Thioamides: Synthesis, Stability, and Immunological Activities of Thioanalogues of Imreg. Preparation of New Thioacylating Agents Using Fluorobenzimidazolone Derivatives

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Imreg (Tyr[1]-Gly[2]-Gly[3]) is a well-known immunostimulant. However, it possesses a short half-life. Stabilized analogues of Imreg were prepared by a regioselective insertion in which peptide bonds at position 1,2 or 2,3 were replaced by thioamide linkages. This was achieved by using new thioacylating agents based on thioacyl-fluoro-*N*-benzimidazolone. The synthesis and properties of these reagents are described herein. This peptide modification enhanced significantly the half-life of the thioanalogues relative to Imreg in blood. The thioanalogues and Imreg were tested in vitro in T and B cell proliferation assays and for their ability to stimulate cytotoxic T-lymphocytes (CTLs). Only thiotyrosyl glycyl glycine **11** displayed some activity as evidenced by a weak stimulation of CTLs. On the basis of this activity and the increased stability, an in vivo immunological evaluation was undertaken. Immunophenotyping of **11** revealed a significant increase in activated CTL and NK cell populations in the spleen. This expansion was also accompanied by a significant stimulation of NK cells and the B cell proliferative response. Thioanalogues of Imreg were generally nontoxic, as exemplified by **11**. The latter is a promising immunostimulant which may be targeted for cancer and viral infections, where CTLs and NK cells play an important role, or as a vaccine adjuvant where stimulation of antibody-producing B cells is important.

Introduction

The tripeptide Imreg, tyrosyl glycyl glycine (TyrGlyGly), possesses immune stimulation properties.[1] It stimulates T cell subsets as evidenced by restoration of delayed-type hypersensitivity and induction of cytokines such as interleukin 2 and interferon γ.[2] Subsequently, Imreg is under clinical evaluation as a treatment to reduce the progression of HIV in AIDS patients.[3] However, the therapeutic utility of this and similar peptides is limited due to their short half-life in plasma.[4] Degradation is primarily due to amide bond cleavage by proteolytic enzymes. Therefore, considerable effort has been undertaken to discover analogues with greater resistance to degradation.

One approach to increase the half-life of Imreg is by the stabilization of the peptide linkage by replacement of peptide bond(s) with the thioamide linkage. Several examples have been reported of thiopeptide analogues containing one or more thioamide substitutions in the peptide backbone.[5] These modified peptides exhibit improved resistance to degradation by proteolytic enzymes.[6] They also demonstrate increased[7] or similar[8] equipotent pharmacokinetic activity relative to their parent structures.[7] In addition, this substitution has been shown to result in inhibitors of peptidyl-propyl isomerase.[9] Recently, we reported the preparation of monothionated analogues of the peptide immunostimulants thymopentin (Arg-Lys-Asp-Val-Tyr)[10] and tuftsin (Thr-Lys-Pro-Arg).[11] These peptides display improved stability and potency. The larger and less electronegative sulfur atom, relative to oxygen, is expected to induce some conformational distortions in the modified peptide. This may alter the secondary structure of the polypeptide chain. As a result, the incorporation of thioamide bonds might provide a promising approach for the preparation of pharmacologically important peptides.

Synthesis of thioamides has been well documented.[12] Conventional preparative methods are essentially based upon conversion of the corresponding carbonyl compound with an oxygen/sulfur exchange reagent. The most often used reagent is phosphorus pentasulfide and derivatives such as Lawesson reagent.[13] The latter was successfully applied for thionation of suitably protected peptides. This method, however, displays lack of reaction site specificity and leads to mixtures of thiopeptides which are difficult to separate.[14] An alternative route for the synthesis of endothiopeptides, thioacylation, has received more attention. It offers the potential advantage of regioselectivity for the introduction of thioamide linkages into oligo peptides. Unfortunately, thioanalogues of the classical acylation reagents are not readily available. For example, thioacyl halides and thioacyl anhydrides are unstable and therefore difficult to manipulate.[15] Most often alkyl thioesters[16] and dithioesters[17] have been employed for thioacylation. However, racemization of the final product was observed.[18] More recently, the reaction of amines with monothiocarboxylic acids activated by benzotriazolyl phosphorus reagents has been described for the preparation of thioamides.[19] This procedure is of limited utility since it affords mixtures of amides and thioamides due to interconversion between the carbonyl and thiocarbonyl intermediates.

