Synthesis and Activity of NAcSerAspLysPro Analogues on Cellular Interactions between T-Cell and Erythrocytes in Rosette Formation

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Analogues of NAcSerAspLysPro (AcSDKP), a natural regulator of hematopoiesis isolated from fetal calf bone marrow, were synthesized. The biological activity of these molecules were evaluated in vitro in the rosette assay, which measures the interaction between human Jurkat T-cells and sheep red blood cells. In this test, the tripeptide SerAspLys was the most efficient. Inhibitory activity was detected at the concentration 10^{-14} M for the analogue and at 10^{-9}M for the parent tetrapeptide. The dipeptide NAcSerAsp still showed activity but at much higher doses (10^{-6} M). Substitution of polar amino acids led mostly to inactive molecules. Thus, replacement of Ser by Ala, or Lys by Orn yielded completely inactive compounds and replacement of Asp by Glu decreased the activity (10^{-6} M). The present study gives an insight into the role of individual amino acids of AcSDKP in the inhibition of the rosette formation which implicates interactions with T-cell CD_2 glycoprotein.

The tetrapeptide NAcSerAspLysPro (AcSDKP) **1** was recently isolated from fetal calf bone marrow and characterized by its ability to inhibit the entry of the hematopoietic stem cells (defined here as colony-forming unit CFU-S) into proliferation following administration of the cytotoxic drug cytosine arabinoside.[1]

Further studies demonstrated that the molecule partially inhibited rosette formation obtained through human Jurkat lymphoma T-cells and sheep erythrocytes interaction.

This interaction results from a recognition process which implicates the $T_{11}T_s$ glycoprotein present on SRBC and several regions of the glycoprotein CD_2 present on T lymphocytes.

In particular, epitope region 1 of CD_2 located between Lys 42 and Lys 56 was shown to modulate rosette formation. This sequence includes the characteristic sequence $^{44}Ser^{45}Asp^{46}Lys$.

Preliminary assay demonstrated that the AcSDKP target was located on the T-cell membrane and this led us to postulate that the inhibition observed in the rosette assay was due to a competition between added peptide and the SDK sequence present on CD_2 for a postulated recognition site located on T-cell membranes.[2]

In order to evaluate the importance of each of the residues of the tetrapeptide AcSDKP for the biological response, we have initiated a structure–activity relationship study. Biological evaluation of the synthetic peptides was carried out comparatively with AcSDKP by their ability to inhibit human lymphoma Jurkat T-cell and sheep red blood cell (SRBC) interactions measured by the number of rosettes formed between the two cells species.[3]

A number of peptides have been synthesized by classical peptide synthetic methods. The basic strategy to identify the minimal sequence that is responsible for the biological activity of a peptide relies on the gradual shortening of its sequence. Thus, the following sequences were prepared: NAcSDK (**2**), SDK (**3**), NAcSD (**4**), SD (**5**), DKP (**6**), and DK (**7**).

We then turned our attention to the side chains of the molecule and substituted each one of the residue for an amino acid bearing similar functions. In the case of serine, we also suppressed the hydroxyl function by substituting serine with alanine. The importance of the aspartyl residue was particularly considered. So the D isomer analogue **11** and the β-branched peptide **12** were prepared. Deacetylated tetrapeptide **13** was also synthesized to check the influence of the acetyl group on the biological activity.

Chemistry

Most of the peptides were prepared stepwise by using mixed anhydride[4] methodology. Peptides **4**, **7–10**, and **12** were synthesized according to the *N*-hydroxysuccinimide ester methodology[5] or both methodologies. Peptide **13** was prepared according to REMA[9] ("Repetitive excess of mixed anhydride") conditions using isopropenyl chloroformate[10] as an activating agent.

For all of the syntheses, the amino acids were protected with the *tert*-butyloxycarboxyl (Boc) groups on the α-amino function, with the benzyloxycarbonyl (Z) group on the side chain amino function and the benzyl group on both side chain and C-terminus carboxylic acids and hydroxyl functions.

Z and benzyl groups were removed by catalytic hydrogenolysis. Boc groups were cleaved by acidolysis with trifluoroacetic acid (TFA). Acetylation of the N-terminus was performed with acetylimidazole on the trifluoroacetate salt of the peptide with triethylamine as a tertiary base.

Whenever the free N-terminus peptides were to be prepared, acidolysis was performed prior to hydrogenolysis. In the case of N-acetylated peptides, catalytic hydrogenolysis was the last deblocking step. The FAB spectra corresponding to the fully protected peptides are reported in Table I. All the sequences having a C-terminus proline benzyl ester show a fragmentation corresponding to the loss of ProOBzl. Deprotected peptides were purified by HPLC on a C_{18} reversed-phase column. The purified peptides were characterized by their mass spectrum (FAB) and amino acid analysis (Table II).

Results and Discussion

The aspartic acid residue is known to be prone to give numerous side reactions[6] either in acidic or basic media. Under the conditions we have used, we have not been able

