δ; [d] CH_3I, CH_3CN; [e] H_2S, C_2H_5OH.

In view of the relative inertness of **13** and the need for strongly basic conditions to obtain useful reaction rates, it became obvious that more reactive dithioesters were needed. Several attempts were made to prepare aryl dithioesters by thionation of Boc-leucine thiophenyl ester[20] with **3** in toluene at reflux[21] or with **4** in THF, but without success. Direct thionation[10b] of Boc-leucine with **4** in toluene at reflux was not successful either. Attention was then focused on preparing dithioesters bearing electronegative ester groups (cyanomethyl, *tert*-butyl carboxymethyl).

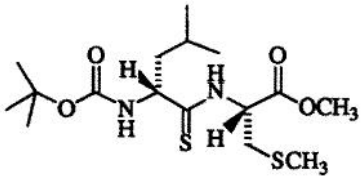
Attempted alkylation of thiopiperidide **12** with iodoacetonitrile in acetonitrile led to a complex mixture of products, and its reaction with *tert*-butyl iodoacetate[22] was sluggish. In contrast, facile alkylation of **12** was achieved with the triflate of *tert*-butyl glycolate (**15**)[23] in acetonitrile or methylene chloride. The crude alkylation product was treated with hydrogen sulfide in ethanol to give the desired dithioester **16**. The dithioester **16** proved to be more reactive than **13**; its thioacylation of methionine methyl ester was usually complete in 15-24 h at room temperature. The thioacylation of methionine methyl ester or its hydrochloride was studied under a variety of conditions and the results are summarized in Table 1. It can be seen that the use of a dipolar aprotic solvent and free amino component help suppress racemization to a certain extent. The use of weakly acidic additives was not beneficial except in the case of acetic acid, but the use of excess acetic acid led only to a modest improvement in the extent of racemization. On the other hand, the reaction of stereochemically pure

endothiodipeptide **9** with TEA (1 eq.) in acetonitrile did not lead to any observed epimerization of the leucine chiral carbon. Assuming that the starting dithioester is stereochemically pure, the above facts point to dithioester racemization, presumably through proton abstraction by the amine, as the sole reason for the formation of the diastereomer Boc-DLeu-ψCSNH-Met-OCH_3 (**14**), a conclusion that has been independently reached by Hartke.[24] Therefore, it is not surprising that the less reactive **13** led to a higher degree of racemization, even when acetic acid was present.

The assumption about the stereochemical purity of the thioacylating agent has not been rigorously verified, but the fact that we prepared two known dithioesters **13** and **17**, by a modification of the literature method,[16] and obtained very close optical rotations to those reported, is consistent with this synthesis of amino acid dithioesters being free of racemization.

In certain instances, the use of amino acid dithioester derivatives has been documented to lead to racemization,[11, 24] but the reactants or reaction conditions do not represent standard examples of what is encountered in peptide synthesis (imidazole as catalyst by Lajoie,[11a] hindered amino terminus of α-aminoisobutyric acid by Jensen,[11b] 1-phenylethylamine by Harke[24]). In other instances, no mention of racemization was made.[8, 16, 17] Therefore, we decided to reinvestigate the thioacylation with **17**, which has been used in the synthesis of thionated enkephalin. In contrast to dithioester **13**, this compound was reported to be a potent thioacylating agent which gave high yields of endothiopeptides. The synthesis of **17** was accomplished by the method described in Scheme 4, using commercially available Boc-Tyr(OBzl)-OH as the starting material. The reaction of **17** with methionine methyl ester hydrochloride was conducted exactly as described in the literature[8] (1 eq TEA, 1:1 ether-ethyl acetate, 26 h at room temperature). The stereochemically pure dipeptides Boc-Tyr(OBzl)-ψCSNH-Met-OCH_3 (**18**) and Boc-DTyr(OBzl)-ψCSNH-Met-OCH_3 (**19**) were prepared by standard procedures as shown in Schemes 5 and 6. Unfortunately, these diastereomers could not be separated on the DNPG chiral column, but their NMR spectra were quite distinct. The methyl ester signal appeared at δ 3.70 in the spectrum of **18** and at δ 3.74 in the spectrum of **19**. The product isolated from the thioacylation reaction with **17** showed both peaks in the ratio of 48:52, respectively. It is clear that significant racemization has occurred. To date, the use of dithioesters has been plagued by racemization and Hartke predicted that the synthesis of endothiopeptides by this route "will most probably be a dead end street".[24] This may be so, but the lower extent of racemization obtained with the more reactive **16** leads us to believe that if a truly potent thioacylating agent could be developed, the problem of racemization might be circumvented.

Considering the position of the thioamide group in **2**, a new route, which relies on the thionation of a resin-bound dipeptide, was tried next (Scheme 7). The resin linked dipeptide **20** was synthesized by conventional solid phase procedures, and was reacted with **4** (2.5 equivalents) in anhydrous THF at room temperature to give resin **21**, as evidenced by IR analysis. Further confirmation of complete thionation was obtained by transesterification with TEA and methanol to give the endothiopeptide **9** as the exclusive product (HPLC and NMR analysis). It is worth noting that thionation of Boc-Lys(Cbz)-benzhydrylamine resin with **3** required heating at 90-100 °C for 8 h in toluene.[25] The resin **21** was deblocked with 4 N hydrogen chloride in dioxane and coupled to Boc-His(Tos)-OH to give resin **22**. As a model, the reaction of **22** with ammonia in CH_3OH-DMF gave a very complex reaction mixture which contained some of the desired **23** (according to FABMS analysis). The discouraging result prompted the discontinuation of work along this route.



