Characterization of Acyl Adenyl Anhydrides: Differences in the Hydrolytic Rates of Fatty Acyl-AMP and Aminoacyl-AMP Derivatives

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An improved procedure has been developed to prepare $RCOOPO_2$-Ado ($R = C_6H_{13}$ and $C_{15}H_{31}$), the intermediate in the enzymatic synthesis of acyl-CoA's. The product has been characterized by spectral methods which, in turn, were used to define the hydrolysis rates of RCO-AMP. When R is a fatty acyl group, the hydrolysis rate is 10-fold slower than when R is aminoacyl. In both cases, the hydrolysis rate is enhanced by Mg^{2+}. We speculate that the rate acceleration is due to intramolecular participation of the second carbonyl group in the aminoacyl residue.

Introduction

The formation of acyl derivatives of coenzyme A is catalyzed in biological systems by a group of ATP-dependent acyl-CoA synthetases (EC 6.2.1.3). In recent years, the genes encoding several of these enzymes have been isolated from eukaryotes. These enzymes have been produced using recombinant DNA techniques, setting the stage for a detailed analysis of the enzyme's structure−activity relationships and host−guest interactions, as well as an assessment of the chemical basis for their catalysis.[1]

The overall process of acyl CoA formation is commonly accepted to occur as follows (eq 1).

$$RCOOH + ATP + Mg^{2+} + CoA\text{-}SH \rightarrow RCOSCoA + AMP + PP_i \quad (1)$$

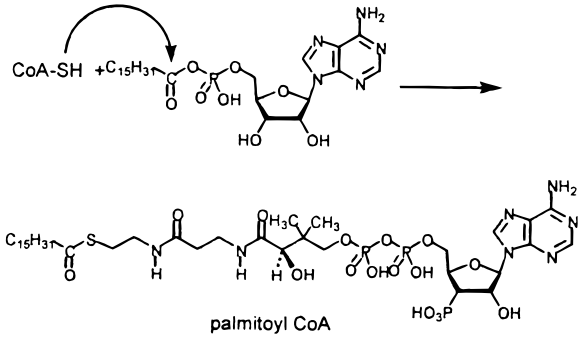
Berg[2] proposed that the activation of the carboxylic acid functional group by coenzyme A involves two steps (eqs 2 and 3):

$$ATP + RCOOH \rightarrow RCO\text{-}AMP + PP_i \quad (2)$$

$$RCO\text{-}AMP + HS\text{-}CoA \rightarrow RCOS\text{-}CoA + AMP \quad (3)$$

The existence of an acyl-AMP intermediate is attractive from the chemical perspective because it offers a solution to an obvious problem: how is RCOOH activated for attachment of the CoA activating group? Activation of the fatty acid carbonyl group for nucleophilic substitution by formation of a mixed phosphoryl carboxyl anhydride obviates the need for direct reaction between RCOOH and multifunctional CoA (Scheme 1). The mixed anhydride is presumably required to esterify the thiol functional group of CoA. In the absence of the mixed anhydride or some other activating group, carboxylate acylation of the thiol would likely fail.

Scheme 1



In contrast, aminoacyl activation by tRNA synthetases occurs by use of adenylate (mixed anhydride) rather than CoA (thioester).[3] The single-step activation seems to be a more primitive activation mechanism than that used for fatty acids. Therefore, we wondered if there were intrinsic chemical differences between the fatty acyl and aminoacyl phosphoryl mixed anhydrides that might explain why amino acid activation by coenzyme A has not been observed in biological systems. To address this question, we improved the standard method used for the synthesis of these mixed phosphoryl anhydrides, fully characterized them chemically and spectroscopically, and then determined their rates of hydrolysis.

Results and Discussion

Improved Method for the Synthesis of Acyl Adenylphosphoryl Anhydrides. Several methods have been used historically to synthesize a limited number of acyl adenylates. Acetyl adenylate was prepared enzymatically by Berg from sodium acetate using acetyl CoA synthetase, ATP, and $MgCl_2$.[2b] Berg also prepared the compound nonenzymatically from acetyl chloride and silver adenylate.[2c] In the same paper, a preparation is reported from adenylic acid and acetic anhydride in aqueous pyridine. A related aqueous pyridine preparation was used to prepare propanoyl adenylate.[4] Talbert

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(1) See, for example, Knoll, L. J.; Schall, O. F., Suzuki, I.; Gokel, G. W.; Gordon, J. I. *J. Biol. Chem.* **1995**, *270*, 20090−20097.

(2) (a) Berg, P. *J. Biol. Chem.* **1956**, *222*, 991, 1015, 1025. (b) Berg, P. *J. Biol. Chem.* **1956**, *222*, 1015. (c) Berg, P. *J. Biol. Chem.* **1956**, *222*, 1025. (d) Berg, P. *Science* **1957**, *129*, 895.

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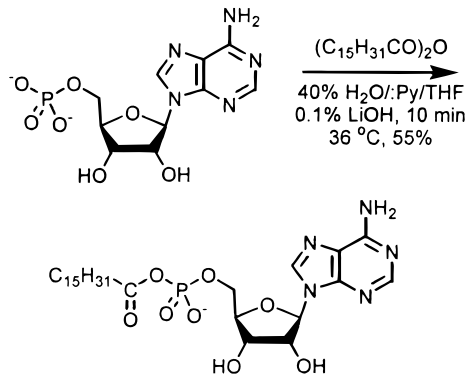
Scheme 2



and Huennekens used dicyclohexylcarbodiimide to couple adenylic acid and butyric acid in aqueous pyridine.[5] Butyroyl adenylate was also prepared by Peng from butyric acid anhydride and adenylic acid in aqueous pyridine.[6] Hexanoyl adenylate was obtained by Whitehouse and co-workers[7] by using an analogous approach from hexanoyl anhydride.

Vignais and Zabin (V−Z) described the standard method used for synthesizing palmitoyl adenyl anhydride.[8] This involves reaction of adenylic acid with a carboxylic acid anhydride (Scheme 2). The V−Z synthesis involves LiOH-mediated reaction of AMP (adenyl phosphate) with palmitoyl anhydride in aqueous solution to afford the mixed anhydride in approximately 50% yield.[10] This reaction occurs in the opposite sense to the one postulated for biological systems, i.e., the phosphate rather than the carboxyl group serves as the nucleophile (cf. eq 2).

There are three obvious reactions that could diminish yield in the V−Z procedure. First, the palmitoyl anhydride may be consumed by reaction with the 6-amino group of AMP. This is an unlikely possibility: we found that 3′,4′-isopropylideneadenosine gave the amide when treated with palmitic anhydride in pyridine under stringent conditions (100 °C for 8 h) and then only as a mixture with the *O*-acylation product. Second, the use of Li^+ rather than Mg^{2+} may limit the efficiency of reaction between phosphate and the anhydride; Mg^{2+} is employed in biological systems[9] (see eq 1). Furthermore, Herschlag and Jencks[10] reported that Mg^{2+} enhances the rate of reaction between pyridyl-phosphates and various oxygen nucleophiles. Hydrolysis of the acyl-AMP derivative is a third possible contributor to poor yield in the V−Z reaction. The rate of hydrolysis should be base-dependent.

