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A PIONEER SYNTHESIS OF CONJUGATES OF A THYMIC PEPTIDE HORMONE AND POLYAMINES

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To synthesize peptidic derivatives of polyamines, sequential reactions were designed for the polyfunctional peptides and polyfunctional amines which utilize specific protection and deprotection. Utilizing these reactions, a thymic nonapeptide, a lymphocyte-differentiating hormone, was coupled with spermidine and spermine to yield, respectively: Glp-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-NH$(CH_2)_3$NH$(CH_2)_4NH_2$; Glp-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-NH$(CH_2)_3$NH-$(CH_2)_4$NH$(CH_2)_3NH_2$. Both conjugates inhibited rather than stimulated incorporation of [^{3}H]-thymidine into DNA at levels 20-fold that of spermine. This activity is less than that based on the percent of the spermine moeity (ca. 20%) in the conjugate.

Research on polyamines has increased, because of implications of their functions in mechanisms of growth. Aigner-Held and Daves (1) reviewed polyamine metabolites and particularly peptidic conjugates. Tabor and Tabor (2) described the isolation from E. coli of a peptidic derivative of spermidine which was characterized as glutathionylspermidine, γ-Gln-Cys-Gly-NH$(CH_2)_3$NH-$(CH_2)_4NH_2$, and which they considered could have a role in growth process. Folkers et al. (3) isolated glutathione might be functional in immune systems. Chan et al. (4) found that spermidine in amniotic fluid is apparently a peptidic conjugate; M.W. 10,000-30,000. They considered polyamine conjugates could function in regulation of embryonic development. Seale et al. (5) prepared highly purified polyamine conjugates from human plasma. Putrescine and spermidine were associated, and a pure peptide of 32 amino acids with a combined molecular weight of 4180 was obtained. Rennert et al. (6) identified six polyamine-conjugated proteins in human amniotic fluid. Three contained spermine, one contained spermidine, and two contained putrescine. They projected specificity for such conjugates in cellular processes. Schindler et al. (7) studied a role of polyamines in embryonal carcinoma cell differentiation. Lutz (8) obtained a uremic peptide containing polyamine and studied its formation and possible role in uremic hypertriglyceridemia.

The synthesis of peptidic conjugates requires the design of sequential reactions of the poly-functional peptides and poly-functional amines which utilize specific protection and deprotection of intermediates to allow the

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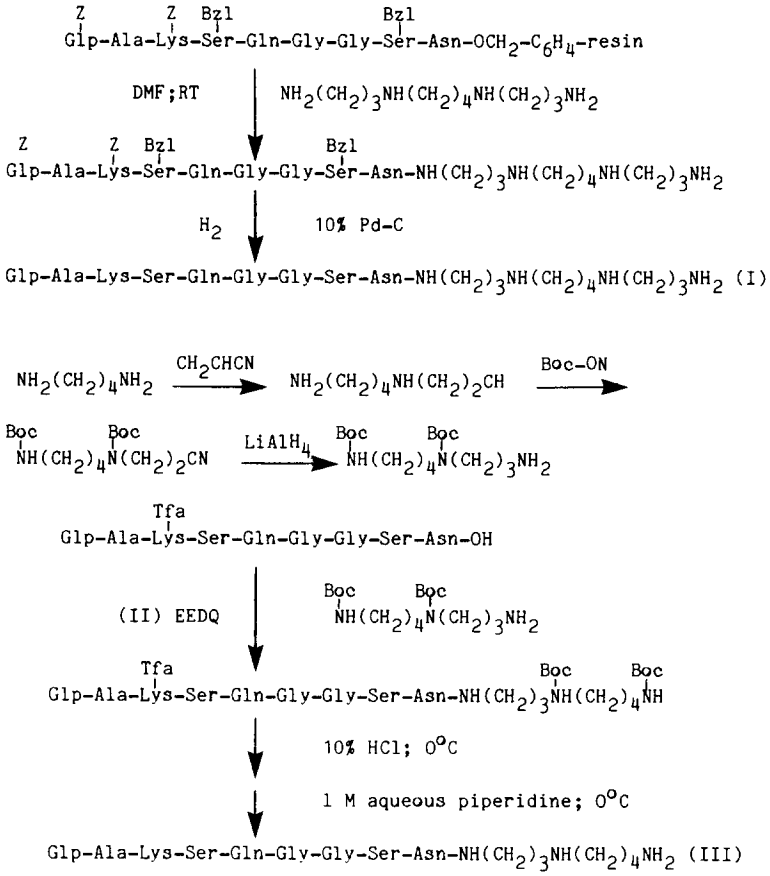

