Investigations on the biosynthesis of the novel antimalarial drug artemisinin in the plant Artemisia annua.

Wallaart, Thorvald Eelco

Summary and Concluding Remarks

The aim of the project as described in this thesis was to gain insight in the biosynthetic pathway of artemisinin in order to be able to increase its content in the plant *Artemisia annua*.

Phytochemical analysis of *A. annua*, of Vietnamese origin, revealed dihydroartemisinic acid and the very unstable and reactive compound dihydroartemisinic acid hydroperoxide as new constituents. These compounds were found to be direct precursors of artemisinin in an *in vitro* reaction. The conversion of dihydroartemisinic acid into dihydroartemisinic acid hydroperoxide was found to be mediated by singlet (excited) oxygen ($^1O_2$), while the conversion of dihydroartemisinic acid hydroperoxide into artemisinin was found to be an oxidation with triplet oxygen ($^3O_2$) which took place spontaneously upon exposure to air. The presence of dihydroartemisinic acid and dihydroartemisinic acid hydroperoxide in *A. annua* and the conditions under which these conversions occurs makes it very likely that these reactions may also take place *in vivo* in *A. annua*. Nevertheless the question how $^1O_2$ is formed *in vivo* in *A. annua* remains. Although $^1O_2$ can be formed in various ways including enzymatic catalysis, it is known that $^1O_2$ is generally formed, in biological systems, by the mechanism of energy transfer from photoexcited compounds. Certain secondary plant products (chromophores) are capable of photosensitizing reactions that involve the transfer of light energy to oxygen. Because certain chromophores are known to be present in *A. annua*, the conditions for a photooxydative (non-enzymatic) conversion of dihydroartemisinic acid into its hydroperoxide seem to be present in the plant. Despite several enzyme assay experiments, we were never able to convert dihydroartemisinic acid into its hydroperoxide enzymatically (unpublished results). Taking these facts in account it has become very likely that in the last part of the biosynthetic pathway of artemisinin, starting with dihydroartemisinic acid no enzymes are involved. Additional indications for the existence of the non-enzymatic conversion of dihydroartemisinic acid into artemisinin were obtained by the increase of the artemisinin level and the simultaneous decrease of the dihydroartemisinic acid level after exposure of *A. annua* plants to a night-frost period. It is known from literature that relatively high levels of $^1O_2$ can be formed in plants under many stress conditions such as tissue damage caused by predators, pathogens or frost. It is stated in literature that the plant uses $^1O_2$ as a protective agent against pathogens and predators. Besides offering protection, $^1O_2$ can also damage the plant itself. It has been demonstrated for certain secondary metabolites of higher plants that they protect the plant against damaging photodynamic reactions by quenching $^1O_2$. This phenomenon is in accordance with our hypothesis that dihydroartemisinic acid is acting as an anti-oxidant by quenching $^1O_2$ yielding artemisinin as a stable end-product in which reactive oxygen is stored.

Although it became very likely that the conversion of dihydroartemisinic acid into artemisinin is a non-enzymatic one, it is still remarkable that a non-enzymatic reaction, consisting of several reaction steps, yields only one stereospecific product, in stead of a racemic mixture which one would expect. This led us to the assumption that the stereochemical configuration of the end product, artemisinin, should already be present in its precursor, dihydroartemisinic acid. Elucidation of the crystal structure of dihydroartemisinic acid by X-ray crystallographic analysis confirmed this hypothesis.

The gained knowledge of the biosynthetic pathway of artemisinin and of the compounds involved in this pathway (artemisinic acid, dihydroartemisinic acid and dihydroartemisinic acid hydroperoxide) gave us the opportunity to start an investigation to find an explanation for the considerable differences in artemisinin levels present between *A. annua's* of different geographical origins. Monitoring of the time course of the levels of artemisinin as well as its precursors in *A. annua's* of different geographical origins yielded insight in the fluxes of and relationships between the precursors of artemisinin. For the first time the *A. annua* plants of different geographical origin were found to belong to different chemotypes. A chemotype with a high artemisinin level was found to have also a high dihydroartemisinic acid level but a relatively low artemisinic acid level. Reversibly, a chemotype with low levels of artemisinin and dihydroartemisinic acid contained a high artemisinic acid level. Because artemisinic acid is considered to be the direct precursor of dihydroartemisinic acid, accumulation of artemisinic acid may indicate the presence of a rate-limiting step in the biosynthetic pathway of artemisinin towards dihydroartemisinic acid. The enzymatic reduction of artemisinic acid into dihydroartemisinic acid is probably a "bottle-neck" in the biosynthetic pathway of artemisinin in the chemotype with high artemisinic acid and consequentially low dihydroartemisinic acid and artemisinin levels. This step should be catalyzed by a postulated reducing enzyme, '*artemisinic acid reductase*'. Because all *A. annua's* except those of Vietnamese origin were found to accumulate artemisinic acid it is evident that the postulated reducing enzyme, '*artemisinic acid reductase*' is rate limiting in these chemotypes. This enzyme was obviously not rate limiting in the *A. annua's* of Vietnamese origin in which dihydroartemisinic acid was found to accumulate. Consequently the *A. annua's* of Vietnamese origin are also the chemotype with the highest artemisinin level. Interference in the biosynthetic pathway of artemisinin by metabolic engineering at the point of the postulated reducing enzyme, '*artemisinic acid reductase*', may yield increased levels of artemisinin in the non-Vietnamese *A. annua's*, in which this step is rate limiting. It is however questionable if this possible obtained increase will significantly exceed the artemisinin levels in the *A. annua's* of Vietnamese origin.

