excellent technical assistance.

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(27) The system of abbreviation for arginine-vasopressin (AVP) analogues is as previously suggested:[10] lower case "d" to indicate deamination of the N-terminal half-cystine, capital "D" to signify the presence of [D-Arg^8], capital "V" for [Val^4]. Where unspecified, optically active amino acids are of the L configuration.
(28) Following completion of these studies the synthesis and some pharmacological properties of [1-(L-2-hydroxy-3-mercaptopropanoic acid)]lysine-vasopressin were reported.[9]

Potential Inhibitors of Collagen Biosynthesis. 4,4-Difluoro-L-proline and 4,4-Dimethyl-DL-proline and Their Activation by Prolyl-tRNA Ligase

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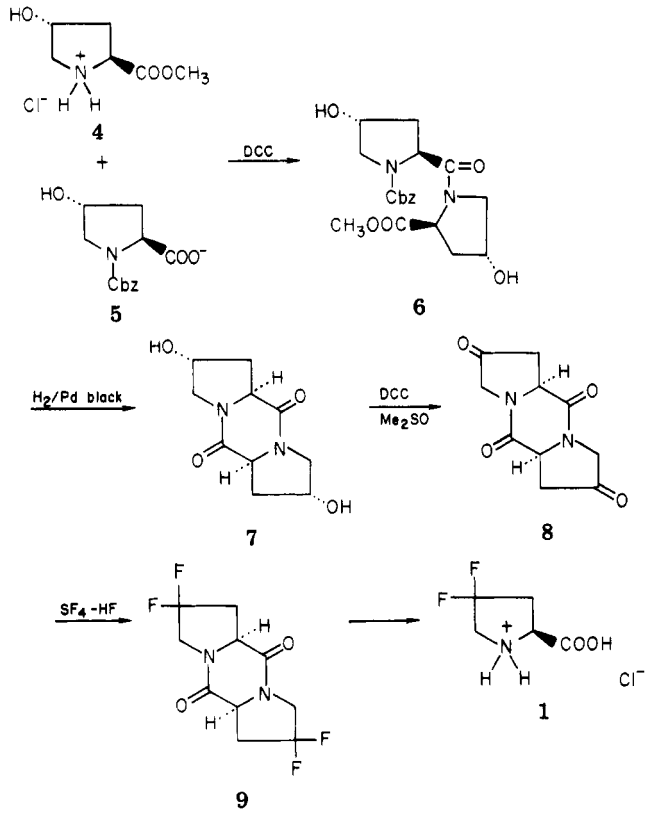
The therapeutic need for selective inhibitors of collagen biosynthesis and the fact that the enzymatic conversion of peptidylproline to peptidyl-4-hydroxyproline occurs almost exclusively in collagen prompted the synthesis of proline analogues bearing geminal blocking substituents at C-4, viz., 4,4-difluoro-L-proline (**1**) and 4,4-dimethyl-DL-proline (**2**). The diketopiperazine (**7**) of 4-hydroxy-L-proline was oxidized with Me_2SO–DCC to the corresponding diketone and the latter fluorinated with SF_4–HF to give the key intermediate, 2,2,7,7-tetrafluorooctahydro-5*H*,10*H*-dipyrrolo[1,2-*a*:1′,2′-*d*]pyrazine-5,10-dione (**9**). Acid hydrolysis of **9** afforded **1**. **2** was prepared by reductive cyclization of α-(β-cyanoethyl)isobutyraldehyde to 5,5-dimethyl-2-piperidone (**13**) which was converted in two steps to 3-chloro-5,5-dimethyl-2-piperidone (**15**). Base-catalyzed rearrangement of **15** gave **2**. The basic dissociation constant of **2** was similar to that of L-proline, while the pK_a of **1** was lower by ~3.5 units. The ability of these proline analogues to be incorporated into procollagen protein was assayed by their ability to stimulate the proline-dependent ATP-PP_i exchange reaction in the presence of prolyl-tRNA ligase. The difluoro analogue **1** stimulated exchange but at a K_m (2.34×10^{-3} M) 100 times greater than that for L-proline itself. The dimethyl analogue **2** did not stimulate exchange but was a competitive inhibitor with an apparent K_i of 1.5×10^{-2} M.

