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AND NUCLEOTIDES

John C. Gilkey
(M.S. Thesis)

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SPECIFICITY IN REACTIONS OF AMINO ACIDS AND NUCLEOTIDES

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ABSTRACT

The 3'-terminal nucleotide triplet pCpCpA, found on all complete transfer RNA molecules, is required for attachment of an amino acid by the amino-acyl synthetase enzymes. A possible explanation for this specificity is that pCpCpA, perhaps because of unique conformation in solution, reacted faster with primordial activated amino acids, so that the amino-acyl synthetases evolved to recognize only this triplet.

A supposedly ideal system was chosen to investigate the above possibility. Investigations showed that the chosen system was unsuitable for the intended purpose.
I. INTRODUCTION

Apparently, all complete t-RNA molecules have the 3'-terminal nucleotide triplet pCpCpA (or CCA). In 1958, Hecht and co-workers\(^1\) found that CCA was required for attachment of an amino acid to the 3'-OH group of the terminal adenosine by the amino-acyl synthetase enzymes. Since this discovery, chemical evolutionists have been asking "Why CCA?". Some possible reasons are: (1) CCA, by pure chance, happened to be the best conformational fit for the first primitive equivalent of a ribosome and/or an amino-acyl synthetase enzyme, or (2) Perhaps CCA is, chemically, better suited for its role in peptide synthesis than all other possible nucleotide triplets; for example, CCA, again perhaps due to some conformational effect, may have reacted faster with primordial "activated amino acids", so that amino-acyl synthetases evolved to recognize only this triplet, or (3) Some combination of (1) and (2) above. General background material, and a review of the problem and of various approaches to its solution may be found in references 2, 3, 4, and 5.

II. THE PROJECT

The initial objective of this project was to determine the rates of coupling of activated amino acids with mono-, di-, and trinucleotides in hopes of finding a correlation between nucleotide sequence and reactivity. To this end were needed:
(1) A coupling reagent capable of esterifying a secondary alcohol in aqueous solution (not such an easy task - see reference 11) with minimum formation of side products.

(2) Appropriately modified substrates, such as N-protection for the amino acid (to prevent peptide formation) and blocking groups for the phosphate and amino functions (if any) of the nucleotide, with the restriction that any modifications must leave the substrates soluble in an aqueous medium, and under the somewhat tenuous assumption that all such modifications would have the same relative effect on the amino acid-nucleotide interactions.

(3) Some means of following the reaction quantitatively.

Kraevskii and co-workers\(^6\) had used a synthetic procedure for synthesis of 2'(3')-O-(amino-acyl) nucleotides which seemed to meet all the above requirements. The reaction scheme was:

\[
\begin{align*}
(\text{A}) & \quad H_3C-\text{BOC}-\text{NH}-C-C-OH \\
(\text{B}) & \quad + \quad \text{DMF} \\
(\text{C}) & \quad \text{r.t., 20 min.} \\
(\text{D}) & \quad \text{r.t., 3.5 hrs.} \\
(\text{E}) & \quad \text{H}_2\text{O}/\text{DMF, 5/1} \\
\end{align*}
\]

Equimolar quantities of tert-butyloxycarbonyl (t-BOC) alanine (A) and N,N'-carbonyldiimidazole (B) were allowed to react in DMF to form t-BOC alanyl imidazolidine (C) (reaction I). This mixture was then added to an aqueous solution of nucleotide (D) to yield a 2'(3')-O-(amino-acyl) nucleotide (E) (reaction II). The products were isolated by paper chromatography, using n-BuOH/HOAc/H\(_2\)O, 5/2/3 (Solvent A).
-3-

Apparently, the t-BOC alanyl imidazolide reacted with the secondary hydroxyl groups in aqueous solution with no detectable formation of side products, as indicated by UV spectra and a test for monosubstituted phosphate. Thus, the reaction scheme seemed to be ideally suited for this project.

III. EXPERIMENTAL SECTION

A. Introduction

Investigations were based upon modifications of the scheme given above. The nucleotide was AMP-5' or (to circumvent possible side reactions - discussed later) a derivative, ammonium methyl adenosine-5'-phosphate (MAMP-5'). In some cases, AMP-5'-8-14C was used to facilitate detection of products (by X-ray autoradiographs) and determination of relative yields (by liquid scintillation counting). The amino acids used were t-BOC alanine and carbobenzoxy (CBZ) glycine. For kinetic studies, ten-microliter aliquots were withdrawn from the imidazolide/nucleotide solution at timed intervals, spotted on Whatman chromatography paper, developed in Solvent A, and analyzed by UV-scanning and, where possible, autoradiography and beta-counting.

