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Solid-phase Synthesis of Peptide Nucleic Acids

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Abstract: Peptide nucleic acids (PNA) were synthesized by a modified Merrifield method using several improvements. Activation by *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in combination with *in situ* neutralization of the resin allowed efficient coupling of all four Boc-protected PNA monomers within 30 min. HPLC analysis of the crude product obtained from a fully automated synthesis of the model PNA oligomer H-CGGACTAAGTCCATTGC-Gly-NH_2, indicated an average yield per synthetic cycle of 97.1%. N^1-benzyloxycarbonyl-$N6^3$-methylimidazole triflate substantially outperformed acetic anhydride as a capping reagent. The resin-bound PNAs were successfully cleaved by the 'low–high' trifluoromethanesulphonic acid procedure.

Keywords: Peptide nucleic acids, PNA, PNA synthesis

Abbreviations:

A, adenine; aeg, 2-aminoethylglycine; C, cytosine; DECA, diethylcyclohexylamine; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; EDCHA, ethyldicyclohexylamine; G, guanine; HATU, *O*-(7-azabenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HDPU *O*-(1,2-dihydro-2-oxo-1-pyridyl)-*N*,*N*,*N*′,*N*′-bis(tetramethylene)uronium hexafluorophosphate; MDCHA, methyldicyclohexylamine; PNA, peptide nucleic acid; PyBOP, benzotriazolyl-tris-pyrrolidino-phosphonium hexafluorophosphate; Rapoport's reagent, benzyloxycarbonyl-*N*′-methylimidazole triflate; T, thymine; TBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

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Peptide nucleic acids (PNA) are novel DNA mimics in which nucleobases are attached via methylenecarbonyl linkers to a peptide backbone consisting of (*N*-2-aminoethyl)glycine units [1–5]. PNA oligomers have potential as therapeutic agents [6, 7], tools in diagnostics [8], and probes in molecular biology [9]. Their assembly can be accomplished by modified Merrifield synthesis [10, 11] employing Boc-Z protected monomers (see Figure 1) in combination with carbodiimide activation. However, for the synthesis of longer PNA oligomers containing all four nucleobases (>15 residues) we have encountered problems with the quality of the products. Therefore the focus of our recent work was to optimize the synthetic protocol for solid-phase PNA synthesis. To this end, we report a study examining rearrangement of the N-terminal residue as a side reaction during the coupling step. We then describe an efficient way to minimize this side reaction using *in situ* neutralization in combination with coupling reagents such as HBTU or PyBop, and we explore optimal coupling conditions by varying solvent, monomer concentration, tertiary bases and additive. Finally, the use of N^1-benzyl-

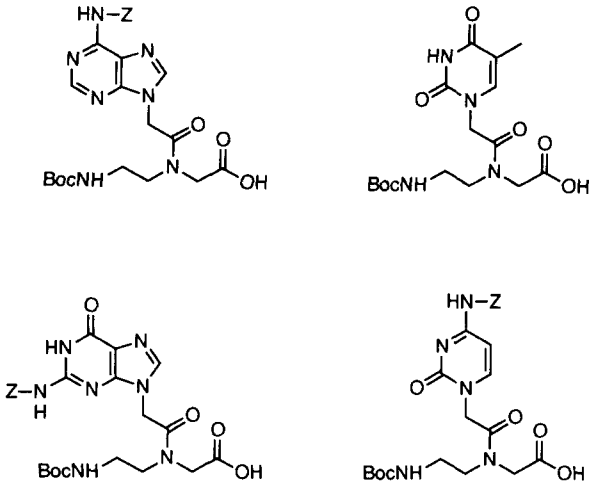

Figure 1 The Boc/Z-protected monomers used in the solid-phase synthesis of PNA oligomers.

oxycarbonyl-N^3-methylimidazole triflate [12] as a capping reagent is compared with the commonly used acetic anhydride.

The carbodiimide-based coupling procedures first described for PNA synthesis require a resin neutralization step prior to the coupling reaction. We realized that under basic conditions the N-terminal residue would undergo the *N*-acyl transfer side reaction displayed in Figure 2. As a result of this side reaction the nucleobase side chain is transferred to the terminal primary amino group. The secondary amino group formed would then react during the coupling step leading to a mixture of structural isomers which might not be chromatographically separable from the desired product and which would not be distinguishable by mass spectrometry.

