Synthesis and analysis of peptide nucleic acid oligomers using Fmoc/acyl-protected monomers

György Kovács,* Zoltán Timár, Zoltán Kupihár, Zoltán Kele and Lajos Kovács *

Department of Medicinal Chemistry, Dóm tér 8, University of Szeged, H-6720 Szeged, Hungary. E-mail: gyorgyi@ovrisc.mdche.u-szeged.hu.
E-mail: kovacs@ovrisc.mdche.u-szeged.hu; Fax: +36 62 54 59 71; Tel: +36 62 54 51 45

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The optimization of PNA oligomer synthesis has been accomplished employing Fmoc/acyl-protected monomers on TentaGel™ and Wang resins. Among the tested activating agents (CMP, BET, HATU) the latter was of choice in solid phase syntheses. "Leakage" of TentaGel™ resin greatly hampers the solution and MS analyses. Synthesis and acyl group deprotection steps have been separately examined using Wang resin. Optimal conditions also worked well on the CPG support. HPLC and MS analyses of the PNA oligomers were carried out under various conditions.

Introduction

Peptide nucleic acids (PNA) are important tools in molecular biology and in drug design owing to their antisense and antigene effects.[1–4] They have the advantage of greater *in vivo* stability and stronger hybridization ability in comparison with native oligonucleotides. However, they enter less easily into the cells and cell nuclei and have low RNAseH activity. PNA conjugates incorporating peptides,[5] oligonucleotides,[6–9] carbohydrates[10] or lipophilic molecules,[11] respectively, might be considered as possible solutions for the above problems.

In order to synthesize PNA oligomers and their conjugates with peptides or oligonucleotides, suitably protected monomers are indispensable and they have to be compatible with both peptide and oligonucleotide synthesis protocols. To date this problem has not been solved as shown in Table 1. The monomers protected by Fmoc (backbone) and acyl (nucleobase) groups (Fig. 1)[12] could fulfil the requirements of PNA–oligonucleotide conjugate synthesis. This combination of protecting groups has not been thoroughly explored,[13–17] the advantages and pitfalls have not been characterized in detail. There are a few reports on optimization of PNA oligomer syntheses,[15,18–22] however, there are so many variables affecting the effectiveness of the synthesis (nature of solid supports and monomers including the protecting groups of the latter, coupling reagents and conditions *e.g.* bases, preactivation, reaction time, mixing sequence, monomer excess, repetition of couplings *etc.*) that it is impossible to simply establish the optimal conditions for the preparation of PNA oligomers. The present report describes our efforts directed towards the elaboration of a PNA oligomer synthesis protocol convenient for the synthesis of PNA–oligonucleotide conjugates and their HPLC and MS analysis. This paper complements and details our preliminary account on this subject.[23]

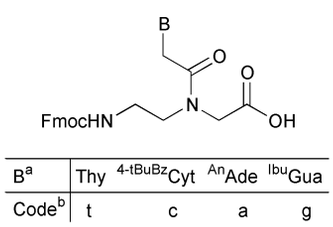

Fig. 1 The structure of Fmoc/acyl-protected peptide nucleic acid (PNA) monomers. a. Thy: thymin-1-yl, $^{4\text{-tBuBz}}$Cyt: N^4-(4-*tert*-butylbenzoyl)cytosin-1-yl, ^{An}Ade: N^6-anisoyladenin-9-yl, ^{Ibu}Gua: N^2-isobutyrylguanin-9-yl. b. Abbreviated notation of unprotected or protected monomer units, depending on the context.

Table 1 Compatibility of protecting group strategies with peptide and oligonucleotide protocols

Protecting groups[a]	Compatibility
Boc/Cbz[b](Benzyl)	Peptide
Fmoc/Cbz[b]	Peptide
Fmoc/Bhoc[c]	Peptide
Fmoc/Mmt[d]	Peptide
Fmoc/acyl	Oligonucleotide
Mmt[d]/acyl	Oligonucleotide

[a] The first abbreviation denotes the protecting group of the backbone while the second one that of the nucleobase. [b] Cbz: benzyloxycarbonyl. [c] Bhoc: benzhydryloxycarbonyl. [d] Mmt: monomethoxytrityl.

