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Modification of guanine residues in PNA-synthesis by PyBOP☆

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Abstract—The phosphonium-type coupling reagent PyBOP, when applied to the synthesis of peptide nucleic acid (PNA) oligomers, was found to form O^4-phosphonium compounds of the nucleobase guanine which can be converted into C^4-modified guanine-derived PNAs by nucleophiles.

Peptide nucleic acids (PNAs) have been derived from oligonucleotides by substituting the phosphate-sugar backbone by *N*-(2-aminoethyl)glycine units.[1] PNAs are resistant to enzymatic degradation by nucleases and peptidases and form very stable duplexes and triple helix structures with complementary DNA or RNA. Therefore, PNAs gained broad attention as interesting oligonucleotide analogs in antisense/antigene experiments and as diagnostic tools.[2]

Several strategies have been developed for the solid phase synthesis of PNAs, using Boc or Fmoc protection of the backbone amino function, and Z, Bhoc or Mmt protection for the exocyclic amino function of the nucleobases. In principal, the activation of the PNA monomers can be performed with several activating reagents known from peptide synthesis. Especially, HATU[3] or PyBOP[4] is often used for the coupling of PNA monomers.[5] Synthesis with guanidinium-derived coupling reagents like HATU is more laborious, because preactivation is needed in order to avoid guadinylation of the N-terminus, a side reaction, well known in peptide synthesis,[6] but also observed in the synthesis of PNAs.[7] When using PyBOP or similar phosphonium compounds (like BOP, PyBroP, PyAOP, etc.), no chain-terminating side reactions at the amino terminus have been detected.[8] Thus, preactivation should not be needed, which is especially useful in automated synthesis. Therefore, in the synthesis of PNAs and PNA-peptide conjugates required in our laboratory in order to study the influence of various peptides on the PNA-evoked antisense activity,[9] we used PyBOP activation for the above mentioned reasons.

Surprisingly, in the PyBOP-mediated syntheses of Ac-oootccttcccaactttgacaoooLPKTGGR-NH_2[10] and related sequences the obtained products showed molecular masses approx. 67 ± 4 Da above the expected masses in mass spectrometry, whereas the synthesis with HATU gave the desired products. Detailed analysis of the acetylated termination sequences by mass spectrometry revealed that the modification occurred at the only guanine residue in the sequence, and was not observed in guanine-free sequences. In order to further characterize the side reaction, a short fragment containing adenine and guanine (Fig. 1) was synthesized using HATU and PyBOP activation, respectively, and cleaved from the resin with (**1**) and without (**2**) the N-terminal Fmoc-group.[11]

Synthesis with HATU gave the desired products with single peaks in HPLC and MALDI-MS in both cases (Table 1 and Supplementary data). On the other hand, the Fmoc-protected product **1**, obtained by PyBOP

Keywords: Coupling reagents; PNA synthesis; Guanine; PyBOP; HATU.

☆ Electronic Supplementary Information (ESI) available: Experimental details for MS and HPLC experiments, analytical data of full-length PNAs and MALDI-MS spectra and HPLC traces of the syntheses with HATU and PyBOP. Abbreviations used: Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *N*-[(dimethylamino)-(1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate N-oxide; HOBt, benzotriazol-1-ol; MALDI-MS, matrix-assisted laser desorption ionisation-mass spectrometry; PNA, peptide nucleic acid; PyBOP, benzotriazol-1-yloxytripyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reversed-phase high performance liquid chromatography.

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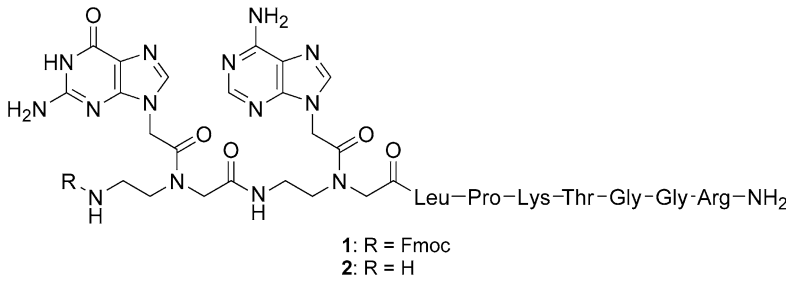


Figure 1. PNA fragments for model synthesis with (**1**) and without (**2**) Fmoc-group.

Table 1. Analytical data for the unmodified PNAs **1** and **2** and the modified PNAs **3** and **4**

Entry	Substance	Activating reagent	N-Terminus	$[M]^+$ calcd	$[M+H]^+$ found	(%)[a]
1	**1**	HATU	Fmoc	1514.74	1515.56	>99
2	**2**	HATU	H	1292.67	1293.57	>99
3	**1**	PyBOP	Fmoc	1514.74	1515.70	83
4	**2**	PyBOP	H	1292.67	1293.65	81
5	**3**	PyBOP	Fmoc	1754.90	1755.01 $[M]^+$	13
6	**4**	PyBOP	H	1359.75	1360.88	18

[a] Purity according to HPLC in the crude product.

activation contained a more hydrophobic impurity (13% according to HPLC) with an increased mass (+239 Da), corresponding to the mass of the O-phosphonium-modified product **3** (Fig. 2 and Table 1). The Fmoc-deprotected product **2** was contaminated (18%) with a more hydrophobic by-product, possessing a mass difference of +67 Da, corresponding to the piperidine-modified compound **4**. The mass difference in this product corresponds to the increased mass observed at the full length PNAs (see above). In both products, obtained by PyBOP activation, truncated sequences were detected, pointing to incomplete couplings.

