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Polymer-assisted solution-phase parallel synthesis of dipeptide *p*-nitroanilides and dipeptide diphenyl phosphonates

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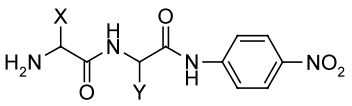
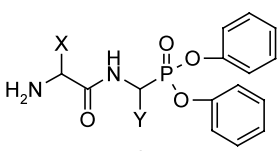
Abstract—This letter describes the parallel synthesis of dipeptide *p*-nitroanilides (**1**) and dipeptide diphenyl phosphonates (**2**), compounds that can be used as substrates and irreversible inhibitors for the rapid profiling of dipeptidyl peptidases. A polymer-assisted solution-phase synthesis was used for a rapid and clean coupling between easily available building blocks.

1. Introduction

Proteases constitute one of the largest superfamilies of enzymes, some of which are among today's most promising drug targets. We are particularly interested in dipeptidyl peptidases from the serine protease family. A recent and exciting development in this family is the design and synthesis of dipeptidyl peptidase IV (DPP IV) inhibitors with potential use in type II diabetes.[1] A first step in understanding the biological function of a particular protease is knowledge of the substrate specificity. This can be investigated by the use of chromogenic or fluorogenic substrates. A well known example of a chromogenic substrate for dipeptidyl peptidases are dipeptide *p*-nitroanilides (**1**, Xaa-Yaa-*p*-NA). This substrate specificity is then often used to design peptide-based inhibitors, e.g. dipeptide diphenyl phosphonates (**2**, Xaa-Yaa^{P}(OPh)$_2$) as irreversible inhibitors of DPP IV.[2]



1



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There is no doubt that a rapid parallel synthesis of both substrates and inhibitors of dipeptidyl peptidases would greatly accelerate medicinal chemistry programs in this field. A few methods for the synthesis of chromogenic or fluorogenic substrates on solid phase are reported. A first approach is the linkage of a *p*-nitroaniline analogue to the solid support, followed by Fmoc/*t*Bu peptide synthesis and cleavage from the resin. The use of *p*-diaminobenzene as linker[3] requires oxidation of the linked amino to a nitro function after cleavage. It is also possible to link an amino-nitrobenzoic acid[4] through the carboxylic acid resulting in a *p*-nitroanilide analogue after cleavage. Both methods suffer from difficult couplings of the first amino acid because of the low nucleophilicity of the aniline. In a second approach the linkage to the resin is done with a part of the peptide. This can be effected through the side chain of an amino acid,[5] limiting of course its use to peptides containing at least one amino acid with a functionalised side chain. A more general linkage is through the backbone nitrogen (BAL)[6] of the penultimate residue, with the carboxylic acid protected as an allyl ester. After Fmoc/*t*Bu peptide synthesis and removal of the allyl ester, the free carboxylic acid can be coupled to an amino acid *p*-nitroanilide. An inversion of the peptide on the resin[7] also affords a free C-terminal carboxylic acid ready for modification to a *p*-nitroanilide. A last approach uses a safety-catch linker that after completion of the peptide synthesis can be cleaved with, e.g. an amino acyl aminomethylcoumarin.[8] To the best of our knowledge a parallel synthetic method for peptide diphenyl phosphonates is not reported.

For our purpose, the above-mentioned solid-phase methods are too lengthy and complex compared to the two-step procedure in solution: i.e. coupling between a protected amino acid and either an amino acid *p*-nitroanilide or an amino acid diphenyl phosphonate,

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followed by a deprotection step. Therefore, we turned our attention to polymer-assisted solution-phase synthesis.[9] This method has several advantages over solid-phase synthesis: the absence of a linker to the solid support removes the necessity of an additional attachment and cleavage step; conventional analytical tools for assessing the reaction can be used; intermediate purifications in a multi-step synthesis are possible; well known reaction conditions can be used and the synthesis scale is unlimited.

