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Inhibition of trypsin and urokinase by Cbz-amino(4-guanidinophenyl)methanephosphonate aromatic ester derivatives: The influence of the ester group on their biological activity

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Abstract—The urokinase plasminogen activator is a trypsin-like serine protease, important in tumor development. Here, we report the synthesis and biochemical evaluation of selective and potent diaryl esters of phosphonic-type inhibitors for urokinase. We have found that the substituted phenyl ester ring has a strong influence on the inhibitory activity of these compounds. This led to the most potent phosphonic inhibitor for uPA synthesized to date.

The urokinase plasminogen activator (urokinase, uPA) is a trypsin-like serine protease, important in many cellular processes such as tumorigenesis, cell proliferation and migration, cell adhesion, angiogenesis, intravasation, and metastasis.[1–3] The primary role of uPA is to convert plasminogen into its active form plasmin, a broad spectrum serine protease which in turn activates matrix metalloproteases, and also degrades several extracellular matrix components, including laminin, collagen type IV, fibrin, and fibronectin.[4] It facilitates the detachment of cancer cells from the primary tumor and their migration within the surrounding tissue into blood and lymph vessels leading to the formation of metastases at distant sites.[5]

Since the urokinase plasminogen activator is involved at major steps in tumor progression, several molecules able to inhibit uPA activity have been synthesized. The common structural motif found in uPA inhibitors is an aromatic moiety substituted by an amidino or guanidino function, which mimics the arginine side chain. Some of these compounds inhibit uPA-mediated processes such as angiogenesis, extracellular matrix degradation, tumor cell adhesion, migration, and invasion.[6–8] Despite the large group of such molecules, only a few potent and selective low-molecular weight uPA inhibitors are presently known.[9,10]

α-Aminoalkylphosphonate diphenyl esters, the phosphonic analogues of naturally occurring amino acids, and their peptidyl-analogues comprise a group of irreversible, slow binding, competitive inhibitors for chymotrypsin-like and trypsin-like serine proteases such as chymase, trypsin, elastase or urokinase.[11–13] However, almost no attention has been paid to the possible enhancement of their activity through the substitution at the phenyl ester ring.[14] Here we describe the synthesis of new Cbz-*N*-protected α-aminoalkylphosphonate diphenyl ester derivatives, aromatic analogues of arginine, as potent and selective inhibitors for urokinase plasminogen activator. The main purpose of this study was to determine how simple modifications in the aromatic ester ring would change the potency of synthesized inhibitors. We have also examined how these modifications influence its selectivity for trypsin.

The synthetic approach is outlined in Scheme 1.[15] In the first step, different aromatic phosphites **1a**–**i** were synthesized using PCl_3 and substituted phenols in refluxing acetonitrile. The crude phosphites **1a**–**i** were used in the next step in the amidoalkylation reaction with benzyl carbamate and 4-nitrobenzaldehyde affording Cbz-*N*-

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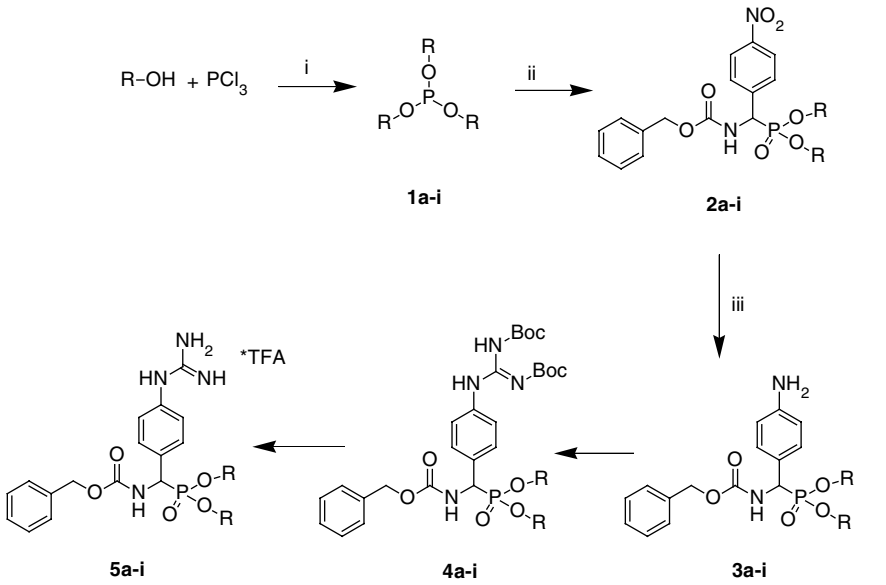


