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New Carbamoylpiperidines as Human Platelet Aggregation Inhibitors

Zhengming Guo,[a] Xiaozhang Zheng,[a] Walter Thompson,[a] Marion Dugdale[b] and Ram Gollamudi[a,*]

[a]*Department of Pharmaceutical Sciences, The University of Tennessee, Memphis, TN 38163, USA*
[b]*Department of Medicine, The University of Tennessee, Memphis, TN 38163, USA*

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Abstract—A series of 3-carbamoylpiperidines (nipecotamides) are designed, synthesized and tested for their inhibitory action against adenosine diphosphate (ADP)-induced aggregation of human platelets. A structure–activity analysis of the bis(nipecotamido)aralkane type showed that a substituent on the piperidine ring should preferably be an amide and that the electronegativity of the carbonyl oxygen and the orientation of the amide group affected activities. Based on the knowledge of factors influencing platelet activation and aggregation, a nitric ester moiety which could release nitric oxide (NO) in situ, is incorporated into the nipecotamide structure. These compounds exhibit increased activity compared to those having no $-ONO_2$ function. They also show stereoselectivity, with the *meso* isomer being approximately twice as potent as the synthetic diastereomeric mixture. Replacement of the $-ONO_2$ function with hydroxyl, ester or alkyl groups considerably diminishes aggregation–inhibitory potential. Nipecotamides are shown here to inhibit the basal and collagen-induced rise in platelet inositol trisphosphate (IP_3) levels, as well as phosphoinositide turnover. A comprehensive mechanism of action is proposed taking earlier results into consideration.

Introduction

Platelets play a major role in thrombus formation particularly in the arterial system. Platelet deposition followed by thrombus formation has been implicated in the growth of atherosclerotic plaques.[1] A high degree of morbidity and mortality is associated with the formation of intravascular thrombi. When vascular endothelium is ruptured, the subendothelial matrix containing a variety of adhesive proteins such as collagen is exposed. Contact with these adhesive proteins activates platelets changing their discoid shape to spherical forms which then convert into tiny spheres. Following adhesion, platelets may undergo a spreading reaction in which multiple contacts are formed between the cell surface and the matrix. By doing so, platelets form a mesh and recruit other platelets at the site of injury to form a hemostatic plug. When sufficiently activated, platelets release their dense granule contents such as adenosine diphosphate (ADP), thromboxane A_2, serotonin, and catecholamines. These agonists, in their own capacity can then activate the surrounding platelets leading to thrombus formation.

Antiplatelet drugs such as aspirin and ticlopidine have been used to prevent acute thromboembolic artery occlusions in cardiovascular diseases and the benefits are evident.[2] However, it is also clear that one of the major problems in clinical use is the association of antithrombotic efficacy of antiplatelet agents with their interference of physiological platelet function in hemostasis. There is therefore a need for more specific and potent antiplatelet agents.

3-Carbamoylpiperidine derivatives (nipecotamides) have been reported to possess structural features that can inhibit platelet aggregation. Compounds in this class inhibit human platelet aggregation induced by ADP,[3] collagen,[4] thrombin,[5] epinephrine,[6] and the stable TxA_2-mimetic U46619 in vitro.[7] They inhibited collagen-induced platelet adhesion and thrombus growth, under simulated physiological conditions, with a shear rate of 1000 s^{-1}.[4] These compounds also inhibited polymer surface-induced clustering of platelets in human whole blood.[8] Nipecotamides inhibited platelet aggregation in Beagle dogs ex vivo.[9] These compounds reduced the deposition of platelets and fibrin on Dacron grafts, which were surgically implanted in exteriorized femoral arteriovenous shunts, in normal male baboons.[10] Nipecotamides also protected mice from thromboembolic

*Corresponding author. Tel.: +1-901-448-6026; fax: +1-901-448-6940.

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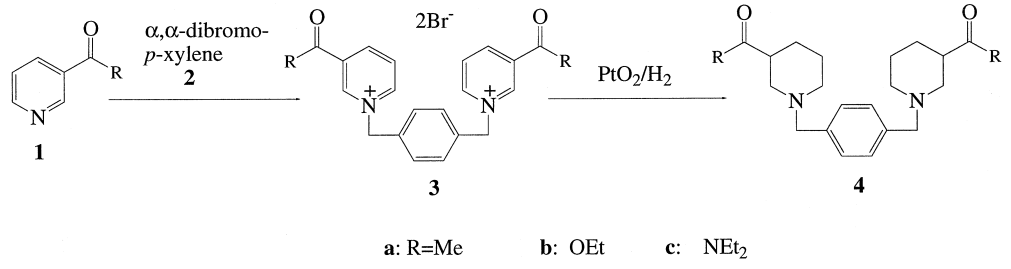
death caused by the intravenous injection of collagen + epinephrine.[11] These molecules thus offer promising leads for the development of drugs inhibiting platelet aggregation. Since they possess several sites with potential for structural manipulation, systematic modification of their structure may yield superior antiplatelet compounds with improved specificity and intensity of action. Nipecotamides have been shown to interact with phospholipids of the platelet membrane, thus stabilizing it against agonist-induced activation.[12a,b] Nitric oxide (NO), synthesized by the endothelial cells diffuses into platelets where, through mechanisms involving activation of intracellular soluble guanilylcyclase and resultant elevation of cyclic guanosine monophosphate (cGMP) levels, it inhibits adhesion and aggregation.[13a,b] Therefore, compounds capable of releasing therapeutic concentrations of NO could offer advantages as potential antithrombotic agents. Thus, nitric ester derivatives of nipecotamides would possess dual functions: (a) the nipecotamide moiety stabilizes the platelet membrane, thereby preventing activation and subsequent aggregation, and (b) the nitric ester segment releases NO, which by itself inhibits platelet adhesion and aggregation. The present report deals with a discussion of the structure–activity relationships of the 3-carbamoylpiperidines and the effect of incorporation of the nitric ester moiety into their structures, on platelet aggregation inhibitory activity.

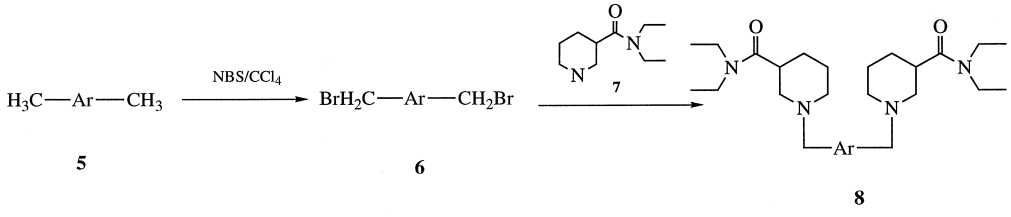
Chemistry

Previously reported methodologies were appropriately modified and adapted for the synthesis of compound **4a** (Scheme 1).[12a,14a] 3-Acetylpyridine **1** was reacted with α,α′-dibromo-*p*-xylene **2** to afford an appropriate quaternary intermediate **3**. The latter was reduced with PtO_2/H_2 to give the final product.

The synthetic procedures used for the compounds with a modified connecting bridge is shown in Scheme 2. Substituted α,α′-dibromomethyl-*p*-xylene (**6**) was prepared from the corresponding substituted dimethyl-*p*-xylene (**5**), followed by condensation with *N*,*N*-diethylnipecotamide (**7**) to afford the final product **8**. In the preparation of compounds **8a**, **8c** and **8l**, the respective starting materials **5** were synthesized as described for **8a**. Compound **8f** was obtained by acetylation of the amine **8e**. The latter was prepared by catalytic reduction of the nitro-substituted product **8d** using PtO_2/H_2. Since α-bromination is a free radical reaction, the reaction conditions are mainly dependent on the electrostatic properties of the aromatic ring. For example, in the synthesis of 2-nitro-α,α′-dibromo-*p*-xylene (**6d**), a refluxing period of 10 h was needed for the completion of the reaction whereas, stirring at room temperature for 24 h was needed in the synthesis of 2,5-dibromomethylfuran (**6h**).



Scheme 1.

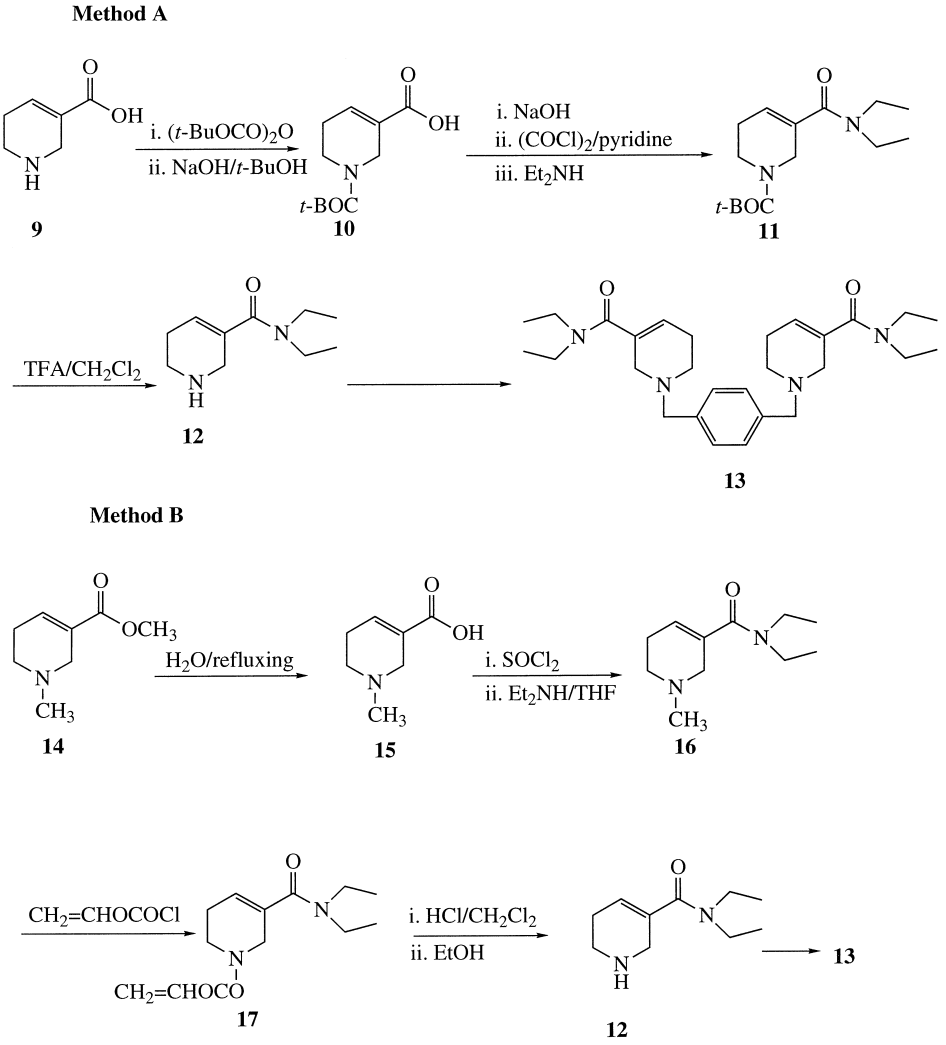


Cpd.	Ar	Cpd.	Ar
8a	2-EtO-*p*-C_6H_3	**8h**	2,5-Furanyl
8b	2-MeO-*p*-C_6H_3	**8i**	2,5-Thiophenyl
8c	2-*n*-C_6H_{13}-O-*p*-C_6H_3	**8j**	2,5-Pyrazinyl
8d	2-NO_2-*p*-C_6H_3	**8k**	α,α′-DiMe-*p*-C_6H_4
8e	2-NH_2-*p*-C_6H_3	**8l**	2-MeOOC-*p*-C_6H_3
8f	2-AcNH-*p*-C_6H_3	**8m**	2-Cl-*p*-C_6H_3
8g	1,4-Naphthyl	**8n**	2-I-*p*-C_6H_3
		8o	2,5-DiMethyl-*p*-C_6H_2

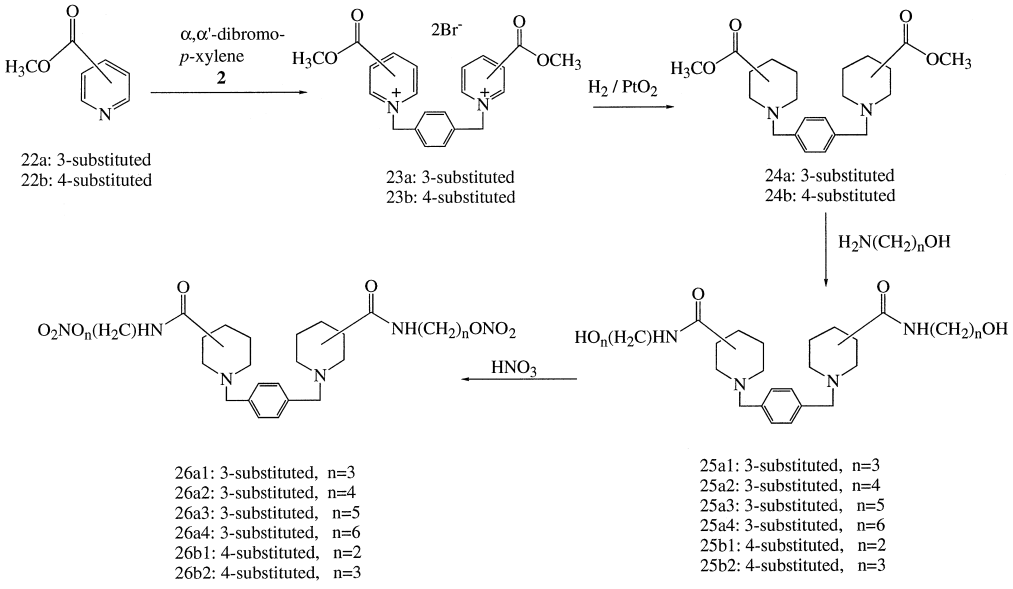
Scheme 2.

