Notes

Studies of the Synthesis, Immunology, and Cytotoxicity of a Cyclic Octapeptide Corresponding to TNF-α-(59–66)[1,2]

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In an attempt to investigate possible binding domains of the tumor necrosis factors (TNF), we have previously synthesized a cyclic hexapeptide corresponding to murine TNF-(127–132) (cTNF-1).[3] In this report, we describe the synthesis and biological activity of another cyclic octapeptide corresponding to human TNF-α-(59–66) (cTNF-2). The design of these cyclic peptides is based on their high sequence homology with corresponding fragments of human TNF-α or TNF-β. Similar to cTNF-1,[3] the cyclic octapeptide cTNF-2 displayed low *in vitro* cytotoxicity against human HeLa and HEP-2 cell lines. The cyclic peptides cTNF-2 and cTNF-1 were then tested for the induction of interleukin-1 (IL-1) production from human peripheral blood mononuclear cells and monocytes *in vitro*. At low concentrations, the IL-1 levels induced by these cyclic peptides were similar to that of recombinant TNF-α. However, the IL-1 production by cTNF-2 stimulation was dose-dependently increased and reached that of a lipopolysaccharide (LPS; 0.1 μg/mL) level. These findings suggest that the fragments corresponding to human TNF-α-(59–66) and murine TNF-(127–132) may represent certain binding domains of the tumor necrosis factors that elicit IL-1 production.

Introduction

Tumor necrosis factors (TNF) are an important class of cytokines which mediate *in vitro* and *in vivo* destruction of tumor cells in response to bacterial endotoxin or certain immunostimulatory agents.[4-8] These active proteins also have regulatory functions in a variety of immune and inflammatory reactions.[5-7] The better known tumor necrosis factors are human tumor necrosis factor (TNF-α), human lymphotoxin (TNF-β), and murine TNF (Mu-TNF). TNF-α is secreted by macrophages[4,9] whereas TNF-β is a product of T cells and B cells.[10]

It was reported that TNF-specific cell-surface receptors are present in several tumor cell lines[11-13] and that different tumor cell lines have different major receptors for TNF-α.[14] TNF-α has about 30% homology in its amino acid sequence with TNF-β (lymphotoxin), a cytokine of 171 residues which has similar cytotoxic activities to many tumor cell lines.[15,16] It has been reported that TNF-α and TNF-β share a common receptor and that the receptor binding domains of TNF-α and TNF-β are similar.[13] Although the X-ray structure of TNF-α was reported recently,[17,18] the "cytolytic sites" which are responsible for the *in vitro* cytotoxic activity of TNF have not been identified. In addition, the binding domains of TNF to peripheral blood mononuclear cells (PBMC) which cause the expression of the diverse immunological activities have not been investigated. One approach to investigating the possible binding domains of TNF-α is by synthesizing fragments of the polypeptide which have high sequence homology with TNF-β,[3,16] on the basis that TNF-α and TNF-β share a common receptor.[13]

In a previous paper[3] we reported the synthesis and cytotoxicity studies of a cyclic hexapeptide corresponding to murine TNF-(127-132) (cTNF-1). The rationale for synthesizing this fragment is based on the fact that it has high sequence homology with human TNF-α-(128–133) and also with human TNF-β-(143–148). The cyclic hexapeptide cTNF-1 displayed weak cytotoxicity on three human tumor cell lines: HEP-2, HeLa, and Colo-205.

In this paper we report the synthesis of a cyclic octapeptide 14 corresponding to human TNF-α-(59–66). This fragment was tentatively chosen as a probe for studying a possible binding domain of TNF-α since it has a 75% sequence homology with human TNF-β-(76–83) (six residues identical) and also has a 100% sequence homology with murine TNF-(59–66) (Chart I). Furthermore, TNF was reported to interact with PBMC and endothelial cell receptors to induce the release of interleukin 1 (IL-1),[19,20] an important cytokine. Thus, in addition to testing the cytotoxicity of the cyclic octapeptide 14 (cTNF-2), we are interested in assaying the immunological activity of the cyclic peptides cTNF-1 and cTNF-2 for the induction of IL-1 from PBMC and monocytes.

