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## COMMUNICATION

## Synthesis of coenzyme A thioesters using methyl acyl phosphates in an aqueous medium†

Cite this: DOI: 10.1039/x0xx00000x

Mohan Pal<sup>a</sup> and Stephen L. Bearne<sup>a,b,\*</sup>Received 00th January 2012,  
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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**Regioselective *S*-acylation of coenzyme A (CoA) is achieved under aqueous conditions using various aliphatic and aromatic carboxylic acids activated as their methyl acyl phosphate monoesters. Unlike many hydrophobic activating groups, the anionic methyl acyl phosphate mixed anhydride is more compatible with aqueous solvents, making it useful for conducting acylation reactions in an aqueous medium.**

Coenzyme A (CoA) thioesters are one of the key substrates for nearly 4% of the known enzymes of metabolic pathways, including enzymes that are targets for the development of pharmaceuticals.<sup>1</sup> Therefore, thioester derivatives of CoA are valuable as analytical reagents for enzyme assays and as probes of enzyme chemistry.<sup>2</sup> Various chemical methods have been described for the synthesis of CoA thioesters by acylation of the thiol with activated carboxylic acid groups, including acid anhydrides,<sup>3,4</sup> acid chlorides,<sup>4,5</sup> the mixed anhydride of ethyl hydrogen carbonate,<sup>6</sup> *N*-hydroxysuccinimide esters,<sup>3,7</sup> and *O*-acylisoureas (e.g., adducts with *N,N'*-dicyclohexylcarbodiimide (DCC) or *N,N'*-carbonyldiimidazole).<sup>8</sup> Typically, these activated carboxylic acids are hydrophobic in character while CoA is very polar, necessitating that acylation reactions be conducted in biphasic systems or at elevated temperatures. Consequently, yields tend to be low due to the activated carboxylic acids often being labile under aqueous conditions and the occurrence of various competing side reactions. Alternatively, the enzymatic acylation of the thiol of CoA using ligases affords an effective synthetic strategy under physiological conditions. Unfortunately, the synthetic utility of enzymatic routes is often limited by substrate specificity and the availability of the required enzymes.<sup>9</sup> Because of the utility of acyl-CoA thioesters in biological chemistry, efficient general strategies for the synthesis of CoA thioesters are sought.

Herein, we report the use of methyl acyl phosphate monoesters in a biomimetic protocol for producing thioesters under aqueous conditions. These activated mixed anhydrides of carboxylic acids act as electrostatically selective anionic electrophiles with methyl phosphate as the leaving group.<sup>10-12</sup> Due to the hydrophilicity of the methyl phosphate group and the stability of the mixed anhydride in water,<sup>13,14</sup> acyl phosphate monoesters have been employed in aqueous acylation reactions.<sup>14-16</sup> Recently, methyl aminoacyl phosphates have been reported to acylate amino acids, producing peptides in water even in presence of other non-amine nucleophiles.<sup>17</sup> Huang and co-workers have reported the use of acyl adenylates in an imidazole-catalyzed acylation reaction to prepare acetyl-CoA and several derivatives of biocytin-CoA.<sup>18</sup> However, adenylation of the acyl group using adenosine 5'-monophosphate and DCC, following the procedure of Berg,<sup>19</sup> is somewhat cumbersome. Considering the reactivity and hydrophilic character of methyl acyl phosphates, we anticipated that such compounds, conveniently prepared from their corresponding acyl chlorides, would serve as acylating agents for the synthesis of thioesters under aqueous conditions.

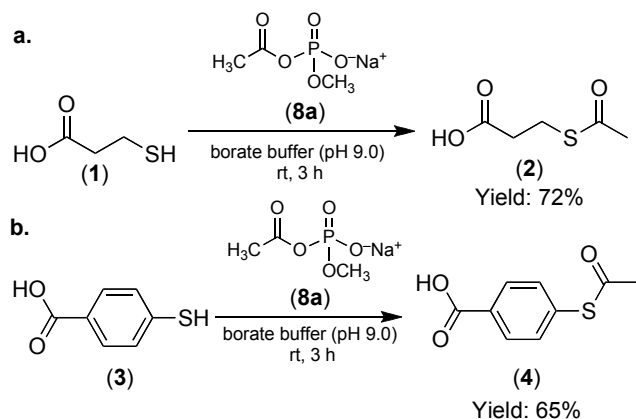
As an initial test of this synthetic strategy, we carried out the acylation of the aliphatic thiol 3-mercaptopropanoic acid (**1**) and the aromatic thiol 4-mercaptobenzoic acid (**3**) with sodium methyl acetyl phosphate (**8a**)<sup>10,15</sup> in borate buffer (pH 9.0) for 3 h at room temperature (Scheme 1). Borate buffer at pH 9.0 was chosen as the reaction medium to promote formation of thiolate anions. <sup>1</sup>H NMR spectra of the purified products showed signals arising from protons present in both the acetyl group and the thiol moiety with the appropriate integration ratios. In addition, ESI-MS analyses confirmed the presence of the acylated products 3-(acetylthio)propanoic acid (**2**) and 4-(acetylthio)benzoic acid (**4**). The possible formation of an anhydride product was ruled out based

