O-Allyl protection in the Fmoc-based synthesis of difficult PNA

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The synthesis of homothymine PNA-oligomers can be plagued by the occurrence of a significant amount of truncation products, probably because on-resin aggregation hinders access during the coupling reactions. The use of low resin loading and the addition of the chaotropic salt KSCN in DMF allowed a partial remedy by conferring enhancements to the coupling yields. However, protection of the imide group by using *O*-allyl-protected thymine Fmoc-t^{All} provided the most significant improvements to the yields, even in cases where the use of non-protected thymine building blocks resulted in 70% truncation products. Deallylation occurs during the TFA cleavage step. Thus, *O*-allyl-protection can be applied in combination with standard protocols used in automated PNA synthesis.

Introduction

Peptide nucleic acid (PNA) is a DNA mimic with a pseudopeptide backbone.[1] PNA is mainly synthesized using methods known from the solid-phase synthesis of peptides.[2,3] By analogy with peptide synthesis, Boc- or Fmoc-groups serve as temporary protecting groups. Permanent protection of the exocyclic amino groups is usually achieved by means of benzyloxycarbonyl (Z),[4] benzyl (Bn),[5] benzhydryloxycarbonyl (Bhoc)[6] and methoxytrityl (Mmt)[7] protecting groups. Boc/Z-protected PNA building blocks have been shown to provide facile access to PNA and PNA conjugates.[8] However, the majority of automated synthesis equipment is not adapted to the repeated usage of corrosive TFA. Automated synthesis is routinely performed using Fmoc/Bhoc-protected building blocks. This strategy has allowed the synthesis of various PNA constructs, including labelled PNA–peptide conjugates,[9–12] PNA's containing acid labile modifications[7] and DNA–PNA conjugates.[13,14] Problems that have been reported to occur in Fmoc-based PNA synthesis include acyl shift reactions during Fmoc-deprotection[14] and side-reactions at nucleobases, such as unwanted reactions at the oxygen of primary amides.[15] We have faced yet another problem during the synthesis of PNA conjugates; the enormous accumulation of truncation products during the synthesis of PNA that contains extended homothymine segments, which are required for the construction of PNA_2–DNA triplex structures.[14] Failed coupling reactions in solid-phase synthesis are often caused by the formation of secondary structure and aggregation.[16,17] We herein present a solution to this problem in PNA synthesis. A new thymine based PNA building block (t) is presented. It is shown that the use of *O*-allyl protection of the thymine (t^{All}, Fig. 1) provided higher synthetic yields and product purities than the use of classical solvent systems known in the synthesis of difficult peptides. It adds to the attractiveness that no changes to the synthesizer protocols were necessary.

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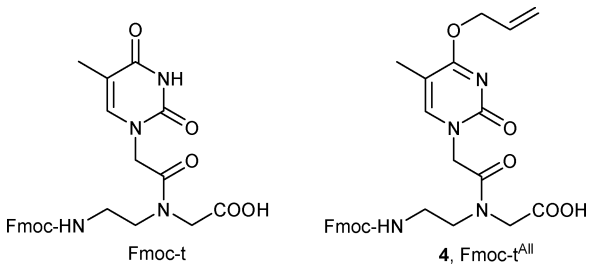


Fig. 1 Fmoc-protected thymidine PNA monomer Fmoc-t and the *O*-allyl protected form Fmoc-t^{All}, **4**. (Fmoc, fluorenylmethoxycarbonyl; All, allyl.)

