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Alternatives to the 4,4'-dimethoxytrityl (DMTr) protecting group

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Abstract—The 9-phenyl- and the 9-(p-tolyl)-xanthen-9-yl groups 2a and 2b are recommended as alternatives to the 4,4'-dimeth-oxytrityl group 1 for the protection of the 5'-hydroxy functions in oligonucleotide synthesis. © 2004 Elsevier Ltd. All rights reserved.



Forty years ago, Khorana and his co-workers introduced¹ the acid-sensitive 4,4'-dimethoxytrityl (DMTr) 1 group for the protection of 5'-hydroxy functions in oligonucleotide synthesis. This protecting group has since been used very widely indeed, especially in the solid phase synthesis of oligonucleotides and their analogues.² Twenty-five years ago, we introduced³ the 9-phenylxanthen-9-yl (Px) group 2a as an alternative to the DMTr protecting group 1. Unlike the corresponding 5'-O-DMTr derivatives (as in 3a), 5'-O-Px derivatives (as in 3b) of the commonly used N-acyl-2'-deoxyribonucleosides crystallise readily.³ Another advantage of the Px group that was apparent from our original study³ was that it is marginally (ca. 33%) more labile than the DMTr group in acetic acid-water (4:1 v/v) solution. This is advantageous because the glycosidic linkages of purine (especially 6-N-acyladenine) deoxyribonucleosides are readily cleaved² under acidic conditions. Nevertheless, the Px group 2a has so far been used much less widely in the solid phase synthesis both of DNA and RNA sequences than the DMTr protecting group 1. One possible reason for this is that the required monomeric building blocks are not commercially available.

In a later study, we demonstrated⁴ that the acid-lability of the Px protecting group could either be increased or decreased by the introduction of electron-donating or electron-withdrawing substituents. Of particular significance in the context of oligonucleotide synthesis was the observation that the introduction of a para-methyl substituent in the 9-phenyl residue (as in 2b) of the Px group increased its acid-lability without compromising its robustness. Thus 5'-O-[9-(p-tolyl)xanthen-9-yl]thymidine (5'-O-Tx-thymidine) **3c** was converted⁴ into thymidine (3; R = H) by treatment with trifluoroacetic acid (TFA) in dichloromethane-ethanol (95:5 v/v) solution at 23 °C ca. 2.5 times more rapidly than the corresponding 5'-O-Px derivative **3b**. We now report a more detailed study of the comparative unblocking rates of the DMTr, Px and Tx protecting groups (1, 2a and 2b, respectively). In the deoxy-series, we chose the 5'-protected 3'-O-acetylthymidine derivatives⁵ 4a-c as model substrates and dichloroacetic acid as the unblocking agent. In order to measure the relative unblocking rates in solution, it is necessary to add a reagent that irreversibly scavenges 'trityl' cations (i.e., DMTr⁺, Px^+ and Tx^+). We have found pyrrole⁶ to be particularly useful for this purpose and have demonstrated its efficacy as a rapid scavenger for Px^+ and $DMTr^+$ cations.^{6,7} The pK_a of pyrrole (-0.27)⁸ is such that it is only partially protonated by dichloroacetic acid $(pK_a 1.25)^8$ and is incompletely protonated both by trichloro- and trifluoro-acetic acids ($pK_a \pm 0.66$ and 0.23, respectively).⁸ Although 'trityl' scavengers may also be

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Table 1. Unblocking of 3'-O-acetyl-5'-protected thymidine derivatives 3

Entry no	Substrate ^a	Acid molarity ^b	Pyrrole molarity	$t_{1/2}$ (s)
1	4 a	0.125	0.375	450
2	4b	0.125	0.375	198
3	4c	0.125	0.375	60
4	4a	0.375	0.50	48
5	4b	0.375	0.50	14
6	4c	0.375	0.50	10
7	4a	0.625	0.75	12
8	4b	0.625	0.75	ca. 4

^a All reactions were carried out in dichloromethane solution at 0 °C with a substrate molarity of 0.025.