Scheme 1. Preparation of compounds **5a**–**i**. Reagents and conditions: (i) acetonitrile, reflux; (ii) benzyl carbamate, 4-nitrobenzaldehyde, AcOH, 80–90 °C; (iii) $SnCl_2/H_2O$, AcOEt, reflux; (iv) *S*-ethyl-*N*,*N*′-di(Boc)-isothiourea, Et_3N, $HgCl_2$, $CHCl_3$; (v) TFA, CH_2Cl_2.

protected amino(4-nitrophenyl)methanephosphonate derivatives **2a**–**i** as the racemic mixtures in good yields.[16]

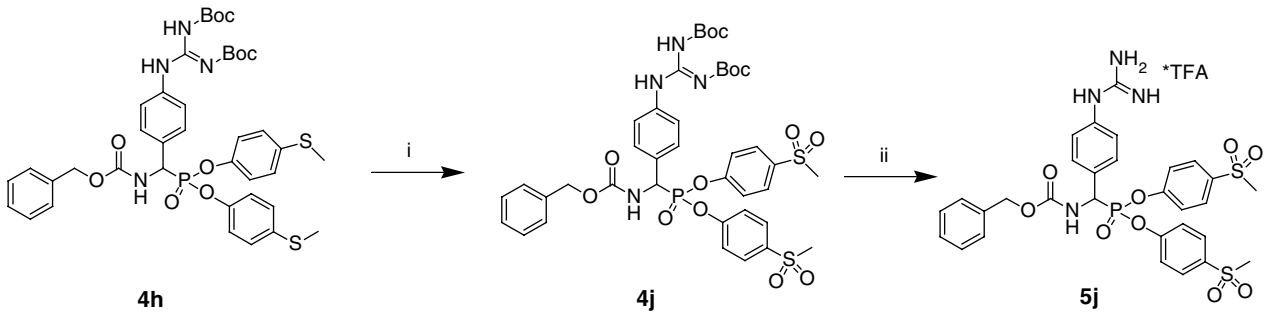
For the reduction of nitro derivatives **2a**–**i**, $SnCl_2$ dissolved in water, as a reductive agent in refluxing ethyl acetate, was applied. The desired aromatic amines **3a**-**i** were obtained in quantitative yields. The crucial, guanylating step was achieved using *S*-ethyl-*N*,*N*′-di(Boc)isothiourea in chloroform, in the presence of Et_3N and $HgCl_2$. Subsequent flash chromatography on Silica gel using $CHCl_3$/ethyl acetate (4:1) as an eluent gave the guanidines **4a**–**i**.

The oxidation of the *S*-methyl groups in compound **4h** into the methylsulfonyl groups was performed in the absolute THF using 30% hydrogen peroxide as an oxidizing agent, followed by the addition of TFA in CH_2Cl_2 (Scheme 2) without isolation of the intermediate product **4j**.

