Effect of Sparsomycin Analogues on the Puromycin-Peptidyl Transferase Reaction on Ribosomes

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Sparsomycin analogues in which the unique $-S(O)CH_2SCH_3$ moiety was replaced by a variety of more easily accessible side chains were evaluated as inhibitors of the peptidyl transferase reaction with bacterial ribosomes. Competitive inhibition of acetyl[^{14}C]phenylalanylpuromycin formation revealed that the sulfur-containing side chain of sparsomycin could be replaced with hydrophobic moieties, whereas complete removal of the $-S(O)CH_2SCH_3$ side chain eliminated the ribosomal binding affinity of sparsomycin. The specificity for the D isomer of *S*-deoxo-*S*-propylsparsomycin has established that the chiral carbon of sparsomycin analogues must be identical with the chirality of D-cysteinol for ribosomal binding.

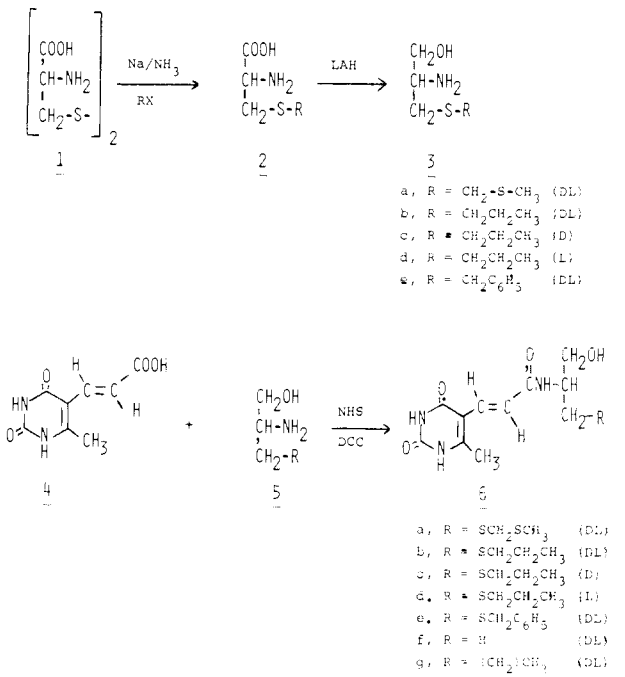
Sparsomycin is a broad-spectrum antitumor antibiotic from *Streptomyces sparsogenes*.[1] Its toxicity to both pro- and eukaryotic cells[2] is a consequence of its ability to inhibit protein synthesis at the ribosome level.[3] Sparsomycin has proved to be a useful tool in the study of the biochemistry and physiology of protein synthesis in living cells, and its antitumor activity has been evaluated in phase I clinical studies.[4] Sparsomycin blocks the peptidyl transferase function of the larger ribosomal subunit. A convenient way to study this ribosomal function is by the use of the antibiotic, puromycin, an analogue of the aminoacyladenosine end of tRNA. Thus, in the formation of polylysylpuromycin[5,6] and acetylphenylalanylpuromycin,[7] sparsomycin acts as a competitive inhibitor of the peptidyl transferase reaction with respect to puromycin.

Although sparsomycin was first isolated in 1962[1] the proposed structure of the antibiotic was not reported until 1970.[8] Because of the unique structural features of the amino alcohol moiety of sparsomycin (Figure 1), a total synthesis of the antibiotic has not yet appeared in the literature. Several related structures have recently been synthesized.[9,10] However, none of these compounds has been tested as inhibitors of ribosomal functions. In a preliminary communication[11] we reported the first active sparsomycin analogue. The present communication reports the preparation of several sparsomycin analogues and an extended study of the structural and stereochemical requirements for their interactions with ribosomes. Specifically, the ability of the sparsomycin analogues to serve as inhibitors of transpeptidation was examined, and the kinetic parameters for the interaction of these analogues with *Escherichia coli* ribosomes are reported.