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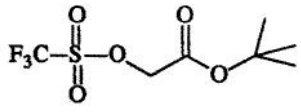

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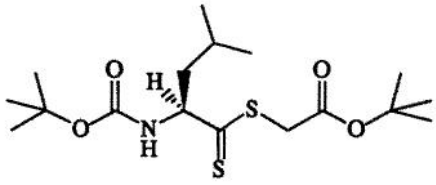

16

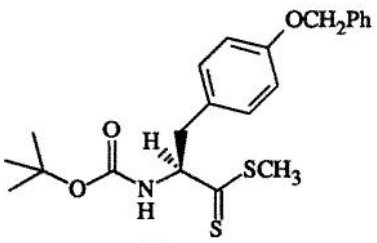

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Table 1. Racemization Studies on the Thioacylation of Methionine Methyl Ester/Hydrochloride with Dithioesters.

Dithioester	Amine	Base	Solvent	Additive	%14[a]
16	HCl·Met-OCH_3	TEA	CH_3CN	--	31
16	HCl·Met-OCH_3	NMM	CH_3CN	--	39
16	Met-OCH_3	--	EtOAc	--	37
16	Met-OCH_3	--	CH_3CN	--	27
16	Met-OCH_3	--	CH_3CN	--	33[b]
16	Met-OCH_3	--	CH_3CN	HOBt	33
16	Met-OCH_3	--	CH_3CN	PCP[c]	33
16	Met-OCH_3	--	CH_3CN	HOAc (1 eq.)	26
16	Met-OCH_3	--	CH_3CN	HOAc (3 eq.)	23
13	Met-OCH_3	--	CH_3CN	HOAc (1 eq.)	29[d]

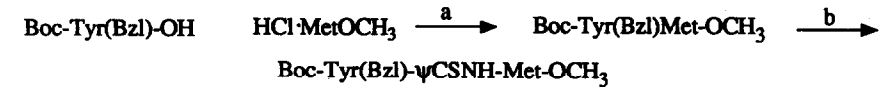
a The product was isolated as a mixture of **9** and **14** by chromatography and analyzed by HPLC to determine **%14**. All numbers were obtained from single experiments.

b This experiment was run as a control using a new batch of **16**. All the entries below were run with the same batch.

c Pentachlorophenol

d This reaction was worked up after 48 h at which time it was far from being complete. Had it been allowed to proceed to completion a higher percentage of **14** would have been obtained.

Scheme 5



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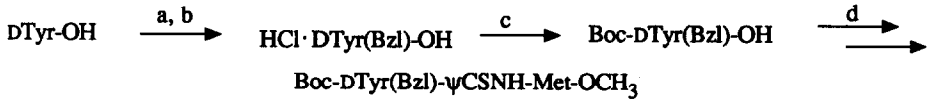
[a] TEA, BOP, CH_3CN; [b] Lawesson's reagent, C_6H_6, Δ.

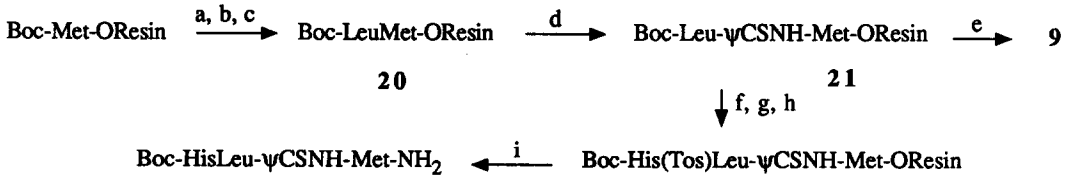
Scheme 6



19

[a] 2 N NaOH, $CuSO_4 \cdot 5H_2O$, $PhCH_2Br$, CH_3OH; [b] aq HCl; [c] BOC-ON, TEA; [d] Scheme 5.

Scheme 7

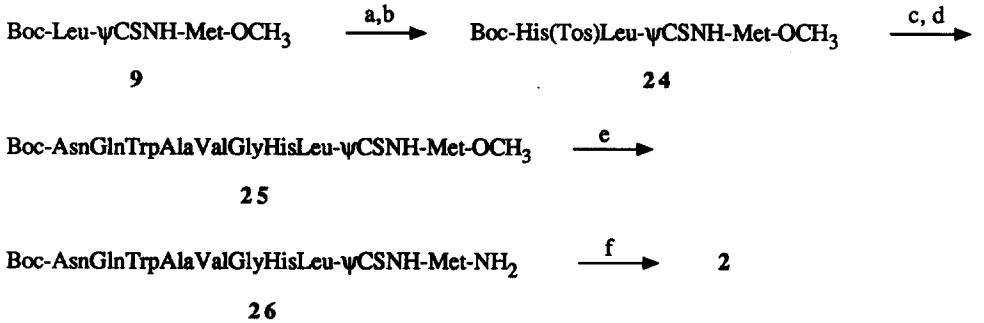


20 **21** **23** **22**

[a] TFA, CH_2Cl_2, 5% EDT; [b] TEA, CH_2Cl_2, [c] Boc-Leu-OH, DCC, CH_2Cl_2; [d] 2.5 eq **4**, THF; [e] TEA, CH_3OH; [f] 4 M HCl-dioxane; [g] TEA, $CHCl_3$; [h] Boc-His(Tos)-OH, DCC, CH_2Cl_2; [i] NH_3, CH_3OH, DMF.

