Determining Viable Protocols for the Derivatisation of Artemisinin into Dihydroartemisinin and into Artesunate

A study commissioned through

Medicines for Malaria Venture (MMV)

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Preface

More than 600 million people, most of them children living in Sub-Saharan Africa, face daily the threat of dying from malaria because effective treatments are not accessible to them. In many malaria stricken areas affordable medicines, such as chloroquine, sulfadoxine–pyrimethamine (SP) and mefloquine, which have been used for many years, are no longer effective, because the parasites have become resistant [19]. Since 2001, WHO has therefore recommended the switch to artemisinin-based combination therapies (ACTs), which provide a rapid and reliable cure with very few side effects [2]. Between 2001 and 2005, 56 countries have adopted these ACTs as first or second line treatment and 29 countries have started deploying them [20]. The main problem with the ACTs is their price. ACTs are 10 to 20 times more expensive than the old monotherapies, which puts them beyond the reach of many people and particularly the poorer section of the population. So there is an urgent need to bring down the costs. Once the price has been reduced, and/or donors can subsidise the ACTs to a point where they can be made available to all those who need them, demand will outstrip production by far, so additional production capacities will also be required.

In order to produce ACTs, Artemisia annua, the plant from which artemisinin comes, has to be grown and extracted. Breeding more productive plants and extracting them in a more efficient manner can bring down some of the costs, which at the moment constitute approximately between 25% and 35% of the total costs. Because of its poor oral availability, artemisinin is not used directly anymore but modified into so-called derivatives like dihydroartemisinin, artesunate and artemether [1], adding another 20–40% to the costs, depending on the type of derivative chosen. Efforts to make this derivatisation as economical as possible are therefore worthwhile.

Derivatisation is either done by companies who extract artemisinin or by pharmaceutical companies who produce ACTs. These companies use their own proprietary protocols and any new entrant into the market will either have to rely on competitors to do the derivatisation or has to do his own research to find a suitable protocol. This situation poses an obstacle, for instance, if newly established extraction companies in Africa are to supply pharmaceutical companies in Africa when neither of them have access to their own or an independent derivatisation unit.

The aim of the study presented here is to provide publicly accessible protocols for the economically and ecologically viable production of dihydroartemisinin and artesunate, thus saving those who would like to enter this field some time and money in their pursuit.

In developing these protocols the following criteria were used: An approach that promised a higher yield was preferred over an approach with a lower yield, between two different agents the more effective agent was preferred over the less effective one and the cheaper agent was given preference over the more

expensive agent and finally, if economic efficiency was the same, preference was given to the less toxic agent.

The division of labour in this study was as follows: Silke Buzzi did the scientific research, Armin Presser acted as scientific advisor, and Michaela von Freyhold suggested and co-ordinated the study and was responsible for the economic considerations. We thank MMV for their support.

Part 1: Determining a Viable Protocol for the Derivatisation of Artemisinin into Dihydroartemisinin

Introduction

Dihydroartemisinin (DHA) is the simplest derivative of artemisinin and one where derivatisation reduces significantly the cost of the treatment, since the reduction in the amount of active substance required for the treatment after derivatisation, a reduction of about 40%, outweighs by far the costs of this modification.

At present the combination dihydroartemisinin-piperaquine is the cheapest option. It is, however, not yet on the official list of ACTs recommended for Africa.

DHA was developed in China about thirty years ago. Since then, a variety of protocols have been developed to perform the derivatisation.

Some protocols for the production of DHA have been described in the scientific literature, but most of these are not optimal for practical purposes.

Preliminary considerations

Choice of solvent and means of reduction

The aim of this part of the study is to determine an economically and environmentally optimised protocol for the chemical modification of artemisinin to dihydroartemisinin.

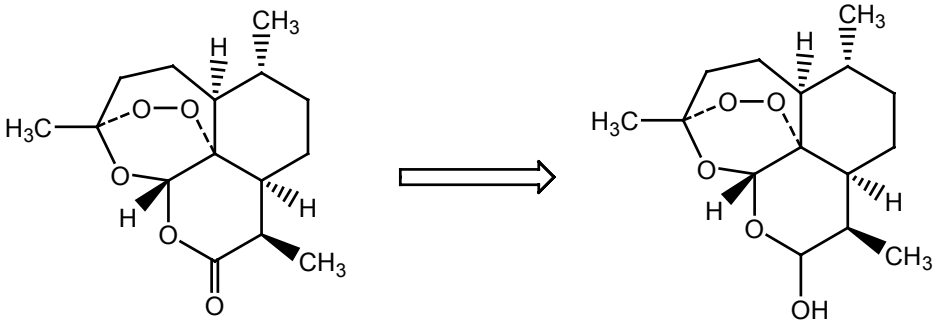
In the literature two methods of reduction are mentioned.

The first method proposes to convert artemisinin to dihydroartemisinin by reduction with sodium borohydride in methanol or ethanol at about 0 to 5°C [1-11]. In the literature only minor differences in the conditions of the reaction are reported, but there are obvious differences in the workup.

The second method shows the reduction with DIBAL-H in dichloromethane at -78°C [12-15]. The disadvantages of DIBAL-H are the smaller yield and the higher prices of both the solvent and the means of reduction.

In the literature THF is also used as a solvent for the reduction of artemisinin, but the use of THF was not considered here, because it is more toxic and expensive than methanol [9].

According to the considerations outlined above, sodium borohydride to be suspended in ethanol or methanol was selected as a base of the study.



Artemisinin *Dihydroartemisinin*

In order to decide between ethanol and methanol, the optimum solubility of artemisinin was investigated.

At room temperature, the concentration of a saturated solution (highest concentration of artemisinin) was shown to be approximately 0.5g/ 10ml for methanol and approximately 0.3g/ 10ml for ethanol.

Judging from these results, methanol is the better solvent and was therefore used in all the following steps. It should be mentioned that it takes a long time (about half a day) until the solution is saturated no matter whether artemisinin is added step by step to the methanol or all at once.

A higher saturation of the solution would be achieved by raising the temperature (40-50°C), but this attempt is of no actual use, because the reaction temperature is at 0 to 5°C and so the additional artemisinin in the solution would precipitate. In an effort to increase the solubility, the artemisinin was pulverised into smaller particles, but no change in solubility could be observed.

Point of departure

Among the protocols that use methanol and $NaBH_4$ the procedure described by Shrimali et al. [1] appeared to be the most promising, claiming a good yield while using the smallest amount of methanol. There are, however, some inconsistencies in this report:

According to Shrimali et al. [1] 10g of artemisinin should be dissolved in 40ml of methanol, but in view of the above mentioned solubility tests this seems to be more than questionable. The small amount of $NaBH_4$, which he added, only 0.25g, is also questionable. Even if, hypothetically, all four H-atoms would react with artemisinin, the amount of $NaBH_4$ involved would simply be too small. $NaBH_4$ also reacts (in parts) with the solvent methanol, and not only with artemisinin and therefore would not be completely available for the reaction of artemisinin to dihydroartemisinin. Experimentation was therefore required to determine the correct proportions.

Experimental section

General procedure

The experimental attempts focused on the following reaction:

Artemisinin suspended in methanol was cooled in an ice bath to about 0 to 5°C. To the cooled solution $NaBH_4$ was added step by step in small amounts over a period of 30 minutes. Afterwards, the solution was stirred vigorous for another hour. The reaction was monitored by TLC. Then the reaction mixture was neutralised (pH 5-6) with a mixture of 30% acetic acid/ methanol and evaporated to dryness under reduced pressure. The white residue was extracted with 50ml ethyl acetate several times. The ethyl acetate extracts were combined, dried with Na_2SO_4, filtered and evaporated to dryness under reduced pressure.

The characterisation of the structure was made by NMR and HPLC.

First attempt

Artemisinin was completely dissolved in methanol at a constant temperature of about 0 to 5°C (table: entry 1, page 14). $NaBH_4$ was slowly added to the artemisinin over a period of 30 minutes until a ratio of 1:1 was reached. During this procedure gas developed and the temperature increased by 1-2 degrees.

To monitor the conversion, a TLC (CH_2Cl_2:MeOH=20:0.5) was made after 1 hour. There was still artemisinin in the reaction mixture and therefore further amounts of $NaBH_4$ were gradually added. After 5 hours, the final ratio of artemisinin to $NaBH_4$ had increased to 1:3, but the TLC did not show any further reduction of artemisinin to dihydroartemisinin. The reaction mixture was neutralised with a mixture of 30% acetic acid/ methanol and evaporated to dryness under reduced pressure. The white residue was extracted with 50ml ethyl acetate five times. The combined ethyl acetate extracts were dried for 30 minutes with Na_2SO_4, filtered and evaporated to dryness under reduced pressure.

A yield of 90% was obtained.

The experiment showed that the long time taken for adding $NaBH_4$ to the reaction mixture was disadvantageous since artemisinin could be found unreacted in the product as shown in figure 1.

In the following tests all the $NaBH_4$ was added step by step in small amounts over a period of only 30 minutes, which led to better results.

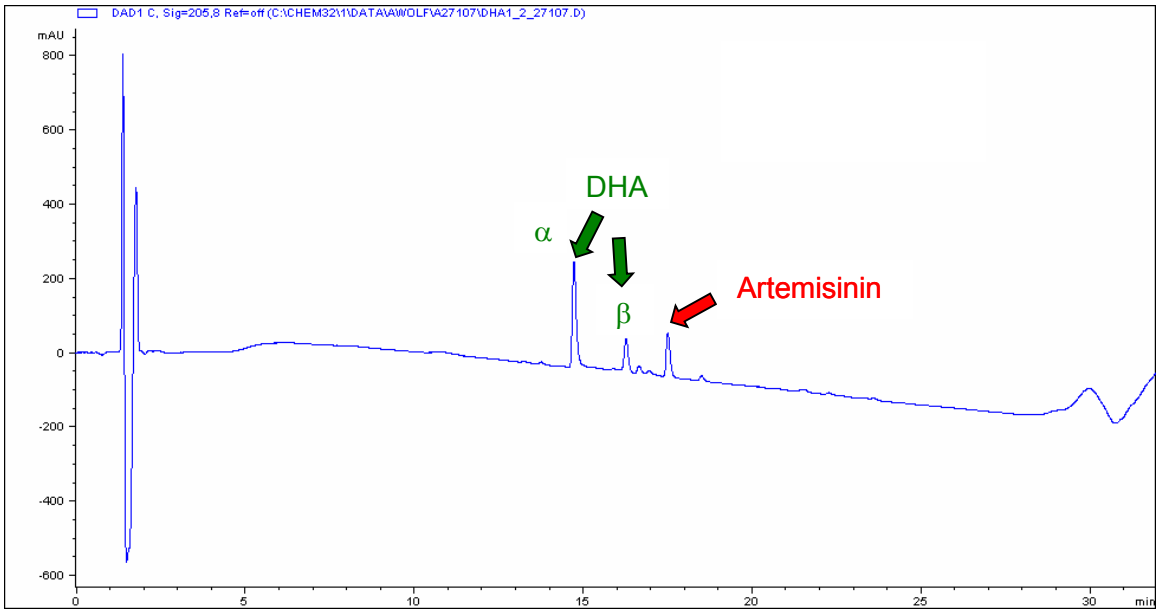


Figure 1: HPLC-curve of the first attempt

Changes in the amount of artemisinin

In the first experiments an unsaturated solution of artemisinin in methanol was used. Although yields were satisfying, the amount of methanol used was more than expected.

