Cell Culture and Immunofluorescence Studies. Murine macrophage-like cell line, J774.2, and Chinese hamster ovary (CHO) cells were maintained as described.[31] Immunofluorescence studies were performed on CHO cells using a mouse monoclonal antibody against tubulin as the first antibody and a rhodamine-conjugated rabbit anti-mouse antibody as the second antibody.[32]

Modelling Calculations. Calculations were performed with MM2 (87) available from the Quantum Chemistry Program Exchange, Department of Chemistry, Indiana University, Bloomington, IN. The following parameters were employed: dielectric constant, 4.0; torsional constants for the 6-1-3-6 atom type angle, V1 = 0.0, V2 = -0.6, V3 = 0.3; torsional constants for the 2-3-9-28 atom type angle, V1 = 0.0, V2 = 5.0, V3 = 0.0.

Acknowledgment. Support through USPHS Grant No. CA 41349 (to C.S.S.) and CA 39821 (to S.B.H.) awarded by the National Cancer Institute, DHHS, and by the Israel Cancer Association (to I.R.) is gratefully acknowledged. The acquisition of major instrumentation at Bryn Mawr was made possible by grants from the Camille and Henry Dreyfus Foundation, the PQ Corp., the W. M. Keck Foundation, and Merck & Co., Inc.

Registry No. **3**, 131760-44-6; **4**, 131896-65-6; **5**, 131760-45-7; **6**, 131896-66-7; **7**, 131760-46-8; **8**, 131896-67-8; (*R*)-**9**, 131760-47-9; (*S*)-**9**, 131760-62-8; (*R*)-**9** methyl ester alcohol, 17392-83-5; (*S*)-**9** methyl ester alcohol, 27871-49-4; (*R*)-**9** methyl ester, 131760-51-5; (*S*)-**9** methyl ester, 131760-52-6; (*R*)-**10**, 131760-48-0; (*S*)-**10**, 131760-63-9; (*R*)-**10** methyl ester alcohol, 27000-00-6; (*S*)-**10** methyl ester alcohol, 13673-95-5; (*R*)-**10** methyl ester, 131760-53-7; (*S*)-**10** methyl ester, 131760-54-8; (*R*)-**11**, 57044-25-4; (*S*)-**11**, 60456-23-7; (*R*)-**11** *p*-methoxyphenyl ether, 71031-04-4; (*S*)-**11** *p*-methoxyphenyl ether, 71048-65-2; (*R*)-**12**, 131760-49-1; (*S*)-**12**, 131760-64-0; (*R*)-**12** azide, 131760-56-0; (*S*)-**12** azide, 131760-55-9; (*R*)-**12** azido benzoate, 131760-58-2; (*S*)-**12** azido benzoate, 131760-57-1; (*R*)-**12** trichloroethoxymethyl ether, 131760-59-3; (*S*)-**12** trichloroethoxymethyl ether, 131760-60-6; (*R*)-**13**, 131760-65-1; (*S*)-**13**, 131760-50-4; **16**, 131760-61-7; *p*-methoxyphenol, 150-76-5.

Supplementary Material Available: Preparation and characterization data for **9**, **10**, **13**, and **16** (4 pages). Ordering information is given on any current masthead page.

Positional Effects of Sulfation in Hirudin and Hirudin PA Related Anticoagulant Peptides†

Marguerite H. Payne, John L. Krstenansky,* Mark T. Yates, and Simon J. T. Mao

Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215. *Received July 30, 1990*

C-Terminal fragment analogues of the leech protein hirudin or the related protein hirudin PA block thrombin's cleavage of fibrinogen. Three series of synthetic peptides were synthesized to study the effects of sulfation in hirudin-derived peptides. Potency of hirudin analogues increased with *p*-(amino)Phe^{63}, *p*-(aminosulfonate)Phe^{63}, and *p*-(sulfate)Tyr^{63} substitution in place of Tyr^{63}. Sulfation of Tyr^{56}, which in hirudin is normally Phe, resulted in a loss of 1 order of magnitude in potency. The sulfation of Tyr^{64} of the hirudin PA related analogue resulted in increased potency as for the hirudin analogue. However, in this series the *p*-(amino)Phe^{64} and *p*-(aminosulfonate)Phe^{64} did not have increased potency. In addition to these positional effects, replacing all the Glu residues with (O-sulfato)Ser yielded an analogue with full antithrombin potency.