The synthetic routes to **13** are shown in Scheme 3. In method A, the ring *N* of guvacine (**9**) was first protected by preparing its *t*-BOC derivative **10**, which was then converted into the amide **11**. After removal of the *t*-BOC group, the amide **12** was treated with α,α′-dibromo-*p*-xylene (**2**) to yield **13**. Since the starting material guvacine[14b] (**9**) is very expensive, an alternate method was designed to obtain compound **13** in quantities sufficient for biological testing. In method B, hydrolysis of arecoline (**14**) afforded the acid **15**, from which the amide **16** was obtained. *N*-Demethylation of the latter afforded an intermediate **17**, which was then converted into **12** from which **13** was obtained as described in method A. The critical step in the synthesis of **13** by method A was the acylation reaction. Initially, isobutyl chloroformate was used to activate the acid **10** by forming a mixed anhydride, but the yield was very low (12%). The major product of the reaction was *N*,*N*-diethyl isobutyl formamide (81%), along with some unreacted acid. The possibility of steric hindrance by the isobutyl group was first entertained and ethyl chloroformamide was used in place of isobutyl chloroformate, but the yield was even lower (<6%). This suggested that the activity of the carbonyl group in *N*′-*t*-butoxycarbonyl-3,4-dehydronipecotic acid **10** was very low due to the formation of an α,β-unsaturated acid. Based on this assumption, a new method was designed. Sodium salt of the acid **10** was converted into a much more reactive intermediate (acid chloride) by treatment with oxalyl chloride in the presence of pyridine. Basic reaction conditions (pyridine, 0–15 °C) were employed to forestall the possible decomposition of the *t*-BOC derivative **10** by the HCl liberated during the reaction. The yield of the amide **11** was 84%. In method B, the critical step was in the removal of the vinyl formate group to form compound **12**. This reaction was carried out using an acid-induced deprotection process.[15] Compound **17** was first reacted with HCl gas to form an unstable adduct, which was then treated with ethanol to afford the acylal. This acylal was so reactive that no acid beyond that produced in the cleavage was required to complete the formation of the product **12** along with the volatile by-products, CO_2 and acetal.

The synthetic strategies of the nitric esters (**25a1**, **a2**, **a3**, **a4**, **b1**, **b2**) is outlined in Scheme 4. As in the preparation



Scheme 3.



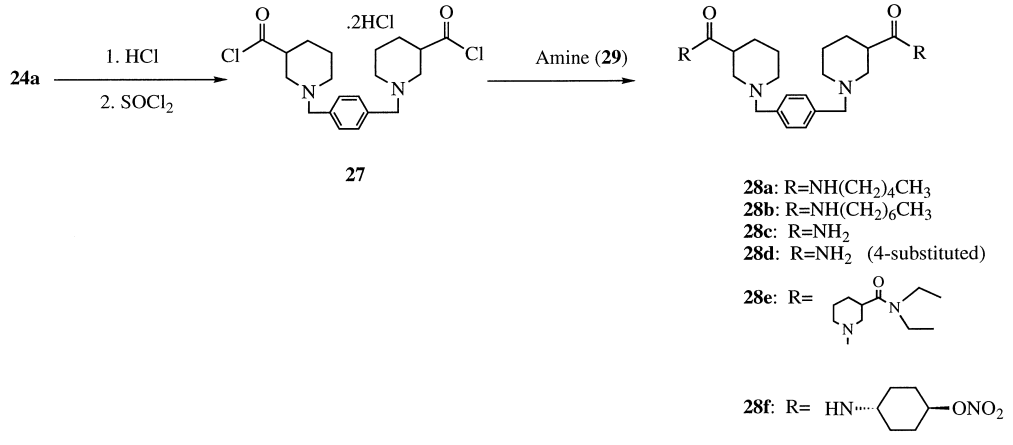
Scheme 4.

of compound **8**, α,α′-dibromomethyl-*p*-xylene (**2**) was condensed with methyl nicotinate (**22a**) (or isonicotinate, **22b**) to afford a pyridinium salt (**23**) which was further converted to the corresponding bis-(nipecotinyl) derivatives (**24**) using catalytic hydrogenation at 60 psi. Heating with the corresponding aminoalcohol converted compound **24** into the *N*-hydroxyalkyl amide **25**. The latter was then reacted with fuming HNO_3 to obtain the nitric ester **26**. With a view to avoid possible nitration of the xylene moiety, selective nitration of **25** was attempted successfully using low temperatures (−10 to −5 °C).

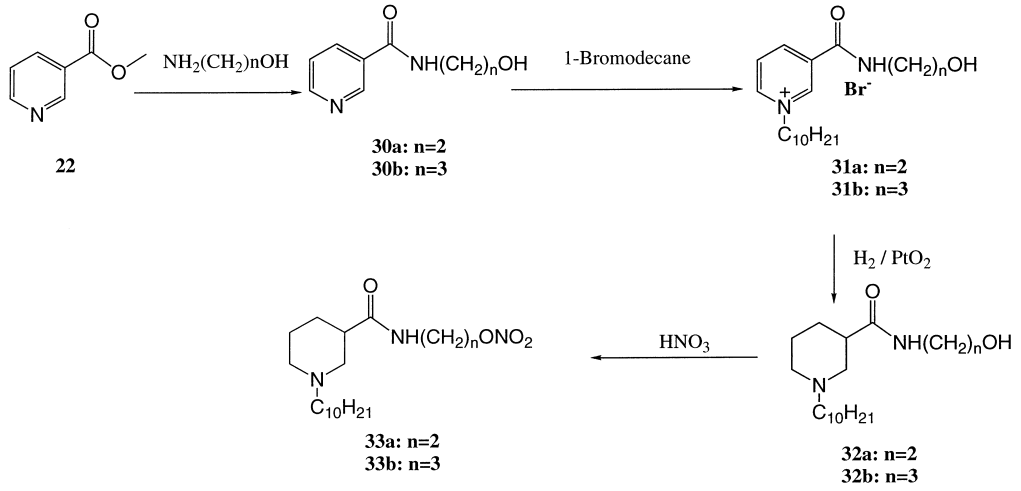
The other strategy employed for the synthesis of bisnipecotamide derivatives is outlined in Scheme 5. Hydrolysis of compound **24a** under acidic conditions followed by treatment with thionyl chloride provided the acyl chloride (**27**). Reaction of the latter with the corresponding amines afforded the target compound series (**28**). In the case of compound **28d**, the starting material was **24b** with a substitutent on the 4-position of the piperidine ring.

The synthetic methods used for the nitric esters of 1-decylnipecotamides (monosubstituted series) (**33**) were similar to those used for the preparation of their bis-counterparts (Scheme 6). However, an unexpected problem was encountered during the alkylation of methyl nicotinate (with bromodecane). The intermediate pyridinium salt (**31**) had a soap-like property and resisted all attempts at purification. An alternative synthetic scheme was therefore designed with a change in the reaction sequence. Instead of using methyl nicotinate (**22a**) as the starting material, the *N*-hydroxylalkyl amide (**30**) was alkylated to provide the pyridinium salt **31**, which was then converted to the corresponding nitric ester (**32**) by catalytic hydrogenation followed by nitration to afford **33**.

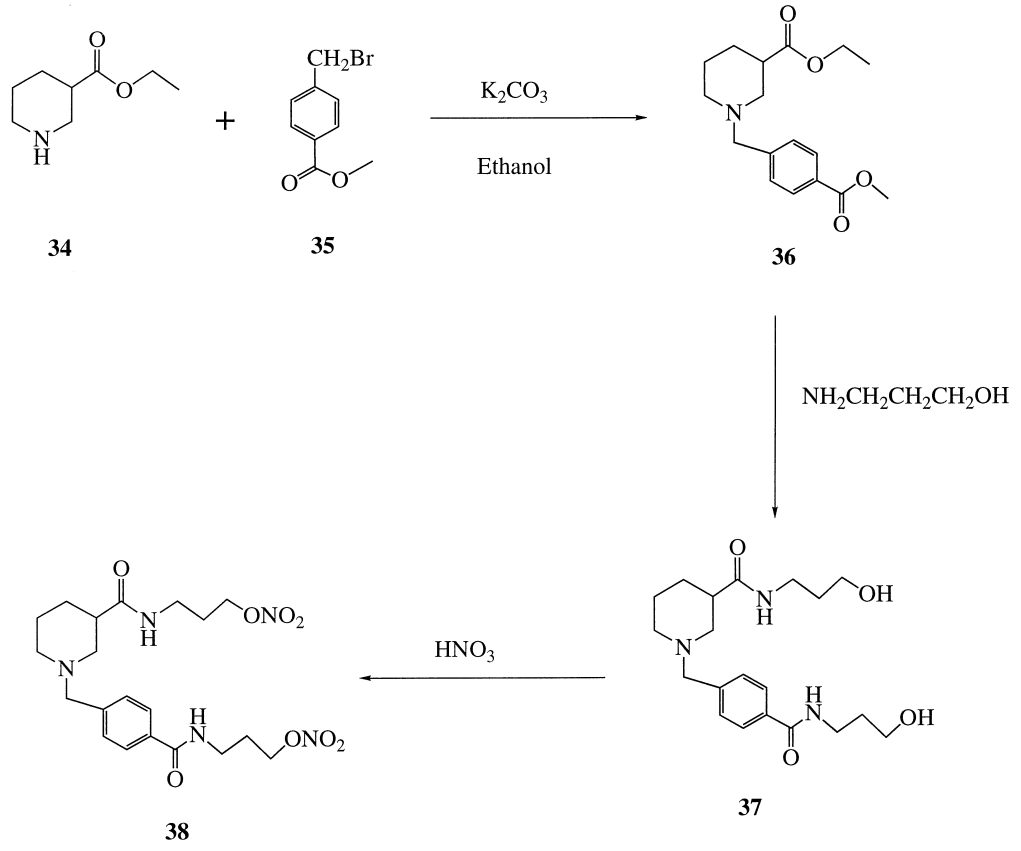
Scheme 7 outlines the synthetic strategy for compound **38** which is a monosubstituted nipecotamide, but with two nitric ester moieties in the molecule. Condensation of ethyl nipecotate (**34**) with 4-(bromomethyl)methylbenzoate (**35**) under basic conditions gave the diester **36**. The target dinitric ester **38** was obtained by the



Scheme 5.



Scheme 6.



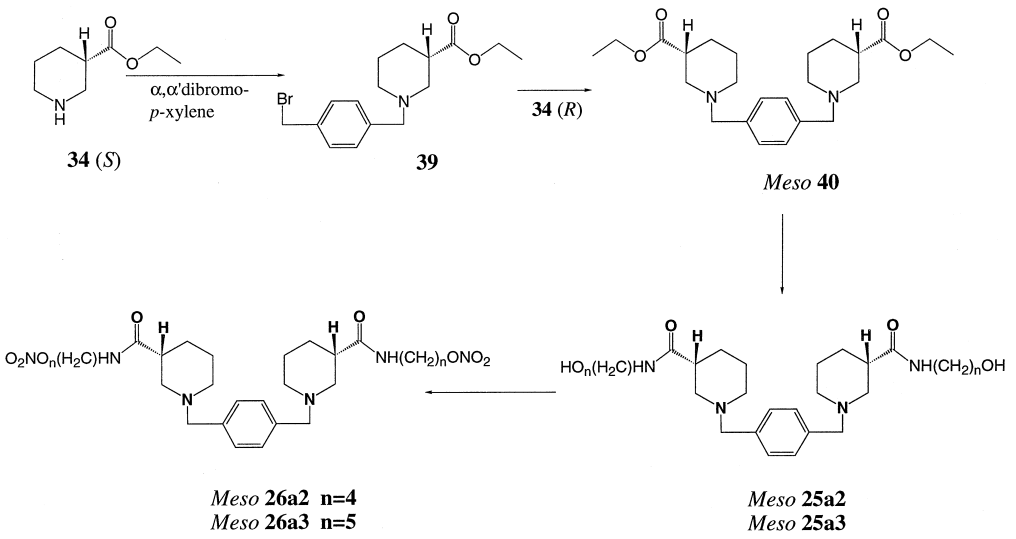
Scheme 7.

treatment of compound **36** with 3-amino-1-propanol followed by nitration with fuming nitric acid at low temperature as discussed earlier.

It was reported earlier that the *meso*-isomer of bis-nipecotamidoaralkanes is more potent than the *R,R*- and *S,S*-enantiomers and the synthetic diastereomeric mixture (d.m.).[7,16] Following this rationale, the two *meso*-nitric esters, **26a2** and **26a3** were synthesized as outlined in Scheme 8. The *S*-isomer of compound **34** was treated with excess α,α′-dibromo-*p*-xylene to give the monobromosubstituted compound **39**. After removal of the excess α,α′-dibromo-*p*-xylene, the reaction mixture was treated with an excess of the *R*-isomer of **34** to produce the *meso* intermediate, **40**. Then following the synthetic procedure similar to that of the diastereomeric mixtures, the *meso*-isomers of compounds **26a2** and **26a3** were obtained from *meso*-**40**.

Results and Discussion

Nipecotamides inhibit the primary phase of platelet aggregation, believed to be related to receptor-associated



Scheme 8.

initial events, as well as the secondary, irreversible phase of aggregation linked to the release reaction. At higher concentration of nipecotamides, both phases, as well as the shape change were totally eliminated. The in vitro platelet aggregation–inhibitory activities of the nipecotamide derivatives are presented in Table 1. The IC_{50} (μM) values represent the mean ± SE of 4–6 individual determinations. Only one or two determinations were made in cases where the compounds appeared to be feebly active (e.g., IC_{50} of 500 μM). The activities of some compounds had to be determined using (95%) ethanolic solutions (total ethanol concentration 0.19%). It has been reported that total ethanol concentration up to 0.19% had no effect on platelet aggregation under identical conditions.[17] We had compared earlier the activities (IC_{50}, μM) of nipecotamides (e.g. **4c**, 44.5) with those of established aggregation inhibitors like chlorpromazine (160.6), propranolol (155.4) and trifluoperazine (224.7).[7b,16] Subsequently, selected concentrations of **4c** were included in each series of determinations as a means of ascertaining the validity of experimental methodology.

Influence of the 3-functional group

The presence of a piperidine-3 substituent appears essential for the antiplatelet activity of carbamoylpiperidines. For instance, when the 3-substituent of compound **20** (IC_{50} 29.4 μM) was replaced with a substituent on the piperidine-4 position resulting in **26b1** (IC_{50} 468 μM), a precipitous decline in the antiplatelet potency was noticed. Likewise, compound **26b2** (IC_{50} 216 μM) was about 1/10th as active as its 3-substituted analogue **26a1** (IC_{50} 22.2 μM).