Boc-deprotection of the guanidine group was achieved by using trifluoroacetic acid (50% solution in methylene chloride) to yield the TFA salts of **5a**–**j**. The structures of all final compounds, as well as the intermediates, were characterized by ^1H, ^{13}C, ^{31}P NMR, and melting point analyses.[17]

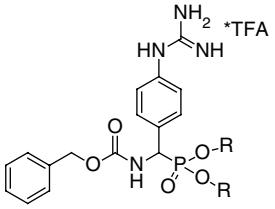
The TFA salts of Cbz-*N*-amino (4-guanidinophenyl) methanephosphonate aromatic esters **5a**–**j** were evaluated for their ability to inhibit the proteolytic activity of urokinase and trypsin using a chromogenic assay.[18] The enzyme and the inhibitor were incubated for 15 min at 25 °C before the addition of the substrate. Longer incubation times did not change the IC_{50} values.

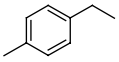
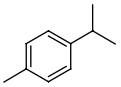
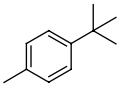
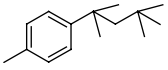
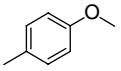
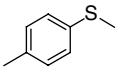
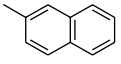
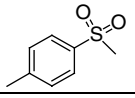
The effects resulting from the substitution of simple aliphatic chain in the phenyl ring at *para* position of Cbz-(4-GuPhg)^P$(OPh)_2$ are noticeable, as seen in Table 1. In general, a comparison of the IC_{50} values clearly shows that the substitution at position 4 has a strong influence on the inhibitory activity of these derivatives, but in most cases it lacks the selectivity. Compound **5h** is the most potent phosphonic uPA inhibitor synthesized to date (8.7 nM toward trypsin and 26 nM toward uPA). The highest selectivity was obtained for **5j** and **5f**, and total selectivity for **5i**. These results are in agreement with the 3D-QSAR studies and the model which predict the existence of



Scheme 2. Reagents: (i) THF, 30% H_2O_2 (this step should be handled carefully, as highly explosive peroxides may be generated); (ii) TFA, CH_2Cl_2.

Table 1. The inhibitory activity of different aromatic esters of α-aminoalkylphosphonates



Compound	R	IC_{50} (μM)		$k_{obsd}/[I]$[b] [M^{-1} s^{-1}]		Selectivity
		uPA	Trypsin	uPA	Trypsin	
5a		0.532	0.112	1450	6870	0.21
5b		0.370	0.176	2080	4370	0.48
5c		7.80	0.017	100	>10,000	0.002
5d		0.208	0.061	3700	>10,000	0.29
5e		0.098	0.088	7850	8750	0.90
5f		0.204	2.31	3800	330	11.3
5g		2.60	0.251	300	3070	0.10
5h		**0.026**	**0.0087**	>10,000	>10,000	0.33
5i		0.455	NI[a]	1700	NI	—
5j		0.053	23	>10,000	30	434

[a] NI, no inhibition after 25 min of incubation with enzyme.
[b] The apparent second-order inhibition rate constant $k_{obsd}/[I]$ was calculated using incubation method under pseudo-first-order reaction conditions (inhibitor concentration $\geqslant 10\times$ enzyme concentration).[19]

large hydrophobic subsite in the uPA structure on the leaving group site.[20] It is possible that one of the substituted aromatic ester rings could attach to this site. This region seems to play a pivotal role in the selectivity of these molecules.

In conclusion, we describe herein the synthesis and the inhibitory activity of new Cbz-*N*-protected derivatives of α-aminoalkylphosphonates with different aromatic ester rings. The compounds obtained are potent inhibitors of urokinase and trypsin even as the racemic mixtures. This leads to the conclusion that such modifications have a strong influence on the inhibitory activity of these derivatives. Further investigation in this field may lead to more potent and selective inhibitors. The next challenge in this area is the synthesis of pure enantiomers of the most potent inhibitors and their peptidyl-derivatives. We are currently at advanced stages in the synthesis of peptides which contain such phosphonic motif at the C-terminal as selective toward several other trypsin-like serine proteases. Irreversible inhibition of u-PA by phosphonate type inhibitors may be advantageous for in vivo activity.[21] However, this needs to be established in the animal model, if u-PA gene knockout mice remain healthy.[22]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.002.

