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ELIZABETH
NOLAN:

Last time we were working on this PKS assembly line that makes acrylide. And just as a review of that, we left off having gone over the domains and module architecture for this assembly line. So recall, each module activates a given monomer. And we can use depictions like this to show how the PKS builds a growing polyketide chain.

OK, and as you saw in recitation last week, the actual structure of one of these synthases is very different than what's depicted by this left-to-right kind of assembly-line depiction there. So you saw some amazing conformational changes of the fatty acid synthase. And they're all different, but just keep that in mind when thinking about these. So this sort of notation is very helpful for us in terms of thinking about how the biosynthesis goes, but it's not an accurate representation of structure.

OK, so where we left off was with looking at how these optional domains can do chemistry on the upstream monomer. And the last thing we're going to do related to this assembly line is, one, ask how is the polyketide released from the assembly line when the biosynthesis is over. And then we'll just do one exercise looking at the macrolide and working backwards. So last time, we were looking at the domain organization to determine what sort of chemistry happens to a given monomer. We can do, effectively, the opposite, looking at a natural product and identify what those monomers and properties of the assembly line are.

OK, so in terms of chain release, there are thioesterase domains. And these domains are involved in chain release from the assembly line. So if you take a look in the final module here, what we see at the end is a TE for the thioesterase.

OK, and so what happens in the case of DEBS is, ultimately, the chain gets transferred to a serine residue on the TE domain. OK, and I'm just going to draw the polyketide like that. And then in this case here, we remember we have the propionyl-CoA from the loading module, so the starter unit.

In this case, what happens is there is a macrocyclization. So we can imagine deprotonation--

oh, excuse me, I forgot the linkage here. So for this TE, we no longer have the growing chain tethered by a Ppant arm. With the TE domain, it's tethered to the serine residue. So it's transferred from the thioester to this serine.

And this here is just the polyketide in between. I'm just abbreviating it. So we can have that. We can have attack and loss. OK, so in this case what we end up with is the TE domain plus a macrocycle.

And so that's how we end up with the structure as shown here. So some TE domains will result in formation of a macrocycle. Some TE domains will catalyze a hydrolytic release. And you get the linear chain.

So you need to look at the natural product structure. And based on that structure, you can make an assessment as to how the TE works. And so that's also shown in this depiction and one other depiction in the notes. So here, the entire chain is drawn. And we're seeing deprotonation donation here and then attack here to give the macrocycle.

So here is the product of this DEBS. And what we're going to do as a last exercise with the PKS is just look at this structure and work through identifying the monomer units and what optional domains acted on each monomer. And basically, where can we start?

So if the thioesterase catalyzes a macrocyclization, that's an easy starting point, because basically, the final monomer needs to be involved there. And we know that the only place we can get a structure like this is from the starter, from that propionyl-CoA. So here, if we just look, we have the monomer from module 0, the loading. And then as we learned last time, each additional unit that gets attached to the growing polyketide gives two carbons, so two carbon units to the growing chain.

So we can work our way around by two carbons, 1, 2. OK, here we have module 1, another two carbons, module 2 here, module 3, module 4, module 5, and here, module 6. So looking at a structure, you can begin to dissect what the assembly line will look like in terms of the number of modules by counting C2 units to the growing chain here.

And then the other thing we can do is look at the functional group status and ask what types of optional domains needed to be there in order to give a given functional group. So for instance, in this case, in module 1 here, we're seeing an OH group. So we know there had to be the

action of a keto reductase to reduce the ketone. Here we see we have this carbonyl, so there was no optional domain.

In this case, what happens? We have a methylene. So that ketone we started with was fully reduced. So in this case, we have the keto reductase, the dehydratase, and the enoyl reductase.

Again, here we can look at this unit. We see an OH, which tells us that there was action of a keto reductase. And here we have another OH, so we have a keto reductase. And in this case, we have none, this final one. And here, I didn't write it, but none in terms of optional domains.

So this can be pretty fun. This is a pretty simple structure, but as structures get more complex, you can map out what are the optional domains there. And maybe you'll see, some other unusual structural features will indicate there is other optional domains beyond these three. And we're going to see some of that as we move into the non-ribosomal peptides. So that's given in the notes if you want to practice on that.

