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Synthesis and properties of cationic 2′-*O*-[*N*-(4-aminobutyl)carbamoyl] modified oligonucleotides

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ABSTRACT

2′-*O*-[*N*-(4-Aminobutylcarbamoyl)]uridine (U_{abcm}) was synthesized and incorporated into oligonucleotides. The oligonucleotides incorporating U_{abcm} formed more stable duplexes with their complementary and mismatched RNAs than those containing 2′-*O*-carbamoyluridine (U_{cm}). The stability of duplex with a U_{abcm}-rG base pair showed higher thermostability than the duplex having unmodified U-rG base pair. The U_{abcm} residue showed enhanced resistance to snake venome phosphodiesterase.

2′-*O*-Modified RNA molecules have been extensively used for gene regulation such as antisense,[1] antigene,[2] and RNA interference (RNAi).[3] 2′-*O*-Modification of RNAs can improve their stability toward hydrolysis[4] and enhance the hybridization affinity for the target RNAs.[5]

As one of the 2′-modified RNAs, several research groups have reported the synthesis and properties of oligonucleotides containing 2′-*O*-carbamoyl and 2′-*O*-*N*-alkylcarbamoyl groups.[6–8] In these studies, it was reported that various functional groups, such as the propargyl group, the dansyl-6-sulphonamidohexyl group and 4-(pyren-1-ylethynyl)phenylmethyl group, could be easily introduced into the 2′-position of RNAs through the carbamoyl group.[6] In addition, we have reported the uridine derivative having the simplest carbamoyl group (U_{cm})[7] to study the intrinsic properties of the carbamoyl modifications and found that the carbonyl could participate in the wobble-type uracil-guanine base pair forming a hydrogen bond with the amino group of guanine at position 2. These results indicated the unique character of carbamoyl modifications for the development of the artificial nucleic acids having useful functional groups and unique base pairing properties. However, we and other groups also found a drawback of the carbamoyl group that the incorporation of the carbamoyl modification decreased the stabilities of the duplexes probably due to the close contact between the carbonyl oxygen of the 2′-*O*-carbamoyl substituent and the O2 of nucleobase.[8]

In this paper, in order to improve the hybridization affinity and the nuclease resistance, we designed new carbamoyl-type modified nucleoside, 2′-*O*-[*N*-(4-aminobutyl)carbamoyl]uridine (U_{abcm}). It is well known that the amino groups incorporating into oligonucleotides neutralize the negative charges of the phosphate backbone. Therefore, it is expected that the oligonucleotides having U_{abcm} form more stable duplexes with their complementary strands than those containing U_{cm}. We report the synthesis of the oligonucleotides having U_{abcm} and the properties of the 2′-*O*-methyl RNA oligomers incorporating U_{cm} and U_{abcm}.

To introduce U_{abcm} into the oligonucleotides, the phosphoramidite unit **5** was synthesized, as shown Scheme 1.

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)uridine **1** was treated with 1.1 equiv of phenyl chloroformate. Subsequently, compound **2** was treated in situ with 1,4-diaminobutane at 0 °C. The NH_2 group was protected with a trifluoroacetyl group to give **3** in 70% yield in three steps. The silyl-protecting group was removed by using 3.5 equiv of triethylamine-tri(hydrofluororide). The triethylammonium salts were removed by treatment with ethoxytrimethylsilane,[9] and the resulting 5′-hydroxyl group was protected with a DMTr group to give **4**. Phosphitylation with chloro-(2-cyanoethoxy)-(*N*,*N*-diisopropylamino)phosphine furnished the phosphoramitite unit **5**.

Before synthesis of the oligonucleotides, we synthesized the dimer **6** incorporating U_{abcm} in the usual manner by using 1*H*-tetrazole as an activator (Scheme 2).[10] However, the coupling yield was surprisingly too low (<3%) to obtain the target dimer as judged by the trityl cation assay.