^b Molarity of dichloroacetic acid.

useful in solid phase synthesis, they are rarely used as released 'trityl' cations are generally washed away.

All the unblocking experiments involving the 5'-protected-3'-O-acetylthymidine derivatives 4a-c were carried out⁹ by treating a 0.025 M solution of the substrate in dichloromethane with an excess each of dichloroacetic acid and pyrrole at 0 °C. In the first series of experiments, 5 mol equiv of acid (i.e., ca. 1% by volume) and 15 mol equiv of pyrrole were used. It can be seen from Table 1 (entries 1–3) that the half-times $(t_{1/2})$ for the removal of the DMTr, Px and Tx protecting groups were 450, 198 and 60 s, respectively. Despite the fact that the Px and especially the Tx groups offer a rate advantage over the DMTr protecting group, the time required for the complete unblocking even of the Tx group is likely to be ca. 8-10 min under these conditions. Although unblocking is likely perhaps to be 4–5 times faster at room temperature, removal, especially of the DMTr protecting group, would still be unacceptably slow. If the quantity of acid is increased to 15 mol equiv (i.e., ca. 3% by volume) and 20 mol equiv of pyrrole is added (entries 4-6), the Px and Tx groups are again removed at significantly faster rates than the DMTr protecting group. Indeed it can be estimated that, under the latter conditions, the times required for *virtually* complete removal of the Px and Tx groups at room *temperature* would be ca. 30 and 20 s, respectively, compared with ca. 1.5 min for the DMTr protecting group. Finally, it can be seen that the unblocking rates of the DMTr and Px groups at 0 °C can be further increased by a factor of 3-4 (entries 7 and 8) by increasing the acid concentration to 0.625 M (ca. 5% by volume) and the pyrrole concentration to 0.75 M. Under these conditions, the unblocking rate of the Tx-protected substrate 4c was too fast to measure with any accuracy.



The 5'-O-DMTr protecting group has also been used widely in the solid phase synthesis of RNA sequences.² As the glycosidic linkages of ribonucleoside derivatives are much more resistant to acidic hydrolysis¹⁰ than those of the corresponding 2'-deoxyribonucleoside derivatives, concomitant depurination should not be a problem during the removal of the 5'-O-DMTr group. A crucial aspect of oligonucleotide synthesis is the choice¹¹ of the protecting group for the 2'-hydroxy functions. The tertbutyldimethyl-silyl (TBDMS)¹² **5**, 1-(2-fluorophenyl)-4-methoxy-piperidin-4-yl (Fpmp)¹³ **6** and (tri-isopropyl-silyloxy)methyl (TOM)¹⁴ **7** groups are among the 2'-protecting groups that have commonly been used in combination with the 5'-O-DMTr group in solid phase oligonucleotide synthesis. We have favoured the use of the Fpmp and related 1-aryl-4-alkoxypiperidin-4-yl protecting groups. Recently, the 1-(4-chlorophenyl)-4ethoxypiperidin-4-yl (Cpep) group 8, which has improved hydrolysis properties (i.e., greater stability at low and greater lability at high pH), has been developed¹⁵ as an alternative to the Fpmp group 6. A unique and most important property of the Fpmp, Cpep and related protecting groups is that their use 15-17 allows chemically- and ribonuclease-stable 2'-protected oligonucleotides to be isolated in a pure state and then converted into fully-unblocked RNA sequences under mild conditions of acidic hydrolysis, such that cleavage and migration of the internucleotide linkages can occur only to a negligible extent. Although the Fpmp and Cpep groups have also been designed to resist hydrolytic cleavage under relatively strong acidic conditions, it is nevertheless desirable that the 5'-protecting group should be removable as rapidly as possible. We have therefore carried out a comparative unblocking study on a group of these 5'-protected (i.e., 5'-O-DMTr, 5'-O-Px and 5'-O-Tx)-2'-O-Cpep-ribonucleoside derivatives 9a-c.