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Table I. Mass Spectral Data of Intermediate Peptides Prior to the Last Deblocking Step

	molecular formula, molecular weight	mass spectrum, m/e
NAcS(OBzl)D(OBzl)K(Z)OBzl (II)	$C_{44}H_{50}N_4O_{10}$, MW 794	MH^+ 795, $MH^+ - Z$, $MH^+ - 90$
BocS(OBzl)D(OBzl)K(Z)OBzl (III)	$C_{47}H_{56}N_4O_{11}$, MW 852	MH^+ 853, $MH^+ - 100$ $MH^+ - 100 - 90$
NAcS(OBzl)D(OBzl)OH (IV)	$C_{23}H_{26}N_2O_7$, MW 442	$M^{+\circ} = 442$[a]
BocS(OBzl)D(OBzl)OBzl (V)	$C_{33}H_{38}N_2O_8$, MW = 590	MH^+, MNa^+, $MH^+ - 100$, $MH^+ - 56$
BocD(OBzl)K(Z)POBzl (VI)	$C_{42}H_{52}N_4O_{10}$, MW = 772	MH^+ 773, $MH^+ - 100$, $MH^+ - Z$
BocD(OBzl)K(Z)OH (VII)	$C_{30}H_{39}N_3O_9$, MW 585	MNa^+, MK^+, $MK^+ - 100$, $MNa^+ - 100$, $MH^+ - 100$
NAcS(OBzl)E(OBzl)K(Z)POH (VIII)	$C_{43}H_{53}N_5O_{11}$, MW 815	MH^+ 816, MNa^+, MK^+, $MH^+ - P$
NAcS(OBzl)D(OBzl)Orn(Z)POBzl (IX)	$C_{48}H_{55}N_5O_{11}$, MW 877	MH^+ 878, $MH^+ - POBzl$, $MH^+ - Z$
NAcAD(OBzl)K(Z)POBzl (X)	$C_{42}H_{51}N_5O_{10}$, MW 785	MH^+ 786, $MH^+ - POBzl$
NAcS(OBzl)dD(OBzl)K(Z)POBzl (XI)	$C_{49}H_{57}N_5O_{11}$, MW 891	MH^+ 892, $MH^+ - Z$, $MH^+ - POBzl$
NAcS(OBzl)D(OBzl)βK(Z)POH (XII)	$C_{42}H_{51}N_5O_{11}$, MW 801	MH^+, MNa^+, MK^+, $MH^+ - P$, $MH^+ - Z$
BocS(OBzl)D(OBzl)K(Z)POBzl (XIII)	$C_{52}H_{63}N_5O_{12}$, MW 949	MH^+, $MH^+ - 100$, $MH^+ - 100 - POBzl$

[a] Spectrum under electronic-impact conditions.

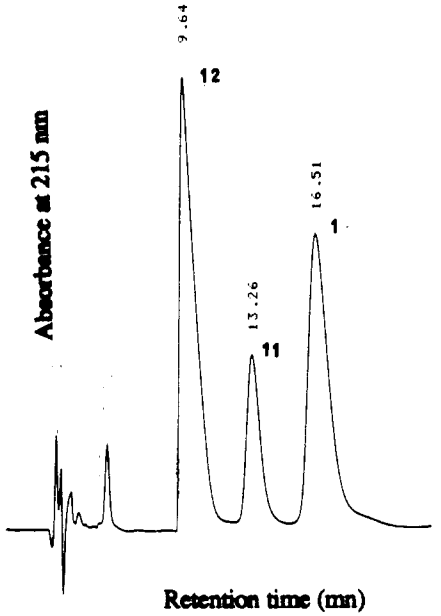

Figure 1. HPLC analysis of isomeric peptides 1 (L), 11 (D), 12 (β) on a C-18 Delta Pak (4 × 250 mm) column: eluent, 4.5% CH_3CN/0.1% TFA/95.4% H_2O. Elution was monitored by measuring the absorbance at 215 nm.

to detect any succinimide derivative.

However, in one instance, hydrogenolysis of the tripeptide S(OBzl)D(OBzl)K(Z)OBzl, an ion in the FAB mass spectrum ($MH^+ + 14$) indicates that a methyl ester was formed as a side product during the hydrogenolysis, presumably via a succinimide intermediate. It is worth noting that the isomeric structures **1**, **11**, **12** have quite different retention times on reverse-phase HPLC as illustrated by Figure 1. β-Isomer **12** was eluted first. This is consistent with the greater acidity constant of the α-carboxylic acid.

Biological evaluation of the peptides are reported in Table III. Deacylation of **1** yields a compound (**13**) with higher activity than the parent sequence. The same observation can be made concerning tripeptides **2** and **3**, the latter being by far the most active derivative (detection of activity at 10^{-14} M). In both cases, deacetylation of the peptide increases the potency of the compound. None of the shorter dipeptide sequences (**5** and **7**) show any inhibitory effect.

The activity of compound **8**, where Asp was replaced by Glu, was reduced (10^{-6} M). None of the isomeric structures (**11** and **12**) showed any inhibitory activity. Compound **9**, where Lys was replaced by Orn, had no activity either. Substitution of Ser by Ala in the parent tetrapeptide led to an inactive product (**10**). This emphasized the role of the Ser residue and was further verified by the lack of inhibitory effect of the tripeptide AspLysPro (**6**) and the weak activity of NAcSerAsp (**4**).

Conclusion

This study shows that the three polar amino acid side chains are essential for the biological activity of the peptides in the biological assay used. Serine seems to be particularly important. It is interesting to note that deacetylation provides compounds with enhanced potency (activity threshold 2 orders of magnitude lower).

More work is under progress to demonstrate any interaction between the peptide backbone and the interaction site on lymphocyte T and to study the conformational influence of the backbone on this bonding. For this study pseudo-peptide analogues are being synthesized.

Experimental Section

Chemistry. All protected amino acids were purchased from Bachem AG or Novabiochem. Melting points were reported uncorrected and taken with a Ernst Leitz Wetzmar apparatus. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Thin-layer chromatography (TLC) was on silica gel precoated plates (60F 254 Merck). The following solvent systems were used: A, dichloromethane/methanol 95/5; B, dichloromethane/methanol 9/1; C, ethyl acetate/hexane 2/1.

UV light, ninhydrin, and (or) Pataki reagent[11] were used for detection. Protected peptides were purified on SDS silica gel 60 (40–60 μm) columns.

All reagents and solvents were of analytical grade. Isopropenylchloroformate was a generous gift from SNPE.