A series of model experiments was designed to investigate whether the *N*-acyl transfer reaction occurred in a PNA oligomer under the conditions of solid-phase peptide synthesis. As a preliminary experiment, the Boc group was removed from a Boc-T-MBHA resin with 50% trifluoroacetic acid (TFA) in DCM. Following a neutralization (5% DIEA in DCM) and washing (DCM) step, the number of primary amino groups was determined by the quantitative ninhydrin reaction [13] to be 0.39 mmol/g. The deprotected H-T-MBHA resin was then treated with 5% DIEA in DCM and time-course studies (see Figure 3) showed a significant decrease in the number of primary amino groups. Thus, after 125 h the quantity of resin-bound amine reacting with ninhydrin had decreased by 50%. This finding is in agreement with the proposed *N*-acyl transfer side reaction. Surprisingly, a significant decrease in accessible primary amino groups was also observed upon treatment with neat DCM or DMF (see Figure 3).

In a second experiment the rearrangement of the phenylethyl amides of the A- and T-monomers, i.e. **1** and **2**, respectively, were monitored. The rate of migration was estimated to be $t_{1/2} \approx 20$ h (**1**) and $t_{1/2} \approx 50$ h (**2**) in 5% DIEA in DCM. By substituting DCM with DMF both migration rates were reduced by a factor of two. An equilibrium between the parent and the rearranged form was established in DCM at a 3:17 (**1**) and 1:19 (**2**) ratio, favouring the rearranged product.

A third experiment was designed to establish whether the *N*-acyl transfer reaction occurs under aqueous conditions. This is of specific interest with regard to purification and handling of PNA oligomers. Two purified PNA oligomers, H-T_5-Gly-NH_2 (PNA-1) and H-Gly-T_5-Gly-NH_2 (PNA-2), were treated with 30% aqueous ammonia for 4 h at room temperature. High performance liquid chromatography (HPLC) analysis of PNA-1 showed two peaks of identical mass, corresponding to the N-terminal isomers. PNA-2 was unaffected by the treatment, demonstrating the intramolecular nature of the reaction and its prevention by extension or capping of the N-terminal amino group. Therefore, when PNA oligomers are to be used in applications under alkaline conditions the N-terminal residue should be blocked or extended (e.g. by acetylation or addition of a glycine residue).

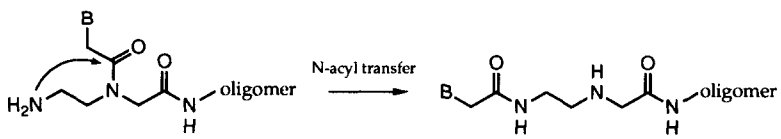

Figure 2 The *N*-acyl transfer reaction, which may occur under neutral and alkaline conditions by attack of the terminal primary amino group on the carboxymethyl nucleobase side-chain (B = nucleobase).

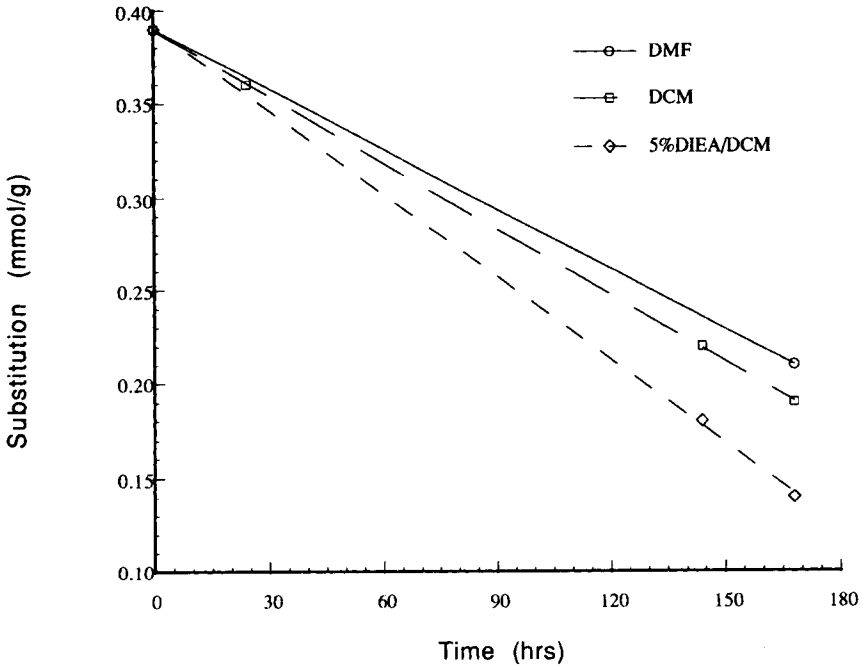

Figure 3 Time courses for the rearrangement of H-T-MBHA resin as measured by the decrease in the number of primary amino groups according to quantitative ninhydrin analysis.

