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Ser-His catalyses the formation of peptides and PNAs

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ABSTRACT

The dipeptide seryl-histidine (Ser-His) catalyses the condensation of esters of amino acids, peptide fragments, and peptide nucleic acid (PNA) building blocks, bringing to the formation of peptide bonds. Di-, tri- or tetra-peptides can be formed with yields that vary from 0.5% to 60% depending on the nature of the substrate and on the conditions. Other simpler peptides as Gly-Gly, or Gly-Gly-Gly are also effective, although less efficiently. We discuss the results from the viewpoint of primitive chemistry and the origin of long macromolecules by stepwise fragment condensations.

1. Introduction

One of the fundamental and largely unsolved questions in the origin of life is the biogenesis of long and specific co-oligopeptide sequences, the gate to enzymes and/or transport proteins. The *N*-carboxy anhydride (NCA) amino acid condensation brings to 10–20 residues long homo- and co-oligopeptides [1–4], but the achievement of longer sequences remains elusive. A possible effective prebiotic route to long polypeptides resides in the condensation of peptide fragments, catalyzed by very simple peptides. In addition to several reports focusing on the catalytic activity of simple peptides [5–10], it was recently shown that seryl-histidine (Ser-His) acts as protease and phosphoesterase [11]. On the basis of microscopic reversibility principle, a protease – e.g., α-chymotrypsin, or Ser-His – can also catalyze the reverse reaction, i.e. the formation of peptide bonds, which is well documented in the literature [12,13] (a similar use of transpeptidases has also been similarly reported [14]).

The availability of the Ser-His might be arguable in an origin of life context. Whereas serine is considered a prebiotic amino acid [15], histidine is not. There are reports, however, on the prebiotic synthesis of histidine [16], so that the formation of Ser-His might be seen as plausible.

We describe how Ser-His and other simple peptides can effectively catalyze the synthesis of peptide bonds with a yield comparable to that of α-chymotrypsin, although in a more sluggish time-scale. We argue here that the repeated fragment condensation may in principle represent a simple gate for the biogenesis of polypeptides. We have also extended the use of Ser-His to the synthesis of peptide nucleic acid (PNA) oligomers. PNAs are nucleic acid analogues, having a polypeptide backbone and purine/pirimidine bases as side chains, being capable of Watson–Crick base pairing with natural nucleic acids [17,18].

2. Materials and methods

2.1. Peptide synthesis

The ethyl or methyl esters of amino acids and peptides (50 mM) were added as DMF solution to the nucleophilic amino acid derivatives (e.g. H-Leu-NH_2 or similar; 50 mM), dissolved in aqueous buffer (generally, 100 mM sodium borate pH 10), so that the final DMF content in the aqueous solution was 6% (v/v). After addition of the catalyst (e.g. Ser-His, or α-chymotrypsin, etc.), and proper incubation time, the reaction was stopped by adding 10% v/v HCl

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1 N. The precipitate was collected by centrifugation (13 000 rpm, 15 min), dissolved in a measured amount of DMF and analysed by HPLC. The supernatant was also analysed by HPLC. The coupling product and the unreacted acyl donor are generally recovered in the precipitate. In contrary, the hydrolized substrate is found in the supernatant. Yields were calculated from HPLC peak areas; each compound was identified by mass spectrometry.

2.2. Synthesis and oligomerization of PNA building blocks

PNA thymine monomer was synthesized according to published procedure, and condensation was carried out as essentially described in the above.

Details on materials and methods are given in the Supplementary material.

3. Results and discussion

As shown in Chart S1 (in the Supplementary material), our approach is based on the Ser-His catalyzed aminolysis of ethyl esters. In particular we show first the formation of peptide bonds by reacting the ethyl ester of *N*-acetyl-phenylalanine (Ac-Phe-OEt), acting as acyl donor, with leucinamide (H-Leu-NH_2) [13]. The formation of the product (Ac-Phe-Leu-NH_2 is followed by reverse phase HPLC and mass spectrometry. This product, being sparingly soluble in water, forms quickly (within 1 minute), and in high yields (>60%), when the catalyst is α-chymotrypsin. The substrate Ac-Phe-OEt is also hydrolyzed to give about 35% Ac-Phe-OH (water soluble). In the following, we will refer to these two competitive reactions as coupling and hydrolysis.

When Ser-His (4 mM) is used instead of α-chymotrypsin (40 μM), we observe the same reactive pattern. After one-day incubation at room temperature, Ser-His brought to the formation of the desired coupling product in 10% yield, which is only six times lower that when α-chymotrypsin is used. Control experiments, without catalyst, or in the presence of imidazole, His, Ser, His plus Ser, and the isomer His-Ser, do not give any reaction in the allotted time-scale (data not shown). Only in particular conditions (1 month, 25 °C) we measured a certain amount of uncatalyzed coupling, which corresponds to about 20% of the Ser-His catalyzed coupling. It is remarkable that His-Ser is not a catalyst for ester aminolysis; on the other hand, Li et al. [11] have already reported the lack of His-Ser hydrolytic activity when compared to Ser-His. The terminal serine amino group and/or the different stereo-electronic arrangement around imidazole ring may possibly explain this observation.

Fig. 1a shows the pH dependence of products for the reaction between Ac-Phe-OEt and H-Leu-NH_2. The formation of Ac-Phe-Leu-NH_2 is significant only at pH > 7, and reaches a plateau after pH 9. On the contrary, the hydrolysis of the substrate constantly increases in basic conditions. If Ser-His concentration is increased from 4 to 16 mM, the yield of coupling product increases by a factor 2.4 (Fig. 1b). Above 16 mM, the yield decreases and hydrolysis becomes favored. Fig. 1c illustrates the time course of Ser-His catalysed reaction between Ac-Phe-OEt and H-Leu-NH_2. The amount of coupling product constantly increases in time, reaching an asymptotic value of about 60% in 30 days. Interestingly, the amount of hydrolyzed substrate does not exceed 40%, and actually decreases after 7 days. The fact that Ser-His catalyzed coupling, although slowly, reaches high conversion yields is particularly important in a primitive fragment condensation scenario (see below). Finally, lower temperature favored the synthesis of the peptide (Fig. 1d), probably due to lower solubility of the product. By contrast, substrate hydrolysis is abundant at high temperature. Longer peptides can be also obtained, as shown in Table 1 (entries 1–6); di-, tri-, and tetra-peptides are formed in yields that depend on the nature of the substrates (0.5–31.5%).

In some cases (entries 4 and 5) the low solubility of acyl donor substrates did not allow the reaction to proceed effectively; but the solubility of the product can also affect the coupling yield (e.g., as in entry 6). When the ethyl ester and the nucleophilic amino groups are present in the same molecule, the substrate can oligomerize, as in the case of thymine PNA building blocks. In fact, Ser-His readily oligomerizes the peptide precursor 1 (Fig. S1) to

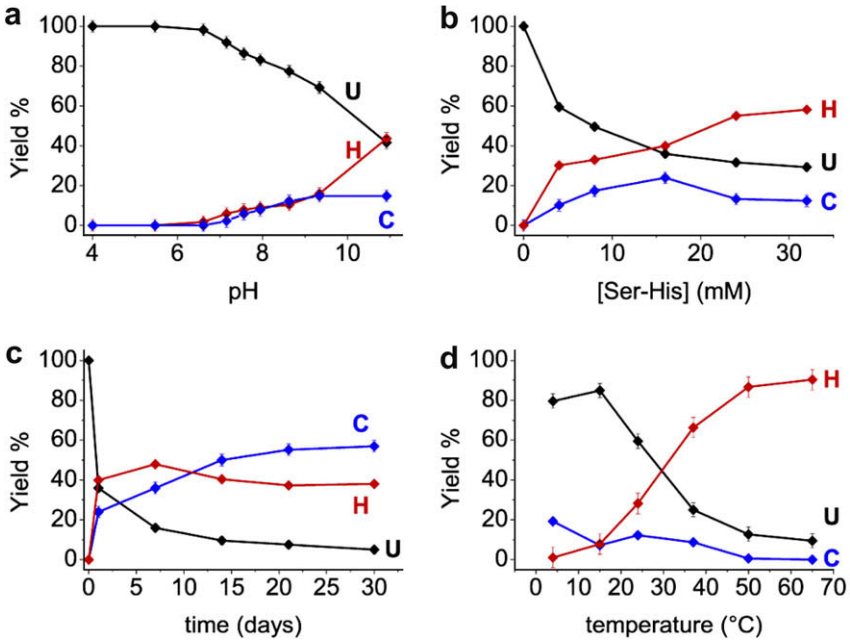

Fig. 1. Reaction between Ac-Phe-OEt and H-Leu-NH_2. The yields of the coupling product (Ac-Phe-Leu-NH_2, "C", blue), hydrolysis product (Ac-Phe-OH, "H", red), and of the unreacted substrate (Ac-Phe-OEt, "U", black) are shown. (a) Dependence on the pH; 4 mM Ser-His in Britton–Robinson buffer, 25 °C/ 3 days. (b) Dependence on the Ser-His concentration: Ser-His in sodium borate (100 mM, pH 10), 25 °C/1 day. (c) Reaction kinetics: 16 mM Ser-His in sodium borate buffer (100 mM, pH 10), 25 °C. (d) Dependence on the temperature: 4 mM Ser-His in sodium borate buffer (100 mM, pH 10), 6 days.

Table 1
Yields of peptides and PNAs, as catalysed by Ser-His and $(Gly)_n$.

Entry #	Catalyst	Conditions	Acyl donor	Free amine	Coupling product	Yield (%)
1	Ser-His	[a]	Ac-Phe-OEt	H-Leu-NH_2	Ac-Phe-Leu-NH_2	13.3
2	Ser-His	[a]	Ac-Phe-OEt	H-Phe-NH_2	Ac-Phe-Phe-NH_2	31.2
3	Ser-His	[a]	Ac-Phe-OEt	H-Leu-Phe-NH_2	Ac-Phe-Leu-Phe-NH_2	27.4
4	Ser-His	[a]	Z-Ala-Phe-OMe	H-Leu-NH_2	Z-Ala-Phe-Leu-NH_2	2.0
5	Ser-His	[a]	Z-Ala-Phe-OMe	H-Phe-NH_2	Z-Ala-Phe-Phe-NH_2	0.5
6	Ser-His	[a]	Z-Ala-Phe-OMe	H-Leu-Phe-NH_2	Z-Ala-Phe-Leu-Phe-NH_2	6.3
7	Ser-His	[b]	PNA monomer (1)	PNA monomer (1)	PNA dimer (2)	6.5[f]
8	Ser-His	[b]	PNA monomer (1)	PNA monomer (1)	PNA trimer (3)	3.6[f]
9	Ser-His	[b]	PNA monomer (1)	PNA monomer (1)	PNA tetramer (4)	9.1[f]
10	Ser-His	[c]	H-Phe-OEt	H-Phe-OEt	H-Phe-Phe-OEt	0.5–3
11	Ser-His	[d]	H-Phe-OEt + H-Trp-OEt	H-Phe-OEt + H-Trp-OEt	H-(Phe)(Trp)-OEt	0.1
12	Gly	[e]	Ac-Phe-OEt	H-Leu-NH_2	Ac-Phe-Leu-NH_2	11.9
13	Gly-Gly	[e]	Ac-Phe-OEt	H-Leu-NH_2	Ac-Phe-Leu-NH_2	13.2
14	Gly-Gly-Gly	[e]	Ac-Phe-OEt	H-Leu-NH_2	Ac-Phe-Leu-NH_2	14.2

[a] 50 mM reactants (each), 4 mM Ser-His, 24 h, 25 °C.
[b] 10 mM PNA monomer; 1.5 mM Ser-His, 35 h, 25 °C (Numbers 1, 2, 3, 4 refer to structures shown in Figure S1).
[c] 270 mM H-Phe-OEt; 22 mM Ser-His, 14 days, 4 °C.
[d] 50 mM reactants (both), 16 mM Ser-His, 7 days, 60 °C.
[e] 50 mM reactants (each), 4 mM Gly (or Gly-Gly; or Gly-Gly-Gly), 15 days, 4 °C.
[f] Calculated from the theoretical 100% yield of that product, as the only product in the mixture; chemical structures are shown in Fig. S1.

give the corresponding PNA dimer 2, trimer 3, and tetramer 4 in moderate yields (Table 1, entries 7–9). Also in this case, the driving force comes from the low solubility of products. Notice that the tetramer is formed in abnormal high yields, when compared with the lower oligomers. In this reaction, Ser-His is about 75 times less effective than α-chymotrypsin. Similarly, dimers of H-Phe-OEt and/or H-Trp-OEt can be obtained, although in lower yields (Table 1, entries 10–11).