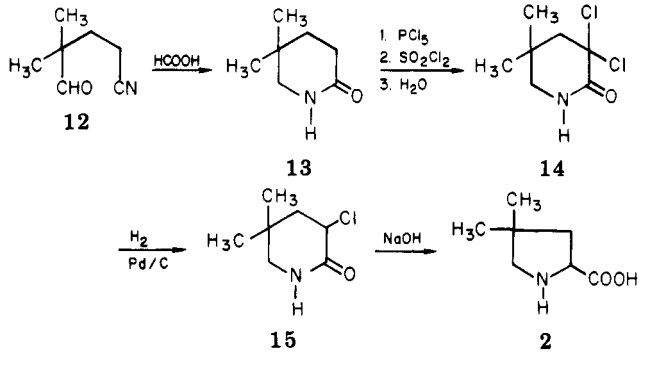
The growing implication of abnormal collagen production in the development of a variety of pathologic conditions[1] including tumor growth[2] suggests the need for selective inhibitors of collagen biosynthesis. Two unique features of collagen which may serve to enhance the selectivity of such inhibition are (a) its low metabolic turnover rate compared to other proteins and (b) the presence of 4-hydroxy-L-proline residues in its peptide structure. The biosynthesis of the latter involves the hydroxylation of the proline moieties which have been incorporated into a collagen precursor, procollagen, by an α-keto glutarate dependent mixed function oxidase, viz., peptidylproline hydroxylase (PPH, E.C. 1.14.11.2; proline 2-oxoglutarate dioxygenase[3]). The exclusiveness of this hydroxylation step suggests that collagen synthesis should be amenable to biochemical manipulation by synthetic analogues of proline wherein the position of hydroxylation is blocked. A number of proline analogues have been reported to be incorporated into proteins in place of L-proline, viz., L-azetidine-2-carboxylic acid,[4,5] 3,4-dehydro-L-proline,[6] thiazolidine-4-carboxylic acid,[7] *cis*-4-hydroxy-L-proline,[8] *cis*-4-fluoro-L-proline,[4,5,9] and *trans*-4-fluoro-L-proline.[9] Microbial studies of the *cis*-4-fluoro-L-proline incorporated into the actinomycin molecule by *S. antibioticus* indicate that it is converted to 4-keto-L-proline.[10] Whether this cis isomer is also converted to 4-keto-L-proline in a mammalian system has yet to be determined.

Although the isomeric 4-monofluoroprolines have been studied extensively, the use of the corresponding geminally disubstituted analogues as inhibitors of collagen synthesis has not been previously explored. 4,4-Difluoro-L-proline (**1**) and 4,4-dimethyl-DL-proline (**2**), the title compounds, represent such proline analogues wherein the position of hydroxylation is blocked by geminal fluoro or methyl substituents. 4,4-Difluoro-L-proline (**1**) on incorporation

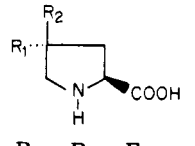
Scheme I



Scheme II



into procollagen was envisioned to directly inhibit PPH by virtue of the presence of an electronegative *gem*-difluoro group at the site of hydroxylation. Alternatively, should metabolic conversion of 1 to 4-keto-L-proline take place by defluorination, the procollagens formed would be expected to be abnormal and thereby retained within the cell as in the case with *cis*-4-fluoroproline. Methyl substitution would, of course, introduce steric factors which could block hydroxylation. Based on this biochemical rationale, the synthesis and biological evaluation of 4,4-difluoro-L-proline (Scheme I) and 4,4-dimethyl-DL-proline (Scheme II) were undertaken.



1, $R_1 = R_2 = F$
2, $R_1 = R_2 = CH_3$
3, $R_1 = OH$; $R_2 = H$

Chemistry. A salient feature of the synthetic route to 1 involved the selective replacement of the carbonyl oxygens in 8 with fluorines using SF_4 as the fluorinating agent (Scheme I).[11] The use of this ketonic diketopiperazine 8 for functional group protection exploits the reported lower reactivity of SF_4 toward amide carbonyls relative to ketonic carbonyls at temperatures below 60 °C. To our knowledge the simultaneous protection of both amino and carboxyl groups of an α-amino acid in such manner has not previously been reported. The synthetic sequence to 1 started with *trans*-4(*R*)-hydroxy-L-(*S*)-proline (3) whose known stereochemistry established the chirality of the α carbon in the final product (1).

The hydroxyproline 3, as its methyl ester hydrochloride 4,[12] was condensed with the dicyclohexylammonium salt of *N*-carbobenzoxy-4-hydroxy-L-proline (5) in the presence of dicyclohexylcarbodiimide (DCC) using the procedure described by Stewart[13] to give 6. Hydrogenolysis of the carbobenzoxy group in 6 with hydrogen/palladium black under neutral conditions[14] gave an intermediate dipeptide ester which spontaneously cyclized to the known diketopiperazine 7.[15] Mild oxidation of 7 with Me_2SO–DCC in the presence of pyridinium trifluoroacetate[16] led, after extensive purification, to the key optically active diketonic tricyclic piperazinedione 8. The electron-ionization mass spectrum (EI–MS) of 8 displayed a molecular ion ($M^{+\cdot}$) at the expected *m/e* of 222 and characteristic fragment ions at *m/e* 194 $[M - CO]^{+\cdot}$ and 167 $[M - HCN - CO]^{+\cdot}$, these fragmentation pathways being verified by the presence of appropriate metastable peaks, and ions at *m/e* 138 and 84. Accurate mass measurements using high-resolution EI–MS substantiated the elemental composition of the molecular ion as well as that of the amine fragment at *m/e* 84, the latter characteristic for this type of diketopiperazine.[17]

Fluorination of 8 with SF_4 in the presence of HF led to the key optically active tetrafluorodiketopiperazine 9 whose mass spectrum displayed a prominent molecular ion ($M^{+\cdot}$) at its expected *m/e* of 266 and a characteristic amine fragment at *m/e* 106. Hydrolysis of 9 with acid gave the desired optically active 4,4-difluoro-L-(*S*)-proline (1). Evidence supporting the structural assignment of 1 was further provided by IR, elemental analysis, and conversion to its corresponding phenylthiohydantoin (10). Both the IR and mass spectral data[18] of 10 were consistent with a phenylthiohydantoin derivative of a difluoro-substituted proline.

