^{31}P NMR study on aminoacylation of 5′-AMP and its analogs

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The progress of aminoacylation of 5′-AMP with amino acids in the presence of several condensing reagents was monitored by ^{31}P NMR. The resulting products were identified on the basis of their ^{31}P NMR chemical shifts. The aminoacylation of 5′-AMP proceeded completely when DCC was used as a condensing reagent under anhydrous conditions. The synthesis of aminoacyl-adenylate analogs substituted with sulfur and a methylene group for the 5′-phosphoryl oxygen and ribosyl 5′-oxygen atoms, respectively, were also examined under various conditions. The ^{31}P NMR analysis of these reactions revealed that the 5′-thiophosphate analogs (aa-AMPSs) substituted with sulfur for the 5′-phosphoryl oxygen were formed as main products by the condensation of 5′-AMPS and amino acids by use of diphenyl phosphorochloridate as a condensing reagent, while 5′-methylenephosphonate derivatives (aa-AMPCs) were formed quantitatively by use of DCC as a condensing reagent.

Introduction

In protein biosynthesis, it is known that aminoacyl-tRNA synthesis is a very important step. This reaction is performed *via* two steps catalyzed by cognate aminoacyl-tRNA synthetases (ARSs) [equilibria (1) and (2), Chart 1]. The first step is ATP-dependent activation of amino acids, giving rise to aminoacyl-adenylates (aa-AMPs), which are extremely unstable but exist as complexes with cognate ARSs.[1] The second involves the terminal 2′- or 3′-aminoacylation of the 3′-CCA end of tRNAs.[2,3] Recent progress in biotechnology makes it possible to synthesize artificial peptides and proteins *via in vitro* and *in vivo* systems.[4] These studies require large quantities of aa-AMP as key intermediates.

$$\text{aa} + \text{ATP} + \text{ARS} \rightleftharpoons \text{aa-AMP-ARS} + \text{PPi} \quad (1)$$

$$\text{aa-AMP-ARS} + \text{tRNA} \rightleftharpoons \text{aa-tRNA} + \text{AMP} + \text{ARS} \quad (2)$$

Chart 1 Biosynthesis of aminoacyl-tRNA.

The chemical synthesis of such unstable aa-AMPs was reported by several laboratories in the 1950s.[5–11] Most of these synthetic strategies involve dehydration between amino acids and 5′-AMP. DeMoss *et al.* prepared aa-AMPs by condensing amino acid chlorides, prepared by the reaction of amino acids with phosphorus pentachloride, with a silver salt of adenosine 5′-phosphate.[6] Some condensing reagents such as dicyclohexylcarbodiimide (DCC),[8–10] ethyl chloroformate[11] and diphenyl phosphorochloridate,[11] have been used to synthesize aa-AMPs.

However, the purity of aa-AMPs was unsatisfactorily less than 80%, suggesting contamination with amino acids and 5′-AMP. It is known that the hydrolysis rate of aa-AMPs is dependent on the pH-value of the solution.[12] At neutral or alkaline pH, aa-AMPs tend to self-oligomerize to give oligopeptides[12,13] along with an imidazole-catalyzed side-reaction giving the 2′- and 3′-amino acid ester derivatives of 5′-AMP.[10b,c,14–16] Because of their inherent extraordinary instability in aqueous solution, isolation of aa-AMPs in pure form is very difficult. Actually, recent work has tried to reveal the inherent nature of aa-AMPs themselves.[17]

Some aa-AMP analogs have been synthesized to stabilize such unstable natural products. Some of them were designed as inhibitors for the aminoacylation reaction of ARSs.[18–20] Others were used for co-crystallization with ARSs and with complexes of ARSs and tRNAs.[21,22] In all cases, however, amino acids or mixed acid anhydride bonds were changed to stable structures such as aminoalkyl phosphoryl[18,19] and pyrophosphate[20] linkages, lacking the ability of aminoacylation of tRNAs. Therefore, it is necessary to synthesize aa-AMP analogs bearing mixed acid anhydride bonds capable of aminoacylation and to disclose the chemical property of such analogs. Since aa-AMPs possess a phosphorus atom, ^{31}P NMR spectroscopy can be applied to the analysis of the stability of these extremely unstable mixed-anhydride compounds. To our surprise, however, there have been only few papers of ^{31}P NMR studies concerning aminoacyl-AMPs to date.

In this paper, we report the detailed analysis of aminoacylation of 5′-AMP and its analogs with amino acids in the presence of several kinds of condensing reagents by means of ^{31}P NMR spectroscopy.

Results and discussion

Synthesis and properties of aminoacyl-adenylates (aa-AMPs)

Among several methods reported for the synthesis of aa-AMPs to date, the Berg method[9] has been frequently used as the hitherto superior one. This method involves condensation of amino acids with 5′-AMP in the presence of an excess of DCC as a condensing reagent. This reaction did not proceed completely. Moreover, at the purification step, there was considerable decomposition of aa-AMPs because of their intrinsic basicity and the nucleophilicity of the amino group of amino acids. As a result, the isolated yields of aa-AMPs were not so high. Later, Moldave *et al.*[10] reported an improved method using N-protected amino acids *via* a two-step reaction involving condensation and deprotection. Consequently, decomposition of aa-AMPs was significantly controlled so that their yields were improved to 60–90%. Subsequent to this paper, however, there has been a hiatus in the publication of reports on the chemical synthesis of aa-AMPs.

