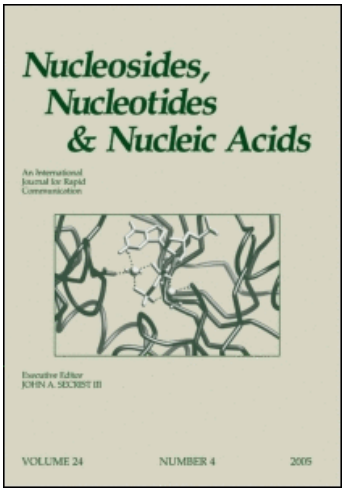

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EFFECT OF MICROWAVE IRRADIATION ON PHOSPHORAMIDITE COUPLINGS ON CONTROLLED PORE GLASS

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□ *The chain extension step in the synthesis of DNA oligomers on controlled pore glass was shown to be higher yielding when the reaction mixture is irradiated with microwaves. Both a commercial thymidine 3′-phosphoramidite building block and a 3′-phosphoramidite of protected 1′-aminomethylthymidine were coupled using dilute solutions that give only partial conversion. In either case, higher coupling yields were observed when microwaves were used. The results of our exploratory experiments suggest that microwave-assisted DNA syntheses might require fewer equivalents of phosphoramidites and/or shorter coupling times than those performed at room temperature.*

Keywords DNA Synthesis; Microwave irradiation; Phosphoramidites; Solid support

INTRODUCTION

The use of phosphoramidites as building blocks for DNA synthesis[1] has led to a synthetic procedure remarkable for its efficiency and reliability.[2] While it is now possible to prepare DNA chains routinely of ≥100 nucleotides in automated machines, the synthetic approach still has some drawbacks, such as the massive excess of phosphoramidites used during coupling (typically between 10 and 40 equivalents for reactions on 0.04–1 μmol scale). Further, couplings performed on planar surfaces, e.g., to generate microarrays in massively parallel fashion,[3,4] often suffer from lower yields than those performed using traditional synthesizers. Finally, manual couplings of custom-prepared phosphoramidites on small scale are frequently lower yielding than automated couplings in high-throughput syntheses employing commercial building blocks, as surface water and

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impurities are harder to exclude in these cases. Thus, unsatisfactory yields can result for the incorporation of precious synthetic building blocks, prepared in multistep syntheses.

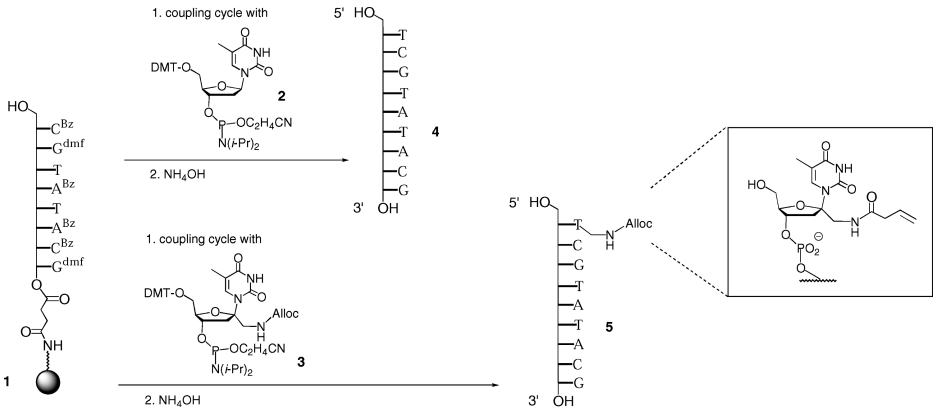
Microwave irradiation has recently been shown to lead, in certain cases, to surprising increases in yield of reactions performed both in solution and on solid support.[5] While this effect is well established for peptide chemistry, and automatic peptide synthesizers with microwave capabilities are now commercially available,[6] the benefit of microwave treatment on DNA and RNA couplings has, to the best of our knowledge, not been demonstrated. Microwave-accelerated reactions in nucleic acid chemistry have been reported,[7] but these do not concern the chain extension step critical for the success of oligonucleotide syntheses.

