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Post-synthetic transamination at position N4 of cytosine in oligonucleotides assembled with routinely used phosphoramidites†

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The commercially available and cheap nucleotide phosphoramidites are routinely used for the oligonucleotide (ODN) assembly. T, isobutyl-dG (ⁱBu dG), benzoyl-dA (^{Bz}dA), acetyl-dC (^{Ac}dC) and benzoyl-dC (^{Bz}dC) derivatives are sufficient to produce orthogonally protected ODNs. Clean and efficient (ca. 30%–70% yield) post-synthetic amination of an ODN assembled with such phosphoramidites was selectively achieved at the N4 position of a singly introduced ^{Bz}dC. Such a method represents a novel and cheap strategy for the user-friendly post-modification of oligonucleotides at the internal position.

Introduction

Synthetic oligodeoxynucleotides (ODNs) have attracted considerable interest because of their capacity to expand intrinsic nucleic acid properties (*i.e.* as carriers of genetic information), and therefore they are being investigated for various applications in medical research (*e.g.* control of gene expression),¹ molecular diagnostics (*e.g.* DNA-based biosensors)² and in biotechnology (*e.g.* catalysts)³ and nanotechnology (*e.g.* origami DNA for nanomaterials).⁴ Chemical modifications in their structure (*e.g.* phosphorothioate, 2'-methoxyethoxy, and locked nucleic acids) have been achieved for these purposes. Considerable efforts have been performed to develop efficient methods for conjugating ODNs with a reporter group, because it can be used to both improve existing ODN properties and to introduce entirely new properties.⁵ The conjugation of ODNs is not trivial because subtle changes in the structure may influence their biological properties. The solid phase synthesis (SPS) represents a suitable approach to prepare oligonucleotide conjugates using the corresponding phosphoramidite derivatives bearing the reporter. Although the on-support conjugation method was reported to be convenient for incorporating multiple reporters because of the large variety of commercially available building blocks, it shows a few limitations. Indeed, the reporter must be stable during the automated oligonucleotide synthesis and subsequent deprotection, the latter being performed under drastic basic conditions. Furthermore,

it requires the preparation of the appropriate phosphoramidite building blocks for introducing non-commercially available modifications.

Post-synthetic strategies can overcome these limitations. In this context, numerous methods have been developed by introducing a reactive group onto ODNs by a subsequent coupling reaction with reporters bearing the corresponding complementary functional moiety.⁶ ODNs are generally conjugated at 5'- or 3'-extremity because of an easier accessibility. Efficient strategies involving oxime and thiol-maleimide conjugations,^{7,8} Cu-catalyzed (or Cu-free) azide-alkyne cycloaddition,^{9,10} Staudinger ligation¹¹ and Diels-Alder reactions¹² have been reported for this purpose.

Conjugation on nucleobases is much less reported. Indeed, the modifications at the nucleobases have to be carefully selected; therefore, the hybridization properties (binding with the target sequence) are not compromised. Most methods involve the reaction at the carbon C-5 of pyrimidine nucleobases primarily *via* an organo-metallic coupling reaction.¹³ The modification on the nucleobases can be achieved using a suitable linker attached to the amino group of adenosine, guanosine or cytosine. The actual methods require using a modified nucleobase bearing a good leaving group at the position N6 of adenosine and guanosine and the N4 of cytosine. For example an alkyl group can be introduced into position 4 of dC or 6 of dG by the reaction of N4-triazolo-dC or O6-pentafluorophenyl-dG with an alkylamino derivative.^{14,15} A major limitation of these methods is the high price of the phosphoramidite derivatives used for introducing the convertible nucleoside.¹⁶ Another elegant method for the functionalization at the internal position is the use of highly nucleophilic alkoxyamines derivatives, which are able to achieve a substitution reaction at the C4 position of cytosine nucleobase embedded

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in an oligonucleotide.¹⁷ However, this method requires a tedious synthesis of the alkoxyamine derivatives. Consequently, there is a strong need for more practical and cheaper methods for the conjugation of ODNs at the amino position.

