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A Convenient Synthesis of Amino Acid *p*-Nitroanilides; Synthons in the Synthesis of Protease Substrates

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Abstract: A method is described for the synthesis of N^{α}-protected bi- and trifunctional amino acid *p*-nitroanilides. The reaction uses phosphorus oxychloride as the condensing agent. The synthesis is simple, rapid, free of racemization and affords yields between 70-90%. The synthesis can be performed not only with amino acid derivatives of the urethane type including acid-labile (Z, Boc) and base-labile (Fmoc, Msc) N^{α}-protective functions or allyl-derived protections, but also with N^{α}-trityl amino acids, albeit in lower yield. The reaction runs in pyridine and its mechanism implies carboxyl activation by formation of a mixed anhydride with phosphorodichloridic acid ($HOPOCl_2$).

INTRODUCTION

Amino acid *p*-nitroanilides are compounds of great interest and are widely used as chromogenic substrates for the determination of the activity of proteolytic enzymes present in body fluids[1].

Their synthesis however, appears as rather troublesome, since *p*-nitroaniline is a very weak nucleophile. As a consequence, the acylation of *p*-nitroaniline has been studied intensively to circumvent this difficulty and several methods are mentioned in the literature.

The common coupling methods used in peptide synthesis are insufficient. The use of DCC[2], DCC/HOBt[3] or the mixed anhydride method[4] gives yields in the range between 30-58%. Acid chlorides[5] and thermal activation[6] have been recommended to cope with the low nucleophilicity of *p*-nitroaniline, but they afford products of questionable optical purity.

As an alternative to *p*-nitroaniline, *p*-nitrophenylisocyanate[7], pure or *in situ* generated from *p*-nitrobenzoic acid through the modified Curtius reaction with diphenyl phosphorazidate[8], are found to be general and efficient methods.

In the recent literature some examples are given in which a monoacylated 1,4-diaminobenzene has been used as a precursor, to give a *p*-nitroanilide following oxidation with sodium perborate. To this end, Burdick *et al.*[9] described a solid phase synthesis of peptide *p*-nitroanilides using *p*-diaminobenzene as a linker in an urethane-type bond to the resin. Reiter[10] described the use of *p*-(Boc-amino)aniline as precursor for *p*-nitroaniline. Apart from the moderate yields which were obtained, the final oxidation step limits the use of this method to peptides not containing methionine, tyrosine, tryptophan and cysteine.

Aminolysis of a weakly activated peptide derivative by *p*-nitroaniline has been studied by Voyer *et al.*[11] They treated a peptide linked to an oxime resin[12] with *p*-nitroaniline; in spite of its low nucleophilicity, aminolysis by *p*-nitroaniline afforded a cleavage yield of 30%; the pure product being obtained in 16% yield.

In the early 1960s, an intensive research was devoted to the applicability of phosphorus compounds as coupling agents in peptide synthesis. Among them, the phospho-azo method[13] and the use of phosphorus pentoxide in diethylphosphite[14] were the most frequently applied methods to synthesize *p*-nitroanilides[15]. However, the obtained yields and the scope of these methods are rather limited. These syntheses appear only to be succesful when the α-amino function is protected with groups which are relatively resistant to acidic reagents (Z-family, Pht, etc.).

Table 1. Physicochemical Properties of N^{α}-Boc-protected Amino Acid *p*-Nitroanilides.

Compound	Yield %	mp °C	$[\alpha]_D$ c = 1 MeOH	TLC	Elemental Analysis (Calc.), %			
					C	H	N	S
Boc-Gly-pNA	80	192	-	0.18[a]/0.49[b]	52.73 (52.88)	5.66 (5.80)	13.85 (14.23)	-
Boc-Phe-pNA	75	150-151	+69.0	0.51[a]/0.70[b]	62.26 (62.33)	5.86 (6.01)	10.67 (10.90)	-
Boc-Ala-pNA	77	168	-58.6	0.31[a]/0.51[b]	54.26 (54.36)	6.13 (6.19)	13.52 (13.58)	-
Boc-Ile-pNA	82	foam	-13.7	0.50[a]/0.70[b]	58.22 (58.11)	7.01 (7.17)	11.56 (11.96)	-
Boc-Nle-pNA	60	foam	-20.8	0.53[a]/0.73[b]	58.42 (58.11)	7.19 (7.17)	11.64 (11.96)	-
Boc-Val-pNA	78	foam	-20.0	0.44[a]/0.68[b]	57.01 (56.96)	6.89 (6.87)	12.13 (12.45)	-
Boc-Arg-pNA.HCl*	91	187	-12.8	0.67[c]/0.70[d]	48.15 (48.29)	6.62 (6.79)	16.80 (16.89)	-
Boc-Tyr(Bzl)-pNA	89	179-181	+82.0	0.57[a]/0.79[b]	65.98 (65.98)	5.79 (5.95)	8.41 (8.55)	-
Boc-Cys(Bzl)-pNA	90	foam	+24.6	0.62[a]/0.81[b]	58.40 (58.45)	5.75 (5.84)	9.45 (9.74)	7.55(7.43)
Boc-Lys(Z)-pNA	84	foam	-8.1	0.18[a]/0.53[b]	59.72 (59.99)	6.32 (6.44)	10.90 (11.19)	-
Boc-His(Bzl)-pNA**	84	138-141	+24.4	0.04[a]/0.28[b]	61.55 (61.70)	6.01 (6.71)	13.91 (13.32)	-
Boc-Glu(OBzl)-pNA	84	foam	-7.9	0.46[a]/0.75[b]	60.05 (60.39)	5.77 (5.95)	9.03 (9.19)	-

[a]CH_2Cl_2/MeOH 98:2 v/v; [b]CH_2Cl_2/MeOH 95:5 v/v; [c]BuOH/AcOH/H_2O 4:1:1 v/v/v; [d]BuOH/pyridine/H_2O 1:1:1 v/v/v; *EA calculated for Boc-Arg-pNA.HCl.0.5AcOH.0.5BuOH; **EA calculated for Boc-His(Bzl)-pNA.*iso*propyl alcohol.

The first syntheses of *tert.*-butyloxycarbonyl amino acid *p*-nitroanilides have been reported by Okada *et al.*[3] for Boc-Met-pNA and Boc-Lys(Z)-pNA, by Shioiri *et al.*[8] for Boc-Leu-pNA, Boc-Lys(Z)-pNA and Boc-Arg(Mts)-pNA and by Noda *et al.*[16] Both Bajusz[17] and Oyamada *et al.*[18] used the phospho-azo method in the synthesis of Boc-Arg-pNA.HCl. Only Oyamada *et al.*[18] obtained high yields (43-94%) after reinvestigation of the phospho-azo method and synthesized *p*-nitroanilides of Z- and Boc-amino acids.

The results of Noda *et al.*[16] and Oyamada *et al.*[18] prompted us to resume our results using phosphorus oxychloride as the condensing agent[19]. In this contribution we present a summary of our results: (1) we found phosphorus oxychloride the reagent of choice for the synthesis of *p*-nitroanilides of all kinds of amino-protected carboxylic acids. (2) Pyridine is the preferred solvent for these condensations. (3) The method is compatible with the use of (the base labile) Fmoc-amino acid derivatives[20], giving access to orthogonally protected *p*-nitroanilides as synthons for virtually each substrate of this class.