Chart I: Synthesis of Peptidic Derivatives of Spermine and Spermidine

acquisition of peptidic derivatives of the polyamines, spermine and spermidine. We have synthesized such conjugates by reactions which may have general application in this field. These reactions and products are depicted in Chart I.

Materials and Methods - Amino acid derivatives were purchased from Peninsula Laboratories, Belmont, California, and Vega Biochemicals, Tucson, Arizona. The amino acid analyses were conducted with a Beckman Amino Acid Analyzer, Model 119. The sample was hydrolyzed in 6N HCl overnight in evacuated sealed tubes at 110°c. The peptide was synthesized on the hydroxylmethyl resin by a solid-phase methodology and a Beckman Peptide Synthesizer Model 990. The α-amino groups were protected by the t-butoxycarbonyl group (Boc), with the exception of pyroglutamic acid, which was protected by carbobenzoxy (Z). The side-chain protecting groups were trifluoroacetyl for Lys in the peptidic derivative of spermine, and carbobenzoxy for the peptidic derivative of spermidine, and benzyl for Ser. A side-chain protecting group was not used for glutamine and asparagine. The coupling reactions were by dicyclohexylcarbodiimide, and generally with a threefold excess of the Boc-amino acid derivatives in CH_2Cl_2 or DMF. N-Hydroxylbenzatriazole was added to minimize side reactions (9).

Synthesis of the Facteur Thymique Serique-Resin (FTS-Resin;Z-Glp-Ala-Lys(Z)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-OCH_2-C_6H_4-resin) - Boc-Asn-OH was coupled to the hydroxymethyl resin as described by Mitchell et al. (10). A solution of Boc-Asn (696 mg; 3 mM) and 1,1'-carbonyldiimidazole (487 mg, 3 mM) in a mixture

of DMF and CH_2Cl_2 (10 ml) was kept at -5°C for 30 min., and was then added to a reaction vessel containing the hydroxymethyl resin (3 g, 1.5 mM); the mixture was stirred at room temperature for 20 hr. The resin was collected on a filter, washed with CH_2Cl_2, DMF, CH_2Cl_2, EtOH, and CH_2Cl_2, and dried, *in vacuo*, overnight. The coupled resin was acetylated with a mixture of pyridine and acetic anhydride [20 ml, 1:1 (v/v)] for 30 min., and collected by filtration, washed with DMF, CH_2Cl_2, i-PrOH, CH_2Cl_2, and dried, *in vacuo*, to give the Boc-Asn-resin (3.2 g, 1.4 mM). Then, there were eight successive cycles of deprotection and coupling to give Z-Glp-Ala-Lys(Z)-Ser(Bzl)-Gln-Gly-Gly-Ser(Bzl)-Asn-OCH_2-C_6H_4-resin.

The Synthesis of FTS-Spermine(Glp-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-$NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) (I) - The protected nonapeptide--resin (1 g, 0.5 mM) was treated with spermine (202 mg, 1 mM) in DMF under nitrogen at room temperature. The reaction was followed by silica gel TLC using a solvent system of n-butanol: pyridine:HOAc: H_2O [5:5:1:4 (v/v)]. TLC showed a major spot (R_f:0.47), after stirring 20 hrs. A longer reaction time did not increase the intensity of this major spot. The mixture was filtered. The resin was washed with DMF, 20% HOAc, and the combined elute was lyophilized to give the crude protected peptidic spermine (174 mg, 0.16 mM) in 32% yield.

