Articles

Novel Inhibitors of Prolyl 4-Hydroxylase

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A series of 5-acyl sulfonamides derived from pyridine-2,5-dicarboxylic acid (15) has been prepared and several members of this series have been shown to be more potent, in vitro, as inhibitors of prolyl 4-hydroxylase than 15. Several chain-extended pyridinedicarboxylic acids have also been prepared and shown to be potent inhibitors of prolyl 4-hydroxylase. The structure–activity in both these series is discussed. The results indicate that the 5-carboxylic acid binding site, in the enzyme, can accept a carboxylic acid or an acyl sulfonamide equally well. This indicates a much greater degree of freedom in this distal carboxylic acid binding site than is predicted by the current theorical model of the active site.

Introduction and Background

Each year 25 000 people die prematurely from fibrotic liver diseases in the United States, where cirrhosis is the ninth leading cause of death.[1] Other fibrotic diseases[2] such as idiopathic and pulmonary fibroses, renal fibrosis, and the diffuse cardiac fibrosis of progressive heart failure also lead to premature death, while the fibrotic component of less severe conditions such as scleroderma and rheumatoid arthritis disable and disfigure many more. Inhibition of prolyl 4-hydroxylase (EC 1.14.11.2) [procollagen-L-proline,2-oxo-glutarate:oxygenoxidoreductase (4-hydroxylating)][3] is of therapeutic interest because the enzyme is essential for the biosynthesis of the collagen deposited during these life-threatening fibroses.[4] During these fibrotic states the involved organ or tissue becomes congested with large amounts of collagen.

Collagenous proteins are characterized by the noncovalent association of three chains into a relatively inert rodlike triple-helix.[5] It is the resistance of these triple-helical rods to proteolysis that is responsible for the inability of the body to degrade the excessive accummulation of collagen.[6] The stability of the triple-helical domains is known[5] to be dependent on the extent of the conversion carried out by prolyl hydroxylase, i.e. the conversion of many of the X-Pro-Gly sequences into X-Hyp-Gly sequences [H-Hyp-OH = (2*S*,4*R*)-4-hydroxyproline] (Scheme I). Insufficiently hydroxylated chains do not form triple-helical domains that are stable at body temperature; therefore they remain gelatinous rather than collagenous proteins and are susceptible to normal catabolism. Inhibition of prolyl hydroxylase should therefore prevent the undesirable accumulation of newly synthesized collagen in fibrotic diseases.

There are, however, a dozen or more collagens[7-9] which are each discrete and defined structural proteins of the extracellular matrix. Several other biologically important macromolecules contain significant collagenous regions such as the C1q component of the classical pathway of complement,[10] the acetylcholine esterase of neuromuscular junction endplate,[11] conglutinin,[12] hepatic mannose binding proteins,[13] pulmonary surfactant apoproteins,[14] and the macrophage receptor for acetyl low density lipoprotein (Ac-LDL).[15]

By inhibiting prolyl hydroxylase, there is also the possible toxic consequence of interfering both with normal collagen turnover and with the biosynthesis of other vital collagenous molecules.

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(8) Gordon, M. K.; Gerecke, D. R.; Dublet, B.; van der Rest, M.; Olsen, B. R. Type XII Collagen. A Large Multidomain Molecule with Partial Homology to Type IX Collagen. *J. Biol. Chem.* **1989**, *264*, 19772-19778.
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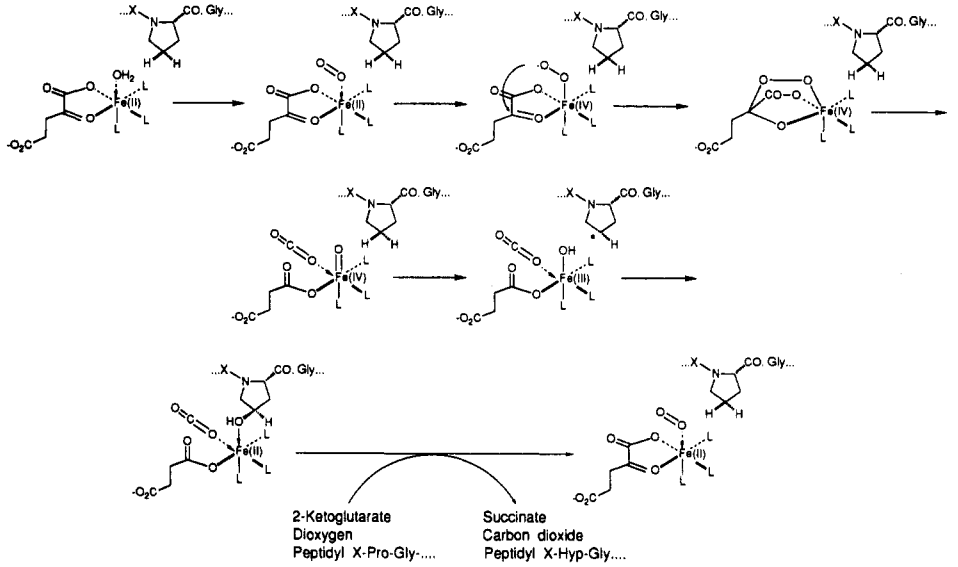
Scheme I. Oxidative Decarboxylation of 2-Ketoglutarate during the 4-Hydroxylation of Peptidylproline



