Supplementary Information

for manuscript entitled

Accelerating Chemical Replication Steps of RNA Involving Activated Ribonucleotides and Downstream-Binding Elements

by

S. R. Vogel, C. Deck & C. Richert

Contents

General. Reagents were the best available grade from Acros (Geel, Belgium) or Aldrich/Fluka/Sigma (Deisenhofen, Germany). Adenosine 5′-monophosphate (5′-AMP) was purchased from Acros (Geel, Belgium), guanosine 5′-monophosphate (5′-GMP) and cytidine 5′-monophosphate (5′-CMP) were from Fluka (Deisenhofen, Germany). 5-Methyluridine-5′-monophosphate was prepared in-house from ribothymidine in analogy to established protocols.[1,2,3,4,5,6] HATU was purchased from Fluka (Deisenhofen, Germany). HOAt (7-aza-1-hydroxybenzotriazole) was from Perseptive Biosystem (Washington, UK). DIEA was from Acros (Geel, Belgium). Dowex 50 WX8-200 cation exchange resin was from Acros (Geel, Belgium). Oligoribonucleotides (**7**, **8a**/**g**, **9, 11**) were purchased from BioSpring (Frankfurt, Germany) in HPLC-purified form and were used without further purification. Universal Support II was purchased from Glen Research (VA, USA). Cyanoethyl-phosphoramidites of 2′-OMe-ribonucleosides of all four protected nucleosides (A^{bz}, C^{tac}, G^{ib}, U) and standard reagents for DNA synthesis were from Proligo (Hamburg, Germany). NMR spectra were recorded on a Bruker AC250 spectrometer at resonance frequencies of 250 and 500 MHz (^{1}H-NMR) and at 101 and 202 MHz (^{31}P-NMR). MALDI-TOF MS spectra were acquired on a Bruker REFLEX IV spectrometer. MALDI spectra of oligonucleotides were acquired in negative, linear mode and spectra of nucleotides were aquired in positive, linear mode. MALDI matrices were mixtures of 2,4,6-trihydroxyacetophenone[7] (0.3 M in EtOH) and diammonium citrate (0.1 M in H_2O) (2:1 v/v).

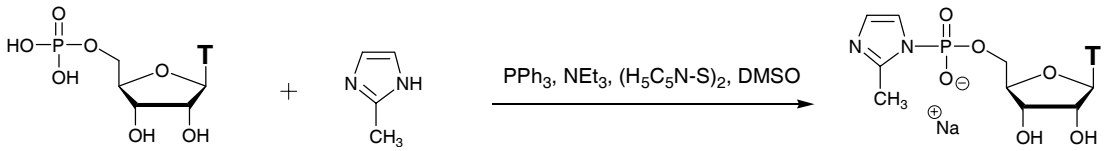
HPLC analyses of activated nucleotides were performed on reversed phase columns using a modification of the conditions of Ferris and coworkers.[8] HPLC analysis of HATU-activated nucleotides was performed on a Nucleosil 120-5 RP C18 (250 x 4.6 mm) reversed phase column (Macherey-Nagel) with HPLC-grade CH_3CN (Fisher Scientific) and water/triethylamine (2 µL TEA per 500 mL water), pH 8.4 at a flow of 1 mL/min and detection at 260 nm. Elution started with 0 % CH_3CN for 5 min, followed by a gradient to 15 % CH_3CN in 25 min. Purities given are based on relative intensity of product peaks in integrated HPLC traces, without correction for the peak of the solvent front or partial hydrolysis during handling and analysis.

Abbreviations: DIEA = diisopropylethylamine; DMAP = 4-(*N*,*N*-dimethylamino)pyridine; HATU = 7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate;[9] HOAt = 7-aza-1-hydroxybenzo-triazole; AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate, GMP = guanosine 5′-monophosphate; TMP = 5-methyluridine 5′-monophosphate; MeIm = 2-methylimidazole residue; OAt = oxyazabenzotriazole residue; tac = tert. butylphenoxyacetyl; THAP = 2,4,6-trihydroxyacetophenone.

