

The Effect of STF and Its Analogs on T Cells with Cellular Immunodeficiency¹⁾

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[Arg³]-STF and [DAla¹]-STF were synthesized by the solution method. STF and its analogs caused an increase in E-rosette forming capacity when incubated *in vitro* with uremic T cells.

Keywords—serum thymic factor; middle molecular substances; uremic patients; rosette formation cell; immunodeficiency

Bach *et al.*^{3,4)} have reported the amino acid sequence of a putative thymic hormone isolated from pig serum,⁵⁾ a nonapeptide (Pyr-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn) called STF. Natural and synthetic STF showed high activity in the rosette test used as a bioassay.

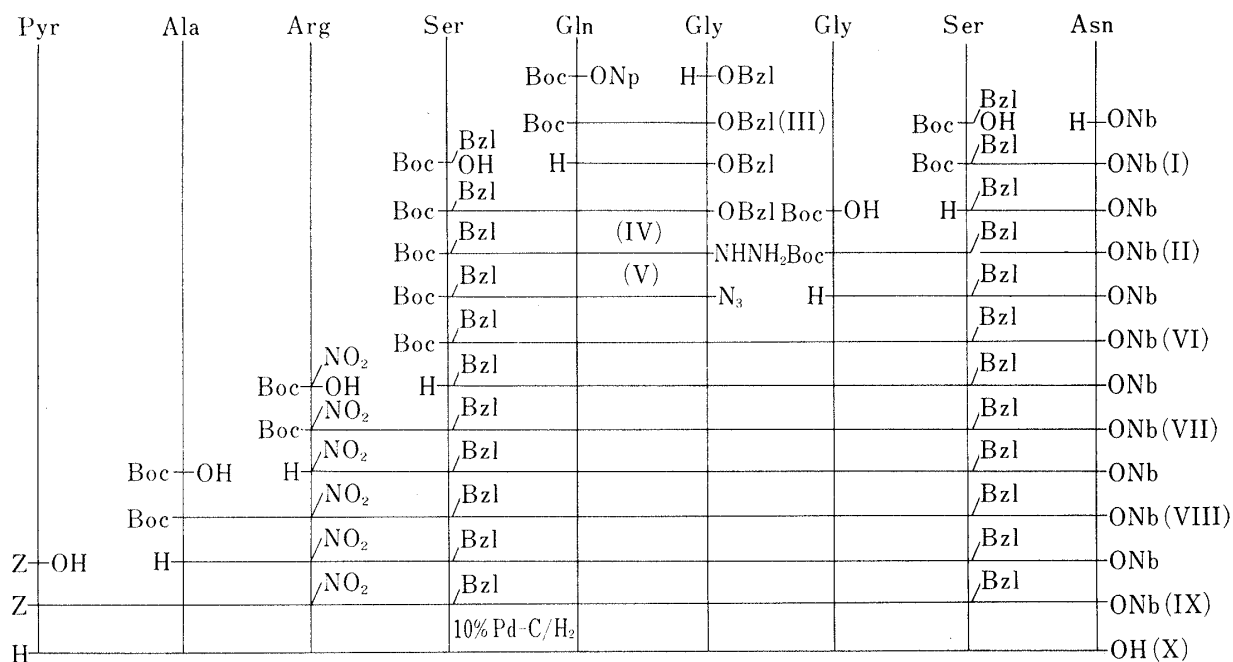


Fig. 1. Synthetic Scheme for [Arg³]-STF

- 1) Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: STF, serum thymic factor; MMS, middle molecular substances; PHA, phytohemagglutinin; WSCI, water-soluble carbodiimide; E, sheep erythrocytes; RFC, rosette-forming cells; DMF, dimethylformamide; HONB, N-hydroxy-5-norbornene-2,3-dicarboximide; TFA, trifluoroacetic acid; GVB²⁺, gelatin veronal buffer; PBS, phosphate-buffered saline; FCS, fetal calf serum; Tos, *p*-toluenesulfonic acid.
- 2) Location: a) *Tsutsumimachi 3-16-1, Sendai, 980, Japan*; b) *Tsutsumidori-amamiyachō 2-21, Sendai, 980, Japan*.
- 3) J.F. Bach, M. Dardenne, J.M. Pleau, and J. Rosa, *J. C. r. hebdomadaire Acad. Sci. Paris*, **283**, 1605 (1977).
- 4) J.F. Bach, M. Dardenne, and J.M. Pleau, *Nature* (London), **266**, 55 (1977).
- 5) J.F. Bach, M. Dardenne, J.M. Pleau, and M. Bach, *Ann. N.Y. Acad. Sci.*, **249**, 186 (1975).