Results and discussion

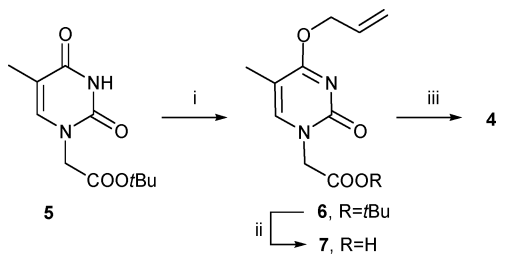
Within an ongoing research project[18] directed towards the development of peptide conjugates that can be switched by hybridization we were in need of PNA–phosphopeptide chimeras such as Gly-t_7-Gln-pTyr-Glu-Glu-Ile-Pro-t_7-Gly **1**, which features two 7-mer homo-t PNA-segments. The automated Fmoc-based synthesis of such chimeras proved difficult. Even double couplings as described by Mayfield and Corey[19] led to the predominant formation of truncation products, which constituted 75% of the crude product (*vide infra*). We considered that the presence of the poly-t PNA stretch may have been the likely cause of this low synthetic yield. Indeed, the Fmoc-based synthesis of t_7-PNA **2** and t_{10}-PNA **3** revealed similar problems in affording the full length products (*vide infra*). Difficult PNA sequences such as very long PNA (>15-mer), homopurine or homopyrimidine PNA have been most successfully prepared by Boc-chemistry.[1,3,20] We reasoned that the repeated exposure to acids such as TFA destroys any secondary structures by protonation of the hydrogen bond acceptors. In seeking an alternative means of secondary structure disruption that would be amenable to Fmoc-solid-phase synthesis, two different approaches were pursued. On the one hand we considered the use of additives such as chaotropic salts and detergents. This approach resembles procedures applied to facilitate the synthesis of difficult peptides.[21] The synthesis of difficult peptides has been most successfully dealt with by coupling building blocks in which amide protecting groups served to prevent hydrogen bonding.[22,23] Thus, on the other hand

we evaluated the coupling of an imide-protected thymine building block.

Building block synthesis

The permanent protection of the O^4-position of thymine was envisioned to disturb the aggregation tendency by the concomitant removal of the hydrogen from the N^3 imide. We chose the allyl group as a protecting group for the O^4-position in thymine (Fig. 1). The allyl group should be stable under Fmoc-cleavage conditions and subsequent coupling reactions. Removal of the allyl group was expected to occur during TFA treatment due to the known acid lability of imino esters.

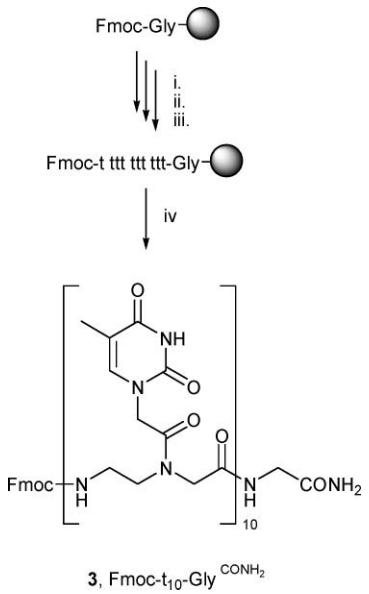
The synthesis of an *O*^{4}-allyl-protected thymine PNA monomer was commenced from the *N*^{1}-alkylated thymine **5** (Scheme 1). The introduction of the allyl group was performed by analogy with Noyori *et al.*'s procedure for the synthesis of *O*^{4}-allyl-thymidine.[24] The O^4-carbonyl oxygen was activated with 2-mesitylenesulfonyl chloride followed by treatment with allyl alcohol. The acid lability of the resulting allyl imidate **6** called for basic *tert*-butyl ester cleavage, which was induced with NaH in DMF.[25] Carbodiimide-mediated coupling of the allyl-protected carboxymethylthymine **7** with the Fmoc-protected aminoethylglycine backbone[2] afforded the desired Fmoc-t^{All} PNA monomer **4** in 32% overall yield.



Scheme 1 Synthesis of the allyl modified thymidine PNA-monomer. i: (a) 2-mesitylenesulfonyl chloride, NEt_3, DMAP, CH_2Cl_2, 5 h; (b) allyl alcohol, DBU, LiCl, NEt_3, 12 h, 80%; ii: NaH, DMF, 1 h, 90%; iii: TCTU, DIPEA, HOBt, Fmoc-aminoethylglycine, DMF, 5 h, 44%. (DBU, 1,8-diazabicyclo-[5,4,0]undec-7-ene; DIPEA, ethyldiisopropylamine; DMAP, *N*,*N*-dimethylaminopyridine; TCTU, 1*H*-6-chloro-benzotriazole-1,1,3,3-tetramethyluronium tetrafluoroborate).