RESULTS AND DISCUSSION

The use of phosphorus oxychloride in peptide synthesis has been described for the first time by Wieland *et al.*[21]

The method implies activation of a protected amino acid with phosphorus oxychloride in pyridine at -15°C (see Figure 1). Aminolysis by *p*-nitroaniline occurs already at low temperatures and reaches completion within 15-30 min. We developed the method with Z-amino acids, since the acid stability of the Z-group precludes the occurrence of side reactions in the phosphorus oxychloride/pyridine reaction mixture. It is essential to use pyridine as the solvent since it catalyses nucleophilic attack[22] and since the solvent acts as a weak base, it protects acid-labile functions against acidolysis allowing extention of the method to more acid-labile (*i.e.* Boc-) protections.

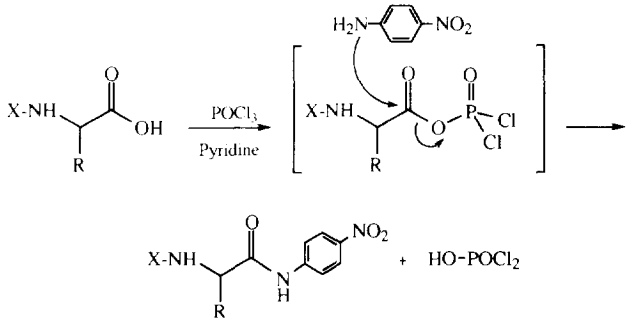

Figure 1. Equimolar amounts of an amino acid and phosphorus oxychloride are required to give *in situ* a mixed anhydride as the activating species. X respresents the α-amino protective group (Aloc, Boc, Fmoc, Trt or Z), R symbolizes the (suitably protected) side chain.

The Boc-amino acid *p*-nitroanilides synthesized in this study are listed in Table 1. The here recorded physicochemical data give good correlation with known data. Trifunctional N^{α}-*tert.*-butyloxycarbonylamino acids require protections which resist acidolysis by trifluoroacetic acid. Benzyl functions usually fulfil this demand, but since their hydrogenolytic removal is evidently incompatible with the presence of nitro groups, rather severe acidolytic conditions (HBr/glacial AcOH) have to be applied. The acid stability of the *p*-nitroanilide bond is however, well documented in the literature: the bond resists 30% HBr in glacial acetic acid[2a], saturated HCl in glacial acetic acid[23], methanesulfonic acid[8], 10% HCl in EtOAc and even neat HF[7a,b].

Orthogonality will be reached if in an α-amino acid the α-amino function is protected with a base-labile group and the side chain with a group removable under mildly acidic conditions. This however, requires good stability of the *p*-nitroanilide bond in media, which attack the base-labile protection. We tested the base-stability

Table 2. Physicochemical Properties of N^{α}-Fmoc-protected Amino Acid *p*-Nitroanilides.

Compound	Yield %	mp °C	$[\alpha]_D$ c = 1 DMF	TLC	Elemental Analysis (Calc), %			
					C	H	N	S
Fmoc-Gly-pNA	89	212-213	-	0.24[a]/0.14[b]	66.02 (66.18)	4.50 (4.59)	9.89 (10.07)	-
Fmoc-Val-pNA	70	189-191	-27.1	0.67[a]/0.48[b]	67.47 (67.96)	5.30 (5.48)	8.86 (9.14)	-
Fmoc-Phe-pNA	88	197-199	+13.1	0.71[a]/0.49[b]	70.95 (71.00)	4.93 (4.96)	8.16 (8.28)	-
Fmoc-Met-pNA	79	183	-39.1	0.68[a]/0.41[b]	63.32 (63.53)	5.06 (5.13)	8.35 (8.55)	6.41 (6.52)
Fmoc-Arg-pNA.HCl*	88	183	-45.5	0.73[c]/0.72[d]	57.51 (57.27)	5.73 (5.89)	13.06 (12.93)	-
Fmoc-Lys(Boc)-pNA	81	116-117	-23.3	0.24[a]/0.25[b]	63.79 (64.80)	6.10 (6.79)	8.80 (8.64)	-
Fmoc-Orn(Boc)-pNA	92	180	-20.5	0.29[a]/0.24[b]	65.36 (65.26)	6.37 (6.66)	9.13 (8.95)	-
Fmoc-Glu(O^{t}Bu)-pNA	83	98	-26.8	0.70[a]/0.47[b]	65.47 (66.04)	5.79 (5.73)	6.52 (7.70)	-
Fmoc-Ser(^{t}Bu)-pNA	79	foam	-36.2	0.76[a]/0.47[b]	66.39 (66.79)	5.70 (5.80)	8.18 (8.34)	-
Fmoc-Tyr(tBu)-pNA	79	93-95 (dec.)	+24.1	0.71[a]/0.51[b]	68.88 (68.86)	5.95 (5.60)	6.60 (7.05)	-
Fmoc-Cys(Trt)-pNA	90	foam	-18.8	0.84[a]/0.58[b]	69.77 (69.88)	4.86 (4.85)	5.54 (5.62)	4.45 (4.28)
Fmoc-His(Trt)-pNA	95	foam	-6.1	0.16[a]/0.10[b]	72.36 (72.35)	4.90 (4.90)	8.93 (9.11)	-
Fmoc-Asn(Trt)-pNA	71	214-216	-25.3	0.71[a]/0.49[b]	73.53 (73.73)	5.05 (5.06)	7.70 (7.82)	-

[a]CH_2Cl_2/MeOH 98:2 v/v; [b]EtOAc/n-hexane 1:1 v/v; [c]BuOH/AcOH/H_2O 4:1:1 v/v/v; [d]BuOH/pyridine/H_2O 1:1:1 v/v/v; *EA calculated for Fmoc-Arg-pNA.HCl.0.5BuOH

Table 3. Physicochemical Properties of Miscellaneous Amino Acid *p*-Nitroanilides.

Compound	Yield %	mp °C	$[\alpha]_D$; c; solvent	TLC	Elemental analysis (Calc), %			
					C	H	N	S
Msc-Arg-pNA.HCl	65	lyophil	-12.4; 1; MeOH	0.45[c]/0.70[d]	38.33 (38.38)	5.27 (5.48)	16.21 (16.78)	6.39(6.40)
Trt-Ala-pNA[a]	26	foam	no data	0.33[e]/0.79[f]	-	-	-	-
Aloc-Val-pNA[b]	78	foam	-21.1; 1; MeOH	0.35[f]/0.71[g]	56.27 (56.07)	5.77 (5.96)	12.93 (13.08)	-
Z-Phe-pNA	86	163	+76.9; 2; DMF	-	-	-	-	-
Z-Arg-pNA.HCl[*]	96	174	-10.7; 1; MeOH	0.69[c]/0.71[d]	51.75 (51.32)	6.05 (5.88)	15.43 (15.05)	-
2HCl.H-Arg-pNA[**]	-	255	+71.1; 1; H_2O	0.38[c]/0.61[d]	34.74 (35.75)	5.11 (6.00)	19.36 (20.84)	-
HCl.H-Tyr(Bzl)-pNA	-	foam	+114.9; 1; MeOH	0.80[c]/0.73[d]	60.88 (60.88)	5.22 (5.21)	9.04 (9.43)	-
HCl.H-Val-pNA	-	264 (dec.)	+64.9; 1; MeOH	0.65[c]/0.68[d]	48.35 (48.27)	5.87 (5.89)	14.98 (15.35)	-
HCl.H-Nle-pNA	-	lyophil	+41.1; 0.53 MeOH	0.74[c]/0.81[d]	50.08 (50.09)	6.48 (6.30)	-	-
HCl.H-Lys(Z)-pNA	-	foam	+39.1; 1; MeOH	0.74[c]/0.72[d]	54.01 (54.02)	5.72 (5.79)	11.96 (12.00)	-
H-Lys(Boc)-pNA	-	94-95	+30.1; 0.76; MeOH	0.73[c]/0.82[d]	55.92 (55.73)	7.15 (7.15)	15.15 (15.29)	-
HCl.H-Orn(Boc)-pNA	-	lyophil	+33.5; 0.37; MeOH	0.74[c]/0.80[d]	51.56 (51.71)	6.70 (6.89)	13.57 (13.79)	-