As ammonolysis of peptide **9** and resin **22** had proven to be difficult, it was decided to modify the first approach and introduce the terminal amide group at a later stage, where it becomes practical to separate the peptide by HPLC (Scheme 8). Clean deprotection of endothiopeptide **9** could be effected with 4 N hydrogen chloride in dioxane or 30% TFA in methylene chloride containing 5% of *m*-cresol. Coupling of the deprotected peptide to Boc-His(Tos)-OH was most satisfactorily achieved by the mixed anhydride method, to give tripeptide **24** in good overall yield. Attempted deprotection of **24** under the conditions used for **9** led only to unidentified decomposition products. Most likely, this problem arises because of the presence of a histidine residue, since simpler endothiotripeptides have been easily deprotected.[16] Also, Kruszynski[26] has encountered similar problems with the deprotection of Boc-His(DNP)Pro-ψCSNH-NH_2, but was able to circumvent them by using neat TFA at 0 °C for a short period of time. This approach was also successful in deprotecting **24**, although some cleavage of the tosyl group was also observed. The deprotected tripeptide was immediately coupled to hexapeptide **5** to give a moderate yield of nonapeptide **25**. The coupling reaction was very slow, but surprisingly the tripeptide His(Tos)Leu-ψCSNH-Met-OCH_3 seemed to be quite stable under the reaction conditions. The nonapeptide **25** was converted to **26** in low yield by the action of methanolic ammonia at 0 °C. Cleavage of the Boc protecting group with TFA at 0 °C gave the desired product **2**, whose structure was confirmed by high resolution FABMS.

Scheme 8



[a] 4 M HCl-dioxane; [b] Boc-His(Tos)-OH, *i*-BuOCOCl, NMM, THF; [c] TFA, 0 °C; [d] **5**, DCC, HOBt, DIEA; [e] NH_3, CH_3OH; [f] TFA, 0 °C.

In conclusion, a thioamide-containing nonapeptide of complex structure has been successfully synthesized, but perhaps the most important contribution of this work is the demonstration that dithioesters of α-amino acids are not suitable for thiopeptide synthesis due to accompanying racemization. The synthesis was complicated by the presence of a histidine residue and an amide carboxy terminus. The nucleophilic and basic conditions needed to generate this terminus are not well tolerated by thioamide peptides.

EXPERIMENTAL

Analytical thin-layer chromatography was done on Whatman silica 60 K6F 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 x 250 mm, 10 μ, C-18, Vydac 218TP column. Semipreparative HPLC was accomplished on Dynamax 300A, C-18, 12 μ, 10 x 350 mm or Vydac 218TP, 15-20 μ, C-18, 22.5 x 250 mm columns. Benzene was dried by azeotropic distillation. *N,N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. Triethylamine and *N*-methylmorpholine were distilled from calcium hydride and stored over potassium hydroxide pellets.

Peptide 5. A suspension of the resin **7** (622.5 mg) and palladium acetate (622.5 mg) in DMF (8.1 mL) was allowed to stir at room temperature for 12 h. A solution of ammonium formate (822 mg) in water (0.8 mL) was added. Gas evolution was observed and the resin turned black. After 2 h at room temperature, the resin was filtered off and washed successively with DMF (2 x 8 mL) and acetic acid (8 mL). The solvents were removed under high vacuum to give a grayish solid. A small portion of this solid was purified by chromatography on a Dynamax 300A, C-18, 12 μ, 10 x 350 cm column using a gradient of 9% CH_3CN-0.1% TFA to 33% CH_3CN-0.1% TFA in 34 min at a flow rate of 3 mL/min. The fraction containing the major peak was lyophilized to give a white solid: low resolution FABMS *m/e* (relative intensity) 824 (14), 802 (MH^+, 100), 702 (42). The crude solid was dissolved in 1M aqueous hydroxylamine (33 mL, pH 9) and stirred at room temperature for 3 h. The pH of the reaction mixture was lowered by the addition of glacial acetic acid and the product was chromatographed on a Vydac 218TP, C-18, 15-20 μ, 22.5 x 250 mm column using a gradient of 15% CH_3CN-0.1% TFA to 65% CH_3CN-0.1% TFA in 50 min at a flow rate of 10 mL/min. The fraction containing the major peak was lyophilized to give 75.8 mg of a white solid: low resolution FABMS *m/e* (relative

intensity) 811 (30), 795 (29), 773 (M^+, 100), 673 (39).

Boc-Leu-ψCSNH-Met-OMe (9). A suspension of Boc-LeuMet-OMe[15] (150 mg, 0.398 mmol) and Lawesson's Reagent (88.7 mg, 0.219 mmol) in dry benzene (1 mL) was heated at reflux under an atmosphere of nitrogen for 2 h. The solvent was removed on a rotary evaporator and the residue was purified by flash chromatography on silica gel (230-400 mesh, 15% ethyl acetate-hexane) to give the product as a yellow oil (122 mg, 78%): IR (neat) 3380, 1745, 1695 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 8.55 (d, 1 H, J = 7 Hz, exchangeable with D_2O), 5.26 (ddd, 1 H, J = 8, 6, 5 Hz), 5.09 (d, 1 H, J = 8 Hz), 4.44-4.29 (m, 1 H), 3.78 (s, 3 H), 2.58-2.46 (m, 2 H), 2.45-2.10 (m, 2 H), 2.09 (s, 3 H), 1.80-1.50 (m, 3 H), 1.43 (s, 9 H), 0.95 (d, 6 H, J = 6 Hz); low resolution FABMS *m/e* (relative intensity) 393 (MH^+, 56), 337 (96), 305 (34), 293 (71), 276 (97), 245 (27). Anal Calcd for $C_{17}H_{32}N_2O_4S_2$: C, 52.02; H, 8.22; N, 7.14; S, 16.30. Found: C, 52.15; H, 8.32; N, 7.13; S, 16.30.