The structures **3** and **4** could be further confirmed by MS/MS-fragmentation. Here, fragments were found which clearly show that the modification is located at the guanine base residue (Figs. 3 and 4). The synthesis of the full-length PNAs led exclusively to modified products, while at the model fragment experiments the expected product was obtained, too. This suggests that the side reaction also takes place during subsequent coupling steps and increases with the number of coupling steps subsequent to the insertion of the guanine-containing PNA monomer.

We propose the following rationale for this side reaction (Scheme 1). Guanine possesses an enolisable keto group, which reacts with the phosphonium ion of the PyBOP reagent, liberating the HOBt leaving group. The thus activated alcohol is substituted by the nucleophile

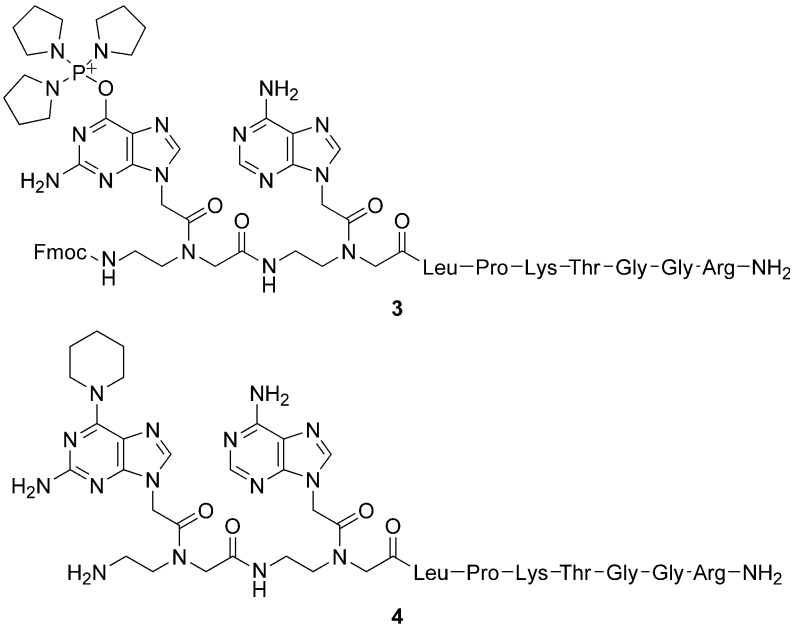


Figure 2. Structures of the modified PNAs **3** and **4**.

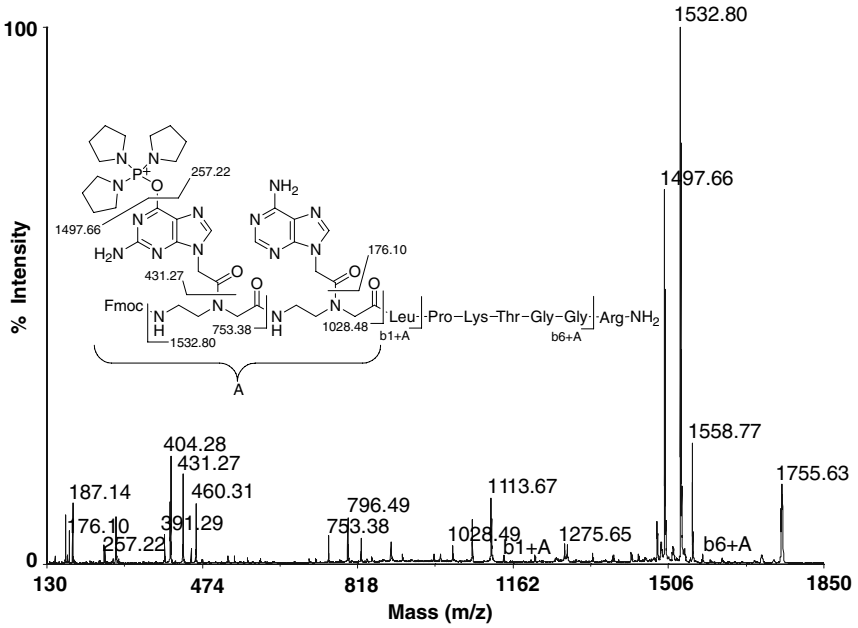


Figure 3. MS/MS-spectrum of PNA **3**, identified fragments are marked in the structure (precursor-ion 1754.75 Da).