[a]Trt-Ala-pNA could not be obtained in a pure form; [b]Aloc-Val-pNA was synthesized from Aloc-Val-OH.DCHA; [c]BuOH/AcOH/H_2O 4:1:1 v/v/v; [d]BuOH/pyridine/H_2O 1:1:1 v/v/v; [e]CH_2Cl_2; [f]CH_2Cl_2/MeOH 98:2 v/v; [g]CH_2Cl_2/MeOH 95:5 v/v; [*]EA calculated for Z-Arg-pNA.HCL.0.5AcOH.0.5BuOH; [**]EA calculated for 2HCl.H-Arg-pNA.2H_2O.

of the *p*-nitroanilide bond in Boc-Ala-pNA with the following reagents: 10, 25 and 50% piperidine in DMF and 25% pyrrolidine in DMF. We found the *p*-nitroanilide bond to be stabile under these conditions; even on prolonged treatment (24 hrs) with 50% piperidine in DMF, no *p*-nitroaniline was set free and the optical rotation (-58.6°) was unaffected. Since the Fmoc-group is removed within 10 min in 25% piperidine in DMF, the *p*-nitroanilide bond cannot be expected to undergo damage during this reaction.

These observations motivated the synthesis of Fmoc-protected amino acid *p*-nitroanilides with acid-labile side chain protections, (Table 2). The choice of Fmoc-amino acids has been made such as to cover all types of side chains (acidic, basic, neutral and polar).

In some instances it is desirable to have at one's disposal a third orthogonality in protective groups. For this purpose, groups of the allyl-family[24] will be very useful. We found *p*-nitroanilides to resist the allyl-deprotection conditions (catalytic allyl-transfer to morpholine in THF). The usefulness of the trityl function as α-amino protective group has also been investigated in these syntheses. We have attempted the preparation of Trt-Ala-pNA, but the anilide was hard to obtain in pure form. Apart from this, the yield was low and the laborious work-up prevented the application of the α-trityl amino acids routinely. It is noteworthy that phosphorus oxychloride activates this carboxyl group also sufficiently to allow conversion into a nitroanilide in spite of the low nucleophilicity of the amine (*p*-nitroaniline) and the deactivating electron release of the trityl group into the carboxyl function. Table 3 summarizes properties of micellaneous *p*-nitroanilides.

Among these, derivatives of arginine *p*-nitroanilide are of special intrest, since they constitute starting material for the synthesis of chromogenic substrates as required by serine proteases with a primary specificity pocket for a guanidinium residue. This category involves enzymes of the blood clotting cascade, which exhibit a rather strict dependency on the presence of arginyl residues for exertion of hydrolytic activity.

We wished therefore to develope a fertile synthesis of Boc-Arg-pNA.HCl and observed that Boc-Arg-OH.HCl can be activated as such with phosphorus oxychloride in pyridine and that the activated compound reacts with *p*-nitroaniline to give the *p*-nitroanilide in excellent yield; the same yield being found in larger scale experiments (up to 200 mmoles).

Table 4. NMR Spectra of some Arginine *p*-Nitroanilides.

derivative	solvent	signals
Boc-Arg-pNA.HCl	CD_3OD	δ = 1.45 (s, 9H, Boc); 1.72 (m, 4H, β-CH_2/γ-CH_2); 3.22 (m, 2H, δ-CH_2); 4.23 (m, 1H, α-CH); 7.84/7.86-8.20/8.22 (dd, 4H, arom pNA).
Z-Arg-pNA.HCl	CD_3OD	δ = 1.60 (m, 4H, β-CH_2/γ-CH_2); 3.20 (m, 2H, δ-CH_2); 4.25 (m, 1H, α-CH); 5.10 (s, 2H, CH_2-benzyl), 7.34 (m, 5H, arom benzyl); 7.82/7.84-8.19/8.21 (dd, 4H, arom pNA).
Fmoc-Arg-pNA.HCl	CD_3OD	δ = 1.64 (m,4H, β-CH_2/γ-CH_2); 3.20 (m, 2H, δ-CH_2); 4.22 (t, 1H, CH-Fmoc); 4.26 (m, 1H, α-CH-Arg); 4.41 (d, 2H, CH_2-Fmoc); 7.29/7.38 (m, 4H,arom Fmoc); 7.68/7.79 (m, 4H, arom Fmoc); 7.82/7.84-8.19/8.21 (dd, 4H, arom pNA).
Msc-Arg-pNA.HCl	CD_3OD	δ = 1.69/1.81 (m, 4H, β-CH_2-Arg/γ-CH_2-Arg); 3.07 (s, 3H, CH_3-Msc); 3.24 (m, 2H, δ-CH_2-Arg); 3.49 (m, 2H, S(O_2)-CH_2-Msc); 4.33 (m, 1H, α-CH-Arg); 4.49 (m, 2H, CH_2-O-C(O)-Msc); 7.86/7.88-8.18/8.20 (dd, 4H, arom pNA).
2HCl.H-Arg-pNA	CD_3OD	δ = 1.72 (m, 2H, γ-CH_2); 2.04 (m, 2H, β-CH_2); 3.24 (m, 2H, δ-CH_2); 4.24 (m, 1H, α-CH); 7.65/7.67-8.14/8.16 (dd, 4H, arom pNA).
2HCl.H-Arg-pNA	D_2O	^{13}C: δ = 24.4 (γ-C); 28.9 (β-C); 41.2 (δ-C); 54.5 (α-C); 121.4, 126.0 (arom pNA, C̲H); 143.6, 144.7 (arom pNA); 157.6 (~NH-C̲(NH)-NH_2.HCl); 169.2 (~C̲(O)-NH~).

The guanidinium group endows arginine *p*-nitroanilide and its N^{α}-acylated forms with water solubility. They nevertheless can be extracted from aqueous solutions with n-butanol and they can be purified by counter current distribution. The tables 4 and 5 summarize NMR-data and distribution coefficients of some arginine derivatives as hydrochlorides. Contact with drying agents like anhydrous sodium sulfate is not recommended, since this may lead to ion exchange and precipitation of less soluble sulfates[25]. The preferred removal of the N^{α}-Boc-group is by acidolysis with hydrochloric acid; the α-ammonium group in the ensuing dihydrochloride can selectively be deprotonated and acylated with all activated carboxylic acid derivatives known (if peptidyl groups have to be introduced, azides are used to prevent racemization).

With the obtained amino acid *p*-nitroanilides we have synthesized (see Figure 2 and 3) the thrombin substrate 2HCl.H-D-Phe-Pip-Arg-pNA[26] and the factor Xa substrate Bz-Ile-Glu(γPi)-Gly-Arg-pNA.HCl[27], both in good overall yield.

Table 5. Partition Coefficients of Arginine *p*-Nitroanilides in BuOH/AcOH/H_2O 4:1:5 v/v/v.

p-nitroanilide	K[a]	R_f[b]
Boc-Arg-pNA.HCl	2.01	0.67
Z-Arg-pNA.HCl	2.31	0.69
Fmoc-Arg-pNA.HCl	5.97	0.73
Msc-Arg-pNA.HCl	1.00	0.45
2HCl.H-Arg-pNA	0.46	0.38

[a]partition coefficient: $K = g = (r_{max} + 0.5)/(n - r_{max} + 0.5)$. r_{max}: fraction with highest concentration of product, n: number of transfers; [b]BuOH/AcOH/H_2O 4:1:1 v/v/v.