2-Methylimdiazolide of Cytidine 5'-Monophosphate (5c)

The synthesis of **5c** was performed as described by Kanavarioti and coworkers.[10]

2-Methylimdiazolide of Ribothymidine 5'-Monophosphate (5t)



A mixture of ribothymidine 5′-monophosphate **4t** (150 mg, 0.44 mmol), 2,2′-bipyridine-1,1′-disulfide (310.2 mg, 1.41 mmol, 3.2 eq), PPh_3 (369.3mg, 1.41 mmol, 3.2 eq) and 2-methylimidazole (582.4 mg, 7.10 mmol, 16 eq) was dried at 0.1 Torr. The mixture was dissolved in anhydrous DMSO (3.7 mL) under argon to give a clear, colorless solution. After adding triethylamine (142 µL, 1.01 mmol, 2.3 eq), the resulting yellow solution was stirred under argon at r.t. for 4 h and then added dropwise to a stirred solution of $NaClO_4$ (0.01 M) in acetone/diethylether (1.4:1, v/v, 100 mL). The precipitate was isolated by filtration and washed twice with acetone/diethylether (1/1, 100 mL), followed by drying at 0.1 Torr to give **5t** (168.0 mg, 90 %) as an off-white solid. ^{1}H-NMR (500 MHz; DMSO-d_6) δ 1.82 (s, 3H); 2.42 (s, 3H); 3.65 (m, 1H); 3.72 (m, 1H); 3.86 (m, 1H); 3.90 (t, J = 4.4 Hz, 1H); 4.07 (t, J = 5.3 Hz, 1H); 5.19 (bs, 1H); 5.41 (bs, 1H); 5.80 (d, J = 6.0 Hz, 1H); 6.71 (t, J = 1.4 Hz, 1H); 7.10 (t, J = 1.3 Hz, 1H); 7.80 (s, 1H); 11.29 (bs, 1H); ^{31}P-NMR (202 MHz, DMSO-d6) δ –9.97.

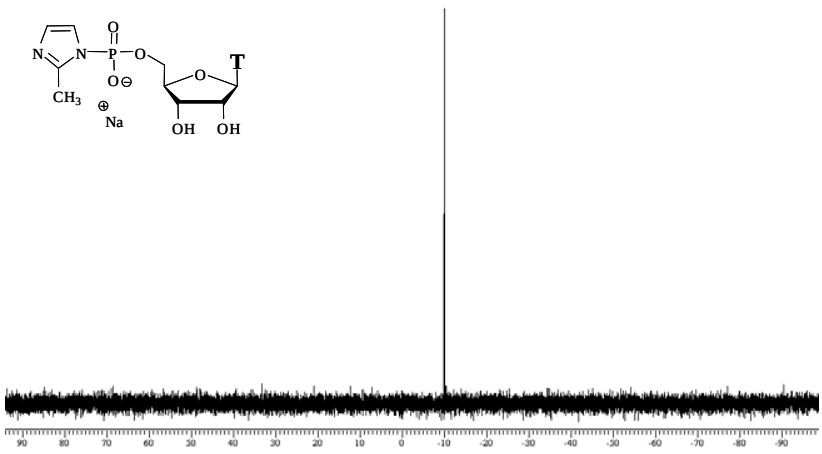
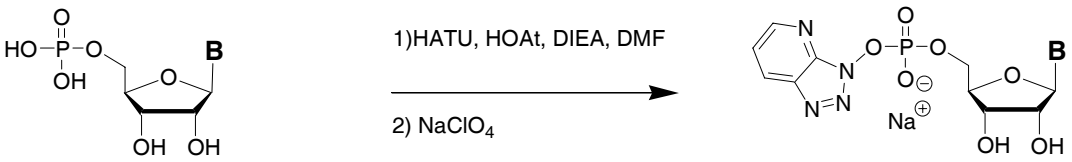


