# Divergent and Linear Solid-Phase Synthesis of PNA Containing Thiazole Orange as Artificial Base

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**Keywords:** Cyanine dyes / DNA / Fluorescent probes / Peptide nucleic acids / Peptide coupling / Protecting groups

Fluorescent nucleobase surrogates provide nucleic acids with interesting properties. We have recently introduced thiazole orange as base surrogate into PNA and found that the so-called FIT (*F*orced *I*ntercalation of *T*hiazole orange) PNA probes signal hybridization by enhancements of fluorescence. Common approaches of modifying nucleobases or introducing nucleobase surrogates draw upon the usage of monomer building blocks that have been synthesized in solution phase. The need to prefabricate a base-modified building block can hold up progress if several base modifications or base surrogates are to be evaluated. Herein, a method for divergent solid-phase synthesis is presented that serves the purpose to facilitate the screening for base surrogates that fluoresce upon hybridization. An Fmoc/Aloc-protected submonomer allowed the application of commonly used Fmoc-based solid-phase synthesis protocols while removal of the fully orthogonal Aloc group enabled the on-resin introduction of base surrogates after the linear chain assembly had been completed. The divergent solid-phase synthesis strategy is automatable, gives overall yields matching those of linear solid-phase synthesis and, most importantly, provides rapid access to any kind of base-modified PNA.


## Introduction

The replacement of nucleobases with artificial chromophores or fluorophores provides DNA with interesting properties that enable studies of DNA–DNA and DNA–protein interactions.[1] In contrast to fluorophores that are appended by means of a flexible tether, fluorescent bases and base surrogates allow measurements of local structure and dynamics of nucleic acids.[2–9] Environmentally sensitive fluorophores can report on binding events such as hybridization and are, thus, principally suited for homogeneous DNA detection.[10–14] Recently, our group and the group of Wagenknecht have introduced intercalator dyes, thiazole orange (TO)[15] and phenanthridinium,[16] respectively, as artificial bases that fluoresce upon hybridization. One of the interesting opportunities provided by such base replacements is to link or even force fluorophores into a specific site of the nucleic acid duplex which under normal circumstances would compete with many other binding sites or even be devoid of binding of fluorophores. For example, the DNA stain thiazole orange (TO) was forced to intercalate next to mismatched base pairs in a PNA–DNA duplex, a disfavored site for intercalation. We termed these conjugates FIT (*F*orced *I*ntercalation of *T*hiazole orange) probes and found that the forced mode of intercalation renders the fluorescence of TO responsive against neighboring base mismatches.[17] The relative ease of introducing base analogues into PNA, a DNA mimic that binds complementary DNA with increased affinity and selectivity,[18,19] has been exploited in many studies aimed at enhancements of hybridization properties,[20–28] and site-selective incorporation of fluorescent or photoactive base analogues.[29–35] In these works, single base modifications were introduced into PNA by coupling pre-fabricated monomer building blocks in a linear solid-phase synthesis. In our explorative study[17] of cyanine dye intercalators as artificial bases in PNA that signal hybridization we considered the need to evaluate several thiazole orange derivatives. This endeavor was motivated by Tanaka's studies of acridine-containing oligonucleotides which highlighted the importance of the linker between the scaffold and the intercalator.[36] We therefore set out to synthesise six different thiazole orange "nucleobases" **1a**–**f** which differed in length and attachment sites (Figure 1). According to "conventional" linear solid-phase synthesis, the evaluation of six different thiazole orange derivatives (**1a**–**f**)[37] would require the synthesis of six preformed thiazole orange monomers, a time-consuming venture that we sought to avoid. Previously, we have developed a submonomer approach which omitted the need to prepare preformed monomer building blocks.[38] However, due to the use of the Boc strategy this strategy required cleavage conditions too harsh for the synthesis of acid-labile PNA–fluorophore conjugates. We now present all details on a rapid and automatable solid-phase synthesis that allows access to the title compounds by employing a divergent Fmoc-based solid-phase synthesis protocol which should be generally applicable to the synthesis of any type of base-modified

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PNA molecule. The efficiency of the divergent solid-phase synthesis is compared to that of the "conventional" linear solid-phase synthesis.

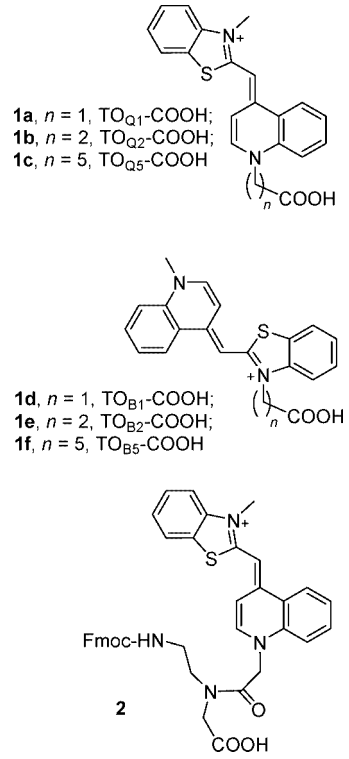
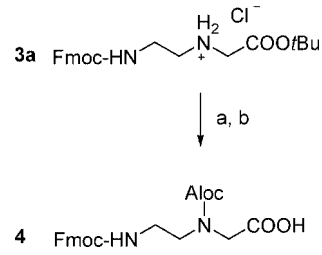



Figure 1. Thiazole orange derivatives for the synthesis of PNA conjugates.

## Results

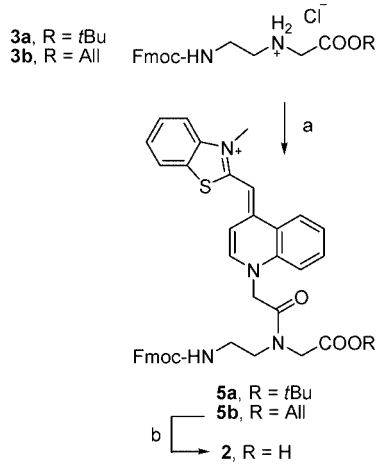
### Synthesis of Building Blocks

PNA synthesis is performed by employing solid-phase strategies that allow the automation of the repetitive process of protecting group removal and building block coupling. Most commonly, the Fmoc group is used as temporary protecting group while the acid-labile benzyhydryloxycarbonyl (Bhoc) group is used for permanent protection of the exocyclic amino groups of nucleobases. In the pursuit of developing a divergent Fmoc-based solid phase we envisioned that an orthogonally protected full-length PNA oligomer would be split into several portions which would then be subjected to on-resin couplings of a particular TO derivative. It was assumed that the use of the Fmoc[39] and the Aloc protecting groups[40] in aminoethylglycine building block **4** in combination with commercially available Fmoc/benzhydryloxycarbonyl-(Bhoc-)protected PNA monomers would allow the selective liberation of the secondary backbone amino group on resin-bound PNA (Scheme 1). In the synthesis of backbone module **4** known Fmoc–Aeg–O*t*Bu[41] **3a** was allowed to react with allyl chloroformate. Subsequent TFA treatment cleaved the *t*Bu ester and furnished **4** in 83% yield after recrystallization.