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17. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 300.13, 75.47, and 121.50 MHz, respectively. Spectroscopic data of all synthesized compounds can be found in Supplementary material. Compound **5a**: White solid, mp 97–100 °C; ^{31}P NMR (DMSO): 14.36 (s); ^{1}H NMR (DMSO): 5.07 (d, J = 12.6 Hz, 1H), 5.15 (d, J = 12.0 Hz, 1H), 5.65 (dd, J = 10.2, 22.2 Hz, 1H), 7.37–7.72 (m, Ar-H, NH, 22H), 8.97 (d, J = 10.2 Hz, 1H), 10.03 (s, 1H); ^{13}C NMR (DMSO): 52.84 (d, J = 158.6 Hz), 66.72, 120.82 (d, J = 3.0 Hz), 120.88 (d, J = 4.5 Hz), 124.46, 125.82 (d, J = 9.1 Hz), 128.51, 128.87, 130.35 (d, J = 6.0 Hz), 132.79, 136.03, 137.08, 150.25 (d, J = 5.3 Hz), 150.56 (d, J = 5.3 Hz), 156.22, 156.46 (d, J = 2.3 Hz), 159.21 (d, J = 15.8 Hz).
    Compound **5b**: White solid, mp 95 °C; ^{31}P NMR (DMSO): 14.36 (s); ^{1}H NMR (DMSO): 2.27 (s, 6H), 5.06 (d, J= 12.0 Hz, 1H), 5.16 (d, J = 12.0 Hz, 1H), 5.59 (dd, J = 10.2, 22.2 Hz, 1H), 6.90–7.69 (m, Ar-H, NH, 20H), 8.92 (d, J = 10.2 Hz, 1H), 9.99 (s, 1H); ^{13}C NMR (DMSO): 20.72, 52.88 (d, J = 158.0 Hz), 66.69, 120.44, 120.47, 120.58, 124.46, 128.50, 128.86, 130.25, 130.63, 132.95, 134.91 (d, J = 10.6 Hz), 135.95, 137.09, 148.13, 156.21.
    Compound **5c**: White solid, mp 163–170 °C; ^{31}P NMR (DMSO): 14.39 (s); ^{1}H NMR (DMSO): 1.15 (t, J = 7.8 Hz, 6H), 2.57 (d, J = 7.2, 15.3 Hz, 4H), 5.07 (d, J = 12.6 Hz, 1H), 5.15 (d, J = 12.6 Hz, 1H), 5.61 (dd, J = 10.2, 22.8 Hz, 1H), 6.93–7.70 (m, Ar-H, NH, 20H), 8.94 (d, J = 10.2 Hz, 1H), 10.08 (s, 1H); ^{13}C NMR (DMSO): 16.12, 27.88, 52.80 (d, J = 158.6 Hz), 66.70, 120.60 (dd, J = 3.0, 8.3 Hz), 124.43, 128.51, 128.86, 129.43 (d, J = 6.8 Hz), 130.26, 132.92, 135.98, 137.09, 141.26 (d, J = 9.8 Hz), 148.23 (d, J = 10.6 Hz), 156.25, 156.46 (d, J = 9.0 Hz), 159.53, 159.74.
    Compound **5d**: White solid, mp 135 °C; ^{31}P NMR (DMSO): 14.37 (s); ^{1}H NMR (DMSO): 1.17 (d, J = 7.2 Hz, 12H), 2.85–2.89 (m, 2H), 5.06 (d, J = 12.6 Hz, 1H), 5.15 (d, J = 12.6 Hz, 1H), 5.61 (dd, J = 10.8, 22.5 Hz, 1H), 6.93–7.70 (m, Ar-H, NH, 20H), 8.93 (d, J = 10.2 Hz, 1H), 10.01 (s, 1H);^{13}C NMR (DMSO): 24.23, 33.25, 52.95 (d, J = 167.6 Hz), 66.71, 120.53, 120.55, 120.66, 124.44, 127.93, 128.03, 128.52 (d, J = 6.0 Hz), 128.86, 130.27, 132.94, 135.96, 137.08, 145.82. 145.95, 148.23 (d, J = 9.1 Hz), 148.53 (d, J = 9.0 Hz), 156.21, 156.49.