Interference in the biosynthetic pathway of artemisinin by metabolic engineering should be started at an earlier key step, which is also fundamentally rate limiting in the *A. annua* of Vietnamese origin. As mentioned before, the part of the biosynthetic pathway between dihydroartemisinic acid and artemisinin is very likely to be not enzymatically catalyzed and the reduction of artemisinic acid into dihydroartemisinic acid is only a rate limiting step in the *A. annua's* of non-Vietnamese origin. The part of the biosynthetic pathway of artemisinin between farnesyldiphosphate and artemisinic acid is for the *A. annua's* of Vietnamese origin the only part left in which rate limiting enzymatically catalysed conversions may be present. With the exception of farnesyldiphosphate, only highly speculative pathways have been proposed with respect to possible precursors of artemisinic acid. Screening of *A. annua* plants for the presence of possible precursors of artemisinic acid yielded amorpha-4,11-diene as most likely candidate. This compound could only be detected in *A. annua* at very low levels indicating that the conversions in this part of the pathway take place very rapidly. This suggests that there are no rate-limiting steps present between amorpha-4,11-diene and artemisinic acid. The remaining step in this part of the pathway is the cyclization of farnesyldiphosphate into the first specific precursor of artemisinin, the already mentioned amorpha-4,11-diene, by a sesquiterpene synthase, in this case *amorpha-*

4,11-diene synthase. The presence of a rate limiting step at this point is supported by literature data describing terpene synthases as enzymes occurring only at low intercellular concentrations and catalyzing reactions rather slow. Overexpression of the gene encoding the sesquiterpene synthase involved in the biosynthetic pathway of artemisinin may result in an *A. annua* variety with higher levels of artemisinin.

Besides this targeted approach to increase the artemisinin production, also a less specific, more general approach can be applied by inducing tetraploidy in *A. annua* plants. Using colchicin we induced tetraploidy in *A. annua* plants of Vietnamese origin. During a vegetation period we monitored the time course of the levels of artemisinin, its direct precursors, the biosynthetically related sesquiterpenes and the essential oil content in the diploid (wild-type) and tetraploid *A. annua* plants. The averaged artemisinin level in tetraploids was 38% higher than that of the wild-type as measured over the whole vegetation period. The averaged biomass of the leaves of the tetraploid plants was however lower in comparison to the wild-type plants. Therefore the artemisinin yield per m^2 tetraploids was decreased by 25%. Although, tetraploid *A. annua* plants can in principle be a useful starting material for a breeding program in order to obtain larger and faster growing plants, which produce higher levels of artemisinin, this has not been realized as yet.

Based on the results it can be concluded that overexpression of the gene encoding the sesquiterpene synthase involved in the biosynthetic pathway of artemisinin is the most straightforward and promising approach to obtain *A. annua* strains with an increased artemisinin production. As terpene synthases in general occur only at low intercellular concentrations, the isolation of these enzymes for a N-terminus determination is very difficult. The significant homology present between the terpene synthases enabled the design of oligonucleotides which were used in a PCR reaction yielding probes for the isolation of terpene synthase encoding cDNA clones. Because *A. annua* contains several terpene synthases, a number of different probes were obtained by PCR. These PCR-probes are a good basis for future research in order to obtain full lenght terpene synthase encoding cDNA clones especially that clone encoding the sesquiterpene synthase involved in the biosynthetic pathway of artemisinin.

Although we did not achieve metabolic pathway engineering in *A. annua*, this goal was reached by inducing tetraploidy in *A. annua* plants, in stead. Of most importance is that crusial parts of the biosynthetic pathway of artemisinin were elucidated and insights were gained about this pathway which were absolutely new. By showing where in the biosynthetic pathway of artemisinin the rate limiting steps (bottle-necks) are to be expected, very direct genetic strategies can be followed to increase the artemisinin production in *A. annua* plants.