We chose to synthesize conformationally restricted cyclic peptides in preference to conventional peptides for the following reasons. A number of cyclic peptide hormone analogues are more potent in biological activities than linear peptide analogues.[21,22] Cyclic peptides are conformationally constrained and have been found to possess several useful features such as increased agonist or antagonist potency, prolonged biological activity, and increased specificity for a particular receptor.[23] Cyclic peptides are more stable to the action of endogenous

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Chart I. Comparison of the Amino Acid Sequences of Murine TNF-(59-66), Human TNF-α-(59-66), Human TNF-β-(76-83), cTNF-2, Murine TNF-(127-132), Human TNF-α-(128-133), Human TNF-β-(143-148), and cTNF-1.

Murine TNF-(59-66)	60 -Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-
Human TNF-α -(59-66)	60 -Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-
Human TNF-β -(76-83)	80 -Tyr-Ser-Gln-Val-Val-Phe-Ser-Gly-
cTNF-2	Cyclo[Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-]
Murine TNF-(127-132)	130 -Lys-Gly-Asp-Gln-Leu-Ser-
Human TNF-α-(128-133)	130 -Lys-Gly-Asp-Arg-Leu-Ser-
Human TNF-β-(143-148)	145 -Gln-Gly-Asp-Gln-Leu-Ser-
cTNF-1	Cyclo[Asp-Gln-Leu-Ser-Lys-Gly-]

proteases than linear peptides,[24] resulting in higher stability and thus prolonged biological activity.[23] Since the *N*- and *C*-terminals of cyclic peptides are blocked, they are more likely to mimic the natural conformation of the binding domains of the parent polypeptide than linear synthetic peptides which have extra *N*- and *C*-terminals. The absence of zwitterionic properties also causes the cyclic peptides to be considerably more hydrophobic, thus allowing them to pass through the cell membrane more readily than linear peptide analogues.

Chemistry

The synthesis of cyclic peptides is involved,[25,26] and the synthesis of a cyclic octapeptide with several bulky protecting groups (before deblocking) is challenging. The strategy for the synthesis of the cyclic octapeptide is outlined in Scheme I. The phenacyl group (OPa) was chosen to protect the *C*-terminal carboxyl since it is stable[27] to trifluoroacetic acid, which is generally used to cleave the Boc groups prior to peptide bond coupling. The OPa group can be selectively removed under the mild conditions (Zn/AcOH) without affecting any other protecting groups prior to synthesizing the pentafluorophenyl esters for the segmental condensation or the cyclization reaction. The nonchiral glycyl residue was chosen as the *C*-terminal residue in order to minimize racemization during the cyclization step.

In order to obtain a higher yield of the linear octapeptide **10**, segmental condensation of the two tetrapeptide fragments **4** and **8** was carried out (Scheme I). The tetrapeptides were synthesized in solution phase by the DCC/HOBt procedure.[28] Selective cleavage of the OPa group of linear peptide **8** afforded the free acid **9**, which was condensed with protected peptide **4** by DCC/HOBt after the removal of the Boc group of **4**. Selective cleavage of the OPa group of octapeptide **10** by Zn/AcOH gave the free acid **11**, which was esterified with pentafluorophenol/DCC to give the active ester **12**. The pentafluorophenyl ester **12**, after the removal of the Boc group, was successfully cyclized at high dilution[3] in a solution of dioxane–pyridine–ethanol maintained at 50–55 °C. This temperature range appeared to be important since higher cyclization temperatures gave lower yields of the desired cyclic monomer. The monomeric structure of the protected cyclic octapeptide **13** was established by fast atom bombardment mass spectrometry (FABMS). Treatment of cyclic peptide **13** with HF removed all the protecting groups, affording the target cyclic octapeptide **14**.

The cyclic octapeptide **14** was purified to homogeneity by semipreparative HPLC using an isocratic system of methanol in 0.1% TFA solution. FABMS employing the positive-ion mode (Ar beam) generated intense molecular ions $[M + H]^+$ (see Experimental Section).

This work again demonstrated the usefulness of the pentafluorophenyl ester method in the synthesis of cyclic peptides. The cyclization temperature appears to be important. We recommend a cyclization temperature range of 80–90 °C for small rings (11–18 membered rings) and 50–60 °C for larger rings.