These findings also dictate that the coupling reactions must proceed rapidly to avoid formation of substantial quantities of rearranged products. After a few preliminary experiments, we decided to conduct a systematic study on the use of HBTU and other uronium or phosphonium salt reagents in combination with *in situ* neutralization (i.e. deprotonation of amino groups in the presence of the activated monomer). Several factors related to coupling efficiency were examined. Concentration of the reactants, choice of base (tertiary amine) for *in situ* neutralization, choice of activator, solvents and additives. Different uronium-type coupling reagents were evaluated by synthesis of three non-selfcomplementary 8-mers of the sequence H-TXTXT$_2$XT-Gly-NH$_2$ where X equalled C (PNA-4), A (PNA-5) or G (PNA-6). The uronium salts tested were the widely used HBTU and TBTU, and two new uronium salts HATU and HDPU. All couplings were performed in DMF/pyridine in the presence of DECA at a total monomer concentration of 0.05 M. All of the uronium salts performed well, but HBTU consistently performed slightly better than the other activating agents. The data in Table I (entries 13–24) show the average coupling yields obtained. Another popular coupling reagent, the phosphonium-based PyBop, was then compared with HBTU in the synthesis of H-CGGACTAAGTCCATTGC-Gly-NH$_2$ (PNA-3) which contains all possible coupling combinations of the four monomers. The sequence was designed so that the purine/pyrimidine ratio changes from low to high from the carboxy to the amino terminus, thereby changing the environment for the couplings. Thus, we believe that the coupling yields obtained in this experiment are as expected for the synthesis of other PNAs under similar conditions. The couplings were again performed in DMF/pyridine in the presence of DECA with a total monomer concentration of 0.10 M. The highest yield was obtained from the reaction employing HBTU, resulting in 61% of PNA-3 as opposed to 40% using PyBop. This corresponded to average coupling yields of 97.1 and 94.7%, respectively (Table I; entries 3 and 6).

The synthesis of PNA-3 was performed utilizing the HBTU strategy in three different solvent systems: DMF, DMF/DMSO and DMF/pyridine (Table I). The DMF/pyridine solvent system resulted in a product yield of 61% versus 55% for DMF and 22% for the DMF/DMSO mixture. The lower yield with the latter solvent systems appears to result from a few incomplete coupling reactions.

By tradition and convenience the choice of base for peptide synthesis is a tertiary amine as it fulfils several needs; it is soluble, it deprotonates the N-terminal amino group, and it does not react with either the coupling reagents or the activated mono-

Table I. Effect of Monomer Concentration, Solvent, Additive, Identity of Activating Reagent and Identity of Tertiary Amine on Coupling Yield During the Synthesis of PNA Oligomers

Entry	PNA oligomer[a]	Monomer conc. (mol/l)	Solvent	Activat. reagent	Additive	Tertiary amine	Average coupl. yield (%)
1	PNA- 3	0.10	DMF	HBTU		DECA	92.7
2	–	–	DMF/DMSO	–		–	91.5
3	–	–	DMF/pyridine	–		–	97.1
4	–	–	–	–	HOBt	–	96.1
5	–	–	–	–	DMAP	–	94.1
6	–	–	–	PyBop		–	94.7
7	PNA- 4	0.20	–	HBTU		DIEA	97.9
8	–	0.10	–	–		–	97.2
9	–	0.05	–	–		–	95.3
10	–	–	–	–		DMAP	93.7
11	–	–	–	–		MDCHA	95.3
12	–	–	–	–		EDCHA	94.8
13	–	–	–	–		DECA	95.0
14	–	–	–	HATU		–	93.9
15	–	–	–	TBTU		–	93.7
16	–	–	–	HDPU		–	94.0
17	PNA-5	–	–	HBTU		–	96.1
18	–	–	–	HATU		–	95.5
19	–	–	–	TBTU		–	94.7
20	–	–	–	HDPU		–	94.3
21	PNA- 6	–	–	HBTU		–	93.0
22	–	–	–	HATU		–	92.4
23	–	–	–	TBTU		–	92.2
24	–	–	–	HDPU		–	92.7

[a] PNA oligomers are H-CGGACTAAGTCCATTGC-Gly-NH_2 (PNA-3), H-TCTCTTCT-Gly-NH_2 (PNA-4), H-TATATTAT-Gly-NH_2 (PNA-5) and H-TGTGTTGT-Gly-NH_2 (PNA-6).

