Molecular Determinants of the Platelet Aggregation Inhibitory Activity of Carbamoylpiperidines

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A series of α,α'-bis[3-(*N*,*N*-dialkylcarbamoyl)piperidino]-*p*-xylenes were synthesized and tested for their inhibitory activity on ADP-induced aggregation of human platelets. A parabolic curve was obtained when log $1/C$ (activity) was plotted against log P (octanol/water partition coefficient). Using this as a model, a new analogue, α,α'-bis-[3-(*N*-methyl-*N*-butylcarbamoyl)piperidino]-*p*-xylene (**3g**), was synthesized with a predicted IC_{50} of 25 μM. When this compound was subsequently evaluated, the IC_{50} was 22.1 ± 5.5 μM, demonstrating the applicability of this model. The amide oxygen of the carbamoyl substituent appeared necessary for activity. Thus, for example, when the amide carbonyl group of **3a** (IC_{50} = 44.5 μM) was reduced to CH_2, the resulting compound **4** had a dramatically reduced activity, IC_{50} = 1565 μM. Compound **3a** was resolved into (+) and (−) enantiomers and a meso (0) diastereomer using fractional crystallization, diastereomeric tartrate formation, and chiral HPLC. Compared to (−)-**3a**, the (+) isomer was 15 times more potent when ADP was the agonist and 19 times more active when collagen was used as the agonist. Molecular modeling of *R*,*R*- and *S*,*S*-**3a** using the SYBYL program was used to examine their interactions with phosphatidylinositol (PI). There was a better fit between PI and the *R*,*R*-**3a** with the energy of interaction being 17.6 kcal/mol less than that of the *S*,*S*-**3a**/PI complex. Although the absolute stereochemistry of individual enantiomers is not known, this study shows that *R*,*R*-**3a** interacts more favorably with PI than does *S*,*S*-**3a** and that (+)-**3a** is a more potent inhibitor of human platelet aggregation than (−)-**3a**. It is postulated that because of their lipophilicity, these compounds penetrate the platelet membrane and are then protonated at the pH of the cytosol. The protonated N then neutralizes the anionic charge on the membrane phosphoinositides, thereby rendering them less susceptible to hydrolysis by phospholipase C. Thus, the determinant parameters for optimum antiplatelet activity in 3-carbamoylpiperidines are (1) the amide carbonyl, (2) appropriate stereochemistry of the 3-substituent and (3) a log P value of about 4.5.

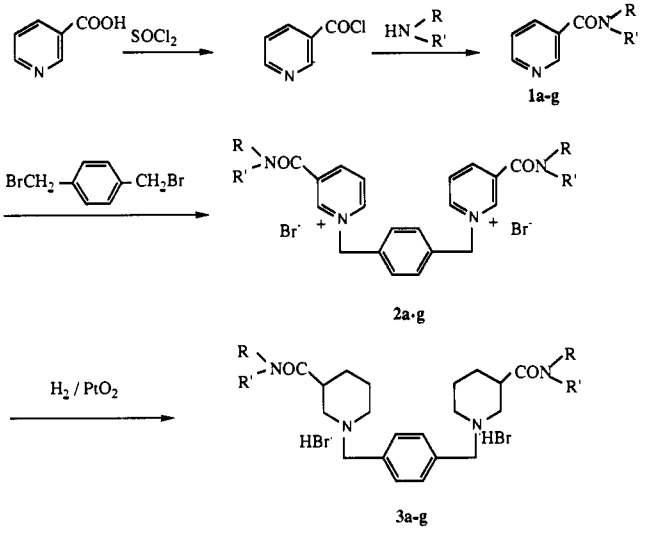
Introduction

Platelets are discoid anucleate cells whose primary functions are hemostasis and thrombosis. Thrombosis and thromboembolism are the leading causes of death and disability in the U.S. When a blood vessel wall is damaged, exposure to collagen in the endothelial lining activates the platelets resulting in the release of proaggregatory agonists such as ADP, thromboxane A_2, serotonin, and catecholamines. These agonists then activate the surrounding platelets leading eventually to thrombus formation. Exposure of blood to artificial surfaces leads almost invariably to the deposition of a layer of adherant platelets often accompanied by activation of the intrinsic coagulation system and the formation of a thrombus.[1-4] All mechanical prostheses carry with them a long-term risk of systemic embolism, and thrombosis induced by contact with biomaterials is a major obstacle in the further development of artificial internal organs.[2,4]

Platelet aggregation inhibitors such as aspirin, dipyridamole, and sulfinpyrazone have been used with limited success in different forms of thromboembolic disease.[5-7] All the currently available antiplatelet agents have limitations with regard to broad clinical utility, side effects, and dosage regimen.[8,9]

Carbamoylpiperidines (structure **3**, Scheme I) constitute an attractive group of compounds known to inhibit platelet aggregation.[10,11] Among compounds of this type, increasing hydrophobicity of the molecule was found to increase potency, and the structural modifications made to date involved alterations in the aralkane or alkane chain connecting the two piperidinyl nitrogens of the molecule.[10,12]

Scheme I



This report describes the effect of the modification of R and R′ on platelet aggregation inhibitory activity, the