The next consideration was to perform the reduction of artemisinin in a more concentrated suspension. The amount of artemisinin in methanol was enhanced from 3g/ 40ml up to 6.6g/ 40ml. The obstacle to raising the concentration of the suspension was a mechanical one. From 5g/ 40ml onwards, a magnetic stirrer was not sufficient, therefore in the work with higher concentrations a mechanical stirrer had to be employed.

Changes in the amount of $NaBH_4$

In the literature different amounts of $NaBH_4$ are mentioned for the reduction of artemisinin to dihydroartemisinin. Some of these are questionable, because it can be theoretically predicted that the amount of $NaBH_4$ is too small. During the investigation different ratios of artemisinin to $NaBH_4$, such as 1:2, 1:2.5 and 1:3, were tested.

Since the same yield is achieved with ratios of 1:2.5 and 1:3; the first-mentioned ratio is the better option.

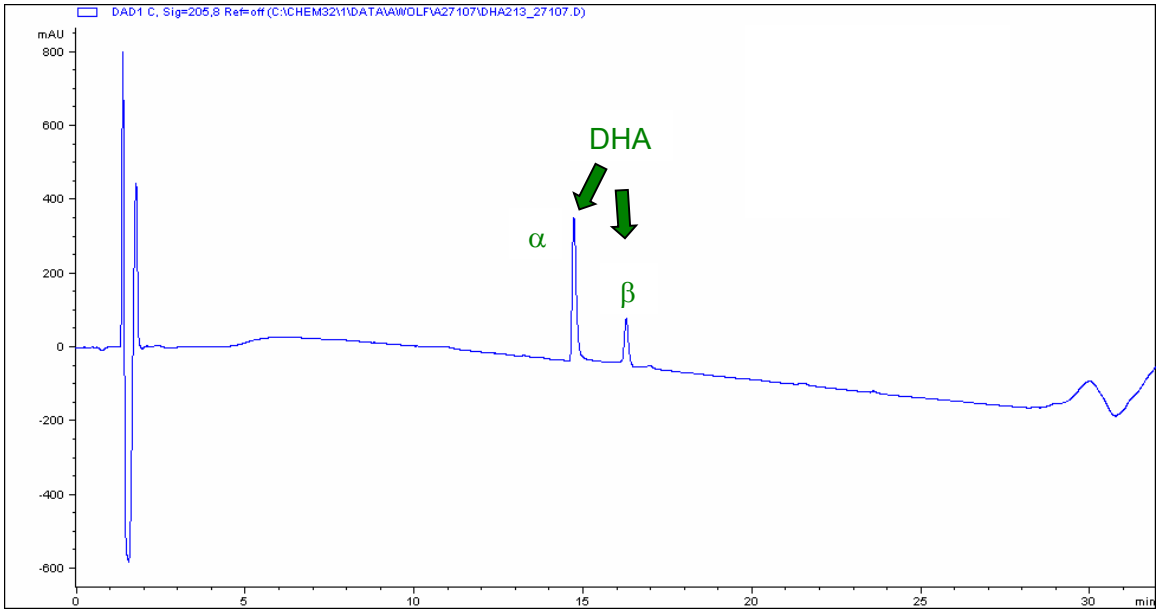


Figure 2: HPLC-curve of an optimised attempt

Two different types of $NaBH_4$ were used, powder and granulate, which both worked equally well. Due to health considerations granulate is the preferred option because less toxic dust emerges during handling. Furthermore, $NaBH_4$ granulate is more stable during storage.

Workup

In the literature two different workups are reported.

In most protocols, the reaction is stopped by neutralisation with acetic acid, the reaction mixture is evaporated, and the dihydroartemisinin in the residue extracted with ethyl acetate. The other method of workup is to precipitate the dihydroartemisinin with cold water after neutralising the reaction mixture with acetic acid.

The role of the acid is to destroy the excess of $NaBH_4$ and alkalic impurities. It should be mentioned that dihydroartemisinin is sensitive to acid conditions, and thus the pH value should not fall below about 5 to 6.
The first method of workup has only one minor disadvantage: the large amount of ethyl acetate necessary for complete extraction of the dihydroartemisinin. The ethyl acetate can, however, be recycled by column-distillation.

The second option for the workup was tried as well. After precipitation, the water of crystallisation could only be removed completely by dissolving the precipitate in dichloromethane and thereafter evaporating it to dryness under

reduced pressure. The yield was relatively low. The trial showed that with a high concentration of dihydroartemisinin in the reaction mixture, precipitation by adding cold water does not work in a satisfying way.

An additional possibility not mentioned in the literature is the use of hydrochloric acid instead of acetic acid for the neutralisation of the reaction mixture. By using hydrochloric acid, the surplus $NaBH_4$ should be destroyed, and the inorganic salts should not dissolve in the extraction agent (EtOAc) but should rather stay in the residue. It needs to be noted, however, that especially with hydrochloric acid, the acid has to be added very slowly in small amounts because this reaction is quite vigorous. In the first attempt, hydrochloric acid with the same pH value as the 30% acetic acid was used, a pH value of 2.

To obtain a reaction mixture with a pH value of 5 to 6, a larger amount of hydrochloric acid was needed compared to the quantity of acetic acid required. As a result, the destruction of excess $NaBH_4$ and alkalic impurities, which had been produced during the reaction, proceeded too slowly and incompletely.

In the next attempt diluted hydrochloric acid (2N in H_2O) was used, which led to a satisfying result.

When working with hydrochloric acid, however, the product, dihydroartemisinin, became a brown instead of a white crystalline powder, which is obviously a disadvantage.

The conclusion from the above trials was that neutralisation with acetic acid and, after evaporation, the extraction with ethyl acetate is more advantageous.

Recrystallisation

Recrystallisation is only necessary if the dihydroartemisinin is to be used directly as a drug. If DHA is only the first step in the production of some other derivative, recrystallisation is not necessary.

In the literature two different solvents for the purification of dihydroartemisinin by recrystallisation are mentioned and both of them were tested.

Firstly, ethyl acetate/ hexane in a ratio of 1:3 and secondly, diisopropyl ether were used. Dihydroartemisinin is brought to suspension with the solvent and heated up to 80-90°C. Afterwards small additional amounts of the corresponding solvent were added to the suspension and heated up to reflux again. In both cases no completely clear solution could be achieved, therefore the residue was eliminated by filtering with a heating funnel. The precipitation took place over night without action of light. Afterwards the precipitate was filtered under suction and dried under reduced pressure. TLC analysis detected in both mother liquors remnants of dihydroartemisinin, which can be recuperated during recrystallisation of the next batch.

During the process of recrystallisation, only minor differences between the two solvents were noted, like the faster precipitation of dihydroartemisinin with diisopropyl ether compared to ethyl acetate/ hexane. Another small difference is the crystal form that is finer with diisopropyl ether than with ethyl acetate.
Since there were no obvious advantages in using diisopropyl ether while there are more problems in the handling of the substance during the production process, ethyl acetate/ hexane appeared to be the better choice as the solvents for recrystallisation.

Recycling of methanol

Apparently, most laboratories working on the derivatisation of artemisinin treat the methanol used in the reaction as a consumable. Recycling of the methanol would be of economical and ecological advantage.

When evaporated methanol was used a second time, the reaction was too vigorous and the temperature increased up to 20°C, caused probably by the rapid destruction of $NaBH_4$, which did not react with the artemisinin anymore.

It was obvious that the evaporated methanol cannot be re-used without clarification, because impurities from the first reduction interfere with the following reduction. Theoretically, these impurities could be different forms of boric acid methyl esters.

There may be three different ways to clarify the used solvent: by addition of chemicals, by introduction of specifically designed polymers that filter out the impurities or by fractional distillation. Before any of these processes can be introduced, however, a more precise knowledge of the target compounds would be necessary. The characterisation of these impurities proved difficult. HPLC and NMR were used to determine the nature of the waste products, but no satisfying answers could be achieved with the available equipment.

Some attempts of chemical clarification of the methanol were nevertheless made:
At first, methanol was redistilled with a Vigreux column (20cm) at 90°C with the addition of solid NaOH and some water (pH 7-8). The base should saponify the esters into non-volatile acids, which should then stay in the distillation flask. When the methanol that had been redistilled in this manner was reused, the result remained unsatisfactory. The transformation of artemisinin to dihydroartemisinin remained incomplete, and the temperature increased too much.

In view of this unsatisfactory result the question arose whether the amount of base (NaOH) might have been under dosed. Therefore 2N NaOH was added to methanol until the pH value of 10, and then distilled with a column again. After redistillation, the methanol was reused for the reduction. This attempt also did not work. The transformation was incomplete, temperature increased and some decomposition products were found.

To try and remedy this situation, a small amount of dextrose, which should build a complex with the ester, was added to the redistilled methanol and stirred over night. This attempt was also unsuccessful.

Another consideration was that, maybe, the problem of the reusability of methanol stemmed from a small amount of water therein and not the esters of borohydride. Therefore a 3Å molecular sieve was added to the redistilled methanol, but this also did not lead to a satisfying result.

The problem of the reusability of methanol could not be solved, and would require more detailed chemical analysis of the spent solvent.
The question arose, whether it was not at least possible to employ the used methanol in the mixture of acetic acid and methanol needed for stopping the reaction.
Evaporated and redistilled methanol was used. In both cases a yield of 90% was obtained, which is lower than the yield with fresh methanol. During scale up this issue deserves to be explored further.

Use of NaOMe

$NaBH_4$ does not only react with artemisinin but, to a limited extent, with methanol also. In order to suppress the hydrolysis of $NaBH_4$ in methanol, the use of NaOMe is recommended [26]. The desired result of such an addition would be to reduce the amount of $NaBH_4$ required for the reaction and maybe even the amount of impurities in the spent solvent.

The test was started with a ratio of artemisinin to $NaBH_4$ of 1:1.5. The ratio of artemisinin to NaOMe was 1:0.02. The reaction conditions and the implementation were as usual. After 1 hour a TLC was made in order to monitor the conversion. There was still artemisinin in the reaction mixture as well as some by-products. Therefore another small amount of $NaBH_4$ was added to the reaction mixture and the ratio of artemisinin to $NaBH_4$ increased to 1:2. An hour later a TLC was performed, but no obvious differences could be seen, and the reaction was stopped.

A second test was made. The ratio of artemisinin to $NaBH_4$ was from the beginning of 1:2 and NaOMe was added in a ratio of 1:0.1 to artemisinin. The TLC showed no obvious difference to the first test and so the reaction was discarded. The reduction remained incomplete, the amount of $NaBH_4$ needed for the reaction could not be reduced, and some by-products developed. A possible explanation could be the high sensitivity of artemisinin to basic compounds.

In order to analyse the incomplete conversion of artemisinin to dihydroartemisinin a HPLC was made. The result is shown below.