For amino acid analysis, peptides were hydrolyzed with constant-boiling hydrochloric acid for 12 h at 120 °C or with TFA/HCl 6 N for 1 h at 150 °C in evacuated and sealed tubes. The analyses were performed on a Waters HPLC apparatus equipped with a RP-C_{18} 5 μm column and a precolumn derivatization with *o*-phthalaldehyde. GABA was used as an internal standard.[12] The concentration of the analyzed sample was calculated. This allows the preparation of samples of appropriate concentrations for the biological assays.

Mass spectra using fast atom bombardment (FAB) techniques were performed on a MS 50 using glycol, thioglycol, or nitrobenzyl alcohol as a matrix.

HPLC purifications were realized on a reverse phase RP-C_{18} column of either Hypersil 10 μm SFCC (7.8 mm × 30 cm), RP18 Merck 7 μm (0.7 mm × 25.7 cm), or Delta Pak (Waters) 5 μm (7.8 mm × 30 cm) using isocratic elution with a mixture of acetonitrile and water containing 0.1% of trifluoroacetic acid. Elution was monitored by following the absorbance at 215 nm.

Biological Assay. Cells. The Jurkat T lymphoma cell line (a generous gift from Dr. These, Institut Pasteur, Paris, France) was maintained in RPMI 1640 medium supplemented with 2 mM glutamine (Gibco, Cergy Pontoise, France), 20 mM HEPES (Eurobio, Paris, France), 1 mM sodium pyruvate (Eurobio), 100 units/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco), and 10% heat-inactivated fetal calf serum (FCS; Eurobio). Cells

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Table II. Physical Properties of Deprotected Peptides 2–13

	molecular formula	molecular weight	mass spectrum	amino acid composition
NAcSDK (2)	$C_{15}H_{26}N_4O_8$	390	MH^+ 391	S 1.0, D 1.0, K 1.0
SDK (3)	$C_{13}H_{24}N_4O_7$	348	MH^+ 349	S 0.8, D 1.0, K 1.2
NAcSD (4)	$C_9H_{14}N_2O_7$	262	MH^+ 263	S 1.0, D 1.1
SD (5)	$C_7H_{12}N_2O_6$	220	MH^+ 221, MNa^+	S 1.0, D 1.1
DKP (6)	$C_{15}H_{26}N_4O_6$	358	MH^+ 359, MH^+ – P	D 1.0, K 0.8
DK (7)	$C_{10}H_{19}N_3O_5$	261	MH^+ 262, MK^+	D 1.0, K 1.0
NAcSEKP (8)	$C_{21}H_{35}N_5O_9$	501	MH^+, MNa^+, MK^+	S 1.1, E 1, K 1.1
NAcSDOrnP (9)	$C_{19}H_{31}N_5O_9$	473	MH^+ 474, MH^+ – P	S 0.9, D 1.1, Orn 1.0
NAcADKP (10)	$C_{20}H_{33}N_5O_8$	471	MH^+ 472	A 1.0, D 1.0, K 1.1
NAcSDDKP (11)	$C_{20}H_{33}N_5O_9$	487	MH^+ 488, MNa^+	S 1.1, D 1.1, K 1.1
NAcSDβKP (12)	$C_{20}H_{33}N_5O_9$	487	MH^+ 488, MNa^+, MK^+	S 1.2, D 1, K 1.1
SDKP (13)	$C_{18}H_{31}N_5O_8$	445	MH^+ 446, MNa^+	S 1.0, D 0.8, K 1.1

Table III. Activity of Peptides on Rosetting of Jurkat T-Cells with SRBC[a]

peptide concn (M)	number of rosettes/100 Jurkat T-cells												
	NAcSDKP (1)	NAcSDK (2)	SDK (3)	NAcSD (4)	SD (5)	DKP (6)	DK (7)	NAcSEKP (8)	NAcSDOP (9)	NAcADKP (10)	NAcSDDKP (11)	NAcSDβKP (12)	SDKP (13)
10^{-3}	32 ± 4 (48)	ND	ND	ND	ND	ND	ND	ND	ND	ND	48 ± 2 (NS)	ND	ND
10^{-4}	33 ± 3 (48)	35 ± 2 (44)	38 ± 3 (40)	39 ± 2 (30)	46 ± 2 (31)	55 ± 3 (NS)	53 ± 3 (NS)	ND	54 ± 6 (NS)	54 ± 5 (NS)	48 ± 4 (NS)	80 ± 4 (NS)	47 ± 2 (25)
10^{-5}	32 ± 3 (48)	37 ± 6 (40)	45 ± 4 (28)	39 ± 4 (30)	55 ± 3.6 (NS)	57 ± 1 (NS)	56 ± 2 (NS)	29 ± 1 (37)	52 ± 2 (NS)	57 ± 4 (NS)	49 ± 2 (NS)	78 ± 4 (NS)	49 ± 2 (22)
10^{-6}	37 ± 2 (40)	38 ± 1 (39)	50 ± 2 (22)	46 ± 3 (18)	55 ± 5.7 (NS)	ND	ND	35 ± 4 (25)	52 ± 3 (NS)	59 ± 4 (NS)	42 ± 2 (NS)	76 ± 11 (NS)	50 ± 2 (20)
10^{-7}	40 ± 5 (35)	45 ± 4 (27)	49 ± 2 (22)	52 ± 0.6 (NS)	58 ± 1.5 (NS)	ND	ND	41 ± 4 (NS)	ND	59 ± 3 (NS)	47 ± 2 (NS)	83 ± 3 (NS)	54 ± 3 (14)
10^{-8}	41 ± 2 (34)	48 ± 4 (23)	40 ± 2 (36)	54 ± 0.6 (NS)	55 ± 6.5 (NS)	ND	58 ± 1 (NS)	42 ± 3 (NS)	ND	63 ± 1 (NS)	47 ± 4 (NS)	83 ± 3 (NS)	56 ± 4 (11)
10^{-9}	48 ± 2 (21)	51 ± 4 (18)	43 ± 1 (32)	51 ± 2 (NS)	64 ± 1 (NS)	ND	ND	43 ± 0 (NS)	ND	64 ± 6 (NS)	51 ± 5 (NS)	ND	ND
10^{-10}	63 ± 2 (NS)	56 ± 3 (10)	43 ± 3 (32)	57 ± 1.5 (NS)	ND	ND	ND	46 ± 4 (NS)	ND	56 ± 7 (NS)	43 ± 3 (NS)	83 ± 8 (NS)	55 ± 1 (13)
10^{-11}	58 ± 3 (NS)	62 ± 5 (NS)	40 ± 1 (36)	58 ± 1.4 (NS)	ND	ND	ND	45 ± 0 (NS)	ND	56 ± 2 (NS)	57 ± 1 (NS)	ND	55 ± 3 (13)
10^{-12}	ND	59 ± 5 (NS)	44 ± 3 (30)	ND	ND	ND	ND	ND	ND	ND	ND	ND	59 ± 4 (NS)
10^{-13}	ND	ND	51 ± 7 (19)	ND	ND	ND	ND	ND	ND	ND	ND	ND	58 ± 4 (NS)
10^{-14}	ND	ND	55 ± 2 (14)	ND	ND	ND	ND	ND	ND	ND	ND	ND	61 ± 4 (NS)
10^{-15}	ND	ND	63 ± 4 (NS)	ND	ND	ND	ND	ND	ND	ND	ND	ND	60 ± 4 (NS)
0	62 ± 5	63 ± 5	63 ± 4	56 ± 3	51 ± 5	54 ± 0.6	56 ± 3	47 ± 2	58 ± 3	60 ± 5	51 ± 5	82 ± 2	63 ± 4