Results and discussion

1. Coupling experiments in the solution phase

For the efficient coupling of PNA monomers, an activating agent is required which works quickly with the lowest possible monomer excess, gives high yields and is economically viable. BET[24] and CMP[25] gave almost quantitative yield while HATU gave only 90% yield in solution phase studies in the model reaction between our t monomer and *tert*-butyl phenylalaninate.[26]

2. Initial coupling studies on the solid phase

A solid phase compatible with peptide and oligonucleotide synthesis protocol was needed for PNA oligomer synthesis. Poly(ethylene glycol) attached to polystyrene (known as TentaGel™) meets the above criteria and the removal of protecting groups and final cleavage from the support can be realized also in one step.[27]

In the following experiments coupling yields were determined by UV absorbance of dibenzofulvene-piperidine adduct.

2.1. CMP and BET. Surprisingly, these activating agents gave low yields in solid phase coupling reactions. Thus, they were no longer utilized in further studies. The reasons of this

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Table 2 Coupling yields (%) on TentaGel™ resin using 4 equiv. monomer excess[a]

	a	c	g	t
a	100	92	98, 100	99, 100, 100
c	83, 100, 86	93, 100, 89	96	100
g	100	100, 93, 100	91, 91	100, 100, 100
t	100	97, 88	92, 83	97, 97, 98

[a] Data taken from the synthesis of sequences **1**–**4** (*cf.* Table 4). Monomers (rows) were coupled to the monomers on the resin (columns).

discrepancy between the solution and solid phase experiments are currently still under investigation.

2.2. HATU. During the synthesis of the first sequences on FmocGly-O-TG resin 2 equiv. of monomer was not sufficient even after prolonged reaction time thus the excess was increased to 4 equiv. resulting in 95% average yield (Table 2). When the monomers were coupled to t the yield was almost quantitative in all cases. The reactions c + a and t + g were the lowest yielding (<90%) while couplings to c or g monomer were in the 91–96% range.

3. Problems with the PNA oligomer synthesis on TentaGel™

The synthesis runs smoothly according to the UV absorbances but there is a problem with the solubility of the product, consequently also with its analysis, and this problem was increased with the length of oligomer.

3.1. Ethylene glycol oligomers in the ESI-MS spectra. In the ESI mass spectra of PNA oligomers with different chain lengths synthesized on TentaGel™ a series of peaks corresponding to the cluster ions $[HO(CH_2CH_2O)_nH + 2NH_4]^{2+}$ was observed. This process can be the result of decomposition of the resin during storage, however, the above peaks were present even after thorough washing. The fracture of TentaGel™ beads resulting in the release of oligo(ethylene glycol) fragments ("leakage" of the support) has been a known phenomenon,[27] however, there are no reports on the fact that this would interfere with the synthesis of oligomers. Probably, the "leakage" causes problems only if the synthesis is performed on a low scale.

Oligo(ethylene glycol) chains make the spectra rather noisy but applying the nano-ESI technique[28] a clear spectrum is observable most likely due to separation inside the gold-coated capillary tube of the nano-ESI-MS interface as is represented by the mass spectra of H-Gly-ggtt-Gly-NH_2 (compound **3**, Fig. 2). The attempted purification of PNA samples by precipitation to separate them from the contaminating oligo(ethylene glycol) was not successful.

