to yield 4.8 g (12 mmol or 30%) of the bicyclic product: ^{1}H NMR (270 MHz) (DMSO-$d_6$/Me$_4$Si) δ 3.32 (s, 3 H, S(O)$_2$CH$_3$) 4.03 (s, 3 H, OCH$_3$) 7.64 (s, 1 H, phenyl H) 7.68 (d, J = 8 Hz, 1 H, phenyl H), 7.76 (d, J = 5 Hz, 1 H, ring H), 8.55 (d, J = 8 Hz, 1 H, phenyl H), 8.67 (d, J = 5 Hz, 1 H, ring H), 8.84 (s, 1 H, ring H); MS, *m/e* (relative intensity) 337 (M$^+$, 100), 308 (28), 228 (84), 209 (56), 129 (83). Anal. (free amine) (C$_{14}$H$_{12}$N$_3$O$_3$SCl) C, H, N, S, Cl.

**B. Preparation of 2-[2-Methoxy-4-(methylsulfonyl)-phenyl]imidazo[1,2-*a*]pyrazine.** The chloro derivative (3.6 g, 9.1 mmol) was added to 195 mL of dimethylformamide followed by 2.7 g of triethylamine. Hydrogenation was carried out at an initial pressure of 60 psi with 1 g of 5% Pd/BaSO$_4$ as catalyst. After the mixture was shaken at room temperature for 1.5 h, 100% of theoretical hydrogen uptake had occurred. The catalyst was removed by filtration, and the reaction mixture was added to 700 mL of ethyl acetate. The ethyl acetate solution was washed four times with 250 mL of saturated NaCl solution, the ethyl acetate solution was dried, and the solvent was removed by rotary evaporation. The product was crystallized from a small volume of ethyl acetate to yield 540 mg (1.78 mmol or 20%) in the first crop: ^{1}H NMR (270 MHz) (DMSO-D$_6$/Me$_4$Si) δ 3.32 (s, 3 H, S(O)$_2$CH$_3$), 4.14 (s, 3 H, OCH$_3$), 7.64–7.70 (m, 2 H, phenyl H), 7.93 (d, J = 5 Hz, 1 H, ring H), 8.57 d, J = 9 Hz, 1 H, phenyl H), 8.64 (m, 1 H, ring H) 8.73 (s, 1 H, ring H); MS, *m/e* (relative intensity) 303 (M$^+$, 100), 274 (34), 209 (39) 194 (75). Anal. (C$_{14}$H$_{13}$N$_3$O$_3$S) C, H, N, S.

# Deletion Sequences of Salmon Calcitonin That Retain the Essential Biological and Conformational Features of the Intact Molecule[†]

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Salmon calcitonin has an amino acid sequences that would allow it to form an amphipathic helix from approximately residue 9 to residue 22. We have synthesized a number of analogues of this peptide hormone with deletions in the carboxyl terminus of this putative amphipathic helix. These analogues include deletions of single amino acid residues at positions 19, 20, 21, or 22 as well as deletions of progressively larger segments starting with residue 19 and including deletions of residues 19 and 20; 19, 20, and 21; or 19, 20, 21, and 22. There is a small decrease in the helical content of these analogues compared with the native hormone, both in the presence and absence of amphiphiles. However, the extent of formation of secondary structure, as measured by circular dichroism, is similar for these deletion sequences as it is for the native hormone. In all cases, there is a large increase in the helical content of the peptide in the presence of dimyristoylphosphatidylglycerol, lysolecithin, or sodium dodecyl sulfate. All of the analogues have hypocalcemic activity in vivo in rats, comparable to the native hormone, except for des-Leu$^{19}$-salmon calcitonin, which is about twice as active as the unmodified hormone. With use of an in vitro assay of adenylate cyclase activation in purified rat kidney membranes, des-Tyr$^{22}$-salmon calcitonin, des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$-salmon calcitonin, and des-Leu$^{19}$Gln$^{20}$,Thr$^{21}$,Thr$^{22}$-salmon calcitonin exhibited about one-tenth the stimulatory activity of the native hormone. Des-Tyr$^{22}$-sCT and des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT were also tested for their activity in inhibiting prolactin release from isolated rat pituitary cells. Both of these analogues exhibited inhibitory activity. Thus, the region of residues 19–22 does not greatly affect either the conformational or the biological properties of salmon calcitonin.