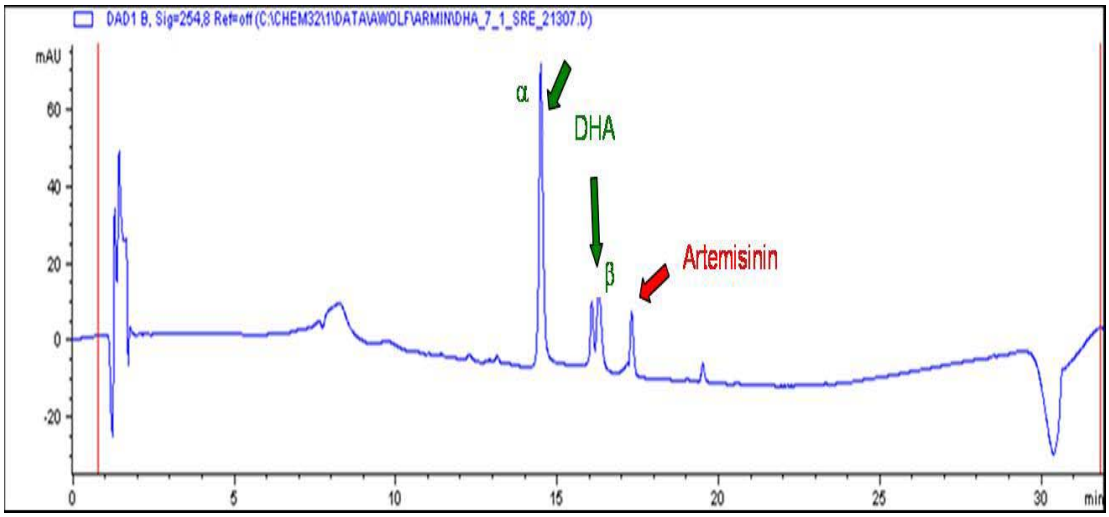


Figure 3: HPLC-curve of the test with NaOMe

Overview of the tests

Entry	Artemisinin	$NaBH_4$	MeOH	Ratio (Artemisinin/ $NaBH_4$)	Yield
1	1.0g [a]	402mg	40ml	1:3.0	90%
2	1.0g	268mg	40ml	1:2.0	91%
3	1.0g	402mg	40ml	1:3.0	95%
4	1.0g [b]	335mg	40ml	1:2.5	85%
5	1.0g [c]	335mg	40ml	1:2.5	99%
6	2.0g	670mg	20ml	1:2.5	95%
7	2.0g [g]	670mg	20ml	1:2.5	0%
8	2.0g [h]	670mg	20ml	1:2.5	0%
9	2.0g [i]	670mg	20ml	1:2.5	0%
10	3.0g	804mg	40ml	1:2.0	95%
11	3.0g	1.2g	40ml	1:3.0	96%
12	4.0g	1.6g	40ml	1:3.0	96%
13	4.0g	1.3g	40ml	1:2.5	96%
14	4.0g [c]	1.6g	40ml	1:2.5	90%
15	4.0g [c]	1.6g	40ml	1:2.5	80%
16	4.0g [d]	1.3g	40 ml	1:2.5	0%
17	4.0g [e]	1.3g	40 ml	1:2.5	88%
18	4.0g [e]	1.3g	40ml	1:2.5	96%
18	4.5g	1.5g	40ml	1:2.5	93%
20	4.5g	1.8g	40ml	1:3.0	93%
21	5.5g	1.85g	40ml	1:2.5	94%
22	6.5g [j]	2.2g	60ml	1:2.5	86%
23	6.6g [f]	2.2g	40ml	1:2.5	95%
24	2.0g [k]	537mg	20ml	1:2.0	0%

[a] long reaction time

[b] precipitation with water

[c] workup with hydrochloric acid

[d] reused MeOH

[e] reused ethyl acetate

[f] mechanical stirrer

[g] redistilled MeOH

[h] redistilled MeOH+ dextrose

[i] redistilled MeOH+ molecular sieve

[j] big attempt with a magnetic stirrer

[k] attempt with NaOMe

Results

The trials have led to a protocol, which produces a high yield of dihydroartemisinin, while the amount of methanol and the amount of $NaBH_4$ needed for the reduction could be reduced.

The acetic acid used to stop the reaction requires a somewhat tedious removal but works better than other alternatives tested.

The extracting agent, ethyl acetate, can be reused after a distillation with a column.

A still unsolved problem is the reusability of methanol. Some methods were tried but without success.

Detailed description of preferred option

material	amount	mmol	Mr
Artemisinin	6.6g	23.4mmol	282.34
$NaBH_4$	2.2g	58.4mmol	37.83
methanol dest.	40ml		

Apparatus:

- three- necked flask
- thermometer
- mechanical stirrer
- ice- bath
- venting

Implementation:

Artemisinin (6.6g) is suspended in methanol (40ml) and cooled in an ice bath to about 0 to 5°C. To the cooled suspension $NaBH_4$ (2.2g) is added step by step in small amounts over a period of 30 minutes. Afterwards, the reaction mixture is stirred vigorously for another hour.

> *Note:* In order to get a better distribution of artemisinin in methanol the reaction mixture should be stirred vigorously with a mechanical stirrer.
> It should be mentioned that some gas develops, and the temperature increases 1-2 degrees as the $NaBH_4$ is added.

The reaction is monitored by TLC (CH_2Cl_2:MeOH=20:0.5) to ensure a complete transformation.

Note: The substances are detected by spraying with molybdatophosphoric acid and by subsequent heating with a heat gun.

Workup:

A mixture of 12ml of 30% acetic acid and 12ml methanol is prepared and added to the solution until the pH value of about 5 to 6 is reached to stop the reaction. Afterwards the neutralised reaction mixture is evaporated to dryness under reduced pressure and finally lyophilised.

Note: Evaporating under high vacuum is necessary to remove the acetic acid completely from the residue. With the evaporator available in the laboratory a faint smell of acetic acid remained on the product, although the quantity involved was below the level of detection by NMR.

The residue is extracted with 50ml ethyl acetate several times (up to seven times) until no dihydroartemisinin can be found in the extracting agent. To control this, a TLC is made.

Note: The ethyl acetate can be recycled by column-distillation. On an industrial scale the amount of extracting agent and the number of extractions required could be reduced by counter-current continuous extraction.

The combined ethyl acetate extracts are dried with Na_2SO_4 (about 15-20g), filtered, and evaporated to dryness under reduced pressure (at the end with an oil pump).

Note: The combined extracts are cloudy, and after drying with Na_2SO_4, they should be filtered until they appear transparent.

6.3grams (95% yield), of a white, crystalline powder are gained, which is, according to NMR analysis, pure dihydroartemisinin (margin of error 1-2%).

The characterisation of the structure is made with NMR and HPLC.

Recrystallisation:

Recrystallisation is only necessary if the dihydroartemisinin is to be used directly as a drug. If it is only the first step in the production of some other derivatives, recrystallisation is not necessary.

Apparatus:

- round- bottom flask
- magnetic stirrer
- oil bath
- reflux condenser
- venting

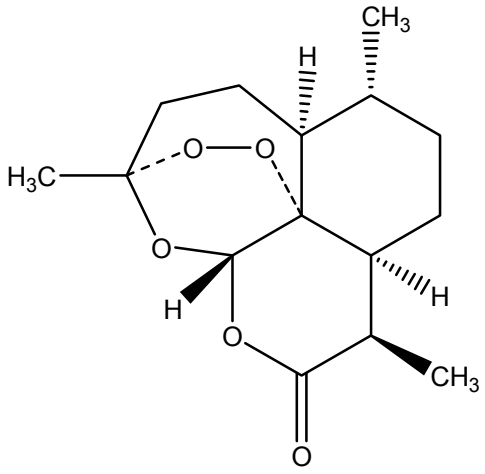
Recrystallisation in Ethyl acetate/ hexane
The product is suspended in hexane and heated up to reflux (80-90°C). Then ethyl acetate is added in small amounts to the suspension and heated up again to reflux. At the end the final ratio of ethyl acetate to hexane is 1:3, about 500ml in total.

The solution was not completely clear and was therefore filtered with a heating funnel. Afterwards, dihydroartemisinin was precipitated over night without action of light and then filtered under suction and dried under reduced pressure (73%; 4.6g).

In the mother liquor there was still some dihydroartemisinin (15-20%; 0.95-1.3g) that should be recovered when the next batch is recrystallised.

> *Note:* Reducing this large amount of solvent used for the recrystallisation is possible but is a technical rather than a chemical problem, which needs to be addressed during scale up.

Analytical results



Name:	**Artemisinin**
Chemical formula:	$C_{15}H_{22}O_5$
Molecular mass:	282.34g/mol
Appearance:	white, crystalline powder
Rf- Value:	0.72 (CH_2Cl_2:MeOH=20:0.5)

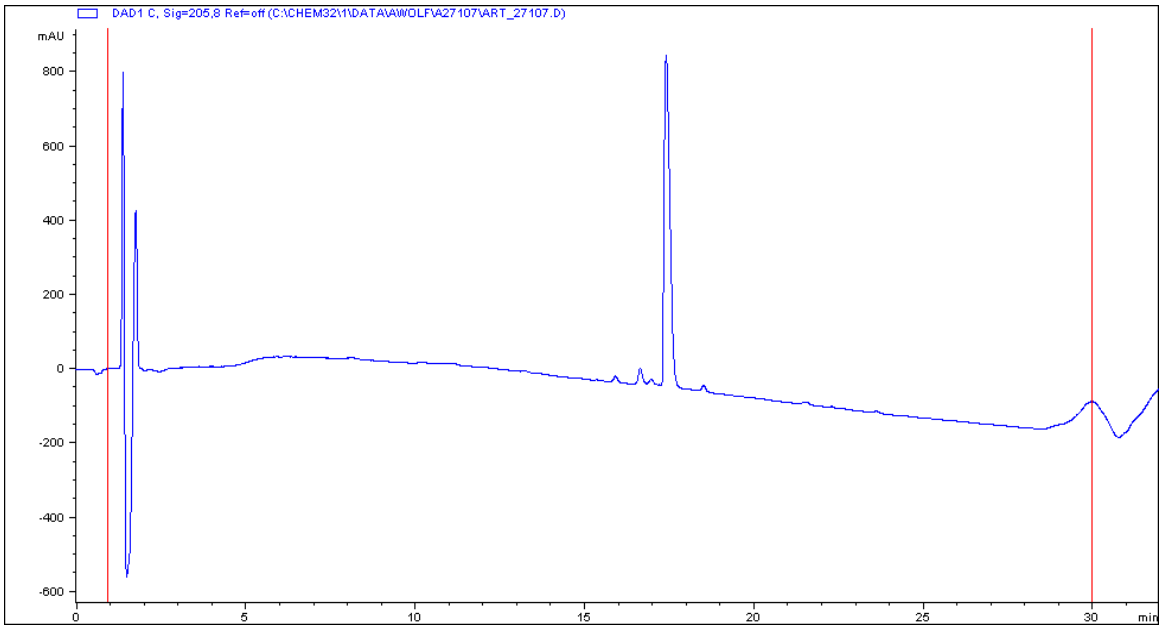
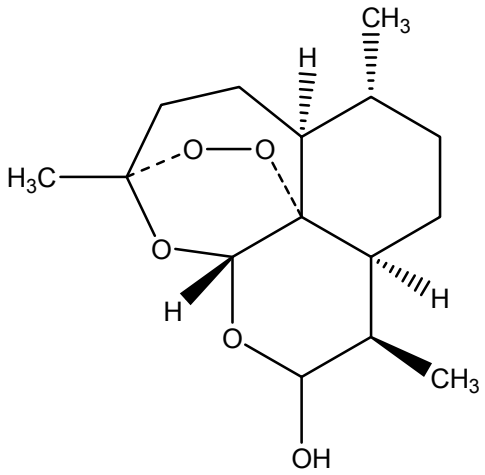


Figure 4: HPLC-curve from artemisinin sample



Name:	**Dihydroartemisinin**
Chemical formula:	$C_{15}H_{24}O_5$
Molecular mass:	284.35g/mol
Appearance:	white, crystalline powder
Rf- Value:	0.51 (CH_2Cl_2:MeOH=20:0.5)
Melting point:	142°C (identical with dihydroartemisinin sample)