The stereochemical integrity of the diketopiperazines 8 and 9 was further adduced by examination of their NMR spectra in Me_2SO-d_6. The central piperazine-2,5-dione ring of tricyclic diketopiperazines has been reported[19] to exist in two stable conformations, viz., the optically active 11a and meso 11b forms for *cyclo*-Pro-Pro (Figure 1). In the optically active forms (*cyclo*-L-Pro-L-Pro, 11a, $R_1 = R_2 =$ H, or as the case may be, *cyclo*-D-Pro-D-Pro) the central piperazine-2,5-dione ring is held in a stable boat conformation by cis–cis ring juncture to the two five-membered rings. In the meso form (*cyclo*-L-Pro-D-Pro, 11b, $R_1 = R_2$ = H) the central ring assumes a more planar conformation imposed by the trans–cis ring junctures. The protons α to the carbonyl groups in 11b are shifted to a higher field due to the shielding effect of the latter in contrast to 11a where such shielding effects are minimal. The lower field absorptions of the α protons for the diketopiperazines 8 and 9 are consistent with an L(*S*),L(*S*) stereochemistry.

4,4-Dimethyl-DL-proline (2) was synthesized as outlined in Scheme II. α-(β-Cyanoethyl)isobutryaldehyde (12) was reductively cyclized in refluxing formic acid to the known 5,5-dimethyl-2-piperidone (13).[20] Chlorination of 13 with PCl_5 and sulfuryl chloride[21] yielded the dichloro lactam

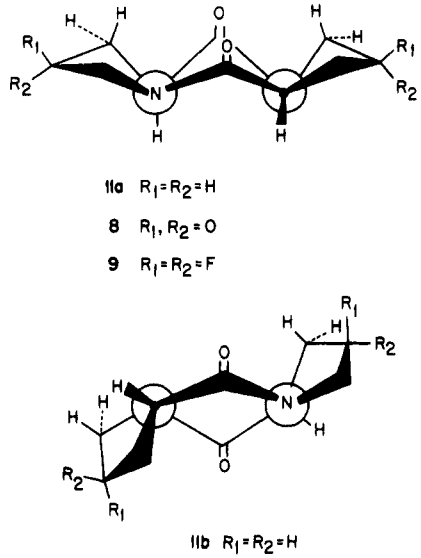


Figure 1. Stereochemical relationship of the optically active (**11a**, 8, and 9) and meso (**11b**) forms of diketopiperazines from proline and its analogues.

14, which on hydrogenolysis gave the monochloro lactam **15**. Hydrolytic rearrangement[22] of the latter gave 4,4-dimethyl-DL-proline (**2**).

The fragmentation patterns of these proline analogues **1** and **2** on chemical ionization mass spectrometry (CI–MS, methane) were entirely consistent with their assigned structure and predictable from the known CI–MS of L-proline itself,[23] viz., presence of a prominent quasimolecular ion (MH^+) and characteristic fragment ions produced by the loss of the elements of H_2O and CO_2H_2 from this quasimolecular ion. Thus, the CI–MS of 4,4-dimethylproline (**2**) displayed a quasimolecular ion (MH^+) and fragment ions corresponding to the loss of 18 and 46 amu from MH^+. The CI–MS of 4,4-difluoroproline (**1**) was more complex due to the loss of the elements of HF superimposed on this basic pattern as evidenced by additional fragment ions at m/e 132, 114, and 86.

The elution patterns of these 4,4-disubstituted prolines **1** and **2** on a cation-exchange column used for the chromatographic separation of amino acids in biological fluids are shown in Figure 2 (Supplementary Material). Their retention times are compared to that of L-proline and 4-hydroxy-L-proline. These amino acids were eluted in order of their respective pK_a values, and, where these were similar, in order of their molecular weights.

The basic dissociation constants determined for **1** and **2** are compared to the pK_a (conjugate acid) reported for L-proline in Table I.[24] The pK_a of the dimethyl-substituted **2** was similar to that of L-proline, but the pK_a of the difluoro-substituted **1** was 3.5 units lower due to the strong inductive effect of the *gem*-difluoro group on the amino nitrogen. Thiazolidine-4-carboxylic acid, where sulfur is substituted for the CH_2 at position 4, had a slightly lower pK_a, while the N-hydroxylated 1-hydroxy-L-proline[25] had the lowest pK_a, viz., 5.81.

Biological Results. Inhibition of collagen biosynthesis by such proline analogues as **1** and **2** requires prima facie that they be activated by the amino acid activating system specific for L-proline for subsequent incorporation into protein. The activation of **1** and **2** by prolyl-tRNA ligase (eq 1) was therefore determined using a partially purified preparation of this enzyme.[26] Although 4,4-difluoro-L-proline (**1**) was activated by this system as evidenced by

$$E + AA + ATP \xrightleftharpoons{Mg^{2+}} E\text{-}AA\text{-}AMP + PP_i \quad (1)$$

Table I. Comparison of pK_a and Kinetic Parameters in the ATP–PP_i Exchange Reaction of Proline and Related Analogues