First, the Berg procedure[9] for the aminoacylation of 5′-AMP **1** with methionine (Met) **2m** to obtain methionyl-AMP **4m** was followed (Scheme 1): 5′-AMP was dissolved in pyridine–H_2O (1 : 4, v/v). To this solution were added 8 M HCl, 25 equiv. of DCC, and **2m** at 0 °C. Progress of this reaction was monitored

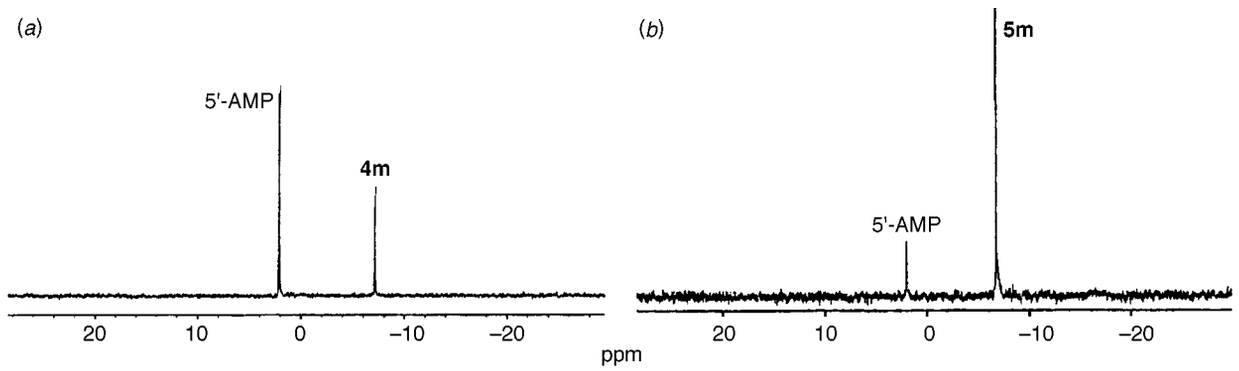


Fig. 1 ^{31}P NMR spectra of the aminoacylation reaction under aqueous conditions: (*a*) The reaction mixture obtained by the reaction of **2m** with 5′-AMP in the presence of 25 equiv. of DCC in 8 M HCl–pyridine (4 : 1) for 1 h; (*b*) the reaction mixture obtained by the reaction of **3m** with 5′-AMP in the presence of 20 equiv. of DCC in H_2O–pyridine (1 : 3) for 1 h.

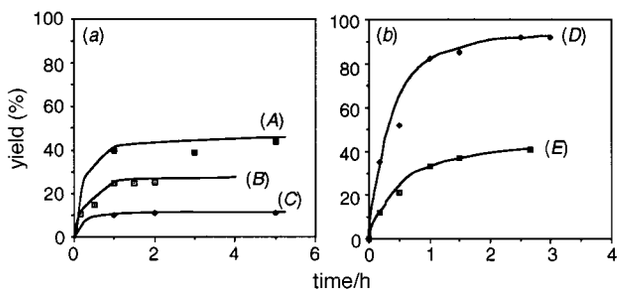
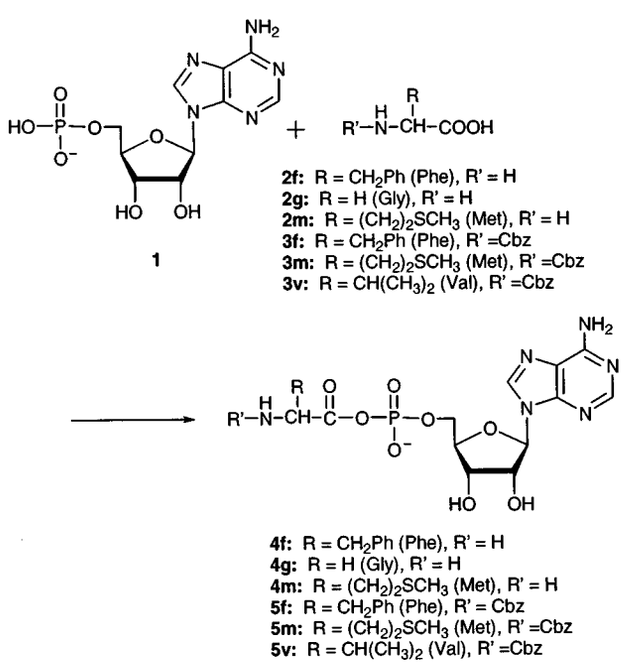


Fig. 2 Rate of formation of aa-AMP derivatives: (*a*) Curves (*A*), (*B*) and (*C*) correspond to **4f**, **4m** and **4g**, respectively. (*b*) Curves (*D*) and (*E*) correspond to **5m** and **5v**, respectively.



Scheme 1

by ^{31}P NMR at 0 °C. The time course of this reaction is shown in Fig. 2. As evidenced by Fig. 1, it turned out that we could directly measure the reaction proceeds. The starting material 5′-AMP showed a single signal at 2.1 ppm in H_2O–pyridine–pyridine-d_5 (16 : 4 : 5, v/v/v). The ^{31}P NMR spectrum measured after 1 h exhibited the resonance signal of a new product at −7.1 ppm along with the signal of AMP at 2.1 ppm [Fig. 1(*a*)]. Since the ^{31}P NMR chemical shifts of pyrophosphate derivatives appear usually around −9 ppm,[23] it was concluded that the new signal could be attributed to Met-AMP **4m**. The reaction was not complete even after several hours, reaching a plateau at about 25% yield after 1 h, and then did not proceed further [Fig. 2(*a*) curve *B*]. In addition, complete decomposition of **4m** was observed after 1 day. Similar DCC-mediated reactions of AMP with phenylalanine **2f** and glycine **2g** were also incomplete and gave the corresponding aa-AMPs **4f**, **4g** in only ≈45% and 10% yield, respectively [Fig. 2(*a*) curves *A* and *C*]. From the above results, it is noted that the efficacy of the reaction was highly dependent on the kind of side chain on the amino acids, and that prolonged reactions led to decomposition of aa-AMPs. Since decomposition of aa-AMPs was caused by the basicity and nucleophilicity of the amino group of the amino acids, the aminoacylation of AMP was carried out according to Moldave's procedure[10b] using an N-protected methionine, Cbz-methionine **3m**. A mixture of **3m** and **1** was dissolved in pyridine–H_2O (4 : 1, v/v) and 20 equiv. of DCC were added at room temperature. ^{31}P NMR analysis [Fig. 1(*b*)] showed that N-protected aa-AMP **5m** was formed in 92% yield after 1.5 h *without formation of any by-products* but the reaction did not reach completion, as seen in Fig. 2(*b*) (curve *D*). A similar reaction using Cbz-valine **3v** gave Cbz-Val-AMP **5v** in 41% yield after 2 h [Fig. 2(*b*) curve *E*]. The distinct difference in yield between **5m** and **5v** seemed to be due to the difference in hydrophobicity and to some steric effect against hydrolysis of aa-AMPs between the side chains of the two amino acids.