Scheme 1. a) Aloc–Cl, $i$Pr$_2$NEt, $CH_2Cl_2$; b) TFA, $CH_2Cl_2$, 83% ($i$Pr$_2$NEt = *N*-ethyl-*N*,*N*-diisopopylamine, TFA = trifluroacetic acid).

The proposed divergent solid-phase synthesis was considered as useful for accelerating the identification of a suitable thiazole orange derivative. After this sub-goal had been completed, it was considered more convenient to apply preformed TO-containing monomers in standard linear solid-phase synthesis. Scheme 2 shows the synthesis of PNA monomer **2** containing TO$_{Q1}$ **1a** as fluorescent base surrogate. It was first attempted to couple TO derivative **1a** to the free amino group of Fmoc–Aeg–O*t*Bu **3a**. Neither the pivaloyl chloride mediated coupling used in standard PNA monomer synthesis[42] nor the optimized PyBOP coupling protocol (vide infra, Scheme 3) gave access to product **5a**. However, the difficult coupling succeeded in 62% yield after replacement of the *t*Bu ester by the sterically less demanding allyl ester in **3b** and usage of PyBOP[43] as coupling reagent. Finally, the allyl protecting group in **5b** was removed by Pd$^0$-catalyzed allyl transfer to *N*-methylaniline[44] which afforded the TO$_{Q1}$–PNA monomer **2** in 73% yield.

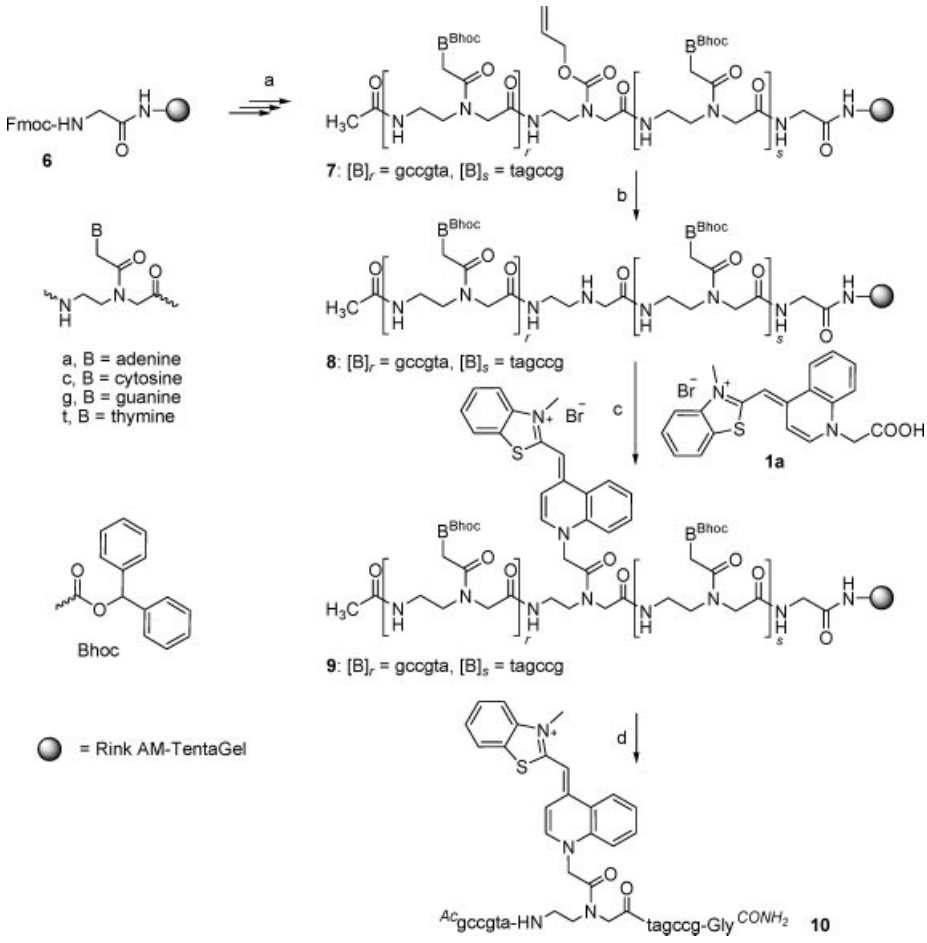



Scheme 2. a) **1a**, PyBOP, PPTS, NMM, DMF, 62%; b) [Pd($PPh_3$)$_4$], $PhNHCH_3$, THF, 73% [PyBOP = (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate, PPTS = pyridinium *p*-toluenesulfonate, NMM = *N*-methylmorpholine].

### Divergent Solid-Phase Synthesis

For the development of a divergent solid-phase synthesis protocol we chose PNA–TO$_{Q1}$ conjugate **10** as synthetic target (Scheme 3). The solid-phase synthesis was commenced from Rink amide resin **6** loaded with Fmoc–gly-




Scheme 3. a) Cycle of 1) piperidine/DMF (1:4); 2) Fmoc–B(Bhoc)–OH or **4**, NMM, PyBop, NMP; 3) $Ac_2O$/lutidine, DMF; b) [Pd-($PPh_3$)$_4$], $Me_2NH{\cdot}BH_3$, $CH_2Cl_2$; c) **1a**, PyBOP, PPTS, NMM, DMF (double coupling); d) TFA, *m*-cresol, $H_2O$, H–Cys–OMe, 8.3% overall yield.