Aqueous ammonia is the historical reagent for the final ODNs deprotection.¹⁸ However, as usually overnight reactions are required, methods as short as possible have been developed. Reddy *et al.* have developed AMA (conc. ammonia/conc. methylamine mixture, 50/50) as an efficient deprotection cocktail, since the deprotection time was drastically reduced to a few minutes.¹⁹ Unfortunately, the benzoyl-dC (^{Bz}dC) residue, classically used for cytosine introduction, was prone to transamination at the N4 position, leading to *ca.* 10% formation of the undesired N4-methylcytosine containing ODN. Similar results were obtained in the early time of the ODNs synthesis, when various alkylamines were used as deprotection reagents leading to *ca.* 5% to 15% of by-product formation with ethylenediamine or 5–13% with butylamine.^{20–22} Clean deprotection was finally achieved by using acetyl-dC (^{Ac}dC) instead of ^{Bz}dC during the oligonucleotide elongation.²³ Nowadays, routine protocols recommend the use of ^{Ac}dC, when using AMA or methylamine as the deprotection reagent or ^{Bz}dC, when performing the deprotection step with conc. ammonia. In this context, we envisioned that ^{Ac}dC and ^{Bz}dC moieties could be seen as orthogonally protected synthons for the chemoselective post-synthetic modification of ODNs. These two differently protected dCs could be easily introduced into oligonucleotide sequences by using the particularly cheap corresponding phosphoramidites. Prompted by a few data reporting the direct conversion of ^{Bz}dC into N4-alkyl dC at the nucleotide level with low to moderate yields (*i.e.* 18% to 40%),^{24,25} we focused on the high-yielding transamination of ODNs containing a single ^{Bz}dC residue.

In the present paper, we report on the use of this concept to introduce various amine derivatives at position N4 of a single cytosine residue. Cyclopropylamine (CPA) was chosen as a model amine as N4-cyclopropylcytosine (^{CPA}dC) is of great interest for studying the charge transport through DNA.²⁶ The aromatic substitution of the benzamide moiety in ^{Bz}dC by cyclopropylamine was found to be efficient and selective for the N4 position of the desired cytosine moiety. When the chemoselectivity of the conjugation reaction was established with the CPA model amine, the methodology was extended to the synthesis of more complex conjugates. For this purpose, various amine derivatives bearing another functional group, such as an amine, an alcohol, a masked aldehyde, an alkyne or an aromatic moiety, were efficiently introduced on medium-sized (12-mer) or longer (30-mer) oligonucleotides. Given the fact that ^{Bz}dC phosphoramidite derivative (see Fig. S1† for structure of routinely used phosphoramidites for ODNs synthesis) is much more cheaper than the commercially available convertible nucleotides,¹⁶ our method represents a cost-effective alternative towards the N4-cytidine modification in ODNs, and therefore it is an inexpensive way to functionalize ODNs at the internal position.

Results and discussion

Cyclopropylamine was first chosen as a model amine to be introduced at the N4 position of cytosine nucleobase, as N4-cyclopropylcytosine (^{CPA}dC) is currently used for studying the charge transfer through DNA.²⁶ The actual method for the preparation of ^{CPA}dC containing oligonucleotides usually involved the use of a 4-thio-uridine phosphoramidite derivative for the introduction of the S-alkyl moiety at position 4 of the uridine nucleobase and a subsequent treatment with CPA at 60 °C for 16 h followed by a RP-HPLC purification.²⁶

As above mentioned, the direct transamination of ^{Bz}dC was reported to proceed with relatively modest yields. To establish the most appropriate conditions for the amination reaction, we performed preliminary assays with both model CPG-linked ODN **1a** (TT^{Bz}CTT-CPG) and cyclopropylamine (see Table 1) by varying the reaction conditions and monitoring the formation of the desired ^{CPA}dC-containing ODN **1b** (TT^{CPA}CTT). The screening of various solvents showed that the effect is quite weak, however, the rate of conversion in NMP was found to be slightly lower than those obtained when using isopropanol as the solvent (see entries 1–3). Tuning of the CPA/DBU/solvent ratio revealed that lower concentrations in DBU afforded slightly better conversions (entries 1, 4 and 5), whereas the absence of DBU gave a far lower conversion (entry 16). In the other hand, a decrease in the CPA concentration was detrimental to the reaction yield (entries 1, 6–8). A longer reaction time (16 h *vs.* 2 h) did not increase the rate of conversion (entries 11–13), while a heating at 60 °C afforded a better rate of conversion (entries 11, 14, 15 and 3, 10). At last, the starting material was completely recovered without CPA and/or DBU (entries 17 and 18), which demonstrated that the heating in