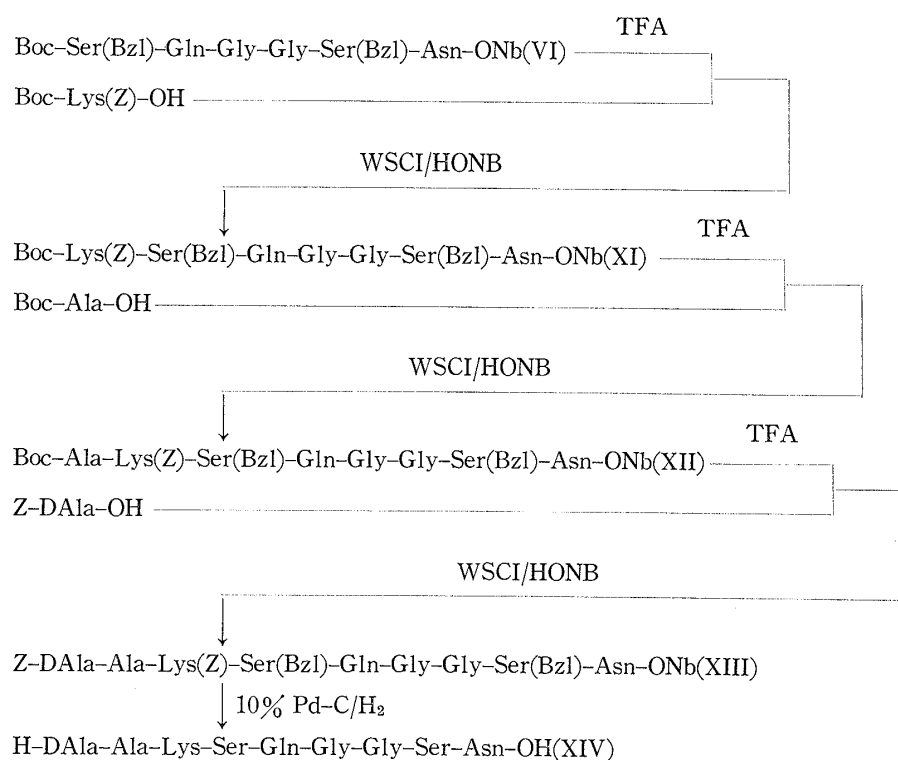
Fig. 2. Synthetic Scheme for (DAla¹)-STF

TABLE I. Effects of STF and Its Analogs on the E-Rosette Formation Inhibiting Effect of MMS

Dose = 10 µg/ml		E-Rosettes (%)
—		78
H-Gly-Gly-His-OH ^{a,b}		41
1)	STF ^a	71
2)	(Arg ³)-STF ^a	74
3)	(DAla ¹)-STF ^a	75

^a) Uremic T cells: incubation was carried out for 30 min at 37° with MMS at a concentration of 3 mg/ml.

^b) Control (purchased from the Protein Research Foundation, Minoh, Osaka).

On the other hand, uremic patients are known to have impaired cellular immunity⁶⁾ and lymphocytes from patients may exhibit decreased *in vitro* reactivity to mitogens.⁷⁾ Uremic plasma also has an inhibitory effect on the transformation of lymphocytes *in vitro*.⁸⁾ The mechanisms of these *in vitro* phenomena are not known. It was found that MMS (MW 500—5000) obtained from dialysates of uremic patients caused suppression of PHA-induced lymphocyte transformation.⁹⁾ It was concluded that metabolites in the molecular weight range of 500—5000 were significant uremic toxins and E-rosette formation inhibition activity may be caused by excess blood concentration levels of these materials.¹⁰⁾

- 6) H. Huber, D. Pastner, P. Kittrich, and H. Braunstainer, *Clin. Exp. Immunol.*, **5**, 83 (1969).
- 7) L.S. Nakhla and M.J. Goggin, *Immunology*, **24**, 229 (1973).
- 8) M.S. Silk, *Invest. Urol.*, **5**, 195 (1967).
- 9) Z. Hanicki, T. Cichocki, M. Sarnecka-Keller, A. Klein, and Z. Komoroska, *Nephron*, **17**, 73 (1976).
- 10) Z. Hanicki, M. Sarnecka-Keller, A. Klein and K. Slizowska, *Clin. Chim. Acta*, **54**, 47 (1974).