Solid-phase PNA synthesis

The different approaches were evaluated in the synthesis of Fmoc-t_{10}-PNA **3** (Scheme 2). Automated solid-phase synthesis was performed on a 2 μmol scale on a glycine-loaded TentaGel Rink amide resin with a loading of either 182 μmol g^{-1}, 101 μmol g^{-1} or 72 μmol g^{-1} glycine. Chain elongation involved the use of TCTU as the coupling reagent, with 2 min preactivation and 30 min reaction time. The Fmoc-protected oligomer, such as **3**, was cleaved from the resin after 90 min exposure to TFA–*m*-cresol–triisopropylsilane–H_2O (85 : 5 : 5 : 5). The crude product obtained upon precipitation with diethyl ether was dissolved in H_2O–MeCN (35 : 65) to assess the purity, based on relative contents as determined by RP-HPLC-MS analysis. Analyzing in the Fmoc-on mode, with the concomitant increased retention of the full-length products, greatly facilitated product identification.



Scheme 2 Solid-phase synthesis. i: piperidine–DMF (1 : 4), (2 × 3 min); ii: 4 eq. Fmoc-t, 3.6 eq. TCTU, 8 eq. NMM in NMP or in DMF and additives as specified, 30 min: iii: Ac_2O–2,6-lutidine–DMF (5 : 6 : 89), (2 × 3 min); iv: TFA–TIS–*m*-cresol–H_2O (85 : 5 : 5 : 5), 90 min. (NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; TCTU, 1*H*-6-chloro-benzotriazole-1,1,3,3-tetramethyluronium tetrafluoroborate; TIS, triisopropylsilane.)

The crude material contained only 28% of the desired Fmoc-protected t_{10}-PNA **3** when the synthesis was performed in NMP without additives using a high, 182 μmol g^{-1}, resin loading (Table 1).

The majority (70%) of the peak area in the HPLC trace (Fig. 2A) was assigned to truncated products at 3–9 min. MS analysis revealed reduced coupling yields after the introduction of the fifth thymine building block. A reduction in the resin loading from 182 μmol g^{-1} to 72 μmol g^{-1} resulted in an increase in the product content from 28% to 38% (Table 1, see also Fig. 2B). A change of the solvent used in the coupling reactions from NMP to DMF allowed for small improvements at both 182 μmol g^{-1} and 72 μmol g^{-1} resin loadings. The addition of detergents such as 0.1 M sodium dodecylsulfate (SDS) or 0.5% Triton X 100 in DMF had virtually no influence. The additive DMSO, which has previously been reported to confer enhancements to the yields in peptide synthesis,[26,27] led to decreases in the coupling yields as evidenced by the 7% increase in the content of truncation products at both 182 μmol g^{-1}(compare Fig. 2C with Fig. 2D) and 72 μmol g^{-1} resin loading. Interestingly, the use of 0.1 M chaotropic salt KSCN in DMF resulted in an increase in the product content from 35% to 40% at 101 μmol g^{-1} resin loading (compare Fig. 2E with 2F).

The test series listed in Table 1 suggests that the employment of low resin loading confers significant improvements to the coupling yields in the synthesis of difficult PNA sequences. However, the coupling of the *O*-allyl-protected thymidine building block **4** in NMP was found to enable even higher yields (Table 2). Despite the use of high resin loading the content of t_{10}-PNA **3** in the crude material was increased from 28% to 48%. The HPLC trace (Fig. 3B) shows that the use of Fmoc-t^{All}, **4** allowed the most

Table 1 Content[a] of truncated products and PNA oligomer **3** in the crude material obtained after solid-phase synthesis

Additive	Solvent	Resin loading/μmol g^{-1}	Truncation products (%)	PNA product (%)
SDS	DMF	182	67	31
Triton X 100	DMF	182	65	32
DMSO	DMF	182	71	26
DMSO	DMF	72	63	35
KSCN	DMF	101	56	40
—	DMF	182	64	31
—	DMF	101	63	35
—	DMF	72	55	42
—	NMP	182	70	28
—	DMF	72	59	38

[a] Based on the ratio of peak areas as determined by HPLC with detection at 260 nm.

