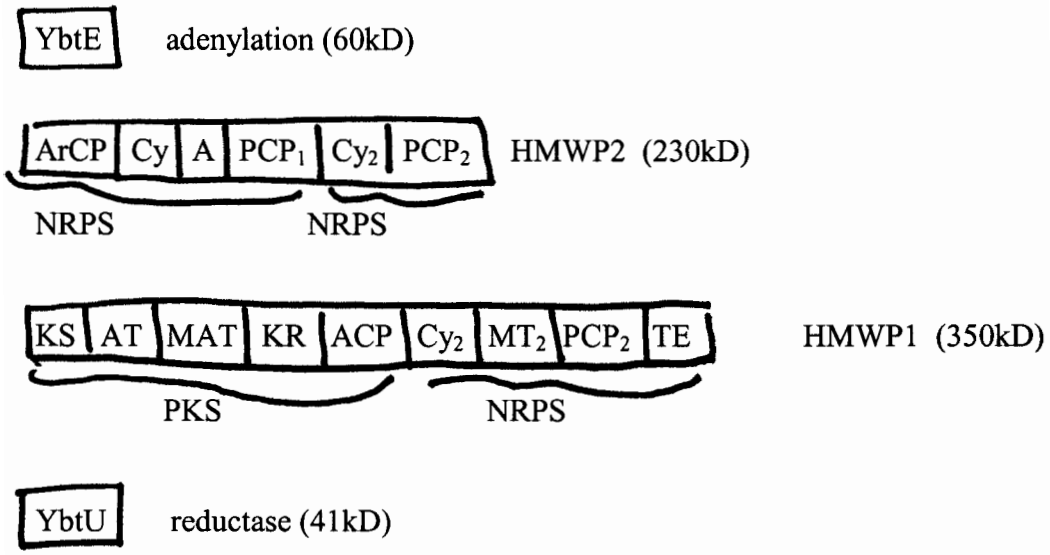
TD3
5.08 – PKS/NRPS pathways and mass-spec techniques for studying them
Reference: Mazur et al. Biochemistry 2003, 42, 13393

Background:
-Yersiniapestis = bacteria that caused bubonic plague
-biosynthesizes yersiniabactin (Ybt), an Fe III chelator
-Ybt strips Fe3+ from host proteins and is used for Y. pestis survival
-Ybt synthesized by hybrid NRPS/PKS system

D= Dalton = g/mol example: 10kD= 10,000 g/mol

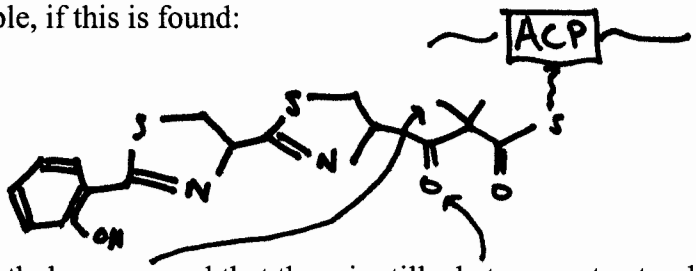


HMWP= high molecular weight protein

-first part (the work of HMWP2) is pretty well understood
-second part less clear, involves
1. dimethylation
2. keto reduction
3. condensation to Cys and cyclization
4. methylation
etc

Question: What order??

Overall Approach:
Use high resolution mass-spec to solve the structure of biosynthetic intermediates.
For example, if this is found:

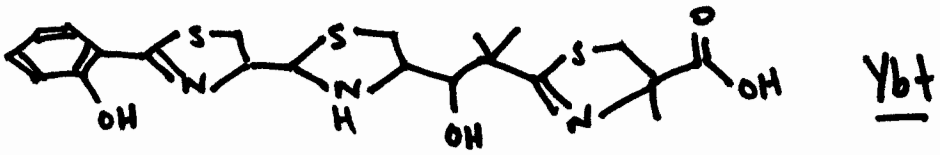


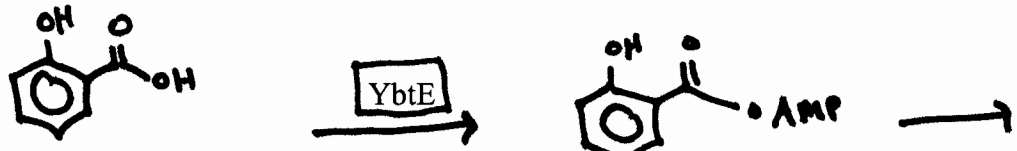
(note dimethyl groups, and that there is still a ketone- not yet reduced to –OH)