Results and Discussion

Synthesis. The synthetic route for the preparation of the pyrimidinylpropenamide sparsomycin analogues is outlined in Scheme I. Thus, the S-alkylated cysteines **2** were prepared by reduction of the desired stereochemical isomer of cystine (**1**) with sodium in liquid ammonia followed by addition of alkyl halide. Reduction of the S-alkylated cysteines **2** with lithium aluminum hydride in tetrahydrofuran gave the corresponding S-alkylated cysteinols **3**. The preparation of *S*-(methylthiomethyl)-cysteinol (**3a**) from the corresponding methyl ester of **2** was as previously reported.[9] The 6-methyl-5-uracilylacrylic acid (**4**) was prepared by previously reported methods.[8,12] Condensation of **4** with the appropriate amino alcohol **3** was accomplished using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in dimethylformamide. The desired pyrimidinylpropenamides (**6a–e**) were isolated in pure form by chromatography on a silica gel column. The preparation of **6f** and **6g** was accomplished by the condensation of **4** with DL-alaninol and DL-2-amino-1-heptanol, respectively. The letters, D and L, in Scheme I refer to the chirality of the asymmetric carbon originating from the stereochemistry of the starting cystines.

Scheme I



Biological. Sparsomycin analogues in which the unique $-S(O)CH_2SCH_3$ moiety was replaced by a variety of more easily accessible side chains were evaluated as inhibitors of the peptidyl transferase reaction with *E. coli* ribosomes. A preliminary account of the effect of the DL-*S*-deoxo-*S*-propyl analogue **6b**[11] indicated that this sparsomycin analogue inhibits the puromycin reaction competitively with a K_i equal to 2×10^{-6} M (Figure 2). A Dixon analysis[13] also indicated competitive inhibition by **6b**, and the K_i of 2×10^{-6} M was in agreement with that obtained from the double reciprocal plot.

The chirality of the asymmetric carbon of sparsomycin has been shown to be identical with the chirality of D-cysteinol.[8] Thus, it was anticipated that only the D isomers of the racemic analogues are capable of ribosomal binding. Synthesis of the D isomer **6c** and the L isomer **6d** provided the first opportunity to assess the stereochemical specificity of sparsomycin binding. Examination of Table I reveals that the D isomer (**6c**) of the *S*-deoxo-*S*-propyl analogue is twice as active as the DL mixture (**6b**). In addition, the L isomer **6d** is inactive even at a concentration two orders of magnitude higher than the K_i value of the D isomer.

A comparison of *S*-deoxosparsomycin (**6a**) and the *S*-deoxo-*S*-propyl analogue **6b** establishes a definite contribution to binding by the sulfur atom of the CH_2SCH_3

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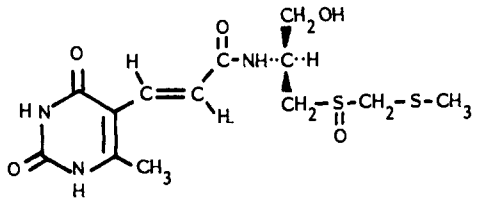


SPARSOMYCIN

Figure 1. Structure of sparsomycin.

