

# Reviews

## From Ergot to Ansamycins—45 Years in Biosynthesis<sup>†</sup>

Heinz G. Floss\*

Department of Chemistry, Box 351700, University of Washington, Seattle, Washington 98195-1700

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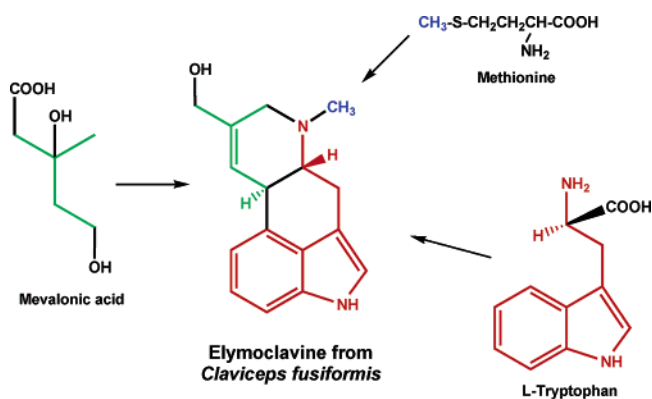
In this review the author traces his scientific career from its beginnings in Germany to his moves to, successively, Purdue University, The Ohio State University, and finally University of Washington. During this time his research progressed from extensive studies on ergot alkaloids, the stereochemistry of enzyme reactions, and tracer studies on antibiotic biosynthesis to its latest emphasis on the molecular biology of ansamycin antibiotics. The formative influence of several mentors and colleagues is acknowledged.

The year 2005 marks the end of my active involvement in scientific research. On such an occasion it is appropriate to look back and review where I came from, how I got to where I am now, and what some of the formative influences were along the way. However, I was cautioned not to become entirely enmeshed in a sentimental journey, and hence, the second part of this review will summarize some of the recent findings from our laboratory.

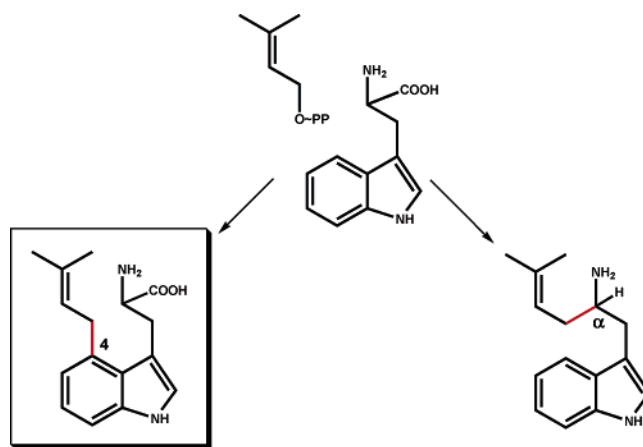
I was born and raised in Berlin, Germany, finishing high school in 1953. How did I become a chemist? Well, as I prepared to enter college, I was undecided between electrical engineering and chemistry. The Technical University Berlin at the time had capped enrollments and required students to pass an entrance examination. I signed up for the exams in both subjects. The one for chemistry was scheduled first; I passed it and never bothered to go to the other one. I have sometimes wondered what I would be doing had the electrical engineering exam been scheduled first, but I think, subconsciously, chemistry was my true preference.

After the basic course of studies in chemistry, I joined the research group of organic chemistry professor Friedrich Weygand for my “Diplomarbeit” (master’s thesis). Weygand was a student of Richard Kuhn (Nobel Prize 1938), who was a student of Richard Willstätter (Nobel Prize 1915), a student of Adolf v. Baeyer (Nobel Prize 1905), who in turn was a student of August v. Kekulé of benzene fame—not bad for pedigree. I studied a rearrangement of diazoketones using radioactive substrates that I synthesized.<sup>1</sup> My work was supervised by one of Weygand’s assistants, Dr. Helmut Simon, just returned from a postdoctorate with Melvin Calvin at UC Berkeley. Simon was later appointed to the faculty of the Technical University Munich and became a lifelong friend.

How did I get involved in natural products biosynthesis? Halfway into my M.S. research, Weygand accepted an offer from the Technical University Munich, and in good German fashion my colleagues and I all migrated with him to Munich. When I arrived there after completion of my M.S. thesis, Weygand was very busy and, given my prior experience, asked me to synthesize several radiolabeled versions of mevalonic acid while he thought about a proper Ph.D. thesis topic for me. Mevalonic acid had just been identified as the precursor of biological isoprene units and was needed for a collaboration with the group of Prof. Kurt Mothes in Halle, East Germany, on the biosynthesis of the ergot alkaloids.



**Figure 1.** Biosynthetic origin of the ergoline ring system of ergot alkaloids.



**Figure 2.** Two possibilities for attachment of the isoprenoid side chain.

One year later, when I had finished all these syntheses, the idea of switching to a new topic for my Ph.D. thesis was rather unappealing, and I asked Weygand whether I could join the collaboration with the Mothes group for my thesis work.

Feeding experiments with the radiolabeled precursors established the biosynthetic building blocks of the ergoline ring system as tryptophan, the methyl group of methionine, and an isoprene unit derived from mevalonic acid (Figure 1).<sup>2</sup> This involvement of an amino acid and an isoprene unit represented a new paradigm, be-

<sup>†</sup> Based on the opening lecture presented at the 46th Annual Meeting of the American Society of Pharmacognosy, Corvallis, OR, July 23–27, 2005.

\* To whom correspondence should be addressed. Tel: (206) 543-0310. Fax: (206) 685-8665. E-mail: floss@chem.washington.edu.

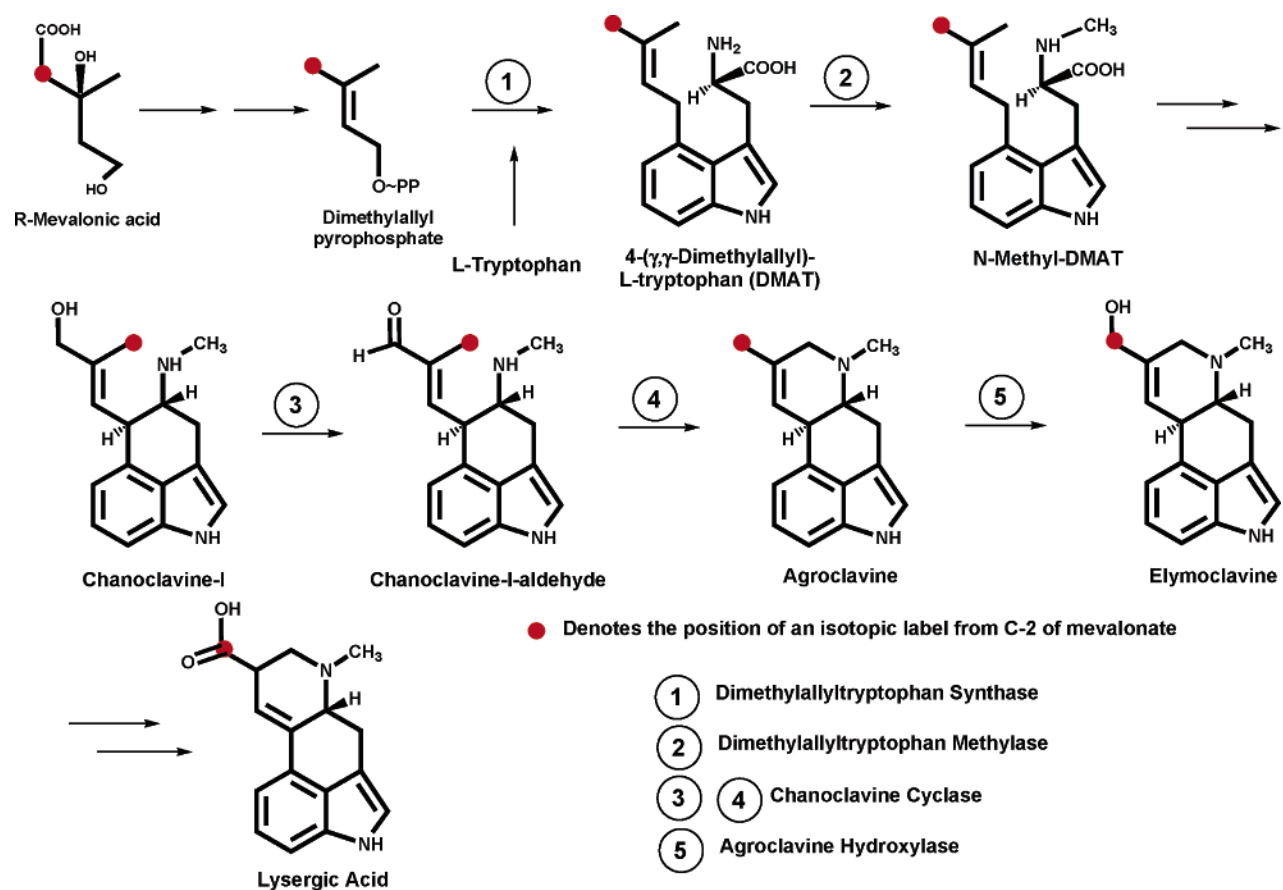


Figure 3. Biosynthetic pathway to the ergot alkaloids.