The ribonucleoside substrates¹⁸ used in this part of the study were the 5'-O-DMTr-, 5'-O-Px- and 5'-O-Txderivatives (**9a–c**, respectively) of 4-*N*-benzoyl-2'-O-[1-(4-chlorophenyl-4-ethoxypiperidin-4-yl]-3'-O-levulinylcytidine **10**. As the rate of cleavage of the Cpep protecting group¹⁵ **8** is virtually constant in the pH range 0.5-2.5, it seemed sensible to use a stronger acid than dichloroacetic acid in the unblocking reactions. These reactions (Scheme 1) were carried out by treating 0.025 M solutions of the substrates **9a–c** with 6.0 mol equiv of trifluoroacetic acid and 15 mol equiv of



Scheme 1. Reagents and conditions: (i) CF_3CO_2H , pyrrole, CH_2Cl_2 , 0 °C.

pyrrole in dichloromethane solution at 0°C. No detectable loss of the 2'-O-Cpep protecting group was observed in any of these experiments and the half-times for the removal of the 5'-O-DMTr, 5'-O-Px and 5'-O-Tx groups from **9a**, **9b** and **9c**, respectively, were found²⁰ to be 42, ca. 6 and ca. 3 s. It would therefore seem to be advantageous to use the Px (or Tx) rather than the DMTr group to protect the 5'-hydroxy functions in solid phase oligoribonucleotide synthesis. Indeed, in our original study¹⁶ involving the use of the 2'-O-Fpmp protecting group in solid phase synthesis, the 5'-hydroxy functions were protected with the Px group. All subsequent work was carried out with 5'-O-DMTr-2'-O-Fpmp-protected monomers as they were commercially available. As in the case of DMTr-protected building blocks, coupling yields obtained with Px (and presumably also with Tx)-protected building blocks¹⁶ can be assayed spectrophotometrically.

In conclusion, we recommend that the 5'-O-DMTr group **1** should be replaced either by the 5'-O-Px or by the 5'-O-Tx protecting group in the solid phase synthesis both of DNA and RNA sequences. In reaching this conclusion, it should be borne in mind that, if solid phase synthesis is to be carried out with 5'-O-Px- or 5'-O-Tx-protected phosphoramidites, and perhaps this is also true for 5'-O-DMTr-protected phosphoramidites, it may be advisable to use a less acidic activating agent than 1*H*-tetrazole (p K_a 4.8),²¹ such as 1-phenylimidazo-lium triflate (p K_a 6.2)²¹ or imidazolium perchlorate (p K_a 7.0).²¹ This should ensure that absolutely no 5'-unblocking occurs during the coupling process, even in the synthesis of RNA sequences when coupling times tend to be relatively long.²¹

Acknowledgements

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4a [1.43 (3H, s), 2.04 (3H, s), 2.32 (1H, m), 2.44 (1H, m), 3.22 (1H, dd, *J* 3.9 and 10.4), 3.74 (6H, s), 4.07 (1H, m), 5.30 (1H, m), 6.22 (1H, dd, *J* 6.0 and 8.5), 6.09 (4H, d, *J* 7.9), 7.12–7.40 (9H, m), 7.53 (1H, s), 11.40 (1H, br s)]; **4b** [1.46 (3H, s), 2.03 (3H, s), 3.36 (1H, m), 2.50 (1H, m), 3.15 (1H, dd, *J* 3.6 and 10.3), 3.20 (1H, dd, *J* 3.0 and 10.3), 4.05 (1H, m), 5.29 (1H, m), 6.20 (1H, dd, *J* 6.1 and 8.3), 7.08–7.45 (13H, m), 7.58 (1H, s), 11.40 (1H, br s)]; **4c** [1.47 (3H, s), 2.02 (3H, s), 2.22 (3H, s), 2.34 (1H, m), 2.45 (1H, m), 3.13 (1H, dd, *J* 3.6 and 10.3), 3.18 (1H, dd, *J* 3.8 and 10.3), 4.04 (1H, m), 5.26 (1H, m), 6.19 (1H, dd, *J* 6.1 and 8.3), 7.12 (7H, m), 7.25–7.44 (5H, m), 7.58 (1H, s), 11.40 (1H, br s)].