3.2. Incomplete removal of isobutyryl (Ibu) protecting groups (presence of $[M + 70 + H]^+$ peaks). Capped (*e.g.* Ac-gtt-Gly-NH_2) and incompletely deacylated sequences (*e.g.* peaks $[M + Ibu + H]^+$) were also present in the MS spectra beside the expected product (Fig. 2, lower panel). To determine the ratio of capped, protected sequences to that of the product an HPLC analysis was needed whereby these compounds could be separated. In order to separate the coupling and acyl deprotection steps, the polystyrene-based, acid sensitive Wang resin was employed because the protected oligomer can be cleaved with TFA and the acyl group deprotection can be realized by NH_3 treatment in solution.

4. Syntheses on Wang resin

Similar results were obtained with 4 equiv. monomer excess during the synthesis of test sequence **1**.[18] As a compromise between monomer excess and coupling efficiency, compound **5** was synthesized using 3 equiv. of monomers resulting in

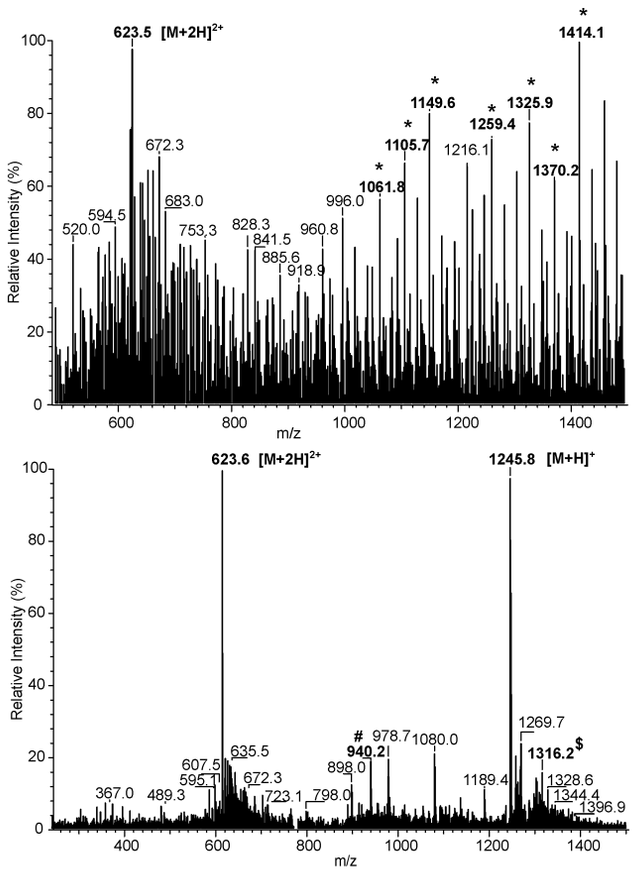

Fig. 2 Mass spectra of PNA oligomer **3** (M_{calc} 1245) synthesized on TentaGel™. Upper panel: ESI-MS spectrum. The values marked with an asterisk denote the cluster ions $[HO(CH_2CH_2O)_nH + 2NH_4]^{2+}$. Lower panel: nano-ESI-MS spectrum. Peaks denoted with # and $ signs correspond to the ions [Ac-gtt-GlyNH_2 + H]$^+$ and [M + Ibu + H]$^+$, respectively.

98% average yield per cycle and HPLC chromatogram of the products (Fig. 3) confirmed that this excess is optimal. It is noteworthy that the highest ever reported coupling yield (99.4%) was achieved at the expense of 7-fold monomer excess.[19]

To find appropriate HPLC conditions for separation of the product from capped sequences and anisoyl derivatives (An–X, X = NH_2, OH originating from the protecting group) the solution of oligomer **5** was analyzed on different HPLC column packings in order to examine the effects of pH of the eluting solution (TEAA, pH 7.0 or dilute TFA, pH ~ 2) and temperature (rt or 55 °C) on the resolution (Table 3 and Fig. 4).

TFA buffer gives better resolution than TEAA as exemplified by the separation of pentadecamer **5** on LiChrospher RP Select B column (Fig. 4, traces A and B). Apparently, this molecular size does not require 300 Å pore size for an efficient separation.