Table 1 The results of the synthesis of aa-AMP derivatives using various condensing reagents

Condensing reagent[a]	Ratio in the reaction mixture[b]		
	5′-AMP **1**	aa-AMP **5**	$(AMP)_2$
DCC	0	100	0
DIC	0	93	7
EDC	20	50	30
TPSCl	100	0	0
^{i}BuO(C=O)Cl[c]			
BOPCl	0	30	70
PSC	30	60	10

[a] All reactions were carried out by use of 1.1 equiv. condensing reagents in pyridine. [b] The ratio of AMP derivatives was estimated by ^{31}P NMR. [c] We were not able to determine the ratio of the products because many signals were observed around −7 ppm.

Since, apparently, competitive hydrolysis occurred during the aa-AMP synthesis described above, and hence the yield did not reach 100%, we tried the aminoacylation of AMP in anhydrous media using various types of condensing reagents. Our results are summarized in Table 1.

When 1.1 equiv. of DCC was added to a solution of **3m** and 5′-AMP (monotributylammonium salt), this reaction was completed in 3 h to give quantitatively **5m** as evidenced by ^{31}P NMR [Fig. 3(*a*)]. A similar aminoacylation of AMP with Cbz-Phe **3f** was also completed in 3 h to give Cbz-Phe-AMP **5f** as an almost single resonance in its ^{31}P NMR spectrum. It was, how-

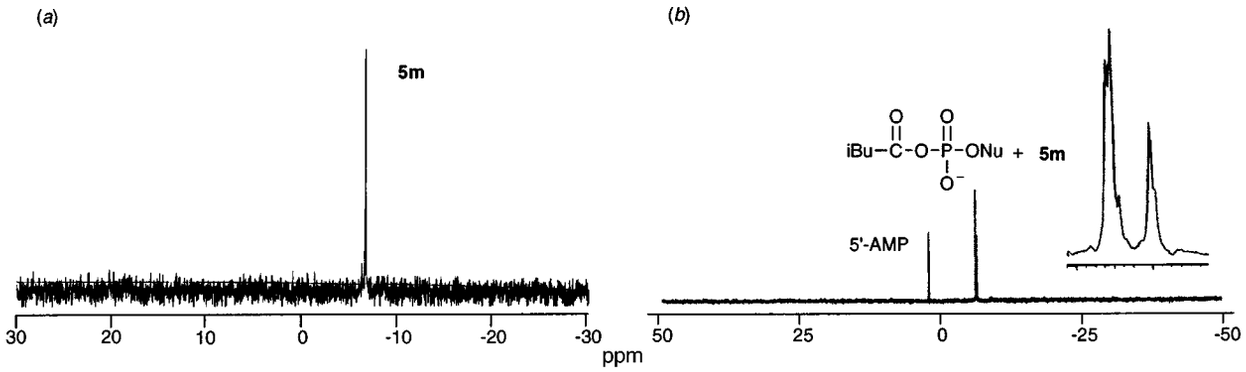


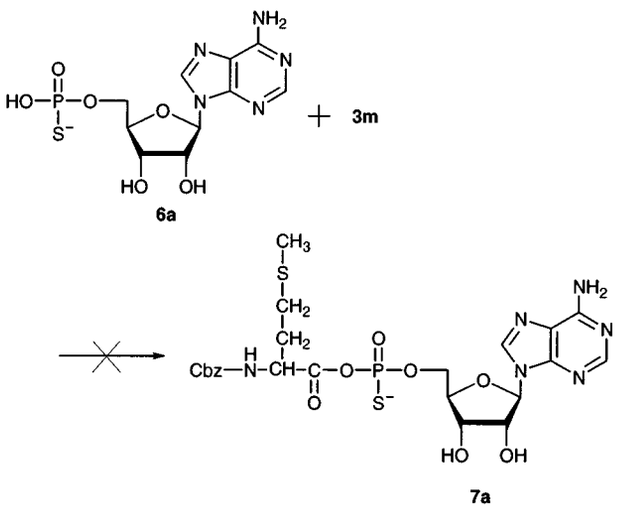
Fig. 3 ^{31}P NMR spectra of the aminoacylation reaction under anhydrous conditions: (*a*) The reaction mixture obtained by the reaction of **3m** with 5′-AMP in the presence of 1.1 equiv. of DCC in pyridine for 1 h. (*b*) The reaction mixture obtained by use of 1.1 equiv. of isobutyl chloroformate in pyridine for 1 h.