Figure S1. ^{31}P NMR ({^{1}H}, DMSO-d_6, 202 MHz)-spectrum of MeIm-T **(5t)**

Synthesis of OAt-Activated Ribonucleotides (6a-t), General Protocol



B = A, C, G, T **6a, 6c, 6g, 6t**

A mixture of the ribonucleoside 5′-monophosphate (0.275 mmol) (free acid for **6a, 6g, 6t** and triethylammonium salt for **6c**), HATU (115 mg, 0.303 mmol), and HOAt (56 mg, 0.413 mmol,) was dried at 0.1 Torr for 2 h. The mixture was dissolved in anhydrous DMF (27.5 mL) under argon to give a clear, colorless solution. To this solution was added DIEA (68 µL, 0.411 mmol), and the reaction mixture was stirred under argon at r.t. for 5 h. Any precipitate found at the end of the reaction time was removed by centrifugation, and the supernatant was used for precipitation by dropwise addition to an ice-cold solution of $NaClO_4$ (0.01 M) in acetone/diethylether (1.4:1, v/v, 500 mL). The precipitate was isolated by filtration and washed twice with acetone/diethylether (1/1, 100 mL), followed by drying at 0.1 Torr to give an off-white solid.

***O*-(Guanosin-5′-*O*-phosphor-5'-*P*-yl) 7-aza-1-oxybenzotriazolide sodium salt (6g)**

Yield: 127.9 mg (92 %). ^{31}P NMR (101 MHz, DMSO-d_6) δ = 1.79 ppm. HPLC t_R = 17 min (49 %). MALDI-TOF MS *m/z* calcd. for $C_{15}H_{16}N_9O_8P$ $[M + H]^+$ 481.3, found 482.2.

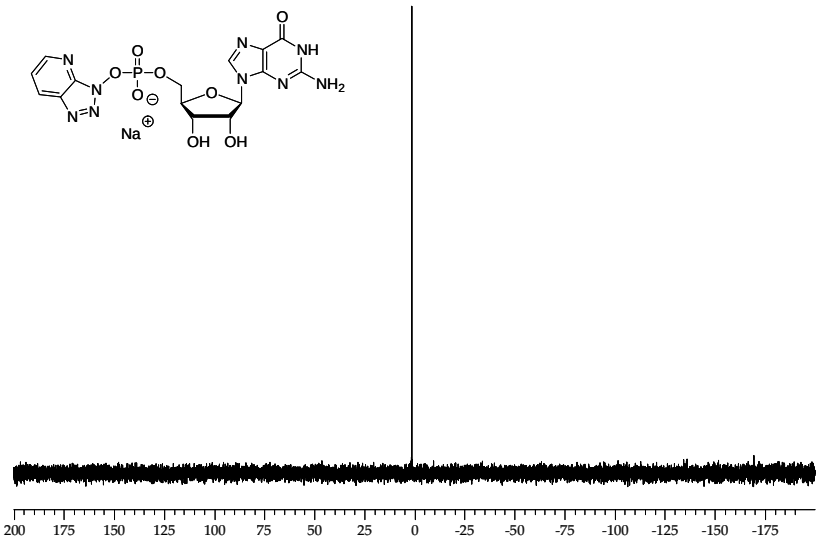


Figure S2. ^{31}P NMR ({^{1}H}, DMSO-d_6, 101 MHz)-spectrum of **6g**.

***O*-(Adenosin-5′-*O*-phosphor-5'-*P*-yl) 7-aza-1-oxybenzotriazolide sodium salt (6a)**

Yield: 113.3 mg (85 %). ^{31}P NMR (101 MHz, DMSO-d$_6$) δ = 1.79 ppm. HPLC t_R = 20 min (58 %). MALDI-TOF MS *m/z* calcd. for $C_{15}H_{16}N_9O_8P$ $[M + H]^+$ 465.3, found 467.8.