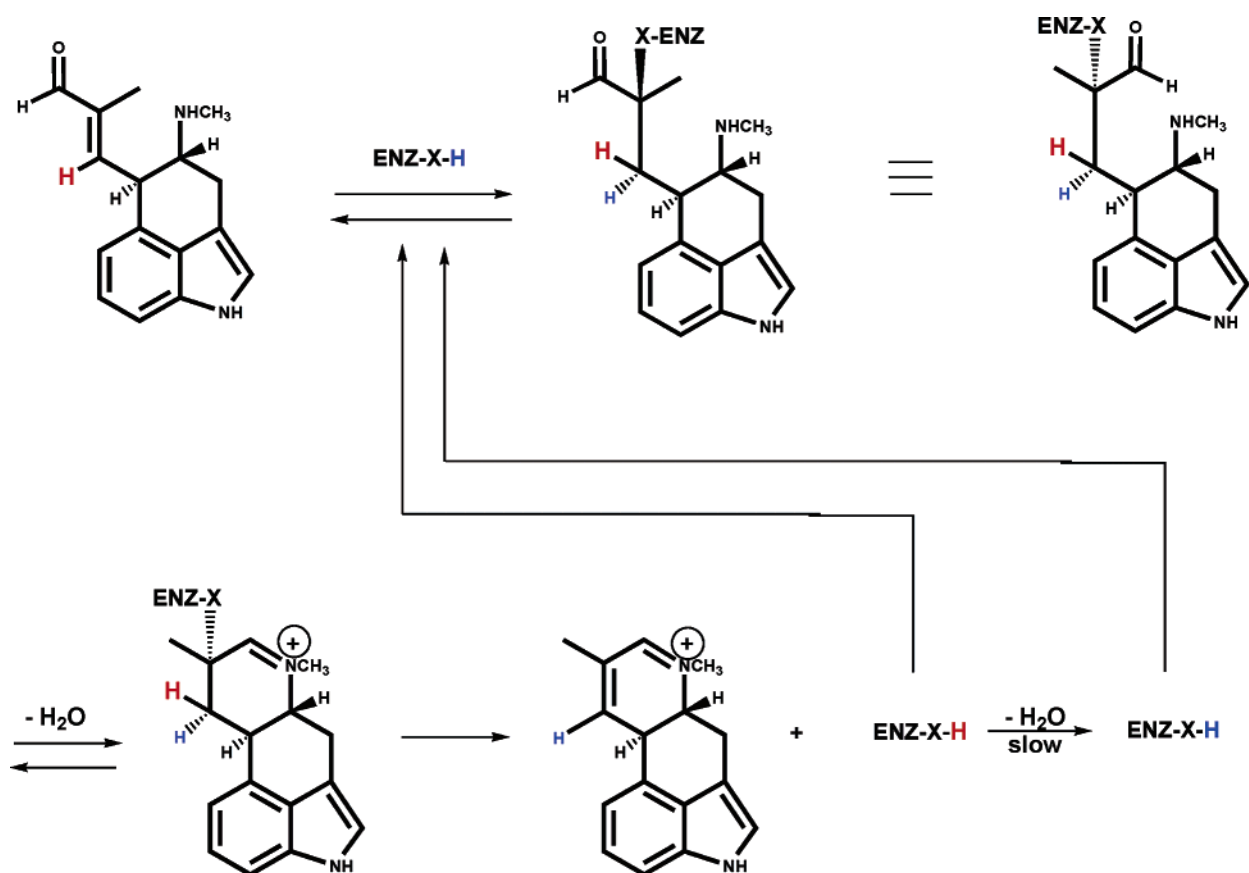
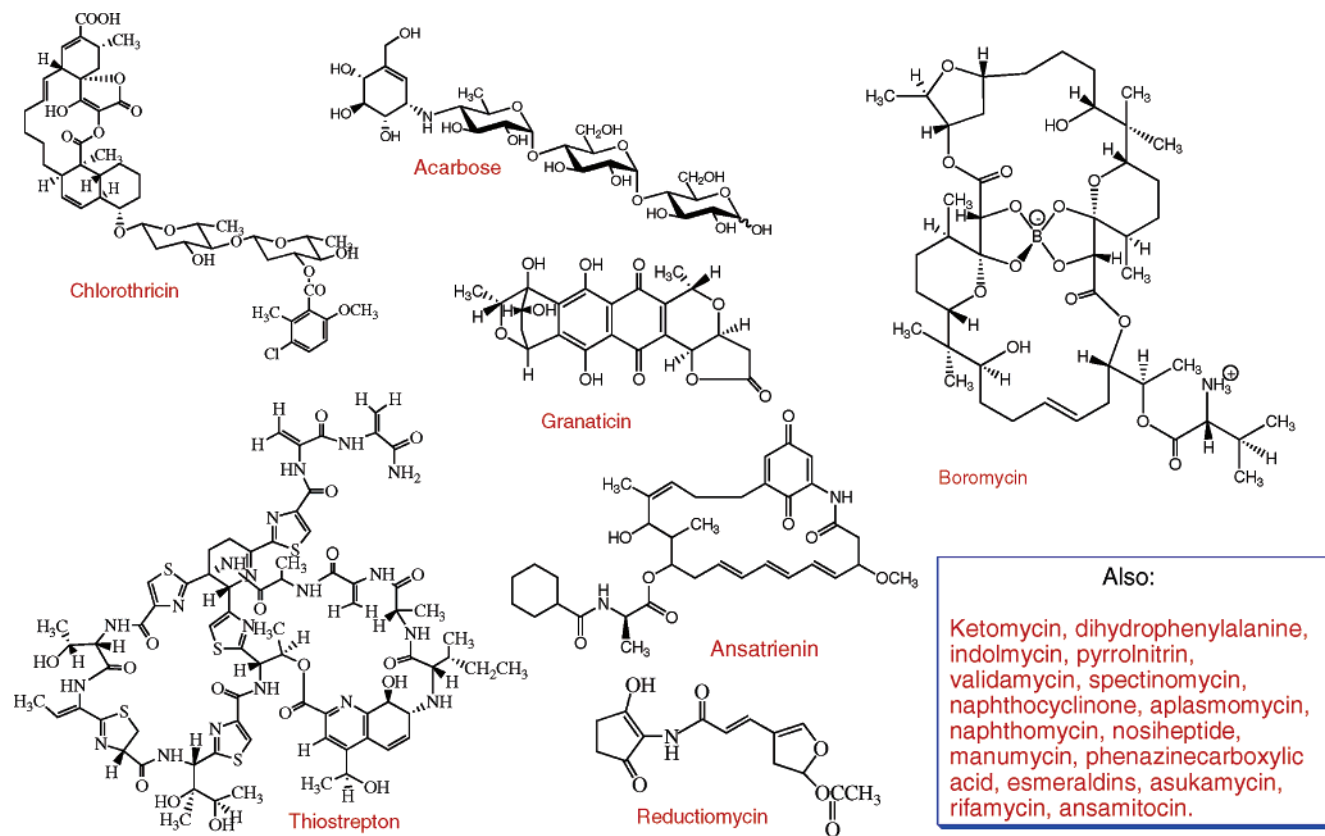
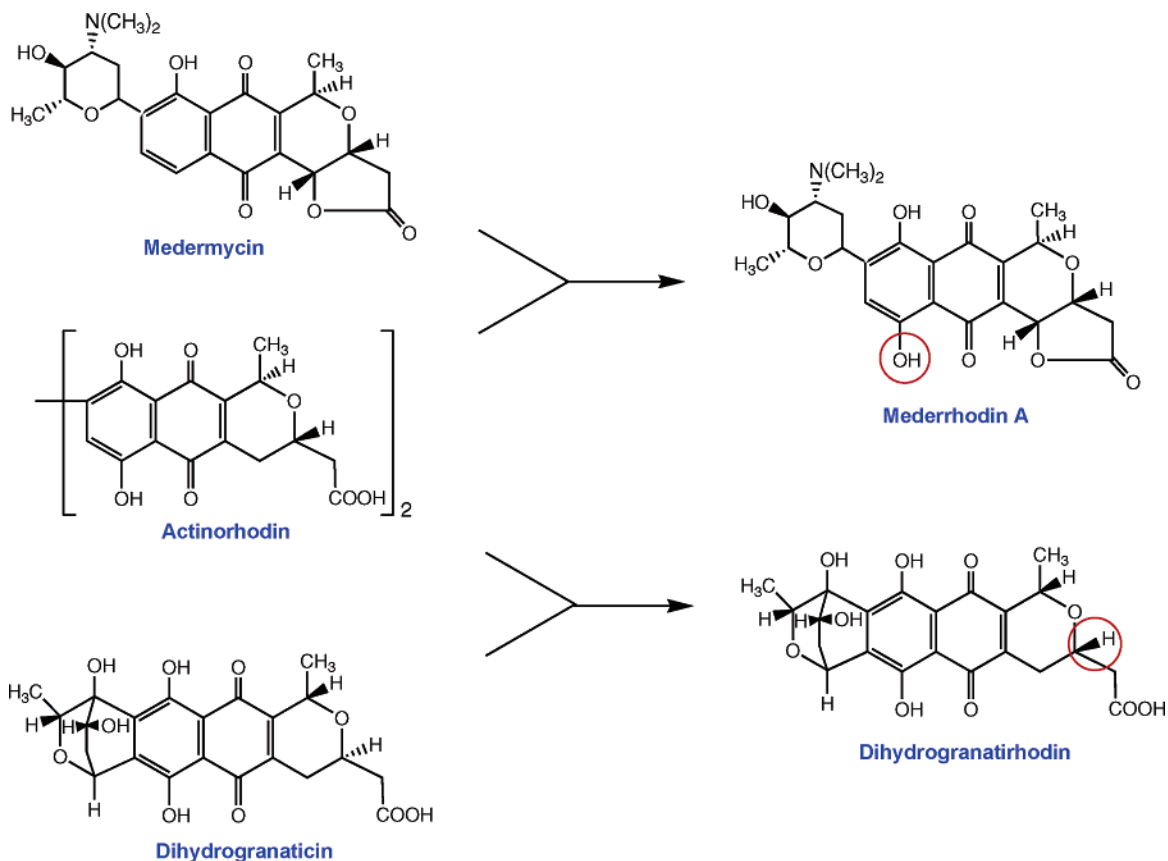


Figure 4. Mechanism of cis-trans isomerization during D-ring closure.