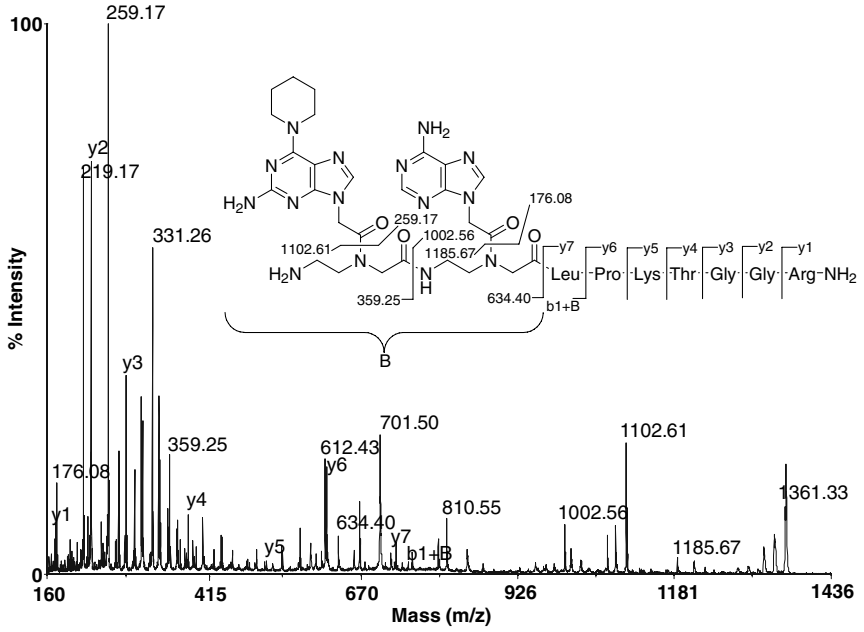
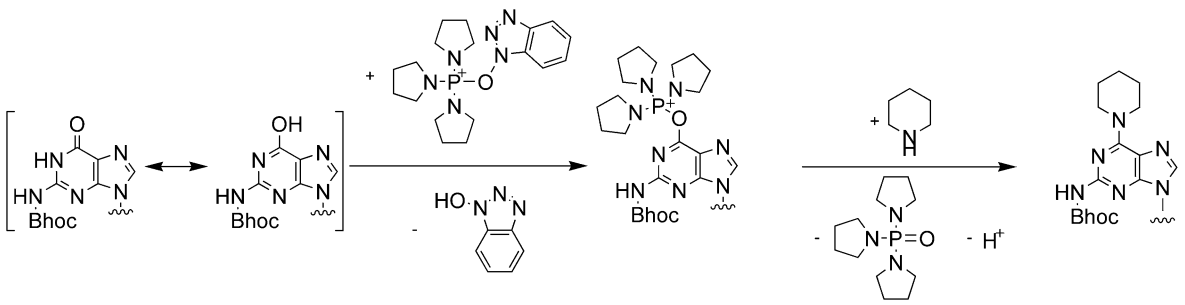


Figure 4. MS/MS-spectrum of PNA **4**, identified fragments are marked in the structure (precursor-ion 1360.57 Da).



Scheme 1. Proposed rationale for the PyBOP-induced side-reaction.

piperidine, yielding the final stable product. Why this reaction does not take place at thymine residues which also possess keto groups remains unclear. Probably the vicinity of the methyl group hinders the attack of the bulky phosphonium reagent.

Our results show, although we suspect this side reaction to be sequence dependent, that care must be taken when synthesizing PNAs with PyBOP activation. In ongoing studies we will investigate the influence of different amines on the phosphonium-evoked modification. This reaction may offer an opportunity to synthesize guanine derivatives modified in position 4 by treatment of the phosphonium intermediate with a variety of nucleophiles. Such modified nucleobases have gained increasing attention in attempts to modulate the hybridization characteristics of PNAs[12] and to insert labels into PNA oligomers by post-synthetic modifications.[13]

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.06.069.

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10. 18mer PNA sequence with ethylene glycol spacer (o, 2-[2-amino-ethoxy]-ethoxy acetyl); PNA-bases = lowercase letters, amino acids = uppercase letters.
11. Synthesis of the PNAs was performed manually using Fmoc/Bhoc protected PNA monomers; resin: TentaGel SRAM (Rapp, 0.22 mmol g^{-1}), HATU couplings: single couplings, 1 min preactivation, 20 min, 5 equiv monomer in NMP (0.25 M), 4.5 equiv HATU in DMF (0.23 M), 5 equiv DIPEA, 5 equiv collidine; PyBOP couplings: single couplings, 20 min, 5 equiv monomer in NMP (0.25 M), 5 equiv PyBOP in DMF (0.25 M), 5 equiv DIPEA, 5 equiv collidine; capping: 4% Ac_2O, 4% DIPEA in DMF, 1 min; Fmoc-cleavage: 2× 20% piperidine in DMF, 30 s/6 min; resin cleavage: 5% m-cresol in TFA, 1 h.
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