With these thoughts in mind, we examined the effects of cation and base on the yield of $C_{15}H_{31}COOPO_2$-Ado (**4**). This compound was selected as a model product because palmitate is among the most abundant fatty acids in eukaryotic cells and because Vignais and Zabin used it in their experiments.

Table 1. Influence of Metal Cation and Base on the Formation of Palmitoyl-AMP Mixed Anhydride (4) from AMP and Palmitic Anhydride[a]

exp no.	additive	equiv of M^+ or base	% yield
1	none		60 ± 2
2	$LiOH·H_2O$	0.5	79 ± 3
3	$LiOH·H_2O$	5.0	0[b]
4	NaOH	0.5	80
5	LiCl	0.5	58 ± 1
6	NaCl	0.5	58
7	$MgCl_2·H_2O$	0.5	56
8	$(i\text{-Pr})_2NEt$	0.5	84
9	Et_3N	0.5	84 ± 2

[a] The reactions were carried out according to the optimized procedure (see Experimental Section). [b] No product was isolated from the reaction mixture.

When neither cation nor base was present, the maximum yield obtained was ~60%. This was accomplished by conducting the reaction at 23 °C rather than 36 °C, increasing pyridine concentration (40 → 50%) so that the reactants were more soluble, and by doing an extractive workup (pH 3, ether) rather than simply precipitating the product with acetone. The cation was not the critical factor in determining yield: substitution of LiCl, NaCl, or $MgCl_2$ for LiOH (all at [salt] = 0.5 M) resulted in a yield of mixed anhydride equivalent to that obtained when both cation and base were absent (Table 1). The type of base used was less important than its concentration: the yield of product was similar (79−84%) if 0.5 M LiOH, NaOH, $(i\text{-Pr})_2NEt$, or Et_3N was used. When 5 equiv of LiOH was employed, no product was obtained (Table 1).

Characterization of the Vignais−Zabin Reaction Products. Previously reported characterizations of the V−Z reaction product, palmitoyl-AMP (**4**), have been limited to three qualitative tests: (i) the reaction of its carbonyl group with hydroxamic acid, (ii) diazotization of its free 6-amino group by reaction with nitrous acid, and (iii) periodic acid cleavage of its vicinal, nonacylated 2′,3′-hydroxyl groups.[11]

A series of spectroscopic studies was performed on 9 compounds to make unequivocal structural assignments to possible V−Z reaction products (Table 2). Compounds **1** and **2** (adenosine and AMP) were used as reference-controls. The fully acetylated derivative **3** of adenosine was used to determine the chemical shift changes induced by acylation of all hydroxyls and the 6-amino group. Acetyl was selected as the acyl residue in this case because its protons are observed as a singlet rather than as a >30 proton multiplet (see also **7**), as would be the case for palmitoyl. The palmitoyl group was isotopically labeled in compounds **5** and **6** to facilitate NMR spectral assignments. Compound **8** was a substrate in the hydrolytic studies described below. Finally, **9** is a ^{13}C-labeled version of **7** in which the ribosyl hydroxyls have been protected so that they could not be acylated.

^1H-NMR analysis revealed that proton chemical shifts for the 6-amino group were more pH-sensitive than those for the C2 and C8 protons of adenylate or for the ribosyl 1′-protons. These pH variations in signal position are illustrated in Table 3 for acetyl-AMP (**7**) in DMSO-d_6. Note that at pH 5.5 the NH_2 signal shifts upfield by 1.5 ppm.

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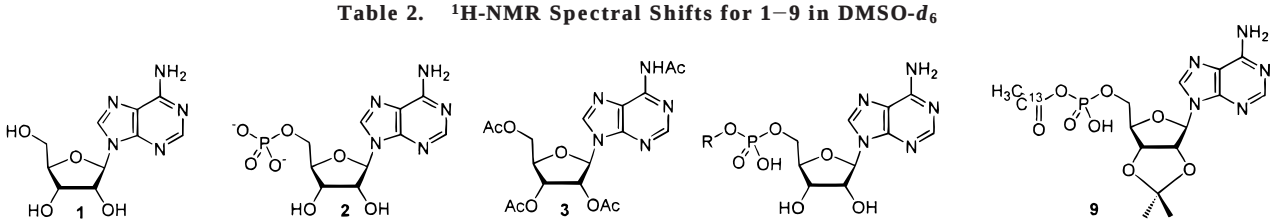
(11) Jencks, W. P. *Methods Enzymol.* **1963**, *6*, 762−766.

Table 2. ^{1}H-NMR Spectral Shifts for 1−9 in DMSO-d_6



4, R = $CO(CH_2)_{14}CH_3$
5, R = $^{13}CO(CH_2)_{14}CH_3$
6, R = $COCD_2(CH_2)_{13}CH_3$
7, R = $COCH_3$
8, R = $CO(CH_2)_5CH_3$

compound[a]	C2-H	C8-H	6-NH_2	H1′	amide	CH_nCO[b]	CH_3
1 adenosine	8.330	8.120	7.650	5.860	−	−	−
2 AMP·H_2O	8.351	8.165	7.482	5.911	−	−	−
	(8.97)	(8.61)	(8.18)	(6.80)	−	−	−
3 adenosine, 2′,3′,5′-*O*-trisacetate, 6-*N*-acetamide	8.691	8.265	−	6.206	9.300	2.597 2.131 2.088 2.058	
4 AMP-palmitic acid anhydride	8.593	8.331	8.680	5.926	−	2.317	0.835
	(9.15)	(8.62)	(8.20)	(6.85)	−	(2.45)	(0.85)
	[8.52]	[8.07]	[7.05]	[6.07]	−	[2.23]	[0.72]
5 AMP-palmitic acid anhydride (1-^{13}C-palmitate)	8.571	8.309	8.651	5.930		2.317	0.834
6 AMP-palmitic acid anhydride (2,2-d_2-palmitate)	8.571	8.309	8.644	5.931		−	0.835
7 acetyl-AMP	8.603	8.341	8.706	5.948	−	2.054	−
8 heptanoyl-AMP	8.589	8.331	8.683	5.933	−	2.325	0.814
9 adenosine-2′,3′-*O*-isopropylidene-5′-*O*-(1-^{13}C-acetate)−6-*N*-(1-^{13}C-acetamide)	8.679	8.152	−	6.141	∼9.1	1.954 2.600	−
	(8.840)	(8.795)	−	(6.656)	(12.363)	(1.899) (2.701)	− −

[a] Concentrations were ∼20 mM. Resonance positions are given in ppm downfield of residual CD_3SOCD_2H (δ2.490) or pyridine (δ7.190). Values in parentheses refer to chemical shifts in pyridine-d_5 solutions; those in brackets refer to solutions in D_2O:H_2O:C_5D_5N:THF-d_8 (0.1:0.03:0.2: 0.67, v/v). [b] For compounds **3** and **7**, shifts refer to the acetyl methyl group. Infrared spectral data are included in the Experimental Section.