Most important, none of these methods display complete reaction site specificity. Decreased purity and overall yield have been observed because of side reac-

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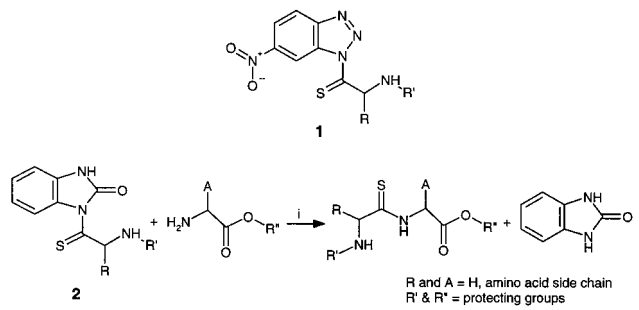
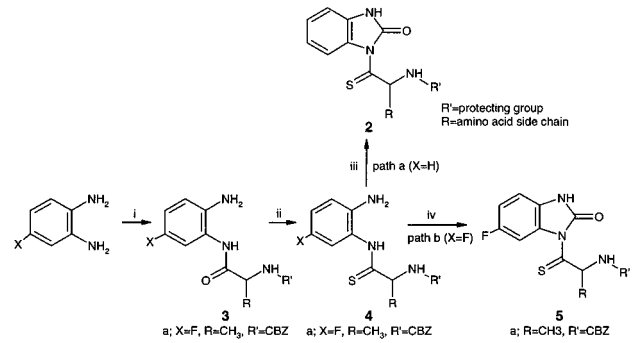
Scheme 1[a]



[a] Reagents and conditions: (i) DMF or CH_2Cl_2, 25 °C, overnight.

Scheme 2[a]



[a] Reagents and conditions: (i) protected amino acid, EDC, CH_2Cl_2; (ii) P_2S_5, THF; (iii) 1,1′-carbonyl ditriazole, Et_3N; (iv) phosgene or triphosgene, CH_2Cl_2.

tions. Furthermore, apparent racemization of the final product is often observed which limits the use of this procedure.

Chemistry

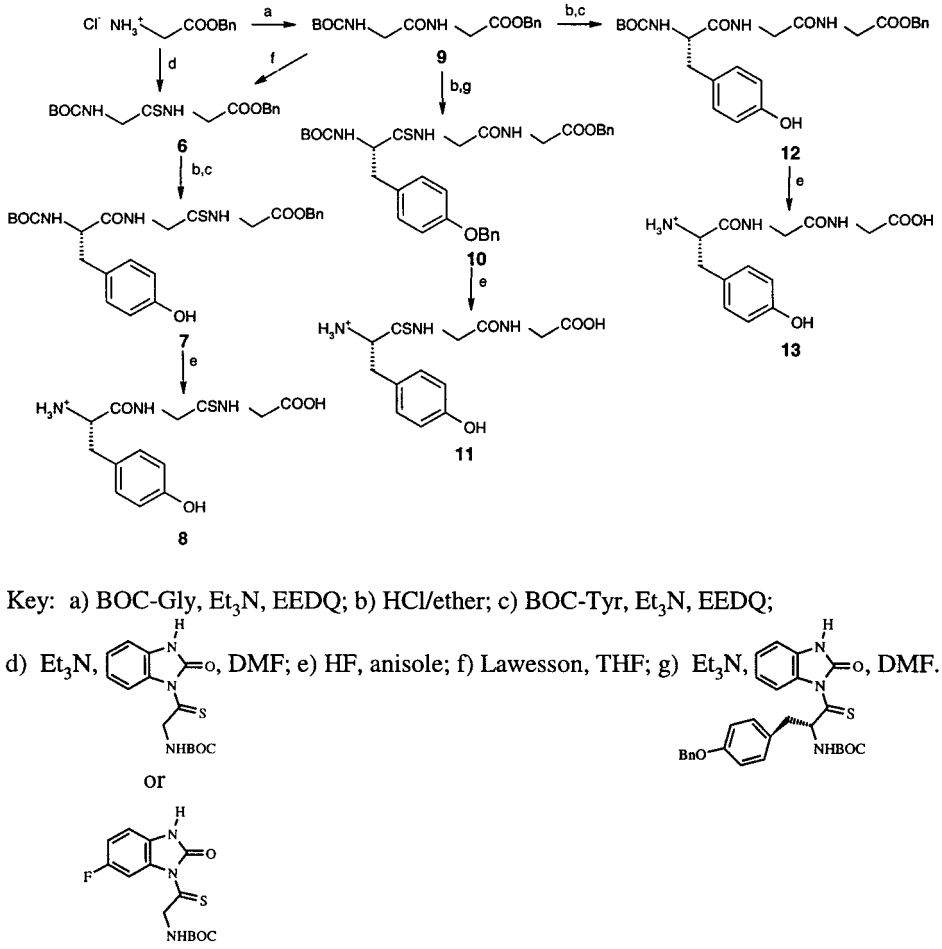
The development of efficient thioacylating agents has received much interest.[20] A recent approach has been reported for monothionation of peptides using thioacylnitrobenzotriazole **1**.[21] Although this is a convenient procedure for the synthesis of thiopeptides, the preparation of these reagents suffers from the formation of acylthioimidate as byproduct. In addition, their preparation requires the cyclization of the precursor nitrothioanilides using nitrous acid generated in situ from $NaNO_2$ and aqueous acetic acid as a solvent. Therefore, this method is limited and incompatible with the presence of acid sensitive or oxidizing groups carried by these substrates. Furthermore, these compounds are precipitated from the reaction mixture as amorphous solids which complicates the determination of their purities especially upon scale-up. To overcome these limitations, a practical approach was developed for thiopeptide synthesis.