mers. The commonly used tertiary amine DIEA is not well-suited in PNA synthesis since it forms insoluble salts of the monomers in commonly used solvents (DMF, pyridine). The salts obviously inhibit automation of the chemistry. Consequently, several organic bases were examined as alternatives to DIEA. DMAP and the three tertiary amines DECA, MDCHA and EDCHA were found to solubilize the monomers adequately. The test sequence H-TCTCT$_2$CT-Gly-NH_2 (PNA-4) was then synthesized using HBTU activation in DMF/pyridine, at a final monomer concentration of 0.05 M and a tertiary amine concentration of 0.10 M. The four syntheses were compared with the synthesis using DIEA. Minor differences in quality and yield were observed when the various bases were interchanged (Table I; entries 9–13).

Entries 7–9 in Table I show the effect of monomer concentration on coupling yield. The concentration was varied from 0.05 to 0.2 M. Acceptable yields were obtained with concentrations of 0.1 M or higher. At lower concentrations (0.05 M), the coupling kinetics were disfavoured leading to an increased risk of competing reactions such as the reaction of the N-terminal amino group with the activating reagent.

It has been demonstrated that certain additives, such as DMAP and HOBt, assist the coupling reaction in peptide synthesis and, therefore, their effect on the synthesis of PNA oligomers was evaluated. PNA-3 was synthesized with addition of HOBt (1 eq. based on activator) or DMAP (0.1 eq.) to the activator (HBTU). DECA was used for *in situ* neutralization and the final concentration of monomer in DMF/pyridine was 0.10 M. Unexpectedly, the additives had a deleterious effect on the quality of the crude PNA as judged by the appearance of late eluting side products in the HPLC chromatograms.

Use of acetic anhydride or *N*-acetylimidazole in the presence of DIEA as a capping reagent, led to a mixture of early and late eluting side products. Furthermore, acetic anhydride capping often resulted in a collapse of the synthesis; e.g. in the case of PNA-3 where only minor amounts of the desired product was detected (see Figure 4 A). Mass spectro-