[a] T lymphocytes (2.10^5 cells) were incubated for 10 min at 4 °C with peptide solution at appropriate concentration. After addition of SRBC (2.10^7 cells) rosetting assays were carried out as described in the Experimental Section. Results are the mean value of three experiments. Each experiment is carried out in quadruplicate. Percent inhibition of rosette formation is in parentheses. ND = not determined. NS = nonsignificant.

were washed three times in RPMI 1640 containing 10% FCS before being used in the rosetting assay. Sheep erythrocytes (SRBC; Institut Pasteur, Paris, France) were washed three times by centrifugation in the same medium).

Rosetting Assay. One hundred microliters of Jurkat cell suspensions (2×10^6 cells/mL) in RPMI/20% FCS was incubated with 200 μL of a solution of the peptide in RPMI for 10 min at 4 °C. Untreated SRBC suspensions (100 μL, 2×10^8 cells/mL) were then added; the samples were centrifuged for 5 min at 200g and incubated at 4 °C overnight. Tubes were gently rocked to resuspend the pellet and rosettes (referred as three erythrocytes bound per lymphocyte) were counted "blind" in triplicate in a hemocytometer. Four determinations of the rosette ratio were carried out for each assay and each assay was done in triplicate (400 T-cells were counted per assay).

Chemistry—Typical Procedures. Boc Acidolysis. The protected peptide was dissolved in a 1/1 mixture of dichloromethane trifluoroacetic acid (10–30 equiv). After stirring for 1–2 h at room temperature, the solvents were evacuated under vacuum after dilution with dichloromethane. The trifluoroacetate salts so obtained were generally soluble in ether. They were thoroughly dried under vacuum over KOH before being used in the acylation step.

Hydrogenolysis. The protected peptide was dissolved in methanol/water 9/1. To this solution was added (about 25% w/w) 10% Pd/C. The reaction was stirred under hydrogen at atmospheric pressure and room temperature for 6–24 h. The catalyst was removed by filtration through a Celite pad which was washed with methanol.

Coupling Reactions. Mixed Anhydride Method. The N-protected amino acid was dissolved in tetrahydrofuran (5 mL/mM) and the solution was cooled to −15 °C. To this solution was added *N*-methylmorpholine (1.2 equiv) and isobutyl chloroformate (1 equiv). After 5 min, the temperature was lowered to −20 °C and the cooled solution of the trifluoroacetate salt along with 1.1 equiv of *N*-methylmorpholine in dimethylformamide was added. The temperature was kept below −10 °C for 1 h and then allowed to reach room temperature. After completion of the reaction (followed by TLC) the reaction mixture was evaporated down. The residue was taken up with ethyl acetate and 5% citric acid. The organic layer was washed with water, 5% sodium bicarbonate, water, and saturated sodium chloride solution and then dried over sodium sulfate. Evaporation of the solvent yielded the crude product which was either purified on silica gel column or crystallized.

Succinimide Ester Method. The salt of the peptide and the *N*-hydroxysuccinimide ester of the N-protected amino acid were dissolved in dimethylformamide. The solution was chilled with an ice bath and 1 equiv of triethylamine was added. After 1 h of stirring, the cooling bath was removed and stirring was maintained for several hours.

The reaction mixture was concentrated in vacuo. The residue was taken up with ethyl acetate and 0.1 N HCl. The organic layer was washed successively with water and saturated sodium chloride solution and dried over sodium sulfate. Evaporation under vacuum yielded a crude product which was purified on a silica gel column.