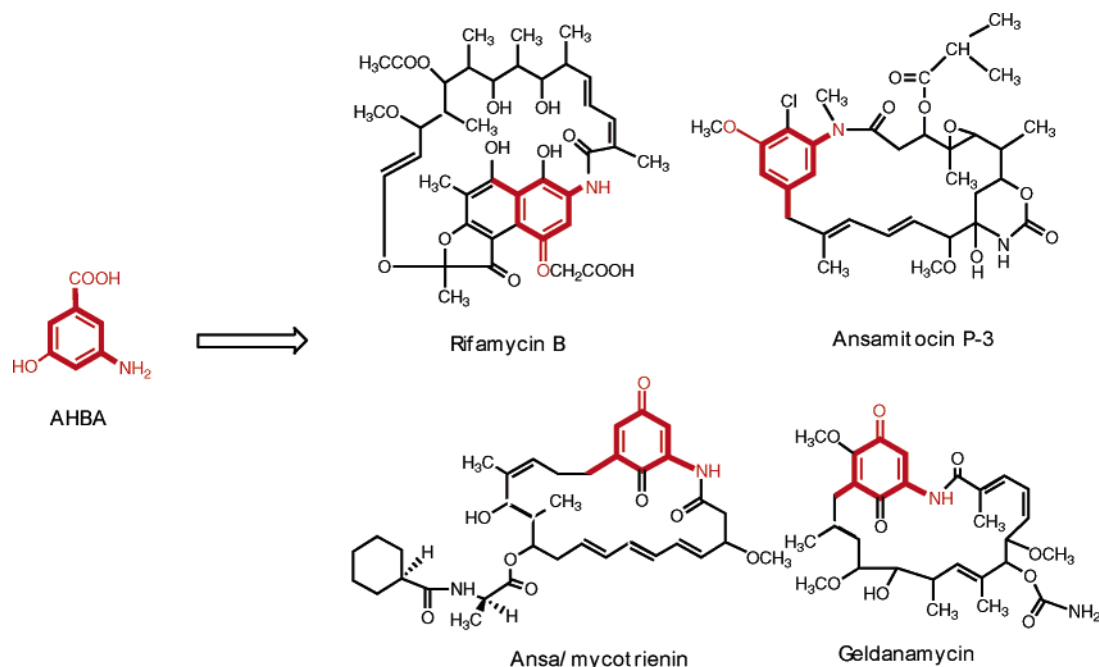
**Figure 5.** Examples of microbial metabolites studied.



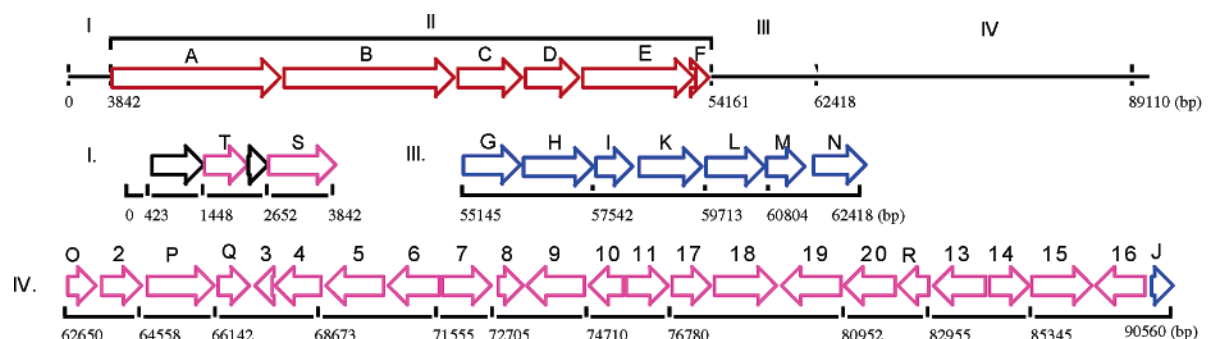
**Figure 6.** First demonstration of genetic engineering of hybrid antibiotics (Hopwood et al., 1985).

cause until then, an axiom of plant physiology had been that alkaloids and terpenoids did not co-occur in nature; subsequently, of course, many other classes of compounds, such as the indole alkaloids, were found to be of mixed amino acid and isoprenoid origin.

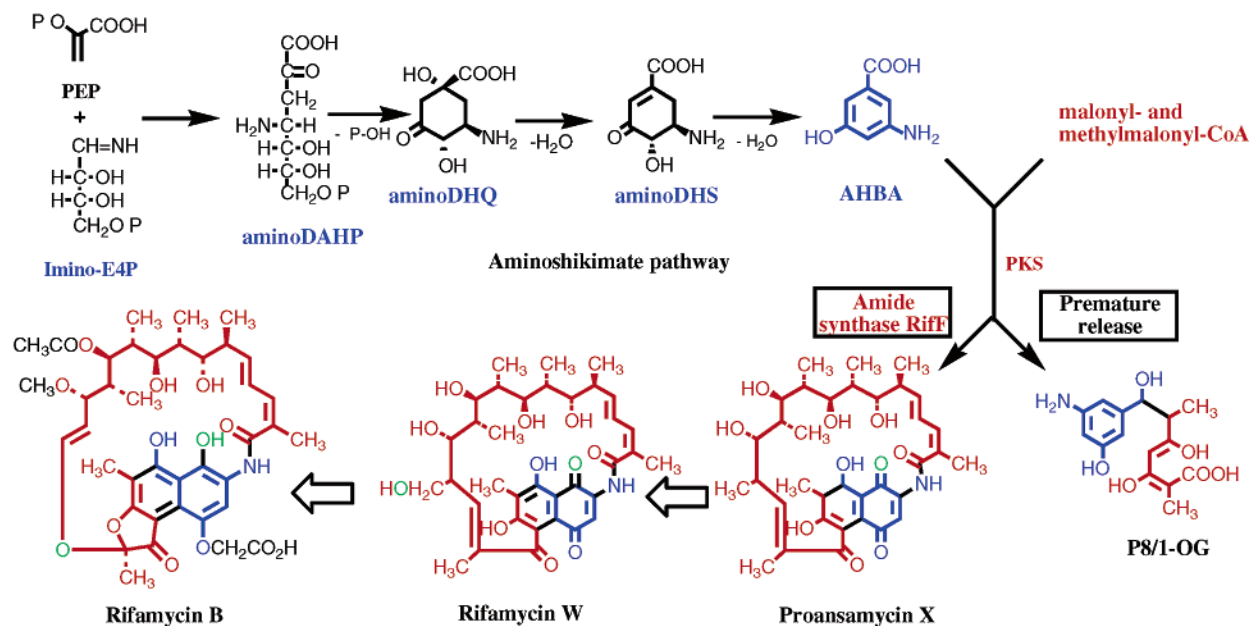
The next question was how the two main building blocks, tryptophan and the isoprenoid precursor dimethylallyl diphosphate, were initially connected. Two plausible possibilities are shown in Figure 2. The direct prenylation at C-4 of tryptophan had the



**Figure 7.** Ansamycin antibiotics with mC<sub>7</sub>N units derived from 3-amino-5-hydroxybenzoic acid (AHBA).



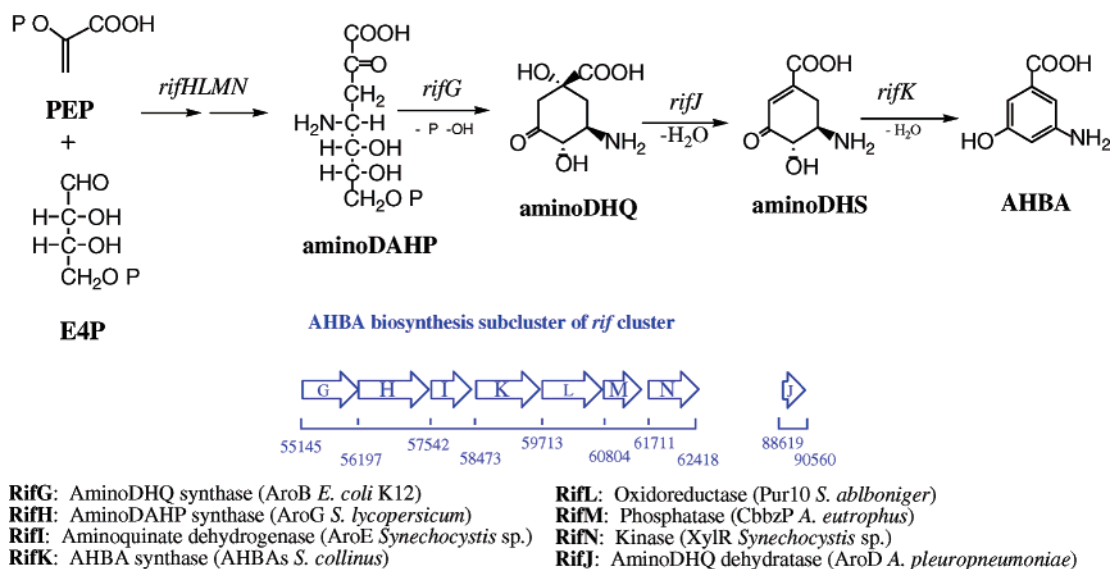
RifA-E: PKS, RifF: Amide synthase, RifG-N: AHBA formation, RifO-T, Orf2-16: Post-PKS modification, regulation