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on the  $^{13}\text{C}$  NMR spectra of the products (see ESI) which showed chemical shifts corresponding to the carbonyl carbon of **2** and **4** at 195.70 ppm and 192.63 ppm, respectively, with the absence of a signal expected for the anhydride carbonyl carbon at  $\sim 167$  ppm. Thioesters **2** and **4** were obtained with isolated yields of 72% and 65%, respectively.

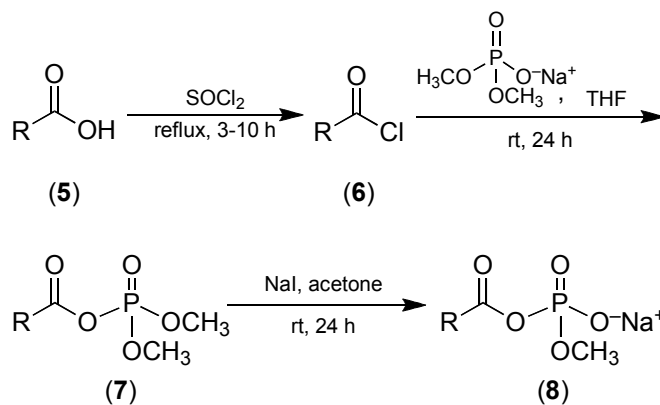
Based on the above results, we envisaged extending the scope of this methodology for the facile preparation of CoA thioesters. For this purpose, we synthesized various sodium methyl acyl phosphates of aliphatic (small, medium, and long chain), aromatic, and unsaturated carboxylic acids (Scheme 2, Table 1). In the first step, acyl chlorides were prepared by refluxing the corresponding carboxylic acids in excess thionyl chloride, which was then distilled off to give a light yellow oil. The acid chloride was then coupled with sodium dimethyl phosphate in dry THF under an inert atmosphere.<sup>15</sup> The resulting dimethyl acyl phosphates (**7a** – **7i**) were obtained as light brown oils and these crude dimethyl acyl phosphates were subsequently reacted with sodium iodide in dry acetone to effect removal of a single methyl group. Of the resulting methyl acyl phosphates, only **8a** and **8e** gave pure white precipitates from acetone similar to that reported for sodium methyl acetyl phosphate;<sup>15</sup> the others required purification. Due to the poor stability of sodium methyl acyl phosphates on silica gel, we purified



**Scheme 1** S-Acylation of (a) 3-mercapto-propanoic acid (**1**) and (b) 4-mercaptobenzoic acid (**3**) with sodium methyl acetyl phosphate.

the other monoesters by solid-phase (C18) extraction using varying concentrations of MeCN/water as the eluent to remove contaminating dimethyl phosphate, as well as minor amounts of methyl phosphate and the parent carboxylic acid arising from hydrolysis. Fractions were analyzed using  $^1\text{H}$  and  $^{31}\text{P}$  NMR spectroscopy, and those fractions containing the pure methyl acyl phosphates were pooled and lyophilized to give white solids of the sodium methyl acyl phosphates (**8b** – **8d** and **8f** – **8i**). Table 1 summarizes the isolated yields for each methyl acyl phosphate synthesized (**8a** – **8i**).