Biological Results and Discussion

The cytotoxicity evaluation of cTNF-1 was reported previously.[3] The cytotoxicity of cTNF-2 was evaluated against two human cell lines: HEP-2 and HeLa. As shown in Table I, cTNF-2 displayed weak cytotoxicity against HEP-2 cells at high concentration (10 μg/mL). The cyclic octapeptide cTNF-2 appeared to have moderate cytotoxic effect against HeLa cells at high concentration (10 μg/mL).

Since the active form of TNF is a trimer,[29] the synthetic fragments of TNF which represent the "cytolytic sites" of this protein may have cytotoxicity indices considerably lower than that of the protein trimer. However, if the synthetic fragment does represent the "cytolytic site" of TNF, a significant increase in cytotoxicity should be observed at high concentration. Thus, at present there is insufficient evidence to support the hypothesis that the fragments corresponding to human TNF-α-(59–66) or murine TNF-(127-132) are the "cytolytic sites" of the tumor necrosis factors. In addition, the *in vitro* cytotoxic mechanism by which TNF kills tumor cells is controversial and unclear. Further studies of the TNF fragments synthesized by us and others should add to our understanding of the "cytolytic sites" of TNF.

The immunological activities of cTNF-2 and cTNF-1 were studied by assaying their abilities to induce IL-1 production. Human PBMC and monocytes were stimulated with either lipopolysaccharide (LPS), recombinant human TNF-α (rTNF-α), cTNF-2, or cTNF-1 in the cultured medium for 24 h.

As shown in Table II, cTNF-2, and cTNF-1 induced statistically significant production of IL-1 by human PBMC compared with medium control. At a concentration of 1 μg/mL, the IL-1 levels induced by these two cyclic peptides were comparable to that of recombinant TNF-α but lower than that of LPS (0.1 μg/mL). The stimulation indices for cTNF-2, cTNF-1, and rTNF-α are 1.78 ± 0.2, 1.64 ± 0.11, and 1.99 ± 0.18, respectively. Similarly, human monocytes were induced by cTNF-2 and cTNF-1 to secrete