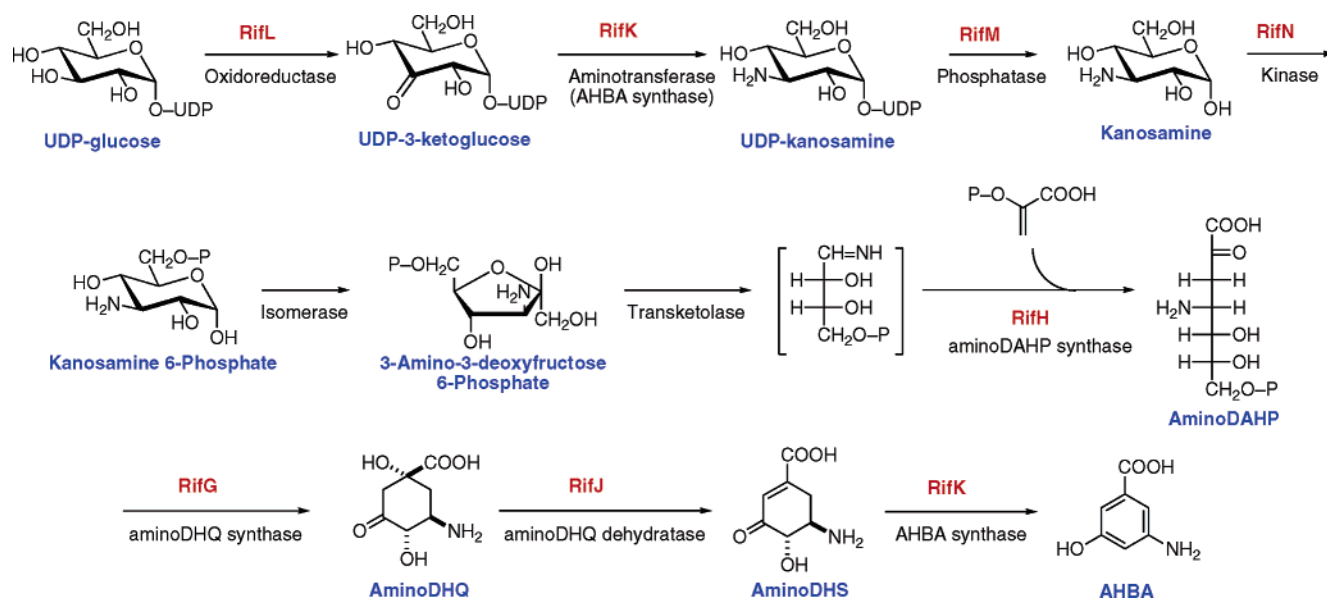
**Figure 8.** Rifamycin biosynthetic gene cluster and pathway.

problem that the 4-position is not the most nucleophilic site in an indole. The alternative, prenylation at the  $\alpha$ -position of the side chain, with simultaneous decarboxylation, circumvents this problem, because in the subsequent attack of a newly generated allylic cation,

formation of a six-membered ring would favor the 4-position. We planned to test these two possibilities by synthesizing both potential products in labeled form. We obtained compound **II** first and upon feeding it to the ergot fungus found radioactivity in the elymoclavine



**Figure 9.** AHBA biosynthesis in *Amycolatopsis mediterranei*.



**Figure 10.** Complete AHBA biosynthetic pathway in *Amycolatopsis mediterranei*.

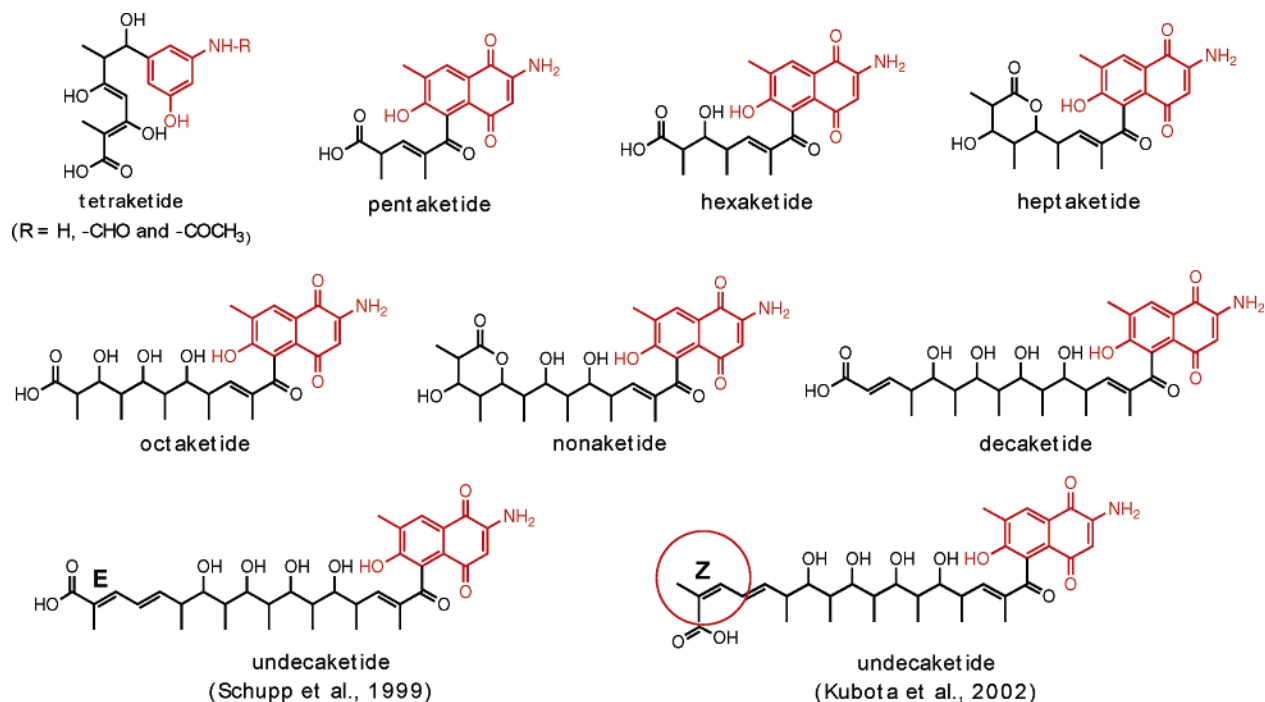
fraction. Weygand reported this result in a lecture he was scheduled to give a few days later, and we published a preliminary communication on it.<sup>3</sup> Simultaneously, the group of Pleninger in Heidelberg addressed the same issue; they obtained compound **I** (DMAT) first and reported its incorporation into elymoclavine.<sup>4</sup> There followed an agonizing six months during which our ergot cultures did not produce alkaloid, but once this problem was overcome, we confirmed that compound **I** was indeed efficiently incorporated into elymoclavine, whereas the radioactivity in the alkaloid fraction after feeding **II** was due to metabolism to its *N*-acetyl derivative with chromatographic properties similar to those of elymoclavine.<sup>5</sup> This blunder could have easily ended my scientific career before it ever started, but Weygand held his hand over me and I was given another chance. Years later, in collaboration with Peter Heinste in at Purdue, we purified the enzyme DMAT synthase to homogeneity, one of the first key enzymes of alkaloid biosynthesis.<sup>6</sup>

The collaboration with Prof. Mothes, a charismatic plant biochemist, and his assistant, Detlef Gröger, was instrumental in awakening my interest in the biosynthesis of natural products. Mothes radiated an infectious enthusiasm for the subject. The collaboration with Gröger, later Professor at the Academy of Sciences Institute for Plant Biochemistry in Halle, lasted for many

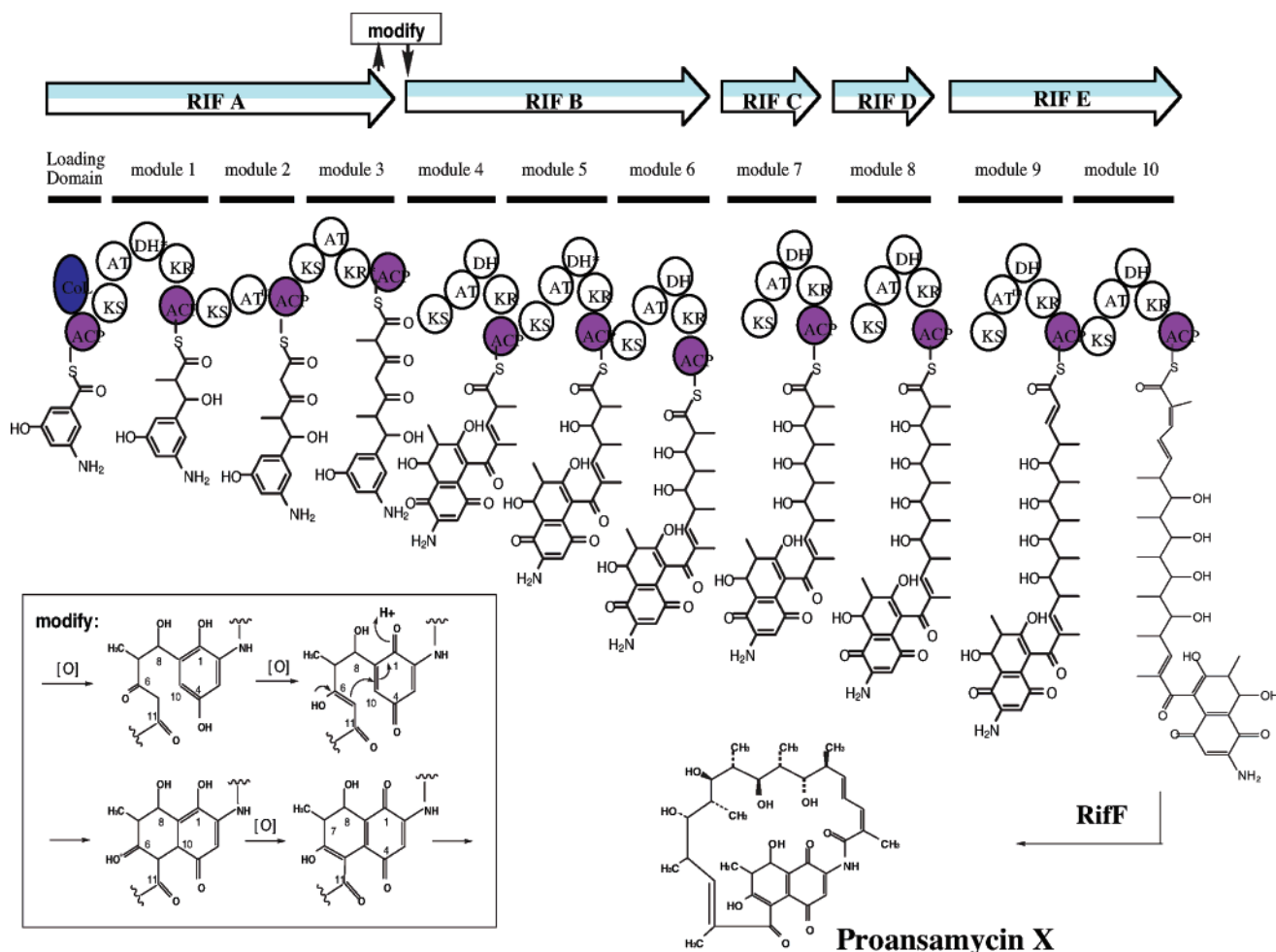
decades, reaching across the iron curtain, and led to a close personal friendship. Our collaborative efforts, and parallel work in the laboratory of Duilio Arigoni at the ETH Zürich, led to the elucidation of the biosynthetic pathway to the ergoline ring system as shown in Figure 3.<sup>7,8</sup> This deceptively simple reaction sequence actually encompasses several mechanistic subtleties. Surprisingly, for example, the methylation of the amino group is the second step after the formation of DMAT,<sup>9</sup> precluding mechanistically plausible pyridoxal phosphate catalysis for the subsequent decarboxylation/C-ring closure steps. Following the fate of an isotopic label from C-2 of mevalonate reveals that up to the stage of DMAT the label is located in the *E* methyl group of the isoprene unit, as predicted by Cornforth's work.<sup>10</sup> In the tricyclic intermediate chanoclavine-I it resides in the *Z* methyl group, but in the tetracyclic alkaloids it again occupies the *E* position. Thus, the biosynthesis involves two *cis-trans* isomerizations at the allylic double bond, one in the formation of ring C and another during D-ring closure.<sup>11</sup>