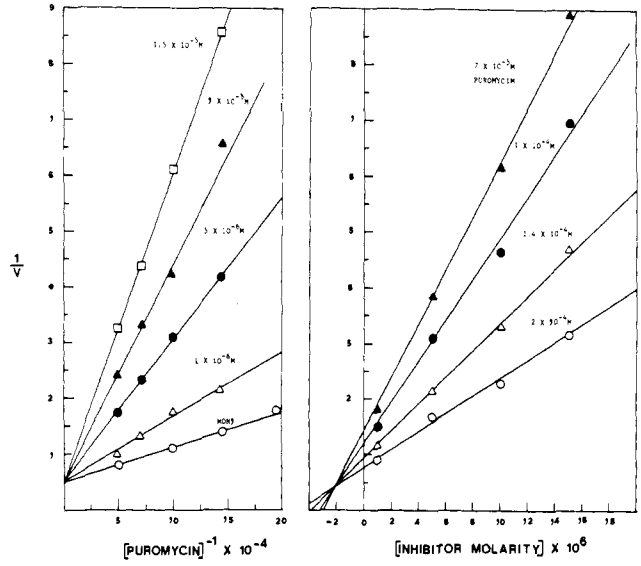


Figure 2. Competitive inhibition of *N*-acetyl[^{14}C]phenylalanylpuromycin synthesis of *E. coli* ribosomes with *S*-deoxo-*S*-propylsparsomycin. The Ac[^{14}C]Phe-tRNA was bound to the ribosomes in a reaction mixture containing 100 mM Tris–Cl (pH 7.5), 100 mM NH_4Cl (pH 7.6), 15 mM $Mg(OAc)_2$, 0.65 mM dithiothreitol, 2.78 A_{260} units of washed *E. coli* ribosomes, 1.2 mM GTP, 63 μg of FWR, 0.35 unit of poly(U), and 20.8 pmol of Ac[^{14}C]Phe-tRNA (464 pCi/pmol). The binding mixture was incubated at 28 °C for 8 min, and the peptidyl transferase reaction was initiated by the addition of 80 μL of the incubation cocktail to 20 μL of puromycin or a mixture of puromycin plus inhibitor. Reactions were incubated at 28 °C for a specified time and product formation was measured as described in ref 15. Initial velocities were recorded as counts per minute of product formed per minute.

moiety of sparsomycin. Thus, replacement of –S– with $-CH_2-$ reduces the binding affinity twofold. The increased activity of the *S*-benzyl analogue **6e** indicates a tolerance for a fairly large hydrophobic group extending into the region occupied by the CH_2SCH_3 moiety of sparsomycin. The hydrophobic character of this binding region may play a significant role in the binding of these analogues. For example, it is particularly noteworthy that the complete removal of a hydrophobic moiety from the sulfur-containing region (compound **6f**) completely abolishes the activity of the antibiotic. These observations are important factors in the design of affinity-labeling analogues since they indicate that the ribosomal binding site should accommodate chemically reactive or photosensitive affinity-labeling moieties in this region.

An assessment of the contribution to binding attributed to the sulfoxide moiety of sparsomycin leads to some interesting observations. For example, a comparison of the K_i values for sparsomycin and the DL-*S*-deoxosparsomycin **6a** leads to the conclusion that the sulfoxide oxygen does not contribute to binding since the K_i for the D isomer of **6a** would be 0.5 μM. In addition, the relative equal binding affinities exhibited by **6b** and the analogue **6g**, lacking both sulfur atoms, indicate relatively little

Table I. Competitive Inhibition of *N*-Acetyl[^{14}C]phenylalanylpuromycin Synthesis by *E. coli* Ribosomes with Sparsomycin Analogues[a]

Compound	R	Stereo Chemistry	Ki(μM)[b]
sparsomycin		D	0.50 ± 0.01
6a	SCH_2SCH_3	DL	1.09 ± 0.13
6b	$S(CH_2)_2CH_3$	DL	2.00 ± 0.19
6c	$S(CH_2)_2CH_3$	D	1.10 ± 0.13
6d	$S(CH_2)_2CH_3$	L	Inactive[c]
6e	$SCH_2C_6H_5$	DL	1.21 ± 0.14
6f	H	DL	Inactive[c]
6g	$(CH_2)_3CH_3$	DL	2.30 ± 0.20

[a] The assay procedure is described in the legend of Figure 2. The K_i values were determined by double reciprocal plots. [b] All active analogues exhibited competitive inhibition. [c] No activity at highest concentration tested (2×10^{-4} M).

