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A convenient synthesis of new α-aminoalkylphosphonates, aromatic analogues of arginine as inhibitors of trypsin-like enzymes

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Abstract—A simple and efficient protocol for the synthesis of new *N*-protected α-aminoalkylphosphonic diphenyl esters—aromatic analogues of arginine—is presented. The crucial, guanylating step was achieved using *S*-ethyl-*N,N′*-di(Boc)isothiourea in chloroform and in the presence of Et_3N and $HgCl_2$. Deprotection of the derivatives obtained was performed using trifluoroacetic acid in CH_2Cl_2 or hydrogenolysis over Pd/C. The products are potent inhibitors of trypsin.

Trypsin-like serine proteases compose a family of enzymes with a wide range of activity. They are known to be involved in many physiological states and pathological disorders. Their uncontrolled activity is very hazardous and often leads to serious diseases like emphysema, cystic fibrosis or cancer development and progression. For example, the key enzyme in tumour growth and metastasis is urokinase plasminogen activator (uPA) belonging to the trypsin-type serine protease family. Under normal physiological conditions this enzyme plays an essential role in angiogenesis processes but its uncontrolled activity results in enormous levels of active plasmin, which facilitates movement of cancer cells.[1]

To date numerous phosphonate- and peptidyl-phosphonate diphenyl esters have been used as effective and selective inhibitors of serine proteases,[2] but only a few papers describe the synthesis of phosphonate analogues of arginine,[3] lysine[4] or their mimetics.[5] After presentation of our preliminary results on the synthesis of such analogues as inhibitors of uPA at the Polish–Austrian–German–Hungarian–Italian Joint Meeting on Medicinal Chemistry (Cracow, October 2003) and during the preparation of this manuscript, an alternative synthesis of phosphonic analogues and their peptide derivatives was reported.[6] However, the yields of most steps were low and some yields of final products did not exceed 5%. Here we report a simple and more efficient method for the synthesis of α-aminophosphonic diphenyl esters as aromatic analogues of arginine.[7]

The overall synthetic approach is outlined in Scheme 1. In the first step, an amidoalkylation reaction was applied for the synthesis of Cbz-*N*-protected α-aminophosphonate diphenyl ester derivatives.[8] These compounds were obtained as racemic mixtures upon condensation of 3-nitrobenzaldehyde (derivatives **a**) or 4-nitrobenzaldehyde (derivatives **b**), benzyl carbamate and triphenyl phosphite. Compound **1a** was obtained in 71% yield and **1b** in 64% yield.[9] Deprotection of **1a**–**b** using 33% HBr in acetic acid gave the corresponding hydrobromide salts of the appropriate α-aminophosphonates, ready to apply in future planned peptide synthesis.[10]

For the reduction of nitro derivatives **1a**–**b**, anhydrous $SnCl_2$ (5 equiv) dissolved in water (20 equiv), as a reductive agent in refluxing ethyl acetate was applied. The desired aromatic analogues **2a** and **2b** possessing an aromatic amine were obtained in quantitative yields as pale yellow solids, which turned brown upon exposure to light.[11] Under these conditions we did not observe removal of the Cbz group.

The Boc-protected guanidine derivatives **3a**–**b** were obtained using *S*-ethyl-*N,N′*-di(Boc)isothiourea (1.1 equiv) in chloroform as guanylating agent, Et_3N

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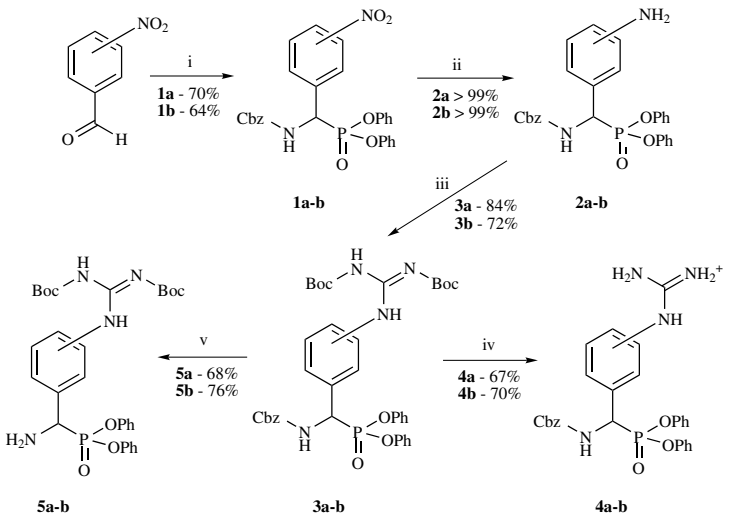