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Table I. Analytical and Physical Data of Novel Carbamoylpyridinium and Carbamoylpiperidine Derivatives

compd	R	R′	yield, %	recryst solvent	mp, °C	bp, °C (mmHg)/n_D^{25}	formula[a]
2b	H	$CH_2C_6H_5$	78	MeOH	249.3–250.0		$C_{34}H_{32}N_4O_2Br_2$
3b	H	$CH_2C_6H_5$	26	MeOH	267.0–268.0		$C_{34}H_{44}N_4O_2Br_2$
1c	CH_3	$CH_2C_6H_5$	71			152–156 (0.05)/1.5852	$C_{14}H_{14}N_2O$
2c	CH_3	$CH_2C_6H_5$	72	EtOH	201.0–202.0		$C_{36}H_{36}N_4O_2Br_2$
3c	CH_3	$CH_2C_6H_5$	33	EtOH/EtOAc	251.5–252.2		$C_{36}H_{48}N_4O_2Br_2$
1d	CH_2CH_3	$CH_2C_6H_5$	77			172–174 (0.10)/1.5757	$C_{15}H_{16}N_2O$
2d	CH_2CH_3	$CH_2C_6H_5$	84	EtOH	256.0–257.0		$C_{38}H_{40}N_4O_2Br_2$
3d[b]	CH_2CH_3	$CH_2C_6H_5$	67	EtOH/EtOAc	285.0–287.0		$C_{38}H_{52}N_4O_2Br_2$
2e	$CH_2CH_2CH_3$	$CH_2C_6H_5$	77	EtOH	257.0–258.0		$C_{40}H_{44}N_4O_2Br_2$
3e[b]	$CH_2CH_2CH_3$	$CH_2C_6H_5$	27	EtOH	276.0–277.0		$C_{40}H_{56}N_4O_2Br_2$
1f	$CH_2C_6H_5$	$CH_2C_6H_5$	65	toluene	80.0–81.0		$C_{20}H_{18}N_2O$
2f	$CH_2C_6H_5$	$CH_2C_6H_5$	72	EtOH	212.0–213.0		$C_{48}H_{44}N_4O_2Br_2$
3f[b]	$CH_2C_6H_5$	$CH_2C_6H_5$	65	EtOH/EtOAc	263.0–264.0		$C_{48}H_{56}N_4O_2Br_2$
3a base[c]	CH_2CH_3	CH_2CH_3	46	EtOAc	122.5–126.0		$C_{28}H_{46}N_4O_2$
4 base[c]	CH_2CH_3	CH_2CH_3	64			213–216 (0.07)/1.5129	$C_{28}H_{50}N_4$
4[c]	CH_2CH_3	CH_2CH_3	83	EtOH/EtOAc	252.8–253.8		$C_{28}H_{54}N_4Cl_4 \cdot 2H_2O$
3g	CH_3	$(CH_2)_3CH_3$	70	PrOH	258.0–258.5		$C_{30}H_{52}N_4O_2Br_2$

[a] The elemental analyses were within ±0.4% of the theoretical values for C, H, N, and halogen when present. [b] Hydrogenation was carried out at 50 °C. [c] See Experimental Section.

functional importance of the 3-amide group, the influence of hydrophobicity, and the stereochemistry of the 3-amide substituent.

Results and Discussion

The synthetic approach for making carbamoylpiperidines (Scheme I) was to convert nicotinic acid into the acid chloride followed by acylation of the appropriate amine. The resulting amides (**1**, Scheme I) were treated with α,α'-dibromo-*p*-xylene to form bis(3-carbamoylpyridinium) salts (**2**, Scheme I) which, upon hydrogenation (PtO_2), afforded structure **3**.[13–15] The analogues prepared along with the methods employed and yields obtained are summarized in Table I.

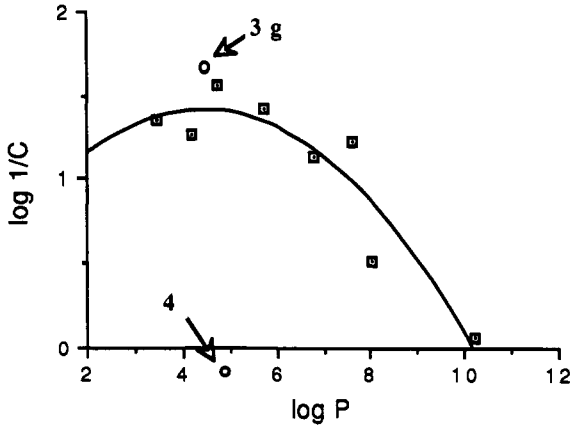

Figure 1. Relationship between platelet aggregation inhibitory activity (log 1/*C*; $C = IC_{50} \times 10^{-3}$; IC_{50} = compound concentration which inhibits ADP-induced aggregation by 50%) and hydrophobicity (log *P*; *P* = octanol/water partition coefficient) of carbamoylpiperidines.

Chiral Resolution. **3a**·2HBr was found to contain three stereoisomeric forms which are designated as **3a**-A, **3a**-B and **3a**-C, respectively. Of these, **3a**-C·2HBr selectively crystallized out from ethanol in the first crop which had isomeric composition of 80% C, 12% A, and 8% B as determined by chiral HPLC using an α_1-acid glycoprotein (AGP) column. Conversion of **3a**·2HBr into the free base i, mp 122.5–126 °C, followed by further purification afforded **3a**-C with 99% purity. The composition of **3a**-A, -B, and -C in the second crop of **3a**·2HBr was 40%, 40%, and 20%, respectively. Conversion of the second crop of **3a**·2HBr into free base ii, mp 160–161.8 °C, followed by recrystallization, resulted in a change in the composition to 54% A and 46% B, with C being lost in the mother liquor. The identity of the low- and high-melting forms of free base as having structure **3a** was confirmed by elemental analysis and desorption chemical ionization mass spectrometry. Low-melting free base i, MS *m/z* 471 $(M + H)^+$, was consistent with the proposed structure. High-melting free base ii, MS *m/z* 471 $(M + H)^+$, was very similar to that of the former, suggesting that both forms of the free base are positional isomers. Interestingly, a diastereomeric tartrate salt formed only with **3a**-B, as evidenced by HPLC of the resulting free base. The filtrate from **3a**-B D-(−)-tartrate contained a mixture of **3a**-A and