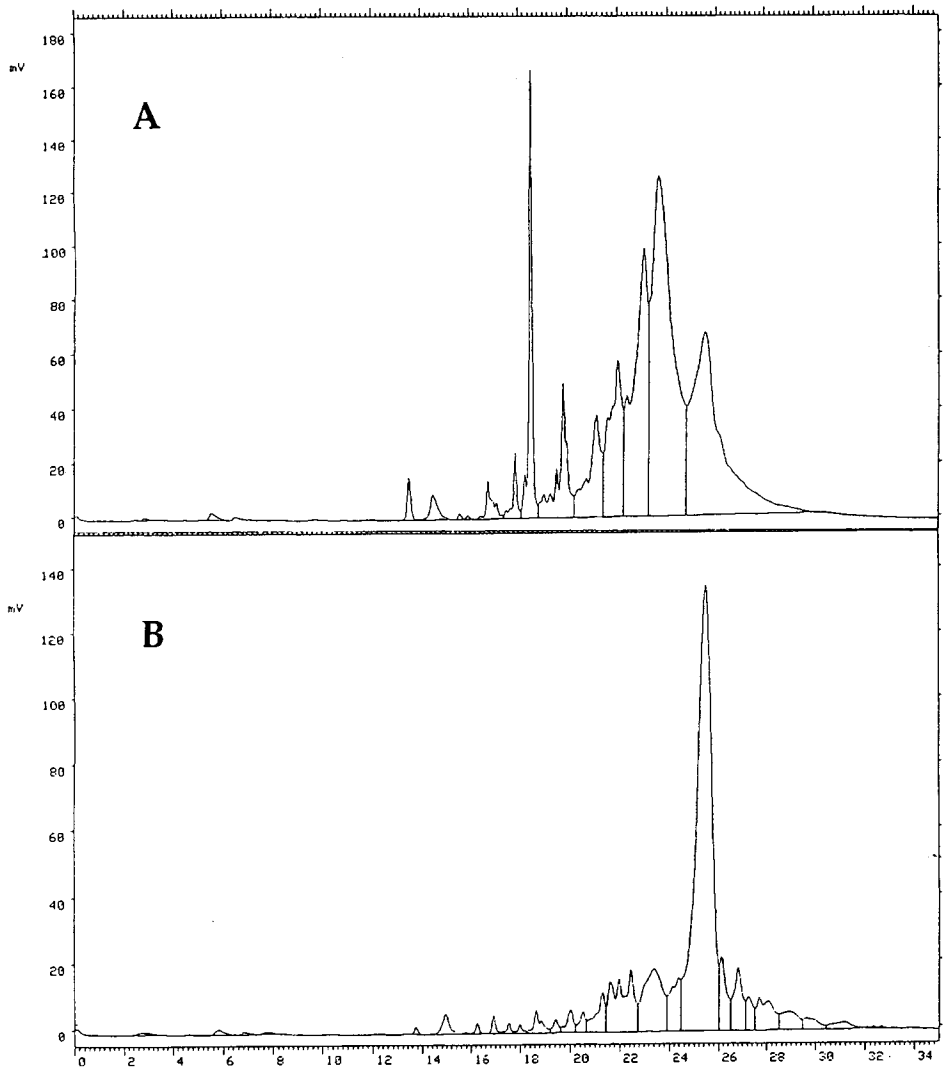

Figure 4 HPLC chromatograms of crude H-CGGACTAAGTCCATTGC-Gly-NH_2 (PNA-3) from syntheses using acetic anhydride (A) and N^1-benzyloxycarbonyl-N^3-methylimidazole triflate (B), respectively, as capping reagents. A C_{18} reverse-phase column was used for the analysis. Buffer A was 0.1 vol% TFA in water and buffer B was 0.08 vol% TFA in CH_3CN. The linear gradient was 0–35% of buffer B in 35 min at 55°C and a flow rate of 1.0 ml/min. The eluents were monitored at 260 nm.

metric analysis indicated the impurities were 'capped' or nucleobase-modified molecules formed by actylation of Boc- or Z-protected amino groups with the acetic anhydride/DIEA mixture. In order to avoid acetylation as a side reaction, we examined N^1-benzyloxycarbonyl-N^3-methylimidazole triflate (Rapoport's reagent) as a capping reagent. This compound would be expected to cap the terminal amino group of an oligomer which had failed to couple as well as to reprotect any nucleobase that might have prematurely been deprotected during Boc removal by TFA treatment. This capping method substantially outperformed acetic anhydride thereby producing HPLC-purified products in high yield (see Figure 4 B).

We have found that the most satisfactory results are obtained if the initial resin loading is 0.1–0.2 meq./g. To achieve this loading, starting resins were derivatized with a limiting amount of PNA monomer followed by a thorough capping with acetic anhydride and drying *in vacuo*. After reswelling the resin overnight in DCM prior to PNA synthesis the first Boc deprotection was typically not quantitative

under the standard deprotection conditions used later in the ongoing synthesis. The cause of this phenomenon is unknown, but it can be reduced by increasing the first deprotection time from 2 × 2 min (95% TFA/*m*-cresol) to 4 × 4 min.

In conclusion, experiments presented here demonstrate that a significant amount of rearranged products can be formed during the solid-phase synthesis of PNA oligomers. Undesired products may arise by an intramolecular *N*-acyl transfer reaction or by inappropriate choice of capping conditions. Therefore the length of time for exposure of the free N-terminal amino group – before and during coupling reactions – should be kept to a minimum and we have found that the combination of *in situ* neutralization and HBTU activation offers a more effective alternative to standard carbodiimide methods in this regard. Furthermore, due to the high reactivity of acetic anhydride or acetylimidazole in the presence of DIEA N^1-benzyloxycarbonyl-N^3-methylimidazole triflate is recommended as a capping reagent.

EXPERIMENTAL PART

Materials

The four PNA monomers, Boc-A(Z)-OH, Boc-C(Z)-OH, Boc-G(Z)-OH and Boc-T-OH were from Millipore. 1% crosslinked *p*-methylbenzhydrylamine resin (0.8 meq. of amino groups/g) and Boc-Gly were obtained from Millipore (Bedford, MA). Other reagents were pyridine (Pierce), DMF (Aldrich), DMSO (Aldrich), acetonitrile (Baker), dichloromethane (Baker), TFA (Pierce), *m*-cresol (Aldrich), TFMSA (Aldrich), DMS (Aldrich), HBTU (Peninsula), TBTU (Millipore), PyBOP (Novabiochem), HATU (Millipore), HDPU (Fluka), DMAP (Aldrich), DECA (Aldrich), MDCHA (Aldrich), EDCHA (Aldrich) and DIEA (Aldrich). The Rapoport reagent was prepared by published methods.