Two early kinetic runs by my predecessor4 (8/1, imidazolide/nucleotide, 25°C and 0°C) and one by me7 (1/1, imidazolide/nucleotide, 0°C) indicated that (1) the reaction was too fast to follow by manual sampling techniques, and (2) two nucleotide-containing products were observed (Kraevskii et al. reported only one product band with AMP-5'). After this, the project rapidly degenerated to the tasks of (i) isolating, purifying, and characterizing the amino acid imidazolide and
the products of the amino acid imidazolide/nucleotide reaction, and
(ii) modifying substrates and reaction conditions in attempts to
slow the reaction and alter product yield and distribution.

B. Isolation, Purification, and Characterization

1. t-BOC-L-alanyl imidazolide (t-BOC-Ala-Im): The initial prepara-
tive scheme involved reaction of equimolar quantities of t-BOC-alanine,
imidazole, and dicyclohexylcarbodiimide (DCC) in methylene chloride
at 0°C for twenty-four hours. Filtration of dicyclohexylurea (DCU)
and addition of hexane to the clear yellow supernate caused separation
of a viscous yellow oil. All attempts to obtain pure imidazolide from
the separated oil by crystallization failed. A small quantity of
white crystals with a melting range of 120-123°C (not the same as the
melting ranges of DCC, DCU, t-BOC-alanine, or imidazole) was isolated
by cooling a saturated hexane/CH₂Cl₂ solution of the yellow oil in a
freezer, causing separation of oil and a white precipitate. Warming
to room temperature redissolved the oil, and the white precipitate
was collected by suction filtration. Subsequent elemental and NMR
analyses indicated that the compound was not the desired imidazolide -
at least not in a chemically pure state. Attempts to recrystallize
the white precipitate only decomposed it. Column chromatography of
the yellow oil on Florisil (a commercial magnesium silicate) yielded
a clear, viscous oil, which became a white crystalline solid after
standing for two months in a vacuum desiccator. The solid decomposed
at about 290°C, characteristic of free alanine. Elemental analysis,
however, showed a composition (C, 46.72%; H, 6.92%; N, 10.83%) which
was not that of t-BOC-Ala-Im, t-BOC alanine, or free alanine.
Attempts to crystallize t-BOC-Ala-Im presumably prepared by reaction I above, using acetone as solvent, also yielded a viscous yellow oil. Chromatography of an oil sample on neutral alumina, using petroleum ether, diethyl ether, and DMF as eluants, yielded only large quantities of imidazole. It is, of course, possible (though highly undesirable) that the pure imidazolide is indeed a liquid at room temperature.

In all of the above preparations, the fact that a reaction had occurred was evidenced by vigorous evolution of CO₂ (see reaction I above). UV spectra of some of the "t-BOC-alanyl imidazolide" samples were almost identical to that of CDI, as would be expected, since models show that the two imidazole rings of CDI cannot be simultaneously coplanar with the carbonyl group - imidazole nitrogen plane. Therefore, the spectrum of CDI is that of the conjugated system comprised of one imidazole ring and the carbonyl moiety, just as in the t-BOC-alanyl imidazolide. This provides some evidence that t-BOC-Ala-Im was indeed present in the reaction mixture.

2. Carbobenzyloxy-glycine imidazolide (CBZ-Gly-Im): To be certain that I was capable of isolating an imidazolide, CBZ-Gly-Im was prepared by the method of Paul and Anderson. Equimolar quantities of CBZ-glycine and carbonyldiimidazole (CDI) were allowed to react in tetrahydrofuran (THF) until cessation of CO₂ evolution. Three volumes of diethyl ether were added and the resulting cloudy solution was placed in a freezer. Eventually, the imidazolide precipitated and was collected by suction filtration. The product was recrystallized from THF/ether, 1/3, filtered, and dried under vacuum. The melting range
of the white imidazolide was 113.5-114°C (Paul and Anderson reported 119-120°C). Elemental analysis - Calculated: C, 60.23%; H, 5.02%; N, 16.22%. Found: C, 60.06%; H, 5.20%; N, 16.30%. An attempt to prepare t-BOC-alanine imidazolide by the above procedure yielded, once again, a viscous oil.

