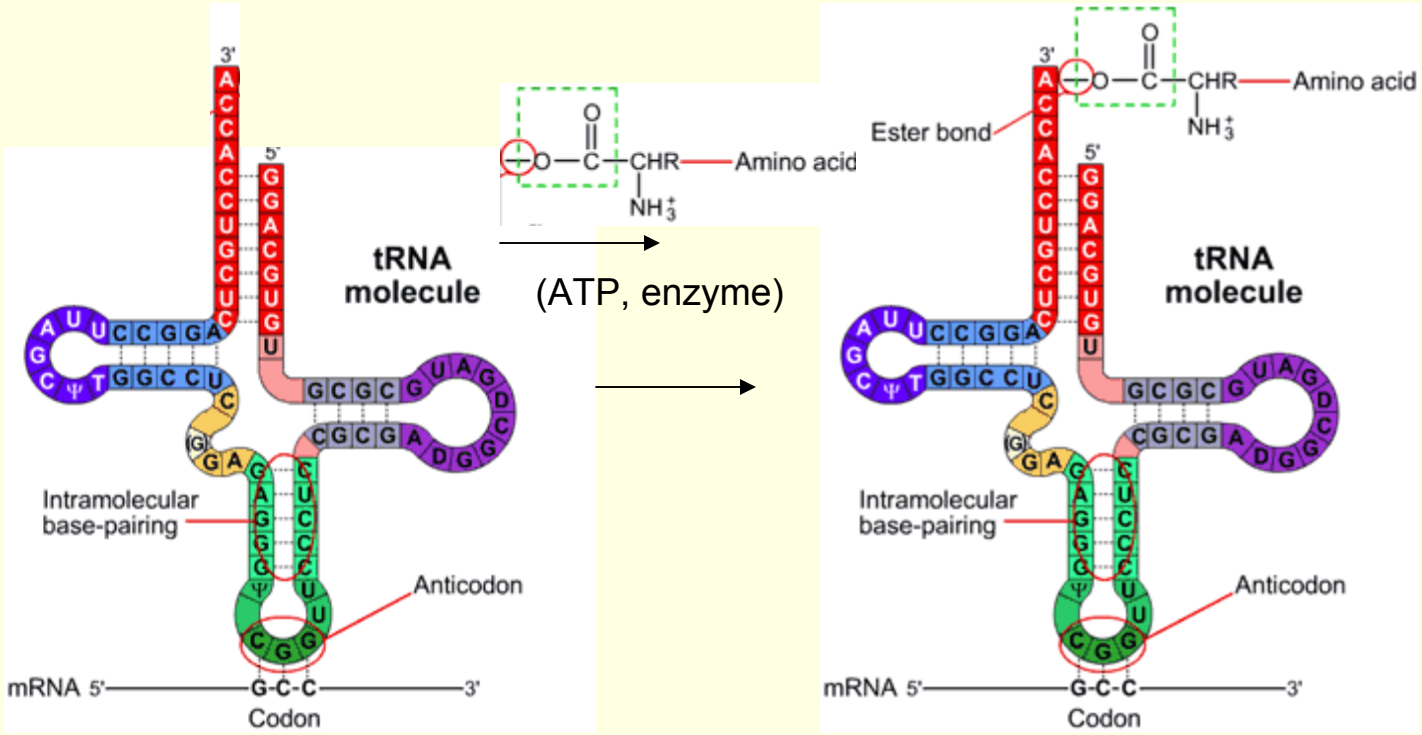
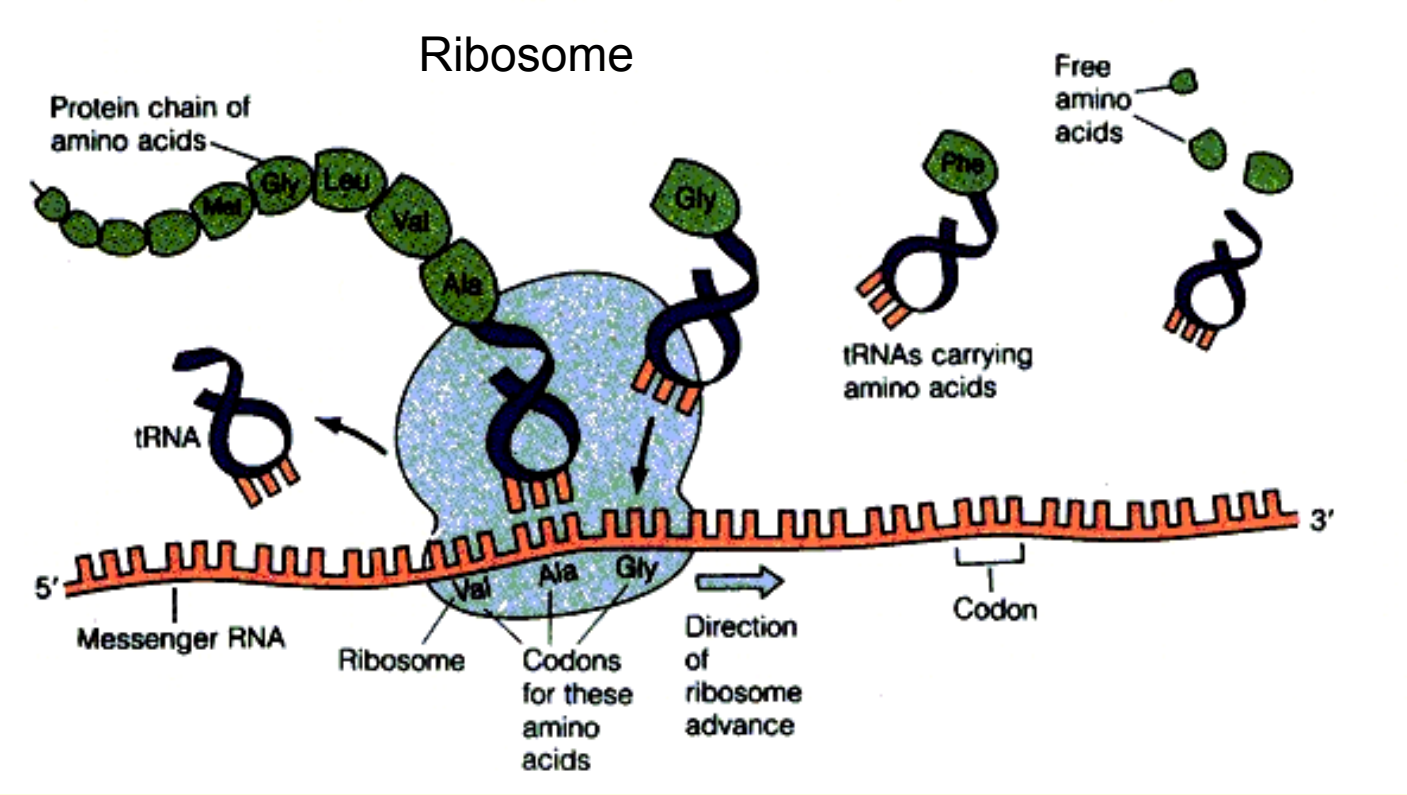
Lanthanum-Directed Acylation of RNA

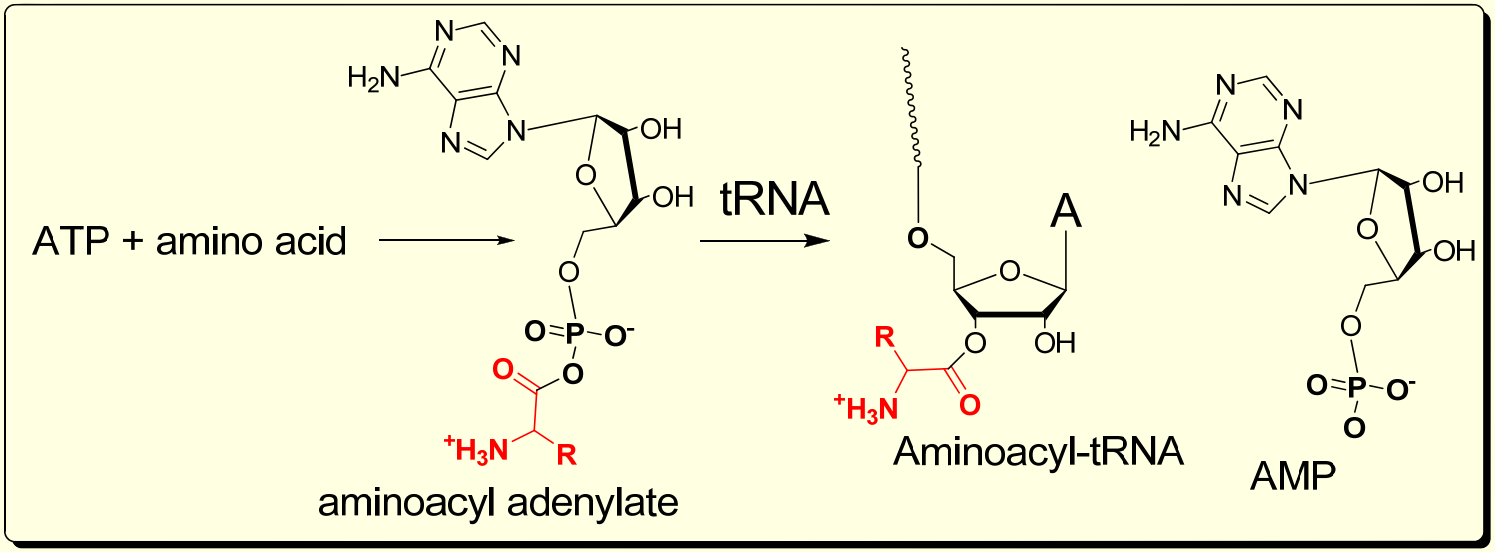


Biochemical protein synthesis



Aminoacylated tRNA

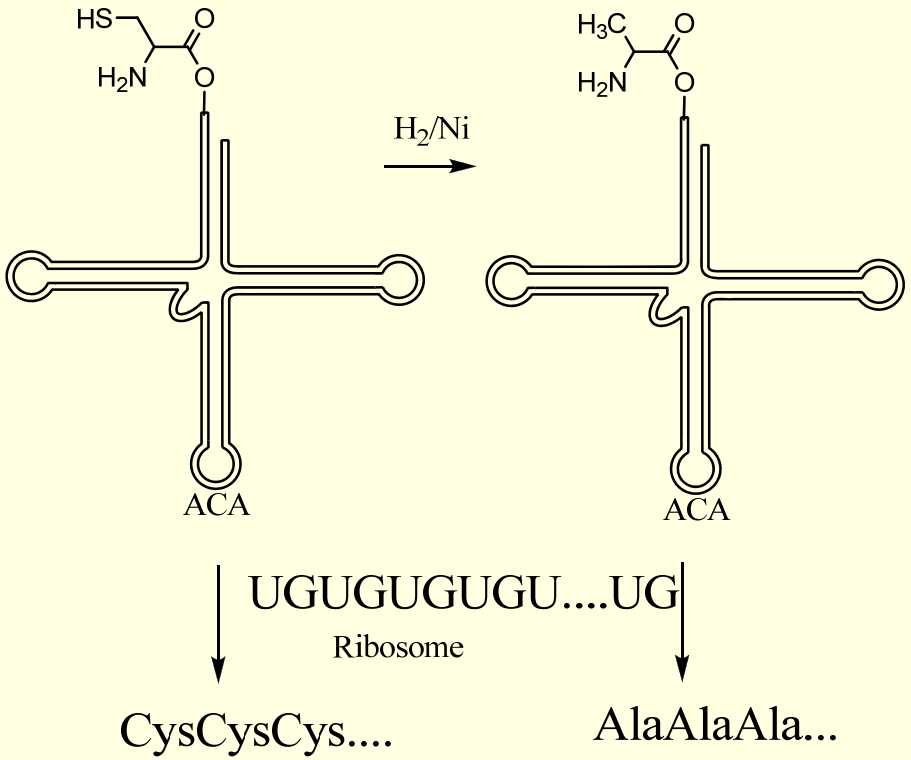
- From aminoacyl adenylate and tRNA



Hoagland “An enzymatic mechanism for amino acid activation in animal tissues” *Biochim. Biophys. Acta* **1955** *16* 288

tRNA alone determines sequence

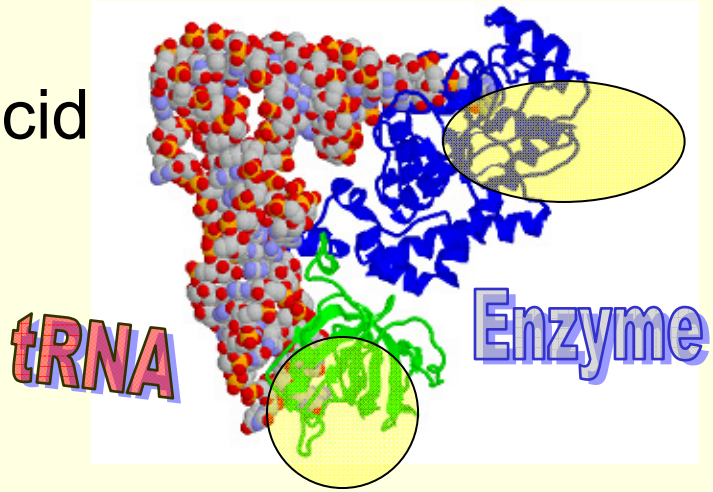
- Misacylation leads to mis-incorporation

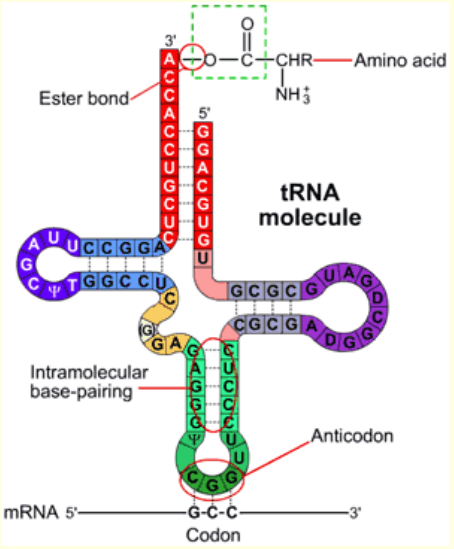


Chapeville et al.: “On the role of soluble ribonucleic acid in coding for amino acids”; *Proc. Natl. Acad. Sci. USA* **1962** *48* 1086.