Introduction

Peptide derivatives of hirudin's C-terminal functional domain are antithrombin agents that bind thrombin at a noncatalytic site, preventing its procoagulant actions.[1] While the lipophilic residues of the hirudin peptides are essential to potent interaction with thrombin,[2] these peptides are anionic and some of their charged residues are involved in direct interactions with thrombin.[3] In particular, the X-ray crystallographic structure of the hirudin–thrombin complex shows that Asp^{55} and Glu^{57} of hirudin are involved in interactions with basic residues on the surface of thrombin.[3] Additionally, sulfation of Tyr^{63} may add some ionic interactions to the overall binding energy of hirudin.[3,4] Hirudin is a member of a family of anticoagulant proteins isolated from bloodsucking leeches. Most known variants of this family of proteins share the hirudin C-terminal amino acid sequence. The only known exceptions are hirudin PA[5] and hirullin P18,[6] which have significant differences in their sequences in this region. Hirullin P18 does not contain a Tyr residue near its C-terminus that could be sulfated as in hirudin although it shares the same functional role in the protein.[7] Hirudin PA contains a sulfated Tyr resiude in its C-terminal functional domain, but its location is shifted by one position relative to hirudin. Structure–activity relationships of the unsulfated hirudin and hirudin PA C-terminal peptide analogues are comparable.[8,9] However, a comparison of sulfated and nonsulfated hirudin PA analogues or sulfated hirudin and sulfated hirudin PA analogues has not been reported.

Sulfate groups are known to be capable of strong interaction with basic residues of proteins. Heparin can bind tightly to specific regions of proteins using almost exclu-

* Present address: Syntex Research, 3401 Hillview Avenue, Palo Alto, California 94303.

† All amino acids are the L enantiomer unless otherwise noted. Abbreviations used include: Boc, *tert*-butyloxycarbonyl; 2-BrZ, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Cha, β-cyclohexylalanine; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, dimethylformamide; HOBT, *N*-hydroxybenzotriazole; Pnh, *p*-aminophenylalanine; Pno, *p*-nitrophenylalanine; SO_3·pyridine, sulfur trioxide pyridine complex; Suc, succinyl; TFA, trifluoroacetic acid.

(1) Krstenansky, J. L.; Mao, S. J. T. *FEBS Lett.* **1987**, *211*, 10.
(2) Krstenansky, J. K.; Owen, T. O.; Yates, M. T.; Mao, S. J. T. *J. Med. Chem.* **1987**, *30*, 1688.
(3) Rydel, T. J.; Ravichandran, K. G.; Tulinksy, A.; Wolfram, B.; Huber, R.; Roitsch, C.; Fenton, J. W. *Science* **1990**, *249*, 277.
(4) Stone, S. R.; Dennis, S.; Hofsteenge, J. *Biochemistry* **1989**, *28*, 6857.
(5) Dodt, J.; Machleidt, W.; Seemuller, U.; Maschler, R.; Fritz, H. *Biol. Chem. Hoppe-Seyler* **1986**, *367*, 803.
(6) Maschler, R.; Steiner, V.; Grutter, M. G.; Fritz, R. Eur. Patent Applic. 1989, EP-A-0347376.
(7) Krstenansky, J. L.; Owen, T. J.; Yates, M. T.; Mao, S. J. T. *FEBS Lett.* **1990**, *269*, 425.
(8) Krstenansky, J. K.; Owen, T. J.; Yates, M. T.; Mao, S. J. T. *Thromb. Res.* **1988**, *52*, 137.
(9) Krstenansky, J. L.; Broersma, R. J.; Owen, T. J.; Payne, M. H.; Yates, M. T.; Mao, S. J. T. *Thromb. Haemostasis* **1990**, *63*, 208.