So with this, we're going to transition into an NRPS and look at the assembly line logic for non-ribosomal peptides. And so this is a slide from last time, where we considered the starter units and extender units for fatty acids and for polyketides. And so in non-ribosomal peptides, we also have starter units and extender units, but in the case of the non-ribosomal peptide, as the name indicates, we're going to be thinking about amino acid monomers. And we're also going to be considering examples where there is aryl acid monomers. So these NRPS assembly lines will form polymers that incorporate amino acid and aryl acid monomers.

And this is another slide from last time that is just summarizing the core domains and then examples of optional domains for the PKS and NRPS. So we learned last time that for PKS, every module will have a KS and a T domain, with the exception of the loading or starter module. That has no keto synthase, because there is no upstream group here.

For NRPS, the core of a module is CAT trio. So condensation domain, or C domain, this is the domain that's going to catalyze peptide bond formation between two of the monomers. We have an adenylation domain. And we'll see this does chemistry similar to the aminoacyl tRNA synthetases. And then we have the T domains that are carrier proteins for the monomers and growing chain.

OK, and then within a given NRPS module, there can also be optional domains. And just two

examples are shown here. So maybe there is an epimerization of an amino acid. Maybe there is a methyl group and there needs to be methyltransferase to put that on.

There is a lot of diversity that comes into these structures on the basis of these optional domains. And just to highlight that, I've presented here a list of possible optional domains you can find in NRPS, or for that matter, a PKS here, so all sorts of things. Look at halogenase, cyclase, reductase. There is tremendous structural diversity that can occur.

OK, so if we consider the NRPS assembly line structure and notation similar to what we did with the polyketide synthases, what do we see? So I'll just draw one with two modules here, although n can indicate more. So initially what we have here is a starting or loading module, OK, so for instance, module 0.

OK, here we have module 1, 2 for extenders. And here we have a thioesterase for chain release. So we'll find that in the final module like what we saw with the polyketide synthase for DEB. So this whole thing can be called an NRPS here.

And what happens in terms of the action of these different core domains-- so A, we have adenylation. OK, and what these domains do are select and activate the amino acid or aryl acid monomers. OK and after these monomers are activated, the A domain also transfers them to the T domain. And we'll go over the chemistry in a minute.

OK, this T domain is like what we saw with the PKS. We can call it a thiolation domain or a peptidyl carrier protein. So these T domains are going to be modified with the Ppant arm, like what we saw for PKS.

We have the C domain, condensation. And so this domain capitalizes peptide bond formation. And I'll just point out here that, in contrast to the keto synthase we saw in PKS-- so we saw the keto synthase doing covalent catalysis via its cysteine residue-- the condensation domains of NRPS are involved in non-covalent catalysis. So that's just an important distinction. The growing chain does not get attached to the C domain here.

And then we have the TE, so thioesterase, as we saw, for chain release. And this can be hydrolytic or macrocyclization. OK, so let's consider just the example of an NRPS that is responsible for synthesizing a tripeptide. So what is the net reaction?

So imagine that we have three amino acid monomers. And I'll just point out here too that beyond knowing an epimerization domain epimerizes an amino acid, you're not responsible for

stereochemistry in terms of the various structures we'll look at going through here. So I'm just not drawing stereochemistry here.

So we have three amino acid monomers. There is going to be some NRPS that's responsible for formation of the tripeptide. And what we'll see is that making a trimer requires three ATPs, so one ATP per amino acid or aryl acid monomer, giving us three AMP plus three P_i here to give us our tripeptide plus three water molecules here.

OK, so how does this happen? How does the NRPS take these monomers and build, say, a tripeptide? We're going to look at the ACV synthetase as a model for this.

And so the ACV tripeptide is important. It forms the backbone of antibiotics of the penicillin and cephalosporin classes. So many of these are used clinically.

So here are the structures of penicillin N and the cephalosporin. So at first inspection, you might not guess that these are effectively built from a tripeptide, but what happens is that a non-ribosomal peptide synthetase, the ACV synthetase, is responsible for forming two amide bonds between the three starters-- or the three monomers. And then there is additional enzymes that are responsible for modifying that peptide scaffold to give, say, this four-five fused ring system or this four-six fused ring system.

OK, so what is the overall reaction of this? So similar to having these three amino acid monomers here, what we have are aminoacyl-tRNA. We have L-cysteine and L-valine. And the synthetase takes these three monomers and makes this molecule here, which is called ACV.

And so if we look at the synthetase in cartoon form, this is the cartoon. So we see a loading module, so just AT. Similar to the PKS, there is no catalytic domain to make a new bond in the loading module because there is nothing upstream.