Aminoacyl tRNA synthetases

- Recognize
  - Specific anticodon
  - Specific amino acid

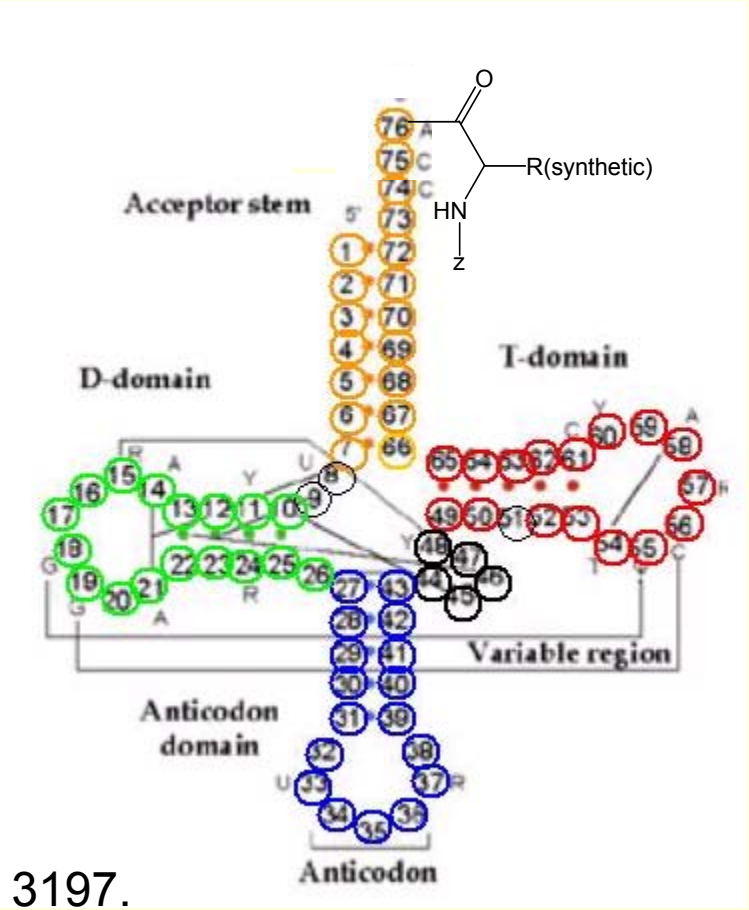




Produces the correct aminoacylated tRNA

Producing mis-acylated tRNA

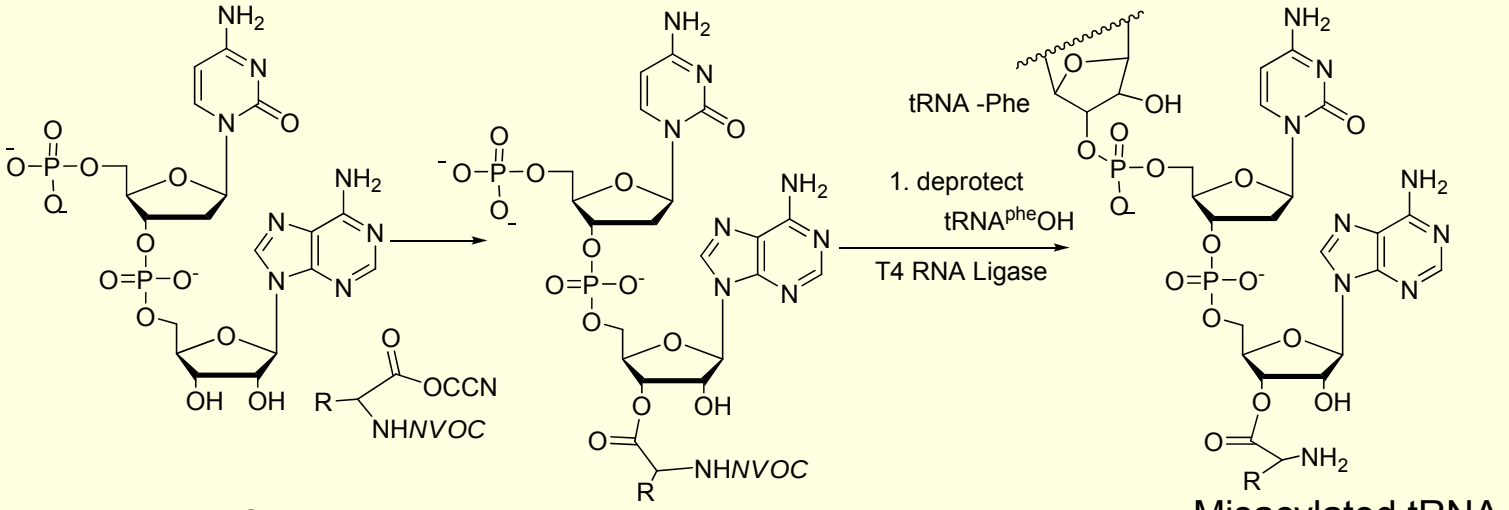
- Exonuclease to remove CpA from 3' terminus
- Synthesize aminoacyl-tRNA
- Ligate to restore 3'-terminus



Hecht et al. *Biochemistry* **1987** *26*, 3197.
Schultz et al. *Science,* **1989** *244***,** 182

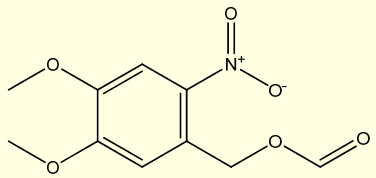
Ligation to RNA by enzyme

- Multiple steps



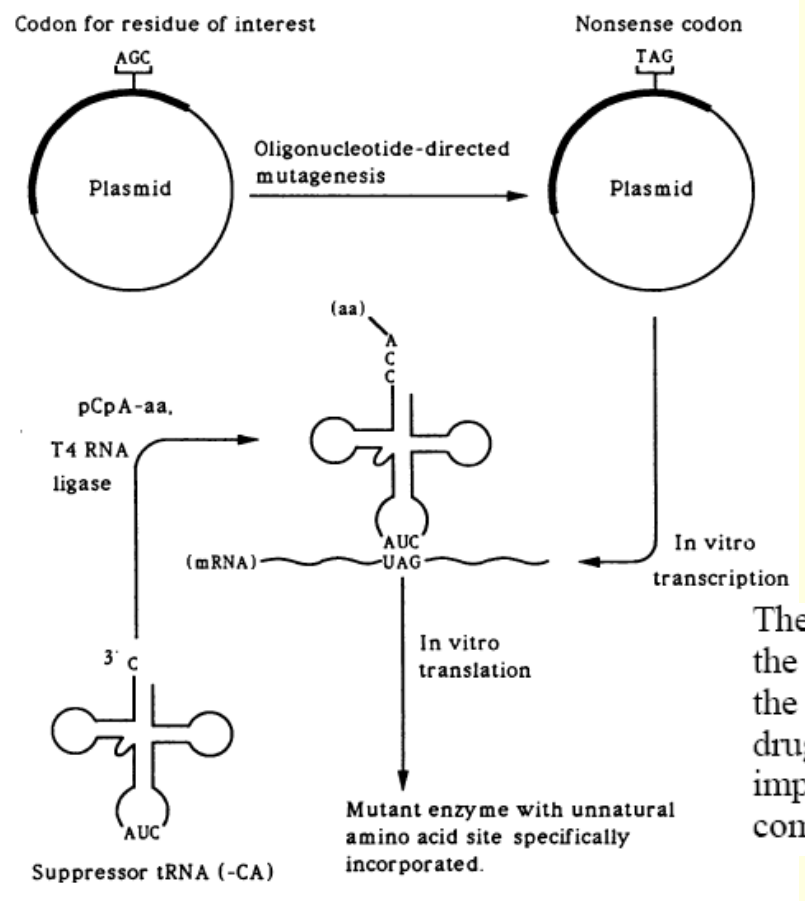
pCpA aminoacyl pCpA Misacylated tRNA

A general and efficient route for chemical aminoacylation of transfer RNAs
S.A. Robertson, J.A. Ellman, and P.G. Schultz
J. Am. Chem. Soc. **1991**,*113*, 2722.



6-Nitroveratryloxycarbonyl
NVOC

Unnatural proteins



Codon suppression
Schultz et al. *Science,* **1989,** *244***,** 182

Mutated synthase
Furter et al., *Protein Sci.,* **1998** *7***,** 419 - in yeast
Schultz et al. *Science* **2001** *292* 498 - in bacteria

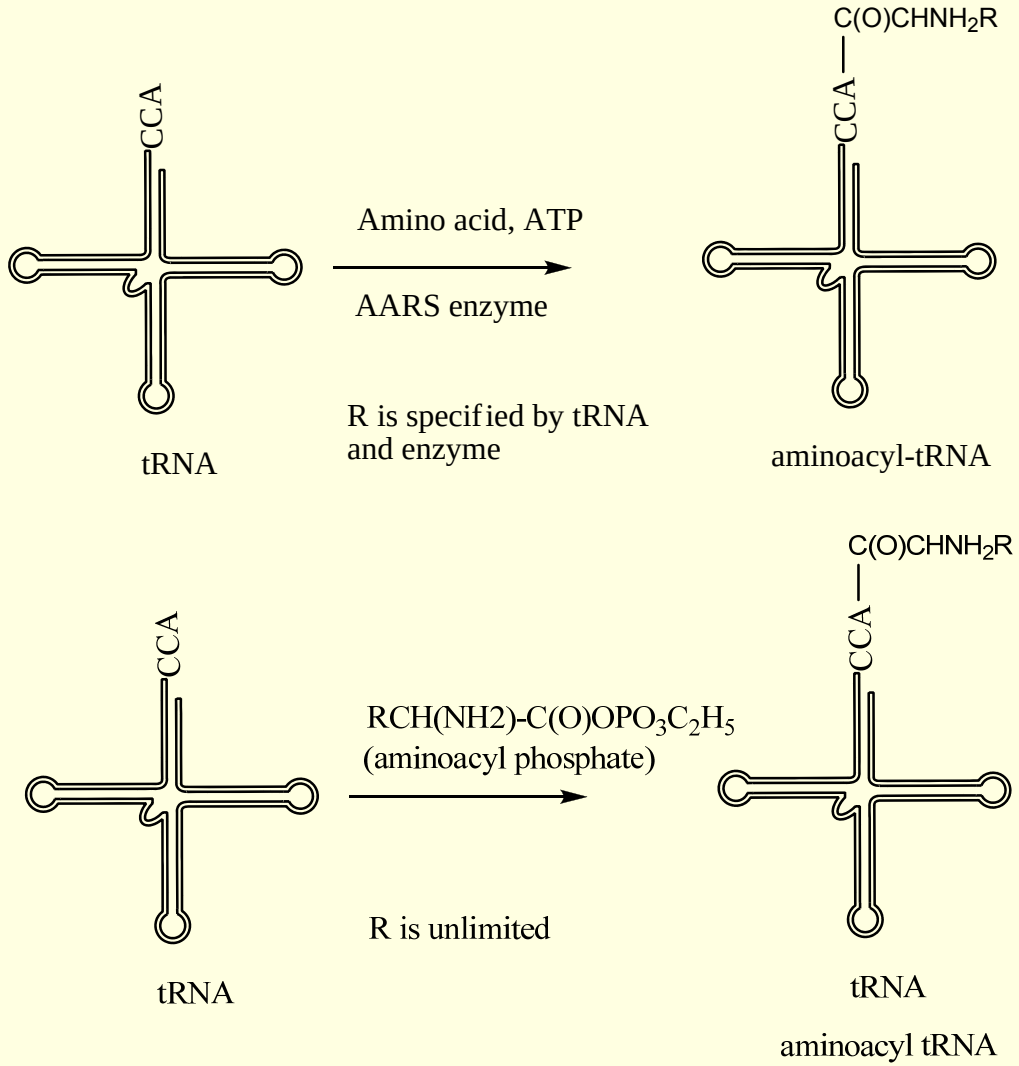
The studies represent key advances in continuing efforts to expand the genetic code beyond that provided by nature. A major goal of the work is to create designer proteins as building blocks for new drugs and materials. But it may also have profound long-term implications--such as making it possible to develop new life-forms composed of customized proteins.

Stuart Borman, C&E News, 2001

Evolution, ribozymes, micelles

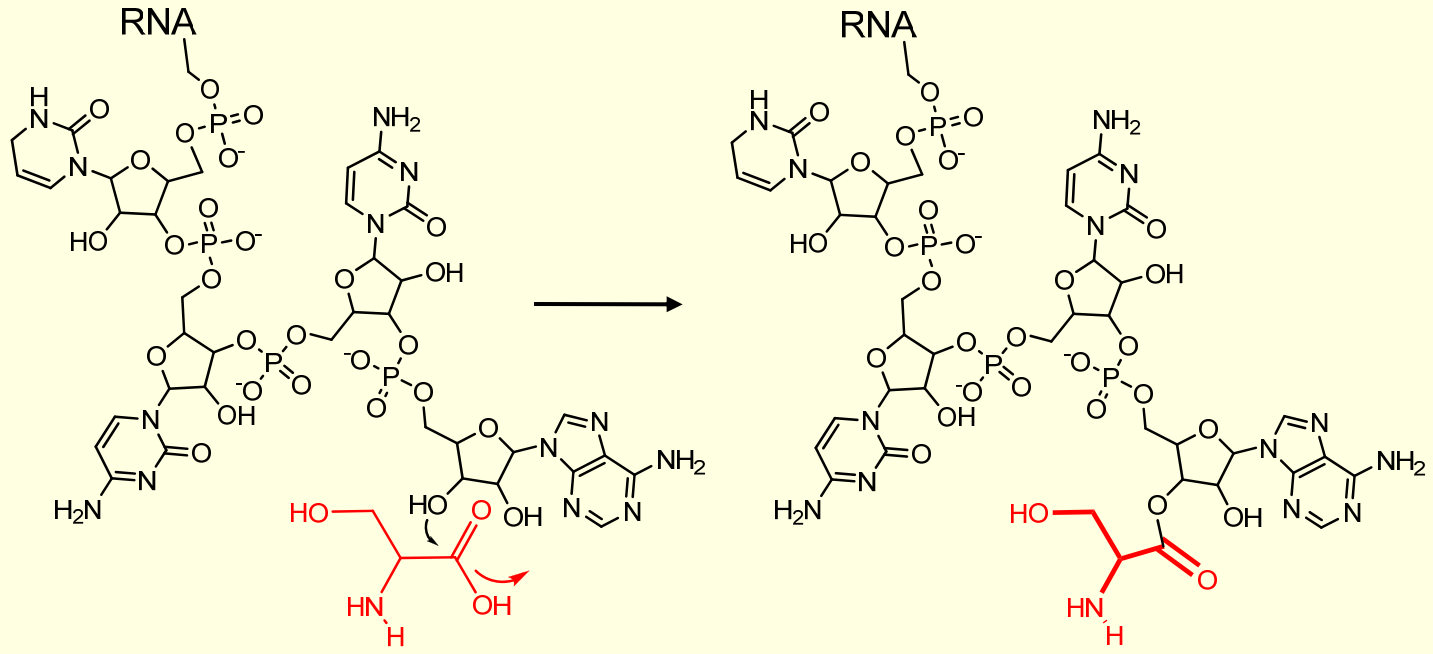
- Bertozzi, Tirrell et al.
  Discovery of Aminoacyl-tRNA Synthetase Activity Through Cell Surface Display of Noncanonical Amino Acids. PNAS **2006**, *103,* 10180. (Screening of a saturation mutagenesis library of the *E. coli* methionyl-tRNA synthetase (MetRS) led to the discovery of three MetRS mutants capable of incorporating the long-chain amino acid azidonorleucine into recombinant proteins with modest efficiency)
- Szostak et al.
  Enzymatic aminoacylation of tRNA with unnatural amino acids PNAS **2006** *103* 4356 "we have found 59 previously unknown AARS substrates. These include numerous side-chain analogs with useful functional properties."
- Suga et al.
  Ribozyme-Catalyzed tRNA Aminoacylation in *Aminoacyl tRNA Synthetases*, **2007** Michael Ibba, editor
- Schultz, P.G. et al.
  An efficient system for the evolution of aminoacyl-tRNA synthetase specificity *Nature Biotechnol.* **2002** *20*, 1044. "we identified three new variants that allow the selective incorporation of amino-, isopropyl-, and allyl-containing tyrosine analogs into a desired protein."
- Sisido, M. et al.
  Simple and quick chemical aminoacylation of tRNA in cationic micellar solution under ultrasonic agitation *Chem. Commun*., **2005**, 4321.
  Micelles: : N-protected amino acid activated ester, tRNA, and a cationic detergent

Seeking a biomimetic route



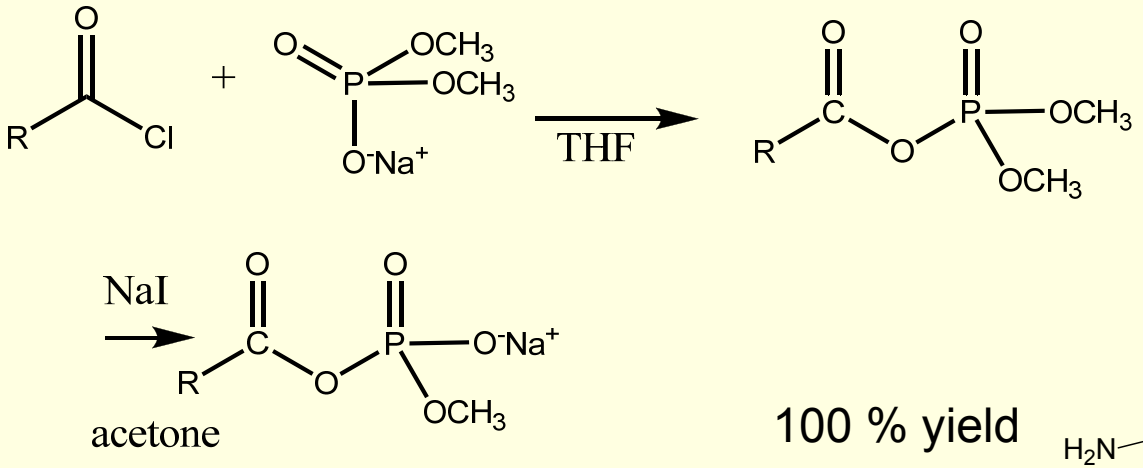
Target reaction

- Activate nucleophile and/or electrophile
- Improve leaving group
- Select 3’ terminal OH
- Detect product