Acetylation Reaction. The salt of the peptide was dissolved in dimethylformamide (2 mL/mM). Acetylimidazole (1.1 equiv) followed by triethylamine (1.1 equiv) was added on an ice bath. The reaction mixture was stirred at room temperature. The reaction evolution was monitored by TLC. The reaction mixture was evaporated down and the residue was taken up with 0.5 N HCl and ethyl acetate. The organic layer was washed with water until the pH was neutral and with saturated sodium chloride solution and dried over sodium sulfate. Evaporation under vacuum yielded a crude product which was purified on a silica gel column or by crystallization.

Synthesis of NAcSerAspLys (2). BocD(OBzl)K(Z)OBzl was prepared according to mixed anhydride methodology: crystallization from ethyl acetate/hexane 1/1; yield 83%; mp 101–102 °C; $[\alpha]_D$ −15° (*c* 1.1, methanol); R_f (A) 0.62, R_f (C) 0.50. Anal. ($C_{37}H_{45}N_3O_9$) C, H, N, O.

After deprotection with trifluoroacetic acid the dipeptide salt was condensed with BocS(OBzl) according to the mixed anhydride methodology. Trituration with ether yielded 81% of an amorphous solid: mp 79–80 °C; R_f (A) 0.50, R_f (C) 0.62. Anal. ($C_{47}H_{56}N_4O_{11}$) C, H, N, O.

After deprotection with trifluoroacetic acid, the trifluoroacetate salt of tripeptide III was acetylated according to the procedure described above: crystallization from AcOEt/hexane; yield 61%; mp 135–137 °C; $[\alpha]_D$ −13° (*c* 1.1 methanol); R_f (A) 0.39, R_f (C) 0.14. Anal. ($C_{44}H_{50}N_4O_{10}$) C, H, N, O.

Hydrogenolysis of NAcS(OBzl)D(OBzl)K(Z)OBzl (II): reaction time 16 h; HPLC purification on a Hypersil C_{18} (24 °C) column; eluent, H_2O/0.1% TFA; flow rate, 4 mL/min; $k' = 3.13$.

Synthesis of SerAspLys (3). Tripeptide III was treated with trifluoacetic acid in dichloromethane for 100 min. Hydrogenolysis of the dry trifluoroacetate salt yielded a crude product purified by HPLC on a Hypersil C_{18} (24 °C) column: eluent, H_2O/0.1% TFA; flow rate, 2 mL/min; $k' = 0.69$.

Synthesis of NAcSerAsp (4). BocS(OBzl)D(OBzl) was prepared from BocS(OBzl)OSu and D(OBzl) according to the method of Wang et al.[7] Deprotection with a solution of 4 N HCl/THF as described by Wang and acetylation according to the procedure described above yielded IV as a yellowish oil which solidified on standing. The solid was triturated with ether: yield 62%; mp 129–131 °C (methanol/ether); $[\alpha]_D$ +13° (*c* 1.2 methanol). Anal. ($C_{23}H_{26}N_2O_7$) C, H, N, O.

Hydrogenolysis of IV provided NAcSD (4), which was purified by HPLC on a Delta Pak (24 °C) column: eluent, H_2O/0.1% TFA; flow rate, 0.2 mL/min; $k' = 1.45$.

Synthesis of SerAsp (5). BocS(OBzl) was coupled with the *p*-toluenesulfonic salt of D(OBzl)OBzl according to the mixed anhydride methodology. Purification was carried out on preparative silica gel plates: yield 93%; mp 53–57 °C (ethyl acetate/petroleum ether); $[\alpha_D]$ −2° (*c* 1.2 methanol). Anal. ($C_{33}H_{38}N_2O_8$) C, H, N, O.

After usual deprotection with trifluoroacetic acid (2 h), hydrogenolysis (22 h) provided SerAsp (5), purified by HPLC on Delta Pak and Nucleosil columns: eluent, H_2O/0.1% TFA; flow rate 0.4 mL/min; $k' = 0.29$.

Synthesis of AspLysPro (6). The dipeptide BocLys(Z)-ProOBzl was prepared from BocLys(Z) and the hydrochloride salt of ProOBzl by using mixed anhydride methodology. The crude product was purified by chromatography on a silica gel column. Elution with dichloromethane/methanol (99/1) gave pure BocLys(Z)ProOBzl: yield 76%; R_f (A) 0.65.

The trifluoroacetate salt was obtained by treatment of BocLys(Z)ProOBzl with trifluoroacetic acid. The dry salt was coupled with BocAsp(OBzl) by using the mixed anhydride methodology. The crude product was purified on a silica gel column. Elution with dichloromethane/methanol (98/2) yielded compound VI homogeneous on TLC: R_f (A) 0.56.

Treatment of compound VI with trifluoroacetic acid was followed by hydrogenolysis (16 h). Purification was by HPLC on an RP-18 Merck column: eluent, 3% CH_3CN 97% H_2O/0.1% TFA; flow rate, 2 mL/min; $k' = 1.4$.

Synthesis of AspLys (7). The *N*-hydroxysuccinimide ester of BocAspOBzl (1 mM) was reacted with Lys(Z) (1.1 equiv) in dimethylformamide. Triethylamine (1.1 equiv) was added on an ice/water bath. After 30 h of stirring at room temperature, ethyl acetate and 0.5 N HCl were added to the reaction mixture. The organic layer was washed with water to neutrality and with brine. The organic layer was dried over sodium sulfate and evaporated. The crude product was purified by chromatography on a silica gel column. Elution was with dichloromethane/methanol 95/5. Homogeneous fractions were pooled and evaporated down. Hydrogenolysis (22 h) then acidolysis with trifluoroacetic acid gave 7 as a white solid after trituration with dry ether. Purification was by HPLC on a Hypersil column: eluent, 1% CH_3CN/99% H_2O/0.1% TFA; flow rate, 1.5 mL/min; $k' = 0.96$.