Clinical evaluation of the net benefit of a prolyl hydroxylase inhibitor in the treatment of life-threatening fibrotic conditions would be very desirable.[16,17] However, no agents are available that would be suitable for such an investigation. In this paper we describe a series of novel acyl sulfonamides that are potent inhibitors, in vitro, of prolyl hydroxylase.

Enzyme Mechanism

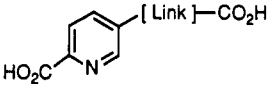
It seems probable that the hydroxylation is carried out by an iron(IV) oxo species and that this species is generated by oxidative decarboxylation of 2-ketoglutarate in the coordination shell of enzyme-bound iron(II) (Scheme I). The exact molecular details of the enzyme reaction are not known with certainty but the active-site chemistry recently proposed by Hanauske-Abel and Guenzler[18] and summarized in Scheme I provides a sound basis for work in this area.

This, the best mechanistic hypothesis available, emphasizes the availability of a binding site for 2-ketoglutarate in the region of the iron(II) held at the catalytic site of the enzyme. The iron(II) is assumed to form a 5-membered chelate with the planar, anionic, bidentate oxo carboxylate ligand while a lipophilic spacer presents the distal carboxylate to an unspecified anion binding-site on the enzyme.

In pyridine-2,5-dicarboxylic acid, the carboxylate group and nitrogen atom of the pyridine ring are thought to chelate with the iron(II) while the 5-carboxylic group is well placed to occupy the distal carboxylate binding site.

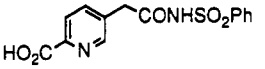
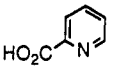
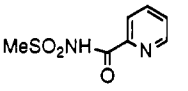
It was considered[19] that ligands which could bind to the iron(II) but could not undergo the catalytically important oxidative decarboxylation would be inhibitors of the enzyme, and this idea lead to the successful prediction, by Hanauske-Abel and Guenzler, of the inhibitory properties of pyridine-2,5-dicarboxylic acid.

Table I. Structure and Biological Data for Chain-Extended Pyridinedicarboxylic Acids



compd no.	link	IC_{50}, μM	comment
4	—CH_2—	9.6	
5		27	*R*, *S* mixture
10		10.6	trans
11		9.6	
15	none	5.5	Aldrich Chemical Co.

Table II. Structures and Biological Data for 6, 16, and 17

compd no.	structure	IC_{50}, μM
6		17
16		100
17[a]		500

[a] We thank Dr. E. R. H. Walker for this compound.

Results and Discussion

On the basis of the successful prediction of the inhibitory activity of pyridine-2,5-dicarboxylic acid, we decided to investigate the 2- and 5-carboxylic acid binding sites. This investigation was carried out (a) by varying the distance between the 2-carboxylic acid chelation site and the 5-carboxylic acid binding site, and (b) by replacing the carboxylic acid group in the 2- and 5-positions with an acid mimic, e.g. acyl sulfonamide.