It has been hypothesized that the capability of nipecotamides to stabilize the platelet membrane may be a consequence of their interaction with membrane phosphoinositides.[12a,18] The presence of a *N*-substituted 3-amide function also appears necessary for activity as seen with **28c** ($IC_{50} \gg 1000$ μM) and **28d** ($IC_{50} \gg$ 1000 μM). An alkyl moiety on the 3-amide *N* seems to enhance activity. Thus, alkylation of the 3-CO-NH_2 group of **28c** ($IC_{50} \gg 1000$ μM) to give **28a** (IC_{50} 153.5 μM) with a *n*-pentyl group enhanced activity more than 6-fold. Ideally, the *N*-substituent should be aliphatic (**4c** IC_{50} 44.5 μM) rather than ring structures as in **28e** (IC_{50} 369 μM) and **28f** (IC_{50} 268 μM).

The 3-amide oxygen of these compounds is capable of forming a H bond with the 3-OH group of the inositol portion of phosphoinositides.[16,18] It then follows that the stronger this H bond, the greater would be the potential for stabilization. The strength of this H bond can be related to the electronegativity of the 3-amide oxygen of the compounds. Thus, the binding affinity would increase with increasing electronegativity. The higher the electronegativity, the better the binding. For example, the relative activities of **4a**, **4b** and **4c** correspond to the order of electronegativity (MOPAC charges)[14c] of their 3-carbonyl *O* atom. Thus, their IC_{50} μM/MOPAC charges are: **4a** (3-$COCH_3$) 2500/−0.276, **4b** ($COOCH_2CH_3$) 756.8/−0.344 and **4c** ($CONEt_2$) 44.5/−0.368. In order to examine the functional importance of the chiral center at the 3-position of the piperidine ring, compound **13** was synthesized. Unlike the 3-carbamoylpiperidines, chirality in the 3-position of compound **13** is eliminated due to the introduction of a double bond at the 3,4-positions of the piperidine ring. It had much lower activity (IC_{50} 1.412 mM) than **4c** (44.5 μM). Because of the conjugation of the amide group with the 3,4-double bond, the electronegativity of the 3-carbonyl oxygen is higher in **13** (−0.373) than in **4c** (−0.368). A possible explanation is that, because of the double bond, the hybridization state of the piperidine 3-carbon is changed from sp^3 to sp^2. This changes the orientation of the 3-amide group. Since the 3-amide group is important for forming hydrogen bonding with membrane phosphoinositides, and also because this binding exhibits stereoselectivity,[7,16] the relatively rigid amide group in **13** may be unfavorably oriented for interacting with the 3-OH group of the inositol portion

Table 1. ADP-induced platelet aggregation–inhibitory activities and partition coefficients of nipecotamides

Compoud	Log P	IC_{50} (μM ± SE)	*n*
4a	2.38	2500	2
4b[a]	4.21	756.8 ± 0.1	1
4c[b]	3.48	44.5 ± 12.7	6
8a	4.07	244.8 ± 94.5	4
8b	3.55	82.6 ± 33.5	5
8c	6.14	38.7 ± 4.8	5
8d	3.22	1070	2
8e	2.44	33.9 ± 2.2	4
8f	2.59	3669	2
8g	4.75	131.5 ± 34.1	4
8h	2.82	1321	2
8i	5.03	271.6 ± 75.5	4
8j	1.25	31 500	2
8k	4.52	142.0 ± 65.7	4
8l	3.57	358.3 ± 91.2	4
8m	4.22	262.5 ± 61.4	4
8n	4.74	166.7 ± 39.1	4
8o	4.52	180.0 ± 41.1	4
13	3.33	1412	2
18[c]	−0.29	476.9 ± 114.0	4
19[d]	2.81	84.8 ± 18.2	4
20[e]	2.11	29.4 ± 4.9	4
21[f]	1.50	825	2
26a1	3.02	22 ± 11.2	4
26a2	3.94	5.3 ± 3.4	4
***meso*-26a2**	3.94	3.1 ± 1.3	4
26a3	4.85	9.8 ± 5.3	4
***meso*-26a3**	4.85	5.7 ± 1.9	4
26a4	5.76	48.4 ± 20.2	4
26b1	2.11	468 ± 380	4
26b2	3.02	216 ± 48.3	4
28a	4.57	177 ± 72	4
28b	6.39	867 ± 412	3
28c	−0.59	≫1000	2
28d	−0.59	≫1000	2
28e	2.66	369 ± 278	4
28f	—	268 ± 58.3	4
33a	4.62	196 ± 90	4
33b	5.07	188 ± 66	4
25a1	0.14	1982 ± 1863	4
25a4	3.15	141 ± 79	4
37	0.09	≫1000	2
38	2.70	528 ± 456	5

[a]α,α′-Bis[3-ethoxycarbonylpiperidino)-*p*-xylene, from ref 12a.
[b]α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene, from ref 16.
[c]α,α′-Bis[3-(*N*-hydroxyethylcarbamoyl) piperidino]*p*-xylene, from ref. 25.
[d]α,α′-Bis[3-(*N*-chloroethylcarbamoyl)piperidino]-*p*-xylene, from ref. 25.
[e]α,α′-Bis[3-(*N*-nitrooxyethylcarbamoyl)piperidino]-*p*-xylene, from ref. 25.
[f]α,α′-Bis[3-nitrooxypiperidino)-*p*-xylene, from ref 25.

of phosphoinositides. In fact, when the 3-D structures of **13** and **4c** were superimposed (figure not included), these two amide groups showed different orientations.

Quantitative structure–activity relationships (QSAR) analysis of the connecting bridge

Since an aromatic ring rather than aliphatic groups connecting the two nipecotoyl moieties showed to confer greater antiplatelet potency, and since it appeared that van der Waal's forces and π interactions may be operative in interactions between nipecotamides and platelet target sites,[12a] efforts were made to examine in detail how the electrostatic, steric and lipophilic properties of the aromatic rings influenced antiplatelet activities. The relationships between biological activities and physicochemical parameters of 15 compounds were examined using QSAR analysis. Molecular volume (MV; a steric factor) and the Hammett constant (σ an electrostatic factor) appeared to significantly influence antiplatelet activity of nipecotamides, as shown in eq 1.

$$\begin{aligned} \text{Log}\,1/\text{C} &= 0.32894\,\text{MV} - 0.00185\,(\text{MV})^2 \\ &\quad - 2.07570\,\sigma - 13.12552 \\ n &= 15; \quad r = 0.9112; \quad F = 23.8209; \quad \text{SE} = 0.36612 \\ \text{C} &= \text{IC}_{50}\,(\mu\text{M}) \times 10^{-3} \end{aligned} \tag{1}$$

IC_{50} = compound concentration inhibiting ADP-induced aggregation by 50%.

Thus, the parameter (σ) which represents the electrostatic properties of substituent groups on the aromatic ring, and the steric parameter (MV) which represents the molecular volumes of the connecting bridges, accounted for 83% variance in the activities of the nipecotamides. Increased activity was found to be associated with an increase in electron density (lower σ value) of the aromatic ring. Further, antiplatelet activity is also governed by steric factors relating to the connecting bridge. When the electrostatic factor (σ) was fixed, the optimum size (MV) of the connecting bridge was equal to 88.9. An exception is compound **8c**, which does not appear to fit in this hypothesis. It has a large molecular volume (MV = 180.9) and an unfavorable electrostatic factor (σ = 0.10). Based on these parameters, this compound should have been inactive ($IC_{50} = 3.15 \times 10^{17}$ μM, calculated from eq 1). Instead, it exhibited a relatively high activity (IC_{50} = 38.7 μM). A unique feature in the structural features of this compound is that it has a long flexible alkyl chain ($-O-C_6H_{13}$) on the aromatic ring. A possible explanation for this anomaly is that a small but deep cavity may be present in the platelet binding site, in addition to the relatively bulky cavity, which can accommodate aromatic rings. Thus, the long flexible alkyl chain substituted on the aromatic ring of **8c** could fit into this small cavity leading to better binding.

Effect of the nitric ester

Nitric oxide decreases the platelets' affinity to the vascular endothelial surface (adhesion) and to each other (aggregation).[13a] In vivo, nitric esters are metabolized to form *S*-nitrosothiols, which serve as precursors for the release of nitric oxide.[22a,b] It seemed appropriate to examine if incorporation of a NO-releasing moiety into the nipecotamide structure would enhance potency due to a dual mechanism of action, since the latter have been demonstrated to prevent platelet shape change.[12b]

Among compounds of the type **26a1** to **26b2** (Scheme 4), the nitric ester function appeared to enhance platelet aggregation–inhibitory potency. Thus, the nitric ester **26a2** (IC_{50} 5.3 μM) was 30-times more potent than **28a**

(IC_{50} 153.5 M) where the $-ONO_2$ group was replaced by a $-CH_3$. Also, replacement of the nitric ester moiety (**26a4** IC_{50} 48.4 μM) with a hydroxyl (**25a4** IC_{50} 141 μM) resulted in a loss of potency; cf. **38** (IC_{50} 528 μM) versus **37** ($IC_{50} \gg 1000$ μM).

1-Alkylnipecotamides **33a** (IC_{50} 196 μM) and **33b** (IC_{50} 188 μM) were prepared to examine if one amide function in the molecule would yield potent compounds, but were abandoned in the light of higher activities of bis-nipecotamidoaralkanes **20** and **26a1**–**26a4** (IC_{50} 5.3–48.4 μM). This preference for two nipecotamide moieties is in accordance with our earlier findings with similar structures but without the $-ONO_2$ group.[3,5,11,12a] In order to examine if dyssymmetric, disubstituted molecules carrying two amide and two $-ONO_2$ functionalities would provide appreciable activity, compound **38** was prepared. Its poor activity (IC_{50} 528 μM) suggested that the bis-nipecotamidoaralkane skeleton, as in **26a1**–**26a4** is the preferred molecular determinant of antiplatelet activity. Among compounds containing structural features deemed desirable for optimum activity (structures **8a**–**f** Scheme 2, **26a1**–**b2** Scheme 4, and **20**), potency appeared to be related in part, to hydrophobicity. The activity (log 1/IC_{50}) of these compounds appeared to be associated with logP (octanol/water partition coefficient) by a parabolic relationship, with the optimum logP of about 4, corroborating our earlier findings.[12a,16] Thus for example, IC_{50} (μM)/logP were: **20**, 29.4/2.11; **26a1**, 22.2/3.09; **26a2**, 5.3/3.94; **26a3**, 9.8/4.85; **26a4**, 48.4/5.76.

Methylene blue inhibits guanylate cyclase and quenches NO by producing the superoxide anion ($O_2^{\cdot -}$), thereby inhibiting the activity of NO.[23,24] Such inhibition of the antiplatelet activity by nitric esters by methylene blue was suggested as evidence for the release of NO.[23] We have demonstrated earlier that the antiplatelet action of nipecotamide nitric esters was reduced significantly when the platelet preparation was preincubated with methylene blue.[25] Also, the release of NO by nitric esters was reported by others.[22a,b] It may therefore be concluded that the effect of nitric ester compounds on platelets may be related in part, to the release of the NO.

We reported previously that bis-nipecotamidoaralkanes exhibited stereoselective antiplatelet properties. Thus the activities decreased from *meso*- > *S,S*-(+)-≃d.m. ≫ *R,R*-(−)-.[7b,16,19,20,21] Stereoselectivity was observed among the nitric esters also. The *meso*-isomer of the nitric ester **26a3** (IC_{50} 5.7 μM) was more potent than the synthetic d.m. (IC_{50} 9.8 μM), and ***meso*-26a2** (IC_{50} 3.1 μM) was more active than its d.m. (IC_{50} 5.3 μM). It should be noted that ***meso*-26a2** was the most potent among the more than 80 nipecotamides synthesized in our laboratory,[8,12,16] including the nitric esters evaluated earlier.[25] In fact, the average IC_{50} value 3.1 μM consisted of individual determinations on four human volunteers, with a range of 1.7–4.3 μM.

The antiplatelet activities of the nitric ester derivatives attests to the benefit of incorporating the nitric ester group into the bis-nipecotamide structure, and corroborates the rationale in their design. These results suggest that (a) the 3-amide functional group is essential for the activity of nipecotamide nitric ester analogues, (b) the 3-nitric ester group by itself, without the amide moiety, is very feebly active, and (c) incorporation of the nitric ester moiety into the bis-nipecotamide structure enhances the activity of the latter, and among these compounds, the disubstituted 3-amides ($-CONR_2$) and their semi-substituted analogues (−CONHR) are approximately equipotent. Finally, it is suggested that these nitric ester derivatives may have a dual function: (1) they spontaneously release nitric oxide, which by itself inhibits platelet adhesion and aggregation, and (2) the nipecotamide part stabilizes the membrane complexes, thereby preventing the platelet activation.

Effects on IP_3 levels and phosphoinositide turnover rate

The basal and 30 s post-collagen (activation) levels of platelet IP_3 were significantly reduced (Fig. 1). Preincubation of platelets with **4c** led to a 64% decline in IP_3 (basal) levels ($P < 0.05$) compared to the control. At 30 s after the addition of collagen, the **4c**-treated platelets had 55% less IP_3 levels than the untreated controls ($P < 0.05$). Although the 48% reduction observed at the 60 s interval was not statistically significant ($P < 0.1$), it suggests a trend that might be significant with a larger sample size. After the 120 s interval (13% reduction), the compound effects on IP_3 levels began to disappear.

A preliminary phosphoinositide turnover study was performed as a time-dependent analysis with a view to identify the time when the effect of **4c** on platelet PIP_2 levels was highest. This was found to be at 30 s after stimulation with collagen, the PIP_2 levels declining by 64% (Fig. 2). A more rigorous examination of the phosphoinositide turnover rates at this time interval was then conducted (Fig. 3). Thus, **4c** reduced the formation of phosphatidylinositol (PI) by 19% ($P < 0.1$), of PIP by 7% and that of PIP_2 by 42% ($P < 0.05$).