Scheme I

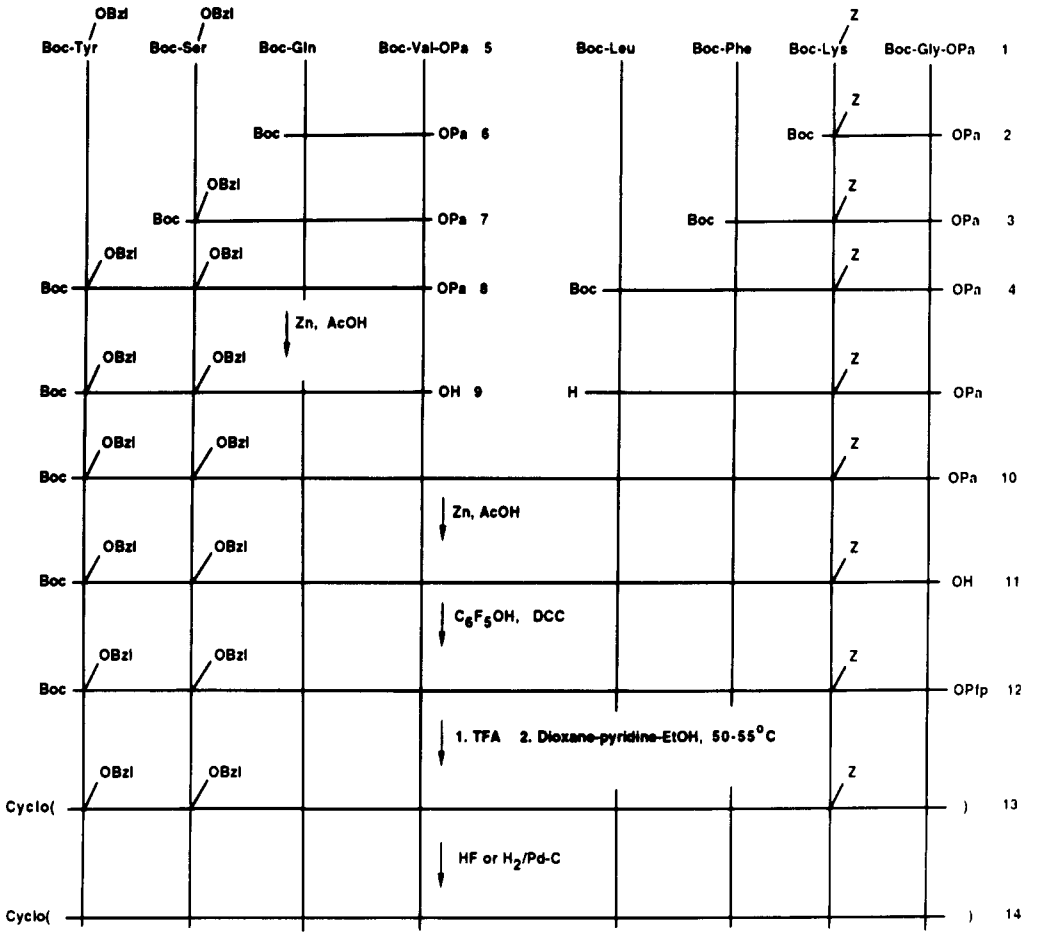

Table I. Cytotoxicity Assay of Cyclic Octapeptide 14 (cTNF-2) by the MTT Method

compound	concn (μg/mL)	cytotoxicity index[a] HEP-2	HeLa
cTNF-2	0.1	0.4 ± 2.9	17.9 ± 7.5
	1.0	7.5 ± 6.2	27.0 ± 3.6
	10.0	19.9 ± 0.8	44.7 ± 2.3
adriamycin	0.1	39.0 ± 3.4	50.6 ± 2.7
	1.0	75.5 ± 1.5	96.6 ± 3.7

[a] Cytotoxicity index = [1 − (OD of treated cells/OD of control cells)] × 100%. Values are the mean ± SD of three experiments.

Table II. Effects of cTNF-2 and cTNF-1 on the Induction of IL-1 from Human PBMC and Monocytes[a]

	IL-1 production (pg/mL) PBMC (2×10^6 cells)		monocytes (1×10^5 cells)
medium only	375 ● 42	medium only	233 ± 163
LPS (0.1 μg/mL)	2317 ● 126*	LPS (0.1 μg/mL)	1259 ± 150*
cTNF-1 (1 μg/mL)	579 ± 101*	cTNF-1 (10 μg/mL)	1047 ± 147*
cTNF-2 (1 μg/mL)	598 ± 128*	cTNF-2 (10 μg/mL)	1102 ● 82*
rTNF-α (1 μg/mL)	741 ± 55*		

[a] The cells in 10% FCS-RPMI 1640 medium were cultured with stimulants for 24 h at 37 °C in a 5% CO_2 incubator. The supernatants were collected and assayed for IL-1 by the ELISA method. Data represent mean ± SE from eight (PBMC) or four (monocytes) experiments; *$p < 0.01$.

significant levels of IL-1 (Table II). In monocytes, the IL-1 levels induced by cTNF-2 (10 μg/mL) and cTNF-1 (10 μg/mL) were equivalent to about 85% of that induced by LPS (0.1 μg/mL). In addition, the IL-1 production by PBMC was dose-dependently increased by cTNF-2 (Figure 1). At a concentration of 10 μg/mL, the IL-1 level induced by cTNF-2 (2616 ± 320 pg/mL) was similar to that induced

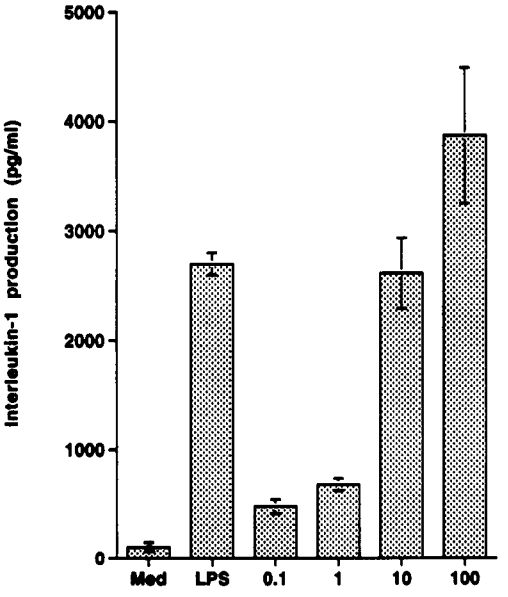

Figure 1. Dose-dependent response of cTNF-2 on IL-1 production by human PBMC. The cells (2×10^6/mL) in 10% FCS-RPMI 1640 medium were stimulated with 0.1, 1, 10, 100 μg/mL of cTNF-2 or with 0.1 μg/mL of LPS for 24 h at 37 °C. Med, culture medium only. Data represent mean ± SE of four replicates; $p < 0.01$.

by 0.1 μg/mL of LPS (2700 ± 100 pg/mL). A higher dose (100 μg/mL) of cTNF-2 gave a further increment of IL-1 production.