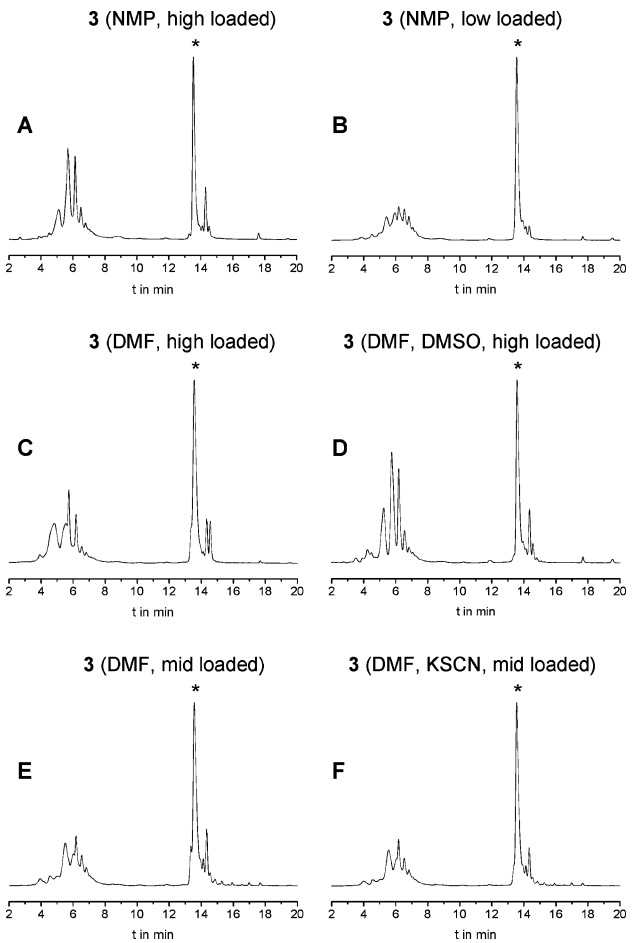


Fig. 2 HPLC traces of crude products **3** that were obtained when NMP was used as the solvent in a synthesis with (A) high and (B) low resin loading, when DMF was used as the solvent with high resin loading (C) without and (D) with DMSO as a cosolvent, and when DMF was used with mid resin loading (E) without and (F) with KSCN as an additive. The asterisk indicates the desired product. (Nucleodur-Gravity C18, 1 ml min^{-1}, 3%–50% buffer B [CH_3CN, 1% H_2O, 0.1% formic acid] in A [H_2O, 1% CH_3CN, 0.1% formic acid].)

