Convergent Strategies for the Attachment of Fluorescing Reporter Groups to Peptide Nucleic Acids in Solution and on Solid Phase

Oliver Seitz* and Olaf Köhler[a]

Abstract: The site-selective conjugation of peptide nucleic acids (PNA) with fluorescent reporter groups is essential for the construction of hybridisation probes that can report the presence of a particular DNA sequence. This paper describes convergent methods for the solution- and solid-phase synthesis of multiply labelled PNA oligomers. The solid-phase synthesis of protected PNA enabled the selective attachment of fluorescent labels at the C-terminal end (3′ in DNA) which demonstrated that further manipulations on protected PNA fragments are feasible. For the conjugation to internal sites, a method is introduced that allows for the on-resin assembly of modified monomers thereby omitting the need to synthesise an entire monomer in solution. Furthermore, it is shown that the application of a highly orthogonal protecting group strategy in combination with chemoselective conjugation reactions provides access to a rapid and automatable solid-phase synthesis of dual labelled PNA probes. Real-time measurements of nucleic acid hybridisation were possible by taking advantage of the fluorescence resonance energy transfer (FRET) between suitably appended fluorophoric groups. Analogously to DNA-based molecular beacons, the dual labelled PNA probes were only weakly fluorescing in the single-stranded state. Hybridisation to a complementary oligonucleotide, however, induced a structural reorganisation and conferred a vivid fluorescence enhancement.

Keywords: DNA recognition · donor–acceptor systems · fluorescence spectroscopy · peptide nucleic acids · solid-phase synthesis

Introduction

A large portion of current approaches towards the diagnosis and therapy of diseases is gene-oriented by its very nature. The successful completion of the human genome project will likely intensify the research efforts aiming at the identification, detection and remedy of disease-related genes or gene sequences. Synthetic compounds that specifically recognise and bind to a specific DNA or RNA sequence of interest are therefore of great value.[1–3] A particularly successful DNA binding agent is a recently developed class of DNA analogues, the peptide nucleic acids (PNA) (Figure 1).[4–9] The complete replacement of the ribose phosphate backbone with an artificial pseudopeptide backbone resulted in the remarkably improved binding to complementary nucleic acid sequences occurring with both high affinity and high selectivity. Despite its name, PNA is not an acid rendering the hybridisation event largely independent of the salt concentration.[10] A particularly attractive feature is that PNAs are not subject to nuclease or protease mediated degradation and are thus highly stable even in living cells. These properties have been advantageously employed for example in pre-gel hybridisations as alternative to Southern hybridisation,[11] for the identification of point mutations by PCR clamping[12, 13] and fluorescently labelled probes,[14, 15] in the fabrication and usage of PNA probe arrays,[16] and in the isolation,[17] blocking[18–21] and restriction[22–24] of genes or mRNAs.

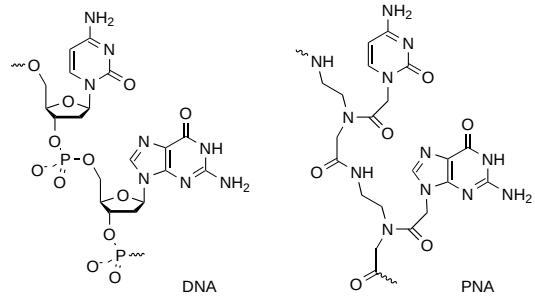

Figure 1. DNA and its analogue PNA.

For applications as diagnostical probes PNAs as well as oligonucleotides have to be equipped with reporter groups.

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However, the highly versatile labelling techniques that involve the use of enzymes such as terminal transferase are not applicable since PNA is not a substrate of any of the known DNA-modifying enzymes. The functionalisation and labelling of PNA, therefore, has to be accomplished by chemical means. Most commonly, fluorophores were appended to the N-terminal amino group of PNA oligomers which corresponds to the 5′-end of oligonucleotides.[25–27] The conjugation to the C-terminal carboxyl group (3′) is difficult since access is blocked during the solid-phase synthesis. An alternative would be the use of internal conjugation sites that could be provided by modified nucleobases or backbones. However, this usually requires the often cumbersome synthesis of modified PNA monomers. It is the aim of the work presented in here to extend the repertoire of the existing methodology available for PNA labelling. It will be shown that the attachment of fluorophores to the C-terminus[28] and to internal conjugation sites[15] is possible by employing convergent strategies and both solution- and solid-phase labelling of PNA will be discussed. Focus of these studies is the rapid synthesis of dual labelled PNA probes[29, 30] which may prove suitable for the sequence specific DNA-detection in homogeneous solution.

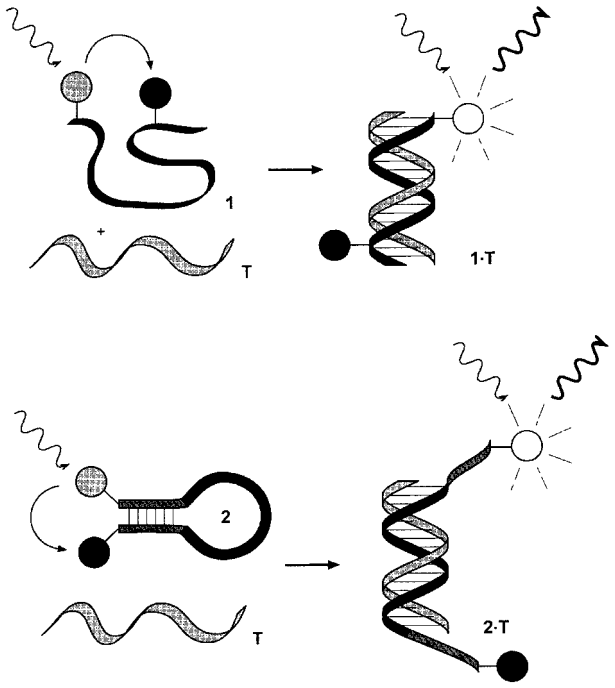

Figure 2. Unhybridised PNA forms intra- or intermolecular associates of unknown structure. The representation of the dual labelled PNA-probe **1** is intended to illustrate that appropriately appended fluorescence donor and fluorescence quencher groups could be located in close proximity. The DNA-based molecular beacons **2** contain target-unrelated arm sequences and are designed to form a stem-and-loop structure. In both **1** and **2**, fluorescence is quenched due to collisional quenching and fluorescence resonance energy transfer (FRET). When the probe sequence anneals to the target sequence **T**, a structural reorganisation increases the donor–quencher distance within the duplexes **1·T** and **2·T** and fluorescence can occur.

Design principles: It was observed that thermal denaturation of single-stranded PNA in contrast to DNA showed a phase transition along with a considerable hyperchromicity, which indicated that base stacking might be a favourable process even in unhybridised PNA.[31] We reckoned that a suitably appended fluorescence donor could be located in close proximity to the fluorescence quencher due to a possible inter- or intramolecular association of PNA single strands (Figure 2). As a result collisional quenching and fluorescence resonance energy transfer (FRET)[32, 33] would diminish the fluorescence of the single-strand **1**. The hybridisation to a complementary nucleic acid (→**1·T**), however, would induce a structural reorganisation that would lead to an increase of the donor–quencher distance. Thus, in the duplex **1·T**, fluorescence would occur serving as a means to detect the complementary nucleic acid in homogeneous solution. This process is similar to the fluorescence dequenching that is observed for structured DNA-based molecular beacons (**2**→**2·T**).[34, 35] Structured molecular beacons based on PNA[36] and PNA/DNA hybrids[37] have also been reported. It is important to note, however, that in the PNA-based approach outlined in here target-unrelated arm sequences, which serve to maintain the structural integrity of the probe, would not be required.

The above-mentioned approach involves the attachment of two reporter groups, a fluorescence donor and a fluorescence quencher. Due to the unknown structure of single-stranded PNA it was unclear how the markers have to be arranged in order to maximise fluorescence quenching of the unhybridised PNA **1**. Hence, a flexible conjugation strategy was desired. An approach, that combines terminal and internal labelling allows for the optimisation of the donor–quencher distance without simultaneously changing the length of the PNA oligomer. For terminal labelling a reporter group can be appended to either the N-terminus or the C-terminus. Both conjugation sites were envisioned to be useful for the approach outlined in Figure 2. The N-terminal amino group is readily accessible during the solid-phase synthesis of PNA and therefore most labelling techniques were focussed on this particular site.[25–27] However, within the framework of a research project aiming at the development of PNA ligation probes, we were also in need for C-terminally modified PNA conjugates. Unfortunately, during solid-phase synthesis the growing PNA oligomer is anchored through this carboxyl group. The liberation of the PNA from the solid phase by routine procedures usually furnishes unprotected oligomers. Since selective modification of the C-terminus are difficult to achieve in the presence of the unprotected exocyclic amino groups of the nucleobases,[38, 39] it was planned to employ protected PNA oligomers. Surprisingly, when we became aware of this synthetic need there had been no method reported for the solid-phase synthesis of protected peptide nucleic acids suitable for further modifications at the C-terminal carboxyl group.[40]

For the attachment of reporter groups to internal positions conjugation sites are required which can be provided by either the nucleobase or the aminoethylglycine backbone. In PNA, the introduction of modified nucleobases or surrogates thereof can be accomplished by applying the well-established methodology of amide bond forming reactions rather than the complex glycosylation reactions that would be necessary for

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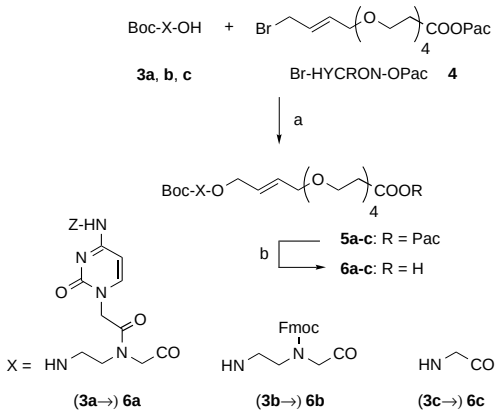
installing the corresponding modification in DNA. A convergent strategy can therefore readily be implemented by employing modified nucleobases. The ease of altering PNA at the nucleobase level had been exploited in several studies.[41–44] However, in all these studies, the non-standard nucleobases were incorporated by using preformed monomeric building blocks. A strategy that would omit the need to synthesise an entire monomer building block in solution would facilitate the rapid synthesis of modified PNA oligomers.[45, 46] As the central building block in this approach an orthogonally protected backbone module such as the Boc/Fmoc-protected aminoethylglycine **3b**[47] would be incorporated during PNA assembly. After removal of the Fmoc group the non-standard nucleobase would be coupled, thereby furnishing the modified monomer on the solid phase.

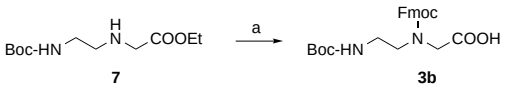
Results and Discussion

Synthesis of building blocks: Currently, two different PNA-building blocks are commercially available, Boc/Z- and Fmoc/Bhoc-protected monomers. The former combine the TFA-removable Boc group for temporary protection with a HF (or TFMSA)-cleavable Z-protecting group for permanent protection of the exocyclic amino groups of the nucleobases.[48, 49] The latter offer a real orthogonal protecting scheme comprised of the base-labile Fmoc group for temporary protection and the acid-labile benzhydryloxycarbonyl group for permanent blockage.[50] Recently, Dts/Z protection was demonstrated to allow for the solid-phase synthesis of fully protected PNA amides.[51] In developing a strategy for the solid-phase synthesis of protected PNA bearing a C-terminal carboxyl group, a linker of general utility was desirable that could enable the use of both types of commercially available PNA building blocks. The allylic HYCRON linker was chosen since the allylic linkage provides a high degree of orthogonality in combination with the Boc- as well as the Fmoc strategy.[52] For the synthesis of preformed starting monomer–HYCRON conjugates such as the cytosine conjugate **6a** a nucleophilic esterification was employed by using the Boc-protected starting monomers **3a** and the allylic bromide **4** (Scheme 1).[53] The subsequent reductive removal of the phenacyl (Pac) ester[54] yielded the Boc-protected HYCRON conjugate **5a**. An analogous reaction scheme provided the conjugates **5b** and **5c** containing Boc/Fmoc-protected aminoethylglycine **3b** and the N-Boc-protected glycine **3c**, respectively. Unfortunately, in all attempts in which a Boc/Z-protected guanosine building block was subjected to the nucleophilic esterification, a complex mixture was obtained with a major product that contained two HYCRON tethers. However, a concomitant allylation of other nucleophilic structures can be avoided by activating the carboxylic group in presence of the anchor alcohol or by constructing the Boc-G^{Z} monomer on the solid phase (see below).



Scheme 1. a) sat. aq. $NaHCO_3$, Bu_4NBr, CH_2Cl_2 (**5a**, 83%; **5b**, 92%; **5c**, 76%); b) Zn, AcOH (**6a**, 79%; **6b**, 94%; **6c**, 89%).

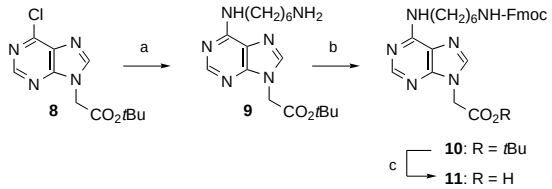
The Boc/Fmoc-protected aminoethylglycine **3b**[47] (Scheme 2) was obtained in a one-pot synthesis starting from the N-Boc-aminoethylglycine ethyl ester **7**,[48] an intermediate



Scheme 2. a) i) 0.4 M NaOH/EtOH (5:8); ii) AcOH; iii) Fmoc-OSu, 91%.

in the synthesis of the conventional Boc/Z-protected PNA monomers. Saponification of the ethyl ester was followed by a careful adjustment to pH 8 and subsequent addition of Fmoc-OSu. Crystallisation furnished the backbone building block **3b** in high purity and high yield.