Table 1 Screening of reaction conditions for the N4-cyclopropyl-amination of model ODN **1a**^a

Entry	Volume of (μL)			Time (h)	Temp. (°C)	Rate conv. ^b
	Solvent	CPA	DBU			
1	200 (Dodecane)	200	40	16	60	59
2	200 (NMP)	200	40	16	60	56
3	200 (iPrOH)	200	40	16	60	60
4	230 (Dodecane)	200	10	16	60	63
5	238 (Dodecane)	200	2	16	60	62
6	300 (Dodecane)	100	40	16	60	60
7	350 (Dodecane)	50	40	16	60	56
8	390 (Dodecane)	10	40	16	60	39
9	200 (iPrOH)	200	40	16	60	59
10	200 (iPrOH)	200	40	16	18	49
11	230 (iPrOH)	200	10	16	60	63
12	230 (iPrOH)	200	10	0.5	60	61
13	230 (iPrOH)	200	10	2	60	63
14	230 (iPrOH)	200	10	16	35	55
15	230 (iPrOH)	200	10	16	18	51
16	240 (iPrOH)	200	0	2	60	40
17	400 (iPrOH)	0	40	2	60	0
18	440 (iPrOH)	0	0	2	60	0

^a See the experimental conditions. ^b The rates of conversion were estimated by RP-HPLC on crude reaction mixture.

isopropanol is not detrimental to the ODN stability. From these assays, we retained iPrOH/CPA/DBU (230:200:10, v:v:v, μL) at 60 °C for 2 h (entry 13) as the standard conditions for the amination reaction. After evaporating the solvent, a subsequent deprotection step was carried out using a classical ammonia treatment at 55 °C for 16 h.²⁷ Under these conditions, the expected ^{CPA}dC containing ODN **1b** was obtained in a conversion rate of 63% (see Fig. 1).

In order to investigate the chemoselectivity of the transamination reaction, five model CPG-linked ODNs containing the commercially available protected nucleotides were synthesized. CPG-linked ODN 5'TTXXT3' with X = Bz dC (ODN **1a**, see Table 2), Ac dC, iBu dG, dm f dG or Bz dA were subjected to forced cyclopropylamination reaction conditions (16 h heating instead of 2 h) followed by a treatment with ammonia. As expected, the treatment of the ODNs containing Ac dC, iBu dG, dm f dG or Bz dA afforded the corresponding deprotected unmodified ODNs except for **1a** (see Fig. S23 in the ESI†). The structures were confirmed by ES-MS measurements and by RP-HPLC analyses for which the products obtained were co-eluted with independently synthesized deprotected ODNs (data not shown). Upon treatment, model CPG-linked ODN **1a** displayed two peaks in a roughly 1:2 ratio as estimated by RP-HPLC (Fig. 1). The fast-eluting peak corresponded to the

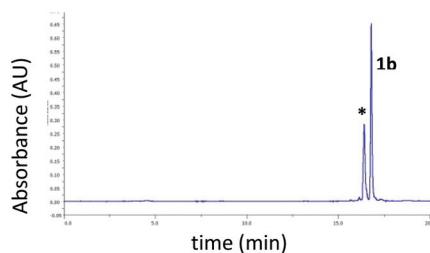


Fig. 1 RP-HPLC analysis of the crude reaction mixture of conversion of CPG-linked ODN **1a** into dC^{CPA} containing ODN **1b**. *Corresponds to unmodified ODN.

Table 2 Transamination by CPA on model ODNs

ODN ^a	Sequences	ESI-MS analysis		
		Calcd mass	Obt. Mass	Rate of conversion ^c
1a, 1b	TTXTT	1484.0	1483.3	63%
2a, 2b	TTGAXAATTGTG	3713.7	3714.2	71%
3a^b, 3b	ATCGGTGXGTTCA	3674.6	3675.2	70%
4a, 4b	TGGATTXGGTAT	3729.6	3730.2	70%
5a, 5b	CATCGTXCCCAG	3604.6	3604.8	51%
6a, 6b	CCXCC	1423.9	1423.3	73%
7a^b, 7b	GGATGXCCAGTT	3699.6	3699.9	70%

^a Compounds **1a–7a** refer to the protected CPG-linked ODN (A = Bz dA; C = Ac dC; G = dm f dG and X = Bz dC). Compounds **1b–7b** refer to the deprotected cyclopropylated ODNs (X = ^{CPA}dC). ^b G = iBu dG instead of dm f dG. ^c Rates of conversion were estimated by RP-HPLC on crude reaction mixture. See Fig. S2–S8† for ESI spectra and HPLC chromatograms.

unmodified deprotected 5'TTCTT3' ODN and the second peak corresponded to the desired N4-cyclopropylated ODN **1b** as confirmed by the LC-MC analysis. These results thus confirmed the chemoselectivity of the cyclopropylamination, which occurred only with the Bz dC containing ODN.