In this communication, the syntheses of two analogs of STF and the results of biological assay of the synthetic peptides in comparison with STF are described (Table I). The solution method for peptide synthesis was used in this investigation. The analogs synthesized were [Arg³]-STF and [Dala¹]-STF.

1) The reason for selecting [Dala¹]-STF was that N-terminal H-Pyr-OH is thought to increase the resistance of this active peptide to enzyme degradation. This was confirmed by replacing the N-terminal residue with H-DAla-OH and testing the resistance of the analog to enzyme degradation.

2) The reason for selecting [Arg³]-STF was that the replacement of the H-Lys-OH of this peptide with H-Arg-OH resulted in a marked increase in basicity, and this is expected to influence the biological activity. The synthetic routes to the analogs are illustrated in Figs. 1 and 2. Table I shows the increases in percentage T cell rosettes on incubation *in vitro* with MMS, lymphocytes and STF analogs. Increased activity for E-rosette formation was observed with the two analogs up to a concentration of 10 µg/ml, and a rapid increase in the E-RFC rate was clearly observed, both of which were comparable to those obtained with STF. On incubation with MMS (3 mg/ml), "total" T cell rosettes ranged from 41% (Table I). However, after incubation with MMS (3 mg/ml) and STF analogs (10 µg/ml), the maximum T cell rosettes ranged from 41 to 71% (Table I).

Experimental

Melting points are uncorrected. Unless otherwise mentioned, Z-groups of the protected amino acids and peptides were deblocked with HBr in AcOH, and Boc groups with TFA. The resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. *Rf*¹ values refer to the Partridge system¹¹⁾ *Rf*² values refer to the system of BuOH-pyridine-AcOH-H₂O (30:20:6:24).¹²⁾ Rotations were determined with an Atago Polax. Amino acid analyses were performed with a JEOL JLC-8AH amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 35–40°. MMS (MW 500–5000) was isolated from dialysates of an uremic patient by ultrafiltration using Amicon Centriflo membranes. STF was purchased from the Protein Research Foundation, Osaka.

Boc-Ser(Bzl)-Asn-ONb (I)—Z-Asn-ONb (4.8 g) was dissolved in AcOH (24.0 ml), anisole (5.0 ml) and 25% HBr in AcOH (24.0 ml). After 40 min at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate was washed with dry ether and dried over KOH pellets in a vacuum. HONB (1.6 g),¹³⁾ Boc-Ser(Bzl)-OH (3.0 g) and WSCI (2.3 g) were added to an ice-cold solution of H-Asn-ONb HBr in DMF (30.0 ml), followed by addition of N-methylmorpholine¹⁴⁾ to keep the solution slightly alkaline. After 24 hr at 4°, the reaction mixture was poured into 1 N NaHCO₃ with stirring. The precipitate thus formed was washed successively with 1 N NaHCO₃, H₂O, 1 N citric acid and H₂O. The precipitate was crystallized from EtOAc. Yield 5.0 g (79%), mp 121–122°, $[\alpha]_D^{25}$ –42.4° (*c*=1.0, DMF), *Anal.* Calcd. for C₂₆H₃₂N₄O₉: C, 57.33; H, 5.92; N, 10.29. Found: C, 57.71; H, 5.97; N, 9.85. *Rf*¹ 0.70, *Rf*² 0.84, single ninhydrin-positive spot.