At the time we took the ergot work as far as we could with the tools then available. However, many questions remained unanswered, and these could and should be tackled, now that the genes for ergot alkaloid biosynthesis have been cloned.<sup>12</sup> In particular, the mechanistic enzymology of C- and D-ring closure merits further





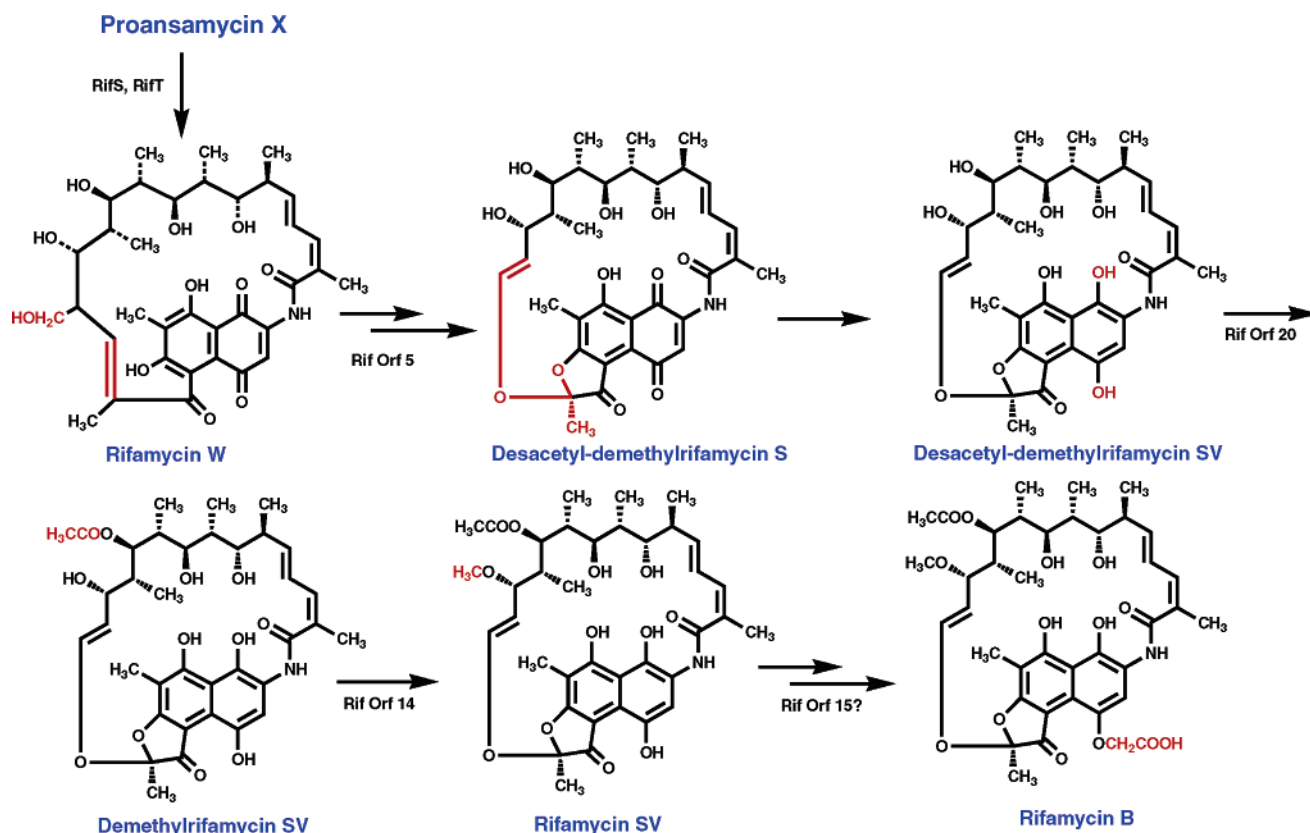
**Figure 11.** Ketides isolated from amide synthase (*rifF*) mutants.



**Figure 12.** Rif-PKS domain organization and proansamycin X biosynthesis.

investigation, as these appear to be intriguing reactions. We did a considerable amount of tracer work on the mechanism of D-ring formation.<sup>13</sup> This revealed that the process is accompanied by an intermolecular transfer of the hydrogen at C-9 of the educt,

chanoclavine-I or its aldehyde, to the same position in a different molecule of the product (Figure 4). Also, this hydrogen is subject to a large primary kinetic isotope effect, implying that it is in competition with another hydrogen at some stage in the process.



**Figure 13.** Post-PKS steps in rifamycin biosynthesis.

Figure 4 summarizes these observations and a possible explanation, but how this reaction works at the molecular/enzymatic level has remained obscure.

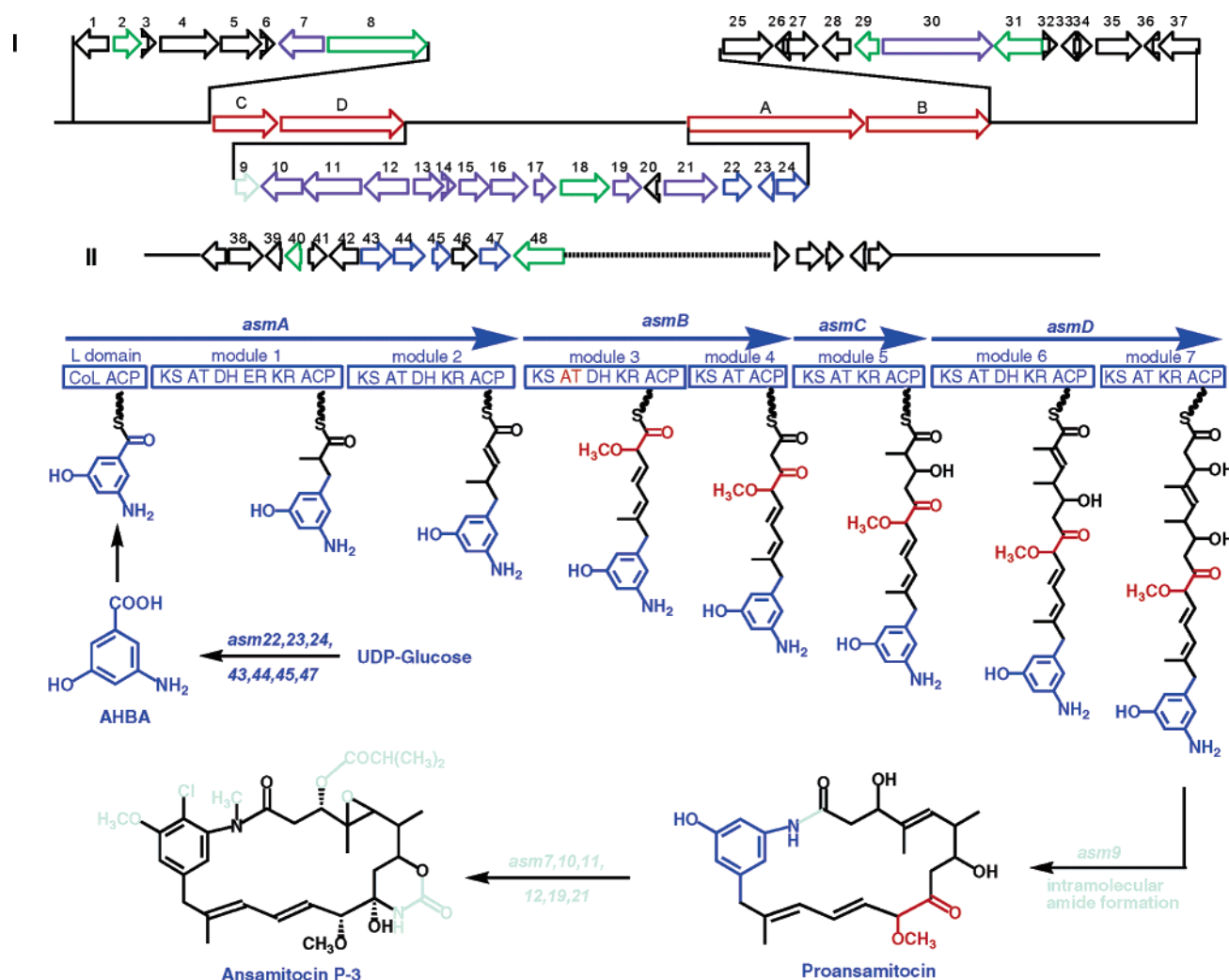
We also studied the regulation of alkaloid formation in fermentations of the ergot fungus, *Claviceps*. Among the regulatory principles demonstrated were (i) catabolite repression by glucose and phosphate, commonly seen in many secondary metabolite fermentations, and (ii) induction of alkaloid synthesis by the substrate, tryptophan, and analogues that do not serve as substrates, a much less common regulatory principle in secondary metabolism.<sup>14</sup> The molecular mechanisms underlying this regulatory principle are unknown. Another important issue is the biosynthetic origin of the ergot alkaloids isolated from higher plants. Higher plants in the family Convolvulaceae (e.g., morning glory) contain hallucinogenic ergot alkaloids, primarily in their seeds (hence the use of ololiuqui = *Rivea corymbosa* seeds in Mexican Indian religious ceremonies).<sup>15</sup> They are synthesized from the same precursors, tryptophan and mevalonic acid, as in the fungus.<sup>16</sup> Since (presumed to be sterile) cell cultures have been reported to produce the alkaloids,<sup>17</sup> it was assumed that the plant itself must be the site of synthesis. However, recent work by the group of Leistner in Bonn has shown that the source is a plant-associated fungus, which can be transmitted only via the seeds of the plant.<sup>18</sup>