0022-2623/91/1834-1184$02.50/0

Table I. Fibrin Clot Inhibition

no.	compound	IC_{50},[a] μM
1	$hirudin_{54-65}$ Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln-OH	3.7[8]
2	hirudin PA_{54-66} Gly-Asp-Phe-Glu-Pro-Ile-Pro-Glu-Asp-Ala-Tyr-Asp-Glu-OH	2.7[8]
3	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH	0.15
4	Suc-Tyr(OSO_3H)-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH	1.7
5	Suc-Phe(*p*-NO_2)-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH	2.8
6	Suc-Phe(*p*-NH_2)-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH	1.3
7	Suc-Phe(*p*-$NHSO_3H$)-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-D-Glu-OH	13.1
8	Suc-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Leu-D-Glu-OH	1.6
9	Suc-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Tyr(OSO_3H)-Leu-D-Glu-OH	0.087
10	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Phe(*p*-NO_2)-Leu-D-Glu-OH	2.8
11	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Phe(*p*-NH_2)-Leu-D-Glu-OH	0.38
12	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Phe(*p*-$NHSO_3H$)-Leu-D-Glu-OH	0.12
13	Suc-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Tyr-D-Glu-OH	1.2
14	Suc-Phe-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Tyr(OSO_3H)-D-Glu-OH	0.30
15	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Phe(*p*-NO_2)-D-Glu-OH	0.71
16	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Phe(*p*-NH_2)-D-Glu-OH	2.9
17	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Phe(*p*-$NHSO_3H$)-D-Glu-OH	2.5
18	Suc-Tyr-Ser-Pro-Ile-Pro-Ser-Ser-Ala-Cha-Ser-OH	>76
19	Suc-Tyr-Ser(OSO_3H)-Pro-Ile-Pro-Ser(OSO_3H)-Ser(OSO_3H)-Ala-Cha-Ser(OSO_3H)-OH	0.21

[a] IC_{50} = molar dose of peptide that results in 50% inhibition of fibrin clot formation relative to a blank control after thrombin added to plasma.

sively ionic interactions.[10] Sulfates or carboxylate groups can form well-defined planar salt bridges with arginine residues that are more stable than unoriented ionic interactions.[11]

Since the sulfation of Tyr^{63} is known to affect the potency of hirudin and peptide analogues of hirudin,[12] the present study was intended to examine the role of sulfates and sulfonamates at various positions of these peptides. Additionally, the ability of sulfate groups to replace carboxylate groups in hirudin C-terminal peptide analogues was explored.

Results and Discussion

The peptides were synthesized by solid-phase methods using N^{α}-Boc chemistry. *p*-Nitrophenylalanine residues were reduced to *p*-aminophenylalanine by catalytic transfer hydrogenation. Sulfated peptides were prepared with sulfur trioxide pyridine complex (SO_3·pyridine) in anhydrous DMF and pyridine.[13,14] All peptides were purified by preparative HPLC and characterized by analytic HPLC, FAB-MS, and amino acid analysis.

Position 56 of hirudin is a key residue for interaction with thrombin. A β-aromatic L-alanyl residue is required at position 56 in hirudin C-terminal analogues for measurable anticlotting activity.[9] X-ray crystallography of the hirudin–thrombin complex shows Phe^{56} penetrating into a depression on thrombin's surface with multiple hydrophobic contacts and a perpendicular stacking with Phe^{34} of thrombin.[3] With such strong hydrophobic interactions the relatively slight loss of activity from charged substituents on the phenyl ring was surprising (Table I). Potency correlates best with the steric bulk and branching of the para-substituted group: phenylalanine, 2′-naphthylalanine, 2′-thienylalanine, and tyrosine have approximately equal potency,[9] substituting –NH– for –O– in tyrosine or tyrosine sulfate reduces potency 10-fold (**3** vs **6**, **4** vs **7**), and sulfation reduces potency 10-fold (**3** vs **4** and **6** vs **7**). Whether the lower potency of the sulfates is from steric bulk, negative charge or both is unclear. The electronegativity of the substituents appears to have little effect (**4**–**6**).