We see a module here, CAT. We have another CAT module here. And then what's this? This is our first example of an optional domain within an NRPS. OK so this E is for epimerization. I mean what we'll see is that the synthetase epimerizes L-valine to D-valine during the synthesis, and then the thioesterase.

So similar to what we did with the PKS, for the NRPS, you can count T domains as a way to identify the modules and to figure out how many monomers are involved. I also just point out-- and this builds upon Colin's comment from last time-- is that this assembly line is responsible

only for the synthesis of a tripeptide, but look at its size. It's greater than 450 kilodaltons. That's quite big-- so a large enzyme, 10 different domains, all just for synthesis of this one tripeptide here.

So what happens? We're going to go over the action of the A domains and the T domains first. And then we'll look at a cartoon in the slides.

So the first points to make are that we need to have loading of the assembly line. So amino acids need to be selected and activated. And that's where these A domains come in.

So what's happening? So we have some amino acid monomers-- so maybe it's the L-cysteine, for instance, or the L-valine-- plus ATP. The A domain does chemistry similar to what we saw with the aminoacyl tRNA synthetases to form an activated intermediate.

So we get an amino adenylate here. And then what happens? So the T domain-- OK, and this T domain must be modified by a PPTase, like what we saw for the PKS, to have the Ppant arm. After we have activation of the amino acid or aryl acid monomer, the A domain is going to assist with transfer of this monomer to the Ppant arm of the T domain here. OK, so we got an aminoacyl-S-T covalently tethered via a thioester linkage.

So one ATP is consumed per monomer loaded. And the ATP PPi exchange assay we discussed back in the translation module for studying the aminoacyl tRNA synthetases is used all the time to study new A domains and ask what amino acid or aryl acid monomers do they activate here. So that assay comes up in this type of work.

So what happens then in terms of formation of a peptide bond, we're going to consider condensation by the C domains. And so let's just imagine-- we're just going to draw two modules. So we have a loading module and then a first extender module. And the T domains have been post translationally modified with the Ppant arm. And the action of the A domains has loaded the amino acids at this stage. OK, so we have some amino acid loaded here. And then we have some amino acid loaded here.

OK, and what happens? We're going to have nucleophilic attack from the alpha amino group onto the upstream monomer and then transfer of this monomer. And this occurs via the action of the C domain. We have R2. And now we have formation of our new peptide bond. Sorry, this is R2 here. And as I noted above, there is no covalent catalysis with the C domain. Somehow it's helping to bring these chains together and to allow this nucleophilic attack to

occur and to allow the monomer to be transferred, but this unit is never transferred to the C domain itself. Yeah?

AUDIENCE: So is the C domain responsible for deprotonating the NH₂? Or is that just always--

ELIZABETH Yeah, I don't-- how this gets deprotonated, I don't know. But this is back to similar, like what

NOLAN: we saw in the ribosome. And somehow, this alpha amino group needs to be deprotonated. And there is something in the environment of this machine that's allowing that to happen, but whether it's the C domain or something else, yeah, I don't know the answer to that.

So let's look at a cartoon of this with this ACV synthase. So here we have on top the synthase loaded with the amino acid monomers. OK, so we see loading module and then two extender modules. We have the amino adipate. So it's not a canonical amino acid, but it's amino-acid-like. We have the cysteine and the valine.

What happens as these condensation reactions occur, we get chain elongation. So this is depicted here in a similar manner to how that PKS assembly line was depicted. So formation of two peptide bonds, and then what happens? Ultimately, we have chain transfer to a serine residue on the thioesterase domain.

And this is a case where the thioesterase domain catalyzes this hydrolytic release. So as opposed to macrocyclization, we're seeing activation of a water molecule and attack, which releases this ACV tripeptide. OK, and I've drawn the ACV tripeptide here to indicate effectively getting to this structure.

So what happens after this tripeptide is released from the assembly line, is that there is additional enzymes that play a tailoring role. So like, for proteins we talk about post-translational modification, for these types of natural products, we talk about post-assembly-line tailoring. And so in this case, there is some enzymes such as IPNF, and non-heme iron enzyme that's responsible for oxygenated cyclization to give the fused ring system characteristics of these beta-lactams like isopenicillin N.

We can look at this in another cartoon form. So here is the holoform. Recall, we called the T domains apo when the serine is not post-translationally modified with the Ppant arm. And the T domains are holo when the Ppant arm has been attached, as indicated by this squiggle.