The side-chain protecting groups were removed by hydrogenolysis using 45mg of 10% palladium on charcoal in 20 ml DMF. When hydrogen uptake ceased, the calatyst was removed by filtration. The filtrate was diluted with water and lyophilized to give the crude product (128 mg 0.15 mM) in 90% yield. The lyophilizate was purified over a Sephadex G-25 column (2.5 x 110 cm). Elution was with 20% HOAc. The major fractions from this gel filtration were combined and purified over semi-preparative ODS-silica cartridges (Radial-Pak, C18, 8 mm x 10 cm, Waters Associates). Elution was linear with aqueous 10-50% of acetonitrile in 0.1 M KH_2PO_4 in 35 min.; absorbance at 210 nm; flow rate, 2.0 ml/min; retention time, 20.5 min..

The yield of the pure FTS-spermine (85 mg, 0.1 mM) was about 20% from the protected nonpeptide resin. This product was homogeneous on TLC in three solvent systems. The data are: R_f^1=0.47; R_f^2=0.64; R_f^3=0.23. The systems were: (v/v), R_f^1, n-BuOH:Py:HOAc:H_2O (5:5:1:4); R_f2, EtOAc:Py:HOAc:H_2O (5:5:1:3); R_f^3,n-BuOH:HOAc:H_2O (1:1:1).

The amino acid analyses gave the following ratios: Asp, 1.08; Ser, 2x0.92; Glu, 2 x 0.84; Gly, 2 x 1.12; Ala, 0.94; Lys, 1.11.

The presence of spermine was confirmed by hydrolysis of ca. 1 mg of product in an evacuated sealed tube in 6N HCl at 110°C, overnight. The hydrolysate was lyophilized and was chromatographed on a cellulose plate, 5 x 20 cm; n-BuOH: Py: HOAc:H_2O = 5:5:1:4 (v/v). The R_f was 0.11 for the "unknown" and authentic spermine. The R_f of the tetradansyl derivatives of spermine after hydrolysis was 0.47 on silica gel plate, (E. Merck); chloroform:2-propanol; 25:1; v/v, which is consistent with the authentic tetradansyl derivative of spermine (Pierce).

Synthesis of the Tfa-Lys^3-FTS (Glp-Ala-Lys(Tfa)-Ser-Gln-Gly-Gly-Ser-Asn-OH) - The synthesis of the protected peptide-resin was conducted as described by Folkers and Wan (11). The protected nonapeptide-resin (2.7 g, 1.3 mM) was treated with anhydrous HF in the presence of *ca.* 10% anisole for 1 hr. at 0°C. The excess HF was quickly removed at reduced pressure. The dried reaction mixture was washed with Et_2O and EtOAc, and then extracted three times with 20% HOAc. The combined extract was lyophilized to give 900 mg of the crude product; yield, 90%. The lyophlized product was purified over G-25 gel-filtration column (2.5 x 110 cm) by elution with 20% HOAc. Amino acid analyses gave the following ratio: Asp, 1.01; Ser, 2 x 0.88; Glu, 2 x 1.0; Gly, 2 x 1.0; Ala, 1.02; Lys, 1.07.

Synthesis of N^2,N^3-Di-tert-butoxycarbonyl Spermidine - This compound was prepared as described by Quick *et al* (12). The 1,4-diaminobutane (17.6 g, 0.2 M) was allowed to react with acrylonitrile (10.6 g, 0.2 M) to afford N-(2-cyanoethyl)-1,4-diaminobutane (22.5 g, 0.16 M) in 80% yield [H^1-NMR: 1.50