    Compound **5e**: White solid, mp 105–108 °C; ^{31}P NMR (DMSO): 16.08 (s); ^{1}H NMR (DMSO): 1.22 (s, 18H), 5.03 (d, J = 12.4 Hz, 1H), 5.13 (d, J = 12.4 Hz, 1H), 5.58 (dd, J = 10.3, 22.7 Hz, 1H), 6.90–7.68 (m, Ar-H, NH, 20H), 8.92 (d, J = 10.2 Hz, 1H), 9.92 (s, 1H); ^{13}C NMR (DMSO): 31.62, 34.62, 52.90 (d, J = 157.6 Hz), 66.73, 120.20 (d, J = 4.1 Hz), 120.29 (d, J = 3.8 Hz), 124.47, 126.97 (d, J = 7.7 Hz), 128.55, 128.87, 130.30 (d, J = 5.7 Hz), 133.02, 135.94, 137.09, 147.94, 148.10, 148.24, 148.31, 148.44, 156.22, 156.48 (d, J = 8.6 Hz).
    Compound **5f**: White solid, mp 106–109 °C; ^{31}P NMR (DMSO): 14.31 (s); ^{1}H NMR (DMSO): 0.68 (s, 18H), 1.30 (d, J = 3.6 Hz, 12H), 1.70 (s, 4H), 5.08 (d, J = 12.6 Hz, 1H), 5.17 (d, J = 12.6 Hz, 1H), 5.62 (dd, J = 10.2, 22.2 Hz, 1H), 6.91–7.71 (m, Ar-H, NH, 20H), 8.95 (d, J = 10.2 Hz, 1H), 10.10 (s, 1H); ^{13}C NMR (DMSO): 31.85, 32.04, 32.47, 38.42, 52.96 (d, J = 158.6 Hz), 56.63, 66.68, 119.96, 119.99, 120.10, 124.36, 127.67, 127.78, 128.46, 128.52, 128.85, 135.27 (d, J = 6.0 Hz), 132.92, 136.00, 137.14, 147.00, 147.13, 147.84 (d, J = 9.1 Hz), 148.15 (d, J = 9.1 Hz), 156.24, 156.52.
    Compound **5g**: White solid, mp 80–85 °C; ^{31}P NMR (DMSO): 14.81 (s); ^{1}H NMR (DMSO): 3.72 (d, J = 3.0 Hz, 6H), 5.07 (d, J = 12.6 Hz, 1H), 5.16 (d, J = 12.6 Hz, 1H), 5.58 (d, J = 10.2, 22.5 Hz, 1H), 6.75–7.69 (m, Ar-H, NH, 20H), 8.92 (d, J = 10.2 Hz, 1H), 9.98 (s, 1H); ^{13}C NMR (DMSO): 52.63 (d, J = 158.6 Hz), 55.93, 66.70, 115.16 (d, J = 12.1 Hz), 121.62, 121.64, 121.79, 124.48, 128.54, 128.87, 130.24, 133.02, 135.94, 137.10, 143.66 (d, J = 4.5 Hz), 144.02 (d, J = 4.5 Hz), 156.21, 156.46, 156.85, 156.94.
    Compound **5h**: White solid, mp 90–93 °C; ^{31}P NMR (DMSO): 16.37 (s); ^{1}H NMR ($CDCl_3$): 2.43 (d, J = 1.1 Hz, 6H), 5.03 (d, J = 12.4 Hz, 1H), 5.12 (d, J = 12.4 Hz, 1H), 5.60 (dd, J = 10.2, 22.7 Hz, 1H), 6.95–7.68 (m, Ar-H, NH, 20H), 8.89 (d,J = 9.7 Hz, 1H), 9.75 (s, 1H); ^{13}C NMR (DMSO): 15.78 (d, J = 3.7 Hz), 52.78 (d, J = 156.4 Hz), 66.78, 121.31, 121.36, 121.49, 124.64, 128.08, 128.55, 128.89, 130.32, 132.84, 137.06, 148.20, 156.15.
    Compound **5i**: White solid, mp 130 °C; ^{31}P NMR (DMSO): 14.81 (s); ^{1}H NMR (DMSO): 5.07 (d, J = 12.6 Hz, 1H), 5.12 (d, J = 12.0 Hz, 1H), 5.79 (dd, J = 10.2, 22.8 Hz, 1H), 7.31–7.95 (m, Ar-H, NH, 26H),