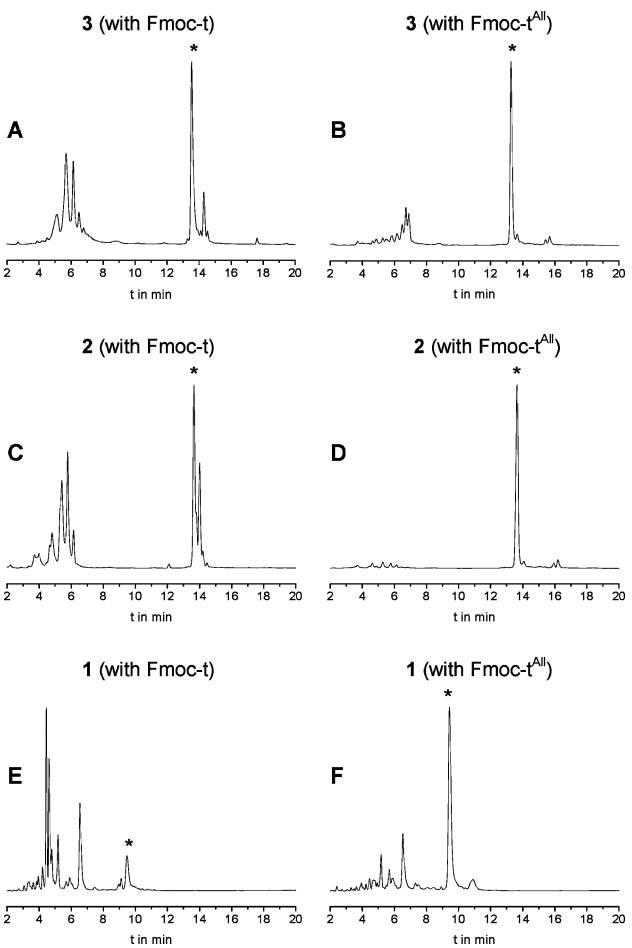


Fig. 3 HPLC traces of the crude products **3** (A, B), **2** (C, D) and **1** (E, F). The asterisk indicates the desired product. Traces B, D and F were obtained when Fmoc-t^{All} building block **4** was used in the synthesis. (Nucleodur-Gravity C18, 1 ml min^{-1}, 3%–50% buffer B [CH_3CN, 1% H_2O, 0.1% formic acid] in A [H_2O, 1% CH_3CN, 0.1% formic acid].)

efficient decrease in the amount of truncation products at 4–8 min. As a result the overall yield of isolated t_{10}-PNA **3** was increased from 4% to 10%. In the synthesis of t_7-oligomer **2**, the use of the Fmoc-t^{All} building block **4** provided a crude material with 68% product content. On the contrary, the content of product **2** was reduced to only 23% when the allyl protection of thymine was omitted. Again, the HPLC traces (Fig. 3C and 3D) provided clear testimony to the improved purity.

The advantage of using t-allyl protection also became apparent when chimera **1** was synthesized. The purity of the crude material

Table 2 Content[a] of product in the crude material and yields[b] of the solid-phase synthesis of homothymine-containing PNA oligomers

Product	Thymine derivative	Content in the crude[a]	Yield[b]
Fmoc-t_{10}-Gly-NH_2, **3**	Fmoc-t	28%	6%
	Fmoc-t^{All}, **7**	48%	10%
Fmoc-t_7-Gly-NH_2, **2**	Fmoc-t	23%	8%
	Fmoc-t^{All}, **7**	68%	14%
Fmoc-Gly-t_7-Gln-pTyr-Glu-Glu-Ile-Pro-t_7-Gly-NH_2, **1**	Fmoc-t	10%	1%
	Fmoc-t^{All}, **7**	48%	4%

[a] Based on the ratio of peak areas as determined by HPLC with detection at 260 nm. [b] The product yield after HPLC purification was determined *via* absorption at 260 nm. The extinction coefficient was $\varepsilon = 114\,000\ M^{-1}cm^{-1}$ for **1**, $\varepsilon = 57\,300\ M^{-1}cm^{-1}$ for **2** and $\varepsilon = 81\,600\ M^{-1}cm^{-1}$ for **3**.

obtained after acidolytic cleavage was increased from 10% to 48% (compare Fig. 3E and 3F). The main deletion products again occurred after the fifth coupling in each of the two PNA-strands. This indicates that allyl protection does not completely solve the problem of reduced coupling yields in the synthesis of long oligo-t PNA.

Conclusions

The collected data suggests that the reduced coupling yields that have been observed in the Fmoc-based solid-phase synthesis of homothymine-containing PNA are most likely caused by on-resin aggregation. Among the approaches known to prevent aggregation in the synthesis of difficult peptides, the use of low resin loading and the addition of the chaotropic salt KSCN in DMF resulted in significant improvements to the coupling yields. Furthermore, we have demonstrated a viable approach that allowed the optimized synthesis of oligothymine-PNA even when high resin loadings were used. A new building block, the O^4-allyl protected t-monomer Fmoc-t^{All} **4** was introduced. Allyl protection of the thymine provided significant improvements to the oligomer purity in cases where the use of non-protected thymine building blocks resulted in 70% truncation products. The overall synthesis time can be shortened because double couplings are rendered unnecessary. In addition, Fmoc-t^{All} **4** can be used in NMP solutions, which provides advantages over DMF solutions which tend to form dimethyl amine. Deallylation occurs during standard TFA-cleavage. Thus, allyl-protection can be applied in combination with standard protocols used in automated PNA synthesis.