The transamination reaction was then investigated with more complex ODNs. When the CPG-linked ODNs – **2a** lacking Ac dC nucleobase or **3a** containing all four nucleotides – were used as the starting materials (Table 2), the cyclopropylamination proceeded cleanly and a late-eluting product was observed by RP-HPLC with a +40 amu mass as measured with the purified product by ESI-MS analysis (Fig. 2b shows the crude mixture of cyclopropylamination of **3a**). This was also observed for other ODNs **4a–7a** (Table 2). Interestingly, a clean reaction on model ODN **6a** (i.e. containing only C nucleobases) further demonstrated the orthogonality of the Ac- and Bz-cytosine protecting groups.

To further assess the nature of the late-eluting +40 amu product, we independently synthesized ^{CPA}dC nucleoside from N4-benzoyl dC nucleoside (see the experimental part). Purified ODN **3b** was then subjected to an enzymatic digestion with a phosphatase alkaline and snake phosphodiesterase cocktail. RP-HPLC analysis of the crude digestion mixture showed five main peaks (Fig. 3b), the first four were the expected unmodified dA, T, dG and dC, and the slow-eluting one (at a retention time (R_t) = 9.8 min) having the same R_t that the independently synthesized ^{CPA}dC (Fig. 3c). The experimental nucleotide composition (dC/dG/T/dA/^{CPA}dC: 2.1/3.2/4.0/1.6/1.1) matched well with the theoretical one (2/3/4/2/1). These results confirmed the efficient and selective transamination by CPA at the N4 position of the single Bz dC residue introduced in the ODN.

As the chemoselectivity of the amination reaction was established, in order to broaden the scope of the reaction, the transamination reaction was applied for the synthesis of more

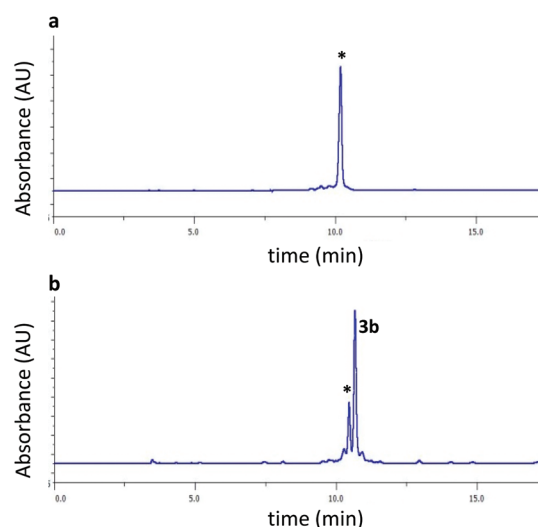


Fig. 2 RP-HPLC profiles of: (a) starting ODN **3a** directly deprotected by ammonia and (b) crude cyclopropylamination reaction mixture of **3b**. *Corresponds to unmodified ODN.