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Table II. Platelet Aggregation Inhibitory Activities and Partition Coefficients of Carbamoylpiperidines (Structure 3, Except for Compound 4)

compd	log P^a	IC_{50}, μM ± SE[b]	log $1/C^c$	n^g
3a	3.478	44.5 ± 12.7	1.352	6
3b	4.176	53.6 ± 5.3	1.271	6
3c	4.718	27.3 ± 3.2	1.564	7
3d	5.756	37.7 ± 4.6	1.424	7
3e	6.794	72.9 ± 20.8	1.138	5
3f	8.034	302.6 ± 60.8	0.519	5
5[d]	7.630	58.7	1.231	
6[e]	10.202	862.2	0.064	
4[f]	4.80	1565.0 ± 459.9	−0.195	4
3g	4.516	22.1 ± 5.5	1.656	6

[a] Octanol/water partition coefficients using PROLOG version 4.1e, CompuDrug Inc. [b] Compound concentration which inhibits ADP-induced platelet aggregation by 50%. [c] $C = IC_{50} \times 10^{-3}$. [d] α,α′-Bis[3-(*N*,*N*-dibutylcarbamoyl)piperidino]-*p*-xylene dihydrobromide. From ref 11 (computed based on the relative values of **3a**). [e] α,α′-Bis[3-(*N*-decylcarbamoyl)piperidino]-*p*-xylene dihydrobromide. From ref 27 (computed based on the relative values of **3a**). [f] α,α′-Bis[3-[(*N*,*N*-diethylamino)methyl]piperidino]-*p*-xylene tetrahydrochloride. [g] *n* = Number of individuals determinations.

3a-B. Also, **3a**-A and **3a**-C did not form diastereomeric salts with either D-(−)-, or L-(+)-tartaric acids. In both cases, the isomers coprecipitated even after three recrystallizations. It was therefore necessary to obtain pure **3a**-A by preparative chiral HPLC of free base ii, although **3a**-B was prepared via tartaric acid salt formation, and **3a**-C by selective crystallization from the (low-melting) free base i.

Circular dichroic spectra of the three isomers thus resolved showed that **3a**-A had a negative Cotton effect at 220 nm and that **3a**-C had a positive Cotton effect at the same wavelength, suggesting that the two are enantiomers. **3a**-B appears to be the meso isomer with no Cotton effect.

Hydrophobicity. Figure 1 depicts the relationship between activity (log $1/C$) and hydrophobicity (octanol/water partition coefficients expressed as log P). This plot was constructed initially for compounds **3a-f**. It showed that the parabolic relationship is statistically significant (eq 1).

$$\log (1/C) = 0.387(\log P) - 0.043(\log P)^2 + 0.545 \quad (1)$$

$$n = 8;\ r = 0.93;\ s = 0.22;\ F_{\alpha=0.01} = 16.7$$

Thus, the single parameter log P accounts for about 87% ($r^2 = 0.87$) of the variance of the activities of these derivatives. The data also suggest that hydrophobicity associated with the 3-substituent plays a significant role in influencing the platelet aggregation inhibitory activity.

From eq 1, the optimum log P value is 4.5. With this parabolic relationship as a model, the derivative **3g** was designed (log P = 4.5) with a predicted log $1/C$ value of 1.6 (IC_{50} = 25 μM). When this compound was synthesized and evaluated, the observed log $1/C$ value was 1.7 (IC_{50} = 22.1 ± 5.5 μM).

The log P values along with the IC_{50} values of all the test compounds are given in Table II. The most active compound in this series was **3g**. The activities decreased in the order Bu, Me > Bz, Me > Bz, Et > Et_2 > Bz, H > Bu_2 > Bz, Pr > Bz_2 > Dec_2.

The remarkable difference in the potencies of compounds **3a** (log P = 3.48; IC_{50} = 44.5 μM) and **4** (log P = 4.80; IC_{50} = 1565 μM) which are structural analogues is noteworthy. Based on the log P value, the activity of compound **4** should be between 27.3 μM (**3c**) and 37.7 μM (**3d**), but instead, it is a much weaker inhibitor. One difference is that the amide carbonyl of **3a** is reduced to a CH_2 group in **4**, illustrating the functional importance of the carbamoyl group. Also, compound **4**, by virtue of its high degree of basicity, would be present mostly in its ionized form in the medium surrounding the platelets. Consequently, penetration of the platelet plasma membrane is impeded.

Table III. Inhibition of Human Blood Platelet Aggregation by *rac*-**3a** and Its Stereoisomers

compd	IC_{50}, μM ± SE ADP-induced[a] (*n* = 5–6)	IC_{50}, μM ± SE collagen-induced[c] (*n* = 4)
rac-**3a**	44.5 ± 12.7	20.2 ± 3.7
3a-A (−)	233.4 ± 52.1	198.8 ± 29.4
3a-B (0)	41.4 ± 11.8	65.7 ± 18.3
3a-C (+)	15.3 ± 3.9[b]	10.7 ± 2.7
propranolol	178.4 ± 17.0	
trifluoperazine	201.8 ± 21.9	
chlorpromazine	155.7 ± 25.5	

[a] See ref 28. [b] Significantly different from **3a**-A, $P < 0.05$. [c] Significant differences, $P < 0.05$, **3a**-C > *rac*-**3a** > **3a**-B > **3a**-A.