From the mechanistic viewpoint, the Ser-His reaction represents an interesting case of organocatalysis. It may proceed through a Ser-*O*-ester intermediate, which in turn reacts with an amine to give the peptide bond. The lack of reactivity displayed by His-Ser, however, may also suggest the involvement of the Ser-His free amino group. Further studies are in progress in our laboratory in order to elucidate the reaction mechanism. Importantly, the effect of histidine on salt-induced peptide formation has been recently reported [19,20].

One advantage of Ser-His, and simple peptides in general, is that they can be active under conditions where large enzymes like α-chymotrypsin are inhibited or denaturated. Thus, in the presence of 50 mM cupric ions, Ser-His is 1.5 times more effective than α-chymotrypsin; typical denaturating conditions (6 M urea) completely inhibit α-chymotrypsin, whereas Ser-His is still active (Fig. S2).

Interestingly, the simpler and certainly more prebiotic Gly, Gly-Gly and Gly-Gly-Gly, act similarly to Ser-His – see entries 12-14 of Table 1 (see also Fig. S3). It is noteworthy, however, that the coupling yield is about 13%, whereas Ser-His, in similar conditions, gave ca. 40% yield. The glycine-based peptide formation catalysis has been reported [9,10,21,22].

The present preliminary work shows the feasibility of using simple peptides for the condensation of peptide fragments. In principle, then, short peptides, which form spontaneously – for example by the NCA condensation – may combinatorially give origin to a library of longer peptides thanks to short-peptide catalysis. Of course, in order to proceed towards longer chains, successive, multiple condensation steps are needed.

The latter goal can be achieved by changing the experimental conditions at each step so that the insoluble products may become soluble, as in the dry-wet or laguna scenario proposed for the origins of life [23,24]. A synthetic analogue of this reaction scheme has been recently presented by us, in which the multiple condensation steps have been carried out by the Merrifield method. In this way, a de novo 44-residue folded protein has been synthesized [25].

In conclusion, short peptides such as Gly-Gly, Gly-Gly-Gly, and Ser-His, that could have been formed before the very biogenesis of specific macromolecules, can act as catalysts for the condensation of activated peptide fragments. The substrates used in this study (protected amino acids or PNAs ethyl esters) should be mainly considered as a model system, in order to provide a proof of principle; but it is conceivable that other activated amino acids (thioesters, for example, which have been extensively investigated in this respect) can react similarly, whereas free amino acids cannot.

Acknowledgments

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Appendix A. Supplementary data

Materials and methods; HPLC, mass spectrometry and NMR characterizations of the products. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.11.052.

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SUPPLEMENTARY MATERIAL

Ser-His catalyses the formation of peptides and PNAs

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SUPPLEMENTARY MATERIAL

1. Materials. *N*-acetyl-phenylalanine ethyl ester (Ac-Phe-OEt), phenylalaninamide hydrochloride (H-Phe-NH_2 · HCl), leucinyl-phenylalaninamide hydrochloride (H-Leu-Phe-NH_2 · HCl), phenylalanine ethyl ester hydrochloride (H-Phe-OEt · HCl), tryptophan ethyl ester hydrochloride (H-Trp-OEt · HCl), *N*-(carbobenzyloxy)-alanyl-phenylalanine methyl ester (Z-Ala-Phe-OMe), seryl-histidine acetate salt (H-Ser-His-OH acetate salt), leucinyl-alanyl-leucinyl-alanine ethyl ester hydrochloride (H-Leu-Ala-Leu-Ala-OEt · HCl), glycyl-glycine (H-Gly-Gly-OH), and glycyl-glycyl-glycine (H-Gly-Gly-Gly-OH) were purchased from Bachem (Bubendorf, Switzerland); all amino acid derivatives have purity > 99%. Potassium carbonate (> 99%) was from Acros. Leucinamide hydrochloride (H-Leu-NH_2 · HCl) (> 99%), α-chymotrypsin from bovine pancreas (code: 27270), trifluoroacetic acid (TFA) (> 99%), glycine (> 99%), serine (> 99%), histidine (> 99.5%), histidyl-serine (His-Ser); dimethyl formamide (DMF) (> 99%), acetonitrile for HPLC (ACN) (> 99.9%), boric acid (> 99.5%), sodium dihydrogen phosphate dihydrate (> 99%), acetic acid (> 99.7%), sodium hydroxide (> 98%), urea (> 99.5%), cupric chloride dihydrate (> 99%); di-*t*-butyl dicarbonate (> 99%); thymine-1-acetic acid (> 98%); ethyl bromoacetate (> 98%); EDC·HCl (*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride) (>98%); triethylamine (>99.5%) were purchased was purchased from Sigma Aldrich. Freshly deionised, 220-nm filtered ultrapure water was obtained by a Milli-Q (Millipore) apparatus.

2. Methods I: peptide synthesis

2.1. General procedure for the synthesis of peptides. The ethyl or methyl esters of amino acids and peptides (50 mM) were added as DMF solution to the nucleophilic amino acid derivatives (e.g. H-Leu-NH_2 or similar; 50 mM), dissolved in aqueous buffer (generally, 100 mM sodium borate pH 10), so that the final DMF content in the aqueous solution was 6% (v/v). After addition of the catalyst (e.g. Ser-His, or a-chymotrypsin, etc.), and proper incubation time, the reaction was stopped by adding 10% v/v HCl 1N. The precipitate was collected by centrifugation (13,000 rpm, 15 minutes), dissolved in a measured amount of DMF and analysed by HPLC. The supernatant was also analysed by HPLC. The coupling product and the unreacted acyl donor are generally recovered in the precipitate. In contrary, the hydrolized substrate is found in the supernatant. Yields were calculated from HPLC peaks' area, combining the amount of each specie in the precipitate and in the supernatant. Compounds' identities were confirmed by mass analysis. (Figures S4-S11). Phenyl-containing peptides can also be quantified by using the following calibration line: HPLC_area (mAU_{265} · s) = 11400 × C_{Phe} (μmoles).

2.2. HPLC analysis. Reaction products were analyzed by analytical reversed phase HPLC using RP Nucleosil C18 column (Macherey Nagel 5 μm, 125 mm × 4 mm) at a constant flow rate of 1 mL/min. Elution was accomplished according to the following gradient: (A) water + 0.1% TFA; (B) ACN + 0.1% TFA: 0' A 97% B 3%; 3' A 97% B 3%; 28' A 40% B 60%; 30' A 3% B 97%; 33' A 3% B 97%; 35' A 97% B 3%; 37' A 97% B 3%. HPLC profiles were detected photometrically at 210 and 265 nm. Reactions rates and product yields were calculated from peak areas of reactants and products.

When needed, the reaction products were isolated after HPLC analysis, by using a slightly modified gradient program: (A) water + 0.1% TFA; (B) ACN + 0.1% TFA: 0' A 97% B 3%; 3' A 97% B 3%; 60' A 40% B 60%; 65' A 40% B 60%; 67' A 97% B 3%; 70' A 97% B 3%; the flow rate was 1 mL/min. After isolation, the samples were concentrated under vacuum and analysed for determining their mass spectrum.

2.3. Mass spectrometry. Each HPLC fraction was solubilized in 5 μL of a 70% acetonitrile solution containing 0.2% TFA. An aliquot of each sample (1 μL) was mixed with the 2,5-dihydroxybenzoic acid matrix aqueous solution (10 mg/mL) in different ratios (1:1, 1:3, 1:5); 1 μL of each mixture was deposited onto a MALDI target plate and allowed to dry on air. MALDI-ToF MS analyses were performed in a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser and operating in reflector mode. Mass data were obtained by accumulating spectra from 200 laser shots with an accelerating voltage of 20 kV. All mass spectra were externally calibrated using a standard peptide (des-Arg-bradykinin m/z 904.4681 - Sequazyme; Applied Biosystems) and specific m/z values of ion matrix (m/z 137.1 and 273.1)

2.4. Reaction catalysed by Ser-His.

2.4.1. Ac-Phe-Leu-NH_2 (from Ac-Phe-OEt and H-Leu-NH_2). Ac-Phe-OEt (50 mM), H-Leu-NH_2 (50 mM) and Ser-His (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Conditions for the pH dependence (Figure 1a): 4 mM Ser-His in Britton-Robinson buffer (40 mM sodium acetate, 40 mM sodium phosphate, 40 mM sodium borate), 25 °C/ 3 days. Conditions for the Ser-His concentration dependence (Figure 1b): Ser-His in sodium borate (100 mM, pH 10), 25 °C/1 day. Conditions for the time dependence (Figure 1c): 16 mM Ser-His in sodium borate buffer (100 mM, pH 10), 25 °C. Conditions for the temperature dependence (Figure 1d): 4 mM Ser-His in sodium borate buffer (100 mM, pH 10), 6 days. Products were identified by HPLC (265 nm) and mass spectrometry (Figure S4).

Ac-Phe-Leu-NH_2: calc. MW 319.4; found MH^+ 320.1913 m/z; HPLC T_R 14.78 min.

Ac-Phe-OEt: calc. MW 235.28; found MH^+ 236.1495 m/z; HPLC T_R 16.89 min.
Ac-Phe-OH: calc. MW 207.23; found MH^+ 208.0798 m/z; HPLC T_R 13.00 min.

2.4.2. Ac-Phe-Phe-NH_2 (from Ac-Phe-OEt and H-Phe-NH_2). Ac-Phe-OEt (50 mM), H-Phe-NH_2 (50 mM) and Ser-His (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Conditions: 1 day at 25 °C. Products were identified by HPLC (265 nm) and mass spectrometry (Figure S5).
Ac-Phe-Phe-NH_2: calc. MW 353.41; found MH^+ 354.2217 m/z; HPLC T_R 15.81 min.

2.4.3. Ac-Phe-Leu-Phe-NH_2 (from Ac-Phe-OEt and H-Leu-Phe-NH_2). Ac-Phe-OEt (50 mM), H-Leu-Phe-NH_2 (50 mM) and Ser-His (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Conditions: 1 day at 25 °C. Products were identified by HPLC (265 nm) and mass spectrometry (Figure S6).
Ac-Phe-Leu-Phe-NH_2 : calc. MW 466.57; found MH^+ 467.331 m/z; HPLC T_R 17.62 min.

