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PAPER

Synthesis of nucleoside analogues in a ball mill: fast, chemoselective and high yielding acylation without undesirable solvents†

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The chemoselective acylation of primary aliphatic amines has been achieved in under ten minutes (and for aromatic amines under 120 min) using vibration ball-milling, avoiding undesirable solvents which are typically employed for such reactions (*e.g.* DMF). Under optimised conditions, the synthesis of amides in the presence of both primary and secondary alcohol functions was achieved in high to excellent yields (65–94%). Overall, the methods described have significant practical advantages over conventional approaches based upon bulk solvents including greater yields, higher chemoselectivity and easier product separation.

Introduction

Mechanochemistry is a well-established technique in materials science[1] but has only recently begun to be developed for molecular inorganic[2] and organic synthesis.[3] The E-factor[4] of organic reactions can be considerably decreased when using vibrational or planetary ball-milling as minimal or even no solvent is required. In addition, improvements in the yield and selectivity can be observed,[5] the reaction times and temperatures can be reduced[6,7] and especially in the context of reactions using inorganic catalysts, expeditious and atom-efficient work-up procedures can be employed.[8]

Ball-milling remains particularly under-exploited for the preparation or derivatisation of biological molecules and their analogues. In this regard, recent reports have described the application of this technique for the preparation or derivatisation of amino acid analogues,[9] peptide linkages,[10] secondary metabolites[11,12] and functionalised nucleosides.[13] The amphiphilic nature of nucleosides and their analogues, coupled with typically high levels of hydration, means that their derivatisation through conventional methods usually requires bulk quantities of purified, high boiling and toxic or carcinogenic solvents such as DMF, DMSO or pyridine. There is considerable scope therefore for reducing the time, energy and toxicity of the materials used in nucleoside chemistry by applying ball-milling. In addition, more generally, it is important to conduct the systematic optimisation of reaction parameters under ball-milling conditions in order to better understand and exploit the technique.

The current work relates to azobenzene-(AB-)derivatised nucleoside and nucleotide mimetics. These have enabled the irradiation-dependent manipulation of several oligonucleotide properties through reversible $E \rightarrow Z$ photoisomerisation.[14] The introduction of AB chromophores into oligonucleotides has most commonly been performed with the *para*-phenylazobenzoyl (*p*-AB-CO-) moiety appended to D-threoninol.[15] However, in our hands, the synthesis of the corresponding *ortho*- and *meta*-AB derivatives of threoninol under literature conditions in DMF met with limited success. In particular, difficulties arose during their isolation due to their susceptibility to photoisomerisation to the *Z*-isomer, their high water solubility and the similar retention characteristics of the multiple side-products and isomers on silica gel in the presence of residual solvent.

Herein, we report the synthesis of *o*-, *m*-, and *p*-AB-derivatised aminonucleoside analogues and propargylamine carried out in a vibration ball mill. Chemoselective acylation of these polyfunctional substrates was rapid, completely avoided the use of DMF, required minimal work-up and thereby avoided undesirable photoisomerisation.

Results and Discussion

N-Hydroxysuccinimidyl (NHS) esters are well established for amine-selective bioconjugation due to the relative insensitivity of such activated esters towards hydrolysis even at alkaline pH.[16] In order to optimise the ball-milling conditions, a model reaction between propargylamine (**1**) and *p*-AB-CO-NHS (**2a**, Fig. 1a) was therefore investigated. The progress of this reaction was monitored using C18 RP-HPLC at 360 nm (Fig. 1b). At this wavelength only those compounds containing the

