

Click–Click–Click: Single to Triple Modification of DNA**

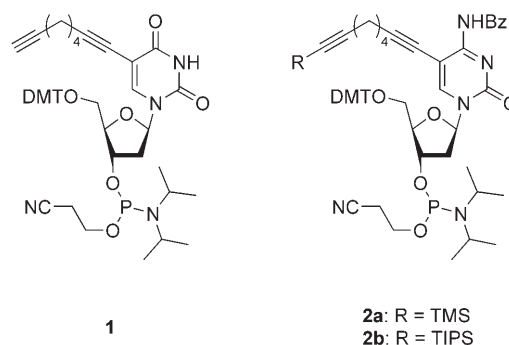
Philipp M. E. Gramlich, Simon Warncke, Johannes Gierlich, and Thomas Carell*

The attachment of labels such as fluorescent dyes or biotin molecules to DNA is of paramount importance for DNA-based molecular diagnostics^[1] and for nanotechnological applications.^[2,3] There is high demand for such modified oligonucleotides, but the chemistry behind the labeling procedures is cumbersome, and the modified oligonucleotides are frequently obtained in only low yields. Presently, the labels are incorporated as the corresponding phosphoramidites^[4] during the solid-phase synthesis of oligonucleotides, which frequently reduces the coupling yield significantly. This method is restricted to labels that can withstand the harsh conditions of DNA synthesis and deprotection. Alternatively, the labels are introduced postsynthetically^[5] by, for example, reaction of the corresponding activated esters with amino-alkyl-modified oligonucleotides.^[6] This method suffers from inefficient coupling yields, making the purification of the labeled oligonucleotides a challenging task.

In a world in which the demand for labeled oligonucleotides is rapidly growing, new methods for the efficient incorporation of multiple different labels are required. Seela and Sirivolu^[7] and our group^[2,8] have recently discovered that the copper(I)-catalyzed version of the azide–alkyne reaction to give triazoles, developed by Meldal et al.^[9] and Sharpless et al.^[10] can be used to functionalize alkyne-modified DNA nucleobases with extremely high efficiency. A critical point is the presence of a sufficient amount of a proper copper(I)-complexing ligand^[11] to prevent the copper-catalyzed cleavage of DNA.^[12] Herein we report that this chemistry can be extended to label oligonucleotides with up to three (and possibly more) different labels. These functionalizations can be achieved either directly on the resin^[13] or in solution after deprotection of the oligonucleotide. The latter method can be used to incorporate extremely sensitive labels with unprecedented efficiency.

The first goal was to establish a method for the introduction of two different labels^[14] during the solid-phase synthesis

of oligonucleotides. We thought that the best way to achieve this goal would be to introduce one free alkyne for the first click reaction and a second TMS-protected alkyne (Scheme 1) for the second click process after removal of the



Scheme 1. Phosphoramidites **1** and **2**. DMT = 4,4'-dimethoxyxytriphenylmethyl, TMS = trimethylsilyl, TIPS = triisopropylsilyl, Bz = benzoyl.

TMS group with mild acid on the resin. To test the feasibility of a click reaction on the resin we prepared a test strand containing the base derived from alkyne **1** and performed the click reaction directly on the resin, followed by DNA deprotection. Comparison of the HPLC trace of the functionalized DNA strand with that of an untreated DNA strand of the same series showed virtually quantitative conversion proving that the click reaction proceeded with extremely high efficiency directly on the controlled pore glass (CPG) support used for DNA synthesis (data not shown).

To introduce two labels, we incorporated the thymidine and the cytidine building blocks **1** and **2a** into oligonucleotides such as ODN-1 and ODN-2 (Table 1) using standard

Table 1: ODNs employed in this study.^[a]

ODN	Sequence
ODN-1	5'-GCGC X GTTTCATT Y GCG-3'
ODN-2	5'-CGC Y ACACGA X CCG-3'
ODN-3	5'-GCGC Z GTTTCATT X GCG-3'
ODN-4	5'-GCGC Y GTT X ATT Z GCG-3'

[a] **X** = DNA nucleotide based on **1**, **Y** = DNA nucleotide based on **2a**, **Z** = DNA nucleotide based on **2b**.