Considering first the spacial orientation of the carboxylic acid group at the 5-position of the pyridine ring, it can be seen from Table I that the potency of compounds 4, 10, and 11 does not change as the carboxylic acid is moved

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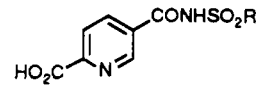
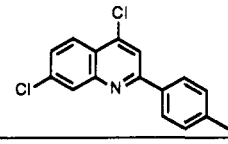
Table III. Chemical and Biological Data for Acyl Sulfonamides



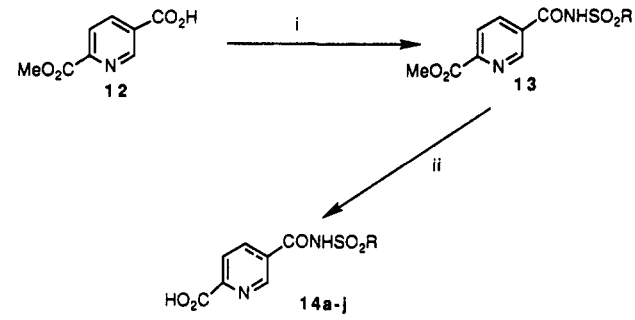
compd no.	R	recryst solvent	mp, °C	formula	anal.	adduct	IC_{50}, μM
14a	Me	ethanol	241–3	$C_8H_8N_2O_5S$	C, H, N	0.2 M NaCl	1.8
14b	Pr^i	ethanol	>250	$C_{10}H_{12}N_2O_5S$	C, H, N	0.75 M NaCl	1.6
14c	phenyl	acetic acid	243–4	$C_{13}H_{10}N_2O_5S$	C, H, N		1.1
14d	benzyl	ethanol	236–8	$C_{14}H_{12}N_2O_5S$	C, H, N		3.0
14e	1-naphthyl	ethanol	>250	$C_{17}H_{12}N_2O_5S$	C, H, N	monohydrate	4.4
14f	8-quinolyl	water	240–3	$C_{16}H_{11}N_3O_5S$	C, H, N	2Na salt·$3H_2O$	1.8
14g	2-(5-chlorothienyl)	not recryst	>250	$C_{11}H_7ClN_2O_5S_2$	C, H, N	2Na salt·$0.5H_2O$	2.4
14h	2-(4,5-dibromothienyl)	not recryst	240–2	$C_{11}H_6Br_2N_2O_5S_2$	C, H, N	2Na salt	8.5
14i	4-methoxyphenyl	not recryst	228–31 (d)	$C_{14}H_{12}N_2O_6S$	C, H, N	Na salt	1.0
14j		not recryst	218–21 (d)	$C_{22}H_{13}Cl_2N_3O_5S$	C, H, N	2Na salt	6.6

away from the pyridine ring. Indeed, only when the large benzyl group is introduced, as in compound **5**, is a small drop in potency observed. Thus, assuming that the compounds bind in the same mode as pyridine-2,5-dicarboxylic acid, the distal carboxylate does not seem to have a fixed binding site. This observation is in contrast to the report[20] that replacement of 2-ketoglutaric acid by any other chain-extended keto acid in the hydroxylation reaction leads to a large drop in the efficiency of the hydroxylation process. This suggests that there is some "flexibility" in the distal carboxylic acid binding site since compounds with a variety of chain-extended structures will inhibit the enzyme. When the 5-carboxylic group of **15** is replaced by a variety of acyl sulfonamides, an increase in potency is observed (Table III). Most of the compounds **14a–j** are more potent as inhibitors of prolyl 4-hydroxylase than **15**. Their inhibitory potency does not depend on the nature of the substituent on the acyl sulfonamide, with alkyl-substituted sulfonamides **14a** and **14b** being equipotent with aryl substituents **14c**, **14e**, **14f**, and **14i**. The distal carboxylate binding site of the enzyme is also able to accommodate substituents with different physiochemical properties. Only when the substituent is very large is a slight fall in potency observed, e.g. **14j** and **14h**. The homologated acyl sulfonamide **6** is statistically equipotent with its carboxylic acid analogue **4**. In contrast, when the carboxylic acid group of the weak inhibitor pyridine-2-carboxylic acid (**16**) (Table II) is replaced with an acyl sulfonamide, as in **17**, a large drop in potency is observed. Thus, in the 2-position an acyl sulfonamide will not replace the carboxylic group, an observation consistent with the iron-chelating role ascribed to the 2-carboxylic moity of pyridine-2,5-dicarboxylic acid.