Table 3. Medium pH Dependence of Adenine ^{1}H-NMR Resonances in AMP-Acetic Anhydride, 7

	chemical shifts[a] for			
pH[b]	H1′	C8-H	C2-H	6-NH_2
2.0	5.943	8.346	8.612	8.786
3.9	5.942	8.343	8.607	8.756
5.5	5.897	8.126	8.424	7.260

[a] [**7**]=20 mM (DMSO-d_6), calibrated against the 2.490 ppm signal of internal CD_3SOCD_2H. [b] Acidity was adjusted with 5% aq HCl.

The ^{1}H-NMR spectral assignments were then used to confirm that the compound obtained from both the original Vignais−Zabin procedure and from our modified method was, in fact, palmitoyl-AMP (**4**, Table 2 and Experimental Section). ^{31}P- and ^{13}C-NMR spectral analyses revealed no cross signal from the ^{13}C nucleus of ^{13}C-enriched palmitoyl-AMP (**5**) to the NH of adenine (see Experimental Section), thus confirming the absence of acylation at the 6-NH_2 group.

The Effects of Cation on Hydrolytic Sensitivity of $C_{15}H_{31}COOPO_2$-Ado. Thin layer chromatographic analysis indicated that palmitoyl-AMP underwent hydrolysis to palmitate and AMP when stirred at ambient temperature in 5:95 (v/v) H_2O:C_5H_5N for 1−2 h. We determined the kinetics of hydrolysis at 37 ± 0.1 °C for both palmitoyl-AMP, **4**, and the more soluble heptanoyl derivative, **8**. To do so, we followed changes in their ^{31}P-NMR spectra because the ^{31}P-resonances of acyl-AMP and 5′-AMP are separated by nearly 12 ppm (Table 4). Both **4** and **8** (25 mM) were studied in 20% DMSO-d_6/80% 150 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (v/v) at pH = 9.1. The plot of [RCOO-PO_2-Ado] (as ln(^{31}P integral)) vs time was linear in both cases over a period of ≥3 half-lives (e.g., Figure 1). From the equations of the best exponential fits, $[C_7AMP] = 90.6e^{-(-0.001588)t}$ and $[C_{16}AMP] = 90.1e^{-(-0.001605)t}$, the hydrolysis rate constants were calculated to be similar: 1.59×10^{-3} min^{-1} for **8** and 1.61×10^{-3} min^{-1} for **4**. This translates to a half-life ($t_{1/2}$) of 438 min for heptanoyl-AMP and 432 min for palmitoyl-AMP under the experimental conditions used. In separate experiments, we added a cation ([$MgCl_2$] = 50, 100, or 150 mM). At [$MgCl_2$] = 100 mM, the hydrolysis rates for both **4** and **8** increased ∼10-fold to $\sim 1.7 \times 10^{-2}$ min^{-1}. As can be seen in Figure 1, incrementally increasing the concentration

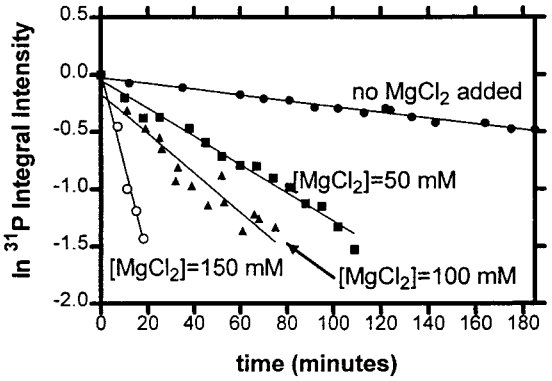


Figure 1. ^{31}P-NMR-monitored hydrolysis at 37 °C of heptanoyl-AMP, **8**. [**8**] = 25 mM. ● [$MgCl_2$] = 0 mM; ■ [$MgCl_2$] = 50 mM; ▲ [$MgCl_2$] = 100 mM; and ○ [$MgCl_2$] = 150 mM; lines (—) represent ln ^{31}P-integral intensity values for the ^{31}P resonances of **8**.

Table 4. ^{31}P-NMR and Hydrolysis Data for Fatty Acyl and Aminoacyl-AMPs[a]

compd no.	compd[b]	additive	K_{hydrol} (min^{-1})	$t_{1/2}$ min	δ(^{31}P) ppm before hydrolysis	δ(^{31}P) ppm after hydrolysis
8	C_6H_{13}CO-AMP	–	1.6×10^{-3}	430	−7.188	4.294
8	C_6H_{13}CO-AMP	50 mM $MgCl_2$	1.2×10^{-2}	58	−7.13	3.88
8	C_6H_{13}CO-AMP	100 mM $MgCl_2$	1.7×10^{-2}	41	−7.126	3.885
8	C_6H_{13}CO-AMP	150 mM $MgCl_2$	8.2×10^{-2}	9	−7.12	3.89
4	$C_{15}H_{31}$CO-AMP	–	1.6×10^{-3}	430	−7.280	4.24
10	Cbz-A-AMP	–	1.4×10^{-2}	49	−7.076	4.240
10	Cbz-A-AMP	100 mM $MgCl_2$	5.0×10^{-2}	14	−7.229	3.706
11	Cbz-A-A-AMP	–	1.9×10^{-2}	37	−7.110	4.194
11	Cbz-A-A-AMP	100 mM $MgCl_2$	7.2×10^{-2}	10	−7.131	3.886

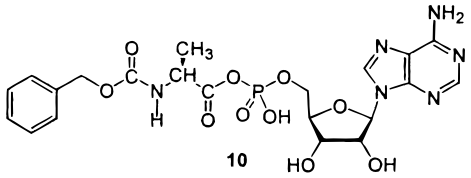
[a] ^{31}P-derived hydrolysis data and chemical shifts were determined in 20% DMSO-d_6:150 mM TAPS (pH = 9.1) solutions. Concentrations of the mixed anhydride species were 15 mM. [b] C7 and C16 stand for heptanoyl and hexadecanoyl, respectively. Cbz and "A" stand for carboxybenzyloxy and alanine, respectively.

of $MgCl_2$ from 50 mM to 150 mM ([**8**] = 25 mM) enhanced the hydrolysis rate correspondingly. Since 4 equiv of a divalent salt should have completely occupied all of the donor sites on **8**, the increase in rate may be a medium effect. Indeed, the largest rate increment is observed between 0 and 50 mM of added $MgCl_2$.