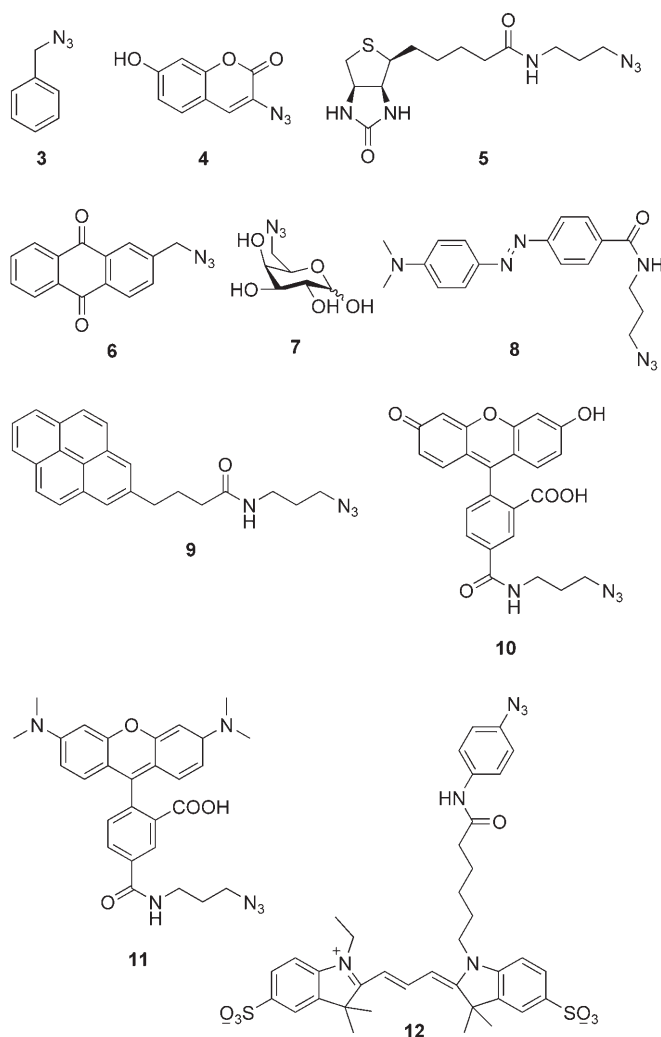
phosphoramidite chemistry. The coupling yields of both phosphoramidites were excellent. After full assembly of the oligonucleotide on the solid support, the resin was dried and the first click reaction was performed by shaking the resin with a solution of CuBr, tris(benzyltriazolylmethyl)amine

[*] Dipl.-Chem. P. M. E. Gramlich, Dipl.-Chem. S. Warncke, Dr. J. Gierlich, Prof. Dr. T. Carell
Department Chemie und Biochemie
Ludwig-Maximilians-Universität
Butenandtstrasse 5-13, 81377 München (Germany)
Fax: (+49) 89-2180-77756
E-mail: thomas.carell@cup.uni-muenchen.de

[**] P.M.E.G. thanks the Cusanuswerk for a PhD scholarship. J.G. thanks the Fonds der chemischen Industrie for a PhD scholarship. We thank Dr. G. Clever for the preparation of the anthraquinone azides and pyrene azides. The work was supported by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (SFB 749 and SFB 486). Support by the Excellence Cluster NIM (Nanoinitiative Munich) is greatly appreciated.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

(TBTA),^[11] sodium ascorbate, and benzyl azide (**3**; Scheme 2). The resin was washed and rinsed with 1% acetic acid to cleave the TMS protecting group on the second



Scheme 2. Azide building blocks used.

alkyne. Then the second click reaction was performed analogously to the first one using azide **8**. The DNA was finally cleaved from the resin, and all protecting groups were removed by exposing the resin to ammonia in H₂O/EtOH (3:1). The MALDI-TOF spectrum obtained for the crude product was in full agreement with the expected mass of the doubly modified oligonucleotide (Table 2, entry 1 and Supporting Information), showing that two stable labels can be introduced into DNA directly on the solid support.