The ergot work also brought me in contact with Varro E. (Tip) Tyler, another ergot researcher and Head of the Department of Pharmacognosy at the University of Washington, who in 1992–1993 spent a sabbatical year at Mothes' institute in Halle. A year later, during my postdoctoral time with Eric E. Conn at the University of California, Davis, he invited me and my family to join them for Thanksgiving in Seattle, and in 1966, when he became Dean of the Pharmacy School at Purdue University, he offered me a faculty position in their Department of Medicinal Chemistry. I went there sight unseen—the idea of asking for a round-trip ticket from Germany just to look the place over seemed quite preposterous—basically on Tip's pledge to do everything in his power to make sure I would never regret this move. Tip was true to his word, and

accepting his offer was the best professional decision I ever made. Outside my family, nobody has had a more profound influence on the direction of my life than Tip Tyler.

The move to Purdue allowed me to expand the scope of our research activities. We became involved in two new areas. One was the stereochemistry and mechanism of enzyme reactions. Over the years we worked on enzymes of the shikimate pathway (e.g., DAHP synthase, EPSP synthase, chorismate synthase, chorismate mutase), pyridoxal phosphate-dependent enzymes<sup>19</sup> (e.g., tryptophan synthase, cysteine synthase, serine dehydratase, aspartate  $\beta$ -decarboxylase, tryptophanase, tyrosinase), chiral methyl groups<sup>20</sup> (e.g., methyltransferases, methylreductase, methane monooxygenase, malonate decarboxylase, berberine biosynthesis), and deoxysugars and cyclitols (e.g., dTDP-glucose dehydratase, dTDP-6-deoxyglucose 2,3-dehydratase, *myo*-inositol phosphate synthase, sedoheptulose 7-phosphate cyclase). In entering this field, I was very much inspired by the elegant work of Prof. Duilio Arigoni at the ETH Zürich. Arigoni, a scientist of intimidatingly sharp intellect and almost impossibly rigorous standards, became a yardstick for my own research; I would always ask myself whether a particular experiment or proof would meet Arigoni's standards. If you choose a role model, you might as well pick a challenging one, even if you can never live up to it.

The other new area was the biosynthesis of various antibiotics, which we initially pursued by feeding experiments with stable isotope-labeled precursors followed by NMR analysis of the products.<sup>21</sup> Figure 5 shows some of the compounds we investigated over the years. Many of these were discovered by the group of Prof. Hans Zährner, a microbiologist at the University of Tübingen, with whom we had a long-standing, fruitful interaction. Through the antibiotics work I also met Prof. Sir David Hopwood of the John Innes Institute in Norwich, the pioneer of Streptomyces genetics, and Prof. Satoshi Omura, President of the Kitasato Institute in Tokyo and a world-renowned antibiotics researcher. In a three-way collaboration we demonstrated in 1985 by the experiments shown in Figure 6 the feasibility of genetically engineering the



**Figure 14.** Ansamitocin biosynthetic gene cluster.

biosynthesis of new hybrid antibiotics.<sup>22</sup> Introduction of some of the genes of actinorhodin biosynthesis into the producer of medermycin gave a new compound, mederrhodin A, with the extra hydroxy group of actinorhodin added to the medermycin structure. Transfer of all the actinorhodin genes into the producer of granaticin gave the hybrid compound dihydrogranatirhodin, with the actinorhodin stereochemistry at one stereocenter and the granaticin configuration at the other. From these simple demonstration cases grew the field of combinatorial biosynthesis, which is now practiced in many laboratories around the world.

The interaction with Hopwood raised my awareness of the power of genetic tools in studies of natural product biosynthesis. After a learning period in the form of a collaboration with Prof. William R. Strohl following my move to The Ohio State University in 1982, we started to use molecular biological approaches in my own laboratory upon my final move to University of Washington in 1987. The primary focus of our work was ansamycin antibiotics, which contain a so-called mC<sub>7</sub>N unit (Figure 7), a structural element known to be derived from the unusual amino acid 3-amino-5-hydroxybenzoic acid (AHBA).<sup>23</sup> We first hypothesized and then demonstrated that AHBA is formed by a new variant of the shikimate pathway, now called the aminoshikimate pathway (Figure 8), in which a nitrogen is introduced in the very first step, the DAHP synthase reaction.<sup>24</sup> We used this information to clone the AHBA synthase gene,<sup>25</sup> which then served as a probe to isolate gene clusters for the biosynthesis of rifamycin B<sup>26</sup> and ansamitocin P-3.<sup>27</sup>

The rifamycins,<sup>28</sup> isolated in 1959 by Sensi and co-workers from the Actinomycete, *Amiclatopsis mediterranei*, show strong antimycobacterial activity, acting by inhibiting the DNA-dependent

RNA polymerase. Semisynthetic derivatives, such as rifampicin, are widely used for the clinical treatment of tuberculosis, leprosy, and AIDS-associated mycobacterial infections. Sequence analysis of the rifamycin (*rif*) biosynthetic gene cluster, in collaboration with the group of Prof. C. R. Hutchinson, showed the presence of candidate genes for all the reactions expected to be necessary for the formation of rifamycin B from AHBA by polyketide chain extension with two acetate and eight propionate units (Figure 8).<sup>26,29</sup> A subcluster of genes shown to be necessary and sufficient for AHBA formation contains the AHBA synthase gene and genes with the expected homologies to the early enzymes of the shikimate pathway, but also three genes of unknown functions, *rifL*, *rifM*, and *rifN*, and, surprisingly, no candidate gene for the provision or introduction of the nitrogen function (Figure 9).<sup>30</sup> This enigma was resolved with crucial input from Prof. John Frost at Michigan State University, who reasoned and then demonstrated that the presumed substrate for the aminoDAHP synthase, the imine of erythrose 4-phosphate, could be formed from 3-amino-3-deoxyfructose 6-phosphate by a transketolase reaction.<sup>31</sup> Further work, some of it unpublished, suggests considerably greater complexity of the AHBA pathway than originally anticipated; formation of an amino sugar, kanosamine, precedes the aminoDAHP synthase reaction, and the pyridoxal phosphate enzyme, AHBA synthase, has a second function in the pathway as the enzyme that, in complex with the oxidoreductase *RifL*, introduces an amino group into UDP-glucose to give UDP-kanosamine. The complete pathway of AHBA formation can now be written as shown in Figure 10.<sup>32</sup>

The *rif* cluster also contains five genes, *rifA–E*, which encode a large modular type I polyketide synthase (PKS). The domain



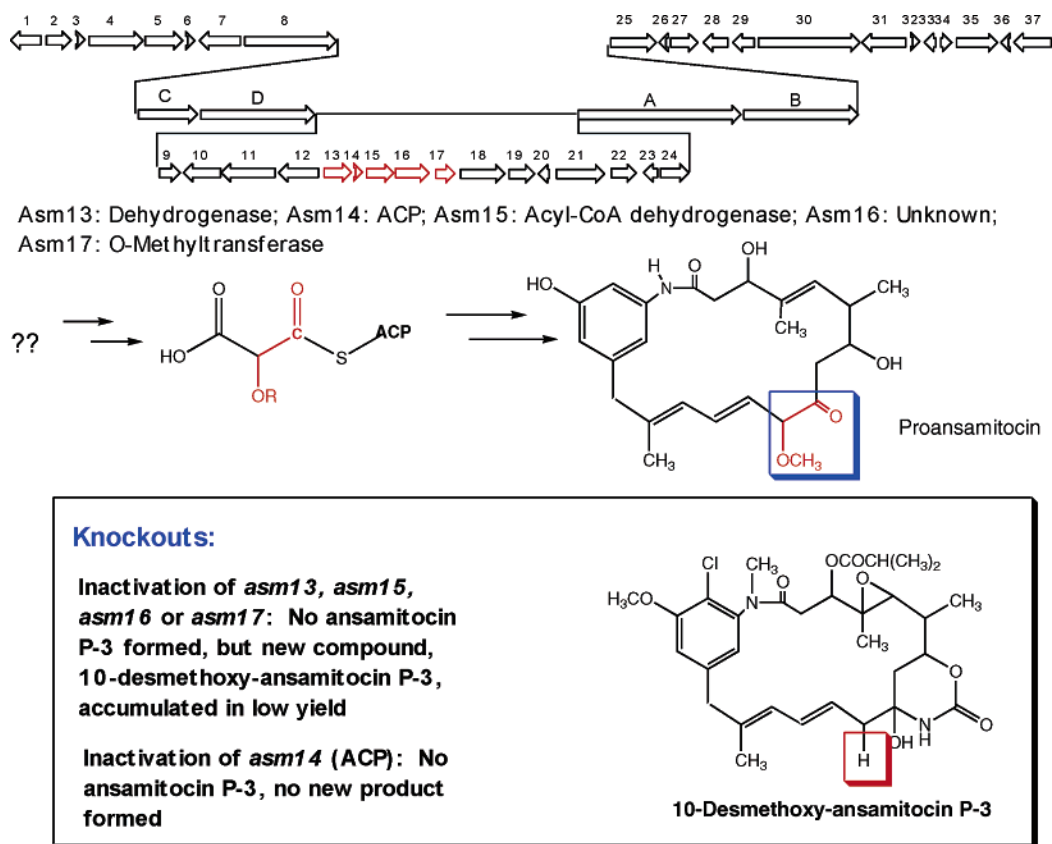


Figure 15. Hydroxy/methoxymalonate biosynthesis genes.