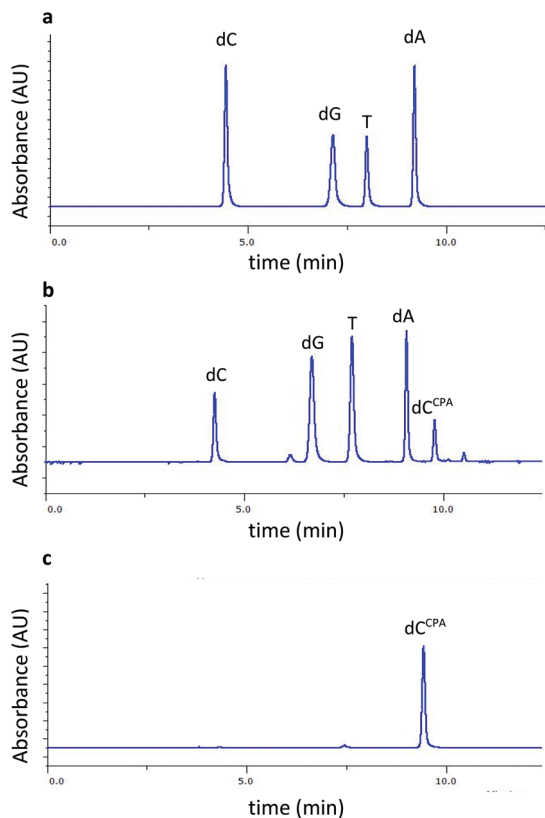


Fig. 3 RP-HPLC traces of (a) equimolar mixture of dA, dC, T and dG; (b) ODN **3b** digested by P1 and PA enzymes; (c) independently synthesized CPA dC .

complex conjugates. The introduction of linkers containing various functional groups such as amines (**c** and **f** linkers), alcohol (**d** linker), masked aldehyde (**e** linker) and alkyne (**g** linker) as well as linkers containing an aromatic (**h** and **i** linkers) or aliphatic moiety (**j** linker) was investigated (Scheme 1 and Table 3). Different sizes of ODN were used including 12-mer (ODNs **3–5** and **7**), 18-mer (ODNs **8**), 24-mer

(ODNs **9**) and 30-mer (ODNs **10**). By using the above procedure, the modified ODNs **3c–10i** could be obtained in two working days from cheap raw materials with satisfactory yields.

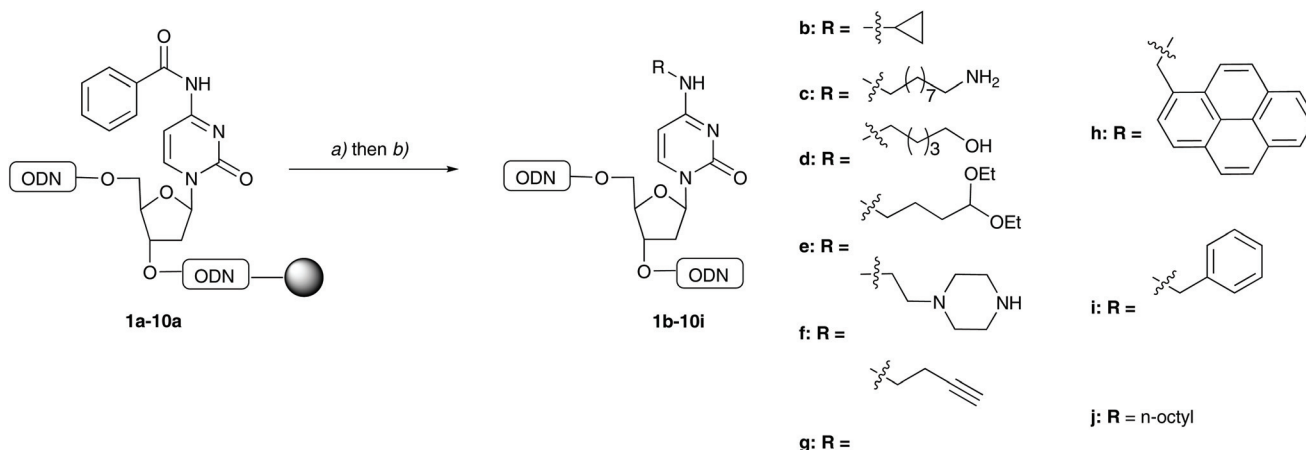
Experimental section

General remarks

ESI mass spectra were recorded on an Esquire 3000 spectrometer from Bruker or on an Acquity UPLC/MS system from Waters equipped with a SQ Detector 2. Oligonucleotides were analyzed in negative mode. All solvents and reagents used were of highest purity commercially available.