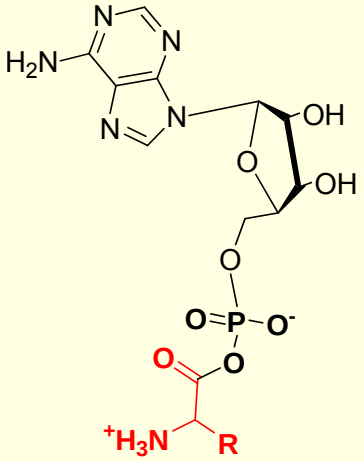


Acyl phosphate monoesters: synthesis

- Couple and monodemethylate



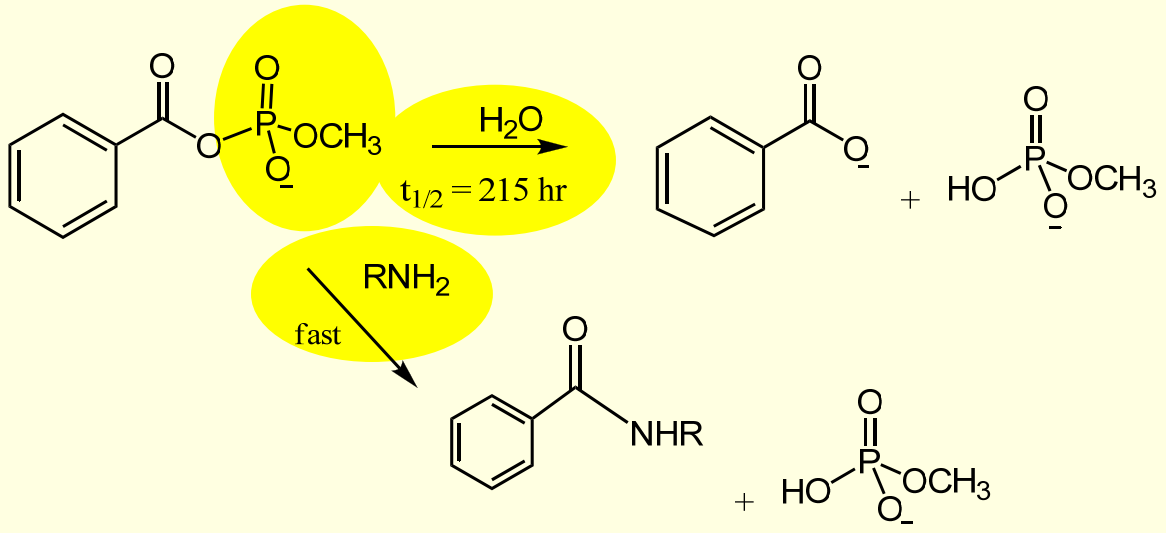
100 % yield



Biomimetic of acyl adenylate

Inherent reactivity

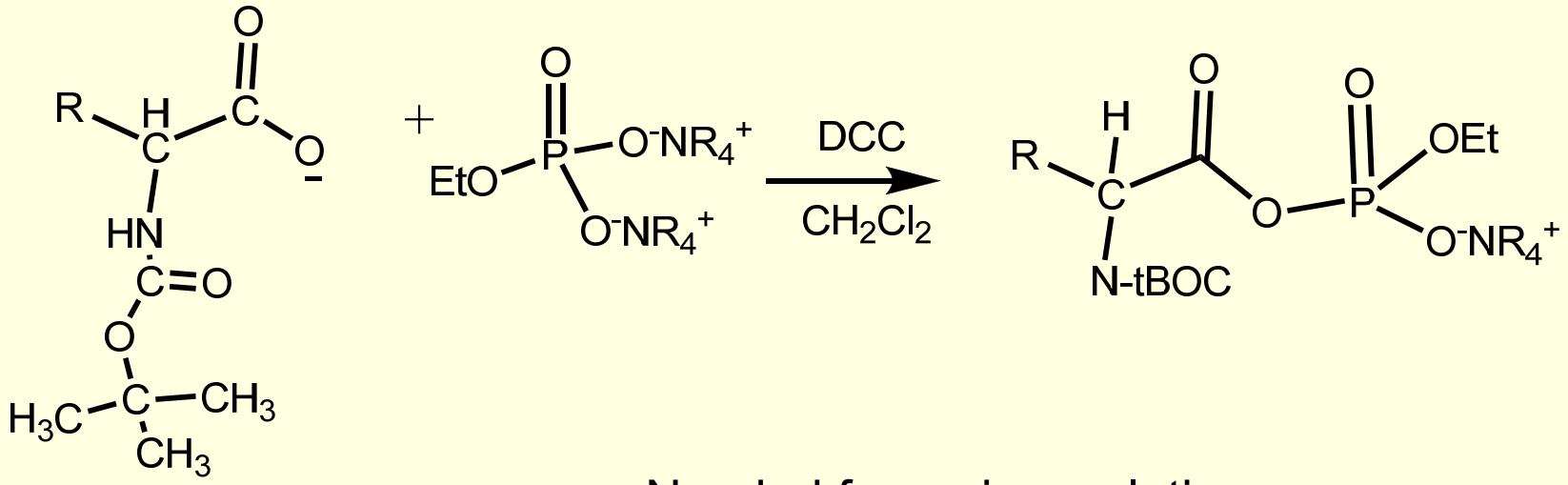
- Anionic leaving group: repelled by anions, attracted by cations
- React rapidly with amines (useful for protein modification)
- React slowly with oxygen nucleophiles – not good for tRNA



W. P. Jencks and J. Carriuolo *J. Biol. Chem.* **1959,** *234,* 1272, 1280.
G. DiSabato and W.P. Jencks, *J. Am. Chem. Soc*. **1961,** *83*, 4400.

Aminoacyl phosphate monoesters

- One step coupling
- N-protection

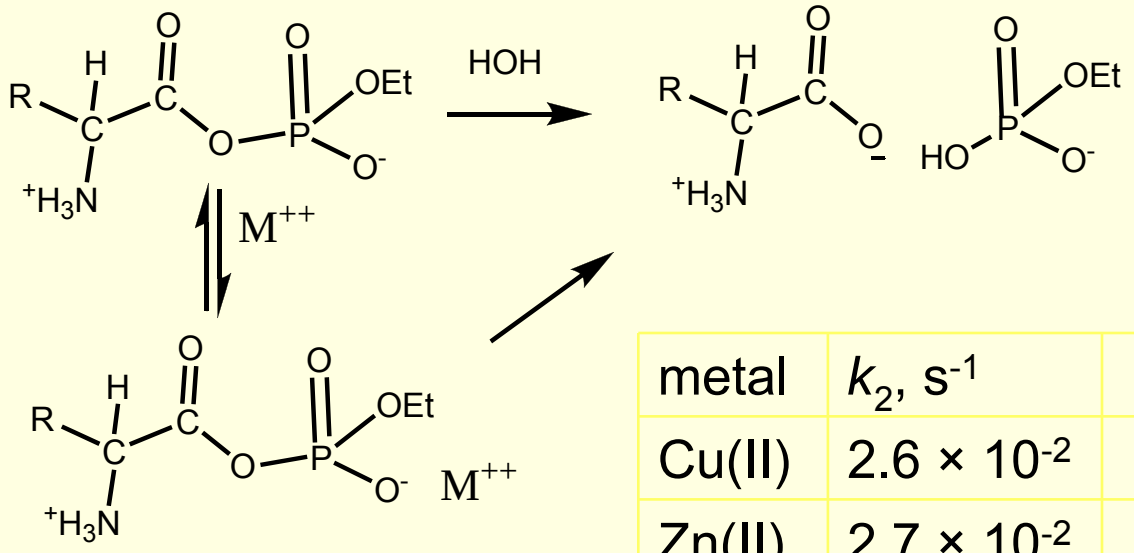


Needed for aminoacylation

R. Kluger, X. Li, and R. W. Loo, *Can. J. Chem.* **1996**, *74* 2395.

Moldave, Castelfranco, and Meister, *J. Biol. Chem.* **1959** *234* 841.

Lewis acid activation



pH 7.0, 25 ° C

Modest acceleration

metal	k_2, s^{-1}	K_1, M^{-1}
Cu(II)	2.6×10^{-2}	250
Zn(II)	2.7×10^{-2}	141
Mg(II)	1.0×10^{-2}	28
Ca(II)	0.9×10^{-2}	15

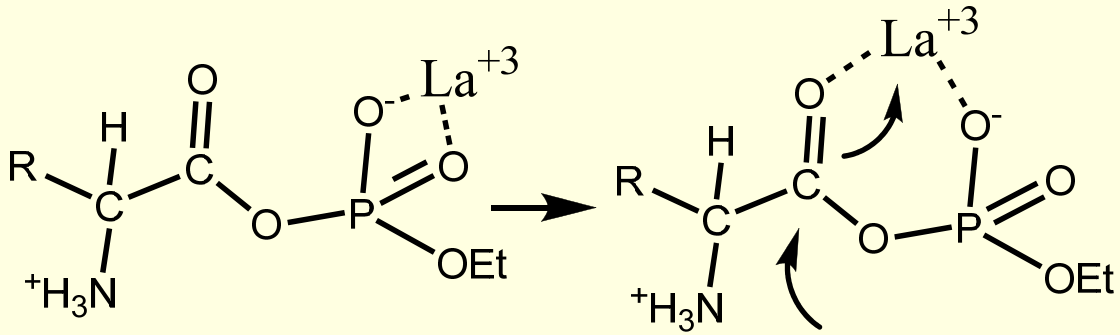
$t_{1/2}$ ~ 400 minutes

$k_{HOH} = 3 \times 10^{-5}\ s^{-1}$

R. Kluger, R. W. Loo, and V. Mazza, *J. Am. Chem. Soc.* **1997** *119* 12089.

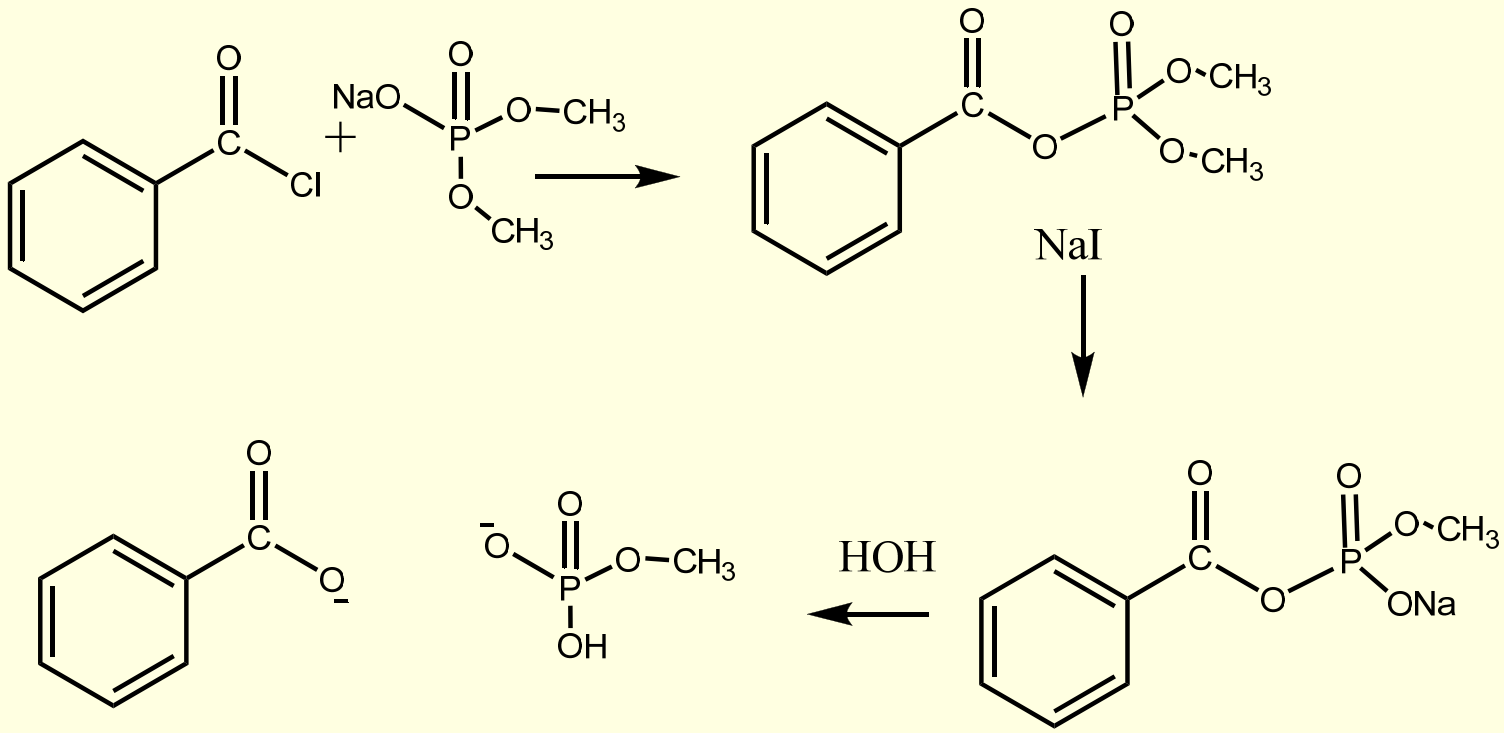
Activation by lanthanides?

- Stable and water soluble
- Hard Lewis acids, loose coordination number and geometry
- Coordinate to phosphates and promote reactions of the unreactive ester



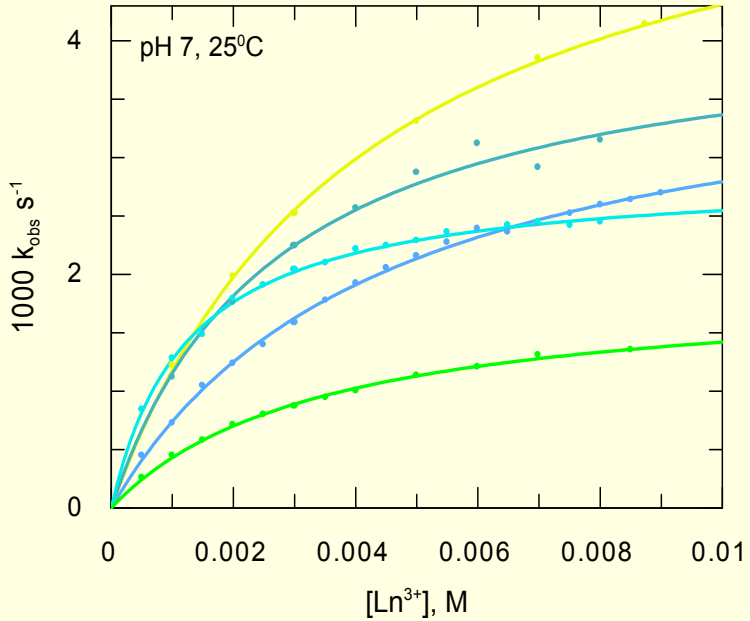
General review: Shibasaki and Yoshikawa *Chem. Rev.*, **2002** *102* 2187

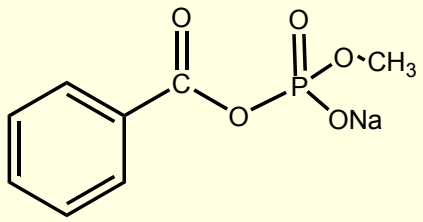
Benzoyl methyl phosphate (BzMP)



Provides spectroscopic change λ_{max}=234 becomes 224

Lanthanides accelerate hydrolysis of BzMP





Metal	K_1, M^{-1}	k_2, s^{-1}	k_2 x K_1
$NdOTf_3$	2×10^{2}	4×10^{-3}	0.88
$EuOTf_3$	4×10^{2}	4×10^{-3}	1.6
$EuCl_3$	2×10^{2}	6×10^{-3}	1.3
$YbOTf_3$	8×10^{2}	2×10^{-3}	2.3
$LaOTf_3$	3×10^{2}	1×10^{-3}	0.53
$MgCl_2$	-	-	8×10^{-6}
no metal		2×10^{-7}	