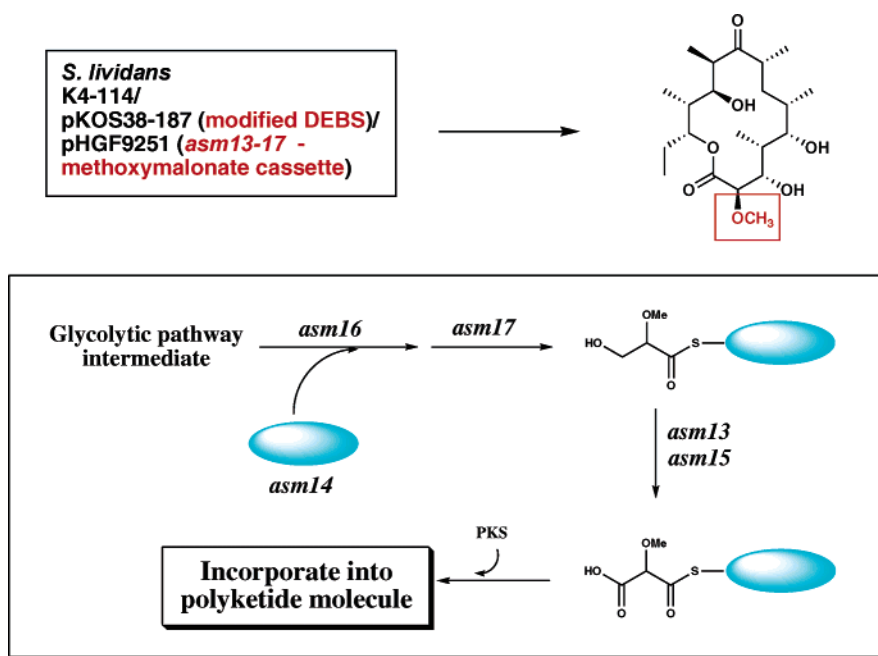
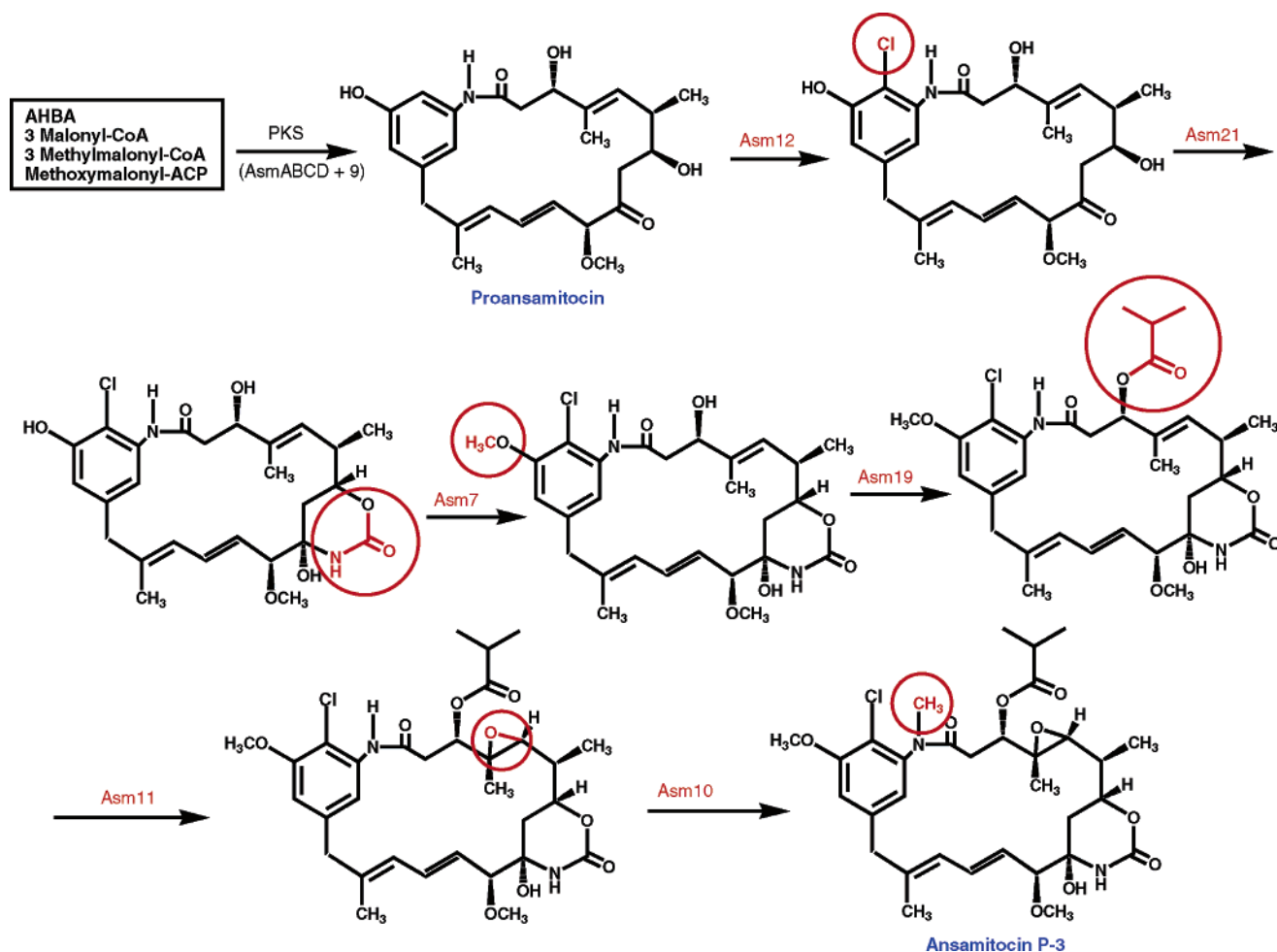


Figure 16. Biosynthesis of methoxymalonyl-ACP catalyzed by the *asm13-17* gene products.

structure of this enzyme corresponds to the expected 10 chain extension modules and their associated modifying functions, but unlike the enzymes catalyzing the formation of macrolactones, such as 6-deoxyerythronolide B,<sup>33</sup> it does not contain a terminal thioesterase domain. Instead, *rifE* is followed by a gene, *rifF*, whose product is homologous to arylamine:acetyl-CoA acetyltransferases from mammalian liver. On the basis of the analogous chemistry it was proposed that this gene product acts as an amide synthase, catalyzing the release of the completely assembled undecaketide from its cognate ACP and its cyclization to an ansa macrolactam,

the postulated proansamycin X. This notion was tested by both our laboratory<sup>34</sup> and that of Schupp at Novartis<sup>35</sup> by inactivation of *rifF*. Instead of just the linear undecaketide, the mutants produced a series of linear ketides ranging from the tetraketide to the undecaketide or, in our experiments, the decaketide (Figure 11). These experiments provided the first direct evidence that type I PKSs indeed operate in a processive fashion, as widely assumed. Furthermore, the results show that the enzyme processes multiple polyketide chains simultaneously and that the modification of the benzenoid to the naphthalenic ring system occurs on the PKS