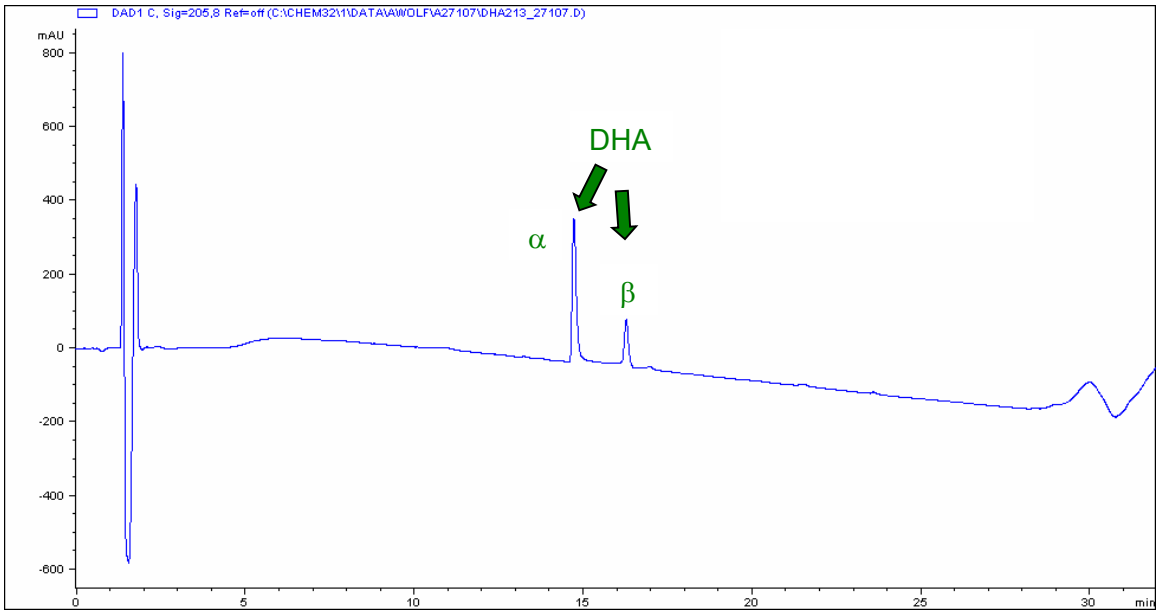


Figure 5: HPLC-curve of self- produced dihydroartemisinin

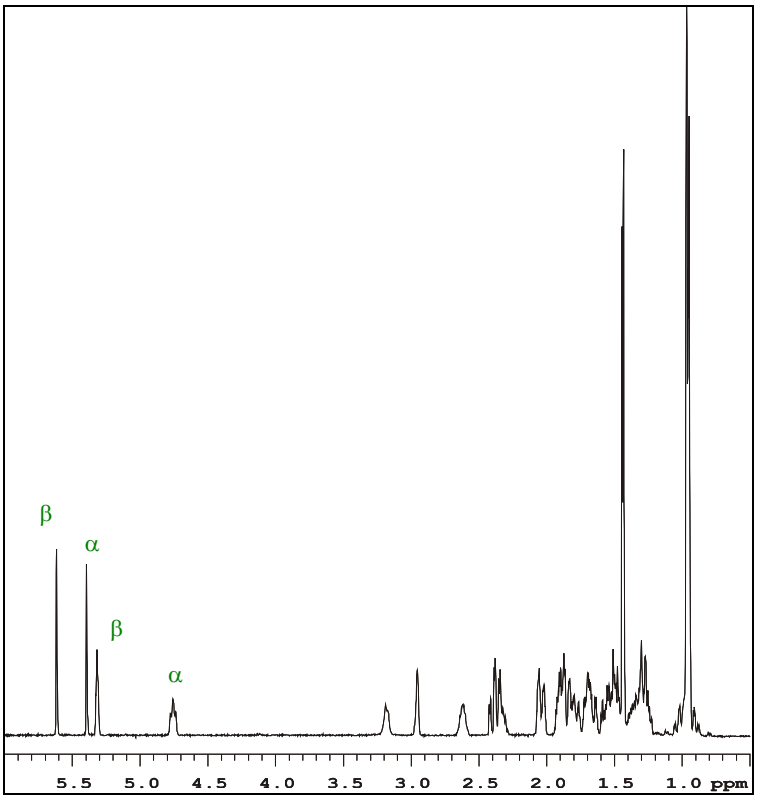


Figure 6: NMR of self-produced dihydroartemisinin

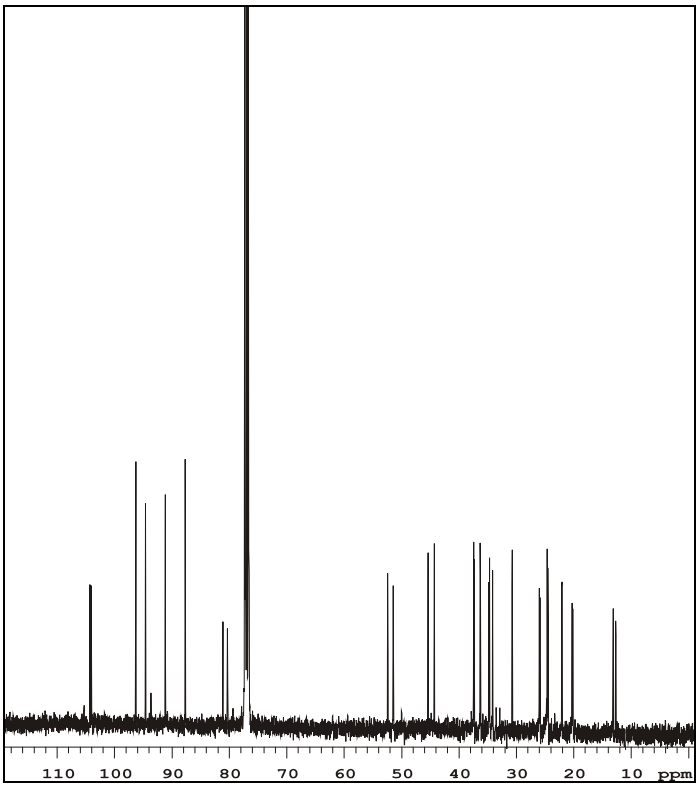


Figure 7: 13C-NMR of self-produced dihydroartemisinin

Methods of measurements

The dihydroartemisinin was characterised using two different methods.
At the beginning a NMR and a HPLC were made of the dihydroartemisinin that came from the Dang Quang Trading Company in Vietnam in order to make a meaningful comparison to the self-produced dihydroartemisinin.

The solvent for the NMR measurements is chloroform D+ 0.03% TMS.
The HPLC measurements are made with a gradient of two solvents, water with 1% formic acid and acetonitrile with 1% formic acid and a flow rate of 0.3ml/min.

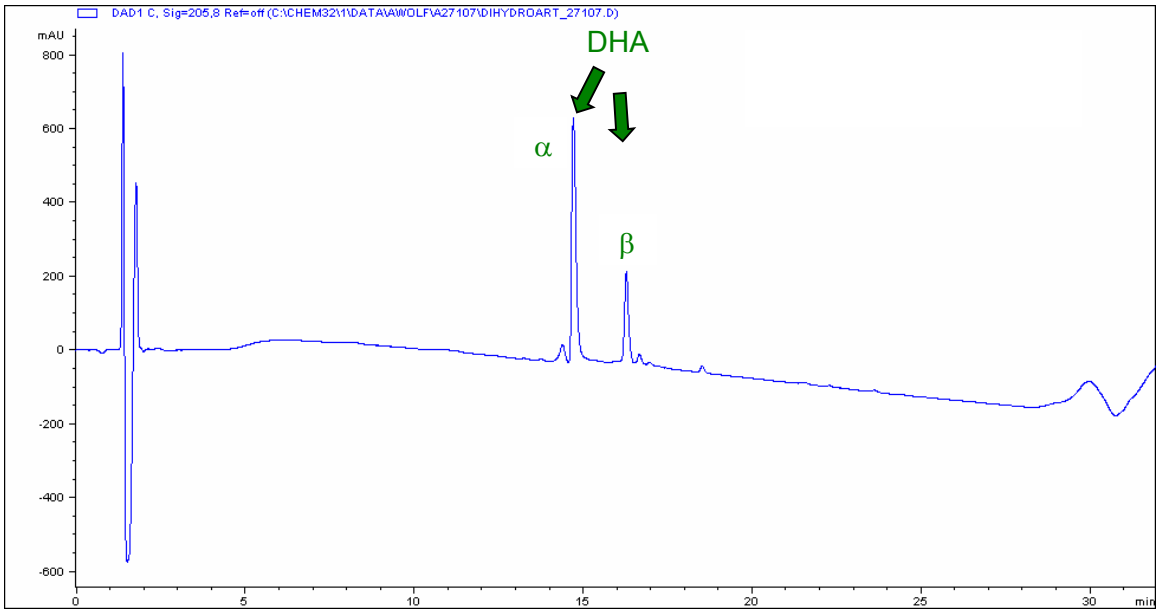


Figure 8: HPLC-curve of dihydroartemisinin sample

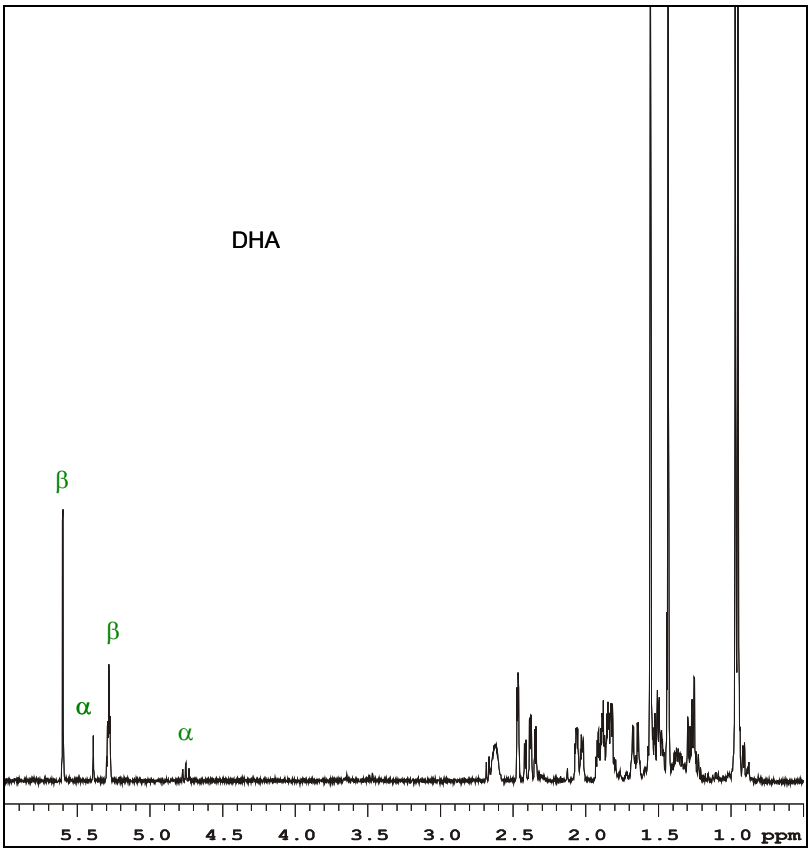


Figure 9: NMR of dihydroartemisinin sample

There is a difference in the spectrum of NMR if the measurement is done immediately after preparing the sample (only β-dihydroartemisinin; figure 10) or after a longer period of time (e.g. 12 hrs; α:β- dihydroartemisinin=1:1; figure 11). The reason for that is the adjustment of equilibrium between the two isomers of dihydroartemisinin, which is dependent on the solvent.

