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Biosynthesis of Rifamycins (Ansamycins) and Microbial Production of Shikimate Pathway Precursors, Intermediates, and Metabolites**

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Is the elucidation of biosynthetic pathways of academic interest only, or are there enough valuable spin-offs to make biogenetic studies attractive for a research laboratory in a pharmaceutical company? In this short review it is demonstrated how an interdisciplinary approach to the biosynthesis of rifamycins resulted in the formulation of a general biosynthetic pathway for the whole group of ansamycins and related antibiotics. 3-Amino-5-hydroxybenzoic acid, a natural amino acid derived from the shikimate pathway has been identified as the starter unit for ansamycins and related antibiotics. The microbial mutants produced for this biogenetic study are very useful for the microbial production of shikimate pathway precursors or intermediates such as D(−)*-ribulose or shikimic acid.*

1. Introduction

The ansamycin antibiotics derive their name from their characteristic molecular structure which consists of an aromatic nucleus and a long handle-shaped aliphatic ansa bridge joining two opposite positions of the nucleus (chromophore). Two types of aromatic units can be distinguished: a naphthalenic ring system (as in the rifamycins, streptovaricins, tolypomycins, halomicins, naphthomycin, actamycin, and rubransarols) or a benzenic ring system (as in geldanamycin, herbimycins, macbecins, ansatrienins, mycotrienins, maytansinoids, and ansamitocins).

The rifamycins, as well as the streptovaricins and tolypomycins, specifically inhibit the synthesis of RNA in bacteria by inactivating RNA polymerase. This occurs at very low concentrations (10^{-8} M). These antibiotics do also exert antiviral and antitumour activity, but only at very high concentrations[1]. The clinical application of the most important semisynthetic rifamycin, rifampicin (see Scheme 1), has been principally in the treatment of tuberculosis in combination with other drugs. Other types of ansamycins such as naphthomycin, geldanamycin, maytansins etc. do not inhibit RNA polymerase but act as antibacterial, antifungal, antiprotozoal, antimitotic, antileukaemic or antitumour agents[1].

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Rifamycin B is produced by *Nocardia mediterranei* in large-scale fermentations (for details see[2]). Rifamycin B is oxidized to rifamycin O with persulfate and can then be easily extracted in a suitable solvent. The glycolic acid residue is hydrolyzed by sulfuric acid in a solvent mixture. The resulting rifamycin S is the most important starting material for the preparation of semisynthetic derivatives such as rifampicin. Rifampicin is prepared from rifamycin S via 3-formyl rifamycin SV.

A comprehensive review of the properties, biosynthesis, and fermentation of rifamycins and other ansamycins has been published recently[2], including a detailed description of the different groups of ansamycins produced by different microorganisms (*Actinomycetes*) and plants (*Maytenus* sp., *Colubrina* sp. etc.). The present overview will therefore concentrate on the most important biosynthetic results (sections 3 to 7) and, as this paper is addressed to the members of a chemical society, discuss the methods of biosynthetic research (section 2) and accentuate the potential application of bacterial mutants and metabolites isolated and identified in the course of our biosynthetic research project (sections 8 to 11).

2. Methods in Biosynthetic Research – General Aspects

Fundamental biosynthetic studies have revealed the existence of several general biosynthetic pathways to natural products: the acetate-malonate pathway yielding polyketides and derivatives thereof, the mevalonate route to isoprenoids, and the shikimate pathway of aromatic amino acid metabolism. In the biosynthetis of rifamycins (ansamycins) two of these major

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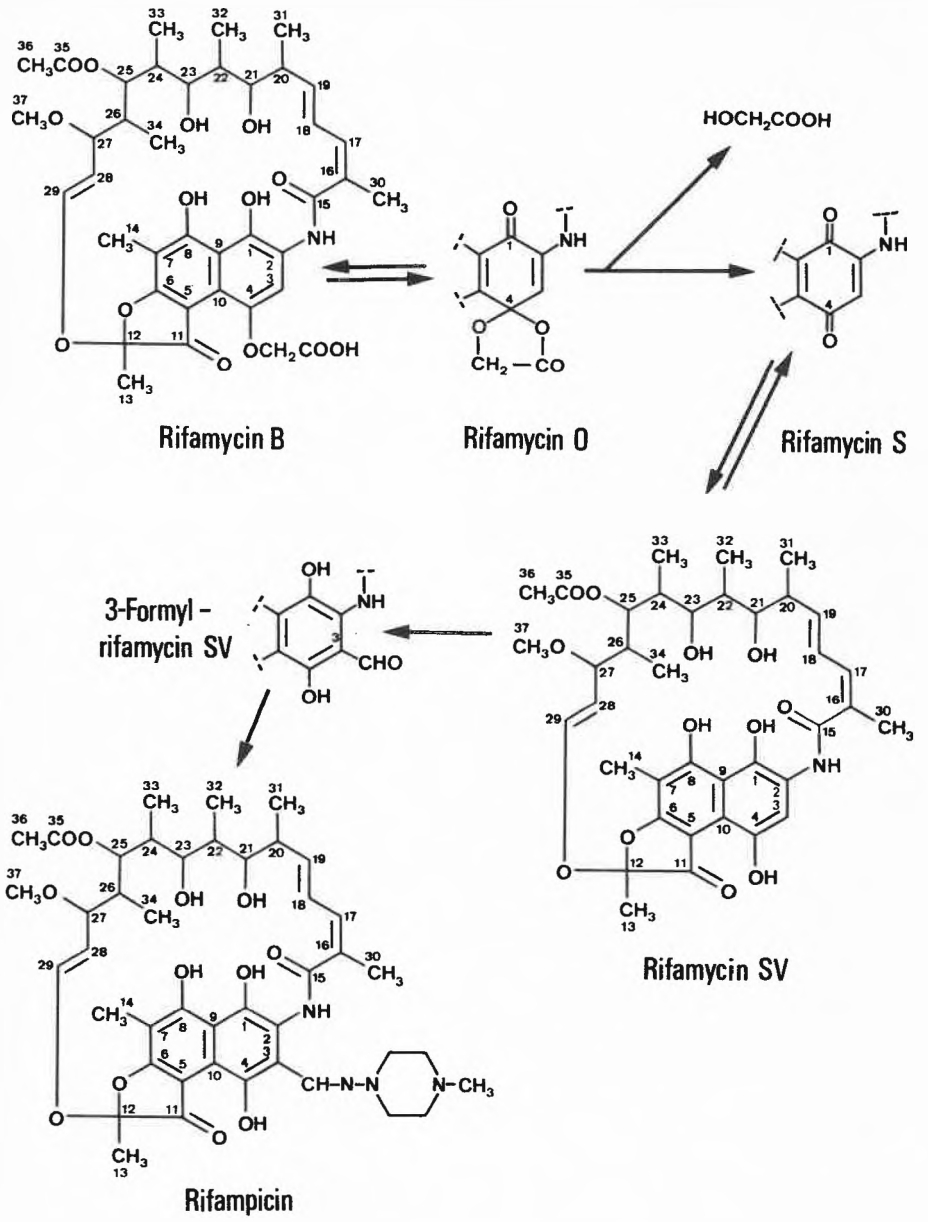


Scheme 1. The most important rifamycins.

pathways are involved: the acetate-malonate pathway (biosynthesis of the ansa chain) and the shikimate pathway (biosynthesis of the chromophore). The shikimate pathway is found in microorganisms and plants but not in animals[3].

