Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2020, **18**, 9632

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

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Received 8th October 2020,
Accepted 3rd November 2020

DOI: 10.1039/d0ob02059a

rsc.li/obc

The commercially available and cheap nucleotide phosphoramidites are routinely used for the oligonucleotide (ODN) assembly. T, isobutyryl-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 ligation[11] and Diels–Alder reactions[12] 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 been 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 *N*4-triazolo-dC or *O*6-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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†Electronic supplementary information (ESI) available: RP-HPLC profiles of all crude reaction products. ESI-MS spectra of purified ODNS. See DOI: 10.1039/d0ob02059a

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 *N*4-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 *N*4-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 *N*4-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 *N*4-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 *N*4-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 *N*4-cyclopropylamination of model ODN **1a**[a]

Entry	Volume of (μL) Solvent	CPA	DBU	Time (h)	Temp. (°C)	Rate conv.[b]
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 $^{5'}$TTXTT$^{3'}$ with X = ^{Bz}dC (ODN **1a**, see Table 2), ^{Ac}dC, ^{iBu}dG, ^{dmf}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, ^{dmf}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 coeluted 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 unmodified deprotected $^{5'}$TTCTT$^{3'}$ ODN and the second peak corresponded to the desired *N*4-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 *N*4-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

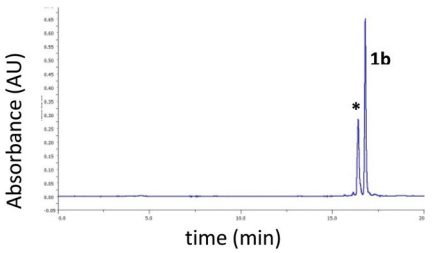


Fig. 1 RP-HPLC analysis of the crude reaction mixture of conversion of CPG-linked ODN **1a** into dC^{CPA} containing ODN **1b**. *Corresponds to unmodified ODN.

Table 2 Transamination by CPA on model ODNs

ODN[a]	Sequences	ESI-MS analysis: Calcd mass	ESI-MS analysis: Obt. Mass	Rate of conversion[c]
1a, **1b**	TT**X**TT	1484.0	1483.3	63%
2a, **2b**	TTGA**X**AATTGTG	3713.7	3714.2	71%
3a[b], **3b**	ATCGGT**X**GTTCA	3674.6	3675.2	70%
4a, **4b**	TGGATT**X**GGTAT	3729.6	3730.2	70%
5a, **5b**	CATCGT**X**CCCAG	3604.6	3604.8	51%
6a, **6b**	CC**X**CC	1423.9	1423.3	73%
7a[b], **7b**	GGATG**X**CCAGTT	3699.6	3699.9	70%

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

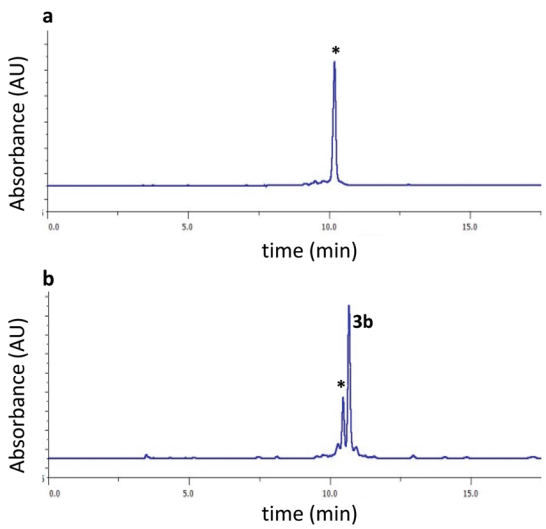


Fig. 2 RP-HPLC profiles of: (a) starting ODN **3a** directly deprotected by ammonia and (b) crude cyclopropylamination reaction mixture of **3b**. *Corresponds to unmodified ODN.