cine. In the subsequent assembly of PNA resin **7**, Fmoc cleavage was achieved by treatment of the resin with piperidine in DMF. The coupling reactions were performed by using commercially available Fmoc/Bhoc-protected building blocks and the backbone module **4** and PyBOP as activation reagent. In this and all other syntheses acetylation was employed in order to block uncoupled amino groups. After completion of the iterative building block assembly, the next task was to accomplish the removal of the Aloc group from fully protected PNA resin **7**. This proved, surprisingly, more difficult than expected. HPLC analysis of crude material obtained after attempted Aloc cleavage and acidolytic release from the solid phase revealed that conventional allyl scavengers such as *N*-methylaniline,[44] dimedon,[40] *N*,*N*-dimethylbarbituric acid,[45] diethyl malonate[46] and tributylstannane[47,48] were inefficient in conveying a palladium(0)-catalyzed allyl transfer. Accordingly, stoichiometric amounts of palladium(0) complex had to be employed. The cleavage of Aloc-protected secondary amines has been reported to be slow and it is hence conceivable that liberation of the high molecular weight secondary amine **8** is even more challenging.[49] Tributyltin hydride is known to confer fast Aloc cleavage rates.[47,48] However, the generation of hydrogen upon addition of the $Pd^0$ catalyst indicated that the transition metal preferably reacted with tin hydride rather than with the hindered Aloc group in **7**. It was, therefore, deemed important to use allyl scavengers that do not react with the $Pd^0$ catalyst. Dimethylamine–borane complex was well suited and Aloc cleavage reached completion within minutes.[50]

The subsequent introduction of carboxyalkyl fluorophores such as **1a** was hampered by their low solubility. As a result the coupling with the relatively unreactive high molecular weight secondary amine **8** had to proceed despite low concentration of the acyl component. A variety of potent coupling reagents such as CIP,[51] DIC,[52] HATU,[53] PyBOP,[43] PyBroP[54] and TFFH[55] was evaluated by HPLC analysis of the crude material furnished upon acid-mediated release, however, with little success. Thiazole orange dissolves at acidic pH and we therefore evaluated coupling reactions in the presence of acidic additives such as pyridinium *para*-toluenesulfonate (PPTS), pyridinium hydrobromide and tetrazole. The addition of PPTS was most efficient in increasing the solubility of TO derivative **1a** in the coupling mixture. The use of this additive buffered with diisopropylethylamine in a PyBOP-mediated coupling

reaction enabled the introduction of $TO_{Q1}$. These observations prompted a following study in which we provided clear evidence that PPTS accelerates the coupling of carboxylic acids and carboxylic acid salts.[56] Interestingly, CIP, PyBroP and HATU, each of them reported as potent coupling reagents, failed to confer significant product formation. A comparison of HPLC traces of crude materials provides evidence for the largely increased yields obtained when using PyBOP (Figure 2A) for coupling of $TO_{Q1}$–COOH as opposed to coupling reactions mediated by PyBroP (Figure 2B). For the release of conjugate **10**, PNA resin **9** was treated with TFA in the presence of *m*-cresol, water and cysteine methyl ester as cation scavengers. HPLC purification furnished **10** in 8% overall yield based on the initial loading of **6** with Fmoc groups. Analytical RP-HPLC, MALDI-TOF mass spectrometry and UV/Vis and fluorescence spectroscopy confirmed the purity and molecular mass of **10** and the integrity of the chromophore.

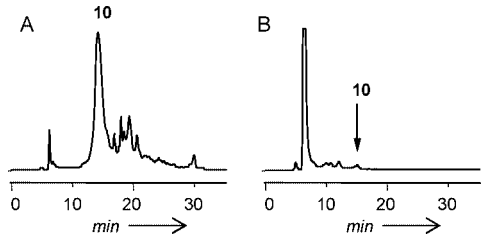
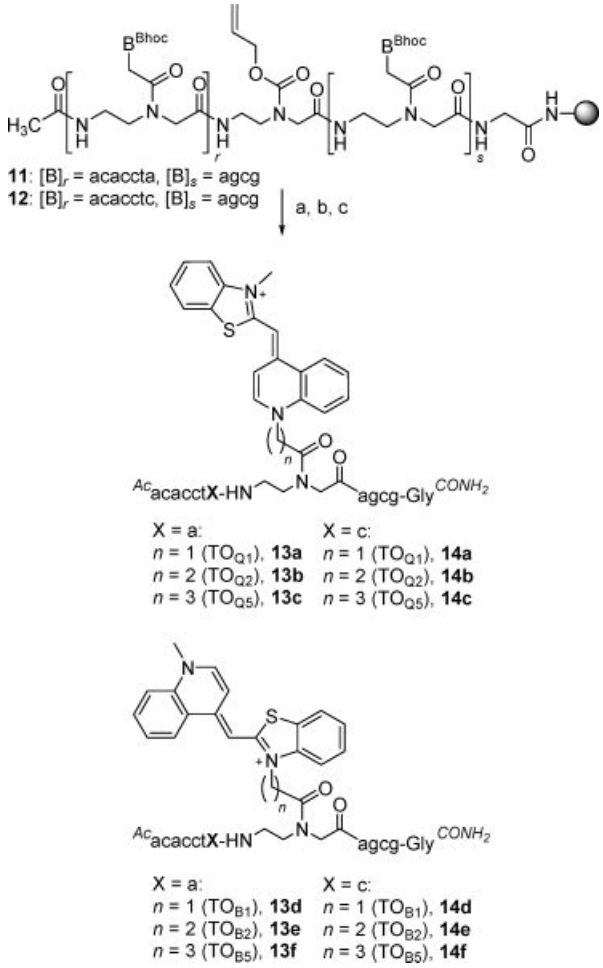



Figure 2. Preparative HPLC trace of crude material **10** obtained when using A) PyBop or B) PyBroP as coupling reagents [HPLC conditions: flow: 6 mL/min; gradient: 0–1 min: 9% MeCN (0.1% TFA) in $H_2O$ (0.1% TFA); 1–31 min: 9–24% MeCN (0.1% TFA) in $H_2O$ (0.1% TFA)].



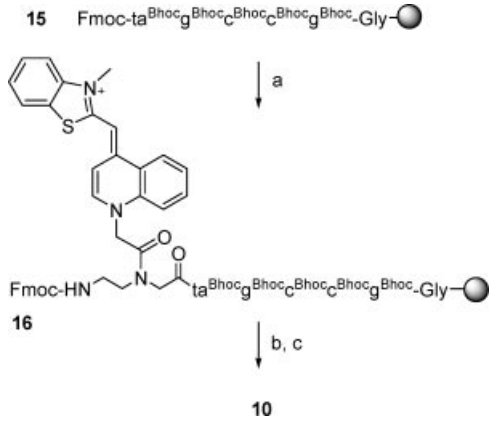

Scheme 4. a) $[Pd(PPh_3)_4]$, $Me_2NH{\cdot}BH_3$, $CH_2Cl_2$; b) **1a**–**f**, PyBOP, PPTS, NMM, DMF; c) TFA, *m*-cresol, $H_2O$, H–Cys-OMe.