Boc-Leucine Piperidide. Under an atmosphere of nitrogen, a solution of Boc-Leucine monohydrate (1.05 g, 4.224 mmol) in anhydrous THF (10 mL) was cooled to -10 °C in an ice-salt bath. Dry NMM (0.465 mL, 4.224 mmol) was added followed by isobutyl chloroformate (0.548 mL, 4.224 mmol). The mixture was stirred for 15 min, then piperidine (0.418 mL, 4.224 mmol) was added. The mixture was allowed to warm to 0 °C over a period of 2 h and then stirred at room temperature for 3 h. The precipitated salts were filtered and washed with THF (10 mL). The filtrate was concentrated. The residue dissolved in ethyl acetate (50 mL) and the solution washed with 1.5 N hydrochloric acid (2 x 20 mL), 5% sodium bicarbonate (2 x 20 mL), saturated sodium chloride (20 mL), and dried over $MgSO_4$. The drying agent was filtered off and the filtrate concentrated under vacuum to give the product (1.195 g, 95%) as a colorless oil: $[\alpha]_D$ = -26° (*c* 0.9, CH_3OH) [lit[16] $[\alpha]_D$ = -23.3° (*c* 1, CH_3OH)]; ^1H NMR (200 MHz, $CDCl_3$) δ 5.34 (d, 1 H, J = 9 Hz), 4.66 (td, 1 H, J = 9, 4 Hz), 3.54 (t, 2 H, J = 5 Hz), 3.50-3.40 (m, 2 H), 1.79-1.48 (m, 9 H), 1.42 (s, 9 H), 0.99 (d, 3 H, J = 7 Hz), 0.91 (d, 3 H, J = 7 Hz).

Boc-Leucine Thiopiperidide (12). This compound was obtained by a procedure similar to the preparation of **9** as an oil in 91% yield after purification by chromatography on silica gel (10% ethyl acetate-hexane). ^1H NMR (200 MHz, $CDCl_3$) δ 5.77 (d, 1 H, J = 9 Hz), 4.92 (td, 1 H, J = 10, 4 Hz), 4.25 (br, 2 H),3.80 (br, 2 H), 1.80-1.56 (m, 9 H), 1.43 (s, 9 H), 1.00 (d, 3 H, J = 6 Hz), 0.92 (d, 3 H, J = 7 Hz).

Boc-Leucine Methyl Dithioester (13). Methyl iodide (3.15 mL, 50.6 mmol) was added to a solution of **12** (1.589 g, 5.06 mmol) in dry acetonitrile (5 mL) and the mixture was stirred under nitrogen for 48 h. The volatiles were removed on a rotary evaporator with protection from moisture and the residue further dried under high vacuum. The residue was dissolved in absolute ethanol (12 mL), cooled to 0 °C and a slow stream of hydrogen sulfide was bubbled through for a period of 1 h. The mixture was stirred at 0 °C overnight and then the solvent was removed *in vacuo*. The residue was triturated with ether (50 mL), filtered and concentrated to give a yellow oil which was chromatographed on silica gel (70 g, 2.2 x 28 cm, 4% ethyl acetate-hexane) to give the product (1.3 g, 93%) as a yellow solid: mp 55-56 °C (lit[16] mp 56-57 °C); $[\alpha]_D$ = -95.5° (*c* 1, CH_3OH) [lit[16] $[\alpha]_D$ = -89.4° (*c* 1, CH_3OH)]; ^1H NMR (200 MHz, $CDCl_3$) δ 5.26 (d, 1 H, J = 9 Hz), 4.94-4.87 (m, 1 H), 2.63 (s, 3 H), 2.80-2.55 (m, 3 H), 1.43 (s, 9 H), 0.97 and 0.95 (2 d, 6 H, J = 6 Hz).

Boc-DLeuMet-OMe.[27] Triethylamine (45 μL, 0.32 mmol) was added to a solution of Boc-D-leucine monohydrate (40.0 mg, 0.16 mmol), methionine methyl ester hydrochloride (32.1 mg, 0.16 mmol), and BOP (71.1 mg, 0.16 mmol) in acetonitrile (1 mL). The mixture was stirred at room temperature for 3 h, and the

solvent was removed on a rotary evaporator. The residue was dissolved in ethyl acetate (15 mL), washed with 1.5 N hydrochloric acid (2 x 5 mL), saturated sodium bicarbonate (2 x 5 mL), 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 (0.5 g, 0.5 x 5 cm, 30% ethyl acetate-hexane) to give a white solid (56.9 mg, 90%): mp 88-89 °C; IR (KBr) 3346, 3238,1750, 1680, 1652 cm^{-1}; ^{1}NMR (200 MHz, $CDCl_3$) δ 6.84 (d, 1 H, J = 8 Hz), 5.86-5.76 (br, 1 H), 4.69 (td, 1 H, J = 8, 5 Hz), 4.20-4.07 (br, 1 H), 3.74 (s, 3 H), 2.54-2.46 (m, 2 H), 2.28-2.10 (m, 1 H), 2.08 (s, 3 H), 2.08-1.90 (m, 1 H), 1.78-1.60 (m, 3 H), 1.45 (s, 9 H), 0.94 (d, 3 H, J = 6 Hz), 0.93 (d, 3 H, J = 6 Hz). Anal Calcd for $C_{17}H_{32}N_2O_5S$: C, 54.23; H, 8.57; N, 7.44; S, 8.50. Found: C, 53.92; H, 8.70; N, 7.34; S, 8.60.