ever, observed that dicyclohexylurea (DCU) formed by the reaction could not be removed completely during the isolation. The use of 1.1 equiv. of diisopropylcarbodiimide (DIC) was added to a solution of **3m** and 5′-AMP in a similar manner, and the reaction resulted in completion within 2.5 h at room temperature, but a small amount of a pyrophosphate derivative $(AMP)_2$ (^{31}P NMR δ_P −9.4 ppm) was obtained in 7% yield. When 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) was employed, a considerable amount of $(AMP)_2$ was observed after 35 min and the yield of **5m** was only 68%. It seems that the reason for the formation of the pyrophosphate derivative is due to the reaction of 5′-AMP with an N-protected aa-AMP derivative. Unexpectedly, it was, however, found that the pyrophosphate derivative was the main product in the case of polymer-supported carbodiimide (PSC) derivative[24] as a condensing reagent. Therefore, since the two-phase reaction using PSC proceeds very slowly and 5′-AMP has low solubility in organic solvents, the main product was a pyrophosphate derivative. Other types of condensing reagents, such as acyl chloride and sulfonyl dichloride were also tested, and the reactions were monitored by ^{31}P NMR, as summarized in Table 1. These condensing reagents were not suitable to give aa-AMPs.

Synthesis and properties of aminoacyl-adenylate analogs

Thiophosphate analogs (aa-AMPSs). In nucleic acid chemistry, it is well known that phosphorothioate derivatives, in which the non-bridged oxygen atom is replaced by a sulfur atom, are useful analogs of phosphate derivatives.[25] Such thiophosphate analogs have a pair of diastereomers arising from the chiral center on the phosphorus atom, and each stereoisomer has a different chemical and biological activity.[26] Adenosine 5′-monothiophosphate has shown interesting behavior with enzymes associated with nucleic acid metabolism.[27] It is interesting to see if thiophosphate analogs of aa-AMP are more or less stable than aa-AMPs. Therefore, we examined the stability of such analogs directly by means of ^{31}P NMR spectroscopy.

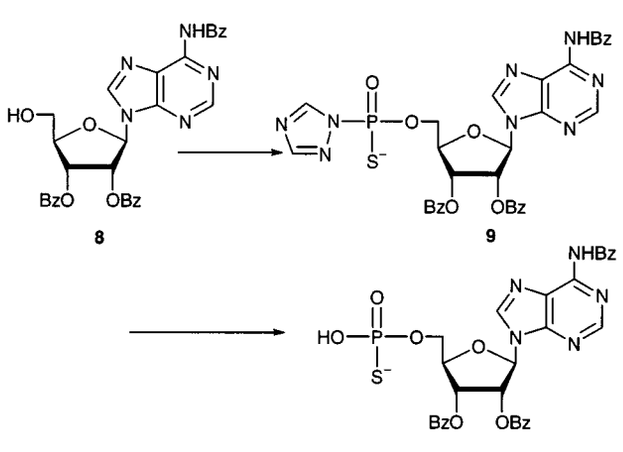
A 5′-AMP analog, adenosine 5′-monothiophosphate, was synthesized by the reaction of adenosine with thiophosphoryl trichloride in triethyl phosphate, as reported previously by Murray and Atkinson.[28] The attempted synthesis of a phosphorothioate analog **7a** of aa-AMP was carried out by use of the same procedure as described in the case of that of aa-AMP. When DCC or DIC was added to a mixture of 5′-AMPS **6a** and **3m**, the formation of Cbz-Met-AMPS **7a** was not observed in the ^{31}P NMR spectrum. Instead, a desulfurization reaction[29] occurred to give 5′-AMP and its aminoacylated product, Cbz-Met-AMP **5m** as main products. When 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl), 5′-AMPS **6a** and **3m** were mixed at the same time, the desulfurization reaction also proceeded. Therefore, two modes for addition of the reagents were



Scheme 2

employed for coupling between **6a** and **3m**. When isobutyl chloroformate was used as a condensing reagent, the aminoacylation occurred without desulfurization. However, the reaction of the condensing agent with the 2′- or 3′-hydroxy group was also observed by ^{31}P NMR.

Therefore, to avoid aminoacylation of the hydroxy group, 6-*N*,2′-*O*,3′-*O*-tribenzoyladenosine **8**[30] was used as the starting material for the synthesis of fully protected aa-AMPS, and the progress of formation of aa-AMPS was investigated by use of ^{31}P NMR. A fully protected 5′-thiophosphate derivative **6b** was synthesized (Scheme 3) by a modification of the method previously reported by Reese *et al.*[31] *via* the triazolide **9**. The 5′-thiophosphate derivative **6b** was allowed to react with