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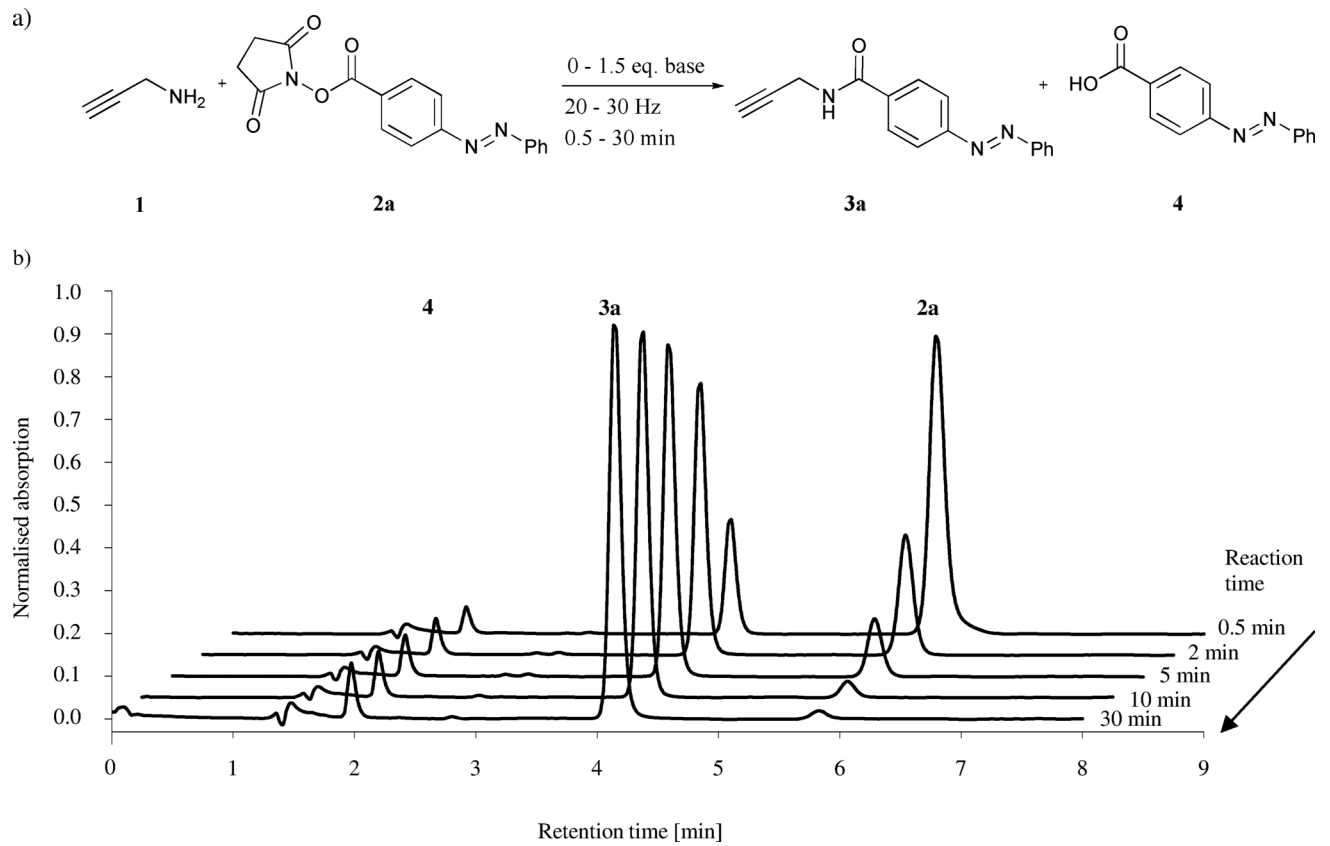


Fig. 1 a) Model reaction of propargylamine (**1**) with *N*-hydroxysuccinimidyl *para*-phenylazobenzoate (*p*-AB-CO-NHS, **2a**) used for optimising the ball-milling conditions; b) typical HPLC chromatograms of the ball-milling reaction between **1** (2 eq.) and **2a** in the presence of DMAP (0.2 eq.) at 28 Hz, monitored at 360 nm at different time points (0.5–30 min).

AB chromophore were detected and isocratic elution readily separated the more hydrophobic NHS-ester (**2a**) from the major product *p*-AB-propargylamide (**3a**) and minor quantities of the hydrolysis product (*p*-AB-CO_2H, **4**).