To test the functionalization of oligonucleotides with labels too unstable to survive the harsh cleavage conditions, we next performed the second click reaction in solution after oligonucleotide deprotection. Treatment of the singly modified ODN-2 (Table 1) with conc. NH₃ in water/ethanol cleaved the DNA from the resin. Under these conditions the base protecting groups and the TMS group on the second alkyne were removed as well. The obtained DNA, bearing

Table 2: Postsynthetic labeling of ODNs 1–4.^[d]

Entry	DNA	Label 1	Label 2	Label 3	Yield [%] ^[a]
1	ODN-1	3*	8*	–	[b]
2	ODN-2	3*	4	–	75 ^[c]
3	ODN-3	5	4	–	67
4	ODN-3	4	6	–	59
5	ODN-3	4	7	–	59
6	ODN-3	3	5	–	70
7	ODN-3	3	4	–	85
8	ODN-3	3	7	–	67
9	ODN-3	3	9	–	66
10	ODN-3	8	4	–	83
11	ODN-3	9	3	–	92
12	ODN-3	9	5	–	62
13	ODN-3	9	4	–	90
14	ODN-3	10	5	–	74
15	ODN-3	8	11	–	58
16	ODN-4	3*	8	7	45 ^[c]
17	ODN-4	3*	7	5	52 ^[c]

[a] Determined by integration of the HPLC trace of the crude product at 260 nm after the last click reaction. [b] n.a. [c] HPLC purification after the click reaction on the resin. [d] Click reaction performed on resin.

one clicked-on modification and one free alkyne, was subjected to the second click reaction in solution (CuBr, TBTA, azide **4**), yielding the doubly modified DNA in excellent yields and purity (Table 2, entry 2).

The previously unmet challenge was to prepare oligonucleotides modified with two sensitive molecules. This can be readily achieved with the building blocks **1** and **2b**, which were incorporated into ODN-3 (Table 1) using standard phosphoramidite chemistry. After deprotection and cleavage of the oligonucleotide from the resin, the first click reaction was performed (using the solution conditions reported above) yielding the singly modified oligonucleotide with the expected high yield of >90% on average and full retention of the TIPS protecting group. For the second click step we cleaved the TIPS protecting group with a solution of tetrabutylammonium fluoride (TBAF) in acetonitrile/DMF (4:1 v/v) without causing any damage to the DNA. The second click reaction in solution yielded the doubly modified oligonucleotides in excellent yields (60–90% over three steps). We performed the double click with a whole series of different labels and always observed excellent yields (Table 2, entries 3–15). It is worth mentioning that in all cases simple precipitation of the product from ethanol after each reaction step was sufficient for purification. Figure 1 shows a typical HPLC chromatogram and a MALDI-TOF spectrum of the crude product obtained after a double modification of ODN-3. For very sensitive applications one final HPLC purification is recommended. In rare cases, such as for Cy3 azide **12**, we found that the linker was cleaved to a small extent, making the development of a more stable linker necessary.

Using the click reaction followed by precipitation of the product from ethanol, it was also possible to modify oligonucleotides with three different labels. To this end, we introduced the building blocks **1**, **2a**, and **2b** into oligonucleotides such as ODN-4 (Table 1). The first click reaction was performed directly on the resin. The singly modified

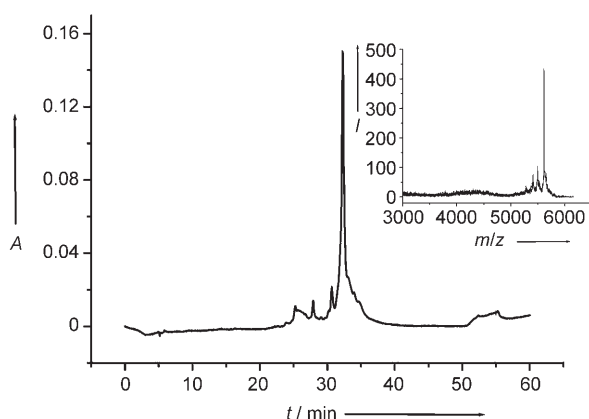
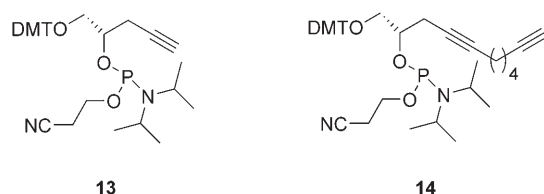


Figure 1. HPLC trace (260 nm) of the crude product ODN-3 modified with **8** and **4** (Table 2, entry 10) and the corresponding MALDI-TOF spectrum (inset).

oligonucleotide was subsequently cleaved from the support under concomitant cleavage of the TMS group and then purified by HPLC. The second click reaction was performed in solution with the expected high yield. Precipitation of the doubly modified oligonucleotide from ethanol, cleavage of the TIPS group with TBAF, and a subsequent third click reaction in solution furnished the desired triply modified oligonucleotides after a final precipitation in yields of about 50% (Table 2, entries 16 and 17).