Conclusions

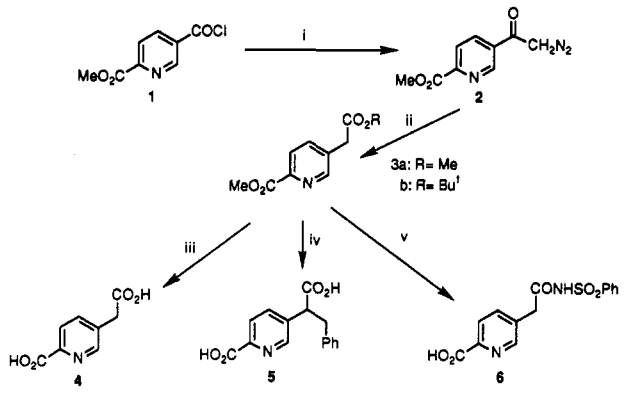
Although pyridine-2,5-dicarboxylic acid may exert its inhibitory activity by occupying the binding site of 2-ketoglutaric acid, as suggested by Guenzler and Hanauske-Abel, the distal carboxylic acid binding site is not as well-defined as they suggest. Although the presence of a 5-substituent has a clearly demonstrable effect on potency, this distal binding site can accommodate an acidic group at a variety of distances from the iron-binding site. The 5-substituent is not constrained to carboxylate and when it is a substituted acylsulfonamide, the substituent

Scheme II[a]



[a] Reagents: (i) DCCl/RSO_2NH_2; (ii) NaOH.

Scheme III[a]



[a] Reagents: (i) CH_2N_2; (ii) AgO; (iii) NaOH; (iv) LDA/BzBr then NaOH; (v) TFA then $PhSO_2NH_2$/DCCl then NaOH.

appears to have no upper limit on its size. Most of these acyl sulfonamides are more potent than pyridine-2,5-dicarboxylic acid as inhibitors of prolyl 4-hydroxylase.

Chemistry

The acyl sulfonamides **14a–j** were made from the known[21] half-ester **12** and the corresponding sulfonamide using a DCCI coupling method. The intermediate esters **13** were not purified but saponified to the corresponding acids (Scheme II). The half ester acid chloride[22] **1** was homologated using the Arndt–Eistert procedure to give

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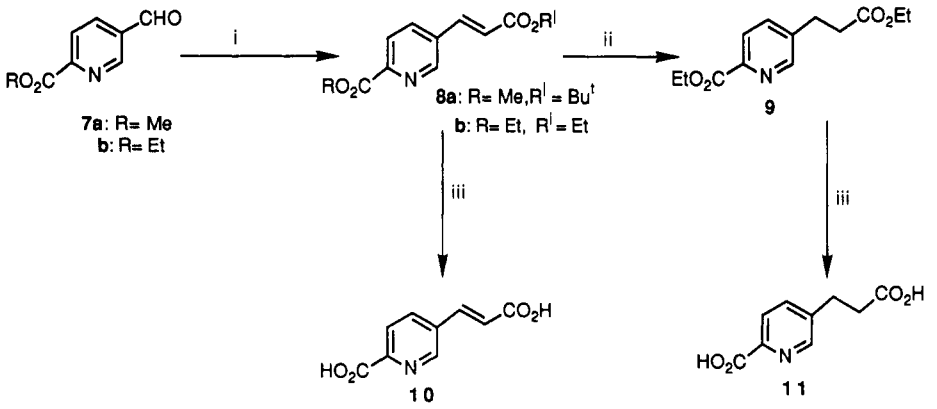
Scheme IV[a]



[a] Reagents: (i) $(EtO)_2CH_2CO_2R^1$/BuLi/THF; (ii) NaTeH; (iii) NaOH.

diesters **3a,b**. Selective deprotection of tert-butyl ester **3b** gave the acid from which the homologated acyl sulfonamide **6** was obtained. Dimethyl ester **3a** was smoothly deprotonated with LDA at the benzylic position and alkylated with benzyl bromide to give **5** (Scheme III). The known[23a,b] aldehydes **7a,b** underwent a Wittig–Horner reaction with triethyl phosphonoacetate or *tert*-butyl diethyl phosphonoacetate to give the corresponding trans α,β unsaturated esters **8a,b**. These esters would not hydrogenate using 30% Pd on carbon. A modest yield of the reduced diester **9** was obtained using sodium hydrogen telluride as reducing agent (Scheme IV).