There is much current interest in "protein engineering", i.e. the design and synthesis of modified peptides or proteins with altered biological activity. Most of these studies involve substituting or deleting single amino acid residues of a native peptide chain. Deletion of amino acid residues that have important functional roles leads to a marked reduction in activity. In the case of salmon calcitonin

(sCT), most of the 32 amino acid peptide is required for activity since removal of several residues from the carboxyl terminus leads to sCT(1–23)-peptide amide that has only 0.25% the potency of the native hormone while the carboxyl terminal segment sCT(12–32)-peptide amide is devoid of activity.[1] A more complete discussion of structure–activity relationships in sCT can be found in a recent review.[2] The results suggest that several regions of the peptide contribute to the activity and/or potency of the

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(1) Epand, R. M.; Stahl, G. L.; Orlowski, R. C. *Int. J. Peptide Protein Res.* **1986**, *27*, 501.
(2) Epand, R. M.; Caulfield, M. P. In *Comprehensive Medicinal Chemistry*; Emmett, J. C., Ed.; Pergamon: Oxford; Vol. 3, in press.

**Table I.** CD and in Vivo Hypocalcemic Activity of sCT and Deletion Peptides (100 $\mu$M Peptides in the Presence or Absence of 1 mM DMPG, 2.5 mM LPC, or 25 mM SDS at 25 °C in 20 mM Pipes, 1 mM EDTA, 0.15 M NaCl, 0.02 mg/mL NaN$_3$, pH 7.40)

| peptide | $-[\theta]_{222}$ (deg cm$^2$ dmol$^{-1}$) | | | | hypocalcemic activity[a] (IU/mg) | partitioning liter. | |
|---|---|---|---|---|---|---|---|
| | no additions | DMPG | LPC | SDS | | $R_f$ | ref |
| sCT | 4160 | 12350 | 10830 | 11410 | 4500 (4103–4900) | 0.58 | 2 |
| des-Leu$^{19}$-sCT | 3640 | 7905 | 8650 | 12000 | 8000 (7112–8850) | 0.39 | 4 |
| des-Gln$^{20}$-sCT | 3725 | 10830 | 11610 | 10330 | 4000 (3536–4416) | 0.48 | |
| des-Thr$^{21}$-sCT | 3600 | 11390 | nd | 11160 | 5500 (4731–6061) | 0.68 | 5 |
| des-Tyr$^{22}$-sCT | 3240 | 7715 | nd | 10850 | 5200 (4645–5650) | 0.50 | 6 |
| des-Leu$^{19}$,Gln$^{20}$-sCT | 3550 | 9000 | 11040 | 12790 | 6400 (5500–7363) | 0.42 | 7 |
| des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$-sCT | 3140 | 7745 | 8050 | 10880 | 7100 (6248–8136) | 0.42 | 8 |
| des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT | 3765 | 7380 | 10750 | 10660 | 6300 (5851–7159) | *b* | 9 |

[a] Value is weighted mean average of four assays with 95% confidence limits given in parentheses. [b] Analogue too water-soluble, purified via HPLC.

hormone. Nevertheless, in the present study we demonstrate that up to four residues of sCT can be deleted without loss of biological activity. The amino acid sequence of sCT is Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH$_2$.

## Results

**Conformational Studies.** CD spectra of proteins and peptides are markedly dependent on secondary structure content. Several peptide hormones, including sCT, become more helical in the presence of anionic phospholipids and detergents.[3] Deletion of one or more amino acids in the region of residues 19–22 of sCT causes some loss of secondary structure (Table I), but the change is relatively modest. There is still a marked increase in secondary structure in the presence of amphiphiles.

**Biological Activity.** Hypocalcemic activity measured in vivo is not decreased for any of these deletion peptides and several of the deletion analogues have markedly higher hypocalcemic activities, even if expressed on a molar basis, than that of sCT (Table I).

Des-Tyr$^{22}$-sCT, des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$-sCT, and des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCt were tested for their ability to activate adenylate cyclase from rat kidney plasma membranes. All of the analogues are capable of activating adenylate cyclase, but the potency is about a tenth that of sCT.