The elucidation of biosynthetic routes to primary or secondary metabolites is a highly interdisciplinary task. In order to obtain conclusive results a combination of the following disciplines is required:

- *Microbiology:*
  a) culturing of the organisms or mutants under study (shake flasks and fermenters), production of biomass for enzymology etc.;
  b) optimization of culture conditions to increase productivity;
  c) supplementation, transformation, and mutasynthesis experiments with possible biosynthetic precursors or intermediates;
  d) biotests for antibiotic activities etc.
- *Genetics:*
  a) induction, selection, and isolation of suitable mutants, e.g. mutants with increased productivity, auxotrophic mutants requiring defined supplements for growth, mutants deficient in the biosynthetic route under study, mutants with altered morphology or colour, antibiotic resistent mutants etc.
  b) genetic mapping of mutations in order to establish gene maps.
- *Biochemistry:*
  a) studies of metabolic enzymes to characterize mutants and pathways;
  b) studies on the regulation of enzymes and pathways;
  c) enzymatic synthesis of substrates and intermediates.
- *Analytical Chemistry:*
  a) isolation and identification of biosynthetic precursors and intermediates produced and excreted by mutants or natural strains→spectroscopic methods IR, UV, MS, NMR etc.;
  b) confirmation of identified structures by derivatization etc.;
  c) evaluation of incorporation experiments with ^{13}C-, ^{14}C-, ^{15}N- or ^{32}P-labeled precursors;
  d) quantitative determination of product titers in fermentation broths or extracts thereof.
- *Organic Synthesis:*
  a) synthesis of enzyme substrates;
  b) synthesis of precursors and hypothetical precursors for supplementation and mutasynthesis experiments (labeled or unlabeled);
  c) derivatization of isolated natural products to support the identification of molecular structures.

All of the five disciplines listed above have made significant contributions to the elucidation of the biosynthetic pathways described in the following sections.

3. Biosynthesis of the Ansa Chain

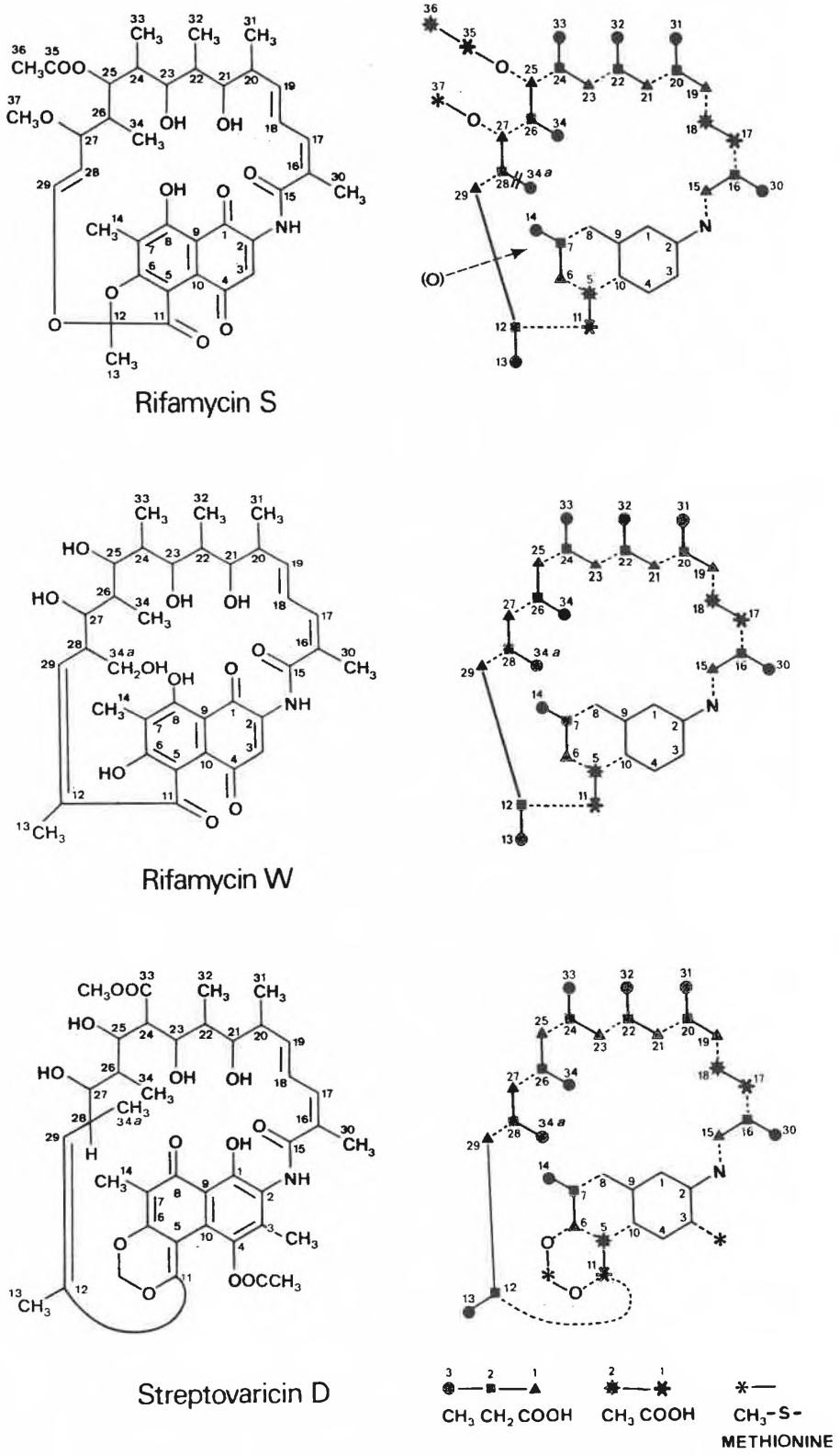
Biogenetic data on ansa chain synthesis are available for rifamycins, streptovaricins, geldanamycin, herbimycins, and ansatrienins.

By incorporation of ^{14}C precursors followed by chemical degradation and by incorporation of ^{13}C precursors combined with nuclear magnetic resonance spectroscopy, it has been shown that the ansa chain of rifamycin S is derived from acetate, propionate, and methionine, as is depicted in Scheme 2[4–6]. The biogenetic origin of 30 of the 37 carbon atoms in rifamycin S was established using this incorporation pattern. The incorporation of acetate (except the O-acetyl group which originates directly from acetyl coenzyme A) and propionate units into rifamycin takes place via malonyl coenzyme A or methylmalonyl coenzyme A, respectively. A seven-carbon amino unit including C1–C4 and C8–C10 of the naphthoquinone part of rifamycin S is not derived from acetate/propionate units. From the labeling pattern it was concluded that in earlier biosynthetic steps a carbon atom 34a (originating from C3 of propionate) was attached to C28. C34a is lost in the later steps of rifamycin biosynthesis. The ansa chain was interpreted as a clockwise condensation of acetate/propionate units with a seven-carbon amino starter unit. C12, C13, and C29 originate from the same propionate unit which is later split off by the introduction of oxygen between C12 and C29.

From a mutant strain of *N. mediterranei* a biogenetic precursor of rifamycin S was isolated and designated rifamycin W[7]. In a transformation experiment it was demonstrated that radioactive labeled rifamycin W was transformed into radioactive rifamycin B via rifamycin S. The structure of rifamycin W (Scheme 2) is in accordance with the biogenetic model derived from the incorporation pattern of

acetate/propionate into rifamycin S. C34a is still present in rifamycin W and the carbons C12, C13, and C29 are not yet separated by the introduction of oxygen. Rifamycin W showed an incorporation pattern for ^{13}C-labeled acetate and propionate identical to the pattern found earlier for rifamycin S[8].

The propionate incorporation pattern for streptovaricin D (Scheme 2) is identical to the pattern for the rifamycins[9]. C34a is still present and C12, C13, and C29 originate from the same propionate unit, as in rifamycin W. The methyl group attached to C3 and the methylenedioxy group between C6 and C11 are derived from methionine[10]. As in the case of rifamycin, we are left with a seven-carbon amino unit which is not derived from acetate/propionate units.