CONCLUSION

Phosphorus oxychloride in pyridine is a powerful reagent to synthesize orthogonally protected *p*-nitroanilides. After selective deprotection, the anilides can serve as synthons in the synthesis of nearly every chromogenic substrate.

EXPERIMENTAL PROCEDURES

General

^1H NMR spectra were recorded on Bruker AM 100 and AM 400 spectrometers. ^{13}C NMR- and heteronuclear multiple quantum coherence (HMQC) ^{13}C NMR spectra were recorded on a Varian Unity-Plus 400 spectrometer. As an internal standard the residual solvent peak was used. Chemical shifts are given in part per million (ppm). Optical rotations were measured on a Perkin Elmer 241 polarimeter in a 10 cm cuvette at room temperature. Melting points were determined with a Büchi melting point apparatus (Tottoli). Elemental analyses were performed on a Carlo Erba Strumentazione EA MOD 1106. TLC was performed on Merck Silicagel $60F_{254}$ plates, and column chromatography on Merck Kieselgel 60 70-230 Mesh ASTM. Spots were detected by UV-fluorescence quenching, ninhydrine (free amino functions), chlorine/TDM[28] (NH groups), Barton[29] (hydrazides), Sakaguchi[30] (arginine residues), Pauly[31] (histidine residues) and sulfuric acid (trityl groups). Pyridine was distilled over KOH pellets and stored on 4Å molsieves. *p*-Nitroaniline was purified by dissolution in hot EtOAc, treatment with active carbon, filtration and dilution with three volumes of toluene. The crystals formed on cooling were filtered off. Z-amino acids were synthesized with benzyloxycarbonylchloride (Z-Cl)[32]. Boc-amino acids were synthesized by the method of Schnabel[33]. Fmoc-amino acids were synthesized by

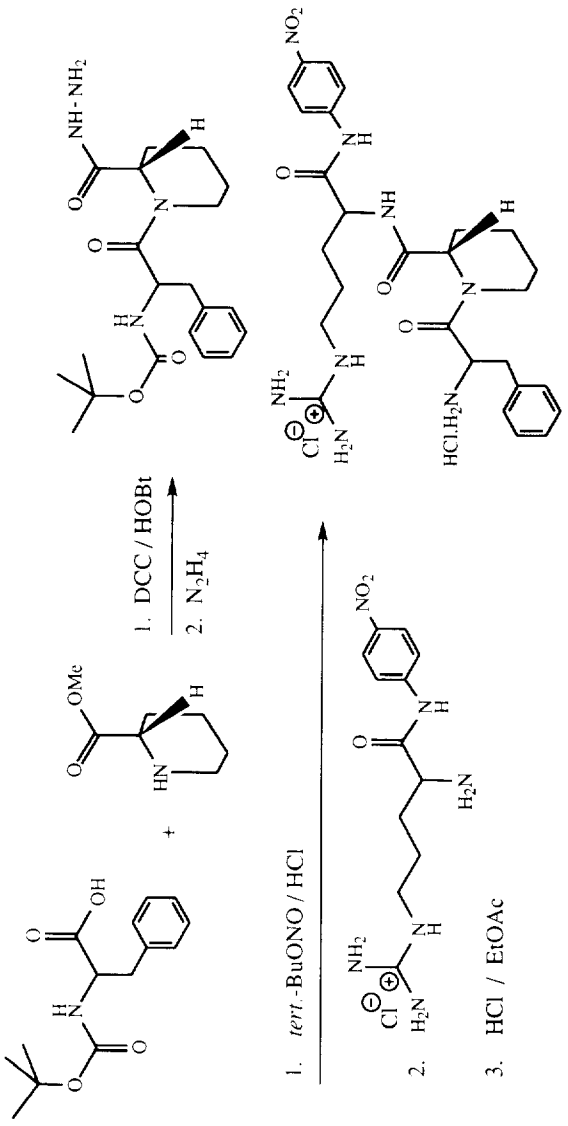

Figure 2. Synthesis of 2HCl.H-D-Phe-Pip-Arg-pNA, a thrombin-specific substrate.

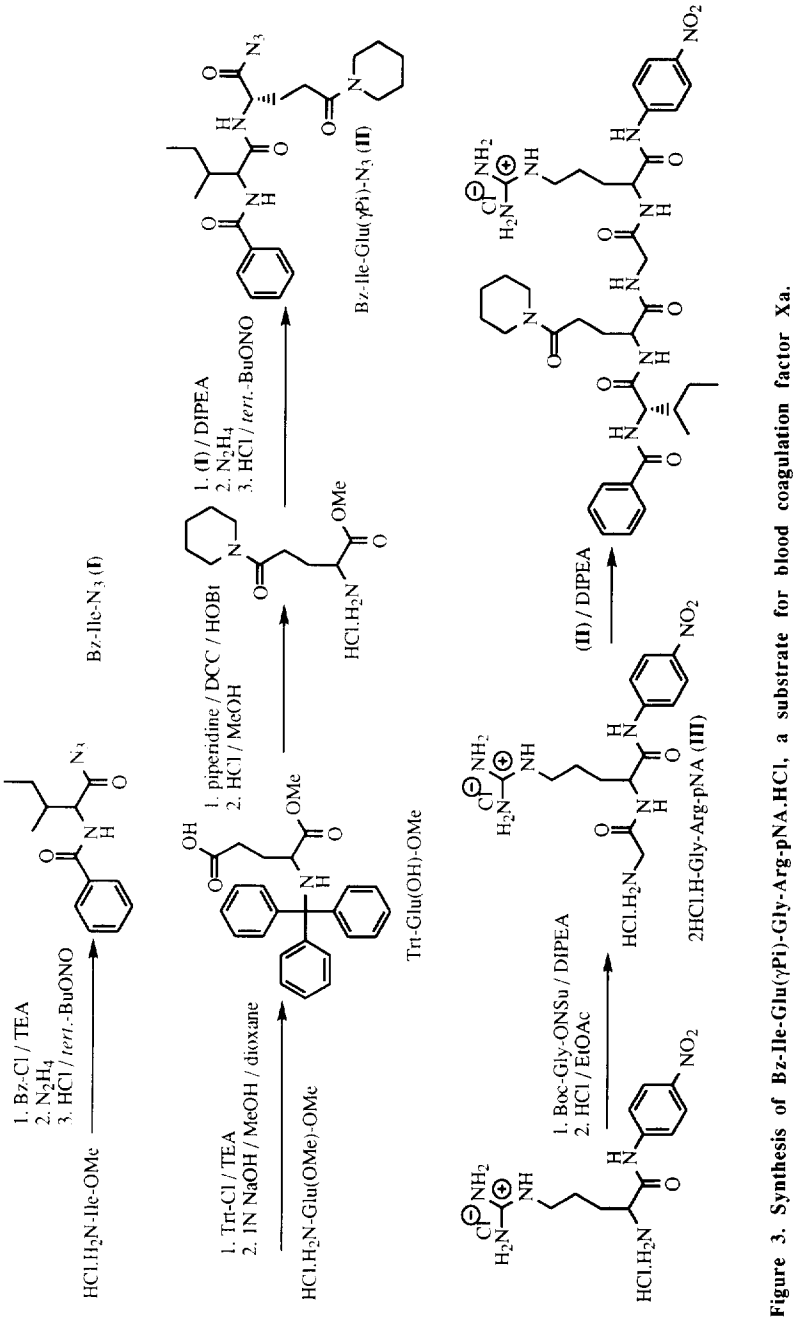

Figure 3. Synthesis of Bz-Ile-Glu(γPi)-Gly-Arg-pNA.HCl, a substrate for blood coagulation factor Xa.

acylation with 9-fluorenylmethyl succinimidyl carbonate (Fmoc-ONSu)[34]. Trityl-Ala-OH was synthesized by the method of Barlos[35]. 2-(Methylsulfonyl)ethyloxycarbonyl (Msc)-Arg-OH.HCl was synthesized with Msc-Cl[36]. The synthesis of allyloxycarbonyl (Aloc)-Val-OH was carried out with Aloc-ONSu (*cf* reference 34). Phosphorus oxychloride was from Merck (Darmstadt, Germany). DL-Pipecolic acid was from Janssen Chimica (Geel, Belgium), D(-)-tartaric acid was from Aldrich (Bornem, Belgium). D-Phenylalanine was a generous gift of Dr. J. Kamphuis (DSM Research, Geleen, The Netherlands). Boc-derivatives of Ile, Cys(Bzl), Glu(OBzl), His(Bzl) and Fmoc-derivatives of Met, Lys(Boc), Glu(O^{t}Bu), Ser(^{t}Bu), Tyr(^{t}Bu), Cys(Trt), His(Trt) and Asn(Trt) were from Bachem (Bubendorf, Switzerland). All other chemicals were of reagent grade.