Boc-Gly-Ser(Bzl)-Asn-ONb (II)—The protected dipeptide ester (I) (1.6 g) was dissolved in TFA (4.0 ml) in the presence of anisole (0.5 ml) and the solution was kept at room temperature for 20 min, then ether was added. The precipitate formed was dried over KOH pellets in a vacuum. The resulting dipeptide ester was condensed with Boc-Gly-OH (0.527 g) in the presence of HONB (0.645 g) and WSCI (0.633 g) essentially as described in the preparation of I. EtOAc was added and the EtOAc solution was washed successively with 1 N NaHCO₃, H₂O, 1 N citric acid and H₂O. The solution was dried over MgSO₄ and concentrated to small volume, then petroleum ether was added to the residue. The precipitate was recrystallized from EtOAc. Yield 1.4 g (76%), mp 143–144°, $[\alpha]_D^{25}$ –38.8° (*c*=1.0, DMF). *Anal.* Calcd. for C₂₈H₃₅N₅O₁₀: C, 55.90; H, 5.86; N, 11.64. Found: C, 55.27; H, 5.89; N, 11.37. *Rf*¹ 0.57, *Rf*² 0.73, single ninhydrin-positive spot.

Boc-Gln-Gly-OBzl (III)—Boc-Gln-ONp (1.1 g)¹⁵⁾ was added to a solution of H-Gly-OBzl Tos (1.0 g) in DMF (10.0 ml), followed by N-methylmorpholine to keep the solution slightly alkaline. After 24 hr at

11) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).

12) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).

13) M. Fujino, M. Wakimasu, S. Shinagawa, C. Kitada, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 539 (1978).

14) G.W. Anderson, F.M. Callahan, and J.E. Zimmerman, *J. Amer. Chem. Soc.*, **89**, 178 (1967).

15) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).

room temperature, the reaction mixture was diluted with 1 N NH_4OH (3.0 ml) with stirring. After 1 hr, the mixture was extracted with EtOAc and washed successively with 1 N NH_4OH , H_2O , 1 N citric acid and H_2O . The solution was dried over MgSO_4 and concentrated to small volume, then petroleum ether was added to the residue. The precipitate thus formed was reprecipitated from EtOAc and petroleum ether. Yield 0.720 g (60%), mp 108–110°, $[\alpha]_D^{25} -63.0^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{19}\text{H}_{27}\text{N}_3\text{O}_6$: C, 56.84; H, 8.35; N, 10.47. Found: C, 56.84; H, 8.71; N, 10.26. R_f^1 0.69, R_f^2 0.76, single ninhydrin-positive spot.

Boc-Ser(Bzl)-Gln-Gly-OBzl (IV)—III (0.700 g) was treated with TFA (2.0 ml) as described above. The resulting dipeptide ester was condensed with Boc-Ser(Bzl)-OH (0.526 g) in the presence of HONB (0.383 g) and WSCI (0.376 g) essentially as described in the preparation of I. The reaction mixture was extracted with EtOAc and washed successively with 1 N NaHCO_3 , H_2O , 1 N citric acid and H_2O . The solution was dried over MgSO_4 and concentrated to a small volume, then petroleum ether was added to the residue. Yield 0.860 g (86%), mp 113–116°, $[\alpha]_D^{25} -9.0^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{29}\text{H}_{38}\text{N}_4\text{O}_8$: C, 61.40; H, 6.71; N, 9.82. Found: C, 60.58; H, 6.54; N, 9.33. R_f^1 0.79, R_f^2 0.89, single ninhydrin-positive spot.

Boc-Ser(Bzl)-Gln-Gly-NHNH₂ (V)—IV (570 mg) was dissolved in MeOH (2.0 ml). Hydrazine hydrate (0.5 ml) was added and the solution was left to stand at room temperature for 24 hr. After evaporation of MeOH, the residue was recrystallized from MeOH and ether. Yield 425 mg (85%), mp 134–135°, $[\alpha]_D^{25} -41.0^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{22}\text{H}_{34}\text{N}_6\text{O}_7$: C, 53.43; H, 6.93; N, 17.00. Found: C, 53.25; H, 6.76; N, 17.41.