Calcitonin is also a potent inhibitor of hormone release from several endocrine glands. This activity is distinct from its direct action on bone and kidney calcium metabolism. The des-Leu$^{19}$-sCT and des-Leu$^{19}$,Gln$^{20}$,Thr2$^1$,Tyr$^{22}$-sCT, like sCT, are capable of inhibiting TRH-stimulated prolactin release from isolated pituitary cells of estradiol-primed rats (Figure 2). All three calcitonin peptides appear to inhibit the prolactin release. Because of the variability in the assay, a quantitative comparison of the potencies cannot be made.

## Discussion

We have measured three activities for this series of deletion peptides: in vivo hypocalcemic activity, in vitro stimulation of kidney membrane adenylate cyclase, and inhibition of prolactin release in isolated pituitary cells. It is striking that despite the deletion of up to four amino acids there is little change in any of these activities.

In the case of the in vivo hypocalcemic activity, the des-Leu$^{19}$-sCT has a somewhat higher activity than that of the intact peptide or the other deletion sequences (Table I). This occurs despite the fact that this analogue has less
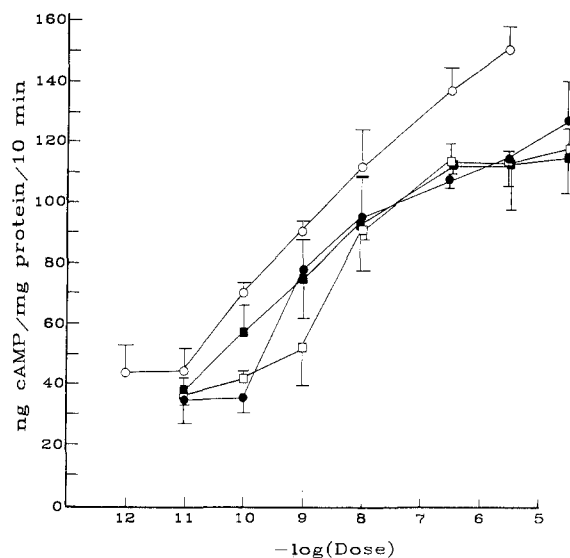


**Figure 1.** Dose–response curves for the activation of rat kidney adenylate cyclase by sCT and deletion analogues. Basal activity is 29 ± 5 ng cAMP/mg protein per 10 min. (O) sCT, (●) des-Tyr$^{22}$-sCT, (□) des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$-sCT, (■) des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT. Error bars represent the standard deviation of triplicate determinations done on the same day.

structure than the native hormone in buffer or in the presence of amphiphiles. We have suggested that some less structured analogues can exhibit enhanced activity as a result of increased conformational flexibility.[10]

The enhanced activity of des-Leu$^{19}$-sCT is not observed with adenylate cyclase stimulation (Figure 1). This is also the case for other analogues of sCT that show equal or enhanced in vivo hypocalcemic potency but decreased adenylate cyclase stimulation compared with the native hormone.[11] The adenylate cyclase activation is similar for

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(4) Orlowski, R. C.; Seyler, J. K. (*Des-19-Leucine*) *Calcitonin*; U.S. Patent 4 639 511, January 27, 1987.

(5) Orlowski, R. C.; Seyler, J. K. (*Des-21-Threonine*) *Calcitonins*; U.S. Patent 4 604 237, August 5, 1986.

(6) Hughes, J. L.; Seyler, J. K.; Liu, R. C. *Synthesis of Biologically Active Peptides*; U.S. Patent 4 239 680, December 16, 1980.

(7) Orlowski, R. C.; Seyler, J. K.; Geever, J. E. (*Des-19-Leucine, 20-Glutamine*) *Calcitonin*; Serial No. 859 527; Filing Date: May 5, 1986.

(8) Orlowski, R. C.; Seyler, J. K. (*Des-19-Leucine, 20-Glutamine, 21-Threonine*) *Calcitonin*; Serial No. 040 963; Filing Date: April 21, 1987.

(9) Orlowski, R. C.; Seyler, J. K.; Flanigan, E. (*Des-19-Leucine, 20-Glutamine, 21-Threonine, 22-Tyrosine*) *Calcitonin*; Filing Date: April 21, 1987.