Scheme 2. Incorporation of acetate, propionate, and methyl from methionine into rifamycin S, rifamycin W, and streptovaricin D.

Based on the identical incorporation patterns, a common progenitor for some of the naphthoquinoid ansamycins was proposed[6, 8].

All the available complementary incorporation data for actamycin[11], geldanamycin[10, 12, 13], herbimycin A[14], and mycotrienins[15] indicate that, as in the rifamycins and streptovaricins, the ansa chain is derived from acetate (or in geldanamycin glycerate/glycolate) and propionate units, whereas the remaining seven-carbon amino unit must be synthesized by a different biosynthetic pathway.

4. The Origin of the Seven-Carbon Amino Unit

All the ansamycins investigated so far contain a seven-carbon amino unit which is not derived from acetate/propionate units. A similar C_7N unit was found in the mitomycins and in ferrimycin A_1. Incorporation studies[5, 16] with rifamycin and D-[1-^{13}C]glucose and D-[1-^{13}C]glycerate led to the hypothesis that the C_7N unit of the ansamycins derives from an intermediate of the shikimate pathway (for details see[2]). [U-^{14}C]Shikimic acid is not incorporated into the rifamycin chromophore, but this is no proof for it not being a precursor of the C_7N unit of rifamycin, as postulated by *Karlsson* et al.[5]. It was later found that shikimate is not able to penetrate into the cells of *N. mediterranei* (rifamycin producer) within a pH range of 4.0–9.0, and even the addition of dimethyl sulfoxide (DMSO) did not improve the uptake of shikimate into the cells[17, 18]. *Karlsson* et al.[5] suggested that 3-dehydroquinate or 3-dehydroshikimate was the most probable precursor for the C_7N unit. The results of the corresponding incorporation experiments with geldanamycin[13] and mitomycin C[19–21] are also in agreement with a shikimate-type origin of the seven-carbon amino unit.

Because of the inability of shikimic acid (and most likely also of its precursors 3-dehydroquinic acid and 3-dehydroshikimic acid) to penetrate into the cells of *N. mediterranei* (and other ansamycin producers) and because the selection of ^{13}C precursors available for further incorporation studies is very limited, we chose a genetic approach for further investigations.

Two aromatic amino acid-deficient mutants of *N. mediterranei* were isolated and characterized. These mutants provide genetic evidence that intermediates of the shikimate pathway must be involved in the biosynthesis of the rifamycin chromophore. The mutant strain *N. mediterranei* A8[22] is auxotrophic for phenylalanine and tyrosine and shows greatly reduced rifamycin production compared with the parent strain N813 (high rifamycin B producer). Although it is phenotypically an aro$^{\ominus}$-mutant, no block in the enzymes of the shikimate pathway was found, but only a block in the transketolase activity. A mixture of pentoses with D(−)-ribulose as

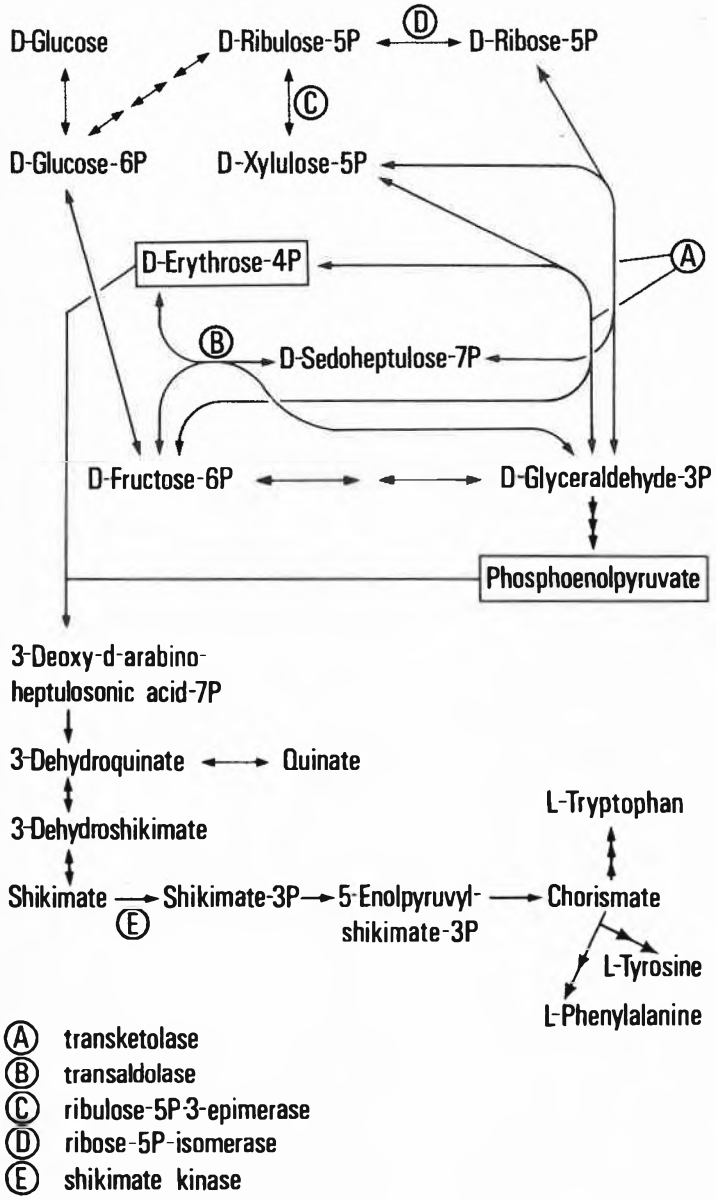
the major product was found to be accumulated by mutant A8 in the culture broth (see section 9). The mutant is slightly leaky. The mutation was shown by reversion to be single-point one. The fact that the production of rifamycin B is dependent on the formation of D-sedoheptulose-7-phosphate (Su7P) and the presence of transketolase activity (see pathway in Scheme 3) shows clearly that the C_7N unit of the rifamycin chromophore must be derived from an intermediate of the shikimate pathway. No other pathway for synthesizing Su7P except the transketolase reaction is known so far. Thus a mutant lacking the transketolase activity is not able to synthesize both D-erythrose-4-phosphate (E4P) and Su7P, and no other pathway except the shikimate one is known which starts from Su7P or E4P and leads to aromatic compounds. The mutant strain *N. mediterranei* A10[23] is auxotrophic for phenylalanine, tyrosine, and tryptophan and produces the same amount of rifamycin B as the parent strain N813, at least under suboptimal conditions. The mutant accumulates shikimic acid and 3-dehydroshikimic acid in the culture broth (see section 10). The enzymatic and auxanographic studies showed that mutant A10 is blocked in one of the enzymatic steps leading from shikimic acid to chorismic acid (see Scheme 3). The missing enzyme is most likely to be the shikimate kinase. As mutant A10 is only defective in the biosynthesis of aromatic amino acids and not in the biosynthesis of rifamycin, it would appear that the C_7N unit of the rifamycin chromophore must be derived from an intermediate of the shikimate pathway not behind shikimate. If one combines the results from the mutants A8 and A10 and from the ^{13}C incorporation experiments[5, 16], the origin of the C_7N unit can be localized between 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) and shikimate.

A number of non-rifamycin-producing ultraviolet mutants derived from strains *N. mediterranei* N813 and A10 were found to accumulate an identical complex of aromatic components instead of rifamycin B[24]. The main component of this complex, product P8/1-OG, was isolated from six of these $P^{\ominus}$-mutant strains and identified spectroscopically as a very early precursor in the biosynthesis of rifamycins (see Scheme 4). The compound was isolated in two forms, either as 2,6-dimethyl-3,5,7-trihydroxy-7-(3′-amino-5′-hydroxyphenyl)-2,4-heptadienoic acid or as its 1,5-lactone.