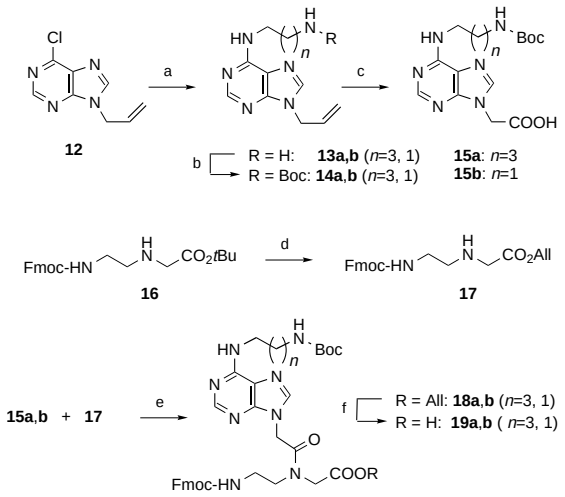
In order to link fluorescent reporter groups to nucleobases additional conjugation sites have to be introduced. Scheme 3 shows the synthesis of the adenine modification **11**. The



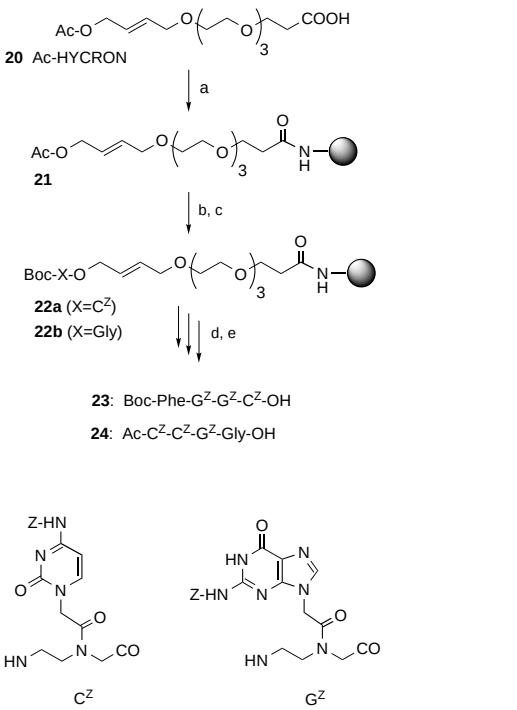
Scheme 3. a) $H_2N(CH_2)_6NH_2$, *n*BuOH, 38%; b) Fmoc-Cl, NMM, CH_2Cl_2, 72%; c) TFA, 95%.

N-Boc protected N^6-alkylamino tether was chosen because it directs the attachment of fluorophores to the major groove of a DNA duplex without detriment to the Watson–Crick base pairing.[55] The synthesis was achieved starting from 6-chloropurine, which was first carboxymethylated to obtain **8**.[56] After separation from the N^7-isomer, **8** was treated with hexamethylenediamine. Subsequent reaction of **9** with Fmoc-Cl blocked the primary amino group and treatment with TFA cleaved the *t*Bu ester to yield **11**.

A rapid synthesis of multiply labelled PNA oligomers is provided by a strategy in which the attachment of the fluorescent reporter groups is performed on the solid phase. When a particular nucleobase modification is intended to be repeatedly used it might be preferable to employ preformed monomer modifications. Scheme 4 describes the synthesis of



Scheme 4. a) **13a**: $H_2N(CH_2)_4NH_2$, *n*BuOH, 42%; **13b**: $H_2N(CH_2)_2NH_2$, *n*-BuOH; b) Boc_2O, DMAP, Pyr (**14a**, 51%; **14b**, 44% based on **12**); c) i) O_3, CH_2Cl_2, MeOH; ii) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*BuOH (**15a**, 58%; **15b**, 71%); d) i) TFA; ii) $SOCl_2$, All-OH, 84%; e) HOBt, DCC, CH_2Cl_2, DMF (**18a**, 76%; **18b**, 84%); f) MeNHPh, $[Pd(PPh_3)_4]$, THF (**19a**, 81%; **19b**, 71%). (DCC = *N*,*N*′-dicyclohexylcarbodiimide).



Scheme 5. a) HBTU, HOBt, NMM, CH_2Cl_2, aminomethyl-polystyrene; b) 1N NaOH/dioxane (1:3); c) Boc-X-OH, DIC, DMAP, d) i) TFA/*m*-cresol (95:5); ii) Boc-B^Z-COOH, HBTU, iPr_2NEt, Pyr; iii) Ac_2O/Pyr; iv) repeat i) – iii); e) $[Pd(PPh_3)_4]$, morpholine, DMSO, DMF (**23**, 36%, **24**, 35% based on aminomethyl-polystyrene). (DIC = *N*,*N*′-diisopropylcarbodiimide, HBTU = *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate, HOBt = 1-hydroxy-benzotriazol, NMM = *N*-methylmorpholine).

Fmoc/Boc-protected adenine monomers suitable for a synthetic strategy in which all reactions including the labelling reactions can be performed on the solid phase. As starting material 6-chloropurine was subjected to a regioselective allylation[57, 58] followed by treatment with tetramethylenediamine or ethylenediamine. Then the primary amino groups of **13a** and **13b** were protected by reaction with Boc_2O. The conversion of **14a** and **14b** to the carboxymethylene derivatives proceeded smoothly by ozonolysis and oxidative workup providing the Boc-protected N^6-aminoalkyl-N^9-carboxymethyladenine **15a** and **15b**. The protected aminoethylglycine unit **17** suitable for the coupling with **15a** and **15b** was synthesised from the known backbone **16**.[59] Treatment of **16** with TFA liberated the carboxyl group, which in a Brenner-type esterification was converted to the allyl ester **17**.[60] This seemingly tedious scheme avoids the δ-lactam formation which can be observed when N-deprotected aminoethylglycine allyl esters are employed. The couplings of the carboxymethylated nucleobases **15a** and **15b** with the backbone **17** were accomplished by activation with DCC[61] and HOBt.[62] Finally, Pd^0-catalysed allyl transfer to *N*-methylaniline furnished the Fmoc/Boc-protected adenine modifications **19a** and **19b**.

Solid-phase synthesis: For the synthesis of the model tetramers **23** and **24** the loading was commenced by coupling the acetylated HYCRON handle **20**[53] to aminomethylated polystyrene resin (Scheme 5). For the determination of the loading yield an NMR assay was developed. A few beads of resin **21** were treated with a mixture of deuterated sodium methoxide in $CDCl_3$ that contained toluene as an internal standard. The acetate formed was quantified by NMR which revealed a quantitative loading reaction. Saponification of the HYCRON-resin **21** liberated the allylic hydroxyl group which was subjected to a esterification using N-Boc protected starting monomers. The subsequent solid-phase synthesis was performed according to the Boc strategy using the loaded resins **22a** and **22b**. In this and all other syntheses acetylation was employed as a means to cap eventually unreacted amino groups. The final detachment was accomplished by a Pd^0-catalysed allyl transfer to the nucleophile morpholine. Purification by silica gel chromatography or size-exclusion chromatography afforded the protected PNA-oligomers **23** and **24** in 36 and 35% overall yield, respectively. It should be mentioned that the protected PNA oligomers were poorly soluble, which might also be responsible for the broad peaks observed in HPLC analysis. MALDI-TOF analysis, however, clearly confirmed the identity of the protected PNAs **23** and **24**.

The determination of the relatively low overall yields reached in the synthesis of **23** and **24** was based on the initial loading of resin **21** with reactive acetyl groups. It thus seemed that the esterification using polymer-bound alcohols did not reach completion. In order to test this hypothesis it was planned to draw on amide bond forming reactions rather than esterification reactions, thereby employing an amino functionalised resin and preformed conjugates of the starting monomer and the HYCRON linker. Hence, HYCRON-conjugates **6a** and **6c** were coupled with the solid support (Scheme 6). Application of the solid-phase synthesis protocol as described above was followed by the Pd^0-catalysed cleavage of the allylic linkage. The subsequent chromato-

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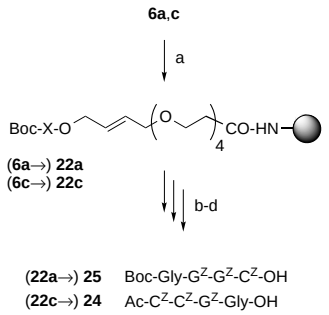
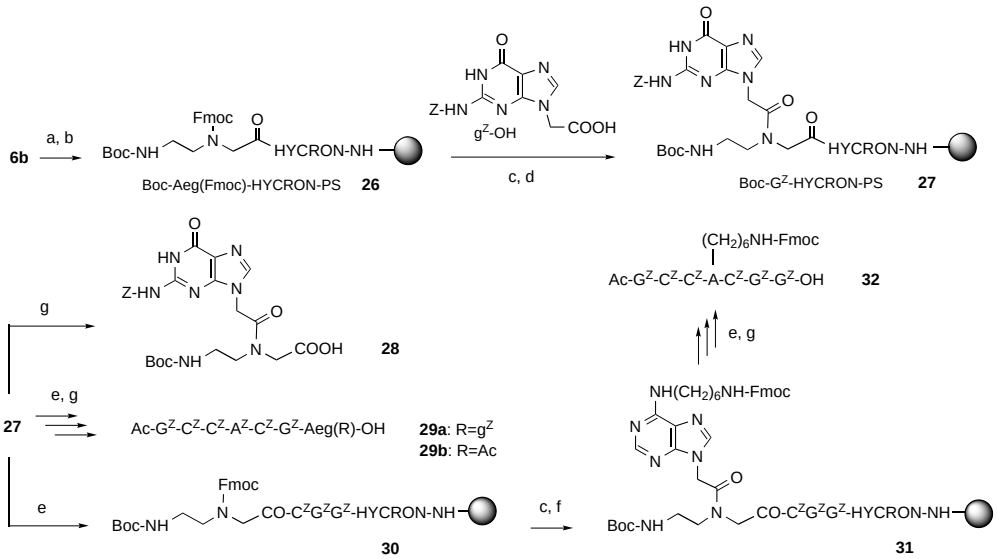


Scheme 6. a) aminomethyl polystyrene, HBTU, *i*Pr_2NEt, HOBt, DMF; b) Ac_2O, Pyr; c) Boc/Z-PNA solid-phase synthesis (see Scheme 5); d) [$Pd(PPh_3)_4$], morpholine, DMSO, DMF (**24**, 59%; **25**, 67%).

graphic purification afforded the protected PNA-oligomers **24** and **25** in 59% and 67% overall yield, respectively. This high overall yield was based on the initial loading of the polystyrene resin with reactive amino groups. It is, thus, noticable that the attachment of the starting monomers through preformed linker conjugates allowed a highly efficient solid-phase synthesis.

It has to be noted that for a general applicability of the above-mentioned strategy a set of at least four different starting monomer–HYCRON conjugates would be required. In addition, the synthesis of conjugates that contain acidic structures such as the guanine imide system has to be performed by acylating esterification, which for the HYCRON anchor is less efficient than nucleophilic esterification. An alternative loading procedure employs the Boc/Fmoc-protected aminoethylglycine conjugate **6b** as a common precursor (Scheme 7). Attachment of **6b** onto the solid support furnished the precursor resin **26**. Treatment with DMF/morpholine liberated the secondary amino group, which subsequently was coupled to carboxymethyl guanine g^Z-OH. The success of this on-resin synthesis[45,46] became apparent when a part of the resulting resin **27** was subjected to the Pd^0-catalysed allyl cleavage. The Boc/Z-protected guanosine monomer was released in a yield of 61% based on the initial loading of resin **26** with Fmoc groups, which exceeds the yield of 52% that can be accomplished in the solution-phase synthesis.[63] For elaboration to the oligomer **29a** resin **27** was extended using the described Boc/Z methodology. The final Pd^0-catalysed cleavage and subsequent HPLC purification afforded the protected 7-mer **29a** in an overall yield of 36%. However, the coupling of the carboxymethyl guanine to the polymer-bound aminoethylglycine did not reach completion and the corresponding acetylated derrivative **29b**, which was easier to purify, was isolated in 39% yield.

The on-resin synthesis of PNA monomers was advantageously applied for the assembly of PNA-conjugate **32**, which provides an conjugation site at an internal position. Starting from resin **26** the linear solid-phase synthesis proceeded as described. Introduction of the aminoethylglycine **3b** yielded the orthogonally protected PNA-backbone resin **30**. After removal of the Fmoc group the adenine modification **11** was coupled by using HATU[64] activation and prolonged coupling times. The photometric determination of the Fmoc loading revealed a quantitative yield for the incorporation of **11**. The subsequent chain elongation was followed by the Pd^0-catalysed cleavage in presence of *N*-methylaniline as an allyl scavenger. As the analysis of **29a** and **29b** had suggested it seemed possible that the on-resin synthesis of the starting guanosine derivative did not reach completion. MALDI-TOF analysis showed two major peaks (Figure 3). One was assigned to the desired product and the other to a product from incomplete coupling of the carboxymethyl guanine g^Z-OH. A compound indicative for a failed introduction of the adenine modification **11** was not detectable by MALDI-TOF MS. This nicely confirmed the Fmoc-loading analysis which had indicated a quantitative yield for this on-resin coupling. The final HPLC purification furnished the PNA heptamer **32**



Scheme 7. a) aminomethyl polystyrene, HBTU, *i*Pr_2NEt, HOBt, DMF; b) Ac_2O, Pyr; c) DMF/morpholine; d) HATU, *i*Pr_2NEt, DMF; e) Boc/Z-PNA solid-phase synthesis (see Scheme 5); f) **11**, HATU, *i*Pr_2NEt, DMF; g) [$Pd(PPh_3)_4$], morpholine (MeNHPh for **32**), DMSO, DMF (**28**, 61%; **29a**, 36%; **29b**, 39%; **32**, 50%).(Aeg = aminoethylglycine, HATU = *O*-(1-oxy-7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate, PS = polystyrene).

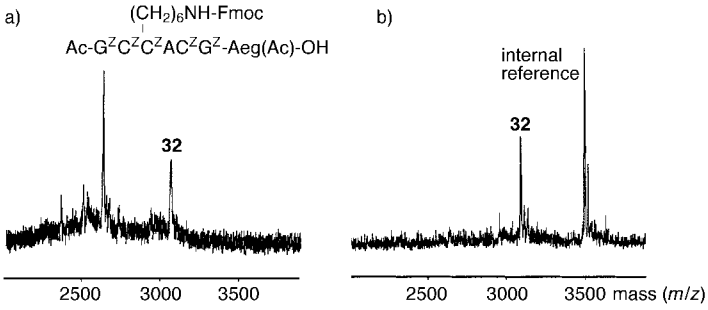
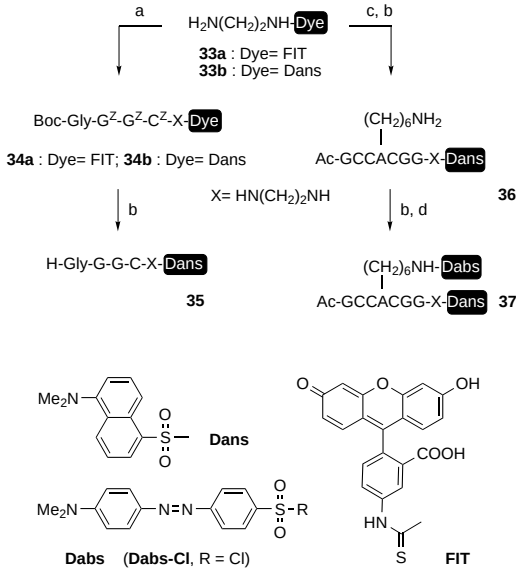


Figure 3. MALDI-TOF-MS of a) crude **32** as obtained after resin cleavage and of b) purified **32** that was mixed with the β-chain of bovine insulin as internal reference.

protected by six Z groups and one Fmoc group in an overall yield of 50% (based on the Fmoc loading of **26**). It can be concluded that the introduction of the adenine modification **11** proceeded smoothly whereas it proved difficult to achieve a quantitative on-resin synthesis of the guanosine monomer (Scheme 7).