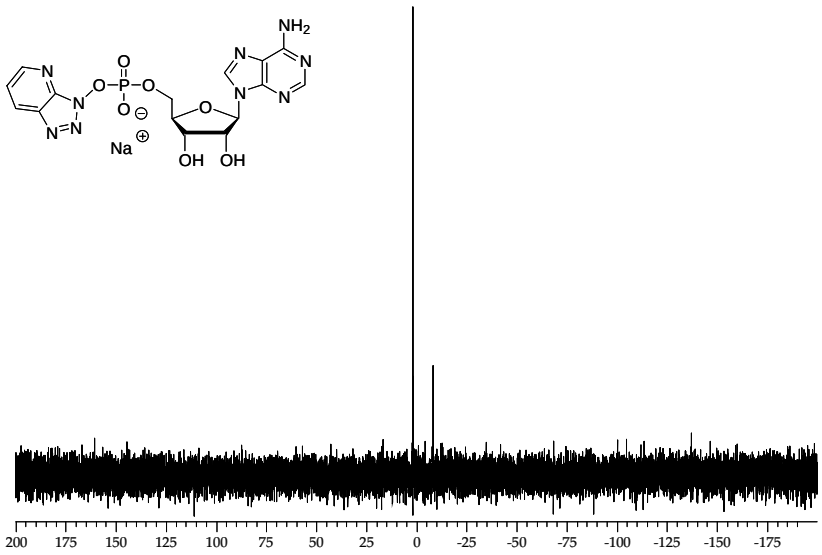


Figure S3. ^{31}P NMR ({^{1}H}, DMSO-d$_6$, 101 MHz)-spectrum of **6a**.

***O*-(Cytidine-5′-*O*-phosphor-5'-*P*-yl) 7-aza-1-oxybenzotriazolide sodium salt (6c)**

Yield: 67.5 mg (53 %). ^{31}P NMR (101 MHz, DMSO-d$_6$) δ = 1.77 ppm. HPLC t_R = 17 min (59 %). MALDI-TOF MS *m/z* calcd. for $C_{15}H_{16}N_9O_8P$ $[M + H]^+$ 441.3, found 443.7.

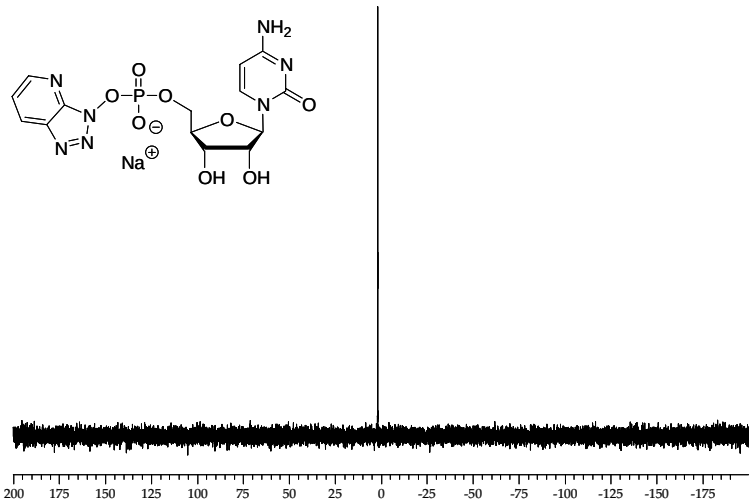


Figure S4. ^{31}P NMR({^{1}H}, DMSO-d$_6$, 101 MHz)-spectrum of **6c**.

***O*-(*Ribo*thymidine-5′-*O*-phosphor-5'-*P*-yl) 7-aza-1-oxybenzotriazolide sodium salt (6t)**

Yield: 84.4 mg (64 %). ^{31}P NMR (101 MHz, DMSO-d$_6$) δ = 1.53 ppm. HPLC t_R = 17 min (50 %). MALDI-TOF MS *m/z* calcd. for $C_{15}H_{16}N_9O_8P$ $[M + H]^+$ 456.3, found 458.6.