**Figure 17.** Pathway of ansamitocin P-3 formation.

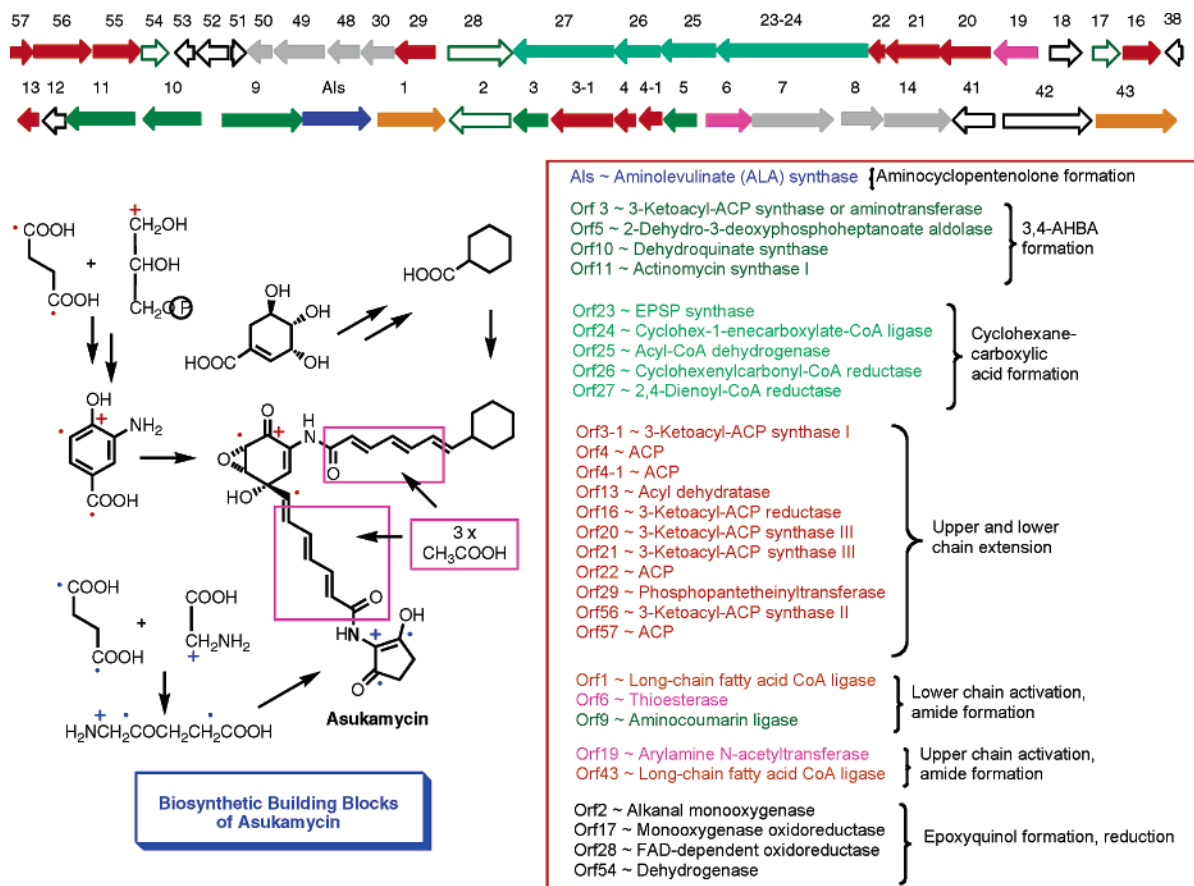
between the third and fourth chain elongation step. Other evidence shows that if this modification reaction does not occur, chain elongation terminates at the tetraketide stage.<sup>36</sup> Since the PKS contains no candidate domains for these modification reactions, they must be catalyzed by (a) separate enzyme(s), and we have recently identified Rif Orf19 as being involved in this process.<sup>36</sup> Our failure to detect the undecaketide was initially assumed to be due to our use of a low-producing parent strain, but closer analysis has now shown that it is due to the fact that in our mutant,<sup>34</sup> unlike that of Schupp's group,<sup>35</sup> the entire RifF protein had been deleted. When we just mutated the arylamine-binding region of the enzyme, the resulting mutant produced the undecaketide in addition to the other ketides.<sup>37</sup> Evidently, the tetra- to decaketide and the undecaketide are released by different mechanisms. Also, our structural analysis of the undecaketide, contrary to the report by the Schupp group,<sup>35</sup> revealed that the terminal double has *Z*, not *E*, configuration,<sup>37</sup> consistent with predictions based on the sequence analysis of the ketoreductase domain of module 10 of the *rif* PKS.<sup>38</sup> The assembly process on the *rif* PKS can thus be pictured as shown in Figure 12.

The *rif* cluster also contains a substantial number of candidate genes for the post-PKS modification reactions leading from proansamycin X to rifamycin B, as well as potential regulatory and transport genes. By mutational analysis, functions were assigned to several of these genes, identifying Rif Orf 5 as being involved in the cleavage of the carbon chain of rifamycin W and formation of the ketal structure,<sup>36</sup> Rif Orf 20 in the 25-*O*-acetylation,<sup>39</sup> and Rif Orf 14 in the 27-*O*-methylation reaction.<sup>40</sup> On the basis of this work, the downstream part of the rifamycin B biosynthetic pathway can be written as shown in Figure 13.

The genes coding for ansamitocin biosynthesis were cloned from *Actinosynnema pretiosum* by the same strategy as used for the *rif*

cluster in a collaboration with the group of E. Leistner at the University of Bonn.<sup>27</sup> The ansamitocins<sup>41</sup> belong to the family of maytansinoids, extremely potent antitumor agents first isolated from higher plants. Since structurally they do not look like typical plant metabolites, it came as no surprise when a few years later the ansamitocins were isolated from an Actinomycete. The maytansinoids bind to tubulin, preventing the assembly of functional microtubules. They proved ineffective in clinical trials, possibly due to dose-limiting toxicity, but still command high interest as "warheads" in tumor-targeted immunoconjugates. Sequence analysis showed that the ansamitocin (*asm*) biosynthesis genes are found in two clusters separated by 30 kb of nonessential DNA. Cluster I contains the majority of the pathway genes and cluster II only some of the genes necessary for AHBA formation (Figure 14). Consistent with the dual function of AHBA synthase, there are two copies of an AHBA synthase gene present, one in cluster I, probably encoding the aromatizing enzyme, and one in cluster II, probably providing the transaminase function.<sup>27</sup>

A peculiarity of the *asm* PKS is the incorporation of an oxygenated chain extension unit, a "glycolate" unit, in the third chain extension step. The precise source of this "glycolate" unit is still not clear, but it appears to originate from an intermediate of glycolysis, possibly 3-phosphoglycerate. The substrate for incorporation of this "glycolate" unit, by analogy with other chain extension units, should be 2-hydroxy- or 2-methoxymalonyl-CoA, but feeding experiments showed that this is not the case.<sup>42</sup> An operon of five genes, *asm13–17*, with *asm14* encoding a dedicated acyl carrier protein (ACP), was found to be responsible for providing the substrate for this particular chain extension reaction, which was therefore deduced to be methoxymalonyl-ACP (Figure 15). Mutational inactivation of *asm13*, *15*, *16*, or *17* abolished



**Figure 18.** Asukamycin biosynthesis: building blocks and genes (M. Petricek, T.-W. Yu, H. G. Floss, and co-workers, unpublished work).

ansamitocin P-3 formation, but resulted in aberrant incorporation of an acetate unit to produce low levels of 10-demethoxy-ansamitocin P-3 (Figure 15). Inactivation of *asm14* showed that this aberrant incorporation of an acetate unit still requires the dedicated ACP.<sup>42</sup> Coexpression in *Streptomyces lividans* of a cassette of *asm13–17* with a plasmid carrying the DEBS genes, modified in the acyltransferase of module 6 to select the substrate for incorporation of a “glycolate” unit,<sup>43</sup> gave in good yield a new analogue of 6-deoxyerythronolide B with a methoxy instead of a methyl group at C-2 (Figure 16).<sup>44</sup> This established that *asm13–17* are sufficient for production of the substrate for this unusual chain extension reaction, allowing us to formulate the reaction sequence as shown in Figure 16.

The further conversion of the initial PKS product, proansamitocin, into the bioactive ansamitocin P-3 requires six enzymatic reactions (Figure 14). Mutational analysis identified the genes responsible for each of these reactions, and phenotypical analysis of the mutants combined with cross-feeding experiments demonstrated the preferred order in which these reactions take place (Figure 17).<sup>45</sup> However, most of the enzymes show relaxed substrate specificity, allowing minor parallel routes through what represents a metabolic grid. Interestingly, the attachment of the ester side chain, which confers bioactivity on the compounds, is not the terminal step, even though maytansinol, the alcohol corresponding to ansamitocin P-3, is a constituent of the fermentation. Although the recombinant acyltransferase *Asm19* is rather promiscuous with respect to the acyl component, it is fairly specific toward the alcohol component and will not acylate maytansinol.<sup>46</sup>

Finally, I want to introduce briefly some as yet unpublished work on another class of antibiotics. The manumycin family of antibiotics,<sup>47</sup> exemplified by asukamycin (Figure 18),<sup>48</sup> also contains a mC<sub>7</sub>N unit, but feeding experiments have shown that it is not derived from AHBA, but from an isomer, 3,4-AHBA,<sup>49</sup> which in

turn arises not from the shikimate pathway, but in an unknown fashion from a C-4 dicarboxylic acid and a three-carbon glycolytic intermediate.<sup>50</sup> The molecule contains two amide-linked unsaturated fatty acid chains, one initiated by cyclohexanecarboxylic acid, the other initiated by 3,4-AHBA and terminating in a 2-aminocyclopentenolone moiety (C<sub>5</sub>N unit), which represents a cyclized molecule of 4-aminolevulinic acid (ALA) (Figure 18).<sup>50</sup>

In collaboration with the group of M. Petricek in Prague we cloned and sequenced the biosynthetic gene cluster for asukamycin (Figure 18) and carried out a limited number of gene inactivation experiments. The results so far establish a number of points. (i) Earlier work in which we had failed to obtain incorporation of a number of potential intermediates between 3,4-AHBA and protoasukamycin<sup>51</sup> had led us to consider the possibility that both polyketide chains of asukamycin are assembled in parallel on a type I PKS and then connected to each other and the C<sub>5</sub>N unit on the enzyme. The gene cluster, however, contains no type I PKS genes, but instead two fatty acid synthases, which thus must be responsible for the biosynthesis. (ii) The conversion of protoasukamycin into asukamycin had been postulated to involve a dioxygenase reaction.<sup>49</sup> However, there is no dioxygenase gene in the cluster, and inactivation of *asu17*, encoding a monooxygenase, abolished this transformation. (iii) The presence of an ALA synthase gene (*als*) confirmed the conclusion from earlier feeding experiments<sup>50</sup> that the ALA for asukamycin biosynthesis is derived from the Shemin pathway, whereas *Streptomyces* generally synthesize their ALA for heme biosynthesis by the C<sub>5</sub> pathway. Inactivation of *als* led to the formation of asukamycin lacking the C<sub>5</sub>N moiety; complementation experiments suggest the possibility that the *als* gene product may catalyze not only the formation but also the cyclization of ALA. Work on the further analysis of this gene cluster is under way in the laboratories of Miroslav Petricek in Prague and Tin-Wein Yu at Louisiana State University.

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