C-terminal and internal PNA labelling in solution: Fully protected PNA oligomers such as **23**–**25**, **29a** and **32** allow for selective modifications at the C-terminal carboxyl group. In order to assess the feasibility of coupling reactions which employ oligomeric PNA as acylating agents, the tetramer **25** was used as a model compound, activated with EDC[65] in presence of HOAt and coupled to the fluorescein–ethylenediamine conjugate **33a**[66] (Scheme 8). After size exclusion chromatography the C-terminally labelled PNA **34a** was obtained in high yield. However, the acid sensitivity of the fluoresceinyl group precluded a complete deprotection of **34a**. Hence, a coupling to dansylethylenediamine **33b** was used for C-terminal labelling of **25**. The successive treatment of **34b** with TFA and trifluoromethanesulfonic acid in TFA removed the Boc and Z groups. HPLC purification furnished the C-terminally dansylated PNA **35** in good yield.



Scheme 8. a) **25**, EDC, HOAt, DMF, CH_2Cl_2 (**34a**, 64%); b) *m*-cresol, Me_2S, TFA, TFMSA (1:1:10:1) (**35**, 60% based on **25; 36**, 66% based on **32**); c) i) **32**, EDC, HOAt, DMF, CH_2Cl_2; ii) DMF/morpholine (1:1); d) 4 equiv Dabs-Cl, sat. aq. $NaHCO_3$, DMF, 50%. (Dabs = 4-(4′-dimethylaminophenylazo)benzene sulfonyl, Dans = 5-dimethylaminonaphthaline-1-sulfonyl, EDC = $Et_2(CH_2)_3N=C=NEt \cdot HCl$, HOAt = 1-hydroxy-7-azabenzotriazol).

In addition to a selective modification of the C-terminus the protected PNA conjugate **32** enables the attachment of reporter groups to the Fmoc-protected primary alkylamino group. First, **32** was coupled to the dansylethylenediamine **33b** as described. The subsequent treatment with DMF/morpholine removed the Fmoc group which was followed by the acidolytic removal of the Z-protecting groups. A second reporter group was attached to **36** by using the liberated primary alkylamino group. The reaction with the fluorescence quenching 4-(4′-dimethylaminophenylazo)benzene sulfonic acid chloride (dabsyl chloride) proceeded in high selectivity forming the dual labelled PNA conjugate **37** in high purity.

The examples presented in Scheme 8 demonstrate that protected PNAs indeed serve as suitable substrates for the attachment of reporter groups to the C-terminal carboxyl group. However, it should be mentioned that although the conjugation reactions proceeded smoothly, the sometimes unpredictable solubility properties of protected PNA oligomers can render work-up and purification procedures cumbersome.

PNA labelling on the solid phase: In the work described above the labelling steps have been performed in solution by using protected PNA oligomers that were obtained by solid-phase synthesis. The conjugation reactions and the subsequent work-up involved can be time-consuming, particularly when more than one reporter group has to be attached. A strategy, in which all reactions including the labelling steps can be performed on the solid phase, is thus desirable. For the on-resin labelling it seemed advantageous to combine internal labelling with N-terminal labelling since access to the C-terminus can be hindered on solid phases. The internal conjugation site would be provided by the N^6-aminoalkyl modified adenine which enabled the efficient conjugation of **36** with the dabsyl moiety (Scheme 8). In the examples presented above Boc/Z-protected PNA monomers were used. Many fluorescent labels, however, are subject to degradation under the conditions required for the removal of the permanent Z-protecting groups. A solution to this problem is offered by the combination of a highly orthogonal blocking group strategy with chemoselective conjugation reactions. Hence, the use of Fmoc- and Bhoc-protecting groups in combination with the HYCRON linkage was fashioned. It enables the application of much milder cleavage conditions in that the removal of all protecting groups would proceed on the solid phase being followed by an on-resin labelling procedure. Finally, Pd^0-catalysed cleavage from the HYCRON resin would provide particularly mild cleavage conditions that leave labile fluorophores unaffected.

First, the solid-phase synthesis of **42** was performed, in order to examine whether the incorporation of the adenine modification **19a**, which contains an unprotected N^6-amino

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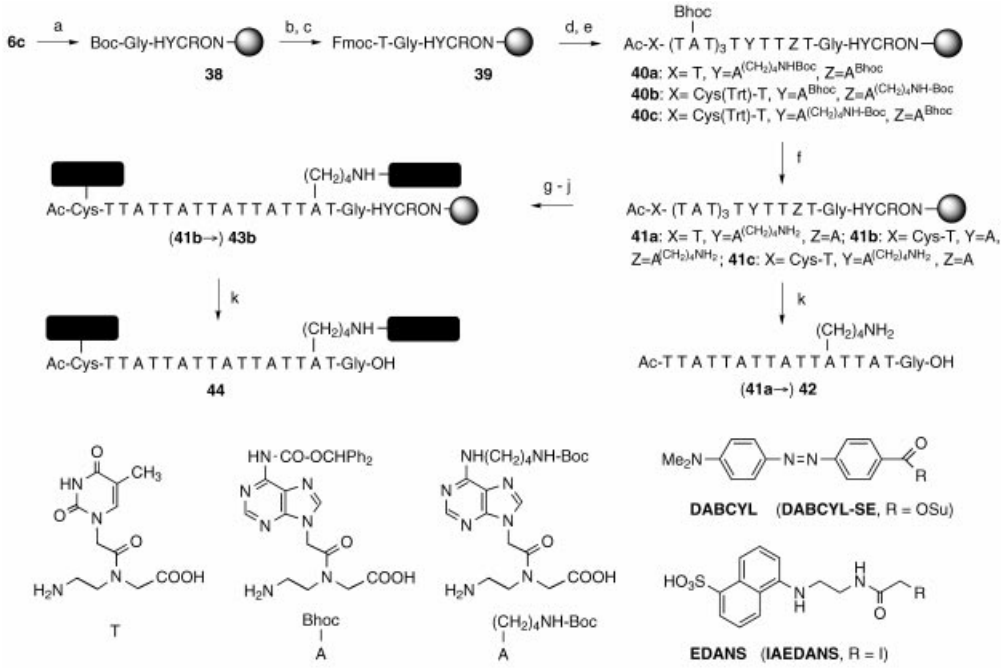
group, into a Fmoc-based solid-phase synthesis would allow for an efficient solid-phase assembly (Scheme 9). Fmoc/Bhoc-protected monomers as illustrated in the inset were coupled using the HYCRON-loaded resin **38**. All coupling reactions succeeded through HATU activation.[64] For the introduction of the internal conjugation site, the Fmoc/Boc-protected N^6-modified adenine building block **19a** was incorporated. After completion of the chain elongation all protecting groups including the Bhoc and Boc groups were removed on the solid phase by treating **40a** with a TFA/ethanedithiol/water mixture. The fully unprotected PNA **41a** remained bound to the solid support, thus, allowing for a simple and efficient removal of scavengers and cleaved by-products. Finally, the Pd^0-catalysed cleavage accomplished the liberation of the PNA-conjugate **42**. After HPLC purification an overall yield of 30% (based on **39**) was reached, demonstrating that the use of the N^6-unprotected derivative **19a** enables the effective synthesis of PNA conjugates with an internal conjugation site.

This result was promising for the fashioning of an on-resin labelling protocol. The solid-phase synthesis of the dual labelled PNA-oligomers **44** and **45** is shown in Scheme 9.

The chain assembly proceeded as described for **42**. After its completion the resin **40b** was prepared for the chemoselective conjugation reactions by which the reporter groups should be appended. The treatment of the fully protected PNA-resin **40b** with TFA/ethanedithiol/water mixture removed all protecting groups including the S-Trityl, N-Boc and Bhoc groups. The unprotected PNA-oligomer **41b**, again, remained attached to the solid phase. After neutralisation the PNA resin **41b** was reacted with the 4-(4′-dimethylaminophenylazo)benzoic acid hydroxysuccinimide ester (DABCYL-SE). This reaction led to acylation of both the primary amino group of the N^6-modified adenine and the cysteine thiol group. The latter was cleaved by subsequent treatment with DMF/piperidine. Since unprotected thiol groups are prone to form disulfides, a preventive dithiothreitol reduction was performed. The liberated thiol group was selectively alkylated by a reaction with 5-(2′-iodoacetamidoethyl)aminonaphthalene sulfonic acid (IAEDANS) furnishing resin **43**. The final Pd^0-catalysed detachment proceeded in presence of morpholine yielding the dual labelled PNA-conjugate **44**. It should be emphasised that although the chemical yield was modest the additional hydrophobicity as conferred by the attachment of the two labels greatly facilitated the purification procedure. For example, HPLC analysis of a material obtained by simple C18-SepPak extraction showed two peaks only (Figure 4). The minor peak resulted from incomplete coupling to the modified adenine as judged by MALDI-TOF analysis. The subsequent RP-HPLC purification furnished **44** and the analogously prepared positional isomer (**41c** → **45**) in high purity. Interestingly, even after repeated HPLC purification, the MALDI-TOF-MS spectra consistently showed a second peak with a m/z ratio that was 133 units lower than the molecular mass. This peak, however, seemed to be associated with a photochemically induced fragmentation by the MALDI-laser since ESI-MS analysis revealed the presence of a single peak with the expected molecular mass.

Hybridisation experiments: Both conjugates the dual labelled PNA-probes **44** and **45** were used in hybridisation experiments targeting the oligonucleotides **46** and **47**. Figure 5 shows the fluorescence spectra of **44** and **45**. It became apparent that the single stranded PNA-conjugates indeed exhibited a quenched EDANS fluorescence for reasons outlined in



Scheme 9. a) i) HBTU, HOBt, iPr$_2$NEt, DMF, amino-Tentagel; ii) Ac$_2$O, Pyr (1:10); b) i) TFA; ii) iPr$_2$NEt/DMF (1:9); c) Fmoc-T, HBTU, HOBt, iPr$_2$NEt, DMF; d) iterative cycles of: i) piperidine/DMF (1:4); ii) Fmoc-B^{Bhoc}-OH, HATU, iPr$_2$NEt, Pyr, DMF; iii) Ac$_2$O, Pyr, DMF; e) i) piperidine/DMF (1:4); ii) Ac$_2$O, Pyr, DMF; f) TFA/ethanedithiol/H$_2$O (95:2.5:2.5); g) 10 equiv DABCYL-SE, DMF/Pyr/NMM (7:1:1); h) piperidine/DMF (1:4); i) 10 equiv dithiothreitol, DMF/H$_2$O/NMM (9:3:1); j) 10 equiv IAEDANS, DMF/H$_2$O/NMM (9:3:1); k) [Pd(PPh$_3$)$_4$], morpholine, DMSO, DMF, 10% (based on **39**).

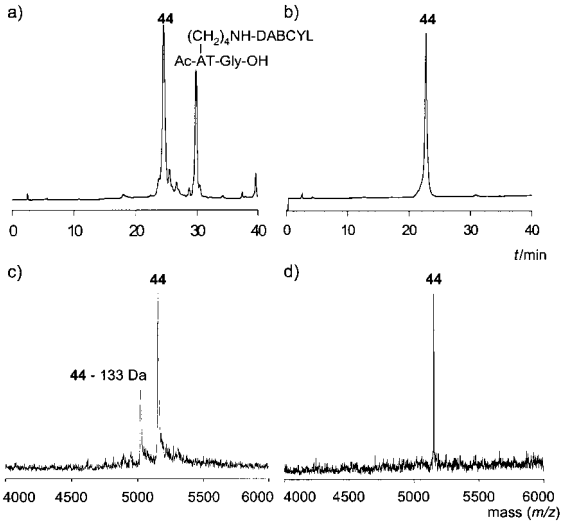


Figure 4. Analytical HPLC trace at $\lambda = 250$ nm of **44** as a) crude material after SepPak C18 extraction and b) after purification (conditions as specified in the experimental part). The c) MALDI-TOF-MS of purified **44** consistently showed a second peak, which presumably originates from fragmentation, with a m/z ratio that was 133 units lower than the molecular mass. The d) ESI-MS trace, however, revealed the presence of a single peak with the expected molecular mass.

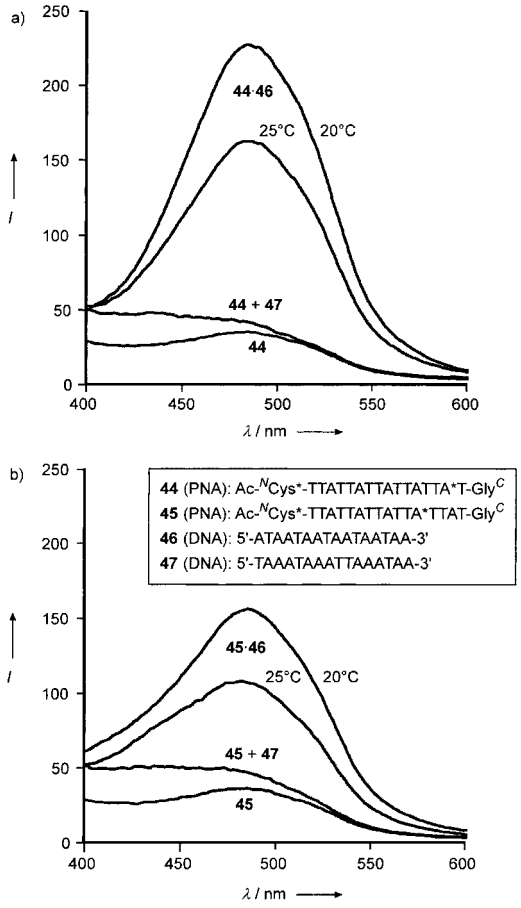


Figure 5. Fluorescence spectra (arbitrary units, calibration based on the fluorescence of single stranded **44**) of the a) PNA-probe **44** and b) PNA-probe **45**. The inset shows the oligomers used (Cys* = Cys(EDANS), A* = A(N^6(CH$_2$)$_4$NH-DABCYL)) Measurement conditions: 100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH 7, 298 K, 1 μM in **44** and **45**, 4 μM in **46** or **47** when added, excitation at 335 nm. The uppermost curves were measured at 1.5 μM probe concentration and 293 K.

Figure 2. Addition of the mismatched oligonucleotide **47** led only to a minor change of the fluorescence spectrum. However, when the complementary oligonucleotide **46** was added at 25 °C the fluorescence spectra changed dramatically. The emission of PNA-probes **44** and **45** was enhanced by a factor of 4.6 and 3, respectively. The fluorescence enhancement was even more pronounced when the hybridisation was allowed to occur at 20 °C. At this temperature, 10 °C below the T_M, presumably 100% of the PNA probes are in the hybridised state leading to a fluorescence intensification by factors of 6.4 and 4.3, respectively. A spectral shift that could be indicative for a hydrophobicity-induced increase of the fluorescence was not detectable. It is thus likely that the mechanism by which the fluorescence increased followed the dequenching process that is observed for equally labelled molecular beacons.