In order to improve the coupling yield, we optimized the conditions of the coupling reactions by measuring the coupling yield at the Tr cation assay step. First, we tested 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42),[11] *N*-phenylimidazolium triflate (*N*-PhIMT)[12] and 5-benzylthiotetrazole (BTT)[13] which

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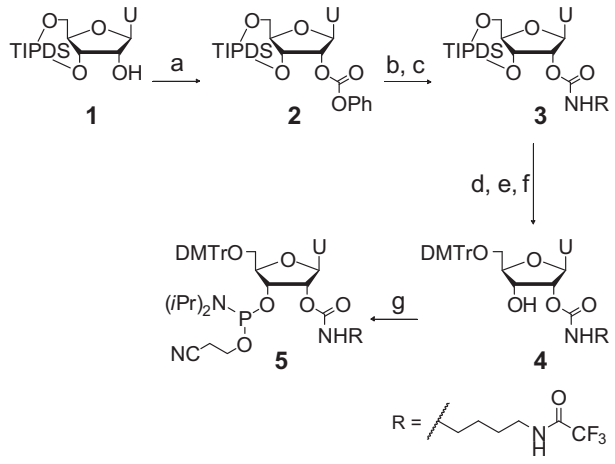
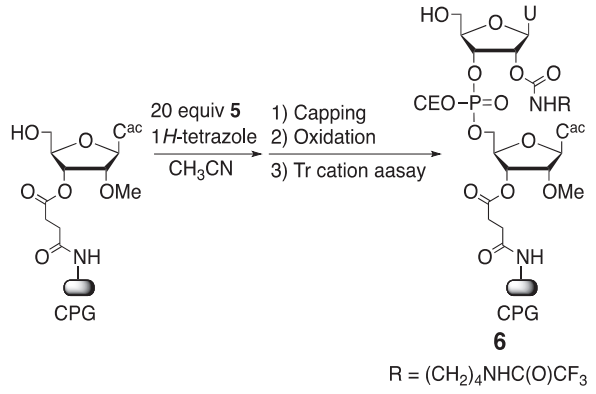


Scheme 1. Synthesis of phosphoramidite unit 5 of U_{abcm}: (a) PhOC(O)Cl (1.1 equiv), pyridine, rt, 3 h; (b) 1,4-diaminobutane (1.2 equiv), pyridine, 0 °C, 3 h; (c) CF_3COOEt (2.0 equiv). TEA (1.0 equiv), EtOH, rt, overnight, 70% (3 steps); (d) TEA-3HF (3.5 equiv), TEA (1.8 equiv), THF, rt, 3 h; (e) TMSOEt (10 equiv), THF, rt, overnight; (f) DMTrCl (1.2 equiv), pyridine, rt, 8 h, 75% (3 steps); (g) $NCCH_2CH_2OPClN(iPr)_2$ (1.2 equiv), $(iPr)_2NEt$ (1.5 equiv), dichloromethane, 1 h, rt, 72%.



Scheme 2. Synthesis of dimer **6**.

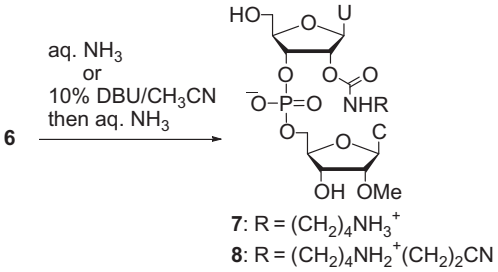
are frequently used stronger activator for the oligonucleotide synthesis, but the coupling yields were not improved even after the reaction time prolonged to 40 min (data not shown).