We then have loading of the amino acid monomers via the action of the A domains. So formation of that aminoacyl AMP or amino adenylate intermediate, so one monomer per

module. We have chain elongation events catalyzed by the condensation domain. We have chain transfer to the TE domain as shown here, chain transfer, and then chain release here, and then post-assembly-line tailoring.

So with that in mind, what we're going to do now is look at another non-ribosomal peptide synthetase. This one synthesizes the backbone of the antibiotic vancomycin. And the structure of vancomycin is shown here. This is an antibiotic that's basically considered one of last resort for bacterial infections. And there is a huge problem of vancomycin resistance in the clinic these days.

So at first glance, this molecule might not look like it's based on a peptide. But then if you look more carefully, you see there is a lot of amide bonds. And there is also some other things going in to get this final structure. So effectively, the backbone of vancomycin is a polypeptide that's a sevenmer.

So within this heptapeptide scaffold, there are two proteinogenic amino acids and five non-proteinogenic amino acids here. And because we have seven amino-acid-type monomers, we need an assembly line that has seven modules, one module per amino acid monomer. And what we'll see is that these seven modules are distributed over three proteins. We have a case of a thioesterase catalyzing hydrolytic release. And then we're going to need to think about what are the other tailoring enzymes involved in giving vancomycin this structure.

So for instance, look here. We see there is this aryl-aryl C-C bond. We see these aryl-ether connections. And we also have these sugars attached. And look, there is also an N-methylation here of leucine 1, so a lot happening.

And the consequence of this post-assembly-line tailoring is that, what's a linear sevenmer polypeptide ends up having an architecture that's described as a dome, so a dome-shaped architecture. And what vancomycin does is that it blocks biosynthesis of the bacterial cell wall by binding to a certain lipid precursor in that. So let's look at the assembly line. And this is just an overview of the tailoring I just told you about. And this is the amino acid sequence in order of the different monomers there and the identities of the non-proteinogenic amino acids.

So here is the assembly line. And if we take a look, we have the loading module, AT. We can count the T domains to give us the modules involved in extension. So there is seven T domains. And look, CAT, CAT, CAT-- we have a number of optional epimerization domains.

And at the end, we see this TE domain.

And so you can walk through and look at each monomer being attached to the growing chain. And then what do we see? What we see happening down here is that when we have the linear polypeptide attached to this module here, what happens is that there is some tailoring happening while the polypeptide is still attached to the assembly line.

So enzymes that are not parts of the assembly line but are involved in the biosynthesis can come in. And sometimes they'll modify the chain when it's still attached to the NRPS or PKS. Or sometimes they do the chemistry after the chain is released. And often, this is a question that people need to sort out experimentally.

So in this case here, we see that there is some oxidative cross-linking that occurs while the chain is still attached to the T domain. So there is formation of the aryl-ether bond and this aryl-aryl bond here. And then after the chain is released in a hydrolytic manner, what happens is the sugars get attached post-assembly-line here. Do you have a question?

AUDIENCE:

Yeah, are the enzymes ever actually in the assembly line, like the optional domains of PKS? Or in this case, is it always such that the enzymes are separate?

ELIZABETH

It will depend on the assembly line. Yeah, so that's something you need to look for in the

NOLAN:

assembly line from the bioinformatics. So in this case, we're only seeing epimerization domains in the assembly line, but there can easily be methyltransferases, or reductases, or cyclases-- any number of possibilities within the assembly line itself there. And these optional domains will work on the upstream monomer.

This is just an example of the tailoring enzymes involved for cross-linking of this vancomycin scaffold. In this case, there are three cytochrome P450 enzymes that are needed in order to make these cross-links. And that chemistry is shown here to get to what's called the vancomycin aglycone, which means that there are no sugars attached.

And I won't draw this one on the board, but you can do a similar exercise with this molecule or any others in terms of identifying the monomer units from the structure for yourself. So if we're looking here, we have effectively the N-terminus, so the starter, and then effectively look at the peptide bonds and work your way through to find the different monomers here. So by doing that, if you're given a natural product, you can figure out how many modules are needed in the assembly line. And you can also make an assessment as to what other types of chemistry

might have to happen.

And I'll just keep in mind, for something like this-- let's just take this for an example with this halogen. You might ask, well, is that part of the monomer? Or is that atom incorporated sometime down the road? OK, those are types of questions people who explore biosynthesis of these molecules think about.