Analogous to molecular beacons, probes **44** and **45** began to fluoresce almost immediately after addition of the nucleic acid target strand. Figure 6 shows that the fluorescence of

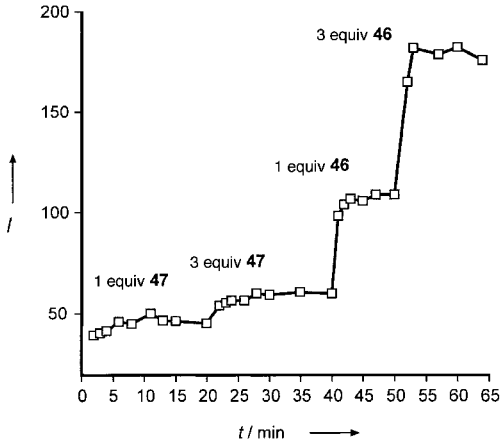


Figure 6. Fluorescent emission of probe **44** after addition of 1 equiv **47** (0 min), 3 equiv **47** (21 min), 1 equiv **46** (40 min) and 3 equiv **46** (51 min). Measurement conditions: 100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH 7, 298 K, 1 μM in **45**, excitation at 335 nm.

PNA-probe **44** reached a plateau five minutes thereafter. The half-maximal enhancement was obtained in less than one minute indicating that hybridisation of the dual labelled probes is a rapid process. Noteworthy the preparatory denaturation or renaturation procedures were not required.

The dual labelled PNA **45** spans an 11-base segment between the two labels. If the hybridisation-induced increase of the fluorescence intensity were a general, sequence-independent phenomenon, the PNA-probe **48**, which accommodates a 10-mer mixed-base segment between the terminally appended dansyl and DABCYL labels, should exhibit a similar fluorescence enhancement. Indeed, addition of the oligonucleotide **49**, a synthetic fragment of the pig-H-Ras gene, led to a fluorescence intensification by a factor of 3 (Figure 7). The PNA-probes **45** and **48** differ in sequence and length. However, the hybridisation of both PNA probes was accompanied by the same fluorescence increase. This result suggests that the fluorescence behaviour of the dual labelled

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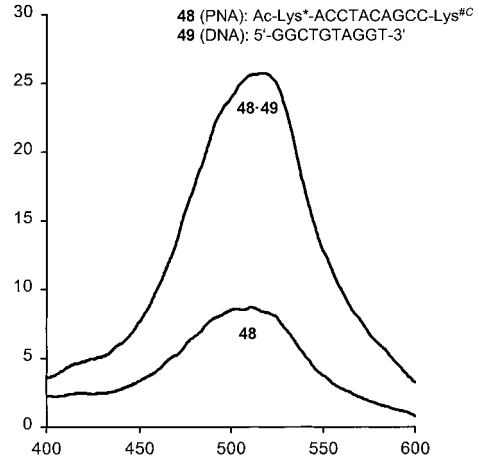

Figure 7. Fluorescence spectra (arbitrary units) of the terminally labelled PNA-probe **48** as single strand and in complex with DNA **49** (Lys* = Cys(Dans), Lys# = Lys(DABCYL)). Measurement conditions: 100 mM NaCl, 10 mM NaH_2PO_4, pH 7, 298 K, 1 μM in **48**, 1 μM in **49** when added, excitation at 330 nm.

PNA-oligomers **44**, **45** and **48** is a generalisable phenomenon that is independent of the sequence context.

In order to determine the DNA binding affinity of the internally modified PNA conjugates **44** and **45**, the temperature-dependent UV absorbance was analysed. This revealed a melting temperature T_M of 30.5 °C for the **44·46** duplex and a T_M of 28.5 °C for the **45·46** duplex (Table 1). A comparison with the T_M of 37.0 °C determined for the hybridisation of unmodified PNA **50** indicated that the introduction of the fluorescent labels in **44** and **45** led to a destabilisation of the duplex with complementary DNA. If the modifications would block parts of the duplex formation, the T_M values of the **44·46** and the **45·46** duplex would correspond to that of unmodified PNAs spanning 14 and 11 base pairs, respectively. The PNA-oligomer **51** spans a 13-base segment when hybridised with DNA **46**. However, the T_M of 27.0 °C for the **51·46** duplex is lower than the T_M of 28.5 °C obtained for the **45·46** duplex, which suggests that the modification distorts duplex geometry but not to the extent that base pairing of the nucleobases located C-terminal of the modification site would be completely prevented.

It is well known that the introduction of fluorescent labels affects the duplex stability particularly when attached to internal nucleobases.[67] However, the PNA backbone confers a substantial stabilisation which compensates for the perturbation introduced by the labelling. Indeed, the melting temperatures of the **44·46** and the **45·46** duplex compared well with the T_M of 30.4 °C for the unmodified DNA·DNA duplex **46·52**. This data supports the notion that PNA through its favorable base pairing properties is able to tolerate modifications that if installed in DNA might result in a greatly limited affinity to nucleic acid targets.

Table 1. Duplex stabilities.

Duplex (with 5′-$(ATA)_5$A-3′, **46**)	Melting temperature[b]
Ac-Cys(EDANS)-$(TTA)_4$TTA*T-Gly^C, **44**[a]	30.5 °C
Ac-Cys(EDANS)-$(TTA)_3$TTA*TTAT-Gly^C, **45**[a]	28.5 °C
Ac-$(TTA)_5$T-Gly^C, **50**	37.0 °C
Ac-$(TTA)_4$T-Gly^C, **51**	27.0 °C
5′-$(TTA)_5$T-3′, **52**	30.4 °C

[a] A* = A(N^6-$(CH_2)_4$NH-DABCYL). [b] Determined as denaturation curves at 1.25 μM probe concentration in a buffered solution (100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH 7).

Conclusion

The convergent strategies elaborated in these studies provided access to multiply labelled PNA conjugates, which are difficult to synthesise by alternative means. Both solution- and solid-phase methods were used. The first solid-phase synthesis of protected PNA enabled a selective functionalisation of the C-terminal carboxyl group as demonstrated by the synthesis of C-terminally dansylated PNA such as conjugates **35** and **37**. For adding new functionality to PNA, non-standard nucleobases such as the modified adenine **11** were introduced. In contrast to previous studies, a strategy was fashioned that allowed for the introduction of the modified nucleobase to occur on the solid phase, thereby omitting the need to synthesise an entire monomer in solution. It has to be emphasised that an analogous modification of oligodeoxyribonucleotides would require a glycosylation reaction to be performed on the solid support, which by current techniques is virtually impossible. In this approach, the key building block was the Boc/Fmoc-protected aminoethylglycine **3b** which was used in combination with the allylic HYCRON linker. Both Boc- and Fmoc-protecting groups were removed while the PNA oligomer remained bound to the polymeric resin. This strategy proved particularly successful for the on-resin labelling of PNA. The alliance of orthogonal protecting group techniques with chemoselective conjugation reactions gave rapid access to dual labelled PNA probes such as **44** and **45**. It was demonstrated that the single-stranded PNA probes were only weakly fluorescing but experienced a substantial fluorescence enhancement when bound to complementary DNA. Applications such as real-time PCR monitoring and real-time RNA detection in living cells could be feasible and benefit from the increased biostability of the PNA-based hybridisation probes.

Experimental Section

General methods: Boc/Z-protected PNA monomers were prepared according to literature procedures.[48] Fmoc/Bhoc-protected PNA-monomers were purchased from Applied Biosystems. Oligonucleotides were custom-made by MWG-Biotech (Ebersberg, Germany). Reactions were carried out at room temperature if no other specifications are given. Solid-phase synthesis was performed manually using 5 mL polyethylene syringe reactors which are equipped with a fritted disc. All column chromatography was performed on SDS 60 ACC silica gel and TLC on E. Merck silica gel 60 F_{254} glass/plastic/aluminium coated plates. The photometric determination of the Fmoc loadings was performed following a literature method.[53] ^{1}H- and ^{13}C NMR spectra were recorded on Bruker AC250, AM400, or DRX-500 spectrometers. The signals of the residual protonated solvent ($CDCl_3$ or $[D_6]$DMSO) were used as reference signals. Coupling constants *J* are reported in Hz. Mass spectra were measured on a Finnigan MAT MS70 spectrometer for FAB-MS, on a PerSeptive Biosystems Voyager for MALDI-TOF-MS and on a Finnigan-MAT LCQ for ESI-MS.

Fluorescence spectroscopy was performed with a Perkin–Elmer LS-50B. Analytical HPLC was performed with a Merck–Hitachi system, for preparative HPLC Rainin SD-300 pumps were used. A Nucleosil C18-HD column (5μ, 250 × 4 mm, Macherey&Nagel) was used for analytical HPLC and a Nucleosil C18-HD column (5μ, 250 × 10 mm) for preparative HPLC. Gradients of solvent A (H_2O, 1 % CH_3CN, 0.1 % TFA) and B (CH_3CN, 1 % H_2O, 0.1 % TFA) were used as specified.

***N*-[2-(*tert*-Butyloxycarbonyl-amino)ethyl]-*N*-fluorenylmethyloxycarbonylglycine, Boc-Aeg(Fmoc)-OH (3b)**: H_2O (3 mL) and 1M NaOH (2 mL) were added to *N*-[2-(*tert*-butyloxycarbonyl-amino)ethyl]-glycine ethyl ester (116 mg, 0.50 mmol) in ethanol (8 mL). After 1 h of stirring the pH was adjusted to pH 8 by addition of acetic acid. Subsequently, Fmoc-OSu (138 mg, 0.41 mmol) was added. After 14 h of stirring the pH was adjusted to pH 8 by addition of 1M NaOH. Again Fmoc-OSu (30 mg, 0.09 mmol) was added. The pH adjustment and Fmoc-OSu addition was repeated until all starting material was converted. Then, acetic acid was added for neutralisation and the volatiles were removed by evaporation in vacuo. The residue was dissolved in $CHCl_3$ (20 mL). The organic phase was washed with 0.5M $KHSO_4$ (10 mL). The aqueous phase was extracted with $CHCl_3$ (5 mL) before the combined organic layers were washed with brine (5 mL). The organic layer was dried over $MgSO_4$, and solvents were evaporated in vacuo. The residue was dissolved in ethylacetate (20 mL) and *n*-hexane was added until a precipate formed. Filtration and repeated washing with *n*-hexane furnished an off-white powder (114 mg, 91 %). ^1H,^{13}C NMR and FAB-MS analysis confirmed the identity of **3b**.[47]

(*E*)-17-[*N*-(2-(*tert*-Butyloxycarbonyl-amino)ethyl)-*N*-[N^4-(benzyloxycarbonyl)cytosine-1-ylacetyl]glycinyloxy]-4,7,10,13-tetraoxa-15-heptadecenoic acid phenacyl ester, Boc-C^Z-HYCRON-OPac (5a): Br-HYCRON-OPac **4** (149 mg, 0.32 mmol), tetrabutylammonium bromide (63 mg, 0.19 mmol) and saturated aqueous $NaHCO_3$ solution (4 mL) were added to Boc-C^Z-OH **3a** (95 mg, 0.19 mmol) in CH_2Cl_2 (4 mL). The two-phase system was vigorously stirred for 14 h. The organic layer was separated and the aqeous phase extracted twice with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, and solvents were evaporated in vacuo. Chromatography ($CHCl_3$/MeOH) yielded a slightly yellowish oil (140 mg, 83 %). R_f ($CHCl_3$/MeOH 95:5) = 0.19; ^1H NMR (two rotamers, 250 MHz, $CDCl_3$): δ = 7.87 (dd, $J_{o,m}$ = 8.6, $J_{o,p}$ = 1.3, 2H, o-Pac), 7.62–7.49 (m, m-Pac, p-Pac, 4H, C^6), 7.47–7.45 (m, 5H, Z^{ar}), 7.43–7.35 (m, 1H, C^6), 5.85–5.75 (m, 2H, HYCRON-$H^{14,15}$), 5.64–5.58 (m, 0.7H, Aeg-H^6), 5.34 (s, 2H, Pac-CH_2), 5.19 (s, 2H, Z-CH_2), 4.99–4.97 (m, 0.3H, Aeg-$H^{6'}$), 4.73 (s, 1.4H, C^{CH_2}), 4.64 (d, J = 4.9, 0.6H, HYCRON-$H^{17'}$), 4.59 (d, J = 4.6, 1.4H, HYCRON-H^{17}), 4.53 (s, 0.6H, $C^{CH_2'}$), 4.30 (s, 0.4H, Aeg-$H^{2'}$), 4.05–3.98 (m, 3.6H, Aeg-H^2, HYCRON-H^{14}), 3.81 (t, J = 6.6, 2H, HYCRON-H^3), 3.63–3.48 (m, 14H, HYCRON, Aeg-H^4), 3.35–3.15 (m, 2H, Aeg-H^5), 2.77 (t, J = 6.5, 2H, HYCRON-H^2), 1.40 (s, 9H, *t*Bu); ^{13}C NMR (two rotamers, 62.5 MHz, $CDCl_3$): δ = 192.09, 175.87, 171.01, 169.33, 167.72, 167.22, 163.06, 162.62, 156.08, 155.48, 152.42, 149.85, 135.11, 134.15, 133.91, 132.56, 131.80, 128.87, 128.64, 128.55, 128.19, 127.76, 125.63, 125.15, 95.10, 79.81, 77.26, 70.68, 70.56, 70.44, 70.40, 69.77, 69.68, 67.80, 66.42, 66.06, 65.83, 65.28, 50.79, 49.39, 49.09, 48.91, 38.69, 36.49, 34.72, 31.44, 21.67, 28.39, 27.86, 27.39, 20.99; $C_{44}H_{57}N_5O_{15}$ (895.95).

(*E*)-17-[*N*-[2-(*tert*-Butyloxycarbonyl-amino)ethyl]-*N*-fluorenylmethyloxycarbonyl-glycinyloxy]-4,7,10,13-tetraoxa-15-heptadecenoic acid phenacyl ester, Boc-Aeg(Fmoc)-HYCRON-OPac (5b): A two-phase system consisting of Boc-Aeg(Fmoc) (1.01 g, 2.29 mmol), Br-HYCRON-Pac **4** (1.15 g, 2.44 mmol), Bu_4NBr (0.72 g, 2.22 mmol), CH_2Cl_2 (20 mL) and saturated $NaHCO_3$ (20 mL) was treated as described for **5a**. Purification was achieved by chromatography (*n*-hexane/EtOAc), which yielded of a colorless oil (1.75 g, 92 %). R_f (*n*-hexane/EtOAc 1:2) = 0.47; ^1H NMR (two rotamers, 250 MHz, $CDCl_3$): δ = 7.89 (d, J = 7.3, 2H, o-Pac), 7.74 (2 × d, J = 7.3, 2H), 7.62–7.25 (m, 9H, Pac^{ar}, $Fmoc^{ar}$), 5.84–5.71 (m, 2H, HYCRON-$H^{14,15}$), 5.34 (s, 2H, Pac-CH_2), 5.09–5,06 (m, 0.5H, Aeg-H^6), 4.80–4.77 (m, 0.5H, Aeg-$H^{6'}$), 4.61 (d, J = 4.5, 1H, HYCRON-H^{17}), 4.56 (d, J = 4.7, 1H, HYCRON-H^{17}), 4.50 (d, J = 6.0, 1H, Fmoc-CH_2), 4.41 (d, J = 6.5, 1H, Fmoc-CH_2'), 4.24 (t, J = 6.0, 0.5H, Fmoc-H^9), 4.17 (t, J = 6.4, 0.5H, Fmoc-H^9), 3.99–3.91 (m, 4H, Aeg-H^2, HYCRON-H^{14}), 3.81 (t, J = 6.6, 2H, HYCRON-H^3), 3.64–3.53 (m, 12H, HYCRON), 3.44–3.41 (m, 1H), 3.27–3.24 (m, 2H), 2.99–2.94 (m, 1H), 2.78 (t, J = 6.5, 2H, HYCRON-H^2), 1.38 (s, 9H, *t*Bu); ^{13}C NMR (two rotamers, 100.6 MHz, $CDCl_3$): δ = 191.94, 170.85, 169.47, 156.17, 156.04, 155.83, 143.76, 143.67, 141.19, 141.15, 134.05, 133.77, 131.75, 131.56, 128.74, 127.63, 127.11, 126.96, 125.73, 125.50, 124.78, 124.73, 119.81, 79.18, 70.59, 70.52, 70.45, 70.40, 70.34, 70.30, 69.59, 69.56, 67.64, 67.45, 66.32, 65.94, 65.03, 64.98, 49.89, 49.51, 48.88, 48.44, 47.10, 38.89, 34.62, 28.28; $C_{46}H_{58}N_2O_{13}$ (846.96).

