An Improved Synthesis of Alkyl AMP Esters

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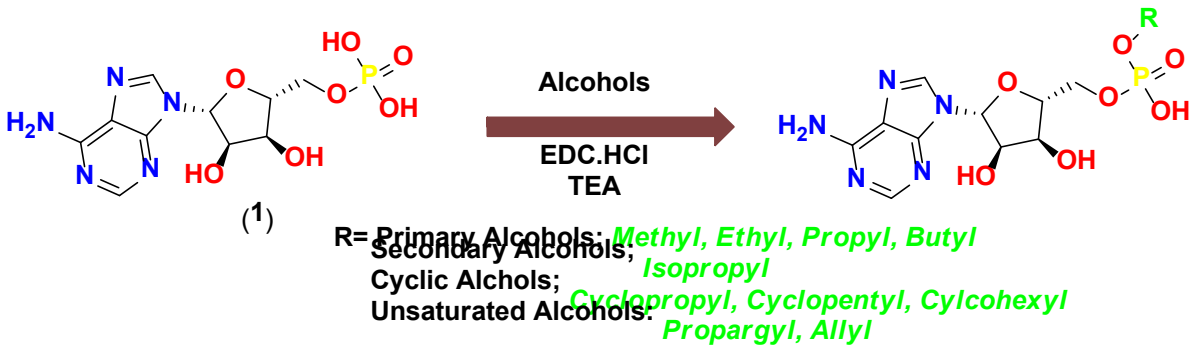
Abstract

Enzymes belonging to the Acyl-CoA/NRPS/Luciferase (ANL) superfamily of enzymes, are significant targets in drug development. One member of this superfamily is Acetyl-CoA Synthetase (ACS). This enzyme is an emerging target for the treatment of various infectious diseases and cancer. Alkyl AMP esters have emerged as potent inhibitors of ACS. Previous methods for synthesizing these esters often involved water-based reactions or necessitate purification through reverse phase prep-HPLC or ion-exchange chromatography. To address these challenges, we developed a new approach utilizing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) to couple 5'-adenylic acid to the corresponding alcohol, utilizing triethylamine as the base. This method yielded primary, secondary, unsaturated, and cyclic alcohols in excellent yields. Importantly, we optimized the reaction conditions to achieve excellent yield and purity without the need for reverse phase prep-HPLC or ion exchange chromatography. Instead, purification was achieved through silica gel chromatography using Biotage ® Sfar spherical silica gel.



Scheme 1: The conversion of 5'-adenylic acid to alkyl AMP ester through use of an alcohol, EDC.HCl, and triethylamine

Introduction

Adenosine 5′-phosphate alkyl esters (AMP esters) are known to potently inhibit acetyl- and acyl-CoA synthetase enzymes[1]. The Acetyl Co-A Synthetase (ACS) enzyme is an emerging target for the treatment of various infectious diseases and cancer[2-5]. These AMP esters have been extensively utilized to solve crystal structures of ACS enzymes in a wide range of organisms. Acetyl-CoA, a pivotal molecule in biology, plays multifaceted roles in cellular energetics, gene

expression regulation, protein post-translational modification, and lipid biosynthesis among other fundamental cellular functions[6-10]. The conversion of acetate to acetyl-CoA occurs via a two-step reaction catalyzed by ACS[6-7]. AMP esters have proven instrumental in elucidating the structures of ANL-family enzymes targeted by antifungal, antiparasitic, antimalarial, antiviral, and antimicrobial agents. For instance, Reger et al. reported the structure of propyl AMP in complex with *Salmonella enterica* ACS[8]. In 2013, Kim et al. provided structural and mechanistic insights into the inhibition of class C β-lactamases through the adenylation of a serine residue, using acyl AMP to solve the structure of β-lactamases[9]. Additionally, Gulick et al., in 2003, reported the structure of propyl AMP bound to coenzyme A[10]. In 1998, Pope et al. elucidated the structure of isoleucyl tRNA synthetase from *Staphylococcus aureus* complexed with an analog of Ile-AMP[11], while Ling et al. in 2012 solved the crystal structure of yeast mitochondrial threonyl-tRNA synthetase (MST1) complexed with an analog of Ser-AMP[12].