2.4.4. Z-Ala-Phe-Leu-NH_2 (from Z-Ala-Phe-OMe and H-Leu-NH_2). Z-Ala-Phe-OMe (50 mM), H-Leu-NH_2 (50 mM) and Ser-His (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Conditions: 1 day at 25 °C. Products were identified by HPLC (265 nm) and mass spectrometry (Figure S7).
Z-Ala-Phe-Leu-NH_2: calc. MW 482.57; found MH^+ 483.3116 m/z ; HPLC T_R 19.01 min.
Z-Ala-Phe-OMe: calc. MW 384.43; found MH^+ 385.2341 m/z; HPLC T_R 19.83 min.
Z-Ala-Phe-OH: calc. MW 370.40; found MH^+ 371.2134 m/z; HPLC T_R 18.19 min.

2.4.5. Z-Ala-Phe-Phe-NH_2 (from Z-Ala-Phe-OMe and H-Phe-NH_2). Z-Ala-Phe-OMe (50 mM), H-Phe-NH_2 (50 mM) and Ser-His (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Conditions: 1 day at 25 °C. Products were identified by HPLC (265 nm) and mass spectrometry (Figure S8).
Z-Ala-Phe-Phe-NH_2: calc. MW 516.59; found MH^+ 517.3274 m/z; HPLC T_R 19.39 min.

2.4.6. Z-Ala-Phe-Leu-Phe-NH_2 (from Z-Ala-Phe-OMe and H-Leu-Phe-NH_2). Z-Ala-Phe-OMe (50 mM), H-Phe-NH_2 (50 mM) and Ser-His (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% v/v of DMF) at room temperature. Conditions: 1 day at 25 °C. Products were identified by HPLC (265 nm) and mass spectrometry (Figure S9).
Z-Ala-Phe-Leu-Phe-NH_2: calc. MW 629.75, found MH^+ 630.425 m/z ; HPLC T_R 21.04 min.

2.4.7. Condensation of bifunctional substrates. Bifunctional substrates having general formula H-X-OEt (50 mM) and Ser-His (16 mM) were dissolved in 0.2 M sodium borate pH 10.5 (6% v/v DMF; 30 % w/v PEG6000) at 60 °C. Conditions: 7 days at 60 °C. The substrate were (*1*) H-Leu-OEt; (*2*) H-Phe-OEt; (*3*) H-Trp-OEt; (*4*) 1:1 mixture H-Phe-OEt and H-Trp-OEt; and (*5*) H-Leu-Ala-Leu-Ala-OEt. Products were identified by HPLC (220 or 265 nm) and mass spectrometry. The following products (in addition to the hydrolized substrate, i.e., H-X-OH) were identified from the reaction mixtures: (*1*) none; (*2*) H-Phe-Phe-OEt (Figure S10); (*3*) none; (*4*) H-Phe-Trp-OEt or H-Trp-Phe-OEt (Figure S11); (*5*) H-Leu-Ala-Leu-Ala-Leu-Ala-Ser-His-OH, and H-Leu-Ala-Leu-Ala-Ser-His-Ser-His-OH (data not shown).
Alternatively, the condensation of H-Phe-OEt was carried out by dissolving H-Phe-OEt (270 mM) and Ser-His (22 mM) in 0.1 M sodium borate pH 10 (6.2% v/v DMF) at 4 °C. Conditions: 14 days at 4 °C. After freeze drying and solubilization in 500 mM sodium borate pH 10/ethanol 80/20 v/v, samples were analyzed by HPLC.

2.5. Reaction catalysed by α-chymotrypsin. Ac-Phe-OEt (50 mM), H-Leu-NH_2 (50 mM) and α-chymotrypsin (40 μM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Conditions: from 1 minute to 1 month, 25 °C. Products were identified by HPLC and mass spectrometry. Yields of this reaction, and negative control (in the absence of catalyst) are given in Figure S12.

2.6. Preparation of Ac-Phe-Leu-NH_2 (from Ac-Phe-OEt and H-Leu-NH_2) with Ser-His and α-chymotrysin in the presence of denaturating or inactivating agents. Ac-Phe-OEt (50 mM), H-Leu-NH_2 (50 mM) and Ser-His (4 or 16 mM) or α-chymotrysin (40 μM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF) at room temperature. Experimental conditions: (a) 16 mM Ser-His or 40 μM α-chymotrysin, seven days, 25 °C; (b) 16 mM Ser-His or 40 μM α-chymotrysin, three days, 25 °C, in the presence of 50 mM $CuCl_2 \cdot 2H_2O$; (c) 4 mM Ser-His or 40 μM α-chymotrysin, three days, 25 °C, in the presence of 6 M urea. Yields are reported in Figure S2.

2.7. Reaction catalysed by Gly, Gly-Gly, and Gly-Gly-Gly. Ac-Phe-OEt (50 mM), H-Leu-NH2 (50 mM) and Gly (or Gly-Gly, or Gly-Gly-Gly) (4 mM) were dissolved in 1.0 mL 0.1 M sodium borate pH 10 (6% of DMF). Products were identified by HPLC (Yields provided in Figure S3).

3. Methods II: PNA synthesis

3.1. Synthesis of the PNA building block. Synthesis of PNA thymine monomer **1** according to Fader et al.,[S1] (Figure S13). Ethylenediamine **5** was mono-Boc protected by reaction with limiting amounts of di-tert-butyl dicarbonate to give pure compound **6** in 91% yield. Compound **6** was further reacted with a limiting amount of ethyl bromoacetate in the presence of K_2CO_3, giving the PNA backbone **7** in 79% yield. Thymidyl-1-acetic acid was then coupled to secondary amine **7**, giving fully protected thymine monomer **8** (61%), which was hydrolyzed to give the building block **1** (97%).

3.2. Oligomerization of PNA thymine monomer with Ser-His. The PNA building block (monomer) **1** (10 mM) was incubated with Ser-His (1.5 mM) or a-chymotrypsin (40 mM) in 200 mM EPPS (sodium salt), pH 8.3, at room temperature, for 35 hours or 25 minutes, respectively. After the completion of the reaction, solvent (water) was removed on rotary evaporator, sample was dissolved in water and purified by HPLC (260 nm, Figure S14), then analyzed by mass spectrometry (Figure S15), and 1H-NMR (Figure S16). Together with the oligomerization products, the ketopyperazine derivative **9** was observed (Figure S17).
Monomer **1**: calc. MW 311.15; found 313.0 m/z; HPLC T_R 4.27 min.
Dimer **2**: calc. MW 576.30; found 579.2 m/z; HPLC T_R 4.86 min.
Trimer **3**: calc. MW 841.45; found 845.3 m/z; HPLC T_R 6.34 min.
Tetramer **5**: calc. MW 1106.60; found 1112.5 m/z; HPLC T_R 10.52 min.

3.3. Oligomerization of PNA thymine monomer with α-chymotrypsin. The reaction was carried out as in the case of Ser-His. α-Chymotrypsin was used at a concentration of 40 μM (1 mg/mL), and reaction was stopped after 25 minutes. A comparison between yields obtained with Ser-His and α-chymotrypsin is given in Table S1.

3.4. HPLC analysis. Reaction products were analyzed by analytical reversed phase HPLC using Varian VariTide RPC 6M column at a constant flow rate of 1.5 mL/min. Elution was accomplished according to the following gradient: (A) water + 0.1% TFA; (B) ACN + 0.1% TFA: 0’ A 98% B 2%; 35’ A 50% B 50%; 40’ A 2% B 98%; 44’ A 98% B 2%. HPLC profiles were detected photometrically at 260 nm.

3.5. Mass and NMR spectrometry. Low-resolution MS analysis was performed on a Bruker Daltonics Esquire 3000 Plus mass spectrometer. NMR analysis were carried out on Varian 400-MHz spectrometer.

5. Table S1, Chart S1 and Figures S1 – S17

Table S1. Oligomerization yields and substrate conversion in the Ser-His (or α-chymotrypsin)-catalyzed reaction of the PNA building block **1**.

Catalyst	[catalyst] mM	Dimer[a] **2**	Trimer[a] **3**	Tetramer[a] **4**	Substrate conversion[b] (%)
Ser-His	1.5	0.38 mg 6.5%	0.2 mg 3.6%	0.51 mg 9.1%	19.0%
α-chymotrypsin	0.04 (1 mg/mL)	0.29 mg 10.3%	0.15 mg 5.2%	0.42 mg 15.3%	30.8%

[a] Calculated from the theoretical 100% yield of that product as the only product in the mixture;

[b] Percent of monomer **1** conversion calculated from the total yield of isolated products (**2**, **3** and **4**).

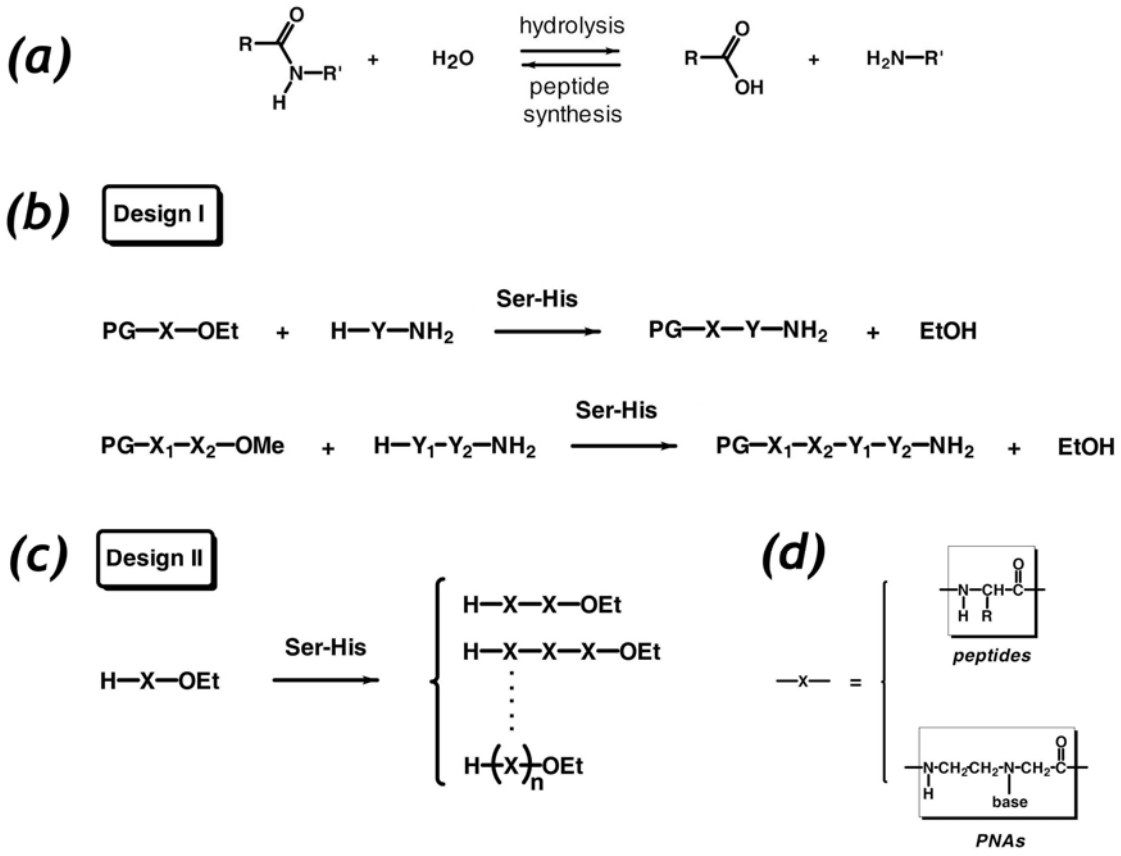

Chart S1. Chemistry of peptide bond formation and cleavage (*a*) and experimental design of coupling (*b*, *c*, *d*). (*a*) The amide (RCONHR', on the left) reacts with water giving the corresponding carboxylic acid (RCOOH) and an amine (R'NH2) (these two compounds are actually ionized in aquous media, not shown). A catalyst that promotes the peptide bond hydrolysis (from left to right), also catalyses the reverse reaction (from right to left). Equilibrium may be shifted to the left if the amide is poorly soluble. Amines can also react with esters, to give a peptide. An aminolysis reaction closely follows the equation (*a*) (from right to left), where the amine (R'NH_2) reacts with a carboxylic ester (RCOOR", not shown) to give the amide (RCONHR'). The alcohol (R"OH, not shown) is released instead of water. (*b*) Design I: one step peptide coupling via ester aminolysis. Peptides, activated as ethyl or methyl esters, and protected at N-terminus by a protecting group (PG), react with nucleophile peptides (protected at *C*-terminus as amide) to give coupling products. (*c*) Design II: multi-steps peptide elongation via ester aminolysis. Bifunctional peptides or PNA building blocks (having free amino groups, and activated at *C*-terminus as ethyl ester) react to give dimers, which in turn may react further to give trimers, tetramers, etc. The oligomerization may occur by one-residue elongation (2 +1 → 3; 3 + 1 → 4; etc.) or by fragment condensation (2 + 2 → 4; etc). Hydrolysis reactions are not shown. (*d*) Details of chemical structure of "X" (i.e., a-amino acids, or PNA monomers)