Experimental

General

All organic starting materials were purchased in analytically pure grade and used without further purification. The Fmoc-t-PNA monomer was purchased from Applied Biosystems. The amino acid building blocks, Fmoc-Gln(Trt), Fmoc-Glu(tBu), Fmoc-Ile, Fmoc-Pro and Fmoc-Tyr[$PO(NMe_2)_2$] were acquired from Senn Chemicals. HPLC-grade acetonitrile was purchased from Acros, DMF from Biosolve. The solid-phase peptide synthesis was performed by using a Respep Synthesizer from Intavis Bioanalytical Instruments AG . Cleavage of the peptide from the resins was performed in 2 ml PET-syringes from Intavis, that were equipped with Teflon filters (pore size 50 μm). The combined cleavage filtrates were concentrated by rotary evaporation and the crude product isolated by ether precipitation. An Agilent 1100 system with a Gravity C18 3μ 125/4 column, multi wavelength detector and coupled ESI-MS was used in RP-HPLC. Eluents A (H_2O : MeCN : HCOOH = 98.9 : 1 : 0.1 (v : v : v)) and B (MeCN : H_2O : HCOOH = 98.9 : 1: 0.1 (v : v : v)) were used in a linear gradient (gradient 1: 0–20 min, 3–50% B in A, or gradient 2: 0–20 min, 3–80% B in A) at a flow rate of 1 mL min^{-1}. The NMR analysis was performed with a Bruker DPX 300 spectrometer.

***O*⁴-Allyl-*N*¹-*tert*-butyloxycarbonylmethylthymine 6.** To a solution of 1.20 g (5 mmol) N^1-*tert*-butyloxycarbonylmethylen-ethymine **5** in anhydrous dichloromethane (70 ml) under argon were added 430 mg (3.5 mmol) DMAP, 1.97 g (9 mmol) mesitylenesulfonyl chloride and 3.47 mL (25 mmol) triethylamine. After 6 h stirring the solvent was removed at reduced pressure and the oil dried for 30 minutes *in vacuo*. The solid was dissolved in allyl alcohol (80 mL). At 0 °C 1.87 ml (12.5 mmol) DBU, 1.2 g (28.4 mmol) lithium chloride and 5.9 ml (42.5 mmol) triethylamine were added slowly. The solution was stirred for 12 h at room temperature and concentrated *in vacuo*. Chromatography (dichloromethane–methanol 55 : 2) and subsequent washing with ethyl acetate–hexane (1 : 1) yielded 1.11 g (4 mmol, 80%) of product **6** as a colorless solid.

R_f: 0.3 (dichloromethane–methanol 55 : 2); δ_H (300 MHz, DMSO): 1.42 (9H, s, CH_3, *tert*-butyl), 1.90 (3H, s, CH_3, thymine), 4.44 (2H, s, CH_2-CO), 4.83 (2H, dt, *J* 5.3 and 1.5 Hz, CH_2-O, allyl), 5.38 (1H, dd, *J* 17.1 and 1.8 Hz, *trans* CH_2=CH, allyl), 5.27 (1H, dd, *J* 10.5 and 1.5 Hz, *cis* CH_2=CH, allyl), 6.03 (1H, ddt, *J* 17.2, 10.5 and 5.3 Hz, C*H*, allyl), 7.80 (1H, s, *H*⁶-thymine); δ_C (75 MHz, DMSO): 11.9, 28.1, 51.1, 67.1, 82.1, 102.9, 118.3, 133.1, 147.2, 155.7, 167.7, 170.1; HRMS (ESI) *m/z* $C_{14}H_{20}N_2O_4$ + H^+ calcd 281.1496 (M + H^+), found 281.1498.