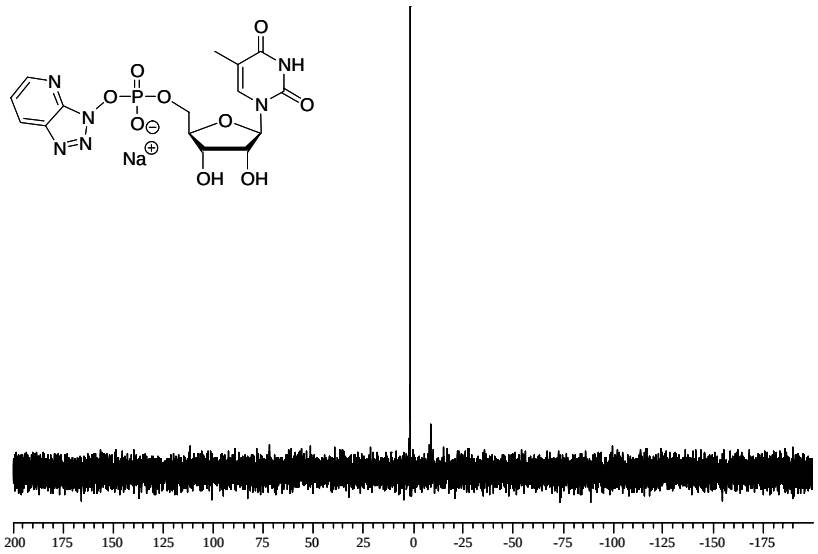


Figure S5. ^{31}P NMR ({^{1}H}, DMSO-d$_6$, 101 MHz)-spectrum of **6t**.

Primer Extension, General Protocol

The oligonucleotides (template, primer and downstream-binding element) were each dissolved in de-ionized water to give stock solutions (1.34 mM). Aliquots (0.5 µL) of stock solutions were added to a polypropylene reaction vessel to give a 268 µM solution of each oligonucleotide in the final assay mixture. Stock solutions of the buffer and the activated nucleotides were also prepared. These were combined to give a final assay volume of 2.5 µL containing the activated monomer(s), HEPES (200 mM), NaCl (400 mM) and $MgCl_2$ (80 mM). Assays were performed at room temperature (24 °C). From the assay solution, samples (0.4 µL) were drawn at chosen intervals and diluted with water (30 µL). The resulting solution was treated with a few beads of Dowex cation exchange resin (NH_4^+-form) for 30 min, followed by MALDI-TOF analysis.

MALDI TOF Analysis, **Kinetic Model and Analysis**. Acquisition of MALDI spectra, the model underlying kinetic analysis, and the details of how rate constants were derived from MALDI data is described in detail in earlier reports.[11,12] Briefly, aliquots of diluted sample solution were spotted on a stainless steel MALDI target plate and dried at 0.1 Torr. These spots were then treated with 0.5 µL of matrix solution (mixture of THAP, 0.3 M in EtOH, and diammonium citrate, 0.1 M in H_2O, mixed 2:1, v/v) and the mixture was allowed to crystallize at room temperature. The target was inserted in the MALDI mass spectrometer and for every data point three spectra with more than 150 shots, each, were acquired. Relative peak intensities for primer **7** and extension products **10a-t** were determined based on peak heights. This data was used for kinetic fits in SlideWrite. For primer conversion, the function f(t) = exp(-a0 * t) was used, where a0 is the rate constant to be determined. The fit for the extended primers was determined using f(t) = a1 * (1-exp(-a0 * t). The pre-exponential factor a1 gives the fraction of the individual product that is formed in competitive reactions.

Data from Extension Assay at 4 °C with or without Downstream-Binding Element 11.