9.06 (d, $J = 10.2$ Hz, 1H), 9.91 (s, 1H); ^{13}C NMR (DMSO): 51.86 (d, $J = 148.0$ Hz), 66.79, 117.25 (d, $J = 15.1$ Hz), 120.82 (d, $J = 6.0$ Hz), 124.59, 126.26 (d, $J = 7.5$ Hz), 127.48, 127.82 (d, $J = 4.5$ Hz), 128.18, 128.51, 128.85, 130.43 (d, $J = 12.1$ Hz), 131.03 (d, $J = 6.0$ Hz), 132.89, 133.76, 136.05, 137.01, 147.90, 147.96, 148.31, 156.17.

Compound **5j**: Pale yellow solid, mp 240 °C; ^{31}P NMR ($CDCl_3$): 14.23 (s); ^{1}H NMR ($CDCl_3$): 3.06 (s, 6H), 4.71–4.81 (m, 1H), 4.85 (d, $J = 12.6$ Hz, 1H), 4.92 (d, $J = 12.6$ Hz, 1H), 6.80 (d, $J = 8.4$ Hz, 2H), 7.08–7.38 (m, Ar-H, 17H), 7.72 (d, $J = 8.7$ Hz, 2H), 9.77 (s, 1H); ^{13}C NMR ($CDCl_3$): 44.32, 54.05 (d, $J = 140.5$ Hz), 65.97, 121.36, 122.72, 128.16, 128.23, 128.79, 129.05, 129.81, 131.33, 134.60, 135.06, 137.55, 156.01, 156.22, 162.41.

18. The inhibitory effects of inhibitors on the enzymatic activity of urokinase-type plasminogen activator (LMW uPA from human urine, Calbiochem) and trypsin (from bovine pancreas, Sigma–Aldrich) were evaluated using Cbz-Val-Gly-Arg-*p*NA as a chromogenic substrate for uPA and Bz-L-Arg-*p*NA for trypsin. The change of absorbance was measured at 410 nm at 25 °C (Biochrom 4060). The assay buffer used was Tris–HCl (pH 8.8) containing 0.1 M NaCl (for urokinase) and HEPES 0.1 M (pH 7.5) containing 0.01 M $CaCl_2$ (for trypsin). The final concentration was 30 IU/ml for urokinase and 200 μM for its substrate, 10 U/ml for trypsin and 100 μM for its substrate. Typical kinetic analysis procedure: 50 μl (for trypsin) or 10 μl (for uPA) of the inhibitor solution in DMSO was added to the buffered enzyme solution (2.05 ml for trypsin and 0.58 ml for urokinase) to initiate the inactivation reaction. After incubation at 25 °C for 15 min, 50 μl (for trypsin) or 10 μl (for uPA) of the substrate solution was added. The change of absorbance was measured for 20 min.
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21. Initial experiments on the stability of the enzyme–inhibitor complex were performed for compound **5a** and trypsin. First, trypsin was inhibited by compound **5a** and the excess of inhibitor was removed by centrifugation using a Millipore Centricon® 10kDa cut-off filter. The enzyme was then incubated in HEPES buffer at pH 7.5 for 7 days. After this time, the enzyme regained 5% of its original activity as compared to the control, which retained 95% of its original activity after 7 days.
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