It is known that macrophages and monocytes are the main producers of cytokines, including IL-1, IL-6, and the tumor necrosis factors. A variety of cell types have been

reported to produce these cytokines.[30,31] LPS-stimulated macrophages or monocytes produced mainly IL-1β, which is an important cytokine, promoting T cell activation as well as activating other macrophages for immunological modulations.[32] Recently, recombinant human IL-1 was shown to have radioprotective and hemopoietic restoration properties. Further, it elicited afferent hormonal signals in murine models.[33,34] Although IL-1 displays both *in vitro* and *in vivo* antitumor activities, the latter activity may require the synergistic action of other cytokines.[35]

The present results showed that both cTNF-2 and cTNF-1 induced statistically significant IL-1 production from human PBMC and monocytes, suggesting that the fragments corresponding to human TNF-α-(59–66) or murine TNF-(127–132) may represent certain binding domains of the tumor necrosis factors that elicit IL-1 production from PBMC and monocytes/macrophages. Although there is little sequence homology between human TNF-α-(59–66) and murine TNF-(127–132), there is some sequence homology between the cyclic peptides cTNF-2 and cTNF-1 (Figure 1), notably, -Leu-X-Lys-Gly- (where X is Phe and Ser, for cTNF-2 and cTNF-1, respectively). It is possible that this small sequence homology may contribute to the observed immunological activities (induction of IL-1) of these two cyclic peptides. Taken together, it is envisaged that the cyclic peptides cTNF-2 and cTNF-1 may be useful as potential immunomodulators under further development.

Experimental Section

All of the protected amino acid derivatives were purchased from Schweizerhall Co., and Sigma Chemical Co. or synthesized in this laboratory according to published procedures. Melting points were determined on a Mel-temp apparatus and were uncorrected. Medium-pressure chromatography was performed using Merck 230-400 mesh silica gel. TLC was performed using Merck silica gel 60 on aluminum sheets. A Sage Model 351 syringe pump was employed in the cyclization reaction. HPLC analyses or semipreparative HPLC purifications were performed on a Beckman 110B solvent-delivery module equipped with a Soma Model S-3702 variable-wavelength UV detector monitoring at 220 nm. The columns used were a Vydac TP-201 (C_{18}, 0.4 × 25 cm, column 1), and a Vydac TP-201 (C_{18}, 1.0 × 25 cm, column 2). Optical rotations were determined on an Optical Activity AA-5 instrument or on a Rudolph Autopol II polarimeter. Mass spectra were taken from a JEOL JMX-HX 110 instrument operating in the FAB mode at Tsing-Hua University. Elemental analyses and amino acid analyses were performed at the Chemistry and Biochemistry Departments, respectively of Cheng-Kung University.

Boc-Lys(Z)-Gly-OPa (2). This compound was prepared as previously described.[3]

Boc-Phe-Lys(Z)-Gly-OPa (3). The dipeptide **2** (3.0 g, 5.4 mmol) in CH_2Cl_2 (4.6 ml) was stirred with TFA (6.0 ml) for 50 min, and the solvents were removed *in vacuo*. Boc-Phe (1.47 g, 5.53 mmol) was treated with HOBt (0.83 g, 6.08 mmol) and DCC (1.71 g, 8.29 mmol) in CH_2Cl_2 (97 ml) at 0 °C for 10 min and then at room temperature for 50 min. The TFA salt prepared above in CH_2Cl_2 (65 mL) was added and the pH adjusted to neutrality by the addition of DIEA. After 105 min the reaction mixture was filtered and diluted with CH_2Cl_2. The combined organic solutions were washed successively with citric acid (10%, 3 × 15 mL), saturated $NaHCO_3$ (3 × 15 mL), and water (3 × 15 mL), dried ($MgSO_4$), and evaporated to give a solid. The crude product was purified by silica gel chromatography using stepwise elution ($CHCl_3$/MeOH, 100:0, 99:1, 98:2) to afford a white solid (3.6 g, 95%): TLC ($CHCl_3$/MeOH, 97:3), R_f 0.31; mp 128–129 °C; $[\alpha]^{33}_D$ −72° (*c* 0.42, $CHCl_3$). Anal. ($C_{38}H_{46}N_4O_9 \cdot 0.5H_2O$) C, H, N.