(10) Epand, R. M.; Epand, R. F.; Orlowski, R. C.; Seyler, J. K.; Colescott, R. L. *Biochemistry* 1986, *25*, 1964.
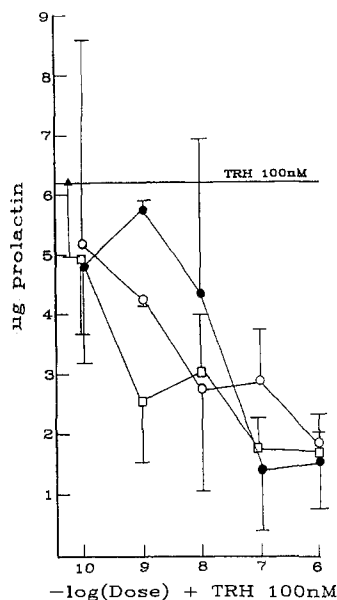
**Figure 2.** Dose–response curves for the inhibition of TRH-stimulated release of prolactin from pituitary cells isolated from estradiol-primed female rats. TRH concentration is 100 nM in all tubes. Prolactin release was measured after 3 h of incubation with the hormones. Basal activity is $0.8 \pm 0.2$ μg of prolactin/culture well. The results are expressed as micrograms of prolactin released/cell culture. Error bars represent the standard deviation of triplicate cell cultures done with the same batch of cells. (O) sCT, (□) des-Tyr$^{22}$-sCT, (●) des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT, (▲) 100 nM TRH alone.

the three deletion peptides tested. There is no progressive loss of activity as additional amino acids are removed. The lack of correlation between adenylate cyclase activation and hypocalcemic activity can occur if only a small increase in cAMP levels is required to trigger a cellular response. The large activation of cAMP production observed in vitro may not be physiologically relevant. In addition, there may be cAMP-independent mechanisms leading to hypocalcemia.

The inhibition of prolactin release by calcitonin is likely to be via a mechanism different from adenylate cyclase stimulation since cAMP promotes pituitary hormone release.[12] The two deletion analogues tested, including des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT, inhibited prolactin release (Figure 2). Thus, two independent activities of sCT, i.e. adenylate cyclase stimulation and inhibition of hormone release, are both retained in these deletion analogues.

It is not known why sCT has more potent hypocalcemic activity in mammals than homologous mammalian calcitonin. This increased activity can be correlated with a greater increase in structure of sCT in the presence of lipids.[3] Mammalian calcitonins have many amino acid replacements compared with sCT. It is therefore possible that sCT can be more readily modified without loss of its affinity for mammalian calcitonin receptors than is often the case for peptide hormones and receptors of the same species. Kaiser and his co-workers demonstrated that a calcitonin analogue with many amino acid substitutions can retain biological activity.[13] In this work, we demonstrate that a large segment of the amphipathic helical region of sCT, residues 19–22, is not required for the biological activity of the hormone.

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(12) Guild, S.; Itoh, Y.; Kebabian, J. W.; Luini, A.; Reisine, T. *Endocrinology* **1986**, *118*, 268.
(13) Moe, G. R.; Kaiser, E. T. *Biochemistry* **1985**, *24*, 1971.

## Experimental Section

**Materials. Lipids.** Dimyristoylphosphatidylglycerol (DMPG) from Avanti Polar Lipids, palmitoyllysophosphatidylcholine (LPC) from Sigma Chemical Co., and sodium dodecyl sulfate (SDS) from Miles Laboratories were all high-purity commercial preparations.

**Peptides.** Synthetic sCT and the deletion analogues were synthesized by using the standard solid-phase methodology[4–6] on a benzhydrylamine resin. Amino acid derivatives were the L isomers and purchased from Bachem, Inc., Torrance, CA. After removal from the resin by treatment with anhydrous hydrofluoric acid and disulfide ring closure, the products were concentrated on SP-Sephadex (C-25), desalted on Sephadex G-25, and purified on CM-52. sCT and the analogues were then further purified by partition chromatography[14] on Sephadex G-25 with the solvent system 1-butanol/95% ethanol/0.2 N ammonium acetate, pH 6.1, in the ratio 4:1:5. The $R_f$ values are reported in Table I.