Experimental Section

Melting points were determined on a Büchi melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Bruker AC250 or Bruker AM200 instrument. All organic extracts were dried over $MgSO_4$ and evaporations carried out under reduced pressure. All microanalyses were within 0.4% of the theoretical value. The inhibitory potency of the compounds against prolyl hydroxylase was determined using the assay described by Cunliffe et al.[24] in which the enzyme-catalyzed conversion of labeled 2-ketoglutarate into labeled succinate is measured. All points were determined in duplicate and IC_{50} values were obtained from six-point dose–response curves by interpolation. The logarithmic standard deviation estimated from the five sets of replicate data ($N = 13$, $\phi = 8$) was $\sigma = 0.14$; a compound is significantly different ($p < 0.05$) in potency to pyridine-2,5-dicarboxylic acid **15** if its IC_{50} values differ by a factor of more than 2.3 ($t_{95\%,\phi=8} = 2.31$). Prolyl 4-hydroxylase obtained from the leg tissue of 17-day-old chick embryos was purified by the method of Kedersha and Berg[25] and appeared homogeneous when examined by SDS/polyacrylamide gel electrophoresis. By using the Cheng and Prusoff[26] equation the IC_{50} of pyridine-2,5-dicarboxylic acid **15**, 5.5 μM, converts to a K_i of 0.96 μM (lit. value = 0.8 μM).[27]

General Preparation of the Acyl Sulfonamides 14a–j. To a suspension of the half ester **1** (0.9 g, 5 mmol) in dichloromethane (100 mL) was added benzenesulfonamide (0.79 g, 5 mmol) followed by DCCI (1.03 g, 5 mmol) and then 4-(*N*,*N*-dimethylamino)-pyridine (0.61 g, 5 mmol) was added. The mixture was stirred for 18 h. The dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The residue was dissolved in saturated aqueous sodium bicarbonate solution and extracted twice with ether. The aqueous phase was acidified to pH 1 with 6 M hydrochloric acid and reextracted twice with ethyl acetate, washed with brine, dried, and evaporated to a solid (1.1 g). The crude ester was stirred at ambient temperature for 1.5 h with N sodium hydroxide (7.8 mL) and then acidified to pH 3 with 2 N hydrochloric acid. The solid was filtered off and recrystallized from ethanol to give **14c** (342 mg), mp = 243–4 °C. Anal. ($C_{13}H_{10}N_2O_5S$) C, H, N. The sulfonamides required to prepare the acyl sulfonamides **14a–d** and **14g–j** are commercially available. See ref 28 for quinoline-8-sulfonamide preparation and ref 29 for naphthalene-1-sulfonamide.

2-Carboxypyridine-5-acetic Acid Hydrochloride (4). To a solution of acid chloride **1** (19.9 g, 100 mmol) in dry tetrahydrofuran (200 mL) at 0 °C was added an ethereal solution of diazomethane (200 mmol in 1 L of ether, from 42.8 g of Diazald). Almost immediately a white solid separated. The mixture was kept at 0 °C for 1 h and the solid (6.5 g) collected. A 3-g portion of this crude diazo ketone **2** was dissolved in methanol (100 mL) and silver(I) oxide (1 g) added. The mixture was stirred and refluxed for 2 h and filtered through Celite, and the filtrate evaporated to give **3a** as an oil (1.2 g): NMR (C_6D_6) δ 8.2 (d, 1 H), 7.9 (d, 1 H), 7.1 (d, 1 H), 3.6 (s, 3 H), 3.3 (s, 3 H), 3.1 (s, 2 H). This dimethyl ester **3a** (1.2 g) was dissolved in methanol (50 mL) and 2 M sodium hydroxide solution (10 mL) added. The mixture was refluxed for 2 h, cooled, and evaporated to dryness. The residue was redissolved in water, acidified with hydrochloric acid, and evaporated to a pale brown solid. Trituration with 2-propanol–ether gave a solid (254 mg): mp 153–5 °C dec; mass measured MH^+ ($C_8H_8NO_4$), theory = 182.0458, found = 182.0464; ^1H NMR (D_2O) δ 8.6–9.3 (m, 3 H), 4.25 (s, 2 H).