(m, 4H); 2.60 (m, 6H); 2.92, (t, 2H)]. The H^1-NMR data are consistent with the reported data. The BOC protecting group was introduced by treatment of N-(2-cyanoethyl)-1,4-diaminobutane (5.03 g, 0.035 M) with 2-(tert-butoxy-carbonyloxyimino)-2-phenylacetonitrile (17.8 g, 0.07 M) (Boc-ON) to give N,N-di-tert-butoxycarbonyl-N(2-cyanoethyl)-1,4-diaminobutane (7.67 g, 0.022 M) in 65% yield. [H^1NMR ($CDCl_3$): 1.12-1.60 (m, 22H); 1.46, (s, 9H); 1.48, (s, 9H); 2.60 (t, 2H); 3.3 (m, 6H) and 4.7 (brs, 1H)]. The nitrile (3.5 g, 0.01M) was reduced to the N^2,N^3 protective spermidine (2.5g, 0.07 M) in 70% yield by $LiAlH_4$ in dry ether [H^1NMR:1.17-1.70 (br, 24H); 2.7 (t, 2H); 3.13 (m, 8H); 4.66 (brs, 1H)].

Synthesis of FTS-Spermidine (Glp-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-$NH(CH_2)_3NH(CH_2)_4NH_2$(III) - The FTS peptide (280 mg, 0.3 mM) was conjugated with the protected (Boc) spermidine (210 mg, 0.6 mM) by the presence of N-ethoxy-carbonyl-2-ethoxy- 1,2-dihydroquinoline (EEDQ) (II) (75 mg, 0.3 mM) in DMF to give the conjugated product (228 mg, 0.18 mM) in 60% yield at room temperature overnight. Water was added and the mixture was lyophilized. Then, the residue was subjected to gel-filtration over G-25. The Boc groups of the conjugated peptide were removed by 10% HCl at 0°C for 30 min. After lyophilization, the tri-fluoroacetyl group on Lys was removed by treatment with 1M aqueous piperidine at 0°C for 2 hours. The crude product was purified over semi-preparative ODS silica cartridges (Radial-Pack, C_{18}, 8 cm x 10 cm, Waters Associate, Milford, MA 01757). Elution was with a linear aqueous gradient of 5-50% of acetonitrile in 0.1 M KH_2PO_4 in 30 min. and the retention time was 10.0 min. Then, the lyophilized product from HPLC-C_{18} column was desalted by Gel-filtration over G-10. On TLC, the R_f was 0.44; n-BuOH:Py: HOAc:H_2O; 5:5:1:4, v/v. The presence of spermidine was confirmed by thin layer chroma-tography of the tridansyl derivative of spermidine after hydrolysis of an aliquot of the product, according to the procedure (13); R_f= 0.66; chloroform: 2-propanol, 10:1; v/v; silica gel plate, E. Merck.

Method of Bioassay - Male intact C57BL/6 mice, which were 21 days old, were obtained from the Charles River Breeding Labs. Inc., (Wilmington, MA).

The assay for the proliferation of lymphocytes, according to Stepien et al. (14) was used to determine activity. The following changes in the assay were made. The RPMI-1640 medium was buffered with 10 mM Hepes (Calbiochem) and 0.3% BSA (Sigma) to a pH of 7.42. An atmosphere of 95% O_2 and 5% CO_2 was used only during the incubation period. All samples to be assayed were dissolved in 100 µl of the RPMI-medium, and the medium was maintained for 30 minutes at 37°C. Two µCi of [^{3}H]-thymidine was added to the cell culture with the samples after 48 hours of incubation.

DISCUSSION

Stepien et al. (14) designed and standardized an assay, based on the stimulation by fractions of the thymus,for the incorporation of [^{3}H]-thymidine into DNA by spleen cells from neonatally thymectomized mice. Fractions were obtained, some of which stimulated the proliferation of lymphocytes, but other fractions inhibited proliferation. From stimulatory fractions, thymones A and B (Folkers et al., 15, 16) and thymone C (Folkers et al., 17.) were isolated. Inhibitory fractions were separately purified, by the guide of inhibition, and spermine and spermidine were identified as the cause of the inhibition. Pure spermidine and spermine inhibited the incorporation of [^{3}H]-thymidine into DNA at levels of 1 and 10 µg/ml, respectively. Kubiak et al. (18) reported on the finding and significance of spermidine and spermine in fraction 5, designed by A. Goldstein, which is an important thymus extract, extensively studied with

goals relevant to immunocompetence. Byrd et al. (19) found that μM levels of spermine and spermidine inhibited, in vitro, parameters of immunity which were based upon : (1) DNA response; (2) the mixed lymphocyte response; and (3) induction of the lymphocyte response.