This means that dimethylation occurs BEFORE ketone reduction

Goal: try to establish order of chemical steps catalyzed by PKS part of HMWP1

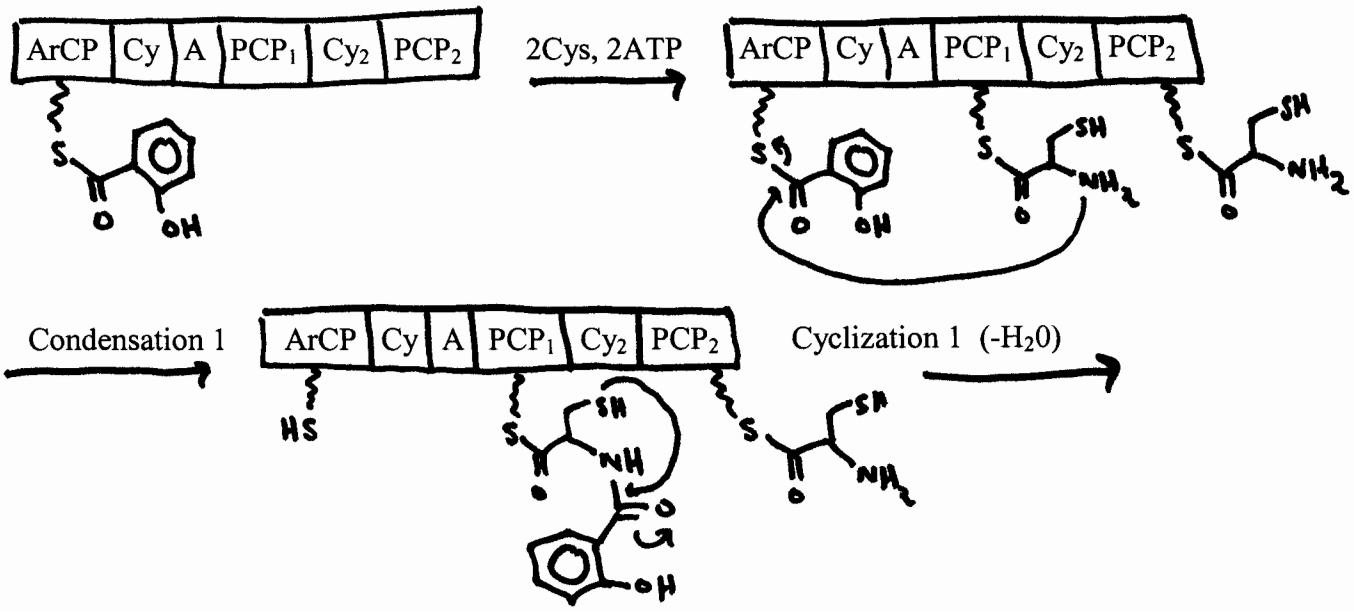
Proposed biosynthesis of Yersiniabactin (Ybt)