***O*⁴-Allyl-*N*¹-carboxymethylthymine 7.** To a solution of 500 mg (1.8 mmol) of compound **6** in dry *N*,*N*-dimethylformamide (15 ml) under argon was added a total amount of 300 mg (7.2 mmol) sodium hydride in two steps. After stirring for one hour the suspension was neutralized to pH 6.0 with concentrated formic acid. Chromatography (dichloromethane–methanol–formic acid 93 : 7 : 1) of the residue obtained after concentration *in vacuo* yielded 360 mg (1.6 mmol, 90%) of the desired product **7** as a colorless solid.

R_f: 0.40 (dichloromethane–methanol–formic acid 93 : 7 : 1); δ_H (300 MHz, DMSO): 1.90 (3H, s, CH_3, thymine), 4.46 (2H, s, CH_2-CO), 4.81 (2H, dt, *J* 5.3 and 1.5 Hz, CH_2-O, allyl), 5.38 (1H, dd, *J* 17.3 and 1.7 Hz, *trans* CH_2=CH, allyl), 5.27 (1H, dd, *J* 10.5 and 1.5, *cis* CH_2=CH, allyl), 6.03 (1H, ddt, *J* 17.2, 10.5 and 5.4 Hz,

C*H*, allyl), 7.81 (1H, s, H^6-thymine), 13.08 (1H, s, COO*H*); δ_C (75 MHz, DMSO): 11.9, 50.5, 67.1, 102.9, 118.3, 133.1, 147.2, 155.8, 170.1; HRMS (ESI) *m/z* $C_{10}H_{12}N_2O_4 + H^+$ calcd 225.0870 ($M + H^+$), found 225.0871.

***N*-[*N*-Fluorenylmethyloxycarbonyl-(2″-aminoethyl)]-*N*-{2-[1′-(*O*⁴-allyl-thyminyl)acetyl]}glycine 4.** To a solution of 50 mg (0.2 mmol) of carboxylic acid **7** in DMF (10 mL) were added diisopropylethylamine (100 μL), 79 mg (0.9 mmol) TCTU and 30 mg (0.2 mmol) *N*-hydroxybenzotriazole. The solution was stirred for 10 minutes before a solution of 73 mg (0.2 mmol) Fmoc-aminoethylglycine in DMF (2 mL) was added. After 5 h the solvent was removed *in vacuo.* Chromatography (dichloromethane–methanol–formic acid 93 : 7 : 1) yielded 53.8 mg (0.2 mmol, 44%) of the Fmoc-t^{All} building block **4** as a colorless solid.

R_f: 0.32 (dichloromethane–methanol–formic acid 93 : 7 : 1); δ_H (300 MHz, DMSO, *cis–trans* isomers): 1.89 (3H, d, *J* 4.9 Hz, CH_3, thymine), 3.11–3.44 (4H, m, CH_2–CH_2, backbone), 3.95–4.33 (4H, m, CH-CH_2 Fmoc, N–CH_2-CO backbone), 4.56–4.75 (2H, m, CH_2-CO, linker), 4.80 (2H, dt, *J* 4.3 and 1.3 Hz, CH_2, allyl), 5.39 (1H, dd, *J* 17.3 and 1.7 Hz, *trans* CH_2=CH, allyl), 5.26 (1H, dd, *J* 10.5 and 1.5 Hz, *cis* CH_2=CH, allyl), 6.03 (1H, ddt, *J* 18.3, 10.5 and 5.3 Hz, C*H*, allyl), 7.33–7.89 (10H, m, C*H* Fmoc and C*H* thymine); δ_C (75 MHz, DMSO, *cis–trans* isomers): 11.9, 31.2, 36.2, 38.7, 42.3, 50.5, 54.0, 67.1, 102.9, 118.3, 125.6, 127.5, 128.0, 128.8, 133.1, 141.2, 142.0, 144.3, 147.2, 155.7, 162.8, 170.1; HRMS (ESI) *m/z* $C_{29}H_{30}N_4O_7 + H^+$ calcd 547.2187 (M), found 547.2196.