We also tested 4,5-dicyanoimidazole (DCI)[14] known as a strong nucleophilic activator. In the activation with DCI for 40 min, the coupling yield increased to 57% (Table1). For further improvement of the reaction, doubling the equivalent of DCI to 160 equiv marginally improved the coupling yield to 62%. Finally, we found that the coupling yield could be increased to 87% by repeating the 40 min coupling twice before the capping. These results indicate that the phosphoramidite having lower reactivity due to the modification at the 2′-position can be efficiently introduced into oligonucleotides by use of DCI.[15]

Next, we tested the deprotection of the protecting groups on the fully protected dimer **6** attached on the solid supports. We first tried the simultaneous removal of the trifluoroacetyl group, the cyanoethyl group and the acetyl group by treatment with aqueous ammonia (Scheme 3). However, we found the formation of considerable amount of *N*-cyanoethylated dimer **8** as the by-product (Fig. 2b). Similar cyanoethylation of the amino groups was also reported by Buchini and Griffey in the synthesis of 2′-*O*-aminoethyl and 2′-*O*-aminopropyl ribonucleotides.[2,16] To prevent the cyanoethylation of the terminal amino group, we treated the oligonucleotides first with 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU)–acetonitrile (1:9, v/v)[2,17] for 1 min to selectively remove the cyanoethyl group on the phosphate, and the subsequent cleavage of the oligonucleotides from the solid supports and the deprotection of the nucleobase and the terminal amino group were performed by treatment with 28% aqueous ammonia. Figure 2a shows the effect of DBU treatment in the deprotection and cleavage of the oligonucleotide having U_{abcm}. The oligonucleotides were characterized by ESI mass.

By using above mentioned coupling and deprotection conditions, we synthesized the 12mer 2′-*O*-methyl RNAs (**ON2**, **ON4** and **ON6** in Table 2) having a 5′-$G_mU^2A_mC_mC_mU^6U_mU^8C_mC_mG_mG_m$-3′ sequence. **ON2** has U_{abcm} at the U^6 position. **ON4** and **ON6** have two U_{abcm} residues at U^6/U^8 andU^2/U^8 positions, respectively. We studied the hybridization properties of them with sequence matched RNA strand 3′-CAUGAAAGGCC-5′ (Table 3). For comparison, we also prepared the 2′-*O*-methyl RNAs such as **ON1**, **ON3** and **ON5** incorporating U_{cm} (Table 2).

As shown in Table 3, **ON1** to **ON6** having U_{cm} or U_{abcm} showed the lower T_m than **ON7** composed of 2′-*O*-methyl RNAs due to the destabilization effect of 2′-*O*-carbamoyl group. While **ON2** incorporating a U_{abcm} at U^6 position showed the T_m of 69 °C which is 4 °C higher than **ON1** having a U_{cm} residue at the same position probably due to the presence of the cationic aminobutyl group. Interestingly, the T_m difference became more significant when two U_{cm} and U_{abcm} groups were incorporated at position U^6 and U^8 as in the case of **ON3** and **ON4**, respectively. In this case, the **ON4** incorporated two U_{abcm} at U^6 and U^8 positions showed T_m of 61 °C that was higher than that of **ON3** incorporating two U_{cm} residues by 18 °C. Similarly, **ON6** having two U_{abcm} group at U^2 and U^8



Scheme 3. Synthesis of dimer $U_{abcm}C_m$ (**7**).

Table 1
Optimization of conditions for coupling reaction of the dimer **6**

Activator (equiv)	Coupling tine (min)	Coupling yield (%)
DCI (80)	40	57
DCI (160)	40	62
DCI (80)	40 × 2	87

Table 2
Oligonucleotide sequences incorporating U_{abcm} or U_{cm}

	Sequences
ON1	5′-*GUACCU*$_{cm}$*UUCCGG*-3′
ON2	5′-*GUACCU*$_{abcm}$*UUCCGG*-3′
ON3	5′-*GUACCU*$_{cm}$*UU*$_{cm}$*CCGG*-3′
ON4	5′-*GUACCU*$_{abcm}$*UU*$_{abcm}$*CCGG*-3′
ON5	5′-*GU*$_{cm}$*ACCUUU*$_{cm}$*CCGG*-3′
ON6	5′-*GU*$_{abcm}$*ACCUUU*$_{abcm}$*CCGG*-3′
ON7	5′-*GUACCUUUCCGG*-3′
ON8	5′-*UU*dT-3′
ON9	5′-$U_{cm}U_{cm}$dT-3′
ON10	5′-$U_{abcm}U_{abcm}$dT-3′

The 2′-*O*-methyl ribonucleotide residues are italicized.