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- 9. The rates of the 5'-unblocking reactions were determined as follows. Pyrrole (0.10-0.21 mL, 1.5-3.0 mmol) was added to a solution of substrate **4a**, **4b** or **4c** (0.10 mmol)and 2',3',5'-tri-*O*-acetyluridine (0.037 g, 0.10 mmol) in dichloromethane (2.0 mL). The stirred solution was cooled to 0 °C and a precooled (to 0 °C) 0.30-1.50 M solution of dichloroacetic acid in dichloromethane (2.0 mL) was added. After appropriate intervals of time, aliquots (0.1 mL) of the reaction solution were removed and basified with 0.7 M methanolic triethylamine. The samples were analysed by HPLC on a Jones C18 reversed phase column. Straight lines were obtained by plotting \log_{10} [% substrate remaining] against time. The times required for 50% unblocking $(t_{1/2})$ are indicated in Table 1.
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- Ribonucleoside substrates **9a–c** were prepared in 86–90% yield from 4-*N*-benzoyl-2'-O-[1-(4-chlorophenyl)-4-ethoxy-piperidin-4-yl]-3'-O-levulinylcytidine¹⁹ **10** and DMTr–Cl, Px–Cl and Tx–Cl, respectively. Their ¹H NMR spectra (360 MHz, in (CD₃)₂SO) are as follows: **9a** [0.96 (3H, t, *J* 7.0), 1.60 (1H, m), 1.83 (3H, m), 2.10 (3H, s), 2.56 (2H, m), 2.72 (2H, m), 2.89 (1H, m), 3.04 (1H, m), 3.13 (1H, m), 3.25 (3H, m), 3.36 (1H, m), 3.47 (1H, dd, *J* 4.1 and 10.7), 3.75 (6H, s), 4.20 (1H, m), 4.86 (1H, m), 5.27 (1H, m), 6.20 (1H, d, *J* 7.4), 6.92 (6H, m), 7.16–7.42 (12H, m), 7.52 (2H, m), 7.64 (1H, m), 8.01 (2H, m), 8.17 (1H, m), 11.38 (1H, br)]; **9b** [0.97 (3H, t, *J* 6.9), 1.64 (1H, m), 1.83 (3H, m), 2.10 (3H, s), 2.53 (2H, m), 2.70 (2H, m), 2.91 (1H, m), 3.03 (1H, m), 3.10–3.42 (6H, m), 4.18 (1H, m), 4.80 (1H, m), 5.18 (1H, m), 6.17 (1H, d, *J* 7.2), 6.93 (2H, d, *J* 9.1),

7.14–7.47 (16H, m), 7.53 (2H, m), 7.64 (1H, m), 8.01 (2H, m), 8.16 (1H, m), 11.38 (1H, br)]; **9c** [0.93 (3H, t, *J* 7.0), 1.65 (1H, m), 1.83 (3H, m), 2.10 (3H, s), 2.26 (3H, s), 2.52 (2H, m), 2.70 (2H, m), 2.92 (1H, m), 3.04–3.42 (7H, m), 4.18 (1H, m), 4.79 (1H, m), 5.17 (1H, m), 6.17 (1H, d, *J* 7.2), 6.93 (2H, d, *J* 9.1), 7.11–7.47 (15H, m), 7.53 (2H, m), 7.64 (1H, m), 8.02 (2H, d, *J* 7.3), 8.17 (1H, m), 11.38 (1H, br)].

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