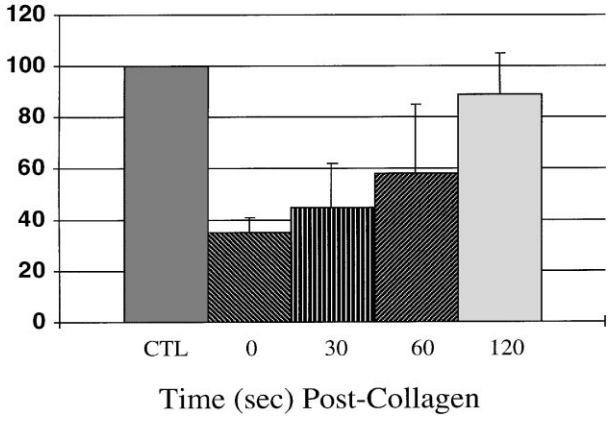


Figure 1. Reduction in IP_3 as a percent of control. Platelets were preincubated for 4 min with **4c** at 37 °C prior to the addition of collagen, $n = 4$.

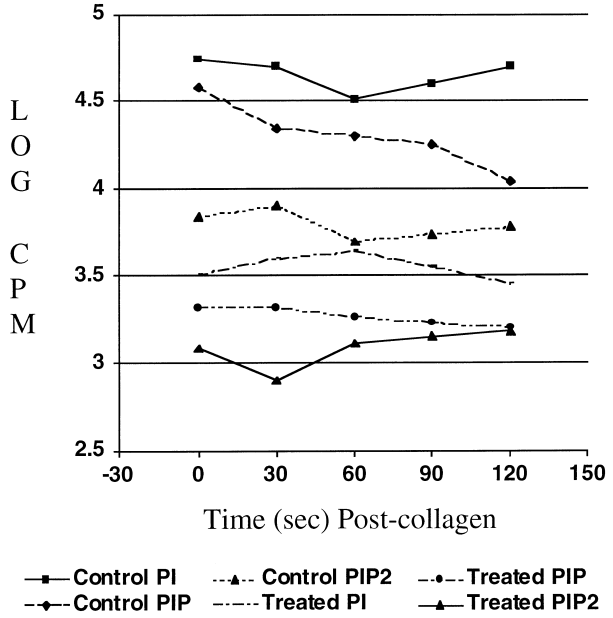


Figure 2. Time-dependence of phosphoinositide turnover and the effect of **4c**, which was preincubated with platelets for 4 min at 37 °C prior to the addition of collagen, $n=4$.

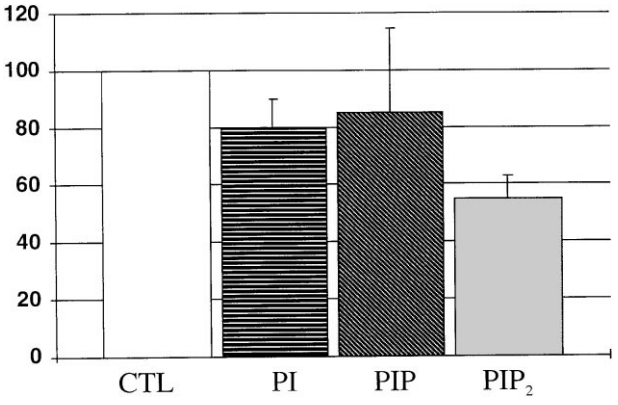


Figure 3. Percent reduction versus control of phosphoinositides in platelets incubated with **4c** at 37 °C. Data obtained 30 s after activation with collagen, $n=4$.

Mechanism of action

Based on our earlier studies and the results presented here, a unified hypothesis on the mechanism of antiplatelet action of nipecotamides is suggested. As reported earlier, by virtue of their lipophilicity, and surface activity, nipecotamides can penetrate the lipid membrane of the platelets.[5,18] The basic piperidine *N* is protonated at the pH of the cytosol to a cation which then interacts with and subsequently neutralizes the charge-density of membrane anionic phosphoinositides, thereby rendering them resistant to hydrolysis by phospholipase-C to the second messengers IP_3 and *s,n*-1,2-diacylglycerol (DAG).[8a,26] As corroborated by the present results, formation of IP_3 is thus reduced. Receptor activation is accompanied by increased phosphoinositide turnover and elevation of the levels of IP_3 and DAG.[27a,b] The present results show that nipecotamides inhibit phosphoinositide turnover, as well as the rise in platelet IP_3 levels. The latter is known to trigger the mobilization of Ca^{2+} from intracellular stores thereby enhancing the cytosolic ionized Ca^{2+} concentrations ($[Ca^{2+}]_i$).[28a,b] We have reported earlier that nipecotamides inhibit in a dose-dependent manner, collagen-induced rise in $[Ca^{2+}]_i$ levels.[21] The formation of DAG and IP_3 is associated with the phosphorylation of two endogenous proteins, the 20 kDa protein, identified as a light chain of myosin (MLC) and the 47 kDa protein, known as pleckstrin, and nipecotamides inhibit both these processes.[29] MLC is causally linked to platelet shape change[30] and contraction,[31] and nipecotamides' blockade of MLC phosphorylation may be associated with their ability to prevent agonist-mediated platelet shape change.[12a,b,21] Further, pleckstrin phosphorylation is closely associated with dense granule secretion.[32] The fact that nipecotamides do block platelet serotonin flux[33] and platelet factor 4 release[34] correlates well with their inhibition of phosphorylation of these two platelet proteins. Additionally, the NO available to the platelets from the compounds reported here, would further enhance their antiplatelet potential.

Experimental

Melting points (mp) were determined on an Electrothermal 9200 apparatus (Electrothermal Engineering Company, Essex, UK) and are uncorrected. Infrared absorption spectra were obtained using a Perkin–Elmer 2000 FT IR instrument (Perkin–Elmer Ltd., Buckinghamshire, UK). Absorption intensities were indicated as strong (s), medium (m) or weak (w). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ARX-300 MHz FT spectrometer (Bruker Instruments Inc., Billerica, MA). Chemical shifts were reported in parts per million (δ) relative to tetramethylsilane (TMS) (δ 0.00). Mass spectra were recorded on a VG Autospec Q instrument (VG Fison Company, Altrincham, UK). Log *P* values were calculated using the PROLOG P program (CompuDrug Inc., Hollan Erno, Hungary) and QSAR was analyzed using the Statgraphics software package (Manugistics, Inc., Rockville, MD). Elemental analyses of the synthetic compounds were performed at Atlantic Microlab Inc., Norcross, GA. Chemicals were purchased from Aldrich Chemical Company, Milwaukee, WI. High performance liquid chromatography (HPLC) was done on a system consisting of a Waters U6K injector, a model 484 tunable UV–VIS detector, a NEC PowerMate SX plus computer with a NEC P5200 printer/plotter and Baseline 810 Chromatography Workstation software. The HPLC columns used for the determination of phosphoinositides were: Bio-Gel HPHT hydroxyaptite (100×78 mm), and Bio-Scale CHT 5-1 (60×10 mm) from Bio-Rad Com. (Hercules, CA). Inositol 1,4,5-trisphosphate (IP_3) levels were determined using the D-*myo*-inositol 1,4,5-trisphosphate [^{3}H] assay system purchased from Amersham Corp. (Arlington Heights, IL). ADP was from Sigma Chemical Co. (St Louis, MO) and collagen (native equine collagen fibrils) was purchased from Nycomed Arzneimittel, Munchen, Germany.

α,α′-Bis(3-acetylcarboxylpyridiniumyl)-*p*-xylene dibromide (3). 3-Acetylpyridine (**1**) (51.0 g, 421 mmol) was reacted with α,α′-dibromo-*p*-xylene (**2**) (52.7 g, 200 mmol). The precipitate was collected and recrystallized from methanol:water (5:1 v/v) to obtain 85.5 g (84.7%) of **3**, mp 272.5–273.6 °C; IR (KBr) 1630 (C=O); ^{1}H NMR (D_2O) (δ ppm) 9.44 (s, 2H, CH:N:C*H*:CCO), 9.01–9.08 (m, 4H, C*H*:CHC*H*:N:CH:CCO), 8.21 (q, 2H, J = 6.5 Hz, C*H*:CHCO), 7.56 (s, 4H, phenyl), 5.95 (s, 4H, phCH_2), 2.73 (s, 6H, $COCH_3$).

α,α′-Bis(3-acetylpiperidino)-*p*-xylene dihydrochloride (4a). Catalytic reduction (0.5 g PtO_2, 60–65 psi, 25 °C) of α,α′-bis(3-acetylcarboxyl pyridiniumyl)-*p*-xylene dibromide (**3**) (7.5 g, 37.0 mmol) in ethanol (250 mL), followed by recrystallization of the product from methanol:ethanol (1:2 v/v) gave 3.5 g of the dihydrobromide salt. The latter was dissolved in 10 mL of water and neutralized with 30% $NaCO_3$. The free base (2.0 g) was extracted with 4×50 mL of ether. The residue was purified by column chromatography on silica gel, using $CHCl_3$:MeOH (99:1, v/v) as the eluent. The solvent was evaporated, the residue dissolved in 100 mL anhyd ether, and dry HCl gas was passed through the solution to afford a solid which was recrystallized from methanol:ether mixture to obtain **4a** (0.45 g, 7.1%), mp 258.2–259.4 °C. Anal. ($C_{22}H_{32}N_2O_2$·2HCl) C, H, N, Cl; IR (KBr) 1708 (C=O); ^{1}H NMR ($CDCl_3$) (δ ppm) 7.25 (s, 4H, Ar*H*), 2.11 (s, 6H, $COCH_3$), 3.50 (s, 4H, ArCH_2), 2.71–2.95 (m, 4H, NCH_2CHCO), 2.57–2.66 (m, 2H, NCH_2CHCO) 1.98–2.22 (m, 4H, CH_2NCH_2CHCO), 1.36–1.91 (m, 8H, $CHCH_2CH_2CH_2N$).

General procedure for the synthesis of bisnipecotamides with different connecting bridges (8a, b, c, d, g, h, i, j, k, l, m, n, o). The synthesis of 2-ethoxy-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (**8a**) is described in detail, as an example.

Step 1. 2-Ethoxy-*p*-xylene (5a). A mixture of 2,5-dimethylphenol (18.3 g, 150 mmol), iodoethane (31.2 g, 200 mmol), K_2CO_3 (27.6 g, 200 mmol), and 150 mL of acetone was refluxed for 48 h, then cooled to room temperature and filtered. After removal of the solvent, the residue was dissolved in 200 mL of ether and washed with 10 N NaOH (2×20 mL). After drying and removing of the solvent, the residue was purified by vacuum distillation to obtain 18.6 g (82.7%) of **6a**, bp_{2mmHg} 86–88 °C; MS (EI) m/z 150 (M+); ^{1}H NMR ($CDCl_3$) (δ ppm) 7.04 (d, J = 7.3 Hz, 1H, Ar-C*H*:$COCH_2CH_3$), 6.69 (d, J = 10.3 Hz, 2H, Ar-C*H*:C*H*), 4.05 (q, J = 6.4 Hz, 2H, OCH_2CH_3), 2.35 (s, 3H, CH_3 C:$COCH_2CH_3$), 2.22 (s, 3H, CH_3C:CH:$COCH_2CH_3$), 1.46 (t, J = 5.9 Hz, OCH_2CH_3).

Step 2. 2-Ethoxy-α,α′-dibromo-*p*-xylene (6a). To a stirred solution of 2-ethoxy-*p*-xylene (**6a**) (7.5 g, 50 mmol) in 200 mL of anhydrous carbon tetrachloride (CCl_4), *N*-bromosuccinimide (21.4 g, 120 mmol) and benzoyl peroxide (0.25 g) were slowly added. The reaction mixture was refluxed for 5 h. After filtration and removal of the solvent, the residue was recrystallized from ether:hexanes (2:1 v/v) to obtain 5.6 g (36.4%) of **7a** mp 97.5–98.9 °C; MS (EI) m/z 308 (M+); ^{1}H NMR ($CDCl_3$) (δ ppm) 7.29 (d, J = 4.9 Hz, 1H, Ar-C*H*:$COCH_2CH_3$), 6.90–6.96 (m, 2H, Ar-C*H*:C*H*), 4.57 (s, 2H, CH_2 C:$COCH_2CH_3$), 4.52 (s, 2H, CH_2C:CH:$COCH_2CH_3$), 4.14 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 1.49 (t, J = 7.0 Hz, OCH_2CH_3).

Step 3. 2-Ethoxy-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)-piperidino]-*p*-xylene dihydrochloride (8a). To a solution of *N*,*N*-diethylnipecotamide (3.50 g, 20 mmol) in 100 mL of THF, K_2CO_3 (4.5 g, 32.0 mmol) was added, followed by the slow addition of a solution of 2-ethoxy-α,α′-dibromo-*p*-xylene (**7a**) (2.0 g, 6.5 mmol) in 40 mL of THF. Stirring was continued for a total of 72 h at room temperature. After filtration and removal of the solvent, the residue was purified by column chromatography on silica gel, using $CHCl_3$:MeOH (24:1, v/v) as the solvent. The free base was dissolved in 200 mL anhydrous ether and dry HCl gas was passed through the solution to afford a solid which was recrystallized from acetonitrile:ether (1:4 v/v) to obtain 2.8 g (74.5%) of **8a**, mp 202.2–204.3 °C (dec.); anal. ($C_{30}H_{50}N_4O_3$·2HCl) C, H, N, Cl; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.25 (d, 1H, J = 5.5 Hz, Ar-C*H*:$COCH_2CH_3$), 6.81–6.87 (m, 2H, Ar-C*H*:C*H*), 4.01 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 3.61 (s, 2H, CH_2 C:$COCH_2CH_3$), 3.48 (s, 2H, CH_2C:CH:$COCH_2CH_3$), 3.30–3.38 (m, 8H, N[CH_2CH_3]$_2$), 1.40 (t, 3H, J = 7.0 Hz, OCH_2CH_3), 1.06–1.19 (m, 12H,[CH_2CH_3]$_2$).

2-Methoxy-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8b). Yield: 43.0%. Mp 211.2–22.7 °C (dec.); anal. ($C_{29}H_{48}N_4O_3$·2HCl) C, H, N, Cl; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.25 (s, 1H, Ar-C*H*:$COCH_3$), 6.84–6.88 (m, 2H, Ar-C*H*:C*H*), 3.82 (s, 3H, OCH_3), 3.58 (s, 2H, CH_2C:$COCH_3$), 3.50 (s, 2H, CH_2C:CH:$COCH_3$).