Syntheses

General method

A protected amino acid (10 mmol) and *p*-nitroaniline (1.38 g, 10 mmol) were dissolved in dry pyridine (30 mL). The clear yellowish solution was cooled to -15°C and phosphorus oxychloride (1.00 mL, 11 mmol) was added dropwise with vigorous stirring. During the addition the reaction mixture coloured deeply red and became turbid in the course of 5 to 20 min. The colour of the suspension slowly changed to orange, the reaction being complete after a total of 10 to 30 min (monitored by TLC). The reaction mixture was then quenched with crushed ice/water (100 mL) and the nitroanilide was extracted into EtOAc (once 50 mL and three times 30 mL). The combined EtOAc layers were washed with saturated $NaHCO_3$ and NaCl (three times 30 mL each). After drying on Na_2SO_4 (except the arginine derivatives!), the EtOAc layer was filtered and evaporated *in vacuo*. The residue was coevaporated successively with toluene, EtOAc and MeOH.

The *p*-nitroanilides of: Boc-Gly-OH, Boc-Phe-OH, Boc-Ala-OH, Boc-Tyr(Bzl)-OH, Boc-His(Bzl)-OH, Z-Phe-OH, Fmoc-Gly-OH, Fmoc-Phe-OH and Fmoc-Met-OH were purified by recrystallization from *iso*-propyl alcohol. The following *p*-nitroanilides were purified by column chromatography (eluens: CH_2Cl_2/MeOH mixtures): Boc-Ile-pNA, Boc-Val-pNA, Boc-Cys(Bzl)-pNA, Boc-Lys(Z)-pNA, Boc-Glu(OBzl)-pNA, Fmoc-Val-pNA, Fmoc-Lys(Boc)-pNA, Fmoc-Glu(O^{t}Bu)-pNA, Fmoc-Ser(^{t}Bu)-pNA, Fmoc-Tyr(^{t}Bu)-pNA, Fmoc-Cys(Trt)-pNA, Fmoc-His(Trt)-pNA, Fmoc-Asn(Trt)-pNA, Trt-Ala-pNA, and Aloc-Val-pNA. The Boc-, Z- and Fmoc-Arg-pNA.HCl were purified by counter current distribution with BuOH/AcOH/H_2O 4:1:5 v/v/v as solvent combination. The water solubility of Msc-Arg-pNA.HCl required a modified work-up procedure. After quenching, the reaction mixture was evaporated *in vacuo* and the oily residue was purified by counter current distribution with the above mentioned solvent combination.

The synthesis of a thrombin substrate: 2HCl.H-D-Phe-Pip-Arg-pNA (Figure 2)

The resolution of DL-pipecolic acid was carried out according to the method of Mende and Lukes *et al.*[37] The complex of D(-)tartaric acid/L(-)pipecolic acid was eluted over a Dowex 50WX2 strongly acidic (H^+-form) ion exchange resin. Pipecolic acid was removed from the resin by elution with 2M NH_4OH and subsequently lyophilized. Mp: 264°C (dec.), $[\alpha]_D$ = -27.1° c = 1 H_2O, R_f (BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.18.

L(-) Pipecolic acid was converted into its methyl ester hydrochloride by the method of Brenner and Huber[38]. Yield: 91%, mp: 164-165°C, $[\alpha]_D$ = -8.8° c =1 MeOH, R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.20.

Boc-D-Phe-Pip-OMe:

To a solution of Boc-D-Phe-OH (18.65 g, 70.31 mmol) in DMF (250 mL) was added: HCl.H-Pip-OMe (12.68 g, 70.64 mmol), HOBt (10.78 g, 70.38 mmol) and NMM (7.73 mL, 70.31 mmol). The reaction mixture was cooled on ice and DCC (15.25 g, 73.91 mmol, 1.05 eq.) was added, the obtained reaction mixture was stirred 1 hr at 0°C and 16 hrs at room temperature. DCU was filtered off and the filtrate was evaporated *in vacuo*. The residue was diluted with EtOAc (300 mL) and washed with 10% citric acid, saturated $NaHCO_3$ and saturated NaCl (three times 75 mL each). After drying on Na_2SO_4 and filtration, the EtOAc solution was evaporated *in vacuo*. The crude dipeptide methyl ester was obtained in 85% yield as an oil and directly used for further synthesis. R_f($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v): 0.88, ^{1}H NMR ($CDCl_3$): δ = 1.36 (m, 6H, CH_2-Pip (3 x 2H)); 1.42 (s, 9H, Boc); 2.99 (m, 2H, β-CH_2-Phe); 3.14 (m, 2H, N-CH_2-Pip); 3.60 (d, 1H, NH); 3.71 (s, 3H, OMe); 4.98 (m, 1H, α-CH-Pip); 5.25 (m, 1H, CH-Phe); 7.19-7.28 (m, 5H, arom Phe).

Boc-D-Phe-Pip-N_2H_3:
To a solution of Boc-D-Phe-Pip-OMe (16.18 g, 41.19 mmol) in MeOH (200 mL), $N_2H_4.H_2O$ (10 mL, 200 mmol, 4.85 eq.) was added and this reaction mixture was left for 3 days at room temperature. The white precipitate was filtered off, dried and recrystallized from *iso*-propyl alcohol. Yield: 10.5 g (65%), $R_f(CH_2Cl_2/MeOH$ 9:1 v/v): 0.55, $[\alpha]_D$ = -78.9° c = 0.54 DMF, mp: 209-212°C, ^1H NMR (CD_3OD): δ = 1.15-1.38 (m, 6H, CH_2-Pip (3 x 2H)); 1.44 (s, 9H, Boc); 2.89-3.04 (m, 3H, β-CH_2-Phe); 3.26 (m, 2H, N-CH_2-Pip); 4.58 (m, 1H, α-CH-Pip); 4.74 (m, 1H, α-CH-Phe); 7.20-7.33 (m, 5H, arom Phe).