Boc-DLeu-ψCSNH-Met-OMe (14). This substance was obtained by a procedure similar to the preparation of **9** as an oil in 93% yield after purification by chromatography on silica gel (benzene followed by 30% ethyl acetate-hexane). IR (Neat) 3302, 1744, 1694 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.65 (d, 1 H, J = 7 Hz), 5.28-5.18 (m, 1 H), 5.02 (d, 1 H, J = 8 Hz), 4.43 (ddd, 1 H, J = 9, 7, 5 Hz), 3.78 (s, 3 H), 2.55-2.47 (m, 2 H), 2.47-2.30 (m, 1 H), 2.25-2.10 (m, 1 H), 2.09 (s, 3 H), 1.90-1.50 (m, 3 H), 1.44 (s, 9 H), 0.95 (d, 3 H, J = 6 Hz), 0.94 (d, 3 H, J = 6 Hz). Anal Calcd for $C_{17}H_{32}N_2O_4S_2$: C, 52.02; H, 8.22; N, 7.14; S, 16.30. Found: C, 52.19; H, 8.36; N, 7.13; S, 16.22.

***tert*-Butyl 2-Hydroxyacetate.** Triethylamine (4.14 g, 41.03 mmol) was added to a solution of 97% formic acid (1.77 g, 38.46 mmol) in ethyl acetate (40 mL). After 5 min *tert*-butyl bromoacetate (5 g, 25.6 mmol) was added and the mixture stirred at room temperature for 24 h. The precipitated salts were filtered off and washed with ether (100 mL). The combined filtrates were washed with half-saturated aqueous sodium chloride (30 mL), dried ($MgSO_4$), filtered and concentrated to give *tert*-butyl 2-formyloxyacetate as a colorless liquid (3.13 g, 76%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.14 (t, 1H, J = 1 Hz), 4.59 (d, 2 H, J = 1 Hz), 1.49 (s, 9 H).

Solid sodium bicarbonate (3.28 g, 39.12 mmol) was added to a suspension of *tert*-butyl 2-fomyloxyacetate (3.13 g, 19.56 mmol) in water (60 mL) and the mixture was stirred at room temperature for 36 h. The reaction mixture was continuously extracted with ether for 12 h and the layers separated. The ether layer was dried ($MgSO_4$), filtered and concentrated to give a yellow liquid which was distilled under vacuum to give a colorless liquid (2.48 g, 96%): bp 69-70 °C/25 torr (lit[23] bp 74-76 °C/25 torr); IR (neat) 3442, 1736 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.02 (s, 2 H), 2.20 (br, 1 H), 1.49 (s, 9 H).

***tert*-Butyl 2-Trifluoromethanesulfonyloxyacetate (15).**[28] Trifluoromethanesulfonic anhydride (1.48 mL, 8.78 mmol) was added to a cold (-22 °C) solution of dry pyridine (0.75 mL, 9.23 mmol) in dry CH_2Cl_2 (31 mL). The mixture turned orange in color and became difficult to stir. The glycolate ester (1.16 g, 8.78 mmol) was added dropwise and the mixture stirred for 5 min at -22 °C then allowed to warm slowly to -10 °C. After warming it to room temperature with a water bath, the mixture was partitioned between hexane (40 mL) and ice cold water (30 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The residue was dissolved in pentane (50 mL) and filtered through silica gel (4 g, 2 x 1.5 cm). The silica was washed with pentane (200 mL) and the combined filtrates concentrated to give a colorless liquid (0.562 g, 26%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.78 (s, 2 H), 1.52 (s, 9 H); high resolution CIMS, *m/e* calcd. MH^+ 265.0355. Found 265.0358.

Dithioester 16. *tert*-Butyl 2-trifluoromethanesulfonyloxyacetate (**15**, 301 mg, 1.14 mmol) was added

to a solution of Boc-leucine thiopiperidide (**12**, 298.5 mg, 0.95 mmol) in dry methylene chloride (5 mL) and the mixture was stirred under nitrogen for 5.5 h at room temperature. The volatiles were removed on a rotary evaporator with protection from moisture and the residue further dried under high vacuum. The residue was dissolved in absolute ethanol (5 mL), cooled to 0 °C and a slow stream of hydrogen sulfide was bubbled through for a period of 40 min. The mixture was stirred at 0 °C overnight and then the solvent was removed *in vacuo*. The residue was triturated with ether (30 mL), filtered and concentrated. The residue was chromatographed on silica gel (25 g, 2 x 21 cm, 6% ethyl acetate-hexane) to give the product (241 mg, 67%) as a yellow oil: $[\alpha]_D$ = -15.9° (*c* 1, CH_2Cl_2), IR (neat) 3352, 1716 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.22 (d, 1 H, J = 9 Hz), 4.89 (td, 1 H, J = 9, 4 Hz), 4.01 (d, 1 H, J = 16 Hz), 3.94 (d, 1 H, J = 16 Hz), 1.80-1.60 (m, 3 H), 1.44 (s, 9 H), 1.43 (s, 9 H), 0.97 (d, J = 6 Hz), 0.95 (d, J = 6 Hz). Anal Calcd for $C_{17}H_{31}NO_4S_2$: C, 54.09; H, 8.28; N, 3.71; S, 16.95. Found: C, 53.93; H, 8.58; N, 4.09; S, 16.79.