Tyr^{63} is sulfated in native hirudin and desulfation increases the dissociation constant 10-fold.[12] The X-ray structure of the desulfato–hirudin thrombin complex shows three thrombin lysines situated near Tyr^{63} which might form ion pairs with a sulfated residue.[3] As expected, both the negatively charged sulfate and sulfonamate are more potent than their neutral precursors and potency decreases with less hydrophilic substituents on the aromatic ring, $-OSO_3^-$, $-NHSO_3^-$ > $-NH_2$ > –OH > $-NO_2$ (**8**–**12**). The aromatic ring at position 63 is also involved in hydrophobic interactions with Pro^{60} of hirudin and Ile^{82} of thrombin[3]; replacement of Tyr^{63} with Glu is tolerated but with a 3-fold loss of potency.[9]

The requirements for position 64 generally favor a lipophilic residue.[7] In hirudin PA the terminal three amino acids of hirudin, Tyr(OSO_3H)-Leu-Gln-OH, are replaced by Ala-Tyr(OSO_3H)-Asp-Glu-OH.[5] This places a polar group in a position where lipophilicity is favored. The X-ray structure shows that both hirudin Leu^{64} and Ile^{59} have close hydrophobic contacts with each other and with thrombin Leu^{65} and Phe^{34}. It would be expected that a charged residue might disrupt this arrangement.[3] In all but one of the compounds studied here more lipophilic residues are more potent: Cha > Phe(NO_2), Tyr > Phe(NH_2), Phe($NHSO_3^-$) (**3**, **13**, **15**–**17**). The one exception is sulfotyrosine (**14**), which is 4 times as potent as tyrosine (**13**) and nearly as effective as β-cyclohexylalanine (**3**). If an alternate binding interaction exists for sulfotyrosine at position 64, for example with thrombin lysines, then access to it must be restricted in a way that excludes the related sulfatoaminophenylalanine analogue.

Of the acidic residues in hirudin's C-terminus, Glu^{57} is most sensitive to substitution.[9] The crystal structure shows the δ-carboxylate of Glu^{57} forming a hydrogen bond with amide of Tyr^{76} in thrombin.[3] Loss of that interaction by substitution with alanine leaves no measurable anticoagulant activity for the C-terminal peptide analogues.[2] In contrast, Glu^{61} and Glu^{62} do not appear to directly interact with thrombin[3] and can be individually replaced by alanine with less than 10-fold loss of potency.[2] Since serine sulfate has the same side-chain length as glutamic acid and carries an equivalent charge, it might effectively replace residues 61 and 62. However, the larger more diffuse charge and tetrahedral nature of sulfates might affect their ability to substitute for carboxylic acid. Compound **19**, in which serine sulfate replaces Glu^{57}, Glu^{61}, Glu^{62}, and Glu^{65} of **3** without loss of potency, shows that sulfates can effectively

(10) DeAngelis, P. L.; Glabe, C. G. *Biochemistry* **1988**, *27*, 8189.
(11) Ichimura, S.; Mita, K.; Zama, M. *Biochemistry* **1982**, *21*, 5329.
(12) Stone, S. R.; Hofsteenge, J. *Biochemistry* **1986**, *25*, 4622.
(13) Martinez, J.; Winternitz, F.; Bodanszky, M.; Gardner, J. D.; Walder, M. D.; Mutt, V. *J. Med. Chem.* **1982**, *25*, 589.
(14) Gilbert, E. E. *Chem. Rev.* **1962**, *62*, 549.