Our early effort in this area resulted in the preparation of thioacylating agents **2**.[11,22] It was possible to undertake the aminolysis of **2** with amino acid or peptide under neutral conditions (Scheme 1). This method is selective, proceeds under mild conditions, and is suitable for solid-phase synthesis. As an application of this approach, we synthesized all possible monothionated analogues of thymopentin[10] and tuftsin.[11] The preparation of **2** is described in Scheme 2, path a (X = H). To shorten the synthesis of **2**, modification of the cyclization step was required. Among the variety of conditions used previously for carbonylation, only 1,1′-carbonylditriazole gave the desired **2** in a reasonable yield. The triazole reagent is not commercially available and is prepared in situ for immediate use. Therefore, the development of an alternative route for the preparation of new thioacylating agents which employs commercially available reagents is desirable. In the first approach (Scheme 2, path a, X = H), it was found that phosgene and triphosgene were too reactive. To overcome such reactivity, we introduced an electron-withdrawing group para to the unacylated amino group. This reduces its nucleophilicity and consequently limits the formation of undesired products. Different electron-attracting groups were examined (Scheme 2, X = F, COOMe, NO_2), and 4-fluoro-1,2-phenylenediamine was chosen as a latent benzimidazolone source.

The modified procedure for the preparation of the new thioacylating agents **5** is described for a typical example in Scheme 2, path b (X = F). Selective coupling was achieved between one of the two amino groups of 4-fluoro-1,2-phenylenediamine and CBZ-Ala-OH in CH_2Cl_2 at 0 °C using EDC. This gave the crystalline anilide **3a** in high yield. Thionation of **3a** with a mixture of phosphorus pentasulfide and anhydrous sodium carbonate in dry THF for 4 h at 0 °C gave thioamide **4a** in 65% yield. The reaction proceeded smoothly with no formation of byproduct. Intramolecular ring closure of **4a** using the commercially available phosgene in toluene reagent or trisphosgene afforded the thiobenzimidazolone derivative **5a** in 70−85% yield. This compound is a stable yellow solid, easy to manipulate, and can be stored for months at 0 °C without decomposition. The approach is compatible with the following functionalities: R = different side chains and R′ = different functionalities.[23] In general, thioacyl-*N*-6-fluorobenzimidazolones are easy to prepare and to scale up. Their behavior is similar to that of thioacylating agents **2**. These compounds reacted with amino acid amides and peptides in high yields. They provide a mild, efficient, and racemization-free route for incorporation of a thioamide linkage into peptides employing available, inexpensive starting materials.

Preparation of Thioimreg. The synthetic route for the preparation of Imreg and its thioanalogues is outlined in Scheme 3. Glycine benzyl ester hydrochloride was coupled with glycine thioacylating agent to give the thiodipeptide **6**. Removal of the BOC group followed by coupling with BOC L-tyrosine using EEDQ as activating agent gave the protected Thioimreg **7**. After cleavage/deblocking with HF/anisole, the target Thioimreg **8** is obtained in reasonable yield. Thioamide **6** may also be synthesized by reacting the dipeptide **9** with Lawesson's reagent in THF. The reaction required reflux conditions, and product yield was lower than in the case of the glycine thioacylating agent. Selective incorporation of the thioamide linkage between tyrosine and glycine was achieved using either **2** or **5** as thioacylating agent. In this case, protected Thioimreg **10** was obtained in a moderate yield by reacting the free amine of **9** with tyrosine thioacylating agent. HF treatment of the resultant product afforded the corresponding deblocked thioamide **11**. Imreg **13** was also prepared for a comparison of its activity and stability with its thioana-

Scheme 3



logues. This was achieved by coupling the amine of **9** with BOC L-tyrosine to give **12** followed by HF cleavage.

Stability Studies

Determination of Serum Half-Life. The stability of Thioimreg **8** and **11** was determined in rat whole blood in comparison to Imreg **13**. The in vitro half-lives of the compounds were determined, following incubation in plasma for variable times, by HPLC. A sensitive HPLC method has been established to separate the compounds from proteins or endogenous materials extracted from rat whole blood. The recovery efficiency of Imreg was found to be 63.0% ± 4 (n = 6), when extracted from rat serum. Recovery of **11** extracted from rat serum was found to be 90.1% ± 5 (n = 6) and that of **8** was determined to be 73.1% ± 3.7 (n = 7).

As predicted, thioanalogues showed a significant improvement in their half-lives when compared with Imreg under the same experimental conditions. The $t_{1/2}$ at 37 °C of compound **8** was greater than 180 min and for **11** was ca. 46 min while Imreg itself was rapidly degraded (<1 min). This result suggests that thioamide substitutions in the peptide backbone at position 1 and 2 of Imreg protected the tripeptide from being digested by proteins found in rat. The data also showed that Thioimreg **8** had virtually a 4-fold longer half-life than thioanalogues **11**. This indicates that the Tyrψ[CSNH]-Gly thioamide bond is more susceptible to degradation than the Glyψ[CSNH]Gly bond. Indeed, previous investigations had indicated that Tyr-Gly bonds are known to be more sensitive to cleavage by proteolytic enzymes than the Gly-Gly fragment.[24]

In vitro studies also included an analysis of the stability of Imreg and its thioanalogues **8** and **11** in rat kidney brush border proteases. The proteolytic digestion of the tripeptides was monitored by HPLC. Similarly, the analysis of the degradation of Imreg and its thioanalogues can be visualized and quantified by the disappearance of the test compound simultaneously with the appearance of degradation products. Again, Thioimreg analogues **8** and **11** showed significant enhancement of their half-lives in the presence of kidney brush border proteases as compared with Imreg. The $t_{1/2}$ values were comparable to that obtained in rat plasma. This further confirms that replacement of amide bonds with thioamides significantly increases the half-life, and compounds such as **8** and **11** may prove superior to Imreg in an in vivo comparison of activity.