By structural comparison with rifamycin S or rifamycin W, product P8/1-OG can easily be recognized as a very early ansamycin precursor containing the C_7N unit and three initial acetate/propionate units of the ansa chain (Scheme 4). 3-Amino-5-hydroxybenzoyl-CoA might act as a starter molecule for the biosynthesis of P8/1-OG and of the rifamycins. To this C_7N starter unit, first a propionate unit (via methylmalonyl-CoA), then an acetate unit (via malonyl-CoA), and finally another propionate unit are added by condensation and decarboxylation. The resulting aromatic triketide is then converted into product P8/1-OG by hydrogenation of the keto group C7 and by enolization of the keto groups C3 and C5. The CoA is then split off (possibly during the excretion of the product). The isolation of product P8/1-OG provides strong evidence that the C_7N starter unit for the ansamycin biosynthesis is 3-amino-5-hydroxybenzoic acid or its CoA derivative.

3-Amino-5-hydroxybenzoic acid has been synthesized (see section 11) and investigated for its ability to induce rifamycin biosynthesis in an appropriate mutant (strain A8) of *N. mediterranei* and was found to be a direct precursor of the C_7N starter unit for the biosynthesis of ansamycins[25]. Supplementation studies with *N. mediterranei* A8 showed clearly that 3-Amino-5-hydroxybenzoic acid (AHBA) can indeed substitute for the C_7N unit. The original rifamycin production capacity of the parent strain N813 can be restored for strain A8 (transketolase$^{\ominus}$ mutant) by supplementation with AHBA. Incorporation experiments with [Carboxy-^{14}C]AHBA and *Streptomyces* sp. E/784 demonstrated that this precursor specifically labels the C_7N unit of actamycin[26], whereas [Carboxy-^{13}C]AHBA was found to label the C_7N units of the mitomycin-type antibiotics porfiromycin[27] and ansamitocin P-3[28].

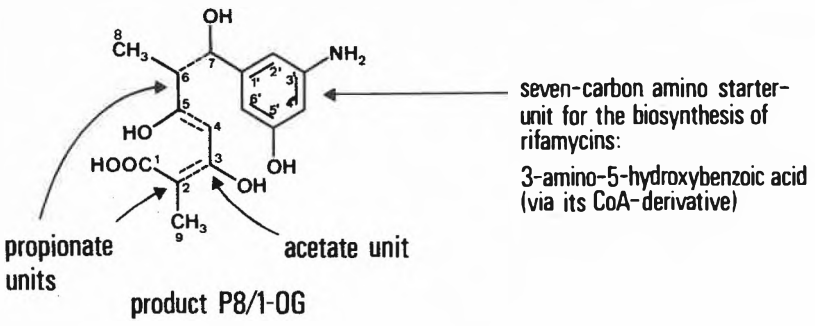
Quite recently Chinese scientists[29] have demonstrated that the nitrogen in AHBA and rifamycin originates predominantly from the amide group of glutamine and that AHBA re-established the synthesis of rifamycin in an inactive mutant *N. mediterranei* rif 2.



Scheme 3. Biosynthetic pathways: pentose shunt, glycolysis, and shikimate pathway.

5. Intermediates in the Biosynthesis of Rifamycins and Streptovaricins

Rifamycin W, the first isolated biogenetic precursor of rifamycin S, has already been mentioned[7, 8] (see Scheme 2). A mu-

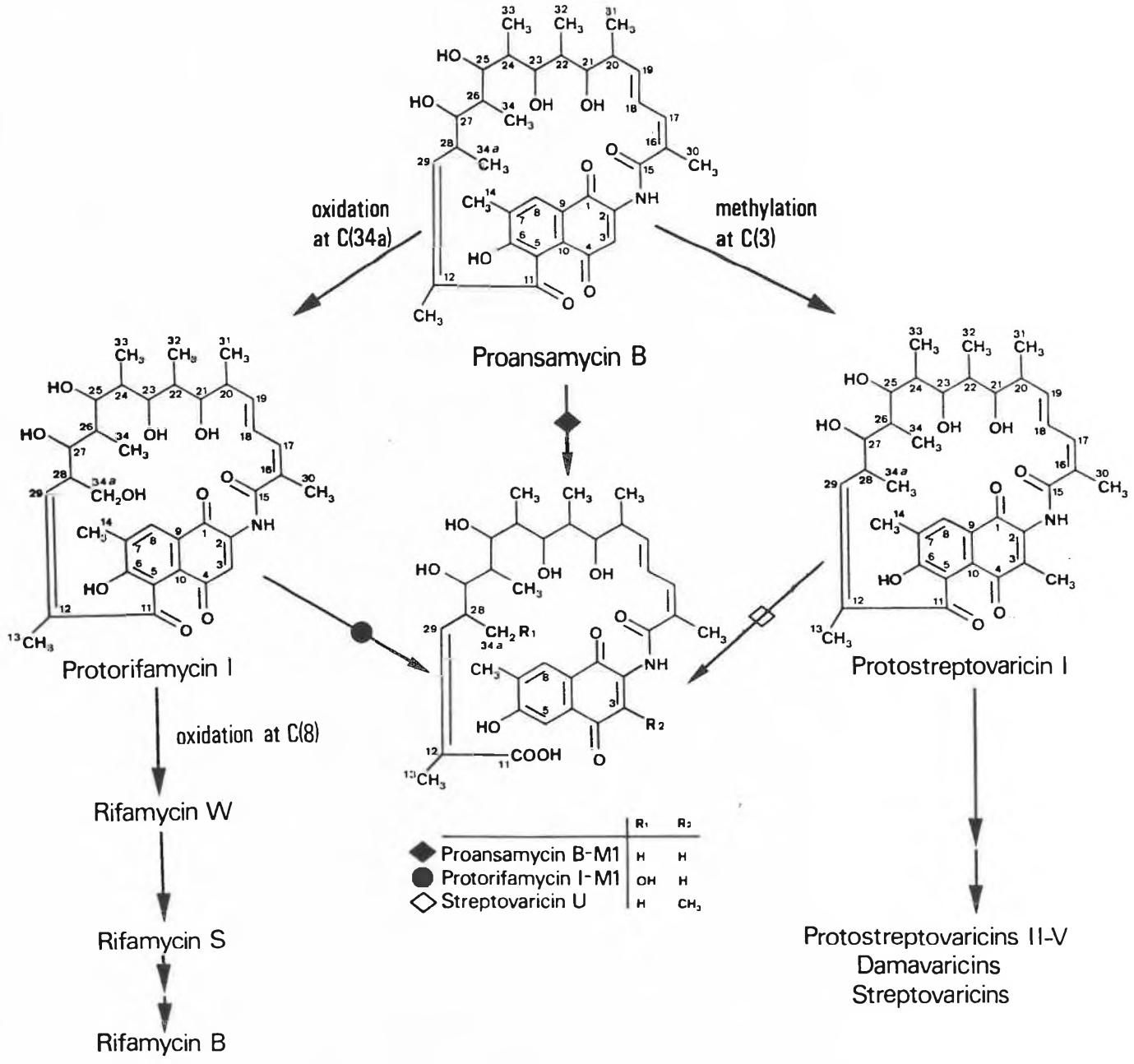


Scheme 4. Structure and biogenetic interpretation of product P8/1-OG.

tant strain *N.mediterranei* F 1/24 was found to accumulate a number of 8-deoxyansamycins[30–32]. The main component of the 8-deoxyansamycin complex, protorifamycin I (8-deoxyrifamycin W), is a direct biogenetic precursor of rifamycin W[30]. Protorifamycin I is structurally related not only to rifamycin W, but also to the protostreptovaricins[33] and damavaricins[34], early precursors of the streptovaricin-type ansamycins. Two hypothetical structures for the common progenitor of the rifamycin-type and the streptovaricin-type ansamycins have been proposed[30], namely «proansamycin A» and «proansamycin B».