Compd	pK_a (NH_2^+)[a]	K_m (or K_i),[b] M	V_{max}, mmol/min/mg
L-Proline	10.65 ± 0.02	5.31 ± 0.30 × 10^{-4}	125.1
		2.33 × 10^{-4} [c]	100[c]
2	10.58 ± 0.03	(1.5 × 10^{-2})	
1	7.09 ± 0.03	2.34 ± 0.06 × 10^{-2}	24.2
Thiazolidine-4-carboxylic acid	6.29 ± 0.03	5.5 × 10^{-2} [c]	101[c]
1-Hydroxy-L-proline	5.81 ± 0.02	(9.8 × 10^{-6})	
trans-4-Fluoro-L-proline			66[c]

[a] Determined by the method of Albert and Sergeant.[24] [b] For Lineweaver–Burk plots, see Figures 4–6, Supplementary Material. [c] Reported by Papas and Mehler.[26]

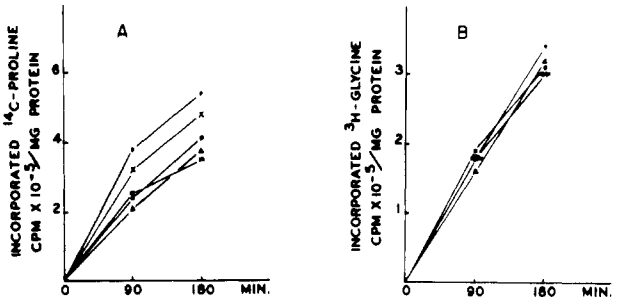


Figure 3. Incorporation of [^{14}C]proline (A) or [^{3}H]glycine (B) into L-929 fibroblast proteins in the presence of 4,4-difluoro-L-proline (**1**): (●—●) controls; (×—×) 0.2 mM; (○—○) 1.0 mM; (□—□) 2.0 mM; (△—△) 5.0 mM.

stimulation of ATP–PP_i exchange, the observed K_m value was 100-fold larger than that reported for L-proline itself and was comparable to the K_m reported for L-thiazolidine-4-carboxylic acid (5.5 × 10^{-2} M) (Table I). 4,4-Dimethyl-DL-proline (**2**) and 1-hydroxy-L-proline[25] did not stimulate ATP–PP_i exchange but competitively inhibited exchange with apparent K_i values of 1.5 × 10^{-2} and 9.8 × 10^{-6} M, respectively.

The ability of 4,4-difluoro-L-proline (**1**) to substitute for L-proline in a collagen-synthesizing system was further suggested by its dose-dependent inhibition of [^{14}C]proline incorporation into the proteins of L-929 fibroblast cells[27] (Figure 3, A). Since the incorporation of [^{3}H]glycine was not inhibited by **1** (Figure 3, B) overall protein synthesis was unaffected. Compared to 3,4-dehydro-L-proline, however, **1** was not as effective an inhibitor in this system, apparently a consequence of itself being less efficiently incorporated into protein than dehydroproline.[28] **1** and **2** were also tested for antitumor activity against P-388 lymphocytic leukemia cells in suspension culture,[29] but they did not significantly inhibit growth of these tumor cells even at concentrations as high as 1.1 mM.

Results and Discussion

In comparing the effect of the cis and trans isomers of 4-fluoro-L-proline on protein biosynthesis in vivo, Bakerman et al.[9b] reported that the cis isomer was inhibitory, although the trans isomer was more readily incorporated into cellular proteins.[9a] The cis isomer was shown to be utilized by a collagen-synthesizing system with the production of abnormal collagens which were retained in the cell.[4,5] Replacement of $-CH_2-$ by $-CF_2-$ at the 4 position

in L-(S)-proline was not expected to introduce major steric effects in the resulting analogue 1 due to the relatively small atomic radius of fluorine and the short C–F bond distance.[30] It was not unreasonable to expect then that 1 might also be incorporated into collagen, once activated by prolyl-tRNA ligase.

On the other hand, the pronounced electronic effect of the $-CF_2-$ group[31] could not be discounted and the proximity of the highly electronegative fluorine atoms β to the amino function was found to significantly lower the pK_a of this amino group (Table I). Studies by Papas and Mehler[25] on the binding requirements of the amino acid site on prolyl-tRNA ligase suggest that the secondary amino group of L-proline is the major locus for binding to this enzyme. Thus, the observed 100-fold larger K_m of 1 in this system may be a consequence of the low basicity of this group. Yet, the decreased basicity of the amino group cannot be the sole factor responsible for the lower affinity of 1 for the enzyme, since L-thiazolidine-4-carboxylic acid with an even lower pK_a is readily incorporated into proteins.[7] While the K_m of the latter is larger, its V_{max} value is comparable to that of proline. The observed V_{max} of 1 which is 20% of that for proline is even lower than that reported for *trans*-4-fluoro-L-proline (66% of L-proline). The disappointing lack of significant inhibition of PPH by 1[28] suggests that unless the proline analogue is more readily incorporated into collagen, inhibition of PPH may not be expected.