Table 3
T_m (°C) and ΔT_m (°C) of 2′-*O*-methyl RNA/ RNA duplexes, 5′-$G_mU^2A_mC_mC_mU^6U_mU^8C_mC_mG_mG_m$-3′/ 3′-CAUGGAAAGGCC-5′, incorporating U_{abcm} or U_{cm}

Me-RNA	U^2, U^6, U^8 =	T_m (°C)	ΔT_m (°C)
ON1	U_m, U_{cm}, U_m	65*	
ON2	U_m, U_{abcm}, U_m	69	+4
ON3	U_m, U_{cm}, U_{cm}	43	
ON4	U_m, U_{abcm}, U_{abcm}	61	+18
ON5	U_{cm}, U_m, U_{cm}	47	
ON6	U_{abcm}, U_m, U_{abcm}	61	+14
ON7	U_m, U_m, U_m	70*	

Conditions: 10 mM phosphate buffer (pH 7.0), 150 mM NaCl, 0.1 mM EDTA, 2 μM duplex.
* Data from Ref. 7.

positions showed T_m of 61 °C that was higher by 14 °C than **ON5** having U_{cm} whose T_m was 47 °C.

These T_m results exhibited that although an increasing number of carbamoyl groups in the oligonucleotide considerably decreased the hybridization stabilities of the oligonucleotide, the cationic residues moderated the destabilization effects of U_{cm} residues.

Next, we evaluated the base discrimination abilities of U_{abcm} (Table 4) by using the singly modified **ON2** and the counter strand RNA 3′-CAUGGXAAGGCC-5′ changing the base of position X to A, U, G and C. As shown in Table 4, **ON2** containing a U_{abcm} showed T_m of 69 °C with fully matched RNA (X = A) which is 3 °C lower than the fully matched duplex of **ON7** without U_{abcm} and same counter-strand RNA whose T_m was 72 °C. On the other hand, in the cases of the mismatched duplexes where nucleotides at position X are U, G and C, the duplexes incorporating U_{abcm} were equally or more stable than the corresponding duplexes incorporating U_m. Especially, the duplex containing G-U_{abcm} was significantly more stable than those incorporating U_m by 5 °C, probably due to the participation of the carbamoyl group to the wobble-type guanine-uracil base pair as suggested in Figure 1.

Finally, we evaluated the stability of the U_{abcm} modified oligonucleotides toward the enzymatic digestion. We synthesized U_{abcm} modified trimer **ON10** (5′-$U_{abcm}U_{abcm}$dT) and digested **ON10** by snake venome phosphodiesterase (SVPDE). The time course of the digestion was compared with the 2′-*O*-methyl modified trimer **ON8** (5′-U_mU_mdT-3′) and the U_{cm} modified **ON9** (5′-$U_{cm}U_{cm}$dT). The results are shown in Figure 3.

Table 4
T_m (°C) and ΔT_m (°C) of 5′-$G_mU_mA_mC_m$ C_m**U^6**$U_mU_mC_mC_mG_mG_m$-3′/3′-CAUGG-**X**AAGGCC-5′

	$U^6 = U_m$ (**ON7**)	$U^6 = U_{abcm}$ (**ON2**)	ΔT_m (°C)
X = A	70*	69	−3
X = U	58	59	+1
X = G	63	68	+5
X = C	57	57	0

Conditions: 10 mM phosphate buffer (pH 7.0), 150 mM NaCl, 0.1 mM EDTA, 2 μM duplex. ΔT_m values were determined from T_m difference of **ON2** and **ON7**.
* Data from Ref. 7.