Synthesis of NAcSerGluLysPro (8). The dipeptide BocLys(Z)Pro was prepared from the *N*-hydroxysuccinimide ester of BocLys(Z) and proline. Two equivalents of proline in water (2 mL) were added to a solution of BocK(Z)OSu (2 mM) in 4 mL of dimethylformamide. Triethylamine (2 equiv) was added. After stirring for 6 h at room temperature, the reaction mixture was concentrated under vacuum. Ethyl acetate and 5% citric acid were added. The aqueous layer was extracted twice with ethyl acetate. The organic layers were pooled, washed with brine, and then dried over sodium sulfate. Evaporation gave an oil homo-

geneous by TLC which was used without purification in the next step.

BocLys(Z)Pro was deprotected with trifluoroacetic acid. The dry trifluoroacetate salt was reacted with BocGlu(OBzl) according to the mixed anhydride methodology. The crude product was purified through two successive chromatographies on silica gel columns using successively dichloromethane/methanol 95/5 and ethyl acetate/methanol 9/1: yield 44%; R_f (B) 0.22.

The trifluoroacetate salt of the tripeptide BocGlu(OBzl)Lys(OBzl)Pro obtained by treatment with trifluoroacetic acid was reacted with the *N*-hydroxysuccinimide ester of BocSer(OBzl). Purification of the crude product yielded BocS(OBzl)Glu(OBzl)Lys(OBzl)Pro with a 78% yield: R_f (B) 0.21.

The trifluoroacetate salt of the tetrapeptide obtained by treatment with trifluoroacetic acid was reacted with acetylimidazole. Compound VIII was purified on a silica gel column: yield 67%; R_f (B) 0.12.

Hydrogenolysis of compound VIII yielded NAcSerGluLysPro (**8**). HPLC, was done on a Delta Pak (3.9 × 150 mm) column: eluent, 4.5% acetonitrile/96.5% H_2O/0.1% TFA; flow rate, 1 mL/min; k' = 3.7.

Synthesis of NAcSerAspOrnPro (9). BocOrn(Z) was prepared according to ref 8 from Orn(Z). Coupling of BocOrn(Z) with the hydrochloride salt of ProOBzl was made according to the mixed anhydride methodology. The compound was purified on a silica gel column using ether/hexane (2/1) as eluent: yield 76%. R_f (A) 0.65, R_f (C) 0.33.

After usual deprotection of BocOrn(Z)ProOBzl with trifluoroacetic acid, the trifluoroacetate salt of the dipeptide was reacted with the *N*-hydroxysuccinimide of BocAsp(OBzl). The tripeptide was purified by chromatography on a silica gel column. The white foam so obtained was very hygroscopic: yield 79%; R_f (A) 0.5.

After usual deprotection of BocAsp(OBzl)Orn(Z)ProOBzl with trifluoroacetic acid, the trifluoroacetate salt was reacted with the *N*-hydroxysuccinimide ester of BocSer(OBzl). The tetrapeptide was purified on a silica gel column using dichloromethane/methanol (98/2) as eluent: yield 90%; R_f (A) 0.5; MS (FAB) *m/e* Calcd 936, Obsd MH^+ 937; MH^+ - Boc, MH^+ - Z, MH^+ - Boc - ProOBzl.

After usual deprotection of BocSer(OBzl)Asp(OBzl)Orn(Z)-ProOBzl with trifluoroacetic acid, the trifluoroacetate salt was reacted with acetylimidazole. The crude tetrapeptide was purified on a silica gel column using dichloromethane/methanol (98/2) as eluent: yield 79%; R_f (A) 0.34.

Hydrogenolysis of tetrapeptide IX yielded NAcSerAspOrnPro (**9**). Purification was by HPLC on a Delta Pak column: eluent, 0.01 M ammonium acetate (pH 3.8); flow rate, 0.8 mL/min followed by desalting.

Synthesis of NAcAlaAspLysPro (10). The tripeptide BocAsp(OBzl)Lys(K)ProOBzl was prepared as described for the synthesis of **6**. Usual deprotection with trifluoroacetic acid yielded the trifluoroacetate salt of the tripeptide, which was reacted with BocAla according to the mixed anhydride method. The crude tetrapeptide was purified by silica gel chromatography using dichloromethane/methanol (98/2) as eluent: yield 76%; R_f (A) 0.3, R_f (C) 0.07.

After usual deprotection of BocAlaAsp(OBzl)Lys(Z)ProOBzl with trifluoroacetic acid, the trifluoroacetate salt was reacted with acetylimidazole (1.6 equiv). The crude product was purified on a silica gel column using dichloromethane/methanol (95/5) as eluent: yield 78%; R_f (A) 0.25.

Hydrogenolysis of tetrapeptide X [NAcAlaAsp(OBzl)Lys(Z)-ProOBzl] yielded NAcAlaAspLysPro (**10**). Purification was by HPLC on a Hypersil (24 °C) column: eluent, H_2O/TFA 0.1%; flow rate, 4 mL/min; k' = 5.3.

Synthesis of NAcSerDAspLysPro (11). The dipeptide BocLys(Z)ProOBzl was prepared as described for the synthesis of **6**. Usual deprotection with trifluoroacetic acid gave the trifluoroacetate salt of the dipeptide, which was reacted with BocDAspOBzl according to the mixed anhydride methodology. The crude tripeptide was purified by column chromatography using dichloromethane/methanol (99/1): yield 70%; R_f (A) 0.61, R_f (C) 0.28.

Usual deprotection with trifluoroacetic acid of the tripeptide BocDAsp(OBzl)Lys(Z)ProOBzl yielded the trifluoroacetate salt, which was reacted with BocSer(OBzl) according to the mixed anhydride method. The tetrapeptide, homogeneous on TLC with different solvent systems, was used as such in the next step: yield 100%; R_f (A) 0.46, R_f (C) 0.13.