The analogue des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT is too soluble in the lower phase to be partitioned. The analogue was further purified via RP-HPLC. The RP-HPLC was performed with a (5.7 × 30 cm) preparative radially compressed column packed with C-18 (Vydac) derivatized silica (pore size 300 Å, 30 μm). The sample, 842 mg was loaded onto the previously equilibrated column (12.5% acetonitrile, (v/v) 0.1% trifluoroacetic acid) and eluted with use of a linear gradient consisting of the equilibrium buffer and the limiting buffer (60% acetonitrile, (v/v) 0.1% trifluoroacetic acid) at a flow rate of 45 mL/min. Fractions of 30 mL each were collected; fractions 98–108 were pooled and lyophilized twice (the second time from 0.5 M acetic acid). The recovered product was converted to the acetate salt by ion-exchange chromatography on a CM-52 column and gel filtered on a Sephadex G-25 (fine) column. The purified peptide was recovered by lyophilization; recovered 236 mg.

For des-Gln$^{20}$-sCT, the amino acid composition is as follows: Arg 1 (1), Asp 2 (2), Thr 5.2 (5), Ser 3.8 (4), Glu 2 (2), Pro 2 (2), Gly 3 (3), Cys 0.85 (1); Leu 5.2 (5), Tyr 0.9 (1), Lys 1.9 (2), His 1 (1), NH₃ 4.3 (4).

For des-Leu$^{19}$,Gln$^{20}$-sCT, the amino acid composition is as follows: Arg 1 (1), Asp 2 (2), Thr 5.3 (5), Ser 3.8 (4), Glu 2 (2), Pro 2 (2), Gly 3 (3), Cys 0.87 (1), Leu 4.2 (4), Tyr 0.9 (1), Lys 1.9 (2), His 1 (1), NH₃ 4.4 (4).

For des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$-sCT, the amino acid composition is as follows: Arg 1 (1), Asp 2 (2), Thr 4.2 (4), Ser 3.7 (4), Glu 2 (2), Pro 2 (2), Gly 3 (3), Cys 0.86 (1), Leu 4.3 (4), Tyr 0.9 (1), Lys 1.9 (2), His 1 (1), NH₃ 4.3 (4).

For des-Leu$^{19}$,Gln$^{20}$,Thr$^{21}$,Tyr$^{22}$-sCT, the amino acid compositions is as follows: Arg 1 (1), Asp 2 (2), Thr 4.3 (4), Ser 3.7 (4), Glu 2 (2), Pro 2 (2), Gly 3 (3), Cys 0.98 (1)8 Leu 4.3 (4), Lys 1.9 (2), His 1 (1)8 NH₃ 4.3 (4).

**Methods. Circular Dichroism (CD).** Peptides were dissolved in 20 mM Pipes, 1 mM EDTA, and 150 mM NaCl containing 0.02 mg/mL NaN₃ and adjusted to pH 7.40 with NaOH. An aliquot of the peptide solution in buffer was added to a dried lipid film of DMPG or LPC or it was mixed with a small volume of SDS in buffer. The DMPG was suspended by vortexing at 40 °C. The sample was then cooled and heated between 10 and 40 °C. All of the peptide solutions with DMPG, LPC, or SDS were visually transparent. CD spectra were obtained with an Aviv Model 61 DS solid-state CD instrument (Aviv Associates, Lakewood, NJ). The CD was measured in a 1-mm sample cell that was maintained at 25 °C with a thermostated cell holder. The scans were corrected for the buffer baseline and multiplied by a constant to obtain the mean residue ellipticity [θ].

**Hypocalcemic Activity.** This activity was determined in vivo by measuring the hormone-induced decrease of blood calcium levels in rats.[15]

Plasma membranes from rat kidneys were prepared as described by Marx and Aurbach.[16] The final purification of the membranes was done by centrifugation through a continuous

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(15) Schwartz, K. E.; Orlowski, R. C.; Marcus, R. *Endocrinology* **1981**, *108*, 831.
(16) Marx, S. J.; Aurbach, G. D. *Methods Enzymol.* **1975**, *38*, 150.

gradient of 36–45% sucrose (w/w).

**Adenylate Cyclase Assay.** Adenylate cyclase activity in the purified kidney membranes was assayed under conditions similar to that described by Neuman and Schneider[17] except that the $MgCl_2$ concentration was 1 mM, 0.5 mM 3-isobutyl-1-methylxanthine was used in place of theophylline, and 0.2 mM [8-$^{14}$C]ATP was used as substrate. The [8-$^{14}$C]cAMP product was isolated by column chromatography by the procedure of White and Karr[18] with [2,8-$^3$H]cAMP as tracer.