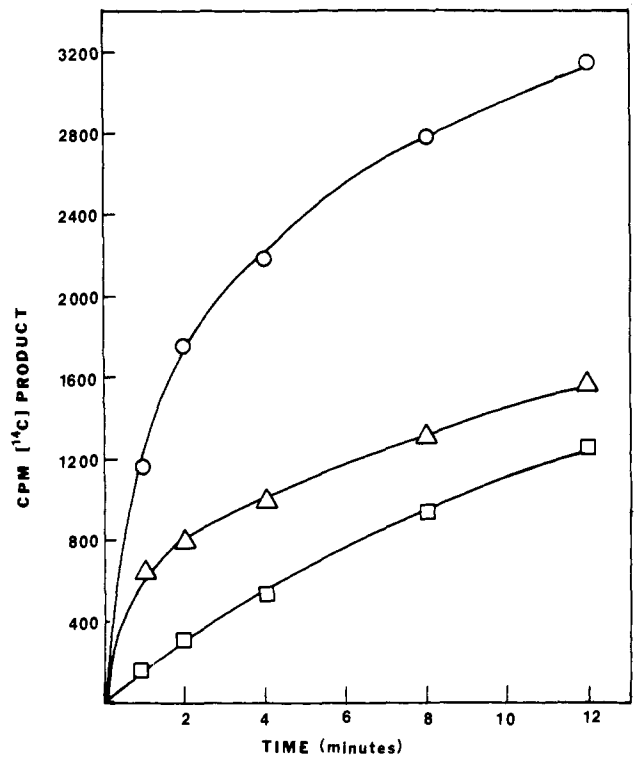
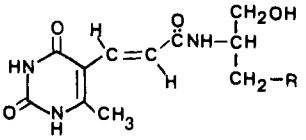


Figure 3. The course of the reaction at 28 °C of Ac[^{14}C]-L-Phe-tRNA as a donor substrate with puromycin as an acceptor substrate with *E. coli* ribosomes. The Ac[^{14}C]-L-Phe-tRNA was bound to the ribosomes as described in the legend to Figure 2. The peptidyl transferase reaction was initiated by addition of 80 μL of the charged ribosome mixture to puromycin (○) and puromycin plus sparsomycin (△). For the incubation experiment (□) the charged ribosome mixture was added to sparsomycin and incubated at 28 °C for 15 min before the addition of puromycin. The concentrations of puromycin and sparsomycin in each experiment were 10^{-4} and 10^{-6} M, respectively. Product formation was as previously described.[15]

binding attributed to the sulfoxide sulfur. However, incubation experiments such as illustrated in Figure 3 demonstrate a significant contribution by the sulfoxide to sparsomycin's ability to inhibit the peptidyl transferase

Table II. Physical Data



cpd	R	Stereo Chemistry	m.p.	%Yield	Mol formula	Anal
6a	SCH_2SCH_3	DL	230-232°	23	$C_{13}H_{19}N_3O_4S$	CHN
6b	$SCH_2CH_2CH_3$	DL	241-244°	19	$C_{14}H_{21}N_3O_4S$	CHN
6c	$SCH_2CH_2CH_3$	D	243-245°	33	$C_{14}H_{21}N_3O_4S$	CHN
6d	$SCH_2CH_2CH_3$	L	242-243°	34	$C_{14}H_{21}N_3O_4S$	CHN
6e	$SCH_2C_6H_5$	DL	209-211°	12	$C_{18}H_{21}N_3O_4S$	CHN
6f	H	DL	266-267°	26	$C_{11}H_{15}N_3O_4$	CHN
6g	$(CH_2)_3CH_3$	DL	248-250°	24	$C_{15}H_{23}N_3O_4$	CHN

reaction. It is seen from Figure 3 that the ability of sparsomycin to inhibit the puromycin reaction is increased if sparsomycin is incubated with the ribosomes prior to the addition of puromycin. A similar observation has been reported by Coutsogeorgopoulos.[14] Preincubation of ribosomes with **6a** does not effect its ability to inhibit the puromycin reaction. These data indicate that the initial reversible sparsomycin–ribosome complex (SR) undergoes an as yet undefined conformational change that increases the tightness with which sparsomycin binds. This change may involve the formation of irreversible complex (SR_{ir}).

$$S + R \underset{K_i}{\rightleftharpoons} SR \overset{k}{\rightarrow} SR_{ir}$$

Since the K_i value in Table I reflects only the dissocation constant of the initial reversible complex, the above conclusion that the sulfoxide moiety of sparsomycin is not involved in initial binding to the ribosome is correct. However, the presence of the sulfoxide may enable the sparsomycin molecule to undergo additional reaction at the binding site which is characteristic of irreversible inhibition.