2-Hexyloxy-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dioxalate (8c). Yield: 44.4%. Mp 186.1–188.3 °C (dec.); anal. [$C_{34}H_{58}N_4O_3$·2$(COOH)_2$] C, H, N; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.23 (d, J = 5.6 Hz, ^{1}H, Ar-C*H*:COC_6H_{13}), 6.82–6.87 (m, 2H, Ar-C*H*:C*H*), 3.95 (t, J = 6.5 Hz, 2H, $OCH_2C_5H_{11}$), 3.60 (s, 2H, CH_2 C:COC_6H_{13}), 3.48 (s, 2H, CH_2C:CH:COC_6H_{13}), 157–1.66 (m, 2H, $OCH_2CH_2C_4H_9$), 1.43–1.54 (m, 2H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 1.30–1.37 (m, 4H, $OCH_2CH_2CH_2CH_2CH_2CH_3$), 0.90–0.98 (m, 3H, $OCH_2CH_2CH_2CH_2CH_2CH_3$).

2-Nitro-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8d). Yield: 83.2%. Mp 219.5–220.9 °C (dec.); anal. ($C_{28}H_{45}N_5O_4$·2HCl) C, H, N, Cl; IR (KBr) 1633 (s); ^{1}H NMR ($CDCl_3$) (δ ppm) 7.78 (d, J = 2.4 Hz, 1H, Ar-C*H*:CNO_2), 7.42 (s, 2H, Ar-C*H*:C*H*), 3.75 (s, 2H, N$CH_2C_6H_4CH_2$), 3.54 (s, 2H, $NCH_2C_6H_4CH_2$).

2-Amino-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8e). 2-Nitro-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene (**8d**) (7.2 g, 14.0 mmol), in 150 mL ethanol was catalytically reduced over PtO_2. After removal of the solvent, the residue was dissolved in 200 mL of ether. Dry HCl gas was passed through the solution and the precipitate was collected

and recrystallized from acetonitrile/ether to yield 7.1 g (90.8%) of **8e**, mp 239.8–241.2 °C (dec.); anal. ($C_{28}H_{47}N_5O_2$·2HCl) C, H, N, Cl; IR (KBr) cm^{-1} 3412 (NH_2), 1636 (CON); ^1H NMR ($CDCl_3$) (δ ppm) 6.91 (d, $J=7.9$ Hz, 1H, Ar-C*H*:CNH$_2$), 6.58–6.62 (m, 2H, Ar-C*H*:C*H*), 4.73 (s, 2H, N*H*$_2$), 3.50 (s, 2H, NC*H*$_2$-C_6H_4-C*H*$_2$-), 3.41 (s, 2H, NCH$_2C_6H_4$C*H*$_2$).

2-Acetylamino-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8f). 2-Amino-α,α′-bis-[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (**8e**) (3.0 g, 6.18 mmol), acetic anhydride (15.0 g, 147 mmol) and pyridine (1 mL) were mixed and stirred for 12 h at 60 °C (until TLC showed no starting material). After removal of the solvent, the residue was dissolved in 100 mL of chloroform and washed with 2×20 mL of 15% $NaHCO_3$, and 2×20 mL of water. The solution was then dried and evaporated, the residue was applied to a silica gel column, and the compound was eluted with $CHCl_3$:MeOH (47:3, v/v). After removal of the solvent, the residue was dissolved in 100 mL anhydrous ether and dry HCl gas was passed through the solution. The precipitate was collected and recrystallized from acetonitrile/ether to obtain 2.10 g (56.6%) of **8f**, mp 227.5–229.6 °C, (dec.); anal. ($C_{30}H_{49}N_5O_3$·2HCl) C, H, N, Cl; IR (KBr) cm^{-1} 3447 (NH), 1689 (CONH), 1634 (CON); ^1H NMR ($CDCl_3$) (δ ppm) 10.87 (s, 1H, CH_3CON*H*), 8.21 (s, 1H, Ar-C*H*:CNH), 6.94–7.05 (m, 2H, Ar-C*H*:C*H*), 3.38–3.59 (m, 4H, C*H*$_2$-C_6H_4-C*H*$_2$), 2.16 (s, 3H, C*H*$_3$CONH).

Compounds **8g**–**8o** were prepared using the synthetic sequence and procedures similar to the ones described above.

α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-1,4-dimethylnaphthalene dihydrochloride (8g). Yield: 37.0%. Mp 186.5–188.4 °C (dec.); anal. ($C_{32}H_{48}N_4O_2$·2HCl) C, H, N, Cl; ^1H NMR ($CDCl_3$) (δ ppm) 8.28–8.32 (m, 2H, CH_2C*H*:C*H*CH$_2$), 7.49–7.53 (m, 2H, C:C*H*:CH:CH:C*H*:C), 7.37 (s, 2H, C:CH:C*H*:C*H*:CH:C), 3.90 (s, 4H, C*H*$_2$CH:CHC*H*$_2$).

α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-2,5-dimethylfuran dihydrochloride (8h). Yield: 4.1%. Mp 232.5–233.7 °C (dec.); anal. ($C_{26}H_{44}N_4O_3$·2HCl) C, H, N, Cl; ^1H NMR ($CDCl_3$) (δ ppm) 6.13 (s, 2H, Ar*H*), 3.52–3.56 (m, 4H, C*H*$_2$ ArC*H*$_2$).

α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-2,5-dimethylthiophene dihydrochloride (8i). Yield: 31.0%. Mp 263.7–65.4 °C (dec.); anal. ($C_{26}H_{44}N_4O_2S$·2HCl) C, H, N, Cl, S; IR (KBr) 1633 (s); ^1H NMR ($CDCl_3$) (δ ppm) 6.13 (s, 2H, Ar*H*), 3.52–3.56 (m, 4H, C*H*$_2$ ArC*H*$_2$).

α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-2,5-dimethylpyrizine dihydrochloride (8j). Yield: 13.4%. Mp 228.5–230.0 °C (dec.); anal. ($C_{26}H_{44}N_6O_2$·2HCl) C, H, N, Cl; IR (KBr) 1631 (s); ^1H NMR ($CDCl_3$) (δ ppm) 8.76 (s, 2H, Ar-*H*), 4.57 (s, 4H, C*H*$_2$ ArC*H*$_2$).

α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-α,α′-dimethyl-*p*-xylene dihydrochloride (8k). Yield: 63.1%. Mp 298.5–300.2 °C (dec.); anal. ($C_{30}H_{50}N_4O_2$·2HCl) C, H, N, Cl; IR (KBr) 1631 (s); ^1H NMR ($CDCl_3$) (δ ppm) 7.23 (s, 4H, Ar-*H*), 3.21–3.51 (m, 10H, N[C*H*$_2$CH$_3$]$_2$, Ar-C*H*CH$_3$), 2.62–3.08 (m, 6H NC*H*$_2$CHCON), 1.38 (s, 6H, Ar-CHC*H*$_3$), 1.06–1.18 (m, 12H, N[CH$_2$C*H*$_3$]$_2$).

2-Methoxycarbonyl-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)-piperidino]-*p*-xylene dihydrochloride (8l). Yield: 56.5%. Mp 298.5–300.2 °C (dec.); anal. ($C_{30}H_{48}N_4O_4$·2HCl) C, H, N, Cl; IR (KBr) 1718 (s), 1627 (s); ^1H NMR ($CDCl_3$) (δ ppm) 7.62 (s, 1H, Ar-C*H*:COOCH$_3$), 7.30–7.35 (m, 2H, Ar-C*H*:C*H*), 3.88 (s, 3H, COOC*H*$_3$), 3.68 (s, 2H, C*H*$_2$C:COOCH$_3$), 3.38 (s, 2H, C*H*$_2$C:CH:COOCH$_3$).

2-Chloro-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8m). Yield: 42.4%. Mp 250.0–252.1 °C (dec.); anal. ($C_{28}H_{45}N_4O_2Cl$·2HCl·0.5H_2O) C, H, N, Cl; IR (KBr) 1718 (s), 1627 (s); ^1H NMR ($CDCl_3$) (δ ppm) 7.34-7.39 (m, 2H, Ar-C*H*:C*H*), 7.16 (dd, $J=$ 6.6 Hz, 1H, Ar-C*H*:CCl), 3.62 (s, 2H, NC*H*$_2C_6H_4CH_2$), 3.47 (s, 2H, NCH$_2C_6H_4$C*H*$_2$).

2-Iodo-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8n). Yield: 70.8%. Mp 198.5–199.8 °C (dec.); anal. ($C_{28}H_{45}N_4O_2I$·2HCl) C, H, N, Cl, I; IR (KBr) 1718 (s), 1627 (s); ^1H NMR ($CDCl_3$) (δ ppm) 7.82 (s, 1H, Ar-C*H*:C-I), 7.21–7.32 (m, 2H, Ar-C*H*:C*H*), 3.50 (s, 2H, NC*H*$_2C_6H_4CH_2$), 3.45 (s, 2H, NCH$_2C_6H_4$C*H*$_2$).

2,-Dimethyl-α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrochloride (8o). Yield: 70.0%. Mp 179.8–280.5 °C (dec.); anal. ($C_{30}H_{50}N_4O_2$·2HCl) C, H, N, Cl; IR (KBr) 1718 (s), 1627 (s); ^1H NMR ($CDCl_3$) (δ ppm) 7.06 (s, 2H, Ar-*H*), 3.42 (s, 4H, C*H*$_2$-Ar), 2.29 (s, 6H, Ar-C*H*3).

***N*-*t*-Butoxycarbonyl 3,4-dehydronipecotic acid (10).** Sodium hydroxide (3 ml, 1 N) was slowly added to a cooled (5–10 °C) suspension of guvacine hydrochloride (**9**) (250 mg, 1.529 mmol) in 3.0 mL of *t*-BuOH. Di-*t*-butyl dicarbonate (349 mg, 1.60 mmol) was then added dropwise (< 25 °C) to this mixture. After stirring for overnight at room temperature, the reaction mixture was diluted with 5 mL of water. After discarding a *n*-pentane extract (3×10 mL) of the reaction mixture, the pH of the aqueous layer (0–2 °C) was adjusted to 2.0 (3 N HCl), followed by extraction with 3×30 mL ethyl acetate. Removal of the solvent (< 30 °C) gave the crude product, which upon recrystallization from EtOAc: petroleum ether (1:4 v/v) afforded 302 mg (87.1%) of **10**, mp 135.1–135.8 °C; MS (EI) *m*/*z*: 227 (M^+); ^1H NMR ($CDCl_3$) δ ppm: 10.08 (s, 1H, COO*H*), 7.19 (s, 1H, C*H*:CCOOH), 4.12 (d, $J=4.1$ Hz, 2H, NC*H*$_2$CCOOH), 3.49 (t, $J=5.6$ Hz, 2H, C*H*$_2$NCH$_2$CCOOH), 2.31–2.33 (m, 2H, C*H*$_2$C:COOH) 1.47 (s, 9H, (C*H*$_3$)$_3$C-); ^{13}C NMR ($CDCl_3$) (δ ppm) 170.50 (*C*OOH), 155.27 (O*C*ON), 140.58 (*C*COOH), 128.38 (*C*H:CCOOH), 80.64 ((CH$_3$)$_3$*C*), 42.75 (N*C*H$_2$CCOOH), 39.12 (N*C*H$_2$CH$_2$ CH), 26.05 (NCH$_2$*C*H$_2$CH).

***N*′-*t*-Butoxycarbonyl-*N*,*N*-diethyl-3,4-dehydronipecotamide (11).** *Procedure 1.* *N*-Methylmorpholine (44.6 mg,

0.44 mmol) was added to *N*-*t*-butoxycarbonyl 3,4-dehydronipecotic acid (**10**) (100 mg, 0.44 mmol) in 10 mL THF, maintaining the reaction mixture at 10–15 °C while stirring. After 15 min, isobutyl chloroformate (57.9 mg, 0.44 mmol) was slowly added (0–5 °C), the stirring continued for 30 min, followed by the addition of *N,N*-diethylamine (32.2 mg, 0.44 mmol) at the same temperature. The reaction mixture was then slowly brought to room temperature and stirred 16 h. The precipitate was filtered off. The solvent was removed from the filtrate and the residue was applied to a silica gel column. Elution with $CHCl_3$:MeOH (99:1, v/v), gave 15 mg of the unreacted acid, 60 mg of a by-product [*N,N*-diethyl isobutyl chloroformamide, MS (EI) *m/z* 145 (M+)] and 15 mg (12.1%) of **11**, as a pale yellow oil, MS (EI) *m/z*: 282 (M+); ^{1}H NMR ($CDCl_3$) (δ ppm) 5.90 (s, 1H, C*H*:CCON), 4.02 (s, 2H, NCH_2CCON), 3.49 (t, J = 5.6 Hz, 2H, CH_2NCH$_2$CCOOH), 3.28–3.48 (q, J = 7.2 Hz, 4H, N(CH_2CH$_3$)$_2$), 2.11–2.30 (m, 2H, CH_2C:CON), 1.41 (s, 9H, (CH_3)$_3$C-), 1.10 (t, J = 7.2 Hz, 6H, N(CH$_2$CH_3)$_2$).

Procedure 2. *N*-*t*-Butoxycarbonyl 3,4-dehydronipecotic acid (**10**) (104 mg, 0.458 mmol) was dissolved in 5 mL of methanol, cooled to 0 °C, and then neutralized with 0.485 mL of 1 N NaOH. The mixture was dried under high vacuum and a suspension of the residue in 10 mL dry benzene was cooled in an ice–water bath. After adding 1 drop of pyridine, 1 mL of 2 M oxalyl chloride was slowly added, stirred for 1 h at 0–15 °C, and for another 2 h at room temperature. The solvent was removed and the dried residue was suspended in 10 mL of THF followed by the addition of a cold (5 °C) solution of diethylamine (109.7 mg, 1.5 mmol) in THF. The reaction mixture was then slowly brought to room temperature and stirred for 12 h. The precipitate was filtered, the solvent was removed, and the residue was applied to a silica gel column. Eution with $CHCl_3$:MeOH (99:1, v/v), gave 15 mg of the unreacted acid and 109 mg (70.3%) of **11**, MS (EI) *m/z* 282 (M+).