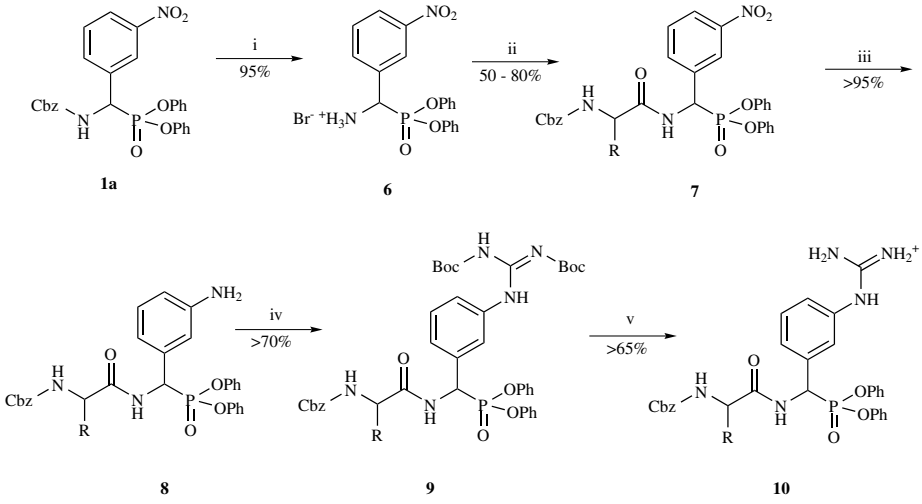
Scheme 1. Preparation of compounds **3a** and **3b**. The conditions are: (i) $P(OPh)_3$, benzyl carbamate, AcOH, 80 °C; (ii) $SnCl_2/H_2O$, AcOEt, reflux; (iii) *S*-ethyl-*N*,*N*′-di(Boc)-isothiourea, Et_3N, $HgCl_2$, $CHCl_3$; (iv) TFA, CH_2Cl_2; (v) H_2/Pd–C.

(3 equiv) and $HgCl_2$ (1.2 equiv) as promoter.[12] The reaction was monitored by TLC until the starting material had disappeared. Compounds **3a** and **3b** were obtained in 84% and 72% yields, respectively. The guanidine derivatives could be additionally recrystallized from a mixture of CH_2Cl_2/hexane (1:2) to give white crystals of **3a** and pearl-white crystals of **3b**.

At this stage two possible methods for deprotection were possible. Hydrogenolysis over Pd/C gives the desired α-aminophosphonates with a free α-amino group (**5a** and **5b** obtained in 68% and 76% yields, respectively), whereas Boc-deprotection of the guanidine group can be achieved by the application of trifluoroacetic acid (50% solution in CH_2Cl_2) to yield the TFA salts of **4a** and **4b** obtained in 67% and 70% yields, respectively.

A similar strategy was applied for the synthesis of several di- and tripeptides with the aromatic analogue of arginine at the *C*-terminal. The typical procedure is outlined in Scheme 2. Compound **1a** was converted into hydrobromide salt **6** in 95% yield using 33% HBr in acetic acid. Coupling of **6** with Cbz-amino acids using DCC/HOBt yielded the desired phosphonopeptides **7** in yields exceeding 50%. Treatment of **7** with $SnCl_2/H_2O$ in refluxing AcOEt reduced the nitro group of the aromatic ring to afford amines **8**. We did not observe removal of the Cbz protection during the reduction of the phosphonopeptides. Further crystallization from *n*-hexane gave pure derivatives. Guanidinylation using *S*-ethyl-*N*,*N*′-di(Boc)-isothiourea in chloroform afforded crystalline, solid products **9**. Finally, removal of the Boc groups was accomplished using TFA/CH_2Cl_2 (1:1).

The inhibitory activity of the compounds described above towards trypsin was determined using a chromogenic assay.[13] As mentioned in a recent paper,[6] these compounds show slow-binding inhibitory behaviour. The enzyme and the inhibitor were incubated for



Scheme 2. Preparation of phosphonopeptides. *R*-Amino acid side chain. The conditions are: (i) HBr/AcOH; (ii) Cbz-amino acid, DCC, HOBt, Et_3N, DMF; (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.

Table 1. Inhibitory activity of new α-aminophosphonate diphenyl esters, aromatic analogues of arginine towards trypsin

Compound	IC_{50} (μM)
4a	0.122
4b	13

10 min at room temperature before the addition of substrate. Our observation proved that longer preincubation times do not seem to change the IC_{50} values. Surprisingly, we have noticed that compound **4a** is a more active inhibitor of trypsin than of uPA (IC_{50} = 1.6 μM, Ref. 6, Table 1).