For shorter sequences better resolution was observed on Jupiter packing with very sharp peaks. However, for longer sequences the product and the anisoyl derivatives could not be separated on this packing (Fig. 4, trace C). The separation was good on the less apolar Vydac column as well but in this case the peaks were broader than on other column packings (Fig. 4, trace D). The retention times were very similar on the above-mentioned packings in TFA solution. The effect of temperature[29,30] was also studied, however, there was no significant improvement in the resolution therefore further separations were carried out at room temperature. In conclusion, the LiChrospher RP Select B column using a TFA-containing eluent provides the optimum conditions for PNA analysis and purification.

Oligomers **3**, **4**, **6**–**8** (Table 4) were synthesized to check the completeness of protecting group removal (it is well known that

Table 3 Comparison of analytical HPLC columns and conditions applied in the analysis of PNA oligomers

Column name[a]	Pore size (Å)	Reverse phase type	End-capping	Eluent[b]	t_R[c] (min)
Jupiter[d]	300	C18	Yes	TFA[e]	17.2
Vydac[f]	300	C4	—[g]	TFA[e]	16.3
LiChrospher RP Select B[h]	60	C18	Yes	TFA[e]	17.6
				TEAA[i]	20.9

[a] Size of columns: 250 × 4.6 mm. [b] Principal component of buffer. [c] Retention time of compound **5** at 25 °C. [d] Phenomenex, Torrance, CA, USA. [e] 0.1% (v/v) Trifluoroacetic acid. [f] The Separations Group, Grace Vydac, Hesperia, CA, USA. [g] Data not available. [h] Merck Ltd. Budapest. [i] 0.1 M Triethylammonium acetate.

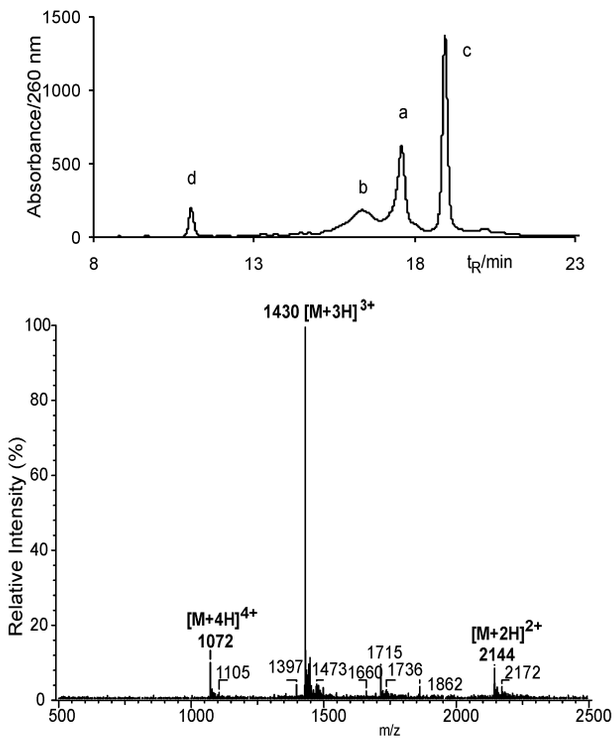

Fig. 3 Upper panel: HPLC chromatogram of the PNA sequence **5** prepared on a Wang resin (crude product, LiChrospher RP Select B column, TFA-containing eluent). Peak a: product, peak c: anisoyl derivatives, peaks b and d: unidentified. Lower panel: ESI mass spectrum (purified product, M_{calc} 4284).