Usual deprotection with trifluoroacetic acid of the tetrapeptide gave the trifluoroacetate salt, which was reacted with acetylimidazole (1.2 equiv). The crude product was purified on a silica gel column using dichloromethane/methanol 98/2 as eluent: yield 84%; R_f (A) 0.25; mp 135–136 ° (ethyl acetate/hexane); $[\alpha]_D$ −27° (*c* 1, methanol).

Hydrogenolysis of tetrapeptide XI NAcSer(OBzl)DAsp(OBzl)Lys(Z)ProOBzl gave NAcSerDAspLysPro. HPLC was done on a Delta Pak column: eluent, 4.5% CH_3CN/95.5% H_2O/0.1% TFA; flow rate, 1 mL/min; k' = 3.7.

Synthesis of NAcSerAspβLysPro (12). The dipeptide BocLys(Z)Pro was prepared as described for the synthesis of **8**. Usual deprotection with trifluoroacetic acid of the dipeptide gave the trifluoroacetate salt, which was reacted with BocAspOBzl. Crude product homogeneous by TLC was used in the next step without purification: yield 90%; R_f (B) 0.18.

Tripeptide BocAspOBzlβLys(Z)ProOBzl (0.4 mM) was deprotected with 3.1 N HCl in THF (3.5 mL). After stirring for 7 h at room temperature, the solvent was evaporated down and the residue was dried overnight over KOH under vacuum. The hydrochloride salt was reacted with the *N*-hydroxysuccinimide ester of BocSer(OBzl). The crude product was purified by chromatography on a silica gel column using dichloromethane/methanol (successively 95/5, 9/1, 8/2): yield 57%.

Usual deprotection with trifluoroacetic acid of the tetrapeptide yielded the trifluoroacetate salt, which was reacted with acetylimidazole. Purification of the crude product on a silica gel column gave XII: yield 54%. Hydrogenolysis of XII yielded NAcSerAspβLysPro (**12**). HPLC was done on a Delta Pak column: eluent, 4.5% CH_3CN/95.5% H_2O/0.1% TFA; flow rate, 1 mL/min; k' = 2.3.

Synthesis of SerAspLysPro (13). The dipeptide BocLys(Z)ProOBzl was prepared according to REMA conditions with isopropenyl chloroformate and diisopropylethylamine as a tertiary base as recommended by the authors[10] for proline coupling. The reaction was run at room temperature except for the activation time (5 min at 0 °C). The dipeptide was purified by column chromatography on silica gel using dichloromethane/methanol (99/1) as eluent: yield 89%; R_f (A) 0.65.

The dipeptide BocLys(Z)ProOBzl (1 mmol) was deprotected as usual with trifluoroacetic acid. After drying, the trifluoroacetate salt in solution in dimethylformamide (2 mL) was reacted with BocAsp(OBzl) (1.5 equiv) activated at 0 °C with isopropenyl chloroformate (1.4 equiv) in the presence of *N*-methylmorpholine (1.5 equiv). After 30 min at 0 °C and 2 h at room temperature, the reaction mixture was worked up as described by Jaoudi et al.[10] The product was purified by column chromatography eluting with ethyl acetate/hexane (2/1): yield 84%, R_f (A) 0.52, R_f (C) 0.23.

The tripeptide BocAsp(OBzl)Lys(Z)ProOBzl (0.5 mM) was deprotected with trifluoroacetic acid as usual. The dry trifluoroacetate salt in solution in dimethylformamide was reacted with BocSer(OBzl) using REMA conditions as above. The workup of the reaction mixture gave 0.430 g of product homogeneous by TLC with three different solvent systems (methylene chloride/methanol 99/1 and 95/5 and ethyl acetate/hexane 2/1). The FAB spectrum of crude XIII showed neither a urethane nor a succinimide derivative: R_f (A) 0.50, R_f (C) 0.18. The tetrapeptide XIII was deprotected first with trifluoroacetic acid (2 h) and then by hydrogenolysis (21 h) according to the general procedure described above.

Free peptide **13** was purified by HPLC on a Hypersil C_{18} (24 °C) column: eluent, 4.5% acetonitrile/95.5% H_2O/0.1% TFA; flow rate, 4 mL/min; k' = 6.25.

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Registry No. **2**, 127103-04-2; **3**, 127103-05-3; **4**, 127103-06-4; **5**, 2543-31-9; **6**, 127103-07-5; **7**, 5891-51-0; **8**, 127103-08-6; **9**, 127103-09-7; **10**, 127103-10-0; **11**, 127103-11-1; **12**, 127129-85-5; **13**, 105464-22-0; II, 127103-12-2; III, 127103-13-3; IV, 127103-14-4;