***N,N*-Diethyl-3,4-dehydronipecotamide (12).** *Method A*. Trifluoroacetic acid (2 mL) was slowly added to a cold (0–5 °C) solution of *N′*-*t*-butoxycarbonyl-*N,N*-diethyl-3,4-dehydronipecotamide (**11**) (200 mg, 0.71 mmol) in 10 mL CH_2Cl_2 with stirring and maintaining the reaction mixture below 15 °C. The reaction was continued for another 12 h at room temperature. The solvent was removed and the residue was treated with 30% aq Na_2CO_3 (pH = 10–12). The mixture was extracted with 4×30 mL chloroform. The extracts were concentrated and the residue was dried to afford an oil, 112 mg (86.8%) of **12**, MS (EI) *m/z*: 182 (M+); ^{1}H NMR ($CDCl_3$) (δ ppm) 5.83–5.89 (m, 1H, C*H*:CCON), 3.47–3.49 (m, 2H, NCH_2CCON), 3.40 (q, J = 7.1 Hz, 4H, N(CH_2CH$_3$)), 2.96 (t, J = 5.7 Hz, 2H, CH_2NCH$_2$CCON), 2.11–2.16 (m, 2H, CH_2C:CON), 1.68 (s, 1H, N*H*), 1.14 (t, J = 7.1 Hz, 6H, N(CH$_2$CH_3)); ^{13}C NMR ($CDCl_3$) δ ppm: 171.33 (*C*ON), 136.21 (*C*CON), 124.91 (*C*H:CCON), 45.99 (N*C*H$_2$CCON), 42.65 (N*C*H$_2$CH$_2$CH), 39.41 (N(*C*H$_2$CH$_3$), 25.19 (NCH$_2$*C*H$_2$CH), 14.03 (N(CH$_2$*C*H$_3$).

***N*-Methyl 3,4-dehydronipecotic acid (15).** Arecoline hydrobromide (**14**) (15 g, 63.5 mM) was dissolved in 50 mL water and the pH was adjusted to 10–11 with 30% $NaCO_3$. The free base was then extracted with 5×50 mL chloroform. After removal of the solvent, 150 mL of water was added to the residue and refluxed for 24 h (until no ester was detected by TLC). After removal of the solvent, the residue was crystallized from water–acetonitrile to yield **15** (8.2 g, 91.4%), mp 224.5–225.3 °C (dec.); MS (EI) *m/z*: 141 (M^+); ^{1}H NMR (D_2O) δ ppm: 6.66–6.72 (m, 1H, C*H*:CCOOH), 3.96 (s, 2H, NCH_2CCOOH), 3.35–3.64 (m, 2H, CH_2NCH$_2$ CCOOH), 2.89 (s, 3H, NCH_3), 2.50 (s, 2H, CH_2C: COOH); ^{13}C NMR (D_2O) (δ ppm) 171.90 (*C*OOH), 132.24 (*C*COOH), 128.90 (*C*H:CCOOH), 52.49 (NCH_2 CCOOH), 50.03 (N*C*H$_2$CH$_2$CH), 42.61 (N*C*H$_3$), 22.68 (NCH$_2$*C*H$_2$CH).

***N′*-Methyl-*N,N*-diethyl-3,4-dehydronipecotamide (16).** *N*-Methyl 3,4-dehydronipecotic acid (**15**) (8.0 g, 56.7 mmol) mixed with 15 mL thionyl chloride was refluxed for 2 h. The excess of thionyl chloride was removed and the residue was added to a cold solution of diethylamine (16.6 g, 226.8 mmol) in THF, maintaining the temperature at −10 to −5 °C. Then, the reaction mixture was slowly brought to room temperature and stirring was continued for another 12 h. The precipitate was filtered off and the filtrate was concentrated. The residue was dissolved in 150 mL of acetone, followed by the addition of a solution of oxalic acid (5.4 g, 60 mmol) in acetone (50 mL). After stirring for 10 min, the precipitate was collected and recrystallized from acetonitrile to obtain 10.2 g (62.8%) of the oxalate salt of **16**, mp 124.1–124.9 °C; MS (EI) *m/z*: 196 (M+); ^{1}H NMR (free base in $CDCl_3$) δ ppm: 5.73 (d, J = 1.7 Hz, 1H, C*H*:CCON), 3.30 (q, J = 7.1 Hz, 4H, N(CH_2CH$_3$)), 3.00 (s, 2H, NCH_2CCON), 2.45 (t, J = 5.7 Hz, 2H, CH_2 NCH$_2$CCON), 2.94 (s, 3H, NCH_3), 2.18–2.20 (m, 2H, CH_2C:CON), 1.05 (t, J = 7.1 Hz, 6H, N(CH$_2$CH_3)); ^{13}C NMR ($CDCl_3$) (δ ppm) 170.78 (*C*ON), 134.17 (*C*CON), 124.03 (*C*H:CCON), 55.07 (N*C*H$_2$CCON), 51.38 (N*C*H$_2$CH$_2$CH), 46.00 (N*C*H$_3$), 41.14 (N(*C*H$_2$ CH$_3$)$_2$), 25.47 (NCH$_2$*C*H$_2$CH), 13.96 (N(CH$_2$*C*H$_3$)$_2$).

***N′*-Vinyloxycarbonyl-*N,N*-diethyl-3,4-dehydronipecotamide (17).** Vinylchloroformate (1.75 g, 16.5 mmol) was added to a cold solution of *N′*-methyl-*N,N*-diethyl-3,4-dehydronipecotamide (**16**) (3.0 g, 15.3 mmol) in methylene chloride (20 mL) while maintaining the temperature between −5 to 0 °C. The reaction mixture was stirred for 10 h at room temperature. The solution was then diluted with 30 mL of methylene chloride, washed with 2×10 mL of 1 N NaOH, followed by 2×10 mL of 1 N HCl and 2×10 mL of water. After removal of the solvent, the residue was applied to a silica gel column, and eluted with ether:hexanes (4:1, v/v), to obtain 1.72 g (44.6%) of **17** as an oil, MS (EI) *m/z* 252 (M+); ^{1}H NMR ($CDCl_3$) (δ ppm) 7.17 (dd, J = 8.5 Hz, 1H, CH$_2$:C*H*O), 5.91 (s, 1H, C*H*:CCON), 4.78 (d, J = 12.8 Hz, CH_aH$_b$:CHO), 4.44 (d, J = 5.2 Hz, CH$_a$*H*$_b$:CHO), 4.15 (s, 2H, NCH_2CCON), 3.57 (t, J = 5.5 Hz, 2H, CH_2 NCH$_2$CCON), 3.33–3.44 (m, 4H, N(CH_2CH$_3$)), 2.22–2.25 (m, 2H, CH_2C:CON), 1.08–1.16 (m, 6H, N(CH$_2$CH_3));

^{13}C NMR ($CDCl_3$) (δ ppm): 169.98 (*C*ON), 153.20 (O*C*ON), 142.70 (CH_2:*C*HO), 133.90 (*C*CON), 124.16 (*C*H:CCON), 96.09 (*C*H_2:CHO), 44.37 (N*C*H_2CCON), 44.22 (N(*C*H_2CH$_3$), 40.18 (N*C*H_2CH$_2$CH), 24.41 (NCH$_2$*C*H_2CH), 13.59 (N(CH$_2$*C*H$_3$).

***N,N*-Diethyl-3,4-dehydronipecotamide (12).** *Method B*. Dry hydrogen chloride was bubbled into a solution of *N*′-vinyloxycarbonyl-*N,N*-diethyl-3,4-dehydronipecotamide (**17**) (1.5 g, 5.9 mmol) in methylene chloride (15 mL) for 5 min. The reaction mixture was stirred for 12 h at room temperature. After removal of the solvent, the residue was dissolved in 20 mL of absolute ethanol and stirred for 2 h at 40–50 °C. The solvent was removed and the residue was recrystallized from ethyl acetate. The resulting solid was dissolved in 15 mL of water and the pH was adjusted to 10–11 followed by extraction with 4×15 mL of chloroform. After removal of the solvent, 0.95 g (88.0%) of **12** was obtained, MS (EI) *m*/*z*: 182 (M+).

α,α′-Bis[3-(*N,N*-diethylcarbamoyl)-3,4-dehydropiperidino]-*p*-xylene dihydrochloride (13). α,α′-Dibromo-*p*-xylene (0.53 g, 2.0 mmol) in 10 mL THF was added dropwise to a stirred suspension of *N,N*-diethyl-3,4-dehydronipecotamide (**12**) (0.80 g, 4.4 mmol) and potassium carbonate (0.7 g, 5.0 mmol) in 30 mL THF, while maintaining the temperature at 0–5 °C. The reaction mixture was then stirred at 24 °C for 72 h. The precipitate was filtered off and the filtrate was concentrated. The residue was applied to a silica gel column and eluted with $CHCl_3$:MeOH (45:1, v/v) to obtain 0.68 g of product. The latter was dissolved in 80 mL anhydrous ether and dry HCl gas was passed through the solution. The precipitated solid was collected and recrystallized from methanol:ether to obtain 0.45 g (41.7%) of **13**, mp 264.5–265.1 °C (dec.); anal. ($C_{28}H_{42}N_4O_2$·2HCl) C, H, N, Cl; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.29 (s, 4H, Ar*H*), 5.80–5.84 (m, 2H, C*H*:CCON), 3.61 (s, 4H, PhCH_2N), 3.14 (s, 2H, NCH_2CCON), 2.59 (t, 2H, J=5.6 Hz, CH_2NCH$_2$CCON), 2.22–2.26 (m, 2H, CH_2C:CON); ^{13}C NMR (D_2O) (δ ppm) 169.63 (*C*ON), 132.42 (*C*CON), 130.98 (NCH$_2$-*C*:CH), 127.18 (*C*H:CCON), 126.67 (NCH$_2$-C:*C*H), 59.35 (N*C*H$_2$-C:CH), 49.71 (N*C*H$_2$CCON), 48.48 (N*C*H$_2$CH$_2$CH), 44.51 (N(C_aH$_2$CH$_3$)$_2$), 40.53 (N(C_bH$_2$CH$_3$)$_2$), 22.07 (NCH$_2$*C*H$_2$CH), 13.82 (N(C_aH$_2$*C*H$_3$)$_2$), 12.19 (N(C_bH$_2$*C*H$_3$)$_2$).

α,α′-Bis[3-(*N*-3-nitrooxylpropylcarbamoyl)piperidino]-*p*-xylene (26a1). The synthesis of **26a1** is described as an example for the synthesis of bisnipecotamides substituted with nitric ester moieties as shown in Scheme 5.

Step 1. α,α′-Bis(3-methoxycarbonylpyridiniumyl)-*p*-xylene dibromide (23a). A solution of 28.8 g (210 mmol) methyl nicotinate (**22a**) and 26.4 g (100 mmol) α,α′-dibromo-*p*-xylene (**2**) in 500 mL of acetone was refluxed for 10 h. The white precipitate was collected via filtration and washed with acetone. Removal of the solvent under vacuum gave 53.6 g (99.6%) of **23a**, mp 198–199 °C (lit. 198.5–199.5 °C).

Step 2. α,α′-Bis(3-methoxycarbonylpiperidino)-*p*-xylene (24a). α,α′-Bis(3-methoxycarbonylpiperidiniumyl)-*p*-xylene dibromide (**23a**) (4.0 g, 7.4 mmol) in 50 mL of 80% methanol was reduced by catalytic hydrogenation (0.2 g of PtO_2, 60 psi) followed by recrystallization of the product from methanol to give 2.3 g (57.5%) of α,α′-bis(3-methoxycarbonylpiperidino)-*p*-xylene dihydrobromide, mp 236.0–237.5 °C (lit. 240.2–242.3 °C).[25] The product was dissolved in 50 mL water, pH adjusted to 10 with 30% Na_2CO_3, and then extracted with ethyl ether (4×20 mL). The combined extracts were washed with water and dried over anhydrous $MgSO_4$. After removal of the solvent, recrystallization of the residue from 20 mL of methanol gave 1.2 g (74.0%) of **24a**; mp 84.0–84.5 °C ^{1}H NMR ($CDCl_3$) (δ ppm) 7.26 (s, 4H, Ar*H*), 3.67 (s, 6H, 2×OCH_3), 3.51 (s, 4H, 2×Ar-CH_2).

Step 3. α,α′-Bis[3-(*N*-3-hydroxylpropylcarbamoyl)piperidino]-*p*-xylene (25a1). α,α′-Bis(3-methoxycarbonylpiperidino)-*p*-xylene (**24a**) (0.94 g, 2.4 mmol) was heated with 3-amino-1-propanol (0.98 g, 13 mmol) at 170 °C for 4 h. The reaction mixture was cooled to room temperature and treated with 5 mL of acetonitrile to give a white solid. Recrystallization of the precipitated solid from methanol:acetonitrile (1:4 v/v) provided **25a1** (0.36 g, 31.6%), mp 161.5–162.5 °C; anal. ($C_{26}H_{42}N_4O_4$) C, H, N; IR (KBr) 3303 (s), 2943 (s), 1643 (s), 1559 (s), 1436 (m) cm^{-1}; ^{1}H NMR (DMSO-d_6) (δ ppm) 7.79 (t, J= 5.6 Hz, 2H, 2×CON*H*), 7.21 (s, 4H, Ar*H*), 4.39 (s, 2H, 2×O*H*), 3.41 (s, 4H, Ar-CH_2).

Step 4. α,α′-Bis[3-(*N*-3-nitrooxylpropylcarbamoyl)-piperidino]-*p*-xylene (26a1). α,α′-Bis[(3-(*N*-3-hydroxylpropylcarbamoyl)piperidino)]-*p*-xylene (**25a1**) (0.53 g, 1.12 mmol) was gradually added to cold fuming nitric acid (3 mL, 30 °C). After stirring the reaction mixture at −10 to −5 °C for 1 h, 30 mL ether was added, stirred at −10 °C for 30 min, the white solid was collected and dissolved in 25 mL of distilled water, the pH was adjusted to 10 with 30% Na_2CO_3, the product was extracted with chloroform (3×25 mL), the organic layer was washed with water (2×20 mL) and then dried over anhydrous $MgSO_4$. The removal of solvent followed by recrystallization from a mixture of chloroform and ethyl acetate provided 0.56 g (88.7%) of **26a**, mp 137.5–138.6 °C; anal. ($C_{26}H_{40}N_6O_8$) C, H, N; IR (KBr) 3263 (s), 2943 (s), 1639 (s), 1619 (s), 1559 (m), 1288 (s) cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.27 (s, 4H, Ar*H*), 4.47 (t, J=6.4 Hz, 4H, 2×CH_2ONO$_2$), 3.53, 3.43 (dd, J= 12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2), 3.33–3.41 (m, 4H, 2×CH_2NH-).