Scheme 3

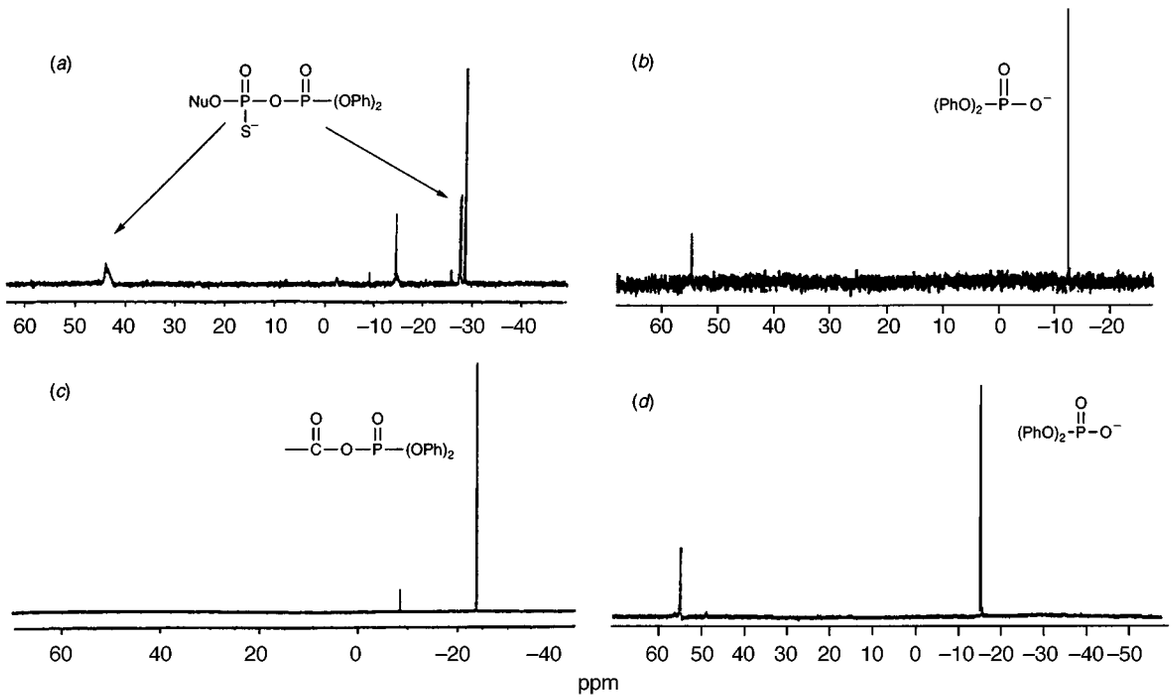
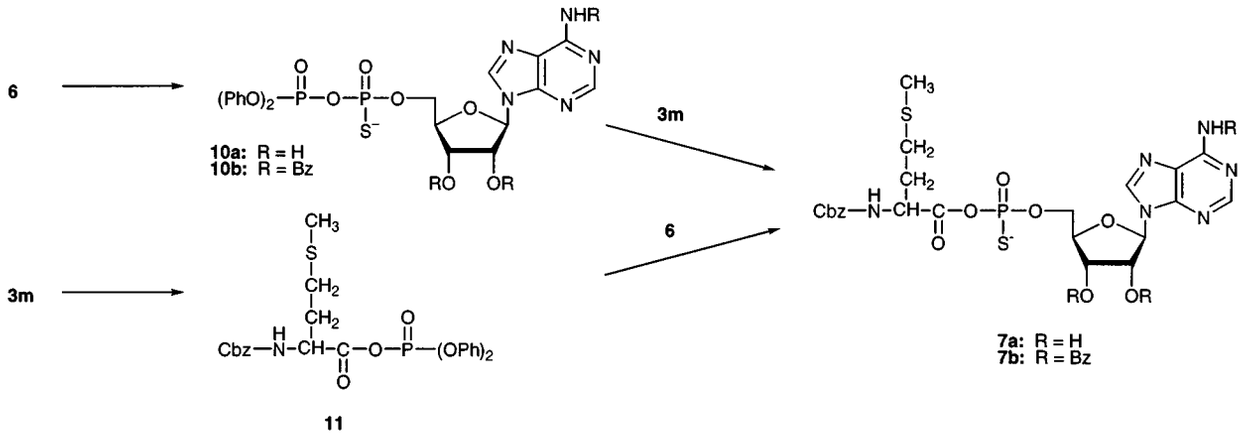


Fig. 4 ^{31}P NMR spectra of aa-AMPS: (*a*) The reaction mixture obtained by the reaction of 5′-AMPS with $(PhO)_2P(=O)Cl$ in pyridine for 30 min. (*b*) The reaction mixture after addition of **3m** in pyridine for 30 min. (*c*) The reaction mixture obtained by the reaction of **3m** with $(PhO)_2P(=O)Cl$ in pyridine for 30 min. (*d*) The reaction mixture after addition of 5′-AMPS in pyridine for 30 min.

5 equiv. of acetic anhydride to check the chemical shift of the resulting acetyl thio-AMP. This reaction gave two new signals around 51 ppm. These two signals were due to a diastereomeric mixture of the acetylated derivative on the basis of the emergence of a chiral center on the phosphorus atom. Next, we investigated the aminoacylation of 5′-AMPS using diphenyl phosphorochloridate [$(PhO)_2P(=O)Cl$] as a condensing reagent, which is commonly used as an activating reagent of thiophosphoryl groups.[32] When 2 equiv. of $(PhO)_2P(=O)Cl$ were added to the solution of compound **6b**, the mixed acid anhydride derivative **10b** was observed in the ^{31}P NMR spectrum showing its characteristic signals around −22 and 49 ppm. A doublet observed around −22 ppm indicated the signal which represents the phosphorus atom of the diphenoxyphosphoryl group that splits (^2J coupling) with the thiophosphoryl phosphorus atom. On the other hand, four signals around 49 ppm indicated the thiophosphate group that couples with the diphenoxyphosphoryl group and further splits due to the presence of the diastereomers. In addition, there was a new broad signal around 56 ppm which seemed to be due to Cbz-Met-AMPS **7b**. When H_2O was added to the reaction, a new signal around 57 ppm was observed as a sharp singlet, suggesting the complete hydrolysis of **7b** to give the AMPS derivative **6b**.