Thioacylation with 16. A typical experiment is described. A solution of methionine methyl ester (0.78 mg, 4.78 x 10^{-3} mmol) and dithioester **16** (2.4 mg, 6.37 x 10^{-3} mmol) in acetonitrile (30 μL) was stirred at room temperature for 20 h. The solvent was removed on a rotary evaporator and the residue was chromatographed on silica gel (0.5 g, 0.5 x 5 cm, 10 mL of 10% ethyl acetate-hexane followed by 5 mL of 30% ethyl acetate-hexane). The fractions containing the desired product were combined and concentrated on a rotary evaporator to give an oil (1.6 mg, 85%). This product was dissolved in methylene chloride and used for HPLC analysis.

Boc-Tyr(O-Bzl) Piperidide. This compound was prepared similar to Boc-leucine piperidide as an oil (84 %) after purification by chromatography on silica gel (30% ethyl acetate-hexane): $[\alpha]_D$ = 2.64° (*c* 1, CH_3OH) [lit[8] $[\alpha]_D$ = 2.5° (*c* 1, CH_3OH)]; IR (neat) 3424, 3294, 1707, 1633 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.42-7.40 (m, 2 H), 7.38-7.35 (m, 2 H), 7.32-7.29 (m, 1 H), 7.09 (d, 2 H, J = 9 Hz), 6.88 (d, 2 H, J = 9 Hz), 5.45 (d, 1 H, J = 9 Hz), 5.04 (s, 2 H), 4.79 (dt, 1 H, J = 9, 7 Hz), 3.52-3.40 (m, 2 H), 3.26-3.20 (m, 1 H), 3.02-2.96 (m, 1 H), 2.89 (d, 2 H, J = 7 Hz), 1.57-1.35 and 1.41 (m overlapping s, 14 H), 1.08-0.99 (br, 1 H).

Boc-Tyr(O-Bzl) Thiopiperidide. This substance was prepared similarly to **14** as an oil in 91% yield. ^1H NMR (200 MHz, $CDCl_3$) δ 7.45-7.29 (m, 5 H), 7.15 (d, 2 H, J = 9 Hz), 6.86 (d, 2 H, J = 9 Hz), 5.94 (d, 2 H, J = 9 Hz), 5.15-4.98 and 5.05 (m overlapping s, 3 H), 4.30-4.14 (m, 1 H), 4.08-3.91 (m, 1 H), 3.56-3.39 (m, 1 H), 3.19-3.00 (m, 3 H), 1.74-1.29 and 1.42 (m overlapping s, 14 H), 0.98-0.79 (m, 1 H).

Boc-Tyr(O-Bzl) Methyl Dithioester (17). This compound was prepared similarly to **13** as a yellow solid in 51% yield: mp 122-123 °C (lit[8] mp 121-122 °C), $[\alpha]_D$ = -5.95° (*c* 1, CH_3OH) [lit[8] $[\alpha]_D$ = -6.2° (*c* 1, CH_3OH)], ^1H NMR (200 MHz, $CDCl_3$) δ 7.45-7.30 (m, 5 H), 7.08 (d, 2 H, J = 9 Hz), 6.88 (d, 2 H, J = 9 Hz), 5.37 (d, 1 H, J = 8 Hz), 5.13-4.98 and 5.03 (m overlapping s, 3 H), 3.19-2.97 (m, 2 H), 2.58 (s, 3 H), 1.39 (s, 9 H).

Boc-Tyr(O-Bzl)Met-OMe. This material was obtained as a waxy solid (93%) by the same procedure used to prepare Boc-DLeuMet-OMe: mp 99-100 °C; IR(KBr) 3332, 1742, 1685, 1657 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.48-7.30 (m, 5 H), 7.12 (d, 2 H, J = 9 Hz), 6.91 (d, 2 H, J = 9 Hz), 6.55 (d, 1 H, J = 8 Hz), 5.04 (s, 2 H), 4.96 (br, 1 H), 4.64 (td, 1 H, J = 7, 5 Hz), 4.31 (q, 1 H, J = 7 Hz), 3.71 (s, 3 H), 3.07 (dd, 1 H, J = 14, 7 Hz), 2.96 (dd, 1 H, J = 14, 7 Hz), 2.44-2.36 (m, 2 H), 2.20-1.82 and 2.05 (m overlapping s, 5 H), 1.42 (s, 9 H). Anal Calcd for $C_{27}H_{36}N_2O_6S$: C, 62.76; H, 7.03; N, 5.43; S, 6.19. Found: C, 62.42; H,

7.14; N, 5.36; S, 5.97.