Reaction conditions were thereby optimised in a 25 mL stainless steel jar containing a 15.0 mm stainless steel ball on a 50 mg scale (of **2a**) by varying the following factors:

1. frequency of milling
2. equivalents of propargylamine and *N*,*N*-dimethyl-4-aminopyridine (DMAP)
3. nature of the base

Influence of milling frequency

We have previously described solvent-free ball-milling for reactions of nucleoside amine and hydroxyl functions at a high vibration frequency (30 Hz).[13a] At lower frequencies (*e.g.* 25 Hz), incomplete reactions were observed even with prolonged reaction times. In the present study, we monitored the reaction between excess propargylamine and the NHS-ester (**2a**) in the presence of DMAP over 30 min over a range of frequencies (20 to 30 Hz).

Consistent with our previous work, increasing the vibration frequency to 28 Hz led to a more rapid reaction (Fig. 2). At 28 Hz, a maximum yield of 95% of the propargylamide **3a** was achieved within 10 min and no further reaction was observed upon further milling. Surprisingly, at 30 Hz, a slower reaction

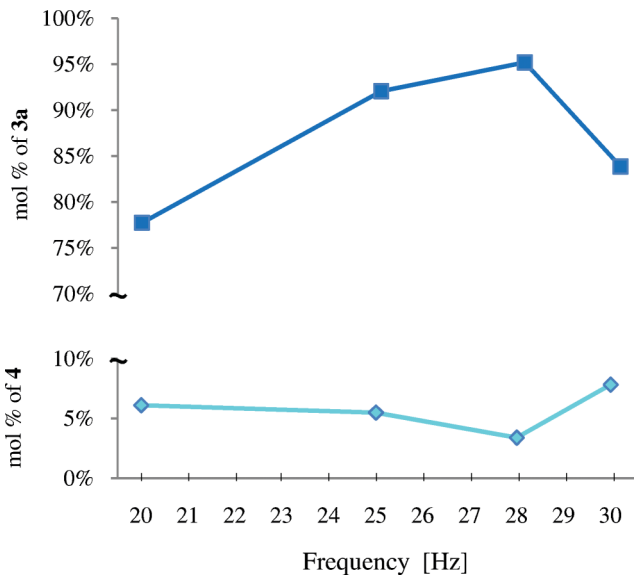


Fig. 2 Rate of reaction to *p*-AB-propargylamide (**3a**) (–■–; after 10 mins) overall level of hydrolysis to *p*-AB-acid (**4**) (–♦–; after 10 mins using different vibration frequencies (2 eq. **1**, 0.2 eq. DMAP, 20–30 Hz).

rate was observed such that after ten minutes there was only 77% consumption of **2a** accompanied by enhanced levels of the corresponding acid (**4**). Although milling at 28 Hz gave the most rapid reaction and highest yield, it is notable that all of the other

Table 1 Influence of the equivalents of propargylamine (**1**) and DMAP on the yield of *p*-AB-propargylamide (**3a**) at 28 Hz

			time (min)					
			1	2	5	10	20	30
entry	**1** (eq)[a]	DMAP (eq)[a]	yield (%) of **3a**[b]					
1	1.0	0.2	23	25	27	27	27	27
2	1.0	0.5	30	41	41	42	43	46
3	1.5	0.2	45	47	53	58	59	69
4	1.5	0.5	45	54	57	70	70	70
5	1.5	1.0	64	79	77	82	82	82
6	1.5	1.5	47	71	84	84	89	89
7	2.0	0	30	46	55	56	60	61
8	2.0	0.2	58	61	77	84	88	91
9	2.0	0.5	70	79	80	88	88	88
10	2.0	1.0	82	55	81	90	91	91
11	2.0	1.5	89	91	92	95	95	95
12	3.0	0	69	72	78	85	88	91
13	3.0	0.2	78	88	91	93	93	93

[a] equivalents quoted relative to **2a**; [b] by HPLC, determined at 360 nm

vibration frequencies investigated gave 90% or greater yield after 30 min (although with higher levels of hydrolysis).

In a seminal work on the variation in energy requirements for a solvent-free Suzuki–Miyaura reaction, Ondruschka and coworkers compared the input for a microwave reactor, a planetary ball-mill and a vibration ball-mill.[17] Related to our observation, in their study a lower yield in the vibration mill at 15 Hz compared with that at 13.3 Hz was observed.