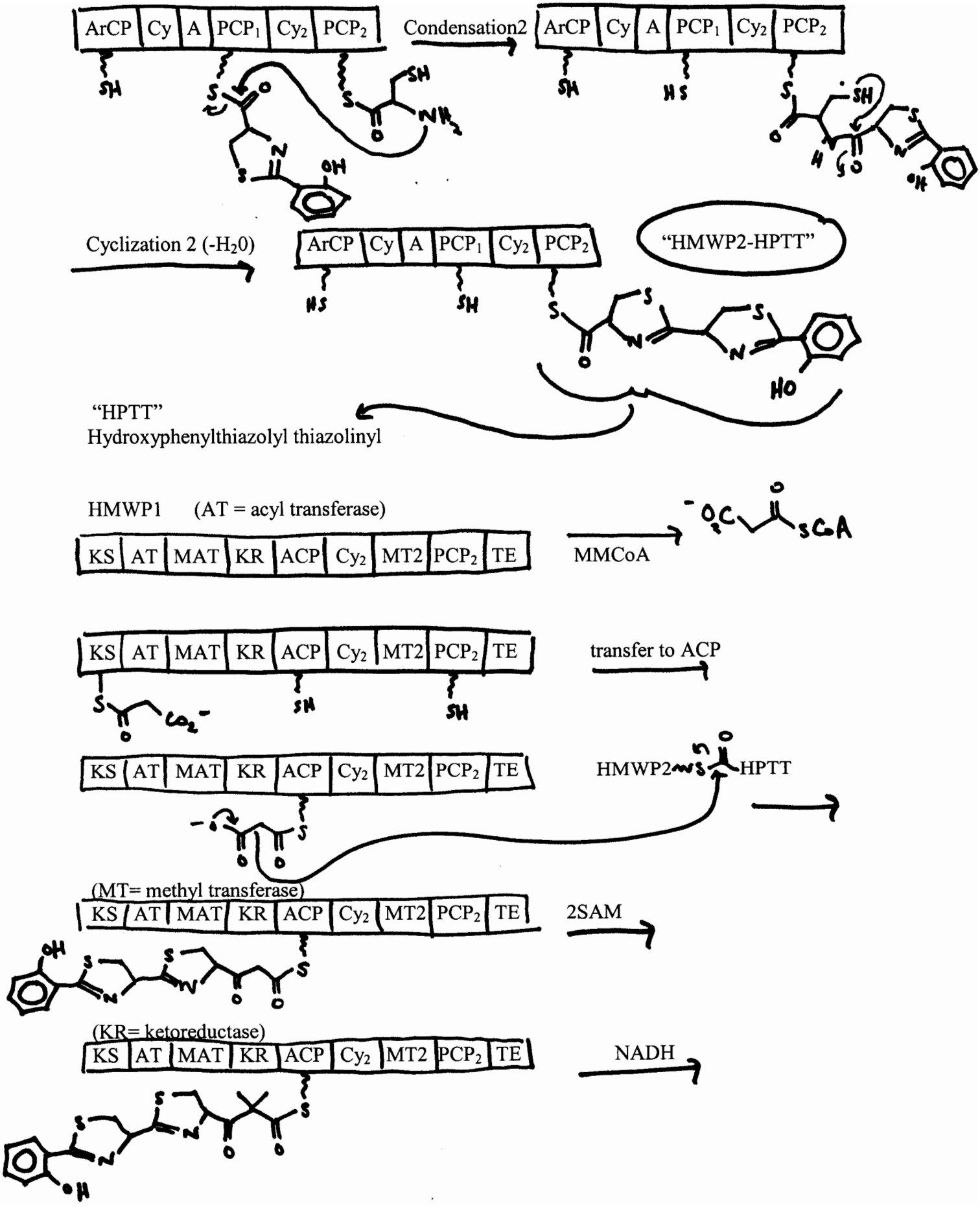


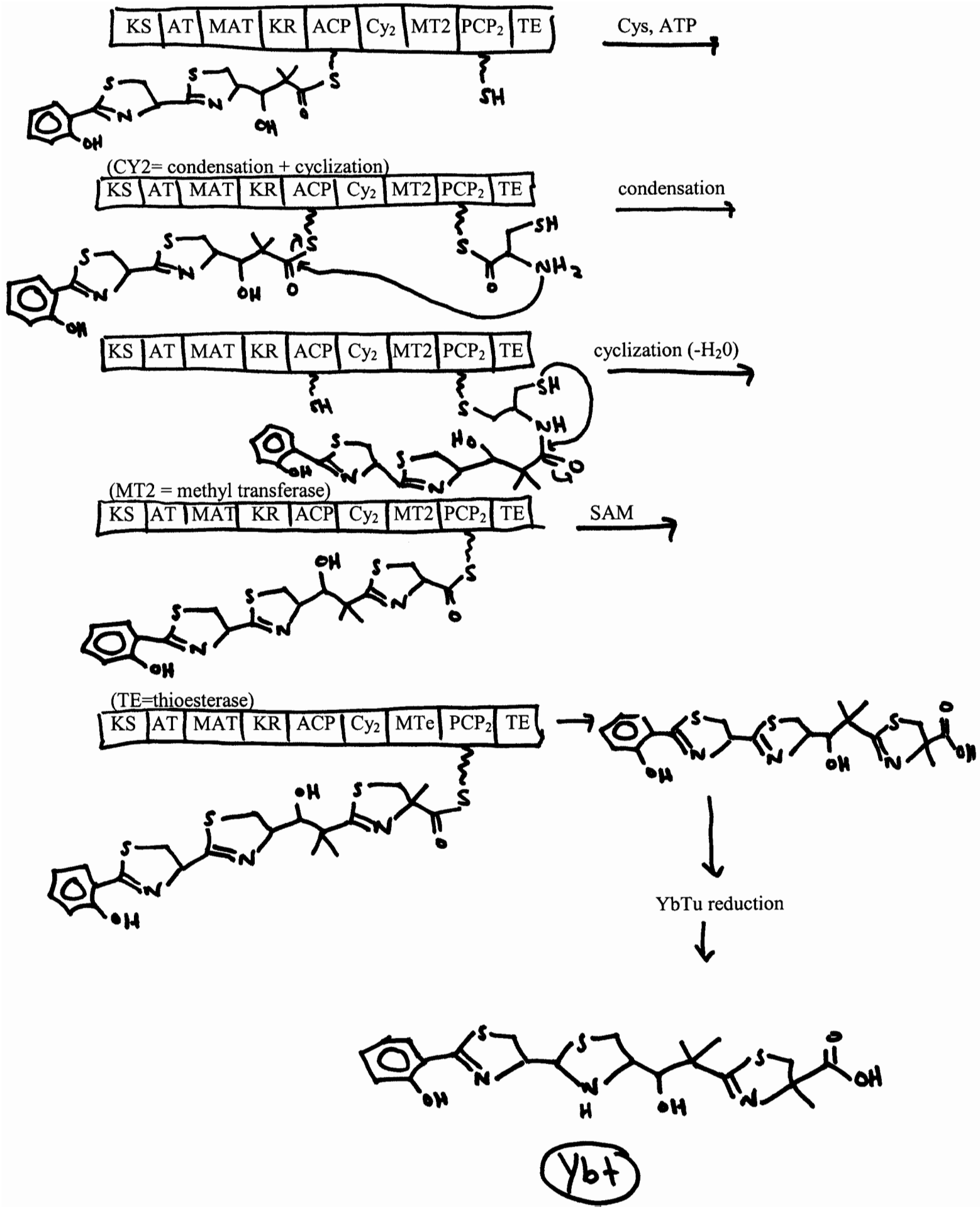


Salicylic acid adenylation

HMWP2 (A = Cys specific adenylation domain) (Cy1= condensation + cyclization)




ArCP Cy A PCP1 Cy2 PCP2
Condensation2
ArCP Cy A PCP1 Cy2 PCP2
Cyclization 2 (-H20)
ArCP Cy A PCP1 Cy2 PCP2
"HMWP2-HPTT"
"HPTT"
Hydroxyphenylthiazolyl thiazolinyl
HMWP1 (AT = acyl transferase)
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
MMCoA
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
transfer to ACP
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
HMWP2
HPTT
(MT= methyl transferase)
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
2SAM
(KR= ketoreductase)
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
NADH


KS AT MAT KR ACP Cy2 MT2 PCP2 TE
Cys, ATP
(CY2= condensation + cyclization)
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
condensation
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
cyclization (-H20)
(MT2 = methyl transferase)
KS AT MAT KR ACP Cy2 MT2 PCP2 TE
SAM
(TE=thioesterase)
KS AT MAT KR ACP Cy2 MTe PCP2 TE
YbTu reduction
Ybt

Steps catalyzed by PKS part of HMWP1
must occur in this order:
-loading of ACP w/ malonyl
-decarboxylation of malonyl
-condensation of malonyl w/ HPTT
-condensation w/ cys on PCP2 of NRPS part