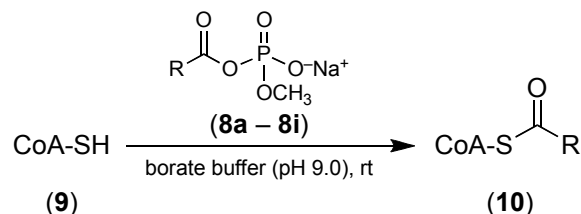
Initially, we examined the S-acylation of CoA by sodium methyl acetyl phosphate (3 eq.) in 15 mL of borate buffer (pH 9.0) for 6 h (Scheme 3). (See ESI for synthetic protocols.) After removal of the water by lyophilization,  $^1\text{H}$  NMR spectroscopy of the crude mixture revealed a downfield shift of the signal corresponding to the protons



**Scheme 2** Preparation of methyl acyl phosphates. Dimethyl acyl phosphates (**7**) were not purified but used directly in the subsequent step.

**Table 1** Preparation of methyl acyl phosphate monoesters

Compound	R group of methyl acyl phosphate	ESI-MS data	Yield (%)
<b>8a</b>	CH <sub>3</sub>	153.0 [M-Na] <sup>-</sup>	76
<b>8b</b>	CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub>	181.0 [M-Na] <sup>-</sup>	34
<b>8c</b>	(CH <sub>3</sub> ) <sub>2</sub> CHCH <sub>2</sub>	195.0 [M-Na] <sup>-</sup>	40
<b>8d</b>	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>6</sub>	237.1 [M-Na] <sup>-</sup>	58
<b>8e</b>	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>14</sub>	349.2 [M-Na] <sup>-</sup>	54
<b>8f</b>		299.1 [M-Na] <sup>-</sup>	23
<b>8g</b>		335.1 [M-Na] <sup>-</sup>	26
<b>8h</b>		243.0 [M-Na] <sup>-</sup>	36
<b>8i</b>		241.0 [M-Na] <sup>-</sup>	32



**Scheme 3** S-Acylation of CoA by methyl acyl phosphates.

on the 9<sup>th</sup> methylene (see ESI for CoA numbering) next to the thiol group of CoA, consistent with S-acylation. Subsequent purification of the acetyl-CoA using solid-phase (C18) extraction gave 90% pure acetyl-CoA (**10a**) in 33% yield. The HRESI-MS for the [M-2H]<sup>2-</sup> ions of **10a** gave a mass of 403.5580, confirming the formation of acetyl-CoA (Table 2).

Similarly, the reaction of sodium methyl butyryl phosphate (**8b**) with CoA gave pure butyryl-CoA (**10b**) in 19% isolated yield; however, we were unable to purify the thioester product from the reaction of sodium methyl isovaleryl phosphate (**8c**) with CoA due to poor conversion (Table 2). Reaction of the medium chain sodium

methyl octanoyl phosphate (**8d**) with CoA yielded octanoyl-CoA (**10d**), which was purified in 26% isolated yield and coupling was confirmed by the HRESI-MS mass for the  $[M-2H]^{2-}$  ion at 445.5997. We also examined *S*-acylation of CoA using an aliphatic long chain (16C) methyl acyl phosphate, sodium methyl palmitoyl phosphate (**8e**). Unfortunately, we did not observe any acylation of CoA with **8e** due to the poor solubility of **8e** in borate buffer. Attempts to enhance the solubility of **8e** in buffer using biphasic conditions with methanol or chloroform as co-solvents, or with sonication, proved unsuccessful. Over time, **8e** was observed to precipitate from the solution.

**Table 2** *S*-acylation of CoA with methyl acyl phosphate monoesters

Compound	CoA-thioester	ESI-MS data	Yield (%)
<b>10a</b>	acetyl-CoA	403.6 $[M-2H]^{2-}$	33 <sup>a</sup>
<b>10b</b>	butyryl-CoA	417.6 $[M-2H]^{2-}$	19
<b>10c</b>	isovaleryl-CoA	–	nd
<b>10d</b>	octanoyl-CoA	445.6 $[M-2H]^{2-}$	26
<b>10e</b>	palmitoyl-CoA	–	nr
<b>10f</b>	ibuprofenoyl-CoA	476.6 $[M-2H]^{2-}$	24
<b>10g</b>	fenoprofenoyl-CoA	494.6 $[M-2H]^{2-}$	40
<b>10h</b>	3-phenylpropanoyl-CoA	448.6 $[M-2H]^{2-}$	26
<b>10i</b>	<i>trans</i> -cinnamoyl-CoA	447.6 $[M-2H]^{2-}$	30