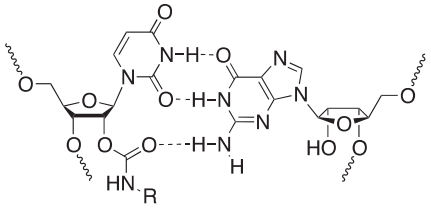


Figure 1. Base pair between guanosine and 2′-*O*-carbamoyl modified uridine. U_{cm}: R = H. U_{abcm}: R = $CH_2CH_2CH_2CH_2NH_3^+$.

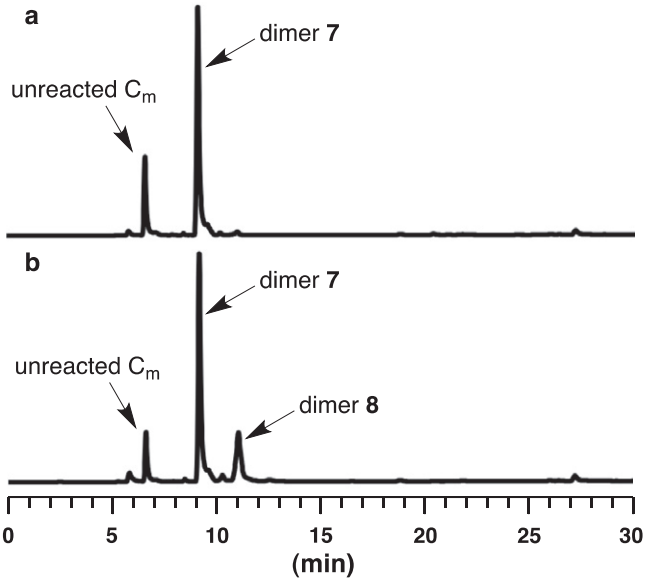


Figure 2. RP-HPLC profiles of the crude mixtures obtained by the treatment with aq ammonia (a) with and (b) without DBU treatment. C_m = 2′-*O*-methylcytidine.

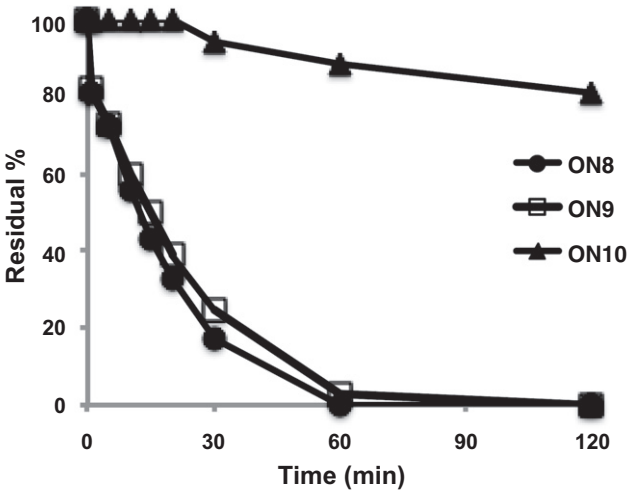


Figure 3. Nuclease resistance of **ON8** (closed circle), **ON9** (open box) and **ON10** (closed trigona) against SVPDE. Hydrolysis of the **ONs** (50 μM) was carried out at 37 °C in 50 mM Tris–HCl buffer (pH 8.5) containing 72 mM NaCl, 14 mM $MgCl_2$ and 2.5×10^{-4} U/ml SVPDE.