Oligonucleotide synthesis

Oligonucleotides were prepared using β -cyanoethylphosphoramidite chemistry on a 3400 DNA synthesizer at 1 μmol scale by using classical conditions. Briefly, 0.25 M ETT in MeCN was used as the activator, 0.02 M iodine solution in THF/Water/Pyridine as the oxidizer, acetic anhydride in THF and 16% *N*-methylimidazole in THF as the cap A and B reagents, 3% TCA in DCM as the deblock reagent and acetonitrile (<30 ppm water) as the washing solvent. Preloaded 500 Å CPG (controlled pore-glass support) was used (loading: 37–45 $\mu\text{mol g}^{-1}$). Otherwise stated, final deprotection was performed by a treatment with 30% NH_4OH at 55 °C for *ca.* 16 h. Oligonucleotides were evaporated to dryness, taken up in 200 μL water and analyzed by RP-HPLC. RP-HPLC analyses were achieved by using a prepacked C18 column (length 250 mm, diam 4.6 mm; particle size 5 μm , porosity 100 Å) at 1 mL min^{-1} flow rate. Eluent A was a 50 mM TEAA solution pH = 7 in water/MeCN (95 : 5, v/v) and eluent B a MeCN/water (9 : 1, v/v) solution. The analyses were monitored at 260 nm. For purification, a 10 mm diameter column was used with a 4 mL min^{-1} flow rate. Gradient was isocratic for 2 min using 4% eluent B, then 4 to 20% B in 15 min.



Scheme 1 Amination reactions of Bz dC containing oligonucleotides: (a) R-NH_2 , DBU, iPrOH , 2h, 60 °C, (b) NH_4OH 28%, 55 °C, 16 h.

Table 3 Reaction extended to various amines and ODN

ODNs ^a	Sequence	ESI-MS analysis		Rate of conv. ^d
		Calcd.	Obt.	
3a, 3c	ATCGGTGXGTTCA	3761.7	3761.8	41%
4a, 4d	TGGATTXGGTAT	3835.8	3834.8	46%
4a, 4e	TGGATTXGGTAT	3777.6	3776.8	45%
4a, 4f	TGGATTXGGTAT	3801.1	3803.0	28%
4a, 4g ^b	TGGATTXGGTAT	3743.5	3742.1	37%
5a, 5h	CATCGTXCCAG	3778.7	3778.3	25%
7a ^c , 7c	GGATGXCCAGTT	3788.5	3788.1	45%
7a ^c , 7j	GGATGXCCAGTT	3773.5	3772.6	38%
8a, 8e	TGAAGCTAGXTAGCTAAG	5689.3	5689.3	42% (30%)
8a, 8i	TGAAGCTAGXAGCTAAG	5635.0	5634.4	45% (30%)
8a, 8j	TGAAGCTAGXTAGCTAAG	5659.8	5659.5	35%
9a, 9h	TTGAGAGACGAXCGCTAGACACTT	7576.0	7575.8	36% (23%)
10a, 10e	GATCGCTAGATTTXGACAGATAGGCTCTCC	9326.8	9325.9	47% (32%)
10a, 10i	GATCGCTAGATTTXGACAGATAGGCTCTCC	9272.0	9271.3	30%

^a ODNs 3a–10a refer to the protected CPG-linked ODN (A = Bz dA; C = Ac dC; G = dmf dG and X = Bz dC). ODNs 3c–10i refer to the functionalized ODNs (X = R dC). ^b Reaction mixture was heated for 16 h. ^c G = iBu dG in 7a. ^d Rate of conversion were estimated by RP-HPLC on crude reaction mixture. Yields in brackets refer to yields of purified ODNs. See Fig. S9–S22† for ESI spectra and HPLC chromatograms.

Procedure for transamination of CPG-linked ODNs

CPG-linked ODN 1a–10a (5 mg) was placed in a vial. Isopropanol (230 μ L), the corresponding amine (200 μ L) and DBU (10 μ L) were then added. Vial was centrifuged to ensure a complete wetting of the beads and heated at 60 °C for 2 h. Isopropanol was removed on a Speedvac apparatus. Then, concentrated ammonia (aq. 28–30%; ca. 1 mL) was added, and the resulting mixture was heated at 60 °C overnight. Ammonia was evaporated on a Speedvac and the solution was diluted by addition of water (1 mL). Aqueous mixture was then filtered and the supernatant was purified by exclusion-size chromatography (NAP25 column; purchased from Fisher: ref. 10186464). Elution of ODNs was monitored by UV. Fractions of interest were gathered and purified by RP-HPLC. ODNs 1b–7b, 10e and 10i were separated from their unmodified counterpart by using the following conditions: 5% B for 2 min, then 5% to 18% B in 15 min. ODNs 3c–9h were separated by using the following conditions: 0% B for 2 min then 0% to 20% in 20 min. Synthesis of ODNs functionalized by pyrene (5h, 9h) was performed using a warm (60 °C) saturated solution of pyrenyl-methylamine in isopropanol.