HPLC was used as well for the characterisation of dihydroartemisinin showing two different peaks in the spectrum. The first possible explanation was that due to the addition of an acid during the measurement an open-chained lactone had developed, but an additional measurement without acid proved this assumption to be wrong. Another reason could be the availability of the two isomers of dihydroartemisinin, α- and β-dihydroartemisinin. To solve this issue, an NMR was made using the same solvent ratio that produced the peaks in the HPLC measurement (70% water and 30% acetonitrile). In this way it could be shown that the two isomers are present in a specific ratio of about 1:2.8 (α/β-dihydroartemisinin; shown in figure 9).

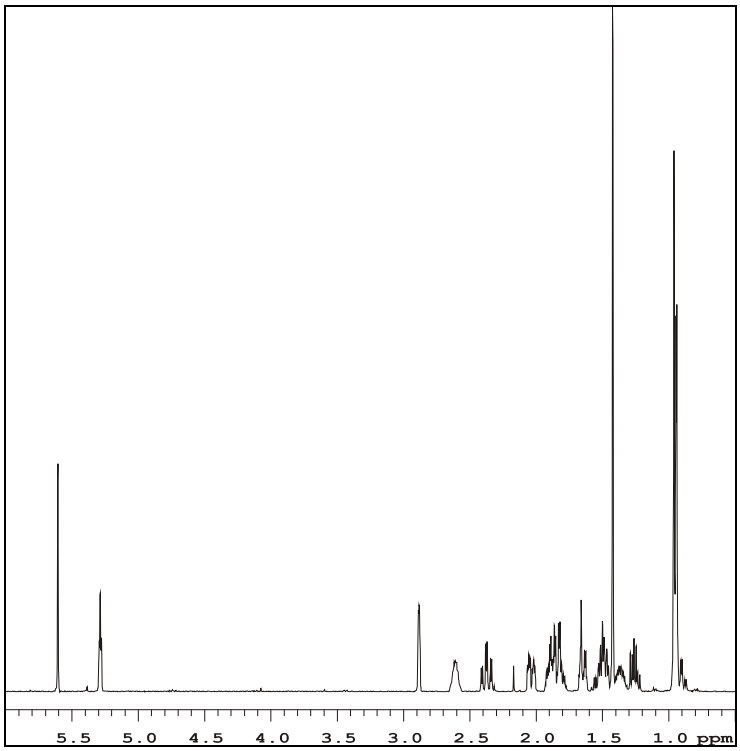


Figure 10: NMR of recrystallised dihydroartemisinin immediately after preparing the sample, β-dihydroartemisinin

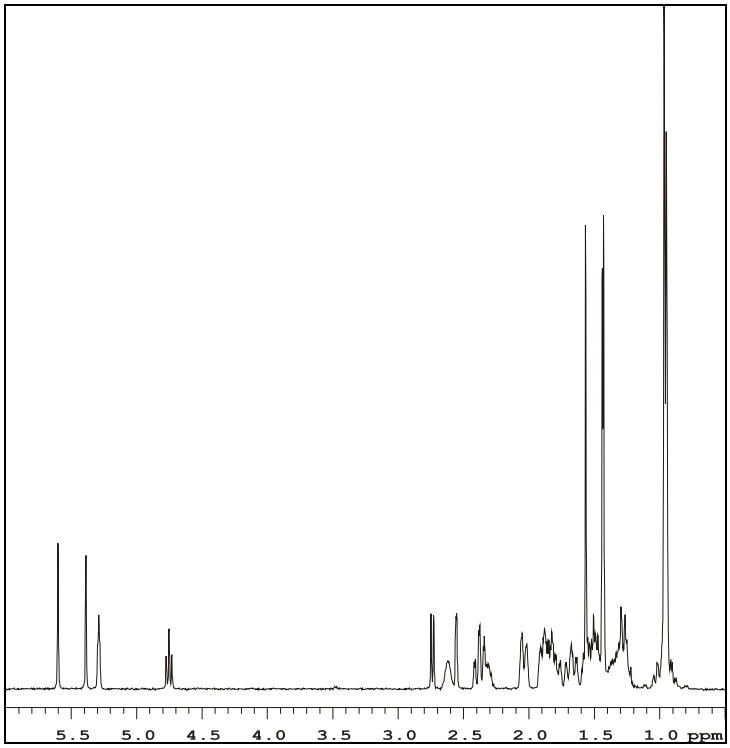


Figure 11: NMR of recrystallised dihydroartemisinin after 12 hours, α/β-dihydroartemisinin

Chemicals used

Acetic acid 30%, (Riedl- de Haën)
Acetonitrile, (Fluka)
Artemisinin, (Dang Quang Trading Company, Vietnam)
Aqua bi-destillata
Chloroform D+0.03% TMS, (Euro-top)
Dichloromethane, (Brenntag)
Dihydroartemisinin, (Dang Quang Trading Company, Vietnam)
Diisopropyl ether, (Fluka)
Ethanol 96%, (Brenntag)
Ethyl acetate dest., (Brenntag)
Formic acid, (Brenntag)
Hydrochloric acid, diluted (2mol/l) p.a., (Merck)
Methanol dest., (Brenntag)
Methanol redest.
Molybdophosphoric acid (Aldrich; in ethanol)
Sodium borohydride
  granulate, 10-40 mesh, 98%; (Sigma Aldrich)
  powder, 98%; (Sigma Aldrich)
Sodium sulphate anhydrous; (Merck)

Equipment used

Magnetic stirrer
IKAMAG RCT
Mechanical stirrer
Heidolph; typ RZR 1
NMR
Variant Unity Inova 400 MHz
HPLC
Analytic, RP
Agilent Zorbax SP- C18; 3.5µm; 2.1x 150mm with Guard Cartridge; flow 0.3ml
Diodes- Array- Detector; (Agilent)

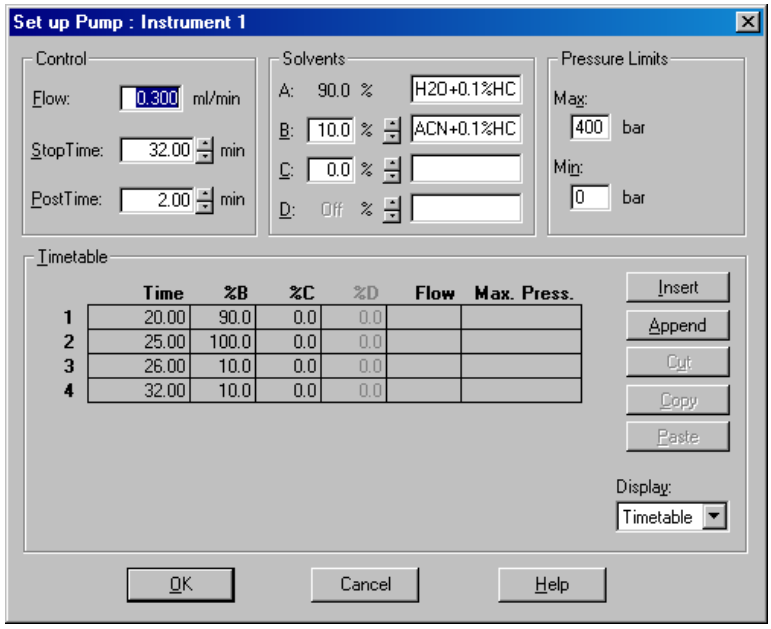
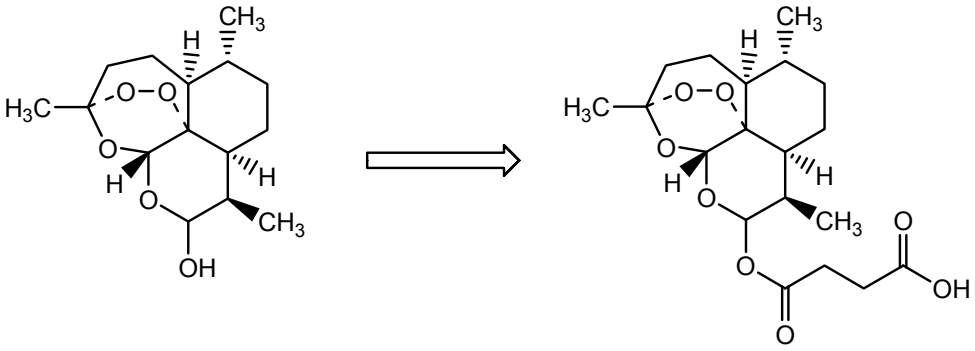


Figure11: screenshot of the setup of the HPLC-method

Part 2: Determining a viable protocol for the derivatisation of dihydroartemisinin into artesunate

Introduction

Since there have been some concerns regarding the shelf life of dihydroartemisinin in warm and humid climate, artesunate and artemether have become the preferred semisynthetic derivatives of artemisinin.
Artesunate, (synonym: dihydroartemisinin hemisuccinate) is synthesized by esterification of artemisinin with succinic acid anhydride in an alkaline medium [21].



Dihydroartemisinin *Artesunate*

Under the synonym artesunic acid, artesunate is dispensed in tablets without any further modification. After treatment with sodium bicarbonate, a salt (sodium artesunate) is obtained, which is more suitable for parenteral or rectal formulations [9]. Object of the study presented here is the artesunic acid.
Artesunate is widely employed in combination therapies (ACTs), with artesunate plus mefloquine and artesunate plus amodiaquine as the most common ACTs.

Preliminary considerations

Artesunate was first prepared by Chinese scientists at the end of 1979 [9].
Outside of Chinese journals, which are sometimes quoted but are not easily accessible, only a few complete protocols for the preparation of artesunate have been published.
Almost all accessible protocols propose very toxic solvents, in which the reaction takes place, such as pyridine and 1,2 dichloroethane. [9, 22]. These solvents are also listed as carcinogenic. Bhakuni et al. [9] describe a single pot conversion of artemisinin to artesunate using dioxane and tetrahydrofuran as solvents, which are equally problematic in terms of occupational and environmental hazards.

Ognyanov at al. [22] claim to address this problem by using acetone as a solvent for the reaction. Haynes [24] has, however, found that: „A patent report of a procedure with triethylamine as a base in acetone, tetrahydrofuran or dioxane giving yields of 92-96% cannot be reproduced.”. Even if this patent could be reproduced, the fact that it has been registered by a pharmaceutical company (Mepha) would pose problems of access to others whishing to set up their own derivatisation unit.
Development of another protocol, which uses a less noxious solvent, therefore became one of the objectives of the present study.

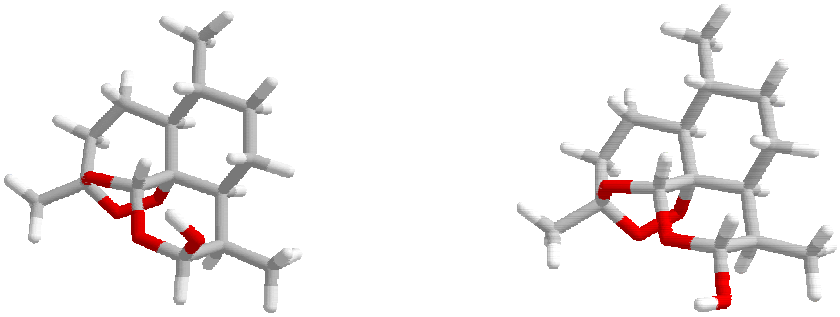
Drawing on experiences with general methods of esterification the trials started with triethylamine, 4-dimethylaminopyridine (DMAP) and ethyl acetate as solvent.