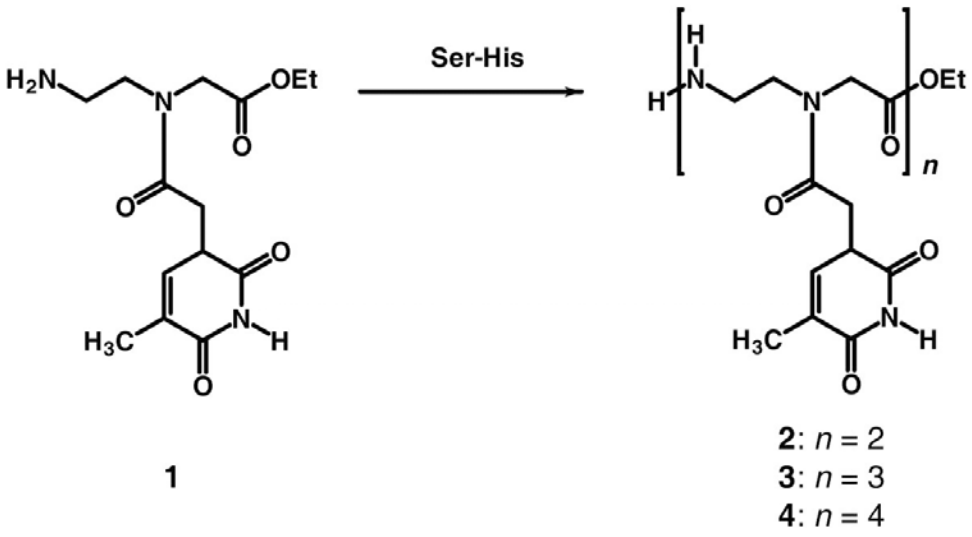

Figure S1. Oligomerization of PNA building block (**1**), the monomer, to give PNA dimer (**2**), trimer (**3**) and tetramer (**4**). Ketopiperazine derivatives are also formed.

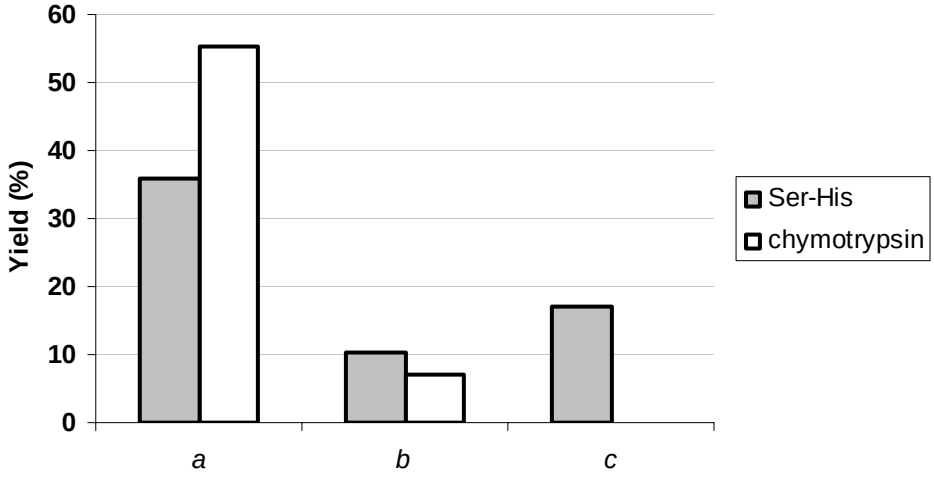

Figure S2. Yield (%) of coupling product (Ac-Phe-Leu-NH_2, from Ac-Phe-OEt and H-Leu-NH_2) under denaturating/inactivating conditions. Experimental conditions: 50 mM reactants (each); Ser-His 16 mM (*a*, *b*) or 4 mM (*c*); α-chymotrypsin 40 μM (*a*, *b*, *c*); seven (*a*, *c*) or three days (*b*) incubation at 25 °C, in 100 mM sodium borate, pH 10. (*a*) Positive control; (*b*) 50 mM Cu(II); (*c*) 6 M urea.

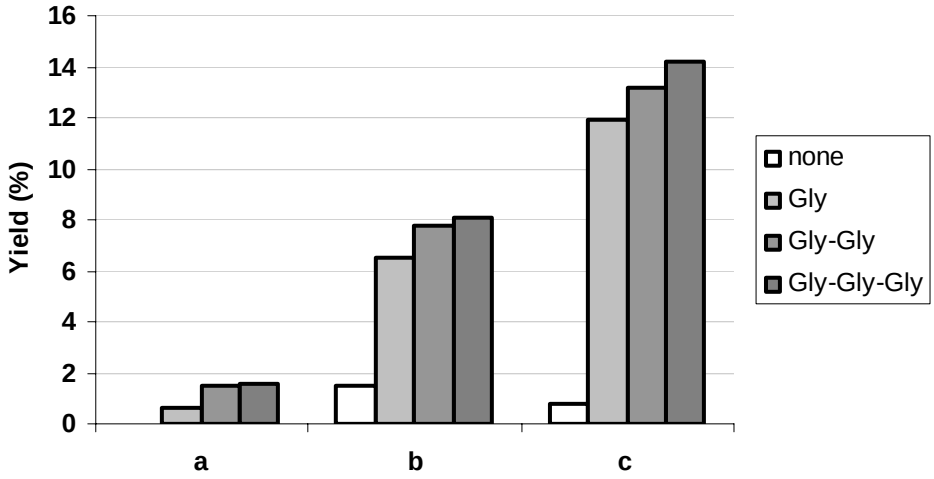

Figure S3. Yield (%) of coupling product (Ac-Phe-Leu-NH_2, from Ac-Phe-OEt and H-Leu-NH_2) under Gly, Gly-Gly, or Gly-Gly-Gly catalysis. Experimental conditions: 50 mM reactants (each); Gly (or Gly-Gly, or Gly-Gly-Gly) 4 mM; one (*a*), or fifthteen (*b*) days incubation at 25 °C (*a*), 37 °C (*b*) or 4 °C (*c*), in 100 mM sodium borate, pH 10.

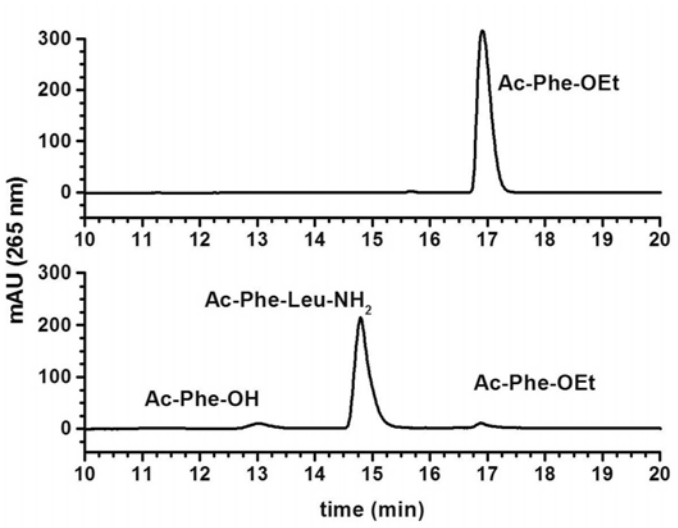

Figure S4a. Synthesis of Ac-Phe-Leu-NH_2: HPLC analysis of crude reaction mixture

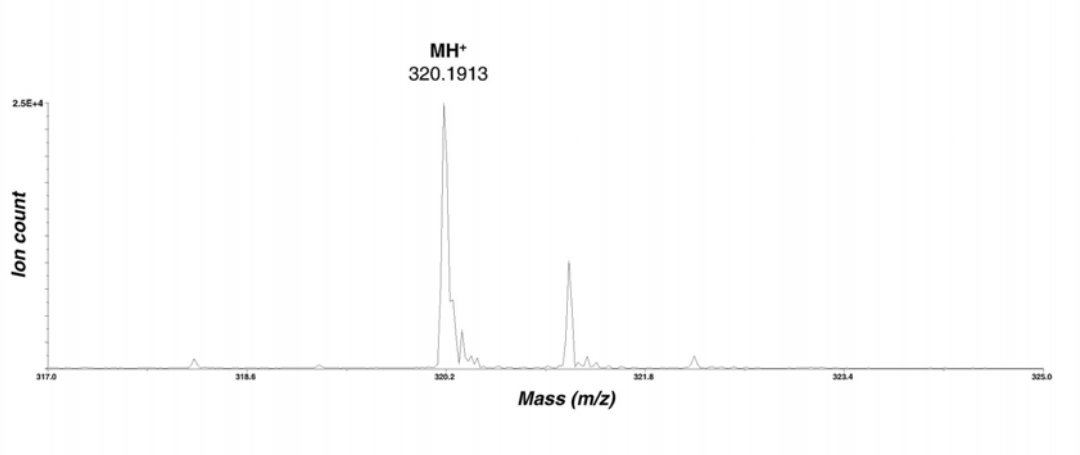

Figure S4b. Mass spectrum of Ac-Phe-Leu-NH_2 (calc. MW 319.40)

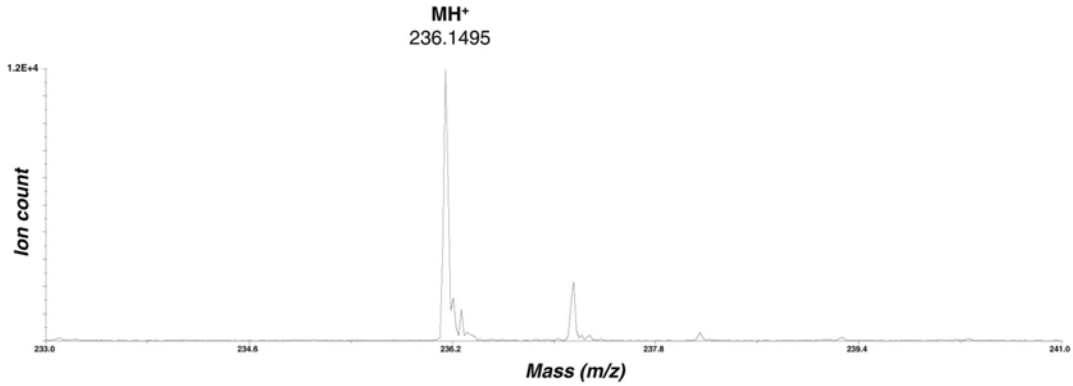

Figure S4c. Mass spectrum of Ac-Phe-OEt (calc. MW 235.28)