Boc-D-Phe-Pip-Arg-pNA.HCl:
Boc-D-Phe-Pip-N_2H_3 (7.8 g, 20.51 mmol) was suspended in DMF (150 mL) and cooled to -30°C. The suspension was acidified with 2.6 M HCl in EtOAc (22 mL, 57.2 mmol, 2.79 eq.) and the reaction mixture became clear. Then *tert.*-BuONO (2.5 mL, 21.01 mmol, 1.02 eq.) was added and the reaction mixture was stirred for 20 min at -25°C. The azide-containing solution was neutralized by DIPEA (9 mL) and 2HCl.H-Arg-pNA (7.60 g, 20.76 mmol) followed with DIPEA (6 mL) were added. This reaction mixture was allowed to stand overnight at 4°C. After evaporation of DMF the residue was diluted with EtOAc/BuOH 1:1 v/v (250 mL) and subsequently washed with 10% citric acid, H_2O, saturated $NaHCO_3$ and saturated NaCl (4 times 100 mL each). The solvent was evaporated *in vacuo*; the oily residue was crystallized from diethyl ether and purified by counter current distribution with the solvent system: BuOH/AcOH/H_2O 4:1:5 v/v/v (K = 10.52).Yield: 12.61 g (89%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.75, $[\alpha]_D$ = -90.0° c = 1 MeOH, mp: 110°C (dec.), ^1H NMR (CD_3OD): δ = 1.33 (s, 9H, Boc); 1.41 (m, 6H, CH_2-Pip (3 x 2H)); 1.67 (m, 2H, γ-CH_2-Arg); 1.86/2.04 (dm, 2H, β-CH_2-Arg); 2.93-3.04 (bm, 4H, β-CH_2-Phe/N-CH_2-Pip); 3.21 (m, 2H, δ-CH_2-Arg); 4.54 (m, 1H, α-CH-Pip); 4.78 (m, 1H, α-CH-Arg); 4.96 (m, 1H, α-CH-Phe); 7.21-7.35 (bm, 5H, arom Phe); 7.90/7.92-8.20/8.23 (dd, 4H, arom pNA).

2HCl. H-D-Phe-Pip-Arg-pNA:
The protected tripeptide was suspended in 2.6 M HCl in EtOAc (200 mL). After stirring for 2 hours the suspension was filtered and washed with EtOAc and diethyl ether. 2HCl.H-D-Phe-Pip-Arg-pNA was purified by counter current distribution with the solvent system: BuOH/AcOH/H_2O 4:1:5 v/v/v (K = 0.92) and obtained as a pale yellow powder in 96% yield. R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.52, $[\alpha]_D$ = -128.7° c = 1 H_2O, mp: 174 °C (dec.), ^1H NMR (D_2O): δ = 1.18 (m, 2H, CH_2-Pip); 1.44 (m, 2H, γ-CH_2-Arg); 1.63 (m, 4H, CH_2-Pip (2 x 2H)); 1.88 (m, 2H, β-CH_2-Arg); 3.13 (m, 2H, β-CH_2-Phe); 3.19 (m, 2H, N-CH_2-Pip); 3.21 (m, 2H, δ-CH_2-Arg); 4.31 (m, 1H, α-CH-Pip); 4.40 (m, 1H, α-CH-Arg); 4.81 (m, 1H, α-CH-Phe); 7.26 (m, 2H, arom Phe); 7.38 (m, 3H, arom Phe); 7.63/7.65-8.18/8.20 (dd, 4H, arom pNA), ^{13}C NMR (D_2O): δ = 23.8, 25.5, 29.0 ($\underline{C}H_2$-Pip); 24.1 (γ-C-Arg); 27.4 (β-C-Arg); 37.9 (β-C-Phe); 41.4 (δ-C-Arg); 45.2 (N-$\underline{C}H_2$-Pip); 52.1 (α-C-Arg); 54.7 (α-C-Phe); 55.5 (α-C-Pip); 121.1, 125.9 (arom pNA, $\underline{C}$H); 129.0, 130.1, 130.5 (arom Phe, $\underline{C}$H); 134.3 (arom Phe); 144.2, 144.3 (arom pNA); 157.6 (~NH-$\underline{C}$(NH)-NH_2.HCl, Arg); 170.6, 173.2, 173.8 (~$\underline{C}$(O)-NH~).

The synthesis of a factor Xa substrate: Bz-Ile-Glu(γPi)-Gly-Arg-pNA.HCl, (Figure 3)
Bz-Ile-OMe:
H-Ile-OH was converted to the methyl ester hydrochloride by the method of Brenner and Huber[38] (HCl.H-Ile-OMe, Yield: quant., R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.65, $[\alpha]_D$ = +26.3° c = 1.30 H_2O, mp: 83°C, Lit[39]: $[\alpha]_D$ = +26.6° c = 2 H_2O, mp: 100.5-101°C). HCl.H-Ile-OMe (18.2 g, 100 mmol) was dissolved in dry pyridine (250 mL) and cooled on ice. To this solution, TEA (31.3 mL, 225 mmol, 2.25 eq.) and Bz-Cl (12.8 mL, 110 mmol, 1.10 eq.) were added. During the addition of TEA the solution became turbid. When the addition of Bz-Cl was complete, the cooling bath was removed and stirring continued for 1 hr. The formed precipitate was filtered off and pyridine was removed under reduced pressure, and by coevaporation with toluene (twice) and MeOH (once). The residue was dissolved in EtOAc (300 mL) and subsequently washed with 2N $KHSO_4$, H_2O, saturated $NaHCO_3$ and saturated NaCl (three times 75 mL each), dried on Na_2SO_4, filtered and evaporated *in vacuo*. The residue was recrystallized from pet. ether 60-80 to yield pale yellow crystals. Yield: 21.1 g (85%), $R_f(CH_2Cl_2/MeOH$ 95:5 v/v): 0.83 (only visible under UV-light), $[\alpha]_D$ = -2.9° c = 1.04 MeOH, mp: 89°C, ^1H NMR ($CDCl_3$): δ = 0.94/1.04 (d, 3H, γ-CH_3-Ile); 0.89/1.08 (t, 3H, δ-CH_3-Ile); 1.26-1.32 (bm, 2H, γ-CH_2-

Ile); 2.04 (m, 1H, β-CH-Ile); 3.76 (s, 3H, OCH_3); 4.81 (m, 1H, α-CH-Ile); 6.70 (m, 1H, NH); 7.48 (m, 3H, arom benzoyl); 7.78 (m, 2H, arom benzoyl).

*Bz-Ile-N_2H_3 (in Figure 3 the corresponding azide is designated with **I**):*
Bz-Ile-OMe (10.0 g, 40.2 mmol) was dissolved in MeOH (100 mL) and $N_2H_4.H_2O$ (10 mL, 200 mmol, 5 eq.) was added. This mixture was left for 3 days at room temperature. After evaporating *in vacuo* the residue was crystallized from di*iso*-propyl ether. Yield: 9.54 g, (95%), R_f(CH_2Cl_2/MeOH 95:5 v/v): 0.25, $[\alpha]_D$ = -16.8° c = 1.25 MeOH, mp: 195-197°C, ^{1}H NMR ($CDCl_3$): δ = 0.92 (m, 6H, γ-CH_3/δ-CH_3-Ile); 1.28 (m, 2H, γ-CH_2-Ile); 1.62 (m, 1H, β-CH-Ile); 4.43 (m, 1H, α-CH-Ile); 7.49 (m, 3H, arom benzoyl); 7.79 (m, 2H, arom benzoyl).