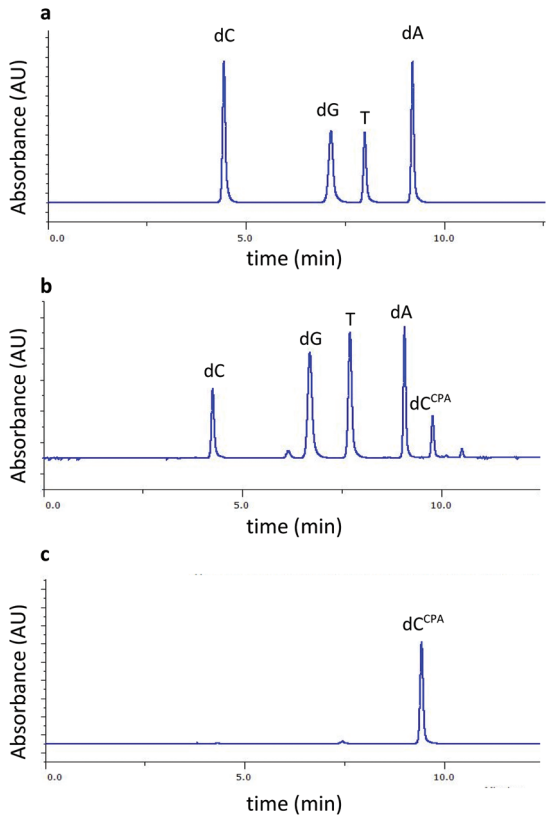


Fig. 3 RP-HPLC traces of (a) equimolar mixture of dA, dC, T and dG; (b) ODN **3b** digested by P1 and PA enzymes; (c) independently synthesized ^{CPA}dC.

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.

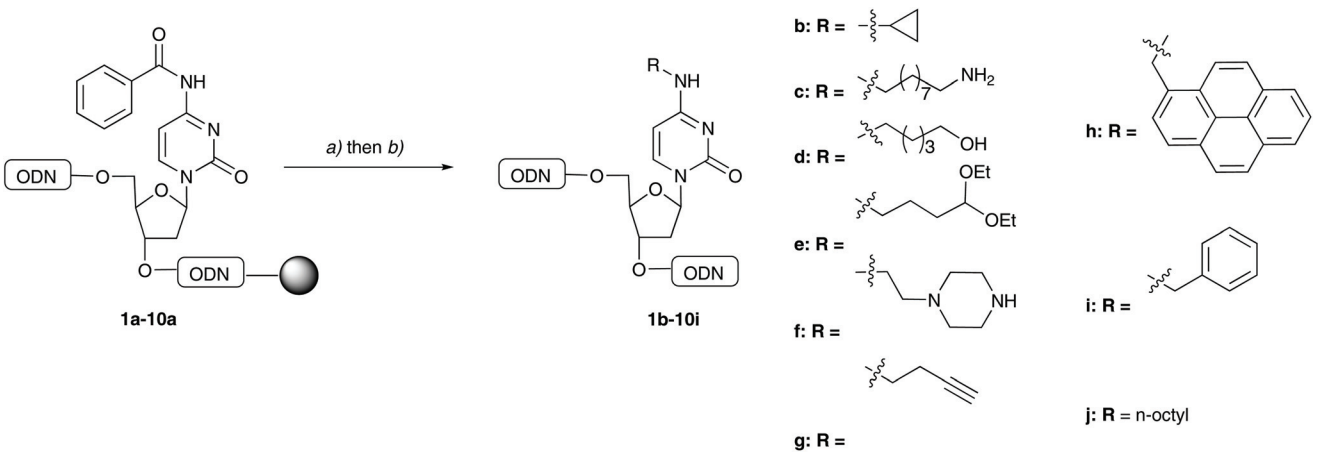
Experimental section

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 μmol 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 Calcd.	Obt.	Rate of conv.[d]
3a, 3c	ATCGGT**X**GTTCA	3761.7	3761.8	41%
4a, 4d	TGGATT**X**GGTAT	3835.8	3834.8	46%
4a, 4e	TGGATT**X**GGTAT	3777.6	3776.8	45%
4a, 4f	TGGATT**X**GGTAT	3801.1	3803.0	28%
4a, 4g[b]	TGGATT**X**GGTAT	3743.5	3742.1	37%
5a, 5h	CATCGT**X**CCCAG	3778.7	3778.3	25%
7a[c], **7c**	GGATG**X**CCAGTT	3788.5	3788.1	45%
7a[c], **7j**	GGATG**X**CCAGTT	3773.5	3772.6	38%
8a, 8e	TGAAGCTAG**X**TAGCTAAG	5689.3	5689.3	42% (30%)
8a, 8i	TGAAGCTAG**X**AGCTAAG	5635.0	5634.4	45% (30%)
8a, 8j	TGAAGCTAG**X**TAGCTAAG	5659.8	5659.5	35%
9a, 9h	TTGAGAGACGA**X**CGCTAGACACTT	7576.0	7575.8	36% (23%)
10a, 10e	GATCGCTAGATTT**X**GACAGATAGGCTCTCC	9326.8	9325.9	47% (32%)
10a, 10i	GATCGCTAGATTT**X**GACAGATAGGCTCTCC	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 μL), the corresponding amine (200 μL) and DBU (10 μL) 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 pyrenylmethylamine in isopropanol.