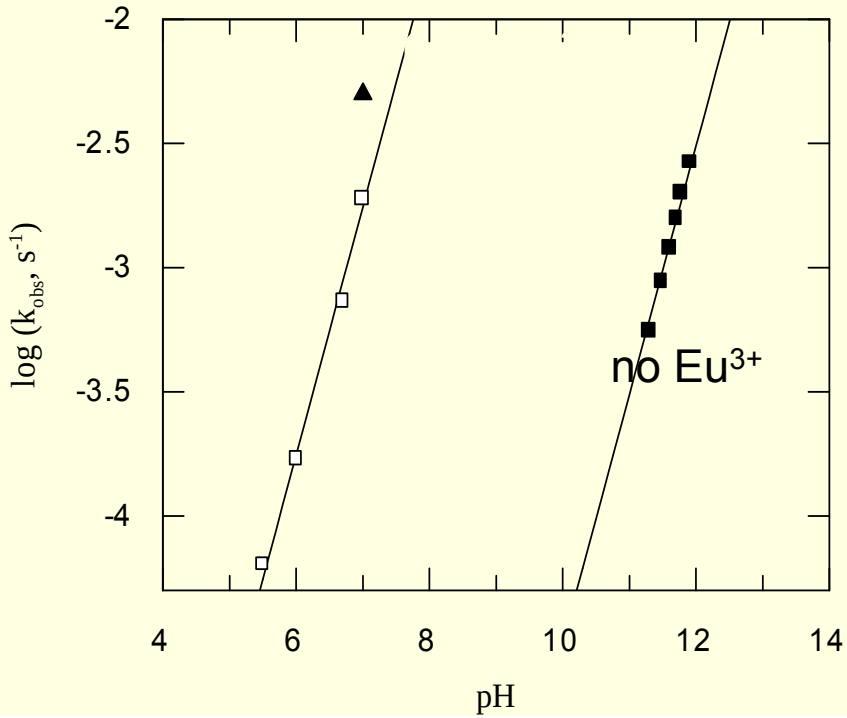
J. Am. Chem. Soc. **2002** *124* 3303

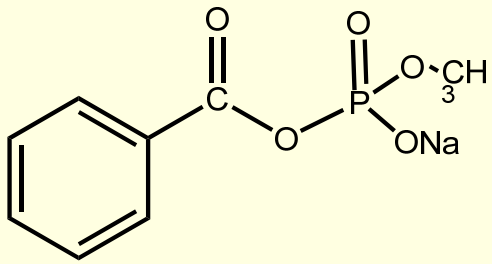
Enhanced electrophilicity of C=O

Metal ion accelerates OH^- catalysis

- First-order in OH^-
- Lanthanides not catalytic at high $[H^+]$
- Internal addition of coordinated hydroxide

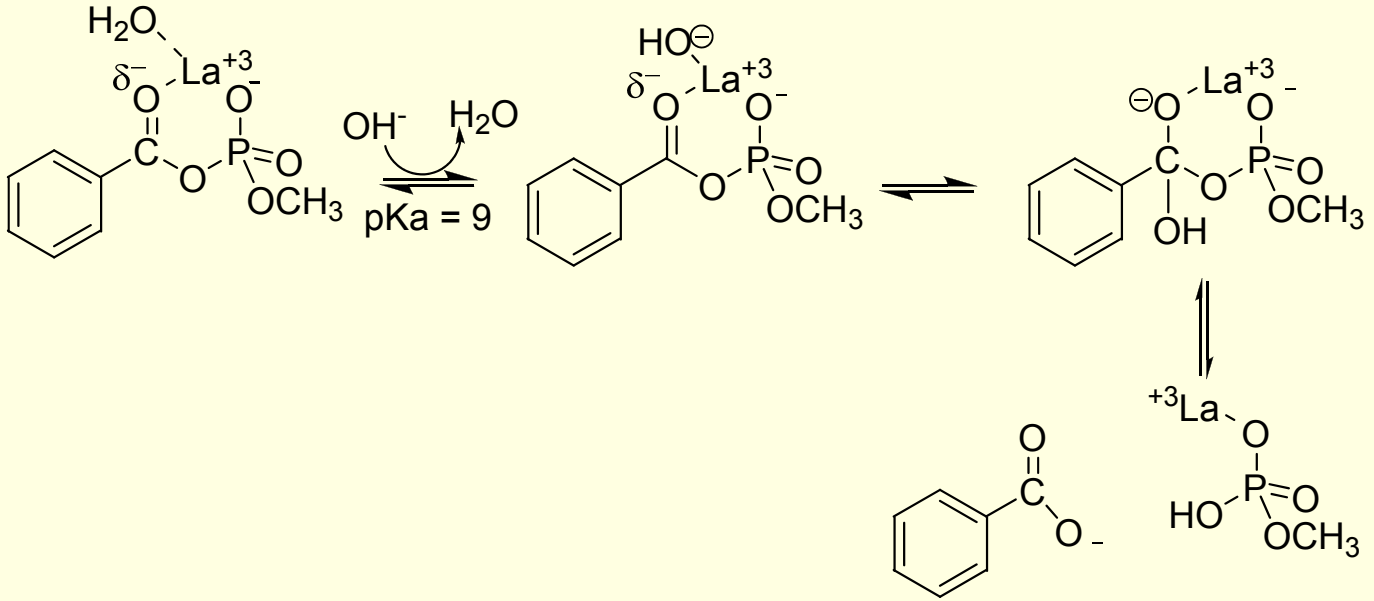
reactant	k, $M^{-1}s^{-1}$
OH	3.4×10^{-1}
$OH(Eu^{3+})$	1.9×10^{4}
$k_{H+} = k_{H+}(Eu^{3+})$	1.9×10^{-6}





Internal addition

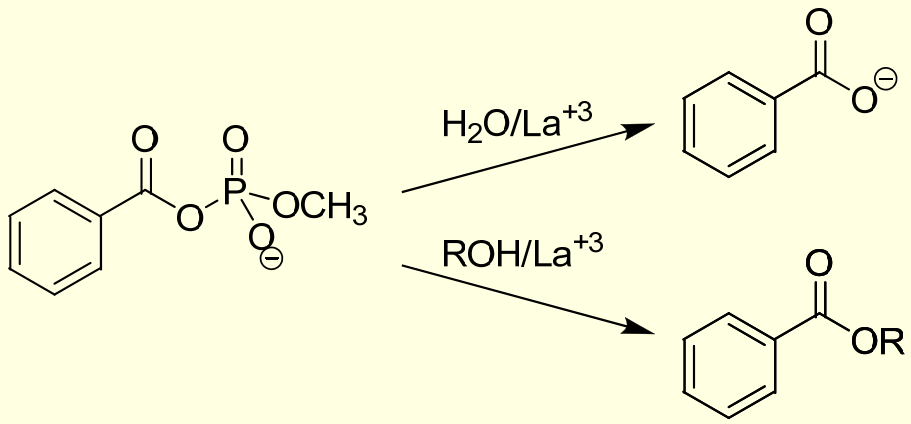
- Reaction between coordinated ligands
- Entropic advantage



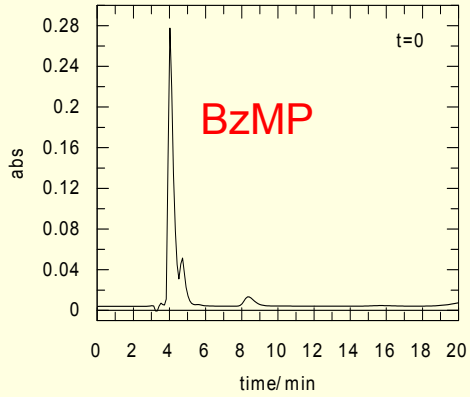
Lisa Cameron, Sheila Wang, and R. K. *J. Am. Chem. Soc*. **2004** *126* 10721.

Refinement: acylation of alcohols

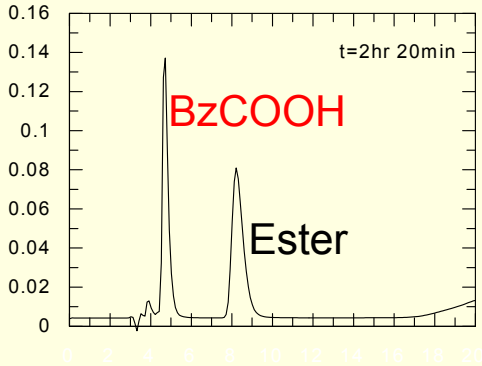
- Does this parallel hydrolysis?



BzMP in 50:50 water:methanol

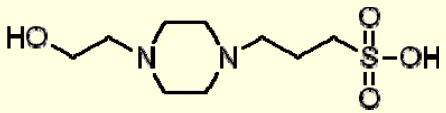


before



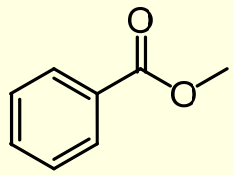
after

10^{-3} M La^{+3}, 10^{-2} M EPPS, pH 8

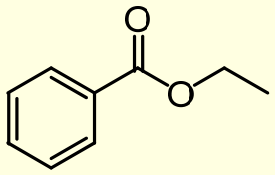


Reactivity – cis diols much better

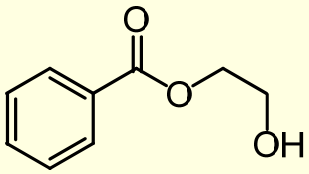
- k_{rel} = 1 for hydrolysis
- Cis or flexible diols are particularly reactive
  - No diester products – only one OH reacts



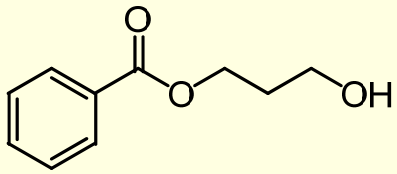
k_{rel} = 20



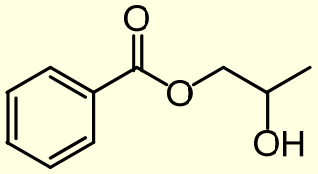
k_{rel} = 0.08



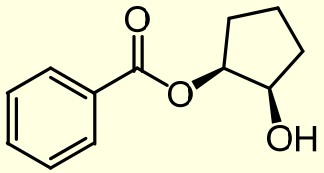
k_{rel} = 170



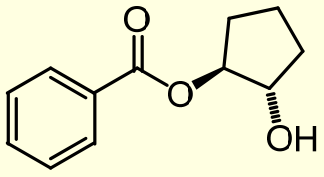
k_{rel} = 17



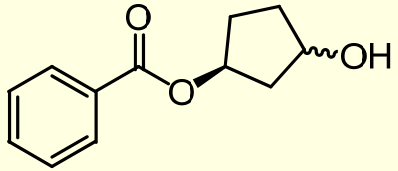
k_{rel} = 82, 11



k_{rel} = 46



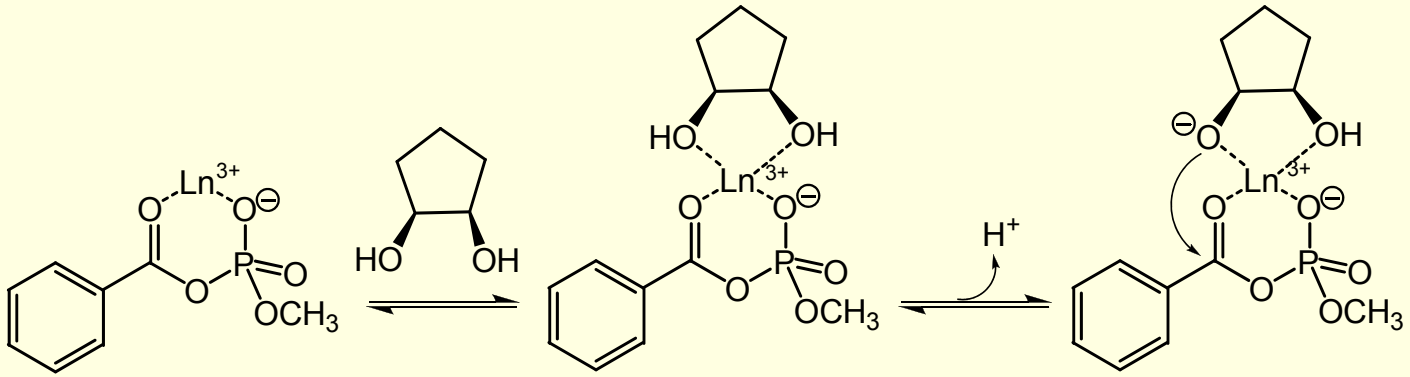
k_{rel} = 0



k_{rel} = 49

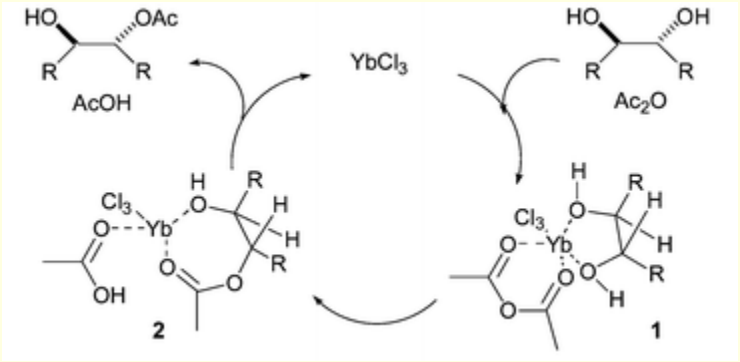
Diol reactions implicate chelation

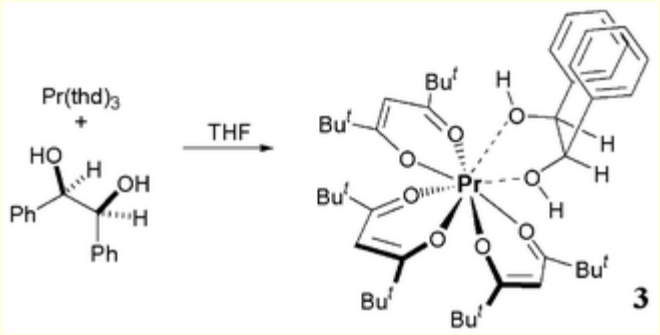
- Indicates kinetically significant nucleophilic coordination
  - Results in a low-entropy reaction



Nonaqueous analogy: monoacylation

- Catalytic monoacylation of diols (1,2; 1,3; 1,4) dichloromethane
- Via bis-bidentate complex

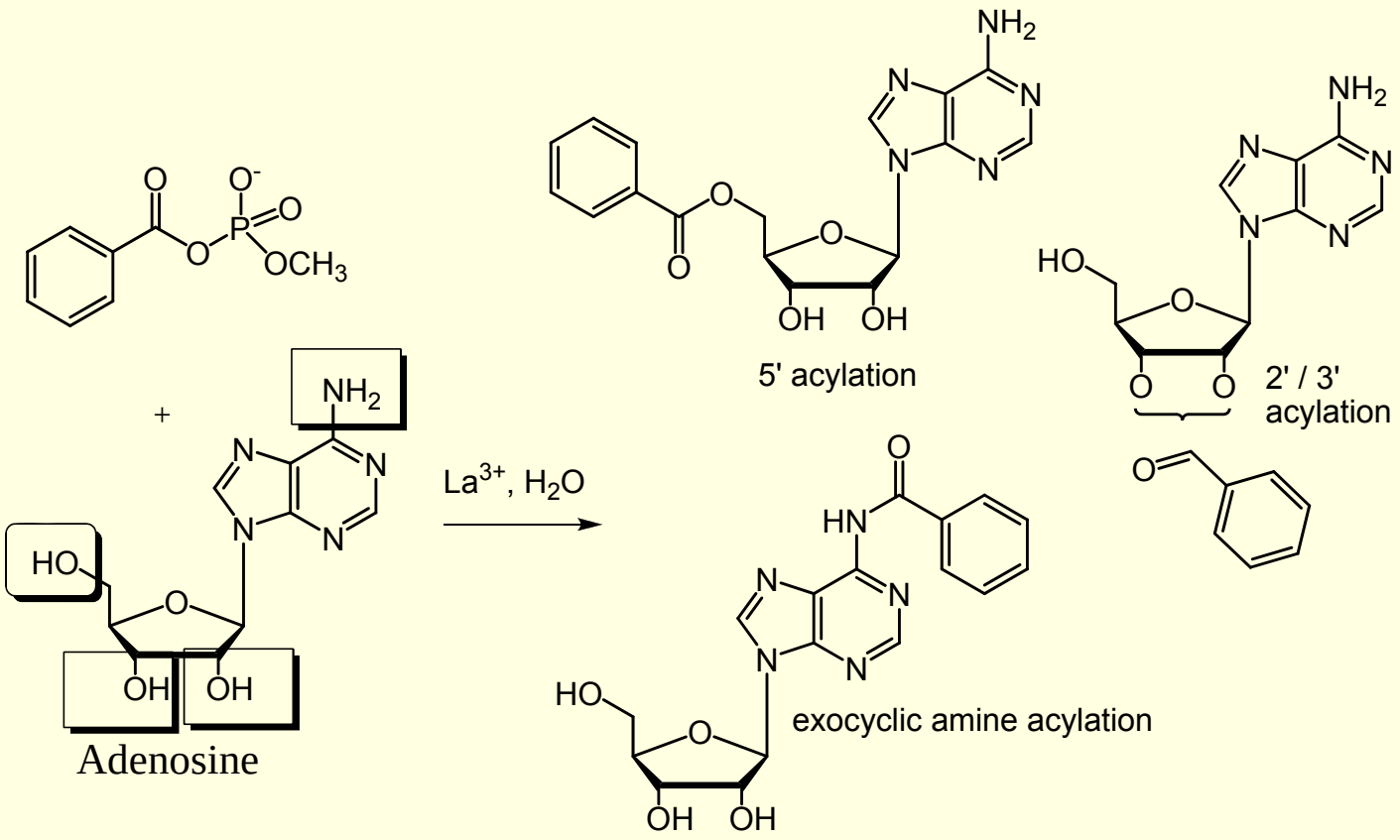




Crystal structure of analogue

P. A. Clarke et al. *Chem. Commun.*, **2003**, 2588 lanthanide (III) salt catalysed monoacylation of symmetrical diols from structural models