Next, an alternative method to synthesize Cbz-Met-AMPS **7a** was tried by use of $(PhO)_2P(=O)Cl$. This phosphorylating reagent reacted with the 5′-thiophosphate derivative **6a** to give a mixed acid anhydride intermediate **10a** [Fig. 4(*a*), Scheme 4] which *in situ* was treated with **3m**. This reaction gave *a new singlet signal* at δ_P 55.4 ppm [Fig. 4(*b*)]. If **7a** is formed, the resonance signal should be observed as a doublet because a pair of diastereomers due to the phosphorus chirality must be formed. However, it is also expected that a single peak would be observed if a rapid acyl-transfer reaction occurred between the geminal oxygen atoms of the thiophosphoryl group of **7a** *via* a 4-membered-ring transition state. To check more carefully if this intramolecular acyl transfer is possible, a different route to **7a** by activation of the amino acid component was tried. Therefore, the carboxy group of **3m** was first activated by treatment with $(PhO)_2P(=O)Cl$ to give the mixed acid anhydride derivative **11**. This mixed acid anhydride showed a characteristic ^{31}P NMR signal at −22 ppm [Fig. 4(*c*)]. After formation of this mixed acid anhydride, **6a** was added. The resulting mixture showed a single resonance signal at δ_P 54.7 ppm which is close to the chemical shift observed in the above experiment using **10a** [Fig. 4(*d*)]. The results of these independent experiments strongly suggested the possibility of such an intramolecular acyl transfer in **7a** that results in the appearance of a single resonance peak or that of superimposition of the two peaks of the diastereoisomers that show almost the same chemical shift. In addition to these spectroscopic data, paper electrophoresis of the mixture obtained in the above reaction revealed that the product moved with decomposition, showing a wide smear with mobility 0.3–0.74 relative to that of 5′-AMPS. As seen by this behavior of **7a**, it was indeed difficult to isolate **7a** because of its extraordinary instability in aqueous solution. It was found that **7a** was quickly hydrolyzed by addition of H_2O to the reaction mixture. It turned out that aa-AMPS is more unstable than aa-AMP because of the stronger acidity of 5′-AMPS than that of 5′-AMP. The pK_{a_2}-values of 5′-AMP and 5′-AMPS are 6.5 and 5.3, respectively.[33] In conclusion, it suggests that the essential factor for stabilization of aa-AMP and its analogs is the leaving ability of the phosphate or thiophosphate group in 5′-AMP and analogs. These results also suggested that hydrolysis of **7a** occurs *via* the attack of water on the carbonyl carbon.

Alkylphosphonate analogs (aa-AMPCs). According to the above discussion, it is expected that, if the modified phosphate group of a 5′-AMP analog has a smaller pK_{a_2} value and thus behaves as a weaker leaving group than the phosphate group of 5′-AMP, the corresponding aa-AMP analog might be more stable than aa-AMP. It has been reported that methylphosphonates have lower pK_a values than phosphate derivatives.[34] 5′-AMPC is the one where a methylene group is substituted for the 5′-bridged oxygen of 5′-AMP. This methylenephosphonate analog was synthesized through 7 steps from 2′,3′-*O*-isopropylideneadenosine by the method previously reported.[35]



Scheme 4

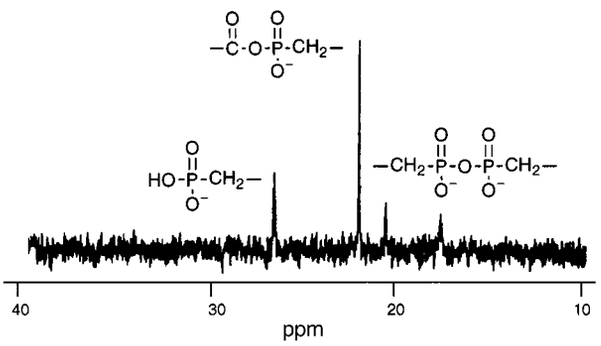
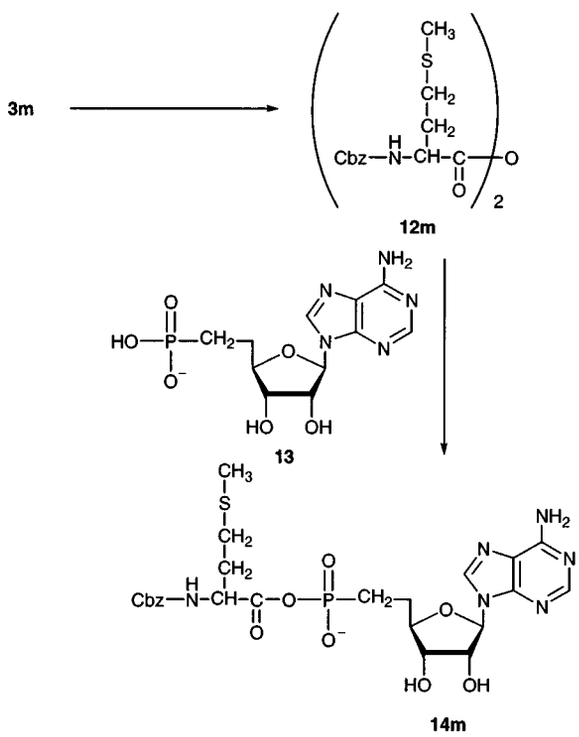


Fig. 5 ^{31}P NMR spectra of the reaction mixture obtained by the reaction of 5′-AMPC with 3 equiv. of **3m** preactivated by DCC in pyridine for 30 min.

When 5′-AMPC was treated with 5 equiv. of acetic anhydride in pyridine, a new signal for the acetyl phosphonate derivative was observed at δ_P 23.0 ppm in pyridine–pyridine-d_5 (4 : 1, v/v). When TPSCl was added to 5′-AMPC in place of acetic anhydride, a pyrophosphonate derivative was observed as a broad signal at δ_P 19.0 ppm.