Boc-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (VI)—II (425 mg) was treated with TFA (1.0 ml) as described above. The resulting tripeptide ester was dissolved in DMF (4.0 ml). N-Methylmorpholine (0.2 ml) and an EtOAc solution (0.5 ml) of azide¹⁶⁾ (prepared from 425 mg of hydrazide and 72 mg of NaNO_2 in 8.0 ml of 1 N HCl containing NaCl at 0°) were added to the ice-cold solution. The reaction mixture was stirred at 4° for 48 hr then at room temperature for 1 hr. The mixture was evaporated down in a vacuum and the residue was poured into cold 1 N NaHCO_3 . To the suspension thus formed, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N NaHCO_3 , H_2O , 1 N citric acid and H_2O . The dried product was recrystallized from EtOAc. Yield 600 mg (73%), mp 184–185°, $[\alpha]_D^{25} -38.7^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{45}\text{H}_{57}\text{N}_9\text{O}_{15} \cdot \text{H}_2\text{O}$: C, 55.09; H, 6.06; N, 12.85. Found: C, 54.93; H, 5.73; N, 12.44. R_f^1 0.66, R_f^2 0.80, single ninhydrin-positive spot.

Boc-Arg(NO₂)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (VII)—VI (600 mg) was treated with TFA (1.0 ml) as described above. The resulting hexapeptide ester was condensed with Boc-Arg(NO₂)-OH (198 mg) in the presence of HONB (135 mg) and WSCI (144 mg) essentially as described in the preparation of I. Yield 425 mg (58%), mp 156–158°, $[\alpha]_D^{25} -39.8^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{51}\text{H}_{68}\text{N}_{14}\text{O}_{18}$: C, 52.57; H, 5.88; N, 16.83. Found: C, 52.55; H, 5.74; N, 16.87. R_f^1 0.62, R_f^2 0.83, single ninhydrin-positive spot.

Boc-Ala-Arg(NO₂)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (VIII)—VII (425 mg) was treated with TFA (1.0 ml) as described above. The resulting heptapeptide ester was condensed with Boc-Ala-OH (70 mg) in the presence of HONB (81 mg) and WSCI (86 mg) essentially as described in the preparation of I. Yield 322 mg (71%), mp 164–165°, $[\alpha]_D^{25} -44.0^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{54}\text{H}_{73}\text{N}_{15}\text{O}_{19}$: C, 52.46; H, 5.95; N, 17.00. Found: C, 52.60; H, 5.55; N, 17.19. R_f^1 0.58, R_f^2 0.71, single ninhydrin-positive spot.

Z-Pyr-Ala-Arg(NO₂)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (IX)—VIII (322 mg) was treated with TFA (1.0 ml) as described above. The resulting octapeptide ester was condensed with Z-Pyr-OH (53 mg)¹⁷⁾ in the presence of HONB (58 mg) and WSCI (62 mg) essentially as described in the preparation of I. The reaction mixture was poured into 1 N HCl with stirring. The precipitate thus formed was washed successively with 1 N HCl, H_2O , 1 N NaHCO_3 and H_2O . Yield 180 mg (53%), mp 190–192°, $[\alpha]_D^{25} -49.1^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{62}\text{H}_{76}\text{N}_{16}\text{O}_{21}$: C, 53.90; H, 5.55; N, 16.23. Found: C, 53.55; H, 5.32; N, 15.93. R_f^1 0.69, R_f^2 0.85, single chlorine-tolidine positive spot.

H-Pyr-Ala-Arg-Ser-Gln-Gly-Gly-Ser-Asn-OH (X)—The fully protected nonapeptide IX (100 mg) was hydrogenated in 50% AcOH (12 ml) over 10% Pd-C for 32 hr. The catalyst was removed with aid of celite. The solution was evaporated to dryness and the residue was dried over KOH pellets in a vacuum. The solution of crude product in 1% AcOH (6 ml) was added to a Sephadex G-15 column (1.8 × 40.0 cm) and eluted with 1% AcOH. Fractions of 5 ml each were collected at a flow rate of 5 ml/10 min with an automatic fraction collector, and the absorbancy of each fraction was determined at 230 nm. The eluates in tubes No. 19 to 26 containing the nonapeptide were pooled, evaporated to dryness in a vacuum and lyophilized. Yield 37 mg (54%), mp 180–196° (dec.), $[\alpha]_D^{25} -42.0^\circ$ ($c=1.0$, H_2O), R_f^1 0.03, R_f^2 0.10, single Sakaguchi-positive spot. Amino acid ratios in the acid hydrolysate: Glu 2.00, Asp 0.92, Ala 0.99, Ser 1.85, Gly 2.32, Arg 0.97.