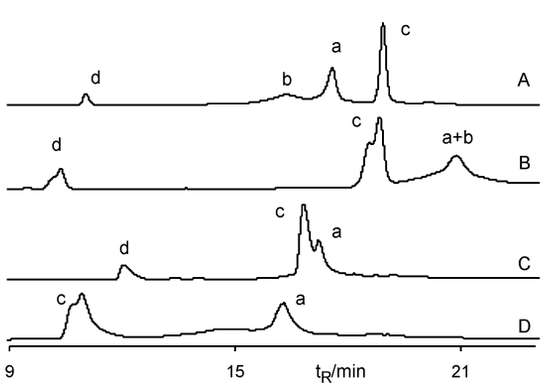

Fig. 4 HPLC chromatograms of the crude oligomer **5** under different conditions. Trace A: LiChrospher RP Select B column, TFA eluent; trace B: LiChrospher RP Select B column, TEAA eluent; trace C: Jupiter column, TFA eluent; trace D: Vydac column, TFA eluent. Peak a: product, peak c: anisoyl derivatives, peaks b and d: unidentified. (*cf.* Table 3).

in oligonucleotide synthesis the rate of deprotection decreases in the order 4-tBuBz > An > Ibu).[31]

The removal was complete in the case of anisoyl and 4-*tert*-butylbenzoyl groups but a small amount of oligomers containing residual isobutyryl groups were detected (Fig. 5).

When the deprotection time was elongated for 48 h the oligomer decomposed. The exocyclic amino group of cytosine with other bases (*e.g.* primary amines) can be converted to the corresponding amine.[15] Indeed, *n*-butylamine, hydrazine or diethylamine furnished complex product solutions even after prolonged reaction times. Thus, the routinely applied ammonia solution was used for cleavage as no better one was found.

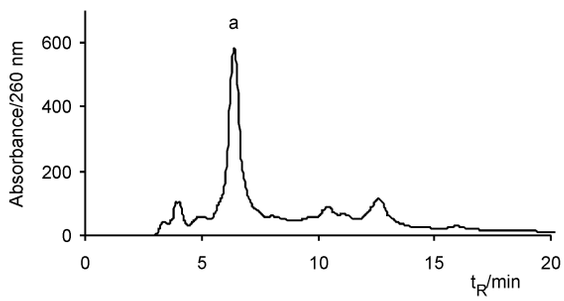

Fig. 5 HPLC chromatogram (Vydac column, TFA-containing eluent) of a crude PNA oligomer (peak a: compound **8**) after detachment from the support (Wang resin) and deprotection.

5. Synthesis on CPG

In order to prepare ON-PNA conjugates Wang resin is not an appropriate support for automated ON synthesis, thus we have turned to controlled pore glass (CPG) that is usually employed for this purpose.

Nonamers **9**, **10** and **11** were prepared on Fmoc-Gly-^{Bz}C-CPG under conditions optimized earlier, where 2′-deoxycytidine unit (^{Bz}C) is a kind of linker that was sacrificed during the cleavage. The HPLC chromatogram of the crude product **9** shows that the synthesis runs smoothly as was expected based on UV absorbances (Fig. 6). Surprisingly, both the ESI- and MALDI MS spectra of the crude product were too noisy due to the capped sequences that were present in low concentration based on HPLC analysis. The unambiguous identification of the product was achieved by MALDI-TOF analysis of the purified sample.

The *C* terminus of this substance is in both carboxy and carboxamide forms as can be seen from the isotopic pattern distribution in the MALDI-TOF-MS due to its high sensitivity. The molecular ions $[M + H]^+$ and $[M - H]^-$ were observed both in positive and negative modes which is also indicative of the presence of carboxy *C* terminus.

Conclusion

The synthesis of PNA oligomers has been elaborated using Fmoc/acyl-protected monomers. Although among the three activating agents (HATU, CMP, BET) the latter two were more effective in solution phase experiments the first one proved to be the best on the solid phase. Due to the "leakage" of TentaGel™ the product was contaminated and this rendered the mass spectrometric analysis difficult. The coupling and protecting group removal steps have been studied separately on Wang resin. Some isobutyryl group-containing PNA sequences remained in negligible amount with the classical (25% aq NH_3,50 °C, 16 h) cleavage condition.