Experimental Section

Materials. Puromycin dihydrochloride was obtained from ICN Pharmaceuticals, Inc.; [^{14}C]-L-phenylalanine was obtained from New England Nuclear. *E. coli* cell paste (B, mid log) was purchased from General Biochemicals. The polynucleotides were obtained from Miles Laboratories, and ATP, GTP, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. Preparation of ribosomes, S-100, factors washable from ribosomes (FWR), and Ac[^{14}C]-L-Phe-tRNA were as previously described.[15]

Methods. Elemental analyses were performed by M-H-W Laboratories, Garden City, Mich. Melting points were determined on a Mel-Temp and are corrected. Nuclear magnetic resonance was determined with a Varian A-60D spectrometer, IR with a Perkin-Elmer 237B spectrophotometer, and UV with a Beckman 25 recording spectrophotometer. Since several compounds were prepared by similar procedures, only one representative example is described in the Experimental Section. Satisfactory elemental analyses (±0.4% of calculated values) are indicated by the elemental symbols in Table II.

S-Alkylated Cysteines. DL-*S*-*n*-Propylcysteine. A solution of DL-cystine (9.69 g, 40.3 mmol) in liquid ammonia (400 mL) was treated with sodium metal until a blue color was retained for several minutes. *n*-Propyl bromide (15 mL, 165 mmol) was added in one portion and the solution was evaporated overnight. The residue was dried in vacuo over concentrated sulfuric acid and then dissolved in water (50 mL) with cooling in an ice bath. The aqueous solution was filtered and adjusted to pH 5 with concentrated hydrochloric acid. The precipitate was collected by filtration and gave 11.7 g (82%) of crude product, mp 225–229 °C. One recrystallization from water gave the analytical sample: mp 229–231 °C; IR (KBr) 3300–2200 (br, COOH), 1615 and 1575 cm^{-1} (C=O); NMR (CF_3COOD–Me_4Si) δ 1.05 (t, 3 H, J = 7 Hz, $-SCH_2CH_2CH_3$), 1.65 (m, $-SCH_2CH_2CH_3$), 2.68 (t, 2 H, J = 7 Hz, $-SCH_2CH_2CH_3$), 3.33 (m, 2 H, $-CHCH_2S-$), 4.58 (m, 1 H, $-CHCH_2S-$). Anal. Calcd for $C_6H_{13}NO_2S$: C, 44.15; H, 8.03; N, 8.58. Found: C, 43.97; H, 7.98; N, 8.54.

S-Alkylated Cysteinols. DL-*S*-*n*-Propylcysteinol. A mixture of lithium aluminum hydride (3.74 g, 98.5 mmol) and tetrahydrofuran (200 mL) was heated under reflux for 2 h under nitrogen. The mixture was cooled in an ice bath and DL-*S*-*n*-propylcysteine (8.39 g, 51.4 mmol) was added in small portions. The mixture was heated under reflux under nitrogen for 12 h. Excess hydride was decomposed by careful addition of water (4 mL), 2 N NaOH (4 mL), and then water (12 mL). The slurry was filtered through a sintered glass funnel and the residue was washed with tetrahydrofuran (150 mL). The filtrate and washings were combined and dried (sodium sulfate). The solvent was removed in vacuo, leaving a pale yellow oil (7.69 g). The oil was distilled in vacuo and gave 5.60 g (73%) of pure product: bp 111–112 °C (0.08 mm); NMR ($CDCl_3$–Me_4Si) δ 0.97 (t, 3 H, J = 7 Hz, $-SCH_2CH_2CH_3$), 1.62 (m, 2 H, $-SCH_2CH_2CH_3$), 2.08 (s, 3 H, $-NH_2$ and $-OH$), 2.47 (m, 4 H, $CHCH_2S-$ and $SCH_2CH_2CH_3$), 2.93 (m, 1 H, $NCH-$), 3.50 (m, 2 H, CH_2OH). Anal. Calcd for $C_6H_{15}NOS$: C, 48.28; H, 10.13; N, 9.38. Found: C, 48.05; H, 10.21; N, 9.40.