Next, we examined the aminoacylation of 5′-AMPC with Cbz-Met **3m** by use of condensing reagents. It was found that a considerable amount of a pyrophosphonate derivative was formed when the amino acid or an appropriately protected 5′-AMPC derivative **13** was, in advance, activated by treatment with $(PhO)_2P(=O)Cl$. When the amino acid **3m** and **13** were added at the same time to 1 equiv. of DCC, the mixed acid anhydride **14m** and the pyrophosphonate derivative were observed (Scheme 5), and the ratio of these compounds was 1 : 3 in favor of the pyrophosphonate derivative. When the amino acid was pre-activated by treatment with DCC, the aminoacylated product was detected as the sole product **14m** without the pyrophosphonylation product. However, this reaction did not go to completion, and the amount of pyrophosphonate derivative produced increased with time. Therefore, 3 equiv. of amino acid relative to **13** were used. It was found that the aminoacylation reaction was complete within 30 min and was not accompanied with any by-products in the case of methionine, glycine and valine. When unprotected derivative of **13** was used as the starting material, it was found that the reaction did not proceed completely and that the pyrophosphonate derivative accumulated gradually. When **13** was treated with 7 equiv. of activated amino acid, the reaction was complete within 30 min without formation of the pyrophosphonate derivative. However, many signals were observed around δ_P 22 ppm, suggesting the concomitant aminoacylation of the 2′- or 3′- hydroxy group of the ribose moiety of **13** (see Fig. 5).



Scheme 5

Conclusions

In this paper, we described the reaction course of aminoacylation of AMP and its analogs under various conditions and also the stability of these aminoacyl-adenylate derivatives by means of ^{31}P NMR spectroscopy. It was found that DCC showed the best result, without pyrophosphorylation by-products. The synthesis of aminoacyl-adenylate analogs bearing mixed acid anhydride bonds was also monitored by ^{31}P NMR. The phosphorothioate-type analogs (aa-AMPSs) were successfully formed by the use of $(PhO)_2P(=O)Cl$ as a condensing reagent. On the other hand, the synthesis of alkylphosphonate-type analogs (aa-AMPCs) was accomplished by preactivation of amino acid derivatives by use of DCC.

Experimental

Pyridine was distilled after being refluxed over toluene-*p*-sulfonyl chloride for several hours, redistilled from CaH_2, and stored over 4 Å molecular sieves. ^{1}H NMR spectra were obtained at 270 MHz with tetramethylsilane as internal standard in $CDCl_3$. ^{13}C NMR spectra were obtained at 67.8 MHz with tetramethylsilane as internal standard in $CDCl_3$. ^{31}P NMR

Table 2 ^{31}P NMR chemical shifts of aa-AMP and aa-AMP analog derivatives

Compound	Chemical shift (ppm)	Solvent
4f	−7.3	[a]
4g	−7.8	[a]
4m	−7.1	[a]
5f	−6.8	[b]
5m	−6.9	[b]
5v	−6.9	[b]
6a	45.2	[c]
6b	56.1	[d]
7a	55.4	[b]
7b	56	[b]
10a	−27.5 (d, *J* 24 Hz), 44 (br)	[b]
10b	−22 (d, *J* 27 Hz), 49 (dd, *J* 24, 26 Hz)	[b]
5′-AMPC **13**	26.7	[b]
$(5'\text{-AMPC})_2$	17.8	[b]
14m	22.2	[b]

[a] Pyridine–0.3 M HCl–pyridine-d_5 (3 : 1 : 1, v/v/v). [b] Pyridine–pyridine-d_5 (4 : 1, v/v). [c] D_2O. [d] $CDCl_3$.

spectra were obtained at 109.25 MHz using 85% H_3PO_4 as external standard. Table 2 shows ^{31}P NMR chemical shifts of the compounds which were observed in these experiments. TLC was performed on precoated glass plates of Kieselgel 60 F_{254} (Merck, No. 5715). Silica gel column chromatography was carried out using Wakogel C-200.

^{31}P NMR study of the reaction of 5′-AMP with amino acids

5′-AMP **1** (83 mg, 0.24 mmol) was dissolved in pyridine–H_2O (3.2 ml; 1 : 4, v/v). To this solution were added 8 M HCl (0.03 ml), DCC (1.29 g, 6.25 mmol) and an amino acid **2** (0.25 mmol), and these solutions were vigorously stirred at 0 °C. The mixture (400 μl) was transferred to an NMR sample tube, and pyridine-d_5 (100 μl) was added. The reaction was monitored at 0 °C by ^{31}P NMR, and the yield of the aminoacyl-adenylate was estimated on the basis of the integration of the signals of **1** and **4**.

^{31}P NMR study of the reaction of 5′-AMP with N-protected amino acids under aqueous conditions

5′-AMP **1** (70 mg, 0.20 mmol) was dissolved in pyridine–H_2O (2.0 ml; 4 : 1, v/v). To this solution were added DCC (825 mg, 4.0 mmol) and an *N*-Cbz-amino acid **3** (0.20 mmol), and these solutions were vigorously stirred at rt. The mixture (400 μl) was transferred to an NMR sample tube, and pyridine-d_5 (100 μl) was added. The reaction was monitored at rt by ^{31}P NMR, and the yield of the aminoacyl-adenylate was estimated on the basis of the integration of the signals of **1** and **5**.

^{31}P NMR study of the reaction of 5′-AMP (monotributylammonium salt) with N-protected amino acids under anhydrous conditions

5′-AMP **1** (monotributylammonium salt) (47 mg, 0.1 mmol) and *N*-Cbz-methionine **3m** (28 mg, 0.10 mmol) were dried by repeated coevaporation three times with dry pyridine, and dissolved in dry pyridine (2.0 ml). To this solution was added a condensing reagent (0.11 mmol), and these solutions were vigorously stirred at rt. The mixture (400 μl) was transferred to an NMR sample tube, and pyridine-d_5 (100 μl) was added. The reaction was monitored at rt by ^{31}P NMR, and the yield of the aminoacyl-adenylate was estimated on the basis of the integration of the signals of **1** and **5m**.