Trt-Glu(OMe)-OMe:
H-Glu(OH)-OH was converted into its dimethyl ester hydrochloride by the method of Brenner and Huber[38] (HCl.H-Glu(OMe)-OMe, Yield: quant., R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.44, $[\alpha]_D$ = +25.8° c = 1.83 MeOH). HCl.H-Glu(OMe)-OMe (21.2 g, 100 mmol) was dissolved in dry pyridine (450 mL) in the presence of TEA (31.3 mL, 225 mmol, 2.25 eq.) which resulted in a viscous turbid reaction mixture. Trt-Cl (34.8 g, 125 mmol, 1.25 eq.) was added portionwise with stirring for 2 hrs at room temperature. After this period the precipitate was filtered off and pyridine was removed under reduced pressure. The residue was coevaporated with toluene (twice) and MeOH (once). The oily residue was diluted with EtOAc (250 mL) and washed with H_2O, saturated $NaHCO_3$ and saturated NaCl (four times 50 mL each). After drying on Na_2SO_4 the solvent was evaporated *in vacuo* leaving a brown-yellow oil containing still some solvent. A sample (5 g) of the crude reaction product was purified by column chromatography on silica gel with CH_2Cl_2 as eluens. The pure product was a pale yellow oil which slowly crystallized. Yield: 2.85 g (64%), R_f(CH_2Cl_2): 0.39, $[\alpha]_D$ = +38.6° c = 1.66 MeOH, mp: 77°C, ^{1}H NMR ($CDCl_3$): δ = 2.12 (t, 2H, γ-CH_2); 2.36 (m, 2H, β-CH_2); 2.62/2.72 (d, 1H, NH (J = 10 Hz)); 3.14 (s, 3H, α-OCH_3); 3.36 (m, 1H, α-CH (J = 10 Hz)); 3.68 (s, 3H, γ-OCH_3); 7.15-7.54 (m, 15H, arom trityl).

Trt-Glu(OH)-OMe:
The hydrolysis of the γ-ester was carried out by a modification of the method described by Amiard *et al.*[40] Trt-Glu(OMe)-OMe (37.2 g, 89.2 mmol) was dissolved in MeOH (360 mL) and dioxane (60 mL). To the clear solution, 1N NaOH (110 mL, 110 mmol, 1.23 eq.) was added portionwise, causing a transient turbidity. When the total amount of base was added the turbidity remained. After 16 hrs of stirring at room temperature, the reaction mixture was neutralized with 1.5N HCl and poured into CH_2Cl_2 (500 mL). The organic phase was extracted with H_2O (twice 75 mL). The aqueous layers were pooled and acidified with 10% citric acid to pH = 4. The precipitating oil was extracted into EtOAc (three times 100 mL) and subsequently washed with H_2O and saturated NaCl (four times 75 mL each). The EtOAc layer was dried on Na_2SO_4, filtered and evaporated *in vacuo*. The residue was a slowly crystallizing oil. Yield: 28.5 g (78%), R_f(CH_2Cl_2/MeOH 95:5 v/v): 0.26 (one spot), $[\alpha]_D$ = +41.1° c = 1.14 MeOH, mp: 120-124°C (lit: $[\alpha]_D$ = +45° c = 2 MeOH, mp: 140-141°C), ^{1}H NMR ($CDCl_3$): δ = 2.04 (m, 2H, γ-CH_2); 2.46 (m, 2H, β-CH_2); 3.18 (s, 3H, α-OCH_3); 3.45 (t, 1H, α-CH (J = 6.7 Hz)); 7.19-7.53 (m, 15H, arom trityl).

Trt-Glu(γ-Pi)-OMe:
Trt-Glu(OH)-OMe (28.0 g, 69.5 mmol), HOBt (11.7 g, 76.4 mmol, 1.10 eq.) and piperidine (6.95 mL, 70.2 mmol, 1.01 eq.) were dissolved in EtOAc (400 mL). This mixture was cooled on ice and DCC (15.0 g, 72.8 mmol, 1.05 eq.) was added. After stirring of one hour at 0°C and 16 hrs at room temperature, DCU was filtered off. The EtOAc solution was subsequently washed with H_2O, 5% citric acid, H_2O, saturated $NaHCO_3$, H_2O and saturated NaCl (three times 75 mL each) and dried on Na_2SO_4. After evaporation *in vacuo* the obtained residue was purified by column chromatography on silica gel with CH_2Cl_2/MeOH 98:2 v/v as eluens. Yield: 27.4 g (84%), R_f(CH_2Cl_2/MeOH 98:2 v/v): 0.52, $[\alpha]_D$ = +56.5° c = 0.84 MeOH, ^{1}H NMR ($CDCl_3$): δ = 1.61 (m, 6H, CH_2-piperidine (3 x 2H)); 2.12 (m, 2H, CH_2-Glu); 2.32 (m, 2H, CH_2-Glu); 2.72 (m, 1H, NH); 3.15 (s, 3H, OCH_3); 3.38-3.57 (broad multiplet, 5H, α-CH-Glu(1H)/N-CH_2-piperidine (2 x 2H)); 7.18-7.46 (m, 15H, arom trityl).

HCl.H-Glu(γPi)-OMe:
Trt-Glu(γPi)-OMe (25.0 g, 53.2 mmol) was dissolved in MeOH (100 mL) and 3.5 M HCl in EtOAc (50 mL, 175 mmol, 3.3 eq.) was added. This reaction mixture was stirred for 40 min at room temperature and subsequently evaporated *in vacuo*. The residue was dissolved in H_2O (130 mL) and washed with diethyl ether (three times 40 mL). The aqueous solution was lyophilized which results in an oil. Yield: 13.1 g (93%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.46, R_f(CH_2Cl_2/MeOH 8:2 v/v): 0.52, $[\alpha]_D$ = +16.2° c = 3.23 MeOH, ^{1}H NMR (D_2O): δ = 1.25 (m, 6H, CH_2-piperidine (3 x 2H)); 1.87 (dd, 2H, β-CH_2-Glu (J = 6.57 Hz)); 2.29 (t, 2H, γ-CH_2-Glu); 3.11 (m, 4H, N-CH_2-piperidine (2 x 2H)); 3.47 (s, 3H, OCH_3); 3.81 (t, 1H, α-CH (J = 6.57 Hz)).

Bz-Ile-Glu(γPi)-OMe:
Bz-Ile-N_2H_3 (1.99 g, 8.0 mmol) was dissolved in DMF (80 mL). This solution was cooled to -20°C and 3.5 M HCl in EtOAc (6.3 mL, 22.1 mmol, 2.75 eq.) followed by *tert.*-BuONO (1.34 mL, 9.6 mmol, 1.2 eq.) were added. After 10 min the azide formation was complete, and the reaction mixture was neutralized by the addition of DIPEA (3.78 mL, 22 mmol). To this solution, HCl.H-Glu(γPi)-OMe (2.14 g, 8.09 mmol, 1.01 eq.) followed by DIPEA (1.38 mL, 8.0 mmol) were added. The obtained reaction mixture was allowed to react at 0°C at pH = 7 to 8 (DIPEA was added during several time intervals to adjust the pH value). After 16 hrs, DMF was removed under reduced pressure and the residue was dissolved in EtOAc (100 mL). This solution was subsequently washed with H_2O, 2N $KHSO_4$, H_2O, saturated $NaHCO_3$, H_2O and saturated NaCl (three times 30 mL each). The EtOAc solution was dried on Na_2SO_4, filtered and evaporated *in vacuo*. The residue was recrystallized from di*iso*-propyl ether. Yield: 2.70 g (76%), R_f(CH_2Cl_2/MeOH 95:5 v/v): 0.25, R_f(CH_2Cl_2/MeOH 92:8 v/v): 0.40, mp: 78-82°C, $[\alpha]_D$ = -21.9° c = 1.25 MeOH, ^{1}H NMR (CD_3OD): δ = 0.53 (t, 3H, δ-CH_3-Ile); 0.65 (d, 3H, γ-CH_3-Ile); 0.88 (m, 1H, γ-CH_2-Ile); 1.04/1.14 (dm, 6H, CH_2-piperidyl (3 x 2H)); 1.23 (m, 1H, γ-CH_2-Ile); 1.59 (m, 2H, β-CH-Ile/β-CH_2-Glu); 1.83 (m, 1H, β-CH_2-Glu); 2.06 (m, 2H, γ-CH_2-Glu); 2.94/3.07 (dm, 4H, N-CH_2-piperidyl); 3.32 (s, 3H, OCH_3); 4.03 (d, 1H, α-CH-Ile); 4.12 (dd, 1H, α-CH-Glu); 7.06 (m, 2H, arom benzoyl); 7.13 (m, 1H, arom benzoyl); 7.46 (m, 2H, arom benzoyl).