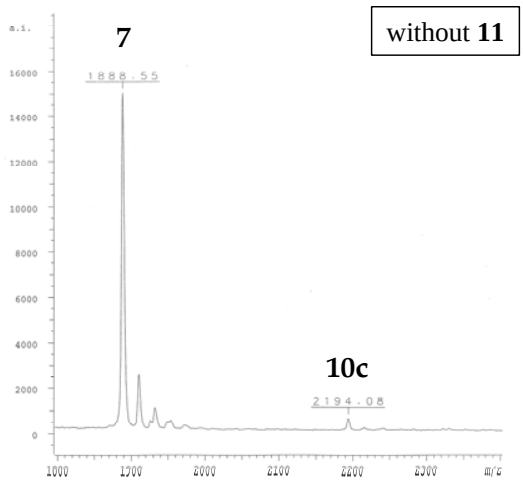


Figure S6. MALDI TOF spectrum from primer extension with template **8g** and primer **7** without downstream-binding element, using **6c** (20 mM) at pH 8.9, after 20 h at 4 °C. Peaks at slightly higher mass than those of the pseudomolecular ion labeled are due to salt adducts (Na^+, K^+, Mg^{2+}).

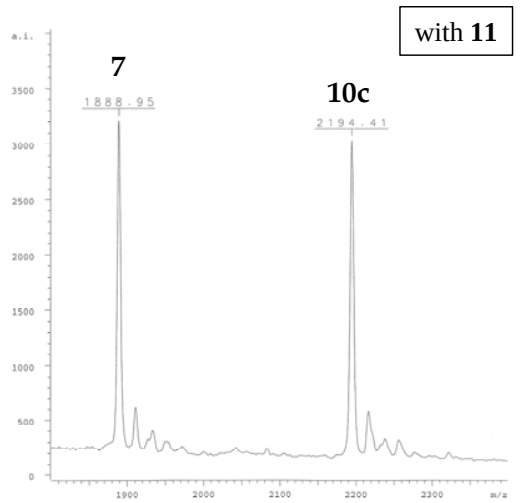


Figure S7. MALDI TOF spectrum from primer extension with template **8g**, primer **7** and downstream-binding trimer **11**, using **6c** (20 mM) at pH 8.9, after 20 h at 4 °C. Peaks at slightly higher mass than those of the pseudomolecular ion labeled are caused by salt adducts (Na^+, K^+, Mg^{2+}).

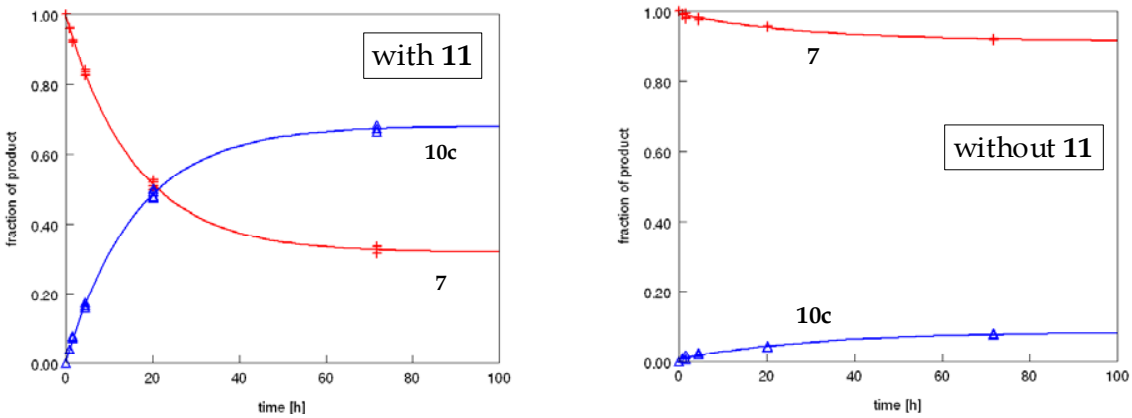


Figure S8. Kinetics for primer extension involving primer **7,** template **8g** and **6c** at 4 °C with or without downstream-binding element **11**. Conditions are those given in the figure legends to Figures S6 and S7.