Table II. Amino Acid Analyses

no.	Ser	Glx	Pro	Ala	Ile	Leu	Tyr	Phe	Pno	% peptide content
3	-	4.06 (4)	1.98 (2)	1.02 (1)	0.95 (1)	-	0.98 (1)	-	-	87
4	-	4.06 (4)	1.97 (2)	1.00 (1)	0.96 (1)	-	1.01 (1)	-	-	82
5	-	4.07 (4)	2.01 (2)	0.98 (1)	0.94 (1)	-	-	-	a	90
6	-	4.09 (4)	1.97 (2)	0.97 (1)	0.95 (1)	-	-	-	-	86
7	-	4.08 (4)	1.98 (2)	1.00 (1)	0.94 (1)	-	-	-	-	84
8	-	4.06 (4)	1.97 (2)	-	0.97 (1)	1.02 (1)	0.99 (1)	1.01 (1)	-	93
9	-	4.06 (4)	2.00 (2)	-	0.95 (1)	1.01 (1)	0.98 (1)	1.01 (1)	-	84
10	-	4.14 (4)	1.95 (2)	-	0.94 (1)	0.99 (1)	0.96 (1)	-	1.02 (1)	98
11	-	4.10 (4)	1.97 (2)	-	0.96 (1)	0.99 (1)	0.98 (1)	-	-	84
12	-	4.06 (4)	1.97 (2)	-	0.96 (1)	1.01 (1)	1.01 (1)	-	-	86
13	-	4.04 (4)	1.96 (2)	1.01 (1)	0.97 (1)	-	0.99 (1)	1.03 (1)	-	90
14	-	4.06 (4)	1.98 (2)	1.00 (1)	0.95 (1)	-	0.99 (1)	1.02 (1)	-	54
15	-	4.06 (4)	2.02 (2)	0.99 (1)	0.95 (1)	-	0.98 (1)	-	1.07 (1)	92
16	-	4.03 (4)	1.96 (2)	0.99 (1)	1.02 (1)	-	1.00 (1)	-	-	80
17	-	4.06 (4)	1.97 (2)	1.01 (1)	0.96 (1)	-	1.00 (1)	-	-	87
18	4.00 (4)	-	1.94 (2)	1.04 (1)	0.98 (1)	-	1.04 (1)	-	-	84
19	4.02 (4)	-	1.94 (2)	1.06 (1)	0.93 (1)	-	1.05 (1)	-	-	81

[a] *p*-Nitrophenylalanine coelutes with β-cyclohexylalanine.

Table III. Physical Data

no.	calcd MW	FAB-MS $(M + H)^+$	FAB-MS $(M - H)^-$	$\epsilon_1 \lambda_1$ max	$\epsilon_1 \lambda_2$ max	% yield	HPLC analytic[c] t_R	HPLC prep gradient
3	1328.6	1329.5		1340 (280)			16.0[e]	29–31[f]
4	1408.6	1409.9	1406.8	424 (265)		41	20.7[e]	10–30[g]
5	1357.6	1358.6		10200 (279)		48	20.3[e]	26.8–30.8[f]
6	1327.6	1328.9		1300 (290)	11600 (236)[h]	78	14.1[e]	19–23[f]
7	1407.6	1408.5	1406.7	982 (279)	15500 (234)	52	13.1[e]	7–12[g]
8	1364.6	1365.5		1340 (280)		42	16.7[e]	28.5–31[f]
9	1444.6	1445.6	1443.1	726 (260)		91	14.2[e]	5–15[g]
10	1409.6	1410.5		10600 (278)		36	18.4[e]	26–30[f]
11	1379.6	1380.4		3750 (291)	21900 (240)[h]	67	11.2[e]	18–22[f]
12	1459.6	1380.6[a]	1458.3	2080 (280)		72	10.3[e]	5–10[g]
13	1322.5	1323.7		1320 (280)		43	12.4[e]	23.5–26[f]
14	1402.5	1402.7	1401.0	994 (260)		82	9.3[e]	0–10[g]
15	1367.5	1368.5		11200 (278)		34	14.3[e]	25.5–28[f]
16	1337.6	1338.2		3240 (290)	21500 (239)[h]	63	17.4[d]	28–20.5[f]
17	1417.5	1337.8[a]	1416.2	2230 (280)	19300 (234)	68	16.4[d]	0–7.5[g]
18	1160.6	1161.6		1360 (280)		48	14.7[e]	26.5–29[f]
19	1480.4	1161.6[b]	1478.8	1340 (280)		34	15.1[d]	5–15[g]