The strong inhibition ($K_i \sim 10^{-5}$ M) of ATP–PP_i exchange by the nitrogen-substituted 1-hydroxy-L-proline with pK_a of 5.81 was unanticipated. The consequence of this in vitro inhibition of proline activation by its *N*-hydroxy analogue is not manifested by toxicity in vivo since doses of this proline analogue as high as 500 mg/kg administered intraperitioneally were nontoxic to mice.[25] 1-Hydroxy-L-proline is also metabolized predominantly to CO_2 in rodents. Further studies are required to assess the role, if any, of this *N*-hydroxyproline analogue on collagen biosynthesis.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus or in a Mel-Temp apparatus and are corrected to reference standards. All solvent evaporations were carried out on a mechanical rotating evaporator using water aspiration. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn., or by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. Spectrometers used were IR, Beckman IR-10; NMR, Varian A-60D; EI–MS, Hitachi Perkin-Elmer RMU-6 (ionization energy, 70 eV; ion source temperatures as indicated). High-resolution EI–MS and CI–MS were provided by Dr. Roger Foltz, Battelle Columbus Laboratories, Columbus, Ohio, using an AEI-MS-902 mass spectrophotometer equipped with an SRIC Model CIS-2 combined CI–EI ion source. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter, and pK_a values were determined using a Beckman Century SS expanded scale pH meter equipped with a Beckman Futura combination electrode and a Mettler digital buret.

***N*-Carbobenzoxy-4-hydroxy-L-prolyl-4-hydroxy-L-proline Methyl Ester (6).** A suspension of powdered 5 (2.2 g, 5.0 mmol) and 4[12] (1.0 g, 5.0 mmol) in 50 mL of $CHCl_3$ was shaken for 1 h. To the cooled (4 °C) reaction mixture, dicyclohexylcarbodiimide (1.03 g, 5.0 mmol) in 30 mL of $CHCl_3$ was added. After stirring for 24 h, the reaction was quenched by pouring the mixture into 300 mL of ice water. The $CHCl_3$ layer was separated and evaporated to dryness and the residue combined with the aqueous layer. Insoluble dicyclohexylurea was removed by filtration and the aqueous filtrate lyophilized. The residue was extracted with acetone (300 mL) and the concentrated extract (120 mL) was passed through a 2 × 32 cm column of silica gel (30–70 mesh). Fractions (100 mL) were eluted with acetone. Recrystallization of fractions 2 and 3 from MeOH–EtOAc–petroleum ether (bp 30–60 °C) (10:130:80) yielded 1.61 g (82.3%) of colorless crystals: mp 151–153 °C; $[\alpha]^{27}_D$ −112.3° (*c* 1.03, H_2O). Anal. ($C_{19}H_{24}N_2O_7$) C, H, N.

Octahydro-2,7-dihydroxy-5*H*,10*H*-dipyrrolo[1,2-*a*:1′,2′-*d*]pyrazine-5,10-dione (7). Hydrogenolysis of 6 (4.2 g, 10.7 mmol) was carried out in MeOH–H_2O (99:1) with H_2/Pd black. Recrystallization of the product from EtOH–Et_2O yielded 2.37 g (99.3%) of 7 as white crystals: mp 243–246 °C; $[\alpha]^{25}_D$ −153.9° (*c* 0.965, H_2O) [lit.[15] mp 245–246 °C; $[\alpha]^{18}_D$ −153.4° (*c* 2.05, H_2O)].[27] Anal. ($C_{10}H_{14}N_2O_4$) C, H, N.

Dihydro-1*H*,5*H*-dipyrrolo[1,2-*a*:1′,2′-*d*]pyrazine-2,5,7,10(3*H*,8*H*,10a*H*)-tetrone (8). To 1.5 g (6.65 mmol) of 7 in 35 mL of Me_2SO (dried over Linde molecular sieves, 4A) were added 1.08 mL of dry pyridine and 0.54 mL of 99% trifluoroacetic acid.[16] Dicyclohexylcarbodiimide (8.34 g, 40.4 mmol) in 30 mL of dry benzene was added slowly and the reaction stirred for 5 days at room temperature. The reaction mixture was then poured into ice water, the insoluble dicyclohexylurea was removed by filtration, and the precipitate was washed thoroughly with water. The combined aqueous filtrates were extracted with benzene (twice) followed by $CHCl_3$ (twice) and the organic layers were back-extracted with water. The combined aqueous layers were evaporated to remove residual solvents and then lyophilized to remove residual Me_2SO. The solid residue was suspended in $CHCl_3$ and filtered. The $CHCl_3$ insoluble solids were dissolved in acetone (2 L), treated with Norite A, and taken to dryness in vacuo. The residue was dissolved in hot CH_3CN and treated with Norite A. Recrystallization from CH_3CN–Et_2O gave 0.95 g (64%) of white plates: mp 296 °C dec; $[\alpha]^{28}_D$ −248.5° (*c* 0.945, H_2O); IR (KBr) 1775, 1760 (C=O), 1665 (amide C=O), 1425 (CN), 1165 cm^{-1}; NMR, δ from Me_4Si (Me_2SO-d_6), 4.93 (t, 2 H, *J* = 9 Hz), 4.01 (d, 2 H, *J* = 19 Hz), 2.74 (d, 2 H, *J* = 19 Hz), 2.85 (d, 4 H, *J* = 9 Hz); EI–MS (300 °C) *m/e* >60 amu (rel intensity > 15%) 222 (100, $M^{+}\cdot$), 194 (19), 167 (19), 166 (16), 138 (58), 84 (61), metastable ions at *m/e* 169.5, 125.5, 115.0, 22.0 (calcd: 169.5, 125.6, 114.7, 21.9); EI–MS (high resolution, 200 °C) *m/e* > 60 amu (rel intensity) calculated for $C_{10}H_{10}N_2O_4$, 222.0641 [found 222.0629 (97, $M^{+}\cdot$)]; calculated for $C_9H_{10}N_2O_3$, 194.0691 [found 194.0685 (9)]; calculated for $C_8H_8NO_3$, 166.0504 [found 166.0484 (25)]; calculated for $C_7H_8NO_2$, 138.0555 [found 138.0546 (57)]; calculated for C_4H_6NO, 84.0449 [found 84.0452 (100)]. Anal. ($C_{10}H_{10}N_2O_4$) C, H, N.