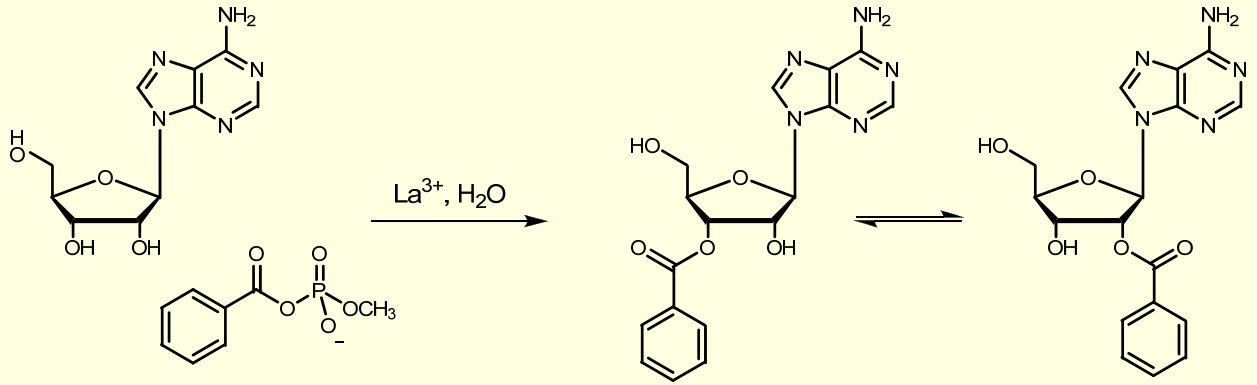
Possible reactions of adenosine + BzMP



Esters from adenosine

adenosine (5×10^{-4} M), BzMP(0.01 M), pH 8.0. EPPS[a]

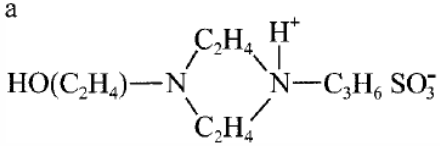
10^3 La^{+3}, M	0.25	0.50	0.75	1.0	1.5	2.0
Time (h)	10.1	3.5	3.5	3.0	3.0	2.5
Yield (%)	44	48	51	55	58	63



BzMP

No acylation in absence of La^{+3}

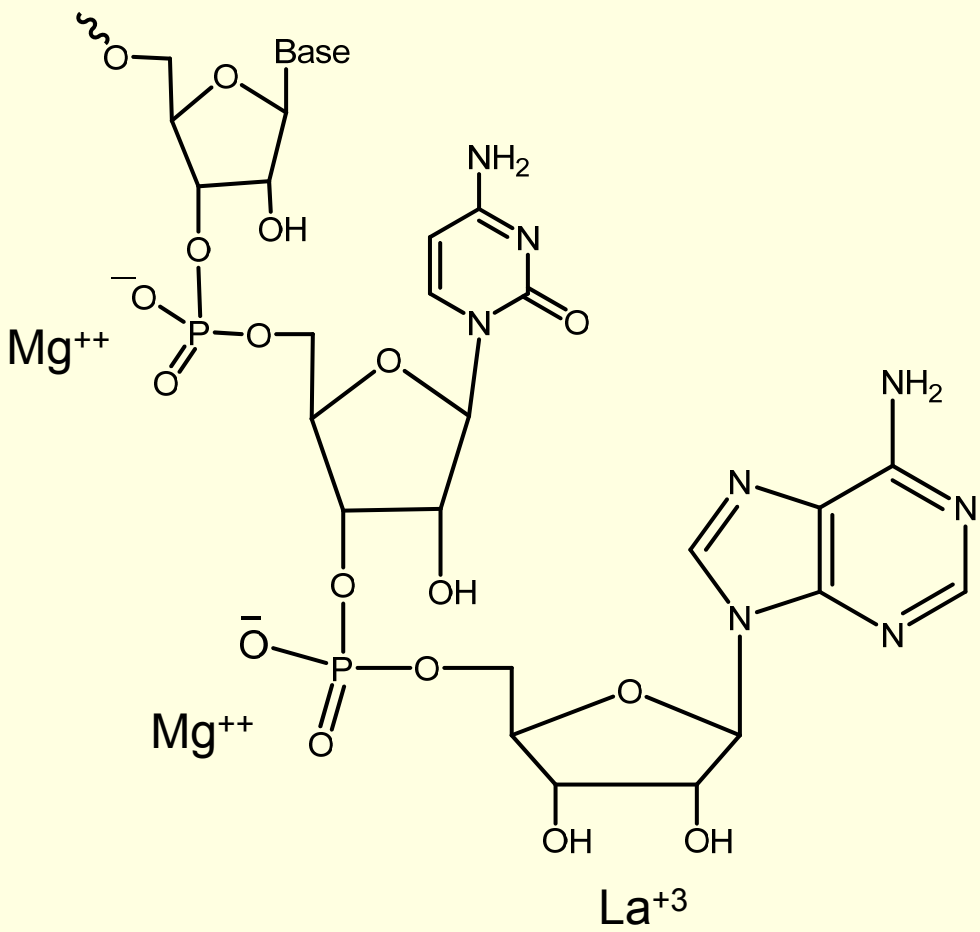
a



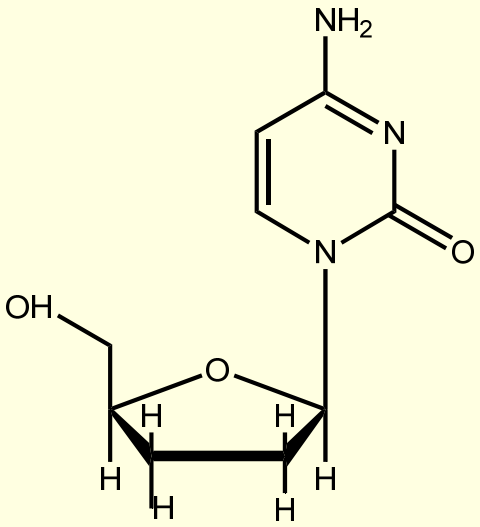
(H)EPPS, pK_A = 8

Extend to RNA

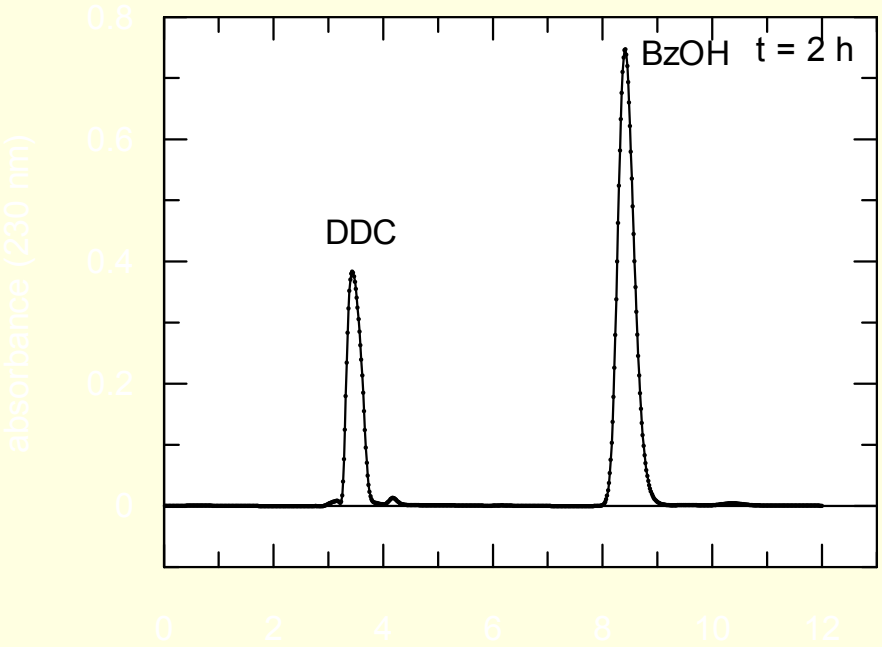
- Mg^{++} binds to phosphates
- Unique diol – binds La^{+3}



Analogue with no cis diol



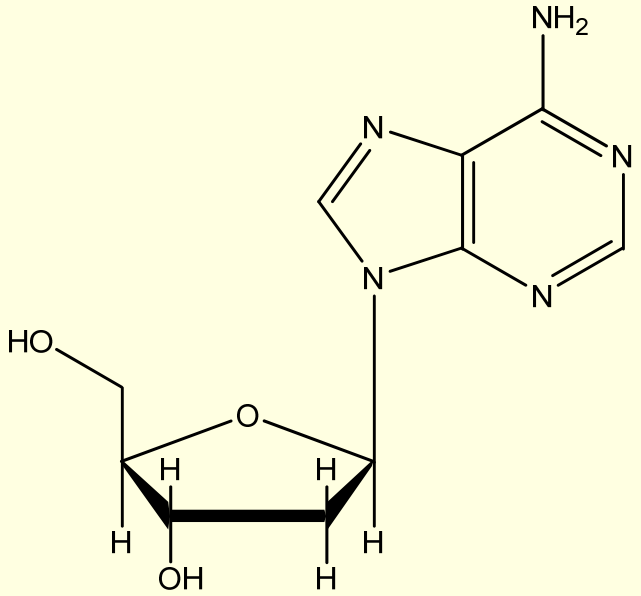
2', 3'-2Dideoxycytidine (DDC)



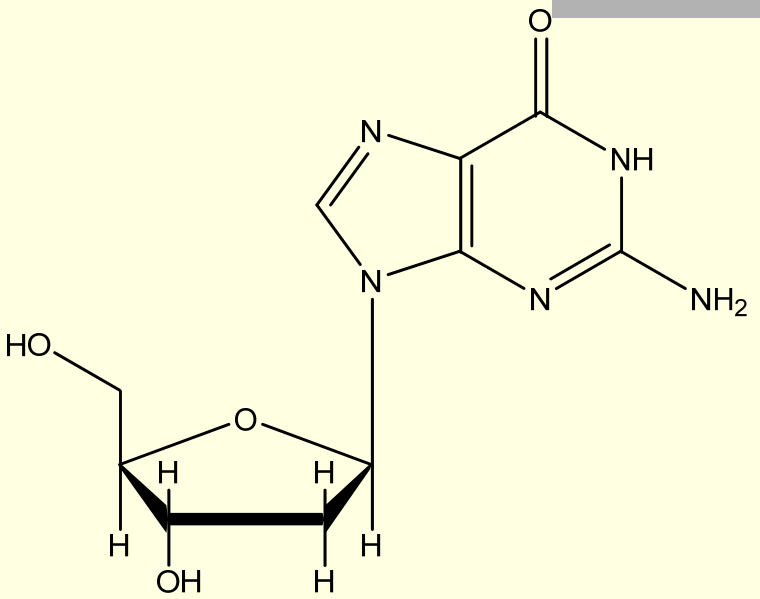
2×10 $^{-3}$ M 2',3'-DDC, 5× 10^{-4} M BzMP, 1 ×10^{-3} M $LaCl_3$, 1× 10^{-2} M EPPS, pH 8.0, room temperature.

Neither OH nor NH_2 react

Deoxynucleosides



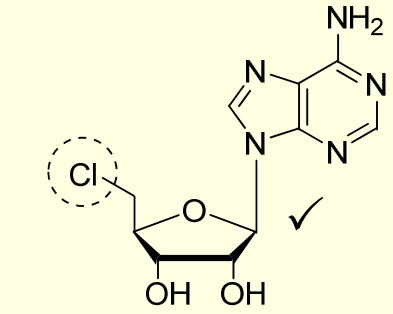
2'-deoxyadenosine



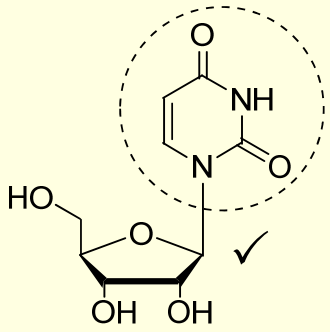
2'-deoxyguanosine

No acylation product with BzMP & La^{+3}

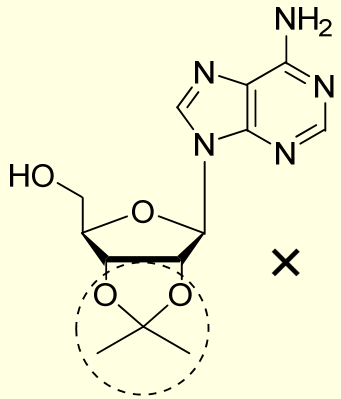
Nucleosides require 1,2-diol



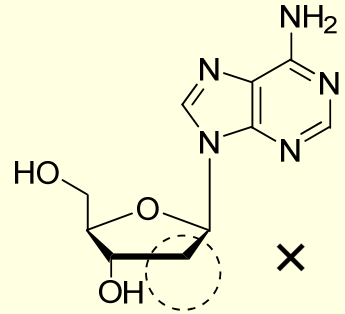
5'-chloro-5'-deoxyadenosine



uridine



2',3'-isopropylidineadenosine



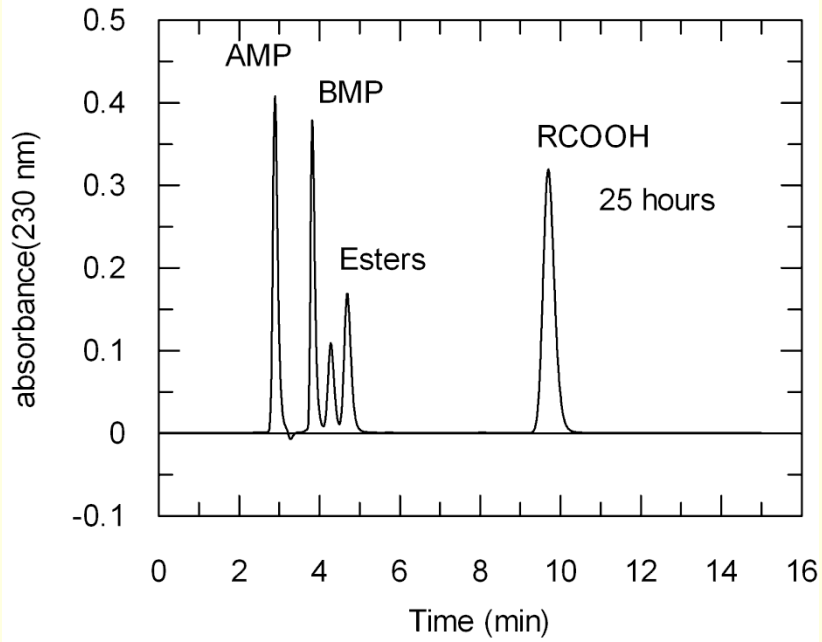
2'-deoxyadenosine

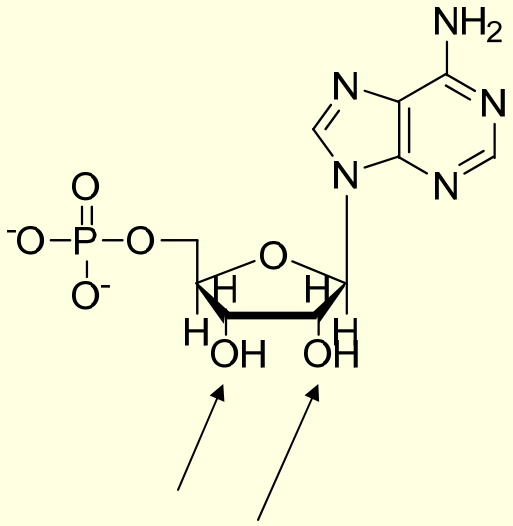
- Analogues with modifications at reaction sites
- Observe: two acylation products or none