The background knowledge on natural peptidic conjugates of the polyamines (cf. Introduction) which implicate functions in mechanisms of growth, in conjunction with the known stimulation of systems of immunocompetence by thymic peptides, and the inhibition of such systems by spermidine and spermine, led us to synthesize peptidic derivatives of spermidine and spermine. The availability of pure peptidic derivatives of spermidine and spermine would allow testing in systems of immunocompetence with an interest of differentiating inhibition and stimulation. It was necessary to pioneer designs of sequential reactions of the polyfunctional peptides and the polyfunctional amines which would allow acquisition of characterized peptidic derivatives of spermidine and spermine.

A characterized conjugate of a peptide and a polyamine from the thymus is not yet known which could be the goal of our design and synthesis. It was appropriate to choose an identified peptide from the field of thymus research including the facteur thymique serique (FTS) of Bach et al. (20), the thymosin of A. Goldstein et al. (21) and thymopoietin of G. Goldstein et al. (22). Since these peptides range in length from 9 to 49 amino acids, and in the absence of established synthesis of peptidic polyamines, it was advisable to choose FTS, because it is a nonapeptide and simplifies design and achievement of conjugating a thymic peptide with spermidine and spermine.

Consequently, we resynthesized FTS as a protected peptide attached to the resin and allowed this complex to react with spermine for cleavage of the protected peptide from the resin as the protected peptide amide of spermine. Catalytic deprotection then yielded the desire conjugate with spermine attached to the C terminal of FTS (FTS-spermine; Glp-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-$NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$).

The corresponding conjugate of FTS and spermidine was achieved by the coupling of Tfa-Lys^3-FTS with the Boc-derivative of spermidine by EEDQ. Then, the removal of the Tfa and Boc groups gave the product, which is spermidine coupled between the N^1 amino group of spermidine with the C terminal of FTS (FTS-spermidine; Glp-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-$NH(CH_2)_3NH(CH_2)_4NH_2$).

These two conjugates were bioassaved for the inhibition or stimulation of the proliferation of lymphocytes (14). In this bioassay, spermidine was more inhibitory than spermine since they inhibited at levels of 1 and 10 μg/ml, respectively. However, both FTS-spermidine and FTS-spermine inhibited (Table 1) at 20 μg/ml, in comparison with spermine as a control at 1 μg/ml.

Table I. Assay Data in Thymidine Incubation

Sample	Level			
	µg/ml	µM	cpm±(SEM)	P
Control	--	--	2410±(400)	--
Spermine	0.1	0.5	2396±(335)	n.s.
"	1	5	517±(206)	<0.001
"	10	50	113±(60)	<0.001
Control	--	--	2109±(489)	--
FTS-Spermine	10	9.6	1617±(138)	n.s.
"	20	19	1304±(216)	0.05>p>0.02
"	100	96	406±(370)	<0.001
Control	--	--	4370±(360)	--
FTS-Spermidine	10	10.1	3737±(708)	n.s.
"	20	20.2	2618±(584)	0.02>p>0.01
"	100	101	137±(22)	<0.001>

Consequently, FTS, said to have the status of a peptide hormone (20), which is active on most T-cell functions, when coupled to spermidine and spermine, yielded an inhibitory conjugate. The content of the spermine moiety in the conjugate is ca. 20% which may be compared with the potency of FTS-spermine which is about 5% that of spermine.

This pioneer design of synthesis of conjugates of the facteur thymique serique with spermidine and spermine will likely be successful for the synthesis of other peptidic conjugates of polyamines.

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