Biological Evaluation

In Vitro Immune Profile. The in vitro activity of Imreg **13** and its thioanalogues, **8** and **11**, was assessed by means of mitogenic proliferation assays. A weak in vitro induction of T (PHA) and B (PWM) cell proliferation (1.5 to 2-fold increase) by the thioanalogues was observed without statistical significance relative to Imreg (data not shown). Only **11** induced a weak

Table 1. Summary of Immunophenotyping Data for Mice Administered Four Doses of **11**

cell subsets		0 mg/kg	50 mg/kg	100 mg/kg	125 mg/kg
	Blood Immunophenotyping **11**[a]				
CD4+CD45+	mean	22.56	23.94	22.9	28.89
	STD	5.8	4.2	5.5	5.14
	P value		0.36	0.45	0.048
NK+	mean	8.0	6.37	5.84	6.29
	STD	1.84	1.25	1.51	1.67
	P value		0.019	0.031	0.11
	Spleen Immunophenotyping **11**				
CD4+CD45+	mean	41.73	43.1	34.63	31.93
	STD	2.44	3.39	6.2	3.4
	P value		0.251	0.008	0.001
TCR+	mean	58.54	59.07	52.63	43.59
	STD	3.18	4.68	3.53	1.69
	P value		0.422	0.008	0.001
CD8+CD45+	mean	4.97	5.77	6.93	5.46
	STD	0.42	1.63	2.57	0.85
	P value		0.16	0.04	0.14
LY5+	mean	38.03	38.56	40.79	50.71
	STD	3.55	5.04	3.48	4.01
	P value		0.43	0.06	0.001
NK+CD3+	mean	2.37	2.70	2.99	3.37
	STD	0.44	0.34	0.83	0.50
	P value		0.024	0.014	0.002

[a] Groups of seven C57BL/6 mice were injected i.p. for four consecutive days with **11**.

stimulation of cytotoxic T-lymphocytes (CTL) compared to our positive control, IL-2 (data not shown).

In Vivo Activity of 11. Since **11** displayed enhanced stability and some in vitro activity, this compound was selected for in vivo evaluation. Therefore, the immunophenotyping experiment was undertaken with normal immune status mice, in the presence or absence of **11,** to determine the increase or decrease in immune cell subset populations, *relative to each other*. Mice were injected intraperitoneally for four consecutive days with 50, 100, or 125 mg/kg of **11**. At the end of the experiment, blood and spleens were collected for determination by flow cytometry of the effects of **11** on the immune cell subsets within these tissues. A summary of the results is presented in Table 1.

In blood, 125 mg/kg of **11** resulted in a significant increase in activated T helper cells (CD4+CD45+, 28%, $P \leq 0.048$). This is accompanied by a significant reduction in NK cells (20−27%, $P \leq 0.02$) at 50 and 100 mg/kg. In spleen, a significant increase in CTL (CD8+CD45+, 39%, $P \leq 0.038$) is observed at 100 mg/kg of **11**. This is accompanied by a significant increase in NK cells (26−42%, all doses, $P \leq 0.05$) and in B cells (33%, 125 mg/kg, $P \leq 0.001$). However, a significant reduction of the relative percentage of activated T helper cells along with TCR (T cell receptor) was observed at 100 and 125 mg/kg ($P \leq 0.008$).

To understand the immunological effect of **11** on cell populations, an important component of the immunophenotyping experiments is the assessment of any changes in the functional activity of immune cells along with changes in their relative numbers, that is, to determine the ability of **11** to stimulate NK cells and lymphoproliferative activities. Therefore, upon sacrifice of the mice at the end of the experiment, a portion of the splenic cell suspension was assayed for lytic activity by the chromium release assay (see Materials and Methods). The NK cell activity is enhanced at all doses of **11**. Further, this increase in functional activity is significant at 100 mg/kg ($P \leq 0.05$) and 125 mg/kg ($P \leq 0.046$) **11**. As reflected by spleen immunophenotyping, a significant decrease of PHA-induced T cell proliferation is observed at 125 mg/kg **11** ($P \leq 0.01$; data are not shown). Further lipopolisaccharide-induced B cell proliferation is enhanced at 125 mg/kg **11** ($P \leq 0.025$; data are not shown).