From «proansamycin B» (34a-deoxyprotorifamycin I or 3-demethylprotostreptovaricin I), protorifamycin I and protostreptovaricin I could easily by synthesized by simple one-step transformations (Scheme 5). In order to isolate such a hypothetical proansamycin, the minor compounds of the 8-deoxyansamycin complex from strain F 1/24 were investigated. None of these postulated proansamycins was found so far, but a metabolite of proansamycin B, designated proansamycin B-M1, has been identified (together with a corresponding metabolite protorifamycin I-M1 derived from protorifamycin I), thus indicating the existence of proansamycin B[31]. An open-chain analogue in the streptovaricin series, streptovaricin U, has also been reported[54].

From strain *N.mediterranei* F 1/24 nine further 8-deoxyansamycins and from a



Scheme 5. Possible pathways for the biosynthesis of protorifamycin I and protostreptovaricin I starting from the hypothetical common progenitor «proansamycin B».

recombinant strain *N. mediterranei* R21 seven novel rifamycins were isolated[31,32,35,36] and a biogenetic tree for rifamycins and streptovaricins was constructed (for details see[2,37]). Mutasynthesis experiments with *N. mediterranei* A8 demonstrated that only AHBA but not 4-substituted 3-amino-5-hydroxybenzoic acids can substitute for the C_7N unit in the biosynthesis of rifamycins[37]. This indicates that the introduction of C3 substituents into rifamycins (e.g. 3-hydroxyrifamycin S) or streptovaricins (3-methyl) or maytansins (3-chlorine) takes place in a later biosynthetic step. Similar results have been reported for mutasynthesis experiments with substituted AHBA and actamycin[38].

6. Transformation Reactions Starting from Rifamycin S

Rifamycin S is a key intermediate in the biosynthesis of a considerable number of rifamycins, namely the rifamycins A, B, C, D, E, SV, L, O, Y, G, R, P, Q, and Verde (for details see[2]). The origin of the glycolic acid moiety in the rifamycins B and L was investigated by ^{14}C-incorporation experiments[39,40] and it was found that only C_3 precursors (glycerol) or sugars (glucose, ribose) were used for the glycolic acid moiety of rifamycin B, but not C_2 precursors such as glycolate, glyoxylate, glycine or ethanol.

From the incorporation data it was speculated that the origin of the glycolic acid moiety is the same for both rifamycins B and L. Rifamycin L was excluded as a precursor of rifamycin B, and rifamycin O was proposed as a common progenitor for both rifamycins B and L. A reinvestigation of the transformation of rifamycin S into the rifamycins B and L gave different results[41,42] (see Scheme 6).

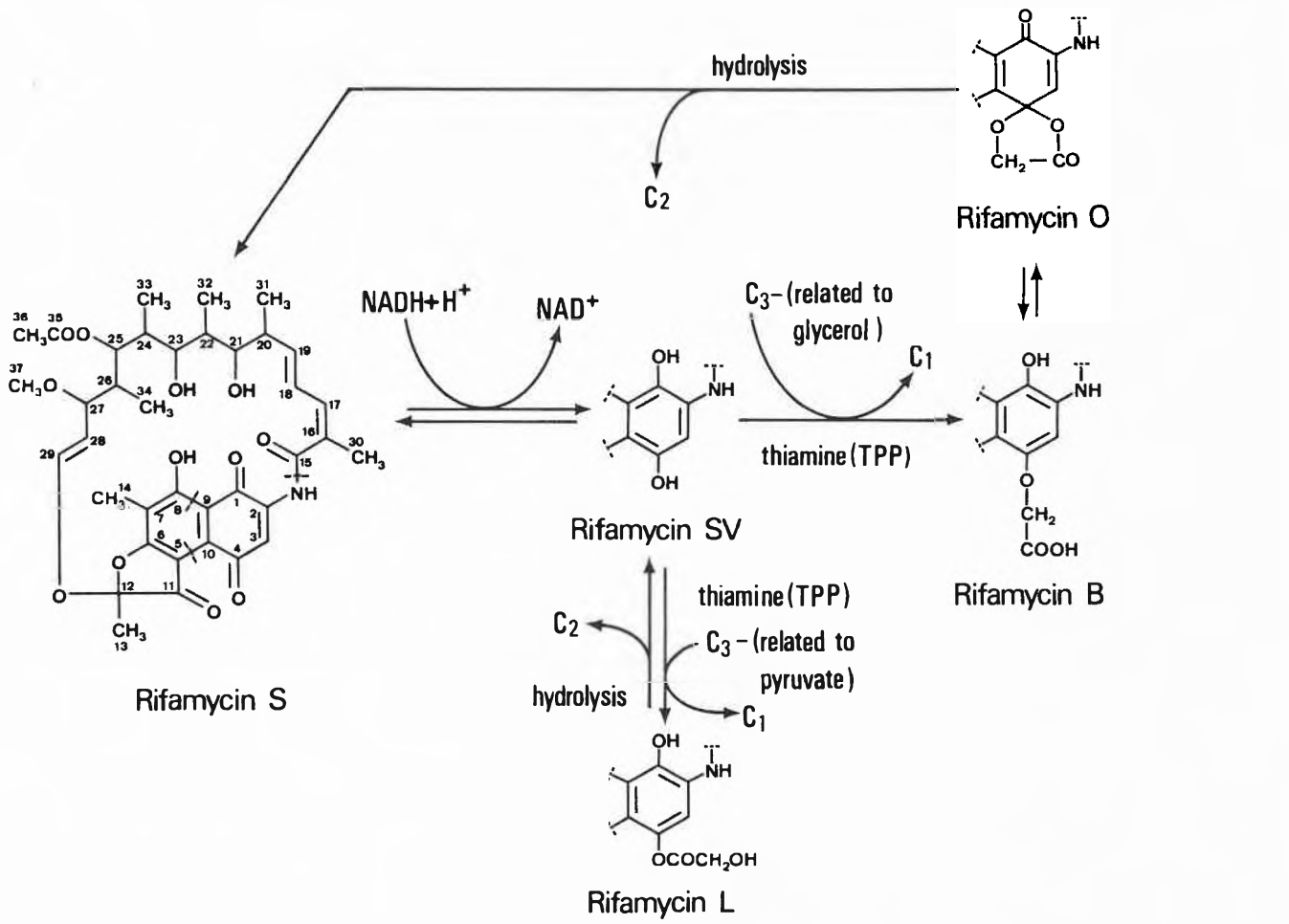
The reduction of rifamycin S to rifamycin SV was found to be NADH dependent, but not specific for the rifamycin biosynthetic pathway. This reduction functions also using *Escherichia coli* cells. Rifamycin O was definitely excluded as a precursor for rifamycins B and L. The transformation of rifamycin S into B and L is completely inhibited by the thiamine antagonist oxythiamine chloride hydrochloride, indicating the participation of thiamine or thiamine pyrophosphate in both transformation reactions. Therefore a thiamine pyrophosphate-dependent decarboxylating enzyme system is postulated for both transformation reactions.

^{14}C incorporation experiments demonstrated that glycerol predominantly labels the glycolic acid moiety of rifamycin B, and pyruvate the glycolic acid moiety of rifamycin L. This indicates that two completely different biosynthetic pathways using different C_3 precursors for the biosynthesis of the glycolate ether rifamycin B and for the glycolate ester rifamycin L must be operating (Scheme 6).