☞ 2’(3’)-diol required for acylation with La^{+3}

Nucleotide reaction

- Forms 2’ and 3’ benzoyl esters of AMP

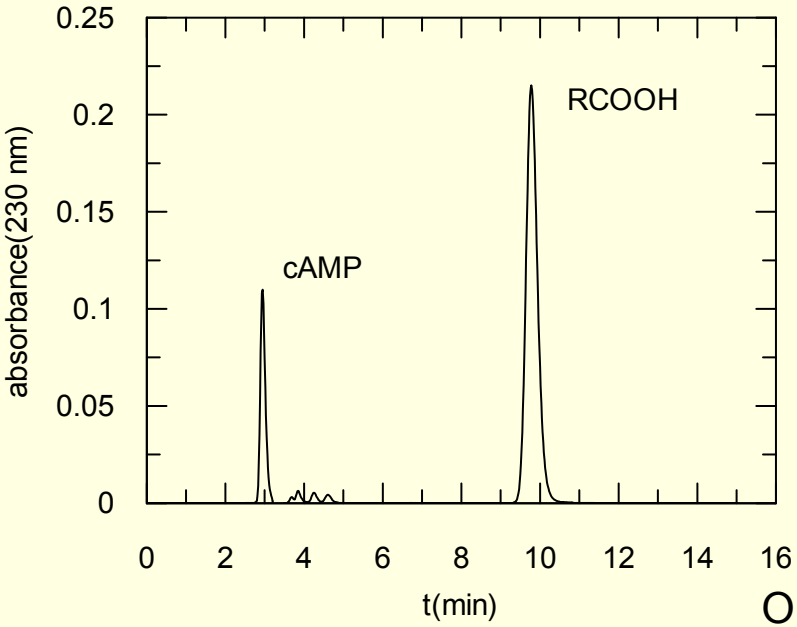


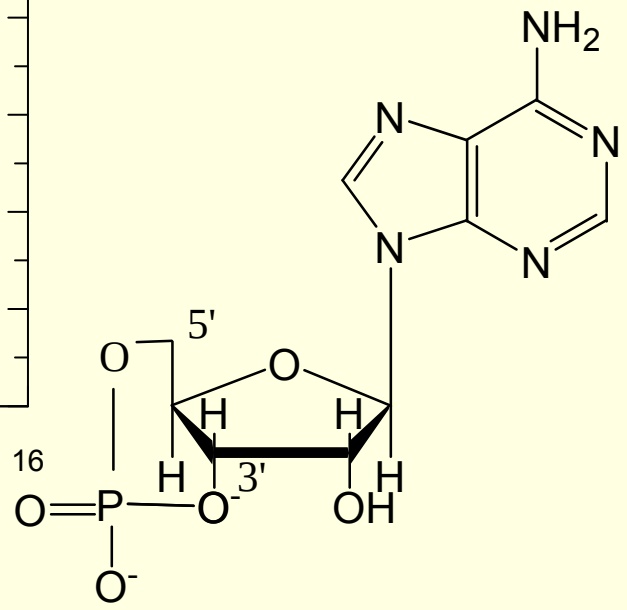


La^{+3}, BzMP

~ 30 % of AMP is acylated

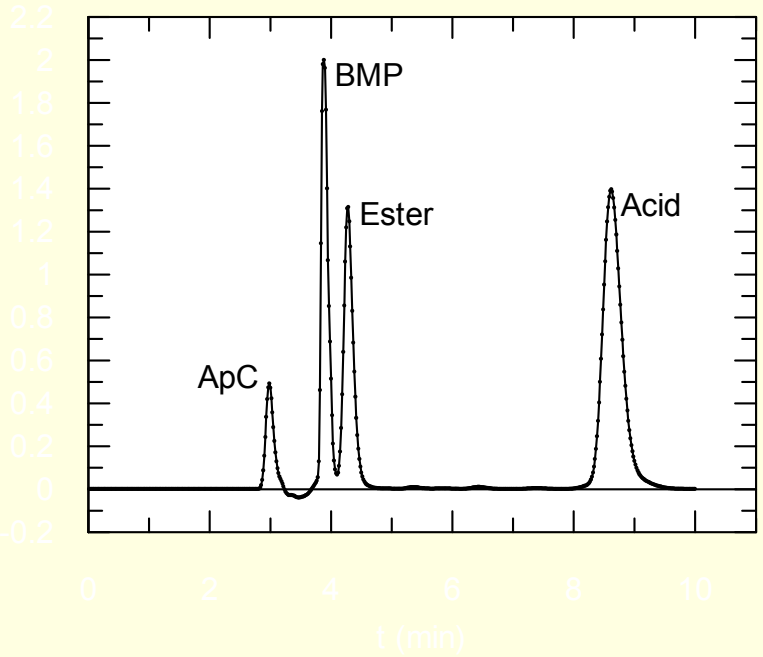
3’5’-cyclic AMP – no reaction



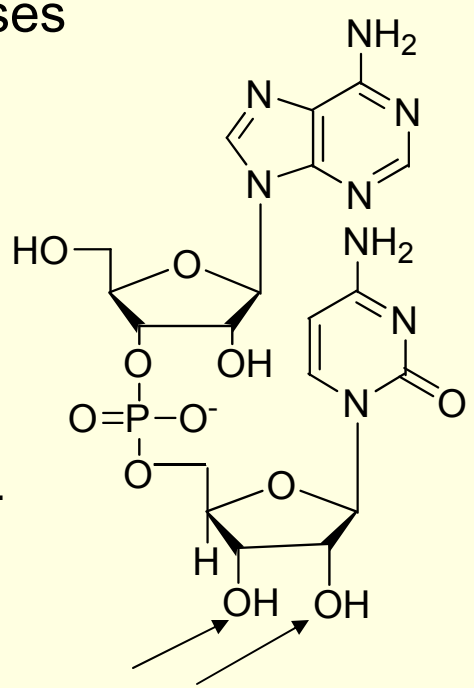


BzMP + La^{+3} etc.

Dinucleotide acylation



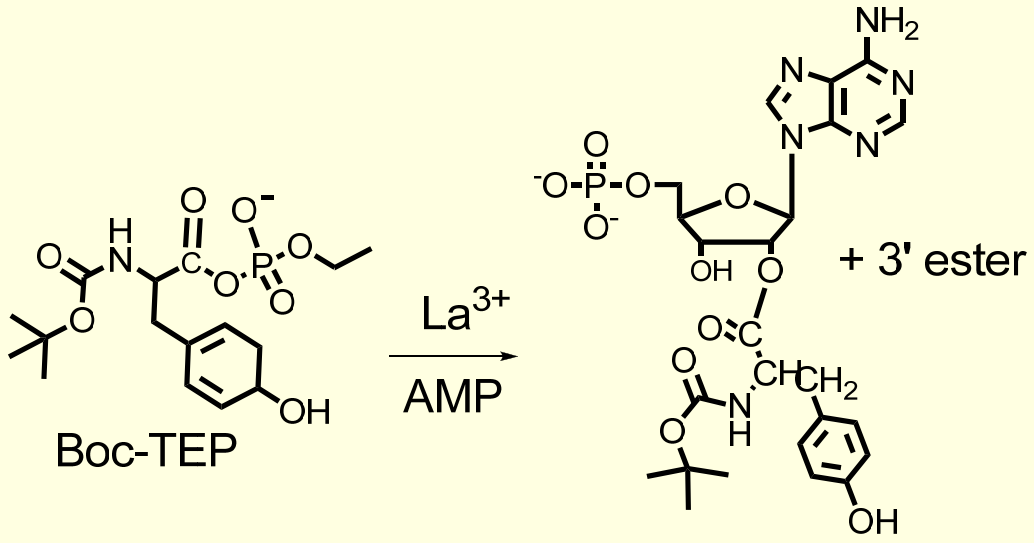
- Monoacylation product
  - Yield: 53 %
- ApC = Terminus of tRNA
- Used in Hecht, Schultz processes



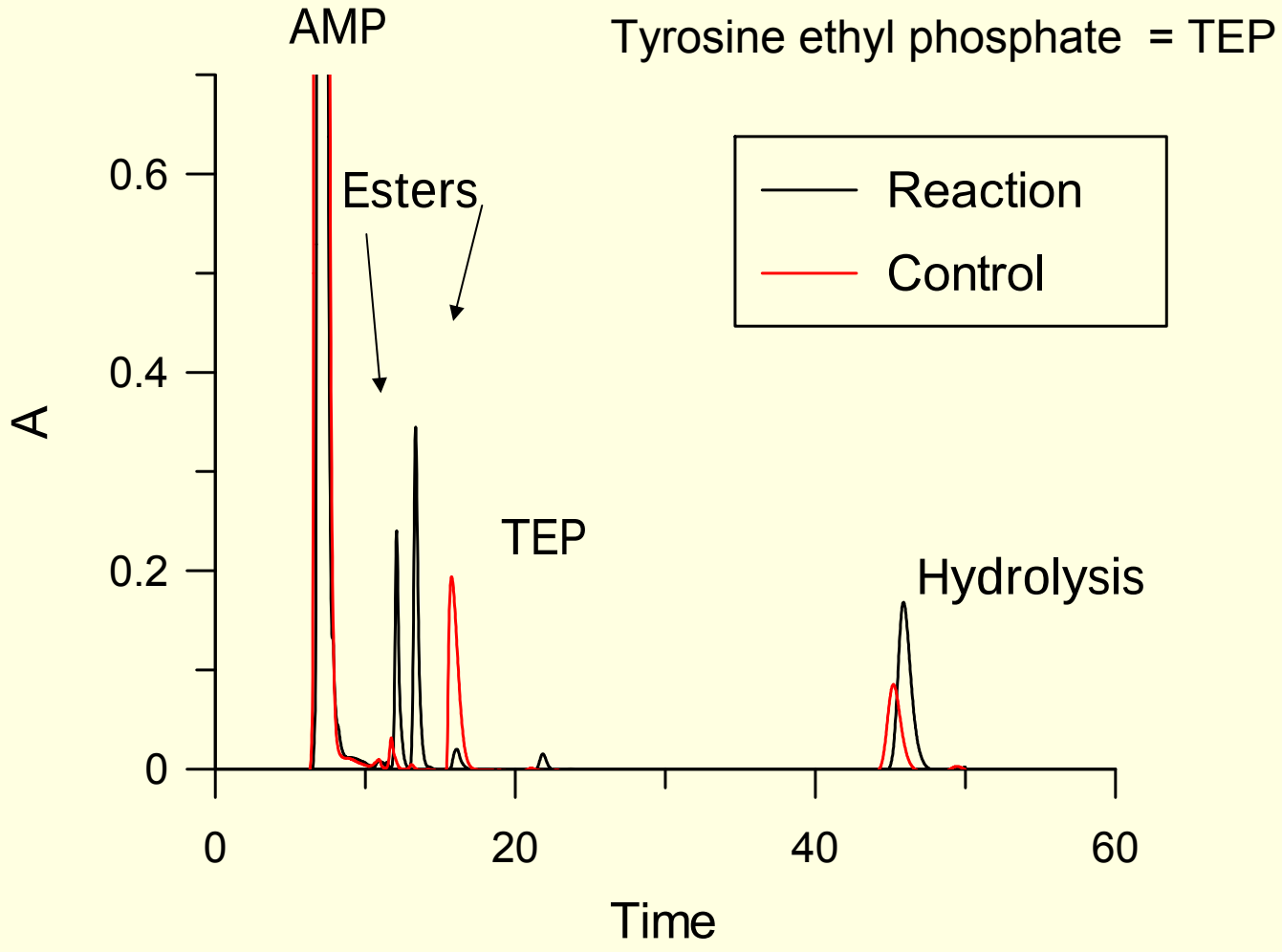
1×10 $^{-3}$ M ApC, 5× 10^{-4} M BzMP, 2 ×10^{-3} M $LaCl_3$, 2.4 h.

Aminoacyl phosphate and AMP

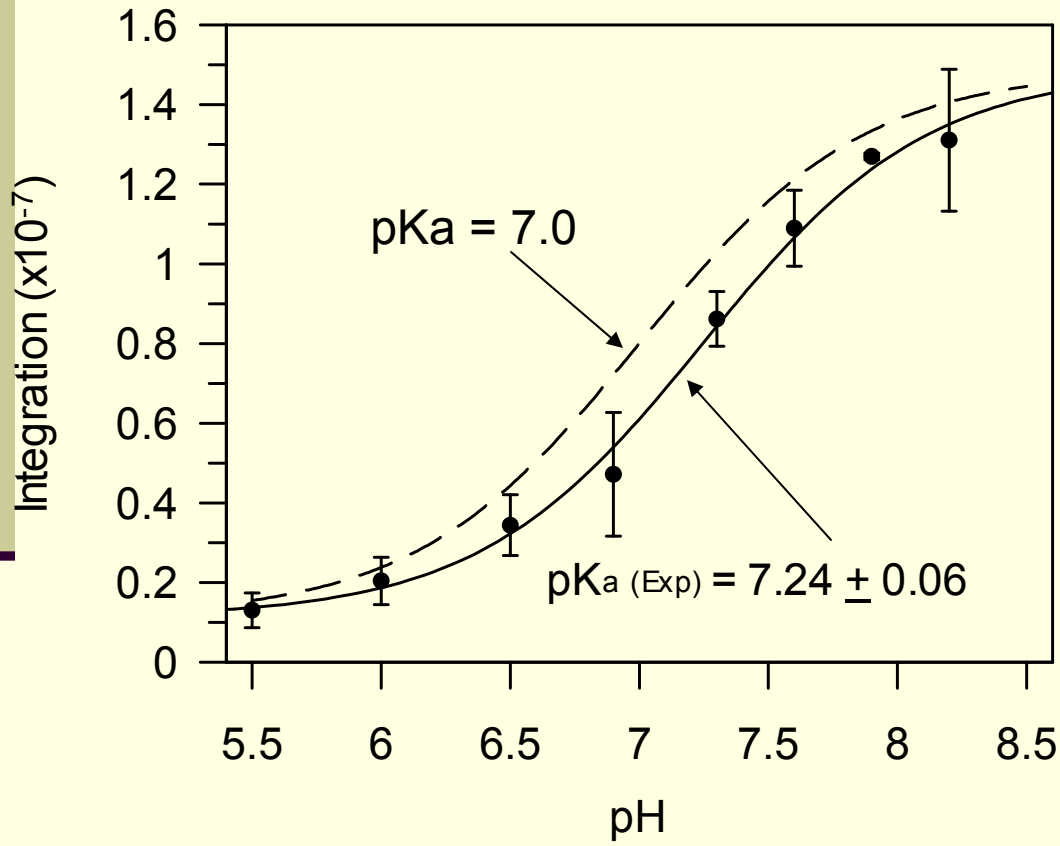
- Tetraethylammonium N-t-BOC tyrosine ethyl phosphate (TEP)
- Esters form (mass spec + HPLC analysis)



HPLC analysis



pH-dependence



$$I = \frac{I_{HOH}[H^+] + I_{OH}K_a}{K_a + [H^+]}$$

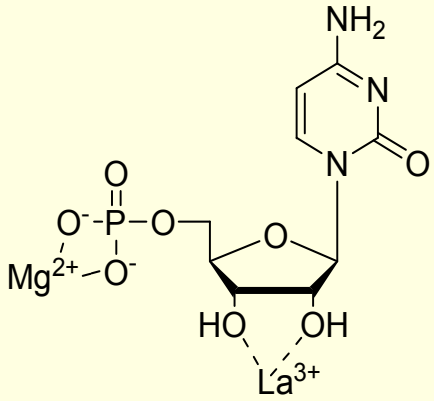
- $pK_a = La^{3+}(HOH)$

Steve Rathgeber

Metal ion synergy/selection

- Larger Ln(III) – more efficient acylation
  - Lanthanide contraction
- Mg^{2+} - no esters
  - Affinity toward PO_4^{2-}
- Mg^{2+} in competition with La^{3+} for PO_4^{2-}
  - Increase in ester formation (8%)