(*E*)-17-[*N*-(2-(*tert*-Butyloxycarbonyl-amino)ethyl)-*N*-[N^4-(benzyloxycarbonyl)cytosine-1-ylacetyl]glycinyloxy]-4,7,10,13-tetraoxa-15-heptadecenoic acid, Boc-C^Z-HYCRON-OH (6a): Zinc (0.1 g, activated by treatment with 1N HCl and subsequent washing with H_2O and glacial acetic acid) was added to a solution of **5a** (51 mg, 56.8 μmol) in glacial acetic acid (2 mL). After 1.5 h of vigirous stirring zinc was removed by filtration over Hyflo. The filtrate was concentrated in vacuo. The residue was repeatedly coevaporated with toluene and purified by chromatography ($CHCl_3$/MeOH/AcOH). Coevaporation with toluene and subsequent drying in vacuo yielded a colorless oil (35 mg, 79 %). R_f ($CHCl_3$/MeOH/AcOH 85:15:1) = 0.50; ^1H NMR (two rotamers, 250 MHz, $CDCl_3$): δ = 7.65–7.52 (m, 1H, C^6), 7.31–7.09 (m, 6H, Z^{ar}, C^6), 5.82–5.72 (m, 2H, HYCRON-$H^{14,15}$), 5.53–5.51 (m, 0.7H, Aeg-H^6), 5.16 (s, 2H, Z-CH_2), 4.99–4.98 (m, 0.3H, Aeg-$H^{6'}$), 4.68 (s, 1.4H, C^{CH_2}), 4.61 (d, J = 3.3, 0.6H, HYCRON-$H^{17'}$), 4.55 (d, J = 4.3, 1.4H, HYCRON-H^{17}), 4.50 (s, 0.6H, $C^{CH_2'}$), 4.28 (s, 0.4H, Aeg-$H^{2'}$), 4.00–3.95 (m, 3.6H, Aeg-H^2, HYCRON-H^{14}), 3.70 (m, 2H, HYCRON-H^3), 3.56–3.45 (m, 14H, HYCRON, Aeg-H^4), 3.29–3.19 (m, 2H, Aeg-H^5), 2.54 (t, J = 5.8, 2H, HYCRON-H^2), 1.36 (s, 9H, *t*Bu); ^{13}C NMR (two rotamers, 62.5 MHz, $CDCl_3$): δ = 169.28, 167.19, 163.40, 156.16, 155.33, 152.58, 150.11, 135.20, 132.34, 131.56, 129.01, 128.61, 128.47, 128.20, 125.74, 125.27, 95.23, 79.83, 70.56, 70.35, 69.72, 69.59, 67.72, 66.61, 65.78, 65.19, 50.76, 49.58, 49.15, 48.85, 38.70, 35.13, 28.39; HR-MS (FAB^+, glycerine/H_2O): m/z: 778.344 $[M+H]^+$, calcd for $C_{36}H_{51}N_5O_{14}$: 778.351.

(*E*)-17-[*N*-[2-(*tert*-Butyloxycarbonyl-amino)ethyl]-*N*-fluorenylmethyloxycarbonyl-glycinyloxy]-4,7,10,13-tetraoxa-15-heptadecenoic acid, Boc-Aeg(Fmoc)-HYCRON-OH (6b): A suspension comprised of **6a** (1.24 g, 1.49 mmol), activated zinc (1 g) and glacial acetic acid (15 mL) was vigirously stirred for 1.5 h. The work-up was performed as described for **6a**. Chromatography ($CHCl_3$/MeOH/AcOH) yielded a yellowish oil (1.0 g, 94 %). R_f ($CHCl_3$/MeOH/AcOH 85:15:1) = 0.58; ^1H NMR (two rotamers, 250 MHz, $CDCl_3$): δ = 7.71–7.67 (m, 2H, $Fmoc^{ar}$), 7.52–7.44 (m, 2H, $Fmoc^{ar}$), 7.36–7.22 (m, 4H, $Fmoc^{ar}$), 5.79–5.73 (m, 2H, HYCRON-$H^{14,15}$), 5.03–5.01 (m, 0.5H, Aeg-H^6), 4.72–4.70 (m, 0.5H, Aeg-$H^{6'}$), 4.55 (d, J = 4.2, 1H, HYCRON-H^{17}), 4.50 (d, J = 4.6, 1H, HYCRON-H^{17}), 4.46 (d, J = 5.8, 1H, Fmoc-CH_2), 4.36 (d, J = 6.5, 1H, Fmoc-CH_2'), 4.19 (t, J = 5.8, 0.5H, Fmoc-H^9), 4.12 (t, J = 6.2, 0.5H, Fmoc-H^9), 3.97–3.87 (m, 4H, Aeg-H^2, HYCRON-H^{14}), 3.69 (t, J = 6.2, 2H, HYCRON-H^3), 3.67–3.50 (m, 12H, HYCRON), 3.38–3.35 (m, 1H), 3.21–3.16 (m, 2H), 2.93–2.91 (m, 1H) 2.54 (t, J = 6.1, 2H, HYCRON-H^2), 1.33 (s, 9H, *t*Bu); ^{13}C NMR (two rotamers, 100.6 MHz, $CDCl_3$): δ = 174.79, 169.80, 169.56, 156.24, 156.01, 143.84, 143.77, 141.30, 141.26, 131.69, 131.50, 127.73, 127.21, 127.06, 125.99, 124.89, 124.83, 119.93, 79.43, 70.68, 70.55, 70.46, 70.37, 70.29, 69.60, 67.79, 67.57, 66.47, 65.12, 49.98, 49.56, 48.90, 48.52, 47.18, 38.93, 34.86, 28.37; HR-MS (FAB^+, glycerine/H_2O): m/z: 715.349 $[M+H]^+$, calcd for $C_{37}H_{50}N_2O_{12}$: 715.344.

(N^6-(1-Aminohex-6-yl)adenine-9-yl)acetic acid *tert*-butyl ester (9): 1,6-Diaminohexane (2.15 g, 18.50 mmol) was added to the 6-chloropurine **8** (0.78 g, 2.90 mmol) in *n*-butanol (15 mL). After 1 h reflux the solvent was removed in vacuo. The residue was purified by chromatography ($CHCl_3$/MeOH 8:2 → $CHCl_3$/MeOH/Me_2NEt 7:3:0.01) yielding a yellowish oil (380 mg, 38 %). R_f ($CHCl_3$/MeOH/$EtNMe_2$ 7:3:0.1) = 0.53; ^1H NMR (250 MHz, $CDCl_3$): δ = 8.36 (s, 1H), 7.79 (s, 1H), 5.97 (t, J = 5.5, 1H), 4.84 (s, 2H, $CH_2COOtBu$), 3.65–3.48 (m, 2H), 3.15–2.99 (m, 2H), 2.70–2.49 (m, 2H), 1.70–1.60 (m, 2H), 1.52–1.39 (m, 15H); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 166.38 (CO), 155.07 (A^6), 153.37 (A^2), 149.18 (A^4), 140.11 (A^8), 119.11 (A^5), 83.42 (CMe_3), 46.92, 44.76 ($CH_2COOtBu$), 32.73, 29.67, 28.00 (*t*Bu), 26.66, 26.53.

***N*-6-(1-(Fluorenylmethoxycarbonyl-amino)hex-6-yl)adenine-9-yl)acetic acid *tert*-butyl ester (10)**: Fmoc-Cl (148 mg, 0.57 mmol) and NMM (66 μL, 0.60 mmol) were added to amine **9** (190 mg, 0.54 mmol) in CH_2Cl_2 (5 mL). After 4 h CH_2Cl_2 (60 mL) and an aqueous pH 4 buffer (70 mL) comprised of 0.5M $KHSO_4$ and sat. $NaHCO_3$ was added. The resulting two-phase system was stirred. The organic layer was separated and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were washed with brine and dried over $MgSO_4$. Chromatography (EtOAc) yielded a white amorphous solid (223 mg, 72 %). R_f (EtOAc) = 0.34; ^1H NMR (250 MHz, $CDCl_3$): δ = 8.37 (s, 1H), 7.80 (s, 1H), 7.74 (d, J = 7.4, 2H, Fmoc-$H^{4,5}$), 7.58 (d, J = 7.3, 2H, Fmoc-$H^{1,8}$), 7.39 (t, J = 7.3, 2H, Fmoc-$H^{3,6}$), 7.30 (t,

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$J = 7.3$, 2H, Fmoc-H^{2,7}), 6.00–5.85 (m, 1H), 5.09–5.00 (m, 1H), 4.84 (s, 2H, $CH_2COOtBu$), 4.40 (d, $J = 7.0$, 2H, Fmoc-CH_2), 4.25 (d, 1H, Fmoc-H^9), 3.70–3.62 (m, 2H), 3.20–3.13 (m, 2H), 1.73–1.64 (m, 2H), 1.53–1.40 (m, 15H); ^{13}C NMR (62.5 MHz, $CDCl_3$): $\delta = 166.16$ (CO), 156.33 (A^6), 154.69 (A^2), 153.08 (Fmoc-CO), 149.06 (A^4), 143.86 (Fmoc-C^{4a,b}), 141.13 (Fmoc-C^{8a,b}), 140.06 (A^8), 127.48 (Fmoc-C^{3,6}), 126.86 (Fmoc-C^{2,7}), 124.90 (Fmoc-C^{1,8}), 119.78 (Fmoc-C^{4,5}), 118.89 (A^5), 83.33 (CMe_3), 66.29, 47.13, 44.60 ($CH_2COOtBu$), 40.75, 29.68, 29.45, 27.83 (*t*Bu), 26.31, 26.20; $C_{32}H_{38}N_6O_4$ (570.68).

(*N*^{6}-(1-(Fluorenylmethoxycarbonyl-amino)hex-6-yl)adenine-9-yl)acetic acid (11): TFA (5 mL) was added to the *tert*-butyl ester **10** (115 mg, 0.20 mmol). After 5 min stirring the solvent was removed in vacuo. The residual TFA was removed by repeated coevaporation with toluene. During the chromatography ($CHCl_3$/MeOH/AcOH) precipitation occured. Elution with DMF and subsequent removal of the solvent in vacuo yielded a yellowish amorphous solid (98 mg, 95%). It was not possible to remove DMF entirely. Since analytical RP-HPLC revealed that no by-products but DMF were present, the material was used for further reactions. R_f ($CHCl_3$/MeOH/AcOH 85:15:1) = 0.4; $t_R = 27$ min (grad: 0 min (0%B) → 40 min (80%B), 1 mL min^{-1}, 50 °C); ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 8.16$ (s, 1H), 8.05 (s, 1H), 7.87 (d, $J = 7.5$, 2H, Fmoc-H^{4,5}), 7.73–7.65 (m, $J = 7.3$, 3H, NH, Fmoc-H^{1,8}), 7.39 (t, $J = 7.3$, 2H, Fmoc-H^{3,6}), 7.32–7.25 (m, 3H), 4.81 (s, 2H, $CH_2COOtBu$), 4.27 (d, $J = 6.9$, 2H, Fmoc-CH_2), 4.19 (d, $J = 6.7$, 1H, Fmoc-H^9), 3.44, 3.35 (2 × brs, incl. H_2O), 2.97–2.92 (m, 2H), 1.73, 1.57–1.54 (m, 2H), 1.39–1.21 (m, 6H); ^{13}C NMR (100.6 MHz, [D$_6$]DMSO): $\delta =$ 170.21 (CO), 156.21 (A^6), 154.56 (Fmoc-CO), 152.45 (A^2), 149.0 (A^4), 144.03 (Fmoc-C^{4a,b}), 141.41 (A^8), 140.82 (Fmoc-C^{8a,b}), 127.69 (Fmoc-C^{3,6}), 127.13 (Fmoc-C^{2,7}), 125.24 (Fmoc-C^{1,8}), 120.19 (Fmoc-C^{4,5}), 118.73 (A^5), 65.23, 46.87, 45.01, 40.26, 29.46, 29.24, 26.26, 26.14; $C_{28}H_{30}N_6O_4$ (514.58).

1-(*N*^{6}-(1-Aminobut-4-yl)adenine-9-yl)prop-2-ene (13a): 1,4-Diaminobutane (3.62 g, 41.1 mmol) was added to a solution of **12** (1.0 g, 5.14 mmol) in *n*-butanol (20 mL). After 1 h stirring at 80 °C the solvent was removed in vacuo. Chromatography ($CHCl_3$/MeOH/H_2O/$EtNMe_2$ 60:30:5:1) and subsequent drying in vacuo furnished a yellowish oil (0.53 g, 42%). Apart from ^{1}H NMR analysis this material was used without further characterisation. R_f ($CHCl_3$/MeOH 3:2) = 0.27; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.20$ (brs, 1H, A^2), 8.09 (s, 1H, A^8), 7.79 (brs, 1H, NH), 6.09–6.00 (m, 1H, All-H^2), 5.16 (dd, $J_1 = 10.3$, $J_2 = 1.0$, 1H, All-H^{3cis}), 5.02 (d, $J = 17.3$, 1H, All-H^{3trans}), 4.77 (d, $J = 5.3$, 2H, All-H^1), 3.80–3.50 (m, NH_2, A$^{N6\text{-}Bu\text{-}1}$, A$^{N6\text{-}Bu\text{-}4}$, H_2O), 1.64 (m, 4H, A$^{N6\text{-}Bu\text{-}2}$, A$^{N6\text{-}Bu\text{-}3}$).

1-(*N*^{6}-(1-Aminoeth-2-yl)adenine-9-yl)prop-2-ene (13b): The reaction of purine **12** (1.00 g, 5.12 mmol) and ethylenediamine (3.09 g, 51.40 mmol) was performed as described above. Chromatography ($CHCl_3$/MeOH/Et_2NMe/H_2O 6:4:1:1) yielded the title compound (1.9 g) which was used without further purification.