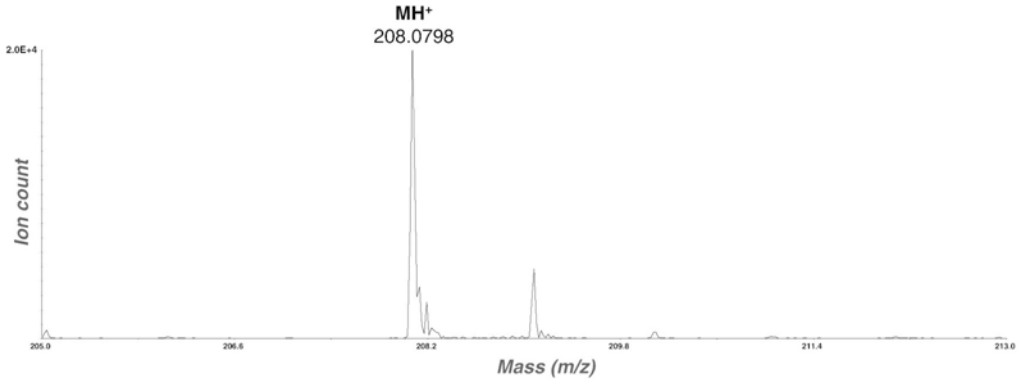

Figure S4d. Mass spectrum of Ac-Phe-OH (calc. MW 207.23)

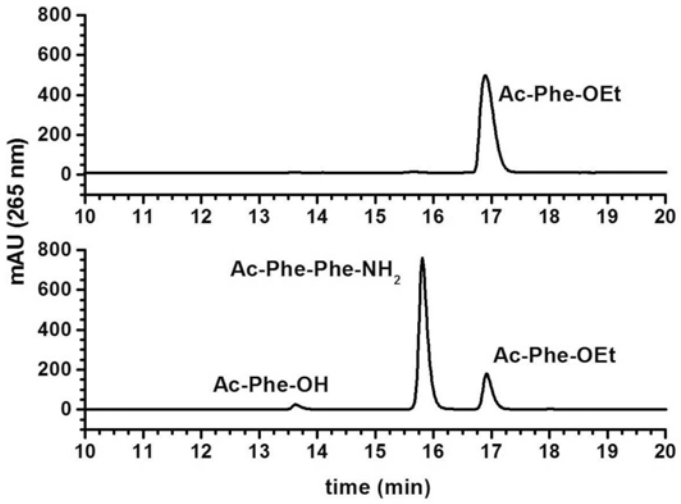

Figure S5a. Synthesis of Ac-Phe-Phe-NH_2: HPLC analysis of crude reaction mixture

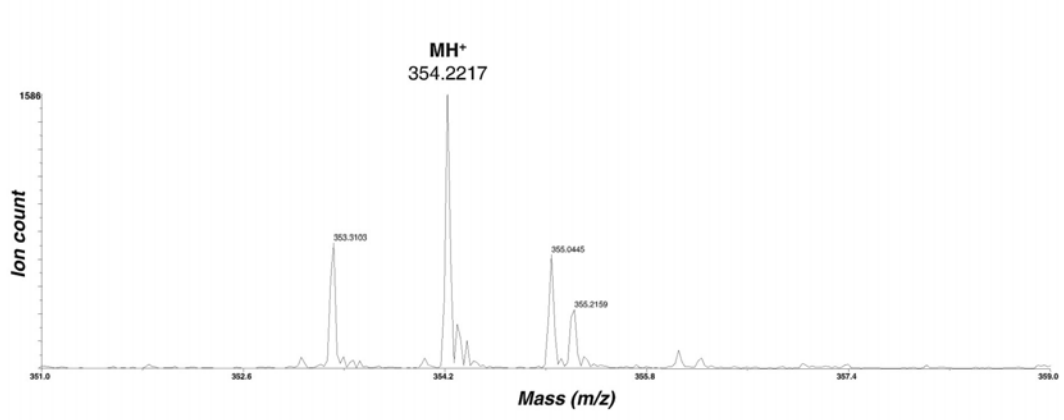

Figure S5b. Mass spectrum of Ac-Phe-Phe-NH_2 (calc. MW 353.41)

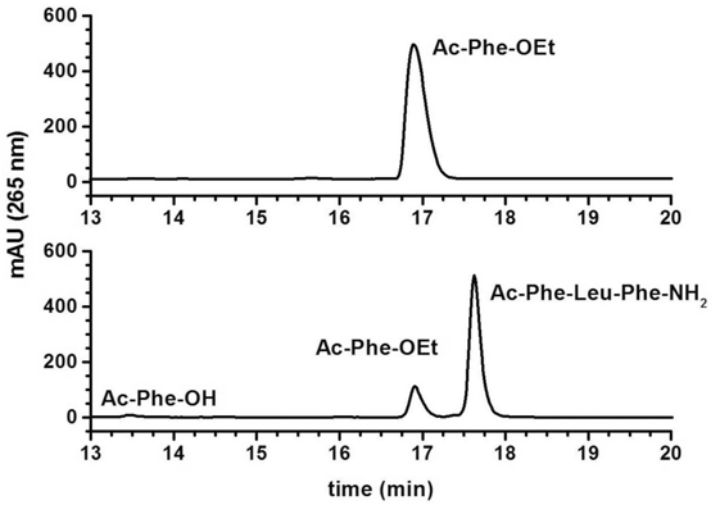

Figure S6a. Synthesis of Ac-Phe-Leu-Phe-NH_2: HPLC analysis of crude reaction mixture

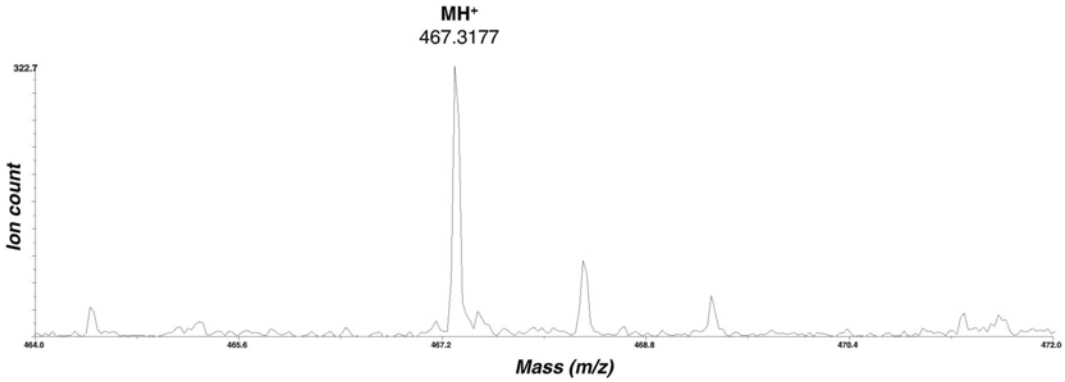

Figure S6b. Mass spectrum of Ac-Phe-Leu-Phe-NH_2 (calc. MW 466.57)

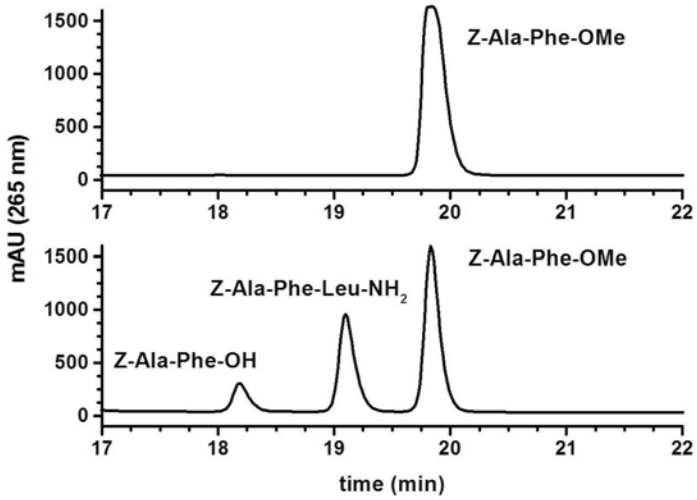

Figure S7a. Synthesis of Z-Ala-Phe-Leu-NH_2: HPLC analysis of crude reaction mixture

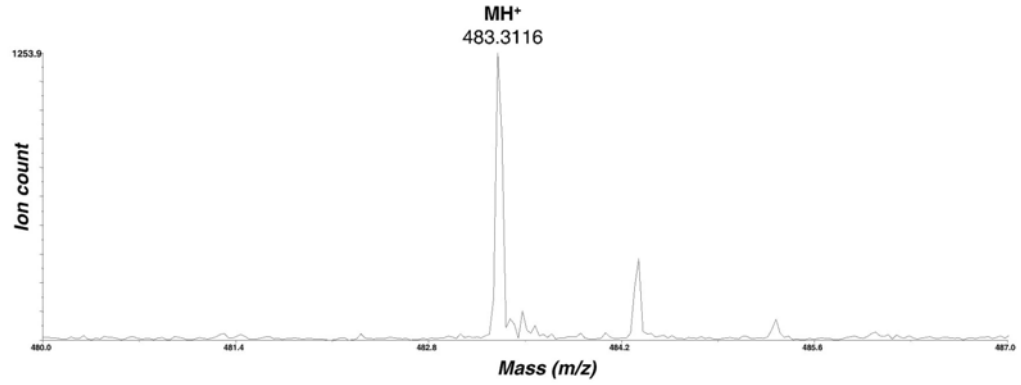

Figure S7b. Mass spectrum of Z-Ala-Phe-Leu- NH_2 (calc. MW 482.57)

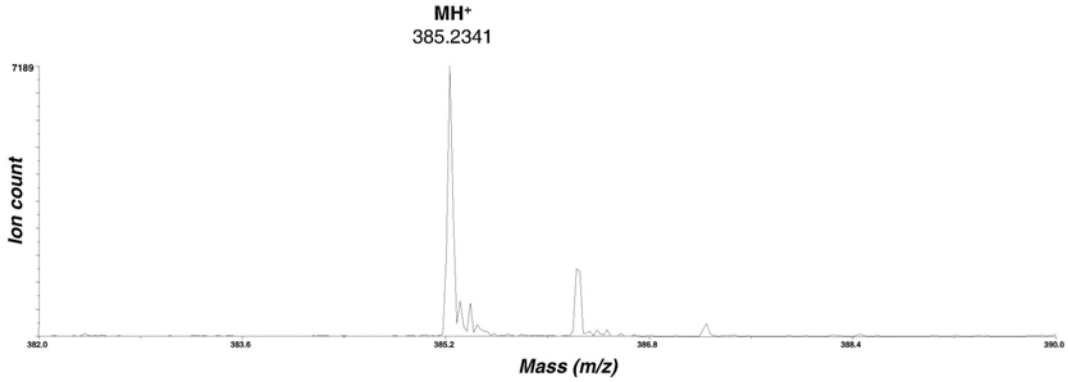

Figure S7c. Mass spectrum of Z-Ala-Phe-OMe (calc. MW 384.43)