Kinetics for Competitive Primer Extension Assay with all-2'-*O*-methyl tetramer 5'-GCUG-3'

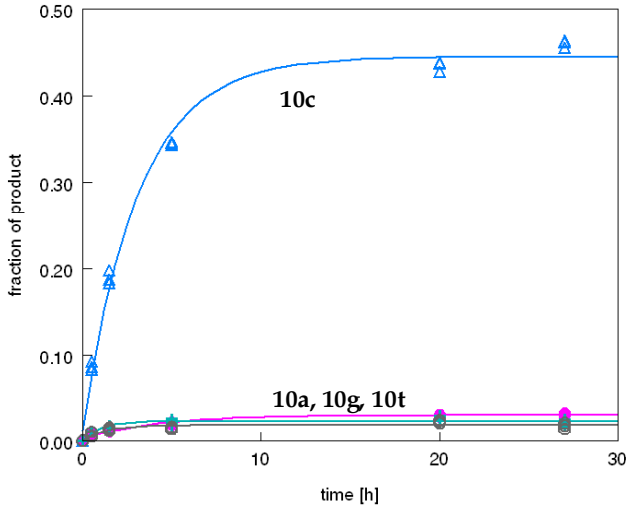


Figure S9. Kinetics for the competitive reaction of RNA primer **7** at pH 8.9 with an equimolar mixture of all four OAt-activated nucleotides **6a-t** on template **8g** in the presence of all-2′-*O*-methyl tetramer (5′-GCUG-3′) as downstream-binding element at room temperature.

References for Supporting Information

1. A. Szemo, A. Szabolcs and L. Ötvös, *J. Carbohydrates Nucleosides Nucleotides* 1980, **7**, 365-379.
2. B. S. Schulz and W. Pfleiderer, *Helv. Chim. Acta* 1987, **70**, 210-218.
3. M. Yoshikawa and T. Kato, *Bull. Chem. Soc. Jpn.* 1967, **40**, 2849.
4. M. Yoshikawa, T. Kato and Takenishi, *Bull. Chem. Soc. Jpn.* 1967, **42**, 3505.
5. S. Bottka and J. Tomasz, *Tetrahedron* 1979, **35**, 2909-2912.
6. T. Ikemoto, H. Akira, H. Hatano, Y. Kitamoto, M. Ishida and K. Nara, *Chem. Pharm. Bull.* 1995, **43**, 210-215.
7. U. Pieles, W. Zürcher, M. Schär and H. E. Moser, *Nucleic Acids Res.* 1993, **21**, 3191-3196.
8. K. J. Prabahar and J. P. Ferris, *J. Am. Chem. Soc.* 1997, **119**, 4330-4337.
9. For the relevant form of HATU, please also see: L. A. Carpino, H. Imazumi, A. El-Faham, F. J. Ferrer, C. Zhang, Y. Lee, B. M. Foxman, P. Henklein, C. Hanay, C. Mügge, H. Wenschuh, J. Klose, M. Beyermann and M. Bienert, *Angew. Chem.* 2002, **114**, 458-461, *Angew. Chem. Int. Ed. Engl.* 2002, **41**, 441-445.
10. A. Kanavarioti, M. W. Stronach, R. J. Ketner and T. B. Hurley, *J. Org. Chem.* 1995, **60**, 632-637.
11. P. Hagenbuch, E. Kervio, A. Hochgesand, U. Plutowski and C. Richert, *Angew. Chem.*, in press (2005).
12. J.A. Rojas Stütz and C. Richert, *J. Am. Chem. Soc.* 2001, **123**, 12718-12719.