**Inhibition of Prolactin Secretion.** Pituitary cells were isolated from estradiol-primed rats as previously described.[19] Prolactin release was stimulated with $10^{-7}$ M TRH. The effect

of varying concentrations of sCT or analogues on prolactin release was measured after 3 h of incubation. Prolactin levels in the culture media were measured by a double-antibody radioimmunoassay using materials and protocols supplied by the National Pituitary Agency of the NIADDK.

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(18) White, A. A.; Karr, D. B. *Anal. Biochem.* 1978, *85*, 451.
(19) Shah, G. V.; Epand, R. M.; Orlowski, R. C. *J. Endocr.* 1988, *116*, 279.

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# Design, Synthesis, and Properties of (4S)-7-(4-Amino-2-substituted-pyrrolidin-1-yl)quinolone-3-carboxylic Acids[†,1]

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The quinolonecarboxylic acids constitute a class of extremely potent and orally active broad-spectrum antibacterial agents. These compounds have been shown to inhibit DNA gyrase, a key enzyme in bacterial DNA replication. The 7-(3-aminopyrrolidinyl)quinolone A-60969 (1) is a particularly potent member of this class and is currently undergoing clinical evaluation. We have studied a series of enantiomerically homogeneous (4S)-7-(4-amino-2-substituted-pyrrolidinyl)quinolones in an effort to utilize the 2-position of the pyrrolidine moiety to improve upon the solubility and pharmacokinetic properties of this class of compounds while still maintaining potent antibacterial activity. We have found that the absolute stereochemistry at the 2-position of the pyrrolidine ring is critical to the maintenance of such activity. In this paper, we report the full details of the asymmetric synthesis and the in vitro and in vivo structure–activity relationships of this series of compounds as well as the physiochemical properties, such as water solubility and log *P*, associated with the structural modifications. We also discuss the pharmacokinetic properties of several of these compounds in mice and the pharmacokinetics of **59**, which has the best overall properties of agents in this study, in dog.

The quinolonecarboxylic acids constitute a class of extremely potent and orally active broad-spectrum antibacterial agents.[2] These compounds have been shown to inhibit DNA gyrase, a key enzyme in bacterial DNA replication.[3] The 7-(3-aminopyrrolidinyl)quinolones **1–3** are
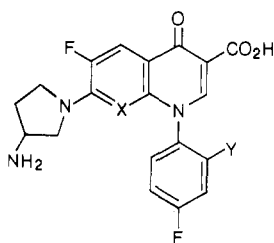


1: X = N; Y = F
2: X = CH; Y = F
3: X = CH; Y = H

particularly potent members of this class; compound **1** (A-60969) is currently undergoing clinical evaluation.[4] Although these (aminopyrrolidinyl)quinolones show excellent antibacterial activity, they usually have very low solubility.

We recently reported that compound **4** is significantly more potent than its enantiomer **5**.[5] We also found that
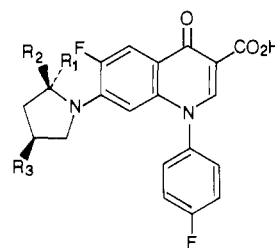


4: R$_1$ = CH$_2$OH; R$_2$ = R$_3$ = H
5: R$_1$ = R$_3$ = H; R$_2$ = CH$_2$OH
6: R$_1$ = R$_2$ = H; R$_3$ = NH$_2$

(1) For a preliminary report of this work, see: Rosen, T.; Chu, D. T. W.; Lico, I.; Fernandes, P. B.; Shen, L.; Pernet, A. *Abstracts of the 27th Interscience Conference on Antimicrobial Agents and Chemotherapy*; New York, NY, October 4–7, 1987; Vol. 115, Abstr. No. 253. Rosen, T.; Chu, D. T. W.; Fesik, S. W.; Cooper, C. S.; Grief, V. J.; Fernandes, P. B.; Shen, L. L.; Pernet, A. G. *Abstracts of Papers*, 193rd National Meeting of the American Chemical Society, Denver, CO, April 5–10, 1987; American Chemical Society: Washington, DC, 1987; MEDI 66. Portions of this work will be presented as part of the International Telesymposia on Quinolones, 1988.
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