Stereochemistry. Phospholipids constitute 70% of the total lipid content of platelet membranes.[16] Of these, PI and phosphatidylserine (PS) are important components of the inner layer.[17] PI is also present in the membranes of intraplatelet organelles and constitutes 5–7% of platelet phospholipids.[18] Earlier work by Quintana et al. demonstrated that compounds of this type interacted with anionic phospholipid films.[19] In monolayer films of different phospholipids on water subphase, these compounds have been found to increase the surface pressure and surface potential with PI and PS but not with phosphatidylcholine (PC), phosphatidylethanolamine (PE), and stearic acid. They postulated that because of their hydrophobic character, these compounds can penetrate the lipid bilayer of the platelet plasma membrane and then, at the pH of the cytosol, are protonated. The cationic N (of the piperidine ring) then neutralizes the anionic charge on PI or PS, thereby rendering them less susceptible to hydrolysis by phospholipase C. This results in diminished generation of inositol 1,4,5-trisphophate (IP_3) and of cytosolic ionized calcium[20] ($[Ca^{2+}]_i$), which is necessary for myosin phosphorylation and subsequent platelet activation. Since the molecules of PI and PS have asymmetric centers, stereoselective binding to xenobiotics seems probable.

To test this hypothesis, we examined **3a** which has two chiral centers and therefore can exist as two stereoisomers and a meso diastereomer. It is one of the most promising inhibitors of platelet aggregation.[10] It was effective in inhibiting human platelet aggregation in vitro,[21] polymer surface-induced platelet clustering of whole human blood

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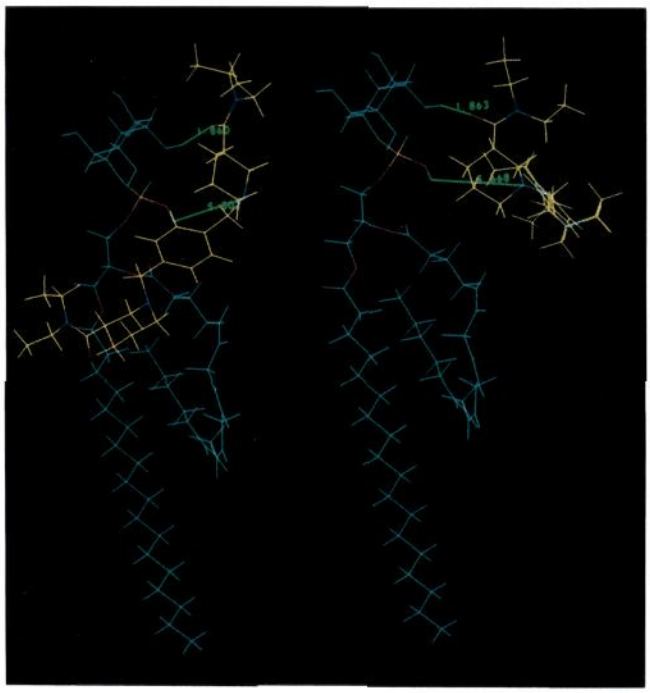

Figure 2. View of the superposition of **3a** enantiomers (yellow) (left, *R,R*-**3a**; right, *S,S*-**3a**) and PI (blue) constructed from the SYBYL fragment library, indicating (top) hydrogen bonding (1.860 Å with *R,R* and 1.863 Å with *S,S*) and (lower) ionic interaction (5.502 Å with *R,R* and 5.668 Å with *S,S*) both in green.

in vitro,[22] platelet aggregation in dogs ex vivo,[23] and platelet deposition on dacron-coated vascular grafts in exteriorized arteriovenous shunts in baboons[24] while showing relatively low toxicity in mice and rats.[25]

Using an α_1-acid glycoprotein chiral HPLC column, **3a** was resolved into three fractions, viz. **3a**-A, **3a**-B, and **3a**-C.[26] The circular dichroic spectra showed a negative Cotton effect at 220 nm with **3a**-A and a positive Cotton effect in the same region with **3a**-C suggesting that the two are enantiomers.[26] **3a**-B with no Cotton effect must be the meso isomer. Work on the determination of the absolute stereochemistry of the individual isomers is in progress.

The platelet aggregation inhibitory potencies of the *rac*-**3a**·2HBr and its stereoisomers were evaluated using ADP and collagen as the agonists (Table III). With ADP as the agonist, **3a**-C was 15 times more potent than **3a**-A and 2.9 times more active than *rac*-**3a**. When collagen was the agonist, **3a**-C was 18 times more potent than **3a**-A and twice as active as *rac*-**3a**. Analysis of mean IC_{50} data obtained using ADP as the agonist was carried out using the Kruskal–Wallis nonparametric analysis of variance and Bonferroni multiple comparisons.[29a] Significant differences at the $P = 0.05$ level show that **3a**-C > **3a**-A. The IC_{50} data obtained by using collagen as the agonist were analyzed by the Friedman nonparametric analysis of variance for blocked data and Bonferroni multiple comparisons.[29b] Significant differences at the $P = 0.05$ level show that **3a**-C > *rac*-**3a** > **3a**-B > **3a**-A. Propranolol, trifluoperazine, and chlorpromazine were included as positive controls to ascertain the proper functioning of our aggregation test system.[27,28]

Although the absolute stereochemical configurations of each isomer fraction are not yet assigned, we constructed molecular models to examine their interaction with PI.

Figure 2 shows the stick model comparison between the complex pairs *R,R*-enantiomer/PI and *S,S*-enantiomer/PI and indicates that there is a better fit between *R,R*-**3a** and PI than there is between the *S,S*-**3a** and PI. It is qualitatively clear that the *R,R*-enantiomer fits better in the proposed active site region selected for PI.