Labeling of oligonucleotides directly at certain bases (here dC and dT) is highly desirable, but the introduction of labels outside the nucleobases, for example, on the phosphates or the sugars is also frequently needed. For this, we prepared the alkyne-bearing nonnucleoside DNA modifiers **13** and **14** (Scheme 3). Click reactions using these building blocks in DNA worked just as efficiently.^[15]



Scheme 3. Nonnucleoside DNA modifiers **13** and **14**.

In summary, we have developed a highly efficient, modular, and robust multiple functionalization protocol for

DNA. The efficiency of the method is based on three observations: 1. The TMS protecting group is quantitatively removed with ammonia during DNA deprotection. 2. The TIPS-protected alkyne is quantitatively retained during this ammonia treatment. 3. The TIPS protecting group can be removed efficiently and mildly. We believe that the chemistry presented here can change the way in which modified oligonucleotides are prepared.

Received: December 11, 2007

Published online: March 28, 2008

Keywords: alkynes · azides · click chemistry · cycloaddition · DNA labeling

- [1] D. M. Hammond, A. Manetto, J. Gierlich, V. A. Azov, P. M. E. Gramlich, G. A. Burley, M. Maul, T. Carell, *Angew. Chem.* **2007**, *119*, 4262; *Angew. Chem. Int. Ed.* **2007**, *46*, 4184.
- [2] G. A. Burley, J. Gierlich, M. R. Mofid, S. T. H. Nir, Y. Eichen, T. Carell, *J. Am. Chem. Soc.* **2006**, *128*, 1398.
- [3] J. J. Storhoff, C. A. Mirkin, *Chem. Rev.* **1999**, *99*, 1849; Q. Gu, C. Cheng, R. Gonela, S. Suryanarayanan, S. Anabathula, K. Dai, D. T. Haynie, *Nanotechnology* **2006**, *17*, R14.
- [4] C. Wagner, H.-A. Wagenknecht, *Chem. Eur. J.* **2005**, *11*, 1871; S. Kodama, S. Asano, T. Moriguchi, H. Sawai, K. Shinozuka, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2685.
- [5] J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13.
- [6] L. M. Smith, S. Fung, M. W. Hunkapiller, T. J. Hunkapiller, L. E. Hood, *Nucleic Acids Res.* **1985**, *13*, 2399.
- [7] F. Seela, V. R. Sirivolu, *Chem. Biodiversity* **2006**, *3*, 509.
- [8] J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond, T. Carell, *Org. Lett.* **2006**, *8*, 3639.
- [9] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057.
- [10] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596.
- [11] T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853.
- [12] C. J. Burrows, J. G. Muller, *Chem. Rev.* **1998**, *98*, 1109; S. Thyagarajan, N. N. Murthy, A. A. N. Sarjeant, K. D. Karlin, S. E. Rokita, *J. Am. Chem. Soc.* **2006**, *128*, 7003.
- [13] F. Seela, V. R. Sirivolu, *Helv. Chim. Acta* **2007**, *90*, 535; C. Bouillon, A. Meyer, S. Vidal, A. Jochum, Y. Chevolut, J.-P. Cloarec, J.-P. Praly, J.-J. Vasseur, F. Morvan, *J. Org. Chem.* **2006**, *71*, 4700.
- [14] V. Aucagne, D. A. Leigh, *Org. Lett.* **2006**, *8*, 4505.
- [15] D. I. Rozkiewicz, J. Gierlich, G. A. Burley, K. Gutsmedl, T. Carell, B. J. Ravoo, D. N. Reinhoudt, *ChemBioChem* **2007**, *8*, 1997.