Boc-Tyr(O-Bzl)-ψCSNH-Met-OMe (18). This compound was prepared similarly to **14** as an oil in 83% yield: IR (neat) 3293, 1742, 1688 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 8.20 (d, 1 H, J = 6 Hz), 7.46-7.30 (m, 5 H), 7.13 (d, 2 H, J = 9 Hz), 6.90 (d, 2 H, J = 9 Hz), 5.22 (br, 1 H), 5.16-5.06 (m, 1 H), 5.03 (s, 2 H), 4.55 (q, 1 H, J = 7 Hz), 3.70 (s, 3 H), 3.19 (dd, 1 H, J = 14, 7 Hz), 3.06 (dd, 1 H, J = 14, 7 Hz), 2.47-2.10 (m, 4 H), 2.05 (s, 3 H), 1.42 (s, 9 H). Anal Calcd for $C_{27}H_{36}N_2O_5S_2$: C, 60.88; H, 6.82; N, 5.26; S, 12.04. Found: C, 60.73; H, 7.07; N, 5.09; S, 12.03.

Boc-DTyr(O-Bzl)-OH. Triethylamine (0.113 mL, 0.81 mmol) was added to a suspension of DTyr(O-Bzl)-OH hydrochloride[29] (100 mg, 0.33 mmol) and 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, 88 mg, 0.36 mmol) in 1:1 acetone-water (2 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 (10 mL), and then washed with ether (4 x 10 mL). The aqueous phase was acidified to pH 2 with 3 N hydrochloric acid and extracted with methylene chloride (3 x 10 mL) and ethyl acetate (2 x 10 mL). The combined extracts were dried ($MgSO_4$), filtered, and concentrated on a rotary evaporator. The residue was recrystallized from ether-hexane to give a white solid (82 mg, 68%): mp 103-105 °C (lit[30] mp for L-enantiomer recrystallized from ethyl acetate-petroleum ether 108-109 °C); $[\alpha]_D$ = -12° (*c* 1, acetone) [lit[30] $[\alpha]_D$ = 11° (for L-enantiomer in acetone)], ^1H NMR (500 MHz, $CDCl_3$) δ 7.43-7.40 (m, 2 H), 7.40-7.35 (m, 2 H), 7.34-7.30 (m, 1 H), 7.10 (d, 2 H, J = 9 Hz), 6.92 (d, 2 H, J = 9 Hz), 5.03 (s, 2 H), 4.92 (d, 1 H, J = 7 Hz), 4.58-4.51 (m, 1 H), 3.13 (dd, 1 H, J = 14, 6 Hz), 3.04 (dd, 1 H, J = 14, 7 Hz), 1.42 (s, 9 H).

Boc-DTyr(O-Bzl)Met-OMe. This substance was obtained as a white solid (77%) by the same procedure used to prepare Boc-DLeuMet-OMe: mp 127-128 °C; IR (KBr) 3340, 1739, 1684, 1660 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.44-7.41 (m, 2 H), 7.40-7.36 (m, 2 H), 7.34-7.30 (m, 1 H), 7.13 (d 2 H, J = 9 Hz), 6.92 (d, 2 H, J = 9 Hz), 6.46 (d, 1 H, J = 8 Hz), 5.04 (s, 2 H), 4.96 (br, 1 H), 4.66-4.62 (m, 1 H), 4.32 (br, 1 H), 3.72 (s, 3 H), 3.01 (d, 2 H, J = 8 Hz), 2.34-2.24 (m, 2 H), 2.09-2.00 and 2.04 (m overlapping s, 4 H), 1.90-1.81 (m, 1 H), 1.42 (s, 9 H). Anal Calcd for $C_{27}H_{36}N_2O_6S$: C, 62.76; H, 7.03; N, 5.43; S, 6.19. Found: C, 62.38; H, 7.13; N, 5.37; S, 5.87.

Boc-DTyr(O-Bzl)-ψCSNH-Met-OMe (19). This compound was prepared similarly to **14** as an oil in 91% yield: IR (neat) 3299, 1740, 1698 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 8.16 (d, 1 H, J = 7 Hz), 7.45-7.31 (m, 5 H), 7.14 (d, 2 H, J = 9 Hz), 6.91 (d, 2 H, J = 9 Hz), 5.28 (br, 1 H), 5.18-5.07 (m, 1 H), 5.04 (s, 2 H), 4.57 (q, 1 H, J = 7 Hz), 3.74 (s, 3 H), 3.11 (d, 2 H, J = 7 Hz), 2.24-1.87 and 2.02 (m overlapping s, 7 H), 1.42 (s, 9 H). Anal Calcd for $C_{27}H_{36}N_2O_5S_2$: C, 60.88; H, 6.82; N, 5.26; S, 12.04. Found: C, 60.51; H, 6.89; N, 4.99; S, 12.07.

Boc-His(Tos)Leu-ψCSNH-Met-OMe (24). Under an atmosphere of nitrogen, dipeptide **9** (114.5 mg, 0.295 mmol) was treated with 4 N hydrogen chloride in dioxane (3 mL) at 0 °C for 5 min and then at room temperature for 25 min. The volatiles were removed on a rotary evaporator, and the residue was dissolved in water (5 mL) and washed with 50% ether-hexane (2 x 2 mL). The aqueous layer was lyophilized to give a yellow oil (97 mg) which was dried over NaOH. This oil was dissolved in anhydrous THF (0.5 mL) and added under nitrogen to a cold (-10 °C) solution of a mixed anhydride which was prepared from BOC-His(Tos)-OH (133 mg, 0.325 mmol), NMM (35.7 μL, 0.325 mmol) and isobutyl chloroformate (42 μL, 0.325 mmol) in anhydrous THF (1 mL). Dry NMM (32.5 μL, 0.295 mmol) was added and the mixture was allowed to warm to