Pyrimidinylpropenamides.[16] **DL-*N*-(1-Hydroxy-3-*n*-propylthio-2-propyl)-(*E*)-3-(1,2,3,4-tetrahydro-6-methyl-2,4-dioxo-5-pyrimidinyl)-2-propenamide.** A mixture of 1.58 g (8.08 mmol) of 6-methyl-5-uracilylacrylic acid (**4**) and 0.95 g (8.25 mmol) of *N*-hydroxysuccinimide was dissolved in 50 mL of dimethylformamide (DMF). The solution was cooled to 0 °C and 1.74 g (8.45 mmol) of dicyclohexylcarbodiimide was added. The solution was stirred at room temperature for 30 h and then heated at 60 °C for 36 h. The mixture was chilled and filtered, and the residue was washed with DMF (10 mL) and ethyl acetate (20 mL). The combined filtrate and washings were evaporated in vacuo to a yellow gum. The product was chromatographed on a column of silica gel (3 × 30 cm) prepared with $CHCl_3$. The column was eluted with chloroform–methanol as follows: (1) 98:2 (2000 mL), (2) 97:3 (2000 mL), (3) 95:5 (500 mL), (4) 95:5 (1250 mL). Fraction 4 gave the pure product as a white powder: 0.51 g; mp 241–244 °C; IR (KBr) 3600–3060 (OH, NH), 1740 and 1680 (C=O), 1617 (C=C), 1538 (amide II), 992 cm^{-1} (trans HC=CH); NMR (CD_3OD–Me_4Si) δ 0.97 (t, 3 H, J = 7 Hz, $SCH_2CH_2CH_3$), 1.53 (m, 2 H, $SCH_2CH_2CH_3$), 2.33 (s, 3 H, $=CCH_3$), 2.60 (m, CH_2SCH_2) overlapping 2.65 (s, total 5 H, OH), 3.10 (m, 1 H, NCH–), 3.67 (m, 2 H, $-CH_2O-$), 4.17 (br, 1 H, NH), an AB quartet centered at δ 7.10 and 7.43 (2 H, J = 16 Hz, HC=CH); UV (EtOH) nm (log ε) 303 (4.45), 271 (4.24). Anal. Calcd for $C_{14}H_{21}N_3O_4S$: C, 51.36; H, 6.46; N, 12.83. Found: C, 51.24; H, 6.24; N, 13.09.

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[1-Deaminopenicillamine,4-threonine]oxytocin, a Potent Inhibitor of Oxytocin[1]

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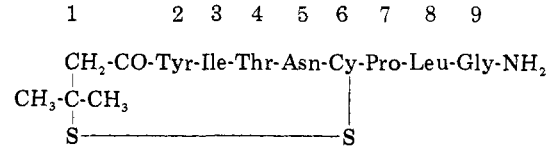
[1-Deaminopenicillamine,4-threonine]oxytocin was prepared in duplicate from *S*-benzyl-3-mercapto-3,3-dimethylpropanoyl-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (I) by removal of the Bzl-protecting groups with Na–NH_3, followed by cyclization of the resulting disulfhydryl compound with $K_3Fe(CN)_6$. The analogue was purified by desalting on Sephadex G-15 in 50% acetic acid and gel filtration on Sephadex G-15. The protected peptide I was synthesized (a) by the solid-phase method and (b) by a combination of solid-phase synthesis and an [8 + 1] coupling in solution. The analogue has no detectable agonist activity in rat vasopressor or isolated rat uterus assays. It has an antivasopressor pA_2 of 6.67 ± 0.09. It is a potent inhibitor of the in vitro oxytocic response to oxytocin and has a pA_2 value of 7.46 ± 0.04. (Material from the repeat synthesis has a pA_2 value of 7.59 ± 0.08.) Thus the substitution of threonine for glutamine in the antagonist [1-deaminopenicillamine]oxytocin (pA_2, 7.14 ± 0.05) has effected a twofold increase in inhibitory potency. [1-Deaminopenicillamine,4-threonine]oxytocin is one of the most potent inhibitors of oxytocin known to date.