2,2,7,7-Tetrafluorooctahydro-5*H*,10*H*-dipyrrolo[1,2-*a*:1′,2′-*d*]pyrazine-5,10-dione (9). The diketone 8 (1.0 g, 4.5 mmol) and 400 μL of H_2O were sealed in a 40-mL monel Parr bomb equipped with a gas inlet. The system was evacuated at 12–15 mmHg for 10–15 min while cooling in a dry ice–acetone bath. Gaseous SF_4 was then allowed to fill the evacuated bomb. After 15 min, the valve to the bomb was closed and the external line cleared. The weight of SF_4 (40.4 g, 0.374 mol) was determined by weight difference. The bomb was shaken for 3 days at room temperature and then cooled in a dry ice–acetone bath and the gaseous contents were released through 5 N NaOH. Residual SF_4 and HF were removed by evacuating the system briefly using a water aspirator. The residual glass was dissolved in CH_3CN, decolorized with charcoal, and taken to dryness. The residue was dissolved in $CHCl_3$ and passed through a 2 × 30 cm column of silica gel (30–70 mesh). The first 200 mL of $CHCl_3$ eluates were discarded. The remaining $CHCl_3$ eluates (800 mL) containing 9 were concentrated in vacuo. Recrystallization of the residue from CH_3CN–Et_2O gave 0.85 g of 9 (70.8%): mp 220–221 °C; $[\alpha]^{28}_D$ −113.3° (*c* 1.15, $CHCl_3$); IR (KBr) 1680, 1660 (amide C=O), 1440 (CN), 1120, 1105 cm^{-1} (CF); NMR δ from Me_4Si (Me_2SO-d_6) 4.80 (t, 2 H, *J* = 9 Hz), 3.90 (m, 4 H); EI–MS (270 °C) *m/e* > 60 amu (rel intensity > 20%) 266 (95, $M^{+}\cdot$), 238 (14), 132 (46), 118 (15), 106 (100), 105 (51), 86 (18); EI–MS (high resolution, 200 °C) *m/e* > 60 amu (rel intensity) calculated for $C_{10}H_{10}N_2O_2F_4$, 266.0678 [found 266.0671 (95, $M^{+}\cdot$)]; calculated for $C_{10}H_{10}N_2O_2F_3$, 247.0694 [found 247.0715 (20)]; calculated for $C_9H_{10}N_2OF_4$, 238.0729 [found 238.0712 (20)]; calculated for $C_4H_6NF_2$, 106.0468 [found 106.0448 (100)]; calculated for $C_4H_5NF_2$, 105.0390 [found 105.0378 (20)]; calculated for C_4H_5NF, 86.0406 [found 86.0396 (9)]. Anal. ($C_{10}H_{10}N_2O_2F_4$) C, H, N, F.

4,4-Difluoro-L-proline (1). The tetrafluoro derivative 9 (2.64 g, 9.93 mmol) was divided in three portions and each portion was suspended in 4.5 mL of 6 N HCl in three reactor tubes. The

reactor tubes were sealed and heated at 110 °C in a micro Carius furnace for 53 h. The acidic solution was extracted repeatedly with $CHCl_3$, decolorized with charcoal, and taken to dryness. The residue was dissolved in H_2O, adjusted to pH 1, and passed through a 2 × 30 cm column of Dowex 50-X8 (NH_4^+, 200–400 mesh). The column was washed with water until neutral and the amino acid eluted with 7 N aqueous ammonia. The fractions containing product were concentrated in vacuo and the combined residues recrystallized from aqueous EtOH to give 1 (1.0 g, 36.7%): mp 252–256 °C dec; $[\alpha]^{29}_D$ −34.5° (c 1.0, H_2O); IR (KBr) 2400–2800 (NH_2^+), 1620 (NH_2^+), 1595 (COO^-), 1145, 1090, 1045 cm^{-1} (CF); CI–MS (methane) m/e (% $\sum_{50}$) 152 (13, MH^+), 132 (9, $MH^+ - HF$), 114 (4, $MH^+ - HF - H_2O$), 116 (35, $MH^+ - COOH_2$); NMR, δ from DDS (D_2O), 4.5 (t, 1 H, J = 8 Hz), 3.87 (t, 2 H, J = 12 Hz), 2.80 (m, 2 H). Anal. ($C_5H_7NO_2F_2$) C, H, N, F.

Phenylthiohydantoin of 1 (10). The phenylthiohydantoin 10 was prepared from 1 by the procedure of Edman[32] and recrystallized from HOAc–H_2O to give 0.2 g (75%) of white crystals: mp 161 °C; IR (KBr) 1760 (C=O), 1600 (ArC=C), 1440 cm^{-1} (C=S); EI–MS (240 °C) m/e > 40 (rel intensity) 268 (100, $M^+\cdot$), 135 (100), 105 (14.7). Anal. ($C_{12}H_{10}N_2OSF_2$) C, H, N, F.