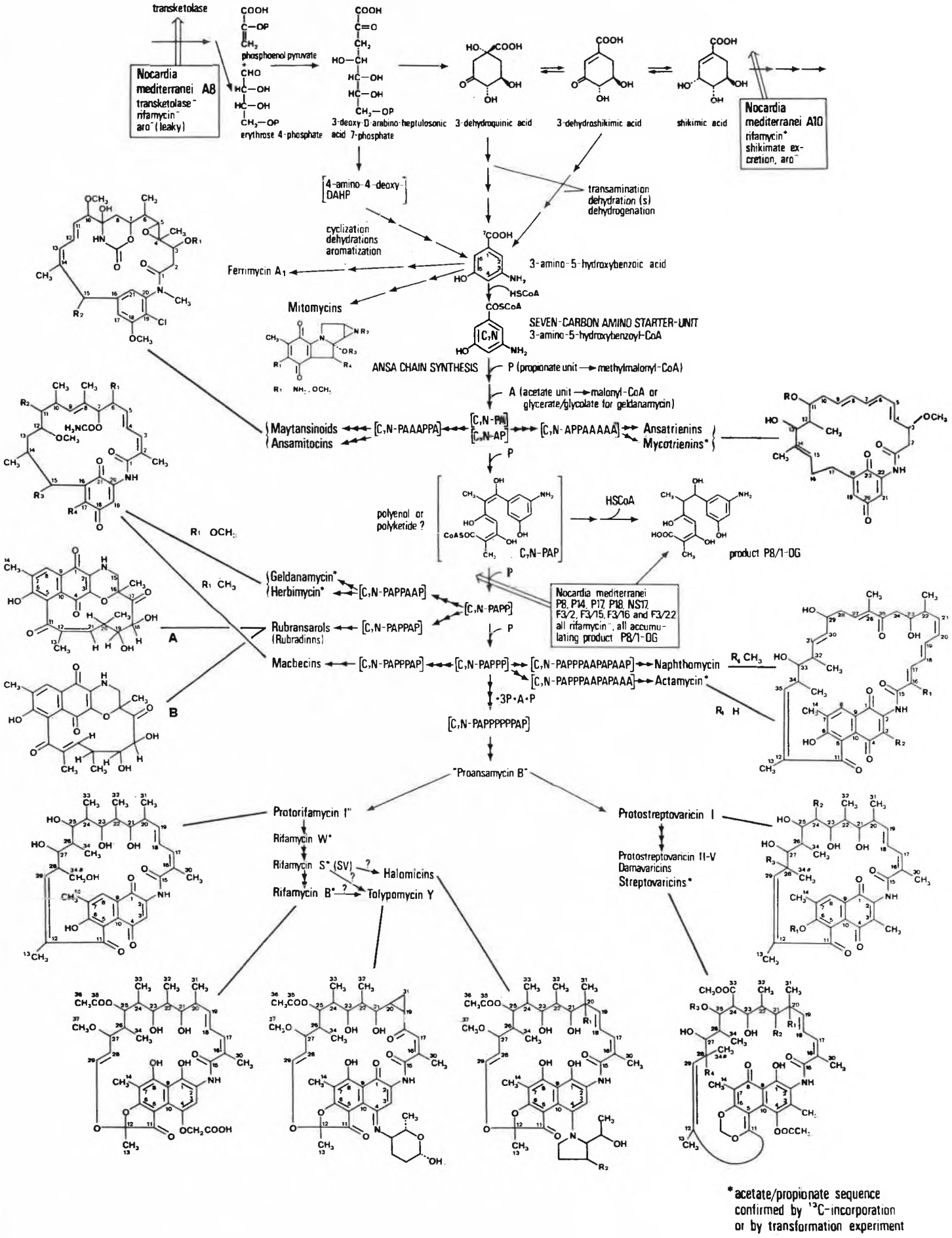
7. A General Pathway for the Biosynthesis of Ansamycins

With all the available biosynthetic data and by analyzing structural analogies, we can depict a biogenetic model for all the known ansamycins (Scheme 7). The C_7N starter unit for the ansamycin biosynthesis is derived from an intermediate of the shikimate pathway between 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) and shikimate. The exact branch point remains to be identified. 3-Amino-5-hydroxybenzoic acid (AHBA) is the direct precursor of the C_7N starter unit which can now be identified as 3-amino-5-hydroxybenzoyl-CoA. The antibiotics of the mitomycin-type containing a C_7N unit similar to that of the ansamycins are derived directly from AHBA (the carboxy group is reduced to a methyl group in one of the following biosynthetic steps). In the case of the antibiotic ferrimycin A_1, AHBA itself is a structural element of the compound[43].

Starting with 3-amino-5-hydroxybenzoyl-CoA, a polyketide chain (ansa chain) is built up by subsequent condensation with propionate and acetate units, via methylmalonyl-CoA and malonyl-CoA, respectively. Different biosynthetic branch points in the poylketide synthesis can be located by analyzing structural analogies of the ansamycin-types (ansa methyl groups indicate a propionate unit!) or by the known incorporation pattern for [^{13}C]acetate and [^{13}C]propionate.



Scheme 6. Revised biosynthetic model for the transformation of rifamycin S into the rifamycins B and L.



Scheme 7. Possible pathways for the biosynthesis of ansamycins starting from shikimate pathway intermediates via 3-amino-5-hydroxybenzoic acid as the direct precursor of the seven carbon amino starter unit.

A first ansamycin branch derived from the sequence C_7N – propionate – acetate (C_7N-PA) leads to the maytansinoids and ansamitocins (whereas the ansatrienins and mycotrienins seem to be derived from the inverse sequence C_7N-AP). The sequence C_7N-PAP is excreted as its deactivated form (product P8/1-OG, see section 4) by nine independent production$^{\ominus}$ mutants of *N. mediterranei*. The following ansamycin types are derived from polyketides behind the sequence C_7N-PAP: geldanamycin and herbimycin, rubradirins (containing the ansamycin moieties rubransarol A and B), macbecins, streptovaricins via protostreptovaricin and damavaricins, rifamycins via «proansamycin B» and protorifamycin I, halomicins and tolypomycin Y, naphthomycin and actamycin. The structural features of AHBA are clearly visible in all these ansamycins.

For tolypomycin Y and halomicins the oxygen function at C4 is replaced by a nitrogen function. The oxygen function originating from C7 of AHBA is lost in ansamitocins, maytansinoids (except colubrinol and colubrinol acetate), ansatrienins, mycotrienins, geldanamycin, rubransarols, naphthomycin, actamycin, protorifamycin I, and protostreptovaricins, but is still present in herbimycins and macbecins. For the naphthalenic ansamycins, this oxygen function must be eliminated during the formation of the left aromatic ring. In later biosynthetic steps leading to the formation of rifamycins, halomicins, tolypomycin Y, damavaricins, and streptovaricins, the oxygen at C8 is again introduced (e.g. protorifamycin I→rifamycin W). The oxygen function at C1 of the chromophore (originating from C2 of AHBA) which is present in most of the ansamycins listed above is still absent in maytansinoids and ansamitocins, thus indicating that this oxygen must be absent in the C_7N starter unit. In some of the ansamycins additional substituents such as chlorine, hydroxy, methoxy, or methyl are introduced into the chromophore in later biosynthetic steps (in positions corresponding to C4 or C6 of AHBA).

8. Genetic and Regulatory Aspects

It has been demonstrated that the final steps in the rifamycin B biosynthesis (protorifamycin I→rifamycin W→rifamycin S→rifamycin B) are chromosomally determined and that the genes are closely linked to each other[44].