2. Results and discussion

As Xaa we chose commercially available amino acids (**3**, Fig. 1) with *tert*-butyloxycarbonyl (Boc) as α-amino protection and acid-labile side-chain protecting groups as mentioned in Tables 1 and 2. These amino acids were reacted with hydroxybenzotriazole (HOBt) and a polymer-bound carbodiimide to afford the activated esters **4**. As Yaa we chose Pro and Ala because DPP II and DPP IV prefer these amino acids at the penultimate position, so that the obtained compounds will give valuable information for our design of inhibitors for the mentioned proteases. Pro-*p*-NA and Ala-*p*-NA (**5**) are commercially available. $Pro^{P}(OPh)_2$ (**6**) was synthesised from the 1-pyrroline trimer.[10] $Ala^{P}(OPh)_2$ (**6**) could be obtained using an amidoalkylation reaction[11] from benzylcarbamate, acetaldehyde and triphenyl phosphite. To each reaction vessel containing **4** a limiting amount of the amine (**5** or **6**) was added. After reaction overnight, addition of a polymer-bound polyamine captured the excess of **4** and HOBt. Filtration and evaporation gave **7** or **8** with purities mentioned in Table 1 or 2, respectively.

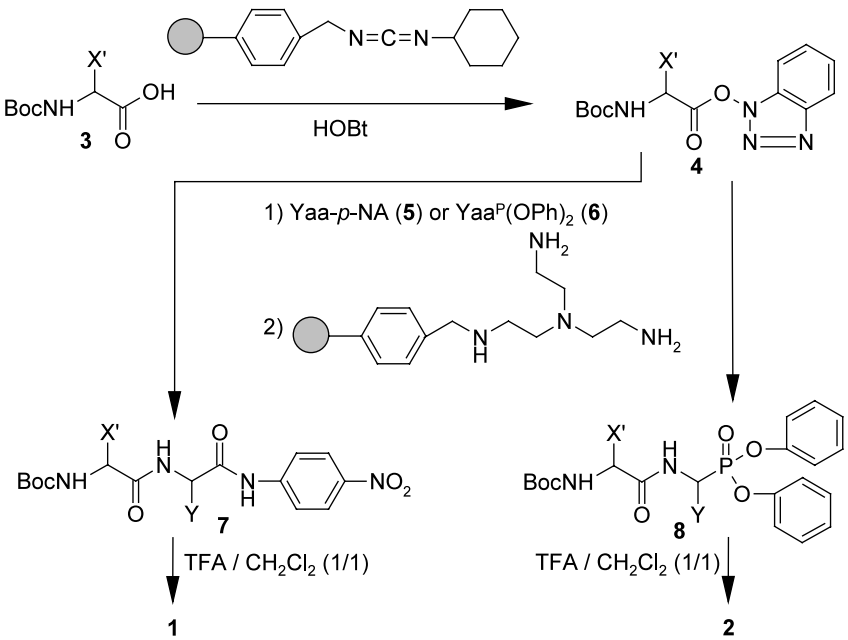

Figure 1.

Table 1. Synthesised dipeptide *p*-nitroanilides

	Pro-*p*-NA			Ala-*p*-NA		
Xaa[a]	Purity[b] (**7**) (%)	Purity[c] (**1**) (%)	Yield[d] (**1**) (%)	Purity[b] (**7**) (%)	Purity[c] (**1**) (%)	Yield[d] (**1**) (%)
Asn	99	94	98			
Asp	90	95[e]	87[e]			
Gly				97	97	86
His	40	91[e]	42[e]	97	96	49
Ile	88	98[e]	10[e]			
Phe	97	99[e]	16[e]	99	95	95
Pro	96	99	47	98	97	94
Ser	84	90	84			
Tyr	91	95	18	98	94	75
Val	88	92[e]	30[e]			

[a] Side chain protection for the synthesis of **7** is as follows: Asn(Trt), Asp(O*t*Bu), His(Boc), Ser(*t*Bu), Tyr(*t*Bu).
[b] The HPLC purity of **7** at 214 nm.
[c] The HPLC purity of **1** at 214 nm after a possible[e] purification by HPLC.
[d] This is the total yield of both coupling and deprotection after a possible[e] purification by HPLC.
[e] Those compounds were purified by preparative reverse-phase HPLC after deprotection.