As shown in Figure 3, the **ON10** modified with U_{abcm} was the most stable to SVPDE among the three trimers. This stability was partially due to the cationic modification that prevents binding of a metal ion that is known to be required for the enzyme to efficiently catalyze the phosphoryl transfer reaction.[18]

In summary, we established the practical protocol for the synthesis of oligonucleotides incorporating U_{abcm}. By using this carbamoyl-type modification, the cationic aminoalkyl group can be readily incorporated to the 2′-position of oligonucleotides. In addition, the U_{abcm} modified oligonucleotide showed improved hybridization affinity in comparison with previously reported U_{cm} keeping its unique base pairing properties. Moreover, the cationic U_{abcm} modification was proved to be effective to increase the enzymatic stability of oligonucleotide. These properties of U_{abcm} suggested its usefulness for the development of functional

oligonucleotides such as nucleic acids drug and oligonucleotide probes.

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References and notes

1. (a) Prakash, T. P.; Bhat, B. *Curr. Top. Med. Chem.* **2007**, *7*, 641; (b) Prakash, T. P.; Kawasaki, A. M.; Wancewicz, E. V.; Shen, L.; Monia, B. P.; Ross, B. S.; Bhat, B.; Manoharan, M. *J. Med. Chem.* **2008**, *51*, 2766; (c) Oeda, Y.; Iijima, Y.; Taguchi, H.; Ohkubo, A.; Seio, K.; Sekine, M. *Org. Lett.* **2009**, *11*, 5582; (d) Sekine, M.; Oeda, Y.; Iilima, Y.; Taguchi, H.; Ohkubo, A.; Seio, K. *Org. Biomol. Chem.* **2010**, *9*, 210.
2. Buchini, S.; Leumaa, C. J. *Eur. J. Org. Chem.* **2006**, *14*, 3152.
3. Bramsen, J. B.; Laursen, M. B.; Nielsen, A. F.; Hansen, T. B.; Bus, C.; Langkjær, N.; Babu, B. R.; Højland, T.; Abramov, M.; Van Aerschot, A.; Odadzic, D.; Smicius, R.; Haas, J.; Andree, C.; Barman, J.; Wenska, M.; Srivastava, P.; Zhou, C.; Honcharenko, D.; Hess, S.; Müller, E.; Bobkov, G. V.; Mikhailov, S. N.; Fava, E.; Meyer, T. F.; Chattopadhyaya, J.; Zerial, M.; Engels, J. W.; Herdewijn, P.; Wengel, J.; Kjems, J. *Nucleic Acids Res.* **2009**, *37*, 2867.
4. Yamada, T.; Okaniwa, N.; Saneyoshi, H.; Ohkubo, A.; Nagata, T.; Aoki, Y.; Takeda, S.; Sekine, M. *J. Org. Chem.* **2011**, *76*, 3042.
5. Saneyoshi, H.; Seio, K.; Sekine, M. *J. Org. Chem.* **2005**, *70*, 10453.
6. (a) Korshun, V. A.; Stetsenko, D. A.; Gait, M. J. *J. Chem. Soc., Perkin Trans. 1* **2002**, 1092; (b) Misra, A.; Dwivedi, P.; Shahid, M. *Russ. J. Bioorg. Chem.* **2009**, *35*, 62; (c) Dioubankova, N. N.; Malakahov, A. D.; Shenkarev, Z. O.; Korshun, V. A. *Tetrahedron* **2004**, *60*, 4617.
7. Seio, K.; Tawarada, R.; Sasami, T.; Serizawa, M.; Ise, M.; Ohkubo, A.; Sekine, M. *Bioorg. Med. Chem.* **2009**, *17*, 7275.
8. (a) Prhavc, M.; Lesnik, A. E.; Mohan, V.; Manoharan, M. *Tetrahedron Lett.* **2001**, *42*, 8777; (b) Pattanaek, R.; Sethaphong, L.; Pan, C.; Prhavc, M.; Prakash, T. P.; Manoharam, M.; Egli, M. *J. Am. Chem. Soc.* **2004**, *126*, 15006.