For the exploration of TO as fluorescent base surrogate in PNA it was chosen to study sequences that spanned a known carcinogenic single base mutation in the *ras* gene. The solid-phase synthesis of PNA oligomers **13** and **14** was performed by applying the divergent strategy described in Scheme 2 (Scheme 4). For coupling of carboxyalkyl fluorophores **1a**–**f** as base surrogate precursors PNA **11** and **12** were split into several portions. In the HPLC purification of PNA–TO conjugates **13** and **14** special care was taken to isolate the samples in very pure form. Overall yields of purified material varied from 3 to 12%.

## Linear Solid-Phase Synthesis

Divergent synthesis allows rapid access to a variety of modifications. Once a suitable TO derivative had been identified, it was deemed more desirable to apply linear solid-phase synthesis protocols which are more convenient to perform and easier to automate than a divergent synthesis. We therefore explored the linear route by employing $TO_{Q1}$ monomer **2** as preformed building block in the synthesis of PNA–$TO_{Q1}$ conjugate **10**.

The linear solid-phase synthesis was performed with an automated synthesizer. The fully protected PNA resin **15** was assembled first by using 5 equiv. of PNA monomer and HCTU[57] (5 equiv.) as coupling reagent in the presence of *N*-methylmorpholine (8 equiv.) in NMP as solvent (Scheme 5). For the introduction of the $TO_{Q1}$ monomer **2** as well as for the subsequent building block double coup-




Scheme 5. a) 1) Piperidine/DMF (1:4); 2) **2**, HCTU, NMM, PPTS, NMP (double coupling); b) cycle of 1) piperidine/DMF (1:4); 2) Fmoc–B(Bhoc)–OH, NMM, HCTU, NMP; 3) $Ac_2O$/lutidine, DMF; c) TFA, *m*-cresol, $H_2O$, H–Cys–OMe, 9% overall yield [HCTU = *O*-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorphosphate].

lings were performed. PPTS was added to the coupling mixture to increase the solubility of **2**. After completion of the linear building block assembly, a final TFA treatment liberated PNA–$TO_{Q1}$ conjugate **10**. HPLC analysis (Figure 3A) of crude **10** and comparison with material obtained by the divergent approach (Figure 2A) suggests that the purity of crude PNA–$TO_{Q1}$ samples produced by the linear solid-phase synthesis is higher than the purity of crude material furnished by the divergent solid-phases synthesis. As a result, purification was easier to perform. The overall yields achieved by linear solid-phase synthesis were comparable to the yields reached in the divergent synthesis (9% in linear synthesis vs. 8% in divergent synthesis). HPLC traces and MALDI-TOF mass spectrometry of purified material (Figure 3B) attested to the purity and molecular mass of **10**.

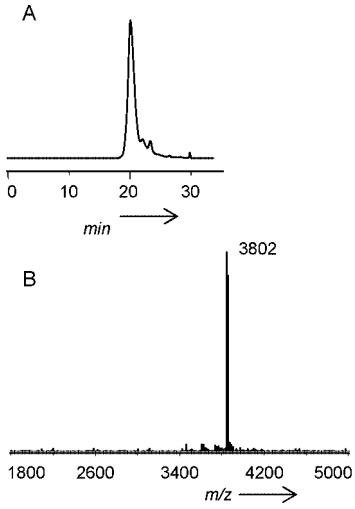



Figure 3. A) Preparative HPLC trace of crude **10** obtained by linear solid-phase synthesis and B) MALDI-TOF-MS of purified **10** [HPLC conditions: flow: 6 mL/min; gradient: 0–1 min: 3% MeCN (0.1% TFA) in $H_2O$ (0.1% TFA); 1–25 min: 3–30% MeCN (0.1% TFA) in $H_2O$ (0.1% TFA)].

## Discussion

Common approaches of modifying nucleobases or introducing nucleobase surrogates draw upon the usage of monomer building blocks that have been synthesized in solution phase. This strategy provided reliable access to a variety of base-modified oligonucleotides and oligonucleotide analogues. However, the need to prefabricate a base-modified building block can hold up progress if several base modifications or base surrogates are to be evaluated. The new orthogonally protected backbone building block Fmoc–Aeg(Aloc)–OH **4** provided rapid access to the desired PNA analogues by using one common PNA precursor for the introduction of 6 different base surrogates in a divergent solid-phase synthesis. The use of the Fmoc group for temporary blockage allowed the application of the Fmoc protocol. The cleavage conditions applied in Fmoc solid-phase synthesis are milder than the strongly acidic conditions required to release PNA oligomers synthesized by Boc chemistry which is of advantage when introducing fluorophores with extended π-systems.

The divergent introduction of thiazole orange derivatives as PNA base surrogates required the Aloc group of **4** to be removed prior to on-resin acylation. However, Aloc cleavage proved slow when using catalytic palladium(0) loads and "conventional" allyl scavengers like morpholine, malonates or barbiturates. The cleavage of Aloc-protected secondary amines has been reported to be slow and it is hence conceivable that liberation of the high molecular weight secondary amine **8** is even more challenging.[58] In order to enhance cleavage rates, either stoichiometric palladium(0) loads or more potent reagents for trapping of the (π-allyl)-palladium(0) intermediate need to be employed. Tributyltin hydride is known to confer fast Aloc cleavage rates.[47,48] However, the generation of hydrogen upon addition of the $Pd^0$ catalyst indicated that the transition metal catalyst preferably reacted with tin hydride rather than with the hindered Aloc group in **7**. It seems, therefore, advantageous to use allyl scavengers such as dimethylamine–borane complex that do not react with the $Pd^0$ catalyst.

The initial attempts to accomplish the on-resin coupling of $TO_{Q1}$–COOH to the relatively unreactive secondary amino group of aminoethylglycine were plagued by the low solubility of thiazole orange in neutral to basic media. However, addition of strong acids would reduce the reactivity of the amine component by protonation. It occurred to us that the use of amine-buffered weakly acidic additives such as PPTS, Pyr·HBr or tetrazole would provide the necessary protons without significant protonation of the amino group to be coupled. PPTS and Pyr·HBr are characterized by similar p$K_A$ values (5.21 and 5.16, respectively). However, PPTS proved best in enhancing the solubility of $TO_{Q1}$–COOH, which suggests a positive effect of the more hydrophobic *para*-toluenesulfonate ion possibly exerted by exchange of the bromide counterion of TO. We speculate that PPTS addition might prove advantageous in the coupling of acyl donor salts in general. The challenges presented by the "difficult" acyl donors used in this study make us confident that the developed divergent solid-phase synthesis protocol is generally applicable, thereby offering new opportunities to rapidly synthesize any type of base-modified PNA.