α,α′-Bis[3-(*N*-4-nitrooxylbutylcarbamoyl)piperidino]-*p*-xylene (26a2). Yield: 23.7%. Mp 124.1–124.9 °C; anal. ($C_{28}H_{44}N_6O_8$) C, H, N; IR (KBr) 3263 (s), 3110 (m), 2943 (s), 1639 (s), 1580 (s), 1290 (s) cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.25 (s, 4H, Ar*H*), 4.46 (t, J=6.4 Hz, 4H, 2×CH_2ONO$_2$), 3.54, 3.42 (dd, J=12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2), 3.26–3.29 (m, 4H, 2×-CH_2NH-).

α,α′-Bis[3-(*N*-5-nitrooxylpentylcarbamoyl)piperidino]-*p*-xylene (26a3). Yield: 46.2%. Mp 129.4–130.0 °C; anal. ($C_{30}H_{48}N_6O_8$) C, H, N; IR (KBr) 3263 (s), 3110 (m), 2943 (s), 1635 (s), 1580 (s), 1290 (s), 850 (s) cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.23 (s, 4H, Ar*H*), 4.45 (t,

J=6.4 Hz, 4H, 2×CH_2ONO$_2$), 3.53, 3.43 (dd, J= 12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2), 3.21–3.29 (m, 4H, 2×CH_2NH-).

α,α′-Bis[3-(*N*-6-nitrooxylhexylcarbamoyl)piperidino]-*p*-xylene (26a4). Yield: 33.7%. Mp 119.3–120.1 °C; anal. ($C_{32}H_{52}N_6O_8$) C, H, N; IR (KBr) 3260 (m), 3110 (w), 2940 (m), 1635 (s), 1580 (s), 1290 (s), 850 (w) cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.23 (s, 4H, ArH), 4.43 (t, J=6.4 Hz, 4H, 2×CH_2ONO$_2$), 3.53, 3.43 (dd, J= 12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2), 3.20–3.29 (m, 4H, 2×-CH_2NH-).

α,α′-Bis[3-(*N*-4-nitrooxylethylcarbamoyl)piperidino]-*p*-xylene (26b1). Yield: 61.9%. Mp 122.5–123.5 °C; anal. ($C_{24}H_{36}N_6O_8$) C, H, N; IR (KBr) 3280 (s), 3110 (m), 2940 (m), 1635 (s), 1585 (s), 1285 (s), cm^{-1}; ^{1}H NMR (DMSO-d_6) (δ ppm) 8.00 (t, J=5.6 Hz, 2H, 2×CONH), 7.22 (s, 4H, ArH), 4.52 (t, J=5.3 Hz, 4H, 2×CH_2 ONO$_2$), 3.29–3.40 (m, 8H, 2×Ar-CH_2, 2×-CH_2NH-).

α,α′-Bis[3-(*N*-4-nitrooxylpropylcarbamoyl)piperidino]-*p*-xylene (26b2). Yield: 63.0%. Mp 140.0–141.0 °C; anal. ($C_{26}H_{40}N_6O_8$) C, H, N; IR (KBr) 3280 (s), 3090 (m), 2940 (s), 1645 (s), 1635 (s), 1580 (s), 1290 (s), 850 (s) cm^{-1}; ^{1}H NMR (DMSO-d_6) (δ ppm) 7.81 (t, J=5.8 Hz, 2H, 2×CONH), 7.22 (s, 4H, ArH), 4.50 (t, J=6.3 Hz, 4H, 2×CH_2ONO$_2$), 3.40 (s, 4H, 2×Ar-CH_2), 3.11 (q, J=6.4 Hz, 4H, 2×-CH_2NH-).

α,α′-Bis[3-(*N*-pentylcarbamoyl)piperidino]-*p*-xylene (28a). The synthesis of **28a** is described as an example for the synthesis of bis-nipecotamidoaralkanes with different substitutents on the amide *N*, as shown in Scheme 6.

α,α′-Bis(3-methoxycarbonylpiperidino)-*p*-xylene (24a). (0.50 g, 1.28 mmol) was refluxed in 16 mL of 1 N hydrochloric acid for 6 h. After removal of the solvent, the residue was dried under vacuum (0.1 mm Hg) for 24 h. The dried white salt was refluxed with 5 mL of thionyl chloride for 2 h. After the removal of the excess thionyl chloride, the residue (crude **27**) was treated with 20 mL of 5% (v/v) amylamine (**29a**) at room temperature for 1 h. The precipitate was collected and recrystallized from methanol to obtain **28a** (0.65 g, 71.0%), mp 145.6–146.4 °C; anal. ($C_{30}H_{50}N_4O_2$) C, H, N; IR (KBr) 3350 (s), 2940 (s), 1630 (s), 1635 (s), 1580 (s); cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.24 (s, 4H, ArH), 3.50, 3.44 (dd, J=12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2), 3.18–3.30 (m, 4H, 2×-CH_2NH-), 0.92 (t, J=6.8 Hz, 6H, 2×-CH_3).

α,α′-Bis[3-(*N*-heptylcarbamoyl)piperidino]-*p*-xylene (28b). Yield: 64.1%. Mp 150.0–150.7 °C; anal. ($C_{34}H_{58}N_4O_2$) C, H, N; IR (KBr) 3350 (s), 2940 (s), 1630 (s), 1635 (s), 1580 (s); cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm), 7.24 (s, 4H, ArH), 3.52, 3.47 (dd, J=12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2), 3.18–3.30 (m, 4H, 2×x-CH_2NH-), 0.92 (t, J= 6.8 Hz, 6H, 2×-CH_3).

α,α′-Bis(3-carbamoylpiperidino)-*p*-xylene (28c). Yield: 84.9%. Mp 250–251.8 °C; anal. ($C_{20}H_{30}N_4O_2$) C, H, N; IR (KBr) 3350 (s), 3140 (m), 2942 (s), 1654 (s), 1420 (m); cm^{-1}; ^{1}H NMR (DMSO-d_6) (δ ppm) 7.25 (s, 2H, 2×CONH), 7.15 (s, 4H, ArH), 6.62 (s, 2H, 2×COHN), 3.32 (s, 4H, 2×Ar-CH_2).

α,α′-Bis(4-carbamoylpiperidino)-*p*-xylene (28d). Yield: 88.6%. Mp 300.5 °C (dec); anal. ($C_{20}H_{30}N_4O_2$) C, H, N; IR (KBr) 3342 (m), 3150 (m), 2930 (m), 1654 (s), 1420 (w); cm^{-1}; ^{1}H NMR (DMSO-d_6) (δ ppm) 7.22 (s, 4H, ArH), 7.15 (s, 2H, 2×CONH), 6.59 (s, 2H, 2×COHN), 3.37 (s, 4H, 2×Ar-CH_2).

α,α′-Bis[3-(3′-*N*,*N*-diethylcarbamoylpiperidinocarbonyl)-piperidino]-*p*-xylene (28e). Crude **27** (1.08 g, 2.3 mmol) was added to a solution of 3-*N*,*N*-diethylcarbamoylpiperidine (1.0 g, 5.4 mmol) in 12 anhydrous pyridine, stirred at room temperature for 18 h. The reaction mixture was poured into 100 g of ice–water and the product extracted with chloroform (3×20 mL). The combined extracts were washed with water, dried over anhydrous $MgSO_4$, and after removal of the solvent, the residue was purified via flash column chromatography to give **28e** (0.76 g, 47.7%) as a foaming solid; anal. ($C_{40}H_{64}N_6O_4$·0.5EtOAc) C, H, N; IR (KBr) 2943 (m), 1639 (s), 1537 (m); 1430 (w), 1278 (w), cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 7.24 (s, 4H, ArH), 4.62, 3.89 (dd, J=11.9 Hz, 11.9 Hz, 4H, 2×Ar-CH_2), 4.11 (q, J=7.1 Hz, 1H, CH$_3$ CH_2OAc).

α,α′-Bis[3-(*N*-trans-4-nitrooxycyclohexylcarbamoyl)-piperidino]-*p*-xylene (28f). Yield: 19.6%. Mp 185.0–186.0 °C; anal. ($C_{32}H_{48}N_6O_8$) C, H, N; IR (KBr) 3270 (m), 2943 (s), 1639 (s), 1541 (m); 1278 (s), 865 (s) cm^{-1}; ^{1}H NMR (DMSO-d_6) (δ ppm) 7.75 (d, J=7.56, 2H, 2×CONH), 7.22 (s, 4H, ArH), 4.93–5.00 (m, 2H, 2×CHONO$_2$), 3.51–3,53 (m, 2H, 2×NH-CH), 3.43, 3.41 (dd, J=12.8 Hz, 12.8 Hz, 4H, 2×Ar-CH_2).

1-Decyl-3-(*N*-nitrooxylethylcarbamoyl)piperidine (33a). Methyl nicotinate (**22a**) (4.0 g, 29.0 mmol) and ethanolamine (3.0 mL) were heated at 160 °C for 4 h. The excess ethanolamine was removed under vacuum (0.05 mmHg, rt, overnight). The residue was stirred with 11.4 mL of 1-bromodecane at 150 °C for 7 h and cooled to room temperature. The resultant viscous oil was treated with hexanes (3×20 mL) to remove excess 1-bromodecane. The upper layer containing hexanes was decanted and the resulting crude **31a** was subjected to catalytic hydrogenation (in 100 mL of 50% ethanol, 0.27 g of PtO_2, 60 psi for 4 h) followed by removal of the catalyst and the solvent. The crude product was dissolved in 100 mL of water and washed with chloroform (3×50 mL). The aqueous layer was adjusted to pH 10 with 30% Na_2CO_3, then extracted with chloroform (4×50 mL). The combined extracts were washed with water and dried over anhydrous $MgSO_4$. After removal of the solvent, recrystallization of the residue from ethyl acetate gave pure **32a** as white crystals (4.02 g, 44.4%); mp 63.0 °C; IR (KBr) 3291 (s), 3150 (w), 2921 (s), 1643 (s), 1559 (m), 1469 (w); 1216 (s), cm^{-1}; ^{1}H NMR ($CDCl_3$) (δ ppm) 8.69 (br, 1H, CONH), 3.74 (t, J=5.2 Hz, 2H, CH_2OH), 3.62 (m, 2H, CONHCH_2), 0.90 (t, J=6.6 Hz, 3H, -CH$_2$CH_3).

1-Decyl-3-(*N*-hydroxylethylcarbamoyl)piperidine (32a). (0.50 g, 1.55 mmol) was gradually added to cold fuming

nitric acid (3 ml, −30 °C). The reaction mixture was stirred at −10 to −5 °C for 1 h, 30 mL of ether was added and stirred at −10 °C for additional 30 min. The mixture was poured into 150 mL of cold 30% Na_2CO_3, the product was extracted with ether (4×50 mL), the extract was washed with water (2×50 mL) and dried over anhydrous $MgSO_4$. Removal of solvent followed by recrystallization from ethyl acetate provided 0.30 g (52.4%) of **33a**, mp 65.7–66.7 °C; anal. ($C_{18}H_{34}N_3O_4$) C, H, N; IR (KBr) 3301 (s), 3150 (w), 2921 (s), 1648 (s), 1618 (s), 1554 (s), 1420 (w); 1280 (s), cm^{-1}; ^1H NMR ($CDCl_3$) (δ ppm) 8.91 (br, 1H, CON*H*), 4.56 (t, J=5.2 Hz, 2H, CH_2ONO$_2$), 3.62 (q, J=5.2 Hz, 2H, CONHCH_2), 0.90 (t, J=6.6 Hz, 3H, -CH_2CH_3).

1-Decyl-3-(*N*-3-nitrooxylpropylcarbamoyl)piperidine (33b). A procedure similar to the one described above for **33a**, was used to prepare 1-decyl-3-(*N*-3-nitrooxylpropylcarbamoyl)piperidine (**33b**), yield 83.0%; mp 45.0–45.6 °C; anal. ($C_{19}H_{36}N_3O_4$) C, H, N; IR (KBr) 3298 (m), 2921 (s), 2852 (m), 1640 (s), 1552 (m), 1468 (w); 1281 (m), cm^{-1}; ^1H NMR ($CDCl_3$) (δ ppm) 8.54 (br, 1H, CON*H*), 4.53 (t, J=5.2 Hz, 2H, CH_2ONO$_2$), 3.38 (q, J=5.2 Hz, 2H, CONHCH_2), 0.90 (t, J=6.6 Hz, 3H, -CH_2CH_3).

1-*N*-3-Nitrooxylpropylcarbamoyl-3-(*N*-3-nitrooxylpropylcarbamoyl)-piperidine (38). To a solution of ethyl nipecotate (**34**, 1.57 g, 10.0 mmol) and K_2CO_3 (2.0 g) in 50 mL ethanol, a solution of methyl 4-(bromomethyl)-benzoate (**35** 2.3 g, 10.0 mmol) in 50 mL ethanol was gradually added. The reaction mixture was stirred at room temperature for 4 days before the removal of the solvent. The residue was dissolved in 20 mL of water and adjusted to pH 10 with 30% of Na_2CO_3, and extracted with toluene (4×20 mL). The combined extracts were washed with water (4×20 mL) and dried over anhydrous $MgSO_4$. Removal of the solvent under reduced pressure gave the crude compound **36** as a colorless oil (2.45 g, 80.0%).