Together, these observations indicate that fatty-acyl-AMP's are sensitive to hydrolysis, that this hydrolysis does not appear to be affected by varying acyl chain length from heptanoyl to hexadecanoyl, and that the presence of a salt containing a Lewis acidic cation accelerates the hydrolytic cleavage. Herschlag and Jencks[4] have also observed a Mg^{2+}-dependent acceleration of the hydrolysis rate in a chemically related but distinct system: they thoroughly studied the reactions of $RCOO^-$ with phosphorylated pyridines, picolines, and morpholines and showed how Mg^{2+} catalyzes the process.

Hydrolytic Sensitivity of Aminoacyl-AMP Derivatives. Aminoacyl adenylates have been prepared previously by De Moss and co-workers[12] who condensed aminoacyl chlorides with silver adenosine 5′-phosphate. Berg also obtained aminoacyl adenylates by reaction of amino acids with ADP in the presence of DCC.[13] Moldave, Castelfranco, and Meister[14] prepared 15 *N*-carbobenzoxyaminoacyl adenylates by a similar, DCC-mediated condensation reaction in 40−81% yields. They noted that the non-nitrogen-protected aminoacyl adenylates "are extremely unstable and rapidly undergo hydrolysis and other reactions including conversion to a compound which appears to be the amino acid ester of the 2′ (or 3′) hydroxyl group or adenosine 5′-phosphate". Kellerman[15] studied the preparation and reactions of benzoyl adenylate and noted that it "is seen to be quite stable in acid and to be considerably more stable in alkali than is acetyl adenylate.... Both are more stable than the aminoacyl adenylates which decompose rapidly at pH 8." In the case of benzoyl adenylate, the rate of hydrolysis was expressed as %-min^{-1} vs pH. The hydrolysis rate reported by Kellerman at 37 °C was ∼0 at pH values between 0 and 8. As the base strength was increased, reaction rate also increased until 100% hydrolysis was observed to occur in about 2 min at pH 13.

In our own studies, we compared the hydrolysis rates of aminoacyl-AMP and fatty-acyl-AMP derivatives. To do so, we prepared Cbz-Ala-AMP (**10**) and Cbz-Ala-Ala-AMP (**11**) by reaction of the *N*-protected amino acid or dipeptide with AMP in the presence of DCC in aqueous pyridine (see Experimental Section). A single ^{31}P-NMR signal was observed at 7.2 ± 0.1 ppm for both **10** or **11**. Hydrolysis of **10** or **11** (25 mM) at 37 ± 0.1 °C was followed in 20% DMSO-d_6 containing 150 mM [3-[[-tris-(hydroxymethyl)methyl]amino]-1-propanesulfonic acid (TAPS) (pH = 9.1) by monitoring the disappearance of the 7.2 ppm signal and the appearance of a new ^{31}P resonance at 4.2 ± 0.1 ppm.



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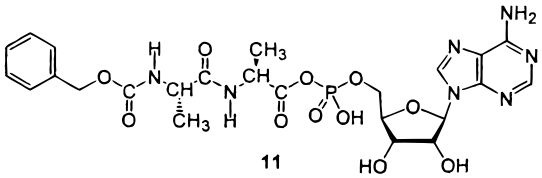

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Remarkably, the hydrolysis of Cbz-Ala-AMP (**10**) and Cbz-Ala-Ala-AMP (**11**) was 10-fold faster than the hydrolysis of **4** or **8** under comparable conditions (Table 4). When $MgCl_2$ was present (100 mM), the hydrolysis rates for **8** and **11** increased 8-fold and 4-fold, respectively.

Mechanistic Considerations. The ∼10-fold difference in hydrolysis rates between aminoacyl and fatty-acyl AMPs could reflect an electron-withdrawing effect of the amide residue relative to the alkyl chain: R′OCONHCHRCOPO$_2$Ado compared to R′COPO$_2$Ado. One way to assess this is to evaluate the Hammett parameters for acetic acid esters. The general formulation is $\log(k/k_0) = \sigma\rho$. For the ionization of acetic acid derivatives,[16] $\sigma_I = -0.03$ for the *n*-hexyl group, +0.26 for $NHCOC_2H_5$, and +0.44 for $OCON(CH_3)_2$.[17] Since the σ_I value for alkyl is essentially 0, and ρ is constant for the identical reaction conditions, the equation $\log(k/k_0) = \sigma\rho$, reduces to $\log(k/k_0) = \sigma_I$. Using this analysis and our data, $\log(1.4 \times 10^{-2}/1.6 \times 10^{-3}) = \log(8.75) = 0.94$. This value is significantly higher than expected for either an amide or carbamate substituent on acetic acid. A simple electronic effect does not appear to account for this modest but significant difference.

We believe that these observed differences in hydrolysis rates are more likely to represent differences in the

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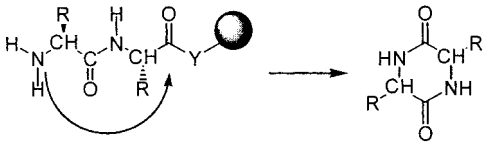
Scheme 3



rates of cleavage of an aminoacyl vs fatty acyl group from AMP rather than differences in the sites of cleavage. There is a single hydrolytic cleavage site in the fatty acyl AMP derivative. In the protected aminoacyl AMP derivative, hydrolysis could, in principle, occur at the urethane of the protecting group, the amide bond of the dipeptide (if present), or at the mixed anhydride link. Gisin and Merrifield noted that the free α-amino group of an anchored dipeptide can undergo the base-catalyzed, intramolecular reaction shown in Scheme 3.[18]

This reaction is unlikely to occur for these aminoacyl-AMP derivatives because there is no free amino group present that could initiate the cyclization. Alternatively, an amino group could be formed by hydrolysis of the Cbz protecting group to afford a dipeptide:

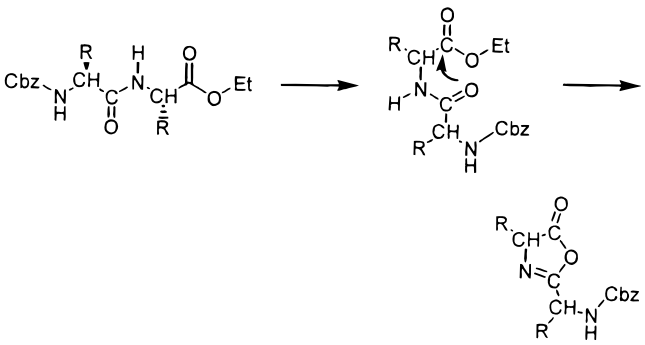
$$PhCH_2OCONHCHMeCONHCHMeCOO\text{-}AMP \rightarrow H_2NCHMeCONHCHMeCOO\text{-}AMP$$

The resulting H-Ala-Ala-AMP derivative could cyclize to give a diketopiperazine intermediate that, in turn, could lead to the observed hydrolytic cleavage product. However, to generate this intermediate, hydrolysis of the urethane link in the Cbz protecting group would have to be faster than hydrolysis of the mixed anhydride (activating group). Even in the unlikely event that urethane hydrolysis was faster than hydrolysis of the mixed anhydride, cyclization by this mechanism would not be possible for **11**.