5,5-Dimethyl-2-piperidone (13). 4-Cyano-2,2-dimethylbutyraldehyde (12) (100 g, 0.8 mol) was heated under reflux in formic acid (200 mL) for 3 days. The excess formic acid was removed by distillation until 150 mL was removed. The residue was diluted with 200 mL of water; the mixture was cooled in ice and neutralized to pH 8 with 6 N NaOH. The aqueous solution was extracted with $CHCl_3$ in a continuous liquid–liquid extractor for 16 h. The $CHCl_3$ extract was then dried over Na_2SO_4, decolorized with charcoal, and taken to dryness. Recrystallization of the residue from CH_2Cl_2–hexane gave 35.5 g (34.7%) of 13 as colorless needles: mp 123.5–123.9 °C. An analytical sample recrystallized from Et_2O and dried at 50 °C under vacuum for 5 h melted at 123.8–124 °C (lit.[20] 124–127 °C). Anal. ($C_7H_{13}NO$) C, H, N.

3,3-Dichloro-5,5-dimethyl-2-piperidone (14). To a cold (0 °C) stirred solution of 13 (12.7 g, 0.10 mol) in 200 mL of $CHCl_3$, PCl_5 (22.9 g, 0.11 mol) was added at such a rate that the temperature never exceeded 7 °C. After the addition was complete stirring was continued for 10 min. Sulfuryl chloride (40.5 g, 0.30 mol) was slowly added and the mixture heated under reflux for 1 h. The $CHCl_3$ was removed by distillation and the solution further concentrated in vacuo to remove traces of sulfuryl chloride. The residue was cooled in ice and diluted with 50 g of ice and 50 g of water. The product was then extracted with $CHCl_3$ in a continuous liquid–liquid extractor for 15 h and the $CHCl_3$ extract was concentrated and passed through a 3 × 40 cm column of silica gel (30–70 mesh). The $CHCl_3$ eluates (200 mL each) from fractions 2–6 were collected and concentrated to give the dichloro lactam 14 which was recrystallized from CH_2Cl_2–hexane to give white crystals (14.1 g, 72%), mp 141–144 °C. Anal. ($C_7H_{11}NOCl_2$) C, H, N.

3-Chloro-5,5-dimethyl-2-piperidone (15). To 9.80 g (0.050 mol) of 14 dissolved in 100 mL of glacial HOAc was added 10% Pd/C (1.5 g) and NaOAc (9.0 g, 0.11 mol) and the mixture hydrogenated at 3.0 kg/cm^2 (42 psi) starting pressure. When hydrogen uptake ceased (2.6 kg/cm^2, 37.5 psi), the catalyst was removed by filtration and the filtrate concentrated in vacuo. $CHCl_3$ (75 mL) and water (75 mL) were added to the filtrate and the aqueous layer was neutralized with 4 N NaOH. The mixture was then extracted in a continuous liquid–liquid extractor for 16 h with $CHCl_3$. After decolorization with charcoal, the $CHCl_3$ extract was concentrated and the residue recrystallized from $CHCl_3$–hexane to yield 7.65 g (96%) of 15, mp 105–107 °C. Anal. ($C_7H_{12}NOCl$) C, H, N.

4,4-Dimethyl-DL-proline (2). A suspension of 15 (3.83 g, 0.020 mol) in 100 mL of 6 N NaOH was heated on a steam bath for 30 min. The solution was cooled and adjusted to pH 8 with concentrated HCl and then concentrated in vacuo until salt formation was observed. Water (50 mL) and $CuCO_3$ (2.7 g, 0.22 mol) were added to the stirred solution and the suspension was digested on the steam bath for 30 min. The solids were collected (saving the filtrate), washed thoroughly with water, and repeatedly extracted with MeOH until no further blue copper complex of the amino acid was extractable. The excess $CuCO_3$ remaining was added to the aqueous filtrate above and the digestion and extraction process repeated. The combined MeOH extracts were concentrated in vacuo to precipitate the blue copper complex of 2. The chloride-free complex was dissolved in 400 mL of hot methanol and 2.9 g (0.02 mol) of 8-hydroxyquinoline was added. The precipitate which formed was removed by filtration and the filtrate concentrated to 200 mL. Water (200 mL) was added, and the mixture was again concentrated to 250 mL to remove MeOH and then thoroughly extracted with Et_2O. The aqueous phase was decolorized with charcoal and concentrated in vacuo to dryness. Recrystallization of the residue from 95% EtOH (25 mL) gave 1.60 g (56%) of white crystals: mp 245–246 °C; IR (KBr) 2250–2800 (NH_2^+), 1615, 1575 (NH_2^+, COO^-), 1380, 1355, 1210, 1185; NMR, δ from DDS (D_2O), 4.23 (t, 1 H, J = 8 Hz), 3.13 (s, 2 H), 2.23 (q, 1 H, $J_{\alpha,\beta}$ = 8 Hz, $J_{\beta,\beta}$ = 14 Hz), 1.83 (q, 1 H, $J_{\alpha,\beta}$ = 8 Hz, $J_{\beta,\beta}$ = 14 Hz), 1.15 (s, 6 H); CI–MS (methane) m/e > 60 (% $\sum_{60}$) 144 (25, MH^+), 126 (2, $MH^+ - H_2O$), 98 (35, $MH^+ - COOH_2$); CI–MS (isobutane) m/e > 60 (% $\sum_{60}$) 144 (50, MH^+). Anal. ($C_7H_{13}NO_2$) C, H, N.