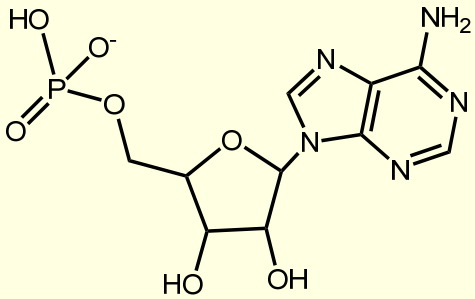
Ion	Atomic number	Ionic radius*, pm	5'CMP ester %
Mg^{2+}	12	72	0
Sc^{3+}	21	75	0.2
La^{3+}	57	116	31
Pr^{3+}	59	113	30
Nd^{3+}	60	111	29
Yb^{3+}	70	99	21



*Housecroft, C. E., Sharpe, A. G., *Inorganic chemistry*, Prentice Hall 2001

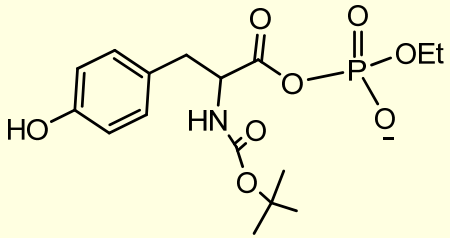
Aminoacylation yields

	BocTEP conversion*, %	Esters % area	BocTyr % area
AMP	66	55	11
GMP	66	58	8
CMP	68	58	10
UMP	62	33	29



AMP

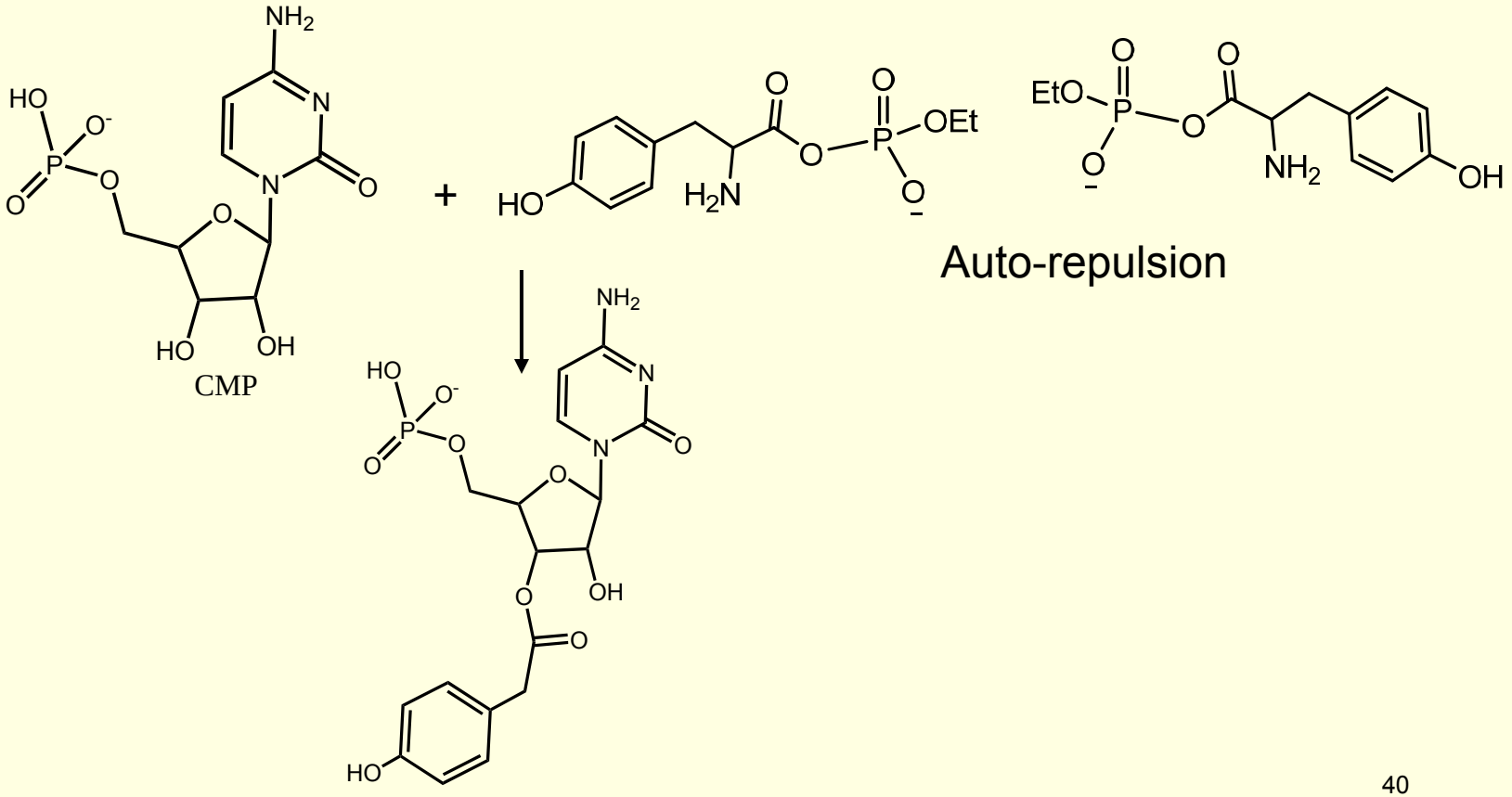
- 1.5 eq BocTEP, pH 8, 2 hours



BOC-TEP

Reaction without N-protection

- CMP + TEP produces ester

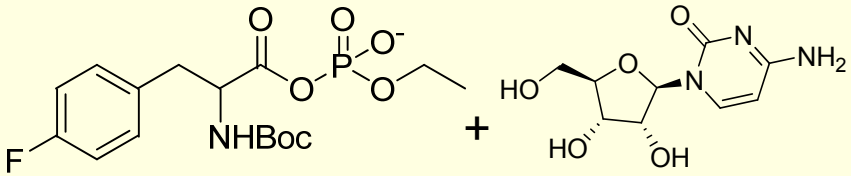


Analysis of reaction with RNA

- HPLC – not enough change to cause separation in macromolecule
- MS requires homogeneous sample, does not identify sites
- Alternative: introduce an unnatural unique signal in the aminoacyl group
- ^{19}F NMR
  - relatively sensitive
  - fluorinated amino acids are available

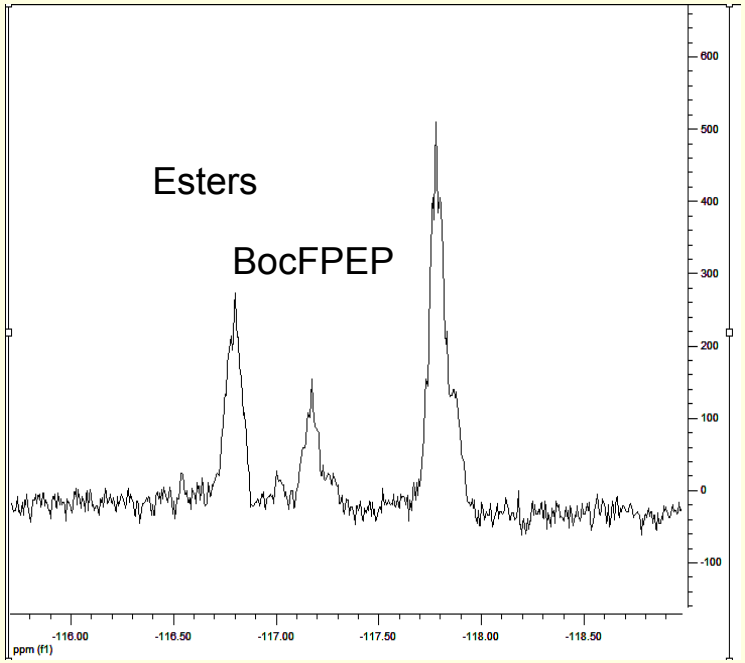
Svetlana Tzvetkova and R.K. *J. Am. Chem. Soc.* **2007**, *127* 15848

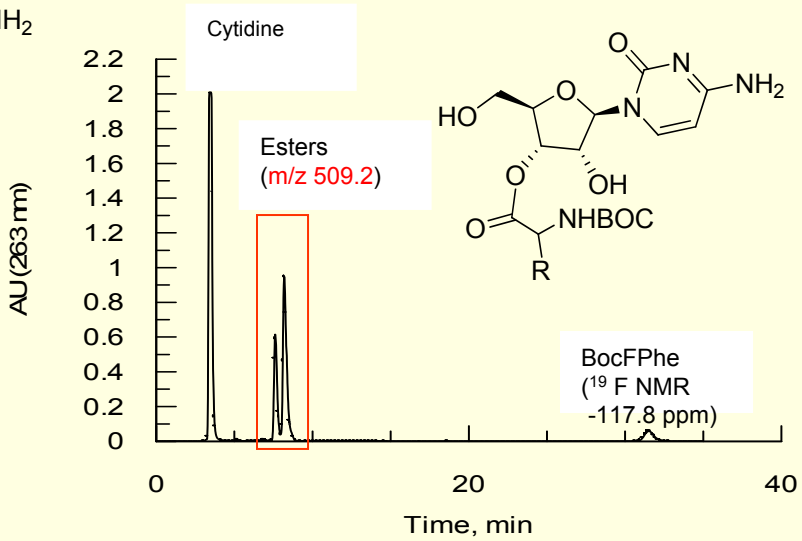
^{19}F NMR – aminoacylation of cytidine



BocFPEP
^{19}F NMR: - 117.2 ppm

NMR





Cytidine aminoacylation 3 minutes

HPLC

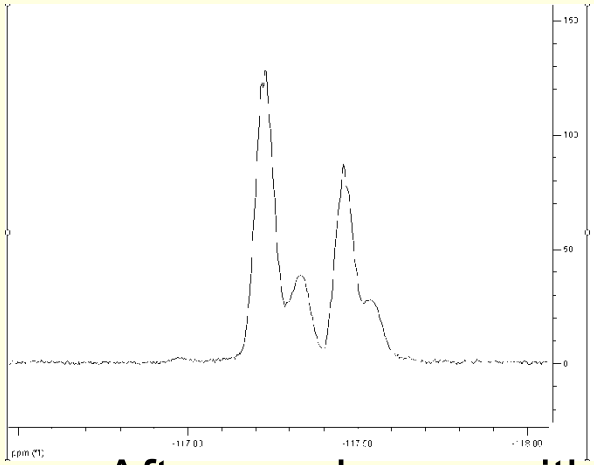
^{19}F NMR (282 MHz)
Reaction mixture quenched with EDTA at 10 sec ($LaCl_3$)

Svetlana Tsvetkova

Reaction with RNA mixture

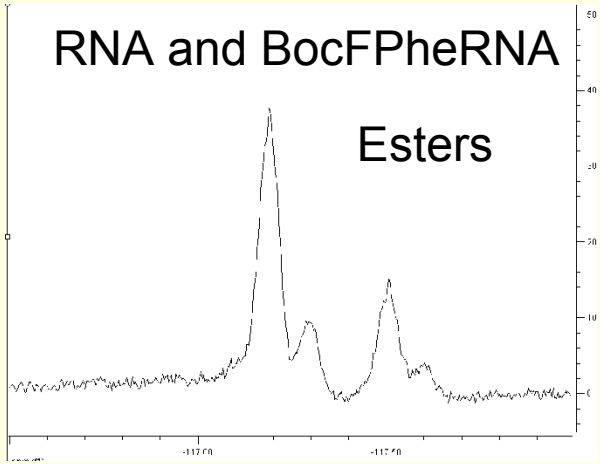
- Lanthanide-catalyzed aminoacylation (diol-specific)
- Mg^{+2} added to block phosphate backbone and keep structure intact

^{19}F NMR (376 MHz, D_2O)



After one hour – without purification

G-25



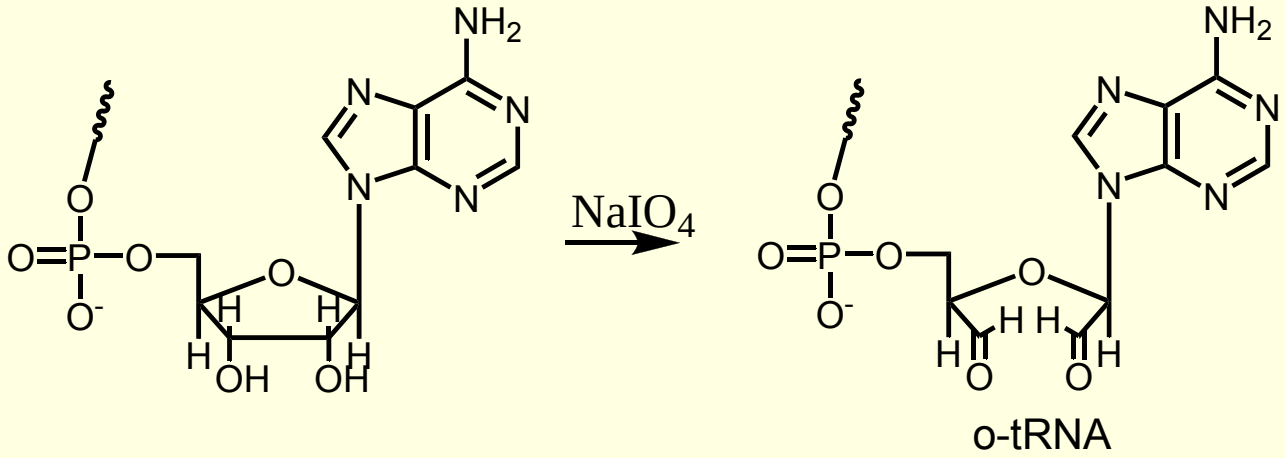
After purification and dilution

RNA mixture, BocFPEP, $LaCl_3$, $MgCl_2$, pH 8; 1 hr

^{19}F peaks are incorporated into RNA

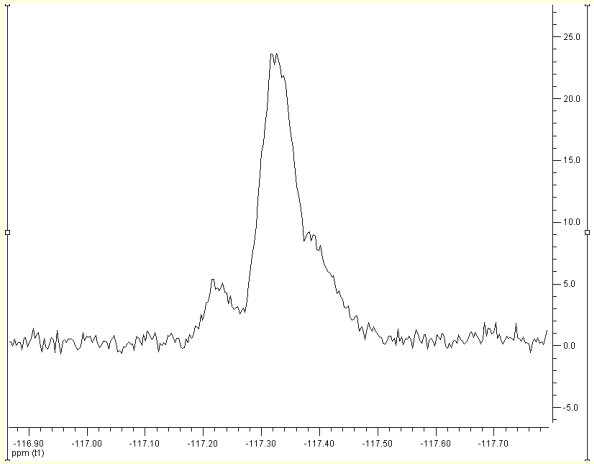
Is reaction at 3'-terminus of tRNA?