General Methods

A Milligen/Biosearch 7500 was replumbed and reprogrammed to automate the PNA synthesis. Analytical HPLC of the PNA oligomers was performed at 55°C on a Deltapak C_{18} reverse-phase column (0.49 × 15 cm, 10 μm spheres, 300 Å pores) with a Waters 490 HPLC system. Unless otherwise stated buffer A was 0.1 vol% TFA in water and buffer B was 0.08 vol% TFA in CH_3CN. A linear gradient of 0–35% of buffer B in 35 min at a flow rate of 1.0 ml/min was used and the eluent was monitored at 260 nm. The molecular weight of each PNA oligomer was determined using a Waters Power-M3 MALDI-TOF mass spectrometer and sinapinic acid as a sample matrix.

Standard Synthetic Protocol

PNAs were synthesized using the custom-made PNA synthesizer at 1 μmol scale employing a resin loaded with 0.10 mmol Gly/g. Unless otherwise stated the following standard single coupling protocol was used: (1) Boc-deprotection with TFA/*m*-cresol (95:5, v/v), 2 × 2 min; (2) washing with DMF/CH_2Cl_2 (1:1, v/v), 1 × 0.5 min; (3) washing with neat pyridine, 1 × 0.5 min; (4) monomers were dissolved in pyridine and base (2 eq. DECA/monomer) to give a final monomer concentration of 0.1 M; HBTU was dissolved in DMF to give a final concentration of 0.085 M; equal volumes of both the monomer and HBTU solutions were mixed in-line to a final concentration of 0.05 M (5 eq. based on the amount of resin) for the monomer; preactivation was done for 1 min; the activated solution was delivered to the reaction column and coupling was allowed to proceed for 15 min; (5) washing with neat pyridine, 1 × 0.5 min; (6) unreacted amino groups are capped by treatment with Rapoport's reagent (100 μl; 10% w/v in DMF), 1 × 5 min; (7) washing with neat pyridine, 1 × 0.5 min; (8) washing with DMF/CH_2Cl_2 (1:1, v/v), 1 × 0.5 min.

Cleavage

The resins were dried in vacuum prior to cleavage. Cleavage of the PNA from the resin was by the 'low–high' TFMSA method [14] and was performed in an Ultrafree microcentrifuge device equipped with a Teflon membrane (Millipore). A stock solution (A) containing 5 ml of TFA-DMS-*m*-cresol (2:6:2, v/v/v) and a stock solution (B) containing TFA- TFMSA (9:1, v/v) were prepared. Low TFMSA was carried out as follows: (1) washing of *ca.* 20 mg resin (dry weight) with neat TFA, 2 × 2 min; (2) after cooling 10 min on ice bath, 100 μl of A and 100 μl of B were added to the resin; the reaction mixture was left at room temperature for 1 h; (3) the filtrate was removed by centrifugation and the resin was washed once with neat TFA. High TFMSA was carried out as follows: a TFMSA-TFA-*m*-cresol (1:8:1, v/v/v) solution was cooled at 0°C for 10 min; 200 μl was added to the resin after 1 h at room temperature, the Ultrafree microcentrifuge tube containing the reaction mixture was spun

and the PNA product was precipitated from the filtrate by the addition of 1 ml of anhydrous ether. The precipitate was washed twice with anhydrous ether, dissolved in 0.1% TFA and then analysed by HPLC and mass spectrometry.

N-Acyl Transfer Reaction

H-T_5-Gly-NH_2 (PNA-1) and Gly-T_5-Gly-NH_2 (PNA-2) were purified by analytical HPLC. The two oligomers were dried in a speed-vac and resuspended in 30% aqueous ammonia for 4 h at room temperature. The samples were again dried in a speed-vac and analysed by analytical HPLC.

Comparison of Uronium-type Activating Agents

In order to compare various coupling agents three H-TXTXTTXT-Gly-NH_2 was synthesized (where all X positions were either C, A or G, i.e. PNA-4, PNA-5 and PNA-6, respectively) using the 'standard synthetic protocol', except that the coupling agent was varied. The coupling agents employed were HBTU, HATU, TBTU or HDPU and the products were compared by analytical HPLC.

Comparison of Phosphonium and Uronium-type Activating Agents.

The 17-residue H-CGGACTAAGTCCATTGC-Gly-NH_2 (PNA-3) oligomer was synthesized using the 'standard synthetic protocol' employing HBTU as a coupling agent. A second synthesis was carried out using the same protocol, except that PyBop was employed as a coupling agent. The products were compared by analytical HPLC.

Comparison of Tertiary Bases

In order to compare the tertiary bases, PNA-3 was synthesized using the 'standard synthetic protocol', wherein the tertiary base was varied with each successive synthesis. The tertiary bases employed were DMAP, DECA, EDCHA or MDCHA. The products were compared by analytical HPLC.