Crude compound **36** (1.18 g, 3.7 mmol) was heated with 3 mL of 3-amino-1-propanol at 170 °C for 4 h. The reaction mixture was cooled to room temperature and the excess reagent was removed by vacuum distillation. The resultant oily residue was treated with 20 mL of ethyl acetate. The precipitated white solid was collected and recrystallized from ethanol and ethyl acetate (1:4, v/v) to give compound **37** as white crystals, mp 128.8–129.7 °C; anal. ($C_{20}H_{31}N_3O_4$) C, H, N; IR (KBr) 3303 (s), 3120 (m), 2958 (s), 1648 (s), 1557 (s), 1266 (m) cm^{-1}; ^1H NMR ($CDCl_3$) (δ ppm) 7.89 (br, 1H, CON*H*), 7.77 (d, J=8.1 Hz, 2 H, Ar*H*), 7.34 (d, J=8.1 Hz, 2H, Ar*H*), 7.19 (br, 1H, CON*H*), 3.70 (t, J=5.3 Hz, 2H, CH_2CH_2 OH), 3.61 (t, J=5.3 Hz, 2H, CH_2CH_2OH).

1-*N*-3-Hydroxylpropylcarbamoyl-3-(*N*-3-hydroxylpropylcarbamoyl)piperidine (37). (0.50 g, 1.32 mmol) was gradually added to cold fuming nitric acid (5 mL, −30 °C). After stirring the reaction mixture at −10 to −5 °C for 1.5 h, 25 mL ether was added, stirred at −25 °C for 30 min, and the upper layer was decanted. The resultant oily salt was dissolved in 20 mL of distilled water, the pH was adjusted to 10 with 30% Na_2CO_3, the product was extracted with chloroform (3×25 mL), the organic layer was washed with water (2×20 mL), and then dried over anhydrous $MgSO_4$. Removal of the solvent, followed by recrystallization from ethyl acetate provided 0.48 g (77.6%) of **38**, mp 84.0–85.0 °C; anal. ($C_{20}H_{29}N_5O_8$) C, H, N; IR (KBr) 3317 (s), 2932 (s), 1636 (s), 1541 (2), 1281 (s) cm^{-1}; ^1H NMR ($CDCl_3$) (δ ppm) 7.91 (br, 1H, CON*H*), 7.76 (d, J=8.1 Hz, 2 H, Ar*H*), 7.34 (d, J=8.1 Hz, 2H, Ar*H*), 6.53 (t, J=5.2 Hz, 1H, CON*H*), 4.60 (t, J=5.3 Hz, 2H, $CH_2CH_2ONO_2$), 4.45 (t, J=5.3 Hz, 2H, $CH_2CH_2ONO_2$).

***meso*-α,α′,-Bis[3-(*N*-5-nitrooxylpentylcarbamoyl)piperidino]-*p*-xylene (26a3).** To a solution of α,α′-dibromo-*p*-xylene (10.99 g, 41.64 mmol) in 140 mL of dichloromethane stirred with 6.92 g of K_2CO_3 at 0 °C, a solution of (*S*)-ethyl nipecotate (**34**, 3.3 g, 20.8 mmol) in 40 mL of dichloromethane was gradually added. The reaction mixture was stirred at room temperature for 3 h and then loaded on to a silica gel column. The column was washed with hexanes to remove the excess α,α′-dibromo-*p*-xylene, followed by elution with ether to collect the monosubstituted intermediate **39**. The ether fraction was concentrated to 150 mL at 20 °C followed by the addition of (*R*)-ethyl nipecotate (**34**, 5.0 g, 31.9 mmol) and 6.92 g of K_2CO_3. The reaction mixture was stirred for 10 h and poured into 100 mL cold water. The organic layer was washed thoroughly with water and dried over anhydrous $MgSO_4$. Removal of the solvent gave ***meso*-40** as a colorless oil (5.13 g, 58.9%).

***meso*-26a3** was prepared by following a procedure similar to that used in the synthesis of **26a1** discussed above, with ***meso*-40** as the starting material. Yield 46.6%. $[\alpha]_D$ 0.0°; mp 130.2–130.7 °C; analytical and spectral data were identical to those of **35a3** obtained above.

***meso*-α,α′-Bis[3-(*N*-4-nitrooxylbutylcarbamoyl)piperidino]-*p*-xylene (26a2)** was also prepared by a similar procedure.

Measurement of platelet aggregation—inhibitory activity

The previously reported turbidimetric procedure was employed in the aggregometric determinations.[16,35] All blood and blood products were handled in plastic ware, except for siliconised glass cuvettes and siliconised metal stir bars. Redistilled water was used to prepare solutions of all compounds which were used as their HCl salts, except for compounds **18**, **20**, **28e**, **33a**, **33b** and **38** which were used in their free base form and dissolved in redistilled 95% ethanol. The minimum concentration of ADP that elicited full biphasic response was used to test the compounds.

Human blood (60 mL) was collected from healthy male and female donors 20–35 years of age. Citrate, 3.2% was added to blood (1:8) to prevent it from clotting. Platelet rich plasma (PRP) was obtained by centrifuging at 1100 *g* for 15 min at 23°C. Using autologous PRP platelet count was adjusted to 250 000–300 000/cm^3. The

plasma was gassed with 5% CO_2 in air to prevent rise in pH and capped.

The plasma (450 μL) was transferred to cuvettes with stir bars in a Marsters constant temperature block. The cuvettes were debubbled and capped with Parafilm. The cuvettes were then transferred to a Payton Associates Dual Channel Aggregometer fitted with a Omniscribe recorder. Control and test cuvettes were treated similarly 1 min apart from each other. The control cuvette received equal volume of the vehicle without the test compound. After stirring at 1100 rpm for 15 s the cuvettes were returned to the Marsters block. After 1.5 min, they were returned to the aggregometer and 15 s later a baseline was recorded 2 min. Thereafter 50 μL of ADP was added and the change in light transmission recorded for another 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.

Determination of inositol 1,4,5-trisphosphate levels

The PRP was centrifuged at 1100 *g* at 23 °C for 15 min and the platelet pellet was resuspended in and washed 3 times with a modified Tyrode's buffer containing EGTA and apyrase (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM glucose, 1 mM EGTA, 0.35% bovine serum albumin and 34 μg/mL apyrase, pH 7.4). The final pellet was suspended in modified Tyrode's buffer with apyrase but without EGTA, and the volume adjusted to give a platelet count of 1×10^9/mL. Aliquots (450 μL) of the platelet suspension were incubated with α,α′-bis[3-(*N,N*-diethylcarbamoyl)piperidino]-*p*-xylene·2HCl (**4c**) at its IC_{50} (collagen) concentration (20.2 μM), or with an equal volume of water, for 4 min at 37 °C followed by the addition of 50 μL of collagen (50 μg/μL). The reaction was quenched at 0, 30, 60 and 120 s after the addition of the agonist, by the addition of 200 μL of 20% (v/v) perchloric acid followed by cooling on ice for 20 min. The proteins were sedimented by centrifugation (2000 *g*, 15 min, 4 °C), and the supernatant decanted and

Elemental analyses.

Compound	Formula	Calculated				Found			
		C	H	N	Cl	C	H	N	Cl
4a	$C_{22}H_{32}N_2O_2\cdot2HCl$	61.53	7.98	6.53	16.51	61.27	7.96	6.45	16.31
8a	$C_{30}H_{50}N_4O_3\cdot2HCl$	61.31	8.92	9.53	12.07	61.18	8.97	9.43	11.95
8b	$C_{29}H_{48}N_4O_3\cdot2HCl$	60.72	8.78	9.77	12.36	60.45	8.67	9.67	12.25
8c	$C_{34}H_{58}N_4O_3\cdot2(COOH)_2$	60.78	8.32	7.46	—	60.83	8.38	7.40	—
8d	$C_{28}H_{45}N_5O_42HCl$	57.13	8.05	11.90	12.05	57.06	8.11	11.88	11.94
8e	$C_{28}H_{47}N_5O_2\cdot2HCl$	60.19	8.84	12.54	12.69	59.95	8.92	12.46	13.08
8f	$C_{30}H_{49}N_5O_3\cdot2HCl$	59.98	8.56	11.66	11.81	59.75	8.44	11.59	11.68
8g	$C_{32}H_{48}N_4O_2\cdot2HCl$	64.74	8.49	9.44	11.94	64.47	8.51	9.39	11.77
8h	$C_{26}H_{44}N_4O_3\cdot2HCl$	58.52	8.69	10.50	13.29	58.42	8.74	10.52	13.35
8i[a]	$C_{26}H_{44}N_4O_2S_1\cdot2HCl$	56.81	8.44	10.19	12.90	56.73	8.47	10.09	12.98
8j	$C_{26}H_{44}N_6O_2\cdot2HCl$	57.23	8.50	15.40	12.99	57.07	8.55	15.34	12.90
8k	$C_{30}H_{50}N_4O_2\cdot2HCl$	63.03	9.17	9.80	12.40	62.92	9.23	9.80	12.35
8l	$C_{30}H_{48}N_4O_4\cdot2HCl$	59.89	8.38	9.31	11.79	59.63	8.34	9.40	11.73
8m	$C_{28}H_{45}N_4O_2Cl_1\cdot2HCl\cdot0.5H_2O$	57.28	8.24	9.54	18.12	57.23	8.23	9.49	18.21
8n[b]	$C_{28}H_{45}N_4O_2I_1\cdot2HCl$	50.23	7.08	8.37	10.59	50.15	6.96	8.30	10.62
8o	$C_{30}H_{50}N_4O_2\cdot2HCl$	63.01	9.17	9.80	12.40	63.11	9.22	9.74	12.34
13	$C_{28}H_{42}N_4O_2\cdot2HCl$	62.32	8.22	10.38	13.14	62.07	8.25	10.47	13.00
25a1	$C_{26}H_{42}N_4O_4$	65.79	8.92	11.80	—	65.91	8.93	11.74	—
25a4	$C_{32}H_{54}N_4O_4$	68.78	9.74	10.03	—	68.72	9.79	9.93	—
26a1	$C_{26}H_{40}N_6O_8$	55.31	7.14	14.88	—	55.27	7.18	14.74	—
26a2	$C_{28}H_{44}N_6O_8$	56.74	7.48	14.18	—	56.81	7.54	14.06	—
26a3	$C_{30}H_{48}N_6O_8$	58.05	7.79	13.54	—	58.20	7.87	13.39	—
26a4	$C_{32}H_{52}N_6O_8$	59.25	8.08	12.95	—	59.49	8.01	12.99	—
26b1	$C_{24}H_{36}N_6O_8$	53.72	6.67	15.66	—	53.77	6.83	15.64	—
26a1	$C_{26}H_{40}N_6O_8$	55.31	7.14	14.88	—	55.38	7.20	14.70	—
28a	$C_{26}H_{40}N_4O_2$	72.25	10.11	11.23	—	72.09	10.13	11.14	—
28b	$C_{30}H_{50}N_4O_2$	73.60	10.54	10.10	—	73.31	10.42	9.98	—
28c	$C_{20}H_{30}N_4O_2$	60.01	8.44	15.63	—	66.79	8.37	15.40	—
28d	$C_{20}H_{30}N_4O_2$	60.01	8.44	15.63	—	66.78	8.51	15.39	—
28e	$C_{40}H_{64}N_6O_4\cdot0.5EtOAc$	68.44	9.30	11.40	—	68.21	9.16	11.73	—
28f	$C_{32}H_{48}N_6O_8$	59.61	7.50	13.03	—	59.63	7.54	12.94	—
33a	$C_{18}H_{34}N_3O_4$	60.48	9.87	11.75	—	60.72	9.92	11.78	—
33b	$C_{19}H_{36}N_3O_4$	61.43	10.04	11.31	—	61.56	10.12	11.37	—
37	$C_{20}H_{31}N_3O_4$	63.64	8.28	11.13	—	63.42	8.29	11.06	—
38	$C_{20}H_{29}N_5O_8$	51.39	6.25	14.98	—	51.13	6.23	14.93	—

[a]S: Calc. 5.83; Found: 5.87.
[b]I: Calc. 18.95; Found: 19.01.

the pH was adjusted to 7.5 with 1.5 M KOH in a 60 mM HEPES buffer. The supernatant was assayed for IP_3 using an Amersham [^{3}H] radioimmuno assay kit.

Phosphoinositide turnover measurement

A platelet suspension (1×10^9 plts/mL prepared as described above) was incubated with [^{3}H] *myo*-inositol for 3 h at 37 °C. The platelets were then washed and resuspended as described above. Aliquots (450 μL) of the platelet suspension were incubated with 20.2 μM **4c** or an equal volume of water for 4 min at 37 °C followed by the addition of 50 μL of collagen (50 μg/μL). For initial experiments intended to find the time course of phosphoinositide turnover, the reaction was quenched at 0, 30, 60 and 120 s by the addition of 750 μL of $CHCl_3$:MeOH:HCl (2:1:0.2), while in subsequent experiments the reaction was terminated in a likewise manner at 30 s.[36] After the addition of 600 μL $CHCl_3$ and 600 μL of an extraction solution (KCl/EDTA), the solutions were placed on ice and shaken immediately for 1 h followed by centrifugation (2500 *g*, 15 min, 25 °C) to separate the layers. The aqueous layer was discarded and the organic layer was evaporated under anhydrous N_2. The residue was reconstituted in 100 μL of $CHCl_3$ and 40 μL aliquots were assayed for phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2). For the time-dependence experiments, the phosphoinositides were separated by HPLC on a Biogel HPHT hydroxyapatite column.[37] Due to the fragile nature of this column packing, the single-time studies were done using a Bio-Scale CHT5-1 column which contains a more durable hydroxyapatite packing. A multistep gradient starting with 1 mM triethylamine phosphate (TEAP) in 54:31:15 THF:ethanol:water mixture, pH 6.5 (initial condition A) and ending with 100% TEAP in the same solvent ratio, pH 6.5 (B) was employed for both analyses. The elution gradient used for the time-dependent assay consisted of initial loading with 100% A for 3 min, increasing linearly over 18 min to 16 mM and maintaining at this molarity for 15 min. The buffer concentration was then increased linearly over 30 min to 100 mM B. The flow rate was 1.0 mL/min. The gradient used with the Bio-Scale CHT-5-1 column consisted of an initial 1 mM TEAP buffer for 5 min at 1.0 mL/min. The buffer concentration was then increased linearly over 18 min to 100 mM (B), while simultaneously increasing (linear) the flow rate to 2.0 mL/min and maintaining at the final conditions for a further 20 min.

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