While the divergent approach was found to bear advantages as far as the rapidity of synthesis is concerned, it is the linear solid-phase synthesis that was demonstrated to provide crude materials of higher purity. In addition, linear solid-phase protocols are easier to automate. The synthesis of preformed TO–PNA monomer **2** enables routine access to PNA oligomers containing $TO_{Q1}$ as fluorescent universal base.

## Conclusions

We have synthesized PNA conjugates, so-called FIT probes,[17] in which thiazole orange replaces a canonical nucleobase. The divergent solid-phase synthesis protocol

developed in this study is expected to provide rapid access to any type of base-modified PNA. The use of an Fmoc/Aloc-protected submonomer allowed the application of the Fmoc strategy and the on-resin introduction of TO derivatives. The applied cleavage conditions are milder than the strongly acidic conditions required to release PNA oligomers synthesized by Boc chemistry which is of advantage when introducing acid-labile fluorophores. This strategy should prove particularly useful in studies aiming for a rapid screening of base analogues or base surrogates in PNA and should therefore offer new opportunities for a more systematic evaluation of PNA base modifications.

## Experimental Section

**General Procedures and Materials:** Manual solid-phase synthesis was performed by using 5-mL polyethylene syringe reactors that are equipped with a fritted disc. Automated solid-phase synthesis was performed with an Intavis ResPep parallel synthesizer equipped with 1-mL reactors. All column chromatography was performed with SDS 60 ACC silica gel and TLC with E. Merck Silica Gel 60 $F_{254}$ plates. $^1$H- and $^{13}$C NMR spectra were recorded with Bruker AC250, DPX 300, AM 300 or AM400 spectrometers. The signals of the residual protonated solvent ($CDCl_3$ or [$D_6$]DMSO) were used as reference signals. Coupling constants are given in Hz. HPLC was performed with a Gilson 321 instrument using RP-C18 columns CC-250/4 NUCEOSIL (100-5) C18-HD for analytical runs and SP125/10 NUCLEODUR C18-gravity (5 μ) for semipreparative runs. Columns were heated to 50 °C. Eluent A (0.1% TFA in water + 1% MeCN) and B (0.1% TFA in MeCN + 1% water) were used in a linear gradient with a flow rate of 1 mL/min for analytical and 6 mL/min for preparative HPLC. Mass spectra were measured with a Finnigan LTQFT spectrometer for FAB-MS and ESI-MS; MALDI-TOF mass spectra were recorded with a Voyager-DE™ Pro Biospectrometry Workstation of PerSeptive Biosystems. DNA was purchased from MWG-Biotech in HPSF quality. Fmoc/Bhoc-protected PNA monomers were purchased from Applied Biosystems. Water was purified with a Milli-Q® Ultrapure Water Purification System (Millipore Corp.). Determination of yields (PNA oligomers): PNA samples were diluted with 500 μL of millipore water, each probe was vortexed for 2 min. To 5 μL of the sample solution was added 995 μL of water. The optical density was measured at 260 nm with a Varian Cary 100 Bio-UV/Vis spectrophotometer by using quartz cuvettes with 10 mm path length. The extinction coefficients were calculated by applying the nearest-neighbour method and oligo calculation at www.Gensetoligos.com.

**Fmoc–Aeg(Aloc)–OH (4):** Fmoc–Aeg-*t*Bu·HCl (990 mg, 2.5 mmol) and allyl chloroformate (400 μL, 4.7 mmol) were dissolved in dry dichloromethane (10 mL). The reaction mixture was kept under argon. To this mixture diisopropylethylamine [609 mg (80 μL), 4.7 mmol] was added slowly within 1 h. After stirring for an additional hour, the reaction mixture was washed with 0.1 M HCl and brine. The organic layer was dried with magnesium sulfate before the solvent was removed in vacuo. The yellowish residue was dissolved in a mixture of dichloromethane (5 mL) and TFA (5 mL). After 30 min of stirring, the volatiles were removed under reduced pressure and the residue was coevaporated with toluene (3 × 20 mL). The residue was dissolved in ethyl acetate (15 mL) and cyclohexane (100 mL) was added. The precipitate was collected by filtration and washed with cyclohexane to yield a colourless powder (880 mg, 83%). $R_f$ ($CHCl_3$/MeOH, 80:20, 1% HCOOH) = 0.84. $^1$H NMR (400 MHz, [$D_6$]DMSO): $\delta$ = 7.71 (d, $J$ = 7.8, 2 H, Fmoc-H$^{4,5}$), 7.56 (d, $J$ = 7.7, 2 H, Fmoc-H$^{1,8}$), 7.30 (t, $J$ = 7.4, 2 H, Fmoc-H$^{3,6}$), 7.21 (t, $J$ = 7.4, 2 H, Fmoc-H$^{2,7}$), 5.74–5.84 (m, 1.0 H, Aloc-H$^2$), 5.19 (d, $J$ = 17.2, 1.0 H, Aloc-H$^{3trans}$), 5.06 (d, $J$ = 10.5, 1.0 H, Aloc-H$^{3cis}$), 4.45 (m, 2.0 H, Aloc-H$^1$), 4.23 (d, $J$ = 6.9, 2.0 H, Fmoc$^{10}$), 4.12 (m, 1.0 H, Fmoc-H$^9$), 3.91 (s, $J$ = 5.2, 2.0 H, Aeg-H$^1$), 3.45–3.41 (m, 2.0, Aeg-H$^3$), 3.28–3.24 (m, 2.0 H, Aeg-H$^2$) ppm. $^{13}$C **NMR (**100.6 MHz, [$D_6$]DMSO): $\delta$ = 173.6, 156.7, 47.5, 144.5, 141.3, 133.8, 128.3, 127.7, 125.7, 120.8, 117.2, 66.2, 66.0, 49.6, 47.9 ppm. HR-MS (FAB$^+$, 3-NBA): *m*/*z* calcd. for $C_{23}H_{25}N_2O_6$ 425.1699; found 425.1713 [M + H]$^+$.