We have recently reported the beneficial effect of microwaves on the rate and cleanliness of reactions involving the removal of Alloc protecting groups and the formation of amide bonds of modified DNA strands on controlled pore glass.[8] Other reactions performed on controlled pore glass, such as Sonogashira coupling reactions[9] on pre-assembled oligonucleotides with a 2′-deoxy-5-iodouridine residue,[10] did not benefit from microwave irradiation.[11] Since heating is not commonly employed to drive phosphoramidite couplings to completion, it was doubtful whether microwave irradiation would have a beneficial or detrimental effect on chain assembly steps generating (modified) oligonuceotide chains.

RESULTS AND DISCUSSION

As mentioned above, employing a more than 10-fold excess of phosphoramidites during chain extension steps is a rather wasteful way of employing one's monomers, even for solid-phase syntheses. Further, generating the 100-mM solutions of phosphoramidites required for standard syntheses can be difficult for modified building blocks with low solubility in common solvents for DNA synthesis (acetonitrile or dichloromethane). We therefore focused our current experiments on coupling reactions involving more dilute solutions of phosphoramidites. Further, we did not take special precautions to remove residual water from reagents and surfaces, in order to simulate difficult coupling conditions that often frustrate syntheses involving small quantities of custom-prepared phosphoramidites. Together, these measures ensured incomplete coupling, as required for detecting differences between yields of control reactions and microwave-assisted reactions. No attempts were made to increase yields, as this would lead to more dificult-to-interpret results

Scheme 1 shows the DNA sequence and the phosphoramidites employed. Oligonucleotide **1** is a protected octamer on long-chain alkylamine controlled pore glass (LCAA cpg) prepared using commercial



SCHEME 1 DNA chain extension reactions studied.

phosphoramidites via conventional automated synthesis. Samples of this support were reacted in conical glass vials for microwave-assisted synthesis with thymidine building block **2** or aminomethylthymidine building block **3**[8] in acetonitrile in the presence of 0.25 M 4,5-dicyanoimidazole as activator and the phosphoramidite concentrations given in Table 1. Phosphoramidite **3** is sterically more demanding than **2** and has shown lower reactivity than its unmodified counterpart in our experience. After phosphoramidite coupling, conventional oxidation and deprotection gave crude products containing extended oligonucleotides **4** or **5**. These crudes

TABLE 1 Results of DNA Chain Extensions Performed with or without Microwave Irradiation[a]

Exp.	Pa[b]/conc. (M)	Heat source	Temperature[c] (°C)	Reaction time (min)	Conversion[d] (%)
1	**2**/0.02	Microwave	30	6	27
	2/0.02	r.t.	22	6	18
2	**3**/0.02	Microwave	30	6	14
	3/0.02	Heat bath	30	6	2
3	**2**/0.05	Microwave	30	6	23
	2/0.05	Heat bath	30	6	9
4	**2**/0.02	Microwave	50	6	35
	2/0.02	Heat bath	50	6	28
5	**2**/0.02	Microwave	30	0.5	25
	2/0.02	Heat bath	50	6	30
6	**2**/0.05	Microwave	30	0.6	22
	2/0.05	Heat bath	40	6	31

[a]Each experiment involved two reactions performed with the same phosphoramidite stock solution and the same solvents and reactants. In some instances, this led to reactions performed twice independently under the same experimental conditions. The differences in the results between these provide a measure of the reproducibility.

[b]Pa = phosphoramidite coupled.

[c]For microwave-assisted reactions, this is the ceiling temperature, reached after the initial burst.

[d]The extent to which **4** or **5** were formed, as determined by MALDI-TOF mass spectrometry.