5-[[(Phenylsulfonyl)carbamoyl]methyl]pyridine-2-carboxylic Acid (6). A suspension of diazo ketone **2** (5 g, 2.6 mmol) in *tert*-butyl alcohol (200 mL) and silver(I) oxide (5 g) was stirred and refluxed for 20 min. The mixture was filtered through Celite and the filtrate evaporated to an oil, **3b** (5 g). This oil was dissolved in trifluoroacetic acid (100 mL), stirred at room temperature for 20 min, and then evaporated to dryness. The residue was dissolved in saturated sodium bicarbonate solution and extracted with ether, and the aqueous phase acidified to pH 4 with dilute hydrochloric acid and reextracted twice with ethyl acetate. The combined organic extracts were dried and evaporated. The

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residue was stirred with ether and filtered to give a solid (1.8 g), mp = 212-4 °C dec. This solid (0.98 g, 5 mmol) was converted to its acyl sulfonamide by the procedure described for general preparation of acyl sulfonamides **14a-j** to afford **6** (210 mg), mp = 176-8 °C. Anal. ($C_{14}H_{12}N_2O_5S$) C, H, N.

5-(α-Carboxyphenethyl)pyridine-2-carboxylic Acid (5). Lithium diisopropylamide (5 mmol) was prepared from a solution of redistilled diisopropylamine (0.7 mL) in dry tetrahydrofuran (50 mL) under argon at -20 °C by adding a solution of *n*-butyllithium (1.6 M in hexane, 3.15 mL). After 10 min at -20 °C the mixture was cooled to -70 °C and diester **3a** (1.0 g, 5 mmol) in tetrahydrofuran (20 mL) added. After 20 min at -70 °C a solution of benzyl bromide (0.75 mL, 11 mmol) in tetrahydrofuran (20 mL) was added. After 1 h at -70 °C the mixture was allowed to warm to ambient temperature and neutralized with glacial acetic acid. The mixture was evaporated to dryness and partitioned between water and ethyl acetate. The organic extracts were washed with brine, dried, and evaporated to an oil. This oil was chromatographed on a Merck silica gel column (Art. 10401) eluting with dichloromethane/ethyl acetate (9:1) to give a colorless oil (800 mg). This oil was dissolved in methanol (10 mL) and 1 N sodium hydroxide (7.5 mL) was added. The mixture was refluxed for 2 h and evaporated to a small volume, and water (6 mL) added. The aqueous phase was extracted with ether, and then the extracts were discarded. The aqueous phase was acidified to pH 1-2 with 2 N hydrochloric acid and extracted twice with ethyl acetate. The combined organic phases were dried and evaporated to a solid. After recrystallization from acetonitrile, **5** was obtained (300 mg), mp = 177-8 °C. Anal. ($C_{15}H_{13}NO_4$) C, H, N.

Ethyl 2-(Ethoxycarbonyl)pyridine-5-propionate (9). To a solution of triethyl phosphonoacetate (3.8 g, 17 mmol) in dry tetrahydrofuran (40 mL) cooled to -60 °C, under argon, was added at this temperature a 1.6 M solution of n-butyllithium in hexane (11.3 mL, 17 mmol). After the addition aldehyde **7b** (3 g, 17 mmol) in dry tetrahydrofuran (80 mL) was added and the mixture kept at -60 °C for 30 min. After warming to room temperature the mixture was evaporated to dryness and the residue partitioned between ether and water. The organic extracts were combined, washed with water, dried, and evaporated to give **8b** (4.1 g) mp = 70-3 °C. A mixture of tellurium metal (1.3 g, 10 mmol), sodium borohydride (0.9 g, 24 mmol), and ethanol was heated to reflux for 30 min to give a purple solution. This was cooled to -20 °C and a deoxygenated solution of glacial acetic acid (1.2 mL) in ethanol (3 mL) was added. The black suspension was stirred at -20 °C for 5 min and a solution of the diethyl ester **8b** in ethanol (25 mL) and dichloromethane (25 mL) added. After warming to room temperature the mixture was filtered through Celite and the filtrate evaporated to dryness. The residue was partitioned between water and ethyl acetate. The combined organic layers were dried and evaporated to dryness. The residue was chromatographed on a Merck silica gel column (Art. 10102) eluting with ethyl acetate/hexane (1:1) to give **9** as an oil (0.7 g). Anal. ($C_{13}H_{17}NO_4$) C, H, N.