The UV-induced mutant *N. mediterranei* A8 (transketolase$^{\ominus}$, rifamycin$^{\ominus}$; ribulose producer) can easily be reverted by UV mutation[22], whereas the mutant *N. mediterranei* A10 (aro$^{\ominus}$; shikimate producer) can not be reverted by UV irradiation[23]. Both auxotrophic mutants A8 and A10 are highly sensitive to lyophilization. With strain A8 the accumulation of ribulose was reduced by a factor of ten after lyophilization and reactivation, probably due to a mutational hot spot at a locus responsible for production, excretion or degradation of ribulose[45]. Phenotypically the culture was still transketolase$^{\ominus}$/rifamycin$^{\ominus}$. With strain A10 the production of shikimic acid dropped to almost zero after lyophilization and reactivation. In this case the effect was due to reversion (backmutation) induced by the lyophilization procedure. After one propagation the population in the culture consisted of 15% revertants (wild-type cells) and 85% cells of phenotype A10. In such a mixed population phenotype A10 was diluted out after several propagations[45]. Similar effects caused by lyophilization of mutants are described throughout the microbiological literature.

In contrast to strains A8 and A10, other blocked mutants such as *N. mediterranei* F 1/24 (protorifamycin I producer) or P$^{\ominus}$-mutants (producing product P8/1-OG) are not affected by lyophilization. DAHP synthetase in *N. mediterranei* N813, the first enzyme of the shikimate pathway, was found to be partially feed-back inhibited by phenylalanin + tyrosine + tryptophan, chorismate, rifamycin B, rifamycin S, rifamycin W, 3-hydroxyrifamycin S, protorifamycin I, and product P8/1-OG. The maximum inhibition for all these inhibitors was only about 30%. A stronger regulatory effect (substrate inhibition) was found for erythrose-4-phosphate which inhibits DAHP synthetase competitively[46].

Propionyl-CoA carboxylase in *N. mediterranei*, the enzyme catalyzing the carboxylation of propionyl-CoA to methylmalonyl-CoA and of acetyl-CoA to malonyl-CoA, was found to be inhibited by citrate or product P8/1-OG and activated by isoleucine, methionine, threonine, and valine[47]. Interestingly, none of the two enzymes DAHP synthetase and propionyl-CoA carboxylase is affected by AHBA.

9. Microbial Production of D(−)-Ribulose

D(−)-Ribulose, a yellowish syrup, is commercially available at extremely high prices and relatively low purity. Generally, the commercial preparations of D-ribulose are produced by isomerization (in pyridine) of D-arabinose and contain about 10–30% of D-arabinose after purification by fractionating crystallization of the non-reacted D-arabinose.

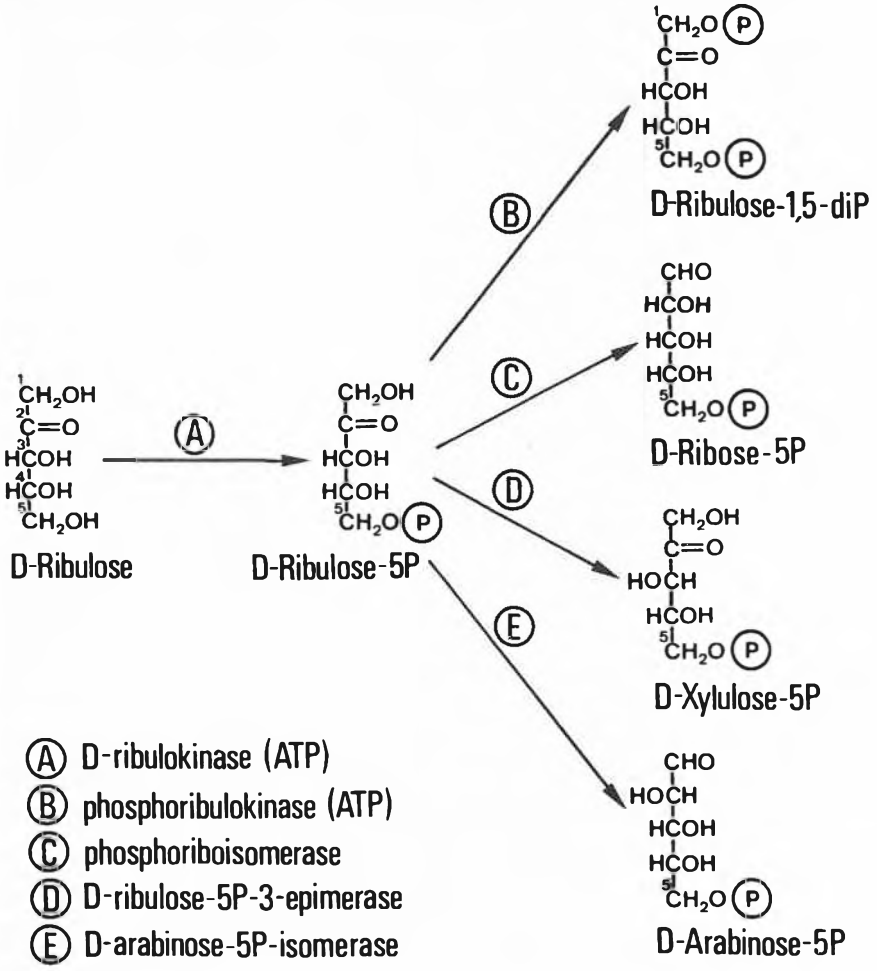
The transketolase$^{\ominus}$ mutant *N. mediterranei* A8 (see sections 4 and 8) accumulates a mixture of pentoses with D-ribulose as the major product and arabinose, xylulose, and xylose as the identified minor products[22]. From the pathways depicted in Scheme 3 one would expect such a mutant to accumulate a mixture of D-ribose-5-phosphate, D-ribulose-5-phosphate, and D-xylulose-5-phosphate. However, prior to excretion into the medium, this hypothetical mixture of pentose-phosphates seems to undergo a series of epimerizations, isomerizations, and dephosphorylations, thus resulting in the predominant accumulation of D-ribulose. In shake flasks (batch cultures) using the industrial production medium 151b[30] titers up to 6–8 g/L D-ribulose can be obtained with strain *N. mediterranei* A8[22,48]. Batch fermentations in 30-liter fermenters with medium 151b yielded titers up to 10–15 g/L of D-ribulose after 8 days with a complete depletion of the glucose (carbon source) present in the medium[48]. By feeding an additional amount of glucose (fed-batch fermentation) the titers can easily be increased up to 20 and more g/L of D-ribulose.

So far, the best results were obtained with a double mutant *N. mediterranei* A8/6 derived from strain A8 by spontaneous mutation[48]. This double mutant has a second block directly in the biosynthesis of rifamycin and revertants of strain A8/6 (transketolase$^{\oplus}$) are still unable to produce significant amounts of rifamycins. In order to preserve the good ribulose productivity, the conservation methods of choice for the strains A8 and A8/6 are agar slants or deep-frozen skim milk suspensions, rather than lyophilizates (see section 8). D-Ribulose of $\geq 95\%$ purity (determined with ribitol dehydrogenase) is obtained from fermentation broths by ion-exchange chromatography[22,48]. D-Ribulose can also be produced in a biotransformation system with glucose, buffer, and mycelium of *N. mediterranei* A8 or A8/6.

Although D-ribulose (Scheme 8) is an interesting chiral compound it is rarely used for chemical synthesis, probably due to its extremely high price. The only derivative available so far is its *o*-nitrophenylhydrazine (see also[22]). On condition that D-ribulose becomes available at high purities and reasonable costs (e.g. when produced by fermentation on a large scale) it would be an excellent starting material for chemical syntheses and enzymatic transformations using enzymes (Scheme 8) from microorganisms (*N. mediterranei*, *E. coli* etc.), plants (spinach) or liver. The products available in one-step or two-step transformations are: D-ribulose-5-phosphate, D-ribulose-1,5-diphosphate, D-ribose-5-phosphate, D-xylulose-5-phosphate, D-arabinose-5-phosphate etc. All these compounds are very important enzyme substrates and intermediates for biochemical research and all of them are very expensive. Specifically labeled [^{13}C, ^{14}C]substrates could be prepared starting from labeled D-ribulose produced in fermentations or transformation systems with *N. mediterranei* A8 or A8/6 and labeled glucose (glucoses of different labeling-types are available).