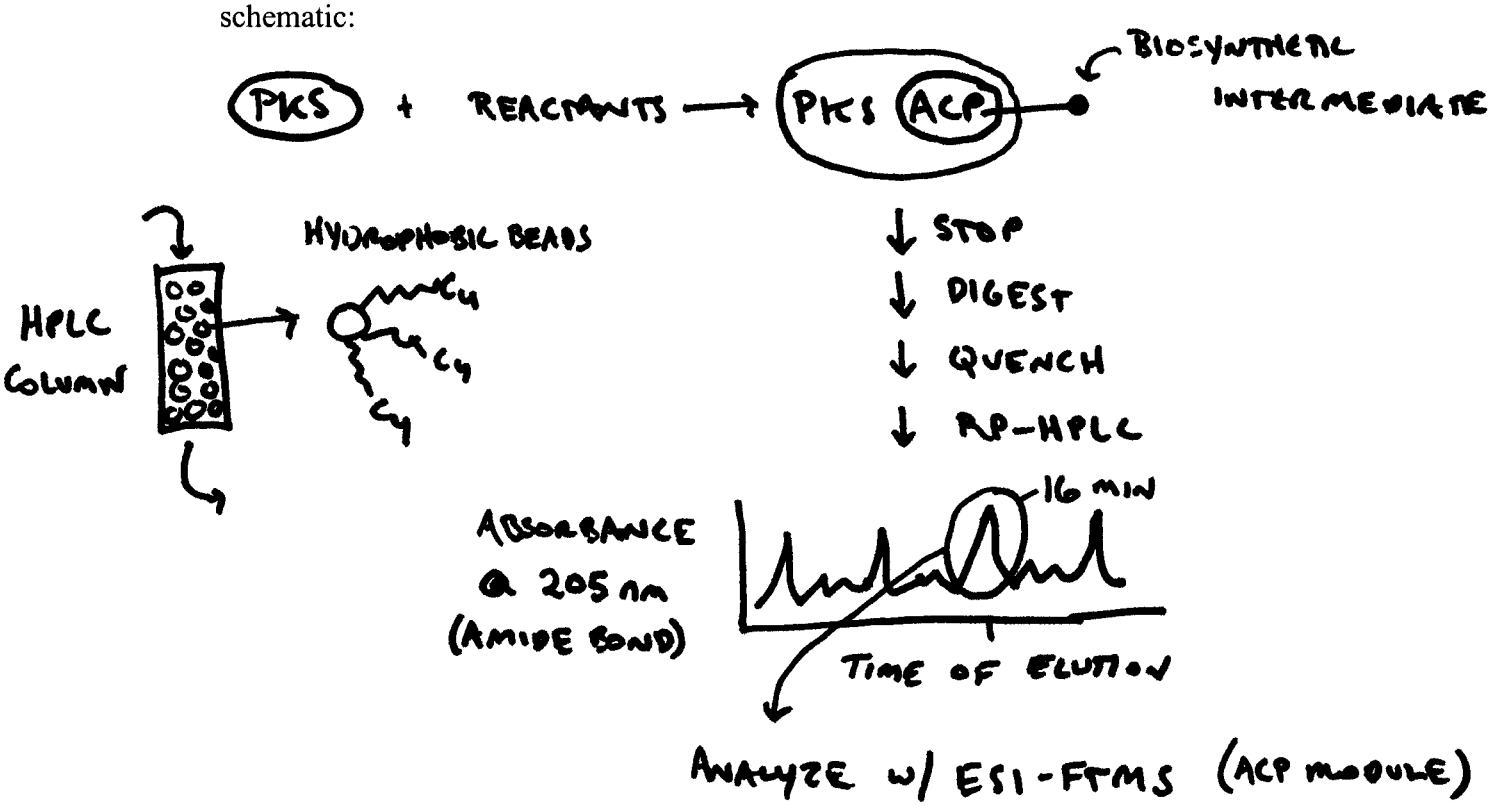
can occur anytime:
-dimethylation
-ketone reduction

Technique 2: Mass spec analysis of the ACP domain of PKS proteins

PKS + reactants -> PKS(ACP)-biosynthetic intermediate
-stop reaction
-add trypsin protease + TPCK (protease inhibitor) at 30degC for 5 min (short)
-quench digestion w/' 10% formic acid
-inject onto RP-HPLC (reverse phase HPLC)
    hydrophobic things stick well, migrate slowly
    hydrophilic molecules stick poorly, migrate quickly
-get complex mixture, ACP comes off at 16-17 minutes, purify this peak
-analyze purified ACP by ESI-FTMS= electrospray ionization fourier transform mass spec

Question: Why do trypsin digest at all? Why not take mass-spec of full-length PKS?