0 °C over a period of 2 h, and then stirred at room temperature for 4 h. The reaction mixture was diluted with ethyl acetate (10 mL) and washed with 0.5 N hydrochloric acid (2 x 2 mL), 5% aqueous sodium bicarbonate (2 x 2 mL), saturated sodium chloride (2 mL) and dried (Na_2SO_4). The drying agent was filtered off and the filtrate concentrated on a rotary evaporator. The residue was purified by flash chromatography over silica gel (230-400 mesh, 2 x 17.5 cm, 30% ethyl acetate-chloroform) to give the product (137 mg, 68%) as a glassy foam: ^1H NMR (200 MHz, $CDCl_3$) δ 8.67 (d, 1 H, J = 7 Hz), 7.93 (d, 1 H, J = 1 Hz), 7.84-7.78 (m, 1 H), 7.36 (d, 2 H, J = 8 Hz), 7.10 (s, 1 H), 7.08 (d, 1 H, J = 9 Hz), 6.04 (d, 1 H, J = 6 Hz), 5.25-5.15 (m, 1 H), 4.71-4.59 (m, 1 H), 4.41-4.32 (m, 1 H), 3.76 (s, 3 H), 3.07 (dd, 1 H, J = 14, 5 Hz), 2.89 (dd, 1 H, J = 14, 6 Hz), 2.56-2.48 (m, 2 H), 2.44 (s, 3 H), 2.40-2.14 (m, 2 H), 2.08 (s, 3 H), 1.74-1.54 (m, 3 H), 1.43(s, 9 H), 0.89 (d, 3 H, J = 6 Hz), 0.84 (d, 3 H, J = 6 Hz); low resolution FABMS *m/e* (relative intensity) 706 (M^+ + 23, 32), 684 (MH^+, 51), 530 (MH^+ - 154, 53), 430 (MH^+ - 254, 19), 264 (100); Anal. calcd. for $C_{30}H_{45}N_5O_7S_3$: C, 52.69; H, 6.63; N, 10.24; S, 14.06. Found: C, 52.93; H, 7.00; N, 9.87; S, 13.92.

Peptide 25. A solution of **24** (8.8 mg, 1.29 x 10^{-2} mmol) in TFA (0.5 mL) was kept at 0 °C for 30 min under nitrogen. The acid was evaporated and the residue was dissolved in methylene chloride (2 mL), concentrated and dried under vacuum. This residue was dissolved in DMF (0.2 mL) and added to a solution of the hydroxybenzotriazole ester generated from **5** (5 mg, 6.47 x 10^{-3} mmol), HOBt·H_2O (2.97 mg, 1.94 x 10^{-2} mmol) and DCC (1.33 mg, 7.12 x 10^{-3} mmol) in DMF (0.5 mL) at 0 °C for 2 h. Diisopropylethylamine (2.24 μL, 1.29 x 10^{-2} mmol) was added and the mixture was stirred for 3 days at 0 °C and 1 week at room temperature. The reaction mixture was diluted with water (15 mL) and washed with methylene chloride (2 x 3 mL). The product was isolated by reverse phase HPLC on a 4.6 x 250 mm Vydac 218TP, C-18, 10 μ column using a gradient of 9% CH_3CN-0.1% TFA to 42% CH_3CN-0.1% TFA in 28 min followed by isocratic elution with 60% CH_3CN-0.1% TFA for 12 min at a flow rate of 1 mL/min. The fraction containing the desired product was lyophilized to give **25** (4.5 mg, 58%) as a fluffy white solid: low resolution FABMS *m/e* (relative intensity) 1185.5 (MH^+, 100).

Peptide 26. A solution of **25** (4.5 mg, 3.80 x 10^{-3} mmol) in absolute methanol (4 mL) was cooled to 0 °C and saturated with ammonia gas. The solution was stirred at 0 °C for 3 days and the solvent was removed *in vacuo*. The residue was dissolved in methanol (4 mL), concentrated, redissolved in methanol (1 mL) and chromatographed on a 4.6 x 250 mm Vydac 218TP, C-18, 10 μ column using a gradient of 18% CH_3CN-0.1% TFA to 45% CH_3CN-0.1% TFA in 28 min followed by isocratic elution with 60% CH3CN-0.1% TFA for 8 min at a flow rate of 1 mL/min. The fraction containing the desired product was lyophilized to give **26** (0.4 mg, 11%) as a sticky solid: low resolution FABMS *m/e* 1170 (MH^+).

Peptide 2. A solution of **26** (0.3 mg) in TFA (0.1 mL) was kept at 0 °C for 20 min and the acid was removed under vacuum while keeping the solution cold. The residue was dissolved in water (0.5 mL) and chromatographed on a 4.6 x 250 mm Vydac 218TP, C-18, 10 μ column using a gradient of 9% CH_3CN-0.1% TFA to 42% CH_3CN-0.1% TFA in 28 min at a flow rate of 1 mL/min. The fraction containing the desired product was concentrated in a vacuum centrifuge to give **2** (0.1 mg) as an amorphous solid: high resolution FABMS calcd MH^+ 1070.5028, found 1070.5020.

Acknowledgment. This research was made possible by contract NO1-CM-17512, awarded by the National Cancer Institute, DHHS.

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