- Oxidize RNA with $NaIO_4$
- Convert diol to dialdehyde

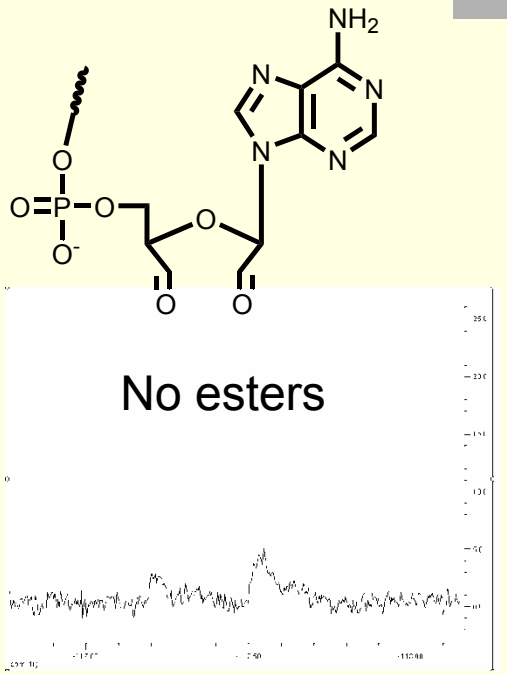


Method*: Nucleic Acids Res.* **1996**, *24*, 4535

o-RNA – no reaction with fluorinated aminoacyl phosphate



G-25



After one hour

^{19}F-NMR

After purification – hydrolysis product remains

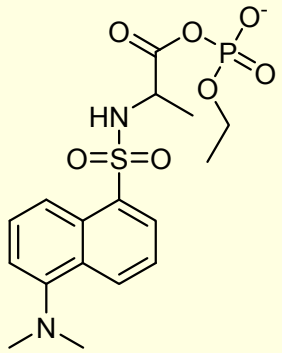
No F in o-tRNA

Establishes that incorporation is at 3' terminus

Fluorescent amino acids

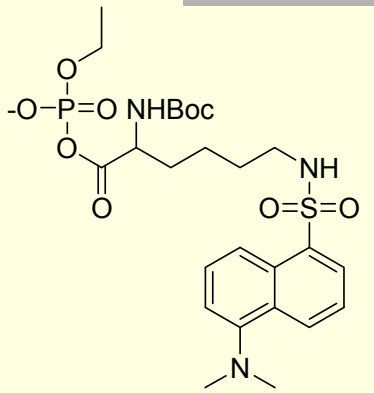
- Why?
  - More sensitive
- DNS
  - Some dansylated amino acids are commercially available
  - Anti-dansyl Ab for further detection

DNS- aminoacyl ethyl phosphates

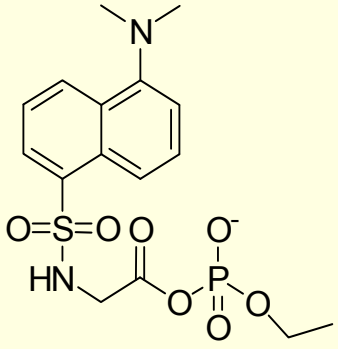


Dansyl-alanyl EP

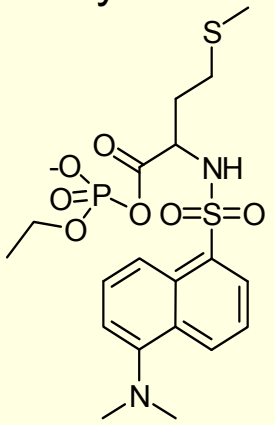
DNS
Excitation at 330 nm
Emission at 550 nm



ε-N-Dansyl-α-N-tBoc-lysyl EP

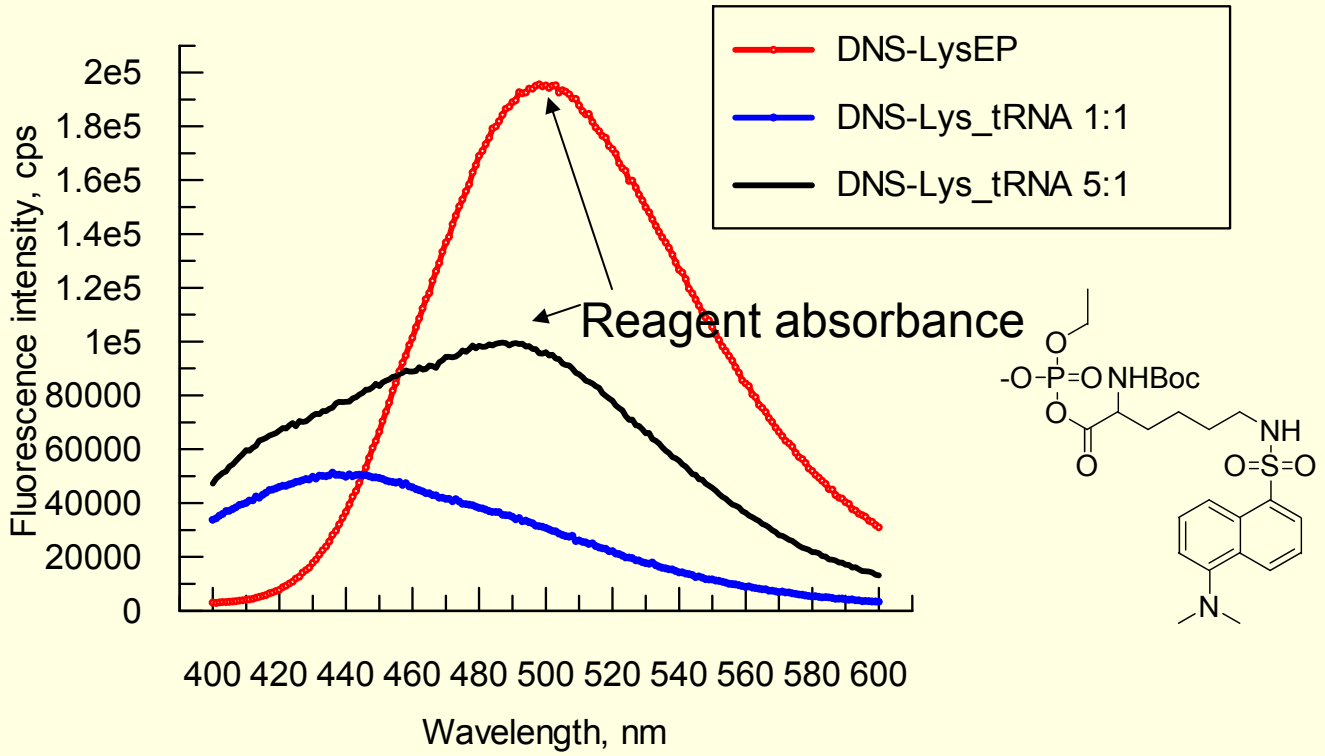


Dansyl-glycyl EP



Dansyl-methionyl EP

Fluorescent aminoacylation of tRNA

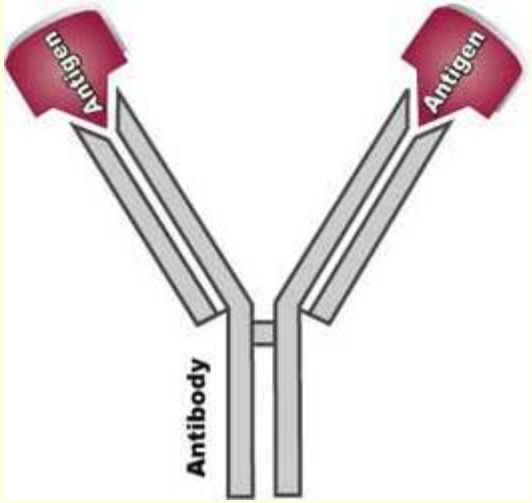


λ_{exc} 437 nm (α–Boc-ε-DNS-lysyl ethyl phosphate)
λ_{max} 500nm (free BocDNSLysEP)

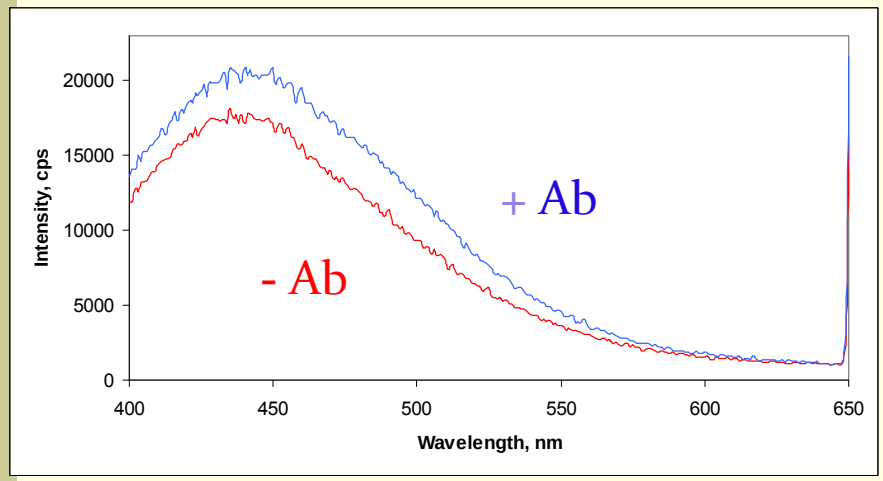
One eq clearly distinct and sufficient

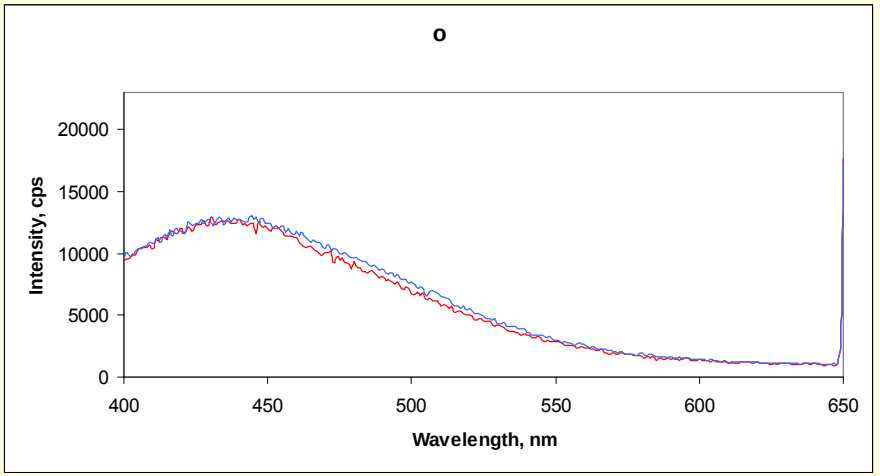
DNS Antibody

- Specific for DNS
- Enhances fluorescence on binding
  - Blue shift when bound (from ~ 520 to ~ 450 nm)
- Max fluorescence enhancement for DNS is about ten x
  - Steric hindrance - less enhancement



Antibody detection of tRNA modification

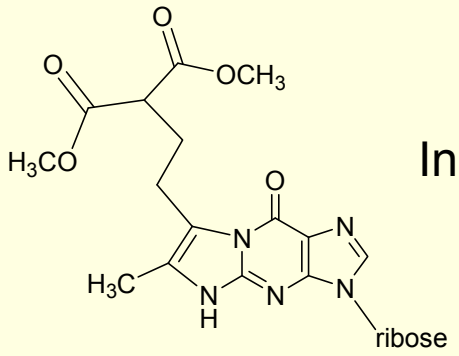




tRNA rxn + Anti-DNS Ab

$\Rightarrow$ Dans-Phe-tRNA forms ester

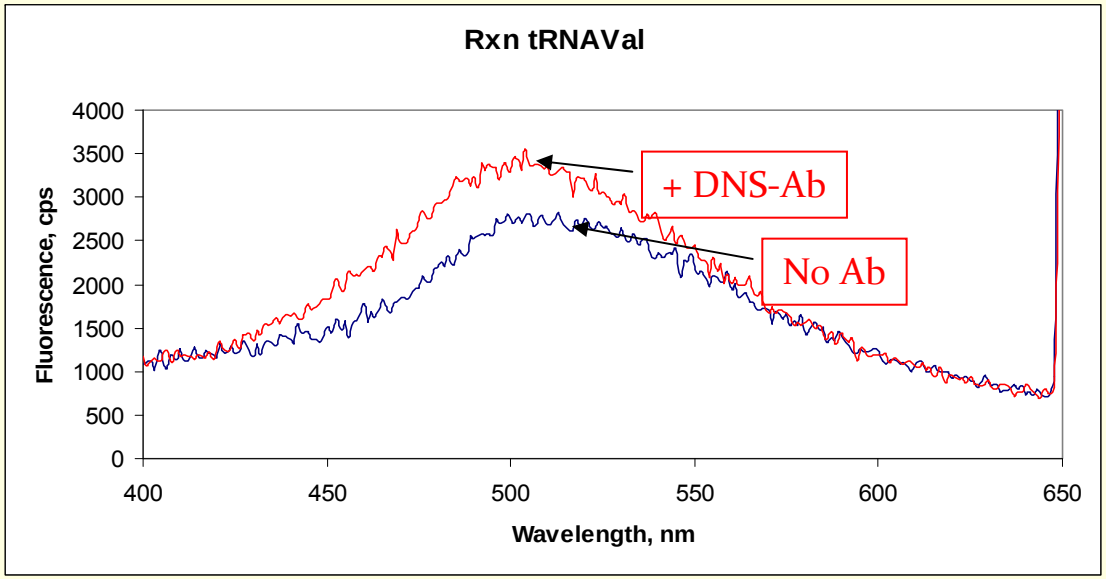
o-tRNA rxn + Anti-DNS Ab



Y

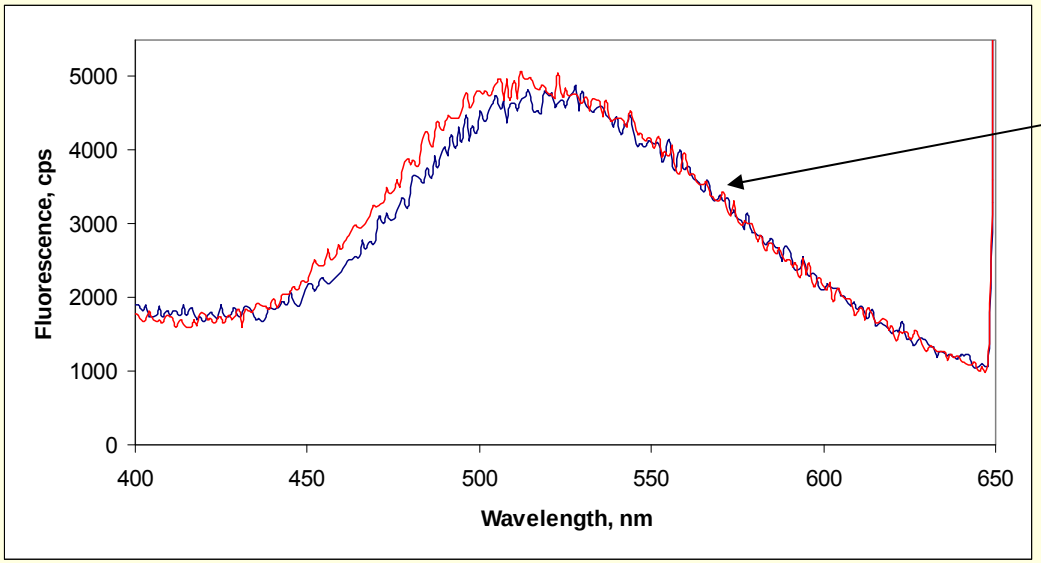
In Phe-tRNA

Val-tRNA antibody detection



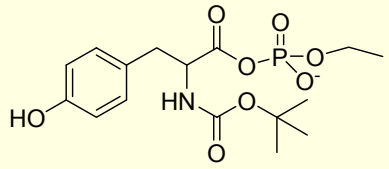
No native fluorescence

o-tRNA with DNS-Val-EP

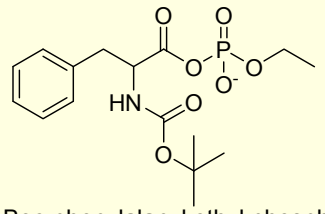


No diol – no reaction – retains DNS fluorescence of reactants only

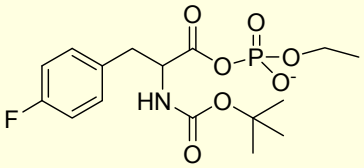
Tested aminoacyl phosphates



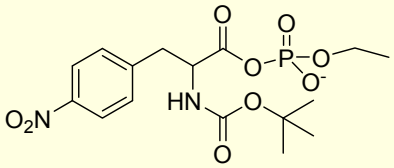
Boc-tyrosyl ethyl phosphate (BocTEP)



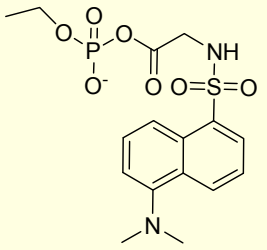
Boc-phenylalanyl ethyl phosphate



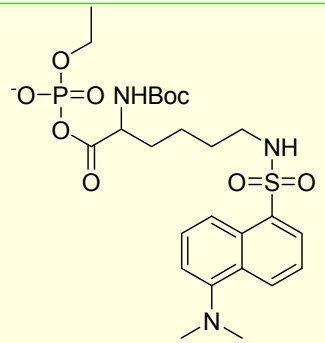
Boc-4-fluorophenylalanyl ethyl phosphate (BocFPEP)



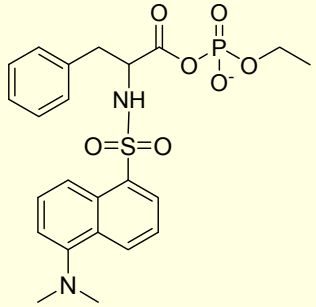
Boc-4-nitrophenylalanyl ethyl phosphate



DNS-glycyl ethyl phosphate (DNSGlyEP)



Boc-DNS-lysyl ethyl phosphate (BocDNSLysEP)



DNS-phenylalanyl ethyl phosphate (DNSPheEP)

Answers: Direct aminoacylation of tRNA

- Activate amino acids - Aminoacyl phosphate monoesters
- React with OH not NH – Lanthanide complexation (Lewis acid)
- Challenges
  - Selecting for 3'-terminal hydroxyls (~75 others!) – diol chelation, Mg^{+2} to block internal OH
  - Detection of product – F NMR and fluorescence