Boc-Leu-Phe-Lys(Z)-Gly-OPa (4). The tripeptide **3** (6.0 g, 8.54 mmol) was deblocked with TFA/CH_2Cl_2 as described above. Boc-Leu (2.78 g, 12.9 mmol) in CH_2Cl_2 (148 mL) was treated with HOBt (1.3 g, 9.58 mmol) and DCC (2.7 g, 13.07 mmol) at 0 °C for 10 min and at room temperature for 50 min. The TFA salt of **3** in CH_2Cl_2 (100 mL) was added and the pH adjusted by DIEA to neutrality. After 2 h the reaction mixture was filtered and worked up as described above for **3**. The crude product was purified by silica gel chromatography using stepwise elution ($CHCl_3$/MeOH, 99:1, 98:2, 97:3) to afford a white solid (6.03 g, 87%): TLC ($CHCl_3$/MeOH, 96:4), R_f 0.30; mp 132–134 °C; $[\alpha]^{33}_D$ −10.2° (*c* 0.30, $CHCl_3$/MeOH, 3:1). Anal. ($C_{44}H_{57}N_5O_{10} \cdot 0.5H_2O$) C, H, N.

Boc-Val-OPa (5). This compound was synthesized from Boc-Val by the procedure described previously.[28] The product was crystallized from EtOAc/hexane to give a white solid (81%): TLC ($CHCl_3$), R_f 0.60; mp 102–103 °C; $[\alpha]^{31}_D$ −3.0° (*c* 0.3, $CHCl_3$/MeOH, 3:1). Anal. ($C_{18}H_{25}NO_5$) C, H, N.

Boc-Gln-Val-OPa (6). Boc-Val-OPa (3.78 g, 11.27 mmol) was deblocked with TFA/CH_2Cl_2 as described above. Boc-Gln (2.78 g, 11.27 mmol) was treated with HOBt (1.68 g, 12.4 mmol) and DCC (3.49 g, 16.91 mmol) and then coupled to the TFA salt of **5** in the presence of DIEA (neutral pH). The reaction mixture was worked up in a similar manner as that described for **3**. The crude product was purified by silica gel chromatography using stepwise elution ($CHCl_3$/MeOH, 99:1, 98:2, 97:3) to afford a white solid (4.31 g, 83%): TLC ($CHCl_3$/MeOH, 94:6), R_f 0.21; mp 146 °C; $[\alpha]^{27}_D$ −35.2° (*c* 0.35, $CHCl_3$). Anal. ($C_{23}H_{33}N_3O_7$) C, H, N.

Boc-Ser(OBzl)-Gln-Val-OPa (7). The dipeptide **6** (1.0 g, 2.16 mmol) was deblocked with TFA/CH_2Cl_2 as described above. Boc-Ser(OBzl) (0.64 g, 2.16 mmol) was treated with HOBt (0.32 g, 2.37 mmol) and DCC (0.67 g 3.24 mmol) and then coupled to the TFA salt of **6** in the presence of DIEA (neutral pH). The reaction mixture was worked up in a similar manner as that described for **3**. The crude reaction product was purified by silica gel chromatography using stepwise elution (same solvent system as described for dipeptide **6**) to afford a white solid (1.14 g, 83%): TLC ($CHCl_3$/MeOH, 92:8), R_f 0.46; mp 162-164 °C; $[\alpha]^{33}_D$ −25.1° (*c* 0.34, $CHCl_3$/MeOH, 3:1). Anal. ($C_{33}H_{44}N_4O_9$) C, H, N.