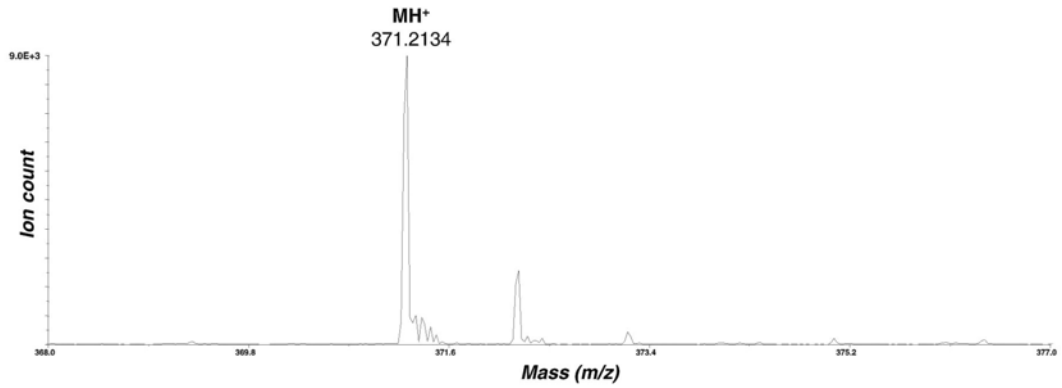

Figure S7d. Mass spectrum of Z-Ala-Phe-OH (calc. MW 370.40)

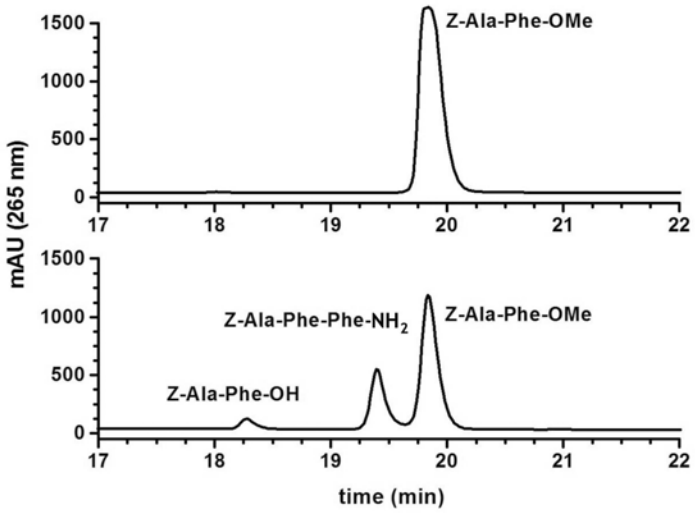

Figure S8a. Synthesis of Z-Ala-Phe-Phe-NH_2: HPLC analysis of crude reaction mixture

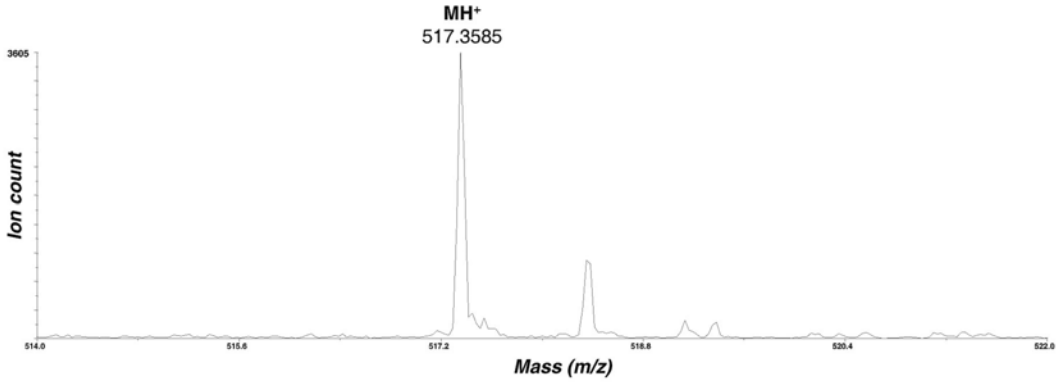

Figure S8b. Mass spectrum of Z-Ala-Phe-Phe-NH_2 (calc. MW 516.59)

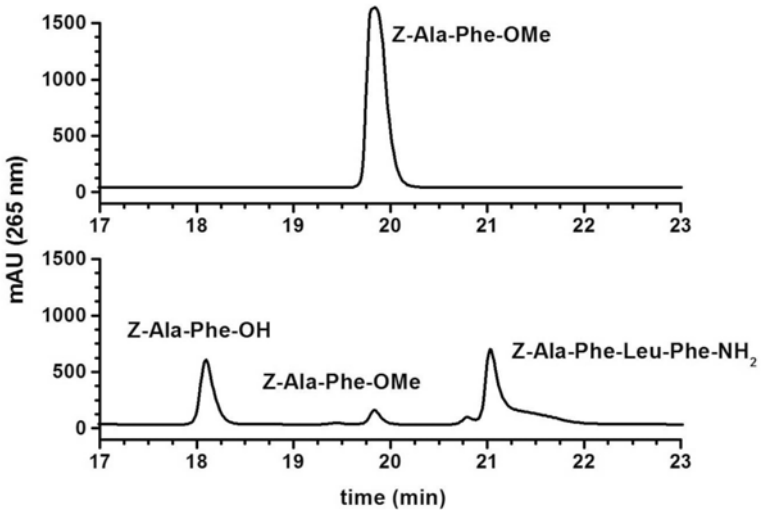

Figure S9a. Synthesis of Z-Ala-Phe-Leu-Phe-NH_2: HPLC analysis of crude reaction mixture

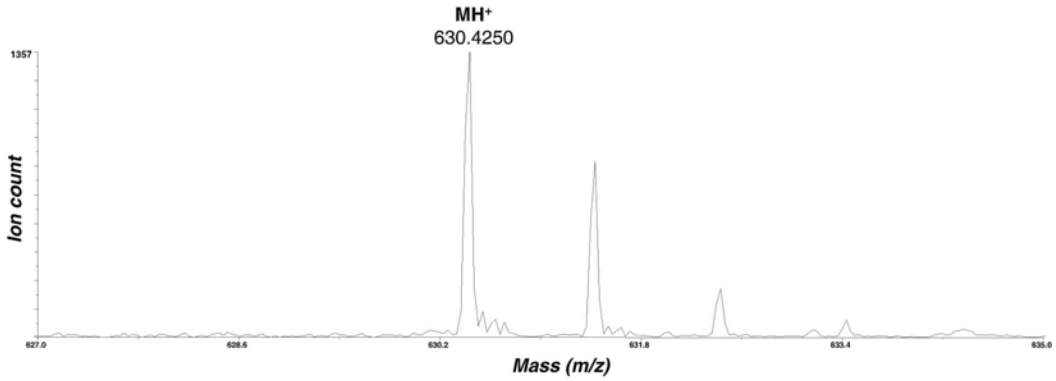

Figure S9b. Mass spectrum of Z-Ala-Phe-Leu-Phe-NH_2 (calc. MW 629.75)

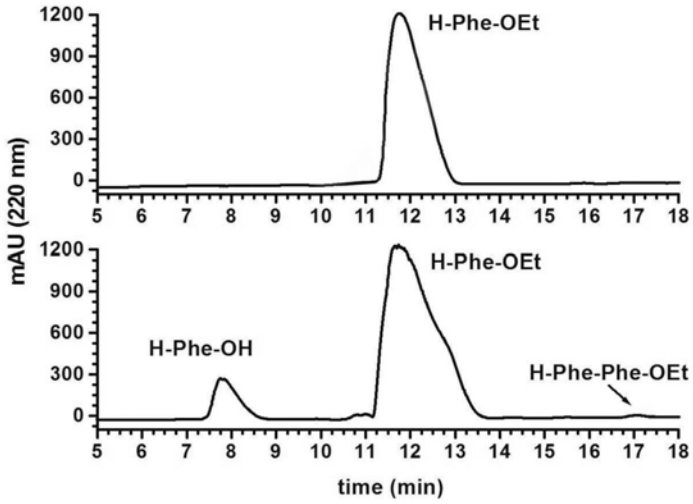

Figure S10a. HPLC analysis of H-Phe-OEt condensation, crude reaction mixture

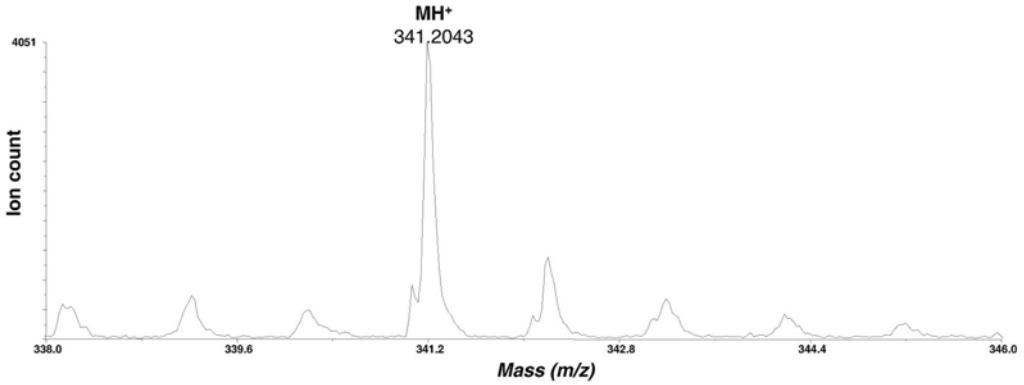

Figure S10b. Mass spectrum of H-Phe-Phe-OEt (calc. MW 340.75)

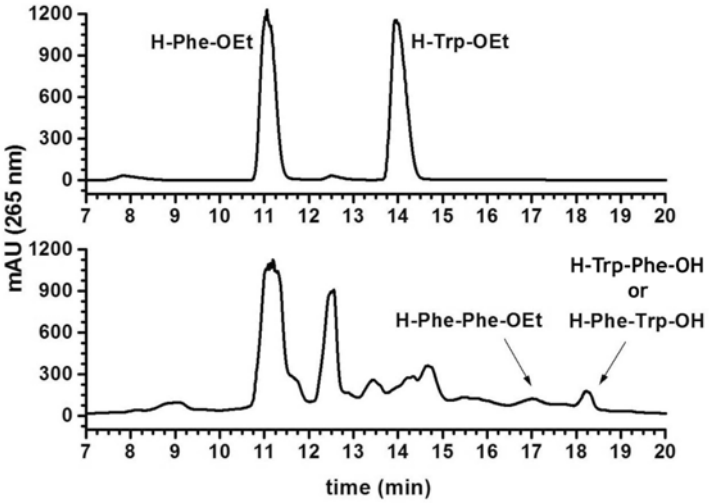

Figure S11a. HPLC analysis of H-Phe-OEt/H-Trp-OEt condensation, crude reaction mixture