**Fmoc–Aeg(TO$_{Q1}$)–OAll (5b):** To TO$_{Q1}$–COOH (100 mg, 0.23 mmol) in dry DMF (2.3 mL) was added PyBOP (145 mg, 0.279 mmol), pyridinium *p*-toluenesulfonate (58 mg, 0.232 mmol) and *N*-methylmorpholine (23 mg, 0.23 mmol). The suspension was stirred under argon until a clear solution was obtained. This solution was added to a solution of amine hydrochloride **3b** (96 mg, 0.23 mmol) and *N*-methylmorpholine (23 mg, 0.23 mmol) in DMF (2.3 mL). The reaction mixture was stirred under argon for 12 h. The volatiles were removed under reduced pressure. To the residue methanol (2 mL) was added. After stirring for 1 h, the precipitate was collected by filtration and washed with methanol (5 mL). The crude product was purified by column chromatography ($CHCl_3$/MeOH, 97:3, 1% formic acid) to yield an orange-colored solid (114 mg, 62%). $R_f$ ($CHCl_3$/MeOH, 80:20, 1% HCOOH) = 0.64. $^1$H NMR (300 MHz, [$D_6$]DMSO/TFA, 10:1.5): $\delta$ = 8.73 (d, $J$ = 8.4, 1 H, Ar-H$^{q5}$), 8.51 (d, $J$ = 7.3, 1 H, Ar-H$^{q2}$), 8.01 (d, $J$ = 7.7, 1 H, Ar-H$^{b1}$), 7.91–7.86 (m, 2 H, Fmoc-H$^{4,5}$), 7.80–7.72 (m, 2 H, Ar-H$^{q7,8}$), 7.68 (m, 1 H, Ar-H$^{b4}$), 7.66–7.63 (m, 2 H, Fmoc-H$^{1,8}$), 7.58–7.56 (m, 1 H, Ar-H$^{b3}$), 7.53–7.48 (m, 1 H, Ar-H$^{b2}$), 7.47–7.44 (m, 1 H, Ar-H$^{q6}$), 7.40–7.37 (m, 2 H, Fmoc-H$^{3,6}$), 7.37–7.33 (m, 2 H, Fmoc-H$^{2,7}$), 7.31–7.28 (m, 1 H, Ar-H$^{q3}$), 6.87 (s, 1.0 H, cyanin-H), 5.98–5.85 (m, 1.0 H, All-H$^1$), 5.46 (s, 2.0 H, methylene-H), 5.33 (d, $J$ = 17.1, 1.0 H, All-H$^{3trans}$), 5.24 (d, $J$ = 10.5, 1.0 H, All-H$^{3cis}$), 4.68 (d, $J$ = 5.3, 2.0 H, All-H$^1$), 4.31 (d, $J$ = 6.6, 2.0 H, FmoC-CH$_2$), 4.22–4.18 (m, 1.0 H, Fmoc-H$^9$), 4.05 (s, 2.0 H, Aeg-H$^1$, 3.98 (s, 3.0 H, Me-H), 3.33 (t, 2.0 H, Aeg-H$^3$), 3.04 (m, 2.0 H), Aeg-H$^2$) ppm. $^{13}$C NMR (300 MHz, [$D_6$]DMSO/TFA, 10:1.5): $\delta$ = 168.7, 166.4, 156.4, 148.5, 144.8, 143.8, 140.8, 140.3, 137.8, 137.7, 133.3, 131.8, 128.3, 128.2, 127.7, 127.1, 126.7, 125.6, 125.2, 124.8, 124.2, 123.7, 122.9, 120.2, 118.6, 117.6, 113.3, 107.5, 88.9, 65.9, 65.7, 54.6, 46.7, 46.6, 34.0 ppm. HR-MS (ESI$^+$, MeOH): *m*/*z* calcd. for $C_{42}H_{39}O_5N_4{}^{32}S^+$ 711.2636; found 711.2636 [M]$^+$.

**Fmoc–Aeg(TO$_{Q1}$)–OH (2):** To a solution of **5b** (90 mg, 0.1 mmol) in THF (5 mL) was added *N*-methylaniline (11.3 mg, 0.1 mmol). The solution was degassed by freeze-thaw-pump cycles. After addition of [Pd(PPh$_3$)$_4$] (12 mg, 0.01 mmol), the mixture was stirred under exclusion of light for 14 h. The solvent was removed in vacuo. To the residue was added methanol (2 mL). The resulting precipitate was collected by filtration. The crude product was purified by column chromatography ($CHCl_3$/MeOH, 97:3, 1% formic acid) to yield an orange-colored solid (62 mg, 73%). $R_f$ ($CHCl_3$/MeOH, 80:20, 1% HCOOH) = 0.28. $^1$H NMR (300 MHz, [$D_6$]-DMSO/TFA, 10:1.5): $\delta$ = 8.74 (d, $J$ = 8.4, 1 H, Ar-H$^{q5}$), 8.50 (d, $J$ = 7.3, 1 H, Ar-H$^{q2}$), 8.02 (d, 1 H, $J$ = 7.8, Ar-H$^{b1}$), 7.90–7.87 (m, 2 H, Fmoc-H$^{4,5}$), 7.86–7.8 (m, 2 H, Ar-H$^{q7,8}$), 7.69 (m, 1 H, Ar-H$^{b4}$), 7.67–7.65 (m, 2 H, Fmoc-H$^{1,8}$), 7.63–7.58 (m, 1 H, Ar-H$^{q6}$), 7.55–7.52 (m, 1 H, Ar-H$^{b3}$), 7.47–7.44 (m, 1 H, Ar-H$^{b2}$), 7.42–7.38 (m, 2 H, Fmoc-H$^{3,6}$), 7.39–7.34 (m, 2 H, Fmoc-H$^{2,7}$), 7.32 (m, 1 H, Ar-H$^{q3}$), 6.89 (s, 1 H, cyanin-H), 5.50 (s, 2.0 H, methylene-H), 4.33 (d, $J$ = 6.7, 2.0 H, Fmoc-CH$_2$), 4.21 (m, 1.0 H, Fmoc-H$^9$), 3.99 (s, 3.0 H, Me-H), 3.89 (s, 2.0 H, Aeg-H$^1$), 3.29 (t, 1.0 H, Aeg-H$^3$), 3.00 (m, 2.0 H, Aeg-H$^2$) ppm. $^{13}$C NMR

(300 MHz, [D$_6$]DMSO/TFA, 10:1.5): $\delta$ = 168.7, 165.8, 158.7, 158.2, 156.4, 148.6, 143.8, 140.8, 137.7, 133.2, 128.2, 128.1, 127.7, 127.1, 126.6, 125.1, 124.8, 124.1, 123.7, 122.9, 120.2, 117.6, 113.8, 113.3, 107.4, 88.8, 65.6, 54.6, 47.4, 46.7, 46.5, 34.0 ppm. HR-MS (ESI$^+$, MeOH): *m*/*z* calcd. for $C_{39}H_{35}O_5N_4{}^{32}S^+$ 671.2328; found 671.2323 [M]$^+$.