Our group recently reported the structural characterization of the reaction and substrate specificity mechanisms of pathogenic fungal ACS enzymes[6]. We synthesized four AMP esters with varying carbon chain lengths from methyl to butyl and screened them against five different fungal organisms: *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Candida albicans*, *Coccidioides immitis*, and *Coccidioides posadasii*. As chemical probes the four alkyl AMP esters provided valuable insight into the enzyme mechanism and substrate specificity[6]. All four AMP esters exhibited activity against the ACS1 enzyme from each of the selected fungi species. Ethyl AMP demonstrated the highest potency, boasting a K_i of 55 nM against *A. fumigatus*, *C. immitis*, and *C. posadasii*, as well as 72 nM and 470 nM against *C. neoformans* and *C. albicans*, respectively. Our previous work primarily focused on the structural characterization of fungal ACS, resulting in the depositing of thirteen crystal structures into the Protein Data Bank. To deepen our understanding of the structure-activity relationship, we aimed to expand the scope of our research by synthesizing additional AMP esters for comparative biological activity analysis.

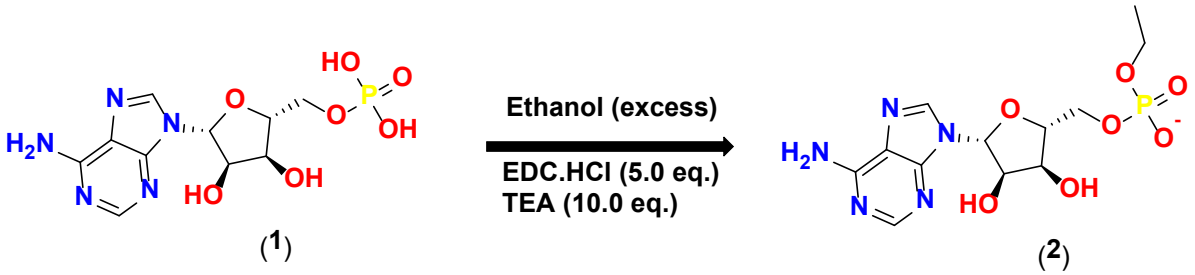
The synthesis of alkyl AMP esters from AMP and an alcohol remains limited in the literature. In 1988, Grayson et al. reported the synthesis of alkyl AMP esters as inhibitors of the ACS1 enzyme in yeast[1]. Their method involved the synthesis of alkyl AMP esters from di(tributylammonium) adenosine 5'-monophosphate and an alcohol using dicyclohexyl carbodiimide (DCC) as the coupling agent. In this way, they succeeded in yielding methyl to hexyl esters; however, it necessitated purification via buffer and ion exchange chromatography using DEAE-cellulose. In 1992, Golz et al. reported the inhibition of plant ACS by alkyl AMP esters[13]. The Golz approach to AMP esters utilized 1 mmol adenosine-5'-phosphate-H_2O (AMP) dissolved in the desired alcohol containing 2 mmol tri-A7-butylamine and 5 mmol DCC. Subsequent purification was performed by water extraction, use of a cation exchange resin, followed by a precipitation to yield the methyl to butyl AMP esters in 50-75% yield. In 1987, Shabarova et al. reported the synthesis of alkyl and aryl AMP esters as well as AMP phosphoamidates using EDC.HCl to couple various nucleotides to simple alcohols and amines utilizing a MES buffer (pH 4.5-5.5) in 70-100% conversion[14]. This approach relied on a purification performed by either reverse phase prep-HPLC or by paper electrophoresis, depending on the compound. In 1983, Toshihiro et al. reported the use of adenosine-3’ and -5’alkylphosphate esters for steady-state kinetic studies of binding and catalysis with ribonuclease T2. These esters were synthesized using 5’-AMP pyridinium salt, triethylamine, an appropriate primary alcohol, and DCC in pyridine. The compounds were purified by aqueous extraction and purification on a DEAE-Sephadex A-25

column, yielding methyl to hexyl esters in varying yields[15]. In summary, several methods have been developed for the synthesis of alkyl AMP esters; however, many procedures require specialized purification methods that vary with each approach. Our group sought to develop a more general method that would utilize few starting materials and allow purification of alkyl AMP esters through normal phase silica gel chromatography.

In our previous work, we synthesized alkyl AMP esters using 5'-adenosine monophosphate disodium salt in excess alcohol with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) as the coupling agent[6]. However, the method was limited by moderate yields (35%-63%), which was attributed to the low solubility of the disodium salt form of adenylic acid in alcohols other than methanol. To improve this synthesis, we opted to use 5'-adenylic acid instead of the previously employed AMP disodium salt. Our refined method also utilizes EDC.HCl as a coupling agent as well as triethylamine as the base. The reaction progress was monitored by reverse phase HPLC, and the desired AMP esters were obtained as ammonium or triethylamine salts, depending on the chosen base for silica gel column basification. Notably, our method introduces the novel use of purification by silica gel chromatography, which can be applied to primary and secondary alcohols without requiring tedious workup procedures. Thus, we have developed a more general and efficient synthesis of these useful chemical probes and inhibitors.