The energy of the *R,R*-enantiomer/PI complex is 17.6 kcal/mol less than that for the *S,S*-enantiomer/PI complex. *R,R*-**3a** interacts with PI (energy of interaction 44.3 kcal/mol), forming a hydrogen bond (between the amide O of **3a** and the 3-OH group of inositol in PI) and an ionic bond (between the protonated piperidinium N of **3a** and the phosphate O of PI). The energy of interaction between PI and *S,S*-**3a** is 61.9 kcal/mol. In the latter case, while hydrogen bond formation is possible, an ionic bond is not possible inasmuch as the piperidinyl N of **3a** is separated from the phosphate O of PI by other atoms.

Studies using X-ray crystallography are under way to determine the absolute stereochemistry of the enantiomers of **3a**. Until such time as the assignment is made, however, one can conclude that the *R,R*-enantiomer binds better to the proposed site and, therefore, is predicted to be enantiomer **3a**-C.

In order to examine the role of the amide group in the 3-carbamoyl substituents, the 3-amide group of **3a** was reduced to CH_2 with $LiAlH_4$. This resulted in a dramatic diminution in activity. That is, the IC_{50} of **4** was 1565 μM compared to 44.5 μM of *rac*-**3a**. The H-bonding between the 3-OH group of inositol and amide O seems essential for activity. In fact, the molecular modeling demonstrates that such a H-bond is possible with **3a** and is not possible with **4**. Also, since reduction of the amide groups introduced two more basic centers into the molecule, poor lipophilicity resulting from a high degree of ionization would interfere with transport across the platelet plasma membrane.

Based on several observations over a 10-year period, a tentative hypothesis on the mechanism of action of the nipecotic acid derivatives is offered. Because of their lipophilicity, these compounds can penetrate the lipid bilayer of the platelet membrane in the form of unionized free bases, and at the pH of the cytosol, the piperidine N is protonated.[12,30,31] The cationic N then neutralizes the

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anionic phosphoinositides of the platelet membrane as well as those of certain organelles.[19,32] The compounds are thus capable of stabilizing membrane complexes of the dense tubular system as well as other membrane complexes sequestering calcium in the platelet.[33] By maintaining or even enhancing the integrity of these membrane complexes, the compounds would inhibit Ca^{2+} release into the platelet cytosol.[21,33] Furthermore, by reducing the response sensitivity of anionic phospholipids,[19] the compounds could block or inhibit the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, thereby reducing or preventing the release of inositol 1,4,5-trisphosphate[34] perceived to act as a second messenger triggering the internal discharge of Ca^{2+} [20,35] which is causally linked to platelet activation. Secondarily, activation of phospholipase A_2 as well as the pathways associated with it are also impeded.[36] In addition, these compounds may also act on specific receptors on the platelet membrane and/or at discrete intraplatelet loci. Such possibilities are being examined.

Conclusions

For 3-carbamoylpiperidines (**3**, Scheme I), the following structural features appear desirable for optimum platelet aggregation inhibitory activity: (1) a log *P* value of 4.5 and (2) appropriate stereochemistry at the 3-position of the piperidine ring. Results obtained so far with compound **3a**, suggest that (+)-CD form is favored over (−)-CD, and based on molecular modeling studies the *R,R* form provides a better conformation than the *S,S*-enantiomer.

Experimental Section

Melting points were taken in glass capillary tubes on a Buchi melting point apparatus and are uncorrected. ^1H NMR spectra of representative compounds dissolved in $CDCl_3$ were recorded on a Varian EM 360L spectrometer. The microanalyses were performed at Galbraith Laboratories, Inc., Knoxville, TN, and the elemental compositions of the compounds agreed within ±0.4% of the calculated values. The chromatographic system consisted of a Waters U6K injector, a Model 600E Powerline multisolvent delivery system, a Model 484 tunable UV/vis detector, a NEC PowerMate SX plus computer, and a NEC P5200 printer/plotter.

Chiral-AGP (α_1-acid glycoprotein) analytical column, 100 × 4.0 mm (5 μm) (ChromTech AB, Norsborg, Sweden), and a chiral-AGP semipreparative column, 150 × 10.0 mm (5.0 μm) (ChromTech), attached to a diol precolumn, 10 × 10 mm (5.0 μm), were purchased from Regis Chemical Co., Morton Grove, IL. Mass spectra were recorded on a Finnigan MAT, TSQ-70 mass spectrometer in the methane chemical ionization mode. Circular dichroic spectra were obtained in a JASCO 500 spectropolarimeter. Compound solutions (approximately 1.0 μg/mL) were made in 0.5 mM phosphate buffer containing 0.5 mM tetrabutylammonium hydrogen sulfate (TBA).

log *P* values were calculated using the PROLOGP program of CompuDrug, Inc. Molecular modeling was performed using the software package SYBYL (Tripos Associates, version 5.4) implemented on an Evans and Sutherland PS390 graphics terminal connected to a minicomputer from SUN Microsystem Inc.

The structures of the enantiomers of **3a** and PI were taken from the fragment library of SYBYL. Original models of compounds were built with standard bond lengths and angles. The energy of each structure was minimized using the MAXIMIN2 program with the Tripos force field.

After minimization, the enantiomers of **3a** were interacted with (*sn*-2-S)-PI using SYBYL to manipulate the molecules so that the hydrogen bond and ionic interactions were aligned. The intermolecular energy of interaction between a pair of molecules was then minimized with MAXIMIN2 with the Tripos force field. The figures were photographed directly from the screen.