Discussion

In this study, stable thioanalogues of Imreg were prepared by the regioselective insertion of thioamide bonds into the peptide backbone of Imreg. This modification protected the tripeptide from proteolytic enzyme degradation as seen by an increase in stability in rat serum and kidney brush border proteases assay. The in vitro immunomodulatory activity of thioanalogues **8** and **11** was examined for their induction of mitogenic T and B cell proliferation and compared with Imreg. It was found that the immune profile of these compounds in mice was similar to that of the parent Imreg. It has been reported that Imreg enhances both human suppressor and helper T cells in a concentration-dependent manner.[2a] Also, it has been shown that TyrGlyGly and TyrGly increase the in vitro proliferation of T cells stimulated by concanavalin A and enhance delayed-type hypersensitivity to recall antigens such as tetanus toxoid.[25] However, our in vitro results indicate that thiopeptides induced a weak T cell proliferation. Only **11** was able to induce a weak stimulation of CTL activity compared to the positive control IL-2.

Immunophenotyping experiments with **11** revealed a significant increase in activated CTL and NK cell populations in the spleen. Also, NK cell functional activity was increased at all doses. Furthermore, **11** augments B cells in the spleen and their proliferation as seen by an increase in response to LPS. Interestingly, it has been reported recently that (Met)^5 enkephalin and its two active metabolites, TyrGlyGly and desMet(Met)^5 enkephalin, increased in vivo/ex vivo natural killer activity and the proliferation of concanavalin. A stimulated T cells, and lipopolisaccharide stimulated B cells in mouse splenocytes.[26]

In conclusion, Thioimreg analogue **11** induces a transient increase in helper T cells and a sustained expansion of CTL, NK, and B cells. Further, this expansion was accompanied by a significant stimulation of NK and the B cell proliferative response. In addition, on the basis of this study, **11** appears to be well tolerated and devoid of any apparent toxicity. Additionally, due to its stability, Thioimreg analogue **11** may offer a clinical potential for the treatment of those diseases, cancer and viral infections, where CTLs and NK cells play an important role or as a vaccine adjuvant where stimulation of antibody-producing B cells is important.

Experimental Section

Progress of reactions and purity of products were determined by analytical TLC on silica gel plates, visualized by UV, iodine vapor, ninhydrin, or Ehrlich's reagent, and by analytical HPLC (Millipore-Waters apparatus) on a 250 × 4.6 mm YMCC4 (5 μm) reverse-phase column with a gradient of 10−50% MeOH/CH_3COONH_4, 0.01 M, pH 6.0, over a period of 50 min; flow rate was 1 mL/min using a UV detector at an absorption of 256 nm for detection. Products were purified by

preparative thin-layer chromatography (PTLC) on 20 × 20 cm silica gel GF uniplates (Analtech 0.25−1.0 mm thickness), by silica gel column (E. Merck 230−400 mesh), and/or by reverse-phase high-performance liquid chromatography (HPLC) using Whatman Partisil 10 ODS (20.250 mm) using the same gradient described above. Final deprotected products were lyophilized from H_2O and often retained as a partial solvate. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer model 21 spectrophotometer, while UV spectra were recorded on Perkin-Elmer 402 spectrophotometer. Proton NMR spectra were obtained on Varian VXR-300 spectrometers. Coupling constants (J) are reported in hertz.

Mass spectra were recorded on a Kratos Ms-50 TA instrument. The FAB (fast atom bombardment) ionization was obtained with a FAB saddle field source operated with xenon atoms at 7 kV. Elemental analyses were performed by Guelph Chemical Laboratories Ltd., Ontario, Canada and are reported in percent. Amino acid analyses were carried out on a Varian 5500 analyzer by BioChem ImmunoSystems after hydrolysis with 6 M HCl, containing 0.1% phenol, at 110 °C for 20 h.

General Procedure for the Preparation of Anilide 3. Coupling of α-*N-CBZ*-L-Amino Acids with 4-Fluoro-1,2-phenylenediamine. To a mixture of α-*N-CBZ*-L-amino acid (11 mmol), 4-fluoro-1,2-phenylenediamine (0.52 mmol), and TEA (1.5 mL; 11 mmol) in CH_2Cl_2 (50 mL) was added EDC·HCl at 0 °C. After the mixture was stirred at this temperature for 2 h and then at room temperature overnight, 5% HCl (25 mL) was added, and the mixture was washed with brine, 5% sodium bicarbonate, and brine and dried over Na_2SO_4. Evaporation of the solvent and crystallization of the residue from CH_2Cl_2/hexanes afforded pure **3**.

α-*N-CBZ*-L-Alanine-2-amino-5-fluoroanilide (3a): yield (94%); R_f = 0.50 (EtOAc/hexanes 1:1); ^{1}H NMR ($CDCl_3$) δ 1.42 (d, 3H, J = 6.6), 3.5 (bs, 2H), 4.39 (m, 1H), 5.02 (s, 2H), 5.52 (d, 1H, J = 4.5), 6.40 (m, 2H), 7.00 (m, 1H), 7.30 (m, 5H), 7.82 (s, 1H), LRMS(FAB) m/z = 332 (MH^+); HRMS calcd for $C_{17}H_{19}FN_3O_3$ (MH^+) 332.1415, found 332.1403.