1-(*N*^{6}-(1-(*tert*-Butyloxycarbonyl-amino)but-4-yl)adenine-9-yl)prop-2-ene (14a): Boc_2O (137 mg, 0.63 mmol) was added to amine **13a** (136 mg, 0.53 mmol) in dry pyridine (3 mL). TLC control after 16 h showed the presence of starting material. Thus, DMAP (20 mg, 0.16 mmol) and Boc_2O (65 mg, 0.3 mmol) were added. After 4 h the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (10 mL) and washed twice with 1M NaH_2PO_4 buffer (pH 5.6). The combined aqueous phases were extracted with CH_2Cl_2 before the combined organic layers were dried over $MgSO_4$. The subsequent chromatography (EtOAc) yielded an amorphous solid (94 mg, 51%). R_f ($CHCl_3$/MeOH 9:1) = 0.27; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.39$ (brs, 1H, A^2), 7.75 (brs, 1H, A^8), 6.09–5.99 (m, 1H, All-H^2), 5.79 (brs, 1H, NH), 5.30 (d, $J = 10.0$, 1H, All-H^{3cis}), 5.19 (d, $J = 17.0$, 1H, All-H^{3trans}), 4.80 (d, $J = 5.8$, 2H, All-H^1), 3.74 (brs, 1H, NH), 3.68 (brs, 3H, A$^{N6\text{-}Bu\text{-}1}$), 3.21–3.18 (m, 2H, A$^{N6\text{-}Bu\text{-}4}$), 1.76–1.70 (m, 2H), 1.64–1.57 (m, 2H), 1.43 (s, 9H, Boc); ^{13}C NMR (125.7 MHz, $CDCl_3$): $\delta = 156.03$, 154.66, 153.26, 148.76, 139.32, 131.70, 118.91, 78.83, 45.67, 39.97, 29.57, 28.35, 26.84, 21.21; $C_{17}H_{26}N_6O_2$ (346.43).

1-(*N*^{6}-(1-(*tert*-Butyloxycarbonyl-amino)eth-2-yl)adenine-9-yl)prop-2-ene (14b): Boc_2O (2.31 g, 10.56 mmol) and DMAP (0.21 g, 1.76 mmol) were added to the raw compound **13b** (1.9 g) in dry pyridine (42 mL). After 19 h a further portion of Boc_2O (1.01 g, 4.58 mmol) and DMAP (0.21 g, 1.76 mmol) were added. After 4 h aqueous work-up was performed as described for **14a**. Chromatography (EtOAc → EtOAc/EtOH 3:1) yielded a yellowish amorphous solid (0.71 g, 44% based on **12**). R_f ($CHCl_3$/MeOH 9:1) = 0.50; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 8.31$ (s, 1H, A^2), 7.73 (s, 1H, A^8), 6.46 (brs, 1H, NH), 6.05–5.89 (m, 1H, All-H^2), 5.26–5.09 (m, $J_{cis} =$ 10.2, $J_{trans} = 16.8$, 3H, NH, All-H^3), 4.73 (d, $J = 6.6$, 2H, All-H^1), 3.74 (brs, 1H, A$^{N6\text{-}CH_2CH_2}$), 3.39–3.34 (m, 2H, A$^{N6\text{-}CH_2CH_2}$), 1.34 (s, 9H, Boc); ^{13}C NMR (62 MHz, $CDCl_3$): $\delta = 156.25$, 154.91, 152.88, 139.76, 131.84, 119.93, 118.80, 79.30, 45.64, 40.75, 28.30; $C_{15}H_{22}N_6O_2$ (318.38).

(*N*^{6}-(1-*tert*-Butyloxycarbonyl-aminobut-4-yl)adenine-9-yl)acetic acid (15a): The ozonolysis of **14a** (0.59 g, 1.70 mmol) was performed as described by Thomson and co-workers.[59] For work-up the reaction mixture was concentrated to ⅓ of the original volume. The precipitate was collected yielding a yellowish powder (346 mg, 58%). R_f ($CHCl_3$/MeOH/AcOH 85:15:1) = 0.17; ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 8.17$ (brs, 1H, A^2), 8.08 (s, 1H, A^8), 7.76 (brs, 1H, NH), 6.81–6.77 (m, 1H, NH), 4.95 (s, 2H, A$^{N9\text{-}CH_2}$), 3.59–3.22 (m, 2H, A$^{N6\text{-}Bu\text{-}1}$), 2.92 (ddd, 2H, A$^{N6\text{-}Bu\text{-}4}$, $J_1 \approx J_2 \approx J_3 \approx 6.3$), 1.59–1.32 (m, 13H, A$^{N6\text{-}Bu\text{-}2}$, A$^{N6\text{-}Bu\text{-}3}$, Boc); ^{13}C NMR (62.5 MHz, [D$_6$]DMSO): $\delta = 169.39$ (COOH), 155.61, 154.55 (Boc-CO, A^6), 152.51 (A^2), 148.97 (A^4), 141.08 (A^8), 118.56 (A^5), 77.35 (Boc), 43.98 (A$^{N9\text{-}CH_2}$), 39.72 (A$^{N6\text{-}Bu\text{-}4}$, A$^{N6\text{-}Bu\text{-}1}$), 28.29 (Boc), 27.04, 26.60 (A$^{N6\text{-}Bu\text{-}3}$, A$^{N6\text{-}Bu\text{-}2}$); anal. calcd for $C_{16}H_{24}N_6O_4$: C 52.7, H 6.6, N 23.0; found: C 52.5, H 6.5, N 22.1.

(*N*^{6}-(1-(*tert*-Butyloxycarbonyl-amino)eth-2-yl)adenine-9-yl)acetic acid (15b): The ozonolysis of **14b** (710 mg, 2.23 mmol) and the subsequent work-up was performed as described above yielding a colorless powder (529 mg, 71%). ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 8.18$ (brs, 1H, A^2), 8.10 (s, 1H, A^8), 7.74 (brs, 1H, NH), 6.94–6.90 (m, 1H, NH), 4.95 (s, 2H, A$^{N9\text{-}CH_2}$), 3.50–3.35 (m, 2H, A$^{N6\text{-}CH_2CH_2}$), 3.16 (m, 2H, A$^{N6\text{-}CH_2CH_2}$), 1.35 (s, 9H, Boc); ^{13}C NMR (100.6 MHz, [D$_6$]DMSO): $\delta = 169.40$ (COOH), 155.72, 154.49 (Boc-CO, A^6), 152.48 (A^2), 149.04 (A^4), 141.30 (A^8), 118.73 (A^5), 77.66 (Boc), 44.00 (A$^{N9\text{-}CH_2}$), 39.90 (A$^{N6\text{-}CH_2CH_2}$), 28.26 (Boc); HR-MS (FAB$^+$, 3-NBA): *m*/*z*: 337.166 [*M*+H]$^+$, calcd for $C_{14}H_{20}N_6O_4$: 337.162.

***N*-(2-(Fluorenylmethoxycarbonyl-amino)ethyl)-glycine allyl ester (17)**: A solution of Fmoc-aminoethylglycine *tert*-butyl ester **16** (1.54 g, 3.66 mmol) in TFA (10 mL) was stirred for 30 min. The TFA was removed in vacuo. The residue was coevaporated with toluene (3 ×). The residue was supended in allyl alcohol (10 mL). At 0 °C thionylchloride (0.90 mL) was added in portions. The mixture was stirred at 100 °C for 1 h. After cooling to room temperature diethylether (25 mL) was added. The precipitate was collected by filtration. Repeated washings with diethyl ether yielded a colorless powder (1.25 g, 84%). The material was used without further purification.

***N*-[2-(Fluorenylmethoxycarbonyl-amino)ethyl]-*N*-[*N*^{6}-(1-(*tert*-butyloxycarbonyl-amino)but-4-yl)adenine-9-yl)acetyl]glycine allyl ester, Fmoc-A$^{(CH_2)_4NH\text{-}Boc}$-OAll (18a)**: HOBt (41 mg, 0.23 mmol) and DCC (48 mg, 0.23 mmol) were added to a solution of the amine **17** (97 mg, 0.23 mmol) and the acid **15a** (84 mg, 0.23 mmol) in a mixture of DMF (1 mL) and CH_2Cl_2 (1 mL). After 20 h stirring the solvent was removed in vacuo. The residue was suspended in $CHCl_3$ (10 mL) and filtrated. The filtrate was washed twice with sat. $NaHCO_3$. The organic layer was washed with 0.1M $KHSO_4$ and with brine. The combined organic layers were dried with $MgSO_4$ before the solvent was removed in vacuo. Chromatography (EtOAc → EtOAc/EtOH 8:1) yielded a colorless amorphous solid (127 mg, 76%). R_f (EtOAc/EtOH 9:1) = 0.4; ^{1}H NMR (two rotamers, 400 MHz, [D$_6$]DMSO): $\delta = 8.12$ (brs, 1H), 7.87 (d, $J = 7.5$, 2H, Fmoc-H^{4,5}), 7.78 (brs, 0.7H), 7.69–7.64 (m, 2.3H), 7.52–7.49 (m, 0.7H), 7.40 (t, $J = 7.5$, 2H, Fmoc-H^{3,6}), 7.32–7.27 (m, 2.3H), 6.81 (brs, 1H), 5.98–5.81 (m, 2H, All-H^2), 5.40–5.16 (m, 3.4H, All-H^3, A^{CH_2}), 5.04 (s, 0.6H, A$^{CH_2'}$), 4.68 (d, $J = 5.5$, 0.6H, All-H$^{1'}$), 4.55 (d, $J = 5.3$, 1.4H, All-H^1), 4.48 (s, 0.6H, Aeg-H$^{2'}$), 4.35 (d, $J = 6.7$, 1.4H, Fmoc^{CH_2}), 4.29–4.19 (m, 2.6H, Fmoc$^{CH_2'}$, Fmoc-H^9), 4.11 (s, 1.4H, Aeg-H^2), 3.58–3.54 (m, 1.4H), 3.46–3.34 (m, incl. H_2O), 3.14–3.10 (m, 0.6H), 2.94–2.90 (m, 2H), 1.58–1.53 (m, 2H), 1.41–1.33 (m, 11H); ^{13}C NMR (two rotamers, 100.6 MHz, [D$_6$]DMSO): $\delta = 172.09$, 169, 25, 168.69, 167.14, 156.46, 156.18, 155.62, 154.43, 152.31, 149.05, 143.88, 141.41, 140.78, 132.26, 127.64, 127.07, 125.11, 120.14, 118.34, 117.88, 77.34, 65.55, 64.91, 47.92, 47.15, 46.76, 43.78, 28.28, 27.04, 26.57, 21.07; $C_{38}H_{46}N_8O_7$ (726.83).

***N*-[2-(Fluorenylmethoxycarbonyl-amino)ethyl]-*N*-[*N*^{6}-(1-(*tert*-butyloxycarbonyl-amino)eth-2-yl)adenine-9-yl)acetyl]glycine allyl ester, Fmoc-A$^{(CH_2)_2NH\text{-}Boc}$-OAll (18b)**: The reaction including amine **17** (174 mg, 0.42 mmol) and acid **15b** (140 mg, 0.42 mmol) was performed as described above. Chromatography (EtOAc → EtOAc/EtOH 8:1) yielded a colorless amorphous solid (245 mg, 84%). The product was used without further characterisation. R_f (EtOAc/EtOH 9:1) = 0.45.

***N*-[2-(Fluorenylmethoxycarbonyl-amino)ethyl]-*N*-[N^6-(1-(*tert*-butyloxycarbonyl-amino)but-4-yl)adenine-9-yl)acetyl]glycine, Fmoc-$A^{(CH_2)_4NH\text{-}Boc}$-OH (19a)**: *N*-Methylaniline (0.56 mL) was added to a solution of allyl ester **18a** (205 mg, 0.28 mmol) in THF (6 mL). The solution was degassed by freeze-thaw-pump cycles. After addition of $[Pd(PPh_3)_4]$ the mixture was stirred for 14 h under exclusion of light. The solvent was removed in vacuo. To the residue was added methanol (5 mL). The resulting precipitate was removed by filtration. The filtrate was concentrated to dryness in vacuo before $CHCl_3$ (20 mL) was added. The solution was washed twice with 0.3 M $KHSO_4$. The aqueous phase was extracted twice with $CHCl_3$. The combined organic layers were washed with brine and dried over $MgSO_4$ before the solvent was removed in vacuo. Chromatography ($CHCl_3$/MeOH/AcOH) and subsequent lyophilisation from benzene yielded an off-white powder (155 mg, 81 %). R_f ($CHCl_3$/MeOH/AcOH 85:15:1) = 0.10; ^{1}H NMR (500 MHz, ^{1}H,^{13}C-COSY, HMQC, [D$_6$]DMSO): δ = 8.14 – 8.12 (m, 1H, A^2), 7.94 (s, 1H, A^8), 7.87 (d, J = 7.5, 2H, Fmoc-H^{4,5}), 7.76 (br s, 1H, NH), 7.66 – 7.62 (m, 2H, Fmoc-H^{1,8}), 7.53 (t, J = 6.8, 0.6H, NH), 7.43 – 7.40 (m, J = 7.4, 2.4H, NH, Fmoc-H^{3,6}), 7.25 (t, J = 7.3, 2H, NH, Fmoc-H^{2,7}), 6.94 (br s, 1H, NH), 5.19 (s, 1.2H, A$^{N9\text{-}CH_2}$), 5.01 (s, 0.8H, A$^{N9\text{-}CH_2'}$), 4.36 – 4.17 (m, 3.8H, Fmoc-CH$_2$, Fmoc-H^9, Aeg-H$^{2'}$), 3.98 (s, 1.2H, Aeg-H^2), 3.57 – 3.52 (m, 1.2H, Aeg-H^4), 3.50 – 3.39 (m, 2H, A$^{N6\text{-}Bu\text{-}1}$), 3.37 – 3.30 (m, 2H, Aeg-H$^{4'}$, Aeg-H^5), 3.16 – 3.10 (m, 0.8H, Aeg-H$^{5'}$), 2.94 – 2.91 (m, 2H, A$^{N6\text{-}Bu\text{-}4}$), 1.60 – 1.52 (m, 2H, A$^{N6\text{-}Bu\text{-}3}$) 1.44 – 1.30 (s, 11H, A$^{N6\text{-}Bu\text{-}2}$, Boc); ^{13}C NMR (two rotamers, 125.6 MHz, ^{1}H,^{13}C-COSY, HMQC, [D$_6$]DMSO): δ = 170.93, 170.47 (Aeg-C^1), 167.47, 167.00 (A$^{N9\text{-}CH_2CO}$), 156.53, 156.16 (Fmoc-CO), 155.65 (Boc-CO), 154.57 (A^6), 152.31 (A^2), 149.29 (A^4), 143.96 (Fmoc-C^{8a,b}), 141.45 (A^8), 140.88 (Fmoc-C^{4a,b}), 127.84 (Fmoc-C^{3,6}), 127.08 (Fmoc-C^{2,7}), 125.18 (Fmoc-C^{1,8}), 120.09 (Fmoc-C^{4,5}), 118.63 (A^5), 77.41 (Boc), 65.64, 65.58 (Fmoc-CH$_2$), 49.24, 47.76, 47.02 (Aeg-C^2, Aeg-C^4), 46.78 (Fmoc-C^9), 43.81, 43.49 (A$^{N9\text{-}CH_2}$), 39.92 (A$^{N6\text{-}Bu\text{-}4}$), 39.63 (A$^{N6\text{-}Bu\text{-}1}$), 38.92, 38.12 (Aeg-C^5), 27.66 (Boc), 26.39 (A$^{N6\text{-}Bu\text{-}3}$), 24.52 (A$^{N6\text{-}Bu\text{-}2}$); HR-MS (FAB, 3-NBA/DMSO, pos): m/z: 687.318 [M+H]$^+$, calcd for $C_{35}H_{42}N_8O_7$: 687.325.