<sup>a</sup>90% pure; nd., not determined; nr., no reaction

Although the anionic methyl phosphate group does not afford sufficient hydrophilicity to promote the solubility of long chain fatty acids, we were able to extend the scope of this methodology further by successfully *S*-acylating CoA with activated aromatic acids. Ibuprofenoyl-CoA (**10f**) and fenoprofenoyl-CoA (**10g**) were synthesized by reaction of sodium methyl ibuprofenoyl phosphate (**8f**) and sodium methyl fenoprofenoyl phosphate (**8g**) with CoA, giving isolated yields of 24% and 40%, respectively. The HRESI-MS of **10f** and **10g** gave masses for the  $[M-2H]^{2-}$  ions of 476.6094 and 494.5912, respectively, thereby confirming product formation (Table 2). Similarly, 3-phenylpropanoyl-CoA (**10h**) was prepared in 26% isolated yield using sodium methyl 3-phenylpropanoyl phosphate (**8h**) as the acylating agent. Finally, we demonstrated the *S*-acylation of CoA using an unsaturated aromatic acid. *trans*-Cinnamoyl-CoA (**10i**) was prepared in 30% isolated yield by reaction of sodium methyl *trans*-cinnamoyl phosphate (**8i**) with CoA.

Yields may decrease with time due to time-dependent hydrolysis of the methyl acyl phosphates. As a representative example, we followed the hydrolysis of methyl ibuprofenoyl phosphate (**8f**) in borate buffer (100 mM, pD = 9.0) (**Fig. 99S**) over 24 h using <sup>1</sup>H and <sup>31</sup>P NMR spectroscopy. After 24 h, ~25% hydrolysis of **8f** occurred yielding ibuprofen and methyl phosphate. On the other hand, <sup>1</sup>H NMR spectroscopy revealed that the product ibuprofenoyl-CoA (**10f**) did not undergo any significant amount of hydrolysis over 24 h under the same conditions (**Fig. 100S**). Consequently, reduced yields may result from hydrolysis of the methyl acyl phosphates, but not hydrolysis of the thioester products.

Based on the <sup>1</sup>H NMR spectra of crude preparations, conversions ranged from 30% to 55% (**Table 1S**) using a 1:1 molar ratio of CoA to methyl acyl phosphate. The conversions may be low due to the hydrolysis of the methyl acyl phosphates. Indeed, we found an increase for the conversion to acetyl-CoA (76%) using a 1:3 molar ratio of CoA to methyl acetyl phosphate. While increased conversions may be obtained using higher equivalents of the methyl acyl phosphates, there is a trade-off with the ease of product purification. We found that using higher equivalents of the methyl acyl phosphate required separation of the product CoA thioester from a greater amount of unreacted methyl acyl phosphate, making purification on the C18 column more difficult. In the present protocol, the C18 column may be used to conveniently purify the CoA thioesters from the remaining reactants without the need to use HPLC. We also note that CoA thioesterification using the various methyl acyl phosphates was highly regioselective. In all the products described, integration of the <sup>1</sup>H NMR spectra revealed only monoacylation. The NMR spectra of the crude thioesters, or of any of the fractions purified on the C18 column, did not show evidence of any side products (other than unreacted CoA and methyl phosphate) arising from acylation of the hydroxyl groups on CoA, as would be expected based on the higher pK<sub>a</sub> (~17) of the hydroxyl groups relative to the thiol of CoA (9.8).<sup>20</sup> Nor did we observe acylation of the less nucleophilic nitrogens of the purine moiety of CoA.

## Conclusions

We have described the use of methyl acyl phosphates to regioselectively *S*-acylate CoA and generate a variety of CoA thioesters under aqueous conditions in the absence of an organic cosolvent. In addition to demonstrating the utility of methyl acyl phosphates as acylating reagents for the thiol of CoA, we have also established synthetic protocols for the preparation of a number of methyl acyl phosphates. Like methyl acetyl phosphate, which can acetylate amino groups located adjacent to cationic sites in proteins,<sup>10-12,15,21</sup> the aliphatic and profen-based methyl acyl phosphates described in the present work should prove useful as protein modification reagents and probes of enzyme active sites.

## Acknowledgements

We thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for support of this research through a Discovery Grant (SLB).

## Notes and references

<sup>a</sup> Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS, B3H 4R2, Canada

<sup>b</sup> Department of Chemistry, Dalhousie University, Halifax, NS, B3H 4R2, Canada

† Electronic Supplementary Information (ESI) available: Materials and methods, synthetic procedures for all compounds and intermediates, analytical and spectroscopic data. See DOI: 10.1039/c000000x/

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