Influence of the amounts of propargylamine and DMAP

N-hydroxysuccinimide is acidic and therefore will protonate both propargylamine and DMAP following the acylation reaction. The lowering of both the reaction rate and the extent of the reaction by such protonation was clearly demonstrated by using only one equivalent of propargylamine in the presence of substoichiometric DMAP (Table 1 entries 1 and 2).

Optimisation of the total amount of base present with different ratios of DMAP and propargylamine was therefore performed. Erratic yields were observed for some reactions during the first two minutes of ball-milling which can be accounted for by inhomogenous mixing. However, clear trends were apparent after five minutes, indicating the influence of DMAP as a catalyst. Thus, using either 1.5 or 2 equivalents of propargylamine, significant enhancements in the rates of reaction were observed, as determined by the yield of **3a** after five minutes, with increasing amounts of added DMAP (Table 1 entries 3–6 and 7–11). Using 1.5 equivalents of DMAP, the reaction was complete within 10 min (entry 11), accompanied by minimal hydrolysis (*ca.* 3%) to **4**.

Finally, we note that using a larger excess of propargylamine with either 0 or 0.2 equivalents of DMAP also gave excellent yields, although the rate of the uncatalysed reaction was slower (Table 1, entries 12 and 13).

Influence of DMAP and other bases

Based upon the use of excess propargylamine and 1.5 equivalents of DMAP (Table 2, entry 1), we next investigated the capacity

Table 2 Influence of the nature of the base on the yield of *p*-AB-propargylamide (**3a**) at 28 Hz, using 2 equivalents of propargylamine, and 1.5 equivalents of base

			time (min)					
			1	2	5	10	20	30
entry	base	pK_a[a]	yield (%) of **3a**[b]					
1	DMAP	9.20	89	91	92	95	95	95
2	DABCO	8.93	56	83	87	90	90	90
3	DBU	11.50	68	75	72	83	83	83
4	Na_2CO_3	10.25	60	76	87	88	89	89

[a] Aqueous pK_a is quoted; [b] by HPLC, determined at 360 nm.

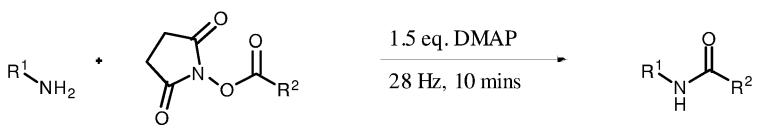
of other dinitrogen bases as well as sodium carbonate to support the reaction. 1,4-Diazabicyclo[2.2.2]octane (DABCO) has proved to be an effective catalyst under solvent-free conditions[6,18] but in our hands, the DABCO-catalysed reaction was neither as rapid nor as high yielding as that using DMAP (Table 2, entry 2). In contrast, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) is more basic than DMAP but has recently been reported to act as a nucleophilic catalyst for acylation reactions.[19] In these reports, the DBU-catalysed reactions were considerably faster and higher yielding than those employing DMAP. However, we observed slower acylation accompanied by higher levels of hydrolysis (up to 15%) leading to lower yields of **3a** (Table 2, entry 3) which may be accounted for by the hygroscopicity of DBU. Overall, in the ball mill, the presence of DMAP provided a significantly faster reaction than DBU and a slight rate enhancement compared with DABCO.