The peptide analogues [1-L-penicillamine]-, [1-D-penicillamine]-, and [1-deaminopenicillamine]oxytocin are potent inhibitors of the oxytocic response in vitro to oxytocin and structurally related compounds.[2,3] Other analogues of oxytocin bearing a 3-mercapto-3,3-dialkylpropanoic acid in the 1 position also inhibit the oxytocic and avian vasodepressor responses to oxytocin.[4,5] The antioxytocic potency increases with increasing size and/or lipophilicity of the alkyl substituent. Inhibitory potencies were determined and expressed as pA_2 values. pA_2 values have been defined[6] as the negative logarithm to the base 10 of the average molar concentration of an antagonist which will reduce the biological response to $2x$ units of agonist to equal the response given by x units of agonist in the absence of antagonist. Among the antagonists of oxytocin containing dialkyl substitutions in the 1 position, [1-(3-mercapto-3,3-cyclopentamethylenepropanoic acid)]oxytocin is the most potent, with a pA_2 value of 7.43 when tested against oxytocin on the isolated rat uterus.[5] The 3,3-diethyl and 3,3-dimethyl (deaminopenicillamine) analogues were reported to have pA_2 values of 7.24[4] and 6.94,[2,3] respectively.

The introduction of threonine into position 4 of oxytocin has been shown to modify its spectrum of pharmacological activities in a remarkable manner. Oxytocic activity relative to oxytocin was significantly enhanced while vasopressin characteristics were diminished. [4-Threonine]oxytocin is thus a highly potent and specific oxytocic agent.[7,8] The enhanced oxytocic activity could result from (a) increased affinity for the smooth muscle receptors, (b) increased intrinsic activity subsequent to binding, or (c) relative resistance to enzymatic inactivation in the vicinity of the receptors. If the enhanced activity is due to greater binding resulting from the presence of the threonine residue at position 4, then, it was reasoned, the substitution of threonine in an oxytocin antagonist might also increase its binding and consequently give rise to an enhancement of its antagonist properties.

In the light of this reasoning it appeared worthwhile to explore the effect of a threonine/glutamine interchange in an oxytocin antagonist in the hope of obtaining a peptide with enhanced antagonist potency. Since it had been shown that [1-deaminopenicillamine]oxytocin is a slightly more effective antagonist than [1-L-penicillamine]oxytocin, their reported pA_2 values being 6.94 and 6.86, respectively,[3] it was decided to substitute threonine for glutamine in the former peptide. Thus [1-deaminopenicillamine,4-threonine]oxytocin was designed according to this rationale. It has the following structure.



We now describe its synthesis (in duplicate) and some of its chemical, physical, and pharmacological properties. The protected peptide precursor of the desired peptide analogue was synthesized (a) by development of the entire acyloctapeptide sequence upon the Merrifield resin,[9,10] using previously described procedures,[7,11] and (b) with the final acylation step conducted in solution, using a dicyclohexylcarbodiimide–*N*-hydroxybenzotriazole preactivation method.[12,13] Deblocking[7,14] and purification[7,15] were carried out by previously described methods.

Results and Discussion

The pharmacological properties of [1-deaminopenicillamine,4-threonine]oxytocin were determined by a variety of biological assays. This analogue has weak antidiuretic

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