Why should there be any difference in the hydrolysis rates of fatty-acyl and aminoacyl-AMPs at the carboxyl group? The carbonyl group of an amino acid might be more hindered than the corresponding functional group of the fatty acid, and therefore hydrolysis would be slower for the former. Our experimental results indicate that the opposite is true. A plausible explanation for the rate difference is that there is anchimeric assistance to hydrolysis of the aminoacyl-AMP derivatives that is not possible for the fatty acyl derivatives. This point is illustrated in Scheme 4. As shown, the second carbonyl group in aminoacyl-AMP can serve as an intramolecular nucleophile to give the oxazolidinone. This is not possible for fatty-acyl-AMPs which lack the second carbonyl group. Thus, the reactivities of the aminoacyl and fatty acyl carboxyl groups would differ. The cyclization shown in the scheme is well-known: it is analogous to that observed when the terminal amino acid of a peptide is cleaved during the Edman degradation. Such a reaction would occur, presumably with the same rate, whether the AMP derivative was attached to a protected amino acid or to a protected dipeptide as in **11**.

(18) (a) Gisin, B. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1972**, *94*, 3102−3106. (b) Barany, G.; Merrifield, R. B. Solid-Phase Peptide Synthesis. In *The Peptides*; Gross, E., Meienhofer, J. Eds.; Academic Press: New York, 1979; Vol. 2, pp 1−284. (c) Pedroso, E.; Grandas, A.; de las Heras, X.; Eritja, R.; Giralt, E. *Tetrahedron Lett.* **1986**, 743−746. (d) Grant, G. A., Ed.; *Synthetic Peptides*; W. H. Freeman & Co.: New York, 1992; p 143.

Scheme 4



Assuming that the different rates of hydrolysis indicate that the aminoacyl AMP carbonyl group is more reactive than the carbonyl group of fatty acyl AMP, we speculate that the reaction of the two derivatives with the thiol of CoA to form the corresponding CoA thioester (see eq 2, above) would not only be equally feasible for both compounds but would be favored from the chemical perspective for aminoacyl AMPs. If so, this may have placed evolutionary pressure on the substrate recognition site of acyl-CoA synthetases to avoid ligation with amino acids.

Experimental Section

^{1}H-NMR spectra were recorded at either 300 or 500 MHz. ^{13}C- and ^{31}P-NMR spectra were recorded at corresponding frequencies. Proton chemical shifts are reported in ppm (δ) downfield from Me_4Si or using residual, nondeuterated, solvent ($CHCl_3$, δ 7.240; CD_3SOCD_2H, δ 2.490; or pyridine-d_4-H, δ 7.190) as a reference signal. Signals are reported in the following order: chemical shift, peak multiplicity (br = broad; s = singlet; d = doublet; t = triplet; m = multiplet), integration, and assignment. Phosphorus and carbon chemical shifts are reported relative to 85% H_3PO_4 (0.00 ppm) and pyridine-d_5 (triplet at 123.44 ppm) as references, respectively. Infrared spectra were recorded on an FT-IR instrument and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined in open capillaries and are uncorrected. High resolution mass spectrometric data were obtained at the Washington University Resource for Biomedical and Bioorganic Mass Spectrometry. TLC analyses were performed using silica gel 60 F-254. Silica gel was used for flash chromatography as originally described by Still et al.[19] All reactions were conducted under dry N_2 or protected from moisture with Drierite. THF and Et_2O were dried and distilled from sodium benzophenone ketyl. Anhydrous solvents were stored over activated molecular sieves (3 Å) and were dispensed by syringe under dry N_2. 1-^{13}C-hexadecanoic acid, 2,2-d_2-hexadecanoic acid, Cbz-Ala-Ala-OH, Cbz-Ala-OH, and deuterated NMR solvents were obtained commercially. All other solvents and reagents were of the best grade available commercially and were used without further purification unless otherwise stated.

Adenine, adenosine (1), adenosine-5′-monophosphate (2), and 2′, 3′-*O*-isopropylideneadenosine were obtained from Aldrich Chemical Co. and were used as received. In some cases, commercial AMP·H_2O was dehydrated by storing over P_2O_5 in vacuo (≤0.05 Torr) overnight.

1-^{13}C-Hexadecanoic Anhydride. 1-^{13}C-Hexadecanoic acid was dissolved in anhydrous Et_2O (30 mL) and stirred while a solution of DCC (865 mg, 4.2 mmol) in Et_2O (20 mL) was added dropwise during 20 min. The resulting white suspension was stirred (12 h) and filtered, and the filtrate was evaporated in vacuo. The residue was crystallized (hexanes) to yield the

(19) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

anhydride (850 mg, 90%) as white needles, mp 62−63 °C (lit.[20] mp for the nonlabeled compound: 64 °C). ^{1}H-NMR: 0.856 (t, 6H, CH_3), 1.230 (broad s, 48H, $(CH_2)_{12}$), 1.623 (m, 4H, CH_2-$CH_2{}^{13}C$═O), 2.410 (q, 4H, $CH_2{}^{13}C$═O, J_{HCC} = 7.2 Hz).

2,2-d_2-Hexadecanoic Anhydride. 2,2-d_2-Hexadecanoic acid was added to Ac_2O (10 mL, 105 mmol) and stirred and heated to reflux for 1.5 h. The solution was cooled, evaporated in vacuo to a light yellow syrup, dissolved in toluene (10 mL), evaporated, and then held under high vacuum (<0.5 Torr) overnight. The resulting waxy solid was crystallized from hexanes to afford the anhydride (840 mg, 88%) as a white solid, mp 63−64 °C. ^{1}H-NMR: 0.855 (t, 6H, CH_3), 1.233 (broad s, 48H, $(CH_2)_{12}$), 1.611 (m, 4H, CH_2CD_2C═O).