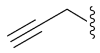
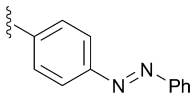
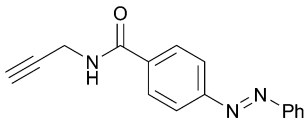
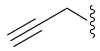
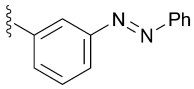
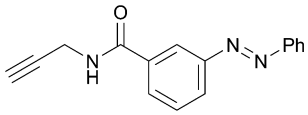
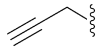
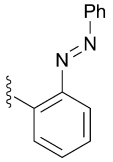
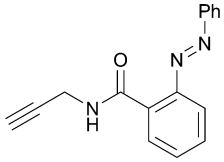
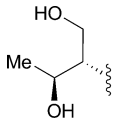
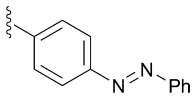
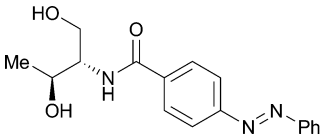
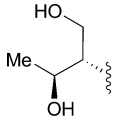
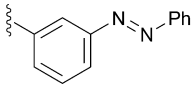
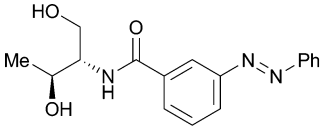
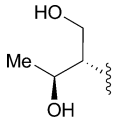
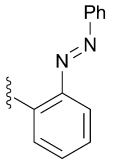
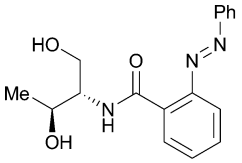
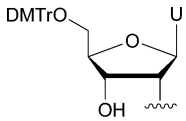
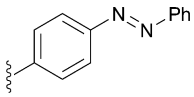
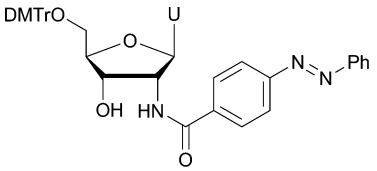
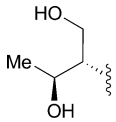
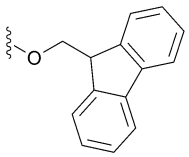
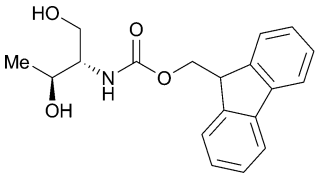
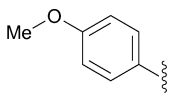
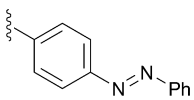
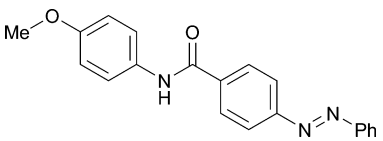
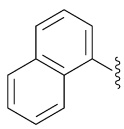
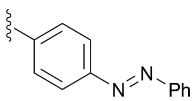
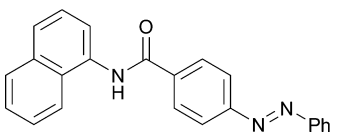
Mayr and coworkers have reported that the nucleophilicity of DABCO towards benzhydrilium ions in acetonitrile is 10^3 times that of DMAP and that the nucleofugalities of the resulting cationic alkylated-DABCO intermediates are *ca.* 10^6 times greater than those of the corresponding DMAP intermediates.[20] No such rate enhancement in the ball mill reaction was observed. Furthermore, the same group have described the carbon basicities of these cationic intermediates which follow the order DBU > DMAP > DABCO.[21] In the ball mill, under conditions in which the concentration of intermediate acyl-ammonium ions were determined by the rate of acylation, we should anticipate the rate order to follow this order of carbon basicities. We therefore assume that under solvent-free conditions, other factors also operate. In particular, the low melting points of DMAP (108–110 °C) and DABCO (156 °C) may mean that the faster rate of reaction with DMAP is a function of the speed with which liquifaction of the reaction mixture occurs—both reactions formed pastes in the early stages of ball-milling but subsequently became powders when the reaction finished.

Sodium carbonate buffers are commonly employed in the acylation of amines by NHS-esters requiring a mixed aqueous-organic solvent system such as those utilised in the post-synthetic labelling of amino-functionalised DNA.[22]

Typically, long reaction times are required and the isolations are therefore often associated with high levels of NHS-ester hydrolysis. Although the addition of anhydrous sodium carbonate gave a reaction which was also slower than that observed in the

Table 3 Preparative-scale reactions under optimised conditions for the synthesis of AB-amides



entry	R^1		R^2		product		yield (%)[a]
1		**1**		**2a**		**3a**	94%
2		**1**		**2b**		**3b**	92%
3		**1**		**2c**		**3c**	90%
4[b]		**5**		**2a**		**6a**	79%
5[b]		**5**		**2b**		**6b**	77%
6[b]		**5**		**2c**		**6c**	83%
7[b]		**7**		**2a**		**8**	90%
8		**5**		**9**		**10**	79%
9		**11**		**2a**		**12**	78%
10		**13**		**2a**		**14**	65%

[a] Isolated yields; [b] 50 μl Ethyl acetate added; U = *N*-1-uracilyl.