Result and Discussion

Optimization Reaction of AMP Esters from Adenylic Acid and Alcohols.



(**1**) (**2**)

Scheme 2: Synthetic route to the synthesis of ethyl AMP, EDC.HCl (5.0 eq.), and Et_3N (10 eq.)

The first phase of optimization aimed to establish a suitable time-frame for the reaction. We decided to utilize ethyl-AMP as the system for our optimization. An excess of both EDC.HCl (5.0 eq) and TEA (10.0 eq) was used to monitor the kinetic profile of the reaction. Under the specified reaction conditions, the progression of the reaction was tracked over time via reverse phase HPLC. After 1 hour, the reaction mixture appeared as a suspension, showing 15% conversion (Table 1, Entry 1). By the 5-hour mark, the reaction became a fully dissolved solution, showing 72% conversion (Table 1, Entry 5). Finally, after 12 hours, reverse phase HPLC showed 100% conversion (Table 1, Entry 11). This phase showed that, even with excess reagents, the reaction requires a minimum of twelve hours to reach quantitative conversion.

Entry	Reaction time (hr)	E-AMP conversion %
1	1	15
2	2	25
3	3	38
4	4	52
5	5	72
6	6	87
7	7	92
8	8	97
9	9	98
10	10	99
11	12	100
12	14	100
13	24	100

Table 1: Optimization of reaction conditions of ethyl AMP by varying reaction time. **Reaction conditions**: RT, EDC.HCl (5.0 eq.), Et_3N (10 eq.). Reaction progress was monitored by reverse phase HPLC.

The second phase of optimization held the 12 h time and 10 eq. TEA constant and varied the quantity of EDC.HCl. No conversion was observed in the absence of EDC.HCl. Increasing equivalents of EDC.HCl achieved increasing levels of conversion. The first equivalent resulted in 24% conversion (Table 2, Entry 1) and the second equivalent achieved 50% conversion (Table 2, Entry 2). The first phase of optimization had noted that the change from suspension to solution had correlated with high levels of conversion. The reaction mixture became a clear solution with 3 equivalents of EDC.HCl, resulting in a conversion of 91% (Table 2, Entry 3). Subsequent increases in EDC.HCl to 4.0 (Table 2, Entry 4) and 5.0 (Table 2, Entry 5) equivalents saw the product conversion rise to 96% and 100%, respectively. Therefore, complete conversion could be achieved after 12 h, using 5.0 eq. EDC.HCl, and 10.0 eq. TEA. However, because using 5.0 equivalents seemed rather inefficient, we sought to use fewer equivalents and simply add a few hours to the reaction time.

When the reaction was stirred for up to 20 hours, the reaction remained a suspension with 1.0 equivalent mole of EDC.HCl, resulting in 61% conversion (Table 3, Entry 1). However, a clear solution was achieved at 2.0 equivalent moles of EDC.HCl, with a conversion of 96% (Table 3, Entry 2). Complete conversion to the product was observed with 3.0, 4.0, and 5.0 equivalent moles of EDC.HCl (Table 2, Entries 3-5). Therefore, if the reaction were run for 20 hr instead of 12 hr, two equivalents of EDC.HCl could be saved.

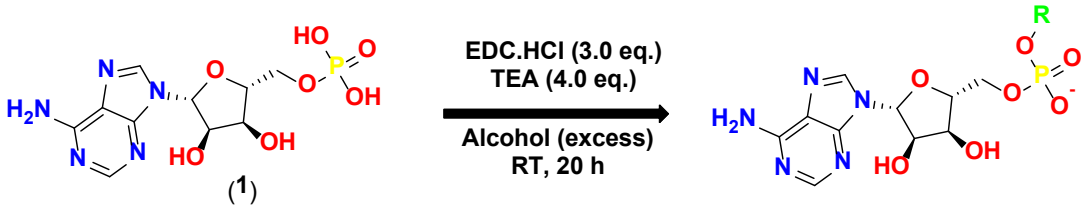
Entry	EDC.HCL eq.	Time	E-AMP conversion %
1	1	12 hr	24
2	2	12 hr	50
3	3	12 hr	91
4	4	12 hr	96
5	5	12 hr	100
6	1	20 hr	61
7	2	20 hr	96
8	3	20 hr	100
9	4	20 hr	100
10	5	20 hr	100

Table 2: Optimization of reaction conditions with ethyl AMP by varying equivalent moles of EDC.HCl at either 12 hr or 20 hr reaction time. **Reaction conditions**: RT, Et3N (10 eq.). Reaction progress was monitored by reverse phase HPLC.