General Procedure for the Preparation of Thioanilides 4. Under a flow of argon, phosphorus pentasulfide (1.70 mmol) was mixed with sodium carbonate (1.70 mmol) in dry THF (100 mL). The mixture was stirred for 1 h at room temperature and then cooled to 0 °C. To this clear solution was added anilide **3** (1.80 mmol), and the reaction was kept at this temperature overnight. To work up, an aqueous solution of sodium tribasic phosphate was added (12%, 7 mL) followed by EtOAc (20 mL) and heptane (20 mL). The organic layer was separated and washed with brine (2 × 30 mL). Solvent was then evaporated, and the residue was purified on silica gel. This gave pure **4** as a pale yellow solid.

α-*N-CBZ*-L-Alanine-2-amino-5-fluorothioanilide (4a): yield (65%); R_f = 0.60 (EtOAc/hexanes 1:1); ^{1}H NMR ($CDCl_3$) δ 1.54 (d, 3H, J = 6.4), 4.01 (bs, 2H), 4.65 (m, 1H), 5.05 (S, 2H), 5.65 (d, 1H, J = 6.8), 6.46 (m, 2H), 7.05 (m, 1H), 7.33 (m, 5H), 9.56 (bs, 1H); LRMS(FAB) m/z = 348 (MH^+); HRMS calcd for $C_{17}H_{19}FN_3O_2S$ (MH^+) 348.1182, found 348.1200.

General Procedure for the Preparation of Benzimidazolone 5. A solution of phosgene in toluene (1.7 mmol) was added to thioanilide **4** (1.8 mmol) in dry THF. The reaction mixture was stirred for 4 h at 25 °C. Insolubles were removed by filtration, and the solvent was evaporated under reduced pressure. The residue was chromatographed on silica gel. This gave pure **5** as a colorless solid.

1-(α-*N-CBZ*-L-Thionoalaninyl)-6-fluorobenzimidazolone (5a): yield (70%); R_f = 0.40 (EtOAc/hexanes 1:1); ^{1}H NMR ($CDCl_3$) δ 1.58 (d, 3H, J = 6.8), 5.10 (S, 2H), 5.92 (d, 1H, J = 7.2), 6.35 (m, 2H), 6.58 (m, 1H), 7.40 (m, 5H), 8.61 (m, 1H), 10.00 (s, 1H), LRMS (FAB) m/z = 374 (MH^+); HRMS calcd for $C_{18}H_{17}FN_3O_3S$ (MH^+) 374.0975, found 374.0986.

Coupling with the Adjacent Amino Acid. Free amino or thioamino acid benzyl ester (2 mmol) was dissolved in $CHCl_3$ (10 mL) together with BOC amino acid (2.2 mmol). After the addition of EEDQ (2.3 mmol), the solution was stirred overnight at room temperature. Insolubles were removed, and the solution was washed successively with 5% HCl (10 mL) and brine (10 mL). The chloroform layer was dried over anhydrous $MgSO_4$ and chromatographed on silica gel.

***N*-BOC-Glycinyl-glycine Benzyl Ester 9:** yield (75%); R_f = 0.30 (EtOAc/hexanes 1:1); ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H), 3.84 (d, 2H, J = 5.77), 4.09 (d, 2H, J = 5.83), 5.19 (s, 2H), 5.20 (bs, 1H), 6.80 (bs, 1H), 7.41 (s, 5H).

***N*-BOC-L-Tyrosinyl-thioglycinyl-glycine Benzyl Ester 7:** yield (62%); R_f = 0.35 (EtOAc/hexanes 2:1); ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H), 2.98 (m, 2H), 4.30−4.56 (m, 5H), 5.19 (s, 2H), 5.38 (d, 1H), 6.70 (d, 2H, J = 5.4), 6.92 (d, 2H, J = 5.5), 7.35 (m, 6H), 8.91 (bs, 1H).

***N*-BOC-L-Tyrosinyl-glycinyl-glycine Benzyl Ester 12:** yield (65%); R_f = 0.15 (EtOAc/hexanes 2:1); ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H), 2.90 (m, 2H), 4.01 (t, 2H), 4.32 (t, 2H), 4.40 (m, 1H), 5.20 (s, 2H), 6.77 (d, 2H, J = 5.3), 7.01(d, 2H, J = 5.5), 7.38 (s, 5H).

Coupling of Thioacylating Reagents with Amino Acid or Peptides. Formation of Thiopeptides 6 and 10. Free amino acid benzyl ester (1 mmol) was dissolved in dry DMF (0.5 mL) at 0 °C under a flow of argon. To this solution, thioacylating agent (1.1 mmol) was added in two portions over a period of 10 min. The resulting mixture was stirred at this temperature for 2 h and then at room temperature overnight. Insolubles were removed by filtration, and the solvent was evaporated under reduced pressure. The remaining residue was dissolved in EtOAc (15 mL) and washed successively with 5% $NaHCO_3$ (20 mL), brine (20 mL), citric acid (20 mL), and brine (20 mL). The organic layer was dried over anhydrous $MgSO_4$, evaporated, and chromatographed on silica gel.