Adenosine-2′,3′,5′-*O*-trisacetate-6-*N*-acetamide, 3. To a solution of adenosine (200 mg, 0.75 mmol) in pyridine (20 mL) was added a solution of Ac_2O (1.52 g, 1.4 mL, 15 mmol) in pyridine (5 mL) during 5 min. The clear solution was heated under reflux for 8 h, cooled, and quenched with EtOH (5 mL). After evaporation of the solvent, the thick, yellow, oily residue was dissolved in minimum $CHCl_3$ and chromatographed (flash, SiO_2, 10% MeOH:$CHCl_3$) to afford **3** (180 mg, 55%) as a yellow gum. IR (CCl_4): 3246−2900, 1752, 1703, 1609, 1589, 1542, 1522, 1470, 1374, 1283, 1225, 1035 cm^{-1}. ^{1}H-NMR: 2.058 (s, 3H, CH_3C═O(O)), 2.088 (s, 3H, CH_3C═O(O)), 2.131 (s, 3H, CH_3C═O(O)), 2.597 (s, 3H, CH_3C═O(NH)), 4.320−4.450 (m, 3H, 5′-Hs + 4′-H), 5.637 (t, 1H, 3′-H), 5.927 (t, 1H, 2′-H), 6.223 (d, 1H, 1′-H), 8.265 (s, 1H, 8-H), 8.691 (s, 1H, 2-H), ∼9.3 (broad, ∼1H, amide-H, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calcd for $C_{18}H_{21}N_5O_8$: 436.1468; found: 436.1455.

Adenosine-5′-phosphoric Hexadecanoic Anhydride (palmitoyl-AMP), 4. AMP (200 mg, 0.58 mmol) was dissolved in 35 mL of 40% H_2O:pyridine (v/v) containing 0.1% (18 mg, 0.43 mmol) of LiOH·H_2O. The resulting clear solution was warmed to ∼40 °C, and a solution of palmitic anhydride (600 mg, 1.21 mmol) in THF (70 mL) was added at once. The initial white suspension turned clear in 1−2 min; the final temperature of the solution was 36 °C. After 10 min, the solution was diluted with acetone (250 mL, −20 °C), and the solution temperature was held at −20 °C. After 15 min, the mixture was filtered, and the last portion of supernatant and solids was combined and centrifuged; the remaining solvent was then decanted. The gellike solid was suspended in H_2O (25 mL) and the pH adjusted to ∼2 with 5% aq HCl. Et_2O (30 mL) was added and the two-phase system stirred vigorously and then centrifuged. The solid was separated, washed successively with ice-chilled acetone (2 × 15 mL) and Et_2O (2 × 15 mL), and then crystallized from H_2O:EtOH (1:15 v/v) to afford, after drying over P_2O_5 at 60 °C and 0.1 Torr overnight, **4** as a white, amorphous powder (185 mg, 55%) mp: ∼175 °C (shrink), 180−182 °C (dec). IR (KBr): 3292, 3127, 2919, 2850, 1758, 1702, 1608, 1560, 1508, 1470, 1420, 1242, 1080, 1030, 817 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 0.835 (t, 3H, CH_3), 1.207 (broad s, 24H, $(CH_2)_{12}$), 1.441 (broad s, 2H, CH_2CH_2C═O), 2.317 (t, 2H, CH_2C═O), 4.085 (broad s, 3H, 4′ and 5′-Hs), 4.166 (t, 1H, 3′-H), 4.563 (t, 1H, 2′-H), 5.926 (d, 1H, 1′-H), 8.331 (s, 1H, 8-H), 8.593 (s, 1H, 2-H), 8.680 (broad s, ∼2H, NH_2, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calcd for $C_{26}H_{44}N_5O_8P$: 586.3006; found: 586.2989.

Adenosine-5′-monophosphoric Hexadecanoic Mixed Anhydride (palmitoyl-AMP), 4. Improved Procedure. 5′-AMP·H_2O (200 mg, 0.548 mmol) was dissolved by magnetic stirring in a solution of 8 mL of 50% H_2O:pyridine (v/v) containing 23 mg (0.57 mmol) of NaOH. A solution of palmitic anhydride (570 mg, 1.152 mmol) in 8 mL of THF was added by pipet in three portions. A white suspension resulted immediately, and stirring was continued at room temperature. After 15 min, the suspension had slowly thinned to an almost clear solution. Et_2O (25 mL) was then added and the two-phase system stirred vigorously for 2 min.[21] After 1−2 min had elapsed, the ether phase was removed by suction using a water aspirator. This procedure was repeated four additional times. Distilled water (3 mL) was added to the resulting whitish gel, and the pH of this suspension was brought to ∼3 with 5% aq HCl. The gel turned into a white suspension and was extracted with Et_2O (3 × 30 mL) by stirring vigorously and separating the top organic layer by suction as before. The white solid was transferred to a 100−200 Å fritted funnel and the remaining aqueous solution filtered. Successive washings with 5% aq HCl (5 mL), acetone (3 × 5 mL), and Et_2O (2 × 5 mL), followed by overnight drying in vacuo (ambient temperature, over P_2O_5), afforded a white powder in 82% yield with properties identical to those of **4**.

Adenosine-5′-phosphoric-(1-^{13}*C*-hexadecanoic) Anhydride, 5. AMP (70 mg, 0.202 mmol) was dissolved in 40% H_2O:pyridine (v/v, 7 mL) containing LiOH·H_2O (8.5 mg, 0.20 mmol). The resulting clear solution was warmed (37 °C), and 1-^{13}*C*-hexadecanoic anhydride (200 mg, 0.40 mmol) in THF (12 mL) was added at once. The initial white suspension turned clear in 1−2 min; the final solution temperature was 36 °C. After 10 min, the solution was poured into acetone (80 mL, −20 °C). After 2 h in a freezer at −20 °C, the suspension was filtered, and the last portion of supernatant and solids were combined and centrifuged; the remaining solvent was decanted. The gellike residue was suspended in H_2O (20 mL), the pH was adjusted to ∼2 (5% aq HCl), Et_2O (20 mL) was added, and the two-phase system was stirred vigorously and then centrifuged. The solid was filtered (100−200 Å fritted funnel) and washed with acetone (−20 °C, 2 × 2 mL) and then with Et_2O (3 × 2 mL). After being dried in vacuo (0.2 Torr, ambient temp, P_2O_5), **5** was obtained (86 mg, 72%) as a white solid, mp ∼175 °C (darken) 179−180 °C (dec). IR (KBr): 3321, 3276, 2920, 2851, 1713, 1607, 1557, 1504, 1470, 1412, 1242, 1140, 1119, 1099, 923, 817 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 0.834 (t, 3H, CH_3), 1.208 (broad s, 24H, $(CH_2)_{12}$), 1.441 (m, 2H, CH_2-$CH_2{}^{13}C$═O), 2.317 (q, 2H, $CH_2{}^{13}C$═O, J_{HCC} = 7.2 Hz), 4.085 (broad s, 3H, 4′ and 5′-Hs), 4.169 (t, 1H, 3′-H), 4.563 (t, 1H, 2′-H), 5.930 (d, 1H, 1′-H), 8.309 (s, 1H, 8-H), 8.571 (s, 1H, 2-H), 8.651 (broad s, ∼2H, $-NH_2$, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calcd for $^{13}C^{12}C_{25}H_{44}N_5O_8P$: 587.3039; found: 587.3022.