***N*-[2-(Fluorenylmethoxycarbonyl-amino)ethyl]-*N*-[N^6-(1-(*tert*-butyloxycarbonyl-amino)eth-2-yl)adenine-9-yl)acetyl]glycine, Fmoc-$A^{(CH_2)_2NH\text{-}Boc}$-OH (19b)**: The reaction including allyl ester **18b** (245 mg, 0.35 mmol) was performed as described above. Two-fold chromatography ($CHCl_3$/MeOH/AcOH) and subsequent lyophilisation from benzene yielded an off-white powder (165 mg, 71 %). However, even after repeated lyophilisation it was not possible to remove DMF entirely. R_f ($CHCl_3$/MeOH/AcOH 85:20:2) = 0.50; ^{1}H NMR (two rotamers, 400 MHz, ^{1}H,^{13}C-COSY, HMQC, [D$_6$]DMSO): δ = 8.12 (br s, 1H, A^2), 7.97 (s, 1H, A^8), 7.87 (d, J = 7.5, 2H, Fmoc-H^{4,5}), 7.69 – 7.64 (m, 3H, NH, Fmoc-H^{1,8}), 7.50 (t, J = 6.8, 0.6H, NH), 7.38 (t, J = 7.4, 2H, Fmoc-H^{3,6}), 7.32 – 7.28 (m, J = 7.3, 2.4H, NH, Fmoc-H^{2,7}), 6.91 (br s, 1H, NH), 5.20 (s, 1.2H, A$^{N9\text{-}CH_2}$), 5.02 (s, 0.8H, A$^{N9\text{-}CH_2'}$), 4.36 – 4.14 (m, 3.8H, Fmoc-CH$_2$, Fmoc-H^9, Aeg-H$^{2'}$), 3.98 (s, 1.2H, Aeg-H^2), 3.53 – 3.51 (m, 3.2H, A$^{N6\text{-}CH_2CH_2}$, Aeg-H^4), 3.33 – 3.30 (m, 1.6H, Aeg-H$^{4'}$, Aeg-H$^{5'}$), 3.16 – 3.13 (m, 3.2H, A$^{N6\text{-}CH_2CH_2}$, Aeg-H^5), 1.35 (s, 9H, Boc); ^{13}C NMR (two rotamers, 100.6 MHz, ^{1}H,^{13}C-COSY, HMQC, [D$_6$]DMSO): δ = 171.00, 170.46 (Aeg-C^1), 167.32, 166.85 (A$^{N9\text{-}CH_2CO}$), 156.42, 156.14 (Fmoc-CO), 155.70 (Boc-CO), 154.47 (A^6), 152.31 (A^2), 149.20 (A^4), 143.87 (Fmoc-C^{8a,b}), 141.64 (A^8), 140.75 (Fmoc-C^{4a,b}), 127.63 (Fmoc-C^{3,6}), 127.08 (Fmoc-C^{2,7}), 125.11 (Fmoc-C^{1,8}), 120.14 (Fmoc-C^{4,5}), 118.52 (A^5), 77.62 (Boc), 65.54 (Fmoc-CH$_2$), 50.00, 47.34 (Aeg-C^2), 47.01, 46.96 (Aeg-C^4), 46.73 (Fmoc-C^9), 43.65, 43.47 (A$^{N9\text{-}CH_2}$), 39.66 (A$^{N6\text{-}CH_2CH_2}$, A$^{N6\text{-}CH_2CH_2}$), 38.70, 37.89 (Aeg-C^5), 28.24 (Boc); HR-MS (FAB$^+$, 3-NBA/DMSO): m/z: 659.301 [M+H]$^+$, calcd for $C_{33}H_{38}N_8O_7$: 659.294.

Solid-phase synthesis by esterification of the starting monomer with the anchor alcohol: A solution of acid **20** (1.14 g, 3.41 mmol), HOBt (0.92 g, 5.11 mmol), NMM (0.75 mL, 6.82 mmol) and HBTU (1.29 g, 3.41 mmol) in CH_2Cl_2 (25 mL) was added to aminomethylpolystyrene (1.24 g, 1.1 mmol per g). After 15 h the resin was washed with CH_2Cl_2 and capped by a 15 min treatment with 16 mL Ac_2O/Pyr (1:3). Washing with CH_2Cl_2 and drying afforded polymer **21** (1.74 g). NMR-in-situ-cleavage assay: Minute amounts of sodium were dissolved in [D$_4$]MeOH (0.5 mL). This solution was added to $CDCl_3$ (3 mL). To the resin **21** (62.4 mg) was added toluene (4.15 μL) and the freshly prepared solution of sodium methylate in $CDCl_3$ (2 mL). After 30 min ^{1}H NMR revealed a toluene/Ac-ratio of 1.28/1 translating into a loading of 0.8 mmol per g.

Loading: Resin **21** was suspended in a solution of 1 N NaOH (3 mL) in 1,4-dioxane (1:3). After 15 min the resin was washed with 1,4-dioxane (4 × 3 mL). This procedure was repeated once. The resin was washed with 1,4-dioxane (3 × 3 mL), 1,4-dioxane/H_2O (1:1, 3 × 3 mL), H_2O (3 × 3 mL) and dry DMF (8 × 3 mL). To the resin was added a 3 – 5-fold excess (based on Ac-loading of resin **21**) of a solution of the symmetrical anhydride of the starting monomers. After 18 h the resin was washed with DMF (6 × 3 mL) and CH_2Cl_2 (4 × 3 mL).

Boc Cleavage: TFA/*m*-Cresol (95:5, 1.5 mL) was added to the resin. After 4 min and washing with CH_2Cl_2 this procedure was repeated once. Finally the resin was washed with CH_2Cl_2 (6 × 2 mL) and DMF (6 × 2 mL).

Coupling: 2.5 equiv of the building block (final concentration ≈ 0.1 M) were added to the resin, which was preactivated for 2 min using 4 equiv HOBt, 2.5 equiv HBTU in a solution of 0.5 M DIPEA in pyridine/DMF (1:4). After 3 h the resin was washed with DMF (5 × 2 mL).

Capping: Ac_2O/Pyr (1:50, 2 mL) was added to the resin. After 3 min the resin was washed with DMF and the procedure was repeated once. Finally the resin was washed with DMF (6 × 2 mL) and CH_2Cl_2 (6 × 2 mL).

Cleavage: A degassed solution of $[Pd(PPh_3)_4]$ in morpholine/DMF/DMSO (1:5:5) was added to the resin. After 16 h the resin was washed repeatedly with DMF. The filtrate was evaporated in vacuo and the residue repeatedly coevaporated with DMF until dryness.

Boc-Phe-G^Z-G^Z-C^Z-OH (23): 31 mg (25 μmol) resin **21** were used. Gradient silica gel chromatography ($CHCl_3$/MeOH/AcOH 85:15:1 → $CHCl_3$/MeOH/AcOH/H_2O 4:2:1:0.35) furnished a white powder (13.5 mg, 36 %). R_f ($CHCl_3$/MeOH/AcOH/H_2O 4:2:1:0.2) = 0.26; MS (MALDI-TOF, DHB, pos): m/z: 1502.11 [M(monoisotop)+H]$^+$, calcd for $C_{70}H_{76}N_{20}O_{19}$: 1501.72.

Ac-C^Z-C^Z-G^Z-Gly-OH (24): 37 mg (30 μmol) resin **21** were used. LH20 chromatography ($CHCl_3$/MeOH 1:1) and subsequent *n*-hexane-mediated precipitation from CH_2Cl_2/MeOH (1:1) and drying in vacuo furnished a white powder (13.6 mg (35 %). R_f ($CHCl_3$/MeOH/AcOH/H_2O 4:2:1:0.5) = 0.5; t_R = 31 min (0 – 40 min: 10 – 80 % B in A, 1 mL min^{-1}, 50 °C); MS (MALDI-TOF, DHB, pos): m/z: 1314.4 [M(average)+H]$^+$, calcd for $C_{59}H_{64}N_{18}O_{18}$: 1314.3.

Solid-phase synthesis by loading preformed starting monomer-HYCRON conjugates: **Boc-Gly-G^Z-G^Z-C^Z-OH (25)**: A solution of conjugate **6a** (80.0 mg, 103 μmol), HOBt (23.6 mg, 150 μmol), DIPEA (35.2 μL, 206 μmol) and HBTU (39 mg, 103 μmol) in DMF (1 mL) was added to aminomethylpolystyrene (62.4 mg, 1.1 mmol g^{-1}). After 15 h the resin was washed with CH_2Cl_2 and capped by a 10 min treatment with Ac_2O/Pyr (1:50, 2 mL). Washing with DMF and CH_2Cl_2 and drying afforded resin **22a** (140 mg). The subsequent synthesis proceeded as decribed using resin **22a** (70 mg). After cleavage the crude material was purified by LH20 chromatography ($CHCl_3$/MeOH 1:1) and gradient silica gel chromatography ($CHCl_3$/MeOH/AcOH 85:20:2 → $CHCl_3$/MeOH/AcOH/H_2O 4:2:1:0.35). Drying in vacuo furnished a white powder (32.2 mg, 67 %). R_f ($CHCl_3$/MeOH/AcOH/H_2O 4:2:1:0.5) = 0.42; t_R = 20 min (0 – 40 min: 25 – 75 % B in A, 1 mL min^{-1}, 50 °C); MS (FAB$^+$, 3-NBA/DMSO): m/z: 1411.82 [M(monoisotop)+H]$^+$, calcd for $C_{63}H_{70}N_{20}O_{19}$: 1411.52.

Ac-C^Z-C^Z-G^Z-Gly-OH (24): A solution of conjugate **6c** (110.0 mg, 240 μmol), HOBt (53.0 mg, 350 μmol), NMM (52.0 μL, 480 μmol) and HBTU (39 mg, 232 μmol) in CH_2Cl_2/DMF (2:1, 1.5 mL) was added to aminomethylpolystyrene (90.0 mg, 1.1 mmol per g). After 15 h the resin was washed with CH_2Cl_2 and capped by a 5 min treatment with Ac_2O/Pyr (1:3, 2 mL). Washing with DMF and CH_2Cl_2 and drying afforded resin **22c** (170 mg). The subsequent synthesis proceeded as decribed using 56 mg of resin **22c**. After cleavage the crude material was purified by LH20 chromatography ($CHCl_3$/MeOH 1:1) and silica gel chromatography ($CHCl_3$/MeOH/AcOH/H_2O 7:2.5:1:0.5). Drying in vacuo furnished a white powder (25.6 mg, 59 %). Analytical data: see above.

Solid-phase synthesis by on-resin synthesis of the starting monomer

Boc-G^Z-HYCRON-PS (27): A solution of conjugate **6b** (0.98 g, 1.37 mmol), HOBt (0.32 g, 2.06 mmol), NMM (0.3 mL, 2.74 mmol) and HBTU (0.52 g, 1.37 mmol) in DMF (7 mL) was added to aminomethylpolystyrene (0.62 g, 1.1 mmol per g). After 15 h the resin was washed with DMF and capped by a 10 min treatment with Ac_2O/Pyr (1:6, 7 mL). Washing with DMF and CH_2Cl_2 and drying afforded resin **26**, displaying a Fmoc-loading of 0.6 mmol per g. To resin **26** (96 mg, 60 μmol) was added DMF/morpholine (1:1, 3 mL). After 45 min the resin was washed with DMF (8 × 3 mL). DIPEA (61 μL, 360 μmol), pyridine (100 μL) and HATU (64 mg, 170 μmol) were added subsequently to a solution of the N^9-carboxymethylene-N^2-(benzyloxycarbonyl)-guanine (61.8 mg, 180 μmol)

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in DMF (1.3 mL). After 5 min of preactivation this solution was added to the resin and shaken for 16 h. Washing with DMF and dry CH_2Cl_2 and drying over P_4O_{10} yielded resin **27** (88 mg). To 19.2 mg of resin **27** was added a degassed solution of $[Pd(PPh_3)_4]$ in morpholine/DMF/CH_2Cl_2 (1:5:3, 10 mL). After 16 h the resin was washed repeatedly with DMF. The filtrate was evaporated in vacuo and the residue repeatedly coevaporated with DMF until dryness. LH20 chromatography was followed by gradient silica gel chromatography ($CHCl_3$/MeOH/AcOH 85:15:1 → $CHCl_3$/MeOH/AcOH/H_2O 70:30:3:10). Suspended silica gel was removed by repeated filtration of a solution of the residue in CH_3CN/EtOH (1:1). Subsequent evaporation of the filtrate yielded building block **28** (4.3 mg, 61 %) which proved identical to an authentic reference (purchased from Applied Biosystems).

Ac-G^{Z}-C^{Z}-C^{Z}-A^{Z}-C^{Z}-G^{Z}-G^{Z}-OH (29a): Starting from resin **27** (22 mg) the linear chain assembly was performed as described above. The crude material obtained after the Pd^0-catalysed cleavage was purified by gradient silica gel chromatography ($CHCl_3$/MeOH/AcOH 80:20:2 → $CHCl_3$/MeOH/AcOH/H_2O 4:2:1:0.5) yielding to fraction A (35 mg) and fraction B (26 mg), which both contained product **29a**. Preparative HPLC of fraction A (4.5 mg) yielded Ac-G^{Z}-C^{Z}-C^{Z}-A^{Z}-C^{Z}-G^{Z}-Aeg(Ac)-OH **29b** (2.0 mg, 39 %) and product **29a** (0.4 mg, 7 %). Preparative HPLC fraction B (4.1 mg) yielded additional product **29a** (2.0 mg, 29 %, overall: 36 %). $t_R = 31$ min (0–40 min: 10–80 % B in A, 1 mL min^{-1}, 50 °C); MS (MALDI-TOF, DHB, pos): *m*/*z*: 2902 [*M*(average)+H]$^+$, calcd for $C_{132}H_{137}N_{43}O_{36}$: 2903.