[a] $(M + H - SO_3)^+$. [b] $(M + H - 4SO_3)$. [c] Vydac 218TP54 (4.6 × 250 mm) C18 column, gradient 1% CH_3CN per minute in 0.1% TFA aqueous buffer at 2.0 mL/min, t_0 = 1.5 min. [d] 5–30% CH_3CN. [e] 15–40% CH_3CN. [f] % CH_3CN gradient over 15 min in 0.1% TFA aqueous buffer. [g] % CH_3CN gradient over 15 min in 10 mM ammonium acetate pH 6.0 aqueous buffer. [h] UV absorbance measured at pH 12, all other compounds measured at pH 2.

replace the carboxylate functionalities.

Experimental Section

Synthesis, Purification, and Characterization. The peptides were synthesized by solid-phase methods on an Applied Biosystems Model 430A peptide synthesizer. Chloromethylated polystyrene resin (Peptides International) was acylated with the cesium salt of Boc-D-Glu(Bzl)-OH to 0.56 mmol/g. N^{α}-*t*-Boc amino acids with side chain protection Glu(Bzl), Ser(Bzl), and Tyr(2-BrZ) (Peptides International) were double coupled via the preformed symmetrical anhydride. Boc-*p*-nitrophenylalanine (Bachem) was single coupled via the HOBT ester because the symmetrical anhydride formed from DCC did not dissolve well in DCM or DMF.

The peptides were cleaved from the resin and deprotected in anhydrous HF containing 5% anisole at -5 °C for 45 min. The HF was removed in vacuo and the peptide extracted from the resin with 50% aqueous CH_3CN. The combined extracts were lyophilized and purified by preparative HPLC on a Beckman (50.8 × 150 mm) C_{18} column at 80 mL/min with a CH_3CN gradient in 0.1% aqueous TFA monitored at 215, 280, or 300 nm. The major peak was collected and lyophilized.

***p*-Aminophenylalanine.** *p*-Nitrophenylalanine in peptides **5**, **10**, and **15** was reduced with 10% Pd/C and ammonium formate in anhydrous methanol under argon for 1 h. The catalyst was filtered, the methanol removed in vacuo and the resulting material purified by preparative HPLC as described above monitored at 280 or 215 nm.

The dissociation constant of *p*-aminophenylalanine in **6** was measured by titration of a 1.0 mM peptide solution in 10 mM TFA (initial pH 2.27) with 0.20 M NaOH. While the free amine absorbs UV at 280–290 nm, the cation does not. Thus, measuring absorbance at 285 nm gives the concentration of free aminophenylalanine. The amine is neutral at pH 6.8 and a fit of the data to the equation $A_{285} = (C\epsilon_{285})(K_a)/(K_a + [H^+])$ yields $K_a = 1.4 \times 10^{-5}$.

Sulfates and Sulfonamates. Successful sulfation of tyrosine, serine, and *p*-aminophenylalanine required anhydrous reaction conditions. The peptide and reaction flask were dried under vacuum and flushed with argon. Dry pyridine and DMF were added in equal volumes, and the flask was kept under argon during the reaction. A 10–30-fold excess of SO_3·pyridine was used to scavenge any residual water and to drive the reaction to completion. After the reaction was quenched with water, the pH was adjusted to 7 with saturated $NaHCO_3$; this not only prevents acid hydrolysis of the sulfate during lyophilization but also yields a tractable powder instead of an oil. The sulfate ester or sulfonamide of a purified peptide is slowly cleaved in acid solution. The rate of hydrolysis is slow enough to allow the peptide to be conveniently analyzed or purified by HPLC in 0.1% aqueous TFA at pH 2. However when acidic solutions of sulfated peptide are frozen and lyophilized, the sulfate is significantly hydrolyzed. Adjusting the pH of the 0.1% TFA HPLC eluent to 7 with ammonium hydroxide solution prior to lyophilization yielded intact sulfate and ammonium trifluoroacetate. To avoid having to remove the ammonium trifluoroacetate a neutral lyophilizable

buffer, ammonium acetate (10 mM, pH 6), was chosen for preparative HPLC of the sulfated peptides.