Boc-Tyr(OBzl)-Ser(OBzl)-Gln-Val-OPa (8). The tripeptide **7** (2.44 g, 3.81 mmol) was deblocked with TFA/CH_2Cl_2 as described above. Boc-Tyr(OBzl) (1.42 g, 3.81 mmol) was treated with HOBt (0.57 g, 4.19 mmol) and DCC (1.18 g, 5.71 mmol) in DMF (13 mL) and then coupled to the TFA salt of **7** (with a further addition of 9 mL of DMF). DIEA was used to adjust the pH to neutrality and the reaction was allowed to proceed at room temperature for 2 h. The reaction mixture was filtered, and the solvents were removed *in vacuo*. Silica gel chromatography using stepwise elution ($CHCl_3$/MeOH, 98:2, 97:3, 96:4) afforded a white solid (3.09 g, 91%): TLC ($CHCl_3$/MeOH, 92:8), R_f 0.40; mp 186–187 °C; $[\alpha]^{32}_D$ −16.2° (*c* 0.28, $CHCl_3$/MeOH, 3:1). Anal. ($C_{49}H_{59}N_5O_{11} \cdot H_2O$) C, H, N.

Boc-Tyr(OBzl)-Ser(OBzl)-Gln-Val-OH (9). The tetrapeptide **8** (0.5 g, 0.56 mmol) was dissolved in glacial acetic acid (39 mL) and water (6 mL) at 0 °C. Zn powder (4.2 g) was added in small portions and the reaction mixture was stirred at room temperture overnight. The mixture was filtered over Celite and the solvents were removed *in vacuo*. The crude solid was washed successively with hexane (3 × 5 mL), EDTA (2%, 2 × 10 mL), and water (2 × 3 mL). The residue was dried *in vacuo* to give a white solid (0.38 g) that was used in the following step without further purification.

Boc-Tyr(OBzl)-Ser(OBzl)-Gln-Val-Leu-Phe-Lys(Z)-Gly-OPa (10). Boc-Leu-Phe-Lys(Z)-Gly-OPa (**4**) (0.84 g, 1.03 mmol) in CH_2Cl_2 (1.0 mL) was stirred with TFA (1.24 mL) for 50 min, and the solvents were removed *in vacuo*. The tetrapeptide **9** (0.8 g, 1.03 mmol) was treated with HOBt (0.17 g, 1.24 mmol) and DCC (0.43 g, 2.08 mmol) in DMF (5 mL) at 0 °C for 10 min and at room temperature for 2 h. The TFA salt of **4** in DMF (4 mL) was added and DIEA was used to adjust the pH to neutrality. After 16 h the solvents were evaporated *in vacuo*, and the product was crytallized from trifluoroethanol/H_2O as a white solid (1.12 g, 74%): TLC ($CHCl_3$/MeOH, 94:6), R_f 0.28; mp 221 °C dec; $[\alpha]^{33}_D$ −9.3° (*c* 0.16, DMF). Anal. ($C_{80}H_{100}N_{10}O_{17} \cdot 1.5H_2O$) C, H, N.

Boc-Tyr(OBzl)-Ser(OBzl)-Gln-Val-Leu-Phe-Lys(Z)-Gly-OH (11). The protected octapeptide **10** (0.5 g, 0.34 mmol) was dissolved in glacial acetic acid (24 mL) and water (3.9

mL) at 0 °C and Zn powder (2.59 g) added in small portions. The reaction mixture was then stirred at room temperature for 16 h, filtered, and evaporated *in vacuo*. The residue was washed successively with hexane (3 × 2 mL), EDTA (2.5%, 2 × 7 mL), and water (2 × 1 mL) to give a solid (0.37 g) which was used in the following step without further purification.

Cyclo[Tyr(OBzl)-Ser-(OBzl)-Gln-Val-Leu-Phe-Lys(Z)-Gly-] (13). The free acid 11 (0.26 g, 0.19 mmol) in DMF (6 mL) was esterified with pentafluorophenol (0.049 g, 0.27 mmol) and DCC (0.079 g, 0.38 mmol) for 6 h. The reaction mixture was filtered, and the solvents were removed *in vacuo* to give a solid. The product was stirred in a solution of CH_2Cl_2 (1.0 mL) and TFA (1.2 mL) for 1 h, and the solvents were removed *in vacuo*. The TFA salt was dried over P_2O_5 under vacuum for 1 h, dissolved in dioxane (25 mL), and injected *via* a syringe pump into a stirred solution of dioxane (500 mL), pyridine (70 mL), and ethanol (6 mL) maintained at 50–55 °C for 8 h. The solvents were removed *in vacuo* to give a solid which was purified by silica gel chromatography using stepwise elution ($CHCl_3$/MeOH, 97:3, 96:4, 95:5) to afford a white solid (0.094 g, 40%), pure to TLC [($CHCl_3$/MeOH, 92:8), R_f 0.23]: mp 238 °C dec; $[\alpha]^{27}_D$ −32.6° (*c* 0.30, $CHCl_3$); MS (FAB, Xe beam) (*m/z*, relative intensity) 1236.5 M^+ (62.6), 1235.6 $[M - H]^-$ (82.4), 1145.5 (14.5), 1127.5 (20.4), 1101.5 (16.9), 155.1 (14.6), 107.0 (100), 42.1 (49.7).