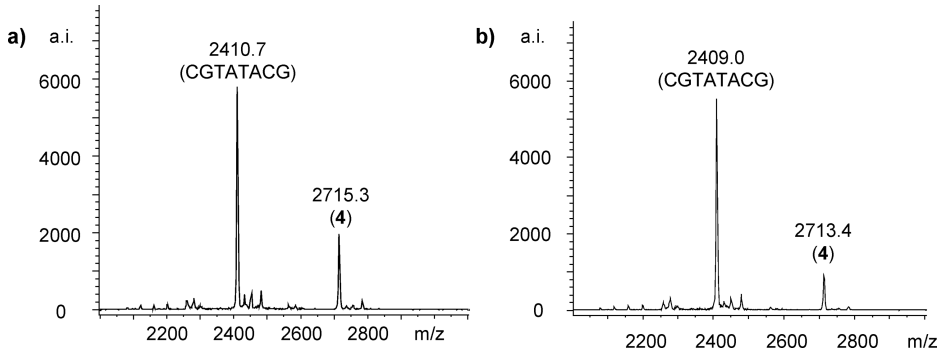


FIGURE 1 MALDI spectra of crudes obtained by coupling **2** to **1** for 6 min, with or without microwave irradiation, followed by oxidation and deprotection. a) Coupling with microwave (30°C ceiling temperature), b) control reaction (22°C).

were analyzed by MALDI-TOF mass spectrometry under conditions that allow for quantitative detection of oligonucleotides.[12]

We first compared whether mild microwave irradiation had any effect on the coupling of either phosphoramidite. Phosphoramidite **2** was coupled to support **1** for 6 min. In the microwave-assisted reaction, very low dosages of microwaves were used, with the power output limited by the ceiling temperature of 30°C set for the instrument. Still approximately twice as much conversion was initially observed compared to the reaction at room temperature (Figure 1). Repeating this experiment, again without carefully excluding moisture, showed between 3% and 32% conversion after 6 min in the microwave-irradiated reactions. On average, a modest increase of 2% in yield over that of the control reaction was observed under these conditions (Entry 1, Table 1). A more significant effect of microwaves was observed for less reactive building block **3**, where the increase in yield was approximately sevenfold (Figure 2, experiment 2 in Table 1).

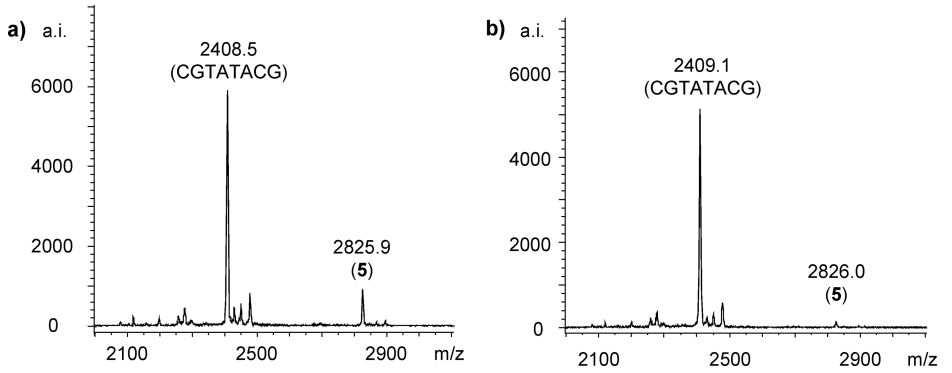


FIGURE 2 MALDI spectra of crudes obtained by coupling 1′-aminomethylthymidine building block **3** to **1** for 6 min, with or without microwave irradiation, followed by oxidation and deprotection. a) Coupling with microwaves (30°C ceiling temperature), b) control reaction at room temperature (22°C).

We then asked whether the increase in yield of extended oligonucleotides **4** and **5** was solely a thermal effect, or potentially due to a true "microwave effect." These experiments involved **2** alone, as the more readily available compound. The control reaction was heated to 30 or 50°C in a conventional heat bath, and microwave-accelerated reactions were performed in parallel with the same temperatures as upper limit of the IR-sensor based feedback-control for microwave irradiations. While the experiment at 30°C, which used a higher phosphoramidite concentration, again showed poorer conversion in the control case, the experiment involving more significant heating, up to 50°C, showed more equal reactivities, with the control reaction achieving 80% of the conversion of the microwave-assisted reaction (experiments 3 and 4 in Table 1). It is therefore possible that the observed effect with the microwaves is largely thermal in nature. Despite the harsher conditions at 50°C, the purity of the crude remained high with few MALDI-detectable side products.