Comparison of Solvents

PNA-3 was synthesized using the 'standard synthetic protocol', wherein the solvent was varied with each successive synthesis. The coupling solvents employed were DMF, DMF/pyridine or DMF/DMSO. The products were compared by analytical HPLC.

Comparison of Capping Methods

PNA-3 was synthesized using the 'standard synthetic protocol', wherein the capping reagent was varied with each successive synthesis. The capping reagents employed were 10% (w/v) of Rapoport's reagent in DMF or 10% (v/v) acetic anhydride with 2% DIEA in DMF. The products were compared by analytical HPLC.

Evaluation of Coupling Additives

PNA-3 was synthesized using the 'standard synthetic protocol', except that a coupling additive was added to the reaction mixture. The additives employed were HOBt or DMAP. The products were compared by analytical HPLC.

N-((*N*-6-Benzyloxycarbonyladenin-9-yl)acetyl)-*N*-(2-ammonioethyl)glycine-((*S*)-phenylethylamide) Trifluoroacetate (1)

1.0 g (1.9 mmol) of adenine monomer was dissolved in 20 ml of DMF/DCM 1:1. DCC (0.55 g) and DhbtOH (0.37 g) were added. After 2 min phenylethylamine (733 ml) was added and the reaction was left stirring overnight. After addition of DCM (100 ml) the reaction mixture was filtered, extracted with 5% $NaHCO_3$ (2 × 100 ml), with saturated $KHSO_4$/water 1/4 (2 × 100 ml), dried over $MgSO_4$ and evaporated. The resulting foam was dissolved in 5 ml TFA and after 5 min 150 ml ether was added. The precipitate formed was isolated by filtration and washed with ether (2 × 50 ml). Yield 0.90 g (73%) (HPLC > 97% pure). ^{1}H-NMR (DMSO-d6): δ8.91 and 8.78 (1 H, 2d), 8.68 (1 H, s), 8.40 and 8.38 (1 H, 2s), 8.12 and 7.81 (3 H, m), 7.6–7.2 (10 H, m), 5.47 and 5.21 (2 H, s), 5.30 (2 H, s), 5.09 and 4.97 (1H, s) 4.37 and 4.11 (2 H, s), 3.84 and 3.61 (2H, m), 3.25 and 3.03 (2H, m), 1.51 and 1.41 (3 H, d); MS(FAB+): Calcd 531.2; found 531.2.

N-(2-ammonioethyl)-*N*-(thymin-1-ylacetyl)glycine((*S*)-phenylethylamide) Trifluoroacetate (2)

The title compound was prepared from 1.2 g (3.1 mmol) of thymine monomer using the procedure for **1**. This gave 1.29 g (82%) of **2** (HPLC > 99% pure). (^{1}H-NMR (DMSO-d6)): δ 11.32 and 11.29 (1 H, s), 8.81 and 8.70 (1H, d), 8.07 and 7.85 (3H, s), 7.21–7.38 (6 H, m), 4.98 (1 H, m), 4.68 and 4.46 (2 H, q), 4.17 and 4.02 (2 H, s), 3.65 and 3.55 (2 H, m), 3.09 and 2.95 (2 H, m), 1.77 and 1.75 (3 H, s), 1.42 and

1.36 (3 H, d). ^{13}C- NMR (DMSO-d6): δ 168.7; 167.5; 164.4; 151.2; 151.1; 144.0; 142.2; 142.0; 128.4; 126.9; 126.8; 126.0; 126.0; 108.2; 49.4; 50.0; 48.6; 48.3; 48.0; 47.8; 45.7; 45.4; 37.3; 37.0; 22.5; 22.4; 12.0. MS(FAB+): Calcd 388.2; found 388.2.

N-(20(Thymin-1-yl-acetyl)-aminoethyl)-*N*-glycine((*S*)-phenylethylamide) (3)

A solution of 0.5 g (1.3 mmol) in 2 M NaOH/methanol 1:1 was stirred overnight. The solution was extracted three times with ethyl acetate. The organic phase was dried over magnesium suphate and evaporated to a small volume. The title compound was precipitated with ether giving 0.48 g (96%) of 3 (HPLC > 99% pure). (^{1}H-NMR (DMSO-d6)): δ 8.27 (1 H, d), 7.49–7.30 (6 H, m), 5.03 (1 H, m), 4.35 (2 H, s), 3.22 (2 H, d), 3.20 (2 H, s), 2.26 (2 H, m), 1.82 (3 H, s), 1.46 (3 H, d). (^{13}C-NMR (DMSO-d6)): δ; 170.3; 166.9; 164.5; 151.0; 144.6; 142.4; 128.3; 126.6; 126.0; 107.9; 52.0; 49.4; 48.5; 47.7; 22.5; 11.9; MS(FAB+): calcd. 388.2; found 388.2.