V, 127103-15-5; VI, 127103-16-6; VII, 63495-82-9; VIII, 127103-17-7; IX, 127103-18-8; X, 127103-19-9; XI, 127129-86-6; XII, 127129-87-7; XIII, 127129-88-8; BocD(OBzl), 7536-58-5; K(Z)OBzl, 24458-14-8; BocD(OBzl)K(Z)OBzl, 33838-60-7; BocS(OBzl), 23680-31-1; BocS(OBzl)OSu, 13650-73-2; D(OBzl), 2177-63-1; BocS(OBzl)D(OBzl), 127103-20-2; D(OBzl)OBzl·TsOH, 2886-33-1; Bock(Z), 2389-45-9; P-OBzl·HCl, 16652-71-4; Bock(Z)P-OBzl, 68280-74-0; BocD(OBzl)OSu, 13798-75-9; K(Z), 1155-64-2; Bock(Z)OSu, 34404-36-9; P, 147-85-3; BocE(OBzl), 13574-13-5; BocE(OBzl)K(Z)P, 127103-21-3; BocS(OBzl)E(OBzl)K(Z)P, 127103-22-4; BocOrn(Z), 2480-93-5; BocOrn(Z)P-OBzl, 127103-23-5; BocD(OBzl)Orn(Z)P-OBzl, 127103-24-6; BocS(OBzl)D-(OBzl)Orn(Z)P-OBzl, 127129-89-9; BocA, 15761-38-3; BocAD-(OBzl)K(Z)P-OBzl, 127103-25-7; Boc-D-D(OBzl), 51186-58-4; Boc-D-D(OBzl)K(Z)P-OBzl, 127103-26-8; BocD-OBzl, 30925-18-9; BocDOBzlβK(Z)P-OBzl, 127103-27-9; BocS(OBzl)DOBzlβK(Z)-P-OBzl, 127103-28-0; BocS(OBzl)-D-D(OBzl)K(Z)P-OBzl, 127103-29-1.

Synthesis and Broad-Spectrum Antiviral Activity of 7,8-Dihydro-7-methyl-8-thioxoguanosine

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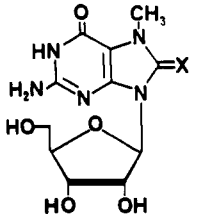
2,6,8-Trichloro-7-methylpurine (3) was converted to 2-chloro-8,9-dihydro-7-methyl-8-thioxopurin-6(1*H*)-one (5) by utilizing the difference in reactivity of the 2-, 6-, and 8-positions in the trichloropurine ring system to nucleophilic displacement. Compound 5 was subsequently glycosylated with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose according to the Vorbrüggen procedure to yield 2-chloro-8,9-dihydro-7-methyl-9-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-8-thioxopurin-6(1*H*)-one (6). Removal of the benzoyl protecting groups, followed by amination of 7 with liquid ammonia at 150 °C, gave 7,8-dihydro-7-methyl-8-thioxoguanosine (2). The structure of compound 2 was confirmed by X-ray crystallographic analysis. Compounds 1 (7,8-dihydro-7-methyl-8-oxoguanosine) and 2 were evaluated for activity in various animal virus infection models. Against banzi, Semliki Forest, and San Angelo viruses in mice, 2 was highly active when administered before virus inoculation.

Introduction

Purine nucleosides are of considerable interest to the medicinal chemist as potential chemotherapeutic agents. Reviews on the antitumor[1,2] and antiviral[3] activity of purine nucleosides have appeared in the literature. Nucleoside derivatives such as 8-bromoguanosine,[4] 8-mercaptoguanosine,[4] and 7,8-dihydro-7-methyl-8-oxoguanosine (1)[5] have been shown to be stimulators of the humoral (B-cell) immune system.[6-9] Robins and coworkers[5] reported the synthesis of 7,8-dihydro-7-methyl-8-oxoguanosine in 1969. Subsequently, we reported a new and facile synthesis of this compound.[10] We have now found that compound 1 exhibits antiviral activity as well.[11] A report from our laboratory on the synthesis[11] and broad-spectrum antiviral activity[12] of another guanosine analogue, 7,8-dihydro-7-thia-8-oxoguanosine, has recently been published. As part of our program for the synthesis of purine nucleoside derivatives as potential antiviral and antitumor agents, the synthesis of 7,8-dihydro-7-methyl-8-thioxoguanosine (2) was pursued and accomplished. This synthesis and the in vivo antiviral activity of the purine nucleoside derivatives 1 and 2 are the subject of this paper.

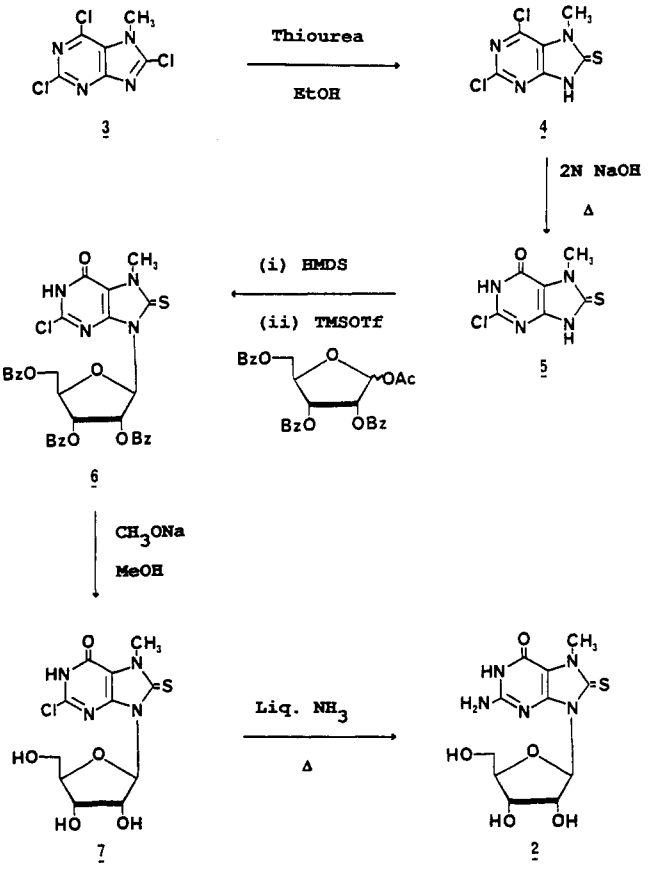


1: X = O
2: X = S

Chemistry

2,6,8-Trichloro-7-methylpurine (3), synthesized by a reported[13] procedure with slight modifications,[14] was the starting compound. The difference in reactivity of the 2-,

Scheme I



3 4 5 6 7 2

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