General procedure for the synthesis of PNA and PNA-peptide chimeras

The solid-phase peptide synthesis was performed on a 2 μmol scale using Fmoc-Gly-loaded TentaGel Rink amide resin. The preloaded resin was suspended in DMF (18 × 200 μL) for 1 min. The automated synthesis was commenced with a deprotection step, which included treatment with 200 μL DMF–piperidine (4 : 1 (v/v)) for 3 min. The reaction was repeated once before washing (10 × 200 μL DMF). For the coupling, 4 equivalents of Fmoc-t^{All} in NMP (final concentration 0.1 M) was preactivated by using 8 equivalents of NMM and 3.6 equivalents of TCTU. This solution was added to the resin after 4 min. The amino acids were coupled by using an excess of 6 equivalents of the monomer (final concentration 0.2 M in NMP), 5.4 equivalents of TCTU and 12 equivalents of NMM. After 30 min the resin was washed (10 × 200 μL DMF). For capping, the resin was treated twice with 200 μL acetic anhydride–2,6-lutidine–DMF (5 : 6 : 89) for 2 min. The resin was washed (10 × 200 μL DMF). Prior to the acidolytic cleavage the resin was washed with dichloromethane (4 × 300 μL). The cleavage solution (1 mL, trifluoroacetic acid–triisopropylsilane–*m*-cresol–water 85 : 5 : 5 : 5) was passed through the syringe reactor for approximately 20 minutes. The filtrate was collected and allowed to stand for 90 minutes in order to complete the removal of the permanent protecting groups. To remove the protecting groups of the phosphotyrosine 10% water was added to oligomer **1** and the reaction mixture was stirred for an additional 12 hours. After concentration under reduced pressure the crude product was precipitated by the addition of diethyl ether. The pellet obtained after centrifugation and disposal of the supernatant was washed with cold diethyl ether and collected by centrifugation.

Fmoc-ttttttt-Gln-Tyr(PO_3H)-Glu-Glu-Ile-Pro-ttttttt-Gly^{CONH2}, 1. 11 mg (2 μmol, 182 μmol g^{-1}) of Fmoc-Gly loaded resin was used. An aliquot (50%) of the crude material was purified by HPLC (gradient 2) to yield 4.6 OD_{260} (39 nmol, 4%); t_R = 9.5 min; (0–20 min: 3–80% B); MALDI-TOF-MS (α-cyano-4-hydroxycinnamic acid); *m/z*: $C_{206}H_{262}N_{65}O_{74}P^+$ calcd. 4860.8 [M(average)], found 4859.9 [M(average)]$^+$.

Fmoc-ttttttt-Gly^{CONH2}, 2. 11 mg (2 μmol, 182 μmol g^{-1}) of Fmoc-Gly loaded resin was used. An aliquot (50%) of the crude material was purified by HPLC (gradient 1) to yield 8.1 OD_{260} (138 nmol, 14%); t_R = 14.0 min; (0–20 min: 3–50% B); MALDI-TOF-MS (α-cyano-4-hydroxycinnamic acid); *m/z*: $C_{94}H_{114}N_{30}O_{31}^+$ calcd. 2158.8 [M(average)], found 2159.1 [M(average)]$^+$.

Fmoc-tttttttttt-Gly^{CONH2}, 3. 11 mg (2 μmol, 182 μmol g^{-1}) of Fmoc-Gly loaded resin was used. An aliquot (50%) of the crude material was purified by HPLC (gradient 2) to yield 8.2 OD_{260} (97 nmol, 10%); t_R = 13.6 min; (0–20 min: 3–50% B); MALDI-TOF-MS (α-cyano-4-hydroxycinnamic acid); *m/z*: $C_{127}H_{156}N_{42}O_{43}^+$ calcd. 2957.1 [M(average)], found 2957.1 [M(average)]$^+$.

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