Chemistry. Representative procedures for preparing a N-substituted nicotinamide (**1**, Scheme I), a bis(3-carbamoylpyridinium)-*p*-xylene (**2**, Scheme I), and a bis(3-carbamoylpiperidino)-*p*-xylene (**3**, Scheme I) are described below.

***N*-Benzyl-*N*-methylnicotinamide (1c).** Thionyl chloride (35.7 g, 0.3 mol) was added dropwise to a cold stirred mixture of 36.9 g (0.3 mol) nicotinic acid, 48.5 mL (0.6 mol) of pyridine, and 15 mL toluene. After the reaction mixture was gradually heated to and maintained at 90 °C for 1 h, 36.4 g (0.3 mol) of *N*-benzylmethylamine in 50 mL of toluene was dispensed gradually into the reaction mixture from a dropping funnel. An additional 80 mL of pyridine was added to trap the liberated acid. The stirred mixture was maintained at 60 °C for 3 h and at 90 °C for 1 h, after which the toluene layer containing the product was separated and washed with 4 × 250 mL of 1 N HCl. The pH of the combined aqueous acidic solution was adjusted to 9.0 with 29% aqueous Na_2CO_3, and the amide was extracted with 4 × 250 mL of toluene. The extract was dried ($MgSO_4$), filtered, and concentrated. The residue was distilled under high vacuum ($bp_{0.050}$ 152–153 °C) to yield 48.0 g of the amide **1c** as a yellowish oil.

α,α′-Bis[3-(*N*-benzyl-*N*-methylcarbamoyl)pyridinium-yl]-*p*-xylene Dibromide (2c). To a stirred solution of 26.6 g of *N*-benzyl-*N*-methylnicotinamide (**1c**, 0.1176 mol) in 350 mL of absolute ethanol was added 15.0 g (0.0568 mol) of α,α′-dibromo-*p*-xylene in 200 mL of hot acetone from a hot water-jacketed dropping funnel. After refluxing for 9 h, the solid reaction product was recrystallized from absolute ethanol to give 29.3 g of **2c**.

α,α′-Bis[3-(*N*-benzyl-*N*-methylcarbamoyl)piperidino]-*p*-xylene Dihydrobromide (3c). Catalytic reduction (0.5 g of PtO_2) of 12.5 g (0.0174 mol) of **2c** in 100 mL of ethanol/150 mL of water (60 psi, ambient temperature) followed by recrystallization of the product from absolute ethanol afforded 4.1 g of **3c**: ^1H NMR ($CDCl_3$) δ 1.70 (bs, 8 H, CH_2), 2.00–2.95 (m, 8 H, CH_2NCH_2), 2.90 (s, 6 H, NCH_3), 3.50 (s, 4 H, $NCH_2C_6H_4$), 4.57 (bs, 4 H, $NCH_2C_6H_5$), 7.30, 7.27 (2 s, 14 H, Ar-H).

α,α′-Bis[3-[(*N*,*N*-diethylamino)methyl]piperidino]-*p*-xylene Tetrahydrochloride (4). A solution of **3a** (8.2 g) in water was adjusted to pH 9.0 with aqueous 29% Na_2CO_3 and extracted with ether. Ether was removed by evaporation, and the residue was recrystallized from ethyl acetate to yield 4.0 g of **3a** free base, mp 122.5–126 °C.

To a solution of $LiAlH_4$ (12.24 g) in anhydrous THF (270 mL) was added **3a** free base (34.5 g) in anhydrous THF (400 mL) dropwise while stirring, and the reaction mixture was refluxed at 66.5 °C for 19 h under N_2. After the mixture was cooled to room temperature, 97 mL of 20% NaOH was added while stirring and maintaining the reaction mixture at 24–29 °C. After vigorous stirring for an additional 20 min the semisolid was filtered off and discarded, and the filtrate was extracted with 3 × 100 mL of THF. The combined extract was dried (anhydrous Na_2SO_4), filtered, and evaporated, and the resulting oil was subjected to fractional vacuum distillation. Free base **4** (20.7 g) was collected

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at $bp_{0.07}$ 213–216 °C: ^1H NMR ($CDCl_3$) δ 0.97 (t, J = 7 Hz, 12 H, CH_3), 1.67 (m, 8 H, NCH_2), 2.44 (q, J = 7 Hz, NCH_2CH_3), 3.46 (d, J = 3 Hz, 4 H, $NCH_2C_6H_4$), 7.27 (s, 4 H, C_6H_4).

A solution of free base **4** (20.0 g) in 500 mL of anhydrous ether was acidified to pH 5.0 with a saturated solution of dry HCl gas in diethyl ether at 0 °C, and the precipitate was recrystallized from a mixture of ethanol–ethyl acetate (2:3) to yield 10 g of **4**.

Synthesis of α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)-piperidino]-*p*-xylene Dihydrobromide (3a). Racemic **3a** was synthesized as described.[13] Briefly, α,α′-dibromo-*p*-xylene (15.0 g, 0.0568 mol) in 310 mL of acetone was added to a solution of *N*,*N*-diethylnicotinamide (23.29 g, 0.1307 mol) in 350 mL of absolute ethanol and refluxed (8.5 h) to yield α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)pyridiniumyl]-*p*-xylene dibromide (18.8 g, 0.0304 mol, mp 267.4–268.1 °C). Hydrogenation (PtO_2/H_2, 60.1 psi) of the quaternary compound afforded **3a** which upon recrystallization from absolute ethanol gave 9.4 g (0.0149 mol) of the "first crop", mp 280–281 °C dec. The filtrate yielded 6.5 g (0.0103 mol) of the "second crop", mp 279–279.8 °C dec.