3. Esters from t-BOC-Ala-Im and CBZ-Gly-Im: Since t-BOC-Ala-Im could not be isolated, ester preparations were attempted to help confirm the presence of the carbonyl imidazolide function. The method used was a modification of that of Staab. For t-BOC-alanyl esters, equimolar quantities of t-BOC alanine and CDI were allowed to react in CHCl₃. Then an equimolar quantity of alcohol (MeOH, n-BuOH, or t-BuOH) and a catalytic amount of NaOMe (for methyl esters) or KO-t-Bu (for other esters) were added, and the solution was allowed to stand for various periods (thirty to twenty-four hours). (Staab noted that reaction times of "a few minutes" were typical for preparation of esters from most acid imidazolides. As shown below, this is not the case for amino acid imidazolides.) Workup entailed removal of imidazole by extraction of the CHCl₃ solution with aqueous 1 N citric acid (chosen because of low CHCl₃ solubility and efficiency of imidazole removal), followed by removal of CHCl₃ under vacuum. The products were in all cases clear viscous oils. Some of the oils solidified and eventually became crystalline after standing for several days. Elemental analyses of the oils showed that none were the desired esters.

As a check of ester preparation from an amino acid imidazolide, CBZ-glycine-n-butyl ester was prepared by dissolving n-BuOH and
CBZ-Gly-Im in THF, followed by a catalytic amount of KO-t-Bu. The reaction was then allowed to proceed for various periods (thirty minutes to twenty-four hours). A preparation was performed using a 2/1 molar ratio of alcohol/imidazolide, with a reaction time of twenty-four hours. For workup, ten volumes of petroleum ether were added to the THF solution, and the mixture was placed in a freezer. The solvent was decanted from the clear crystals (imidazole) which formed. The solvent was then extracted twice with distilled water and evaporated by a stream of dry nitrogen, yielding large quantities of white, cottony crystals. The melting range of the crystals was 45-45.5°C. The product was pure by TLC (Eastman silica gel, acetone), and elemental analysis showed a composition of C, 63.52%; H, 7.23%; N, 5.24%. Calculated: C, 63.40%; H, 7.17%; N, 5.28%. The product was certainly CBZ-Gly-n-Bu ester. Workup of a "thirty-minute" reaction mixture yielded mostly impure CBZ-Gly-Im, indicating that a much longer reaction time was required.

4. Products of the imidazolide/nucleotide reaction (reaction II): Reasonable quantities (about fifty mg each) of the product t-BOC-alanyl AMP-5' derivatives probably could have been obtained by running about 100 paper chromatograms, but I saw no reason to be so wasteful of time, materials, and patience (both mine and that of my colleagues), so I developed a system using analytical TLC. The procedure was fairly simple. The t-BOC-alanyl imidazolide/nucleotide reaction mixture (see reaction II, above) was allowed to react for ten minutes at 0°C, spotted on small TLC strips along with t-BOC-alanine, imidazole, and AMP-5', and chromatographed in various solvent systems. The results
were analyzed by UV scanning, iodine adsorption, and ninhydrin spray. The TLC strips were prepared from 20 x 20 cm Brinkmann MN-polygram cel 300/N₂₅₄ cellulose plates, and 20 x 20 cm Eastman 6060 silica gel plates with fluorescent indicator. Cellulose TLC strips gave only two UV-absorbing spots and so were abandoned quickly. Silica gel strips gave three well-separated UV-absorbing spots (unreacted AMP-5' at about Rₚ 0.30, "products" at about Rₚ 0.75 and Rₚ 0.85), when used with the solvent system acetone/HOAc/H₂O, 7/1/2 (Solvent 1S). Basic (NH₄OH-containing) solvents, and non-hydroxylic solvents alone gave no separation, while pure water carried all derivatives with the solvent front.

Preparative TLC usually allows application of about 50 mg total material per 20 x 20 cm plate, but upon switching from analytical to preparative (1 mm) silica gel plates, overloading (characterized by severe tailing) occurred when more than 10 mg total material per plate was applied. My extraction procedure proved to be too inefficient to separate such small quantities of very polar compounds (such as the AMP-5' derivatives) from large quantities of silica gel.

Column chromatography of an imidazolide/nucleotide reaction mixture on silica gel using solvent 1S gave no detectable separation of the AMP-5' derivatives.

Although this preparative-scale work with the nucleotide/imidazolide reaction products was unsuccessful, fairly good evidence (see below) as to the identity of these products has been obtained using the small quantities available from paper chromatography.
C. Modification of Substrates and Reaction Conditions

To help prevent the formation of (or at least further destabilize) an intermediate amino-acyl adenylate, the methyl ester of the phosphoric acid moiety of AMP-5' was prepared by the procedure of Khorana et al., giving MAMP-5'. MAMP-5' was also found to be quite soluble in DMF (unlike AMP-5'), so hydrolysis of imidazolide by water could be avoided. MAMP-5' was used in several imidazolide/nucleotide reactions. The results, while not unambiguous, were somewhat interesting.