Additionally, our preliminary results showed that these compounds are potent inducers of apoptosis in human cancer cell lines; these results will be published separately in due course.

In conclusion, we have presented an alternative route for the synthesis of novel protected α-aminophosphonic diphenyl esters, aromatic mimetics of arginine, which appears to be more efficient if compared to current methods presented in the literature. The compounds obtained are potent inhibitors for trypsin-like enzymes even as racemic mixtures. The new challenge in this area is the synthesis of optically pure α-aminophosphonates of Cbz-$Arg^{P}(OPh)_2$, Cbz-$HomoArg^{P}(OPh)_2$, Cbz-(3-$GuPhg)^{P}(OPh)_2$, Cbz-(4-$GuPhg)^{P}(OPh)_2$. We are currently at advanced stages in the synthesis of pure enantiomers of these phosphonic analogues of arginine.

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9. ^{1}H, ^{13}C NMR and ^{31}P NMR spectra were recorded at 300.13, 75.47 and 121.50 MHz, respectively.
   1a: White crystals, mp 150 °C [Found C, 62.36; H, 4.70; N, 5.30; P, 5.80. $C_{27}H_{23}N_2O_7P$ requires C, 62.55; H, 4.47; N, 5.40; P, 5.97%]; ^{31}P NMR ($CDCl_3$): 17.31 (s); ^{1}H NMR ($CDCl_3$): 5.12 (q, *J* = 12.1 Hz, 2H), 5.67 (dd, *J* = 8.8, 23.2 Hz, 1H), 6.16 (dd, *J* = 6.0, 9.0 Hz, 1H), 6.94–8.35 (m, Ar–H, 19H); ^{13}C NMR ($CDCl_3$): 52.36 (d, *J* = 155.8 Hz), 67.91, 120.20 (dd, *J* = 4.4, 12.4 Hz), 122.96 (d, *J* = 6.1 Hz), 123.56, 125.75 (d, *J* = 3.0 Hz), 128.28, 128.57 (d, *J* = 11.1 Hz), 129.83, 129.92, 134.19, 135.65, 136.72, 148.44, 150.07, 155.6.
   2a: White, pale yellow solid, mp 120 °C [Found C, 66.51; H, 4.97; N, 5.80; P, 6.32. $C_{27}H_{25}N_2O_5P$ requires C, 66.39; H, 5.16; N, 5.73; P, 6.34%]; ^{31}P NMR ($CDCl_3$): 15.72 (s); ^{1}H NMR ($CDCl_3$): 3.88 (s, 2H), 5.09 (dd, *J* = 12.2, 28.7 Hz, 2H), 5.48 (dd, *J* = 9.9, 21.8 Hz, 1H), 5.93 (d, *J* = 7.7 Hz, 1H), 6.62–7.38 (m, Ar–H, 19H); ^{13}C NMR ($CDCl_3$): 52.9 (d, *J* = 158.7 Hz), 67.51, 115.00 (d, *J* = 7.2 Hz), 115.65, 118.63 (d, *J* = 6.64 Hz), 120.51 (dd, *J* = 4.3, 6.4 Hz), 125.35 (d, *J* = 4.0 Hz), 128.22, 128.28, 128.56, 129.69 (d, *J* = 8.1 Hz), 129.84, 135.18, 136.03, 146.51, 150.14 (d, *J* = 9.5 Hz), 155.55 (d, *J* = 9.0 Hz).
   3a: White crystals, mp 86-88 °C [Found C, 62.11; H, 6.21; N, 7.45; P, 4.40. $C_{38}H_{43}N_4O_9P$ requires C, 62.46; H, 5.93; N, 7.67; P, 4.24%]; ^{31}P NMR ($CDCl_3$): 15.22 (s); ^{1}H NMR ($CDCl_3$): 1.43 (s, 9H), 1.49 (s, 9H), 5.07 (dd, *J* = 12.2, 33.45 Hz, 2H), 5.55 (dd, *J* = 9.8, 22.3 Hz, 1H), 6.04 (d, *J* = 3.1 Hz, 1H), 6.89–7.73 (m, Ar–H, 19H), 10.38 (s, 1H), 11.60 (s, 1H); ^{13}C NMR ($CDCl_3$): 28.17, 52.73 (d, *J* = 160.0 Hz), 67.56, 79.75, 83.87, 120.53 (d, *J* = 4.1 Hz), 121.56, 122.71, 124.50, 125.38 (d, *J* = 4.1 Hz), 128.27, 128.56, 129.50, 129.73 (d, *J* = 3.6 Hz), 134.86, 136.00, 137.44, 150.15, 153.36, 155.44, 161.40, 163.4.