Resolution of 3a on Chiral-AGP Column. The mobile phase consisted of 0.025 M phosphate buffer (PB) containing 0.025 M tetrabutylammonium (TBA) hydrogen sulfate, pH 6.50. The flow rate was 0.4 mL/min with the analytical column and 3.6 mL/min when the semipreparative column was used.

Chiral Resolution. Fractional Crystallization of 3a-C. A suspension (pH 9.0) of **3a** "first crop" (8.2 g) in aqueous 29% Na_2CO_3 was shaken with ether, the extract was evaporated, and the residue was recrystallized from 15 mL of ethyl acetate to yield 4.0 g **3a** free base i, mp 122.5–126 °C: methane DCI MS [M + H]$^+$ 471; fragmentation pattern consistent with proposed structure;[28] ^1H NMR ($CDCl_3$) δ 1.12 (t, J = 7 Hz, 12 H, CH_3), 1.70 (bs, 8 H, NCH_2), 3.33 (q, J = 7 Hz, 8 H, NCH_2CH_3), 3.52 (s, 4 H, $NCH_2C_6H_4$), 7.30 (s, 4 H, C_6H_4). Anal. ($C_{28}H_{46}N_4O_2$) C, H, N. Isomeric purity by HPLC 91% peak C. This was further purified by dissolving 2.5 g in boiling EtOAc (12.0 mL) and bringing it slowly to room temperature. After 2 days, the separated precipitate was recrystallized a third time from EtOAc to obtain 0.1 g of peak C of isomeric purity by HPLC 99%.

Similarly, a solution of the "second crop" of **3a** (31.6 g) in aqueous Na_2CO_3 was extracted with ether, and the free base was recrystallized once from 140 mL of EtOAc to give 10.9 g of **3a** free base ii, mp 160–161.8 °C. Anal. ($C_{28}H_{46}N_4O_2$) C, H, N. Isomeric purity by HPLC, 54% peak A, and 46% peak B.

Resolution of 3a-B via Diastereomeric Salt Formation. The free base ii (3.0 g, 0.00637 mol) and D-(−)-tartaric acid (1.92 g, 0.0128 mol) were dissolved in 50 mL of warm absolute ethanol and kept at room temperature for 3 days. The precipitate was recrystallized from aqueous 91% ethanol. The resulting **3a** (−)-tartrate was dissolved in 1.0 mL of water and made alkaline (pH 9.0) with aqueous 29% Na_2CO_3, and the mixture was extracted three times with ether. The combined ether layers were washed repeatedly with water and evaporated under reduced pressure. The resulting solid was recrystallized from ethyl acetate to give 8.9 mg of peak B, isomeric purity by HPLC 97%.

Preparative Resolution of 3a-A Using a Chiral HPLC Column. An 80-μL aliquot of a solution (10 mg/mL) of the **3a** free base ii in the HPLC mobile phase was injected into the semipreparative chiral-AGP column, and the eluant from peak A was collected. This was repeated until 115 mg of the free base was resolved. The resulting eluant (2.5 L) was concentrated on a Rotavapor to 100 mL. The pH was adjusted to 9.0 with aqueous 29% Na_2CO_3 and extracted four times with 200-mL portions of ether. The ether layer was washed three times with water and then dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure (N_2). The residue was kept at 5 °C for 4 days, and the resulting semisolid was recrystallized with EtOAc to yield 19.15 mg of peak A of 95% isomeric purity.

ADP-Stimulated Platelet Aggregation. Reagents, methodology, and the turbidimetric procedure employed in the aggregometric determinations were reported previously.[11,12,27] Briefly, blood was obtained from male donors, 21–33 years of age, who had fasted overnight and affirmed abstinence from all medications, alcohol, tobacco, and caffeine for at least 1 week prior to donation. The minimal concentration of adenosine diphosphate (ADP) eliciting full biphasic aggregation (8.16 ± 0.19 μM for 64 plasma samples) was determined using platelet-rich plasma (PRP) from each donor immediately prior to the determination of aggregation inhibition induced by the test compounds. With the exception of disposable siliconized glass cuvettes and siliconized metal stir bars, only plastic items were used to handle blood and plasma. PRP was prepared by centrifugation of citrated blood (blood/citrate ratio 8:1, 3.2% sodium citrate) in a Beckman J-6B centrifuge, 120*g*, 15 min, 23 °C, and recovering the upper PRP layer. Simultaneously, platelet poor plasma (PPP) was prepared in a similar manner using a Sorvall GLC-2B centrifuge, 1100*g*. The platelet count of PRP was adjusted to 250 000–300 000 platelets/mm^3 using autologous PPP. The plasma was gassed with 5% CO_2–95% air (v/v), capped, and held at 37 °C until dispensed (450 μL/cuvette) to cuvettes with stir bars in a Marsters constant temperature (37 °C) block. As rapidly as possible, bubbles were removed from the PRP, and each cuvette was capped with Parafilm to minimize CO_2 loss and pH rise. Test compounds were reconstituted to appropriate concentrations in redistilled 95% ethanol. Solubility permitting, 95% ethanol–water (1:1, 1:2, or 1:4) was used in the aggregation system. Total ethanol concentration up to 0.190% was reported to be without effect on platelet aggregation.[37] Control cuvettes received equal volumes of the appropriate vehicle without test compound. Test compound was added to PRP after 15 s of stirring (1100 rpm) in a Payton Associates Dual Channel Aggregometer equipped with a Fisher Dual Channel OmniScribe recorder and transferred back to the Marsters constant temperature block 15 s after the addition of compound. After 1 min and 45 s, the cuvette was returned to the aggregometer and the agonist (ADP, 50 μL) was added after 4-min incubation with compound. Changes in light transmission associated with platelet shape change and aggregation were recorded for an additional 5.5 min. The (control) cuvette containing the vehicle-treated PRP was initiated 1 min after the (treated) cuvette containing plasma treated with the appropriate test compound and followed the same sequence of events. Inhibition of aggregation (IC%) was expressed as the difference in maximum pen responses of the paired treated and control cuvettes as a percent of the control response. The IC_{50} (concentration of test compound effecting 50% inhibition of aggregation) was determined by linear regression of IC% on log concentration of test compound.