DMAP-catalysed reaction, nearly 90% conversion was observed (Table 2, entry 4) compared with 61% in the absence of the base (Table 1, entry 7).

Preparative-scale reactions and chemoselective acylation

To test the effectiveness of this solvent-free process further, the optimised DMAP-catalysed conditions determined above were applied to preparative-scale reactions using *para*- (**2a**), *meta*- (**2b**) and *ortho*- (**2c**) AB-esters (Table 3, entries 1–3). In contrast to the solution-phase reactions in DMF, it was gratifying to note that the less reactive *o*-AB- and *m*-AB-esters also completely reacted in under 10 min to the corresponding propargylamides (**3a–c**) upon ball-milling. These products could be isolated readily in excellent yields following extraction.

Applying the same reaction conditions to the preparation of D-threoninol AB-derivatives (**6a–c**), was found to give incomplete reactions even after 30 min ball-milling at 28 Hz.

Solvent-assisted grinding (often called liquid-assisted grinding, or LAG) has been used to improve ball-milling conversions in both co-crystallisations[23] and metal complexations.[24] In the present case, the addition of stoichiometric quantities of ethyl acetate significantly enhanced the rates and yields to give **6a–c** with only minor amounts of the side-products observed by TLC analysis. We ascribe these less polar, AB-containing side-products to the reaction of the activated esters at both the amine and hydroxyl functions, since they gave negative ninhydrin tests. Ethyl acetate was then added to the reaction vessel and the resulting solution applied to a short silica gel column. Pure products were isolated following isocratic elution with ethyl acetate in high yields. Although this work-up and isolation procedure required larger volumes of solvent compared with those used for **3a–c**, ethyl acetate is categorised as a preferred solvent according to the Pfizer solvent selection guide.[25]

Likewise, using solvent-assisted grinding, the protected 2′-amino-2′-deoxynucleoside analogue (**7**) completely reacted with minimal side-products. The corresponding *p*-AB adduct (**8**) was isolated with excellent yields (Table 3, entry 7).

The Fmoc protecting group is widely applied for the solid-phase synthesis of peptides and often requires mixed solvent systems and a problematic work-up. Even though it is thermally sensitive, the NHS ester (**9**) reacted selectively with D-threoninol to give **10** in high yields (Table 3, entry 8).

Finally, the acylation of aromatic amines using solvent-free ball-milling was investigated (Table 3, entries 9 and 10). The relative reactivities of *p*-anisidine (**11**) and naphthylamine (**13**) compared with the aliphatic amines were found to be consistent with Cline and Hanna's report of NHS-ester aminolysis in 1,4-dioxan. In this study, *p*-anisidine was found to be approximately an order of magnitude more reactive than aniline and half as reactive as benzylamine.[26] It is perhaps noteworthy that the time for the reaction to complete between *p*-nitrobenzoyl-NHS and aniline at 24.6 °C was reported to be 60 days. Using ball-milling, reaction times of 20 or 120 min were required to effect the complete reaction to the corresponding anisidide (**12**) or napthylamide (**14**), respectively.

Conclusions

We have demonstrated that, under optimised conditions, the acylation of amines using NHS esters can be performed in equal or greater yields than by conventional DMF-based approaches; indeed the motivation for this work was that efficient isolation from DMF of *ortho*- or *meta*-AB D-threoninol adducts was not feasible in our hands.

A survey of the ball-milling reaction conditions in terms of frequency, ratio of reactants and the nature of the additional base on a small-scale enabled preparative-scale reaction conditions to be developed without a significant difference between the isolated yield and the conversion observed using HPLC. We note that a distinct advantage of using ball-milling for reactions involving photosensitive groups is that exposure to incident UV-Vis radiation is minimised.

The optimisation of solvent and temperature conditions solely for the purpose of rendering substrates and reagents soluble is often required in nucleoside and nucleotide chemistry using molecular solvents. Solvent-free methods have considerable scope to facilitate this chemistry in general, reducing both the E-factor and the amount of energy required, as well as providing higher yields, faster processes and easier product isolation.

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