OK, so with that in mind, let's take a look at some examples. And the questions are, what kind of assembly line is this? How many monomers? And maybe there will be some extra questions as we go. So here we have an assembly line that's required to make an antibiotic called daptomycin. And a company down the street in Lexington called Cubist has done a lot of work on this natural product.

So how many monomers are here? Yeah, 13, right-- so count these T domains based on what's seen here. How many optional domains?

AUDIENCE: Three.

ELIZABETH And then what else do we see? So we see that this assembly line is divided over three
NOLAN: proteins, effectively, here. And similar to what we saw with DEBS, when we have a break in the cartoon, that indicates a new polypeptide chain. What's missing?

AUDIENCE: Loading module.

ELIZABETH Yeah, there is no loading module here, right, no AT at the beginning. So what's going on? So
NOLAN: in this case, I haven't shown you a structure. It highlights there is always exceptions to the rule.

What happens here is that the loading module actually loads a fatty acid, so not a standard monomer for NRPS. So that fatty acid has to come from somewhere. And you can think about discussions here as to where that may have come from. Look at how big this is, 624 kilodaltons, 783, 256-- we're on the order of 1.5 megadaltons. This is huge for a 13-mer natural product.

What about this one? What do we see here? So this is a natural product-- this makes the natural product produced by *Streptomyces* that has insecticidal activity. And it kills parasitic worms.

But anyhow, what kind of natural product is produced by this assembly line? We have a polyketide, right? How many modules? [INAUDIBLE] the T domains.

AUDIENCE: [INAUDIBLE]

ELIZABETH Yeah, 13 again, right-- four proteins, 13 modules, so how many unmodified beta ketones?

NOLAN: What would you want to look for for a modified beta ketone?

AUDIENCE: [INAUDIBLE]

ELIZABETH Exactly, no optional domains-- so how many of those? Yeah, right, so two modules, we have

NOLAN: one here and then one over here with no optional domains.

What about this one? This is for a molecule called bleomycin. JoAnne is an expert on the mechanism of this molecule. What's going on?

OK, there is a lot going on. This one is very complicated. But in terms of making an assessment about the type of biosynthetic logic, what do we see here?

AUDIENCE: [INAUDIBLE]

ELIZABETH Right, so what we see is that there is both non-ribosomal peptide synthesis happening and

NOLAN: polyketide biosynthesis happening in this assembly line. And that tells us that the product metabolite is a PKS-NRPS hybrid. OK, so what do we see? We see all of these CAT trios which are indicative of non-ribosomal peptide biosynthesis. And then what's happening here? We have a module that's using polyketide machinery. And then we go back to non-ribosomal-peptide-based logic here.

We have many proteins, right? So this assembly line is divided over many proteins. And look, we see that even some of the modules are divided up. So for instance, this CAT trio is divided between two proteins. So you may not have all domains of a module on a given protein.

AUDIENCE: What happens if you have two C domains in a row?

ELIZABETH So where do you see two C domains in a row?

NOLAN:

AUDIENCE: Between BlmV and BlmX.

ELIZABETH Five and--

NOLAN:

AUDIENCE: Is that actually in a row?

ELIZABETH Yeah, so then that's the question. Are they actually in a row?

NOLAN:

AUDIENCE: Further down, four Cy cyclases without any C domain.

ELIZABETH Yeah, so that's actually where I was going next. So what's going on with the Cy without a C

NOLAN: domain? So what's happening-- and we'll probably, if there is time, go over an example of this on Friday-- is that Cy, so these cyclization domains are a variant on a condensation domain. And what they do is, they both catalyze formation of the peptide bond and then they catalyze-- after that, they catalyze formation of a heterocycle.

So if you recall, I believe we looked at the structure of yersiniabactin during the first lecture on these. It has a number of heterocycles. And those form by this Cy domain. And we can see that here in the structure.

So what I've done on this slide is just present to you the structures, so the natural products that result from these different assembly lines. And if we take a look at the bleomycin, what do we see here? We have these two heterocycles that are fused together. And those are formed via the action of these two cyclization domains down here.

So effectively, these originate from cysteine. So cysteines, and serines, and threonines can end up forming structures like these if there is the appropriate type of domain. This molecule is extremely complicated here. And so it's a good puzzle to look at it and try to sort out what are the monomers in it in here. Does anyone know what this does, bleomycin?