**Solid-Phase Synthesis of Labelled PNA**

**Loading of Novagen TGR Resin:** The resin (500 mg, 0.29 mmol/g) was washed (3 × DCM, 3 × DMF, 3 × DCM and 3 × DMF). The resin was allowed to swell in DMF (10 mL) for 30 min. For preactivation PyBOP (301.2 mg, 0.58 mmol) and NMM (87.7 mg, 0.87 mmol) were added to a solution of Fmoc-protected glycine (172.3 mg, 0.58 mmol) in DMF (5.8 mL). After 3 min, the mixture was added to the resin. After 4 h, the resin was washed (3 × DMF, 3 × DCM, 3 × DMF). For capping, the resin was treated with a solution of $Ac_2O$/pyr (1:4; 5 mL). After 5 min, the procedure was repeated once. The resin was washed (3 × DMF, 3 × DCM, 3 × DMF and 5 × DCM) and finally dried in vacuo.

**Divergent Solid-Phase Synthesis:** Resin **6** was allowed to swell in DMF (2 mL). After 30 min, the resin was washed (3 × 2 mL DMF, 3 × 2 mL DCM, 3 × 2 mL DMF).

**Fmoc Cleavage:** DMF/piperidine (4:1, 2 mL) was added to the resin. After 3 min, the resin was washed with DMF. This procedure was repeated twice. Finally, the resin was washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL).

**Coupling:** The resin was suspended in a solution of 4 equiv. of Fmoc-protected building block in 0.125 M NMM in DMF (0.1 M final building block concentration) which was preactivated for 2 min by addition of 4 equiv. of PyBOP. After 2 × h, the resin was washed (3 × 2 mL DMF, 3 × 2 mL DCM, 3 × 2 mL DMF).

**Capping:** $Ac_2O$/pyridine (1:10, 2 mL), 2 × 5 min. The resin was washed (3 × 2 mL DMF, 3 × 2 mL DCM, 3 × 2 mL DMF).

**Aloc Removal:** Resin **7**, **11** or **12** was aliquoted to correspond to 1 μmol based on the initial Fmoc load of resin **6**. A degassed solution of [Pd(PPh$_3$)$_4$] and 8 equiv. of dimethylamine–borane complex in dry DCM (2 mL) was added to the resin. After 20 min, the solvent was removed by filtration. The procedure was repeated once and the resin was washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL).

**Thiazole Orange Coupling:** To 6 μmol of the thiazole orange derivative ($TO_{Q1}$–COOH, $TO_{Q2}$–COOH, $TO_{Q5}$–COOH, $TO_{B1}$–COOH, $TO_{B2}$–COOH or $TO_{B5}$–COOH) in DMF (60 μL) was added PPTS (1.5 mg, 6 μmol) and PyBOP (3.1 mg, 6 μmol). After vortexing, NMM (0.65 μL, 8 μmol) was added to the resulting solution. After vortexing, this mixture was added to the resin. After 12 h of shaking, the solvent was removed by filtration. The thiazole orange coupling was repeated once (coupling time: 2 h). Finally, the resin was washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and again DMF (3 × 2 mL) and DCM (5 × 2 mL).

**Cleavage:** The dried resin was suspended in a solution of cysteine methyl ester hydrochloride (5 mg, 29.1 μmol) in TFA/*m*-cresol/$H_2O$ (37:2:1, 1 mL) and was shaken for 3 h. The resin was washed with TFA (200 μL). The combined filtrates were concentrated in vacuo.

**Purification:** To the concentrated cleavage solution was added cold diethyl ether. The precipitate was collected by centrifugation and disposal of the supernatant. The residue was dissolved in water and precleaned by using a water-equilibrated Sep-pak® C18 cartridge. Coloured eluates obtained upon gradient elution (1 × AcN/$H_2O$, 20:80, 0.1% TFA; 1 × AcN/$H_2O$, 40:60, 0.1% TFA; 1 × AcN/$H_2O$, 80:20, 0.1% TFA; 1 × AcN/$H_2O$, 80:20, 0.1% TFA; 2 mL each) were analyzed by HPLC and MALDI-TOF/MS and purified by semipreparative HPLC. Determination of yields: Purified PNA was dissolved in 500 μL of water. An aliquot of 5 μL was diluted to 1 mL and the optical density was measured at 260 nm by using a quartz cuvette with a 10-mm path length. The sample concentration was calculated by using oligo calculation at www.gensetoligos.com and approximating $\varepsilon$ = 9400 Lmol$^{-1}$ for thiazol orange. [$\varepsilon_{260}$(TO) ≈ $\varepsilon_{260}$(thymine)].

**$^{Ac-N}$gccgtaAeg($TO_{Q1}$)tagccg$Gly^{CONH2}$ (10):** 13.8 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 10.5 (83 nmol, 8.3%); $t_R$ = 19.1 min (0–1 min: 9%B; 1–31 min: 9–24%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{156}H_{189}N_{76}O_{40}S^+$ 3801; found 3802 [M(average)]$^+$.

**$^{Ac-N}$acacctaAeg($TO_{Q1}$)agcg$Gly^{CONH2}$ (13a):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 10.2 (84 nmol, 8.4%); $t_R$ = 24.9 min (0–1 min: 5%B; 1–31 min: 5–19%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{145}H_{175}N_{72}O_{34}S^+$ 3502; found 3500 [M(average)]$^+$.

**$^{Ac-N}$acacctaAeg($TO_{Q2}$)agcg$Gly^{CONH2}$ (13b):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 4.8 (39.9 nmol, 4.0%); $t_R$ = 26.5 min (0–1 min: 9%B; 1–41 min: 9–29%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{146}H_{177}N_{72}O_{34}S^+$ 3516; found 3514 [M(average)]$^+$.

**$^{Ac-N}$acacctaAeg($TO_{Q5}$)agcg$Gly^{CONH2}$ (13c):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 2.4 (20.5 nmol, 2.1%); $t_R$ = 30.6 min (0–1 min: 9%B; 1–41 min: 9–29%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{149}H_{183}N_{72}O_{34}S^+$ 3558; found 3557 [M(average)]$^+$.