Table 4 PNA sequences synthesized and MS data

Sequence[a]	M_{calc}	Peaks observed (ESI-MS)[b]
H-Gly-cgg act aag tcc att gc-Gly-NH_2 (**1**)[c]	4714	–[d]
H-Gly-ag tcc att gc-Gly-NH_2 (**2**)[c]	2814	–[d]
H-Gly-ggtt-Gly-NH_2 (**3**)[c]	1245	1245.8 $[M + H]^+$; 623.6 $[M + 2H]^{2+}$
H-Gly-aacc-Gly-NH_2 (**4**)[c]	1183	1184.2 $[M + H]^+$; 592.9 $[M + 2H]^{2+}$
H-Gly-gaa cat cat ggt cgt-Lys-OH (**5**)[e]	4284	2144.0 $[M + 2H]^{2+}$; 1430.0 $[M + 3H]^{3+}$; 1072.0 $[M + 4H]^{4+}$
H-Gly-a-Gly-NH_2 (**6**)[c]	406	406.9 $[M + H]^+$
H-Gly-g-Gly-NH_2 (**7**)[c]	422	423.0 $[M + H]^+$
H-Gly-g_5-Lys-OH (**8**)[e]	1658	1657.7 $[M + H]^+$; 830.3 $[M + 2H]^{2+}$
Ac-cat ggt cgt-Gly-NH_2 (**9**)[f]	2565	2565.97 $[M + H]^+$; 1283.48 $[M + 2H]^{2+}$, 856.15 $[M + 3H]^{3+}$
H-Gly-cat ggt cgt-Gly-NH_2 (**10**)[f]	2580	2581.28 $[M + H]^+$; 1291.08 $[M + 2H]^{2+}$
H-cat ggt cgt-Gly-NH_2 (**11**)[f]	2523	2524.03 $[M + H]^+$; 1262.5 $[M + 2H]^{2+}$

[a] PNA oligomer (one-letter lower-case abbreviations) and amino acid (three-letter codes) sequences have been depicted from *N* to *C* termini. *C* termini of PNA oligomers cleaved from the support with aq. NH_3 solution are both in carboxamide and carboxy forms but for simplicity only the former one is denoted. [b] Peaks obtained with the ESI technique are given for the most intensive monoisotopic peaks. For MS spectra obtained with the MALDI technique (compounds **9**–**11**) the values are given for the smallest monoisotopic peak. [c] Synthesized on TentaGel™ S–OH 130 resin. [d] Obscured by oligo(ethylene glycol) fragments. [e] Synthesized on Wang resin. [f] Synthesized on CPG support.

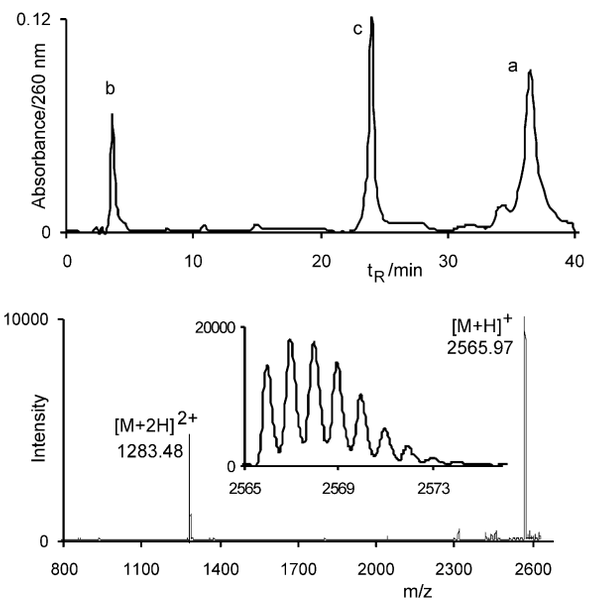

Fig. 6 Upper panel: HPLC chromatogram of the PNA sequence **9** prepared on a CPG support (crude product, LiChrospher RP Select B column, TFA-containing eluent). Peak a: product, peak c: anisoyl derivatives, peak b: 2′-deoxycytidine. Lower panel: MALDI mass spectrum (purified product, M_{calc} 2565). Inset: isotopic distribution of the molecular ion.