   1b: White solid, mp 160–161 °C [Found C, 62.58; H, 4.48; N, 5.48; P, 6.04. $C_{27}H_{23}N_2O_7P$ requires C, 62.55; H, 4.47; N, 5.40; P, 5.97%]; ^{31}P NMR ($CDCl_3$): 13,66 (s); ^{1}H NMR ($CDCl_3$): 5.09 (q, *J* = 12.1 Hz, 2H), 5.65 (dd, *J* = 9.0, 23.4 Hz, 1H), 6.13 (dd, *J* = 5.7, 8.8 Hz, 1H), 6.91–8.19 (m, Ar–H, 19H); ^{13}C NMR ($CDCl_3$): 52.60 (d, *J* = 155.93 Hz), 67.89, 120.22 (dd, *J* = 4.3, 11.8 Hz), 123.94, 125.79, 128.27, 128.58 (d, *J* = 9.7 Hz), 129.05 (d, *J* = 5.6 Hz), 129.92, 135.67, 141.65, 147.96, 149.78 (d, *J* = 9.1 Hz), 155.45.
   2b: White, pale yellow solid, mp 176–178 °C [Found C, 66.23; H, 5.25; N, 5.68; P, 6.32. $C_{27}H_{25}N_2O_5P$ requires C, 66.39; H, 5.16; N, 5.73; P, 6.34%]; ^{31}P NMR (DMSO): 17.38 (s); ^{1}H NMR (DMSO): 5.02 (dd, *J* = 12.5, 26.3 Hz, 2H), 5.13 (s, 2H), 5.28 (dd, *J* = 10.1, 20.1 Hz, 1H), 6.47–7.30 (m, Ar–H, 19H), 8.61 (d, *J* = 10.1 Hz, 1H); ^{13}C NMR (DMSO): 52.97 (d, *J* = 159.3 Hz), 66.52, 114.06, 120.84 (t, *J* = 4.6 Hz), 121.07, 125.63 (d, *J* = 6.6 Hz), 128.37 (d, *J* = 3.5 Hz), 128.84, 129.87 (d, *J* = 6.3 Hz), 130.26, 137.24, 149.24, 150.58 (q, *J* = 11.0 Hz), 156.44 (d, *J* = 8.5 Hz).
   3b: White, pearl crystals, 117–118 °C [Found C, 59.48; H, 6.50; N, 7.17; P, 4.05. $C_{38}H_{43}N_4O_9P \times 2H_2O$ requires C, 59.52; H, 6.18; N, 7.31; P, 4.05%]; ^{31}P NMR ($CDCl_3$): 15.39 (s); ^{1}H NMR ($CDCl_3$): 1.50 (s, 9H), 1.53 (s, 9H), 5.10 (dd, *J* = 12.2, 25.0 Hz, 2H), 5.53 (dd, *J* = 9.7, 22.0 Hz, 1H), 5.79 (d, *J* = 6.9 Hz, 1H), 6.90–7.64 (m, Ar–H, 19H), 10.36 (s, 1H), 11.60 (s, 1H); ^{13}C NMR ($CDCl_3$): 28.16 (d, *J* = 7.2 Hz), 52.41 (d, *J* = 154.0 Hz), 67.59, 79.75, 83.87, 120.49 (t, *J* = 3.3 Hz), 122.44, 125.40, 128.27 (d, *J* = 5.7 Hz), 128.58, 128.80 (d, *J* = 8.5 Hz), 129.74, 130.36, 135.96, 137.31, 150.01 (d, *J* = 11.6 Hz), 155.41, 153.43, 159.57, 163.49.
10. During peptide synthesis we discovered that the hydrobromide salt of the *p*-nitro derivative is highly unstable in alkaline medium—even addition of Et_3N prevents peptide formation. The Cbz-protected derivative is more stable, but after 12 h in weak-alkaline solution (DMSO–Et_3N) we observed, by ^{31}P NMR monitoring, formation of a degradation product. This abnormal behaviour and probable mechanism leading to this instability, is currently the subject of additional investigations.
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13. The inhibitory effects of inhibitors on the enzymatic activity of trypsin from bovine pancreas were evaluated using *N*-α-benzoyl-L-arginine 4-nitroanilide as a chromogenic substrate. The change of absorbance was measured at 410 nm at room temperature (Biochrom 4060). The assay buffer used was HEPES 0.1 M (pH = 7.5) containing 0.01 M $CaCl_2$. The final concentration was 0.15 μM for trypsin and 100 μM for its substrate.