***N*-BOC-Thioglycinyl-glycine Benzyl Ester 6:** yield (70%); R_f = 0.45(EtOAc/hexanes 1:1); ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9H), 4.22 (d, 2H, J = 5.80), 4.46 (d, 2H, J = 5.00), 5.22 (bs, 1H), 5.25 (s, 2H), 7.40 (s, 5H), 8.62 (bs, 1H).

***N*-BOC-*O*-benzyl-L-thiotyrosinyl-glycinyl-glycine Benzyl Ester 10:** yield (61%); R_f = 0.3 (EtOAc/hexanes 2:1); ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 3.10 (m, 2H), 4.01 (m, 2H), 4.30 (m, 2H), 4.60 (m, 1H), 5.20 (s, 2H), 5.25 (s, 2H), 6.90 (d, 2H, J = 5.4), 7.10 (d, 2H, J = 5.4), 7.25−7.45 (m, 10H), 8.30 (bs,-1H).

General Procedure for Removal of Protecting Groups of 7, 10, and 12 and HPLC Analysis. The fully protected peptides (1 mmol) were deblocked with 90% HF (5 mL) in the presence of anisole, ethylmethyl sulfide, and thioanisole (1:1:1 v/v, 0.8 mL) in an ice bath for 1 h. The excess HF was removed by evaporation in vacuo at 0 °C, the residue was treated with 10% AcOH (3 mL), washed with ether (35 mL), and then dissolved in water (20 mL). The resulting solution was then lyophilized and purified by HPLC to give the expected product. The HPLC analysis revealed that compounds **8** and **11** were >99% pure. The mobile phase was 10−50% MeOH/NH_4OAc 0.01 M, pH 6.0; pressure was 2000 psi; and flow rate was 1 mL/min. For **8**: t_R = 26.59 min; for **11**: t_R = 28.80 min.

L-Tyrosinyl-thioglycinyl-glycine 8: yield (63%); R_f = 0.35 (EtOH/NH_4OH/H_2O 8:1:1); UV λ_{max} (50% aqueous ethanol) 267 nm; amino acid analysis Gly (1.92), Tyr (1.00); ^{1}H NMR (DMSO) δ 2.50 (m, 1H), 2.86 (m, 1H), 3.38 (m, 1H), 4.11 (d, 2H, J = 2.8), 4.34 (s, 2H), 6.67 (d, 2H, J = 8.36), 7.01 (d, 2H, J = 8.52), 8.40 (bs, 1H); ^{13}C NMR (DMSO) δ 39.94, 48.75, 49.86, 55.66, 115.56, 127.19, 130.66, 156.49, 169.71, 172.40, 197.59; LRMS(FAB) m/z = 312 (MH^+); HRMS calcd for $C_{13}H_{18}N_3O_4S$(MH^+) 312.1018, found 312.0998.

L-Thiotyrosinyl-glycinyl-glycine 11: yield (57%); R_f = 0.40 (EtOH/NH_4OH/H_2O 8:1:1); UV λ_{max}(50% aqueous ethanol) 268 nm; amino acid analysis Gly (2.00), Tyr (0.96); ^{1}H NMR (DMSO) δ 2.84 (m, 1H), 2.99 (m, 1H), 3.80 (m, 2H), 4.11−4.33 (m, 3H), 6.69 (d, 2H, J = 8.52), 7.05 (d, 2H, J = 8.30), 8.41 (t, 1H, J = 5.55), 9.32 (bs, 1H); ^{13}CNMR (DMSO) δ 40.59, 41.00, 48.14, 59.73, 115.57, 125.70, 130.77, 156.79, 167.22, 171.28, 200.52; LRMS (FAB) m/z = 312 (MH^+); HRMS calcd for $C_{13}H_{18}N_3O_4S$ (MH^+) 312.1018, found 312.1033.

L-Tyrosinyl-glycinyl-glycine (Imreg) 13: ^{13}C NMR (DMSO) δ 40.30, 42.10, 42.30, 48.10, 115.27, 127.30, 130.40, 156.10, 168.20, 171.88, 172.80.

HPLC Instruments. HPLC results were obtained with a Waters HPLC system (Waters Ltd., Mississauga, ON) consisting of a multisolvent delivery system model 600, automatic injector 712 Wisp, UV (Water UV 486) and diode array detector (model 996), Waters data 740 module (system management Millenium 2010 Version 1.1) to analyze the results.

Reagents and Materials. HPLC grade solvents were obtained from EM Science (BDH Inc., Toronto, ON). Reagent grade solvents were purchased from J. T. Baker (Montréal, QC). HPLC grade water was prepared from a Millipore System (Milli-Q). Imreg was purchased from Sigma Chemicals (St. Louis, MO). Male Sprague−Dawley rats were purchased from Charles River Canada Inc. (St. Constant, QC). The protein content of enzymes used was determined by means of the Bradford protein assay kit (Bio-Rad, Melville, NY).