Synthesis of N4-cyclopropylaminecytosine (CPA dC)

N4-Benzoylcytosine (100 mg, 302 μ mol) was suspended in neat cyclopropylamine (4 mL) and heated at reflux temperature overnight. The crude reaction mixture was then evaporated to dryness. For analytical purposes, only a few milligrams were purified by RP-HPLC (isocratic at 0% B for 2 min, then 0 to 45% in 15 min) and lyophilized as white flakes. Cytosine (tR = 7.71 min) was separated from of N4-cyclopropylaminecytosine (tR = 10.68 min) in 79% conversion rate based on HPLC. ¹H NMR (D₂O, 500 MHz): δ ppm 7.83 (H₆, 1H, d, J = 7.6 Hz); 6.28 (H₁, 1H, t, J = 6.6 Hz); 6.06 (H₅, 1H, d, J = 6.6 Hz); 4.44 (H₃, 1H, mult.); 4.06 (H₄, 1H, mult.); 3.84 (1 $\times$ H₅, 1H, d $\times$ d, J = 3.6 $\times$ 12.4 Hz); 3.76 (1 $\times$ H₅, 1H, d $\times$ d, J = 3.6 $\times$ 12.4 Hz); 2.71 (CH,

CP ring', 1H, br.); 2.44 (1 $\times$ H₂, 1H, d $\times$ d $\times$ d, J = 4.1 $\times$ 6.5 $\times$ 14.2 Hz); 2.31 (1 $\times$ H₂, 1H, mult.); 0.86 (CH₂, CP ring', 2H, br.); 0.62 (CH₂, CP ring', 2H, br.). ¹³C NMR (D₂O, 500 MHz): δ ppm 168.2; 157.6; 141.5; 96.2; 96.0; 86.6; 86.1; 70.5; 61.2; 39.2; 6.6; 6.0.

Enzymatic digestion by P1 and PA

PA (reference: P7923; batch SLBW7179) and P1 (reference N8630; batch SLBX9772) enzymes were purchased from Sigma-Aldrich (St-Louis, USA). ODN (50 nmol) was dissolved in 1 mL TRIS buffer (TRIS 100 mM; MgCl₂ 5 mM, NaCl 100 mM, pH = 9.0). Then 10 μ L of P1 phosphodiesterase solution (nuclease P1 from *Penicillium citrinum*; dissolved in 1 mL of the aforementioned buffer; 1.4 mg mL⁻¹; phosphodiesterase activity: 477 units per mg) and 10 μ L of phosphatase solution (from bovine intestinal mucosa; dissolved in 1 mL of the aforementioned buffer; 10 mg mL⁻¹; >4000 units per mg) were added, and the resulting solution was heated at 37 °C overnight. RP-HPLC analysis revealed an incomplete enzymatic digestion. 5 μ L of both enzymes were added and the solution was heated again at 37 °C overnight. Solution was then heated at 90 °C for 10 min, cooled and centrifuged. Supernatant was then analyzed and purified. Gradient was: isocratic at 0% eluent B (2 min), then 0 to 30% B in 15 min.

Conclusion

In conclusion, these results confirmed that acetyl and benzoyl are efficient orthogonal protecting groups for protection of the N4 of cytosine residue embedded in ODN. The amination reaction occurred only on the cytosine nucleobase protected with the benzoyl group, whereas the other nucleobases remained unaffected under these conditions. The amination reaction was performed with various amines as well as with ODNs of different length (from 5-mer to 30-mer). As numerous amines

are commercially available, various groups could be easily introduced by a post-synthetic modification of a CPG-linked ODN assembled with T, ^{iBu}dG, ^{Bz}dA, ^{Ac}dC and only one ^{Bz}dC phosphoramidites. Our protocol thus enables an easy post-synthetic labelling of ODNs of various length in the internal position without the use of sophisticated phosphoramidites and/or a tedious multistep synthesis. It should be noted that the moderate yields are counterbalanced by the cheapness and easiness of this reaction. Due to the very low price of these reactants, we believe that it could be a user-friendly alternative to the expensive and sensitive commercially available phosphoramidites.

Conflicts of interest

They are no conflicts to declare.

Acknowledgements

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