*Bz-Ile-Glu(γPi)-N_2H_3 (in Figure 3 the corresponding azide is designated with **II**):*
Bz-Ile-Glu(γPi)-OMe (1.50 g, 3.37 mmol) was dissolved in MeOH (40 mL) and N_2H_4.H_2O (0.84 mL, 16.9 mmol, 5.0 eq.) was added; the obtained reaction mixture stand for 3 days at room temperature. After this period, the reaction mixture was evaporated to dryness and the residue was recrystallized from di*iso*-propyl ether. Yield: 1.23 g (82%), R_f(CH_2Cl_2/MeOH 92:8 v/v): 0.23, mp: 171°C, $[\alpha]_D$ = -24.1° c = 0.71 MeOH, ^{1}H NMR (CD_3OD): δ = 0.64 (t, 3H, δ-CH_3-Ile); 0.70 (d, 3H, γ-CH_3-Ile); 0.99 (m, 1H, γ-CH_2-Ile); 1.17/1.29 (dm, 6H, CH_2-piperidyl (3 x 2H)); 1.36 (m, 1H, γ-CH_2-Ile); 1.69 (m, 2H, β-CH-Ile/β-CH_2-Glu); 1.78 (m, 1H, β-CH_2-Glu); 2.16 (m, 2H, γ-CH_2-Glu); 3.06/3.18 (dm, 4H, N-CH_2-piperidyl); 4.13 (m, 2H, α-CH-Ile/α-CH-Glu); 7.18 (m, 2H, arom benzoyl); 7.26 (m, 1H, arom benzoyl); 7.61 (m, 2H, arom benzoyl).

Boc-Gly-Arg-pNA.HCl:
Boc-Gly-ONSu[41] (8.30 g, 30.2 mmol) and 2HCl.H-Arg-pNA (11.2 g, 30.5 mmol, 1.01 eq.) were dissolved in DMF (250 mL), the reaction was started by the addition of DIPEA (11.0 mL, 61.4 mmol, 2.03 eq.). After 2.5 hrs of stirring at room temperature, the solvent was removed under reduced pressure. The residue was diluted with BuOH (200 mL) and subsequently washed with H_2O, saturated $NaHCO_3$, H_2O and saturated NaCl (three times 50 mL each). After these washings the BuOH phase was evaporated *in vacuo* and the residue was crystallized in diethyl ether. Yield: 11.1 g (75%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.71, second spot: R_f: 0.52 (HONSu). This crude material was used in the deprotection of the N^{α}-Boc-function.

*2HCl.H-Gly-Arg-pNA (designated in Figure 3 as compound **III**):*
Boc-Gly-Arg-pNA.HCl (11.1 g, 22.8 mmol) was dissolved in AcOH (100 mL) and 3.5 M HCl in EtOAc (100 mL) was added. The obtained reaction mixture was stirred for 2 hrs at room temperature. After acid quenching with *tert.*-BuOH (50 mL), the solvent was removed *in vacuo*. The residue was coevaporated with *tert.*-BuOH (once 50 mL) and MeOH (twice 50 mL) and was subsequently dissolved in H_2O (350 mL). The aqueous solution was washed with CH_2Cl_2 (three times 50 mL) and diethyl ether (50 mL) and lyophilized. This product

was purified by counter current distribution with BuOH/AcOH/H_2O 4:1:5 v/v/v as solvent system (K = 0.62). Yield: 8.19 g (84%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.20 (single spot), $[\alpha]_D$ = -16.7° c = 0.71 MeOH, ^{1}H NMR (D_2O): δ = 1.58 (m, 4H, γ-CH_2/β-CH_2-Arg); 2.92 (m, 2H, δ-CH_2-Arg); 3.62 (s, 2H, CH_2-Gly); 4.21 (m, 1H, α-CH-Arg); 7.27/7.37-7.80/7.89 (dd, 4H, arom pNA).

Bz-Ile-Glu(γPi)-Gly-Arg-pNA.HCl:

Bz-Ile-Glu(γPi)-N_2H_3 (1.0 g, 2.25 mmol) was dissolved in DMF (25 mL) and cooled to -20°C. At this temperature 3.5 M HCl in EtOAc (1.78 mL, 6.23 mmol, 2.77 eq.) followed by *tert.*-BuONO (0.32 mL, 2.70 mmol, 1.20 eq.) were added. After 10 min azide formation was complete and the reaction mixture was neutralized by the addition of DIPEA (1.06 mL). To this neutral solution, 2HCl.H-Gly-Arg-pNA (0.954 g, 2.25 mmol, 1.0 eq.) and DIPEA (0.39 mL, 2.23 mmol, 1.0 eq.) were added and the obtained reaction mixture was allowed to react at 0°C for 16 hrs. When coupling was complete, the reaction mixture was evaporated *in vacuo* and the residue was purified by counter current distribution with the solvent system BuOH/AcOH/H_2O 4:1:5 v/v/v (K = 4.18). Yield: 1.69 g (94%). R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.54, $[\alpha]_D$ = -31.7° c = 0.56 MeOH, ^{1}H NMR (CD_3OD): δ = 0.93 (t, 3H, δ-CH_3-Ile); 0.99 (d, 3H, γ-CH_3-Ile); 1.28/1.71 (dm, 2H, γ-CH_2-Ile); 1.45/1.56 (dm, 6H, CH_2-piperidyl (3 x 2H)); 1.63 (m, 3H, β-CH-Ile(1H)/γ-CH_2-Arg(2H)); 1.87/2.15 (dm, 2H, β-CH_2-Arg); 2.03 (m, 2H, β-CH_2-Glu); 2.52 (m, 2H, γ-CH_2-Glu); 3.21 (m, 2H, δ-CH_2-Arg); 3.36/3.43 (m, 4H, N-CH_2-piperidyl (2 x 2H)); 3.94 (s, 2H, CH_2-Gly); 4.35 (m, 2H, α-CH-Ile(1H)/α-CH-Glu(1H)); 4.55 (m, 1H, α-CH-Arg); 7.43-7.53 (m, 3H, arom benzoyl); 7.84-7.89 (m, 4H, arom benzoyl(2H)/arom pNA(2H)); 8.16/8.19 (d, 2H, arom pNA), ^{13}C NMR (CD_3OD): δ = 11.3 (δ-C-Ile); 16.1 (γ-$\underline{C}H_3$-Ile); 23.1 (γ-C-Arg); 25.4, 26.3, 27.4, 27.9, 30.2 (β-C-Arg, γ-$\underline{C}H_2$-Ile, β-C-Glu, $\underline{C}H_2$-Pip); 37.5 (γ-C-Glu); 41.9 ($\underline{C}H_2$-Gly); 43.9, 44.0 (β-C-Ile, δ-C-Arg); 47.7 (N-$\underline{C}H_2$-Pip); 49.9 (α-C-Glu); 55.0 (α-C-Arg); 60.5 (α-C-Ile); 120.7, 125.7 (arom pNA, $\underline{C}H$); 128.7, 129.5, 132.9 (arom benzoyl, $\underline{C}H$); 135.2 (arom benzoyl); 144.8, 145.7 (arom pNA); 158.7 (~NH-$\underline{C}$(NH)-NH_2.HCl, Arg); 170.8, 171.8, 172.4, 172.5, 174.4, 174.6 (~$\underline{C}$(O)-NH~).

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