Boc-Lys(Z)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (XI)—VI (600 mg) was treated with TFA (1.0 ml) as described above. The resulting hexapeptide ester was condensed with Boc-Lys(Z)-OH (348 mg) in the presence of HONB (135 mg) and WSCI (144 mg) essentially as described in the preparation of I. Yield 419 mg (57%), mp 150–152°, $[\alpha]_D^{25} -36.7^\circ$ ($c=1.0$, DMF), *Anal.* Calcd. for $\text{C}_{59}\text{H}_{75}\text{N}_{11}\text{O}_{18}$: C, 57.78; H, 6.16;

16) T. Kato, N. Mitsuyasu, M. Waki, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **41**, 2480 (1968).

17) H. Gibian and E. Killieger, *Ann. Chem.*, **640**, 145 (1961).

N, 12.57. Found: C, 57.75; H, 5.90; N, 12.45. Rf^1 0.82, Rf^2 0.95, single ninhydrin-positive spot.

Boc-Ala-Lys(Z)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (XII)—XI (300 mg) was treated with TFA (1.0 ml) as described above. The resulting heptapeptide ester was condensed with Boc-Ala-OH (80 mg) in the presence of HONB (75 mg) and WSCI (85 mg) essentially as described in the preparation of I. Yield 320 mg (76%), mp 145–146°, $[\alpha]_D^{25}$ -50.0° ($c=1.0$, DMF), *Anal.* Calcd. for $C_{62}H_{80}N_{12}O_{19} \cdot H_2O$: C, 56.69; H, 6.92; N, 12.79. Found: C, 56.36; H, 5.90; N, 12.31. Rf^1 0.69, Rf^2 0.87, single ninhydrin-positive spot.

Z-DAla-Ala-Lys(Z)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-ONb (XIII)—XII (258 mg) was treated with TFA (0.7 ml) as described above. The resulting octapeptide ester was condensed with Z-DAla-OH (45 mg) in the presence of HONB (40 mg) and WSCI (43 mg) essentially as described in the preparation of I and IX. Yield 251 mg (70%), mp 170–173°, $[\alpha]_D^{25}$ -39.3° ($c=1.0$, DMF), *Anal.* Calcd. for $C_{68}H_{83}N_{13}O_{20} \cdot H_2O$: C, 57.53; H, 6.04; N, 12.82. Found: C, 57.69; H, 5.79; N, 12.38. Rf^1 0.42, Rf^2 0.56, single ninhydrin-positive spot.

H-DAla-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH (XIV)—XIII (150 mg) in 50% AcOH was hydrogenated in the usual manner for 24 hr. The hydrogenated product in 1% AcOH (6 ml) was added to a Sephadex G-15 column (1.8 $\times$ 40.0 cm) and eluted with 1% AcOH. Fractions of 5 ml each were collected at a flow rate of 5 ml/10 min with an automatic fraction collector, and the absorbancy of each fraction was determined at 230 nm. The eluates in tubes No. 11 to 19 containing the nonapeptide were pooled, evaporated to dryness in a vacuum and lyophilized. Yield 61 mg (59%), mp 190–198° (dec.), $[\alpha]_D^{25}$ -38.0° ($c=1.0$, H_2O), Rf^1 0.01, Rf^2 0.07, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Lys 0.96, Asp 0.90, Glu 1.00, Ser 2.08, Gly 1.98, Ala 0.96, DAla 0.96.

E-Rosette Formation—Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient¹⁸⁾ for T cell rosette formation. Isolated lymphocytes were adjusted to a concentration of 5×10^5 /ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 5%. Sheep erythrocytes were washed with PBS and a suspension (1×10^6 /ml) was prepared. Lymphocytes were suspended in GVB²⁺ or FCS (1.0 ml) and incubated for 30 min at 37° with MMS (3 mg) and STF analogs (10 μ g). Lymphocytes were washed with GVB²⁺ and centrifuged for 10 min at 1500 rpm, then suspended in GVB²⁺ or FCS (1 ml). The suspension was mixed with sheep erythrocytes (0.5 ml) and then incubated for 18 hr at 4°. The mixture was centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding three more sheep erythrocytes was determined. Monocytes or polymorphonuclear cells forming rosettes were excluded (Table I).

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18) R. Harris and E.O. Ukaejiofo, *Brit. J. Haematol.*, **18**, 229 (1970).