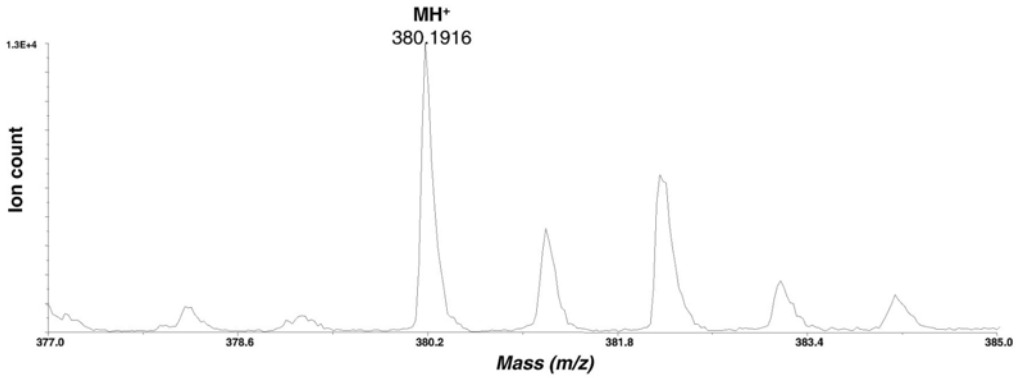

Figure S11b. Mass spectrum of H-Phe-Trp-OEt (or H-Trp-Phe-OEt) (calc. MW 379.75)

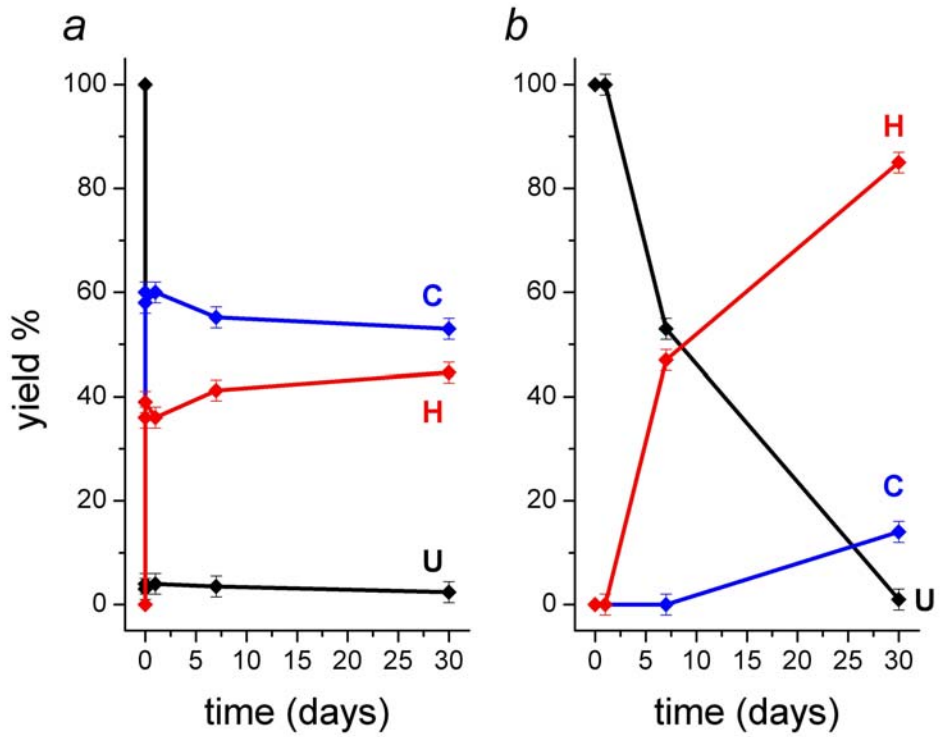

Figure S12. Time course of the reaction between Ac-Phe-OEt and H-Leu-NH_2, in the presence of (*a*) 40 μM α-chymotrypsin; and (*b*) no catalyst. Black line (U): unreacted Ac-Phe-OEt; blue line (C): coupling product (Ac-Phe-Leu-NH_2); red line (H): hydrolyzed substrated (Ac-Phe-OH).

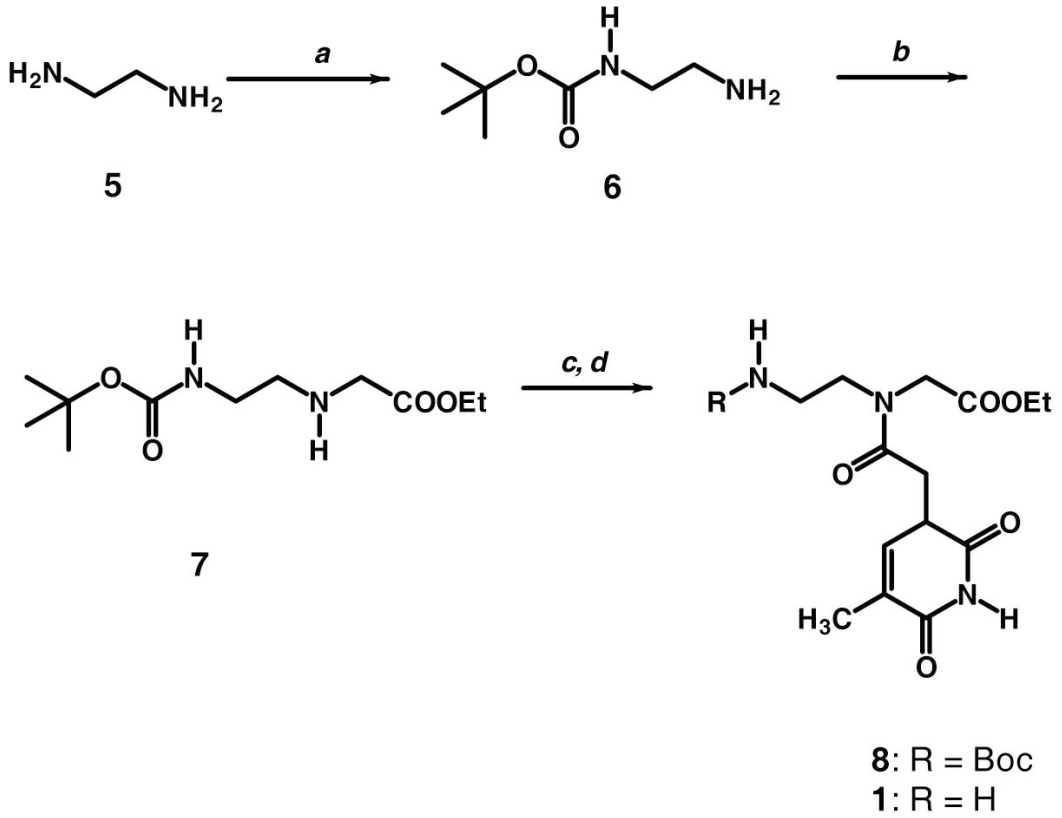

Figure S13. Synthetic route for the preparation of the PNA building block **1**. Conditions: (*a*) $(BOC)_2O$, THF, 0°C → RT, 4h; 91%; (*b*) $BrCH_2COOEt$, K_2CO_3, CH_2Cl_2; 79%; (*c*) Thymine acetic acid, EDC*HCl, Et_3N, DMF, 61%; (*d*) TFA: H2O 1:1, RT, 45 min; 96% .

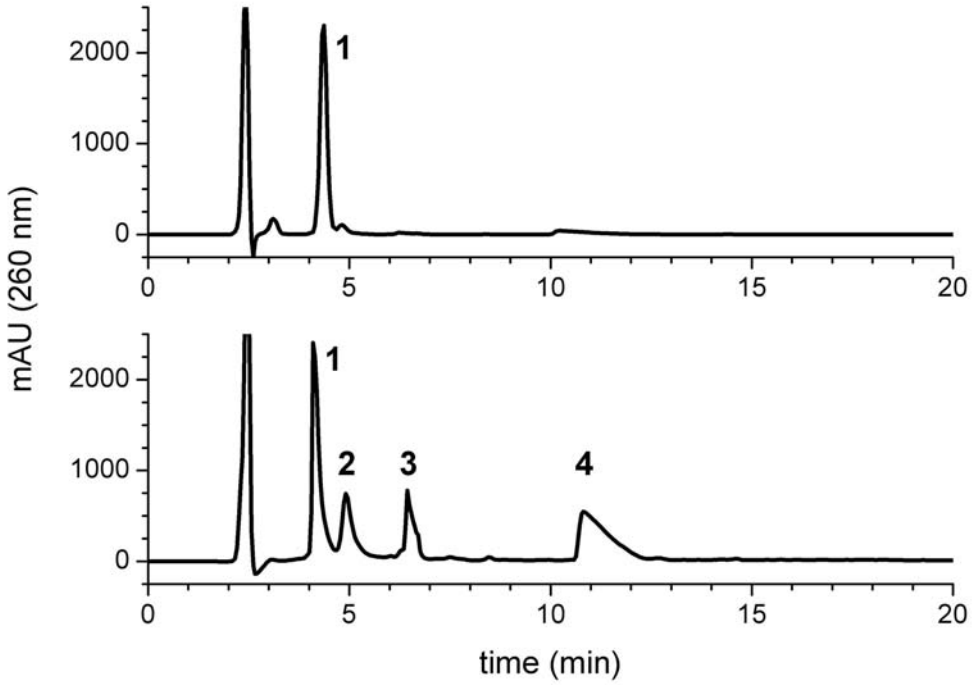

Figure S14. HPLC analysis of PNA oligomers.

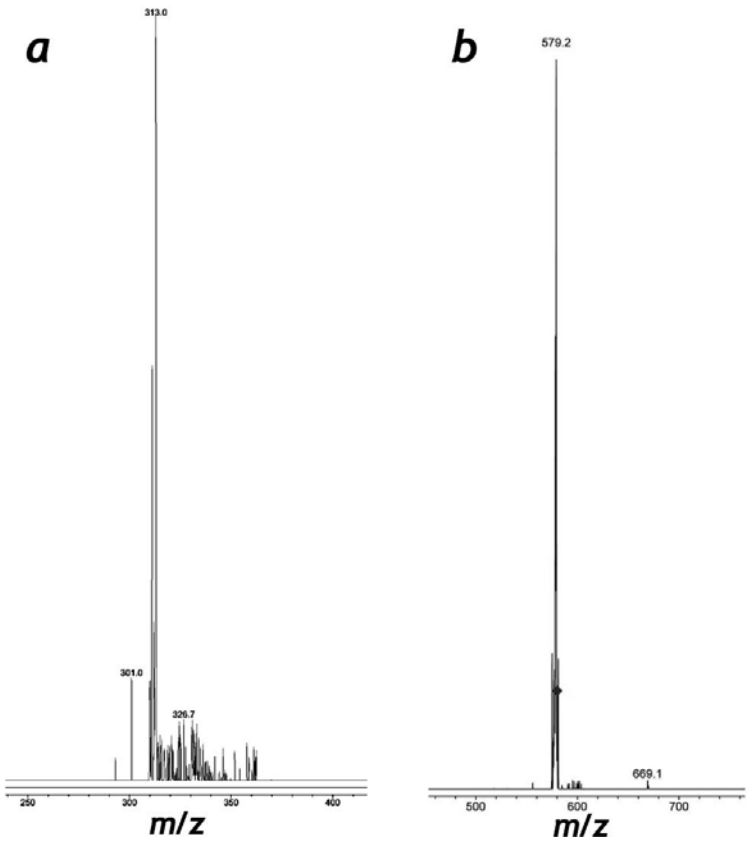

Figure S15a-b. Mass spectra of PNA monomer (a) and dimer (b).