The selective sulfation of *p*-aminophenylalanine or serine in a peptide also containing tyrosine was made possible by tyrosine's lower reactivity toward complexed sulfur trioxide. *p*-Aminophenylalanine and serine were completely sulfated by SO_3·pyridine in anhydrous DMF and pyridine under argon within 1 h while sulfation of tyrosine in similar peptides required up to 24 h to complete. For example the sulfation of *p*-aminophenylalanine in 11 (pyridine–DMF solution 20 mM in peptide, 300 mM in SO_3·pyridine at room temperature for 1.0 h) gave 91% 12 and only 9% disulfated material by HPLC. Similarly HPLC of the sulfation of the four serines in 18 (pyridine–DMF solution 14 mM in peptide, 660 mM in SO_3·pyridine at room temperature for 1.0 h) showed 58% 19, 19% sulfated tyrosine, and 23% incomplete sulfation of serine (combined areas of four peaks).

The sulfated peptides were purified by preparative HPLC on a Rainin (21.4 × 250 mm) C_{18} column at 40 mL/min with a CH_3CN gradient in 10 mM ammonium acetate buffer at pH 6.0. The major peak was collected and lyophilized and then twice dissolved in deionized water and relyophilized to remove ammonium acetate.

Identity and purity for the sulfated peptides and precursors were assayed by analytic HPLC, FAB-MS on a VG Analytical ZAB2-SE instrument, and amino acid analysis (Table II and Table III). Sulfation at the *p*-aminophenylalanine residue rather than the tyrosine in 12 and 17 was confirmed by comparing their ^1H NMR and UV spectra with those of 3–7.

Hydrolysis. Since loss of sulfate can be a potential problem in the purification and storage of peptides containing sulfotyrosine, the rates of acid hydrolysis of purified sulfotyrosine in 14 and *p*-sulfoaminophenylalanine in 17 were compared. Samples of each peptide were dissolved in 10 mM TFA, pH 2.0, to make 250 μM solutions. The disappearance of sulfated peptide and appearance of desulfated material at room temperature were measured by HPLC (Vydac 218TP54 4.6 × 250 mm C18 column, gradient 5–30% CH_3CN/25 min in 0.1% TFA aqueous buffer at 2.0 mL/min) over 125 days. A fit of the data to the equation $C = C_0 \exp(-kt)$, where C is the concentration of sulfated peptide determined by HPLC at time t, C_0 the concentration at time 0, and k the rate constant for hydrolysis at pH 2, gives $k = 4.8 \times 10^{-8}$ s^{-1} for 14 and 9.5×10^{-8} s^{-1} for 17. This corresponds to less than 1% loss of sulfate after 24 h and $t_{1/2}$ of 167 and 84 days, respectively.

Anticoagulant Assay. Inhibition of fibrin clot formation was determined as previously described.[1] In brief, 50 μL (0.2 pmol) of bovine thrombin (Sigma) was added to the wells of a microtiter plate (Falcon) containing 50 μL of a solution of the synthetic peptide to be tested (0–25 nmol). After a 1-min agitation and an additional incubation for 10 min at 24 °C, 100 μL of diluted human plasma (1:10) in 0.1% EDTA was added and vortexed for 20 s. The turbidity of the solution was monitored by an autoreader (EL 309, Bio-Tek Instruments) at 405 nm at 5-min intervals. All of the above reagents were diluted in an assay buffer containing 0.12 M sodium chloride, 0.01 M sodium phosphate, 0.01% sodium azide, and 0.1% bovine serum albumin, pH 7.4.

Registry No. 3, 129521-72-8; 4, 131791-94-1; 5, 131791-95-2; 6, 131791-96-3; 7, 131831-88-4; 8, 131791-97-4; 9, 131791-98-5; 10, 131831-77-1; 11, 131791-99-6; 12, 131831-89-5; 13, 131831-78-2; 14, 131792-00-2; 15, 131792-01-3; 16, 131792-02-4; 17, 131792-03-5; 18, 131792-04-6; 19, 131792-05-7.