The final phase of optimization phase held the time constant at 20 hours and the equivalents of EDC.HCl constant at 3.0 eq, while varying the quantity of triethylamine required for the full conversion of the acid to the ester. It was noted that without the addition of triethylamine the reaction mixture remained a suspension after 20 hours (Table 4, Entry 1) but did produce 32% conversion. Triethylamine presumably enhances the solubility of the acid. With the addition of 1.0, 2.0, and 4.0 equivalents of triethylamine (Table 4, Entries 2-4), the reaction mixture became a clear solution and showed 90%, 95%, and 100% conversion, respectively. Based on these findings, it was concluded that 4.0 equivalent of triethylamine is sufficient for achieving complete conversion of the acid to the ester. Therefore, the preferred optimized reaction condition was determined as follows: EDC.HCl (3.0 eq.), triethylamine (4.0 eq.), with a reaction time of 20 hours.

Entry	TEA eq.	E-AMP conversion %
1	0	32
2	1	90
3	2	95
4	4	100
5	6	100
6	8	100
7	10	100

Table 3: Optimization of reaction conditions of ethyl AMP by varying equivalent moles of triethylamine **Reaction conditions**: RT, EDC.HCl (3.0 eq.), 20 hr. Reaction progress was monitored by reverse phase HPLC.



(**1**)

Scheme 3: Optimized Reaction Conditions are corresponding alcohol (excess)**,** EDC (3.0 eq.), Et_3N (4.0 eq.), 20 hr

After determining the optimal reaction conditions, we explored the reactivity of various primary alcohols. Methyl AMP (**3**) produced an outstanding yield of 90% after purification. Ethyl (**2**), propyl (**4**), and butyl (**5**) AMP followed suit with isolated yields of 83%, 78%, and 73%, respectively. Allyl (**10**) and propargyl (**11**) alcohols, containing alkene and alkyne functional groups respectively, yielded 75% and 80%. Isopropyl alcohol (**6**), a secondary alcohol, yielded 70% with a reaction time of 144 hours. Synthesis with cyclic alcohols such as cyclopropanol (**7**) and cyclopentanol (**8**) resulted in yields of 74% and 65% respectively but their kinetics were slower. Cyclopropanol (**7**) reacted notably faster than either isopropanol (**6**) or cyclopentanol (**8**), achieving 100% conversion in 72 hours compared to isopropanol's (**6**) 144 hours to reach 88% conversion, and cyclopentanol's (**8**) 144 hours to reach 80% conversion. This discrepancy is attributed to the reduced steric hindrance of cyclopropanol due to its ring structure, facilitating a faster S_N2 reaction compared to the more hindered isopropanol and cyclopropanol. Attempts with *tert*-butanol (**9**), a tertiary alcohol, were unsuccessful due to its sterically hindered structure, impeding an S_N2 reaction. Three compounds, the cyclopropyl (**7**), cyclopentyl (**8**), and propargyl (**11**) esters were previously unreported in the literature. The resulting AMP esters were synthesized either as ammonium salts or triethylammonium salts, depending on the base used during the chromatography.

Compound	Alcohol (R)	AMP conversion (%)	Time (hr)	Isolated Yield (%)
3	Methyl	100	20	90
2	Ethyl	100	20	83
4	Propyl	100	20	78
5	Butyl	100	24	73
6	Isopropyl	88	144	70
7	Cyclopropyl	100	72	74
8	Cyclopentyl	80	144	65
9	*Tert*-butyl	0	144	-
10	Allyl	100	24	75
11	Propargyl	100	24	80

Table 4: Optimized reaction conditions with primary, secondary, and tertiary alcohols, showing AMP ester conversion ratio (%), time (hr), and the isolated yield (%) for each compound.

Conclusion

The use of 5'-adenylic acid, EDC.HCl, triethylamine, and an alcohol produced a much easier synthetic method for the synthesis of alkyl AMP esters than previous methods. The use of the desired alcohol as both the reactant and the general solvent for the reaction produced an operationally more simple procedure that requires fewer reagents. Triethylamine and most common alcohols are low boiling point compounds that can often be removed by evaporation. Finally, the products can be purified entirely by normal phase silica gel chromatography without necessitating a work-up procedure. This method proved successful even for alcohols that are not able to be removed by evaporation. Finally, the exact identity of the salt can be specified based upon the base used during chromatography without the need for an ion-exchange column. This method will allow synthetic chemists to produce these highly valuable alkyl AMP esters in a more efficient manner.

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