Adenosine-5′-phosphoric (2,2′-d_2-hexadecanoic) Anhydride, 6. AMP (70 mg, 0.202 mmol) was dissolved in 40% H_2O:pyridine (v/v, 7 mL) containing LiOH·H_2O (8.5 mg, 0.20 mmol). The resulting clear solution was warmed to ∼37 °C, and a solution of 2,2′-d_2-hexadecanoic (palmitic) anhydride (200 mg, 0.40 mmol) in THF (12 mL) was added at once. A white suspension immediately resulted. This initial suspension became clear during 2−3 min. The final temperature of the solution was 36 °C. After 10 min, the solution was poured into acetone (80 mL, −20 °C), and the temperature was adjusted to −20 °C. After 1 h in a −20 °C freezer, the suspension was filtered. The resulting gellike material was dissolved in H_2O (2.5 mL), the pH was adjusted to ∼2 (5% aq HCl), Et_2O (20 mL) was added, and the two-phase system was stirred vigorously and then centrifuged. The solid was separated and washed with acetone (0 °C, 2 × 5 mL) and then with Et_2O (2 × 5 mL). After being dried in vacuo (0.2 Torr, ambient temp, P_2O_5), **6** (96 mg, 80%) was obtained as a white solid, mp 176−181 °C (darken, shrink). IR (KBr): 3292, 133, 2921, 2851, 2773, 2360, 2344, 1756, 1654, 1649, 1609, 1558, 1508, 1471, 1420, 1323, 1240 1100, 1067, 1034 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 0.835 (t, 3H, CH_3), 1.207 (broad s, 24H, $(CH_2)_{12}$), 1.428 (m, 2H, CH_2CD_2C═O), 4.083 (broad s, 3H, 4′ and 5′-Hs), 4.169 (t, 1H, 3′-H), 4.563 (t, 1H, 2′-H), 5.931 (d, 1H, 1′-H), 8.309 (s, 1H, 8-H), 8.571 (s, 1H, 2-H), 8.644 (broad s, ∼2H, NH_2, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calculated for $C_{26}(^2H)_2(^1H)_{42}N_5O_8P$: 588.3131; found: 588.3124.

Adenosine-5′-monophosphoric Acetic Anhydride (acetyl-AMP), 7. AMP (300 mg, 0.86 mmol) was dissolved in 40% H_2O:pyridine (v/v, 27 mL) containing LiOH·H_2O (36 mg, 0.86 mmol). The resulting solution was warmed to 40 °C, and a solution of acetic anhydride (214 mg, 200 μL, 2.1 mmol) in THF (50 mL) was added all at once. The resulting white suspension quickly turned clear (t = 27 °C). After 10 min, the solution was poured into acetone (−20 °C, 300 mL). After 15 min in a

(20) *CRC Handbook of Chemistry and Physics*; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 1993−1994; pp 3−359.

(21) This procedure removes most of the pyridine which may otherwise foster hydrolysis of the carboxylic phosphoric anhydride.

freezer at −20 °C, the suspension was filtered, and the residual white gel was washed with acetone (10 °C, 20 mL) and Et_2O (15 mL). The resulting gellike solid was dissolved in H_2O (2.5 mL) and adjusted to pH = 2 (5% aq HCl). Absolute EtOH (−20 °C, 200 mL) was added, and the suspension was concentrated in vacuo to ∼100 mL (water bath temperature ∼35 °C) and then allowed to stand (rt) overnight. Filtration yielded **7** (180 mg, 54%) as an amorphous, white powder (R_f = 0.43 in *n*-BuOH:acetone:HOAc: 5% NH_4OH:H_2O [9:5:3:3:1]; mp ∼183 °C (darken), 205 °C (foam, dec). IR (KBr): 3439−3119, 1945, 1757, 1698, 1608, 1561, 1508, 1476, 1421, 1372, 1331, 1243, 1088, 1015, 925, 824 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 2.054 (s, 3H, CH_3C═O), 4.100 (m, 3H, 4′ and 5′-H's), 4.185 (m, 1H, 3′-H), 4.571 (t, 1H, 2′-H), 5.948 (d, 1H, 1′-H), 8.341 (s, 1H, 8-H), 8.603 (s, 1H, 2-H), 8.706 (broad s, ∼2H, $-NH_2$, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calcd for $C_{12}H_{16}N_5O_8P$: 390.0815; found: 390.0803.

Adenosine-5′-monophosphoric Heptanoic Anhydride (heptanoyl-AMP), 8. AMP (140 mg, 0.404 mmol) was dissolved in a solution of 14 mL of 40% H_2O:pyridine (v/v) containing $LiOH·H_2O$ (20 mg, 0.480 mmol). The resulting clear solution was warmed to ∼40 °C, and a solution of heptanoic anhydride (198 mg, 0.8 mmol, 2 equiv) in 24 mL of distilled THF was added in one portion. A very light white suspension quickly formed and disappeared almost immediately. After 10 min at 40 °C, the solution was poured into 200 mL of acetone held at −20 °C, resulting in precipitation of a gel. After 1 h in a freezer at −20 °C, the suspension was filtered. The resulting gellike material was dissolved in 1 mL of distilled water and the pH adjusted to ∼2 with 5% aq HCl. The white suspension was poured into a centrifuge tube, mixed, and washed with Et_2O (3 × 4 mL, centrifuged and decanted each time). After the third washing, the aqueous phase was also decanted, and the residual solid was placed in a fritted funnel (100−200 Å) and further washed with acetone (3 × 5 mL) and Et_2O (2 × 5 mL) and air-dried to yield a white powder. After being dried overnight in vacuo (<0.2 Torr, rt) over P_2O_5, 142 mg (71%) of the product was collected (mp turned light brown ∼168 °C, then shrunk at 177−178 °C and darkened. R_f = 0.68 in in *n*-BuOH: acetone:H_2O:5% NH_4OH: AcOH [9:5:3:2:2 (v/v)] on SiO_2. ^{1}H-NMR (DMSO-d_6): 0.814 (t, 3H, CH_3), 1.201 (broad s, 6H, $(CH_2)_3$), 1.444 (m, 2H, CH_2-CH_2C═O), 2.325 (t, 2H, CH_2C═O), 4.097 (broad m, 3H, 4′ and 5′-H's), 4.172 (t, 1H, 3′-H), 4.564 (t, 1H, 2′-H), 5.933 (d, 1H, 1′-H), 8.331 (s, 1H, 8-H), 8.589 (s, 1H, 2-H), 8.683 (broad s, ∼2H, NH_2, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calcd for $C_{17}H_{26}N_5O_8P$: 460.1597; found: 460.1586.