Two runs were performed in an aqueous reaction medium at 0°C, as in previous runs with AMP-5' (see references 4 and 6), with 1:1 molar ratios of t-BOC-alanyl imidazolide to nucleotide. One was analyzed by chromatography on silica gel with Solvent 1S, the other by paper chromatography with Solvent A. Both preparations gave only one product detectable by UV absorption. This suggested that perhaps formation of the second product had been blocked by the methyl group. However, in a previous run with AMP-5'-8-14C and a 1/1 imidazolide/nucleotide ratio, while only one product could be visualized by UV absorption, the second product showed up in an autoradiogram.

Three runs were performed in DMF alone; all had t-BOC-alanyl imidazolide/nucleotide molar ratios greater than unity (ranged from 2-4). One was analyzed by 1 mm silica gel TLC with Solvent 1S and showed a single UV-absorbing product. Two others were analyzed by paper chromatography in Solvent A and showed two UV-absorbing products. After twenty-four hours of reaction, an aliquot of one of the paper-chromatographic analyzed reaction mixtures was chromatographed with Solvent A on Whatman #3 paper. The product bands were eluted with
water, and UV spectra were taken between 200-300 nm. The spectra were identical to that of AMP-5' (and MAMP-5'), suggesting that no substitution had occurred on the adenine amino group of either product, as observed by Kraevskii. This, plus the knowledge that neither product could be an amino-acyl adenylate (far too unstable to survive chromatography with Solvent A), indicated that the second product may be a 2'- and 3'-0,0'-di (amino-acyl) ester of MAMP-5'. By analogy, the same conclusion was reached regarding the second product observed with AMP-5'. As shown below, fairly good evidence has been obtained in support of this hypothesis. (Note: The silica gel/Solvent IS system may not be capable of resolving the product MAMP-5' derivatives.)

Another interesting observation was that, in the above runs performed in DMF, even in the absence of a high H2O concentration and in the presence of a large excess of imidazolide, the t-BOC-alanyl imidazolide/nucleotide reaction did not appear to go to completion (not all MAMP-5' was converted to ester products).

D. Indirect Identification of the Products of Reaction II

For reasons outlined above, the two nucleotide-containing products observed for the reactions of t-BOC-Ala-Im with either AMP-5' or MAMP-5' were thought to be the 2' or 3'-0-(amino-acyl) monoester and the 2'- and 3'-0,0'-(amino-acyl) diester of each nucleotide. This possibility was investigated by simply determining the relative molar ratios of t-BOC-alanine to nucleotide for each product.

General procedure: Reaction sequences I and II, above, were used to obtain the t-BOC-alanine containing nucleotide products. All reactions were performed at room temperature, with a t-BOC-Ala-Im/
nucleotide molar ratio of 10/1 and reaction times of ~10 min (sequence I) and 2.5-3 hours (sequence II). In all runs, the total solution volume for reaction II was 600 \( \lambda \), with the solvent composition being either DMF/H\(_2\)O, 1/5, or pure DMF (see below). In a typical run, a 200 \( \lambda \) aliquot of the reaction mixture was spotted on each of two Whatman 3 mm sheets; in some cases, a 10 \( \lambda \) aliquot of a solution of pure nucleotide was spotted alongside the reaction mixture band. The sheets were then developed with Solvent A, and the desired bands (the "product" bands, and in some cases unreacted AMP-5' and unreacted t-BOC-alanine from hydrolysis of unreacted t-BOC-Ala-Im during development) were eluted with water. The molar ratios of t-BOC-alanine to nucleotide in the "product" eluates of each run were then determined by either of two methods.

**Method 1:** Two runs were performed using MAMP-5' as the nucleotide and pure DMF as solvent. The eluates of the product bands (one at \( R_f 0.75 \), the other at \( R_f 0.80 \), hereafter referred to as "upper" and "lower", respectively) were brought to 2 ml in small volumetric tubes. Ten \( \lambda \) aliquots of each eluate were brought to 1.01 ml with 0.01 N HCl, and UV spectra were taken between 200-300 nm to determine the concentration (and therefore the moles) of MAMP-5' in each eluate (\( \epsilon_{\text{max}} = 1.50 \times 10^4 \) at pH = 2, for MAMP-5'). Two ml of 1 N HCl were then added to each eluate, and the resulting mixtures were extracted with two 2 ml portions of diethyl ether to remove any unbound t-BOC-Ala. (Preliminary investigations of chromatographed reaction mixtures using ninhydrin spray indicated that the t-BOC-alanine band from the hydrolysis of unreacted t-BOC-Ala-Im was partially overlapping the lower...
product band, for both AMP-5' and MAMP-5' derivatives.) The extracted solutions were kept at 100°C in a steam bath for about six hours to hydrolyze the 2' and 3'-OH ester linkages of the products (this procedure also removes the adenine and phosphate moieties from the ribose). The solutions were then evaporated to dryness, and the moles of alanine in each were determined with a Beckman amino acid analyzer by the U.C. Berkeley Chemistry Department's microanalysis laboratory. The results were:

<table>
<thead>
<tr>
<th>moles amino acid</th>
<th>moles nucleotide</th>
<th>for</th>
<th>Run 1</th>
<th>Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>upper</td>
<td>lower</td>
<td>Run 1</td>
<td>0.871</td>
<td>0.833</td>
</tr>
<tr>
<td>lower</td>
<td></td>
<td>Run 2</td>
<td>2.42</td>
<td>2.16</td>
</tr>
</tbody>
</table>

As expected, one product ("upper") had an amino acid/nucleotide ratio near unity, while the other ("lower") product had an amino acid/nucleotide ratio near 2, providing fair support for the mono- and diester hypothesis.

Method 2: One run (Run 3) was performed using $^3$H-AMP-5' and $^{14}$C-t-BOC-alanine (both supplied by Schwartz/Mann) as substrates (both pure by chromatography and autoradiography), with DMF/H$_2$O, 1/5 as solvent. In two other runs (Runs 4 and 5), $^3$H-MAMP-5' and $^{14}$C-t-BOC-alanine were the substrates, with pure DMF as solvent. The $^{14}$C-t-BOC-alanine specific activity in Run 4 was about four times that of Runs 3 and 5. The eluates of the "unreacted nucleotide" and "unreacted t-BOC-alanine" bands were brought to 1 ml. The concentration of nucleotide was determined by UV absorbance measurements, and the $^{14}$C-t-BOC-
alanine concentration was determined using an amino acid analyzer (see Method I, above). One hundred \( \lambda \) samples of each eluate were applied to small strips of paper, and the papers were burned in a Packard Tri-Carb tritium/carbon-14 sample oxidizer, which collected the \( ^3\text{H} \) as \( \text{H}_2\text{O} \) in scintillator, and the \( ^{14}\text{C} \) as a carbonate salt, also in scintillator. The oxidized samples were beta-counted with a Packard scintillation counter; this data, plus the concentrations determined above, allowed calculation of the specific activity of the reactants. The lower product eluates were acidified with an equal volume of \( 1\text{ N HCl} \) and extracted with diethyl ether (see Method 1). All product eluates were evaporated to about 100 \( \lambda \), applied to small strips of paper, and oxidized and counted for \( ^3\text{H}- \) and \( ^{14}\text{C}- \) activity, as for the unreacted nucleotide and \( \text{t}-\text{BOC}-\text{alanine} \) samples. This data, plus the specific activities of the substrates, allowed determination of the amino acid/nucleotide ratios. The results were:

<table>
<thead>
<tr>
<th>moles amino acid</th>
<th>moles nucleotide</th>
<th>for</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Run 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>fraction</td>
<td></td>
<td>upper</td>
<td>0.837</td>
<td>0.927</td>
<td>0.914</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lower</td>
<td>1.82</td>
<td>1.49</td>
<td>1.87</td>
</tr>
</tbody>
</table>

Again, the experimental results provided fair support for the mono- and diester hypothesis. No good explanation has been found for the extremely low ratio of the lower product band of Run 4.

The average values of the amino acid/nucleotide ratios for Methods 1 and 2 were thus: 0.876 for the upper product bands, and
1.95 for the lower product bands. These values were fairly close to the ratios expected for the 2'(3')-t-BOC-alanyl mono- and diesters, respectively, of the nucleotides. In the author's opinion, one may safely conclude that the upper product bands were 2'- or 3'-O-(t-BOC-alanyl) nucleotides, and the lower product bands 2'- and 3'-O,0'- (t-BOC-alanyl) nucleotides.

IV. CONCLUSION

The work contained in this thesis has shown that the scheme of reactions I and II, above, is not a good approach to establishing the chemical uniqueness, if any, of the CCA end-group. At present, the author is not certain that a good approach to the problem exists at all.

V. ACKNOWLEDGEMENTS

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VI. REFERENCES

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