Preparation of Whole Blood (Rat) Stability Assay. Freshly harvested blood samples were spiked with tripeptide compounds and incubated at 37 °C for approximately 3.5 h. Blank rat whole blood samples were processed as the negative controls. Blood samples (500 μL) were removed at specific time intervals and centrifuged at 4 °C, 5200 rpm, for 10 min. A 100 μL sample of the serum was transferred to eppendorf vials. The protein was precipitated with the addition of 200 μL of acetonitrile and 13 μL of acetic acid, vortex-mixed, and centrifuged (4 °C, 13 000 rpm, for 10 min), and an aliquot of the supernatant was evaporated to dryness. The samples were then reconstituted in the HPLC initial mobile phase, and 25 μL was injected into HPLC for analysis.

Preparation of Kidney Brush Border Membranes Assay. (a) Membrane Preparation. All operations were carried out at −4 °C. The cortex was carefully dissected from fresh kidneys obtained from euthanized male Sprague−Dawley rats. Membranes were prepared as described by Maeda et al.[27] Final protein content was determined to be 1.1 mg per mL of homogenate preparation.[28] Enzymatic assay markers (aminopeptidase M and δ-glutamyl transpeptidase) were used to standardize membrane preparations.

(b) Extraction in Kidney Homogenates. The proteolytic digestion of the tripeptides by kidney membrane proteases was monitored. A stock solution of 1.0 mg of compound was prepared in 0.01 M PBS, pH 7.4. An aliquot (800 μL) was mixed with an equal amount of kidney homogenate preparation. At zero time, 100 μL (all samples were aliquoted in duplicate) was removed, and the remaining solution was incubated at 37 °C. A blank solution was prepared as the negative control. The enzymatic activity was stopped after each sample was aliquoted by placing the tube in boiling water for 2 min. The aliquots were then filtered on an HV 0.45 μm filter (13 mM) Millipore Waters. A 10 μL sample of the filtrate was then injected into the analytical column for analysis.

Calculations of Data Analysis. Recovery was calculated using the following equation:

$$\frac{\text{area of STD cpds in spiked serum} \times \text{concn of serum}}{\text{area of STD cpds in stock solution} \times \text{concn in stock solution}} \times 100$$

Splenocyte Cell Suspension. Splenocyte cell suspensions were prepared by homogenization of spleens from 6- to 8-week-old female C57BL/6 (H-2^b) in tissue culture medium [RPMI 1640 (Gibco, Burlington, Canada), supplemented with 10% fetal calf serum (Hyclone, Logan) and 2 μM L-glutamine]. The homogenate was centrifuged on a density gradient (Lympholyte M, Cedarlane, Hornby, Canada) (800 g, 20 min), and the mononuclear leukocytes were collected, washed 3 times in phosphate buffer saline (PBS), and resuspended in RPMI for evaluation of cell viability by the use of trypan blue.

Mitogenic Proliferation. Splenocytes mitogenic proliferation was assayed by incubation of 2×10^5 splenocytes with T cell mitogen, PHA (Gibco, Burlington, Canada), or B cell mitogen, LPS (Oakville, Canada), in 5% CO_2 in a humidified incubator at 37 °C for 72 or 120 h, respectively. They were pulse-labeled with tritiated (^3H)-thymidine for the last 18 h of the culture. Cells were then collected by filtration and newly synthesized DNA was measured by determining the uptake of ^3H-thymidine.

NK Cell Activity Assay. NK cell activity was assayed by incubation of 5×10^5 splenocytes for 4 h with sodium chromate-labeled ($^{51}CrO_4$; Amersham, Oakville, Canada) NK-sensitive YAC-1 cells. After the incubation, chromium in the lysate was quantified, and the percent specific release or lysis was calculated using the expression

$$\text{percent specific lysis} = \frac{(\text{ER} - \text{SR})}{(\text{TR} - \text{SR})} \times 100$$

where ER = experimental release of ^{51}Cr, TR = total release of ^{51}Cr, and SR = spontaneous release of ^{51}Cr.

Immunophenotyping Assay. Female, 6- to 8-week-old, C57BL/6 mice were injected intraperitoneally for four consecutive days with **11** at different concentrations. Mice were sacrificed on day five by cardiac puncture. Gross pathological observations were recorded at the end of the experiment. Blood and spleens were collected, and cell suspension was prepared and lysed in ACK buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3) for 5 min. The cells were washed 3 times in phosphate-buffered saline (PBS), pH 7.4, and resuspended in tissue culture medium. The cells were then incubated for 45 min on ice with fluorescent isothiocyanate (FITC) or phycoerythrin (PE) conjugated cell surface marker according to the manufacturer's (Gibco/BRL, Cedarlane, Boehringer Mannheim) recommendation. The cells were then washed in PBS, fixed with 1% paraformaldehyde, and analyzed with a Coulter XL flow cytometer. Analysis of the cell subsets was undertaken by determination of standard cell surface markers which were as follows: CD3 (T cells), TCR (T cell receptor), CD4 (T helper), CD8 (T cytotoxic/suppressor), CD45 (tyrosine phosphatase; activation marker), CD11b (macrophage), NK (NK cells), and Ly5 (B cells).

Statistical Analysis. The immunophenotyping data are presented as ± standard error. The means are compared using an unpaired Student's *t* test. The differences are considered significant when $P \leq 0.05$.

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