Ion-Exchange Chromatography. A Beckman Spinco Model 120 amino acid analyzer was used with UR-30 cation-exchange resin, column size 0.9 × 56 cm, flow rate 75 mL/h. A mixture containing 1.0 μmol of each of the amino acids was applied to the column; L-proline and *trans*-4-hydroxy-L-proline served as standard markers. (See Figure 2, Supplementary Material.)

In Vitro Enzyme Inhibition and Growth Inhibition Studies. The prolyl-tRNA ligase[26] isolated from *Escherichia coli* B had a specific activity 1 × 10^3 units/mg of protein. One unit of prolyl-tRNA ligase activity is defined as the amount of enzyme required to stimulate the exchange of 1 μmol of [^{32}P]pyrophosphate into ATP under the assay conditions used.[26] K_m values for L-proline and 4,4-difluoro-L-proline (1) were determined using the Lineweaver–Burk relationship. Inhibition of L-proline stimulated exchange was determined at fixed inhibitor concentrations, viz., 25.0 mM 4,4-dimethyl-DL-proline (2) (12.5 mM of L isomer) and 6.25 or 12.5 mM *N*-hydroxy-L-proline. Apparent K_i values[33] were determined from the slopes of the double reciprocal plots of L-proline in the presence and absence of inhibitor using the value of K_m/V_{max} determined for L-proline.

The effect of varying concentrations of 1 on the incorporation of [^{3}H]glycine and [^{14}C]proline into TCA precipitable proteins of L-929 fibroblasts was determined according to Kerwar et al.[34] PPH activity was assayed by the method of Hutton et al.[35]

Growth inhibition of P-388 lymphoid leukemia cells in tissue culture was evaluated using varying concentrations of 1 and 2 using published procedures.[29]

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Supplementary Material Available: Ion-exchange chromatographic behavior of 4,4-difluoro-L-proline (1) and 4,4-dimethyl-DL-proline (2) (Figure 2); Lineweaver–Burk plots (Figures 4–6) of the ATP–PP_i exchange reaction with prolyl-tRNA ligase and 4,4-difluoro-L-proline, 4,4-dimethyl-DL-proline, and 1-hydroxy-L-proline (4 pages). Ordering information is given in any current masthead page.

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Inhibitors of *Bacillus subtilis* DNA Polymerase III. Structure–Activity Relationships of 6-(Phenylhydrazino)uracils

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6-(Phenylhydrazino)uracils inhibit the replication-specific enzyme DNA polymerase III of *Bacillus subtilis* by forming a strong, reversible complex with template–primer DNA and enzyme. The phenyl ring interacts with a hydrophobic enzyme site which, on the basis of structure–activity relationships of substituted analogues, appears to possess the following characteristics: (1) planarity or near-planarity; (2) a finite capacity to accommodate bulky substituents; and (3) location near the domain of the enzyme active site. A mutant DNA polymerase III, derived from a mutant strain of *B. subtilis* selected for resistance to 6-(*p*-hydroxyphenylazo)pyrimidines, is resistant only to inhibitors bearing *p*-hydroxy or amino groups and is hypersensitive to inhibitors containing nonpolar substituents; these results suggest the existence of mutable, secondary regions of the binding site which interact with para substituents and, thus, influence the strength of the primary phenyl–enzyme interaction.

6-(Arylazo)pyrimidine antimicrobials selectively inhibit replicative DNA synthesis in gram-positive bacteria by inhibiting, specifically, the replication-specific enzyme, DNA polymerase III (pol III).[1-3] The azo compounds are not active per se but are activated in vivo by reduction to hydrazino (H_2) forms.[4] The arylhydrazinopyrimidines act, in part, as analogues of purine deoxyribonucleoside triphosphates by pairing with pyrimidine bases in template DNA. The specific purine character of an inhibitor is dictated by the substitution pattern of its pyrimidine ring; for example, 6-(*p*-hydroxyphenylhydrazino)uracil (H_2-HPUra, 1) is competitive with deoxyguanosine 5′-triphosphate (dGTP), pairing specifically with cytosine in the template, and 6-(*p*-hydroxyphenylhydrazino)-2-amino-4-pyrimidone (H_2-HPiCyt, 2) is competitive with deoxyadenosine 5′-triphosphate (dATP), pairing specifically with thymine in the template[4] (see Figure 1). Although H_2-HPUra and H_2-HPiCyt mimic dGTP and dATP, they do not act simply as conventional substrate analogues. They actually inhibit by reacting reversibly with an inhibitor-specific binding site on the enzyme, linking template *and* enzyme in a strong, catalytically inactive ternary complex.[5,6] A model for the ternary complex is depicted in Figure 2.

Examination of the properties of drug-resistant enzymes[6,7] isolated from spontaneous *Bacillus subtilis* mutants resistant to HPUra and HPiCyt has yielded valuable information on the nature of the inhibitor binding