Cyclo[Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-] (14). The protected cyclic octapeptide 13 (0.03 g) was mixed with anisole (0.75 mL) and stirred with anhydrous HF (ca. 1 mL) at 0 °C for 30 min. The solvents were removed *in vacuo*, and the residue was dried in a vacuum dessicator over P_2O_5 for 2 h. The solid obtained was dissolved in a minimum amount of water and washed with ether, and the aqueous layer was lyophilized. The product obtained was purified by semipreparative HPLC [column 2, isocratic, MeOH/0.1% TFA (50:50)] to afford, after lyophilization, a white powder (0.008 g; 35%), pure to HPLC [column 1, isocratic, MeOH/0.1% TFA (50:50); t_R = 5.7 min]. However, a small peak with a retention time of 3.2 min could not be removed by repeated purification: $[\alpha]^{33}_D$ −53.6° (*c* 0.14, MeOH/H_2O, 1:1); mp 156–158 °C dec; MS (FAB, Ar beam) (*m/z*, relative intensity) 945.4 $[M + Na]^+$ (27.3), 923.5 $[M + H]^+$ (100), 216.2 (21.2), 136.1 (35.2), 129.1 (37.4), 120.1 (68.9), 101.0 (24.4), 91.0 (26.1), 86.1 (47.0), 84.0 (64.7), 72.1 (43.2); amino acid analysis, Ser 0.94, Gln 1.04, Gly 1.00, Val 0 .91, Leu 1.02, Tyr 0.95, Phe 0.97, Lys 1.05.

Cytotoxicity Studies. The cyclic octapeptide 14 (cTNF-2) was assayed for its ability to inhibit the growth of two human tumor cell lines: HeLa (cervical carcinoma) and HEP-2 (oesophagus carcinoma). The method used in this assay is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) procedure, as described previously.[3]

Interleukin-1 Assay. Human peripheral blood mononuclear cells (PBMC) from healthy donors supplied by the Taichung Blood Center were prepared from heparinized whole blood by Ficoll-paque (Pharmacia, Piscataway, NJ) gradient separation. The cells were washed four times with RPMI-1640 medium. Two million PBMC (containing 5-8% monocytes) were suspended in a RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 2 μM glutamine, 25 units/mL penicillin, and 25 μg/mL streptomycin in all experiments. The monocytes were collected by scraping the adherent PBMC from culture dishes after 1 h of cell culture. PBMC or monocytes (1×10^5 cells/mL) were incubated with cTNF-1, cTNF-2, recombinant TNF (rTNF), or lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) for 24 h at 37 °C in an atmosphere of 5% CO_2. Supernatants from the cell culture were collected and stored at −70 °C. The IL-1 production from each sample was determined by using an IL-1β ELISA kit (Cistron, Pine Brook, NJ). Samples were added to the wells of microtiter plates precoated with monoclonal anti-IL-1β antibody. After incubation, the unbound components from the samples were removed by washing. Following the binding of polyclonal rabbit antibody to bound IL-1, goat anti-rabbit IgG conjugated with horseradish peroxidase was added to each well. The color product developed after the addition of substrate was measured with a spectrophotometer (Dynatech MR700) at 450 nm. The IL-1 value was estimated from a standard curve.

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References

(1) Abbreviations generally follow the IUPAC–IUB recommendation as published: in *J. Biol. Chem.* **1989**, *264*, 668–673. Other abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DCC, 1,3-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; HOBt, 1-hydroxybenzotriazole; OPa, phenacyl; TFA, trifluoroacetic acid.
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