9. Song, Q.; Jones, R. A. *Tetrahedron Lett.* **1999**, *40*, 4653.
10. Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859.
11. Michael, L.; Andreas, W. PCT Int. WO 2,006,116,476, 2006.
12. Hayakawa, Y.; Kawai, R.; Hirata, A.; Sugimoto, J.; Kataoka, M.; Sakakura, A.; Hirose, M.; Noyori, R. *J. Am. Chem. Soc.* **2001**, *123*, 8165.
13. Welz, R.; Müller, S. *Tetrahedron Lett.* **2002**, *43*, 795.
14. Vargeese, C.; Carter, J.; Yagge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic Acids Res.* **1998**, *26*, 1046.
15. (a) Wang, Z.; Siwkowski, A.; Lima; F, W.; Olsen, P.; Ravikumar, V. T. *Bioorg. Med. Chem.* **2006**, *14*, 5049; (b) Lackey, G. J.; Mitra, D.; Somoza, M. M.; Cerrina, F.; Damha, M. J. *J. Org. Chem.* **2009**, *131*, 8496; (c) Op de Beeck, M.; Madder, A. *J. Am. Chem. Soc.* **2011**, *133*, 796.
16. Griffey, R. H.; Monia, B. P.; Cummins, L. L.; Freier, S.; Greig, M. J.; Guinosso, C. J.; Lesnik, E.; Manalili, S. M.; Mohan, V.; Owens, S.; Ross, B. R.; Sasmor, H.; Wancewicz, E.; Weiler, K.; Wheeler, P. D.; Cook, P. D. *J. Med. Chem.* **1996**, *39*, 5100.
17. (a) Ohkubo, A.; Noma, Y.; Aoki, K.; Tsunoda, H.; Seio, K.; Mitsuo, S. *J. Org. Chem.* **2009**, *74*, 2817; (b) General procedure for the deprotection of Uabcm modified oligonucleotides:
An $^{ac}C_m$-loaded CPG solid phase support (1 μmol) having a succinyl linker was treated with 3% TCA in CH_2Cl_2 (1 mL) for 1 min at room temperature. After washing [CH_3CN 1 mL × 3] and drying under reduced pressure, the condensation of the $^{ac}C_m$-loaded solid phase support with phosphoramidite unit **5** was performed as follows: (1) coupling [phosphoramidite unit **5** (19 mg, 20 μmol) and 4,5-dicyanoimidazole (DCI) (10 mg, 80 μmol) in dry CH_3CN (400 μL)] for 40 min, (2) washing [CH_3CN 1 mL × 3], (3) drying, (4) coupling [phosphoramidite unit **5** (19 mg, 20 μmol) and 4,5-dicyanoimidazole (DCI) (10 mg, 80 μmol) in dry CH_3CN (400 μL)] for 40 min, (5) washing [CH_3CN 1 mL × 3], (6) drying, (7) capping [2.5% phenoxyacetic anhydride and 5% 1-methylimidazole in THF-pyridine 1 mL] for 5 min, (8) washing [CH_3CN 1 mL × 3], (9) oxidation [0.02 M I_2 in THF–pyridine–H_2O 1 mL] for 1 min, (10) washing [CH_3CN 1 mL × 3], (11) drying, (12) 3% TCA in CH_2Cl_2, (13) washing [CH_3CN 1 mL × 3], (12) drying. Subsequently, the CPG solid phase support was treated with 10% DBU in CH_3CN (1 mL) for 1 min. After washing [CH_3CN 1 mL × 3] and drying under reduced pressure, the solid phase support was treated with 28% aqueous ammonia at room temperature for 24 h. The CPG support was removed by filtration and washed with H_2O (500 μL × 3). The filtrate was purified by reversed phase HPLC to give $U_{abcm}C_m$ **7** in 35% yield.
$U_{abcm}C_m$ **7**; ESI-mass *m*/*z* Mass calcd for $[C_{24}H_{36}N_7O_{14}P+H]^+$ 678.2, found 678.5.
18. Teplova, M.; Wallace, S. T.; Tereshko, V.; Minasov, G.; Symons, A. M.; Cook, P. D.; Manoharan, M.; Egli, M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14240.