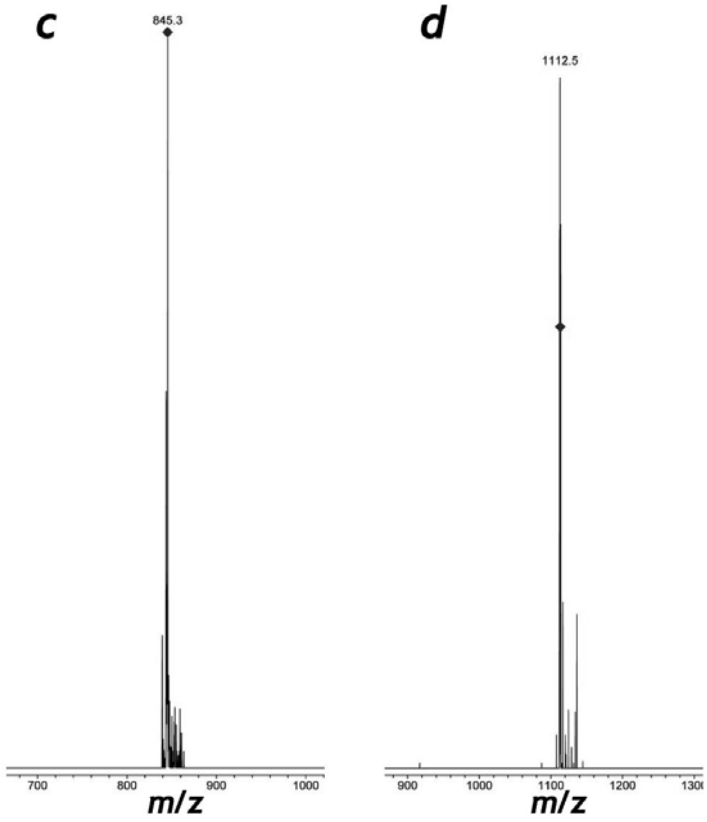

Figure S15c-d. Mass spectra of PNA trimer (a) and tetramer (b).

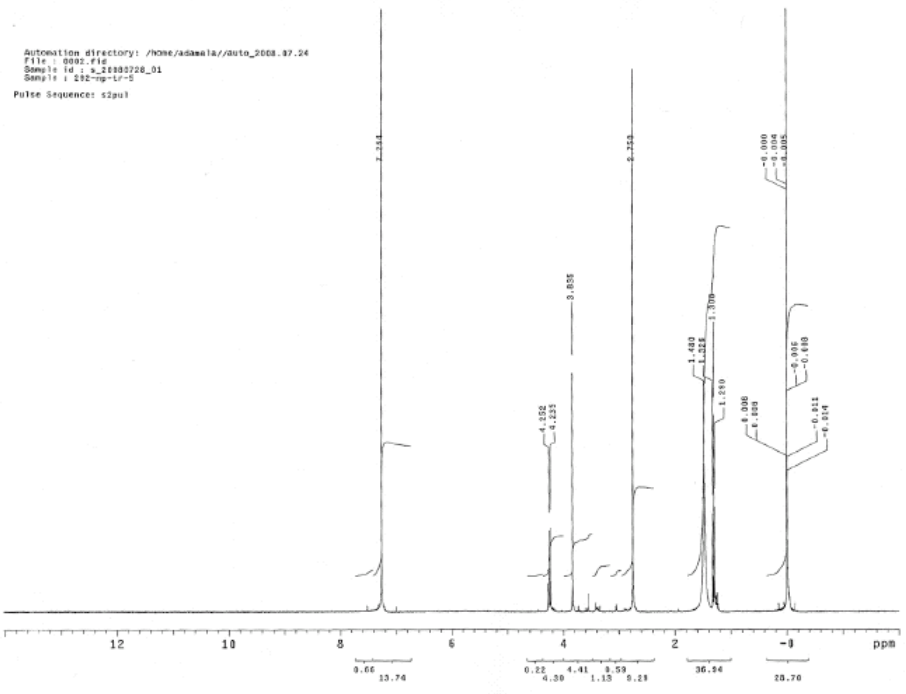

Figure 16a. ^{1}H-NMR spectrum of PNA monomer.

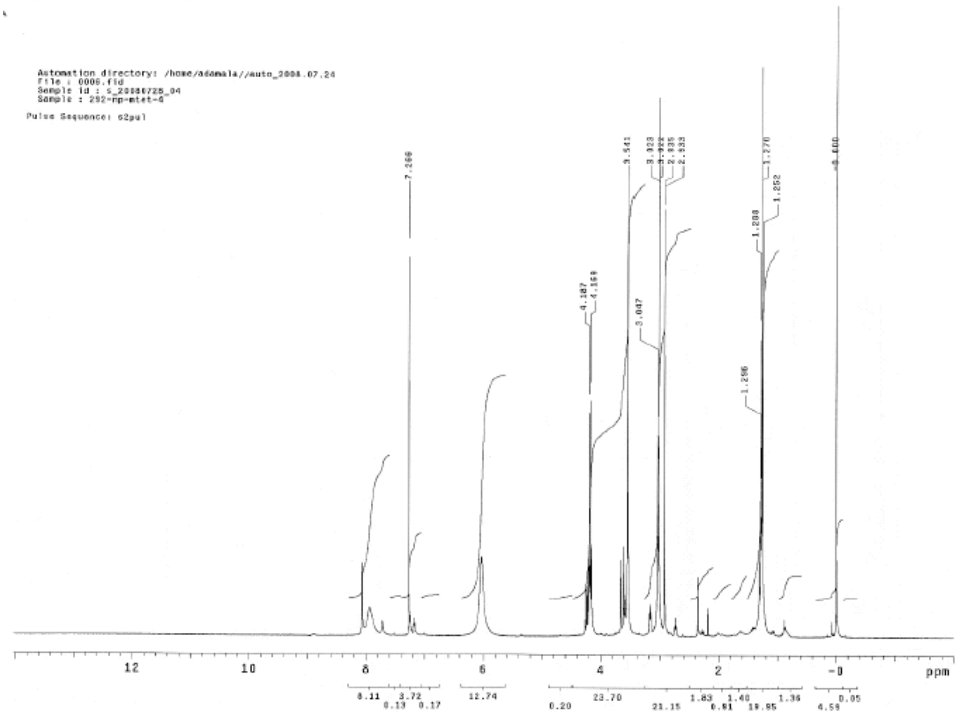

Figure 16b. ^{1}H-NMR spectrum of PNA dimer.

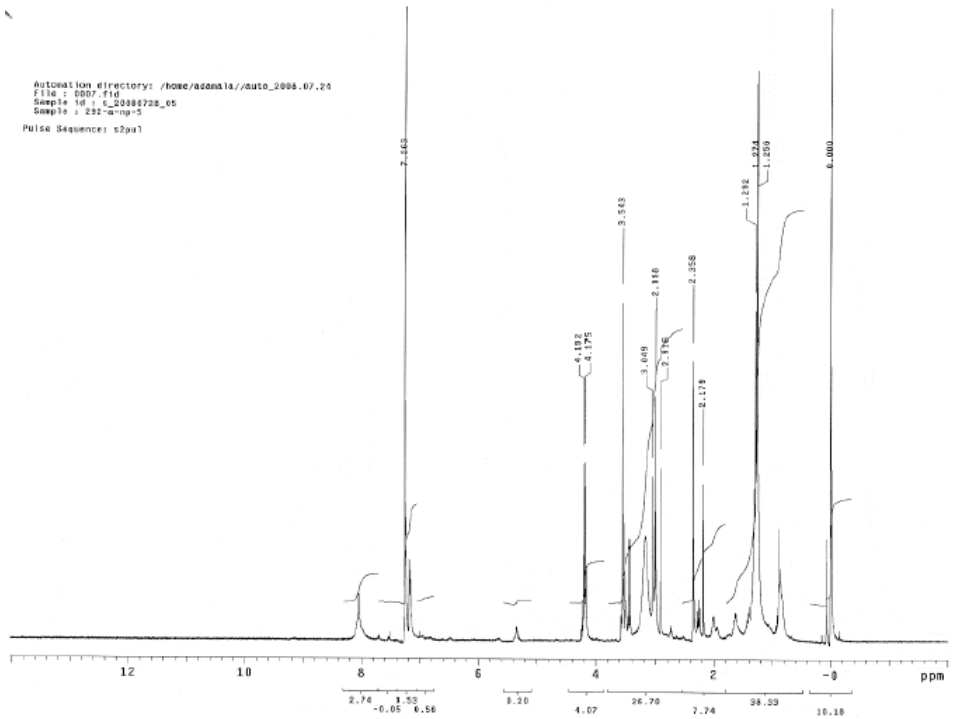

Figure 16c. ^{1}H-NMR spectrum of PNA trimer.

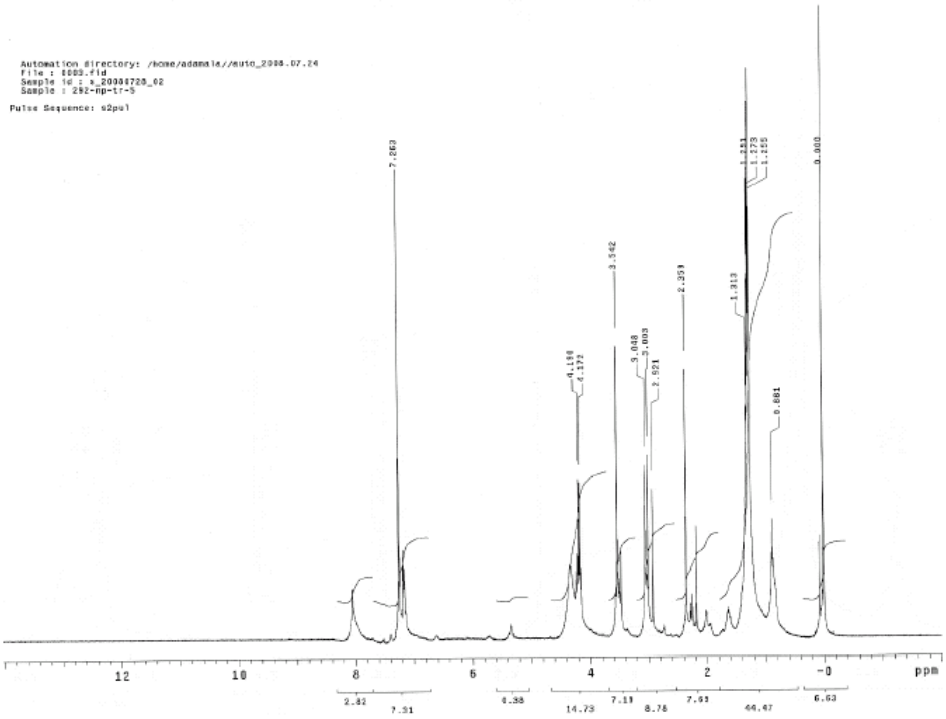

Figure 16d. ^{1}H-NMR spectrum of PNA tetramer.

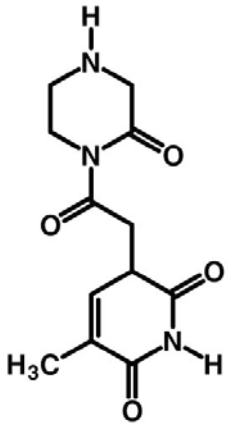

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Figure S17. Structure of the ketopiperazine derivative

5. References

(S1) Fader, L. D.; Boyd, M.; Tsantrizos, Y. S. Backbone Modifications of Aromatic Peptide Nucleic Acid (APNA) Monomers and Their Hybridization Properties with DNA and RNA. *J. Org. Chem.* **2001**, *66*, 3372-3379.