Table 2. Synthesised dipeptide diphenyl phosphonates

	$Pro^P(OPh)_2$			$Ala^P(OPh)_2$		
Xaa[a]	Purity[b] (**8**) (%)	Purity[c] (**2**) (%)	Yield[d] (**2**) (%)	Purity[b] (**8**) (%)	Purity[c] (**2**) (%)	Yield[d] (**2**) (%)
Ala	58[e]	96	36	76[e]	91	51
Asn	87[e]	99	59	80	77	23
Asp	66[e]	98	48	53[e]	91	32
Gly	64[e]	94	61	78	94	60
His	31[e]	100[f]	7[f]	30[e]	100[f]	27[f]
Ile				85	97	21
Lys	66[e]	100[f]	51[f]			
Phe	83[e]	95	58	89	97	13
Pro				90	95	55
Ser	68[e]	84	60	56[e]	83	18
ThiaPro	75[e]	95	41			
Tyr				90	95	65
Val	69[e]	90	41	94	95	13

[a] Side chain protection for the synthesis of **8** is as follows: Asn(Trt), Asp(O*t*Bu), His(Boc), Lys (Boc), Ser(*t*Bu), Tyr(*t*Bu).
[b] The HPLC purity of **8** at 214 nm before a possible purification[e] by preparative TLC.
[c] The HPLC purity of **2** at 214 nm after a possible[f] purification by HPLC.
[d] This is the total yield of both coupling and deprotection after a possible[f] purification by HPLC.
[e] Those compounds were purified by preparative TLC before deprotection.
[f] Those compounds were purified by preparative reverse-phase HPLC after deprotection.

The *p*-nitroanilides **7** were generally more than 95% pure and could be deprotected without further purification. The diphenyl phosphonates **8** were somewhat less pure, probably because of a lower stability, and some needed purification using preparative TLC before proceeding to deprotection. Deprotection with 50% trifluoroacetic acid (TFA) in dichloromethane afforded the target compounds **1** and **2**. Only 8 of the 35 synthesised compounds needed preparative HPLC purification in order to assure the more than 90–95% purity needed for biological evaluation. Generally it was noted that intermediate purification of the protected dipeptides prevented purification of the final compounds.

Concludingly we can state that the dipeptide *p*-nitroanilides **1** and the dipeptide diphenyl phosphonates **2** could be easily and rapidly obtained using polymer-assisted solution-phase synthesis, starting from products that are either commercially available or easily accessible.

3. General synthetic procedure

Parallel synthesis was performed using the Quest 210 Organic Synthesizer (Argonaut Technologies). Boc-protected amino acids, *N*-cyclohexylcarbodiimide,*N*′-methylpolystyrene resin (PS-Carbodiimide) and tris-(2-aminoethyl)-amine polystyrene resin were purchased from Novabiochem. The amino acid *p*-nitroanilides were from Bachem.

Protected amino acid (**3**) (0.375 mmol), HOBt (0.425 mmol) and PS-Carbodiimide (0.75 mmol) were added to a dry reaction vessel. Dichloromethane (4 ml) was added and the mixture was stirred for 10 min prior to the addition of the amine compound (**5** or **6**) (0.25 mmol), dissolved in 0.8 ml of dichloromethane. Diphenyl 1-aminoethane phosphonate hydrobromide, alanine *p*-nitroanilide hydrochloride and proline *p*-nitroanilide hydrobromide were prior to their use converted into their free base form by basic extraction. Diphenyl pyrrolidine-2-phosphonate hydrochloride was used as such: coupling was mediated by adding an equivalent amount of triethylamine to the reaction mixture. After stirring at room temperature overnight the polymer-bound polyamine (1.5 mmol) was added and stirring was continued for 5 h. The reaction mixture was filtered and the amide product (**7** or **8**) was collected in the filtrate. The resins are washed two times with 4 ml of dichloromethane. The combined fractions were evaporated under reduced pressure. The purity of the compounds was checked by TLC and reverse phase HPLC. Some compounds were purified by preparative TLC as indicated in Tables 1 and 2. The protected compounds were dissolved in 4 ml of a TFA/dichloromethane (1:1) mixture. The solution was stirred for 3 h and the volatile part was removed under reduced pressure. The series of the diphenyl alanine phosphonates and the *p*-nitroanilides were treated with dry diethyl ether, the precipitated products were washed with ether and lyophilised from *tert*-butanol/water (4:1). The series of the diphenyl proline phosphonates were lyophilised immediately after evaporation of the volatile part.

Characterisation of all compounds was done with ^{1}H NMR, mass spectrometry and analytical reverse-phase HPLC.

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