**$^{Ac-N}$acacctaAeg($TO_{B1}$)agcg$Gly^{CONH2}$ (13d):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 2.8 (23.2 nmol, 2.3%); $t_R$ = 24.1 min (0–1 min: 5%B; 1–31 min: 5–19%B). MALDI-TOF-MS (sinapinic acid): *m*/*z*, calcd. for $C_{145}H_{175}N_{72}O_{34}S^+$ 3502; found 3503 [M(average)]$^+$.

**$^{Ac-N}$acacctaAeg($TO_{B2}$)agcg$Gly^{CONH2}$ (13e):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 4.5 (37.3 nmol, 3.7%); $t_R$ = 20.1 min (0–1 min: 14%B; 1–31 min: 14–29%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{146}H_{177}N_{72}O_{34}S^+$ 3516; found 3516 [M(average)]$^+$.

**$^{Ac-N}$acacctaAeg($TO_{B5}$)agcg$Gly^{CONH2}$ (13f):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 8.8 (72.8 nmol, 7.3%); $t_R$ = 22.6 min (0–1 min: 14%B; 1–31 min: 14–29%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{149}H_{183}N_{72}O_{34}S^+$ 3559; found 3558 [M(average)]$^+$.

**$^{Ac-N}$acacctcAeg($TO_{Q1}$)agcg$Gly^{CONH2}$ (13a):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 13.7 (118.3 nmol, 11.8%); $t_R$ = 25.5 min (0–1 min: 5%B; 1–31 min: 5–19%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{144}H_{175}N_{70}O_{35}S^+$ 3478; found 3478 [M(average)]$^+$.

**$^{Ac-N}$acacctcAeg($TO_{Q2}$)agcg$Gly^{CONH2}$ (14b):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 8.7 (71.6 nmol, 7.2%); $t_R$ = 22.9 min (0–1 min: 14%B; 1–31 min: 14–29%). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{145}H_{177}N_{70}O_{35}S^+$ 3492; found 3491 [M(average)]$^+$.

**$^{Ac-N}$acacctcAeg($TO_{Q5}$)agcg$Gly^{CONH2}$ (14c):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 4.3 (37.4 nmol, 3.7%); $t_R$ = 22.8 min (0–1 min: 9%B; 1–41 min: 9–29%B). MALDI-TOF-MS (sinapinic acid): *m*/*z* calcd. for $C_{148}H_{183}N_{70}O_{35}S^+$ 3534; found 3532 [M(average)]$^+$.

**$^{Ac-N}$acacctcAeg($TO_{B1}$)agcg$Gly^{CONH2}$ (14d):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 3.8 (33.9 nmol, 3.4%); $t_R$ = 20.5 min

(0–1 min: 14%B; 1–31 min: 14–29%). MALDI-TOF-MS (sinapinic acid): *m/z* calcd. for $C_{144}H_{175}N_{70}O_{35}S^+$ 3478; found 3479 [M(average)]$^+$.

**$^{Ac–N}$acacctcAeg(TO$_{B2}$)agcgGly$^{CONH2}$ (14e):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 10.9 (97.1 nmol, 9.7%); $t_R$ = 20.3 min (0–1 min: 14%B; 1–31 min: 14–29%B). MALDI-TOF-MS (sinapinic acid): *m/z* calcd. for $C_{145}H_{177}N_{70}O_{35}S^+$ 3492; found 3494 [M(average)]$^+$.

**$^{Ac–N}$acacctcAeg(TO$_{B5}$)agcgGly$^{CONH2}$ (14f):** 4.7 mg (ca. 1 μmol) of resin **6** was used. $OD_{260}$ = 9.2 (79.7 nmol, 8.0%); $t_R$ = 23.1 min (0–1 min: 14%B; 1–31 min: 14–29%B). MALDI-TOF-MS (sinapinic acid): *m/z* calcd. $C_{148}H_{183}N_{70}O_{35}S^+$ 3535; found 3535 [M(average)]$^+$.

**Linear Solid-Phase Synthesis:** Linear solid-phase synthesis was performed by using an Intavis ResPep parallel synthesizer equipped with 1-mL reactors. Resin **6** (ca. 2 μmol) was allowed to swell in DMF (2 mL). After 30 min, the resin was transferred to the synthesizer reactor. The resin was washed (2 × 180 μL DMF).

**Fmoc Cleavage:** DMF/piperidine (4:1, 100 μL) was added to the resin. After 2 min, the procedure was repeated once. The resin was washed with DMF (1 × 180 μL, 3 × 100 μL and 1 × 180 μL).

**Coupling of Standard PNA Building Blocks:** A preactivation vessel was charged with a 0.6 M HCTU solution in NMP (12 μL), a 4 M NMM solution in DMF (4 μL), and a 0.6 M PNA monomer solution in NMP (27 μL). After 8 min, 40 μL of preactivation solution was transferred to the resin. After 30 min, the resin was washed (2 × 180 μL DMF).

**Coupling of Fmoc–Aeg(TO$_{Q1}$)–COOH (2):** A preactivation vessel was charged with a 0.6 M HCTU solution in NMP (12 μL), a 4 M NMM solution in DMF (4 μL), and a solution of **2** in NMP (0.6 M **4**, 0.6 M PPTS, 27 μL). After 8 min, 40 μL of preactivation solution was transferred to the resin. After 30 min, the resin was washed (2 × 180 μL DMF).

**Capping:** $Ac_2O$/2,6-lutidine (9:11, 100 μL), 3 min. The resin was washed (3 × 200 μL DMF, 3 × 100 μL DMF).

**Cleavage:** A solution of cystine methyl ester hydrochloride (5 mg, 29 μmol) in TFA/*m*-cresol/$H_2O$ (37:2:1, 1 mL) was passed through dried resin in 40 min. The resin was washed with TFA (500 μL). The combined filtrates were concentrated in vacuo.

**Purification:** Performed as described for divergent solid-phase synthesis.

**$^{Ac–N}$gccgtaAeg(TO$_{Q1}$)tagccgGly$^{CONH2}$ (10):** 9.4 mg (ca. 2 μmol) of resin **6** was used. Fmoc–Aeg(TO$_{Q1}$)–COOH (**4**) and the subsequent building block were double coupled. $OD_{260}$ = 22.2 (176 nmol, 8.8%); $t_R$ = 21.3 min (0–1 min: 1%B; 1–25 min: 3–30%B). MALDI-TOF-MS (sinapinic acid); *m/z* calcd. for $C_{156}H_{189}N_{76}O_{40}S^+$ 3801; found 3802 [M(average)]$^+$.

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