Collagen-Stimulated Platelet Aggregation. Aequorin was obtained from Friday Harbor Photoproteins, Friday Harbor, WA. Ethylene glycol tetraacetate (EGTA), prostaglandin E_1 (PGE_1), albumin (bovine, fraction V, powder), and dextrose were obtained from Sigma Chemical Co., St. Louis, MO. Collagen was obtained from Nycomed Arzneimittel, GmbH, Munchen, Germany. Fibrinogen (grade L lyophilized powder) was purchased from Kabi Vitrum, Franklin, OH. Dimethyl sulfoxide (DMSO) was obtained from Pierce Chemical Co., Rockford, IL. The platelet ionized calcium aggregometer (PICA), Chrono-Log Corp., Ltd., Havertown, PA was used in all assays.

The basic procedure of Yamaguchi et al.[38] was followed. Blood was collected and PRP prepared as described for ADP-stimulated aggregation and 1.0 M citric acid, 0.009 of the volume of PRP, was added. Platelets were collected (800*g*, 15 min), resuspended in and washed (800*g*, 15 min) with HEPES-buffered saline, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 0.1% bovine albumin, 0.5% glucose, 3.8 mM HEPES), with added EGTA (5 mM) and PGE_1 (1 μM). The platelet pellet was resuspended in 80 μL of the same buffer and added to 20 μL of 3 mg/mL aequorin solution in a 1.5-mL Eppendorf centrifuge tube. Six 1-μL aliquots of DMSO were added at 90-s intervals with brief, gentle mixing on a vortex mixer, and the platelet suspension was incubated 2 min after the last addition. One milliliter of HEPES-buffered saline was added, gently mixed, and again incubated for 2 min. The platelets were pelleted (1000*g*, 30 s), resuspended in the same buffer, and pelleted again. The final pellet was resuspended in HEPES-buffered saline to which were added 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The platelet

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count was adjusted to (3-4) × 10^5 platelets/μL.

The PICA was used to obtain aggregation data as follows. Fibrinogen (1.2 μg) in 100 μL of buffer was added to 0.9 mL of platelet suspension. The cuvette was placed in the PICA, 37 °C, with stirring at 1100 rpm. After 15 s, the vehicle (95% ethanol) or test compound in vehicle was added, and 1 min later the agonist (collagen, 5 μg in 5 μL of buffer) was added. Aggregation was recorded for 5 min, the IC% and IC_{50} being determined as described for ADP-stimulated aggregation. Luminescence data, related to calcium flux, were obtained in parallel from the above experiment and will be reported elsewhere.

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Synthesis and Antiviral Activity of Methyl Derivatives of 9-[2-(Phosphonomethoxy)ethyl]guanine[1]

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A number of methyl derivatives of 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG, 1) have been synthesized and tested in vitro for anti-herpes and anti-human immunodeficiency virus (HIV) activity. Among these analogues, (*R*)-2′-methyl-PMEG [(*R*)-3] and 2′,2′-dimethyl-PMEG (7) demonstrated potent anti-HIV activity in the XTT assay with EC_{50} values of 1.0 and 2.6 μM, respectively. The corresponding (*S*)-2′-methyl-PMEG [(*S*)-3] was found to be less potent against HIV. In addition, the (*R*) and (*S*) enantiomers of 9-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine (HPMPG, 8) were prepared for comparison of biological activity, and shown to be active and equipotent against herpesviruses, but inactive against HIV.

Introduction

With the increasing worldwide problems of viral diseases, particularly acquired immunodeficiency syndrome (AIDS),[2] there continues to be a significant need for safe and effective antiviral agents. Several approaches including chemical synthesis, natural products screening, and biotechnology have been utilized to identify compounds having antiviral activity.[3] Since the discovery of selective antiviral agents such as acyclovir (ACV)[4] and zidovudine (AZT),[5] nucleoside analogues have become one of the major classes of compounds that might meet the need. Many of these analogues are converted to their corresponding triphosphates in vitro and in vivo, and terminate elongation of polynucleotide synthesis or destabilize the structure of DNA or RNA when incorporated into the viral genome. Alternatively, the triphosphates may act as inhibitors of viral polymerase and inhibit viral replication. Preferential interaction of the nucleoside triphosphate analogue with viral enzymes instead of host enzymes is an important factor in determining the ultimate selectivity of these nucleoside derivatives.

Recently, analogues of phosphorylated nucleosides have been investigated for their potential as antiviral agents.[6] (*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]adenine, ((*S*)-HPMPA), an acyclic nucleotide analogue reported by Holy and De Clercq,[7] is a representative of a new structural type of antiviral agent which possesses broad spectrum antiviral activity. Acyclic phosphonate nucleotides[8] are analogues of monophosphorylated nucleosides in which the furanose ring is replaced with an acyclic side chain and the $POCH_2$ unit of the monophosphate is replaced with a bioisostere, PCH_2O. These modifications lead to molecules which are chemically more stable than nucleoside monophosphate derivatives: nucleotide analogues such as HPMPA are not prone to enzymatic or chemical hydrolysis of the phosphonate group or to cleavage of the purine or pyrimidine base from the side chain. In cells, mono-

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