Microwaves provide instantaneous heating, not slowed down by the limited conductivity of the vessel and (unstirred) solution. We therefore asked whether microwave-induced heating leads to similar yields as thermally activated couplings after shorter reaction times. For good measure, the reaction times were dropped by at least one order of magnitude, and the ceiling temperature was set below that for the heat bath-promoted reactions. The entries for the last two experiments listed in Table 1 show the results for these coupling reactions. Despite the much shorter reaction time and the milder reaction conditions (30°C ceiling temperature), the microwave-assisted reaction gave only modestly lower conversion in either case.

Together, the results of this exploratory study suggest that microwave-accelerated reactions can lead to more efficient chain assembly cycles during DNA syntheses, when compared to reactions performed at room temperature. The effect of the microwave heating appears to be particularly beneficial for syntheses performed with small quantities of phosphoramidites of less reactive unnatural nucleosides, such as **3**. For these, separate manual couplings at higher dilution, such as those performed here, are not unusual, as they save the extra solution required to fill the void volume of DNA synthesizer channels and thus some precious compound. Further, RNA syntheses, which continue to produce lower coupling yields than DNA syntheses, may benefit from reactions performed with microwave irradiation. Also, massively parallel syntheses of DNA chains on microarrays or "DNA chips,"[13] particularly when used for gene synthesis,[14] may benefit from more rapid and more efficient chain extension steps. Since microwave-based heating has the advantage of being almost instantaneous and easy to dose, it might be suitable for machines producing microarrays, enhancing the likelihood of uniformly high-yielding chain extensions across a surface. Needless to say, more detailed studies are needed to corroborate

our findings and to better quantify the effect of (microwave-based) heating on chain extension steps during DNA synthesis.

EXPERIMENTAL PART

General

Phosphoramidites (dA^{Bz}, $dC^{Bz,}$ dG^{dmf}, dG^{dmf}cpg, dT), DCI activator solution (0.25 M 4,5-dicyanoimidazole in CH_3CN), oxidizer solution (1 M iodine in H_2O/pyridine/THF 2:21:77; *v/v*), amidite diluent (CH_3CN), and TCA deblock solution (3% trichloroacetic acid in CH_2Cl_2; *v/v*) were purchased from Proligo (Hamburg, Germany). Reactions were performed in tapered pyrex vials similar to those sold through the manufacturer of the microwave apparatus (model *Discover*, CEM Inc., Kamp-Lintfort, Germany).

Chain Extension

To a sample of cpg **1** (5 mg, approx. 0.15 μmol loading) was added a solution of phosphoramidite **2** (1.4 mg, 1.8 μmol) or phosphoramidite **3** (1.6 mg, 1.8 μmol) in CH_3CN (19 μl), a solution of DCI activator (19 μl) and CH_3CN (95 μl for fivefold dilution, 38 μl for twofold dilution). Microwave-assisted reactions were performed in a CEM *Discover* microwave apparatus with a power limit of 200 W, the temperature ceilings given in Table 1, and 2 bar as limiting pressure. Cooling was provided by compressed air ventilating the microwave chamber during irradiations. Control reactions were performed at room temperature or in a heat bath at the given temperature. All assays were run in parallel, using the same phosphoramidite stock solution, reactants, and solvents, and the same type of vial for control and microwave-assisted reaction. After coupling, the cpg was washed with CH_3CN (2 × 40 μl), treated with oxidizer solution (100 μl, 15 min), and washed again with CH_3CN (3 × 40 μl) and CH_2Cl_2 (2 × 40 μl). The cpg was then treated with deblock solution (2 × 40 μl, 15 min each), rinsed with CH_2Cl_2 (3 × 40 μl), and dried at 0.1 Torr. The oligonucleotides were deprotected and cleaved from cpg by treating with 30% aqueous ammonia at r.t. over night.

MALDI-TOF Analysis

MALDI-TOF spectra of crude products were recorded on a Bruker REFLEX IV in linear, negative mode, using a mixture of 2,4,6-trihydroxyacetophenone (0.3 M in EtOH) and diammonium citrate (0.1 M in H_2O) (2:1 *v/v*) as matrix and co-matrix.[15] Conversion values given in Table 1 are based on peak heights determined with XMass (Bruker Daltonics, Leipzig, Germany).

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