Like dihydroartemisinin artesunate also has two isomers, α and β, but only the α-isomer is wanted and used in therapy. It should be mentioned that dihydroartemisinin, which is produced by reduction of artemisinin, resulted as β-isomer. The α-isomer of dihydroartemisinin has not been isolated in a solid phase yet.

Dihydroartemisinin is unstable in solution and, depending on the solvent, an adjustment of equilibrium between the two isomers is reached. This makes it possible that succinic anhydride can react with dihydroartemisinin via the α-isomer.

As shown in the figure below, the semiacetal OH-group in the β-isomer is sterically hindered and therefore it is quite impossible for succinic anhydride to attack there. On the other hand, the OH-group of the α-isomer is equatorial and interacts less with neighbouring groups, so the succinic anhydride can easily attack [21, 23].

Another consideration for the occurrence of the α-isomer is higher stability and thermodynamically favoured conformation compared to the β-isomer [23].
This is the reason why only α-artesunate is received by esterification of dihydroartemisinin with succinic anhydride according to this protocol.



β-dihydroartemisinin *α-dihydroartemisinin*

Figure 12: energy minimum computations of α- and β- DHA using the MM+ force field

Experimental section

General procedure

The experimental attempts focused on the following reaction conditions:

Dihydroartemisinin suspended in ethyl acetate was cooled in an ice bath. Afterwards triethylamine was added and the mixture was stirred vigorously. To the cooled suspension succinic anhydride was added step by step in small amounts over a period of 30 minutes. After further 10 minutes the ice bath was removed and the solution was stirred for two to three hours at room temperature. The reaction was monitored by TLC. Water (cooled) was added to the reaction mixture and then neutralised (pH=5) with 2N H_2SO_4. The two phases were separated in a shaking funnel and the aqueous phase was extracted three times with ethyl acetate. The combined ethyl acetate extracts were washed once with water. Afterwards, the extract was dried with Na_2SO_4, filtered and evaporated to dryness under reduced pressure.
The characterisation of the structure was made by NMR and HPLC.

First attempt

The reaction took place under anhydrous conditions and ethyl acetate was dried over a 3Å molecular sieve.
Dihydroartemisinin was dissolved in ethyl acetate and cooled in an ice bath. Then 4-dimethylaminopyridine (DMAP) and triethylamine were added to the solution. After a few minutes succinic anhydride was added step by step in small amounts over a period of 30 minutes. The ratio of artesunate to triethylamine was 1:1, of artesunate to DMAP 1:0.05 and of artesunate to succinic anhydride 1:2.
After another 10 minutes the ice bath was removed and the reaction mixture was stirred for four and a half hours at room temperature.

In order to control the conversion, a TLC was made every hour (CH_2Cl_2:MeOH=20:1; CH_2Cl_2:MeOH=20:0.5). At the end the reaction time was 5 hours.
To stop the reaction, a solution of 2N H_2SO_4/ ethyl acetate equal to a ratio of 1:1 was added and the reaction mixture was stirred for about 10 minutes. The two phases were separated in a shaking funnel. The aqueous phase was washed twice with ethyl acetate. Then the combined ethyl acetate extracts were washed three times with 2N H_2SO_4, twice with 1N $NaHCO_3$ and twice with water (pH value should be neutral).
To monitor the workup a TLC of both phases, the aqueous and the organic, was made. The TLC showed that the product was in the aqueous and not in the organic layer. This could be put down to the fact that the salt of the free acid had developed during the extraction. Therefore, the aqueous phase was acidified with 2N H_2SO_4 (pH=1) and then extracted three times with ether and once with ethyl acetate. The combined organic layers were washed with water once, dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure.

A yield of 73% was obtained.

A possible explanation for the low yield could be the many steps of extraction during the workup, caused by the unexpected situation that the salt of the product was found in aqueous phase. A positive effect might be that the phase change could be a form of purification.

Changes in the use of reagents

In the first attempt ethyl acetate, which had been dried over a 3Å molecular sieve, was used as solvent. Triethylamine was added equimolar to dihydroartemisinin. DMAP was added in a ratio of 1:0.05 as a catalyst and succinic anhydride as a reagent in a ratio of 1:2. The first reactions were thus made under anhydrous conditions.
In following tests it could be shown that anhydrous conditions are unnecessary and ethyl acetate can be used without drying.
In addition it could be proved that the reaction also works without DMAP (dimethylaminopyridine) as a catalyst. There are no obvious differences in yield or in reaction time.
In one trial DMAP was successfully used without the presence of triethylamine. Since DMAP is especially dangerous in terms of occupational health, this method of esterification was dismissed.

Anhydrous tetrahydrofuran was also tested as a solvent. A yield of 66% was obtained. The received residue was not white but yellow. Furthermore, some dihydroartemisinin was found in the product after the normal reaction time of five hours. Consequently, in all the following tests only ethyl acetate was used as the solvent for the esterification of dihydroartemisinin to artesunate.

In conclusion, the esterification of dihydroartemisinin to artesunate with succinic anhydride can be achieved with ethyl acetate as the solvent. Triethylamine is

added in a ratio of 1:1.1 to dihydroartemisinin and succinic anhydride in a ratio of 1:2. The reaction works without a catalyst.

Changes in the amount of dihydroartemisinin

In the first attempts 500mg of dihydroartemisinin were brought to suspension in 15ml of ethyl acetate. The amount of the solvent was relatively large in comparison to the amount of dihydroartemisinin. In addition, the dihydroartemisinin was not completely dissolved although the reaction mixture clarified during the reaction. This could be due to the fact that artesunate is more soluble in ethyl acetate than dihydroartemisinin.
This result led to the conclusion that the esterification of dihydroartemisinin with succinic anhydride also works in a concentrated suspension.
The amount of dihydroartemisinin in ethyl acetate was enhanced from 500mg/ 15ml up to 10g/ 35ml.

It should be noted, however, that in all but one of the trials self produced dihydroartemisinin prior to recrystallisation was used. Recrystallised artesunate can be– depending on the recrystallisation conditions– more voluminous and would then require correspondingly higher amounts of ethyl acetate.

Changes in the amount of succinic anhydride

During the investigations different amounts of succinic anhydride were tested in order to find the smallest necessary amount for the best yield.
There is no obvious difference in yield using various ratios of dihydroartemisinin to succinic anhydride, such as 1:2, 1:2.5 and 1:3.
Obviously, the smallest ratio is economically and environmentally the most advantageous.

Changes in the workup

In the first attempt the reaction was stopped by adding a solution of 2N H_2SO_4/ ethyl acetate in a ratio of 1:1. The two phases were separated in a shaking funnel after 10 minutes of stirring the reaction mixture. The aqueous phase was washed twice with ethyl acetate. The combined ethyl acetate extracts were washed three times with 2N H_2SO_4, twice with 1N $NaHCO_3$ and twice with water.
To control the workup, a TLC was made, which showed that the product was not in the organic but in the aqueous phase. There is a great likelihood that the salt of the free acid was built during the extraction. Therefore, the aqueous phase was acidified and the free acid was extracted by ether and ethyl acetate as mentioned above.
Due to the many extraction steps the yield was only 73%.

In the next tests the extraction steps were reduced in order to cut down the amounts of extracting agents and thus the costs.
In addition, the free acid was extracted with ethyl acetate and not with ether from the aqueous phase. The advantage is that the same solvent, ethyl acetate is used for the reaction and the workup. Therefore, only one solvent has to be purified after the reaction. This would definitely reduce the costs.

This workup has, however, still many extraction steps, which make it a very time consuming procedure.
Bhakuni et al. [9] use another workup for the one pot conversion of artemisinin to artesunate, which was tested herein.
The reaction was stopped by adding cooled water. The reaction mixture was neutralised (pH=5) with 2N H_2SO_4 after a short time of stirring. After separating the two phases in a shaking funnel the aqueous phase was extracted three times with ethyl acetate. The combined ethyl acetate extracts were washed with water. Then the extract was dried over Na_2SO_4, filtered and evaporated to dryness under reduced pressure.
The yield could be increased (95-100%) and the amount of chemicals, which were needed for this workup could be reduced, making it the most suitable method.

Recrystallisation

The purification of artesunate by recrystallisation is only necessary if artesunate is directly used as a drug, not if it is one step in the production of some other derivatives.

Two different options of recrystallisation were tested. The solvents were ethyl acetate and hexane. The only difference between the two options was the sequence of adding the solvents.
For the first option artesunate was dissolved in ethyl acetate and heated up to reflux. Then small additional amounts of the corresponding solvent hexane were added to the solution and heated up to reflux again. The final ratio of ethyl acetate to hexane is 1:1. A yield of 65% was obtained.
For the second method artesunate was brought to suspension with hexane and heated up to 80-85°C. Afterwards small amounts of ethyl acetate were added until the solution became clarified and was then heated up to reflux again. The final ratio of hexane to ethyl acetate is 1:2. A yield of 62% was obtained.
In both cases the precipitation took place over night without action of light. Afterwards the precipitate was filtered under suction and dried under reduced pressure.
TLC analysis detected in both mother liquors remains of artesunate (about 30%), which can be recovered during the recrystallisation of the next approach.

There are only minor differences in the ease of handling and the results of the two methods of recrystallisation.
An advantage of the first method is the smaller amount of solvents needed for recrystallisation. The crystal form of the precipitate is finer than the one obtained from the second method.

The advantage of the second method is that the transition from the milky suspension to the clear solution is easier to monitor and the danger of using too much solvents does not arise.

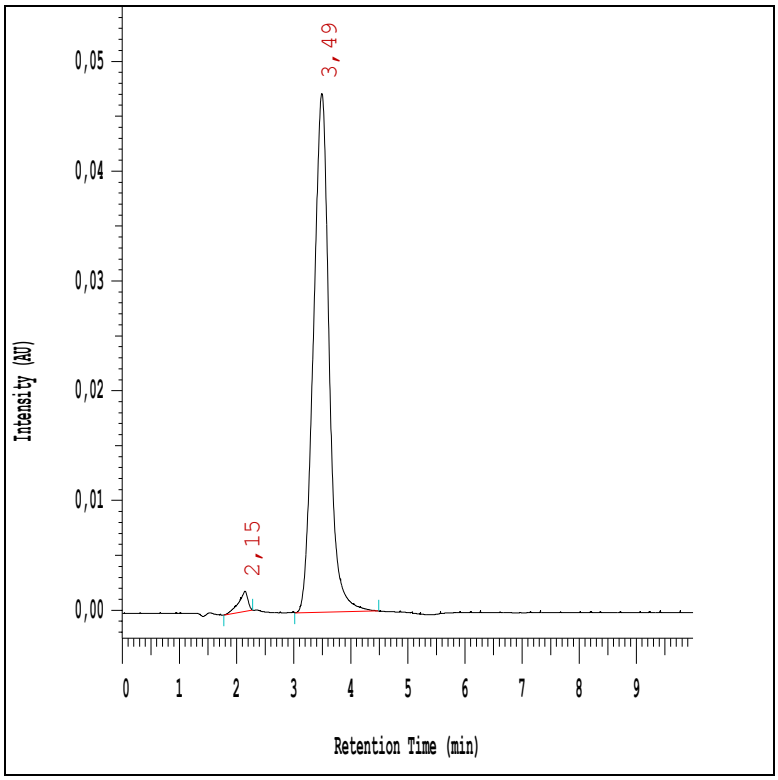


Figure 13: HPLC-curve of recrystallised artesunate

Recycling of ethyl acetate

The reusability of ethyl acetate is economically and ecologically important.