Triethylammonium 6-*N*-benzoyl-2′,3′-di-*O*-benzoyladenosine 5′-*O*-thiophosphate 6b

To a dry THF solution (40 ml) of 1,2,4-triazole (2.07 g, 30 mmol), dried by repeated coevaporation with dry pyridine and toluene, were added triethylamine (1.28 ml, 30 mmol) and thiophosphoryl trichloride (1.0 ml, 10 mmol) at 0 °C. The mixture was stirred at rt for 1 h. To the mixture was added a solution of 6-*N*-benzoyl-2′,3′-di-*O*-benzoyladenosine **8** (1.15 g, 2 mmol, dried by repeated coevaporation with dry pyridine) in THF (20 ml). After being stirred for 3 h, triethylamine (3.0 ml) and H_2O (20 ml) were added to the mixture. The mixture was diluted with CH_2Cl_2 (100 ml) and pyridine (50 ml), washed three times with 0.5 M triethylammonium hydrogen carbonate, and the aqueous layer was back-extracted twice with CH_2Cl_2. The organic layer and washings were combined, dried over Na_2SO_4, filtered, and concentrated to give crude triethylammonium 6-*N*-benzoyl-2′,3′-di-*O*-benzoyladenosine 5′-*O*-thiophosphorotriazolide **9** as a yellowish oil. These crude products were not further purified, and the next reaction was carried out. The formation of **9** was determined by ^{31}P NMR: δ_P ($CDCl_3$) 47.17 and 47.39.

The crude **9** was treated with 80% AcOH (50 ml) at rt for 12 h. AcOH was removed by evaporation, and the residue was dissolved in pyridine (50 ml) and H_2O (100 ml) and washed three times with ether (50 ml), and the organic layer was back-extracted twice with H_2O. The aqueous layer and washings were extracted three times with $CHCl_3$, and the organic layer was back-extracted twice with 0.5 M triethylammonium hydrogen carbonate. The organic layer and washings were combined, dried over Na_2SO_4, filtered, and concentrated to give **6b** (1.10 g, 71%) as a yellowish foam: δ_P ($CDCl_3$) 56.12; δ_H 1.27–1.32 (9H, t, *J* 7.3 Hz, CH_3 of Et_3NH^+), 3.16–3.18 (6H, q, *J* 7.3 Hz, CH_2 of Et_3NH^+), 3.82–4.44 (2H, m, 5′-H_2), 4.66 (1H, m, 4′-H), 6.07–6.08 (1H, m, 3′-H), 6.21–6.24 (1H, m, 2′-H), 6.70 (1H, d, $J_{1',2'}$ 6.6 Hz, 1′-H), 7.18–8.21 (13H, m, ArH of Bz), 8.67 (2H, d, ArH of Bz), 8.73 (1H, s, 2-H), 9.21 (1H, s, 8-H); δ_C 8.58 (CH_3 of Et_3NH^+), 45.77 (CH_2 of Et_3NH^+), 65.17 (d, $^2J_{POC}$ 11.0 Hz, 5′-C), 73.55 and 75.26 (2′- and 3′-C), 83.79 (d, $^3J_{POCC}$ 8.5 Hz, 4′-C), 85.46 (1′-C), 122.64, 124.00, 128.08, 128.08, 128.09, 128.18, 128.28, 128.39, 128.46, 128.66, 128.98, 129.67, 129.72, 132.52, 133.42, 133.51, 133.73 (4-C of Bz), 136.93, 142.68, 148.86, 149.38, 152.13, 152.54, 164.72, 165.16, 165.26 (C=O of Bz); MS (FAB$^+$) Calc. for $C_{37}H_{41}N_6O_9PS$: *M*, 777.24. Found: 777 (M + H), 676 (M − Et_3N + H).

^{31}P NMR study of the reaction of 5′-AMPS with N-protected amino acids

Method A. 5′-AMPS **6a** (73 mg, 0.20 mmol) and *N*-Cbz-methionine **3m** (28 mg, 0.10 mmol) were dried by repeated coevaporation three times with dry pyridine, and dissolved in dry pyridine (2.0 ml). To this solution was added a condensing reagent (0.11 mmol), and the solution was vigorously stirred at rt. The mixture (400 μl) was transferred to an NMR sample tube, and pyridine-d_5 (100 μl) was added. The reaction was monitored at rt by ^{31}P NMR, and the yield of the aminoacyl-adenylate was estimated on the basis of the integration of the signals of **6a** and **7a**.

Method B. 5′-AMPS **6a** (73 mg, 0.20 mmol) was dried by repeated coevaporation three times with dry pyridine, and dissolved in dry pyridine (2.0 ml). To this solution was added a condensing reagent (0.11 mmol), and the solution was stirred at rt. After 1 h, *N*-Cbz-methionine **3m** (28 mg, 0.10 mmol) was added to this solution. The mixture (400 μl) was transferred to an NMR sample tube, and pyridine-d_5 (100 μl) was added. The reaction was monitored at rt by ^{31}P NMR, and the yield of the aminoacyl-adenylate was estimated on the basis of the integration of the signals of **6a** and **7a**.

^{31}P NMR study of the reaction of 5′-AMPC with N-protected amino acids

N-Cbz-methionine **3m** (14 mg, 0.05 mmol) was dried by repeated coevaporation three times with dry pyridine, and dissolved in dry pyridine (500 μl). To this solution was added

DCC (30 mg, 0.15 mmol). After being stirred for 1 h, 5′-AMPC **13** (19 mg, 0.05 mmol, dried by repeated coevaporation with dry pyridine) was added. The mixture (400 μl) was transferred to an NMR sample tube, and pyridine-d_5 (100 μl) was added. The reaction was monitored at rt by ^{31}P NMR, and the yield of the aminoacyl-adenylate was estimated on the basis of the integration of the signals of **13** and **14**.

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