Synthesis of *N*4-cyclopropylaminecytosine (^{CPA}dC)

*N*4-Benzoylcytosine (100 mg, 302 μmol) 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 *N*4-cyclopropylaminecytosine (tR = 10.68 min) in 79% conversion rate based on HPLC. ^{1}H NMR (D_2O, 500 MHz): δ ppm 7.83 (H_6, 1H, d, J = 7.6 Hz); 6.28 ($H_{1'}$, 1H, t, J = 6.6 Hz); 6.06 ($H_{5'}$, 1H, d, J = 6.6 Hz); 4.44 ($H_{3'}$, 1H, mult.); 4.06 ($H_{4'}$, 1H, mult.); 3.84 (1 × $H_{5'}$, 1H, d × d, J = 3.6 × 12.4 Hz); 3.76 (1 × $H_{5'}$, 1H, d × d, J = 3.6 × 12.4 Hz); 2.71 (CH, CP ring', 1H, br.); 2.44 (1 × $H_{2'}$, 1H, d × d × d, J = 4.1 × 6.5 × 14.2 Hz); 2.31 (1 × $H_{2'}$, 1H, mult.); 0.86 (CH_2, CP ring', 2H, br.); 0.62 (CH_2, CP ring', 2H,br.). ^{13}C NMR (D_2O, 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; $MgCl_2$ 5 mM, NaCl 100 mM, pH = 9.0). Then 10 μL 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 μL 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 μL 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 centrifugated. 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 *N*4 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

This work was supported by Labex ARCANE (ANR-11-LABX-0003-01) and CBH-EUR-GS (ANR-17-EURE-0003). The Nanobio-ICMG platform (FR2607) is acknowledged for providing facilities for synthesis and purification of oligonucleotides and mass spectrometry analyses. The authors thank R. Guéret, A. Durand and L. Fort for running ESI-MS analysis. Pr B. Elias is also acknowledged for the fruitful discussions.