Adenosine Deaminase Inhibitors: Synthesis and Structure–Activity Relationships of Imidazole Analogues of *erythro*-9-(2-Hydroxy-3-nonyl)adenine

Gloria Cristalli,* Alessandra Eleuteri, Palmarisa Franchetti, Mario Grifantini, Sauro Vittori, and Giulio Lupidi†

Dipartimento di Scienze Chimiche, and Dipartimento di Biologia Cellulare, Università di Camerino, 62032 Camerino, Italy.
Received May 25, 1990

A series of *erythro*-1-(2-hydroxy-3-nonyl)imidazole derivatives have been synthesized and evaluated for adenosine deaminase (ADA) inhibitory activity, in order to introduce simplifications in the ADA inhibitors *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1a) and 3-deaza-EHNA (1c). Opening the pyrimidine or pyridine ring of EHNA or 3-deaza-EHNA respectively led to compounds which are still ADA inhibitors. The most potent compound was *erythro*-1-(2-hydroxy-3-nonyl)imidazole-4-carboxamide (5, $K_i = 3.53 \times 10^{-8}$ M), which provided potential donor and acceptor sites for hydrogen bonding. Lack of one of this sites could account for the order of potency of all compounds examined in this series. Opening the same ring in adenosine and in 3-deazaadenosine led to fully inactive compounds. These results support the hypothesis of the existence, at or near the enzyme active site, of a hydrophobic region able to bind the *erythro*-nonyl moiety.

Deamination of adenosine and 2′-deoxyadenosine to form inosine and 2′-deoxyinosine is catalyzed by adenosine deaminase (ADA). In the last decade, inhibitors of adenosine deaminase have aroused interest as potential codrugs for use in combination with certain anticancer or antiviral agents which are adenosine analogues.[1] Inherited deficiency of ADA results in a block in development of the lymphoid system, causing severe combined immunodeficiency disease (SCID).[2] ADA inhibitors may be used to mimic the effect of the genetic deficiency of the enzyme and also in the chemotherapy of lymphoproliferative disorders and in the immunosuppressive therapy (i.e. in graft rejection).

Among the ADA inhibitors, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1a), a semi-tight-binding inhibitor ($K_i = 1.6 \times 10^{-9}$ M), is advocated as a possible inhibitor of choice for use with such nucleosides and was preferred to coformycin and deoxycoformycin, two extremely potent naturally occurring inhibitors ($K_i = 10^{-11}$–10^{-12} M).[3] In order to investigate which structural parameters in the purine moiety of EHNA were critical for inhibitory activity, we synthesized and tested the deaza analogues of EHNA, 1b–e (Table I).[4-6]

We demonstrated that isosteric monosubstitution of the pyrimidine nitrogens by carbons can be tolerated at the enzymatic binding site. In fact, 3-deaza-EHNA (1c) was found to have an inhibitory activity comparable to that

† Dipartimento di Biologia Cellulare.

(1) Glazer, R. I. *Cancer Chemother. Pharmacol.* **1980**, *4*, 227.
(2) Van Der Weyden, M. B.; Kelly, W. N. *Life Sci.* **1977**, *20*, 1645.
(3) North, T. W.; Cohen, S. S. *Pharmacol. Ther.* **1979**, *4*, 81.
(4) Antonini, I.; Cristalli, G.; Franchetti, P.; Grifantini, M.; Martelli, S.; Lupidi, G.; Riva, F. *J. Med. Chem.* **1984**, *27*, 274.
(5) Lupidi, G.; Cristalli, G.; Marmocchi, F.; Riva, F.; Grifantini, M. *J. Enzyme Inhib.* **1985**, *1*, 67.
(6) Cristalli, G.; Franchetti, P.; Grifantini, M.; Vittori, S.; Lupidi, G.; Riva, F.; Bordoni, T.; Geroni, C.; Verini, M. A. *J. Med. Chem.* **1988**, *31*, 390.

0022-2623/91/1834-1187$02.50/0