Rearrangement of 1 and 2

3.5 mmol of **1** was dissolved in 4 ml of 5% DIEA in DCM or 4 ml of 5% DIEA in DMF. The solutions of **2** were made analogously and the rearrangement was followed by HPLC (10 µl injections). Buffer A was 0.1 vol% TFA in water and buffer B was 0.1 vol% TFA in 90 vol% CH_3CN/water. For **1** the linear gradient was 20–30% of buffer B in 30 min at a flow rate of 1.5 ml/min. For **2** the linear gradient was 0–100% of buffer B in 35 min at a flow rate of 1.5 ml/min.

Procedure Used in H-T-MBHA Resin Time–Course Studies (Figure 3)

A Boc-T-MBHA resin (0.8 g; approx. substitution = 0.5 mmol/g) was placed in a manually operated solid-phase reaction vessel and treated with 50% TFA in DCM (3 × 1 and 1 × 30 min) to remove the N-terminal Boc group. Subsequently, the resin was washed with DCM (6 × 1 min), neutralized with 5% DIEA in DCM (3 × 2 min), and washed with DCM (6 × 1 min). Quantitative ninhydrin analysis [13] of six replicate samples using an extinction coefficient of 15000 $M^{-1}cm^{-1}$ indicated a substitution of 0.39 mmol primary amino groups per gram resin. The resin was divided into three portions. The first portion was subjected to treatment with 5% DIEA in DCM for seven days and the substitution was regularly checked by ninhydrin analysis. The two other portions were treated in a similar way with DCM and DMF, respectively.

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REFERENCES

1. This work was presented in part at the 3rd International Symposium on Solid Phase Synthesis, Oxford, UK, 31 August–4 September, 1993. L. Christensen, R. Fitzpatrick, B. Gildea, B. Warren and J. Coull in: *Innovations and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., SPCC (UK) Ltd, Oxford, UK, in press.
2. P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt (1991). Sequence selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science 254*, 1497–1500.
3. M. Egholm, O. Buchardt, P. E. Nielsen and R. H. Berg (1992). Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone. *J. Am. Chem. Soc. 114*, 1895–1897.
4. M. Egholm, P. E. Nielsen, O. Buchardt and R. H. Berg (1992). Recognition of guanaine and adenine in DNA by thymine and cytosine containing peptide nucleic acids (PNA). *J. Am. Chem. Soc. 114*, 9677–9678.
5. M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden and P. E. Nielsen (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules. *Nature 365*, 566–568.
6. J. C. Hanvey, N. C. Peffer, J. E. Bisi, S. A. Thomson, R. Cadilla, J. A. Josey, D. J. Ricca, C. F. Hassman, M. A. Bonham, K. G. Au, S. G. Carter, D. A. Bruckenstein, A. L. Boyd, S. A. Noble and L. E. Babiss (1992). Antisense and antigene properties of peptide nucleic acids. *Science 258*, 1481–1485.
7. P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt (1993). Peptide nucleic acids (PNA). Potential antisense and antigene agents. *Anti Cancer Drug Design 8*, 53–63.
8. H. Ørum, P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt and C. Stanley (1993). Single base pair mutation analysis by PNA directed PCR clamping *Nucl. Acids Res. 21*, 5332–5336.
9. P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt (1993). Sequence specific inhibition of DNA restriction enzyme cleavage by PNA. *Nucl. Acids Res. 21*, 197–200.
10. R. B. Merrifield (1963). Solid-phase synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc. 85*, 2149–2154.
11. B. Merrifield (1986). Solid-phase synthesis. *Science 232*, 341–347.

12. B. E. Watkins and H. Rapoport (1982). Synthesis of benzyl and benzyloxycarbonyl base-blocked 2'-deoxyribonucleosides. *J. Org. Chem. 47*, 4471–4477.
13. V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield (1981). Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem. 117*, 147–157.
14. J. P. Tam, W. F. Heath and R. B. Merrifield (1986). Mechanism for the removal of benzyl protecting groups in synthetic peptides by trifluoromethanesulfonic acid–trifluoroacetic acid–dimethyl sulfide. *J. Am. Chem. Soc. 108*, 5242–5251.