The PNA oligomer synthesis runs smoothly on CPG and this support is a promising candidate for the preparation of oligonucleotide–PNA conjugates with a suitable linker.

The best HPLC resolution of oligomers could be achieved with TFA solution on a LiChrospher RP Select B column.

In our experience it is not worthwhile to measure mass spectra of the crude samples because these spectra are rather noisy from the capped sequences even if the latter are present in low concentration.

Our Fmoc/acyl-protected PNA monomers, with certain limitations, can successfully be applied in the preparation of PNA oligomers and their conjugates. Alternatively, other possible methodologies should be evaluated for nucleobase deprotection and consecutive detachment from the solid support. Studies addressing these issues are in progress.

Experimental

General

Abbreviations: controlled pore glass (CPG); TentaGel™ S-OH 130 (TG-OH); triethylammonium acetate (TEAA); retention time (t_R); 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU); *N,N*-diisopropylethylamine (DIPEA); 2-bromo-3-ethylthiazolium tetrafluoroborate (BET); 2-chloro-1-methylpyridinium iodide (CMP, Mukaiyama reagent). TG-OH was obtained from Merck (catalogue no. 814786, capacity: 0.2–0.3 mmol g^{-1}, particle size: 130 μm), 500 Å CPG (~40 μmol g^{-1}) preloaded with 2′-deoxycytidine from ChemGenes Corporations (Ashland, MA, USA), whereas HATU was from PE Biosystems (Warrington, UK). Other chemicals were purchased from Aldrich, Fluka, Merck or Bachem. Anhydrous solvents were prepared as described.[32] DMF refers to an anhydrous solvent, and lutidine to the 2,6-isomer. HPLC chromatography was performed on an HP1050 (room temperature) or on an HP 1090 (55 °C) or on a Shimadzu LC 10 (in case of compounds **9**–**11**) instruments under the following conditions: (a) column packings: Rutin RP column C18, 300 Å, 250 × 4 mm (supplier: BST Ltd. Budapest, Hungary), LiChrospher RP Select B column C18, 60 Å, 250 × 4 mm (supplier: Merck Ltd. Budapest, Hungary), Vydac C4, 300 Å, 250 × 4.6 mm, Jupiter C18, 300 Å, 250 × 4.6 mm; (b) detection: 260 nm; (c) flow rate: 1.0 mL min^{-1}; (d) eluents: A: 0.1 M aq TEAA (pH 7.0), B: acetonitrile–0.1 M aq TEAA (pH 7.0) 8 : 2 (v/v); A: 0.1% aq TFA (pH 2.0), B: 0.1% in TFA acetonitrile–water 8 : 2 (v/v); (e) gradient: 5–35% (v/v) B in A during 30 min or 5–22.5% (v/v) B in A during 40 min (in case of compounds **9**–**11**). UV: PE Lambda 10 instrument. Mass spectrometry: Finnigan MAT TSQ 7000, electrospray (ESI) or nanoelectrospray[28] or Bruker Reflex III MALDI [(in case of compounds **9**, **10** and **11**) reflectron positive mode in 2–3 kDa window using sinapic or 2,5-dihydroxybenzoic acid matrices (the latter is better than sinapic acid in the 1–2 kDa region)]. Calculated molecular weights for peptide nucleic acids are given for the most intensive monoisotopic peaks.

HPLC peaks were identified by MS analysis of the fractions obtained after purification of the product.

Solution phase coupling studies

Reagents used in all coupling experiments have been utilized as stock solutions in anhydrous DMF if not stated otherwise.