10. Microbial Production of Shikimic Acid

(−)-Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) is commercially available at high prices. Generally shikimic acid is extracted from the fruit of *Illicium anisatum* (anis star) or *Illicium religiosum* (in Japanese shikimi-no-ki)[3].



Scheme 8. Enzymatic synthesis of important enzyme substrates starting from D-*ribulose.*

and prephenate. All these compounds are interesting enzyme substrates and intermediates for biochemical research.

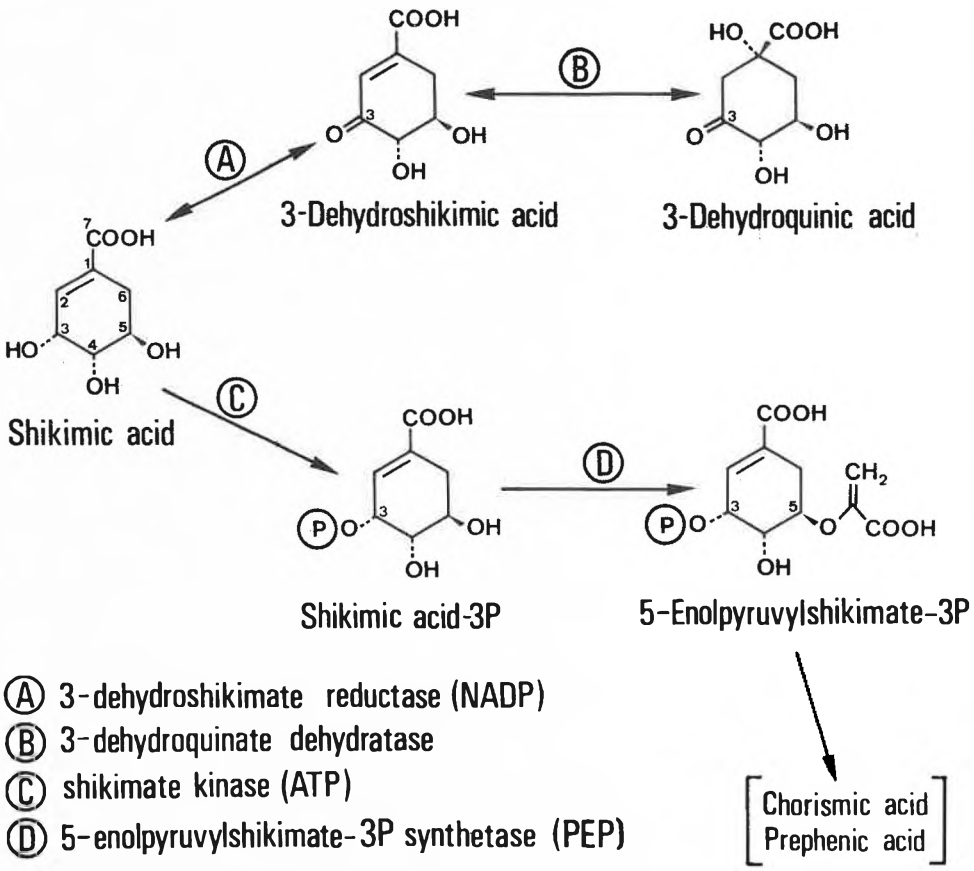
Specifically labelled [^{13}C, ^{14}C]shikimate and derivatives thereof could be prepared by fermentations or biotransformations with *N. mediterranei* A10 and labeled glucose. The incorporation pattern for the individual carbon atoms of glucose into shikimate is well known[3]. The best conservation methods for strain A10 are agar slants or deep-frozen skim milk suspensions, rather than lyophilizates (see section 8).

11. Synthesis and Microbial Production of 3-Amino-5-hydroxybenzoic Acid

3-Amino-5-hydroxybenzoic acid (AHBA) is not yet commercially available, probably due to the fact that the synthesis of AHBA by the routes of classical aromatic chemistry starting from 3,5-dinitrobenzoic acid, and using partial reduction to differentiate the nitro groups, takes at least five reaction steps with rather moderate overall yields[25,43,49]. However, since the discovery of the key role of AHBA in the biosynthesis of ansamycins and mitomycins (see sections 4 and 7), several attempts have been made to improve the synthesis of the compound[50–52]. One method displaced one of the two nitro groups in 3,5-dinitrobenzoic acid by methoxide ion, and achieved a 77% overall yield of AHBA in three steps[50]. AHBA can also be synthesized by direct amination of 3,5-dihydroxybenzoic acid with ammonia and

The aro$^{\ominus}$ mutant *N. mediterranei* A10 (see sections 4 and 8) accumulates shikimate and traces of 3-dehydroshikimate in the culture broth[23]. In shake flasks (batch cultures) using the industrial production medium 151b[30] titers up to 1–1.5 g/L of shikimic acid can be obtained with strain *N. mediterranei* A10[23,48]. Batch fermentations in 30-liter fermenters with medium 151b yielded titers up to 2 g/L of shikimic acid with a complete depletion of the glucose present in the medium[48]. We have calculated that a strain optimization by blocking the rifamycin production of strain A10 directly at the biosynthetic branch point for the C_7N unit (see sections 4 and 11) would channel all the substrate flux for the shikimate pathway into the production of shikimate and result in shikimate titers up to 5 g/L.

Although shikimic acid (Scheme 9) is an interesting chiral compound, it is, like ribulose, rarely used for chemical syntheses, probably also due to its high price. However, it would be a good starting material for enzymatic transformations using enzymes from microorganisms (e.g. *E. coli*) or plants (e.g. *Pisum sativum*). The products available by simple transformations are (Scheme 9): 3-dehydroshikimic acid, 3-dehydroquinic acid, shikimate-3-phosphate, 5-enolpyruvyl-shikimate-3-phosphate, and possibly also chorismate



Scheme 9. Enzymatic synthesis of important enzyme substrates starting from shikimic acid.

ammonium chloride at 170–180 °C under pressure[51, 52]. In addition, synthetic methods for carboxy ^{13}C or ^{14}C labelled AHBA[50] and some AHBA-derivatives for biosynthetic research[37, 38, 51] have been published.

As an alternative to chemical synthesis the microbial production of AHBA could be envisaged. $P^{\ominus}$-mutants derived from *Nocardia mediterranei* N813 or A10 (see section 4) were found to accumulate a complex of aromatic compounds with product P8/1-OG as the major component. One of the minor components of this complex must be AHBA, as can be concluded from cofermentation experiments with strains A8 and P14[25]. AHBA was also detected in the culture medium of the mitomycin producer *Streptomyces verticillatus*[53]. Thus mutants (derived from ansamycin high producing strains) blocked in the AHBA activating enzyme system (AHBA→AHBA-CoA) should accumulate considerable amounts of AHBA. Indeed, Chinese workers have recently described a $P^{\ominus}$-mutant *Nocardia mediterranei* rif 1 accumulating AHBA[29]. Such a mutant could for instance be used for the biosynthesis of uniformly labeled AHBA. AHBA (unlabeled or labeled) could be used as a supplement for the C_7N starter unit in the screening for novel ansamycins in order to increase the initial ansamycin titers in natural low producing strains. AHBA does not seem to increase the rifamycin production in high producing strains such as *N. mediterranei* N813[25] or *N. mediterranei* NG12-4[29].

12. Concluding Remarks

Biosynthetic research projects such as the one presented in this review can result in a variety of valuable achievements:

- useful information on biosynthetic pathways, on the nature of enzymes and intermediates involved, and on the regulation of biosynthetic enzyme sequences;
- identification of novel natural compounds of the primary and secondary metabolism;
- identification of new leads for chemical syntheses;
- isolation of mutants suitable for the microbial production of labeled or unlabeled metabolites.

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