Adenosine-2′,3′-*O*-isopropylidene-5′-*O*-(1-^{13}*C*-acetate)-6-*N*-(1-^{13}*C*-acetamide), 9. 2′,3′-*O*-Isopropylideneadenosine (150 mg, 0.48 mmol) was dissolved in pyridine (8 mL)and 1-^{13}*C*-acetic anhydride (300 mg, 276 μL, 2.88 mmol) was added. The mixture was stirred at 90 °C for 2.5 h, and the solution was allowed to cool overnight while stirring. EtOH (5 mL) was added, and the mixture was evaporated to afford a nearly colorless syrup. EtOH (15 mL) was added, the mixture was evaporated, and the resulting gum was dissolved in minimal $CHCl_3$ and then purified by Chromatotron chromatography (Al_2O_3 rotor, 4 mm thickness, 5:95 (v/v) MeOH:$CHCl_3$). Product **9** (R_f = 0.23) was isolated (73 mg, 33%) from late fractions as a white foam. IR (CCl_4): 3229, 3119, 2992, 2938, 2361, 2343, 1707, 1674, 1660, 1609, 1587, 1559, 1542, 1470, 1372, 1258, 1212, 1106, 1078 cm^{-1}. ^{1}H-NMR: 1.379 (s, 3H, *i*-Pr-CH_3), 1.601 (s, 3H, *i*-Pr-CH_3), 1.954 (d, 3H, $CH_3{}^{13}C$═O(O), J_{HCC} = 6.9 Hz), 2.600 (d, 3H, $CH_3{}^{13}C$═O(NH), J_{HCC} = 6.6 Hz), 4.286 (m, 2H, 5′-Hs), 4.493 (m, 1H, 4′-H), 5.036 (dd, 1H, 3′-H), 5.455 (dd, 1H, 2′-H), 6.141 (d, 1H, 1′-H), 8.152 (s, 1H, H-8), 8.679 (s, 1H, H-2), 9.1 (broad s, ∼1H, amide-H, exchanged in D_2O). HRMS (FAB, $[M + H]^+$) calculated for $(^{13}C)_2(^{12}C)_{15}H_{21}N_5O_6$: 394.1637; found: 394.1622.

Adenosine-5′-monophosphoric *N*-(Carboxybenzyloxy)-alanyl Mixed Anhydride (Cbz-alanyl-AMP), 10. By a procedure identical to that for the preparation of **11** (see below), AMP (340 mg, 0.98 mmol), *N*-Cbz-Ala-OH (225 mg, 1.02 mmol), water (1.2 mL), pyridine (5.2 mL), concentrated HCl (90 μL, 1.1 mmol), and DCC (5.15 g, 25 mmol dissolved in 6 mL of pyridine) yielded mixed anhydride **11** as a white powder in 55%. ^{1}H- and ^{31}P-NMR integrals indicate that the powder consists of a mixture of AMP and Cbz-Ala-AMP in an approximately 3.5:6.5 ratio. ^{1}H-NMR (DMSO-d_6): 1.097 (d, 3H, $-CH_3$, J_{HH} = 6.6 Hz), 4.069 (m, 3H, 4′- and 5′-H's), 4.166 (t, 1H, 3′-H), 4.217 (m, 1H, O═C−C*H*-CH_3), 4.579 (t, 1H, 2′-H), 5.005 (s, 2H, $PhCH_2$), 5.933 (d, 1H, 1′-H), 7.322 (m, 5H, phenyl), 7.844 (broad s, 2H, NH_2, exchanged with D_2O), 8.220 (d, 1H, amide-NH, exchange with D_2O), 8.412 (s, 1H, 8-H), 8.532 (s, 1H, 2-H). ^{31}P-NMR: −7.076 (s).

Adenosine-5′-monophosphoric (*N*-carboxybenzyloxy-alanine)-*N*-alanine Mixed Anhydride (Cbz-alanyl-alanyl-AMP), 11. AMP (340 mg, 0.98 mmol) and *N*-Cbz-Ala-Ala-OH (300 mg, 1.02 mmol) were suspended in water (1.2 mL) and dissolved by addition of 5.2 mL of pyridine. Concentrated HCl (90 μL, 1.1 mmol) was added with a microsyringe causing some fumes to form. The clear solution was cooled to −10 °C in an acetone:ice bath, and then a solution of DCC (5.15 g, 25 mmol) in 6 mL of pyridine was added in one portion and stirred vigorously. After 1 h the cold bath was removed, and the reaction was allowed to warm to room temperature. The light white suspension thickened considerably with time. After 2.5 h (rt), the thick yellow suspension was filtered through a fritted funnel and the filtrate added to 150 mL of acetone held at −50 °C (dry ice:acetone bath). The resulting precipitate was quickly centrifuged in 30-mL batches while keeping the remaining suspension cold (−50 °C). The collected solids were transferred onto a 15−20 μm fritted funnel, washed with acetone (3 × 10 mL) and Et_2O (2 × 10 mL), dried for 5−10 min, and placed in vacuo (≤0.05 Torr) overnight to afford a white powder (305 mg, 51%). ^{1}H- and ^{31}P-NMR integrals indicate that the powder consists of a mixture of AMP and Cbz-Ala-Ala-AMP in an approximately 3:7 ratio. ^{1}H-NMR (DMSO-d_6): 1.025 (d, 3H, CH_3, J_{HH} = 6.6 Hz), 1.193 (d, 3H, CH_3, J_{HH} = 6.6 Hz), 4.072 (m, 4H, 4′- and 5′-H's, and O═CC*H*CH_3), 4.176 (t, 1H, 3′-H), 4.267 (m, 1H, O═CC*H*CH_3), 4.584 (t, 1H, 2′-H), 5.007 (s, 2H, $PhCH_2$), 5.935 (d, 1H, 1′-H), 7.331 (m, 5H, phenyl), 7.883 (broad s, 2H, NH_2, exchanged with D_2O), 8.235 (m, 2H, amide-NH's, exchange with D_2O), 8.402 (s, 1H, 8-H), 8.515 (s, 1H, 2-H). ^{31}P-NMR: −7.110 (s).

Hydrolysis Kinetics. Hydrolysis reactions were conducted in 5 mm NMR tubes containing the mixed anhydride species at a concentration of 25 mM. The solvent was a mixture of 20% DMSO-d_6 and 80% 150 mM aqueous TAPS buffer (v/v) at pH = 9.1. The temperature was held at 37.0 ± 0.1 °C. In those experiments in which $MgCl_2$ was present, its concentration in the final solution was 50 mM, 100 mM, or 150 mM. The ^{31}P signal, relative to external (sealed glass capillary) 85% H_3PO_4 was monitored. Samples were obtained as frequently as could be managed experimentally, typically 10−15 min between data points. A minimum of 15 data points was collected for each run except at [$MgCl_2$] = 150 mM which occurred too rapidly for this to be practical.

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