The first consideration was to use the ethyl acetate evaporated from previous attempts without purification. However, the transformation of dihydroartemisinin to artesunate was not complete with used ethyl acetate. In addition there were some water drops in the reaction mixture, which could be seen at the bottom of the two-necked flask immediately after stopping the magnetic stirrer. The reaction mixture became, however, clarified, which could be put down to the fact that the impurities produced during the reaction are soluble in water. It has to be pointed out that water should not be present during an esterification.

The next consideration was to dry the used ethyl acetate with Na_2SO_4 in order to remove the water. At the beginning the reaction seemed to work but after the usual reaction time there was still some dihydroartemisinin in the reaction mixture meaning that the transformation was still incomplete. Even though the reaction was worked up, the yield was only 78% and there was still some dihydroartemisinin residue left.

Another alternative was to redistill the used ethyl acetate with a Vigreux column (20cm) at 75°C. The impurities and the water should stay in the distillation flask. At the beginning of the distillation the apparatus was moistened by water drops. Therefore, the water in the apparatus had to be removed first, which took a very long time and led to a considerable loss of ethyl acetate. Afterwards the redistilled ethyl acetate could indeed be used for esterification of dihydroartemisinin to artesunate. The problem of the distillation of used ethyl acetate can be solved during scale up with other technical equipment such as a bigger Vigreux column.

In summary, it could be shown that the ethyl acetate used for the chemical modification of dihydroartemisinin to artesunate can be recycled and reused. There is, however, a need to improve the distillation process of the ethyl acetate (e.g. by using better distillation equipment).

Overview of the tests

Entry	DHA	Ethyl acetate	Ratio (DHA/ artesunate)	Yield
1[a,d]	0.5g	15ml	1:2.0	73%
2[a,b,d]	0.5g	15ml	1:2.0	66%
3[a,c,d]	0.5g	15ml	1:2.0	68%
4[a,d]	0.5g	15ml	1:2.5	74%
5[a,d]	0.5g	15ml	1:3.0	78%
6[a,d]	0.5g	15ml	1:3.0	70%
7[a,d]	0.5g	15ml	1:3.0	Quant.
8[d]	0.5g	15ml	1:3.0	58%
9	0.5g	15ml	1:3.0	Quant.
10	0.5g	15ml	1:3.0	89%
11	0.5g	15ml	1:2.5	83%
12	1.0g	15ml	1:2.5	Quant.
13[e]	0.5g	15ml	1:2.5	Quant.
14	2.0g	15ml	1:2.5	Quant.
15[f]	0.5g	15ml	1:2.5	0%
16	3.0g	15ml	1:2.5	Quant.
17	4.0g	15ml	1:2.5	97%
18	4.0g	15ml	1:2.0	96%
19[g]	2.0g	15ml	1:2.0	84%
20[h]	2.0g	15ml	1:2.0	78%
21[c]	4.0g	15ml	1:2.0	92%
22	10.0g	35ml	1:2.0	95%
23	4.0g	15ml	1:2.0	95%
24[g]	4.0g	15ml	1:2.0	Quant.

[a] absolute ethyl acetate

[b] absolute tetrahydrofuran

[c] bought dihydroartemisinin

[d] with DMAP

[e] only DMAP

Quant.: quantitative (100%)

[f] used ethyl acetate

[g] redistilled ethyl acetate

[h] used ethyl acetate dried over Na_2SO_4

Results

This work has led to a protocol for the esterification of dihydroartemisinin to artesunate with succinic anhydride and triethylamine.

Ethyl acetate, which is less toxic than the solvents mentioned in the literature, is used for the reaction and also for the workup. The solvent can be reused after distillation with a Vigreux column, which makes it more economic. On an industrial scale the purification of ethyl acetate could be optimised even further.
It could also be shown, that there is no need at all of a catalyst (such as DMAP), which is of economical and environmental advantage.

Detailed description of the preferred option

The following option gives the highest yield with the lowest use of inputs and is therefore economically and environmentally optimal.

material	amount	mmol	Mr
dihydroartemisinin	10g	35.17	284.35
triethylamine	5.4ml	38.68	101.20
succinic anhydride	7.04g	70.33	100.10
ethyl acetate	35ml		

Apparatus

- two- necked flask
- magnetic stirrer
- ice- bath
- venting

Implementation

Dihydroartemisinin (10g) is suspended in ethyl acetate (35ml) and cooled in an ice bath. Afterwards, triethylamine (5.40ml) is added and the mixture is stirred vigorously. To the cooled suspension succinic anhydride (7.04g) is added step by step in small amounts over a period of 30 minutes. After further 10 minutes the ice bath is removed and the solution is stirred for two to three hours at room temperature.

The reaction is monitored by TLC (CH_2Cl_2:MeOH=20:1) to ensure a complete transformation.

> *Note:* The substances are detected by spraying with molybdatophosphoric acid and by subsequent heating with a heat gun.

Workup

Water (50ml, cooled) is added to the reaction mixture and then neutralised (pH=5) with 2N H_2SO_4. The aqueous phase is extracted three times with about 20 ml of ethyl acetate until no artesunate can be found in the extracting agent. To control this, a TLC is made. The combined ethyl acetate extracts are washed once with water. Afterwards, the extract is dried with Na_2SO_4, filtered and evaporated to dryness under reduced pressure (with an oil pump at the end).

The yield is 12.86g (95%). A white crystalline powder is obtained, which is, according to NMR analysis, pure artesunate (margin of error 1-2%).

The protocol allows producing about 128.6 kg of artesunate from 100 kg of dihydroartemisinin. Taking both protocols proposed here together, it is possible to produce about 122.75 kg of artesunate from 1 kg of artemisinin.

The characterisation of the structure is made by NMR and HPLC.

Recrystallisation

Recrystallisation is only necessary if artesunate is used directly as a drug. If it is only one step in the production of some other derivatives, recrystallisation is not necessary.

Apparatus:

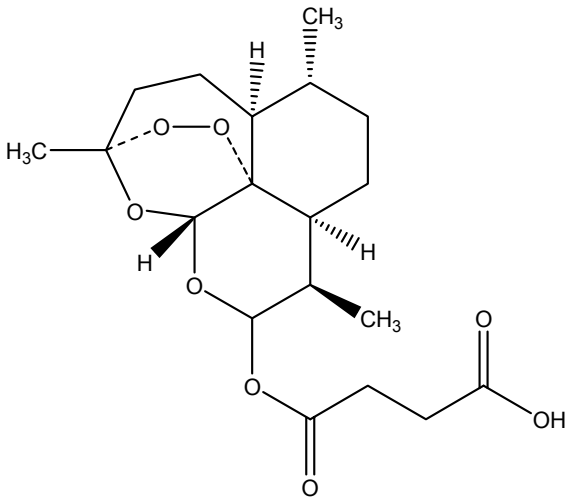
- round - bottom flask
- magnetic stirrer
- oil bath
- reflux condenser
- venting

Recrystallisation with ethyl acetate/hexane:
First artesunate is dissolved in ethyl acetate and heated up to reflux (80-85°C). Then hexane is added in small amounts to the solution and heated up to reflux again. At the end the final ratio of ethyl acetate to hexane is 1:1, about 50ml in total.

Recrystallisation with hexane/ethyl acetate:
The product is suspended in hexane and heated up to reflux (80-85°C). Afterwards, ethyl acetate is added in small amounts to the suspension until the whole artesunate is in solution and heated up to reflux again. At the end, the final ratio of hexane to ethyl acetate is 1:2, about 60ml in total.
Afterwards artesunate is precipitated over night without action of light, filtered under suction and dried under reduced pressure.

In the mother liquor there was still some dihydroartemisinin that should be recovered when the next batch is recrystallised.

Analytical results



Name:	**Artesunate**
Chemical formula:	$C_{19}H_{28}O_8$
Molecular mass:	384.42g/mol
Appearance:	crystalline, white powder
Rf-Value:	0.38 (CH_2Cl_2:MeOH=20:1)
Melting point:	135–137°C (133-135°C artesunate sample)