AUDIENCE: [INAUDIBLE]

ELIZABETH Well, so it's an anticancer antibiotic here. It can intercalate into DNA. And these heterocycles
NOLAN: are important for that. And then it causes strand breaks.

And I've actually learned recently it's also used for, like, treating arts. So it will kill HPV that causes warts. Anyhow, all of these compounds have interesting activities, which is one reason why they can be of interest.

So with the logic in place, where we're going to close this module is thinking about how folks study these in lab. So say you want to figure out the biosynthesis of a molecule like daptomycin or bleomycin, what is it that one needs to do? And something just to keep in mind with this right off the bat, is that these are huge. So some of these examples here, if you take a look at the sizes, they're, like, comparable to the prokaryotic ribosome.

That's a huge protein assembly. And that presents a limitation from the standpoint of doing experimental work, because trying to overexpress or produce these assembly lines in something like *E. coli* is typically just unreasonable. And in terms of a native producer organism, say, something like *Streptomyces*, we may or may not know conditions that cause the organism to make the natural product, so conditions that cause it to express this machinery, and then even if it made at a-- in an amount that's useful.

So what happens? What are we going to do as experimentalists? So as I said, we need to keep in mind that these machines are enormous. And so we need to take this into account during experimental design.

And these days, bioinformatics drives a lot of the studies. So rather than first finding a natural product and determining its structure and then hunting down the protein machinery, a wealth of genomes are becoming available. And so you can use bioinformatics to search for PKS or NRPS gene clusters.

And then you can make some assessment as to what type of molecule these gene clusters might be responsible for making. So bioinformatics plays a huge role. And it allows us to predict the domains, to predict their locations, and predict their boundaries here.

So as I just said, overexpression of a complete assembly line is generally not feasible. So what do people do? People will typically express individual domains or maybe di-domains and study those in the test tube. So you can imagine PCR amplifying an A domain or a T domain, or maybe the A and T domain together, and then creating some plasmid that allows you to express that in *E. coli*. So there is a lot of overexpression.

The proteins need to be purified, so maybe something like affinity chromatography that we've spoken about before. And then a key point is that, in order to have any of this chemistry work, these T domains need to be post-translationally modified by the Ppant arm. And if you're overexpressing a T domain from *Streptomyces* or some organism in *E. coli*, you can pretty

much assume there is no PPTase in *E. coli* that's going to do this for you. So you need to do that after the fact.

And so there needs to be a PPTase. And what we'll see is that there is a PPTase from *B. subtilis* called SFP that's very promiscuous. It will basically modify any T domain. And so experimentally, this is what people use, because often, one has no clue what the endogenous PPTase is here, so SFP to the rescue.

In terms of activity assay, so once you have your domains or di-domains purified, what happens? This is the typical flow. So the first is to characterize the A domains and to ask, what amino acid or aryl acid is activated by the A domain and what is the selectivity? And by getting that information, you have a good clue as to what monomer a given module is responsible for.

And the ATP-PPi exchange assay we discussed in the context of the aminoacyl tRNA synthetases is commonly employed. So this is where we use the radiolabeled ATP and took into reversibility there. So go back and review that assay as needed. There will be some examples of this in the problem set.

So once the A domain activity is known in terms of preferred monomer, the next question is, will that A domain transfer the amino acid monomer to a given T domain? So you design assays to look for transfer of the activated monomer to the post-translationally-modified T domain here. So in these assays, there is a lot of work with radiolabels, with HPLC, and mass spec.

So once these T domains are loaded, you can look for peptide bond formation. So imagine you have an isolated T domain from a loading module that you've stuck the amino acid on and then you have this guy, the next question is, does the C domain catalyze bond formation reaction? And again, we'll see there is a lot of use of radiolabels, HPLC, SDS-PAGE here.

And then you know, there is the question of the TE domain and the TE domain catalyzing chain release. So it's quite systematic in terms of how you work through from identifying an assembly line to then teasing apart the various activities of the different domains and different modules.

And so where we'll close this module on Friday is with looking at the experiments that were done for the biosynthesis of an iron chelator produced by *E. coli* and working through basically you know, how was it that this NRPS was found? What were the experiments done to identify

the different activities of the different domains? And it's really that work that has served as a foundation and a paradigm for many, many further studies of these systems here. And so with that, we'll close for today. And there is no class Wednesday, so I'll see you on Friday.