To a 0.196 M solution of t monomer in DMF (10.2 μL, 2.0 μmol) one of the following solutions was added:

1. 0.77 M solution of HATU (2.6 μL, 2.0 μmol), 0.6 M solution of lutidine (3.7 μL, 2.2 μmol) and 0.4 M solution of DIPEA (5.0 μL, 2.0 μmol).

2. 0.78 M solution of BET (2.8 μL, 2.2 μmol), 0.4 M solution of DIPEA (11.0 μL, 4.4 μmol).

3. 0.51 M solution of CMP (4.3 μL, 2.2 μmol) and 0.4 M solution of DIPEA (11.0 μL, 4.4 μmol).

After 2 min preactivation, to each of the above solutions *tert*-butyl phenylalaninate (3.0 μmol) in DMF (6.0 μL) was added and the reaction mixture was made up to a total volume of 27.5 μL with DMF. After 5 min, 5 μL samples of the reaction solution were analyzed by HPLC (Rutin column, 1–80% (v/v) eluent B in A during 47 min) at 220 nm. Yields were determined by a calibration curve [peak area (5 μL solution, 220 nm) = f(concentration of t monomer)].

Solid phase synthesis (Tables 2 and 4)

Fmoc deprotection was performed with 20% (v/v) piperidine–DMF (1.5 mL) for 5 min, followed by washing with DMF (2 × 1 mL). 100 μL of this solution was added to EtOH (2 mL) and its absorbance was measured at 290 nm.[33]

The accuracy of the sample collection and absorbance determination is ±2.4% based on data from six independent measurements. [0.700; 0.717; 0.713; 0.718; 0.681; 0.730, where $\bar{x} = 0.7098$ and $\sigma = 0.0171$].

Each coupling was followed with capping with a DMF solution (1.5 mL) containing 5% (v/v) Ac_2O and 6% (v/v) lutidine for 5 min.

Coupling conditions in initial studies on TentaGel™ and Wang resins: monomer (0.1 M soln)–HATU–DIPEA–lutidine = 4 : 3.6 : 4.4 : 4.4 equiv. in DMF for 20 min after 2 min preactivation. Optimal coupling conditions: monomer–HATU–DIPEA–lutidine = 3 : 2.7 : 3.3 : 3.3 equiv. in DMF for 20 min after 2 min preactivation applied on Wang and CPG supports. PNA oligomers were synthesized manually using a home-built shaker in 3 mL Whatman syringes equipped with a 1 μm PTFE filter (catalogue no. 6984–0310) on 25–50 mg of resin on which the first unit was Gly (TG-OH, 0.25 mmol g^{-1} or H-^{Bz}C-CPG, 40 μmol g^{-1}) or Lys (Wang, 0.5 mmol g^{-1}, synthesis scale: 12.5–25 μmol). Cleavage: 25% aq NH_3, 16 h, 50 °C (TG-OH and CPG); in the case of Wang resin first a mixture of TFA and 1,3-dimethoxybenzene 9 : 1 (v/v) (1 mL) was added and it was allowed to react for 3 h. Subsequently, it was treated with aq NH_3 in the same way as above.

Using 3 equiv. monomer excess on Wang resin the following coupling yields (%) were achieved: 96 (a + a); 100 (a + c); 100, 100, 96, 100 (a + t); 94, 99, 95, 94 (c + a); 100, 100, 100 (c + g); 93, 100 (g + g); 100, 100, 96, 100 (g + t); 100, 94, 100, 100 (t + c); 100 (t + g). During synthesis on TentaGel™ there was no problem with the remaining 7 couplings which were not studied in detail on TentaGel™ resin: (a + g); (c + t); (g + a); (g + c); (t + a); (t + t) couplings, only (c + c) was a bit low (93, 100, 89%). (a + c), for example, represents coupling of monomer *a* to *c* that is connected to another monomer or amino acid on the support.

Acknowledgements

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