Notes and references

1 A. Khvorova and J. K. Watts, The chemical evolution of oligonucleotide therapies of clinical utility, *Nat. Biotechnol.*, 2017, **35**, 238–248.
2 J. Liu, Z. Cao and Y. Lu, Functional Nucleic Acid Sensors, *Chem. Rev.*, 2009, **109**, 1948–1998.
3 S. K. Silverman, DNA as a Versatile Chemical Component for Catalysis, Encoding, and Stereocontrol, *Angew. Chem., Int. Ed.*, 2010, **49**, 7180–7201.
4 W. Su, V. Bonnard and G. A. Burley, DNA-Templated Photonic Arrays and Assemblies: Design Principles and Future Opportunities, *Chem. – Eur. J.*, 2011, **17**, 7982–7991.
5 R. L. Juliano, X. Ming and O. Nakagawa, The Chemistry and Biology of Oligonucleotide Conjugates, *Acc. Chem. Res.*, 2012, **45**, 1067–1076.
6 Y. Singh, P. Murat and E. Defrancq, Recent developments in oligonucleotide conjugation, *Chem. Soc. Rev.*, 2010, **39**, 2054–2070.
7 N. Spinelli, O. P. Edupuganti, E. Defrancq and P. Dumy, New method to prepare peptide-oligonucleotide conjugates through glyoxylic oxime formation, *J. Org. Chem.*, 2004, **69**, 8544–8546.
8 A. Sanchez, E. Pedroso and A. Grandas, Easy introduction of maleimides at different positions of oligonucleotide chains for conjugation purposes, *Org. Biomol. Chem.*, 2012, **10**, 8478–8483.
9 A. H. El-Sagheer and T. Brown, Click chemistry with DNA, *Chem. Soc. Rev.*, 2010, **39**, 1388–1405.
10 M. Merkel, K. Peewasan, S. Arndt, D. Ploschik and H.-A. Wagenknecht, Copper–Free Postsynthetic Labeling of Nucleic Acids by Means of Bioorthogonal Reactions, *ChemBioChem*, 2015, **16**, 1541–1553.
11 M. Arthur and M. Etheve-Quelquejeu, Covalent Organic Functionalization of nucleic acids, in *Chemistry of Organo-Hybrids: Synthesis and Characterization of Functional Nano-Objects*, ed. B. Charleux, C. Coperet and E. Lacite, Wiley, Hoboken, 2015, pp. 272–298.
12 A. H. El-Sagheer, V. V. Cheong and T. Brown, Rapid chemical ligation of oligonucleotides by the Diels–Alder reaction, *Org. Biomol. Chem.*, 2011, **9**, 232–235.
13 E. Defrancq and S. Messaoudi, Palladium mediated labelling of nucleic acids, *ChemBioChem*, 2017, **18**, 426–433.
14 Y.-Z. Xu, Q. Zheng and P. F. Swann, Synthesis of DNA Containing Modified Bases by Postsynthetic Substitution. Synthesis of Oligomers Containing 4-Substituted Thymine: *O*4-Alkylthymine, 5-Methylcytosine, *N*4-(Dimethylamino)-5-methylcytosine and 4-Thiothymine, *J. Org. Chem.*, 1992, **57**, 3839–3845.
15 H. Gao, R. Fathi, B. L. Gaffney, B. Goswami, P.-P. Kung, Y. Rhee, R. Jin and R. A. Jones, 6-*O*-(Pentafluorophenyl)-2′-deoxyguanosine: A Versatile Synthon for Nucleoside and Oligonucleotide Synthesis, *J. Org. Chem.*, 1992, **57**, 6954–6956.
16 Catalogue prices of one of the main phosphoramidite providers were *ca.* 10 US$ per mmol for BzdC phosphoramidite *vs*. *ca.* 1350 US$ for *O*4-triazolo-dU phosphoramidite or 2950 US$ per mmol for 4-thio-dU-phosphoramidite.
17 L. Gjonaj and G. Roelfes, Selective chemical modification of DNA with alkoxy- and benzyloxyamines, *Org. Biomol. Chem.*, 2015, **13**, 6059–6065.
18 M. J. Gait, in *Oligonucleotide synthesis a pratical approach*, IRL Press, Oxford, 1984.
19 M. P. Reddy, N. B. Hanna and F. Farooqui, Fast cleavage and deprotection of oligonucleotides, *Tetrahedron Lett.*, 1994, **35**, 4311–4314.
20 P. S. Miller, M. P. Reddy, A. Murakami, K. R. Blake, S.-B. Lin and C. H. Agris, Solid-phase syntheses of oligodeoxyribonucleoside methylphosphonates, *Biochemistry*, 1986, **25**, 5092–5097.
21 H. Weber and H. G. Khorana, Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. Chemical synthesis of an icosadeoxynucleotide corresponding to the nucleotide sequence 21 to 40, *J. Mol. Biol.*, 1972, **72**, 219–249.
22 R. I. Hogrefe, M. M. Vaghefi, M. A. Reynolds, K. M. Young and L. J. Arnold, Deprotection of methylphosphonate oligonucleotides using a novel one-pot procedure, *Nucleic Acids Res.*, 1993, **21**, 2031–2038.
23 M. P. Reddy, F. Farooqui and N. B. Hanna, Elimination of transamination side product by the use of dCAc methylphosphonamidite in the synthesis of oligonucleoside

methylphosphonates, *Tetrahedron Lett.*, 1996, **37**, 8691–8694.
24 H. Salo, P. Virta, H. Hakala, T. P. Prakash, A. M. Kawasaki, M. Manoharan and H. Lönnberg, Aminooxy funtionalized oligonucleotides: preparation, on-support derivatization, and postsynthetic attachment to polymer Support, *Bioconjugate Chem.*, 1999, **10**, 815–833.
25 T. A. Abramova, S. V. Vasileva, T. M. Ivanova, G. V. Shishkin and V. N. Silnikov, Monomers for oligonucleotide synthesis with linkers carrying reactive residues: I. the Synthesis of deoxynucleoside derivatives with methoxyoxalamide groups in heterocyclic bases, *Russ. J. Bioorg. Chem.*, 2004, **30**, 224–230.
26 F. Shao, M. A. O'Neill and J. K. Barton, Long-range oxidative damage to cytosines in duplex DNA, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 17914–17919.
27 It should be noted that cleavage of ODNs from the support could occur during the treatment with amines. However, no loss of the material happen as the crude mixture is recuperated after evaporation of the amine in ammonia for subsequent deprotection step.