Ac-G^{Z}-C^{Z}-C^{Z}-A$^{(CH_2)_6NH\text{-}Fmoc}$-C^{Z}-G^{Z}-G^{Z}-OH (32): Starting from resin **26** (20 mg) the guanosine base was coupled as decribed for **27**. The linear chain assembly was performed until incorporation of **3**. The resin was dried yielding **30** (41 mg). To resin **30** (16.4 mg) was added DMF/morpholine (1:1, 2 mL). After 60 min the resin was washed with DMF. The adenine modification **11** (9.0 mg, 19.2 μmol) was preactivated for 5 min by dissolving in DMF (200 μL) and adding NMM (4.4 μL) and HATU (6.1 mg, 16.0 μmol) before the resulting mixture was added to the resin. After 13 h the resin was washed with DMF and CH_2Cl_2 and dried. The Fmoc group was removed from 0.3 mg of the 16.0 mg of resin **31** obtained and the deprotection quantified. The Fmoc loading was 0.4 mmol per g. The chain assembly was completed and the Pd^0-catalysed cleavage was performed as described. C18-SepPak-filtration and preparative HPLC was followed by drying in vacuo to afford a yellowish powder (7.3 mg, 50 %). $t_R = 15.5$ min (0–30 min: 50–90 % B in A, 30–35 min: 90 % B in A, 1 mL min^{-1}, 50 °C); MS (MALDI-TOF, DHB, pos): *m*/*z*: 3091 [*M*(average)+H]$^+$, calcd for $C_{145}H_{154}N_{44}O_{36}$: 3090.

Boc-Gly-G^{Z}-G^{Z}-C^{Z}-NHCH$_2$CH$_2$NH-FITC (34a): A 50 mM HOBt solution (100 μL, 5 μmol) in DMF and a 50 mM EDC solution (50 μL, 2.5 μmol) in CH_2Cl_2 was added to a solution of protected PNA **25** (1.9 mg, 1.35 μmol) in DMF (500 μL). After addition of the amine **33a** (1.3 mg, 2.84 μmol) the solution was stirred for 40 h. Sephadex-LH20 chromatography ($CHCl_3$/MeOH 1:1) furnished a bright yellow solid (1.6 mg, 64 %) after drying in vacuo. MS (MALDI-TOF, DHB, pos): *m*/*z*: 1843.5 [*M*(average)+H]$^+$, calcd for $C_{88}H_{87}N_{23}O_{23}S$: 1843.8.

H-Gly-G-G-C-NHCH$_2$CH$_2$NH-DANS (35): Analogously to **34a** protected PNA **25** (2.0 mg, 1.41 μmol) was subjected to the coupling with dansylamine **33b**. Sephadex-LH20 chromatography ($CHCl_3$/MeOH 1:1) furnished a yellow powder (3.3 mg), which was dissolved in TFA (4 mL). After 15 min the TFA was removed by evaporation in vacuo. To the residue was added dimethylsulfide (300 μL), *m*-cresol (300 μL), TFA (3 mL) and trifluoromethanesulfonic acid (300 μL). After 2.5 h precipitation was induced by the addition of cold diethyl ether (40 mL). The precipitate was purified by size-exclusion chromatography (Biogel P2, 0.05 M NH_4HCO_3) and preparative HPLC. Lyophilisation yielded a yellow solid (1.0 mg, 60 % based on **25**). $t_R = 18$ min (0–40 min: 0–50 % B in A, 1 mL min^{-1}, 50 °C); (FAB$^+$, NBA): *m*/*z*: 1206.53 [*M*(monoisotop)+Na]$^+$, calcd for $C_{48}H_{61}N_{23}O_{12}S$ Na: 1206.45.

Ac-G-C-C-A$^{(CH_2)_6NH_2}$-C-G-G-NHCH$_2$CH$_2$NH-DANS (36): A 30 mM DMF/HOAt solution (25 μL) and the amine **33b** were added to PNA-oligomer **32** (0.4 mg, 0.13 μmol). Subsequently, a 15 mM solution (20 μL) of EDC in CH_2Cl_2 was added. After 25 h of stirring HPLC purification afforded 1 mg material, which was dissolved in DMF/morpholine (1:1, 1 mL). The resulting solution was stirred for 50 min before DMF (3 mL) was added. To the residue obtained after evaporation in vacuo was added a TFMSA/TFA/Me_2S/*m*-cresol-mixture (2:10:1:1, 1 mL). After 3 h the solution was concentrated in vacuo before addition of cold diethyl ether (10 mL). The precipitate was purified by HPLC. Lyophilisation yielded a fluffy powder (0.2 mg, 66 %). $t_R = 18.5$ min (0–30 min: 0–25 % B in A, 30–40 min: 25–80 % B in A, 1 mL min^{-1}, 50 °C); MS (MALDI-TOF, DHB, pos): *m*/*z*: 2337 [*M*(average)+H]$^+$, calcd for $C_{96}H_{125}N_{47}O_{23}S$: 2338.

Ac-G-C-C-A$^{(CH_2)_6NH\text{-}Dabs}$-C-G-G-NHCH$_2CH_2$NH-DANS (37): A 0.1 M $NaHCO_3$ solution (40 μL) and a 30 mM dabsyl chloride solution (10 μL) in DMF were added to oligomer **36** (0.2 mg, ≈0.08 μmol). After 16 h the mixture was passed through an water-equilibrated RP18-SepPak cartridge and washed with water. Elution with CH_3CN/H_2O (6:4) and drying in vacuo yielded a red solid (0.1 mg, ≈50 %). $t_R = 20$ min (0–30 min: 0–25 % B in A, 30–40 min: 25–80 % B in A, 1 mL min^{-1}, 50 °C); UV/Vis: $\lambda_{max} = 269$, 462 nm; $C_{110}H_{138}N_{50}O_{25}S_2$ (2624.75).

Ac-TTATTATTATTA$^{(CH_2)_4NH_2}$TTAT-Gly-OH (42): The Boc-Gly-HYCRON conjugate **6c** was coupled as described above to Tentagel (500 mg, 0.29 mmol per g) to yield resin **38** (535 mg). CH_2Cl_2/TFA (1:1, 2 mL) was added to the resulting resin (135 mg). After 50 min the resin was washed with CH_2Cl_2 and with DMF. This resin was treated with a solution of Fmoc-T (56 mg, 0.11 mmol) in DMF (1 mL), to which HOBt (19 mg, 0.11 mol), NMM (24 μL, 0.22 mmol) and HBTU (19 mg, 0.11 mmol) were added. After 3 h the resin was washed with DMF (5 ×) and was subjected to capping as described. Final washing with CH_2Cl_2 and subsequent drying in vacuo furnished resin **39** (148 mg), which contained 0.19 mmol Fmoc g^{-1} resin. Resin **39** (33 mg, 5 μmol) was used for the synthesis of **41**.

Fmoc removal: DMF/piperidine (4:1), 1 × 1–2 min, 1 × 8 min, 7 washings with DMF.

Couplings: The resin was suspended in a solution of 20 μmol Fmoc-protected building block in 0.266 M NMM in DMF (2 % pyridine, 150 μL) which was preactivated by addition of HATU (7.6 mg, 20 μmol). After 1.5 h the resin was washed 5 × with DMF.

Capping: Ac_2O/pyr (1:50, 1.5 mL), 10 min, 7 washings with DMF. The final Fmoc cleavage was monitored to reveal a loading with 2.9 μmol Fmoc. The N-terminus was acetylated by a subsequent capping step.

Removal of Bhoc- and Boc-protecting groups: The dried resin (46 mg) was suspended in TFA/ethanedithiol/H_2O (95:2.5:2.5, 1.5 mL) and shaken for 15 min. After filtration the resin was washed with CH_2Cl_2 (3 ×), the TFA mixture (1.5 mL) was added again and the resin was shaken for further 40 min. The procedure was repeated for another 5 min until the CH_2Cl_2 filtrates proved colorless. The resin was washed with CH_2Cl_2 (5 ×), pyridine (5 ×) and DMF (5 ×).

Cleavage: The Pd^0-catalysed cleavage was performed as described for **23**. After 16 h the resin was filtered and washed excessively with DMF. The combined filtrates were concentrated to dryness in vacuo. Traces of DMSO were removed by repeated coevaporation of DMF.

Purification: A solution (7 mL) of 0.1 % TFA in H_2O was added to the residue. The precipitate was removed by filtration. The volatiles were removed from the filtrate in vacuo and to the residue was added 5 % MeOH in H_2O (2 mL). After removal of the precipitate by filtration, the filtrate was passed through a H_2O equilibrated Kromafix C18 cartridge. Elution with H_2O (5 mL) and subsequent evaporation yielded the residue (10.8 mg). RP-HPLC purification (Nucleosil 5-100, C18 HD, 250 × 10 mm, 3.5 mL min^{-1}) of 7.7 mg and subsequent lyophilisation yielded a white fluffy powder (4.5 mg, 27 % based on the TFA salt of **42**). $t_R = 16$ min (0–40 min: 0 % → 40 % B in A, 1 mL min^{-1}, 50 °C); MS (MALDI-TOF, DHB, pos): *m*/*z*: 4494 [*M*(average)+H]$^+$, calcd for $C_{184}H_{235}N_{81}O_{57}$: 4494.

Ac-Cys(EDANS)-TTATTATTATTATTA$^{(CH_2)_4NH\text{-}DABCYL}$T-Gly-OH (44): Starting from resin **39** (19.5 mg, 2.9 μmol) the chain assembly (building blocks: Fmoc-T, Fmoc-A^{Bhoc}, Fmoc-Cys(Trt) and **19a**) and the removal of the Trt-, Boc- and Bhoc-groups was performed as decribed for **42**. Before attachment of the reporter groups the resin **40b** was washed with pyridine (4 ×). To the resin was added DABCYL-SE (2.5 mg, 6.8 μmol) in a DMF/pyr/NMM solution (7:1:1, 90 μL). After 15 min a further DABCYL-SE (1.4 mg, 3.8 μmol) was added. The suspension was shaken for 4 h. The resin was washed with DMF (5 ×) and treated with DMF/piperidine (4:1). After 5 min the resin was washed with DMF (5 ×). The resin was supended in a solution of dithiothreitol (10 μmol) in a mixture of DMF/H_2O (3:1, 250 μL) and NMM (20 μL). After 15 min the resin was washed with degassed DMF/H_2O (3:1) and a solution of IAEDANS (4.0 mg, 9.2 μmol) in DMF/H_2O

(3:1, 200 μL) containing 5% NMM. After 4 h the resin was washed with DMF/H_2O (3:1, 5 ×) and with DMF (5 ×).

Cleavage: see **23**.

Purification: The combined DMF filtrates obtained after the cleavage were concentrated in vacuo. Repeated coevaporation with DMF yielded a solid residue. H_2O was added and the precipitate removed by filtration. The concentrated filtrate was applied on a C18-SepPak cartridge, which was "activated" before by passing through MeOH followed by water equilibration. First H_2O (5 mL) was eluted, then CH_3CN/H_2O (4:1, 5 mL). The red-colored eluent obtained after addition of CH_3CN/H_2O (3:1, 5 mL) was collected and lyophylised. Two-fold semipreparative HPLC (Nucleosil 5-100, C18 HD, 250 × 10 mm, 0–30 min: 10% → 40% B in A, 3.5 mL min^{-1}) yielded 25.2 OD_{260}. $t_R = 22$ min (0–40 min: 10% → 40% B in A, 1 mL min^{-1}, 50 °C); ESI-MS (nanospray, pos): *m*/*z*: 5153.0, calcd for $C_{216}H_{267}N_{87}O_{63}S_2$: 5154.0; UV/Vis ($H_2O$): $\lambda_{max} = 265$, 485 nm.

Ac-Cys(EDANS)-TTATTATTATTA$^{(CH_2)_4NH\text{-}DABCYL}$TTAT-Gly-OH (45): Starting from resin **39** (19.5 mg, 2.9 μmol) the synthesis was performed as described for **44**. A yield of 16.3 OD_{260} was obtained. $t_R = 23$ min (0–40 min: 10% → 40% B in A, 1 mL min^{-1}, 50 °C); ESI-MS (nanospray, pos): *m*/*z*: 5153.0, calcd for $C_{216}H_{267}N_{87}O_{63}S_2$: 5154.0; UV/Vis ($H_2O$): $\lambda_{max} =$ 265, 485 nm.

Ac-Lys(Dans)-ACCTACAGCC-Lys(DABCYL)-NH_2 (48): Fmoc-Lys-(DABCYL) (purchased from Molecular Probes, Leiden, Netherlands) was coupled to the Tentagel-resin loaded with the Rink-linker. The subsequent solid-phase synthesis was performed in 3 μmol scale with a Multisyntech Syro synthesizer and with the methods as described for **42**. At the N-terminal end Fmoc-Lys(Boc)-OH was coupled, the Fmoc group was removed and the liberated amino group subsequently acetylated. Cleavage from the solid support was performed using TFA/ethanedithiol/thioanisol/H_2O (92.5:2.5:2.5:2.5). The purification of the PNA–DABCYL conjugate was performed by solid-phase extraction followed by HPLC (see above) which furnished 10.8 OD_{260}. To the purified PNA-DABCYL conjugate (2.1 OD_{260}, 65 nmol) in CH_3CN/H_2O (4:6, 48 μL) DIPEA (4.2 μL) was added. Within 30 min a solution of 5-dimethylaminonaphthaline-1-sulfonyl chloride in 40% CH_3CN/H_2O (0.16 M) was added in four portions (1 μL). The mixture was vortexed for 1 h. The purification proceeded by chromatography on a C18-SepPak cartridge as described for **44**. The fraction eluting with CH_3CN/H_2O (4:6) was collected. HPLC analysis showed a single peak. Lyophilisation yielded 1.4 OD_{260}. $t_R = 14$ min (C18-PPN (Macherey&Nagel), 250 × 4 mm, 0–2 min: 10% B in A, 2–40 min: 10% → 50% B in A, 1 mL min^{-1}, 50 °C); MS (MALDI-TOF, DHB, pos): *m*/*z*: 3440.5 [*M*(average)+H]$^+$, calcd for $C_{146}H_{184}N_{66}O_{34}S$: 3440.6.

Hybridisation experiments: Stock solutions of the PNA probes (50 pmol μL^{-1} in CH_3CN/H_2O (1:1)) and the DNA-targets (400 pmol μL^{-1} in H_2O) were prepared. The PNA probe solution (20 μL) was added into a quartz cuvette and diluted with aq. buffer (100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH 7, 980 μL). The fluorescence spectrum (excitation at 335 nm) was recorded at 298 K sample temperature. DNA target was added (10 μL) and the fluorescence spectrum was measured after 10 min. In an alternative experiment (uppermost curves in Figure 5) the PNA probe solution (30 μL) was diluted with the buffer (970 μL) and fluorescence spectra (excitation at 335 nm) were measured at 293 K sample temperature before and after addition of DNA target (10 μL).

Hybridisation kinetics: To the aq. buffer (100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH 7, 980 μL) was added an aliquot of the stock solution of PNA-probe **44** (50 pmol μL^{-1} in CH_3CN/H_2O (1:1), 20 μL). Oligonucleotide **47** was added (400 pmol μL^{-1} in H_2O, 2.5 μL) and the emission (298 K, excitation at 335 nm) at 485 nm was monitored. After 21 min further **47** (400 pmol μL^{-1} in H_2O, 7.5 μL) was added, after 40 min **46** (400 pmol μL^{-1} in H_2O, 2.5 μL) was added and after 51 min further **46** (400 pmol μL^{-1} in H_2O, 7.5 μL).

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