2-Carboxypyridine-5-propionic Acid (11). Compound **9** (251 mg, 1 mmol) was dissolved in methanol (10 mL) and 1 N sodium hydroxide (4 mL, 4 mmol) was added. The mixture was stirred at room temperature for 3 h and evaporated to dryness, and the residue dissolved in the minimum volume of water. The pH of the solution was adjusted to 4 with dilute hydrochloric acid and then extracted twice with ethyl acetate. The combined extracts were dried and evaporated to a white solid. This solid was boiled with absolute ethanol and filtered, and the filtrate evaporated to yield **11** (60 mg), mp >250 °C. Anal. ($C_9H_9NO_4Na_2$) C, H, N.

2-Carboxypyridine-5-propenoic Acid (10). A solution of *tert*-butyl dimethyl phosphonoacetate (1.12 g, 50 mmol) in dry tetrahydrofuran (10 mL) was cooled under argon to -60 °C and a 1.6 M solution of *n*-butyllithium in hexane (3.2 mL) added. After the addition, a solution of aldehyde **7a** (0.83 g, 50 mmol) in dry tetrahydrofuran (20 mL) was added. The mixture was allowed to warm to ambient temperature for 1 h and evaporated to dryness, and the residue partitioned between ether and water. The combined organic extracts were dried and evaporated to yield **8a** (1 g), mp 120-3 °C. **8a** (0.4 g) in trifluoroacetic acid (15 mL) was stirred at room temperature for 30 min and then evaporated to dryness. The residue was dissolved in saturated sodium bicarbonate solution and extracted with ether, and the aqueous phase acidified to pH 4 with 2 M hydrochloric acid. The aqueous phase was extracted twice with ethyl acetate. The combined organic extracts were dried and evaporated to a solid (0.25 g), mp = 220-3 °C. This solid was stirred with 1 N sodium hydroxide solution (3.4 mL) for 30 min at room temperature, diluted with an equal volume of water, and extracted with ether. The aqueous phase was acidified to pH 4 with 2 M hydrochloric acid and the solid filtered. Recrystallization from aqueous dimethylformamide gave **10** (89 mg), mp = 271-3 °C dec. Anal. ($C_9H_7NO_4$) C, H, N.

2-[(Methylsulfonyl)carbamoyl]pyridine (17). This compound was prepared by the method described for the general preparation of acyl sulfonamides, but using pyridine-2-carboxylic acid (2.46 g, 20 mmol) as starting material gave **17** (2.3 g, 58%), mp = 105-8 °C. Anal. ($C_7H_8N_2O_3S$) C, H, N.

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Novel Inhibitors of Prolyl 4-Hydroxylase. 2. 5-Amide Substituted Pyridine-2-carboxylic Acids

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A series of 5-[(arylcarbonyl)amino]- and 5-(arylcarbamoyl)pyridine-2-carboxylic acids has been prepared and tested for activity as inhibitors of the enzyme prolyl 4-hydroxylase (EC 1.14.11.2). All the analogues prepared were inhibitors of the enzyme in vitro, the best compounds being equipotent with the known inhibitor pyridine-2,5-dicarboxylic acid (**9**). Like **9** these amidic analogues were not active in a cultured embryonic chick tendon cell model, considered to be a predictor of in vivo activity. The activity of the amides is not consistent with the model described for the mode of action of **9** with the enzyme and aspects of this are discussed.

Introduction

The reasons for our interest in inhibitors of prolyl 4-hydroxylase (EC 1.14.11.2), which is critically important in the biosynthesis of collagen, have been discussed in an earlier work.[1] A model of the mode of action of prolyl hydroxylase has been described[2] and has been used by these authors to predict that pyridine-2,5-dicarboxylic acid

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