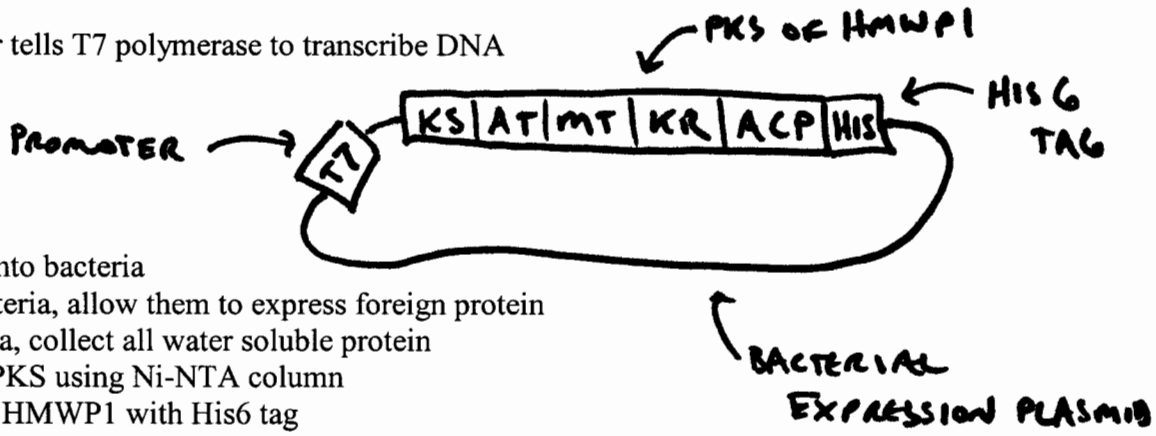
schematic:



Technique 1: Expression, purification and reconstitution of PKS part of HMWP1

recombinant protein expression

-put DNA for PKS part of HMWP1 in bacterial expression plasmid with T7 promoter and His6 tag
T7 promoter tells T7 polymerase to transcribe DNA



-transform into bacteria
-culture bacteria, allow them to express foreign protein
-lyse bacteria, collect all water soluble protein
-purify out PKS using Ni-NTA column
-get PKS of HMWP1 with His6 tag

To load PKS with molonyl CoA:
combine:
2.5 microM PKS (purified)
1.5 mM Malonyl-CoA (big excess)
wait 20min at 30degC

To stop reaction spin through dialysis tubing (big proteins stay above semi-porous membrane, small molecules go through)
-all excess M-CoA is seperated from PKS protein

To load PKS with HPTT and reconstitute activity of all modules:
combine:
2.5 microM HMWP2 (purified protein, same as for HMWP1)
2.5 microM YbtE (purified protein)
2.5 microM PKS (purified)
1mM cysteine
0.375 mM SAM
0.375 mM NADPH
1mM salicylic acid
1mM malonyl CoA
5mM ATP
wait 20 min at 30degC

Stop reaction as above, spin thorugh semi-porous membrane to remove small particles

Note: this is an *in vitro* study, is therefore subject to error

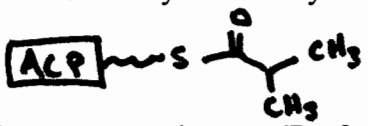
Step 1: Validate that mass spec technique works using controls
Test out method on:

PKS by iteslf-> get ACP mass of 11342 Da
(matches calculated mass for ACP, amino acids 1797-1896)

PKS + malonyl CoA -> get masses for ACP and ACP-malonylCoA (+86Da)

PKS + everythin except SAM -> get mass for ACP-acetylCoA (11342 + 42 Da)
(results from decarboxylation of malonyl)

PKS + everything -> get mass for ACP-caetyl + dimethyl CoA (11342 + 70 Da)



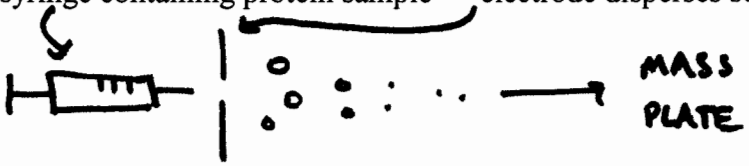
PKS + everything + CD3-SAM -> same as above + 6Da for $(CD_3)_2$ instead of $(CH_3)_2$

Controls all look good
-thioesters are stable to this method of analysis
-resolution of technique at least 6 Da
-technique also gives yields (relative peak intensities reflect relative product amounts)

See reference: Mazur et al. Biochemistry 2003, 42, 13393 **Figure 3A**
for a graph showing stability of malonyl loaded intermediate over time. peak intensities show relative product amounts.