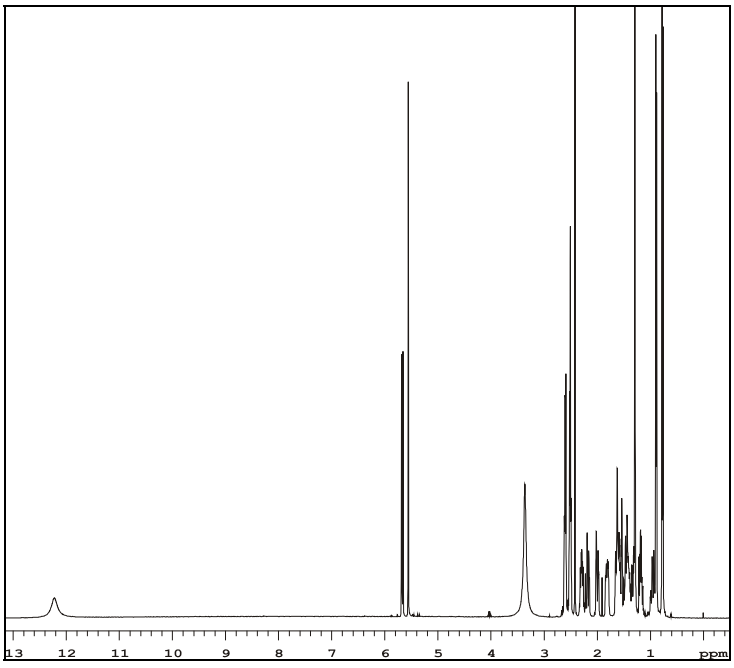


Figure 14: 1H-NMR of self-produced artesunate

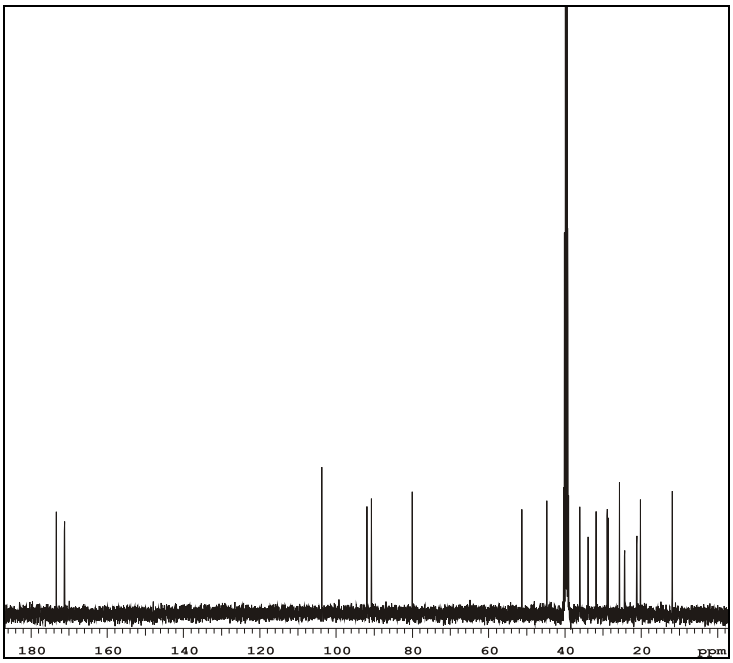


Figure 15: 13C-NMR of self-produced artesunate

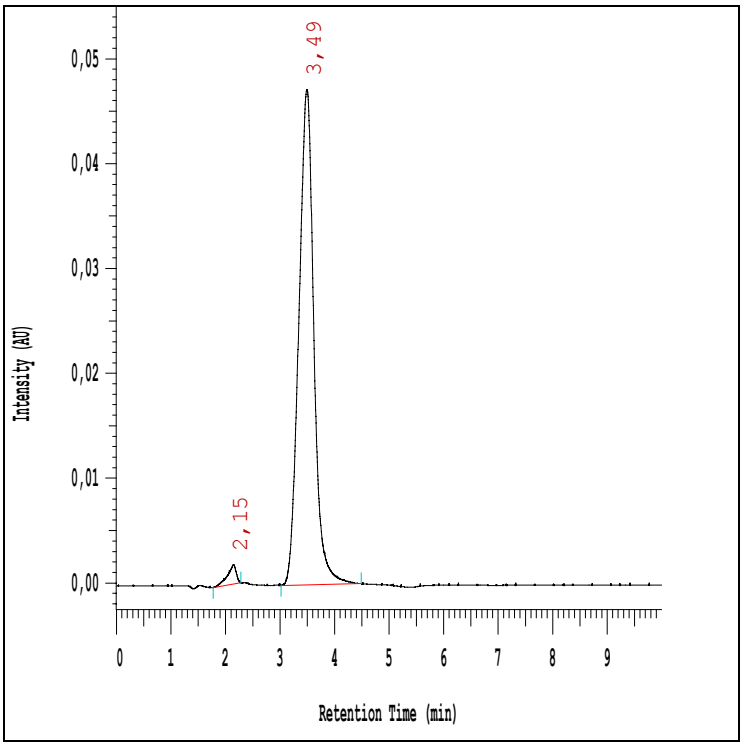


Figure 16: HPLC-curve of self-produced artesunate

Methods of measurements

The characterisation of artesunate was made with different methods.
In order to make a meaningful comparison of the self-produced artesunate with the one send from Botanical Development Ltd., NMR and HPLC measurements were made at the beginning.
The solvent for the NMR measurements is DMSO+0.03% TMS (dimethylsulphoxide).
The HPLC measurements are made with acetonitrile mixed with bidistillated water in a ratio of 4:1 as the solvent system and a flow rate of 0.3ml/min (see "equipment used").

Particularly the NMR was of importance in order to confirm that only the α-isomer was received. Haynes et al. [23] described precisely the difference between the two isomers of artesunate, which made the characterisation straightforward. (see figure 17, 18)

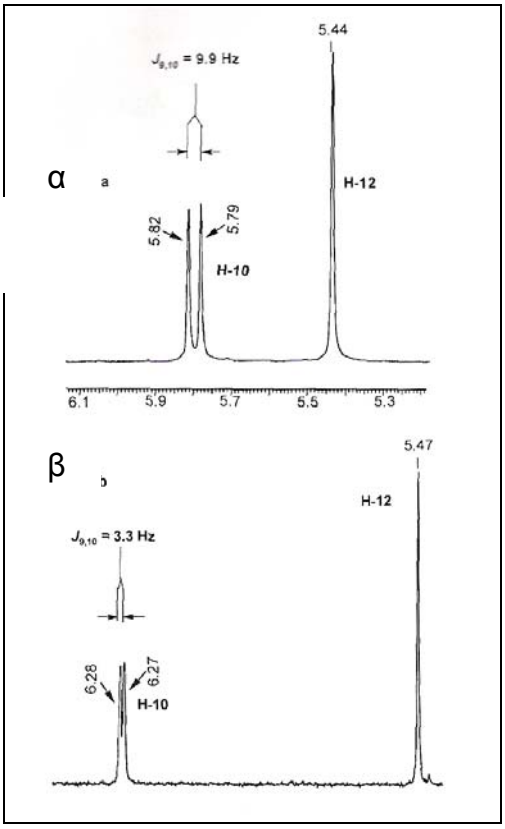


Figure 17: differences in the 1H-NMR between α-and β-isomer of the signal due to H-10 [23]

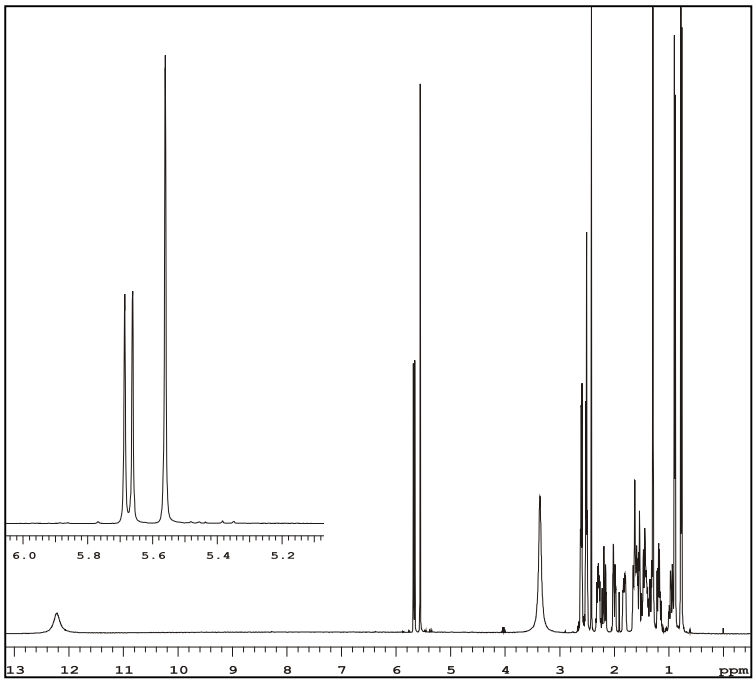


Figure 18: NMR of self-produced artesunate

Some changes were made in the HPLC method (see details below in “equipment used”). Artesunate was analysed isocratically.
The first peak in the HPLC-curve, which is shown below, is acetonitrile and the second one is artesunate. Some impurities (a few percent) can be found in individual samples (before recrystallisation; see figure 20), which were not further characterised in this work.

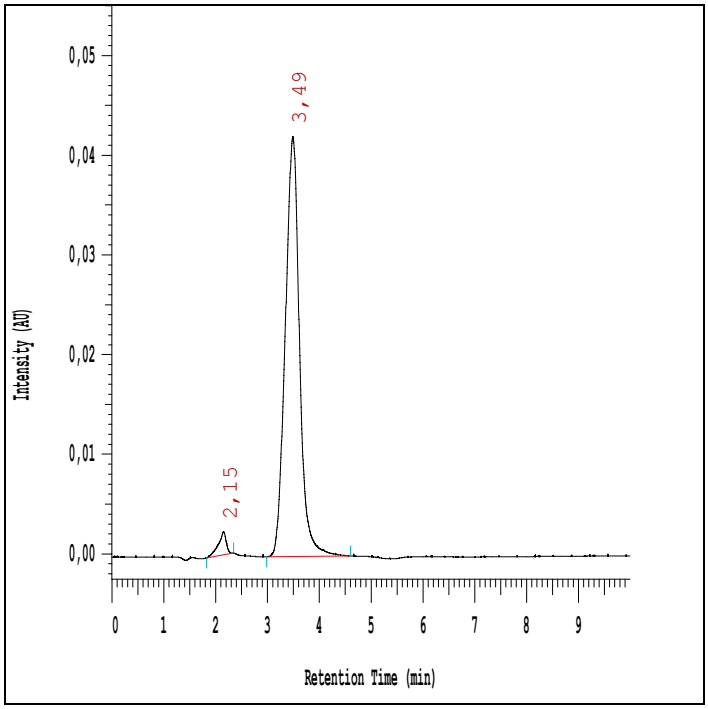


Figure 19: HPLC-curve of artesunate sample

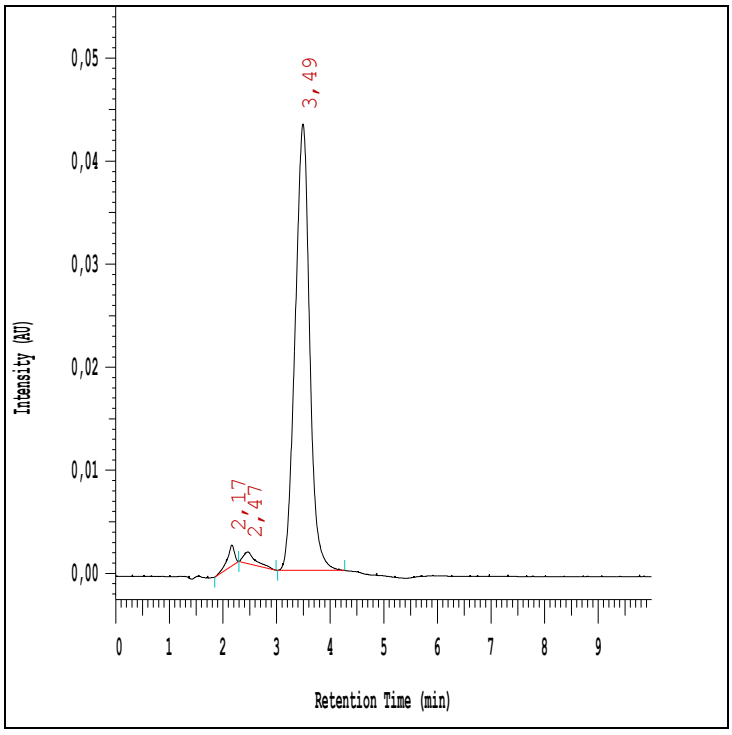


Figure 20: HPLC-curve of preferred option showing artesunate prior to recrystallisation

Chemicals used

Acetonitrile (Fluka)
Artemisinin (Dang Quang Trading Company, Vietnam)
Artesunate (Botanical Development Ltd., UK)
Aqua bidestillata
Dichloromethane (Brenntag)
Dihydroartemisinin (Dang Quang Trading Company, Vietnam; Botanical Development Ltd., UK)
Dimethylaminopyridin, DMAP (Sigma)
Dimethylsulphoxide (Euro-top)
Ether(Brenntag)
Ethyl acetate dest. (Brenntag)
Methanol dest. (Brenntag)
Molybdophosphoric acid (Aldrich; in ethanol)
Sodium bicarbonate (Merck)
Sodium borohydride
granulate, 10-40mesh, 98% (Sigma Aldrich)
Sodium sulphate anhydrous (Merck)
Succinic anhydride (Fluka)
Sulphurid acid (Merck)
Tetrahydrofuran (Fluka)
Triethylamine (Fluka)

Equipment used

Magnetic stirrer
- IKAMAG RCT

NMR
- Variant Unity Inova 400 MHz

HPLC
- Interface: Merck Hitachi D6000A
- Pump: Merck Hitachi L6200A
- Detector: Merck Hitachi L4250 UV-VIS
- Column: LiChrosphere 100, RP-18 (5µm, 125×3nm)
- Loop: 20µl
- Solvent system:acetonitrile:bid.water= 4:1
- Flow: 0.3ml/min
- Run time: 10min
- Wave length: 224nm

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