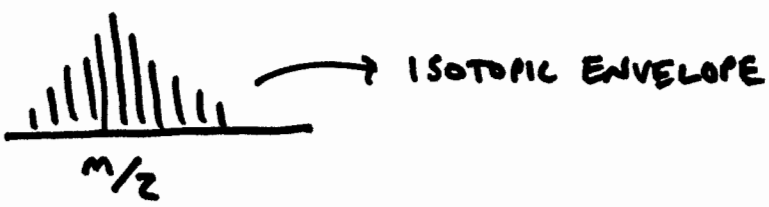
Technique 2:
ESI-FTMS (for more in depth discussion, see review BBA 1636 (2003) pages 1-10

syringe containing protein sample electrode disperses solution into a fine spray



"coulombic explosion" (repulsion of charged molecules overcomes droplets surface tension)

data is displayed as m/z- mass to charge ratio
Ex: insulin has a MW = 5732 Da
its specturm has 4 major peaks: 956 (m/6) 1147 (m/5) 1434 (m/4) 1912 (m/3)
Software calculates the mass of parent from m/z peaks
Each peak usually looks like: isotopic envelope, because proteins contain various isotopes in various combinations (^2H, ^{13}C, etc...)



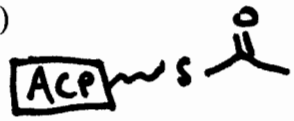
Step 2: the real experiment

PKS + HMWP2 + YbtE + all else

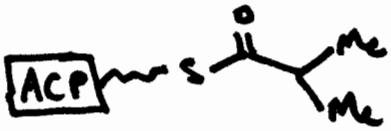
incubate 20 min at 30degC

partial digest, RP-HPLC purify out ACP, mass-spec (ESI-FTMS)

Found:
ACP by itself (11342 Da)
+ 42Da (decarboxylation)

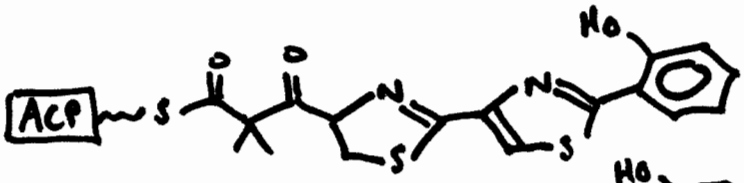


+70Da (dimethylated)

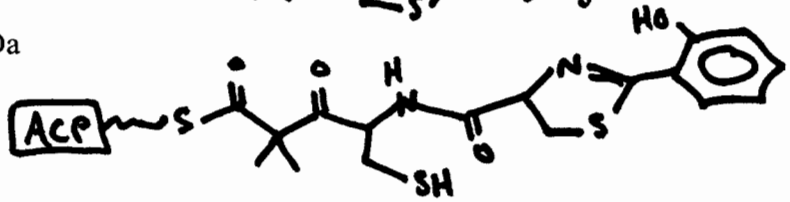


+198 Da ??
+225 Da ??

+ 358Da



+ 378 Da



How to double check these structure assignments? Substitute CD_3-SAM for SAM. If +358 and +378 intermediates really have diMe, should see +6 increase in mass. Similar check were done with D_2-cys, and D_6-salicylic acid

See Mazur et al. Biochemistry 2003, 42, 13393 **Figure 5 A + B**
to see the shift in mass of intermediates when CD_3 labeled SAM is used

Some conclusions:
+358 Da already dimethylated but not reduced. probably air oxidized from thiazoline to thiazole (probably does not occur *in vivo*)
therefore- ketone reduction occurs AFTER dimethylation and condensation (HPTT to malonyl)

+378Da dimethylated but not yet cyclized
therefore, dimethylation occurs BEFORE cyclization

Also note: no monomethyl (only dimethyl) product detected.
-2 methylation events tightly coupled, probably second is faster than the first

Step 3: More experiments
Question: when does dimethylation occur? before or after condensation?

Expt 1: leave out HMWP2-HPTT
-no mehtylation detected
Expt 2: leave out SAM
-decarboxylation malonyl detected but very little condensation product observed

Apparently contradicting!
Model: condensation is reversible
methylation occurs after condensation and methylation traps the condensation product

For the overall MODEL derived from this work, see Mazur et al. Biochemistry 2003, 42, 13393 **Scheme 1**
Remember that this model represents partitioning *in vitro*